CHAPTER II

General Materials and Methods
1.0 Materials and their sources

**Tissue culture chemicals** like powdered HEPES and M-199 medium were purchased from Sigma Chemical Co., USA. Other chemicals like bovine serum albumin (BSA), penicillin-G, RPMI-1640, sodium bicarbonate, streptomycin etc., were also procured from Sigma Chemical Co., USA. Fetal Calf Serum (FCS) was purchased from Gibco-BRL Ltd., USA.

**Luciferase activity** chemicals like luciferase assay lysis buffer were procured from Promega (Madison, USA), LUC-expressing episomal vector pGL-αNEOαLUC was generously provided by Marc Ouellette, University Laval, Canada.

**Ornithine decarboxylase chemicals**, namely benzethonium hydroxide, dithiothreitol (DTT), L-ornithine, pyridoxal phosphate were all purchased from Sigma Chemical Co., USA.

**Signal transduction reagents** like staurosporine, okadaic acid (OKA), dibutyl cyclic adenosine monophosphate (dBcAMP) and phorbol myristate acetate (PMA) were purchased from LC Laboratories, Boston, USA. LPS (lipopolysaccharide) from *Escherichia coli* (serotype 0111:B4) was obtained from Sigma Chemical Co., USA. The protein kinase C (PKC) inhibitor, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine hydrochloride (H7), was purchased from Calbiochem Corp. (La Jolla, California).

**Antibodies** used against all MAP-kinase family members; anti-c-Jun, anti-c-Fos, ERK1, ERK2, pERK1, pERK2, p38, pp38 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Bio-Rad (Hercules, CA). The super-shift assays were performed using antibodies against NF-κB family proteins (p50, p65 and c-rel) and IRF-1.
(M-20) were purchased from Santa Cruz Biotechnology Inc, California, USA. G418 was purchased from Sigma Chemical Co., USA.

**Electromobility Shift assay chemicals:** T4 polynucleotide kinase was purchased from Promega (Madison, USA). dsDNA oligonucleotide primers for NF-κB, AP-1, and IRF-1 were procured from Bangalore Genei.

**Chemicals:** Sodium dodecyl sulphate (SDS), tris [hydroxymethyl] aminomethane (Tris), dimethyl sulfoxide (DMSO), ammonium persulphate (APS), acrylamide, N-N'- methylene-bis-acrylamide, coomassie brilliant blue R-250, coomassie blue G-500, CaCl₂, formamide, 2-mercaptoethanol, ethidium bromide, formaldehyde, 3-(N-Morpholino) propane sulfonic acid (MOPS), diethylpyrocarbonate (DEPC), phenol, phenyl methyl sulphonyl fluoride (PMSF), Denhardt's reagent, salmon sperm DNA, DNase, N,N,N',N'-Tetramethylethylenediamine (TEMED), Triton-X-100, TRI- reagent for RNA isolation, protease inhibitor cocktail, 3-tert-butyl-4-hydroxyanisole (BHA), butylated hydroxy toluene (BHT), mannitol, cytochalasin B, glutathione (GSH), PD98059, SB203580, poly(dI:dC) were all purchased from Sigma Chemical Co., USA.

**Analytical grade:** Chemicals that were purchased locally are listed below: methanol, glycerol, ethanol, chloroform, HCl, isoamyl alcohol, isopropanol, KCl, KH₂PO₄, MgCl₂, acetic acid, NaCl, NaH₂PO₄, Na₂HPO₄, NaOH, boric acid, ethylenediaminetetraacetate (EDTA), hydrogen peroxide (H₂O₂), NEDD (N- (1-Naphthyl ethylene diamine dihydrochloride), sulphanalimide, orthophosphoric acid, scintillation cocktail 'O' etc.

**Radioactive chemicals:** ¹⁴C-ornithine (Specific activity 51.6 mCi/m mole) and [³H] thymidine (Specific activity 89.4 Ci /mmol) were purchased from New England Nuclear, Dupont, USA. (γ-³²P) ATP (Specific activity 5000 Ci/mmole) and (α-³²P) dCTP (Specific
activity ~3000 Ci/mmol) were purchased from Amersham Biosciences Limited, Hongkong.

**Filters and membranes:** Membrane filters of 0.22 μm pore size was purchased from Millipore, USA. Other filter papers were purchased from Whatman, USA. PVDF membranes were purchased from Amresco, USA. Hybond N+ membranes were purchased from Amersham Biosciences, USA.

