CHAPTER 2

The PPE protein Rv1168c of *Mycobacterium tuberculosis* augments transcription from HIV-1 LTR promoter via binding to Toll-like receptor 2

This work has been recently submitted as a revised manuscript in a peer reviewed international journal.
2.1 Introduction

Approximately 33.3 million people are living with the human immunodeficiency virus type 1 (HIV-1) World-wide and about 2.6 million people were newly infected in 2009 (WHO, 2009a). In addition, there is a deadly syndemic interaction between the HIV and tuberculosis (TB) caused by Mycobacterium tuberculosis (Kwan et al., 2011). 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^+T cell count (WHO, 2009b). HIV/M. tuberculosis co-infection results in remarkably higher mortality (Diedrich et al., 2011, Lawn et al., 2002). Due to the high incidence 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 (Williams et al., 2005).

Cells of the monocyte-macrophage lineage play an important role in the transmission and pathogenesis of HIV (Coleman et al., 2009, Roy et al., 1988) in addition to CD4^+T cells. Infected monocytes can differentiate into macrophages and may form long-lived reservoir for the virus (Brown et al., 2006). On the other hand, the macrophages form a replicative niche for M. tuberculosis (Orenstein et al., 1997). 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 (Orenstein et al., 1997). 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 proinflammatory microenvironment produced by the activated macrophages may increase HIV-replication (Garrait et al., 1997).

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 (Moriuchi et al., 1998, Zhang et al., 1995, Kitaura et al., 2001, Toossi et al., 1999, Toossi et al., 1993). The purified protein derivative (PPD), ManLAM and culture filtrate protein of *M. tuberculosis* (Toossi et al., 1999, Bernier et al., 1998) have been shown to enhance transcriptional activity of HIV-1 long terminal repeat (LTR), the sole promoter element of HIV (Bernier et al., 1998, Kitaura et al., 2001). The expression of viral genes is regulated by several host transcription factors such as the Sp family, nuclear factor kappa B (NF-κB) family, activator protein 1 (AP-1) proteins, nuclear factor of activated T cells (NFAT), and CCAAT enhancer binding proteins (X/EBP) family members by binding to the LTR that display different levels of sequence conservation (Kilareski et al., 2009). 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 (Kilareski et al., 2009). 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 (Mingyan et al., 2009). Activation of monocytes by LPS, IL-6 or TNF-α results in enhanced HIV replication, a process that well correlates with NF-κB activity (Molina et al., 1989, Pomerantz et al., 1990, Koyanagi et al., 1988). Induction of these proinflammatory cytokines during mycobacterial infection have been therefore
postulated to be one of the important factors that drive hyper-transcription from LTR promoter (Kitaura et al., 2001, Zhang et al., 1995, Cohen et al., 1997, Wahl et al., 1998). However, several studies have suggested that additional mycobacterial factors may also be responsible for increased transcription from LTR promoter (Ghassemi et al., 2000, Shattock et al., 1994). 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 macrophages and binding of these transcription factors to the specific LTR-regions, can alter the levels of HIV-1 LTR-driven gene expression (Bernier et al., 1998, Ghassemi et al., 2000, Shattock et al., 1994, Pereira et al., 2000). 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 (Cole et al., 1998). Many of these proteins are found to be differentially expressed in *M. tuberculosis* under different conditions (Delogu et al., 2006, Voskuil et al., 2004). These proteins are proposed to be responsible for generating antigenic diversity and may also interfere with the host immune responses (Brennan et al., 2002, Ramakrishnan et al., 2000, Choudhary et al., 2003, Li et al., 2005, Abdallah et al., 2006). Few recent studies indicate that some of these proteins can modulate the
macrophage innate effector signaling pathways (Basu et al., 2007, Nair et al., 2009, Bansal et al., 2010). 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.

It has been reported earlier that one of the PPE proteins, Rv1168c (PPE17) is a highly immunodominant antigen detected during active TB infection (Khan et al., 2008b). 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 (Cole et al., 1998, Bacon et al., 2004, Muttucumaru et al., 2004, Betts et al., 2002, Schnappinger et al., 2003). Rv1168c is found to be over-expressed in macrophages infected with various clinical isolates of M. tuberculosis (Homolka et al., 2010). Since some of the PPE family proteins are shown to be present in the cell surface (Nair et al., 2009, Sampson et al., 2001), it is speculated that Rv1168c may be exposed to the cell surface and could modulate the host immune responses by interacting with the macrophage surface components. In the present study, it has been examined whether Rv1168c interacts with monocyte/macrophage and modulate 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.


2.2 Materials and methods

2.2.1 Cloning expression and purification of recombinant Rv1168c and Rv1196 proteins:

Rv1168c was cloned from a bacterial artificial chromosome (BAC) library contig Rv71 (C2) of *M. tuberculosis* kindly provided by Dr. Shekhar C. Mandey CDFD Hyderabad. The primers were appended with XhoI and HindIII enzyme sites in forward and reverse primers respectively in order facilitate cloning in matching sites in the bacterial expression vector pRSET A. The sequence of the designed primers was F 5′ GACTCGAGATGGATTTCACAATTTTT 3′ (XhoI) and R 5′ GCAAGCTTCTAGCCGGCGGCGGGTGACCGCAGT 3′ (HindIII). After PCR amplification of the Rv1168c ORF, the amplified fragment of expected size was purified by using PCR purification kit as per the manufacturer's protocol (Qiagen GmBH, Germany). Direct digestion of the PCR products with the appended restriction enzymes often results in inefficient digestion leading to inefficient ligation. To avoid this, the PCR amplified products of the Rv1168c ORFs were initially ligated into pGEM-T Easy vector (Promega, Madison, USA). The ligation mix was then transformed into competent DH5α cells and the recombinants were picked up by blue-white screening. The putative positive clones were grown over night in Luria Bertani (LB) medium at 37°C with constant shaking at 200 rpm. The plasmid DNA was isolated and digested with appropriate restriction enzymes to confirm the presence of inserts of expected size. Once the clones were confirmed, large scale restriction digestion was carried out to obtain fragments with the cohesive ends for the respective restriction enzymes. The fragments were purified by using Qiagen Gel Extraction Kit and ligated to a T7 polymerase driven *E. coli* expression
vector pRSET A (Invitrogen, Carlsbad, CA, USA) in the correct reading frame so that recombinant proteins have an N-terminal 6xHis-tag to facilitate purification on metal affinity purification columns. The recombinant expression vectors were then transformed into BL21(DE3)pLysS cells for expression and purification of the recombinant protein.

The mutant Rv1168c proteins with truncated N-terminal (Rv1168cΔN) and truncated C-terminal (Rv1168cΔC) were generated by using specific primers to amplify different regions of the Rv1168c gene from BAC library contig C2. For Rv1168cΔN forward primer 5′ ATGGATCCATGGCGCTGCGCCAGACTTTATGAACGTGAC 3′ (BamHI) and reverse primer 5′ ATAGCTTCTAGCCGCGCGCGGTGACC 3′ (HindIII), were used to amplify Rv1168c region from amino acid 175 to 346 and for Rv1168cΔC forward primer 5′ ATGGATCCATGGATTTCACAATTTTTACGCGGG 3′ (BamHI) and reverse primer 5′-ATAAGCTTCTACGGGTGCGATCGGC-3′ (HindIII) were used to amplify Rv1168c region from amino acid 1 to 173. The amplified products were then cloned in pRSET A vectors. The clones were confirmed by PCR, sequencing and checked for protein expression.

