CHAPTER- III

MATERIALS AND METHODS

3. Materials and Methods

3.1 Reagents

All cell culture materials were obtained from Gibco™, Thermo Fisher Scientific, Grand Island, NY and HiMedia, India. Leishmania LPG monoclonal antibody (Cat No. #MA-83817) and mouse monoclonal β-Actin antibody (Cat No. AM4302) were procured from Thermo Fisher Scientific, Rockford, IL. Horseradish peroxidase-conjugated anti-rabbit (Cat. No. #A9169) and anti-mouse (Cat. No. #A9044) secondary antibodies were purchased from Sigma-Aldrich, St. Louis, MO. Anti-GFP antibody (Cat. No. #BB-AB0065), anti-Myc antibody (Cat. No. #BB-AB0045), anti-FLAG antibody (Cat No. #BB-AB0015), anti-His antibody (Cat. No. #BB-AB0010) and pBBL-κB luciferase plasmid (Cat. No. #BB-V0035) were procured from Bio Bharati Life Sciences Pvt. Ltd., India. TLR4 specific inhibitor Cli-095 (Cat. No. #tlrl-cli95) was purchased from InvivoGen, San Diego, CA. Phorbol 12-myristate 13-acetate (PMA; Cat. No. #P8139), TRI-Reagent (Cat. No. #T9424), Parthenolide (Cat. No. P0667) and Lipopolysaccharides from *E. coli* (Cat. No. L4391) were procured from Sigma-Aldrich, St. Louis, MO. iScript™ cDNA synthesis kit (Cat. No. #1708841) and Clarity ECL western substrate (Cat. No. 1705060) was obtained from Bio-Rad Laboratories, Hercules, CA. Lipofectamine-3000 Transfection reagent (Cat. No. L3000008) and subcellular protein fractionation kit (Cat. No.78840) were purchased from Thermo Fisher Scientific, Carlsbad, CA. TLR4-set siRNA/shRNA/RNAi Lentivector (Human) siRNA Lentivector (Cat. No. i024620) was procured from Applied Biological Materials Inc., Richmond, BC, Canada. H2DCFDA (Cat. No. D399) and ActinGreen™ 488 ReadyProbes™ Reagent (Cat. No. #R37110) were procured from Thermo Fisher Scientific, Life Technologies Corporation, Eugene, OR. Vectashield anti-fade mounting medium containing DAPI (Cat. No. #H-1200) was procured from Vector Laboratories, Burlingame, CA. Giemsa's Stain (Cat. No. GRM945) was purchased from HiMedia Laboratories, India. Different gene-specific primers were procured from Imperial Life Science (P) Limited, India and Integrated DNA Technologies. We purchased Fetuin-A/AHSG (Cat. No. #5258S) and Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cat. No. #9101S) antibodies from the Cell Signaling Technology, Danvers, MA. Phospho-

NF-κBp65 (Ser-536) antibody (Cat. No. sc-101752) and Fetuin-A siRNA (Cat. No. sc-39442) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The TNF-α (Cat. No. A11534), and TGF-β (Cat. No. A2124) antibodies were procured from the ABclonal, Woburn, MA. The GFP-Trap Agarose was purchased from ChromoTek GmbH, Planegg-Martinsried, Germany. QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Cat. No. 210515) was purchased from Agilent Technologies, Santa Clara, CA. We have obtained Lipofectamine™ RNAiMAX Transfection Reagent (Cat. No. 13778075), and JC-1 Dye (Cat. No. T3168) were procured from Invitrogen, Thermo-Scientific, Grand Island, NY. Millicell EZ SLIDES, Amicon®Ultra-15 Centrifugal Filter Units [Ultracel-30, Ultracel-50, and Ultracel-100] (Cat. No. UFC9030, UFC9050, UFC9100) from EMD-Millipore, Darmstadt, Germany. Halt^{TM} Protease and Phosphatase Inhibitor Cocktail (Cat. No. #78441), and BCA kit were purchased from Thermo-Scientific, Grand Island, NY. PCR Master Mix 2X (#K0171) was purchased from Thermo Fisher Scientific, Lithuania. We procured Steady-Glow Luciferase Assay System (Cat. No. #E2510) from Promega, Madison WI. Trans-well inserts containing 5.0 μm pores and 0.4 μm pores (Cat. No. 3421,3379) were purchased from Corning, NY. The pcDNA3.1-CD14-His plasmid and pEF-BOS hMD-2 plasmid were kind gifts of Prof. Liliana Schaefer (Goethe University, Frankfurt, Germany), and Prof. Mateja Maneck Keber (National Institute of Chemistry, Ljubljana, Slovenia) respectively. We received pcDNA3-TLR4-YFP plasmid, as a generous gift from Prof. Doug Golenbock (Addgene plasmid#13018; http://n2t.net/addgene:13018;RRID: Addgene_13018). FITC-Annexin V apoptosis detection kit (Cat. No. #556547), was procured from BD Biosciences, 14 New Bond St, Bath,UK. Fluorescein 5(6)-isothiocyanate (FITC) (Cat. No. #46950) and Propidium iodide solution (Cat. No. #P4864) were purchased from Sigma-Aldrich, Darmstadt, Germany.

