Materials and Methods
5.1 ANIMALS

Female BALB/c mice, 4-6 week of age, kept in the ICGEB animal house under pathogen free environment were used following approval from the Institutional Animal Ethics Committee.

5.2 MATERIALS

Fluorescence-tagged antibodies against mouse or human CD80, CD86, CD54, CD40, H-2D^d, I-A/E, HLA-ABC, HLA-DR-DP-DQ, IFN-γR, IL-12Rβ, IL-10R and isotype controls were from BD Biosciences (San Jose, CA, USA). Recombinant mouse GM-CSF was from R&D Systems (Minneapolis, MN). Dichlorofluorescin diacetate (DCFH-DH) and DiLC18 dye was obtained from Molecular Probes (Eugene, OR). ELISA kits were from eBioscience (San Diego, CA) or BD Biosciences. Antibodies to different signaling molecules and luminol kits for chemiluminescence detection were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TLR2 ligand, Pam3CSK4, was purchased from Invivogen (San Diego, CA). Kit for the isolation of M. tb RNA were obtained from Qiagen (Valencia, CA). MicroEnrich™ kit and poly(A) tailing kit were procured from Ambion (Austin, TX). Amplification of cDNA from poly(A)^+ tailed M. tb RNA was carried out using the Creator™ SMART™ kit (Clontech, CA). RPMI-1640 medium, Hanks Balanced Salt Solution (HBSS), lipofectin and Trizol were purchased from Invitrogen life technologies (NY, USA). Fetal Calf serum (FCS) was purchased from Hyclone (UT, USA). Middlebrook 7H9 liquid media, 7H11 agar, Albumin-Dextrose-catalase (ADC), Oleic acid-Albumin-Dextrose-Catalase (OADC) supplement, Luria Bertani (LB) broth media and LB agar were obtained from Difco-Becton-Dickinson (NJ, USA). Phorbol 12-myristate 13-acetate (PMA) was procured from Sigma Aldrich (St.
Materials and Methods

Restriction enzymes, T4 DNA ligase and DNA ladder were obtained from MBI Fermentas (USA). Mammalian and bacterial electroporation cuvettes, and protein molecular weight markers were from Bio-Rad (Philadelphia, PA, USA). Gel extraction kit, midiprep Plasmid DNA isolation kit, Ni²⁺-NTA agarose and RNAProtect regent were taken from Qiagen (Valencia, CA). Microbeads, MACS® columns and nylon mesh filters were obtained from Miltenyi Biotech (Germany). Nitrocellulose membrane (Hybond C pure) was procured from Amersham (Arlington Heights, IL). Fine chemicals, primers and antibiotics used in the study were procured from Sigma Aldrich (St. Louis, USA).

*M. tb* H37Rv and *M. bovis* BCG were the kind gifts from Dr. Pawan Sharma, Immunology group, ICGEB, New Delhi, India. *E. coli* strains and plasmid DNA used in the study and the sources from which they have been obtained are listed below in Table 5.1 and Table 5.2 respectively.

### Table 5.1: *E. coli* strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Features</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F⁻ φ80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(×10, mK⁺) phoA supE44 thi-1 gyrA96 relA1 λ⁻</td>
<td>Invitrogen Inc., Carlsbad, CA, USA.</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 mupG</td>
<td>Invitrogen Inc., Carlsbad, CA, USA.</td>
</tr>
<tr>
<td><em>E. coli</em> M15[pREP4]</td>
<td>Derived from a strain K12, Na⁺, str⁻, rif⁶, thi⁻, lac⁺, ara⁻, gal⁻, mtl⁻, F⁻, recA⁺, uvr⁻, lon⁻ [pREP4 KanR⁺]</td>
<td>Qiagen, Valencia, CA, USA, USA</td>
</tr>
<tr>
<td><em>E. coli</em> BL21(DE3)</td>
<td>F⁻ ompT hsdSb(φB⁻ mB⁻) gal dem araB::T7RNAP-tetA</td>
<td>Novagen, Madison, WI</td>
</tr>
</tbody>
</table>
Table 5.2: Plasmids used in the study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Feature/Application</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEMT®Easy</td>
<td>To clone PCR products; blue white screening for recombinants</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>pFLAG-CMV™-6a</td>
<td>Shuttle vector for <em>E. coli</em> and mammalian cells; expression vector that is a derivative of pCMV5</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>pQE31</td>
<td>High level expression of 6xHis-tagged proteins in <em>E. coli</em></td>
<td>Qiagen, Valencia, CA</td>
</tr>
<tr>
<td>pET28b</td>
<td>For cloning and expression of recombinant proteins in <em>E. coli</em></td>
<td>Novagen, Madison, WI</td>
</tr>
</tbody>
</table>

5.3 METHODOLOGY

5.3.1 Culturing and Maintenance of THP-1 cells

THP-1 is a human monocytic leukemia cell line that was derived from a 1 year old boy with acute monocytic leukemia (Tsuchiya *et al.*, 1980). THP-1 cells were routinely maintained as suspended cells in RPMI 1640 media (Invitrogen life technologies, NY, USA) supplemented with 1.46 g/l L-glutamine, 2.3 g/l HEPES (Gibco BRL, Gaithersburg, MD), 2.0 g/l NaHCO₃ and 100 U of penicillin per ml–100 μg of streptomycin per ml with 10% (vol/vol) fetal calf serum (HyClone Laboratories, Logan, Utah/ UT, USA) at 37°C in 5% CO₂. Viability was checked by Trypan Blue exclusion assay and 99% viable cultures were used for all the studies.

5.3.2 Differentiation of THP-1 cells

THP-1 cells are non-adherent and have to be treated with PMA (Phorbol 12-myristate 13-acetate) to differentiate them into macrophages. After differentiation, THP-1 cells start adhering to the surface and stop dividing further, forming a monolayer.
For the induction of cell differentiation into adherent cells i.e. macrophages, cells were treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, USA) for 16 hours. After incubation, non-adherent cells were removed by aspiration, and the adherent cells (that showed characteristic macrophage like appearance after differentiation) were washed twice with RPMI. These differentiated cells were then used for all further studies.

5.3.3 Cultivation of E. coli

E. coli strains were stored at -70°C as glycerol stocks (25% glycerol) and were cultured in Luria Bertani (LB) medium containing either ampicillin (100 µg/ml) or kanamycin (25 µg/ml) or other antibiotic as required.

