

Proline-Proline-Glutamic Acid (PPE) Protein Rv1168c of *Mycobacterium tuberculosis* Augments Transcription from HIV-1 Long Terminal Repeat Promoter^{*,§}

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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- κ 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.

Cells of the monocyte/macrophage lineage are shown to play a role in the pathogenesis of human immunodeficiency virus (HIV). The occurrence 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 show here that the *M. tuberculosis* proline-proline-glutamic acid (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- κ B (NF- κ B) 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 the NF- κ B signaling pathway. *In silico* docking analyses and mutation experiments have revealed that the N-terminal domain of Rv1168c specifically interacts with LRR motifs 15–20 of TLR2, and this site of interaction is different from that of Rv1196 protein (LRR motifs 11–15), indicating that the site of interaction on TLR2 dictates the downstream signaling events leading to activation of NF- κ B. This information may help in understanding the mechanism of pathogenesis of HIV-1 during *M. tuberculosis* co-infection.

Approximately 33.3 million people are living with the human immune deficiency virus type 1 (HIV-1) worldwide, 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)³ caused by *Mycobacterium tuberculosis* (2, 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 a long lived reservoir for the virus (9). However, 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 are also a source of increased HIV production in co-infected patients (10). Incubation of HIV-infected peripheral blood mononuclear cells with pleural fluid from individuals with TB induced more replication compared with the pleural fluid obtained from healthy controls and was dependent on TNF- α and IL-6 indicating that an overt proinflammatory microenvironment produced by the activated monocytes/macrophages 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, 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 expressions of viral genes are regulated by several host transcription factors such as

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³ The abbreviations used are: TB, tuberculosis; CAT, chloramphenicol acetyl transferase; LRR leucine-rich repeats; PDTc, pyrrolidinedithiocarbamate; EIA, enzyme immunoassay; PPE, proline-proline-glutamic acid; PE, proline-glutamic acid; PMA, phorbol 12-myristate 13-acetate; DIG, digoxigenin.

the Sp family, nuclear factor- κ B (NF- κ B) family, activator protein 1 (AP-1) proteins, nuclear factor of activated T cells, and CCAAT enhancer-binding protein family members by binding to the LTRs 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- κ B proteins are known to be one of the major modulators of the HIV-1 LTR in all cell types and are 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 correlates well with NF- κ B activity (20–22). Induction of these proinflammatory cytokines during mycobacterial infection has therefore been postulated to be one of the important factors that drive hyper-transcription from the LTR promoter (13, 14, 23, 24). However, several studies have suggested that additional mycobacterial factors may also be responsible for increased transcription from the 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-terminal 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). A 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 trans-activation.

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 overexpressed in macrophages infected with various clinical isolates of *M. tuberculosis* (44). Because 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/macro-

phage surface components. In this 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 LRR motifs 15–20 of TLR2 and activates NF- κ B signaling cascades that leads to HIV-1 LTR hyper-activation in a Tat-independent manner.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Rv1168c (rRv1168c) and Rv1196 (rRv1196) PPE Proteins—The full-length Rv1168c, Rv1196, and Rv1168c truncated genes were cloned and expressed, and the recombinant proteins were purified as described earlier by us (37, 39). Briefly, the ORFs were cloned in-frame with His₆ 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 until the absorbance at 600 nm reached about 0.5. The cultures were induced with isopropylthiogalactoside, and the His-tagged recombinant proteins were purified using TALON resin (Clontech). The Rv1168c Δ C and Rv1168c Δ N mutants were cloned in-frame in pRSET vector for affinity purification using histidine tags. To remove LPS contamination, the recombinant proteins were treated with 10% (v/v) polymyxin B-agarose (Sigma; binding capacity 200–500 μ g of LPS/ml) for 1 h 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, National Institutes of Health, from Dr. Barbara K. Felber and Dr. George N. Pavlakis. The cells were cultured in complete RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FBS, antibiotic/antimycotic (1 $\times$, containing penicillin G, streptomycin, and amphotericin B), 2 mM L-glutamine, and 10 mM HEPES (all from Invitrogen) and maintained at 37 °C and 5% CO₂ in a humidified incubator. HEK293 cell line was maintained in Dulbecco's modified Eagle's (DMEM) high glucose medium (Hyclone) containing 10% FBS, antibiotic/antimycotic (1 $\times$), 2 mM L-glutamine, and 10 mM HEPES (all from Invitrogen).

FITC Labeling—FITC-labeled rRv1168c (rRv1168c-FITC), FITC-labeled rRv1168 Δ N (rRv1168 Δ N-FITC), or FITC-labeled rRv1168 Δ C (rRv1168 Δ C-FITC) was prepared by incubating the recombinant protein with FITC using a commercially available FITC antibody labeling kit from Pierce following the manufacturer's protocol.

Biotinylation of rRv1168c—Biotinylation of recombinant protein was carried out using a commercially available biotinylation kit from Pierce, as described earlier (37). Briefly, rRv1168c was incubated with a 5-fold molar excess of Sulfo-NHS-biotin reagent (sulfo-succinimidyl-2-[biotinamido] ethyl-1,3-dithiopropionate) for about 1 h at room temperature. The nonreacted reagent was removed from the biotinylated protein sample by desalting using Amicon ultracentrifugal filter units.

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Biotinylation of the protein was confirmed by enzyme immunoassay (EIA) using streptavidin conjugated to horseradish peroxidase (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- κ B-binding sites (pDkB-HIV-CAT), were kind gifts from Debashish Mitra (NCCS, Pune, India) and AIDS Research Program, Division of AIDS, NIAID, National Institutes of Health.

Transient Transfection—Transfection in THP-1 cells was carried out using liposome-mediated transfection. DMRIE-C transfection reagent (Invitrogen) 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 the manufacturer's instruction. Briefly, 0.5 million cells were seeded in a 12-well plate in Opti-MEM (Invitrogen). One μ g of DNA and 3 μ l of DMRIE-C reagent were separately incubated in 50 μ l of Opti-MEM each for 15 min. The two preparations were mixed slowly and incubated for 30 min with intermittent mixing. The complex was added to the cells dropwise in a culture plate. Complete medium with 20% FBS was added after 5–6 h post-transfection. The cells were further incubated with various concentrations of rRv1168c protein, 10 h after transfection, and CAT/ β -gal reporter assay was carried out 36 h after treatment with the recombinant protein. The negative-control scrambled siRNA, TLR2 targeting siRNA (sense, 5'-GCCUUGACCUGU-CCAACAtt 3', where the lowercase letters represent two deoxy bases that serve as overhangs for the cleavage by dicer) were purchased from Ambion Inc. (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 24 h post-transfection.

Treatment of BF-24 Cells with the Recombinant Rv1168c/Rv1168 Δ C Protein—BF-24 cells were seeded as 1 million per well and either left untreated or treated with various concentrations of rRv1168c/rRv1168 Δ C protein. Untreated cells were used to check for the basal level expression of HIV-1 LTR. After stimulation, cells were incubated for 36 h 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 h) were treated with various concentrations of rRv1168c/rRv1168 Δ C, and CAT activity was measured after 36 h.

CAT and β -Galactosidase (β -Gal) Reporter Assay—The CAT and β -gal activity was measured using the CAT and the β -gal reporter assay kits from Roche Applied Science following the manufacturer's protocol. Briefly, the cells were harvested and centrifuged at 4000 rpm for 10 min at 4 °C. The pellet was washed twice with PBS and suspended in 500 μ l of Lysis buffer. After incubation at room temperature for 30 min, the lysate was centrifuged at 10,000 rpm for 10 min at 4 °C. Protein was estimated by BCA method (Pierce), and 50–100 μ g of protein lysate was added into a microplate (Roche Applied Science) precoated either with anti-CAT or anti- β -gal antibody (Ab). The plate was incubated at 37 °C for 2 h and washed five times with wash buffer. Anti-CAT-digoxigenin (DIG) Ab or anti- β -

gal-DIG Ab was added, and the plate was further incubated for 1 h. The plate was again washed five times with wash buffer, and anti-DIG-peroxidase (anti-DIG-POD) was added and incubated for 1 h at 37 °C. The plate was washed, and 200 μ l of POD substrate (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt) was added. The plate was incubated at room temperature until the color developed. The reading was taken on an ELISA reader at 405 nm (490 nm reference wavelength).

