Materials and methods

2.1: Bacterial strains and plasmids transformation

The *Escherichia coli* strains DH5α (Invitrogen, Carlsbad, CA, USA) and XL-1 Blue (Stratagene, Santa Clara, CA, USA) were used for propagation and construction of all plasmid constructs. For each transformation, 10-50 ng of DNA was added to 100 µl of chemically competent cells [363] and incubated on ice for 20 minutes, followed by heat shock at 42°C for 90 seconds and incubation on ice for 2 minutes. The cells were allowed to recover in 1 ml Luria-Bertani broth (LB broth: 1% Bacto-Tryptone, 1% NaCl and 0.5% Bacto-Yeast extract) or SOC (2% Bacto-Tryptone, 0.5% Bacto-Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl2, 20mM glucose) broth and then incubated for 45 minutes at 37°C with shaking (200-250 rpm). Cells were plated on LB-agar plate containing appropriate antibiotics and incubated at 37°C overnight to select the transformants.

2.2: Isolation of plasmid DNA from *E. coli*

Large-scale plasmid DNA isolation were carried out using QIAGEN Midi Kit (QIAGEN) as per the manufacturer’s protocol or by standard alkaline lysis method [363]. The cells were first re-suspended in solution P1 (50 mM glucose, 25 mM Tris-HC1 (pH 8.0), 10 mM EDTA [pH 8.0]) and then lysed with solution P2 (1% SDS, 0.2 N NaOH); SDS breaks apart the lipid membranes and
solubilizes cellular proteins, NaOH denatures DNA (both plasmid and genomic). Solution P3 (3.0 M potassium acetate, pH 4.8) was then added to neutralize the acidic pH. The chromosomal DNA renatures as single stranded DNA, gets entangled and is trapped in the precipitate while plasmid DNA remains in solution. Potassium acetate precipitates SDS from the solution as KDS, along with the cellular debris. The precipitated cellular debris and genomic DNA is removed by centrifugation while plasmid DNA remains in solution. Plasmid DNA in the supernatant is precipitated using isopropanol. The precipitate containing the plasmid DNA is pelleted down by high speed centrifugation and washed with 70% ethanol to remove excess salts and re-suspended in dH$_2$O.

2.3: Polymerase chain reaction (PCR)

The general PCR reaction mixture has the following components:

DNA template (10 ng for plasmid/genomic DNA template, 50 ng for crude yeast plasmid), 10 µl of 10x PCR buffer, 2 µl of 10 pM forward primer, 2 µl of 10 pM reverse primer, 2 µl of 10 mM dNTP mixture, 8 µl of DMSO (only when *M. tuberculosis* genomic DNA is used as template), 1 µl of DNA polymerase (Taq DNA polymerase Sigma, St. Louis, USA), dH$_2$O to 100 µl

The PCR reaction was performed with the following cycling parameters:

One cycle of 1 minutes initial denaturation of template DNA at 95°C, 30 cycles of a 30 second template denaturation at 95°C before each extension step, 30 seconds at the annealing temperature, and an extension step at 72°C with time
calculated depending on length of amplification product (1 minute for each kb of DNA to amplify) and a final extension of 10 minutes at 72°C.

2.4: Restriction enzyme digestion of DNA

The analytical DNA digestions were performed in a final volume of 20-50 µl, at 37°C for 8 hours with the restriction enzymes and buffers procured from New England Biolabs (NEB, Massachusetts, USA). Each analytical digestion used 2 µg plasmid DNA, with 10 units of enzyme per 50 µl of reaction mixture. Bovine serum albumin (BSA) is used in the reaction mixture when recommended.

2.5: Agarose gel electrophoresis

Agarose gels (1% w/v in 1XTAE [40 mM Tris-acetate pH 7.5, 2 mM EDTA]) were prepared and supplemented with ethidium bromide (1 µg/ml). The percentage of agarose in the gels was determined depending on the size of the DNA fragments to be resolved. Gels were generally run at 120 V in 1X TAE buffer, and the DNA was visualized on a UV trans-illuminator.

2.6: DNA extraction from agarose gel

Following agarose gel electrophoresis, the DNA-containing gel slices were excised under UV light. The DNA was extracted from these gel slices using
Qiaquick Gel Extraction Kit (QIAGEN), following the protocol provided by the manufacturer.

2.7: Ligation reaction

The ligation reactions were generally carried out as follows:

Molar ratio of restriction enzyme digested vector DNA: digested insert DNA = 1: 2-5, 1 µl 10X ligation buffer (NEB), 1 µl T4 DNA Ligase (400 U/µl) (NEB), dH₂O to 10 µl

Ligation was carried out for 12 hours at 16°C and the whole of the reaction mixture was used for transformation of chemically competent *E. coli* cells.

2.8: Yeast two-hybrid screening

Yeast two-hybrid principle is a technique developed by Stanley Fields [364] to identify protein-protein interaction. This technique is based on the modular structure of eukaryotic transcription factors, which consist of a DNA-binding domain (DBD) and a transactivation domain (AD) separated by a linker. The DBD recognizes and binds to a specific sequence in the promoter region of a gene, and the AD interacts with the RNA polymerase II enzyme (RNA pol II) to initiate gene transcription. Neither of these sub-domains alone can induce transcription. In yeast two-hybrid system, both the DBD and AD of a yeast transcription factor (e.g. GAL4) are tethered to a protein of interest (Pr1 and Pr2), resulting in two hybrid proteins (Pr1-DBD and Pr2-AD). Physical
association between these proteins Pr1 and Pr2 in the yeast cells which are genetically engineered to express both these hybrid proteins, brings the DBD and AD into proximity, thereby reconstituting the active transcription factor (Figure 2.1). The DBD of this functionally complemented transcription factor binds to its cognate recognition site on the promoter region of the reporter gene and the AD interacts with RNA polymerase II, driving transcription of the reporter genes (usually Nutritional markers). The reporter proteins so produced enables selection of yeast auxotrophic cells that harbour a pair of interacting proteins. This system has been applied to chart the first drafts of genome-wide protein networks in a broad range of organisms [365-369].

