Chapter 3

Materials and Methods

3.1. Materials

Roswell Park Memorial Institute medium (RPMI-1640) (with and without phenol red) and Dulbecco's modified Eagle's medium (DMEM) (with and without phenol red) were purchased from Sigma Chemical Company (St. Louis, MO). Fetal calf serum and dextran-coated charcoal stripped fetal calf serum (DCC-FCS) were procured from Biological Industries (Kibbutz Beit Haemek, Israel). 0.22 μm membrane filters were obtained from Millipore (Billerica, MA).

Deoxy-ribonucleotide (dNTP) mix, magnesium chloride ($MgCl_2$), and pGEM-T_{Easy} sequencing vector were purchased from Promega (Madison, WI). Taq DNA polymerase was obtained from New England Biolabs (Beverly, MA), while Superscript II First strand synthesis kit and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). 100 bp DNA ladder, 1 kb DNA ladder, and 6X DNA loading dye were obtained from MBI Fermentas (Ontario, Canada). Synthetic oligonucleotides were obtained from Sigma GENOSYS (Bangalore, India) or Microsynth (Germany). MinElute™ Gel extraction kit was purchased from Qiagen (GmbH, Hilden).

CB-X protein assay kit was purchased from G-Biosciences (St. Louis, MO). Ammonium persulphate (APS) and N, N, N′, N′ - tetramethylene-diamine (TEMED) were obtained from Sigma Chemical Company (St. Louis, MO). Rainbow™ protein markers, nitrocellular membranes, and enhanced chemiluminescnence detection reagent were procured from Amersham Biosciences (Piscataway, NJ). Anti-estrogen receptor α/β, anti-estrogen receptor-α, and anti-actin antibodies were purchased from Calbiochem (Darmstadt, Germany). Anti-phospho CREB, anti-total CREB, anti-phospho ERK, and anti-total ERK were obtained from StressGen Biotechnologies (Victoria, BC). Anti-Bcl-2, anti-Bax, anti-Bad, and anti-Cytochrome C antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA). Anti-histone dimethyl lysine antibody was procured from Upstate (VA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Ambion (Austin, TX). Anti-Clusterin antibody was a kind gift from Dr.C.Yan Cheng of the Population Council, New York, USA. Secondary anti-mouse antibody conjugated to Alexa fluor 488 was purchased from Molecular Probes (Eugene, OR). Secondary anti-

mouse and anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) were obtained from Jackson Immunoresearch (Cambridgeshire, UK).

Chloroform, isopropyl alcohol, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate, sodium chloride, glycine, acetic acid, hydrochloric acid, sulphuric acid, Tris, Tris-HCl, potassium chloride, di-potassium hydrogen phosphate, formaldehyde, phenol, hydrogen peroxide, and methanol were obtained from Merck (Mumbai, India). Ethanol was purchased from Fluka Chemie GmbH (Buchs, Switzerland).

17 β-Estradiol (cyclodextrin-encapsulated), estradiol conjugated to BSA (E2-BSA), E2-BSA conjugated to FITC (E2-BSA-FITC), BSA-FITC, propidium iodide, Lipopolysaccharide from *S.typhosa*, Histopaque 1077, PD 98,059, Bisiondoleylmaleimide (BIM VIII), Verapamil, Pimozide, EGTA, EDTA, Phorbol myristate acetate (PMA), nigericin, amiloride, and aminoguanidine were purchased from Sigma Chemical Company (St. Louis, MO). Fluo-3acetoxymethyl ester (Fluo-3-AM), 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), 5-(and -6) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), SNARF (5-(and -6)-carboxy SNARF®1-AM), Sodium Green™ tetracetate, and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). Ketamine was purchased from Neon Pharma (Mumbai, India), while Xylocaine 2% was purchased from AstraZeneca (Bangalore, India).

siRNA against ER-α, ER-β, and Bcl-2 were obtained from Dharmacon (Lafayette, CO), while the Cy3-labeled negative control siRNA was purchase from Ambion (Austin, TX). The siRNA transfection reagent, TranspassR2 was procured from New England Biolabs (Ipswich, MA). The Vybrant apoptosis detection system was purchased from Promega (Madison, WI). Alexa fluor 488 labeled dead *E.coli* particles were obtained from Molecular probes (Eugene, OR). Enzyme linked immunosorbent assay (ELISA) kits for detection of IL-1β, IL-4, IL-6, IL-8, IL-12, IFN-γ, and TNF were obtained from BD Biosciences (NJ, USA).

All other chemicals used in this study unless otherwise mentioned were purchased from Sigma Chemical Company (St. Louis, MO).