For large scale expression of the recombinant Rv1168c protein (rRv1168c) and the mutant proteins, Rv1168cΔN and Rv1168cΔC from pRSET A in BL21(DE3)pLysS, a single isolated recombinant colony was inoculated in 5 ml of LB medium containing ampicillin (100 µg/ml) and chloramphenicol (35 µg/ml) and incubated at 37°C overnight with constant shaking at 200 rpm. Around 5 ml of the cultures were inoculated in 500 ml of LB medium containing ampicillin (100 µg/ml) and chloramphenicol (35 µg/ml) at 37°C with
constant shaking until the culture reached mid-log growth phase ($A_{600}=0.4 - 0.6$), at this point 1 ml culture was taken out as uninduced control, and rest of the culture was induced with 1 mM IPTG solution and further incubated in the shaker at 37°C for 3-4 hours. After induction, cells were harvested by centrifugation and resuspended in 50 ml of lysis buffer (100 mM Tris pH 7.5, 100 mM NaCl, 5% glycerol and 0.5% sodium lauroyl sarcosine) with 0.1 mM PMSF and disrupted using a sonicator. After another round of centrifugation at 10,000 x g the supernatant was collected. The supernatant was allowed to bind to TALON resin (Clontech, Palo Alto, CA, USA) for 1 hour, which is an immobilized metal affinity chromatography (IMAC) resin, designed for the purification of recombinant 6xHis–tagged proteins. The resin was washed with 500 ml of wash buffer (100 mM Tris pH 7.5, 100 mM NaCl, 5% glycerol and 15 mM Imidazole). The protein was eluted with wash buffer containing 200 mM imidazole. The eluates were analyzed by loading onto a 10% SDS gel. The fractions containing protein were pooled and dialyzed against dialysis buffer (100 mM Tris pH 7.5, 100 mM NaCl and 5% glycerol) to remove imidazole as it interferes with protein estimation and other downstream processes. The eluates were analyzed by loading onto a 10% SDS-PAGE gel followed by Coomassie Blue staining. The protein concentration was estimated following bicinchoninic acid (BCA) method of protein estimation using the Micro BCA™ Protein Assay Kit from Thermo Scientific (Rockford, IL, USA). To remove any endotoxin contamination, purified rRv1168c protein was incubated with 10% v/v polymyxin B-agarose (Sigma-Aldrich; binding capacity, 200 to 500 μg of LPS from *E. coli* serotype O128:B12/ml) for 1 hour at 4°C. After incubation, the agarose beads were removed by centrifugation and the supernatant was filter sterilized and used to stimulate macrophages. Endotoxin content of the recombinant protein was
also measured by using limulus amebocyte lysate assay (E-toxate kit from Sigma-Aldrich, St. Louis, MO) and the endotoxin content in the recombinant protein was found to be very low (< 0.0001 EU/ml). This endotoxin-free recombinant protein preparation was used for further experiments.

Rv1196 was cloned in a similar fashion in pRSET as described earlier (Nair et al., 2009). A vector using the BAC library contig Rv7 and the primers used were F 5’ GTGGATCCATGGTGATTTGCGGCGTTACCA 3’ (BamHI) and R 5’ GCAAGCTTCTAGCCGCGCCGCGGAGAAT 3’ (HindIII). In brief, the PCR product was directly cloned in the intermediate vector pGEM-T Easy (Promega) and confirmed by sequencing. The full-length gene was then sub-cloned in the bacterial expression vector pRSET A (Invitrogen) in frame with a six N-terminal histidine tag. The recombinant PPE18 (rPPE18) protein was purified using TALON resin as described earlier by Nair et al. (2009). The recombinant protein was dialyzed against several changes of PBS. To remove endotoxin contamination, the protein was incubated with 10% v/v polymyxin B-agarose. The preparation had a very low endotoxin content (< 0.05 EU/ml) as measured by the E-toxate (Limulus amebocyte lysate) kit (Sigma-Aldrich). The protein concentration was estimated using bicinchoninic acid method of protein estimation using the Micro BCA™ Protein Assay Kit from Thermo Scientific (Rockford).

2.2.2 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,
USA 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% fetal calf serum (FCS), antibiotic-antimycotic (1X), 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. The HEK293 cells were obtained from National Centre for Cell Science, Pune, India and maintained in DMEM high glucose medium (Hyclone) containing 10% FCS, antibiotic-antimycotic (1X), 2 mM L-Glutamine and 10 mM HEPES (all from GIBCO, Carlsbad, CA) at 37°C and 5% CO₂ in a humidified incubator.

2.2.3 HIV-1 LTR-chloramphenicol acetyltransferase (CAT) constructs

Full-length HIV-1 LTR promoter cloned upstream of chloramphenicol acetyltransferase (CAT) reporter gene (HIV-1 LTR-CAT) construct and the mutant construct having mutation in both the NF-κB binding sites (pDkB-HIV-CAT) were kind gifts from Debashish Mitra, (NCCS, Pune, India) and AIDS Research Program, Division of AIDS, NIAID, NIH, USA from Dr. Gary Nabel and Dr. Neil Perkin (Nabel et al., 1987).

2.2.4 Transient transfection of THP-1 cells

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-galactosidase (β-Gal) (Clontech) constructs. Briefly, 0.5 million cells were seeded in a 12 well plate in Opti-MEM (Invitrogen). Next, 1 μg DNA and 3 μl DMRIE-C reagents were 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 then added to the cells drop-wise on the culture plate. Complete medium with 20% FBS was added after 5-6 hours. Ten hours post-transfection, the cells were incubated with various concentrations of rRv1168c protein and CAT/β-Gal reporter assay was carried out 36 hours after treatment with rRv1168c.

2.2.5 CAT and β-Gal reporter assay

The CAT and β-gal activity was measured using CAT and the β-gal reporter assay kits from Roche Applied Science (Indianapolis, IN, USA) following the manufacturer’s protocol. Briefly, the cells were harvested and centrifuged at 4000 rpm for 10 minutes at 4°C. The pellet was washed twice with PBS and suspended in 500 μl of lysis buffer. After incubation at room temperature for 30 minutes, the lysate was centrifuged at 10,000 rpm for 10 minutes at 4°C. Protein was estimated by BCA method of protein estimation using the Micro BCA™ Protein Assay Kit from Thermo Scientific (Rockford) and 100 μg protein lysate was added into the microplate (Roche Applied Science) pre-coated either with anti-CAT or anti-β-Gal antibody (Ab). The plate was incubated at 37°C for 2 hours 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 hour. The plate was again washed five times with wash buffer and anti-DIG-peroxidase (Anti-DIG-POD) was added and incubated for 1 hour at 37°C. The plate was washed and 200 μl of POD substrate (ABTS) was added. The plate was incubated at room temperature till the color developed. The reading was taken on Ultra Microplate Reader EL808 (Biotek Instruments Inc.) at 405 nm (490 nm reference wavelength).
2.2.6 Treatment of BF-24 cells with the recombinant PPE proteins

One million BF-24 cells were seeded per well in 24-well tissue culture plate and either left untreated or treated with various concentrations of either rRv1168c or rRv1168cΔC or rRv1196 protein. After 36 hours, cells were harvested and CAT activity was measured following the method as described in 2.2.5. In some experiment PMA differentiated BF-24 macrophages (10 ng/ml for overnight followed by rest for 24 hours) were treated with various concentrations of rRv1168c and CAT activity was measured after 36 hours.

2.2.7 TLR2 siRNA

The negative-control scrambled siRNA, TLR2 targeting siRNA (sense, 5’-GCCUUGACCUGUCCAACAtt 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) (Khan et al., 2008a). 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. The cells were then incubated with 0.3 and 3 µg/ml of purified recombinant either the full-length Rv1168c or Rv1168cΔC protein and CAT reporter assay was carried out 36 hours after treatment with the protein.

2.2.8 Estimation of TNF-alpha cytokine production by enzyme immunoassay (EIA)

The TNF-α cytokine in various macrophage culture supernatants was quantified by two-site sandwich EIA (BD Biosciences Pharmingen, San Diego, CA). In brief, 96-well
polyvinyl chloride microtiter plates were coated with purified capture Ab against TNF-α at 1:250 dilutions in coating buffer (0.1 M carbonate buffer, pH 9.5) and were incubated overnight at 4°C. The plates were washed with PBS and blocked with 10% FBS in PBS followed by incubation with various culture supernatants overnight at 4°C. After washing with wash buffer (PBS-T, 1X PBS containing 0.05% Tween-20), plates were incubated with biotin conjugated detection Ab against TNF-α followed by incubation with streptavidin conjugated to horseradish peroxidase (HRP). The HRP activity was detected using a chromogenic substance o-phenylenediamine tetrahydrochloride (Sigma-Aldrich) in citrate-phosphate buffer (pH 5.4) and H₂O₂ (1 µl/ml). The reaction was terminated using 1 N H₂SO₄, and the absorbance value was measured at 492 nm. Standard curve for the cytokine was obtained using the recombinant standard protein provided in the kit.