3.2. Parasite culture and maintenance

Pathogenic morph of *Leishmania donovani* (MHOM/IN/1983/AG-83) is regularly maintained in BALB/c mice in the Animal House Facility of the Department of Zoology, West Bengal State University, Barasat, West Bengal, India, following the previously published protocol [1] as per the guidelines of the Institutional Animal

Ethics Committee (Ref. 1394/GO/Re/S/10/CPCSEA). *L. donovani* promastigotes were cultured in M199 medium supplemented with 10% heat-inactivated FBS along with penicillin (100 U/mL), and streptomycin (100 µg/mL) at 24˚C. Promastigotes were sub-cultured twice a week by maintaining 5×10^6 promastigotes/ml. THP-1 monocyte cell line (Cat. No. #TIB-202, ATCC, USA) was a kind gift from Dr. Rupak Mukhopadhyay, Department of MBBT, Tezpur University, and were cultured in RPMI-1640 containing penicillin (100 U/ml) and streptomycin (100 mg/ml) and supplemented with 10% FBS in a humidified 5% CO2 environment at 37˚C. Confluent THP-1 monocytes were differentiated into macrophages by the treatment with PMA (5 ng/ml) for 48 h following our previously described method [2]. For in vitro studies, THP-1 macrophages were infected with unlabeled or FITC-labeled *L. donovani* promastigotes at the ratio of parasite/ macrophage, 10:1, for 4 h. Intracellular parasite numbers were determined by Giemsa staining or flow cytometry.

3.3. FITC labelling of *L. donovani* promastigotes

L. donovani promastigotes in the mid log phase were incubated with 0.1% FITC in 50 mM Carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH: 8.0), at room temperature for 1 hour. Unbound FITC was thoroughly washed with 1X PBS and the parasites were then suspended in DMEM medium containing 2% Heat-inactivated BCS. THP1 monocyte-derived macrophage cells and human PBMC derived macrophages were then infected with FITC-labelled promastigotes at 37˚C by maintaining the ratio of 1:10 (macrophage/parasite). After infection, cells were washed with 1X PBS and further incubated with 0.02 % Trypan Blue in 1X PBS for 5 min to quench fluorescence associated with membrane-bound uninternalized *L. donovani* promastigotes and that allows selective quantification of internalized FITC- labelled parasites.

3.4. Isolation of peripheral blood mononuclear cells and staining

Human or mouse PBMC were isolated by using gradient Histopaque 1077 (Sigma-Aldrich) following the manufacturer's instructions. Briefly, PBMC's (2×10^6) were

sub-cultured into the human IgG-coated Millicell EZ Slide (8 well) in the presence of recombinant human M-CSF (50 ng/ml). Non-adherent cells were periodically removed followed by the addition of fresh RPMI-1640 medium containing recombinant human M-CSF (50 ng/ml) and 10% FBS every 12 h. PBMC's were allowed to differentiate into macrophages for 7 days. Cells were then incubated with unlabeled or FITC-labeled parasites at a parasite/macrophage ratio of 10:1. The intracellular parasite load was determined by Giemsa staining or flow cytometry. The experimental protocols were approved by the Institutional Ethics Committee, Tezpur University, Assam, and IAEC, West Bengal State University, Barasat, West Bengal, India (Approval number: WBSU/IAEC/CP/1, dated 03.03.2015).