3.2. Methods

3.2.1. Cell culture techniques

3.2.1.1. Cell lines and cell culture

THP-1 acute monocytic leukemia cell line (TIB-202) was purchased from American type culture collection (ATCC) (Manassas, VA). These suspension cells were maintained in culture at 37°C in RPMI-1640 medium supplemented with 10% FCS. They were sub-cultured when the cell density reached ~1X10^6 per mL. To induce differentiation of these monocytic suspension cell cultures to adherent macrophage phenotype, they were subjected to treatment with PMA at a concentration of 10 ng/mL for 36 h. Forty eight hours prior to experimentation, the cells were transferred to phenol-red free RPMI-1640 medium supplemented with 10% dextran-coated charcoal stripped FCS. This was performed to remove all traces of exogenous estrogens as phenol red in culture medium is known to be a weak estrogen (1) and FCS contains multiple steroid hormones which are removed upon stripping with dextran-coated charcoal.

MCF-7, a breast carcinoma cell line was obtained from ATCC (Manassas, VA). They were maintained in culture at 37°C in RPMI-1640 medium supplemented with 10% FCS and were routinely sub-cultured when the cells reached a confluency of around 80%.

3.2.1.2. Peripheral blood monocyte isolation and macrophage differentiation

Peripheral blood (30 mL) was collected by venipuncture from healthy male volunteers after obtaining an informed consent and in accordance to the regulations of the Institutional Human Ethics Committee (National Institute of Immunology, New Delhi, India). The peripheral blood mononuclear cell (PBMC) population was isolated by density gradient centrifugation using Histopaque 1077, where, human whole blood was layered on Histopaque 1077 and centrifuged at 400 x *g* for 35 min at 25°C. The mononuclear cell population was isolated from the plasma-histopaque interface, and the monocytes were further purified by washing off the non-adherent cells after incubating the total PBMC for 1 h at 37°C. The homogeneity of the obtained

population was determined by analyzing cells immunostained with an antibody against CD14 conjugated to FITC and the purity obtained was approximately 85% monocytes, the remaining being lymphocytes. The monocytes were further cultured in the presence of human AB serum for 7 days to allow differentiation to macrophages. At the end of 7 days post-isolation, greater than 95% of cells in culture are monocytes, with the majority of lymphocytes undergoing neglect induced death.

3.2.1.3. Protocol for propagation and maintenance of *Leishmania major* promastigotes

Leishmania major strain (MHOM/Su73/5ASKH) was a kind gift from Dr. Satyajit Rath, Immunobiology Laboratory, National Institute of Immunology, India. *L.major* promastigotes were cultured at 23°C in modified DMEM (DMEM (1 L) supplemented with sodium bicarbonate (3.7 g), HEPES (5.96 g), hemin (5 mg), biotin (1 mg), adenine (13.36 mg), xanthine (7.6 mg), triethanolamine (0.5 mL), and tween 80 (40 mg)) supplemented with 10% FCS. It is known that long term culture of *L.*major promastigotes results in loss of their virulence (2). Hence, to maintain the virulence of these parasites, they were propagated in mice footpad. Towards this end, the stationary phase *L.major* promastigotes were resuspended in Hank's balanced salt solution and $2x10^6$ promastigotes were injected into the footpad of female BALB/c mice. 6 weeks post-infection, the infected footpad was dissected and the lesion harvested. The obtained lesion was minced and resuspended in modified DMEM supplemented with 10% FCS and placed in 23°C incubator to allow differentiation of intracellular amastigotes to promastigotes. This cycle of harvesting promastigotes from footpad lesions was performed every 6 weeks to maintain the virulent phenotype of this parasite.

3.2.2. Biochemical and cell biology techniques

3.2.2.1. Assay for cell viability by propidium iodide dye exclusion method

Propidium iodide (PI) is a DNA intercalating fluorescent dye which is excluded by viable cells with intact membranes, however, dead and dying cells with damaged membranes take up the dye. To assess viability, cells after appropriate

treatment were harvested by centrifugation at 250 x *g* for 5 min following which they were resuspended in 1x PBS (pH 7.5). PI was added at a final concentration of 1 μg/mL and incubated for 5 minutes following which the cells were pelleted by centrifugation and washed once with PBS. These cells were analyzed for uptake of PI by either flow cytometry in FL2 channel (570 nm) or by fluorescence microscopy using a G2A filter block.

3.2.2.2. Assay for cell viability by Trypan blue dye exclusion method

Trypan blue is a diazo vital stain which selectively colours the dead cells blue that can be visualized under light microscope. Equal volumes of cell suspension and 0.4% trypan blue dye were mixed and incubated at room temperature for 5 min. 10 μL of stained cells were loaded on to a hemocytometer and a count of the number of viable and dead cells were made. This procedure was carried out routinely to ensure that cell viability is >95% before plating cells for experiments.