**Kits used:** Chemiluminescence kit for Western blots was purchased from Santa Cruz Biotechnology Inc, California, USA.

**X-ray film:** Hyperfilm for autoradiography was purchased from Amersham Biosciences, USA. X-ray film developer and fixer were purchased from Kodak, India Photographic Company Ltd.

**Leishmania strains:** *L. donovani* (MHOM\IN\1983\AG83) was used in the present study.

**Macrophage cell line:** A murine macrophage cell line J774A.1 (American Type Culture Collection, Rockville, Maryland) was used in this study.

**Animals:** BALB/c mice were procured from National Institute of Nutrition, Hyderabad, India. The University level Ethics committee cleared the work done on animals.

### 2.0 Culture media

All the media prepared were sterilized before use and handled under aseptic conditions.

#### 2.1 Growth media for Leishmania promastigotes

##### 2.1.1 M-199 medium

11.0 g of M-199 and 130 mg each of pencillin-G and streptomycin were dissolved in 800 ml of autoclaved double distilled H₂O. The pH was adjusted to 7.4 by adding solid HEPES. The final volume was adjusted to 1 liter by adding 900 ml of autoclaved double distilled H₂O and 100 ml of heat-inactivated fetal calf serum (FCS). The media was then
sterilized by filtering it through a membrane filter of 0.22 μm pore size. The sterility of the medium was checked by keeping an aliquot at 22°C for three days. Rest of the medium was stored at -4°C.

### 2.1.2 RPMI 1640 medium

16.4 g of RPMI 1640, 2 g of NaHCO₃, 0.06 g of pencillin-G, 0.1 g of streptomycin were dissolved in 800 ml of double distilled water. The pH of solution was adjusted to 7.5 with 5N NaOH and volume was adjusted with double distilled water up to 900 ml. To this 100 ml of heat inactivated FCS was added and the media was sterilized by passing through a 0.22 μm membrane filter and stored at 4°C.

### 3.0 Culturing Leishmania

#### 3.1 In vitro

Promastigotes were maintained in M-199 with 10% FCS at 22°C. Subculturing was done every fourth day when the promastigotes attained stationary phase of growth. Promastigotes from freshly transformed AG83 amastigotes were cultured in M-199 with 30% FCS. A small aliquot of stationary phase culture was added to fresh media at each subculture. The amount of inoculum for different strains of *Leishmania* during subculturing varied from 10-20% of the total volume.

#### 3.2 In vivo

##### 3.2.1 In BALB/c mice

The virulent strain of *Leishmania* AG83 was maintained in BALB/c mice by passaging every 3-4 weeks. Infected spleen of BALB/c mice was removed, aseptically, hand homogenized under sterile conditions and suspended in M-199 with 30% FCS. This suspension was incubated at 22°C for 48 to 72 h. Freshly transformed promastigotes were checked under the microscope and counted. The suspension was centrifuged at 1000 rpm
for 10 min at 4°C to remove splenic debris and the promastigotes were pelleted down at 5000 rpm for 15 min at 4°C. The pellet was resuspended in PBS (pH 7.4) at a concentration of $10^8$ cells/ml. 100 µl of this freshly transformed promastigotes ($10^8$ cells/ml) were injected in the tail vein of 2-4 weeks old mice.

4.0 Maintainence of the macrophages

A murine macrophage cell line (J774A.1) was used in this study:

4.1 Cell culture: J774A.1

The macrophages (J774A.1) were maintained at 37°C in RPMI-1640 medium with 10% FCS in a CO₂ incubator (5% CO₂). The macrophages were seeded onto tissue culture plates (90 mm or 60 mm) at a density of $1 \times 10^6$ cells/plate and incubated for 24 h before being used for the requisite assay.