Western Blotting for p50 and p65 NF- κ B Transcription Factors—The nuclear p50 and p65 transcription factors were detected by Western blotting. The nuclear extracts were prepared from Nonidet P-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 a p50 or p65 (Santa Cruz Biotechnology) transcription factor. The membrane was next incubated with anti-rabbit immunoglobulin G (IgG) coupled to HRP (Sigma). Bound enzyme was detected by chemiluminescence following the manufacturer's protocol (GE Healthcare). Equal loading of protein was confirmed by Ponceau S Red stain (Sigma).

Estimation of TNF- α —The TNF- α cytokine in various macrophage culture supernatants was quantified by two-site sandwich EIA as described earlier (48) (Pharmingen). 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 μ g) were incubated for 30 min at room temperature with 1 ng of 32 P-end-labeled NF- κ B consensus binding sequence 5'-AGTTGAGG-GGACTTTCCAGG-3' (50) in binding buffer (20 mM HEPES, 0.5 mM DTT, 1 mM MgCl₂, 1 mM EDTA and 5% glycerol) containing 2 μ g of poly(dI-dC). The DNA-protein complex was resolved on 7% native gel in 1 $\times$ 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 unlabeled probe. The gel was dried at 80 °C for 1 h 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 (1 $\times$ PBS with 0.5% FBS) and incubated either with medium alone or with a 2- or 15-fold excess recombinant Rv1196 protein for 30 min on ice followed by incubation with 10 μ g/ml biotin-labeled Rv1168c (Rv1168c-biotin) for another 30 min on ice. Cells were washed three times with staining buffer and incubated with streptavidin-FITC (Sigma) for 30 min on ice. Cells were washed, and cell-bound fluorescence was analyzed with the BD FACSVantage SE (BD Biosciences) using CellQuest data analysis software (BD Biosciences).

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 (LRRs 10–15), and Mut4 (LRRs 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 Lipofectamine 2000 (Invitrogen) 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 μ l of lysis buffer (1% Nonidet P-40, 20 mM Tris-Cl, pH 7.4, 10% v/v glycerol, 150 mM NaCl, 20 mM NaF, protease inhibitor mixture). After centrifugation at 12,000 rpm for 15 min 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). 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 h, the 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.

***M. smegmatis* Culture and Transformation**—*M. smegmatis* mc²155 bacteria were grown in Middlebrook 7H9 medium supplemented with 10% albumin-dextrose-catalase (HiMedia, India), 0.5% glycerol, and 0.05% Tween 80 (7H9-albumin-dextrose-catalase-Tween 80). To prepare the competent cells, the culture was allowed to grow until mid log phase. The culture was centrifuged at 3000 rpm for 10 min, washed four times with 10% glycerol, and resuspended in 1/100th of the culture volume in sterile deionized water, 100 μ l aliquots of the cells were snap-frozen and stored at –80 °C until 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 (*Rv1168c* Δ N) was generated by cloning nucleotide sequence representing amino acids 175–346 from BAC contig Rv71(C2). The C-terminal truncated mutant (*Rv1168c* Δ C) was generated by cloning the 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 μ g of DNA was added. The cells were incubated on ice for 10 min and transferred to a prechilled 1-mm gap width cuvette. Electroporation was performed following standard procedure (51). One ml of Middlebrook 7H9-ADCT was added immediately, and the cells were allowed to grow at 37 °C for 4 h to allow the expression of antibiotic resistance genes. The transformants

were selected on 7H9-ADCT agar plates containing 50 μ g/ml kanamycin and 50 μ g/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. smegmatis* Rv1168c) or *Rv1196* (*M. smegmatis* Rv1196), *Rv1168c* Δ N (*M. smegmatis* Rv1168 Δ N) or *Rv1168c* Δ C (*M. smegmatis* Rv1168 Δ C), or the backbone vector (*M. smegmatis* pVV16) at 10 multiplicities of infection. After 4 h, cells were treated with gentamicin to a final concentration of 200 μ g/ml to inhibit the growth of the extracellular bacteria. After 36 h of infection, whole cell extracts were prepared, and CAT expression was measured by ELISA.

Protein-Protein Docking Studies—The N-terminal region (2–171 amino acids) of the *Rv1168c* protein sequence showed nearly 35% sequence identity with the B chain of the 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 the *Rv1168c* protein N-terminal region. The sequence alignment for N-terminal *Rv1168c* and 2G38 chain B was carried out by ClustalW2 software. The alignment was subsequently used for homology modeling using Modeler 9 version 1 (52, 53). To verify the generated *Rv1168c* models, the latter were submitted to Structural Analysis and Verification Server (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 water (56) using steepest descent algorithm. The energy-minimized structure was used for docking with the human TLR2 structure. The crystal structure of TLR1-TLR2 heterodimer (code 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 that buries maximum surface area upon complex formation. We used a protein structure alignment database⁴ 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 means $\pm$ S.D. 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.

⁴ A. Sali and T. L. Blundell, unpublished observations.

Rv1168c PPE Protein Activates HIV-1 LTR Transcription

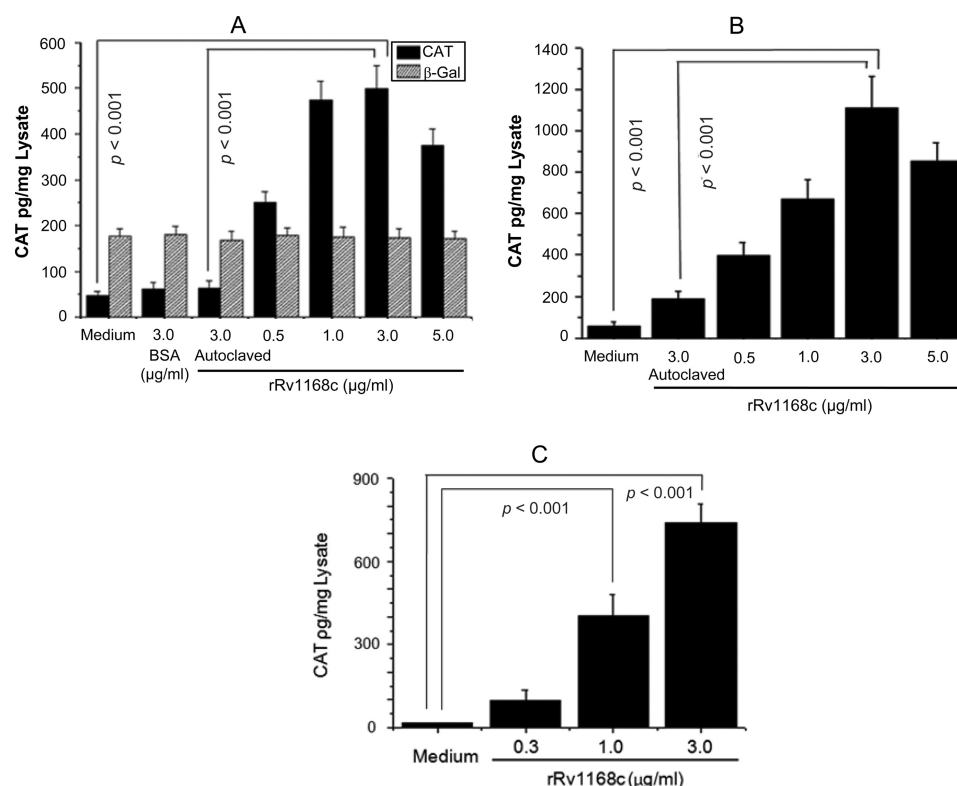


FIGURE 1. rRv1168c protein activates HIV-1 LTR in THP-1 cells. *A*, THP-1 cells were co-transfected with HIV-1 LTR-CAT and β -galactosidase (used as internal control of transfection efficiency) constructs, and after 10 h of transfection, cells were either left untreated or treated with different concentrations (0.5, 1.0, 3.0, and 5.0 μ g/ml) of rRv1168c or BSA. After 36 h, 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 μ g/ml) of recombinant Rv1168c protein. Whole cell extracts were prepared after 36 h 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 means $\pm$ S.D. of three independent experiments.