5.3.4 Preparation of M. tb H37Rv and M. Bovis BCG stocks for infection studies

M. tb H37Rv was grown in Middlebrook 7H9 liquid medium (Difco-Becton-Dickinson, NJ, USA) supplemented with albumin/dextrose/catalase (ADC) (Difco-Becton-Dickinson, NJ, USA) at a final concentration of 5 g/l, 2 g/l and 0.003 g/l, respectively, along with 0.05% Tween 80. The culture was harvested at an O.D$\text{595}$ of 0.5, concentrated to small volume and stocks were prepared in small aliquots. Aliquots were frozen at -85°C and viable bacteria were enumerated by plating serial dilutions of stocks on 7H11 agar (Difco-Becton-Dickinson, NJ, USA) supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase) (Difco-Becton-Dickinson, NJ, USA) and 0.5% glycerol. These stocks were then used for all the infection studies. M. bovis BCG strain was grown in Middlebrook 7H9 liquid medium supplemented with albumin/dextrose/catalase (ADC) (Difco-Becton-Dickinson, NJ, USA) at a final concentration of 5 g/l, 2 g/l and 0.003 g/l, respectively, along with 0.1% Tween 80 and 0.4% glycerol. The stocks were prepared and stored using similar protocol as used for the preparation of stocks of M. tb H37Rv.

5.3.5 Infection of THP-1 cells for the enrichment of M. tb genes

THP-1 (human acute monocytic leukemia cell line) cells were grown to the cell density of 1-2 x 10$^6$ cells/ml in a 75 cm$^2$ flat bottom tissue culture flask and stimulated with 50
ng/ml PMA (Sigma Chemical Co. USA) for 16 h to differentiate them into macrophages. PMA-stimulated cells were then washed extensively with RPMI to remove any undifferentiated cells as well as residual PMA. The cells were then scraped from the flask, suspended in the small volume of RPMI supplemented with 10% FCS and transferred to a falcon for infection with H37Rv. This was done to ensure that maximum cells were infected with the bacteria. Freshly prepared H37Rv stocks were used for the infection. Aliquots were thawed and vortexed vigorously. These were then suspended in RPMI and added to the PMA differentiated THP-1 cells at an infection ratio of 10 bacilli per macrophage. The phagocytosis of bacteria was allowed for 4 hours at 37°C in 5% CO₂ after which cells were washed twice to remove any extracellular bacteria. The infected cells were then seeded back in culture in 75 cm² flat bottom tissue culture flask for 24 hours and 5 days (120 hours) respectively.

5.3.6 Identification and enrichment of M. tb genes expressed at day 1 and day 5 of infection in macrophages

A broad outline of the methodology used to identify the genes expressed by M. tb inside the macrophages (PMA differentiated THP-1 cells) is depicted in the Flowchart 5.1. At the end of respective incubation period, i.e. day 1 and day 5 of infection, THP-1 cells were scraped from the flask, washed with PBS and lysed with 10% NP-40 (a non-ionic detergent). This led to the release of intracellular bacteria from the cells following which the bacterial cells were pelleted. The bacterial pellet was treated with DNase and RNase to remove all mammalian (macrophage) RNA and DNA so as to avoid the contamination from host nucleic acids. This was followed by bacterial lysis in the presence of RNAprotect® Bacteria reagent (Qiagen). The bacteria were lysed as per manufacturer’s instructions (Qiagen) provided for the enzymatic lysis and proteinase K digestion of bacteria. Total RNA was then purified from the bacterial lysate using RNeasy Mini Kit (Qiagen).

From this total RNA preparation, M. tb RNA was further enriched using the MICROBEnrich™ kit (Ambion) that selectively depletes all mammalian RNA from the mixtures containing human, mouse or rat and bacterial RNA. It involves the removal of
18S rRNA, 28S rRNA as well as poly-adenylated mRNAs from the complex host-bacteria RNA mixtures. RNA was enriched as per the manufacturer's instructions. Briefly, purified RNA was incubated with the capture oligonucleotide mix in binding buffer for 60 min. Magnetic beads, derivatized with an oligonucleotide that hybridizes to capture oligonucleotide and to the polyadenylated 3' ends of eukaryotic mRNAs were then added to the mixture and allowed to hybridize for 15 min. The magnetic beads, with 18S rRNA, 28S rRNA and poly-adenylated mRNAs attached, were pulled to the side of the tube with a magnet. The enriched bacterial RNA present in the supernatant was collected and precipitated using ethanol. Further, bacterial mRNA was enriched using bacterial mRNA purification kit.

The enriched RNA preparation was poly(A) tailed using *E. coli* Poly(A) Polymerase and ATP (Using Poly(A) tailing kit from Ambion, Texas, USA). At each step we confirmed the presence of *M. tb* RNA by PCR based amplification of house keeping genes *Rv2244* or *Rv3601c* and the absence of macrophage RNA (β-actin) in the RNA preparation.

The poly(A) tailed RNA was then amplified using the Creator™ SMART™ Kit (Clontech, CA, USA) as per manufacturer's instructions. This employed long-distance PCR (LD-PCR) (Barnes 1994; Cheng *et al.*, 1994) to preferentially enrich for full-length cDNAs from limited amount of RNA. The kit works on a SMART (Switching Mechanism At 5' end of RNA Transcript) protocol. A modified oligo(dT) primer (CDSIII/3' PCR primer) primes the first strand synthesis reaction, and the SMART IV Oligo serves as a short, extended template at the 5' end of the mRNA (Figure 5.1). When the reverse transcriptase (RT) reaches the 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART IV Oligo, which has an oligo(G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. RT then switches templates and continues replicating to the end of the oligonucleotide. The resulting full-length ss cDNA contains the complete 5' end of the mRNA, as well as the sequence complementary to the SMART IV Oligo, which then serves as a universal priming site.
(SMART anchor) in the subsequent amplification by LD-PCR. This results in the amplification of only full-length cDNA clones.

The amplified products obtained using LD-PCR were ligated into pGEMTEasy cloning vector (Promega, Wisconsin, USA) followed by the transformation of competent E. coli DH5α cells. Colonies obtained were partially sequenced to get the identity of the amplified genes. Following the identity of genes, fresh primers were designed to amplify these genes from M. tb genomic DNA and amplicons were cloned into the mammalian expression vector pFLAG-CMV-6a (Sigma Chemical Co. USA) using the cloning strategy as described in the section 5.3.7.

![Diagram of cDNA synthesis using LD-PCR](image)

**Figure 5.1: Synthesis of cDNA using Long Distance PCR (LD-PCR)
(Adapted from Creator™ SMART™ cDNA library construction Kit user manual, Clontech Laboratories Inc. USA)**
Flowchart 5.1: Identification of genes expressed inside macrophages by \textit{M. tb} at day 1 and day 5 of infection

**Infection of THP-1 cells**

PMA stimulated THP-1 cells were infected with H37Rv at an MOI of 10:1 (bacteria : THP-1 cells) for day 1 and day 5 respectively.

