Materials and Methods:

Materials:
RPMI 1640 medium, Dulbecco’s Modified Eagle Medium (DMEM), fetal calf serum (FCS), HEPES, all other chemicals used for cell culture medium, Trizol Reagent and Superscript II reverse transcriptase kit were from Invitrogen Life Technologies (Carlsbad, NY, USA). QIAexpressionist protein expression and purification kit including Ni2+-NTA-Agarose; Gel purification kit and all primers used for amplification of c-DNA were from Qiagen (Valencia, CA, USA). E. Coli lipopolysaccharide (LPS), 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal), Isopropyl-Thio-D-Galactopyranoside (IPTG), Reduced glutathione, Ampicillin, Chloramphenicol, O-phenylenediamine dihydrochloride (OPD), E-TOXATE kit, Acrylamide, Ammonium per sulphate, agarose DNA grade, β-Mercaptoethanol, Boric acid, Bovine Serum Albumin, Bromophenol blue, Freund’s incomplete adjuvant, Glutathione agarose, Griess reagent, Dimethyl sulfoxide, 3,3’-Diaminobenzidine tetrahydrochloride hydrate (DAB), EDTA, Glucose, Imidazole, PMSF, Polymyxin B-agarose beads, Sodium bicarbonate, Sodium orthovanadate, Sodium fluoride, Tween-20, Aprotinin, Pepstatin, Leupeptin, Iodoacetamide, MEK-1 inhibitor PD98059 and p38 inhibitor SB203580 were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Bacto Tryptone, Yeast Extract and Bacto agar were from DIFCO (San Diego, CA, USA). Dithiothreitol (DTT) was from Amersham Biosciences, (Uppsala, Sweden). IL-1β, IL-6, IL-12p40 and TNF-α ELISA Ready-Set-Go kits were obtained from eBioscience (San Diego, CA, USA). Trypsin-EDTA and Opti-MEM were obtained from Gibco BRL. Recombinant mouse IFNγ was purchased from R&D System (Minneapolis, MN, USA). All antibodies used for western blotting and Enhanced Chemiluminescence (ECL) kit were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 100 bp, 1kb ladders and dNTP mix were obtained from Promega Inc. (Madison, WI, USA). All the restriction enzymes were from New England Biolabs (Ipswich, MA, USA).
Bacteriomatch™ Two Hybrid System Reporter Kit including reporter strains and bacterial two-hybrid vectors, bait plasmid (pBT), target plasmid (pTRG) and control plasmid pair pBT-LGF2 and pTRG-Gal11p were purchased from Stratagene (San Diego, CA, USA).

**List of plasmid vectors used**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQE 30</td>
<td>Qiagen (Valencia, CA, USA)</td>
</tr>
<tr>
<td>pGEX4T1</td>
<td>Amersham Biosciences, (Uppsala, Sweden)</td>
</tr>
<tr>
<td>pBT</td>
<td>Stratagene (San Diego, CA, USA)</td>
</tr>
<tr>
<td>pTRG</td>
<td>Stratagene (San Diego, CA, USA)</td>
</tr>
</tbody>
</table>

**List of bacterial strains used**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>Recombination minus strain for plasmid Propagation (Invitrogen Life Technologies, NY, USA)</td>
</tr>
<tr>
<td>E. coli M15</td>
<td>Expression of His-tagged protein (Qiagen, CA, USA)</td>
</tr>
<tr>
<td>E. coli BL21 pLys S</td>
<td>Expression of GST-tagged protein (Merck, Darmstadt, Germany)</td>
</tr>
<tr>
<td>E. coli XL1 Blue MRF'</td>
<td>Used for propagating plain pBT, pTRG and all recombinant Bacterial-2-hybrid plasmids (Stratagene, CA, USA)</td>
</tr>
<tr>
<td>E. coli R1</td>
<td>Used as reporter strain for cotransformations and as an assay strain for B2H assay (Stratagene, CA, USA)</td>
</tr>
</tbody>
</table>
Bacterial Growth Media and Solutions

**LB (Luria-Bertani) medium (1000 ml):** 10 gm bacto-tryptone, 5 gm yeast extract and 10 gm NaCl (Sterilized by autoclaving).