3.2.2.3. Assay for detection of apoptosis by Annexin-V/PI staining

The Vybrant apoptosis assay kit was used to perform Annexin-V/PI staining as described previously (3). The assay is based on the principle that apoptotic cells show loss of membrane asymmetry by exposing phosphatidylserine on the outer surface of the plasma membrane for which Annexin-V, a phosphlipid binding protein, shows high affinity. Hence, Annexin-V conjugated to Alexa fluor 488 binds to phosphatidylserine exposed on apoptotic cells, while propidium iodide binds to nucleic acids of all non-viable cells including necrotic and apoptotic cells. Thus, flow-cytometric analysis of Annexin-V/PI stained cells reveals distinct cellular populations, with the viable cells displaying little or no fluorescence; the early apoptotic cells show green fluorescence of Annexin-V conjugated to Alexa fluor 488; the late apoptotic cells display both green and red fluorescence, while necrotic cells show red fluorescence.

The cells after appropriate treatment were harvested by centrifugation at 250 x *g* for 5 min and were given two washes with ice-cold 1X PBS following which they were resuspended in 100 μL of ice-cold 1X Annexin binding buffer (50 mM HEPES,

700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4). 5 µL of Annexin-V conjugated to Alexa fluor 488 and 1 µL of working solution of PI (100 µg/mL) were added to the 100 µL cell suspension. Cells were incubated for 15 min at room temperature. Following this, 400 µL of 1X Annexin binding buffer was added to dilute the sample. The samples were placed on ice. The fluorescence was measured by flow cytometry in FL1 and FL2 channels for Annexin-V-Alexa fluor 488 and PI fluorescence respectively.

3.2.2.4. Immunocytochemistry

a. Immunostaining in fixed cells:

The cells were fixed with 4% formaldehyde for 20 min, following which two washes were given with ice-cold PBS. Permeabilization and blocking were performed simultaneously by incubating the formaldehyde fixed cells in PBS containing 0.1% saponin and 3% normal goat serum for 30 min. The cells were washed once with ice-cold PBS. The permeabilized cells were incubated with the primary antibody at an appropriate dilution for 1 h at room temperature following which three washes with ice-cold PBS was given. These cells were then incubated with fluorophore conjugated secondary antibody (IgG) for 1 h at room temperature following which three washes with ice-cold PBS were given. The nuclei were stained with Hoechst 33342 at a concentration of 1 µg/mL for 2 min at room temperature. The staining was then visualized under a Nikon TE2000E fluorescence microscope using appropriate filter blocks. Image acquisition was carried out using a high-resolution Retiga *Exi* camera (Q-imaging, Surrey, BC, Canada) and subsequent image analysis was performed on Image-Pro Plus software v5.5 (Media Cybernetics, Silver Spring, MD). Alternatively, the fluorescence staining was detected by flow-cytometry (BD-LSR, Beckton Dickinson, NJ, USA) using an air-cooled argon ion laser (488 nm) at appropriate florescence channels. Subsequent data analysis was performed on WinMdi software (Microsoft, v 2.9).

b. Immunostaining in Live cells:

Immunostaining on live cells was performed by harvesting and resuspending cells in ice-cold PBS. The cells were then incubated with an appropriate dilution of the primary antibody for 1 h at 4°C following which two washes were given with ice-

cold PBS followed by incubation with fluorophore labeled secondary antibody at appropriate dilution for 30 min at 4°C. The fluorescence was then visualized under a fluorescence microscope or analyzed by flow cytometry.

3.2.2.5. siRNA transfection

THP-1 macrophages and human peripheral blood monocyte derived macrophages were transfected with SMARTpool Bcl-2 siRNA (15 pmol), or ER-α siRNA (100 pmol), or ER-β siRNA (100 pmol), or with negative control siRNA (15 pmol or 100 pmol) using TranspassR2 transfection reagent. Prior to transfection, the cells were depleted of serum by washing 2x with serum-free media. The transfection complex was prepared by diluting 0.5 µL of transfection reagent A and 1.0 µL of transfection reagent B to 400 µL of serum-free media and siRNA's were added to the mix at an appropriate concentration and incubated for 20 min at room temperature. The formed transfection complexes were transferred gently using a large bore pipette tip to 10^5 cells/well grown in 24 well plates and incubated for 6 h, following which fresh complete medium was added. Transfection efficiency was estimated by observing Cy3-fluorescence of the negative control siRNA with a Nikon TE2000E fluorescence microscope using a tetramethyl rhodamine filter (530-580 nm). For all transfections, target protein knockdown was assessed 24 h after transfection by probing extracts of transfected cells on Western blots using appropriate antibodies.