2.2.9 MTT assay

BF-24 cells were treated with different concentrations of rRv1168c in the absence or presence of TNF-α inhibitor. After 24 hours, MTT (Sigma-Aldrich) was added at a concentration of 5 mg/ml and the cells were incubated further for 4 hours. The cells were lysed overnight using 100 µl of lysis buffer (20% SDS and 50% Dimethylylformamide). After an overnight incubation at 37°C, the absorbance was measured at 570 nm using Ultra Microplate Reader EL808 (Biotek Instruments Inc.).

2.2.10 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 Labelling Kit from Pierce Chemical Company (Rockford) and following the manufacturer’s protocol.

2.2.11 Biotinylation of rRv1168c

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

2.2.12 Preparation of nuclear extracts

For preparing nuclear extract, about 5 million cells were centrifuged and the cell pellet was washed with ice cold PBS. To the pellet, 250 μl of ice-cold cytoplasmic extraction buffer (30 mM Tris pH 7.5, 10 mM Magnesium acetate, 1% NP-40, 1 mM Sodium orthovanadate) containing cocktail of protease inhibitors (2 μg/ml Leupeptin, 3 μg/ml Aprotenin and PMSF) were added. The cell suspension was kept on ice and vortexed intermittently for 2-3 minutes to rupture the plasma membrane. The lysates were centrifuged for 10 minutes at 10,000 rpm at 4°C to pellet the nuclei. The nuclear pellets were washed with cytoplasmic extraction buffer and centrifuged to remove any residual cytoplasmic material. 50 μl of ice-cold nuclear extraction buffer (10 mM HEPES pH 8,
25% glycerol, 10 mM MgCl₂, 420 mM NaCl, 1mM EDTA and cocktail of protease inhibitors) was added to the pellet (containing nuclei) and incubated on ice for 45-60 minutes with intermittent vortexing and then centrifuged for 20 minutes at 14,000 rpm at 4°C to remove insoluble debris. The supernatants (nuclear extracts) were collected and dialyzed against PBS buffer. The dialyzed preparations were centrifuged and the supernatants were carefully removed and transferred to pre-chilled fresh microfuge tube. The freshly prepared nuclear extracts were used for protein estimation by bicinechoninic acid method using the Micro BCA™ Protein Assay Kit from Thermo Scientific (Rockford) following the manufacturer’s protocol.

2.2.13 Western blotting for p50 and p65 subunits of NF-κB transcription factors

In order to detect p50 and p65 NF-κB levels in the nuclear extracts, equal amounts of freshly prepared extracts were separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane following electroblotting for 6-8 hours at a constant voltage of 45V. Following electrophoretic transfer, the nitrocellulose membranes were washed gently with PBS-T three times and blocked with 5% fat free milk prepared in PBS-T for 4 hours at room temperature with constant slow shaking. The membrane was washed with PBS-T and incubated with rabbit antibody to either p50 or p65 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 2% fat free milk in PBS-T at room temperature for 4 hours. The membrane was washed 5 times with PBS-T for 5 minutes each on a rocker and incubated with anti-rabbit immunoglobulin G (IgG)-HRP conjugate (Sigma-Aldrich) diluted 1:10000 in 2% fat free milk in PBS-T for 1 hour. The membrane was washed and the bound enzyme was detected by chemiluminescence using ECL-plus detection reagent kit.
following the manufacturer's protocol (GE Healthcare, Little Chalfont, UK). Equal loading of protein was confirmed by Ponceau S Red stain (Sigma-Aldrich).

### 2.2.14 Electrophoretic mobility shift assay (EMSA)

The freshly prepared nuclear extracts (as described in section 2.2.12) were used to perform EMSA to check the DNA-binding activity of NF-κB. Briefly, nuclear extracts (10 µg) were incubated for 30 minutes at room temperature with 1 ng of ^32^P–end-labelled NF-κB consensus binding sequence 5'AGTTGAGGGGACTTTCCCAGG-3' (Wagner et al., 2002) in a binding buffer (20 mM HEPES pH 7.9, 0.5 mM DTT, 1 mM MgCl\textsubscript{2}, 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 (FujiFilm) overnight. Visualization of the radioactive bands was carried out using a STARION image scanner (FujiFilm FLA-9000).

### 2.2.15 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 rRv1196 protein for 30 minutes on ice followed by incubation with 10 µg/ml biotin labeled rRv1168c (Rv1168c-biotin) for another 30 minutes on ice. Cells were washed three times with staining buffer and incubated with streptavidin-FITC (1:1000) (Sigma-Aldrich) for 30 minutes on ice. The fluorescence was measured by flow cytometry in BD
FACSVantage SE (Beckton Dickinson, San Jose, CA) using CellQuest data analysis software (Beckton Dickinson).

2.2.16 Immunoprecipitation assay

The FLAG-tagged wild-type (WT) TLR2 as well as TLR2 ectodomain deletion mutants like TLR2-Mut3 lacking LRR 10–15 domain or the TLR2-Mut4 lacking LRR 15–20 domain 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. Cells were then 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 and protease inhibitor cocktail) to prepare whole cell lysates. After centrifugation at 12000 rpm for 15 minutes at 4°C, the supernatants were collected and incubated with TALON immobilized with Rv1168c at 4°C overnight on a rotating platform. The beads were washed extensively with lysis buffer and boiled in Laemmli sample buffer for 10 minutes. Samples were separated on 10% SDS-polyacrylamide gel and then electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare). The membrane was washed and blocked with 5% fat free milk prepared in PBS and incubated for 4 hours at room temperature with gentle shaking. After washing with PBS-T, the membrane was incubated with mouse monoclonal anti-FLAG antibody (Sigma-Aldrich) diluted in PBS-T and incubated for 1 hour at 37°C. The membrane was again washed 3 times with PBS-T and incubated with anti-mouse IgG-HRP conjugate (Sigma-Aldrich) diluted at 1:10000 in 2% fat free milk in PBS-T for 1
hour at 37°C. The membrane was washed and the bound enzyme was detected by chemiluminescence using ECL-plus detection reagent kit following the manufacturer's protocol (GE Healthcare).

2.2.17 Flow cytometry

To examine Rv1168c binding, THP-1 cells (about 10^6/well) were incubated with increasing concentrations of rRv1168c conjugated to biotin (Rv1168c-biotin) for 60 minutes on ice. The fluorescence was measured by flow cytometry using Partek PAS-III (Partec, Germany) and analyzed by Flowjo 7.6.1 flow cytometric analyses program. In some experiment, 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 fluorescence was measured by flow cytometry in FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest (Becton Dickinson) data analysis software. For analysis of binding of Rv1168c to HEK293 cells transfected with either the Flag-tagged wild-type TLR2 (WT-TLR2) or the Flag-tagged TLR2-Mut3 (lacking LRR 10~15 domain) or the Flag-tagged TLR2-Mut4 (lacking LRR 15~20 domain) construct, the cells were incubated with 10 µg/ml of biotinylated Rv1168c at 4°C for 30 minutes. The cells were washed with staining buffer and treated with streptavidin-FITC conjugate (Sigma-Aldrich), diluted at 1:1000 in staining buffer. The fluorescence was measured by flow cytometry in FACS Vantage flow cytometer (Becton Dickinson).
2.2.18 Generation of rRv1168c-specific polyclonal antibody

The antibody to the rRv1168c protein was generated in BALB/c mice maintained in the animal house facility of National Institute of Nutrition, Hyderabad. The experiments were conducted following the institutional rules approved by the animal ethics committee of National Institute of Nutrition, Hyderabad. In brief, mice were immunized with 15-20 µg of rRv1168c in incomplete Freund’s adjuvant. Two booster doses of the rRv1168c (15-20 µg) in incomplete Freund’s adjuvant were injected in 15 days interval. Mice were sacrificed after 45 days and sera were collected. The sera were checked for the Rv1168c specific antibody by Western blotting and EIA.