5.0 Isolation and quantitation of lipophosphoglycan (LPG) from leishmanial promastigotes

5.1 Isolation of LPG

Late log phase promastigotes were harvested by centrifugation at 5000 rpm for 15 min at 4°C, washed with PBS (pH- 7.4) and finally resuspended the pellet in a solution containing 2.5 ml chloroform: methanol (3:2) and 0.5 ml of 4 mM MgCl₂. The mix was sonicated and then centrifuged at 15,000 rpm for 5 min at 4°C. The solid interphase was collected and the above step was repeated. 2.5 ml of 4 mM MgCl₂ was added to the solid interphase and it was sonicated. The mix was centrifuged at 15,000 rpm for 5 min at 4°C and the pellet was collected. The above step was repeated again. 0.5 ml of chloroform:methanol (1:1) was added to the pellet. The solution was sonicated and centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was discarded. 3 ml of
chloroform:methanol:double distilled water (10:10:3) was added to the pellet. The solution was again sonicated and then centrifuged at 15,000 rpm for 5 min at 4°C. The pellet was collected and the above step was repeated again. 2.5 ml of ESOAK (420 ml double distilled water, 420 ml ethanol, 140 ml ethyl ether, 28 ml pyridine and 0.476 ml ammonia solution) was added to the pellet. The solution was sonicated and then centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was collected. 2.5 ml of ESOAK was added to the pellet and the above steps were repeated twice more. All the supernatants, which contained LPG, were pooled together. The solution was put in a speed vac at low temperature for removing the ESOAK solution. The LPG thus obtained was dissolved in the minimum quantity of double distilled water and quantitated before use (Orlandi Jr., and Turco, 1987).

5.1.1 Quantitation of LPG

LPG was quantitated using the orcinol method. This method estimates the total hexoses in a sample. Briefly, the test volume was made to 500 µl with double distilled water and 4.5 ml of orcinol solution was added to it (8 ml of a 1.6% orcinol solution in 60 ml of 60% H₂SO₄). The mixture was heated at 80°C for 20 min, cooled and the absorbance was measured at 540 nm. The amount of LPG was quantitated from the standard curve, which was made with glucose (Rao and Pattabiraman, 1989).

6.0 Ornithine decarboxylase assay

J774A.1 macrophage cells were plated in 60 mm tissue plate at a density of 1x 10⁶ cells/ml. After 48 h of incubation at 37°C in CO₂ incubator, the treated macrophages were scraped off in phosphate-buffered saline (pH 7.4), centrifuged at 5000 rpm for 15 min at 4°C, and resuspended in harvest buffer (50 mM Tris, 10 µM EDTA and 2.5 mM DTT, pH-7.5). The cells were homogenized in an all-glass homogenizer and centrifuged at 5,000
rpm for 10 min at 4°C to pellet the lysed cells. 200 µl of the supernatant was added to 50 µl of the reaction mixture containing 200 µM pyridoxal phosphate, 12.5 mM DTT, 250 mM Tris (pH-7.5), 200 µM L-ornithine and 3 µCi of 14C- ornithine (Seely, Pegg and Poso, 1982). The mixture was incubated at 37°C for 1 h in an airtight tube and the evolved CO2 was trapped in 100 µl of methyl benzethonium hydroxide. The reaction was stopped after 1 h by adding 5N H2SO4 to the reaction mix and again incubating the tubes at 37°C for 30 min. The trapped radioactivity was counted in a toluene based scintillation cocktail fluid in a scintillation counter (1217 Rackbeta, LKB). The results were expressed in nm/hr/mg protein.

7.0 Nitric oxide assay in murine macrophage cell line, J774A.1

A murine macrophage cell line, J774A.1 (5 x 10^5 cells/well) were plated in 96 well micro titre plates and kept in a CO2 incubator (5% CO2) for 12 h at 37°C. J774A.1 was preincubated with specific inhibitors for the indicated period of time at 37°C. Followed by treatment with LPS (1 µg/ml) or LPG (10 µg/ml) for 1 h and incubated at 37°C for 24 h. The samples were then harvested for nitrite concentration, after 24 h supernatants were assayed using an automated procedure based on Griess reagent as described by Stuehr and Nathan (1989). In brief, 100 µl of culture supernants were mixed with 100 µl of Griess reagent (1% sulfanilamide, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 2.5% H3PO4) and incubated at room temperature for 30 min. The absorbance was measured at 540 nm (Green et al., 1982). These results are representative of triplicate samples. Nitrite levels were determined using sodium nitrite (NaN02) as a standard.
8.0 Protein estimation

The protein content of the samples were measured by the following two methods:

(i) Lowry (ii) Bradford method.

8.1 Lowry's method

The protein content was measured using the method of Lowry et al (1951). Different aliquots of the sample were taken and made up to 200 μl with double distilled water. One ml of alkaline copper reagent was added to the sample (1ml of 1% sodium carbonate in 0.1 N sodium hydroxide). The tubes were allowed to stand at room temperature for 10 min and then 300 μl of Folin-Ciocalteau reagent was added to them. The tubes were again allowed to stand for 30 min and the absorbance of the sample was measured at 600 nm against the blank. A standard curve was made with bovine serum albumin (Fraction V) and the protein concentration in the sample was determined by extrapolating from the standard.

