CHAPTER 2

General Materials and Methods
1.0 Materials and their sources

Cell culture chemicals: Powdered M-199 and RPMI-1640 medium were purchased from Sigma Chemical Co., USA. Other chemicals like bovine serum albumin (BSA), penicillin, streptomycin etc. were also purchased from Sigma Chemical Co., USA. Fetal Calf Serum (FCS) was purchased from Gibco-BRL Ltd., USA. Bacto-tryptone, bacto-yeast extract and agar-agar required for E. coli culture were purchased locally.

General chemicals: Sodium dodecyl sulphate (SDS), ammonium per sulphate (APS), isopropylthio-β-D-galactoside (IPTG), Coomassie Blue G-500, Sephadex G-50, dextran sulphate, tris hydroxymethyl aminomethane (tris), acrylamide, N-N’-methylene-bis-acrylamide, CaCl₂, dimethyl sulphoxide (DMSO), formamide, 2-mercaptoethanol were purchased from Sigma Co., USA.

Analytical grade and molecular biology grade chemicals that were purchased locally are listed below: acetic acid, acetone, ammonium acetate, butanol, chloroform, ethanol, glucose, glycine, glycerol, H₂O₂, HCl, isopropyl alcohol, isopropanol, KCl, KH₂PO₄, MgCl₂, methanol, NaCl, NaH₂PO₄, Na₂HPO₄, NaOH, potassium acetate, scintillation cocktail ‘O’.

Molecular biology reagents: Restriction enzymes, ligases, other modifying enzymes etc. were purchased from MBI Fermentas, Promega, New England Biolabs, USA.

Radioactive Chemicals: Tritiated Thymidine [³H] was purchased from Amersham Biosciences Limited, Hong Kong.

Antibiotics: Ampicillin, chloramphenicol, nalidixic acid, penicillin and streptomycin were purchased from Sigma Chemical Co., USA.

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

E. coli cells: Different strains of E. coli were procured from different sources. e.g: BL-21 was from Novagen Inc, USA; DH5α was from Gibco BRL, Ltd., USA.

Leishmania strains: L. donovani (MHOM/IN/1983/AG83) was kindly provided by Prof. A.N. Bhaduri, Indian Institute of Chemical Biology (IICB), Calcutta.

Molecular weight markers: DNA markers were prepared from lambda DNA (Sigma Co. USA) and digested with different endonucleases (Hind III). 1 Kb DNA ladder was obtained from MBI Fermentas Inc, MD, USA. Pulse Field Gel Electrophoresis (PFGE) markers were purchased from BIO-RAD Laboratories, CA, USA.
Kits used: RT PCR Kit and QIA quick Gel Extraction kit was purchased from Qiagen, Germany, Chemiluminescence Kit for Western was purchased from Santa Cruz Biotechnology Inc, California, USA, cytokine ELISA kit was purchased from BD Pharmingen.

X-Ray film: Films for autoradiography was purchased from Kodak (from Sigma Chemical Co. USA). X-Ray film developer and fixer were purchased from Kodak, India Photographic Company Ltd.

Antibody conjugates: Following antibody conjugates were purchased from Sigma Immunochemicals. Anti-rabbit goat horse radish peroxidase (HRPO) and anti-mouse goat HRPO. Mac 1 anti CR3 antibodies were from BD Pharmingen.

Immunization chemicals: Isotyping reagents and Alum were kindly provided by Dr. Om Singh, National Institute of Immunology, New Delhi.

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

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

2.1 Growth medium for bacterial culture

2.1.1 Luria-Bertani (LB)
10 g of Bacto-tryptone, 5 g of Bacto-yeast extract and 5 g of NaCl were dissolved in 800 ml of double distilled H₂O and the pH was adjusted to 7.5 with 5N NaOH. The volume was adjusted to 1000 ml and the medium was sterilized by autoclaving for 15 min at 15 lbs pressure.

2.1.2 LB-agar plate
To each litre of LB, 15 g of agar-agar was added and sterilized by autoclaving. The media was cooled to 60°C and the desired antibiotic was added and was poured into 90 mm plates (25-30 ml per plate). The plates were kept for at least 30 min under the laminar flow for solidification and drying (Sambrook et al., 1989).