**Isolation of intracellular bacteria from THP-1 cells**

Infected THP-1 cells were scraped from the flask at the respective time points and lysed with 10% NP40 to release intracellular bacteria. Intracellular bacterial cells were then pelleted.

**Isolation of bacterial RNA**

Bacterial pellet was lysed in presence of RNAProtect™ Bacteria reagent (Qiagen) using enzymatic lysis and Proteinase K digestion. RNA extraction was done using RNeasy Mini kit from Qiagen.

**Enrichment of Bacterial mRNA and amplification**

Bacterial RNA was further enriched using MICROBEnrich Kit™ (Ambion). Enriched mRNA was then Poly A tailed and amplified using ClontechSMART Kit™ (Clontech, CA, USA).

**Identification of amplified genes by Cloning**

The amplified DNA was then cloned into pGEMTEasy vector (Promega) and transformed in competent \textit{E. coli} cells. Colonies were sequenced to obtain the identity of the amplified genes.

**Cloning of identified genes into mammalian expression vector**

Fresh primers were then used to amplify these genes from H37Rv genomic DNA and then cloned into mammalian expression vector, pFLAG CMV6a (Sigma).
5.3.7 Cloning of the genes identified at day 1 and day 5 of infection in a mammalian expression vector, pFLAG-CMV™-6a

The genes identified at day 1 and day 5 of infection (five day 1 genes and five day 5 genes) were cloned in pFLAG-CMV-6A expression vector (Sigma-Aldrich, St. Louis, MO). pFLAG-CMV-6a expression vector is a shuttle vector for *E. coli* and mammalian cells. The promoter-regulatory region of the human cytomegalovirus drives transcription of FLAG-fusion constructs. A simplified plan used for the cloning is depicted in the Flowchart 5.2

**Flowchart 5.2: A generalized plan used to clone genes in pFLAG-CMV-6a vector**

Specific DNA fragment corresponding to each gene was amplified from H37Rv genomic DNA using the respective primer pairs.

PCR product was purified after resolving it on an agarose gel, digested with restriction enzyme(s), and again resolved and purified by gel extraction.

pFLAG CMV6a, a mammalian expression vector, was digested with corresponding set of restriction enzyme(s), and purified.

The restricted PCR product was ligated with the vector DNA digested with the similar enzymes and transformed in *E. coli*.

The colonies obtained were henceforth screened for the positive clone by PCR followed by the sequencing to confirm the positive clone.

5.3.7.1 Preparation of insert and vector DNA for cloning

5.3.7.1.1 PCR amplification of the genes

The full-length genes were amplified from H37Rv genomic DNA with Taq polymerase (Biotools, Madrid, Spain) using the respective forward and reverse primers designed for the particular gene. Individual PCR assays were standardized for optimum Mg²⁺ concentration (1.0 mM - 4.0 mM), annealing temperature, primer concentration (0.5 μM -
1.0 μM) and were performed as three - step reactions. 25 μl reaction mixtures containing 1X PCR buffer (Biotools, Madrid, Spain); 1.0 - 2.5 mM MgCl₂ as required; 0.2 mM dNTPs; 0.5 μM - 1 μM each of forward and reverse primer; 0.5 U of Taq DNA polymerase and template (20 ng of mycobacterial DNA) was subjected to PCR amplification. Initial denaturation was done at 95°C for 15 minutes. Annealing temperature was usually kept 5°C below the Tₘ of primers. Annealing temperature was increased for a PCR if multiple bands appear and decreased if there was no band. Extension temperature was kept at 72°C. The primer sequences as well as PCR conditions used for the amplification of respective genes are provided in Table 5.3

5.3.7.1.2 Restriction digestion of the PCR products and pFLAG-CMV-6a vector DNA

For restriction analysis, the PCR products were fractionated on a 0.8 % - 1.2 % agarose gel in 1X TBE containing 0.5 μg/ml of ethidium bromide at 50 V until the desired resolution was achieved. The desired fragment was excised using a sterile blade on an UV Trans-Illuminator and the DNA was purified by commercially available gel extraction kit (Qiagen, USA) as per the manufacturer's guidelines. An aliquot of purified DNA (~ 2 μg) was digested with the required restriction enzymes according to the conditions specified by the manufacturer. Simultaneously, pFLAG-CMV-6a (Sigma-Aldrich, St. Louis, MO) vector DNA was also digested with the required restriction enzymes. The digested PCR fragments as well as vector DNA were purified using gel extraction kit.

5.3.7.2 Ligation

25 - 50 ng of linearized vector and insert fragment were mixed in varying molar ratios (usually 1:3, vector: insert respectively) and incubated with 1X ligation buffer and 1.0 Weiss unit of T4 DNA ligase enzyme (MBI Fermentas, USA). The ligation reaction was carried out at 4°C for 16 hours. Aliquots of these were subsequently used to transform E. coli DH5α or Top10 competent cells.
5.3.7.3 Transformation of cells

5.3.7.3.1 Preparation of electrocompetent cells

Primary cultures of *E. coli* strains were grown by inoculating single colony in 5 ml LB media and incubated overnight at 37°C at 220-225 rpm. One percent inoculum of the primary culture was inoculated in 100 ml of LB and grown till OD₆₀₀ of 0.5. The culture was kept on ice for 30 min and centrifuged at 3000 g for 15 minutes in a pre-cooled rotor. The pellet was resuspended gently in 100 ml of ice-cold sterile 10% glycerol. The cells were harvested at 3000 g for 15 min in a pre-cooled rotor. The pellet was similarly washed twice with decreasing volumes of ice-cold 10% glycerol (once with 50 ml of glycerol followed by 25 ml glycerol). The pellet was finally resuspended in 1 ml of chilled glycerol. Aliquots of 100 µl each were made in sterile vials and frozen in an ethanol bath kept at -80°C till further use. The efficiency of competent cells was checked by transforming them with the super-coiled vector DNA.

5.3.7.3.2 Transformation

The ligation mixtures (usually 5 - 10 µl) were mixed with 100 µl competent cells and transferred to a chilled 0.1 cm cuvette (Bio-Rad, Philadelphia, PA, USA). The cells were electroporated using Gene Pulser II (Bio-Rad) and immediately transferred to the sterile tube containing 900 µl LB broth. Electroporation conditions that were used for transformation were standardized at 1.8 kV, 25 µF. The cells were then incubated for 1 hour at 37°C for recovery. Aliquots of the recovered cells were plated on LB agar plates containing the appropriate antibiotics. In addition to the ligation mix, negative control transformation was done without any DNA and a positive control transformation was done with an intact plasmid.