**LB agar:** LB medium containing 15 gm/litre agar (Sterilized by autoclaving).

**Chloramphenicol stock solution:** 34 mg/ml in 100% ethanol, filter sterilized and stored in aliquots at -20°C until use.

**Ampicillin stock solution:** 100 mg/ml in double-distilled water, filter sterilized and stored in aliquots at -20°C until use.

**Kanamycin stock solution:** 25 mg/ml in double-distilled water, filter sterilized and stored in aliquots at -20°C until use.

**X-Gal Indicator Plates (per Liter):** 1 Liter of LB agar was autoclaved, cooled to 55°C and supplemented with, 1 ml of 30 mg/ml chloramphenicol, 1 ml of 12.5 mg/ml tetracycline, 1 ml of 50 mg/ml kanamycin, 1 ml of an 80 mg/ml stock solution of XGal, 1 ml of 200 mM stock solution of β- galactosidase Inhibitor (PETG), 0.5 ml of 1M IPTG. Finally poured into petri dishes and stored in a dark, cool place.

Buffers and Solutions for Western Blotting

**Lysis buffer:** 50 mM HEPES (pH 7.5), 10 mM EDTA, 10% (v/v) Glycerol, 0.5% Triton X-100 along with protease and phosphatase inhibitors as applicable.

**Transfer buffer:** 25 mM Tris base, 250 mM Glycine, 0.01% SDS (Sodium Dodecyl Sulfate) and 20% Methanol.

**1X PBS (Phosphate-Buffered Saline):** 150 mM NaCl, 2.7 mM KCl, 100 mM Di-Sodium hydrogen phosphate (Na₂HPO₄) and 2 mM Sodium di-hydrogen phosphate (NaH₂PO₄).

**Blocking buffer:** 5% (w/v) Bovine Serum Albumin in 1X PBS-T.

**Wash buffer (PBS-T):** 0.1% (v/v) Tween-20 in 1X PBS.

Primary and secondary antibody solutions were prepared in a 1% solution of BSA in wash buffer and blots were developed by Enhanced Chemi-luminescence (ECL) kit.
Reagents for silver staining
Fixing solution: methanol: acetic acid: water (50:5:45)
Sensitizing solution: 0.02 % sodium thiosulfate
Developing solution: 0.04% formaldehyde in 2% sodium carbonate
Stopping Solution: 1% acetic acid

Solutions for Flow Cytometric Analysis
Wash Buffer: 0.5% BSA in PBS
Fixing Solution: 4% Paraformaldehyde solution in PBS

Buffers for plasmid DNA isolation
Solution I: 50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA.
Solution II: 0.2 N NaOH and 1% SDS.
Solution III: 3 M potassium acetate and 11.5% glacial acetic acid.
Tris-EDTA buffer: 10 mM Tris.HCl pH 8.0 and 1 mM EDTA.
5X TBE (Tris-Borate-EDTA) Buffer: 0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3.

Buffers for Protein Purification by Ni-NTA affinity chromatography (under denaturing conditions)
Lysis Buffer: 50 mM NaH2PO4, 300 mM NaCl and 10 mM imidazole. The pH of the solution adjusted to 8.0 with NaOH.
Buffer A: 100 mM NaH2PO4, 10 mM Tris-Cl and 8 M Urea. The pH of the solution adjusted to 8.0 with NaOH.
Buffer B: 100 mM NaH2PO4, 10 mM Tris-Cl and 8 M Urea. The pH of the solution adjusted to 6.3 with HCl.
Buffer C: 100 mM NaH2PO4, 10 mM Tris-Cl and 8 M Urea. The pH of the solution adjusted to 5.9 with HCl.
Buffer D: 100 mM NaH2PO4, 10 mM Tris-Cl and 8 M Urea. The pH of the solution adjusted to 4.5 with HCl.
Buffers for protein purification by glutathione sepharose affinity chromatography

**Wash Buffer I:** 1X PBS (Phosphate-Buffered Saline): 150 mM NaCl, 2.7 mM KCl, 100 mM Di-Sodium hydrogen phosphate (Na₂HPO₄) and 2 mM Sodium di-hydrogen phosphate (NaH₂PO₄), pH 7.4.