8.2 Bradford method

Protein concentration in cell extracts was measured by Bradford assay (Bradford, 1976). 100 μl of standards (ranging from 2-10 μg) and samples were taken and 900 μl of Bradford reagent was added to each. Bradford reagent was prepared by mixing 100 mg of coomassie brilliant blue G-250 in 50 ml of 95% ethanol, to which 100 ml of 85% phosphoric acid was mixed and the volume was made up to one litre with double distilled water. BSA was used as standard.

After mixing the Bradford reagent to the samples and standards, the tubes were kept at room temperature for 5 min and the absorbance was read at 595 nm. The standard curve was plotted and the protein concentration of the samples was computed (Murray et al., 1990).
9.0 Electroporation of *Leishmania*

An appropriate number of cells were grown to mid-late log phase (~2 x 10^7). For each experiment, 1 x 10^8 cells are needed. An appropriate number of cells are spun down in a sterile centrifuge tube at 3000 rpm for 10 minutes at 4°C. The supernatant poured off and the pellet was resuspended in 1 ml of phosphate buffer saline (PBS). The contents were transferred to a new tube and the spin was repeated at 3000 rpm for 10 min and at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml of electroporation buffer (EP buffer) [21 mM HEPES (pH 7.5), 137 mM NaCl, 5 mM KCl, 0.7 mM NaH2PO4, 6 mM Glucose]. The cells were spun down as above. The supernatant was removed and the pellet was again resuspended in EP buffer to 2 x 10^8 cells/ml. 0.4 ml of cells were transferred to the cuvette (BTX 2 mm gap electroporation cuvettes) and placed on ice. DNA was added to the cuvette with a sterile pipette (up to 50 µg for episomal). The cuvettes were kept on ice for 10 min. The cells were zapped using the following BTX settings: 450 V/cm and 500 µF capacitance. After shock, the cuvettes were again kept on ice for 10 min. The cells were transferred to tissue culture flask containing 10 ml of M-199 (20% FCS) without selection antibiotic. The cells were allowed to grow overnight at 22°C. The cell concentration was determined on the following day.

After 24 h drug was added to both the control and the transfected cells [G418 (40 µg/ml)] in case of neomycin resistance gene. Next day, 300 µl aliquot was taken from each flask and added to a new flask containing 5 ml media with drug. All the flasks were kept at 22°C for a week or two till control cells die and transfectants start growing. Later transfectants were subcultured as usual with above drug.
9.1 Luciferase activity

*Leishmania* cells expressing the luciferase gene were harvested by centrifugation, washed, and diluted from $10^1$ to $10^7$ parasites in a luciferase assay buffer (Promega). The samples were lysed with a lysis buffer (5X) at a final concentration of 25 mM Tris pH 7.8, 2 mM DTT, 10% glycerol and 1% Triton-X-100, incubated at room temperature for 30 min, and then kept at -80°C until assayed. An aliquot of 5 μl of parasite lysates was thawed completely, mixed with 20 μl of luciferase assay buffer (20 mM tricine, 1.07 mM (MgCO$_3$)$_4$ . Mg(OH)$_2$ . 5 H$_2$O, 2.67 mM MgSO$_4$, 0.1 mM EDTA, 200 μM ATP, 33.3 mM DTT) and transferred to a luminometer plate (Barbeau, Bernier, Dumais et al., 1997). Luciferase activity was measured using tube scintillation counter. Light output was measured for 20 s/well after a 2-5 s delay. Values were expressed as RLU (relative light units). To measure the luciferase activity in intracellular amastigotes isolated from infected macrophages, macrophage cells were washed to remove non-internalized promastigotes and incubated with the luciferase lysis buffer as described above.

10.0 Isolation of nucleic acid

10.1 Isolation of RNA from *Leishmania* promastigotes

All the glassware and plastic ware were washed with Diethylpyrocarbonate (DEPC) water (0.1% DEPC) and allowed to stand at 37°C for 2 h. They were then rinsed several times with sterile water and the glassware was then heated at 100°C for 15 min followed by autoclaving. RNA electrophoresis tank and gel tray were cleaned with sodium dodecyl sulphate (SDS) solution, rinsed several times with double distilled water, dried with ethanol and then filled with 3% H$_2$O$_2$. After 10 min at room temperature, the tank and tray were rinsed thoroughly with double distilled water and treated with DEPC water. All the working solutions were made in DEPC water.
Total RNA was isolated from the *Leishmania* LUC-transfectants using TRI-reagent (Sigma) according to manufacturer's instructions. The isolated RNA was stored in DEPC water in small aliquot at -80°C.