3.5. Flow cytometric analysis

L. donovani promastigotes were labeled with FITC following the previously described method [3]. Briefly, promastigotes were incubated with 0.1% FITC in 50 mM carbonate buffer, pH 8.0, at room temperature for 1 h. Unbound FITC was washed with PBC and used for infection. Subsequent to infection, cells were washed and incubated with 0.02% trypan blue in PBS for 5 min to quench fluorescence associated with membrane-bound uninternalized promastigotes [4] and that allows selective quantification of internalized FITC labeled parasites. Flow cytometric data were acquired using a BD FACSLyric^{TM} Flow Cytometry System (BD Biosciences) equipped with three laser configurations (488, 405, and 640 nm) and the BD FACSuiteTM Software for acquisition and analysis. The green fluorescence signal was collected using a photomultiplier tube (PMT) after passing through a 527 (± 32) nm band-pass filter. The PMT amplifier was calibrated regularly using standard fluorospheres BD^{TM} CS&T beads. Viable cells were gated based on side-angle light (SSC) scatter and forward angle light scatter (FSC). Single interrogated viable cells (Singlets) were further gated on the basis of FSC-height versus FSC-area. FITC fluorescence of 1×10^4 single viable cells per sample, were analyzed and high fluorescent cells were gated. The cytometric parameters were set to provide accurate discrimination between non-fluorescent (or self-fluorescent) negative cells and positive FITC-fluorescent cells on a FITC-LOG

(gain 1, voltage ranging from 400 to 600 V depending on the signal) versus SSC density plot to estimate the proportion of infected cells.

3.6. Sub-cellular protein fractionation

Control uninfected and *L. donovani*–infected THP-1 macrophages were harvested using cell scraper and centrifuged at 500×g for 5 min at 4°C. Cell pellets were subjected to subcellular protein fractionation using a subcellular protein fractionation kit following the manufacturer's instructions. Briefly, the cell pellet was resuspended with ice-cold PBS, centrifuged, and after removing the supernatant, ice-cold cytoplasmic extraction buffer containing protease and phosphatase inhibitors was added according to the pellet volume and the manufacturer's protocol. The pellet was incubated in cytoplasmic extraction buffer for 10 min at 4°C with gentle mixing. After centrifugation, supernatant containing cytoplasmic fraction was immediately transferred to pre-chilled tube. The ice-cold membrane extraction buffer containing protease and phosphatase inhibitors was then added to the pellet, vortexed vigorously for 5 sec and incubated for 10 min at 4°C with gentle mixing. The supernatant containing membrane fraction was then collected into pre-chilled tube after centrifugation at 3000×g for 5 min at 4°C.

3.7. Immunoblotting

Upon termination of incubations, Control and *L. donovani*–infected macrophage cells were washed twice with ice-cold 1X PBS and harvested using cell scraper. For the preparation of whole-cell lysates, RIPA buffer (140 mM NaCl, 10mM Tris, 1.0% Triton-X-100, 0.1% sodium deoxycholate, 0.1% SDS) with Halt™ protease and phosphatase inhibitors was used. Harvested cells were resuspended in RIPA buffer, vortexed in every 10 min for 30 min in ice, followed by sonication of the cell samples followed by centrifugation at 13,000 rpm for 15 min at 4˚C, supernatant was collected. Protein concentrations in the supernatants were estimated by method of Lowry et al., 1951 [5]. Equal amounts of protein samples were resolved in 10 or 12% SDS–PAGE at 80 volts for stacking gel and resolving gel until the dye-front reached the end of the gel. A polyvinylidene difluoride