**Wash Buffer II:** 500 mM NaCl, 2.7 mM KCl, 100 mM Di-Sodium hydrogen phosphate (Na₂HPO₄) and 2 mM Sodium di-hydrogen phosphate (NaH₂PO₄), pH 7.4

**Elution Buffer:** 10 mM Reduced glutathione, 50 mM TrisCl, pH 8.

Buffers for ELISA:

**Coating Buffer:** 100 mM NaHCO₃, 100 mM Na₂CO₃, pH 9.8.

**Substrate buffer:** 100 mM Sodium Citrate, pH 4.5 by using citric acid (addition of 30% H₂O₂ at the time of adding substrate).

Buffers for preparation of cytoplasmic and nuclear extracts:

**Lysis (cytoplasmic) buffer**

10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA, 1 μg/ml Aprotinin, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin, 1 mM PMSF, 20 μM Sodium Vanadate, 20 μM Sodium fluoride.

**Nuclear extraction buffer**

20 mM HEPES pH 7.9, 400 mM NaCL, 1 mM EDTA pH 8.0, 1 mM EGTA, 1 μg/ml Aprotinin, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin, 1 mM PMSF, 20 μM Sodium Vanadate, 20 μM Sodium fluoride.

Methods:

**Cloning of mpt70 gene**

The open reading frame Rv2875 encoding MPT70 was PCR amplified from genomic DNA of *M. tuberculosis* H37Rv (kind gift from the NIH-NIAID funded "TB
Materials and Methods

Vaccine Testing and Research Materials Contract at the Colorado State University, Ft. Collins, CO, USA) using following primers:

Forward:
mpt70f 5’-CGCGGATCCGGCGATCTGGTGGGCCCGG-3’
Reverse:
mpt70r 5’-CGGGGTACCTTACGCCGGAGGCATTAGCAC-3’
(BamHI and KpnI sites are underlined respectively)

Composition of PCR mixture and PCR conditions were:

**PCR mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double distilled water</td>
<td>40 µl</td>
</tr>
<tr>
<td>10X Taq DNA Polymerase buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 µM mpt70f</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 µM mpt70r</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 µl</td>
</tr>
<tr>
<td>DMSO</td>
<td>2.5 µl (5%)</td>
</tr>
<tr>
<td>Template</td>
<td>2 µl (0.5-1 ng)</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 µl (2.5 U)</td>
</tr>
</tbody>
</table>

**PCR conditions**

- **Initial Denaturation**: 94°C, 15 minutes
- **30 cycles of:**
  - Denaturation: 94°C, 1 minute
  - Annealing: 60°C, 1 minute
  - Extension: 72°C, 1 minute
- Followed by:
  - **Final Extension**: 72°C, 10 minutes
PCR products were analysed by 1% agarose gel electrophoresis. PCR amplified fragment was gel purified using "Qiagen gel purification kit" according to protocol given with the kit.

Preparation of *E. coli* competent cells and their transformation, isolation of plasmid DNA from *E. coli* cells by alkaline lysis method, plasmid DNA preparation and construction and manipulation of recombinant DNA were carried out as described in *Molecular Cloning: A Laboratory Manual (Third Edition)* (Sambrook *et al.*, 2000).