3.2.2.6. SDS-PAGE and Western blot

Whole cell extracts were prepared by treating cells with lysis buffer (0.125M Tris, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol), and protein estimation was performed using CB-X protein assay kit as per manufacturer's protocol. Lysates were resolved on 12% SDS-PAGE gel, following which Western transfer was performed onto nitrocellular membranes using a BioRad Western transfer apparatus. The blots were incubated with 5% blotto (non-fat dry skimmed milk) in 0.05% PBS-Tween 20 for 1 h to block non-specific binding sites following which they were incubated for 1 h with primary antibody at an appropriate dilution prepared in 1% blotto in 0.05% PBS-Tween-20. The blots were washed 3x with 0.05% PBS-Tween-20 at 5 min intervals following which they were incubated for 1 h with secondary

antibody at an appropriate dilution. The immunoreactivity was detected by enhanced chemiluminescence using an ECL detection kit (Amersham Biosciences) and were recorded on X-ray films after appropriate exposure and development. It is important to note that the blots for probing phosphorylated proteins were performed using 1% BSA as blocking agent instead of blotto.

3.2.2.7. Sub-cellular fractionation

Sub-cellular fractionation of THP-1 macrophages was performed after lysis with hypotonic buffer. THP-1 macrophages after appropriate treatment were allowed to swell for 10 min in hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) followed by homogenization with 50 strokes using a Dounce homogenizer. More than 90% cellular lysis was ensured by visualizing under a light microscope, and immediately after lysis, the mitochondrial membranes were stabilized by addition of 2.5x mitochondrial stabilization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, 2.5 mM EDTA, pH 7.5) to a final concentration of 1x. The homogenate was centrifuged at 1300 x *g* for 15 min to isolate the nuclear fraction. The post-nuclear fraction was further centrifuged at 17,000 x *g* for 15 min in an ultracentrifuge (Beckman Optima XL-100K ultracentrifuge) to isolate the mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000 x *g* for 1 h to obtain the membranous fraction as a pellet and the supernatant obtained was the cytosol. The homogeneity of the obtained fractions was determined by probing for fraction specific proteins by Western blotting.

3.2.2.8. Intracellular free Ca^{2+} assay

Cytosolic free Ca^{2+} was measured using the fluorescent Ca^{2+} indicator Fluo3-AM. THP-1 macrophages were harvested and resuspended in Kreb's buffer (118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, 1.5 mM $CaCl_2.2H_2O$). Fluo3-AM was added at a final concentration of 0.5 μM alongwith 1 μM Pluronic acid F-127 to aid in dispersal of the dye. The cells were subjected to constant mixing by end-to-end rotation and incubated with the dye for 20 min at room temperature following which the cells were pelleted and resuspended in fresh Kreb's buffer and incubated for further 15 min to allow complete de-

esterification of the dye. Basal fluorescence was measured in a fluorimeter (BMG Fluostar Optima spectrofluorimeter) at an excitation of 480 nm and an emission of 520 nm. Appropriate treatments were initiated and kinetic fluorescence measurements were performed with the temperature being maintained at 37°C. At the end of each experiment, a calibration was performed to convert the fluorescence values into absolute calcium concentration using the following formula:

$$[Ca^{2+}]_i = K_d\,[(F-F_{min})/(F_{max}-F)]$$

where, K_d is the dissociation constant of Ca^{2+}-Fluo3-AM complex (325 nM), and F represents the fluorescence intensity of cells, F_{max} represents the maximum fluorescence (obtained by treating cells with 1 μM Ca^{2+} ionophore A234187 in the presence of 4 mM $CaCl_2$), and F_{min} corresponds to the minimum fluorescence (obtained by treating cells with 4 mM EGTA).

3.2.2.9. Measurement of intracellular pH

Intracellular pH measurement was performed using the long-wavelength fluorescent pH indicator carboxy SNARF-1 AM. THP-1 macrophages were resuspended in serum-free and phenol-red free RPMI-1640 medium (10^6 cells/mL) and incubated at room temperature for 15 min with SNARF-1 AM at a final concentration of 1 μM. The cells were washed once in fresh serum-free media and incubated for 20 min for complete de-esterification of intracellular acetoxymethyl esters. *In situ* calibration of SNARF-1 AM was performed to determine the pK_a of the dye at 37°C by using the ionophore Nigericin (10 μM), which maintains the intracellular pH the same as that of the controlled extracellular medium in a buffer containing high-K^+. Appropriate groups were subjected to different treatments and fluorescence measurements were commenced in a spectrofluorimeter (Perkin Elmer, Waltham, MA, USA) followed by kinetic analysis. The pH was calculated from the fluorescence measurements using the following formula:

$$pH = pK_a - \log\,[\{(R-R_B)/(R_A-R)\}\ X\ (F_{B(\lambda 2)}/F_{A(\lambda 2)})]$$

where pK_a of carboxy SNARF-1 AM is 7.5 at 37 °C. R is the ratio of fluorescence intensities (F) measured at two emission wavelengths, 580 nm (λ1) and 640 nm (λ2), with fixed excitation at 514 nm. The subscripts A and B represent the limiting values

at the acidic and basic endpoints of the titrations. Na^+ free and HCO_3^- free buffer were prepared as described by Khaled et al., (4).