(PVDF) membrane was activated in 100% methanol for 2 min at RT and the proteins in the gel was transferred the activated PVDF membrane by wet transfer method at 80 volts for 2 to 3 h based on the size of the protein of interest. Blocking of blotted PVDF was done in Tris-buffer saline with Tween-20 buffer (TBST, composition 20 mM Tris (pH 7.5), 150 mM NaCl and 0.1% Tween 20) containing 5% BSA for 1-2 h at RT. The blots were then incubated with respective primary antibodies (1:1000 dilution) at 4°C overnight followed by 3 washes with TBST for 5 min each. The blot was then incubated with secondary antibody (anti-Mouse, dilution 1:20,000, anti-Rabbit, dilution 1:20,000 or anti-goat, dilution 1:20,000) for 2 h at RT followed by 3 washes with TBST for 10 min each and 2 washes with TBS for 5 min each. Protein bands were visualized using Clarity ECL Western Chemiluminescent Substrate (Bio-Rad Laboratories, USA) incubation and images were captured in a Chemidoc XRS+ System (Bio-Rad Laboratories, USA) using Image Lab Software. Bands were quantified by densitometry and normalized to those of β-actin.

3.8. RNA extraction and Semi-quantitative RT-PCR

Total RNA was extracted from different incubations using TRI reagent according to the manufacturer's instructions. RNA was treated with DNase I and reverse transcribed using the iScript™Reverse Transcription Supermix. We used 2X PCR Master mix for semi-quantitative RT-PCR using gene specific primers. Expression of these transcripts was normalized to the expression level of human 18s rRNA gene [6]. Primers used in our study for RT-PCR are listed in Table 3.1.

Table 3.1: List of primers and oligos used in this study

SL No	Oligo Name	Strand	Sequence(5´-3´)
1	mTNF-α	Forward	GGCAGGTCTACTTTGGAGTCATTG
		Reverse	ACATTCGAGGCTCCAGTGAATTCG
2	mTGFβ	Forward	CCTCCCCCATGCCGCCCTCG
		Reverse	CCAGGAATTGTTGCTATATTTCTG

3	mIL-1β	Forward	AGGGCTGCTTCCAAACCTTTGACC
		Reverse	CCAGTTGGGGAACTCTGCAGACTC
4	mIL6	Forward	ATGGGTCTCAACCCCCAGCTAGT
		Reverse	GCTCTTTAGGCTTTCCAGGAAGTC
5	mβ actin	Forward	GTACTCTGTGTGGATCGGTGG
		Reverse	AGGGTGTAAAACGCAGCTCAG
6	hTNF-α	Forward	CCAGGGACCTCTCTCTAATCA
		Reverse	TCAGCTTGAGGGTTTGCTAC
7	hIL-1β	Forward	GGTGTTCTCCATGTCCTTTGTA
		Reverse	GCTGTAGAGTGGGCTTATCATC
8	hMCP-1	Forward	TCATAGCAGCCACCTTCATTC
		Reverse	CTCTGCACTGAGATCTTCCTATTG
9	hCCL5	Forward	CTGCCTCCCCATATTCCTCG
		Reverse	TCGGGTGACAAAGACGACTG
10	hTGFβ	Forward	CACCCGCGTGCTAATGG
		Reverse	ATGCTGTGTGTACTCTGCTTGAACT
11	hIL-10	Forward	TCTCCGAGATGCCTTCAGCAGA
		Reverse	TCAGACAAGGCTTGGCAACCCA
12	h18srRNA	Forward	GTCTGTGATGCCCTTAGATG
		Reverse	AGCTTATGACCCGCACTTC
12	hTLR4	Forward	GAGGCAGCAGGTGGAATTGTATCGC
		Reverse	CTGTTTGCTCAGGATTCGAGGCTTTTCC
13	hFet-A	Forward	CCTGCTCCTTTGTCTTGC
		Reverse	CGGACTGGAGGAACCAC

3.9. Cell viability assay

MTT assay was performed following the method described previously [2]. Briefly, control and treated THP-1 macrophages in 96 well plates were incubated with MTT for 4 h at 37°C. On termination of incubations, formazan crystals formed in cells were dissolved in acidic isopropanol and incubated further for additional 30 min at

37°C. Cytotoxicity was measured spectrophotometrically at 570 nm with Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland). Absorbance values were blanked against acidic isopropanol and the absorbance of cells exposed to medium only (without any treatment) were taken as 100% cell viability (control).