**Expression and Purification of His-MPT 70**

For the expression of recombinant His-MPT 70 in *Escherichia coli*, a 20 ml of LB culture containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin was inoculated by a stab of glycerol stock of *E. coli* M15 cells and allowed to grow overnight for 18 hours. Then 1% inoculum was inoculated into a large volume (2000 ml) of LB broth and allowed to grow at 37°C at 180 rpm in a shaker until O.D._600nm_ reached 0.5. Then cells were induced by addition of IPTG to a final concentration of 1 mM and kept at 37°C for 3 hours in shaker. After 3 hours cells were pelleted and weight of the biomass noted. The cells were then resuspended in 10 ml of phosphate buffer pH 8.0 per gram of the biomass. Then the cells were lysed by sonication in the presence of protease inhibitors 1 µg/ml of Aprotinin, Pepstatin A and Leupeptin each and 1 mM PMSF. The suspension was then centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was kept aside and the weight of the pellet noted. The pellet was dissolved in 4 ml of Buffer A per gram of the pellet at room temperature overnight. The urea lysate was then centrifuged at 13,000 rpm for 30 minutes at room temperature. Then the supernatant was taken and passed through 0.2µm filter and allowed to bind to Nickel-NTA resin in the ratio of 4:1 (Lysate: resin) at room temperature for 4 hours. The lysate-resin slurry was then poured into a column (Biorad, Richmond, CA, USA) and flow through was collected, then the column was washed successively with 50 column volumes (volume of the resin) of Buffer B and Buffer
C and fractions were collected. Then elution was done using 10 column volumes of Buffer D and fractions were collected. The fractions were run in 12\% SDS-PAGE and the pure fractions were pooled together. From the pooled fractions urea was removed by dialysing against 10 mM Na$_2$HPO$_4$, 10 mM TrisCl and 2 M Urea buffer, pH 8.0. The dialysed protein was concentrated by passing through centricon filter of 5000 Mw cut off and stored at -80\(^\circ\)C.

**Expression and Purification of GST-MPT 70**

For the expression of recombinant GST-MPT 70 in *Escherichia coli*, a 20 ml of LB culture containing 100 \(\mu\)g/ml of ampicillin and 34 \(\mu\)g/ml of chloramphenicol was inoculated by a stab of glycerol stock of BL21(DE3) pLysS cells and allowed to grow overnight for 18 hours. Then 1\% inoculum was inoculated into a large volume of 2000 ml LB broth and allowed to grow at 37\(^\circ\)C at 180 rpm in a shaker until O.D.\(_{600nm}\) reached 0.5. Then cells were induced by addition of IPTG to a final concentration of 0.1 mM and kept at 37\(^\circ\)C for 2 hours in shaker. After 2 hours cells were pelleted and weight of the biomass noted. The cells were then resuspended in 10 ml of phosphate buffer pH 7.4 per gram of the biomass. Then cells were lysed by sonication in presence of protease inhibitors 1 \(\mu\)g/ml of Aprotinin, Pepstatin A and Leupeptin and 1 mM PMSF. The suspension was then centrifuged at 13,000 rpm for 30 minutes at 4\(^\circ\)C. The supernatant was passed through 0.2 \(\mu\)m filter and allowed to bind to Glutathione sepharose resin (Amersham Biosciences, Uppsala, Sweden) in the ratio of 4:1 (Lysate: resin) at 4\(^\circ\)C for 3 hours. The lysate-resin slurry was then poured into a column (Biorad, Richmond, CA, USA) and flow through was collected, then the column was washed successively with 100 column volumes each (volume of the resin) of Wash buffer I and Wash Buffer II and fractions were collected. The column was washed again with 10 volumes of Wash Buffer I. Then elution was done using 10 column volumes of Elution Buffer and fractions were collected. The fractions were run in 12\% SDS-PAGE and the pure fractions were pooled together. The pooled fractions were dialysed against PBS, pH 7.4. The dialysed protein was
concentrated by passing through centric filter of 5000 Mw cut off and stored at -80°C.