### 11.0 Electrophoresis of nucleic acids

#### 11.1 Denaturing RNA gel electrophoresis

All precautions were taken to avoid RNase contamination. All the solutions were prepared in DEPC treated double distilled water. 2.0 g of Seakem ME agarose was added to 176 ml of DEPC treated water. The agarose mix was boiled and cooled to 50-55°C followed by addition of 20 ml of 10X MOPS (0.2 M MOPS, 0.05 M sodium acetate, 10 mM EDTA, pH 7.0 adjusted with freshly prepared NaOH solution) and formaldehyde (added to a final concentration of 0.22 M from 12.3 M or 37% stock under the fume hood). It was mixed thoroughly and the gel was casted quickly. RNA samples (~2 µl) were added to 8.8 µl of sample buffer (50% deionized formamide, 2 M formaldehyde, 1 X MOPS, 1 µl of 1 mg/ml ethidium bromide solution) and heated to 65°C for 15 min and the contents were immediately quenched on an ice bath. Dye buffer (1-2 µl) (5% sterile glycerol, 0.1% bromophenol blue and xylene cyanol FF) was added to the mixture. The samples were loaded into the slots of gel, which was submerged under running buffer (1X MOPS, 0.22 M formaldehyde solution). The gel was run over night at low voltage (20-30 volts) or for 4 h at 90 V.

### 12.0 Transfer of nucleic acid from gel to nylon membrane

#### 12.1 Northern blot neutral transfer

The transfer of RNA from formaldehyde agarose gels to nylon membrane was also achieved by the capillary method as described by Sambrook et al., 1989. The RNA samples
were separated on a denaturing RNA gel electrophoresis (section 11.1). Gels containing formaldehyde were less rigid than the native one so they were handled carefully. The gel was placed in glass dish containing 0.05 M NaOH in 1X SSC for 15 min with gentle agitation. The NaOH solution should be freshly made for this step from pellets. The gel was rinsed in several volumes of 10X SSC for 20 min, which facilitate removal of formaldehyde from gel. This step was repeated once more. The RNA was transferred to nylon membrane in 10X SSC solution by capillary transfer assembly. A sponge was placed in a baking dish and adequate volume of 10X SSC was added until the sponge was fully saturated. A filter paper (larger than size of the gel) was placed above the sponge and saturated with 10X SSC. After neutralization, the gel was placed on the top of the filter paper in an inverted position so that the under side was now uppermost and also no air bubbles remained trapped under the gel. Parafilm was kept over areas of sponge and filter paper not covered by gel. Presoaked nylon membrane of the same size of the gel was layered on the gel so that no air bubble remained trapped under the membrane. 2-3 pieces of presoaked 3 mm Whatmann filter papers were kept over the membrane. Paper towels were placed on the filters with a flat plate and a load on the top. The DNA was kept for transfer, overnight.

Next day, the blot was carefully removed with the gel and the marker positions were carefully marked. After completion of transfer, the membrane was briefly rinsed in 2X SSC for 10 min and RNA was cross linked to membrane by in a Stratagene UV cross linker. The membrane can be used immediately for Northern hybridization or kept in a sealed plastic bag until hybridization.
13.0 Labeling the probe for Northern hybridization

13.1 Promega Prime-a-Gene Labeling System

Approximately 1-25 ng of DNA to be labeled was taken in a total volume of 1-5 μl in a 0.5 ml eppendorf tube. The DNA was heated at 95-100°C for 2 min. The tube was rapidly chilled in an ice bath. Immediately, 10 μl labeling buffer (5X), 2 μl of mixture of unlabeled dNTPs, 2 μl of nuclease-free BSA, 5 μl (50 μCi) of [α-32P] dCTP and 5 units of Klenow fragment were added to the tube having denatured DNA. The final volume was made upto 50 μl with nuclease free water. The tube was incubated at room temperature for a minimum of 1 h. The reaction was stopped by heating the tube at 95-100°C for 2 minutes and subsequently chilling in an ice bath.