3.10. TLR4 silencing and forced expression in THP-1 macrophages

THP-1 macrophages were transfected with human TLR4/control siRNAs or human TLR4 overexpression/control plasmids using Lipofectamine 3000 Transfection Reagent following the manufacturer's protocol. The transfection mixture was prepared in Opti-MEM and added to the cells in each well and incubated for 6 h at 37°C. The RPMI-1640 media containing 20% FBS was then added and cells were kept for additional 42 h in transfection medium. After 48 h, media was replaced with fresh RPMI-1640 growth media containing 1% FBS. TLR4 silenced or overexpressed macrophages were infected with either unlabeled or FITC-labeled *L. donovani* promastigotes for 4 h at 37°C and subjected to downstream analysis.

3.11. Measurement of ROS production

THP-1 macrophages were incubated with DCFDA solution (25 µM, 100 µl/well) diluted in Kreb's Ringer Buffer solution for 45 min at 37°C in dark. The Kreb's Ringer Buffer was then removed and RPMI-1640 medium (150 µl/well) was added with or without treatment for 1 h. Medium was removed from each well and cells were washed with PBS (150 µl/well). The cell lysis buffer (150µl/well) was added to each well and incubated for 10 min. Cellular ROS levels was measured in Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland) at Ex/Em = 492/517 nm.

3.12. Fluorescence microscopic imaging

Fluorescence microscopic analysis was performed on THP-1 macrophages infected with FITC-labeled *L. donovani* promastigotes. Briefly, THP-1 macrophages were infected with FITC labeled promastigotes followed by washing and incubation with

0.02% trypan blue solution for 5 min to quench the fluorescence associated with uninternalized membrane-bound promastigotes. Cells were then stained with lysotracker red, washed with PBS and fixed with 4% paraformaldehyde for 10 min followed by mounting with Vectashield anti-fade mounting medium containing DAPI for nuclear staining [7]. Images were taken using inverted fluorescent microscope (Leica, DMi8, Germany) using LAS X software.

3.13. Macrophage migration assay

THP-1 macrophage migration assay was performed using trans-well inserts containing 5.0 μm pores (Cat. No. #3421, Corning, NY). The serum starved control and treated THP-1 cells ($0.5x10^6$ cells) were placed in the upper chamber of 5.0 μm trans-well inserts and 10% FBS was added to the media placed in the lower chamber of 24 well plates and allowed for its migration for 6 h. The upper surface of the membrane was gently scrubbed with a cotton swab, and the cells that migrated to the lower membrane surface were fixed with 2.5% glutaraldehyde for 10 min and stained with 0.5% crystal violet solution for 2 h. The bright field images of migrated cells were captured by a microscope (Leica DMi8, Germany).

3.14. F-actin accumulation study by ActinGreen488 staining

Differentiated THP-1 macrophage cells were sub-cultured into the Millicell EZ Slide, 8 well (Merck) chambered slide. After termination of treatment, culture medium was removed from each well and then cells were fixed with 4% paraformaldehyde in PBS and kept for 30 min at room temperature. Cells were washed twice with PBS and permeabilized by 0.2% Triton-X-100 in PBS for 15 min at room temperature. Cells were then washed twice with PBS and ActinGreen 488 (Thermo) was added following to manufacturer's protocol. Cells were then incubated with staining solution for 30 min in dark. After removing the stain solution, cells were washed twice with PBS and mounted in Vectashield anti-fade mounting medium containing DAPI for nuclear staining. Images were taken using inverted fluorescent microscope (Leica, DMi8, Germany).

3.15. Co-immunoprecipitation study

Differentiated THP-1 macrophage cells were co-transfected with Human FLAG-MD-2, TLR4-YFP; Myc-CD14 plasmids using Lipofectamine-2000 following manufacturer's protocol. THP-1 cells were incubated with *L. donovani* promastigotes by maintaining a ratio of 1:10 (macrophage/parasite). On termination of incubation, cells were harvested in CHAPS buffer containing Protease and Phosphatase Inhibitors and cell lysates were used for immunoprecipitation study. Briefly, 100 µg of protein was incubated overnight at 4 °C with 2 µg of anti-GFP antibody. Around 50 µl of Protein G Sepharose was then added to each tube and incubated at 4°C for 4 h followed by centrifugation at 10,000 g. Immuno-complex was resuspended in 500 µl of 1% Triton X-100 in PBS followed by washing with PBS. Immuno-precipitate was boiled in 4X sample buffer at 90°C for 10 min, vortexed thoroughly and centrifuged at 10,000 g for 10 min at room temperature. Supernatant was isolated and resolved in 10% SDS-PAGE followed by immunoblotting with anti-Myc antibody or anti-FLAG antibody. Anti-GFP antibody was used as loading control for each sample.