5.3.7.4 Screening for the clone of interest

The colonies obtained were streaked on a fresh LB agar plate with appropriate antibiotic. *E. coli* templates for PCR were prepared by boiling cell scrapings from LB plates in 0.1% Triton X -100 for 10 minutes. The debris was pelleted and the supernatant was used as a template for PCR reactions to identify the presence of insert. The plasmid DNA was then
isolated from the positive clone and sequenced to confirm the identity as well as the correct sequence of the clone. Following the identification of positive clone, respective glycerol stocks were prepared and stored at -80°C.

All the constructs were verified by automated DNA sequencing (at Labindia Instruments Pvt. Ltd., Gurgaon, Haryana, India).

**5.3.7.5 Plasmid DNA isolation**

Plasmid DNA was isolated and purified with the help of plasmid DNA isolation kits (Qiagen Plasmid Midi Kit,) as per the protocol provided. Briefly, 100 ml of overnight grown cultures were harvested and were resuspended in 4 ml of buffer P1, followed by lysis with 4 ml of buffer P2. High molecular weight DNA and bacterial cell debris were differentially precipitated with 4 ml of buffer P3 and removed by centrifugation at 13,000 g for 30 minutes at 4°C. The supernatant was passed through the equilibrated membrane column (Qiagen-tip 100) and was washed twice with ethanol-containing wash solution. Finally the plasmid DNA was eluted with 5 ml of buffer QF.

**5.3.8 Cloning of Rv1483, Rv3416, Rv0353 for expression as His-tagged protein**

The ORF corresponding to Rv1483, Rv3416 and Rv0353 were PCR amplified from *M. tb* H37Rv genomic DNA using the respective forward and reverse primers. The primer sequences and the conditions used for amplification are provided in the Table 5.4. The amplified DNAs were A-tailed and cloned into pGEMT-Easy vector (Promega, USA). The inserts were excised by digesting the clone in pGEMTEasy with *BamHI and HindIII*. The excised inserts were directionally cloned into the pQE31 expression plasmid (Qiagen, Valencia, CA) digested with *BamHI and HindIII* followed by the transformation in *E. coli* M15 [pREP4] (Qiagen) strain. The clones resulted in expression of respective fusion proteins (Rv1483 – 25.6 kDa, Rv3416 – 11.6 kDa, Rv0353 – 14.1 kDa) with a N-terminal 6x His-tag. The proteins were expressed and purified from *E. coli* M15 [pREP4] strain as described in sections 5.3.23.1, 5.3.23.2 and 5.3.23.3
5.3.9 **Cloning of *Rv2463* for expression as *His*-tagged protein**

*Rv2463* coding sequence was amplified from *M. tb* H37Rv genomic DNA using Rv2463F and Rv2463R primers, digested with *HindIII* and cloned into the *HindIII* site of pET28b (Novagen, Madison, WI) generating Rv2463pET28b which expresses Rv2463 protein (394 amino acids) along with 6x *His*-tag at N-terminal resulting in a 42.8 kDa protein. Rv2463 was purified from *E. coli* BL21(DE3) strain as described in section 5.3.23.4. The primer sequences and the conditions used for amplification are provided in the Table 5.4.

5.3.10 **DiLC18 staining of H37Rv and detection of bacteria present intracellularly**

DiLC18 (Molecular Probes, Eugene, USA) is a fluorescent carbocyanine dye with a very long (18 carbon) alkyl tail, which gives it an, overall, lipophilic character. It has absorption maxima of 549 nm and emission maxima of 565 nm. This dye binds to mycobacteria irreversibly and therefore can be used to trace internalized bacteria after phagocytosis using flow cytometry. For H37Rv staining, a fresh working solution of 50 μM DiLC18 dye (from a stock of 10 mM dye prepared in DMSO) in 300 mM sucrose (in PBS) was prepared. The requisite amount of H37Rv was suspended in 500 μl PBS together with the freshly prepared working solution of DiLC18 so as to make the final concentration of DiLC18 as 10 μM. The bacteria was then incubated with the dye at 37°C for 2 hours in dark. After incubation, bacteria were harvested at 3000 g for 10 min and washed twice with PBS to remove any unbound dye. Finally, labelled bacteria were suspended in PBS for further use.

PMA stimulated THP-1 cells were infected with DiLC18-labelled bacteria as described above. The cells were harvested at day 1, day 3 and day 5 of infection. The cells were washed with the FACS wash buffer and were fixed using 2 % para-formaldehyde. The cells were then acquired on FACS calibur (BD Biosciences) and analysed using the cellQuest Pro software.
Materials and Methods

5.3.11 Enrichment of dendritic cell precursors from the mouse bone marrow

5.3.11.1 Isolation of Bone Marrow cells

Female Balb/c mice, 4-6 weeks of age, were sacrificed by either putting them in a chloroform chamber or by cervical dislocation. Mice were wiped with alcohol to sterilize them. The body wall was cut and mice were pinned on to a dissection board. The tibia and femur bones were carefully dissected out and put into a petri-dish containing HBSS media. With the help of forceps, bones were teased to remove all the attached tissue. A sharp cut was made at either end of the bones. Following this, 10-15 ml of HBSS was flushed through the bones with a 27-gauge needle to take out the bone marrow into the media. A homogenous suspension of cells was prepared by passing the media containing bone marrow cells through an 18-gauge needle. This suspension of cells was then centrifuged at 700 g for 10 min. After decanting the supernatant, cell pellet was re-suspended in RBC lysis buffer (0.9% Ammonium chloride, 10 mM Tris-Chloride - pH 7.5; 5 ml for 2 mice) for 3-4 min. Thereafter, HBSS was added (four times the volume of RBC lysis buffer used) to dilute the cell suspension in RBC lysis buffer and the cell suspension was centrifuged at 700 g for 10 min. The supernatant was discarded and cell pellet was washed twice with the same volume of HBSS. The cell pellet was then suspended in 1 ml of chilled RPMI and passed through a sterile nylon mesh filter (Miltenyi Biotech, Germany) to remove the tissue pieces.

5.3.11.2 Negative selection of leukocyte precursors from bone marrow cells by MACS®

Leukocyte precursors in the bone marrow were enriched by negative selection using Magnet Assisted Cell Sorting (MACS®).

Principle of MACS® Technology

MACS® Technology is based on magnetic labeling of cells with the help of MACS microbeads conjugated to the relevant specific antibody. MACS microbeads are super paramagnetic particles of 50 nm in diameter, composed of biodegradable matrix. The magnetically labeled cells are separated over a MACS column placed in a MACS separator, a strong permanent magnet. When MACS column is placed in a MACS
separator and a labeled cell suspension is passed through it, the MACS column matrix is provided a magnetic field strong enough to retain cells labeled with minimal amounts of magnetic microbeads. The unlabeled cells pass through the column and can be collected as the unlabeled fraction while the retained cells are eluted from the MACS column after removal from the magnet.