13.2 Removal of Unincorporated Radioactivity from DNA probe

Sephadex G-50 spin column removes all the unincorporated dCTPs from the probe DNA. 2 g of Sephadex G-50 in 100 ml of TE pH 8.0 was added and allowed to swell overnight and autoclaved. One ml syringe was plugged with siliconised glass wool at bottom and filled with Sephadex G-50 up to 1 ml mark position. The syringe was hung in conical falcon tube and centrifuged at 3000 rpm for 3 min at room temperature. This column was washed three times with 100 μl TE. After final wash, labeled DNA was loaded on top of the column and it was again centrifuged at 3000 rpm for 3 min. The unincorporated nucleotides remained in the column and labeled purified DNA came out in the collection tube. The purified DNA probe was stored at -20°C until further use.
13.3 Measurement of incorporation of $\alpha^{32}P$ in the labeling reaction by DE81 binding assay

After the labeling reaction is stopped, 1 µl of reaction mix was spotted on two DE81 filter papers. Both the filters were dried. One filter was put in 10 ml of toluene based scintillation fluid in a scintillation vial. The second DE81 paper was washed 6 times (5 min each wash) in 0.5 M sodium phosphate buffer pH 6.8. The filter was then washed twice with double distilled water for 2 min each. It was washed in 95% ethanol and air-dried. These washings were done to remove unincorporated counts. The air-dried filter was put in 10 ml of toluene base scintillation fluid. Counts (cpm) were taken. The unwashed count was total count and washed on was incorporated count. Percentage of incorporation (incorporated cpm/total cpm) x 100 was calculated.

14.0 Hybridization of Northern Blot

The UV cross linked Northern blots were soaked in 2X SSC for a while and transferred to hybridization plastic bag and 50 µl per cm$^2$ of the membrane prehybridization solution (50% deionized formamide, 0.2X SDS, 5X SSC and 4X Denhardtts reagent and 100 µg/ml denatured sonicated salmon sperm DNA) was poured into the bag. The prehybridization was carried out at 42°C for 3-4 h in a shaking water bath. The probe DNA was denatured in boiling water bath for 10 min and chilled on ice for at least 10 min before adding to the prehybridization bag (10$^6$ cpm/ml of probe was used for hybridization), mixed well and hybridization was carried out for 16-18 h at 42°C. The hybridization buffer was removed and the membrane was washed in excess amount of wash solution I (2X SSC, 0.1% SDS) at room temperature for 15 min followed by another wash for 30 min. It was then washed in wash solution II (1X SSC, 0.1% SDS) at 58°C for 30 min. At this stage, the
blot was monitored for background counts and if high, the washing was done again with wash solution II.

The damp membranes were wrapped in a plastic wrap, not allowing the membranes to dry. It was exposed to an X-ray film and kept at -80°C. The X-ray film was developed after the required exposure time.

15.0 **Stripping probes from the membrane**

Hybridized labeled probes can be washed off from the membrane, which can be used for rehybridization.

15.1 **Stripping of mixed hexamer probe**

The hybridized blot was washed twice with 0.4N NaOH for 15 min each at 42°C. It was washed again with neutralizer (0.1X SSC, 0.1% (w/v) SDS, 0.2M Tris-HCl (pH 7.5)) for 20 min at room temperature. The filter was dried by pressing between two 3 mm Whatmann filters and it was stored in a sealed bag.

16.0 **Developing autoradiogram**

The autorad cassette was taken out from -80°C and kept in the dark room to come to room temperature. In the dark room, the exposed X-ray film was washed in developer for 3-4 min and transferred in water. It was washed in water for 3-4 min. The film was then kept in fixer for 10-15 min. The fixer was washed off with gentle flow of water for a while. The film was air dried (Sambrook et al., 1989).

17.0 **SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

A discontinuous buffer system is required for SDS-PAGE, where electrophoresis is carried out in vertical gels. Polyacrylamide is a polymer of acrylamide and N,N’-methylene-bis-acrylamide. This polymerization was catalyzed by N-N'-N'-tetramethyl
ethylenediamine (TEMED). Acrylamide solution (30%) was prepared by dissolving 29% (w/v) acrylamide and 1% (w/v) bis acrylamide in double distilled water. The protein resolution limit of polyacrylamide gel varies with the concentration of acrylamide present in the gel.

<table>
<thead>
<tr>
<th>Acrylamide Concentration</th>
<th>Linear range of Separation (KD)</th>
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<tbody>
<tr>
<td>15%</td>
<td>12-34</td>
</tr>
<tr>
<td>10%</td>
<td>16-68</td>
</tr>
</tbody>
</table>

Bio Rad’s “mini Protein II electrophoresis cell” and Bangalore Genei made “regular dual model” were used during protein analysis, which has 8.0 X 7.3 cm and 16 X 14 cm gel size respectively. The former has gel thickness of 0.75 mm and later has a thickness of 1.0 mm.