3.2.2.10. Assay for intracellular Na^+ measurement

Intracellular Na^+ measurement was performed using the fluorescent Na^+ indicator Sodium Green™ tetracetate. THP-1 macrophages were resuspended in phenol-red free RPMI-1640 medium and incubated with Sodium Green™ at a final concentration of 1 μM for 20 min at room temperature. The cells were washed once with fresh serum-free media to remove excess probe following which kinetic fluorescent measurements were commenced in a spectrofluorimeter (BMG Fluostar Optima) at an excitation of 480 nm and emission of 520 nm. *In situ* calibration to determine the dissociation constant (K_d) of the dye at 37°C was accomplished by using the indicator dye in solutions of precisely known free Na^+ concentration in the presence of the pore forming antibiotic gramicidin (10 μM). Intracellular Na^+ was calculated using the following formula:

$$[Na^+]_{free} = K_d \{(F-F_{min})/(F_{max}-F)\}$$

where, K_d of the dye is 5.7 mM at 37°C, F is the fluorescence of the experimental sample, F_{min} is the fluorescence in the absence of Na^+ and F_{max} is the fluorescence under saturating concentrations of Na^+ in the presence of gramicidin (10 μM).

3.2.2.11. Detection of intracellular reactive oxygen species generation

The generation of reactive oxygen species in macrophages was detected by fluorimetry using the fluorescent dye CM-H_2DCFDA, which can detect hydrogen peroxide, hydroxyl radical, peroxyl radical, and peroxynitrite anion (5, 6). To perform the assay, THP-1 macrophages were washed and resuspended in serum and phenol-red free RPMI-1640 medium and incubated at room temperature for 30 min in the presence of CM-H_2DCFDA at a final concentration of 1 μM. Subsequently the cells were washed once with fresh media to remove the excess probe and fluorescence measurements were commenced on a spectrofluorimeter (BMG Fluostar Optima) at an excitation of 480 nm and an emission of 520 nm. Appropriate treatments were initiated and time-kinetic measurements were carried out and the values obtained

were represented as arbitrary fluorescence units and comparisons were made against the untreated control samples. Exogenous addition of hydrogen peroxide to cells was used as a positive control for the assay.

3.2.2.12. Measurement of intracellular nitric oxide (NO) generation

Nitric oxide (NO) generation within the macrophage was detected using the fluorescent NO-sensitive probe DAF-FM diacetate (7). THP-1 macrophages were harvested and resuspended in serum and phenol-red free RPMI-1640 medium and incubated at room temperature for 30 min in the presence of 1 μM DAF-FM diacetate dye. The cells were washed once with fresh medium to remove the excess probe and kinetic fluorescent measurements were performed on a spectrofluorimeter (BMG Fluostar Optima) at an excitation of 480 nm and emission of 520 nm. Time kinetic measurements were performed after appropriate treatment and the values were represented as arbitrary fluorescence units with the comparisons being made against the fluorescence of the control cells. SNAP (S-nitroso-N-acetylpenicillamine), a photoactivatable nitric oxide donor (8) was used as positive control in the assay.

3.2.2.13. Measurement of phagocytic ability of macrophages

The phagocytic ability of macrophages was determined by monitoring the uptake of Bioparticles® Alexa fluor 488 labeled dead *E.coli* (Molecular Probes, Eugene, OR). $2X10^5$ THP-1 macrophages were plated per well in a 24 well plate. Alexa fluor 488 labeled dead *E.coli* particles were opsonized with an opsonizing reagent obtained from Molecular Probes. These opsonizing reagents are derived from purified rabbit polyclonal IgG antibodies that are specific for *E.coli*. These opsonized bioparticles® were transferred to the macrophage culture at an multiplicity of infection (MOI) of 1:10, i.e., 10 bacteria per macrophage. The plates were briefly centrifuged at 250 x *g* to allow the bacteria to settle at the bottom of the plate and were then transferred to an incubator maintained at 37°C and 5% CO_2 in air for 1 h. The culture medium was aspirated to remove excess unbound bacteria and the cells were washed 3x with ice-cold PBS. To eliminate fluorescence from non-phagocytosed bacteria adhering to the macrophage membrane, 0.25 mg/mL Trypan blue was added and incubated for 10 min to quench the fluorescence of extracellular

bacteria. The cells were washed once with ice-cold PBS and the uptake of labeled bacteria was analyzed by flow-cytometry.