2.2.19 Cloning of Rv1168c and Rv1196 in pVV16 shuttle vector

In order to express Rv1168c and Rv1196 in M. smegmatis, the ORFs were cloned in a shuttle vector pVV16 which has the replication origins for both E. coli and mycobacterium species. Rv1168c was amplified from pRSET A clone (forward primer 5'-GCAATATTCATATGGATTTCACAATTTTTCCGCC-3' and reverse primer 5'-GCATAAGCTTCCTAGCCGGCGGGGTACCCG-3') and Rv1196c was amplified from pRSET A clone (forward primer 5'-GCATTCAACATATGGATTTCGGGGCGTTAC-3' and reverse primer 5'-CGTAAAGCTTCGCGCCGCGGAG-3') The digested mixture was separated on an agarose gel and the Rv1168c and Rv1196 bands at the expected size were cut and purified. The purified ORFs were ligated to pVV16 double digested with BamHI and HindIII. The N-terminal truncated mutant Rv1168c (Rv1168cΔN) was generated by cloning nucleotide sequence representing amino acids 175-346 from BAC contig
Rv71(C2) clone using the forward primer, 5′-ATCTAATGACATATGGCGCTGCGCAGACTTTATGAACTGAC-3′ (NdeI) and reverse primer, R- 5′ ATGGATCCGGCGGCGGCGGCGGTGACC-3′ (BamHI). The C-terminal truncated mutant (Rv1168cΔC) was generated by cloning nucleotide sequence representing amino acids 1-173 from BAC contig Rv71 (C2) clone using the forward primer, 5′-ATACTAGACATATGGGATTTCAACTTTTCCGGCGG-3′ (NdeI) and reverse primer, 5′-ATGGATCCGGAGTTGCGATCGGCGC-3′ (BamHI). The amplified products were ligated to pVV16 vector. All the clones were confirmed with PCR, restriction digestion and sequencing.

2.2.20 Mycobacterium smegmatis culture and transformation

*Mycobacterium smegmatis* mc2155 bacteria were grown in Middlebrook 7H9 medium supplemented with 10% ADC (HiMedia, India), 0.5% Glycerol and .05% Tween 80 (7H9-ADC-T). 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 cold sterile 10% glycerol and re-suspended 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 full-length Rv1168c, Rv1168cΔN, Rv1168cΔC and Rv1196 genes cloned in pVV16 shuttle vector and the pVV16 backbone vector were transformed into the *M. smegmatis* mc2155. Prior to transformation, the cells were thawed on ice and 1 μg of each 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 keeping voltage at 1500V, capacitance at 25 μF and resistance at 1000 Ω.
(Delogu et al., 2004) using GenePulser Xcell electroporator (Biorad). One ml of Middlebrook 7H9-ADC-T 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-ADC 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 or anti-His Ab.

2.2.21 Infection of BF-24 cells

BF-24 cells contain a stably integrated HIV-1 LTR promoter sequence driving CAT reporter gene. The cells were infected with M. smegmatis strains harboring either the Rv1168c (M. smeg-Rv1168) or Rv1196 (M. smeg-Rv1196) or Rv1168cΔN (M. smeg-Rv1168ΔN) or Rv1168cΔC (M. smeg-Rv1168ΔC) or the backbone vector (M. smeg-pVV16) at 10 multiplicity of infection (MOI). After 4 hours, cells were washed with complete growth medium containing gentamicin (Gibco BRL) to a final concentration of 100 μg/ml to inhibit growth of the extracellular bacteria. After 36 hours of infection, whole cell extracts were prepared and CAT expression was measured by ELISA as described in section 2.2.5.

2.2.22 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 Mycobacterium 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/) (Eswar et al., 2006, Marti-Renom et al., 2000). 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 (Laskowski et al., 1993) and VERIFY-3D (Morris et al., 1992) 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 (Laskowski et al., 1993) was applied with SPC (Simple Point Charge) water (Sorgen et al., 2002) 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) (Jin et al., 2007) 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 (Ritchie et al., 1999) by keeping TLR2 as the static molecule and Rv1168c protein as the mobile molecule. The docking was performed 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. The PSA (Sali and Blundell, unpublished) is used to calculate the solvent accessible surface area of the molecules. HBOND program
(Mizuguchi et al., 1998) was used to identify H-bonds at the molecular interface. The spdbv 3.7 version software (Guex, 1996) was used to identify salt bridges and van Der Waals interactions.

2.2.23 Statistical analysis

Data were expressed as mean ± SD of at least 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.
2.3 Results

2.3.1 The recombinant Rv1168c (rRv1168c) protein activates HIV-1 LTR-driven chloramphenicol acetyl transferase (CAT) expression in THP-1 cells

The SDS-PAGE analysis of the purified protein revealed it to be essentially a homogenous preparation of rRv1168c protein of approximately 40 kDa along with the poly-His tag (Fig. 2.1). To examine whether rRv1168c increases HIV-1 LTR transcription, human monocytic THP-1 cells were co-transfected with a chloramphenicol acetyltransferase (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, the cells were either left untreated or treated with various concentrations of purified rRv1168c 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. 2.2A). CAT was found to be maximally expressed when rRv1168c was used at 3 µg/ml final concentration (Fig. 2.2A, compare bar 1 with bar 11, \( p < 0.001 \)). 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%), the BF-24 cells (THP-1 cells containing a stably integrated HIV-1 LTR promoter sequence driving CAT reporter gene) was also used to assess the effect of Rv1168c on HIV-1 LTR transcription (Schwartz et al., 1989). 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. 2.2B). 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 (Nair et al., 2009). The denatured rRv1168c protein failed to increase HIV-1 LTR activity as indicated by near control levels of CAT gene expression (Fig. 2.2A; compare bar 5 with bar 11, \( p < 0.001 \) and Fig. 2.2B; compare bar 2 with bar 5, \( p < 0.001 \)). These observations confirmed 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 rRv1168c also in PMA-differentiated BF-24 macrophages (Fig. 2.3, compare bar 3 and bar 4 with bar 1, \( p < 0.001 \) in both the cases).
Figure 2.1. Expression and purification of recombinant Rv1168c. 

A. Rv1168c cloned in pRSET A vector was transformed into BL21(DE3)pLysS cells and liquid broth culture was prepared from a single transformed colony. The culture was induced with 1 mM IPTG. The uninduced (-) and induced (+) samples were collected at different time points and boiled in Laemmli sample buffer and SDS-PAGE was performed. The gel was stained with Coomassie Blue stain.

B. The induced culture was harvested after 4 hours and the cells were lysed in lysis buffer. The lysate was incubated with TALON resin. The resin was washed with wash buffer and bound protein was eluted using elution buffer. Different elution fractions (lane 1 to lane 6) were collected and SDS-PAGE was performed and stained with Coomassie Blue stain.
Figure 2.2. The rRv1168c protein activates HIV-1 LTR in THP-1/BF-24 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 either different concentrations (0.5, 1.0, 3.0 and 5.0 μg/ml) of native rRv1168c or 5.0 μg/ml autoclaved rRv1168c protein. After 36 hours, whole cell extracts were prepared and the CAT and the β-galactosidase activity in the cell extracts were 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 either different concentrations (0.5, 1.0, 3.0 and 5.0 μg/ml) of rRv1168c or 5.0 μg/ml autoclaved rRv1168c protein. Whole cell extracts were prepared after 36 hours of treatment and ELISA was performed to measure the levels of CAT gene expression. The results are shown as the mean ± SD of three independent experiments.
Figure 2.3. Rv1168c increased CAT activity in PMA-differentiated BF-24 macrophages in dose-dependent manner. PMA-differentiated BF-24 cells were treated with different concentrations (0.3, 1.0 and 3.0 μg/ml) of rRv1168c protein. After 36 hours, whole cell extracts were prepared and the CAT activity in the cell extracts was measured by ELISA. The results are shown as the mean ± SD of three independent experiments.
2.3.2. *Rv1168c* increases nuclear factor-kappa B (NF-κB) levels in THP-1 cells