2.1.3 SOB/SOC
20 g of Bacto-tryptone, 5 g of Bacto-yeast extract and 0.5 g of NaCl were dissolved in 800 ml of double distilled H₂O. 10 ml of 0.25 M KCl was added to the above solution and the pH was adjusted to 7.0 with a few drops of 5N NaOH. The volume was made up to one liter and the medium was sterilized by autoclaving. Before inoculation of bacteria in SOB,
10 ml of 1M sterilized MgCl₂ was added to it. SOC is the above autoclaved medium supplemented with 10 ml of 1M sterilized MgCl₂ and 10 ml of 2M filter sterilized glucose (Sambrook, et al., 1989).

2.1.4 Additives used in the bacterial growth media

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Final Conc.</th>
<th>Storage</th>
</tr>
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<tbody>
<tr>
<td>Ampicillin</td>
<td>ddH₂O</td>
<td>50-100μg/ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>EtOH</td>
<td>34 μg/ml</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>ddH₂O</td>
<td>20 μg/ml</td>
</tr>
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</table>

2.2 Growth media for Leishmania promastigotes

2.2.1 M-199 medium

11.0 g of M-199 and 130 mg each of penicillin 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 900 ml with autoclaved double distilled H₂O and 100 ml of heat-inactivated FCS was added to it to the final volume of 1 litre. The media was then sterilized by filtering it through a 0.22 μm membrane filter. The sterility of the medium was checked by keeping an aliquot at 22°C for three days. Rest of the medium was stored at -20°C.

2.2.2 RPMI 1640 medium

16.4 g of RPMI 1640, 2 g of NaHCO₃, 0.06 g of penicillin, 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 micron 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 on 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.

3.3 Culturing of J774A.1 macrophages cells

J774A.1 macrophages cells were maintained in RPMI – 1640 with 10% FCS at 37°C in the CO$_2$ incubator (5% CO$_2$). Sub culturing was done at 72 h. when the cells reached confluence.

4.0 Isolation of nucleic acids

4.1 Isolation of RNA from Spleen

All the glassware and plastic ware was washed with 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 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 RT, 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 spleen of immunized mice using TRI-reagent (Sigma) according to manufacturer's instructions. The isolated RNA was stored in DEPC water in small aliquots at –80°C.

4.2 Isolation and purification of plasmid DNA from *E. coli* using miniprep method

*E. coli* cells from 1.5 ml of O/N grown culture were collected in eppendorf tubes and LB was removed following centrifugation. The pellet was resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, sterilized by
autoclaving and stored at 4°C). It was vortexed for 1 min at high speed to completely disperse the cells and 200 μl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added to the above. The solution was mixed well by inverting the tube. 150 μl of freshly prepared solution III (For 2 ml: 1.2 ml of 5M potassium acetate, 0.23 ml glacial acetic acid and 0.57 ml H₂O) was added to this tube, briefly vortexed and kept on ice for 5 min. It was centrifuged at 12,000 rpm for 5 min at 4°C and the supernatant was saved. One volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed well by inverting the tube. The tube was centrifuged at 12,000 rpm for 2 min at 4°C and the upper aqueous phase was saved. 2 volumes of ethanol and 2 μl of DT40 were added. The tube was kept for 2 min at RT and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was drained out and the pellet was washed with 70% ethanol. The pellet was dried and dissolved in 100 μl of TE. RNase was added to a final concentration of 50 μg/ml and the tubes incubated at 37°C for 1h. The solution was then phenol extracted and the plasmid was precipitated (section 5.0). The DNA pellet was resuspended in TE and the concentration of plasmid DNA was measured spectrophotometrically (Sambrook et al., 1989).

5.0 Precipitation of DNA

Plasmid DNA and small DNA fragments were precipitated by 3 M Sodium acetate (pH 5.2). It was added to the DNA solution to a final concentration of 0.3 M and mixed well. 2 volumes of ice cold ethanol and 1-2 μl of DT40 (20 mg/ml) were added. The tubes were kept on ice for 15-30 min and the DNA was recovered by centrifugation at 4°C for 15-20 min at 12,000g. The supernatant was carefully removed and the pellet was washed with 70% ethanol. The pellet was dried and dissolved in TE.