The desired cell population can be purified from a heterogenous cell suspension in two ways:

**Positive Selection:** Cells of interest are magnetically labeled with MACS microbeads. The heterogeneous cell population containing the cells of interest (labelled with microbeads) is passed through a MACS column placed in a MACS separator. The flow-through fraction contains the unlabeled cells as the negative fraction depleted of labeled cells. The column is removed from the separator and the retained cells are eluted as the enriched positively selected fraction.

**Negative Selection:** In this, non-target cells are magnetically labeled with MACS microbeads. Undesired cells are retained in a MACS column placed in a MACS separator. The target cells pass through the column as the enriched, unlabelled fraction, depleted of non-target magnetically labelled cells.

To enrich the leukocyte precursors from the bone marrow cells, microbeads conjugated to anti-CD90, anti-CD45R (B220), anti-CD19, anti-MHC class II were added to the cell suspension of bone marrow cells obtained. These beads were added to remove T lymphocytes, B lymphocytes, and all antigen presenting (MHC class II positive) cells from the bone marrow cells. Bone marrow cells together with the microbeads were incubated at 4°C on a nutator. After 30 min of incubation, the cell suspension was passed through a pre-wet MACS column (MS column, Miltenyi Biotech). The flow-through contained lymphocyte and I-A depleted leukocyte precursors, which were then washed once with HBSS.
5.3.11.3 **Generation of Dendritic cells (DCs) from leukocyte precursors**

Leukocyte precursor cells, negatively selected from the bone marrow cells, were counted using haemocytometer, after diluting cells with trypan blue dye (1:10). Cells were cultured at a density of 1.5 - 2 x 10⁶/ml in RPMI 1640 medium containing 10% FCS, 0.05 M 2-mercaptoethanol, 1 mM sodium pyruvate with 15 ng/ml of GM-CSF (R&D Systems, Minneapolis, MN) for 3 days in a 6-well cell culture plate. It has been shown previously in our lab that this method gives a homogenous population of cells that is 99% DCs with negligible contaminating monocytes or macrophages (Latchumanan *et al*., 2002). These DCs differentiated in the presence of GM-CSF are also referred as GM-CSF DCs.

5.3.12 **Enrichment of mouse peritoneal macrophages**

Female Balb/c mice, 4-6 weeks of age, were euthanized by carbon dioxide narcosis. They were pinned to dissection board and body wall was wiped with alcohol. A sharp cut was made in the body wall carefully so as not to cut the peritoneum present beneath the skin layer. 6-8 ml of RPMI 1640 media was injected into the peritoneal cavity using a 26 gauge sterile needle. The media containing mouse peritoneal macrophages was drained back carefully from the peritoneal cavity using a 18 gauge needle. The cells were pelleted and washed once with RPMI. Enriched macrophages were cultured in RPMI 1640 medium containing 10% FCS, 0.05 M 2-mercaptoethanol and 1 mM sodium pyruvate and used for further experiments.

5.3.13 **Enrichment of T lymphocytes from H37Rv infected mice**

Mice were infected intravenously with 3 - 4 x 10⁶ H37Rv/mouse for two weeks. Mice infected with H37Rv were sacrificed by cervical dislocation and dissected to remove the spleen. The spleen was kept in HBSS media in a petridish. It was cut into small pieces using fine forceps and then crushed between the sterile frosted slides to form the homogenate. The spleen homogenate was then centrifuged at 1000 g for 10 min. The pellet obtained was suspended in RBC lysis buffer to remove RBCs and incubated at room temperature for 5 min followed by a spin at 1000 g for 10 min. The cell pellet was washed twice with HBSS. It was then suspended in 5 ml of RPMI and passed through a
sterile nylon mesh filter (Miltenyi Biotech, Germany) to remove the tissue pieces. The cell suspension obtained was first depleted of adherent cells by two rounds of panning over plastic plates (5 ml of cell suspension/plate in RPMI medium containing 20% FCS) for 1 hr at 37°C in a humidified incubator. The top layer containing non-adherent cells was collected from the plates while adherent cells on the plate were discarded. Splenic T cells from the non-adherent cell population were enriched using MACS. Non-adherent cell suspension was incubated with mouse anti-CD11c, anti-CD11b, anti-I-A^d, anti-CD19 and anti-CD45R coated magnetic beads for 30 min at 4°C on a nutator to remove contaminating DCs, macrophages, MHC-II^+ cells and B lymphocytes, respectively followed by separation through MACS columns. The purity of the resulting negatively selected population of T cells obtained in the flow-through was 95-98% as determined by the surface staining with CD90. The percentage of I-A^+ cells was less than 0.05%.

5.3.14 Infection of mice with *M. tb* H37Rv and enrichment of *M. tb* Antigens

Groups of naïve mice (n = 5/group) were infected intravenously with 4 x 10^6 *M. tb* H37Rv/mouse via the tail vein. One group of mice was sacrificed 24 hour later and lung homogenates were plated onto 7H11 agar plates (supplemented with OADC) to confirm infection. One group of mice was sacrificed at day 3 and another group was sacrificed at day 15 of infection. At both day 3 and day 15 of infection, lungs were homogenized in the presence of RNAprotect® reagent (Qiagen). Total RNA from the lung homogenate was isolated using Trizol reagent (Invitrogen) as per manufacturer’s instructions and subjected to RT-PCR for the detection of day-1 and day-5 antigens at both times post-infection.

5.3.15 Transfection of cells

DCs were transfected with 10 μg (for 5 x 10^6 cells) of pFLAG-CMV-6a (vector control) or pFLAG-CMV-6a expressing individual genes by electroporation using Gene Pulser II (Bio-Rad). Electroporation conditions that were used for transfection were standardized at 0.25 kV, 960 μF for a pulse wave of 40 msec. Prior to the transfection, the cells were washed with plain RPMI (without FCS) containing 10 mM glucose to remove all traces
of fetal calf serum as presence of serum in the cells reduces transfection efficiency. Cells were suspended in plain RPMI medium with 10 mM glucose and the high purity plasmid DNA (10 µg for 5 x 10^6 cells; prepared using Plasmid Midi Kit) was added to cells. 1% DMSO was added to the suspended cells just prior to electroporation. Following electroporation, cells were transferred to 6-well tissue culture plates containing appropriate volume of RPMI with 10% FCS and incubated for 36 hour for the optimum expression of genes.

For standardization of optimum plasmid concentration and cell number ratio as well as the optimum time of transfection, cells were transfected with plasmid expressing Green Fluorescent Protein (GFP) at different concentrations and for different time periods (12 hour to 48 hour). Subsequently, GFP expression was monitored by flow cytometry. It was observed that transfecting the cells for 36 hours gave maximal expression of GFP and hence all transfections were carried out for this time period.