HIV-1 LTR is a well characterized transcription regulatory element (Kilareski *et al*., 2009, Pereira *et al*., 2000, Bernier *et al*., 1998). 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 (Molina *et al*., 1989, Bernier *et al*., 1998, Nabel *et al*., 1987, Mingyan *et al*., 2009, Pomerantz *et al*., 1990, Koyanagi *et al*., 1988), it was first examined whether *Rv1168c* treatment could increase the nuclear NF-κB levels in THP-1 cells. Therefore, nuclear extracts were prepared cells either left untreated (control group) or treated with different concentrations of rRv1168c (0.3 - 3 µg/ml) and these nuclear extracts were used to check the specific DNA-binding activity of the NF-κB complex by EMSA using NF-κB consensus oligonucleotide probe labeled with [γ-32P]-ATP. The EMSA result shown in Fig. 2.4 indicates that the level of NF-κB is increased in rRv1168c-treated group in a dose-dependent manner (Fig. 2.4; lanes 3 to 5). The control group showed very little DNA-binding activity of NF-κB (Fig. 2.4; lane 2). Homologous cold competition abrogated NF-κB DNA-binding activity confirming the specificity of the DNA-protein complex (Fig. 2.4, lane 6). The EMSA result reveals that the intensity of the DNA-protein complex corresponding to the NF-κB is increased by Rv1168c in THP-1 macrophages.
Figure 2.4. The rRv1168c protein increases DNA-binding activity of NF-κB in THP-1 cells. 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. The results are representative of three independent experiments.
Since p50 and p65 factors play important roles in the activation of HIV-1 LTR (Mingyan et al., 2009, Asin et al., 2001), next the expression profile of the p50 and the p65 NF-κB in the nuclear extracts prepared from Rv1168c-treated THP-1 cells was examined 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. 2.5). 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.

**Figure 2.5. The rRv1168c protein increases nuclear p50 and p65 NF-κB levels.**
Nuclear extracts prepared from rRv1168c-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 antibody respectively. Ponceau S staining was performed to check equal protein loading. The results are representative of three independent experiments.
2.3.3 Rv1168c-mediated activation of LTR is dependent on NF-κB transcription factors

Since in the previous section, rRv1168c was found to increase nuclear NF-κB levels (Fig. 2.4, Fig. 2.5), it was next investigated 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 10 hours post-transfection, cells were treated with 10 µM pyrroloidine dithiocarbamate (PDTC), a known inhibitor of NF-κB (Schreck et al., 1992), followed by incubation with rRv1168c (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. 2.6A; compare bar 7 with bar 3 and bar 9 with bar 5, \( p < 0.001 \) in both the cases).

The NF-κB transcription factor is known to be retained in cytoplasm by IkBα protein and its activity is regulated by its release from IkBα complex after phosphorylation and degradation of IkBα (Karin et al., 2002). Therefore, the THP-1 cells were co-transfected with HIV-1 LTR-CAT and phosphorylation-defective IkBα (ΔIkBα) to sequester NF-κB in the cytoplasm and examined HIV-1 LTR trans-activation after treatment 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. 2.6B indicates that Rv1168c-induced HIV-1 LTR activation is abrogated in the group transfected with ΔIkBα when compared with the control group that received pRc/CMV backbone vector alone.
(Fig. 2.6B; compare bar 5 with bar 2, \( p < 0.01 \) and bar 6 with bar 3, \( p < 0.001 \) in both the cases). These results further suggest an important role of NF-kB in the activation of HIV-1 LTR by Rv1168c.
Figure 2.6. 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 (β-Gal, used as internal control for 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 rRv1168c 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). Ten hours after transfection, cells were then treated with 0.3 and 3.0 µg/ml of rRv1168c and after 36 hours of protein treatment, whole cell extracts were prepared and the CAT and the β-Gal expression was measured by ELISA. The results are shown as the mean ± SD of three independent experiments.
2.3.4. Binding of NF-κB with HIV-1 LTR is necessary for the activation of 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) (Nabel et al., 1987) followed by treatment with rRv1168c (0.3 µg/ml and 3 µg/ml) for 36 hours. The results indicate that expression of the CAT reporter gene is significantly down-regulated in the group transfected with pDkB-HIV-CAT as compared to the group transfected with wild-type HIV-1 LTR (Fig. 2.7, compare bar 5 with bar 2 and bar 6 with bar 3, \( p < 0.001 \) for both the cases). All these results confirm a definite role of NF-κB in the Rv1168c-mediated activation of HIV-1 LTR promoter.
Figure 2.7. Rv1168c-mediated activation of HIV-1 LTR is inhibited when NF-κB sites in the HIV-1 LTR were mutated. 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). Next the cells were treated with 0.3 and 3 µg/ml of rRv1168c after 10 hours of transfection and CAT ELISA was performed after 36 hours of protein treatment. The results are shown as the mean ± SD of three independent experiments.
2.3.5 *Rv1168c can still activate HIV-1 LTR even when TNF-α production by Rv1168c is inhibited*

The NF-κB transcription factors are induced by stimuli such as proinflammatory cytokines and bacterial toxins (Rothwarf *et al.*, 1999). Thus, it is possible that the recombinant Rv1168c protein activates the proinflammatory cytokine such as TNF-α to activate HIV-1 LTR via an autocrine and paracrine manner through NF-κB (Toossi *et al.*, 1999, Duh *et al.*, 1989). To test the hypothesis, it was first investigated whether rRv1168c could induce TNF-α cytokine production from THP-1/BF-24 cells. When these cells were treated with various concentrations of rRv1168c for 48 hours, higher levels of TNF-α cytokine was produced upon stimulation with Rv1168c in both BF-24 cells (Fig. 2.8A) and THP-1 cells (Fig. 2.8B) in concentration-dependent manner.
Figure 2.8. The rRv1168c protein activates TNF-α induction in BF-24/THP-1 cells. BF-24 (A) or THP-1 (B) cells were treated with different concentration of rRv1168c and after 48 hours TNF-α cytokine secreted in the medium was measured by two-site sandwich EIA using kit from BD Pharmingen (BD Biosciences Pharmingen, San Diego, CA). LPS (5 µg/ml) was used as positive control of TNF-α induction.
As TNF-α can activate HIV-1 LTR transcription targeting the NF-κB (Duh et al., 1989, Toossi et al., 1999), it was 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-trifluoromethyl-phenyl)-1H-indol-3-ylmethyl)-amino)-ethyl)-amino)-methyl)-chromen-4-one, diHCl}, a pharmacological inhibitor of TNF-α [Calbiochem, San Diego, CA, (He et al., 2005)] 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 (Fig. 2.9A) without affecting the viability of cells as indicated by MTT assay (Fig. 2.9B). Interestingly, rRv1168c was found to activate HIV-1 LTR even in the presence of the specific TNF-α inhibitor (Fig. 2.10). These data indicate that rRv1168c can still activate HIV-1 LTR even when TNF-α production by Rv1168c is inhibited.
Figure 2.9. TNF-α induction by Rv1168c is inhibited when cells were treated with a specific TNF-α inhibitor. BF-24 cells were treated with different concentrations of rRv1168c in the absence or presence of the small molecular inhibitor of TNF-α {6, 7-Dimethyl-3-((methyl-(2-(methyl-(1-(3-trifluoromethyl-phenyl)-1H-indol-3-ylmethyl)-amino)-ethyl)-amino)-methyl)-chromen-4-one, diHCl}. After 36 hours, culture supernatants were harvested and TNF-α production was measured by two-site sandwich EIA (A) and the cell cytotoxicity was measured by MTT assay (B). For MTT assay, MTT (Sigma-Aldrich) was added in various cultures as 1 mg/ml and incubated for 4 hours. The cells were lysed overnight using 100 µl of lysis buffer (20% SDS and 50% DMF) and the absorbance determined at 550 nm as described earlier (Khan et al., 2008). Results are expressed as mean ± SD of three independent experiments.
Figure 2.10. The rRv1168c protein-mediated activation of HIV-1 LTR is independent of TNF-α. BF-24 cells were treated with 0.3 and 3 µg/ml rRv1168c protein in the absence or presence of 5 µM 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. After 36 hours, CAT expression was measured by ELISA. Results shown are mean ± SD of three independent experiments.
2.3.6 *Rv1168c is present in the insoluble cell wall fraction*