**In Vitro Protein Interaction Assay (GST and His ‘pull-down’ assay)**

The Ni-NTA agarose beads bound to purified His-MPT 70 protein (20 μg) or to just the His-GFP (20 μg) were incubated with 100 μg of THP1 or J774 cell lysate prepared in 0.15 M PBS, pH7.4. Eight μl of 25 mM DSP was added to the reaction tubes and kept at room temperature for 30 minutes. The reaction was stopped by addition of 5 μl of 1M Tris-HCl, pH 8.0. Beads were centrifuged at 2,000 rpm for 1 min and supernatant was discarded. This was followed by washing four times with PBS. All bound protein was eluted by adding 20 μl of elution buffer and boiling the beads in 2X SDS-PAGE gel loading buffer (20% v/v glycerol, 4% w/v SDS, 0.125 M Tris/HCl, pH 6.8, 4% v/v 2-β-mercaptoethanol) and resolved on 12% SDS-PAGE for subsequent visualization by silver staining.

Similarly, the purified GST-MPT 70 protein (20 μg) or just the GST protein (10 μg) bound to Glutathione- sephasose-4B beads were incubated with THP1 or J774 cell lysate. Rest of the experiment was performed in the same way as mentioned for His-MPT 70.

**Mice Immunization**

His-MPT 70 protein prepared as above was used for immunization of mice. To investigate the immunogenicity of MPT 70 in mice, groups of 4-6 weeks old Balb/c and C57BL6 mice were immunized (10 animals each) intraperitoneally with 100 μg of purified His-MPT 70 protein with 250 μl Freund’s incomplete adjuvant. Mice were again boosted twice with 100 μg of His-MPT 70 with Freund's incomplete adjuvant intraperitoneally on 4th week and 8th week after primary immunization. Bleeds were collected a day before primary immunization and at every two weeks after primary immunization. The immunization schedule is shown in Figure A.
**Materials and Methods**

**Fig. A: Schematic representation of immunization of mice.**

**Seroanalysis by ELISA using His-MPT70 as capture antigen**

Titers of anti-MPT70 antibody in the sera of immunized mice were determined by sandwich ELISA. The 96-well ELISA plate was coated with His-MPT70 (500 ng per well) in coating buffer for overnight at 4°C. Next day the plate was washed thrice with PBST and blocked with 5% BSA for 2 hours. The plate was again washed and then incubated with two-fold serial dilutions of the individual mouse serum samples for 90 minutes at room temperature. The plate was washed as earlier and then incubated with anti-mouse-IgG HRP (1:2500) for 60 minutes at room temperature followed by washing five times with PBST. Then, 50 μl of 0.5 mg/ml of OPD substrate (prepared in 0.1 M citrate buffer) was added to each well and incubated for 10 minutes. The reaction was terminated with 2 M H₂SO₄ and the plate was read at an optical density (OD) of 490 nm.

**Culture of Mammalian Cells**

Murine macrophage cell line RAW264.7 transformed with Abelson murine leukemia virus, originally obtained from ATCC, was routinely maintained in Dulbecco’s Modified Eagle Medium containing 2 mM glutamine, 100 U/ml of penicillin and streptomycin and 10% fetal bovine serum at 5% CO₂ in a humidified atmosphere at 37°C. Murine macrophage cell line J774, obtained from ATCC, was routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 U/ml of penicillin and streptomycin and 10% fetal bovine serum at 5% CO₂ in a humidified atmosphere at 37°C. Primary macrophages cell cultures were established with cells isolated from the wild-type mice and cultured in standard
RPMI 1640 media supplemented with 10% heat inactivated FBS. All experiments were performed at 5% CO₂ in a humidified environment at 37°C.

**Peritoneal macrophage isolation**
Thioglycolate – elicited peritoneal exudates cells were harvested from the wild-type C57BL/6 mice five days after intraperitoneal injection of 2 ml of 2% sterile thioglycolate. Peritoneal exudates cells were plated in the cell-culture grade Petri dishes overnight and non-adherent cells were washed away by the culture media. Adherent macrophage cells were used for the experiments.