RESULTS

Recombinant Rv1168c Protein Activates HIV-1 LTR-driven Chloramphenicol Acetyltransferase Expression in THP-1 Cells—To examine whether rRv1168c increases HIV-1 LTR transcription, human monocytic THP-1 cells were co-transfected with a 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 h, cells were either left untreated or treated with various concentrations of purified recombinant Rv1168c protein. 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. 1*A*). CAT was found to be maximally expressed when rRv1168c was used at 3 μ g/ml final concentration. Therefore, all the subsequent experiments were carried out using 3 μ g/ml rRv1168c protein. Because 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. 1*B*). To rule out possible LPS contamination in the recom-

binant protein preparation, the HIV-1 LTR-CAT-transfected THP-1 and BF-24 cells were also treated with 3 μ g/ml 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. 1, *A* and *B*). 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. 1*C*) as well as in human monocyte-derived macrophages (data not shown).

Rv1168c-mediated Activation of LTR Is Dependent on NF- κ B 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- κ B is one of the most important transcription factors responsible for HIV-1 LTR transcription (17, 20–22, 62, 63), we first examined whether NF- κ B 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 h post-transfection, cells were treated with 10 μ M pyrrolidine dithiocarbamate (PDTC), a known inhibitor of NF- κ B (64), followed by incubation with Rv1168c (0.3 and 3 μ g/ml). Rv1168c-mediated trans-activation of HIV-1 LTR was found to be strongly inhibited by PDTC, suggesting a possible

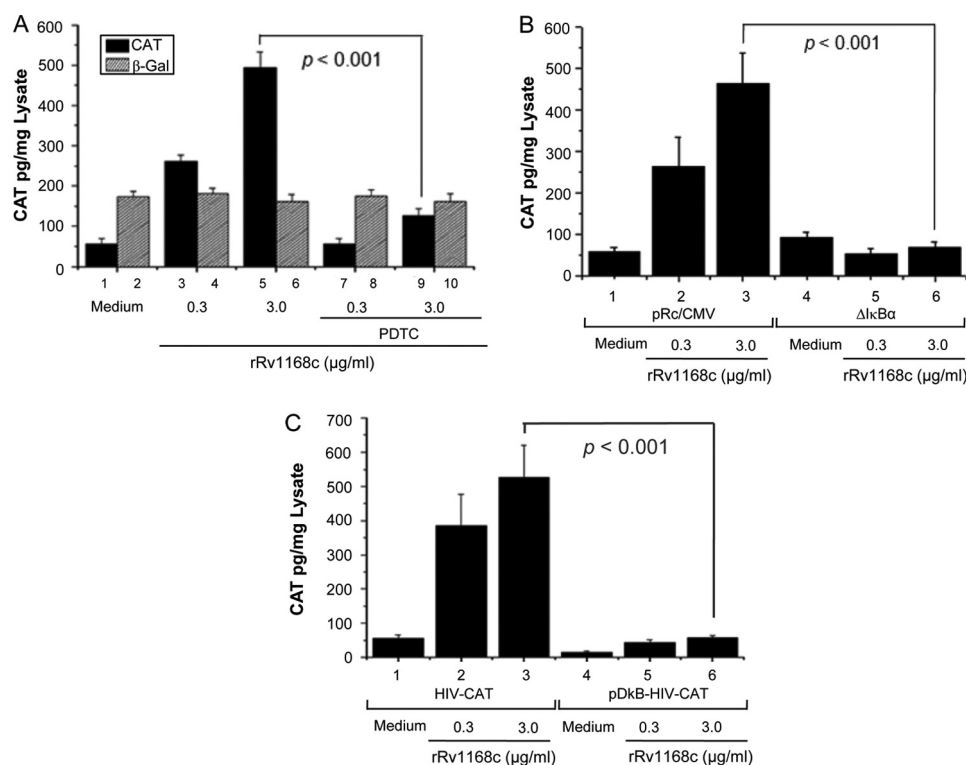


FIGURE 2. Activation of HIV-1 LTR by rVv1168c is mediated through NF- κ B. A, THP-1 cells were transfected with HIV-1 LTR-CAT and β -galactosidase (used as internal control of transfection efficiency) constructs. After 10 h, cells were either left untreated or pretreated with 10 μ M PDTC for 1 h and then treated with 0.3 and 3.0 μ g/ml of rVv1168c. CAT and β -gal expression levels were determined by ELISA after 36 h of Rv1168c treatment. B, THP-1 cells were co-transfected with HIV-1 LTR-CAT and Δ IkB α phosphorylation-defective mutant (Δ IkB α) 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 μ g/ml Rv1168c; 10 h after transfection and after 36 h whole cell extracts were prepared, and the CAT 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 the NF- κ B-binding sites of HIV-1 LTR-CAT). Next, the cells were treated with 0.3 and 3 μ g/ml Rv1168c, and CAT ELISA was performed after 36 h of transfection. The results are shown as the mean $\pm$ S.D. of three independent experiments.

role of NF- κ B in the activation of LTR by Rv1168c (Fig. 2A, compare bar 7 with bar 3 and bar 9 with bar 5).

The NF- κ B transcription factor is known to be retained in cytoplasm by I κ B α protein, and its activity is regulated by its release from the I κ B α complex after phosphorylation and degradation of I κ B α (65). We therefore co-transfected THP-1 cells with HIV-1 LTR-CAT and phosphorylation-defective I κ B α (Δ I κ B α) to sequester NF- κ B in the cytoplasm and examined HIV-1 LTR trans-activation after treatment the cells with rVv1168c. The control group received the backbone vector (pRc/CMV) along with the HIV-1 LTR-CAT construct. After 10 h post-transfection, all the groups were treated with 0.3 and 3 μ g/ml of rVv1168c. The CAT assay was performed 36 h 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 Δ I κ B α 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- κ B in the activation of HIV-1 LTR by Rv1168c. To further underscore the role of NF- κ B in the Rv1168c-mediated activation of HIV-1 LTR, THP-1 cells were transfected with an HIV-1 LTR construct where the NF- κ B-binding sites were mutated (pDkB-HIV-CAT) (62) and followed by treatment with rVv1168c (0.3 and 3 μ g/ml) for 36 h. The results indicate that expression of the CAT reporter gene is significantly down-regulated in the

group transfected with pDkB-HIV-CAT as compared with the group transfected with wild-type HIV-1 LTR (Fig. 2C, compare bar 5 with bar 2 and bar 6 with bar 3). All these results confirm a definite role of NF- κ B in the Rv1168c-mediated activation of the HIV-1 LTR promoter.