6.0 Transformation of plasmids into *E. coli*

6.1 Preparation of competent cells

One healthy colony of *E. coli* DH5-α or BL-21 was picked up from the LB-Agar plate and grown O/N in a 37°C shaker waterbath in 10 ml of LB media. 1% inoculum was added to 50 ml of SOB from the overnight grown culture. The SOB was incubated at 37°C with shaking till the OD₆₀₀ of the culture became 0.37-0.4. The cells were centrifuged at 4,000 rpm for 10 min at 4°C. This step onward all steps were performed on ice under aseptic conditions. The medium was drained and the pellet was resuspended in 10 ml of 0.1M CaCl₂ (filter sterilized) very gently. The cells were pelleted down again and the pellet
was resuspended in 2 ml of ice cold 0.1M CaCl₂. The cell suspension was kept on ice for 1.5h – 2h to make the cells competent for transformation (Sambrook et al., 1989).

6.2 Transformation of plasmid into competent cells

The desired amount of plasmid DNA (usually not more than 1 µg) was added to 200 µl of competent cell suspension, mixed well and kept on ice for 30 min. A heat shock was given for exactly 90 sec at 42°C. The tube was rapidly transferred to ice and allowed to chill for 1-2 min. 800 µl of chilled SOC was added and mixed well. The tube was incubated at 37°C for 45 min. The cells were recovered by brief centrifugation and streaked on a LB-agar plate containing the appropriate antibiotic. The plate was incubated O/N in 37°C incubator and colonies appeared on the plate after 24 h (Sambrook et al., 1989).

6.3 Determination of the efficiency of transformation

10 ng of vector plasmid (eg. PET-17b) was added to 100 µl of competent cells to which 400 µl of SOC was added after heat shock at 42°C for 90 s. Different aliquots of SOC-competent cell mix were taken and streaked onto LB-agar plates containing ampicillin. The transformation efficiency was calculated as number of transformants obtained /µg of DNA used. Usually ~10⁷ transformants/µg of DNA is considered as a good transformation efficiency.

7.0 Restriction digestion of DNA

The reaction mixture for restriction digestion of a particular enzyme(s) was determined according to the manufacturer’s specification. The DNA was subjected to restriction digestion with one enzyme (single digestion) or two enzymes (double digestion). Double digestion was performed in two different ways depending on the enzyme and the buffer compatibility. When only one component (salt concentration) of two reaction buffer differed, the DNA was first cleaved with the restriction endonuclease that required lower salt concentration. Afterwards, the concentration of the reaction was adjusted to approximate the reaction conditions of the second restriction endonuclease. The other way of double digestion was to incubate the DNA with two required restriction endonucleases simultaneously depending on the buffer compatibility, according to the manufacturer’s specifications. If the enzymes and buffers were incompatible for the double digestion, the DNA was first cleaved with one endonuclease, phenol-chloroform extracted, and precipitated. It was then taken for the second restriction endonuclease digestion.
8.0 Electrophoresis of nucleic acids
8.1 Preparation and running of agarose gel for DNA electrophoresis

The gel tray was cleaned and open sides were sealed with plastic tape. 0.5X TBE (0.045 M Tris-borate, 0.001 M EDTA) was prepared and agarose was added according to the size of the DNA fragment to be separated. Agarose in 0.5X TBE mixture was boiled and cooled to 60°C; ethidium bromide was added to a final concentration of 0.5 µg/ml and mixed thoroughly. The gel comb was set on the gel tray. The molten agarose was poured on the sealed tray up to 3-5 mm thickness. Any bubbles formed were quickly removed with a pipette. It was kept for 30-45 min at RT to solidify the agarose. The comb and the plastic tape were removed. The gel with the tray was kept in the electrophoresis tank containing 0.5X TBE buffer. The buffer level in the tank was just enough to cover the gel to a depth of about 1 mm.

The DNA sample was mixed with 6X gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% w/v sucrose) on a piece of parafilm and was loaded into the slots of submerged gel very carefully. The gel tank lid was closed and the electrical leads were attached so that DNA migrates towards the anode. 1-5 volt/cm potential difference was applied. The gel was run until the bromophenol blue and xylene cyanol FF migrated to the appropriate distance. The gel was removed from the tank. The DNA bands were visualized in UV light.

9.0 Purification of DNA fragments from agarose gel
9.1 Purification of DNA by QIA quick Gel Extraction kit (Qiagen, Germany)

The gel piece containing the band of interest was taken in the column and treated according to manufacturer’s instructions. The eluted DNA was checked on an agarose gel.