3.2.2.14. Cytokine ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed to detect cytokine secretion from human THP-1 macrophages upon activation with LPS. The ELISAs were performed according to manufacturer's protocol. Briefly, THP-1 macrophages were subjected to various treatments and after appropriate time interval the cell culture supernatants were harvested. The capture antibodies for the individual cytokines were diluted 1:250 in coating buffer (0.1 M Sodium carbonate, pH 9.5) and 100 µL was aliquoted into each well of a 96 well ELISA plate (BD biosciences). The plates were incubated at 4°C for 16 h following which three washes with 0.05% PBS-Tween-20 were given. Blocking was performed using 200 µL of assay diluent (PBS with 10% FCS) per well for 1 h at room temperature following which 100 µL of appropriately diluted standards and samples were added and incubated for 2 h at room temperature. A total of 5 washes with 0.05% PBS-Tween-20 were given and the plates were subsequently incubated with 100 µL of detection antibody and streptavidin-HRP for 1 h at room temperature following which 5 washes were given. 100 µL of tetramethylbenzidine (TMB) substrate was aliquoted into each well and incubated for 15 min at room temperature in dark following which 50 µL of stop solution (2N H_2SO_4) was added to terminate the reaction. The absorbance was read at 450 nm and the cytokine levels in the samples were derived based on the OD_{450} values obtained with standards of known concentration.

3.2.2.15. *Leishmania major* infection of human macrophages *in vitro*

Human THP-1 macrophages were plated at a density of $2X10^5$ cells per well in a 24 well plate and appropriate treatments were given. The stationary phase *L.major* promastigotes were opsonized with 1% human AB serum in PBS for 5 min at 37°C following which one wash was given with phenol-red free RPMI-1640 medium. The *L.major* promastigotes were added to the macrophage culture at a macrophage: parasite ratio of 1:10 or 1:50 and incubated for 6 h at 37°C following which the unbound parasites were removed by giving 3 washes with warm RPMI-1640 medium.

Fresh complete medium was added and the plates were transferred to 37°C for further incubation. The percentage infection was monitored at appropriate time intervals post-infection by staining the cells with Syto Green 11 nucleic acid dye and the parasite nuclei were visualized by fluorescence microscopy.

3.3. Animal experiments

3.3.1. Bilateral oophorectomy and sham surgery in mice

Bilateral oophorectomy, the surgical removal of both the ovaries, was performed in mice to simulate a condition of estrogen depletion. All procedures in mice were performed after obtaining approval from the Institutional Animal Ethics Committee (National Institute of Immunology, New Delhi). Female BALB/c mice were used in the study.

General anesthesia:

Hair from the skin overlying the left and right dorsal flanks were removed using electrically operated razor. The skin overlying the abdomen was sterilized by wiping with 70% ethanol. Ketamine (100 mg/kg) and xylocaine (2%) (20 mg/kg) were mixed and administered intraperitoneally. The mice were returned to the cage and the onset of anesthetic effect was monitored. The mice were considered to be in surgical anesthesia when there was loss of palpebral reflex, righting reflex, and toe pinch reflex. Respiratory rate and heart rate were monitored continuously.

Bilateral oophorectomy:

The anesthetized mice were operated under strict aseptic conditions inside a laminar flow hood. The mouse was placed over layers of sterile tissue paper and the skin overlying the dorsal flanks was sterilized by wiping with 70% ethanol. The flank was palpated gently to identify the kidney, and an incision (~ 5 mm) was made using a pair of scissors on the overlying skin which penetrated the skin, sub-cutaneous tissue and the muscle layer with the parietal peritoneum being exposed and intact. The para-ovarian pad of fat was identified through the intact peritoneum and a small incision was made on the peritoneum overlying it. The ovarian tissue along with the fallopian tube was mobilized and delivered through the incision site. The ovary was

released from the para-ovarian pad of fat as well as from the peritoneal reflections while care was taken to avoid injury to the ovarian vessels. A ligature was tied around the distal end of the fallopian tube including the ovarian vessels following which the ovary was excised. Hemostasis was secured before the stump of the tube was pushed back into the peritoneal cavity. The peritoneum was closed by continuous sutures using 2-0 silk. The same protocol was followed to perform oophorectomy on the contralateral side. The muscular layer and skin were closed together using surgical clips.

Sham surgery:

Sham surgery was performed on mice as described above except that the ovary and tubes after being delivered from the incision site were pushed back into the peritoneum in an intact state.

Post-operative care:

The mice were returned to a cage and were kept under a 100W bulb light source to prevent hypothermia. Care was taken to ensure that the eyes are kept covered. The respiratory rate and heart rate were monitored till the mice regained complete consciousness. They were fed ab-limitum post-operatively. Metronidazole (20 mg/kg) was added to the drinking water and the mice were fed this medicated water for 5 days post-operatively. On the 7^{th} post-operative day, the health of the wound was observed and the surgical clips were removed from the skin.

3.3.2. *L.major* infection in mouse footpad

$5x10^5$ *L.major* promastigotes were cultured in 5 mL modified DMEM supplemented with 10% FCS. At the end of 5 days of culture, the stationary phase promastigotes were harvested and resuspended in Hanks balanced salt solution at a cell density of $4x10^7$/mL. The cell suspension was aspirated into a 1 mL syringe and 50 µL was injected into the footpad of mice. The mice were returned to the cage and fed ab-limitum. The onset and progression of cutaneous lesion was monitored at 2 weekly intervals by observing an increase in the thickness of the footpad.