**Figure 2.1: An outline of Yeast two hybrid system to detect interaction between two proteins.** Each of the genes that code for the two proteins of interest (Pr1 and Pr2 in the figure) is fused to either the DBD or AD of the transcription factor and then the pair of hybrid gene is expressed in yeast. Interaction of Pr1 and Pr2 reconstitutes the transcription factor, and activates reporter gene transcription.
In contrast to protein interaction mapping methods based on affinity purification (GST pulldown, co-immunoprecipitation and Tandem affinity purification (TAP)), where a tagged bait protein pulls down complexes of endogenously expressed proteins, Yeast two-hybrid screening relies on exogenous expression of hybrid bait and prey proteins. The sensitivity of yeast two-hybrid system is primarily attributable to high-fold amplification of positive signals \textit{in vivo} (i.e., transcriptional, translational, and enzymatic). Because the two-hybrid assay is performed \textit{in vivo}, the proteins are more likely to be in their native conformations, which may lead to increased sensitivity and accuracy of detection. The higher sensitivity of the two-hybrid assay allows to pinpoint residues critical for protein interactions and also allows to evaluate the relative strength of the interactions of protein variants [370]. Protein interactions with dissociation constants (Kd) above \(~70\ \mu\text{M}\) can be detected using a GAL4-based yeast two-hybrid assay [370]. However, the sensitivity of yeast two hybrid assay comes at the cost of large number of false positives during screening. A number of factors like GAL4 domain coupled to the test protein pair, size and orientation of the test proteins, the length and flexibility of the linker connecting them to the GAL4 domain can hinder the association of the test proteins resulting in false negative interactions. Exogenously expressed fusion proteins may also adopt altered conformations. A mammalian protein may not correctly undergo post-translational modification in yeast (e.g., missing phosphorylation) that may either inhibit normally occurring interactions or may facilitate non-native interactions. Exogenously expressed bait and prey fusion proteins may also interact merely due to their high physical concentration in the cell. Another class
of false positives are interactions between proteins that might never be present in the same cell at the same time. The combined effect of all these errors may reduce the overall confidence of the yeast two-hybrid assay. However, the quality of yeast two-hybrid data has been shown to be equivalent to the data generated by alternative approaches like co-affinity purification followed by mass spectrometry (AP/MS) [365]. Therefore, all interactions detected by yeast two-hybrid should be confirmed by high confidence assays like GST pulldown or co-immunoprecipitation of the endogenous proteins. In general, yeast two-hybrid systems provide a sensitive method for detecting relatively weak and transient protein interactions. Though such interactions may not be biochemically detectable, but may be critical for proper functioning of complex biological systems [371, 372].

Yeast two-hybrid screening was performed according to the manufacturer’s instructions for MATCHMAKER 2 Hybrid System (Clontech Laboratories, Mountain View, California, USA). This System features the yeast strain AH109 (MATa) which has three reporters ADE2, HIS3, and MEL1 under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes (Figure 2.2). The Strain Y187 (MATα) contains a LacZ reporter gene under the control of the GAL1 UAS.
Figure 2.2: Reporter constructs in yeast strains AH109 and Y187. Strain AH109 includes the ADE2, HIS3, MEL1/LacZ reporter genes which are under the control of three completely heterologous GAL4-responsive UAS and promoter elements - GAL1, GAL2, and MEL1 respectively. Strain Y187 contains the LacZ reporter gene under the control of the GAL1 UAS.

These promoters yield strong and specific responses to GAL4. The ADE2 reporter provides a strong nutritional selection; HIS3 selection reduces the incidence of false positives and allows to control the stringency of selection [373]. MEL1 and LacZ, which encodes α-galactosidase (α-Gal) and β-galactosidase (β-Gal) respectively, can also be used as colorimetric markers. Because α-galactosidase is a secreted enzyme, it can be assayed directly by blue-white screening on X-α-Gal indicator plates, while LacZ being an intracellular enzyme can be assayed by X-Gal agarose overlay assay.
2.8.1: Yeast two-hybrid vectors

The plasmids provided in the matchmaker yeast two-hybrid kit marketed by Clontech were used in this study. The pGBK T7 plasmid encodes the DNA Binding Domain (DBD) of yeast transcriptional factor GAL4 with a Multiple cloning site (MCS) at the C-terminus of GAL4 BD and carries yeast nutritional marker that allows it to grow in synthetic media lacking tryptophan. This plasmid also confers kanamycin resistance in bacteria. The pGBK T7-53 (Clontech) encodes for murine p53 fused with GAL4 DNA-BD while pGAD T7-T (Clontech) encodes for the large T-antigen of SV40 fused with GAL4 AD. The p53 and the large T-antigen interact with each other in a yeast two-hybrid assay [47, 48] and are used as positive control in interaction mating. The pGBK T7-Lam (Clontech) encodes a fusion of the DNA-BD with human lamin C and is used as a control for fortuitous interaction between bait and unrelated proteins coded by AD/library plasmid. The pCL1 (Clontech) plasmid encodes the full-length, wild-type GAL4 protein and is used as a positive control for α-galactosidase and β-galactosidase assays.

2.8.2: Cloning of ESAT-6 in yeast two hybrid bait vector pGBK T7

The Rv3875 (ESAT-6) open reading frame (ORF) was amplified from the genomic DNA isolated from H37Rv strain of M. tuberculosis, which was kindly provided by Dr. Shekhar C. Mande, CDFD Hyderabad. The primers were designed to clone the Rv3875 ORF in frame with the GAL4
BD of the yeast two-hybrid bait vector pGBKT7. The primers were appended with Ndel and BamHI restriction enzyme sites in forward and reverse primers to facilitate cloning in the matching sites on the bait vector. The primers used were as follows:

**Forward primer**

5′ GGAATTCCATATGATGACAGAGCAGCAGTGGAATTTCG3′  (Ndel)

**Reverse primer**

5′ CGCGGATCCCTATGCGAACATCCCAGTGACGTTG 3′  (BamHI)

The Rv3875 ORF was amplified using PCR (primer annealing temperature 50°C, extension time 20 second and 35 cycles), and the amplicon (288 bp) was purified using PCR product purification kit as per the manufacturer's protocol (QIAGEN). The purified Rv3875 fragment was initially ligated into pGEM-T Easy vector (Promega, Madison, USA). The ligation mixture was then transformed into ultra-competent DH5α cells and the recombinants were selected by blue-white screening. The putative positive clones were grown over night in Luria Bertani (LB) medium containing 100 μg/ml of ampicillin (amp) at 37°C with constant shaking at 200 rpm. Plasmid DNA was isolated and screened for the presence of Rv3875-specific insert by PCR using the same set of primers that were used for initial PCR amplification. Large scale restriction digestion of pGEMT-ESAT-6 clone was carried out with Ndel and BamHI to obtain ESAT-6 fragments with cohesive ends for ligation with pGBKT7 vector digested with the same set of enzymes. The ligation mixture was transformed into DH5α competent cells and plated onto LB kanamycin plate. The positive clones were confirmed by sequencing.
2.8.3: Amplification of prey library

Human Leukocyte Matchmaker cDNA Library cloned in the yeast two hybrid prey vector pACT2 was purchased from Clontech. This library has $3.5 \times 10^6$ independent clones with an average cDNA insert size of 2.0 kb, with a size range of 0.4-4.0 kb. The premade Matchmaker Library was amplified to obtain enough plasmid for library screening in yeast. The library was plated on 300 LB amp plates (containing 100 μg/ml of ampicillin) of 150 mm size at a density of approximately 20000 colony forming units (CFU) per plate to represent three times the size of the original library. These plates were incubated at 37°C for 24 hours. For plasmid isolation, these colonies were scraped into LB medium containing ampicillin (100 μg/ml), pooled into a two litre flask and incubated at 30°C for 4 hours at 200 rpm. One third of the pooled sample was used for plasmid isolation using QIAGEN Plasmid Giga Kit following the manufacturer’s protocol. The rest was stored for further use at -80°C with glycerol added to a final concentration of 25%.