Rv1168c Increases Nuclear NF- κ B Levels in THP-1 Cells—The results presented in Fig. 2 indicate a critical role of NF- κ B in the HIV-1 LTR activation by Rv1168c. Therefore, we next investigated whether Rv1168c treatment could increase the nuclear NF- κ B levels in treated cells. Nuclear extracts were prepared from untreated control THP-1 cells (control group), and the cells were treated with different concentrations of rVv1168c (0.3–3 μ g/ml), and these nuclear extracts were used to check the specific DNA binding activity of the NF- κ B complex by EMSA using NF- κ B consensus oligonucleotide probe labeled with [γ - 32 P]ATP. The EMSA result shown in Fig. 3A indicates that the level of NF- κ B is increased in the Rv1168c-treated group in a dose-dependent manner (Fig. 3A, lanes 3–5). The control group showed very little DNA binding activity of NF- κ B (Fig. 3A, lane 2). Homologous cold competition abrogated NF- κ B DNA binding activity confirming the specificity of the DNA-protein complex (Fig. 3A, lane 6). Similar results were obtained when the deoxyoligonucleotide probe containing the HIV-1 LTR-specific NF- κ B DNA-binding site was used for EMSA (data not shown).

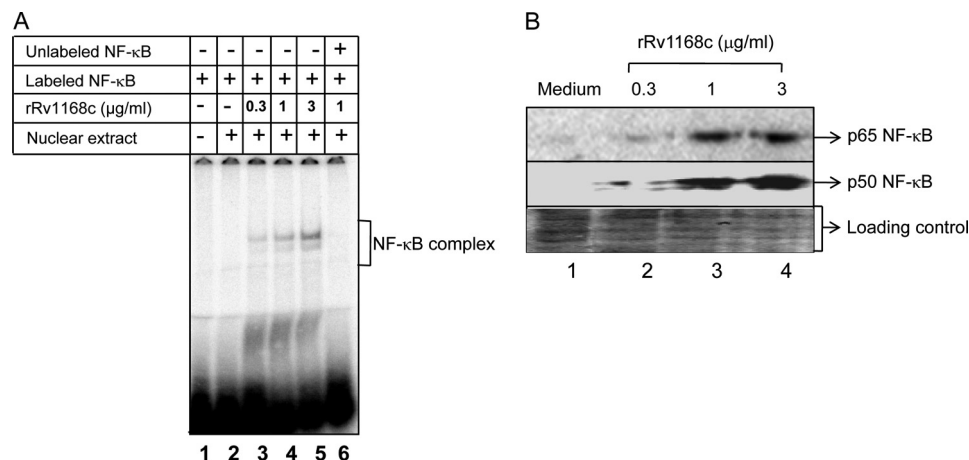


FIGURE 3. rRv1168c protein activates NF-κB induction in THP-1 cells. A, THP-1 cells were treated with various concentrations of rRv1168c protein for 1 h. Cells were harvested, and nuclear extracts were prepared. The DNA binding activity of NF-κB complex was measured by EMSA. B, nuclear extracts prepared 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.

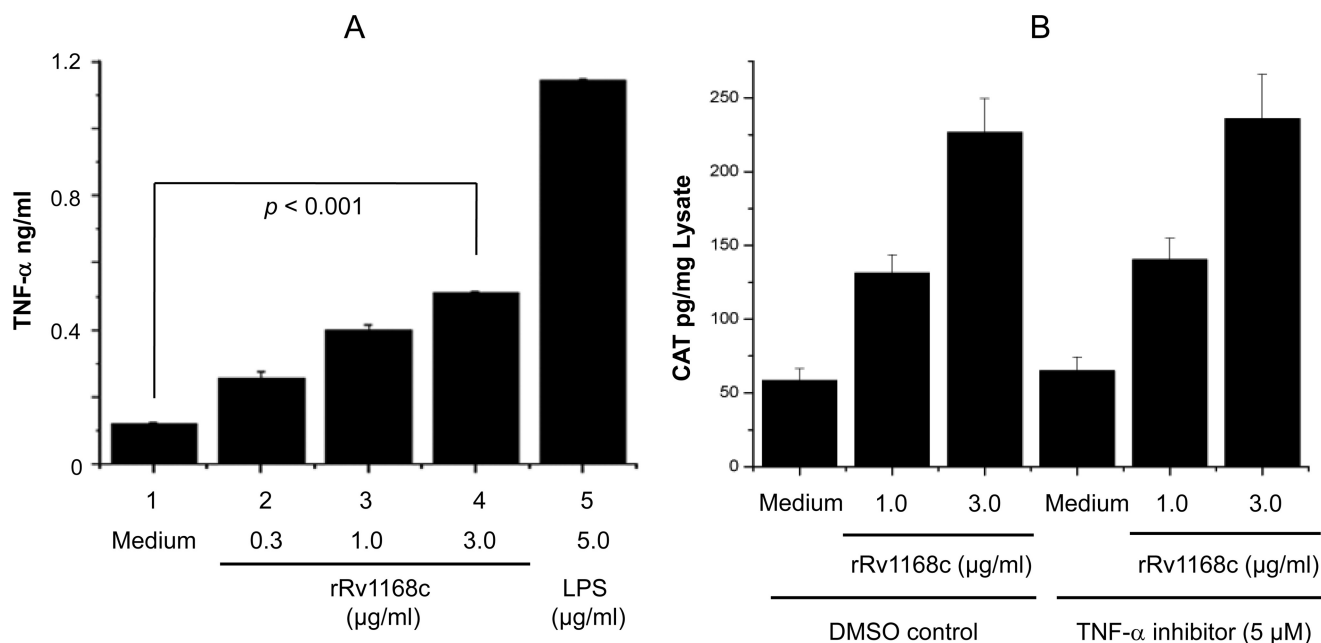


FIGURE 4. rRv1168c protein-mediated activation of HIV-1 LTR in BF-24 cells is independent of TNF-α. A, BF-24 cells were treated with different concentrations of Rv1168c, and TNF-α secreted in the medium was measured by EIA. LPS (3 μg/ml) was used as a positive control of TNF-α induction. B, BF-24 cells 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)-chromen-4-one, diHCl) dissolved in DMSO. The CAT expression was measured by ELISA. Results shown are means ± S.D. of three independent experiments.

Because 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 that 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 transcription

targeting the NF-κB (67), we next examined whether Rv1168c-mediated HIV-1 LTR trans-activation 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-trifluoromethyl-phenyl)-1H-indol-3-ylmethyl)-amino)-ethyl)-amino)-methyl)-chromen-4-one, diHCl, a pharmacological inhibitor of TNF-α (Calbiochem) (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. 1, A and B). 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.

Rv1168c Interacts with TLR2 to Induce NF- κ B-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 overexpressed Rv1168c in *M. smegmatis* using pVV16 under the control of *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 *M. smegmatis* is a nonpathogenic 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 *M. smegmatis* peptide database 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 because Rv1168c could be detected in the insoluble cell wall fraction (supplemental Fig. 2), we hypothesized that Rv1168c is surface-exposed on *M. tuberculosis* and is therefore possibly available for interactions with some surface receptor(s) on the monocyte/macrophage cells to activate the downstream NF- κ B 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 min on ice and measured the surface-bound fluorescence using flow cytometry. The result indicated that Rv1168c protein could strongly bind to the macrophage surface in a concentration-dependent manner (supplemental Fig. 3).