The cleaned glass plates were set and sealed on the vertical stand to cast the gel. The gel solution was prepared according to the desired final concentration of acrylamide using a 30% stock solution. The gel solution composed of acrylamide mixture at desired percentage, 0.375 M Tris (pH 8.8), 0.1 % SDS and 0.1 % ammonium persulphate (APS). The solution was mixed well after adding TEMED (8 µl for 10 ml of solution) and quickly poured between the plates. 0.5 to 1.0 ml of water-saturated butanol was layered on the resolving gel solution and kept for at least 40 min at room temperature for polymerization. Butanol was washed off once polymerization had occurred. The comb was fixed on the resolving gel and the stacking gel solution was poured onto the resolving gel. The stacking gel solution was prepared by combining 5 % acrylamide (from 30 % stock), 0.125M Tris (pH 6.8), 0.1% SDS and 0.1 % APS and polymerization was catalyzed by TEMED. It was kept for at least 30 min at room temperature for polymerization. The gel was fixed on the electrophoresis apparatus and running buffer (25 mM Tris, 250 mM glycerine and 0.1%
SDS) was added to the electrophoresis tank. The protein samples were prepared by mixing with an equal volume of 2X gel loading buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.125 M Tris pH 6.8 and 0.004 % bromophenol blue). The mixture was boiled for 10-15 min in a boiling water bath. Samples were loaded in slots and run at 8 volt/cm till the dye head moved upto the resolving gel. The voltage was increased to 15 volt/cm till the dye reached the bottom of the gel (Sambrook et al., 1989).

17.1 Preparation of cell lysates

Stimulated cells (1x 10^6/sample) were washed twice with ice-cold TBS (50 mM Tris-HCl (pH 7.4), 400 mM NaCl, 1 mM sodium orthovandate) and harvested with a plastic scraper. The cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovandate, 10 mM NaF, 1 mM DDT, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 µg/ml each of leupeptin and aprotinin) by incubating on ice for 30 min. Lysate was then centrifuged at (15,000 x g) at 4°C for 10 min, and supernatants was transferred to fresh tubes and stored at -80°C till required. Protein concentration of the lysates was determined using a colorometric assay against a BSA standard (Bradford, 1976).

18.0 Visualizing the proteins in SDS-PAGE

18.1 Staining with coomassie brilliant blue (R250)

When electrophoresis was over, the gel was removed from the plates and transferred to 15 times the gel volume of staining solution (0.25% w/v Coomassie brilliant blue R-250, 25% methanol and 10% acetic acid). The gel was incubated for 4 h to overnight at RT with gentle shaking.
18.2 Destaining the gel

The gel was transferred from the staining solution to excess volume of destaining solution (25% methanol and 10% acetic acid) and kept at RT with shaking. 2-3 changes of destaining solution made the protein bands clearly visible with no background stain. The sensitivity of this staining method was 0.1-0.5 mg protein per lane (Sambrook et al., 1989).

19.0 Western blot analysis:

19.1 Transfer of the protein from the gel to the membrane

10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run with protein sample along with rainbow marker. The gel was washed with 10-15 ml of transfer buffer (3.03 g Tris, 14.4 g glycine, 200 ml methanol, volume was made up to one liter with double distilled water).

A piece of nitrocellulose membrane was cut to the same size of the gel. The membrane was soaked for 5 min in double distilled water and then put in transfer buffer. If PVDF membrane was used, the membrane was initially dipped in methanol followed by wash in double distilled water for 5 min and then in transfer buffer for 20 min. Two 3 mm Whatmann filter papers, slightly bigger than the gel and two fiber pads were also soaked in transfer buffer. The transfer sandwich was assembled in the following order: fiber pad – filter paper – gel – membrane – filter – fiber pad. Any bubble between gel and membrane was removed. The transfer sandwich was clamped in the gel holder cassette and was immersed in transfer buffer in the transfer tank.

Proteins move from the cathode to the anode when potential is applied. Transfer was performed at low voltage (22 volts) overnight or at high voltage (65 volts) for 1.5-2 h. After the transfer was over, a portion of the membrane was stained with Ponceau-S or
Amido-black to check the transfer. Bio Rad’s mini trans-blot electrophoresis transfer cell was used for this purpose.

19.2 Antibody blotting to the membrane

Nitrocellulose filter was incubated with TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk for at least 2 h to block nonspecific protein binding. The blocked membrane was washed thrice for 10 min each with TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20).