2.8.4: Yeast strain maintenance, recovery from frozen stocks, and routine culturing

All the yeast strains were stored at -80°C in yeast peptone dextrose medium (YPD) containing 20 g/L Difco peptone, 10 g/L Yeast extract, Glucose 20 g/L supplemented with 25% glycerol. Transformed yeast strains were grown on appropriate synthetic dropout (SD) medium (Clontech) to maintain the plasmid under selection pressure. To prepare glycerol stock cultures of yeast, a sterile inoculation loop was used to scrape an isolated colony from the agar plate.
Cells were re-suspended in 200-500 μl of YPD medium in a 1.5 ml micro-centrifuge tube and vortexed vigorously to thoroughly disperse the cells. Sterile 50% glycerol was added to a final concentration of 25% and mixed thoroughly before freezing at -80°C. A small portion of the frozen glycerol stock is streaked onto an YPD agar plate and incubated at 30°C until yeast colonies reach ~2 mm in diameter (3–5 days) and was used as working stock. Fresh working plates were prepared from the frozen stock every 1–2 month intervals. To prepare liquid overnight cultures, fresh (< 1 month old) colonies were used from the working stock plate. One large colony was used (2–3 mm diameter) per 5 ml of medium. Cells in the medium were dispersed by vigorously vortexing the suspension for ~1 minute and subsequently incubating at 30°C for 16-18 hours with shaking at 230-270 rpm till the absorbance at 600 nm ($A_{600}$) reaches >1.5. For mid-log phase culture, the overnight culture was transferred into fresh medium to an $A_{600}$ of approximately 0.2-0.3 and incubated at 30°C for about 3-5 hours with shaking (230-250 rpm) till the $A_{600}$ of the culture is about 0.4-0.6.

### 2.8.5: Transformation of yeast strain

One colony (diameter 2-3 mm, < 4 weeks old) from a freshly grown plate was inoculated into 3 ml YPDA medium in a sterile 15 ml culture tube and incubated at 30°C with shaking at 250 rpm for 16-20 hours. 5 μl of this culture was added to 50 ml of YPDA in a 250 ml flask which was then incubated with shaking (200 rpm) at 30°C until $A_{600}$ reaches 0.15-0.3 (16-20 h). The cells were then pelleted by centrifugation at 700g for 5 minutes at room temperature and re-suspended
in 100 ml of fresh YPDA and incubated at 30°C with shaking till $A_{600}$ reaches 0.4-0.5. The yeast cells were collected by centrifugation at 700g for 5 minutes at room temperature, the supernatant was discarded and the cell pellet was re-suspended in 30 ml of sterile deionized H$_2$O. These cells were again pelleted down by centrifugation at 700g for 5 minutes at room temperature and re-suspended in 3 ml of 1.1X TE/LiAc (made by mixing 1.1 ml of 10X TE Buffer with 1.1 ml of 1 M LiAc (10X) and made the volume upto 10 ml using sterile deionized H$_2$O). This cell suspension was pelleted down in two 1.5 ml eppendorf tubes and each pellet was re-suspended in 600 μl of 1.1xTE/LiAc.

For transformation, plasmid DNA (100 ng for small scale, 15 μg for large scale) was combined with salmon sperm carrier DNA (50 μg for small scale and 200 μg for large scale) in a pre-chilled, sterile tube (1.5 ml for small scale and 15 ml for library scale). The competent yeast cells were added to this mixture with gentle flicking (50 μl for small scale and 600 μl for large scale) followed by addition of PEG/LiAc (PEG 3350 40%, TE buffer 1X and LiAc 0.1 M) to this mixture (500 μl for small scale and 2.5 ml for large scale) and incubated at 30°C for 45 minutes. After the incubation, sterile DMSO was added to this mixture (20 μl for small scale and 160 μl for large scale) and the tubes were placed in a 42°C water bath for 20 minutes. The transformed cells were collected by centrifugation at 700g for 5 minutes and the pellet was re-suspend in 2X YPDA and further incubated at 30°C with shaking for 90 minutes. The cells were then pelleted by centrifugation at 700g for 5 minutes and re-suspended in 0.9% (w/v) NaCl solution (1 ml for small scale and 15 ml for large scale). Appropriate dilutions of the cell suspensions were plated on synthetic defined (SD) selection
plates to select for transformants. For library scale transformation, the transformation mixture was plated on hundred SD/-Leu plates (150 mm). After five days of growth, colonies were scraped in 5 ml of 1X YPDA and pooled. The pooled samples were pelleted and re-suspended in 30 ml of freezing solution (Glycerol 65%, MgSO$_4$ 100 mM and Tris-HCl 25 mM). This re-suspended pellet was aliquoted in such a that each aliquot contains approximately three times the total number of independent clones in the library and stored in liquid nitrogen till further use.

2.8.6: Test for auto-activation of yeast two-hybrid reporters by bait

Auto-activation of reporter genes by the DNA-DBD and AD fusion constructs were examined by independently transforming these constructs into AH109 and screening them for interaction reporter activation. AH109-pGBKT7-ESAT-6 was plated onto SD/-Trp/X-β-Gal, SD/-Trp/-His and SD/-Trp/-Ade. The prey constructs (AH109-pACT2-prey) was plated on SD/-Leu/X-β-Gal, SD/-Leu/-His, SD/-Leu/-Ade. Growth on SD plate and colour development was an indicator of auto-activation by bait or prey constructs. Positive and negative controls were also carried out in parallel.

2.8.7: Protein extraction from yeast (Urea/SDS method)

Proper expression of bait fusion protein in AH109 transformed with bait plasmid is a prerequisite for the bait to be used in yeast two-hybrid screening. Protein
extracts made from yeast cells transformed with the bait vector were immunoblotted to detect expression of the bait fusion protein. Pelleted cells from a 5 ml culture of transformed AH109 (with the bait plasmid pGBKT7-ESAT-6) were grown in SD/-Trp media and analyzed for protein expression by immunoblotting using anti-c-Myc monoclonal antibody (Ab) (Clontech). As a negative control, 5 ml culture of untransformed yeast colony grown in YPD medium was used, The pGBKT7-p53 plasmid of the matchmaker system was used as a positive control. The cells were re-suspended in 200 μl of pre-warmed (to 60°C) complete-cracking buffer containing Urea 8 M, SDS 5% w/v, Tris-HCl [pH6.8] 40 mM, EDTA 0.1 mM, Bromophenol blue 0.4 mg/ml, β-mercaptoethanol 10%, protease inhibitor solution [Sigma-Aldrich]). Each cell suspension was then transferred into a 1.5-ml screw-cap micro-centrifuge tube containing 200 μl of glass beads. Samples were heated at 70°C for 10 minute and vortexed vigorously for 1 minute. Debris and unbroken cells were pelleted in a micro-centrifuge at 14,000 rpm for 5 minutes at 4°C and the supernatants were transferred to fresh 1.5 ml tubes and placed on ice (first supernatants). The pellet was re-suspended again in 200 μl of cracking buffer and placed in a 100°C water bath for 3-5 minutes and subsequently vortexed vigorously for 1 minutes. Debris and unbroken cells were pelleted by centrifugation at 14,000 rpm for 5 minutes at 4°C and the supernatant was combined with the first supernatant. Samples were boiled briefly in 1X loading buffer and immediately loaded onto a 10% Tricine SDS-PAGE gel and immunoblotted to detect expression of c-Myc tag contained on GAL4-ESAT-6 fusion protein.
2.8.8: Screening a cDNA library with the yeast two-hybrid system