Various studies have indicated that TLR2 is the most predominant receptor recognized by the *M. tuberculosis* components (71) and that the TLR2 could play an important role to modulate macrophage signaling cascades during *M. tuberculosis* infection (37, 72–74). TLR2-specific signaling is found to be essential in *M. tuberculosis*-mediated activation of HIV-1 LTR both *in vitro* and *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). Because 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 overexpressing 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 h of transfection. We then carried out a pulldown assay using whole cell extracts prepared from these HEK293 cells transfected with either pCDNA3.1 or TLR2 or TLR4 overexpression 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- κ B to LTR DNA, 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- κ B DNA binding activity by EMSA and HIV-1 LTR activity by estimating CAT expression levels by ELISA. The results shown in Fig. 5, C and D, indicate that blocking the binding of Rv1168c with TLR2 by pretreating cells with anti-TLR2 mAb results in poorer NF- κ B 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 3 with 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- κ B 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 h post-transfection, the cells were treated with rRv1168c for another 36 h. Depletion of surface TLR2 expression by siRNA was confirmed by flow cytometry (supplemental Fig. 5). Consistent with our previous observations with neutralizing antibody (Fig. 5, C and D), we found that silencing of TLR2 expression on the BF-24 cell surface resulted in strong diminishment of nuclear NF- κ B activity when treated with rRv1168c (Fig. 5E, lane 5). However, in the negative control siRNA-transfected BF-24 cells, a significant amount of DNA binding activity was detected (Fig. 5E, lane 3). As expected, the levels of NF- κ B DNA binding activities were well correlated with the LTR-driven CAT gene expression where the CAT expression levels 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). However, 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- κ B-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- κ B 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* overexpressing Rv1168c (*M. smegmatis* Rv1168c), and HIV-1 LTR activity was measured at 36 h post-infection. The control group was infected with *M. smegmatis*

Rv1168c PPE Protein Activates HIV-1 LTR Transcription

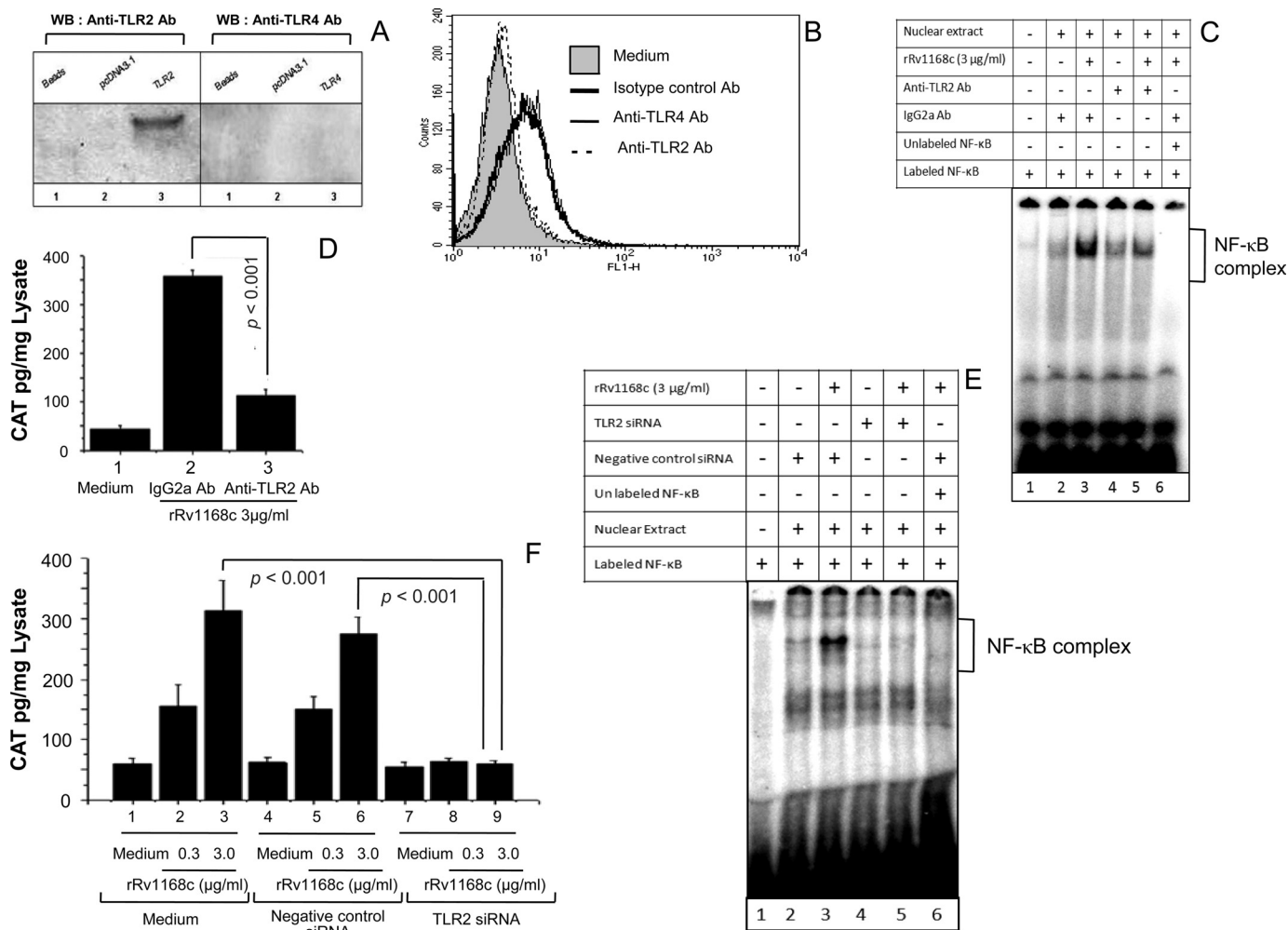


FIGURE 5. TLR2 plays an important role in the Rv1168c-mediated activation of NF-κB and HIV-1 LTR in BF-24 cells. A, lysates from HEK293 cells transfected with either the TLR2 overexpression plasmid or the TLR4 overexpression plasmid or the backbone vector (pcDNA3.1) were incubated with rRv1168c immobilized with TALON resin. The bound protein was eluted and loaded onto a denaturing 10% SDS-polyacrylamide gel and immunoblotted with mouse anti-TLR2 Ab or anti-TLR4 Ab. The membrane was then incubated with anti-mouse IgG-HRP conjugate, and the blot was visualized by chemiluminescence. WB, Western blot. 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 rRv1168c (3 μg/ml) followed by incubation with streptavidin-FITC. The binding of rRv1168c was measured using FACS Vantage flow cytometer. In another experiment, BF-24 cells were either left untreated or pretreated with 10 μg/ml of either anti-TLR2 monoclonal Ab or IgG2a isotype control Ab for 1 h and then incubated with 3 μg/ml of rRv1168c. Cells were harvested either after 1 h to measure NF-κB induction in the nuclear extracts of various groups by EMSA (C) or after 36 h to measure CAT gene expression in the whole cell extracts by ELISA (D). The results are shown as the means ± S.D. of three independent experiments. E, BF-24 cells were transfected with either TLR2-specific siRNA or negative control scrambled plasmid, and after 24 h of transfection, the cells were either left untreated or treated with 3 μg/ml of rRv1168c. Protein extracts were prepared 1 h post-treatment, and EMSA was performed. Results shown are representative of three independent experiments. F, in another experiment, BF-24 cells were either treated with medium or transfected with either TLR2-specific siRNA or negative control scrambled plasmid, and after 24 h of transfection, the cells were either left untreated or treated with 0.3 and 3 μg/ml of rRv1168c for 36 h. Whole cell extracts were prepared to measure CAT expression level by ELISA. The results are shown as the means ± S.D. of at least three independent experiments.

harboring the backbone vector alone (*M. smegmatis* pVV16). Infection of BF-24 cells with *M. smegmatis* Rv1168c as compared with the *M. smegmatis* pVV16 resulted in significant enhancement of LTR-driven CAT expression (Fig. 7A, compare bar 3 with bar 1). Above, 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. smegmatis* Rv1168c), the 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. smegmatis* Rv1168c-induced CAT expression level in the TLR2-siRNA-transfected group was significantly reduced when compared with that of the group transfected with negative control siRNA or treated with medium alone (Fig. 7C, compare bar 9 with bars 6 and 3). These results cumulatively indicate that Rv1168c is capable of interacting 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