10.0 Cloning and expression of open reading frame F (ORFF) from the LD1 locus

PCR cloning was done with B30 cDNA clone using following pair of primers. 3EorfF (5’-CCGCTCGAGCTCGCCGTTGGTGAGCTGCA3’) and 5BorfF(5’TCGGATCCAAATGCAAAGCGACGCACG 3’). The 950 bp PCR product obtained was digested with XhoI and BamHI and ligated into pET-17b vector digested with XhoI and BamHI. The ligated product was transformed into E. coli BL21 cells. Transformants were analyzed by restriction digestion and the positive clone was sequenced to confirm the presence of ORFF insert in proper reading frame. The positive clone was designated pET17bF-C14.
The positive recombinant pET17bF-C14 plasmid was transformed into *E. coli* BL-21 cells. BL-21 cells with the construct was amplified by growing on LB media supplemented with 50 µg/ml ampicillin and 34 µg/ml chloramphenicol. 15 ml of BL21-orF cells were grown to log phase (OD₆₀₀ = 0.6 to 1.0) and IPTG was added to a final concentration of 0.4 mM. The culture was grown at 37°C in a shaking water bath and 1 ml aliquots were collected at each hour. The same number of cells from each aliquot was loaded on an SDS-PAGE gel and checked for recombinant protein expression (pET-system manual, Novagen, 1992).

### 11.0 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’-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>
</tr>
</thead>
<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 have 8.0 X 7.3 cm and 16 X 14 cm gel size respectively. The former has gel thickness of 0.75 mm and latter 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% APS. The solution was mixed well after adding TEMED (8µl 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 RT 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), 0.125 M Tris (pH 6.8), 0.1% SDS and 0.1% ammonium persulphate and polymerization was catalyzed by TEMED. It was kept for at least 30 min at RT for polymerization. The gel was fixed on the electrophoresis apparatus and running buffer (25mM Tris, 250 mM glycine 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 up to the resolving gel. The voltage was increased to 15 volt/cm till the dye reached the bottom of the gel (Sambrook et al., 1989).

11.1 Visualizing the proteins in SDS-PAGE

11.1.1 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 hours to O/N at RT with gentle shaking.

11.1.2 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 μg protein per lane (Sambrook et al., 1989).

11.2 Drying the SDS-PAGE gel

The stained gel was placed on a 3 mm Whatmann paper and covered with plastic wrap so that no air bubble remained in between the gel and the plastic wrap. The covered gel was placed on a vacuum dryer and kept for 2.5 h at 70°C under vacuum. The dried gel on the filter paper was preserved.

12.0 Purification of the Induced Recombinant Protein

The pET – system was supplied with His-Bind™ metal chelating resin. The resin column was used to purify the induced fusion protein exploring the affinity of the fused part of the recombinant protein to the resin. With this resin, the protein was purified either in native or in denatured form.
12.1 Purification of recombinant ORFF by urea gradient

From 250 ml of induced E. coli culture, cells were collected and resuspended in 10 ml of TNE. 10 mg of lysozyme was added to the above and mixed well by inverting the tube. It was then kept on ice for 30 min. The lysed cells were centrifuged at 4000 g for 10 min at 4°C and pellet and supernatant fractions were separated. The induced protein was in the pellet fraction. The pellet was washed again with 10 ml of TNE. The pellet was resuspended in 2 M urea (in TNE). There was a further centrifugation at 4,000g for 10 min at 4°C. The 2 M urea washing step was repeated. Likewise, the pellet was washed twice with 2 M, 4 M and 8 M each of urea and after every wash, the supernatant was saved. During the 8 M urea wash, 1 mM DTT was added. 10 µl from each wash was run on SDS-PAGE and the right fraction containing the induced recombinant protein was identified and collected. The positive fraction(s) was dialysed extensively against slowly decreasing concentrations of urea in PBS and finally dialysed against PBS.

Each pellet was resuspended in a minimum volume of double distilled water and 2µl of each was checked by SDS-PAGE.

13.0 Protein Concentration and Purification by Using Centricon:

Centricon-10 and centricon-30 (Amicon) were used which had 10 kD and 30 kD cut off filters. The diluted protein sample was concentrated through centricon filters by centrifuging the assembly at 6,000 g. Other contaminating proteins were also separated from the induced protein (depending on size) by these filters.