Yeast mating is a convenient method of introducing two different plasmids into the same host cell [374, 375]. One large, freshly transformed (with the bait plasmid pGBK7-ESAT-6) colony of AH109 was inoculated into 50 ml of SD/–Trp media and grown overnight (16-24 hours) at 30°C with shaking at 250-270 rpm. When $A_{600}$ of the culture was > 0.8, the cells were pelleted down by centrifugation at 1000g for 5 minutes and the supernatant was discarded. The pellet was re-suspended at an cell density of $1 \times 10^9$ cells/ml in fresh SD/-Trp media by vortexing. Frozen library aliquot was thawed with gentle vortexing at room temperature and about 10 µl of this library sample was stored on ice for library titering. The AH109 [bait] culture was mixed with 1 ml library culture in a 2 L sterile flask so as to outnumber the prey cells in a ratio of 10:1. About 45 ml of 2X YPDA/Kan was added to this cell mixture and incubated at 30°C overnight. After 24 hours of mating, the mating mixture was pelleted down by centrifugation at 1000g for 10 minutes and the mated cell pellet was re-suspended in 10 ml of 0.5X YPDA/Kan. Hundred µl of a 1:10,000, 1:1,000, 1:100, and 1:10 dilution of the mating mixture was plated on SD/–Leu, SD/–Trp, and SD/–Leu/–Trp plates in triplicate for determining the mating efficiency. The remaining mating mixture was plated on ~50 large (150-mm) SD/–Ade/–His/–Leu/–Trp [Quadruple Dropout (QDO)] plates, at approximately $\sim 2 \times 10^4$ – $2 \times 10^5$ cells per plate as higher cell density will abrogate the selection pressure on QDO plates. The colonies that appear on these QDO plates after 8 days of incubation at 30°C were passaged about 5 times on fresh QDO plates to dilute away any non-specific library plasmid. True Ade+, His+ colonies are robust and
these colonies can grow to more than 2 mm in diameter. Glycerol stock of these colonies were made and stored in -80°C freezer for long term storage. Colonies on the QDO plates were inoculated into 5 ml of liquid SD-Trp-Leu-Ade-His media and incubated at 30°C for 24 hours with shaking at 200 rpm. The cells in this culture were used for bait plasmid isolation using the method of Robzyk & Kassir [376]. Prey cDNA was amplified by PCR using primers encompassing the cDNA insert in pACT2 and sequenced. The cDNA sequences were identified using PSI-BLASTX searches (National Center for Biotechnology Information).

2.8.9: Isolation of yeast plasmid

The method of Robzyk & Kassir [376] was used for efficient plasmid isolation from yeast clones. About 1.5 ml overnight culture of yeast (grown in SD selection media) was harvested in a micro-centrifuge at 5000 rpm for 5 minutes. This pellet was re-suspended in 100 μl of STET (8% Sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA, 5% Triton X-100), along with 0.2 g of 0.45 mm glass beads and vortexed vigorously for 5 minutes. This was followed by addition of another 100 μl of STET and vortexing for 1 minute. This tube was then placed in a boiling water bath for 3 minutes, cooled on ice for 1 minute and the cell debris pelleted down in a micro-centrifuge at 13000 rpm for 10 minutes at 4°C. About 100 μl of this supernatant was transferred to a fresh tube containing 50 μl of 7.5M ammonium acetate and incubated at -20°C for 1 hour. This was centrifuged for 10 minutes at maximum speed at 4°C to pellet down residual
chromosomal DNA, large RNA species, and other impurities which inhibit *E. coli* transformation. Then 100 µl of this supernatant was added to 200 µl of ice cold ethanol to recover DNA by centrifugation. The DNA pellet was washed with 70% ethanol, and re-suspended in 20 µl autoclaved H₂O and 10 µl of this was used to transform 100 µl of competent XL1-blue competent cells which was then plated on LB agar medium containing appropriate antibiotic.

### 2.8.10: X-Gal Agarose overlay assay

The X-Gal Agarose overlay assay was used to screen for expression of LacZ, an intracellular reporter enzyme expressed in diploid yeast strain that harbour an interacting pair of proteins from bait and prey plasmids. The agarose overlay solution is a buffered solution containing DMF and SDS to permeabilize the cells and β-mercaptoethanol is used to preserve activity of the protein. Freshly made agarose overlay solution (0.5 M Potassium Phosphate Buffer pH 7.0, 6% Dimethyl Formamide (DMF), 0.1% SDS and 5 mg/ml low-melting agarose) was boiled to dissolve the agarose and cooled to approximately 55°C, then 0.5 mg/ml X-α-Gal and 1 drop of β-mercaptoethanol (about 50 microliters per 100 ml) was added to this solution. About 8-10 ml of this warm overlay solution was used to cover the surface of each plate on which colonies had been spotted or streaked. After the overlay solution cools and solidifies, the plates were incubated at 30°C. The colour develops in few hours, depending on the strength of the inducer. A diploid yeast strain containing pGBK7-p53 and pGADT7-T (by mating AH109-pGBK7-p53 and Y187-pGADT7-T) was used as positive
control, while a yeast strain containing pGBK7-Lam and pGADT7-T (by mating AH109-pGBK7-Lam with Y187-pGADT7-T) was used as a negative control.

2.9: Purification of ESAT-6:β2M complex

For purification of ESAT-6:β2M complex, ESAT-6 was cloned in the first multiple cloning site (MCS) of the duet vector pETDuet-1 (Novagen EMD, Darmstadt, Germany) in frame with N-terminal 6X Histidine-tag (His-tag) using the following set primers;

Forward primer
5`GCCAGGATCCGATGACAGAGCAGCAGTGGAATTTCGC3` (BamHI)
Reverse primer
5`CGATAAGCTTCTATGCGAACATCCCAGTGACGTTGC3` (Hind III)

β2M was cloned in the second MCS of pETDuet-1 using the set of primers given below

Forward primer
5`TATACATATGTCTCGCTCCGTGGCCTTAGCTG3` (NdeI)
Reverse primer
5`ATTGAGATCTATGATGCTGCTTACATGTCTCGATCCCAC3` (BglII)

β2M was also cloned in the second MCS of pETDuet-1 with the first 20 amino acids (signal sequence) deleted from N-terminus of β2M using the following set of primers.
Forward primer

5’AGCTCATATGATCCAGCGTACTCCAAAGATTCAAGTTTACTC3’  (NdeI)

Reverse primer

5’ATGCTTAATTAAATGATGCTGCTTACATGTCTCGATCCCAC3’  (PacI)