PMA stimulated THP-1 cells or peritoneal macrophages were transfected with 10 µg (for 5 x 10^6 cells) of pFLAG-CMV-6a (vector control) or pFLAG-CMV-6a expressing individual genes by using Lipofectin® transfection reagent (Invitrogen life technologies, NY, USA) as per manufacturer’s instructions.

5.3.16 Processing of cells

Following transfection for 36 hours, cells (DCs or PMA stimulated THP-1 cells or peritoneal macrophages) were infected with 1 MOI H37Rv for 48 hours. Modulations in the surface densities of key activation markers such as co-stimulatory molecules, MHC molecules and cytokine receptors were investigated using flow cytometry. Cells were stained for the expression of surface markers such as MHC class I and class II molecules, CD80, CD86, CD40, CD54, IL-12R, IL-10R, IFN-γR as described in the section 5.3.17. The stained cells were acquired using FACS Calibur (Beckton & Dickinson). The data were analyzed using CellQuest Pro software.
The supernatants collected after the infection of these transfected cells were monitored for the presence of various cytokines using sandwich ELISA.

For the analysis of different signaling molecules involved, gene transfected DCs or THP-1 cells or mouse peritoneal macrophages were stimulated with the TLR-2 ligand, Pam3Csk4, for various times and cytoplasmic extracts were prepared at the indicated time points as described in the section 5.3.18.1. These extracts were then western blotted and probed for molecules such as SOCS1, iNOS2 and GAPDH (used as a loading control). The culture supernatants from these experiments were again used for monitoring cytokine levels.

For detection of NO levels, mouse peritoneal macrophages or PMA stimulated THP-1 cells were transfected with day 1 or day 5 antigens and stimulated for 24 hours with 1 μg/ml LPS. NO levels were detected in culture supernatants using Griess Reagent system as per manufacturer’s guidelines. (Promega).

In parallel, for experiments involving recombinant proteins, four antigens (Two day 1 antigens and two day 5 antigens) were expressed in E. coli as His-tag recombinant proteins as described in the section 5.3.23. The cells were stimulated by the exogenous addition of these proteins followed by H37Rv infection/TLR-2 stimulation as done in case of cells transfected with antigens. Further experiments involving modulations in the activation of surface markers on DCs and macrophages, cytokine profiles in DCs, T-cell response experiments and SOCS1 as well as iNOS2 levels were monitored as described in other sections.

5.3.17 Analyses of cell surface markers by FACS
Approximately 0.5-1 x 10^6 dendritic cells/PMA differentiated THP-1 cells were harvested at 700 g for 10 min and the culture supernatant was discarded. All the further steps were carried out at 4°C and in dark. The cell pellet was washed once with FACS wash buffer and then suspended in 100 μl of wash buffer containing either a respective primary antibody directly conjugated to a fluorochrome such as Fluorescein
isothiocyanate (FITC) or Phycoerythrin (PE); or a biotin conjugated primary antibody at a dilution of 1:1000 or as recommended by manufacturer. The cells were then incubated on ice for 30 min and washed twice with 1 ml of FACS wash buffer. In case of fluorochrome tagged primary antibody, cells were fixed with 300 µl of fixing buffer for 15 min followed by washing with wash buffer and acquired on FACS Calibur (BD Biosciences). In case of biotin conjugated primary antibody, cells were further incubated for 30 min at 4°C in dark with streptavidin-PE or streptavidin-FITC beads diluted 1:1000 in FACS wash buffer. Cells were washed twice with 1 ml of FACS wash buffer and fixed. They were then acquired on FACS Calibur. The data were plotted and analyzed using CellQuest software.

5.3.18 Analyses of signaling intermediates by western blotting

5.3.18.1 Preparation of cytosolic extracts

At the end of incubation of respective time point, cells (either DCs or THP-1 cells or peritoneal macrophages) were harvested at 10,000 g for 10 seconds and the supernatant was discarded. Cell pellet was chilled on ice and washed once with ice-cold PBS followed by lysis in a cytoplasmic lysis buffer (buffer containing 10 mM HEPES (pH 7.9); 10 mM KCl; 0.1 mM EDTA (pH 8.0) and 0.1 M EGTA with freshly added Sodium vanadate, 0.1 M PMSF and 2 µg/ml each of aprotinin, leupeptin and pepstatin). Approximately 100 µl of buffer was used to dissolve a cell pellet of about 3 x 10^6 cells. Cells suspended in the lysis buffer were incubated on ice for 30 min. Following incubation, 0.5% Nonidet P-40 was added to the cell suspension in lysis buffer. Cells were then vortexed briefly twice within the interval of 2-3 min. The suspension was centrifuged at 10000 g for 5 min at 4°C. The supernatant was designated as the cytosolic extract. All the reagents used and steps carried out were strictly at 4°C to prevent any denaturation of proteins in the extract.

5.3.18.2 Polyacrylamide Gel Electrophoresis of Proteins

The Polyacrylamide gel electrophoresis (PAGE) of proteins was performed in the presence of 0.1% SDS in the gels in a Mini Protean III™ apparatus (Biorad). Total
protein concentration in cytoplasmic extracts was estimated using Bradford reagent. 20 μg of protein or as required was mixed with an equal volume of 2X SDS-PAGE sample buffer (4% SDS, 0.2% Bromophenol blue, 20% glycerol, 100 mM Tris-Cl (pH 6.8), 4% β-mercaptoethanol) and denatured in a heating block at 100°C for 10 min. The boiled extracts were then separated on appropriate percentage of SDS-Polyacrylamide gel. A parallel set of samples were run simultaneously on a separate SDS-PAGE and probed for control antibodies (e.g. GAPDH) for normalizing loading errors. Following the run, gels were electro-blotted using the Bio-Rad Mini-transblot apparatus.