3.3.3. Histopathological examination of cutaneous leishmaniasis lesion

The cutaneous lesion developed in the *L.major* infected footpad and tissue from the corresponding region of normal footpad was harvested for histopathological examination. The tissue was fixed in 4% formaldehyde for 24 h following which the tissue was dehydrated by incubating it with ascending concentrations of alcohol (50%, 70%, and 100% ethanol for 1 h each). Subsequently, tissue clearing was performed by incubating with xylene for 1 h following which paraffin embedding was performed. The embedded tissue was cut into multiple sections of 5 μm thickness using a microtome. The paraffin sections were then coated on slides and deparaffinization was carried out by treating with xylene. Subsequently, hematoxylin-eosin staining was performed and the slides were visualized under a light microscope.

3.4. Molecular biology techniques

3.2.1. Total RNA isolation

Total RNA was isolated from cells using TRIzol reagent following the manufacturer's protocol. Briefly, $2x10^6$ cells were harvested by non-enzymatic cell dissociation buffer and washed once with PBS. The cell pellet was lysed with 1 mL ice-cold TRIzol reagent. The lysate was centrifuged at 12,000 x *g* for 10 min at 4°C to pellet down cellular debris, polysaccharides, and high molecular weight DNA. The supernatant was gently decanted into a fresh microcentrifuge tube and 200 μL of chloroform /mL of TRIzol was added and the tube was shaken vigorously for 15 s. The mixture was incubated at room temperature for 2-3 min before centrifugation at 12,000 x *g* for 15 min at 4°C. This resulted in the separation of the mixture into a lower organic phase and an upper aqueous phase. The aqueous phase containing the RNA was gently aspirated and transferred into a fresh microcentrifuge tube and 500 μL of isopropanol /mL of TRIzol reagent was added to precipitate the RNA. The mixture was centrifuged at 12,000 x *g* for 10 min at 4°C to isolate the RNA as a pellet. The supernatant was discarded and the pellet was washed once with 70% ethanol, centrifuged and the pellet was air-dried and re-dissolved in appropriate quantity of nuclease-free water. The purity ($A_{260}/A_{280} > 1.8$) and concentration (A_{260}

X dilution factor X 40) of the obtained RNA was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}).

3.4.2. First strand synthesis by reverse transcription

First strand synthesis of mRNA into cDNA was performed using First strand cDNA synthesis kit from Invitrogen following manufacturer's protocol. Briefly, 4 μg of total RNA was denatured at 65°C for 5 min in the presence of Oligo dT_{16} and dNTPs and incubated at 42°C for another 2 min with DTT, $MgCl_2$, and RNaseOUT in 10 X reverse transcription buffer. 1 μL/reaction of the Superscript Reverse Transcriptase enzyme was added to the denatured RNA and incubated at 42°C for 50 min. The enzyme was denatured by heating at 70°C for 15 min. The reaction was completed by a quick high-speed centrifugation and the complementary RNA strand degraded by incubating with RNaseH for 20 min at 37°C. The preparation was stored at -70°C.

3.4.3. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify specific nucleotide sequences from cDNA derived from human macrophages. The reaction consisted of

Gene	Forward primer	Reverse primer
ER-α	5′-GTGGGAATGATGAAAGGTGG-3′	5′-TCCAGAGACTTCAGGGTGCT-3′
ER-β	5′-TGAAAAGGAAGGTTAGTGGGAACC-3′	5′-TGGTCAGGGACATCATCATGG-3′
Bcl-2	5′-GTGGAGGAGCTCTTCAGGGA-3′	5′-AGGCACCCAGGGTGATGCCA-3′
Mcl-1	5′-CGGCAGTCGCTGGAGATTAT-3′	5′-GTGGTGGTGGTTGGTTA-3′
Bfl-1	5′-AGCTCAAGACTTTGCTCTCCACC-3′	5′-TGGAGTGTCCTTTCTGGTCAACAG-3′
iNOS	5′-GGCCTCGCTCTGGAAAGA-3′	5′-TCCATGCAGACAACCTT-3′
Actin	5′-GTGGGGCGCCCCAGGCACCA-3′	5′-CTCCTTAATGTCACGCACGATTTC-3′

Figure 3.1. The table shows the forward and reverse primers designed against specific genes used for amplifying products using PCR.

an initial denaturation at 94°C for 4 min, followed by 20-30 cycles of denaturation at 94°C for 30 s, annealing at primer specific temperature for 30 s, and extension at 68-

72°C for 45 s – 1 min. A final extension at 68-72°C for 10 min was performed. Relative expression of specific genes in cells subjected to different treatments was determined by semi-quantitative PCR. The optimal number of cycles required for achieving a linear amplification of serially diluted template was determined, which was then used with other samples to quantify the expression of specific genes. The PCR products were resolved on 1-2% agarose gel containing ethidium bromide and visualized under ultraviolet illumination. The specific primers used are shown in Figure 3.1.