The confirmed clone containing both the inserts was transformed in *E. coli* BL21 (DE3) cells and primary inoculum was prepared by scrapping colonies from a single transformed plate. The primary culture was inoculated into 800 ml of Terrific broth (TB broth) (12 g Tryptone, 24 g Yeast extract, 4 ml Glycerol, 0.17 M KH$_2$PO$_4$, 0.72 M K$_2$HPO$_4$ and the volume was made to 1000 ml with H$_2$O) containing 100 μg/ml of ampicillin. The culture was grown in a shaker incubator set at 37°C till the absorbance $A_{600}$ reaches about 0.5. Expression of recombinant protein was induced by incubating the culture for 3 hours in a shaker incubator in the presence of 1 mM isopropylthiogalactoside (IPTG). Cells were harvested by centrifugation and the pellet was re-suspended in 40 ml lysis buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 mM PMSF). The cell suspension was sonicated and the lysate centrifuged at 25000 rpm for 30 minutes. The soluble recombinant protein present in supernatant was allowed to bind 5 ml bed volume of Ni-NTA resin (QIAGEN) pre-equilibrated with lysis buffer for 45 minutes. This protein bound Ni-NTA slurry was loaded on a disposable column and was washed with 500 ml of wash buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 30 mM imidazole, 1 mM PMSF). The resin bound proteins were eluted using elution buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 M imidazole) in 1 ml fractions. Eluted protein samples were loaded and separated on a 16% Tricine SDS-PAGE gel and visualized by Coomassie or
silver staining. The eluted protein fractions were pooled and dialyzed against one litre of dialysis buffer (50 mM Tris-HCl, pH 8.0) (SnakeSkin Dialysis Tubing, cut-off 10000 MW from Thermo Scientific, Illinois, USA), the dialysis buffer was changed two times every one hour with a final buffer change in which the dialysis was confirmed overnight. The concentration of the purified protein was estimated using Micro BCA Protein Assay Kit (Thermo Scientific) following the manufacturer’s protocol. The dialyzed protein samples were concentrated using Amicon Ultra 10000 MW cut-off protein concentrator units (Millipore, Massachusetts, USA).

2.10: Glutaraldehyde cross-linking to confirm protein-protein interaction

Two proteins which physically interact can be covalently cross-linked by a variety of cross-linkers. The formation of cross-links between two distinct proteins is a direct and convincing evidence of their close physical proximity. Glutaraldehyde is one of the commonly used cross-linking reagents. Cross-linking action of glutaraldehyde is characterized by its reaction with ε-amino groups of lysine residues. For Glutaraldehyde cross-linking, 100 µg of interacting proteins were treated with 5 µl of 2% freshly prepared Glutaraldehyde solution for different time-points at 37°C in 100 µl of phosphate buffer, pH 8.0. The cross-linking reaction was terminated by addition of 10 µl of 1 M Tris-HCl, pH 8.0. Cross-linked proteins were solubilized by addition of equal
volume of 2X laemmli loading buffer. Cross-linked proteins were visualized on a 16% Tricine SDS-PAGE gel.

### 2.11: Purification of His-tagged ESAT-6 protein under denaturing conditions

ESAT-6 was cloned with a N-terminal 6X His-tag in the of pET23a (Novagen) using the following set of primers

**Forward primer**

5′GGAATTCCATATGCATCACCATCATCATCATCATGACAGAGCAGCAGTGGAATTTCGCGG3′ (NdeI)

**Reverse primer**

5′CCCAAGCTTCTATGCGAACATCCCAGTGACGTTGCCTTC3′ (HindIII)

His-tagged ESAT-6 cloned in pET23a was transformed in *E. coli* BL21 (DE3) cells and plated on LB-amp plates. The colonies on this plate were scrapped out in LB media containing ampicillin and left on shaker incubator at 37°C for 2 hours. This primary culture was inoculated into 800 ml Terrific Broth containing 100 μg/ml of ampicillin at 37°C till the A₆₀₀ reaches about 0.5. Expression of ESAT-6 was induced by adding IPTG to a final concentration of 1 mM and left on shaker incubator at 37°C for another 4 hours. Cells were harvested by centrifugation and the cell pellet was re-suspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1 mM PMSF). This cell suspension was sonicated and the lysate was centrifuged at 12000 rpm for 30 minutes, the supernatant
was discarded and the pellet (inclusion body containing ESAT-6) was used for ESAT-6 purification. The pellet was washed three times in Triton X-100 containing lysis buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1% Triton X-100) and again washed 3X times in lysis buffer alone. The washed inclusion body pellet was re-suspended in 20 ml of denaturation buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 8M urea) and sonicated to solubilise inclusion bodies and then centrifuged at 25000 rpm for 45 minutes. The supernatant was added to 5 ml of Ni-NTA (QIAGEN) beads pre-equilibrated with denaturing buffer and incubated for 45 minutes with gentle mixing. The slurry was loaded onto a disposable column and the bound protein was renatured back in column by sequentially washing with buffers having decreasing concentration of urea. The beads are first washed with 100 ml of 6M urea wash buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 6M urea), followed by 25 ml 5 M urea buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 4 M urea), then 25 ml 4M urea buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 4 M urea), followed by 25 ml 3 M urea buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 3M urea), and then with 25 ml 2 M urea buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 2 M urea), and next with 25 ml 1 M urea buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1 M urea), and a final wash with 100 ml of native buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl and 20 mM imidazole).The refolded protein is eluted out in elution buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl and 1 M imidazole) as 1 ml fractions. Eluted samples were loaded onto a 16% Tricine SDS-PAGE gel to determine the purity and to confirm the presence of the recombinant protein. The eluted protein fractions were pooled and dialyzed against one litre of dialysis buffer (50
mM Tris-HCl, pH 8.0) (SnakeSkin Dialysis Tubing, cut-off 3000 MW), the
dialysis buffer was changed twice every hour with a final buffer change in which
dialysis was continued overnight. The concentration of purified protein was
estimated using Micro BCA Protein Assay Kit. The dialyzed protein samples
were then concentrated using Amicon Ultra 3000 MW cut-off protein
concentrator units (Millipore) to a concentration not more than 500 ng/μl, as
ESAT-6 tend to precipitate out of solution at high concentrations. The
concentrated protein was aliquoted and stored in liquid Nitrogen till further use.

2.12: Purification of His-tagged mutant ESAT-6 protein

Mutant ESAT-6 with the last 6 amino-acids deleted from the C-terminal was
amplified from the M. tuberculosis genomic DNA by PCR and cloned in pET23a
(Novagen) with an N-terminal 6X His-tag using the set of primers given below,

Forward primer
5′GGAATTCCATATGCATCACCATCATCATCATGACAGAGCAGCAGTGGA
ATTTCGCGG3′ (NdeI)

Reverse primer
5′CGCGGATCCCTAGTTGCCTTCGGTCGAAGCCATTGCC3′ (BamHI)

The clones were confirmed by sequencing and transformed into E. coli BL21
(DE3) cells. Primary inoculum was prepared by scrapping colonies from a single
transformed plate and inoculated into 800 ml Terrific Broth containing 100 μg/ml
of ampicillin. The culture was grown in a shaker incubator set at 37°C till the
absorbance $A_{600}$ reached about 0.5. Expression of recombinant protein was induced by incubating the culture in the presence of 1 mM IPTG for 3 hours in a shaker incubator at 37°C. Cells were harvested by centrifugation and re-suspended in 40 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 mM PMSF), sonicated and the lysate was centrifuged at 25000 rpm for 30 minutes. The soluble recombinant protein present in the supernatant was allowed to bind to 5 ml bed volume of Ni-NTA resin (QIAGEN) which was pre-equilibrated with lysis buffer for 45 minutes. This protein bound Ni-NTA slurry was loaded on a disposable column and washed with 500 ml of wash buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 30 mM imidazole, 1 mM PMSF). The resin bound protein was eluted using elution buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 M imidazole) in 1 ml fractions. Eluted samples were loaded onto a Tricine SDS-PAGE gel to determine the purity of the recombinant protein. The eluted protein was pooled and dialyzed against one litre of dialysis buffer (50 mM Tris-HCl, pH 8.0) (SnakeSkin Dialysis Tubing, cut-off 3000 MW). The dialysis buffer was changed twice every one hour and with the final buffer change, dialysis was continued overnight. The concentration of the purified protein was estimated using Micro BCA Protein Assay Kit. The dialyzed protein samples were concentrated using Amicon Ultra 3000 MW cut-off protein concentrator units.
2.13: Purification of glutathione-S-transferase (GST)-tagged ESAT-6 protein