Various studies have established that some of the PPE family proteins are localized in the cell surface (Nair *et al.*, 2009, Sampson *et al.*, 2001, Gey van Pittius *et al.*, 2006, Newton *et al.*, 2009). Interestingly, Rv1168c was over-expressed in *M. smegmatis* using pVV16 under the control of hsp60 promoter as described elsewhere (Nair *et al.*, 2009, Delogu *et al.*, 2004), it was found that Rv1168c was predominantly present in the insoluble cell wall fraction (Fig. 2.11) but not in the culture filtrate. It will be pertinent to mention here that *M. smegmatis* is a non-pathogenic mycobacterium and its genome does not have most of the PE/PPE genes including Rv1168c (Gey van Pittius *et al.*, 2006). Again, a BLAST search of the Rv1168c sequence against the *M. smegmatis* peptide database (http://blast.jcvi.org/cmrblast/) failed to identify any significantly similar entry matching the Rv1168c protein sequence. Many PPE proteins are known to be surface localized (Nair *et al.*, 2009, Sampson *et al.*, 2001, Gey van Pittius *et al.*, 2006, Newton *et al.*, 2009) and since Rv1168c could be detected in the insoluble cell wall fraction (Fig. 2.11), it was 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-κB signaling cascades that eventually drive the HIV-1 LTR transcription.
Figure 2.11. Rv1168c is present in the cell wall fraction of *M. smegmatis*. *M. smegmatis* bacteria were transformed with either backbone vector (pVV16) alone or with plasmid construct harboring Rv1168c (pVV16-Rv1168c). The transformed cells were selected in 50 μg/ml hygromycin and 50 μg/ml kanamycin. At OD 0.5, cells were harvested after a heat shock at 40°C for 30 minutes. About 50 million cells were washed with PBS-Tween 20 (PBS-T) and then sonicated vigorously. The insoluble pellet was washed with PBS-T three times and boiled with SDS loading dye. The pellet rich fractions were separated on 10% SDS-PAGE and immunoblotted onto nitrocellulose membrane. The membrane was incubated with mouse anti-Rv1168c antibody followed by incubation with anti-mouse HRP. Bands were detected by chemiluminescence using the manufacturer's protocol.
2.3.7 *Rv1168c* specifically interacts with TLR2 receptors

To detect any interaction of Rv1168c with the surface receptors, the THP-1 cells were incubated with titrating concentrations of FITC conjugated rRv1168c (Rv1168c-FITC) for 60 minutes on ice. After washing the cells extensively, the surface-bound fluorescence was measured using flow cytometry. The result indicated that rRv1168c protein could strongly bind to the macrophage surface in concentration-dependent manner (Fig. 2.12).

![Figure 2.12. Rv1168c binds to the THP-1 cell surface in concentration-dependent manner.](image)

FITC labelled rRv1168c (Rv1168c-FITC) was prepared by incubating the rRv1168c protein with FITC using a commercially available FITC Antibody Labeling Kit from Pierce following the manufacturer’s protocol. THP-1 cells were incubated with different concentrations (1, 3 and 10 µg/ml) of Rv1168c-FITC for 1 hour on ice. Data were acquired by Partek PAS-III (Partec, Germany) and analyzed by Flowjo 7.6.1 flow cytometric analyses program.
Various studies have indicated that TLR2 is the most predominant receptor recognized by *M. tuberculosis* components (Jo et al., 2007) and that the TLR2 could play an important role to modulate macrophage signaling cascades during *M. tuberculosis* infection (Nair et al., 2009, Sanchez et al., 2010, Pecora et al., 2006, Sweet et al., 2006). 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 proviral genome failed to transcribe genes under the control of the LTR promoter (Bafica et al., 2003, Equils et al., 2003). Since the rRv1168c protein was found to bind strongly with THP-1 cells (Fig. 2.12), it was next investigated whether Rv1168c specifically recognizes the TLR2 and targets the TLR2-induced signaling to activate HIV-1 LTR. To identify the receptor involved in the binding of rRv1168c on THP-1 cells, an inhibition assay was performed where THP-1 cells were pre-treated with 10 µg/ml of isotype-matched (IgG2a) control Ab or anti-TLR2 mAb or anti-TLR4 mAb and incubated with biotin conjugated rRv1168c followed by incubation with streptavidin-HRP. It was found that anti-TLR2 mAb strongly inhibited rRv1168c binding on THP-1 cells (Fig. 2.13). In contrast, pre-incubation of cells with either anti-TLR4 mAb or isotype-matched control Ab did not show any inhibitory effect on binding of Rv1168c to THP-1 cells (Fig. 2.13). These observations suggest that TLR2 is the possible interacting partner of Rv1168c.
Figure 2.13. Rv1168c interacts with TLR2 receptors on THP-1 cell surface. 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 Rv1168c was measured using FACS Vantage flow cytometer.
2.3.8. The Rv1168c targets the TLR2 pathway to activate NF-κB signaling and HIV-1 LTR promoter activity

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, next BF-24 cells were treated 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. 2.14A and 2.14B 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. 2.14A, compare lane 5 with lane 3) with concomitant inhibition of transcription of the CAT reporter gene from the LTR promoter (Fig. 2.14, compare bar 3 with bar 2, \( p < 0.001 \)).
Figure 2.14. Rv1168c targets the TLR2 receptor-mediated signaling to activate NF-κB and HIV-1 LTR. A. 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 rRv1168c. Cells were harvested either after 1 hour to measure NF-κB induction in the nuclear extracts of various groups by EMSA or B. 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.
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 (Fig. 2.15). Consistent with the previous observations with neutralizing antibody (Fig. 2.14A and 2.14B), it was 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. 2.16A, lane 5). However, in the negative control siRNA-transfected BF-24 cells significant amount of DNA-binding activity was detected (Fig. 2.16A, 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. 2.16B, 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. 2.16B, compare bar 9 with bar 6 and bar 3, p <0.001 for both the cases). These results suggest that Rv1168c mainly targets the TLR2 to induce NF-κB-dependent activation of HIV-1 LTR in monocyte/macrophages.
Figure 2.15. Specific knockdown of TLR2 on the surface of BF-24 cells by TLR2-specific siRNA. BF-24 cells were transfected with either the negative-control siRNA (BF-24-Negative control) or with TLR2-specific siRNA (BF-24-TLR2-siRNA) using lipofectamine 2000. After 24 hours, surface expression of TLR2 receptors were checked by flow cytometry (BD FACSVantage SE from Beckton Dickinson) using anti-TLR2 Ab from Imgenex (San Diego, CA, USA).
Figure 2.16. Silencing of TLR2 abrogates Rv1168c-mediated NF-κB and HIV-1 LTR activation. A. BF-24 cells were 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 rRv1168c. Protein extracts were prepared 1 hour post-treatment and EMSA was performed. Results shown are representative of three independent experiments. B. 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 hours of transfection, the cells were either left untreated or treated with 0.3 μg/ml and 3 μg/ml of rRv1168c for 36 hours. Whole cell extracts were prepared to measure CAT expression level by ELISA. The results are shown as the mean ± SD of at least three independent experiments.
2.3.9. The PPE protein Rv1196 does not activate HIV-1 LTR