3.4.4. Agarose gel electrophoresis

DNA fragments were resolved on 1-2% agarose gel containing 0.5 μg/mL ethidium bromide in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.1). The samples were mixed with 6X loading dye containing bromophenol blue, and the samples were resolved by applying a voltage of ~5-7 V/cm. The resolved DNA fragments were visualized under ultraviolet illumination and the relative band size was determined by comparison against a DNA ladder with bands of known sizes. When required, images were acquired using a UVP Gel Documentation system.

3.4.5. Elution of DNA from agarose gel

To elute DNA from agarose gel, the samples were loaded on a gel (1-1.8%) cast with low melting point agarose (LMP agarose). The samples were resolved and visualized under UV transilluminator, and the band of interest was excised quickly using a scalpel blade. The volume of gel slice was quantitated by weighing and the DNA eluted using MinElute Gel Extraction kit (Qiagen) as per manufacturer's protocol. Briefly, the gel was solubilized by incubating it with buffer QG at 50°C for 10 min. The solubilized gel was loaded onto binding columns and centrifuged at 12,000 x *g* for 1 min. The flow-through was discarded and the column was washed once with buffer PE containing ethanol. The DNA bound to the column was eluted using the elution buffer provided with the kit, or alternatively with nuclease-free water. The concentration of the obtained DNA was estimated by measuring the absorbance at 260 nm (A_{260}) and using the following formula: DNA concentration = A_{260} X 50 X dilution factor.

3.4.6. Sub-cloning of PCR products into pGEM-T_{Easy} vector

The DNA fragments eluted from the agarose gel were cloned into pGEM-T_{Easy} vector which allows efficient sequencing using the sequencing primers for T7 and SP6 promoters. 3 μL of eluted DNA (1 μg/μL) was ligated with 1 μL of pGEM-T_{Easy} vector in the presence of 1 μL of T4 DNA ligase in a 10 μL reaction volume. The reaction was allowed to proceed at 4°C for 16 h following which 8 μL of the ligation mix was used to transform DH5-α strain of *E.coli* following standard protocols (9). The transformation mix was spread onto LB-agar plates containing appropriate ampicillin (100 μg/mL) and the blue-white selection reagent (40 μL/plate) (Sigma chemical company). The plate was incubated at 37°C for 12 h following which the white colonies were picked up for screening for presence of the gene of interest.

3.4.7. DNA sequencing

DNA was sequenced by the di-deoxy method (10) at the DNA sequencing facility of Department of Biochemistry, University of Delhi, South Campus, New Delhi, India.

3.5. Densitometry and statistics

Densitometric measurements for quantitation of signals on immunoblots or ethidium bromide stained agarose gels were performed using a UVP Gel Documentation instrument, and the acquired data was analyzed on LabWorks image analysis and acquisition software (UVP, v.4.0.0.8). Data from at least 3 experiments were quantitated to arrive at the average value of the signal. All measurements were normalized to internal loading controls. To determine statistical significance, the data was analyzed by Student's T test and the values were expressed as mean±SEM. The values were considered to be significantly different at $p<0.05$.

3.6 Instrumentation

Optiphot fluorescence microscope, E600W fluorescence microscope and T2000E Confocal microscope C1 were from Nikon (Tokyo, Japan).

Multitemp III water bath and EPS 500/400 power supply were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Gyratory water bath shaker was purchased from New Brunswick Scientific Co., Inc (Edison, NJ).

Centrivac and Biofuge table top centrifuge were from Heraeus (Allerod, Denmark).

μ- Quant microplate reader was from Bio-tek Instruments Inc. (Winooski, VT).

Protean II polyacrylamide gel system and Mini Trans blot system were from Bio-Rad Laboratories (Hercules, CA).

Submarine DNA electrophoresis system was procured fro Bangalore Genei (Bangalore, India).

Laminar flow hoods were purchased from Kartos Ltd. (New Delhi, India).

Eppendorf 5810R centrifuge was purchased from Eppendorf (Hamburg, Germany).

LS50B flourimeter was from Perkin Elmer Biosystems (Norwalk, CT). Fluostar Optima fluorimeter was from BMG labtech (Offenburg, Germany)

BD-LSR flow-cytometer was from Bectinson Dickinson Biosciences (San Jose, CA).

Peltier Thermal Cycler- 200 was purchased from MJ research (Waltham, MA).

Doc-It Gel Documentation system was procured from UVP Bio Imaging System Incorporation (Upland, CA).

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