**Silver Staining of Polyacrylamide Gels**
After the gel was run, the proteins were fixed by incubating the gel in the fixing solution for 30 minutes. The gel was rinsed with water (2 changes, two minutes per change) and then left in water for one hour on a shaking platform. Extended washing was done to eliminate yellowish background usually observed after over developing of the gel. The gel was sensitized with sensitizing solution for 2 minutes and the solution discarded with a quick rinse of the gel with two changes of water (10 seconds each). Chilled silver nitrate solution (0.1% AgNO₃) was added and the gel was kept for 30 minutes at 4°C in dark. Silver nitrate solution was discarded and the gel was quickly rinsed with two changes of water (30 seconds per each change). The gel was developed with developing solution and as soon as it turned yellow, it was replaced with fresh solution. When sufficient degree of staining was obtained, the developing solution was discarded and replaced by stop solution to quench the staining. The gel was then washed several times with water and dried.

**Bacterial-2-hybrid assay**
One-step bacterial two hybrid system was utilized to identify interactors of MPT 70. Two-hybrid systems utilize a bait protein and a prey protein, one or both of which can be a known protein, to identify protein interactions. The interaction between the two proteins occurs within a living cell (in this case, *E. coli*) which
can be visualized by the use of a reporter system like production of an enzyme whose activity can be measured (Fig. B).

![Diagram](image)

**Fig. B:** Schematic diagram of bacterial two-hybrid system based on RNA polymerase recruitment. αN, N-terminal domain of RNA polymerase α subunit; bait and target are interacting polypeptides fused to λcl and αN-subunit of RNA polymerase.

The full length *mpt70* gene (*Rv2875*) was cloned into pBT plasmid by Restriction Digestion of *mpt70* gene from recombinant pQE30 vector and ligation into pBT vector for the bacterial-2-hybrid screen. The *mpt70* gene was excised by RE enzymes BamHI and SalI and ligated into BamHI and Xhol digested pBT vector. The ligation mixture was transformed into the *E. coli* XL-1 blue MRF strain and plated on LB- agar plates with kanamycin and chloramphenicol. The clones were
Materials and Methods

screened for the gene by PCR using their respective primers and confirmed by sequencing.

![Diagram of plasmid vectors](image)

**Fig. C:** Vector map of (a) pBT vector, (b) pTRG vector, positive control (c) pBT-LGF2 and (d) pTRG-GAL11p vectors.

Five hundred ng of bait plasmid containing the clone of interest (mpt70- pBT) was cotransformed into *E. coli* R1 strain along with 500 ng of the human lung cell cDNA library obtained from Clontech. The transformation mixture was plated on an X-Gal indicator plates containing kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), tetracycline (12.5 μg/ml), X-Gal (80 μg/ml), IPTG (25 μM), and β-galactosidase inhibitor (200 μM) for selection of double transformants. Plasmids pBT-LGF2 (λcl-LGF2 fusion) and pTRG-GAL11p (RNAPα subunit-Gal11p fusion) provided the positive controls, whereas empty pBT and pTRG plasmids served
as negative controls for all in vivo interaction studies (Fig. C). IPTG was added for inducing protein expression, X-Gal as indicator for β-galactosidase activity due to protein-protein interaction and an X-Gal inhibitor, PETG to decrease photo-induced degradation of X-Gal. After growing the transformation plate at 30°C overnight, colonies were checked for blue colour expression. Any colonies showing blue colour were patched onto a new plate for confirmation of blue colour production. Plasmid was later extracted from the putative positive colony and transformed back into E. coli R1 strain. This transformation mix was now plated on LB agar containing only Chloramphenicol or Tetracycline and the colonies obtained were grown to again obtain individual plasmids of bait and prey clones after checking that they did not grow in presence of the other antibiotic. Finally, these plasmids were again cotransformed and plated onto an X-Gal indicator plate to detect expression of blue colour due to interaction of the proteins. This process of iteration was done until all colonies were obtained (Fig. D).
Materials and Methods