For purification of GST-tagged recombinant protein, Rv3875 (ESAT-6) was amplified from \textit{M. tuberculosis} H37Rv genomic DNA, with primers designed to clone Rv3875 ORF in frame with GST ORF in pGEX-4T-1 (GE Healthcare, Little Chalfont, United Kingdom), the primers were appended with EcoRI and NotI enzyme sites in forward and reverse primers to facilitate cloning in matching sites in the pGEX-4T-1. The primers used were:

Forward primer
5′ACGAGAATTCATGACAGAGCAGCAGTGGAATTTCGC3′ (EcoRI)
Reverse primer
5′GATTGCGGCCGCCTATGCGAACATCCCAGTGACGTTGC3′ (NotI)

A confirmed clone of pGEX-4T-1 ESAT-6 was transformed into \textit{E. coli} BL21 (DE3) cells and plated on LB plates containing ampicillin (100 \mu g/ml). The colonies were scrapped out in LB medium containing ampicillin (100 \mu g/ml) and left on shaker incubator at 37°C for 2 hours. This primary culture was inoculated into 800 ml Terrific Broth containing 100 \mu g/ml of ampicillin. The culture was left in a shaker incubator set at 37°C till the A600 reaches about 0.5. Expression of GST-ESAT-6 was induced in the presence of IPTG to a final concentration of 1 mM at 37°C for 4 hours with shaking. Cells were harvested by centrifugation and the pellet was re-suspended in 40 ml of lysis buffer (1x PBS, 1 mM PMSF, 1X protease inhibitor cocktail [Roche, Penzberg, Germany]). The cell
suspension was sonicated and the lysate was centrifuged at 25000 rpm for 30 minutes. The supernatant was added to 1 ml of equilibrated Glutathione agarose beads (Clontech) and after 45 minutes, the beads were washed with 100 ml of lysis buffer. The beads were re-suspended in 1ml of lysis buffer. About 50 μl of these re-suspended beads were mixed with equal amount of 2X SDS loading dye, heated at 90°C for 10 minutes and loaded on 10% Tricine SDS-PAGE gel to check for purity and presence of protein. The amount of protein bound to GST beads was estimated using Micro BCA Protein Assay Kit (Thermo Scientific) following manufacturer’s instructions. GST alone was also purified from the empty pGEX-4T-1 vector using the same protocol.

2.14: Mammalian cell culture

THP-1 cells were obtained from National Centre for Cell Science, Pune, India. The cells were cultured in complete RPMI-1640 medium (Hyclone) containing 10% FBS, Antibiotic-Antimycotic (1X, containing Penicillin G, Streptomycin, Amphotericin B), 2 mM L-Glutamine and 10 mM HEPES and maintained at 37°C and 5% CO₂ in a humidified incubator. KG-1 cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 20% FBS, Antibiotic-Antimycotic (1X, containing Penicillin G, Streptomycin, Amphotericin B), 2 mM L-Glutamine and 10 mM HEPES and maintained at 37°C and 5% CO₂ in a humidified incubator.
2.15: GST pulldown assay to confirm protein-protein interaction

GST pulldown assay was carried out to confirm protein-protein interaction experiment. In GST pulldown assay, GST-ESAT-6 was immobilized on glutathione-resin and was used to pulldown β2M from whole cell protein extract of Human acute monocytic leukemia cell line THP1. The whole cell protein extract prepared in lysis buffer containing 1x PBS, 1% Triton X-100, 1 mM PMSF, 1X protease inhibitor cocktail mixture was pre-cleared by incubating with GST-bound Glutathione (100 μg of GST-bound glutathione beads per 2000 μg of mammalian lysate) for 2 hours at 4°C with end-to-end mixing. The GST beads were removed by centrifugation at maximum speed for 2 minutes at 4°C in a microcentrifuge and the supernatant (the pre-cleared cell lysate) was divided into two microcentrifuge tubes, containing 30 μg of Glutathione bound GST protein and GST-ESAT-6 fusion protein. The tubes were incubated for 2 hours at 4°C with end-to-end mixing followed by centrifugation at 1000g for 2 minutes in a microcentrifuge to collect the beads. The beads were then washed four times each in 1 ml of ice-cold GST lysis buffer and collected by centrifuging at maximum speed for 1 minute. The supernatants were discarded and the proteins bound to these beads were eluted by boiling the beads at 90°C for 5 minutes in 50 μl of SDS loading dye. These samples were then resolved in a 16% Tricine SDS-PAGE and immunoblotted using rabbit anti-β2M antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and goat anti-rabbit IgG (Immunoglobulin G) secondary antibodies conjugated to horseradish peroxidase (HRP).
2.16: Purification of ESAT-6:CFP-10 complex and the mutant ESAT-6ΔC:CFP-10 complex

ESAT-6 ORF was amplified from \textit{M. tuberculosis} H37Rv genomic DNA using the set of primers given below and cloned in the first MCS of duet vector pETDuet-1 (Novagen) in frame with N-terminal 6X His-tag. The primers used were;

Forward primer

\begin{verbatim}
5`GCCAGGATCCGATGACAGAGCAGCAGTGGAATTTCGC3`
\end{verbatim}  \hspace{1cm} \text{BamHI}

Reverse primer

\begin{verbatim}
5`CGATAAGCTTCTATGCGAACATCCCAGTGACGTTGC3`
\end{verbatim}  \hspace{1cm} \text{Hind III}

The mutant ESAT-6ΔC (ESAT-6 with C-terminal 6 amino acids deleted) was also cloned in the first MCS of pETDuet-1 vector using the following primers.