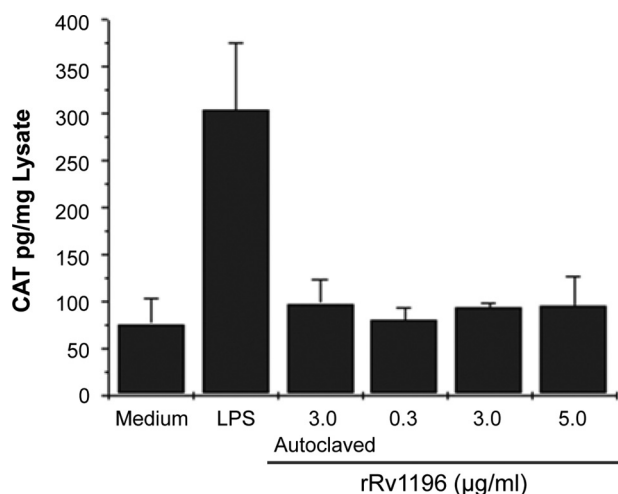


FIGURE 6. **Rv1196 does not activate HIV-1 LTR in BF-24 cells.** BF-24 cells were either left untreated or treated with various concentrations of native recombinant Rv1196 (0.3, 3, and 5 μ g/ml) or autoclaved recombinant Rv1196 (3 μ g/ml) protein. Bacterial LPS (5 μ g/ml) was used as positive control. After 36 h, cell extracts were prepared, and CAT expression was measured by ELISA. The results are shown as the means $\pm$ S.D. of three independent experiments.

increased transcription from the HIV-1 LTR promoter. However, another PPE protein Rv1196 was also found to be surface-exposed and to interact with TLR2 (37), but it failed to activate HIV-1 LTR.

Rv1168c Specifically Interacts with LRR Motifs 15–20 of TLR2—In this study, we have observed that although Rv1168c interacts with TLR2, in a way similar to Rv1196 (37), it triggers predominantly a proinflammatory type of signaling with increased NF- κ B activity and TNF- α cytokine induction unlike Rv1196 which was found to inhibit proinflammatory cytokine production by interacting with the LRR motifs 11–15 (37). Therefore, we speculated that the sites of interaction of these two proteins are different because of 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 pulldown assay carried out using mutant TLR2 (Mut3), where LRR motifs 11–15 are deleted (Fig. 8B), Rv1168c showed interaction. These results suggest that Rv1168c binds to the TLR2 domain in a region other than LRR motifs 11–15 to elicit a proinflammatory type of signaling.

We next generated Rv1168c protein either with deletions in the N-terminal region (Rv1168c Δ N; with an intact C-terminal fragment containing 175–346 amino acid residues) or with deletions in the C-terminal region (Rv1168c Δ C; with an intact N-terminal fragment containing 1–173 amino acid residues) to determine the domain of Rv1168c important for enhancement of LTR-driven transcription. We observed that the rRv1168c Δ N did not bind to the THP-1 cells, whereas the C-terminal truncated Rv1168c protein (Rv1168c Δ C) with an intact N-terminal domain could bind to these cells (Fig. 8C).

We used computational docking studies (as described under “Experimental Procedures”) to understand how Rv1168c interacts with TLR2. Out of $\sim$ 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 LRR motifs 15–20 of TLR2 (Fig. 8D). To confirm whether Rv1168c indeed binds to the TLR2-LRR motifs 15–20 domain, HEK293 cells were transfected with wild-type or TLR2-Mut3 lacking the LRR motifs 11–15 domain or TLR2-Mut4 lacking LRR motifs 15–20 domain (78), and binding of Rv1168c with these TLR2 mutants was 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 motifs 15–20 (supplemental Fig. 6), although 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 region containing motifs 15–20 is important in triggering the downstream signaling events leading to activation of NF- κ B.

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* Overexpressing the Deletion Mutant—We found that purified truncated Rv1168c protein with an intact N-terminal domain containing 1–173 amino acid residues (rRv1168c Δ C) was able to sufficiently activate HIV-1 LTR-driven transcription to the extent similar to that of the purified full-length protein (Fig. 9, bars 4 and 5). In line with our earlier observations, activation of HIV-1 LTR by rRv1168c Δ C 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 to 173 is crucial to activate the TLR2-triggered proinflammatory signaling and HIV-1 LTR trans-activation. The full-length protein when presented in the context of the whole *Mycobacterium* in *M. smegmatis* (*M. smegmatis* Rv1168c) was also able to activate HIV-1 LTR (Fig. 7). Similarly, the N-terminal region was also able to activate NF- κ B and therefore the HIV-1 LTR-driven CAT gene expression almost similar to the levels observed by the full-length protein (Fig. 10, A, compare lane 6 with lane 4, and B, compare bar 5 with bar 3). Similar results were also obtained when we measured the levels of TNF- α in these cells (Fig. 10C, compare bar 5 and bar 3).

To determine whether any role was played by the C-terminal domain, we generated truncated Rv1168c with an intact C-terminal fragment containing 175–346 amino acid residues and overexpressed in *M. smegmatis* (*M. smegmatis* Rv1168 Δ N), and we 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- κ B as compared with full-length Rv1168c or Rv1168 Δ C (Fig. 10A, compare lane 5 with lanes 4 and 6). These observations were also well correlated with the HIV-1 LTR-driven expression of the 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

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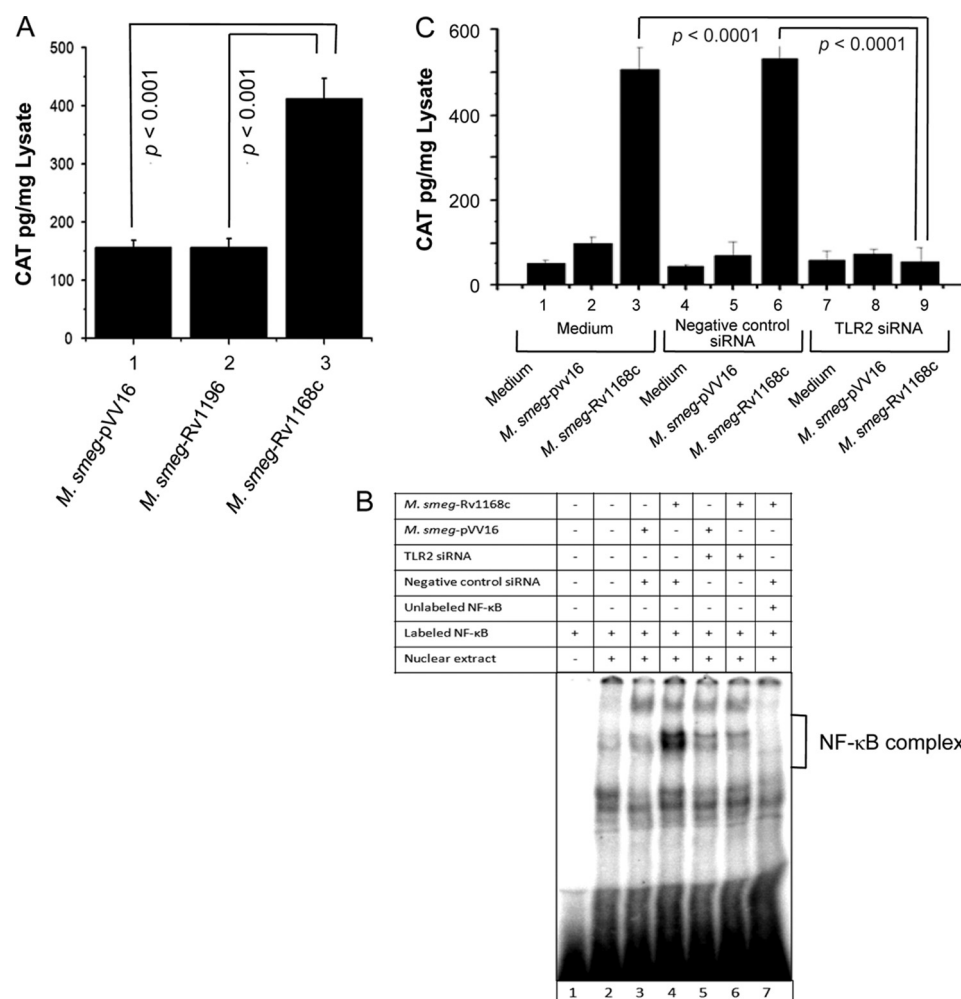