Fig D: Schematic representation of Bacterial-2-Hybrid Assay

Nitrite and cytokine determination
Materials and Methods

Nitrite accumulation, an indicator of NO production, was measured by using the Griess reagent. Briefly, 50 µl aliquots of culture supernatants were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance at 540 nm was measured in an automated microplate reader. The nitrite concentration (in micromolar units) was calculated from a NaNO₂ standard curve. Concentrations of TNFα, IL-1β, IL-12 and IL-12p40 in cell culture supernatants were determined by ELISA using commercial ELISA kits (eBiosciences, San Diego, CA, USA) for each cytokine, according to the manufacturer’s instructions.

Cellular Fractionation Preparation for western blotting
5x10^6 J774 cells were plated per well in a 6-well tissue culture plate (Nunc, Denmark) in 2ml of complete medium. Cells were stimulated with 20 µg/ml of MPT70, or 1 µg/ml of LPS, or both, for 0, 15, 30, 60 and 120 minutes. After stimulation, cells were harvested and lysed in 200 µl of lysis buffer for 20 minutes at 4°C, 5 µl of 10% NP40 was added and the lysate was vortexed vigorously. Then, the suspension was centrifuged at 5,000 rpm and the supernatant was stored as cytoplasmic extract; the nuclear pellet was washed and suspended in 30µl of nuclear extraction buffer and kept on ice for 40 minutes with intermittent vortexing. Then the suspension was centrifuged at 13,000 rpm and the supernatant was ‘nuclear extract’.

Polyacrylamide Gel Electrophoresis of Proteins
The polyacrylamide gel electrophoresis (PAGE) of proteins was performed in the presence of 0.1% SDS in the gels. Protein samples were prepared by mixing with equal volume of 2X SDS-PAGE sample buffer and boiling in a sand bath for 10 min. Gels were run at a constant current of 60 mA per gel. Following the run, gels were either electro-blotted onto the nitrocellulose membrane Hybond-C (Amersham Biosciences, Uppsala, Sweden) or stained with 0.25% Coomassie blue R-250 in 50% methanol and 10% acetic acid.
**Western Blotting**

For western blotting of proteins mini Trans-blot Electrophoretic Cell (Biorad, Richmond, CA, USA) was used to transfer the proteins from gel onto nitrocellulose membrane. The apparatus for electro-blotting was assembled according to the manufacturer's instructions. Electro-blotting was performed at a constant current of 150 milli-Amperes for 2 hrs; the membrane was then incubated in blocking buffer for 2 hrs with gentle shaking at room temperature. After that, the blocking solution was discarded and after washing thrice with wash buffer the blot was incubated with an appropriate dilution of primary antibody for another 2 hrs with gentle shaking. Thereafter, the blot was washed thrice with wash buffer for 5 min each. After washing, the blot was incubated with horseradish peroxidase conjugated secondary antibody solution for 1 hr. The blot was washed as described above and processed using Enhanced Chemiluminescence (ECL) kit. The densitometric analysis of the blots was done using ImageJ software.

**Flowcytometry**

For flow cytometric analysis, 1x10^6 J774 cells were taken and incubated with 50 μg of MPT 70 protein on ice for 2 hrs. After incubation, cells were washed with plain RPMI-1640 medium thrice and centrifuged at 2000 rpm for 5 minutes at 4°C, the supernatant was discarded and the cell pellet resuspended in 200μl of FACS buffer and incubated with 5μl of anti- MPT 70 antisera (1:200 dilution) for 2hrs on ice. Cells were again washed thrice as before and stained with anti-CD11b-PE antibody for 1 hr on ice in dark. Cells were washed and stained with anti- IgG2a-FITC antibody for 1 hr on ice in dark. The cells were then washed twice with FACS staining buffer. The cells were then acquired and analysed on the FACS Calibur (Beckton-Dickinson) using Cell Quest software.