3.16. Affinity purification for TLR4 and LPG

HEK-293 cells were transfected with human pcDNA3-TLR4-YFP plasmid using Lipofectamine 3000 following the manufacturer's protocol. After 48 hours of transfection, cells were harvested in RIPA buffer containing protease and phosphatase inhibitors and subjected to lysis. Cell lysate was used for affinity purification of TLR4 by GFP-Trap Agarose (Cat No. #gta-10, Chromotek) following the manufacturer's instruction. Briefly, cell lysate (100 µg of protein) was incubated for 1 h at 4°C with 25 µl of bead slurry. GFP-tagged TLR4 protein was then eluted in 50 µl acidic elution buffer (200 mM glycine, pH 2.5) and then pH was neutralized by the neutralization buffer. Purified protein was subjected to 10% SDS-PAGE followed by silver staining for visualization.

L. donovani promastigotes were harvested in RIPA buffer and subjected to sonication followed by the vigorous vortexing for complete cell lysis. Parasites cell

lysate was subjected to first 50 kDa followed by 100 kDa molecular weight cutters and the collected fractions were pooled representing 50-100 kDa was subjected to immunoaffinity chromatography using NHS-Activated Sepharose 4 Fast flow (GE healthcare) as a matrix. Briefly, NHS-activated Sepharose 4 Fast flow (GE Healthcare) was coupled with 500 µg of anti-LPG antibody for 4 h at room temperature, packed in a column, and blocked with 0.1 M Tris, pH 8.0 for overnight at 4°C. Unbound antibodies were washed with five column volume of PBS, pH 7.4. The column was incubated with 50-100 kDa molecular cutter fractions overnight at 4°C and then washed with five column volume of PBS, pH 7.4. LPG was eluted by washing the column with five column volume of 0.1 M glycine, pH 2.0 followed by immediate neutralization of pH by 1M Tris-Cl, pH 9.0 and then subjected to 10% SDS-PAGE and silver staining.

3.17. Isothermal Titration Calorimetry (ITC)

To study the thermodynamics of LPG and TLR4 interaction, Isothermal Titration Calorimetry assay was performed following a method described previously [8]. Briefly, TLR4 protein (wild type and mutated LRRs, 1 µM) was taken in binding buffer in the cell as titrate and LPG (100 µM) was taken in the same buffer in the syringe as the titrant (molar ratio of titrate: titrant=1:100). Step-wise injections of equal volumes of titrant were made into the cell and heat changes were measured in a MicrocaliTC 200 system (Malvern Panalytical, UK). The heat change data were plotted against molar ratio of titrant to analyze the binding parameters and curve fitting was performed using a multisite model with reiterative $\chi 2$ -test.

3.18. Arginase activity assay

Briefly, after incubations, cells were lysed with 0.1% Triton-X-100. 25mM Tris-Hcl were added to the cell lysate after 30 minutes of incubation on a shaker. Enzyme activation was done by heating cell lysates for 10 mins at 56°C. Cell lysates were then incubated with 0.5 M L-Arginine (p^H 9.7) for 15-20 mins at 37°C for arginine hydrolysis. This reaction was stopped by adding H_2SO_4 (96%), H_3PO_4(85%), H_2O (1/3/7), v/v/v, alpha-iso-nitroso propiophenone was then added to the cell lysates for

30 minutes and samples were heated at 95°C.Urea concentration of each sample was measured at 540 nm in Varioskan LUX Multimode Microplate Reader. Concentration of protein was quantified by using BCA method.