5.3.18.3 Western Blotting

For western blotting of proteins, mini Trans-blot Electrophoretic Cell (Bio-Rad) was used to transfer the proteins from gel onto nitrocellulose membrane (Hybond C pure, Amersham, IL). The apparatus for electro-blotting was assembled according to the manufacturer’s instructions. Electro-blotting was performed at a constant current of 150 mA for 1 hour. The membrane was subsequently immersed in blocking buffer and kept for overnight incubation at 4°C. Following blocking, blot was washed twice with the wash buffer with gentle shaking for 10 min each at room temperature. The blot was incubated with an appropriate dilution of the respective primary antibody for 2 hours at room temperature with gentle shaking. Thereafter, the blot was washed three times with wash buffer for 5 min each with shaking. After washing, the blot was incubated with horseradish peroxidase conjugated secondary antibody for 2 hours. The blot was again washed three times with wash buffer as done before. Blot was then processed using Luminol Chemiluminescence kit (Santa Cruz Technologies, USA) for the detection of the bands. The blot was exposed to Kodak Xomatic autoradiography film (eBioscience, San Diego, CA) for different times (15 sec to 3 min) till a clear profile of the specific band of interest was visible followed by developing and fixing of the exposed film in Kodak ready-made solutions available for the specific purpose.
5.3.19 Antigen specific T-cell response: Co-culture of DCs with enriched T lymphocytes

For measuring antigen specific T cell responses, 3 x 10⁶ DCs were either transfected with vector plasmid alone or plasmid expressing gene of interest or stimulated by the addition of purified protein for 36 hours. These DCs were then infected with 1 MOI H37Rv for 48 hours following which, they were co-cultured with T cells (1:1) enriched from the spleen of H37Rv infected for 48 h. Following incubation, the culture supernatants were collected and cytokines levels were monitored using ELISA.

5.3.20 Estimation of cytokines: ELISA

Culture supernatants from stimulated as well as infected DCs and mouse peritoneal macrophages were analyzed for IL-12p40, IL-17 and IL-6 levels. DC-T cell co-cultures were analyzed for IFN-γ, IL-10, IL-17 and IL-12p40 levels employing a sandwich ELISA (eBiosciences). Protocols followed were as per manufacturer’s instructions. Samples were diluted to obtain values within the linear range of the standards.

5.3.21 Measurement of intracellular Reactive Oxygen Species (ROS)

Intracellular ROS levels in DCs and macrophages were measured by flow cytometry using the redox-sensitive dye, Dichloro-dihydrofluorescein diacetate (DCFH-DH), (Vaquero et al., 2004). DCs/PMA stimulated THP-1 cells were transfected with the vector alone or with antigen expressing plasmid for 36 hours followed by infection with M. bovis BCG for 30 min, 1 hour and 2 hour at an MOI of 1. Thirty minutes prior to the end of each incubation period, approximately 1 x 10⁶ cells were incubated with 10 μM DCFH-DA dye in dark at 37°C in serum free RPMI medium. The non-fluorescent DCFH-DA readily diffuses into the cells where it is hydrolyzed to the polar derivative DCFH, which is oxidized in the presence of H₂O₂ to the highly fluorescent DCF. Cells were thoroughly and quickly washed twice with pulse spin for 10 sec and immediately acquired using FACS Calibur (BD Biosciences). The data were plotted and analyzed using CellQuest Pro software.
5.3.22 Intracellular survival of *M. tb* H37Rv in DCs and macrophages

Antigen transfected DCs/PMA stimulated THP-1 cells were infected with 1 MOI of *M. tb* H37Rv for 48 hours. Extracellular bacteria were removed by treatment with gentamycin (100 µg/ml) for 2 hours after infection. Infected cells were then scraped and harvested. The supernatant was removed and the pellet was resuspended in PBS for washing. Cells were again pelleted down and were resuspended in PBS (100 µl PBS/ 3 x 10^6 cells). 1% NP-40 was added to the cells for the lysis following which they were vortexed for 2 min after every 5 min. This was done three times to ensure the complete lysis of host cells. This lysed solution containing intracellular mycobacteria was serially diluted and plated onto 7H11 agar culture plates. Three-four weeks later, plates were scored for Colony Forming Units (CFU).

5.3.23 Recombinant expression of proteins in *E. coli*

Rv1483, Rv3416 and Rv0353 were cloned in pQE31 (Qiagen) vector, while Rv2463 was cloned in pET28b (Novagen, Madison, WI) as described in sections 5.3.8 and 5.3.9 and expressed as His-tagged recombinant proteins in *E. coli* following standard procedures. The expression of Rv1483 was observed in the soluble fraction, while expression of the other three proteins was observed as inclusion bodies. Proteins expressed as soluble fraction and inclusion bodies were purified by batch method with Nickel affinity column under native and denaturing conditions respectively. Protein concentration was estimated by both Bradford and SDS-PAGE.

5.3.23.1 Expression and Purification of recombinant Rv1483 by nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography

A glycerol stock of Rv1483 cloned in pQE31 bacterial expression vector (Qiagen) in *E. coli* M15 [pREP4] strain was used to inoculate 15 ml LB broth medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and incubated at 37°C at 200 rpm for 16 hours. 1 L of freshly prepared and autoclaved LB broth (containing 100 µg/ml Ampicillin and 25 µg/ml Kanamycin) was inoculated with 1% primary culture and induced at O.D_{600}= 0.5 with 1 mM IPTG for 5 hours at 37°C at 200 rpm. Test expression cultures revealed
that the protein was expressed as soluble fraction as well as in inclusion bodies. For the ease of protein purification and use in cell culture, protein was purified from the soluble fraction. Induced cells were harvested by centrifugation at 3000 g for 20 minutes at 4°C (SORVALL centrifuge, SLA-600 rotor). Cell pellet was resuspended in lysis buffer (5 ml/g of fresh weight) with freshly added 1 mg/ml of lysozyme and 1.7 mg/mL of PMSF and incubated on ice for 30 min. The cell suspension was then sonicated at the rate of five bursts of 30 seconds each at amplitude of 40% (Vibra-Cell SONICS Newtown, CT, USA) till the clear lysate appeared. The cell lysate was then centrifuged for 15 min at 10,000 g, 4°C to pellet the cell debris. The supernatant was collected and mixed with equilibrated Ni-NTA beads (1 ml of 50% Ni-NTA slurry/3 ml of cell lysate). All subsequent purification steps were carried out at 4°C. This supernatant with Ni-NTA beads was incubated overnight at 4°C on a nutator. The lysate-Ni-NTA mixture was loaded onto a small purification column (20 ml volume) (Bio-Rad), washed with 5 mL of wash buffer (Qiagen) containing 20 mM imidazole. At the end, the protein was eluted with five column volumes of elution buffer containing 250 mM imidazole, with a maximum flow rate of 1 ml/minute. The elutions were collected in 0.5 ml fractions. Samples from all the collected fractions were analyzed on 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 followed by destaining with solution containing 40% methanol and 10% acetic acid. The fractions containing a single band at 25.6 kDa representing the protein of interest with minimal contamination with any other protein were pooled. Imidazole was removed from the pooled fractions by dialyzing the protein against 10 mM NaH₂PO₄ buffer (pH 8.0) using a dialysis membrane at 4°C overnight. The protein was then stored frozen in small aliquots at −20°C until use. The concentration of the purified protein was estimated by Bradford’s method using Bovine Serum Albumin (BSA) as a standard.