Forward primer

\begin{verbatim}
5`GCCAGGATCCGATGACAGAGCAGCAGTGGAATTTCGC3`
\end{verbatim}  \hspace{1cm} \text{BamHI}

Reverse primer

\begin{verbatim}
5`GTGTAAGCTTCTAGTTGCCTTCGGTCGAAGCCATTG3`
\end{verbatim}  \hspace{1cm} \text{Hind III}

The CFP-10 ORF was amplified from pMH406 (kindly provided by David Sherman of Seattle Biomedical Research Institute and this plasmid contains the
RD1 region of *M. tuberculosis*) and cloned in the second MCS of both the pETDuet-1 vectors having wild-type ESAT-6 and mutant ESAT-6 in the first MCS. The primers used are given below-

Forward primer

5′AGCACATATGGCAGAGATGAAGACCGATGC3′  NdeI

Reverse primer

5′ATGCGGTACCTCAGAAGCCCATTTGCGAGGAC3′  KpnI

The positive clones were confirmed by DNA sequencing and transformed into *E. coli* BL21 (DE3) cells and the primary inoculum was prepared by scraping colonies from a single transformed plate. The primary culture was inoculated into 800 ml Terrific Broth containing 100 μg/ml of ampicillin. The culture was grown in a shaker incubator set at 37°C till the absorbance $A_{600}$ reached about 0.5. Expression of the recombinant protein was induced by adding IPTG to a final concentration of 1 mM and further incubating for 3 hours in a shaker incubator at 37°C. Cells were harvested by centrifugation and re-suspended in 40 ml lysis buffer, sonicated and the lysate was centrifuged at 25000 rpm for 30 minutes. The soluble recombinant protein present in supernatant was allowed to bind a 5 ml bed volume of Ni-NTA resin (QIAGEN) that has been pre-equilibrated with lysis buffer for 45 minutes. The resin bound protein was loaded on a disposable column and washed with 500 ml of wash buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 30 mM imidazole, 1 mM PMSF). The resin bound protein was eluted using elution buffer (50 mM Tris-HCl [pH 8.0], 300
mM NaCl, 1 M imidazole) in 1 ml fractions. Eluted samples were loaded onto a 16% Tricine-SDS-PAGE gel to determine the purity and also to confirm the presence of recombinant ESAT-6:CFP-10 and the mutant ESAT-6ΔC/CFP-10. The eluted fractions were pooled and dialyzed against one litre of dialysis buffer (50 mM Tris-HCl [pH 8.0]) (SnakeSkin Dialysis Tubing, cut-off 10000 MW). The concentration of the purified protein was estimated using Micro BCA Protein Assay Kit. The dialyzed protein samples were concentrated using Amicon Ultra 10000 MW cut-off protein concentrator units.

2.17: Purification of His-tagged CFP-10 protein

CFP-10 ORF was amplified from pMH406 (kindly provided by David Sherman of Seattle Biomedical Research Institute) and cloned in pET23a (Novagen) with a C terminal His-tag using the set of primers given below;

Forward primer

5` GATCCATATGGCAGAGATGAAGACCGATGC3` NdeI

Reverse primer

5` GTATCTCGAGGAAGCCCATTTGCGAGGACAGC3` XhoI

The clones were confirmed by sequencing and transformed into E. coli BL21 (DE3) cells and primary inoculum was prepared by scrapping colonies from a
single transformed plate. The primary culture was inoculated into 800 ml Terrific Broth containing 100 μg/ml of ampicillin. The culture was grown in a shaker incubator set at 37°C till the absorbance $A_{600}$ reached about 0.5. Expression of recombinant protein was induced by adding IPTG to a final concentration of 1 mM and further incubated for 3 hours in a shaker incubator at 37°C. Cells were harvested by centrifugation and re-suspended in 40 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 mM PMSF), sonicated and the lysate was centrifuged at 25000 rpm for 30 minutes. The soluble recombinant protein present in the supernatant was allowed to bind to 5 ml bed volume of Ni-NTA resin (QIAGEN) pre-equilibrated with lysis buffer for 45 minutes. This protein bound Ni-NTA slurry was loaded on a disposable column and was washed with 500 ml of wash buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 30 mM imidazole, 1 mM PMSF). The resin bound protein was eluted using elution buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 M imidazole) in 1 ml fractions. Eluted samples were loaded onto a Tricine SDS-PAGE gel to confirm the presence and purity of recombinant protein. The eluted fractions were pooled and dialyzed against one litre of dialysis buffer (50 mM Tris-HCl, pH 8.0) (SnakeSkin Dialysis Tubing, cut-off 3000 MW), the dialysis buffer was changed two times every one hour with a final buffer change in which dialysis was continued overnight. The concentration of the purified protein was estimated using Micro BCA Protein Assay Kit. The dialyzed protein samples were concentrated using Amicon Ultra 3000 MW cut-off protein concentrator units.
2.18: Cloning of ESAT-6 in pEGFP-C1

Rv3875 (ESAT-6) was amplified from the genomic DNA of *M. tuberculosis* strain H37Rv and cloned in frame with the C-terminal of the EGFP coding ORF in pEGFP-C1 (Clontech) using the set of primers described below:

Forward primer

5’ ATGCAGATCTATGACAGAGCAGCAGTGGAATTTCGC 3’   BglII

Reverse primer

5’ ATGCCTGCAGCTATGCGAACATCCCAGTGACGTTGC 3’   PstI

2.19: Surface plasmon resonance (SPR) binding assay

The SPR-based biosensors are used for characterization of molecular interactions in terms of both affinity and chemical kinetics. SPR assay experiments involve immobilizing one of the molecules of a binding pair on the sensor chip surface and injecting a series of concentrations of its partner across the surface. Changes in the refraction index at the surface due to interactions between the two molecules are detected and recorded as RU (Resonance Units). Curves generated from the RU trace are evaluated using a curve-fitting algorithm which compares the raw data to well-defined binding models. This allows determination of a variety of thermodynamic constants, including apparent binding and disassociation constants. Direct interaction of β2M with ESAT-6 was monitored using a BIACORE 3000 Biosensor (Biacore AB, Uppsala, Sweden). β2M purified from human urine (Sigma-Aldrich) (dialysed...
against 10 mM acetate buffer, pH 3.5) was coupled to the flow cells of the sensor chip (Carboxy-Methylated Dextran chip (CM5), Biacore AB) using 100 mM N-hydroxysuccinimide and 400 mM N-Ethyl-N'-(dimethylaminopropyl) Carbodiimide, until an appropriate level of coupling (400 resonance units) was achieved. Deactivation after immobilization was achieved using 1 M ethanolamine (pH 8.5). All binding studies were performed in 75 mM phosphate buffered saline, pH 7.5 using a 5 μl/minute flow rate at 25°C. A control flow cell was also prepared under identical conditions in the absence of the protein using acetate buffer. Recombinant His-tagged ESAT-6 were dialysed against running buffer and injected in different concentrations into the flow cell coupled with β2M.

2.20: Isolation of mouse peritoneal macrophage

About 4-6 week old C57BL/6 mice were injected intraperitoneally with 1 ml of 4% Thioglycolate. After 4 days of injection, mice were sacrificed using CO₂ asphyxiation and the macrophages were harvested by flushing the peritoneum with ice cold Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Thermo Scientific, Massachusetts, USA). The peritoneal exudate cells (PECs) were seeded into 60 well plates and the macrophages were allowed to adhere to the bottom of the culture plates. The non-adherent cells were removed by flushing the plates with DMEM and the adherent cells were maintained in fresh complete DMEM high glucose medium containing 10% FBS, Antibiotic-Antimycotic (1X, containing Penicillin G, Streptomycin, Amphotericin B), 2 mM L-Glutamine and
10 mM HEPES (All from GIBCO, Carlsbad, CA) and maintained at 37°C and 5% CO₂ in a humidified incubator.