Primary antibodies were diluted (1:100) in TBST and applied to the filter for at least 2 h with constant shaking at room temperature. The blots were washed with TBST thrice and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted up to 1:5000 in TBST) for 1 h with constant shaking at room temperature. The membrane was then washed thrice with TBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) for 10 min each. The blot was now ready for developing.

19.3.0 Immunoblotting detection

Immunoblotting detection was done by autoradiography.

19.3.1 Colour developing method by Chemiluminescence

Horseradish peroxidase (HRPO) conjugated antibodies were used in the blot; chemiluminescence kit supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA) was used. Equal volumes of reagent A (luminol) and reagent B (oxidizing agent) were mixed and spread over the blot uniformly. The solution was drained off after 1-2 min and the membrane was covered with a plastic wrap. In the dark room, a piece of Hyperfilm (from Amersham Biosciences) was exposed to the membrane for a maximum of 1 min. The film was developed by standard method as described earlier. Autoradiograms for the
phosphorylated proteins were analyzed by using model FLA 5000 imaging densitometer (Fuji, Japan).

20.0 Electrophoretic mobility shift assays (EMSA)

For the preparation of nuclear extracts (Schreiber, Marthias, Muller and Schaffner, 1989; Chaturvedi, La Pushin and Aggarwal, 1994) the treated cells were washed twice with ice-cold PBS before resuspending in 1 ml cold hypotonic “low salt buffer” buffer A (20 mM HEPES (pH 7.9), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.2% Triton X-100, 0.2 mM PMSF, 0.4 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.2 mM DTT). The cells were allowed to swell on ice for 20 min then centrifuged at 13,000 x g at 4°C for 15 min and resulting nuclear pellet was resuspended in 30 μl ice-cold “high salt buffer” buffer B (20 mM HEPES (pH 7.9), 20% glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.2% Triton X-100, 0.1 mM PMSF, 0.2 μg/ml leupeptin, 0.5 μg/ml aprotinin, and 0.1 mM DTT) by occasionally tapping the nuclear pellet on ice for 1 h. The nuclear extract was centrifuged at 13,000 x g for 10 min at 4°C and the supernatant was collected and stored in aliquots at -80°C until use. The protein content of the extract was measured by the method of Bradford (Bradford, 1976). Binding reactions were initiated by incubating nuclear extract (4 to 6 μg protein) with double stranded poly (dI-dC) (1 μg/μl)(Pharmacia Biotech, St. Albans, UK), under specific salt / pH conditions in a binding buffer (20 mM HEPES (pH 7.9), 3.4% glycerol, 1.5 mM MgCl₂, 1.0 mM DTT) and 1.0 ng/μl of 5’ end ³²P-labeled dsDNA oligonucleotide in a total volume of 60 μl. Double stranded DNA (dsDNA, 10 ng) was labeled with [gamma-³²P] ATP and T4 polynucleotide kinase in a kinase buffer (New England Biolabs, Beverley, MA). This mixture was incubated for 30 min at 37°C, and the reaction was stopped with 5 μl of 0.2 M EDTA. The
labeled oligonucleotide was extracted with phenol/chloroform and passed through G-50 spin column. The dsDNA oligonucleotide probes used in the present work are: the consensus NF-κB binding site corresponding to (5'-'AGTTGAGGGGACTTTCCCAGGC-3') and double stranded mutated oligonucleotide, (5'-AGTTGAGGCGACTTTCCCAGGC-3') was used to examine the specificity of binding of NF-κB to the DNA, for IRF-E in the iNOS promoter, (5'-CACTGTCAATATTTCACCTTCAAT-3') oligonucleotides were purchased from Bangalore Genei (Bangalore, India) and the dsDNA oligonucleotide, for AP-1 consensus sequence (5'-CGCTTGATGAGTCAGCCGGAA-3'), oligonucleotide was kindly gifted by Dr. Shekhar Reddy, John Hopkins University, Baltimore, USA. The specificity of binding was examined by competition with the unlabeled corresponding dsDNA oligonucleotide by adding simultaneously with the labeled probe. The resultant DNA-Protein complexes were resolved from the free labeled DNA by electrophoresis in non-denaturing 8% (w/v) polyacrylamide gels. The gels were subsequently dried and autoradiographed. Visualization and quantitation of radioactive bands were conducted by PhosphorImager (Fuji film BAS-1800, Japan) using Image Quant software.

21.0 Statistical analysis

Statistical analysis was performed by standard t-test (Fisher et al., 1957). Student's test was performed on the data and a probability value (P) of less than 0.05 was considered significant.