5.3.23.2 Expression and Purification of recombinant Rv0353 by nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography

A glycerol stock of Rv0353 cloned in pQE31 bacterial expression vector (Qiagen) in E. coli M15 [pREP4] strain was used to inoculate 15 ml LB broth medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and incubated at 37°C at 200 rpm for 16 hours.
1 litre of freshly prepared and autoclaved LB broth (containing 100 μg/ml ampicillin and 25 μg/ml kanamycin) was inoculated with 1% primary culture and induced at O.D_{600}= 1.0 with 1 mM IPTG for 5 hours at 37°C at 200 rpm.

Test expression cultures revealed that majority of the expressed protein fractionates into inclusion bodies and a negligible amount was observed into the soluble fraction. Hence Rv0353 was purified from the inclusion bodies under denaturation conditions and then refolded. The induced culture was harvested by centrifugation at 3000 g for 20 minutes at 4°C. Cell pellet was resuspended in lysis buffer (5 ml/g of fresh weight) together with 1 mg/ml of lysozyme and 1.7 mg/mL of PMSF and incubated on ice for 30 min. The cell suspension was then sonicated at the pulse rate of 10 seconds each for 20 min at amplitude of 40% (Vibra-Cell SONICS Newtown, CT, USA). The lysate was centrifuged for 15 min at 10,000 g at 4°C. The supernatant was discarded and pellet was solubilised using Buffer B (Qiagen) (4 ml of Buffer B/gram of pellet) containing 8 M Urea. The resuspended pellet was incubated at room temperature on a nutator for 16 hours. The suspension was then centrifuged at 10,000 g for 30 min at room temperature. The supernatant was taken and allowed to bind to 50% Ni-NTA slurry in the ratio of 4:1 (Lysate: resin) at room temperature for 2 hours with gentle mixing. The lysate-resin slurry was then loaded onto the 20 ml column (Biorad) and flow-through was collected. The column was washed successively with 6 column volumes (volume of the resin) of Buffer C (Qiagen). The elution was done using 0.5 column volumes of Buffer D and the fractions were collected. The fractions were run on a 12% SDS-PAGE and the pure fractions were pooled together. From the pooled fractions, urea was removed by dialyzing against 10 mM NaH₂PO₄ buffer (pH 8.0) using a dialysis membrane. The stepwise dialysis was carried out using the buffers containing gradually reducing concentrations of urea (7 M to 0.5 M urea and further with no urea). The dialysed protein was stored in small aliquots -20°C until use.
5.3.23.3 Expression and Purification of recombinant Rv3416 by nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography

A glycerol stock of Rv3416 cloned in pQE31 bacterial expression vector (Qiagen) in E. coli M15 [pREP4] strain was used to inoculate 15 ml LB broth medium containing 100 µg/ml Ampicillin and 25 µg/ml Kanamycin and incubated at 37°C at 200 rpm for 16 hours. 1 litre of freshly prepared and autoclaved LB broth (containing 100 µg/ml Ampicillin and 25 µg/ml Kanamycin) was inoculated with 1% primary culture and induced at O.D 600 = 1.0 with 1 mM IPTG for 5 hours at 37°C at 200 rpm.

Test expression cultures revealed that majority of the expressed protein fractionates into inclusion bodies and a negligible amount was observed into the soluble fraction. Hence Rv3416 was purified from the inclusion bodies under denaturation conditions followed by refolding. The induced culture was harvested by centrifugation at 3000 g for 20 minutes at 4°C. Cell pellet was resuspended in lysis buffer (5 ml/g of fresh weight) together with 1 mg/ml of lysozyme and 1.7 mg/mL of PMSF and incubated on ice for 30 min. The cell suspension was then sonicated at the pulse rate of 10 seconds each for 20 min at amplitude of 40% (Vibra-Cell SONICS Newtown, CT, USA). The lysate was centrifuged for 15 min at 10,000 g at 4°C. The supernatant was discarded and the pellet was solubilised using modified Buffer B (4 ml of Buffer B/gram of pellet). The resuspended pellet was incubated at room temperature on a nutator for 16 hours. The suspension was then centrifuged at 10,000 g for 30 min at room temperature. The supernatant was taken and allowed to bind to 50% Ni-NTA resin in the ratio of 4:1 (Lysate: resin) at room temperature for 2 hours with gentle mixing. The lysate-resin slurry was then loaded onto the 20 ml column (Biorad) and flow-through was collected. The column was washed successively with 6 column volumes (volume of the resin) of modified Buffer C. The elution was done using 0.5 column volumes of modified Buffer D and the fractions were collected. The fractions were run on a 12% SDS-PAGE and the pure fractions were pooled together. From the pooled fractions, urea was removed by dialyzing against 10 mM NaH₂PO₄ buffer (pH 8.0) using a dialysis membrane. The stepwise dialysis was carried out using the buffers containing gradually reducing
concentrations of urea (7 M to 0.5 M urea and further with no urea). The dialysed protein was stored in small aliquots -20°C until use.

5.3.23.4 Expression and Purification of recombinant Rv2463 by nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography

A glycerol stock of Rv2463 cloned in pET28b (Novagen, Madison, WI) bacterial expression vector in *E. coli* BL21(DE3) was used to inoculate 15 ml LB broth medium containing 25 μg/ml kanamycin and incubated at 37°C at 200 rpm for 16 hours. 1 litre of freshly prepared and autoclaved LB broth (25 μg/ml Kanamycin) was inoculated with 1% primary culture and induced at O.D₆₀₀ = 1.0 with 1 mM IPTG for 5 hours at 37°C at 200 rpm. Test expression cultures revealed that majority of the expressed protein fractionates into inclusion bodies and a negligible amount was observed into the soluble fraction. Hence Rv2463 was purified from the inclusion bodies under denaturation conditions and refolded using protocol as described for the purification of Rv3416 from inclusion bodies. The eluted fractions were run on a 10% SDS-PAGE and the pure fractions were pooled together. From the pooled fractions, urea was removed by dialyzing against 10 mM NaH₂PO₄ buffer (pH 8.0) containing 5 mM β-mercaptoethanol using a dialysis membrane with a cut-off value of 2 kDa. The stepwise dialysis was carried out using the buffers containing gradually reducing concentrations of urea (7 M to 0.5 M urea and finally with no urea). The dialysed protein was stored in small aliquots -20°C.

The composition and preparation of the buffers used during protein purification from native as well as denaturing conditions are mentioned in the Appendix I.

5.3.24 Statistics

Student’s t test was carried out for all experiments. P < 0.05 for different groups was considered as significant.
Table 5.3: Primer pairs and PCR conditions used for the cloning of genes in pFLAG-CMV-6a vector.

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<th>PRODUCT SIZE</th>
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Table 5.4: Primer pairs and conditions used in the cloning of genes to express the desired proteins as His-tag proteins.

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