14.0 Protein Estimation: Bradford Method

100 µl of standards and samples were taken and 5 ml 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 liter with double distilled water.

After mixing the Bradford reagent to the samples and standards, the tubes were kept at RT 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).

15.0 Raising antibodies to ORFF

Antibodies to recombinant ORFF was raised in our laboratory. The procedure followed is described below.
15.1 Immunization schedule (for serum preparation)

1 mg of antigen (in PBS) was emulsified with equal volume of Complete Freund’s Adjuvant (CFA) and 100 µg of adjuvant peptide N-acetyl muramyl-L-alamyl-D-isoglutamine. Emulsification was done by passing the antigen-adjuvant mixture repeatedly through a syringe. Emulsification was done so that a drop when placed on the surface of saline solution did not disperse. This emulsion was injected subcutaneously at 4-6 sites into a rabbit, injecting 800 µl at each site. Subsequent booster dose was given after three weeks of immunization. In this booster dose, 500 µg of antigen emulsion with Incomplete Freund’s Adjuvant (ICFA) (without adjuvant peptide) was injected at 1-2 sites. Subsequent boosters were given at 2-3 weeks interval with 500 µg or less amount of antigen with ICFA (Lane and Narlow, 1988).

15.2 Serum preparation

Test bleeds were normally done from the marginal ear vein of rabbit. Exsanguination was done for the collection of large amount of serum. After collection of blood, it was allowed to clot for 30-60 min at 37°C. The clot was saved carefully and placed at 4°C O/N. Serum was collected from the clot by removing insoluble materials by centrifugation at 10,000 rpm for 10 min at 4°C. The serum was stored at -20°C for long time or at 4°C for a few months (Lane and Narlow, 1988).

15.3 Purification of antibodies

The serum collected was diluted 2-3 times with PBS (pH 8.0). A packed column of protein A sepharose was equilibrated with PBS (pH 8.0). Diluted serum was passed through the column. The column was washed 4-5 times with PBS (pH 8.0). The antibodies were eluted by washing the column with 0.1 M citric acid (pH 3.0). Fractions of 0.5 ml were collected in eppendorf tubes, which contained 50 µl of 2M Tris-HCl (pH 8.0) to restore the pH of the antibody fractions. All the fractions were read for absorbance at 280 nm and the protein containing fractions were pooled (Lane and Narlow, 1988). The column was regenerated by washing first with 10 ml of 3M sodium thiocyanate (NaSCN) and then with PBS (pH 7.3). The antibody containing fractions were dialyzed against PBS (pH 8.0) and stored at -20°C with 0.002% sodium azide. The 5th and the 6th bleed of anti-ORFF were used for further experiments.
16.0 Western blotting

16.1 Transfer of the protein from the gel to the membrane

10% 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, then 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) O/N 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.

16.2 Antibody blotting to the membrane

After transfer, the membrane was marked with a graphite pencil. The membrane was washed with 1X PBS (pH 7.0) (with 0.05% Tween 20) thrice, 5 min each on a rocker. The membrane was blocked either with 5% non-fat dry milk in PBS (pH 7.0) or with 3% BSA in PBS pH 7.0 at RT for 1 h to O/N with constant shaking. The blocked membrane was washed thrice for 10 min each with PBS pH 7.0 containing 0.05% Tween-20.

The membrane was incubated in a 1:100 dilution of purified antibody in PBS (pH 7.0) with 0.05% Tween 20 for 1 h with constant shaking at RT. In this step with antibody, 0.5% BSA may also be added. The membrane was then washed thrice for 10 min each with PBS (pH 7.0) (with 0.05% Tween 20). It was incubated with 1:8000 dilution of HRPO conjugated antibodies in PBS (pH 7.0) (with 0.05% Tween 20) for 1 h at RT with constant shaking. The membrane was then washed thrice with PBS (pH 7.0) (with 0.05% Tween 20) for 10 min each. The blot was now ready for developing.
17.0 Immunoblotting detection
17.1 Colour developing method by Chemiluminescence method

When HRPO conjugated antibodies were used in the blot, chemiluminescence kit supplied by Pierce (Rockford, USA) 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 Kodak X-ray film was exposed to the membrane for a maximum of 1 min. The film was developed by standard method as described earlier.