FIGURE 7. Infection of BF-24 cells with *M. smegmatis* (*M. smeg.*) strains overexpressing Rv1168c resulted in increased HIV-1 LTR transcription, and this involves the TLR2-triggered signaling. A, BF-24 cells were infected with either *M. smegmatis* bacteria overexpressing Rv1168c (*M. smegmatis* Rv1168) or *M. smegmatis* strains overexpressing Rv1196 (*M. smegmatis* Rv1196) or *M. smegmatis* harboring the backbone vector (*M. smegmatis* pVV16) at 1:10 multiplicities of infection. After 36 h of infection, whole cell extracts were prepared, and CAT expression was measured by ELISA. The results are shown as means $\pm$ S.D. 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 h of transfection, the cells were either left uninfected or infected with either *M. smegmatis* pVV16 or *M. smegmatis* Rv1168c. Cells were harvested either after 4 h to measure the NF- κ B-DNA binding activity by EMSA or cultured for another 36 h, and CAT expression in the whole cell extracts was measured by ELISA (C). The results are shown as means $\pm$ S.D. of at least three independent experiments.

active domain and is required for elicitation of the proinflammatory 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 with 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 a 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 the host's proinflammatory 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 proinflammatory 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 overexpressed 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

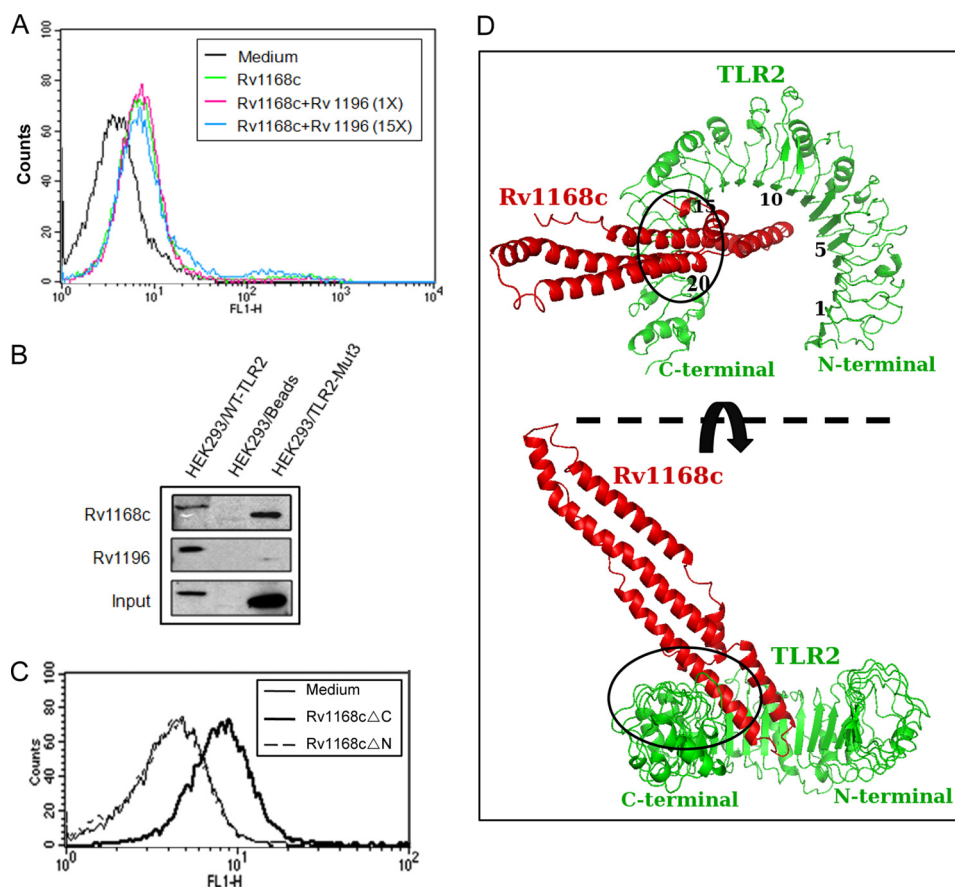


FIGURE 8. Rv1168c interacts with LRR motifs 15–20 of TLR2, a region different from Rv1196. A, THP-1 cells were pretreated with 5- or 15-fold excess concentrations of Rv1196, washed, and then treated with biotinylated Rv1168c protein followed by incubation with streptavidin-FITC. The binding of Rv1168c 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 immunoblotting 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 μ g/ml of either Rv1168 Δ N-FITC or Rv1168 Δ C-FITC for 1 h on ice, and the fluorescence was measured by flow cytometry. D, molecular modeling 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 LRR motifs 15–20 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.

replication. The novelty of this study is that we demonstrate a direct role of PPE protein Rv1168c in activating 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 a significant decrease in HIV-1 LTR trans-activation by rRv1168c. Interestingly, Rv1168c is found to be present in the insoluble cell wall fraction when overexpressed in *M. smegmatis*, and infection with this strain (*M. smegmatis* 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, which 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- κ B transcription factors are activated downstream of the TLR2-induced signaling (36, 88). The NF- κ B factors interact with HIV-1 LTR DNA-binding sites and mediate LTR trans-activation (17, 63). Binding of NF- κ B 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- κ B DNA binding activity downstream of the TLR2 as inhibition of this signaling pathway by either treating cells with PDTC or overexpressing phosphorylation-defective I κ B α 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- κ B-binding sites. Pretreatment of BF-24 cells with anti-TLR2 mAb or silencing of the TLR2 by TLR2-specific siRNA prevented rRv1168c-mediated activation of NF- κ B in these cells which eventually leads to decreased HIV-1

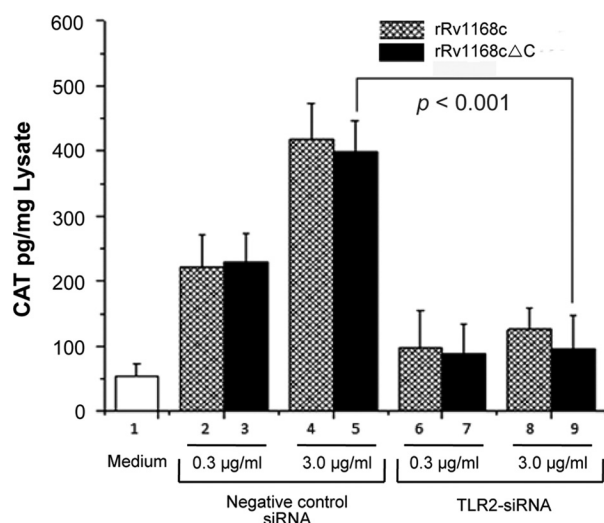


FIGURE 9. N-terminal domain of Rv1168c is essential for activation of HIV-1 LTR in BF-24 cells, and this requires TLR2. BF-24 cells were transfected with either TLR2-specific siRNA or negative control scrambled plasmid, and after 24 h of transfection, cells were treated with 0.3 and 3.0 $\mu\text{g/ml}$ of either full-length rRv1168c or rRv1168c ΔC protein for 36 h. Cells were then harvested, and whole cell extracts were prepared to measure CAT expression by ELISA. Results are means $\pm$ S.D. of at least three independent experiments.