Recently, it has been reported that the PPE protein of *M. tuberculosis*, Rv1196 (known as PPE18) is surface exposed and predominantly binds to TLR2 and modulates macrophage signaling cascades (Nair *et al.*, 2009) like Rv1168c. Therefore, next it was investigated whether Rv1196 also increases LTR activity similar to Rv1168c. Rv1196 protein was purified as described in section 2.2.1 and the recombinant protein preparation was analyzed using SDS-PAGE. The SDS-PAGE analysis of the purified protein revealed it to be essentially a homogenous preparation of rRv1196 protein of approximately 45 kDa along with the poly-His tag (Fig. 2.17A). Interestingly, Rv1196 had no effect on HIV-1 LTR trans-activation (Fig. 2.17B) which is likely due to its inherent ability to inhibit NF-kB activation (Nair *et al.*, 2011).
Figure 2.17. Rv1196 does not activate HIV-1 LTR in BF-24 cells. A. Rv1196 cloned in pRSET A vector was transformed into BL21(DE3)pLysS cells and liquid broth culture was prepared from a single transformed colony. The culture was induced with 1 mM IPTG. The induced culture was harvested after 4 hours and the cells were lysed in lysis buffer. The lysate was incubated with TALON resin. The resin was washed with wash buffer and bound protein was eluted using elution buffer. Different elution fractions (lane 1 to lane 7) were collected and SDS-PAGE was performed. The gel was stained with Coomassie Blue stain. B. 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 three independent experiments.
2.3.10 Rv1168c activates HIV-1 LTR and requires 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, BF-24 cells were infected 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. 2.18; compare bar 3 with bar 1, *p* < 0.001). In the earlier section, it was observed that the recombinant Rv1196 protein was ineffective to trans-activate HIV-1 LTR (Fig. 2.17B). 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. 2.18; bar 2). Thus, Rv1168c but not Rv1196 when presented in the context of a heterologous *M. smegmatis*, can activate HIV-1 LTR transcription (Fig. 2.18, compare bar 3 with bar 2, *p* < 0.001).
Figure 2.18. BF-24 cells show enhanced HIV-1 LTR transcription when infected with \textit{M. smegmatis} strains harboring Rv1168c. BF-24 cells were infected with \textit{M. smegmatis} strains harboring Rv1168c (\textit{M. smeg}-Rv1168c) or Rv1196 (\textit{M. smeg}-Rv1196) or the backbone vector (\textit{M. smeg}-pVV16) at 1:10 MOI. 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.
In order to verify whether Rv1168c could interact specifically with TLR2 in context of the mycobacterial cell wall (M. smeg-Rv1168c) and enhance the NF-κB and HIV-1 LTR activity, BF-24 cells were transfected either with the scrambled siRNA or with TLR2-specific siRNA to suppress TLR2 expression and then infected with M. smeg-Rv1168c or M. Smeg-pVV16. Formation of NF-κB-DNA complex was abrogated when TLR2 expression in BF-24 cells was suppressed by using TLR2-specific siRNA (Fig. 2.19A, 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. 2.19B, compare bar 9 with bar 6 and bar 3, \( p < 0.0001 \) in both the cases). 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 (Nair et al., 2009), fails to activate HIV-1 LTR.
Figure 2.19. TLR2 receptor is required to activate NF-κB and HIV-1 LTR in BF-24 cells when infected with *M. smegmatis* over-expressing Rv1168c (*M. smeg*-Rv1168c). 

A. BF-24 cells were transfected with either siRNA targeting 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 B. cultured for another 36 hours and CAT expression in the whole cell extracts was measured by ELISA. The results are shown as mean ± SD of at least three independent experiments.
2.3.11 Rv1168c specifically interacts with LRR 15~20 domain of TLR2

In the present study, it has been observed that although Rv1168c interacts with TLR2, in a way similar to Rv1196 (Nair et al., 2009), 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 proinflammatory cytokine production by interacting with the LRR 11~15 region (Nair et al., 2009). Therefore, it was 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. 2.20A) 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, Rv1168c showed interaction (Fig. 2.20B). These results suggest that Rv1168c binds to the TLR2 domain in a region other than the LRR 11~15 to elicit a pro-inflammatory-type signaling.
Figure 2.20. The Rv1168c protein and the Rv1196 protein interact with TLR2 at different LRR domain stretches. 

A. THP-1 cells were pre-treated with 5 or 15 fold excess concentrations of Rv1196, washed and then treated with biotinylated Rv1168c (10 µg/ml) protein followed by incubation with streptavidin-FITC. The binding of Rv1168c was measured using FACSVantage 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 antibody. 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 antibody (lower panel). The results are representative of three independent experiments.
Next, the domain of Rv1168c important for its interaction with macrophage was identified. For this, the recombinant Rv1168c truncated proteins either with deletion in the N-terminal region (Rv1168cΔN; with an intact C-terminal fragment containing 175-346 aa residues) or with deletion in the C-terminal region (Rv1168cΔC; with an intact N-terminal fragment containing 1-173 aa residues) (Fig. 2.21A and Fig. 2.21B) were generated. It was observed that the recombinant Rv1168cΔN (rRv1168cΔN) did not bind to the THP-1 cells, whereas the C-terminal truncated recombinant Rv1168c protein (rRv1168cΔC) with an intact N-terminal domain could bind to these cells (Fig. 2.22).
Figure 2.21. Purification of recombinant Rv1168cΔC and Rv1168cΔN fragments. Rv1168cΔC (A) and Rv1168cΔN (B) were cloned in pRSET A and transformed into BL21(DE3)pLysS cells. Liquid broth culture was prepared from single transformed colonies. The cultures were induced with 1 mM IPTG. The induced cultures were harvested after 4 hours and the cells were lysed in lysis buffer. The lysate was incubated with TALON. The resin was washed with wash buffer and bound protein was eluted using elution buffer. Different elution fractions (lane 1 to lane 7) were collected and run on a 10% SDS-PAGE and stained with Coomassie Blue stain.
Figure 2.22. The N-terminal but not the C-terminal domain of Rv1168c strongly interacts with THP-1 cells. The THP-1 cells were either left untreated or incubated with 10 µg/ml of either Rv1168ΔN-FITC or Rv1168ΔC-FITC for 1 hour on ice and the fluorescence was measured by flow cytometry. The results are a representative of three independent experiments.
The computational docking studies (as described in the materials and methods section 2.2.22) were used 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, The Rv1168c was predicted to interact with the 15~20 LRR region of TLR2 (Fig. 2.23). 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 (Meng et al., 2003) and binding of Rv1168c with these TLR2 mutants were compared by flow cytometry. The wild-type and the mutant TLR2 proteins were found to be expressed in HEK293 cells as determined by immunoblotting using anti-FLAG mAb (Meng et al., 2003) (Fig. 2.24A). When the interaction of Rv1168c with these cells was checked, it could be observed that Rv1168c was unable to bind to the HEK293 cells expressing the TLR2-Mut4 that specifically lack LRR 15~20 domain (Fig. 2.24B) whereas it could interact with HEK293 cells expressing either the full-length TLR2 or the TLR2-Mut3 as also indicated in our pull-down assay experiment in Fig. 2.20B. Therefore, it appears that TLR2 LRR 15~20 domain is important in triggering the downstream signaling events leading to activation of NF-κB.
Figure 2.23. The N-terminal domain of Rv1168c interacts with 15~20 LRR region of TLR2. Molecular modelling of the interaction between the LRR domain of TLR2 (green) and the Rv1168c (red) was carried out 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. The two models depict two different orientations (top, front view) of the TLR2-Rv1168c complex, 90° with respect to each other.
Figure 2.24. Rv1168c specifically binds to LRR 15–20 domain of TLR2. A. HEK293 cells were transfected with either the Flag-tagged wild-type TLR2 (WT-TLR2) construct or with the TLR2-Mut3 lacking LRR 11–15 domain or the TLR2-Mut4 lacking LRR 15–20 domain. After 24 hours of transfection, to check the expression of WT-TLR2, TLR2-Mut3 and TLR2-Mut4, the cells were lysed and subjected to immunoblotting using mouse monoclonal anti-FLAG antibody followed by anti-mouse IgG-HRP. Blots were developed by chemiluminescence using ECL-plus detection reagent kit following the manufacturer's protocol (GE Healthcare). B. HEK293 cells over-expressing the Flag-tagged either wild-type TLR2 (WT-TLR2) or TLR2-Mut3 lacking LRR 11–15 domain or TLR2-Mut4 lacking LRR 15–20 domain were incubated with biotynilated Rv1168c. The binding of biotynilated Rv1168c was assessed by flow cytometry using streptavidin-FITC. Results shown are representative of three independent experiments.
2.3.12 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