18.0 Preparation of soluble antigen from promastigotes

*L. donovani* promastigotes strain AG83 (MHOM/IN/AG83/1983) were harvested and washed thrice with PBS, pH 7.2 at 4°C followed by four sonications at 22 micron for 1 min each using an ultrasonicator. The lysate was centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was collected and the protein was estimated by Bradford method as described above. This soluble antigen (SA) was used in ELISA, immunizations etc.

19.0 Enzyme linked immunosorbent assay (ELISA)

The polystyrene microtiter plates were coated O/N at 4°C with 100 ng of *L. donovani* (AG83) soluble antigen or 100 ng of recombinant ORFF in 50 µl of carbonate buffer (pH 9.2). The plates were washed thrice with PBS, pH 7.2, 0.1% Tween-20 (PBS-T). The wells were then blocked by incubation for 1-2 h at 37°C with 200 µl of PBST containing 3% BSA and 5% milk to prevent non-specific binding. After the wells had been washed 3X with PBS (0.1% Tween-20), they were incubated with 100 µl of sera diluted in PBS with 1% BSA for 2h at 37°C. The wells were then washed with PBS thrice and incubated with 50 µl of 1:2000 diluted HRPO conjugated anti-mouse IgG (Biorad, Hercules, CA) (in PBS) for 1h at 37°C and then rinsed thrice with PBST. After incubation for 30 min at 37°C with 50 µl of appropriate substrate solution, colour developing was done.

19.1 Colour development

O-phenylenediamine was used as a substrate when HRPO conjugated secondary antibody was used. 5 mg of o-phenylenediamine was dissolved in 10 ml of freshly prepared citrate phosphate buffer (0.1 M citric acid, 0.2 M Na₂HPO₄, pH 5.0) and 5 µl of 30% H₂O₂ was added to it. 50 µl of this solution was added per well of the microtitre plate. The
reaction was stopped with 50 μl of H₂SO₄ (5N) and the OD read at 492 nm using an ELISA plate reader (Model 550 Microplate reader, Bio-Rad).

19.2 Cytokine ELISA assays:

The concentrations of IFN gamma, IL-4 and IL-12 in culture supernatants were determined as described previously (Walker et al, 1999). Briefly spleen cells were isolated from vaccinated BALB/c mice and resuspended in RPMI 1640 medium supplemented with 10% FCS and incubated in 96 well flat bottomed plate (Nunc, Roskilde, Denmark) at a density of 5 X 10⁵ per well and cultured with rORFF (1 μg/well). After 48 h, supernatants were collected and diluted serially and cytokine concentrations were quantitated using ELISA. ELISA was performed using Opt EIA kit (Pharmingen, SanDiego, CA) for cytokine analysis. Briefly 96-well Immulon-4 plates were coated with capture antibody in 0.1 M carbonate buffer and incubated O/N at 4°C. The plates were washed thrice with PBS (pH 7.2), 0.1% Tween-20 (PBS-T) and then blocked with 200μl of assay diluent (PBS with 10% FBS) and incubated at RT for 1 hour to prevent non-specific binding. The plates were washed thrice and 100 μl of standard or diluted culture supernatants in assay diluent were added and incubated for 2 hours. Plates were washed five times with PBST and 100μl of working detector (Detection antibody + Avidin-HRP) to each well. Incubated for 1 h at RT and then washed with PBST. 100μl of TMB substrate was added to each well and plates were incubated in dark for 30 min. Reaction was stopped using 50 μl of 2N H₂SO₄ and OD taken at 450 nm. Cytokine concentrations were determined using standard curve generated from recombinant cytokines supplied with the kit.