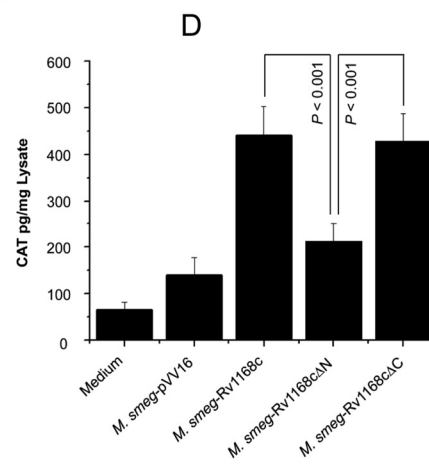
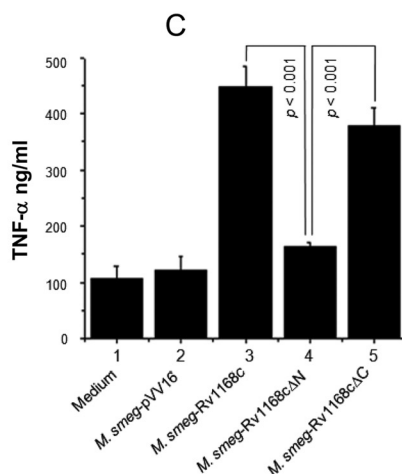
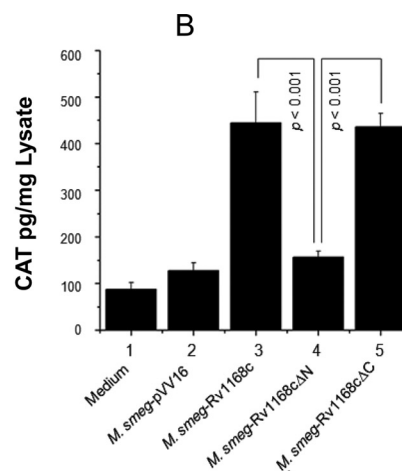
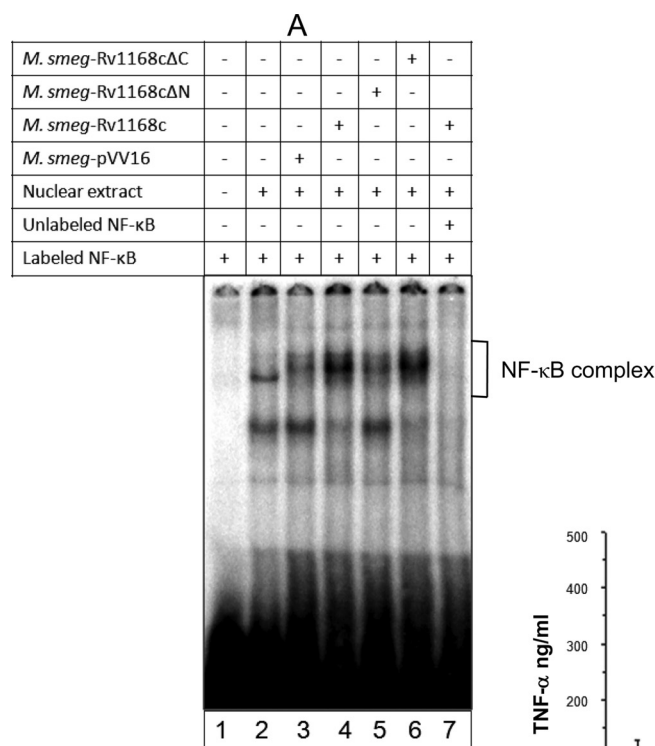


FIGURE 10. *M. smegmatis* bacteria harboring the N-terminal domain deletion mutant of Rv1168c (*M. smegmatis* Rv1168c ΔN) fails to trigger NF- κB activity and HIV-1 LTR-driven CAT gene transcription in BF-24 cells. BF-24 cells were either left uninfected or infected with *M. smegmatis* pVV16 or *M. smegmatis* Rv1168c or *M. smegmatis* Rv1168c ΔN or *M. smegmatis* Rv1168c ΔC at multiplicities of infection of 1:10. Cells were either harvested after 4 h to measure the NF- κB activity by EMSA (A) or cultured for another 36 h either to measure CAT expression in the whole cell extracts by ELISA (B) or to estimate TNF- α cytokine secreted in the culture supernatants by sandwich EIA (C). D, PMA-differentiated BF-24 macrophages were either left uninfected or infected with *M. smegmatis* pVV16 or *M. smegmatis* Rv1168c or *M. smegmatis* Rv1168c ΔN or *M. smegmatis* Rv1168c ΔC , and CAT expression was measured by ELISA after 36 h. Results are means $\pm$ S.D. of at least three independent experiments.

BF-24 cells was also found to be TLR2-dependent as observed using recombinant Rv1168c because silencing of the TLR2 gene expression by specific siRNA prevented *M. smegmatis* Rv1168c-mediated activation of NF- κ B 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 amino acid residues) and presented to BF-24 cells in the context of whole mycobacterium using *M. smegmatis* (*M. smegmatis* Rv1168 Δ N), there was no significant increase in the DNA binding activity of NF- κ B as compared with 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-terminal region containing 1–173 amino acid residues) when presented to BF-24 cells in the context of the whole bacterium using *M. smegmatis* (*M. smegmatis* Rv1168 Δ C) could trigger a strong NF- κ B signaling similar to that of the full-length Rv1168c overexpressed in *M. smegmatis* (*M. smegmatis* Rv1168c). Similarly, BF-24 cells infected with *M. smegmatis* Rv1168 Δ N had a very negligible increase in HIV-1 LTR transcription and TNF- α production. In contrast, infection of BF-24 cells with *M. smegmatis* Rv1168 Δ C 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- κ B signaling and increased HIV-1 LTR trans-activation 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- κ B transcription factors. The Rv1196 protein is found to specifically interact with the LRR motifs 11–15 of TLR2 (37) and triggers an anti-inflammatory signaling, whereas binding of Rv1168c with the TLR2 motifs 15–20 results in induction of proinflammatory signaling leading to activation of NF- κ B 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 that conveys disparate signaling cues downstream from the cytoplasmic stem of the TLR2 receptor resulting in differential NF- κ B 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 of the possible mechanisms on how the TLR2-LRR domain can influence HIV-1 LTR transcription in monocyte/macrophages by regulating the NF- κ B signaling cascades positively or negatively, and some of the mycobacterial PPE proteins can influence the LTR-driven transcription by modulating the TLR2-NF- κ B signaling. Whereas we demonstrated a role for Rv1168c in trans-activating the HIV-1 LTR promoter through activation of the NF- κ B signaling cascades, regulation of this promoter by other signal-activated transcription factors cannot

be ruled out. The TLR2-LRR-specific proinflammatory signaling during opportunistic infections may influence HIV-1 LTR trans-activation in a similar fashion (90). Although in this study we used the THP-1 cell line as a model for cells of the monocyte/macrophage lineage, our data indicate that site-specific interaction of mycobacterial protein(s) with the TLR2 ectodomain is crucial to dictate the downstream signaling events that eventually lead to HIV-1 LTR hyperactivation.

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 long term (92). These CD14⁺CD16⁺ monocytes are also known to be more proinflammatory and play a greater role in infections than the majority of the classical CD14⁺CD16[−] monocytes that may also get infected by HIV (93). In patients with HIV-1 infection, the CD14⁺CD16⁺ 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). However, 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 monocyte/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 purified protein derivative (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 about five times less efficiently with *M. tuberculosis* than the CD14⁺CD16[−] monocytes/macrophages, both these subsets can produce significant amounts of TNF- α upon *M. tuberculosis* infection (96). True to this proposition, we found that Rv1168c can stimulate production of significant amounts of TNF- α in BF-24 cells and can also activate the HIV-1 LTR independent of TNF- α production. Because 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 the HIV-1 LTR promoter in *M. tuberculosis*/HIV-1 co-infected individuals.

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