In the earlier section, it was shown that the rRv1168cΔN 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. 2.22). When BF-24 cells were incubated with the purified truncated Rv1168c protein with an intact N-terminal domain containing 1-173 aa residues (rRv1168cΔC), it was observed that the purified truncated rRv1168cΔC protein was able to sufficiently activate HIV-1 LTR driven transcription to the extent similar to that of the purified full-length protein (Fig. 2.25, 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. 25, 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 the TLR2. This suggests that the region encompassing amino acids from 1-173 is crucial to activate the TLR2-triggered proinflammatory signaling and HIV-1 LTR trans-activation.
Figure 2.25. The N-terminal domain of Rv1168c encompassing amino acids from 1-173 is crucial to activate 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 shown as mean ± SD of at least three independent experiments.
The full length protein when presented in the context of the whole mycobacterium in *M. smegmatis* (*M. smeg*-Rv1168) was shown to activate HIV-1 LTR (Fig. 2.18). Similarly, the N-terminal region when presented in the context of whole mycobacterium (*M. smeg*-Rv1168cΔC) (Fig. 2.26) was also able to activate NF-κB and therefore the HIV-1 LTR driven CAT gene expression almost similar to the levels observed by the full length protein (Fig. 2.27A, compare lane 6 and lane 4; Fig. 2.27B, compare bar 5 and bar 3). Similar results were also obtained when the levels of TNF-α was measured in these cells (Fig. 2.27C, compare bar 5 and bar 3).

To determine if any role played by the C-terminal domain, the truncated Rv1168c with an intact C-terminal fragment containing 175-346 aa residues was generated and over-expressed in *M. smegmatis* (*M. smeg*-Rv1168ΔN) (Fig. 2.26) and infected the BF-24 cells. It was 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. 2.27A, 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. 2.27B, compare bar 4 with bars 3 and 5, *p* < 0.001 in both the cases) as well as in its ability to stimulate TNF-α production (Fig. 2.27C, compare bar 4 with bars 3 and 5, *p* < 0.001 in both the cases). These data indicate that the N-terminal region of Rv1168c is the functionally active domain and is required for elicitation of the proinflammatory signaling pathway and HIV-1 LTR trans-activation.
Figure 2.26. Expression of Rv1168cΔC and Rv1168cΔN in *M. smegmatis*. *M. smegmatis* bacteria were transformed with either pVV16-Rv1168cΔC or pVV16-Rv1168cΔN plasmid constructs. The transformed cells were selected in 50 μg/ml hygromycin and 50 μg/ml kanamycin. At OD 0.5, cells were harvested after a heat shock at 40°C for 30 minutes. About 50 million cells were washed with PBS-T and then sonicated vigorously. The lysate was boiled with Laemmli buffer and separated on 10% SDS-PAGE and immunoblotted onto nitrocellulose membrane. The membrane was incubated with mouse monoclonal anti-His antibody (Sigma-Aldrich) followed by incubation with anti-mouse HRP. The membrane was washed and the bound enzyme was detected by chemiluminescence using ECL-plus detection reagent kit following the manufacturer's protocol (GE Healthcare).
**Figure 2.27.** *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 trans-activation 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*-Rv1168cΔC at MOI of 1:10. Cells were either harvested after 4 hours to measure the NF-κB activity by EMSA (A) or cultured for another 36 hours B. either to measure CAT expression in the whole cell extracts by ELISA or C. to estimate TNF-α cytokine secreted in the culture supernatants by ELISA. The results are shown as mean ± SD of at least three independent experiments.
2.4 Discussion

Cells of the monocyte/macrophage lineage are known to play an important role in the transmission and pathogenesis of HIV (Gartner et al., 1986, Coleman et al., 2009, Kilareski et al., 2009, Doherty et al., 1999). Infected monocytes can differentiate into monocyte-derived macrophages (MDMs) and may form a long-lived reservoir for the virus (Brown et al., 2006, Garcia-Blanco et al., 1991, Igarashi et al., 2001). MDMs can also be infected after differentiation and are found to be more susceptible to new infection (Rich et al., 1992). HIV-1 replication is shown to be markedly up-regulated in monocytes/macrophages during pulmonary tuberculosis (Orenstein et al., 1997, Hoshino et al., 2002, Downing et al., 1995). Interestingly, HIV-1 replication was found to be increased in the lung regions infected with \textit{M. tuberculosis} compared to regions infected with HIV-1 alone (de Noronha et al., 2008).

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 (Shen et al., 2004, Selwyn et al., 1989, Thomas, 2006). In AIDS patients, once TB is established HIV-1 replication is enhanced (Zhang et al., 1995, Kitaura et al., 2001, Toossi et al., 1999, Toossi et al., 1993) because of the indirect effects of host’s proinflammatory immune response against \textit{M. tuberculosis} infection or due to the direct effects of mycobacterial components that modulate the signal transduction cascades of the macrophages (Zhang et al., 1995, Kitaura et al., 2001, Cohen et al., 1997). Interestingly, in many cases neutralizing antibodies against various proinflammatory cytokines did not abrogate HIV-1 transcription induced by \textit{M. tuberculosis} (Wahl et al., 1998, Ghassemi et al., 2000, Shattock et al., 1994). It was
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 (PAMPs) by innate immune receptors like the TLR(s) could be an important event in the modulation of macrophage innate-signaling during mycobacterial infection (Nair et al., 2009, Basu et al., 2007, Jo et al., 2007). Importantly, the TLR2 is shown to interact with a number of mycobacterial components and modulates macrophage innate-signaling cascades (Basu et al., 2007, Jo et al., 2007). The TLR2 receptors are thought to play critical roles to enhance LTR-directed transcription and HIV-1 expression by the mycobacteria (Bafica et al., 2003, Equils et al., 2003). In a transgenic mouse model stably integrated with HIV-1 provirus it has been shown that the activation of HIV-1 replication due to M. avium is highly abrogated in TLR2−/− transgenic mice. In splenic cells cultured from these transgenic mice it was shown that M. tuberculosis- and M. avium-mediated activation of HIV-1 LTR was completely abrogated in the cells deficient in TLR2. Thus, it was proposed that TLR2 is exclusively responsible for the activation of HIV-1 during M. tuberculosis infection (Bafica et al., 2003).

Although several PPE proteins are found to be over-expressed during infection (Nair et al., 2009, Sampson et al., 2001, Gey van Pittius et al., 2006, Newton et al., 2009, Singh et al., 2005) and are shown to modulate macrophage signaling cascades (Nair et al., 2009,
Basu et al., 2007, Jo et al., 2007), 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 direct role of a PPE protein Rv1168c is demonstrated in activating HIV-1 LTR transcription in monocyte/macrophage cells that targets the TLR2 receptor. It was observed 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 transactivation 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. Tat 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 (Basu et al., 2007, Zhou et al., 2010). The NF-κB factors interact with HIV-1 LTR DNA-binding sites and mediate LTR transactivation (Mingyan et al., 2009, Bernier et al., 1998). 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 (Jacque et al., 1996, Wahl et al., 1999,
Jagodzinski et al., 2001). In this study, it was 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 Rv1168c-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 Rv1168c (Rv1168ΔC) 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 Rv1168c protein.

It has been recently shown that the PPE protein, Rv1196 interacts with TLR2 and modulates innate-effector signaling in macrophages (Nair et al., 2009). However, in this study, enhancement of HIV-LTR transcription by Rv1196 was not observed presumably because of its inability to activate NF-κB signaling pathway (Nair et al., 2011). In 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 rRv1168c. 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 (truncated protein 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. 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 (Nair et al., 2009) and triggers an anti-inflammatory signaling, while binding of Rv1168c with the TLR2 15~20 domain results in induction of proinflammatory 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 (Bafica et al., 2003, Equils et al., 2003), 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, a role of Rv1168c in trans-activating HIV-1 LTR promoter through activation of NF-κB signaling cascades is demonstrated here, however, 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 (Wahl et al., 1999). Though in the present study, the THP-1 cell line is used as a model for cells of monocytes/macrophage lineage, the 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 hyper-activation. Since viral replication and multiplication are critically dependent on the LTR promoter transcription, it is likely that Rv1168c (PPE17) may play an augmenting role in trans-activating HIV-1 LTR promoter albeit indirectly in *M. tuberculosis*/HIV-1 co-infected individuals.