2.21: Assay for effect of soluble proteins on MHC-I antigen presentation

Hypertonic cytoplasmic loading of ovalbumin (OVA) followed by osmotic lysis of pinosomes containing OVA was used to introduce soluble antigens into the Major histocompatibility complex (MHC) class-I pathway of antigen presentation [377, 378]. In brief, peritoneal macrophages from C57BL/6 (H-2Kᵇ) mice were incubated with various soluble recombinant proteins for 2 hours. After washing, cells were further incubated in hypertonic serum-free DMEM containing 0.5 M sucrose, 10% polyethylene glycol 800, 10 mM HEPES and 10 mg/ml OVA for 10 minutes at 37°C. After washing and incubation in isotonic serum-free DMEM for 5 minutes, the cells were further incubated for 3 hours to allow OVA antigen processing and presentation. These cells were then fixed with 1% paraformaldehyde (Sigma-Aldrich) and directly probed for the presence of OVA-derived SIINFEKL peptide (OVA 257-264) on MHC-I using a phycoerythrin (PE) conjugated antibody that recognises SIINFEKL peptide bound to MHC-I (H-2Kᵇ) (eBiosciences, San Diego, CA, USA). The PE fluorescence was detected in the FL2 channel of flow cytometer (BD FACSaria, Beckton Dickinson, San Jose, CA) and the data was analyzed using Flowjo software (Tree Star Inc., Ashland, OR, USA).
2.22: Nucleofection of KG-1 cells

Nucleofection (Lonza Cologne GmbH, Cologne, Germany) is a method of electroporation used to introduce DNA into difficult to transfec cell lines. For every transfection reaction, $2 \times 10^6$ KG-1 cells were collected by centrifugation at 2000 rpm for 5 minutes at room temperature and the pellet was re-suspended in 100 µl Nucleofector Solution R at room temperature. This cell suspension was mixed with 2 µg DNA and transferred into nucleofection cuvette provided in the Nucleofection kit. This cuvette was placed into the nucleofector Cuvette Holder and the program optimised for KG-1 cells was initiated (V-001). Once the program was completed, 500 µl of Iscove's Modified Dulbecco's Medium (IMDM) was immediately added into the cuvette and carefully transferred into a 12-well plate containing 1.5 ml IMDM per well. β2M expression on cell surface was examined after 24 hours by flow cytometry using PE-conjugated anti-human β2M antibody.

2.23: Nucleofection of THP-1 cells

THP-1 (1-2 x 10⁶) cells were pelleted down at 2000 rpm for 3 minutes at room temperature, and the cell pellet was re-suspended in 100 µl Nucleofector Solution V (Lonza Cologne GmbH) at room temperature. This cell suspension was combined with 0.5 µg of pEGFP-C1 or pEGFP-C1-ESAT-6 DNA and then transferred into nucleofection cuvette provided in the Nucleofection kit. This cuvette was placed into the Nucleofector Cuvette Holder and the program
optimised by the manufacturer for THP-1 cells (V-001) was initiated. Once the nucleofection is complete, 500 µl of the RPMI-1640 medium was immediately added into the cuvette and gently transferred into a 12-well tissue culture plate containing 1.5 ml RPMI-1640 medium per well (with 10% FBS and antibiotic-antimycotic). Twenty four hours post nucleofection, β2M expression on cell surface was examined by flow cytometry using PE-conjugated anti-human β2M antibody.

2.24: Fluorescence-activated cell sorting (FACS)

For flow cytometry, about 1 x 10⁶ cells were incubated on ice with 100 µl of blocking buffer containing 1% rabbit serum diluted in FACS staining buffer (1x PBS containing 1% BSA and 0.1% sodium azide) in 96 well U bottom plate. The cells were washed three times with FACS staining buffer and incubated on ice with 100 µl of appropriate antibodies (either as a fluorochrome-conjugated primary antibody or in successive steps of unlabelled primary antibody and fluorochrome-conjugated secondary reagents) diluted in FACS staining buffer for 60 minutes on ice. After thorough washing with FACS staining buffer, the cells were fixed in 1% paraformaldehyde. Cell-bound fluorescence was measured on FACS Aria (Beckton Dickinson, San Jose, CA) and the data was analyzed using either FlowJo (TreeStar) or CellQuest data analysis software (Beckton Dickinson). PE-conjugated anti-human β2M antibody (BD Pharmingen, cat no 551337) and PE-conjugated anti-human HLA-ABC antibody
(BD Pharmingen, cat no 560168) were used to stain and compare surface β2M and HLA.

2.25: Labelling of recombinant ESAT-6 and ESAT-6:CFP-10 complex with FITC

FITC-labelled ESAT-6 or ESAT-6:CFP-10 was prepared by incubating purified recombinant protein with FITC using a commercially available FITC Labeling Kit from Pierce (Rockford) following the manufacturer’s protocol.

2.26: Confocal studies for localization of recombinant ESAT-6 and ESAT-6:CFP-10 in endoplasmic reticulum (ER) using ER tracker

ER-Tracker dye (Molecular Probes, Oregon, USA) can specifically localize to the endoplasmic reticulum (ER) of live-cells and hence used to visualize ER. For ER localization studies, 1 million cells were incubated with FITC labelled ESAT-6 or ESAT-6:CFP-10 for about 100 minutes, and the ER-Tracker dye (has an excitation and emission maxima of 374 and 430-640 nm) was added to these cells at a concentration of 1 µM and incubated for 20 minutes. The cells were washed three times in 1X PBS and then fixed with 3% paraformaldehyde for 10 minutes. The cells were again washed three times with 1X PBS and
mounted on coverslip and visualized under LSM 510 META confocal microscope.

In some experiments, ER localization was also confirmed using antibodies to calreticulin (an ER resident protein). For this, cells were incubated with FITC-labelled ESAT-6 or ESAT-6:CFP-10 for 120 minutes and then washed with 1x PBS and fixed with 3% paraformaldehyde for 10 minutes. The cells were then washed with 1X PBS and permeabilized with 0.1% Triton-X 100 (in PBS) for 10 minutes at room temperature. After washing, the cells were blocked with 1% BSA diluted in PBS and incubated at 4°C for 45 minutes with mouse anti-calreticulin antibody in PBS at 1:100 dilution (BD Pharmingen, New Jersey, USA). The cells were then washed three times with 1X PBS and incubated with 1000 times diluted secondary anti-mouse antibody conjugated to Alexa568 [Invitrogen, California, USA]). Cells were washed 3 times in 1X PBS, mounted on coverslip and imaged on LSM 510 META Confocal microscope.

2.27: Immunoprecipitation assay

THP-1 monocytes were treated with 20ng/mL of PMA for 12 hours followed by 24 hours rest. These cells were scrapped and washed with ice cold PBS and lysed with 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 supernatant was collected and after overnight incubation with recombinant protein, primary
antibody was added and incubated for 2 hours at 4°C on a rotating platform. After this incubation Protein A/G-Agarose beads was added to this mixture and incubated at 4°C on a rotor for 1 hour. Protein A/G-Agarose beads were collected by centrifugation at 1,000xg for 30 seconds at 4°C, the supernatant was discarded and the pellet washed 3 times with 1.0 ml PBS. After the final wash pellet was resuspended and boiled in 40 µl of 2X electrophoresis sample buffer. Samples were separated on 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 appropriate combinations of primary and HRP-conjugated secondary antibodies. 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).

2.28: 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.