20.0 Splenocyte proliferation assay

Spleens were removed from mice under aseptic conditions on a sterile dish containing RPMI 1640 media. Single cell suspensions were prepared by grinding the spleen with disk bottom of the plunger of 10 ml syringe. 5-10 ml of RPMI 1640 media was added to it and the contents were mixed to homogeneity. The dish was kept undisturbed for two minutes and the clear supernatant was pipetted out slowly. Cells were pelleted by centrifugation at 4°C at 250 g (Sorvall RC-5 centrifuge, HB-4 rotor) for 10 min. The pellet containing erythrocytes and splenocytes were collected. Washing the pellet once with 0.9% ammonium chloride lysed erythrocytes. The remaining cells were resuspended to a density of 2.5 x 10⁶ cells/ml in RPMI 1640 containing 10% FCS and 0.05 μM 2-mercaptoethanol,
then divided into 200 μl aliquots (5 x 10^5 cells) in 96-well plates. After addition of 1, 5, 10μg recombinant ORFF antigen (for rORFF vaccine) and 1μg (for DNA vaccine) to each well, the cells were incubated for 3-days at 37°C in atmosphere containing 5% CO₂ and 95% humidity. Proliferation was measured by incorporation of 0.5 μCi of [³H]-thymidine over the final 16 h of the 3 days of culture. The plates were incubated further for 16 h after which the cells were harvested on glass fibre filters. PhD Cell Harvester, Cambridge Technology was used for the purpose. The filter papers were taken in scintillation vials, air dried, scintillation fluid was added and counts were taken after 24 h. Stimulation indices (S.Is) were calculated as the ratios of [³H]-thymidine incorporation in the presence of antigen versus the non-stimulated (medium alone) control. All assays were performed in triplicate, with four mice representing each group.

21.0 Immunization and Protection studies in BALB/c mice

Female BALB/c mice (4-6 weeks old) were obtained from the National Institute of Nutrition, Hyderabad, India. All mice were used at ages ranging from 6-8 weeks. Animals were used in accordance to the Institutional guidelines and the relevant committee duly approved the use of animals for this work. All immunizations were given intramuscularly (i.m) at the mid point of left thigh muscle. rORFF and Soluble antigen (50 μg each) were adsorbed overnight on 50 μl alum on a rotary shaker at 4°C before immunization. IL-12 DNA, pcDNA3.1 and F/pcDNA 3.1 were isolated by endotoxin free plasmid isolation kit (Qiagen). 100 μg of each plasmid DNA was given intramuscularly in 50 μl of PBS. For protection studies mice were challenged intravenously (i.v) in the tail vein with L. donovani AG83 promastigotes. Organ parasite load was determined 4 and 8 weeks after challenge.

21.1 Determining antibody levels in immunized mice

Serum was collected from each of the immunized group of animals before infection and after infection and checked for presence of anti-ORFF antibodies by ELISA and Western as described earlier.

21.2 Determining organ parasite load

After 4 weeks and 8 weeks of challenge infection, mice were euthanized and liver and spleen touch biopsies were microscopically examined after fixing and staining the slides with Giemsa. In order to quantitate levels of infection, Leishman Donovan units (LDU) were calculated as: (number of amastigotes /number of organ nuclei) X weight of organ in milligrams. Protection studies were performed using 8 mice per group. 4 mice
from each group were sacrificed for determination of parasitemia both at 4 weeks and 8 weeks.

**22.0 Qualitative reverse transcription PCR (RT-PCR)**

Total RNA was isolated from the spleen of immunized mice using TRI-reagent (Sigma) according to Manufacturer's instructions. RT-PCR was performed using one-step RT-PCR kit (Qiagen) using 3 µg of each of the RNA sample. The primer sequences were as follows:

IFN-γ: sense-TCAGGAAGCGGAATAAGGAGTC
antisense-TCAAGTCACTTGAGACACTGC
IL4:senseTACCAGGAGCCATATCCACG
antisense-GAGTCTCTGCAGCTCCATGAG
Glyceraldehyde-3-phosphate dehydrogenase (GAP):
sense-GATGACATCAAGGTGGT
antisense-TCTTGCTCAGTGCTCCTTGCTG.

Each primer pair amplified across an intron. PCR conditions were as follows: Reverse transcription for 30 min at 50°C followed by an initial PCR activation step for 15 min at 95°C. This step was followed by a 3 step cycling consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, followed by extension at 72°C for 1 min. Number of amplification cycles for IL-4 and IFN-γ was 40 and 25 cycles for GAP. There was a final extension step at 72°C for 10 min. After amplification, 10 µl of each sample was applied to a 2% agarose gel and the products were visualized by ethidium bromide staining. GAP was used as a control for the amount of cDNA used in each sample.

**23.0 Statistical analysis**

Statistical analysis was performed by standard t-test (Fisher et al., 1957).