3.19. Luciferase reporter assay

The THP-1 macrophages (2×10^5 cells/well) were transfected with κB-luciferase or AP1-luciferase or IRF3-luciferase plasmids (0.1 µg/well) using Lipofectamine 3000 Transfection Reagent following manufacturer's protocol. Briefly, 0.3 µl of Lipofectamine 3000 Transfection Reagent and 0.1 µg of respective plasmids were added separately into 10 µl of Opti-MEM media, both solutions were mixed and incubated for 10 min. The transfection mixture was added to the cells containing complete growth media without antibiotics. After incubation at 37°C for 6 h, the culture medium was changed to growth media containing 20% FBS. After 48 h of transfection, cells were washed and used for parasite infection for 4h. THP-1 cells were then lysed and luciferase activity was measured using Steady-Glo Luciferase Assay System with the help of Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland).

3.20. Animal husbandry and treatments

Healthy BALB/c mice were procured from West Bengal Livestock Development Corporation Limited, Kalyani, West Bengal (Reg. No. 10012031000104) and maintained *ad libitum*, in West Bengal State University experimental animal facility for carrying out all the experimental work in this study. Prior to the experiment, animals were kept for acclimatization for 14 days under laboratory condition. On day 1 of 28 days infection model, BALB/c mice were injected with 10^7 stationary-phase *L. donovani* promastigotes through *i.v* administration. Treatment groups for the animals mentioned below as: Group I: Naive/ uninfected Control (n=4), Group II: *L.donovani* infected Control (n=4), Group III: *L.donovani* infected Control Vehicle /PBS (n=4) (*i.p* administration once in a week for 28 days experiment), Group IV: *L.donovani* infected Cli-095 injected group(n=4)(*i.p* administration of Cli-095, 1.5mg/kg/bw for once in a week for 28 days experiment), Group V:

L.donovani infected LPS injected group(n=4)(*i.p* administration of LPS, 100µg/mice for once in a week for 28 days experiment), Group VI: *L.donovani* infected LPS+Cli-095 injected group(n=4) (i.p administration of LPS, 100µg/mice with *i.p* administration of Cli-095,1.5mg/kg/bw once in a week for 28 days experiment), Group VII: *L.donovani* infected TLR4-ASO injected group (n=4)(*i.v* administration of TLR4-ASO, 1µM/mice in lateral tail vein once in a week for 28 days experiment), GroupVIII: *L.donovani* infected LPS+TLR4-ASO injected group (n=4) (*i.p*administration of LPS, 100µg/mice for once in a week and *i.v* administration of TLR4-ASO, 1µM/mice in lateral tail vein once in a week for 28 days experiment). The IAEC (Institutional Animal Ethics Committee) guidelines had been followed during performing the experiments (Approval number: WBSU/IAEC/CP/1, dated 03.03.2015).

3.21. Immunohistofluorescence staining

Immunostaining was performed on cryosections of mouse liver tissue using specific antibodies. Briefly, OCT embedded tissue was cryo sectioned at 15 µm thick, fixed with cold methanol, blocked with 1% BSA and incubated overnight with anti-pNF-κB (S536) antibody (1:1400 dilution) and Anti-Leishmania A2 antibody (1:1000 dilution). Signal was visualized by subsequent incubation with Alexa Fluor 594 conjugated anti-mouse IgG antibody (1:200 dilution) and mounted in Vectashield antifade mounting medium containing DAPI (Cat. No. #H-1200; Vector Laboratories, Burlingame, CA) for nuclear staining. Images were captured by a fluorescence microscope (Leica DMi8, Germany) and analysis was performed using LASX software.

3.22. Parasite burden analysis

For *in vivo* parasite burden determination, BALB/c mice were injected with 10^7 stationary-phase *L. donovani* promastigotes through *i.v* administration. Infection was assessed by removing the liver and spleen from infected mice after completion of 28 days, parasite burdens were determined in Giemsa-stained impression smears and the data are represented in LDU. Infection was determined by measuring the liver

and spleen parasite burden by the conventional Leishman–Donovan unit (LDU) method [12].

3.23. Cytotoxicity assay

Cell cytotoxicity was measured by MTT assay following the method described previously [2]. Briefly, promastigotes (2 x 10^5 cells) were cultured cells into a 96-well plate in the presence or absence of varying concentrations of the IMPA compounds (0.01µM-10µM); for 24 h. Briefly, control and treated *L. donovani* promastigotes in 96 well plates were incubated with MTT for 4 h at 25°C. On termination of incubations, formazan crystals formed in cells were dissolved in acidic isopropanol and incubated further for additional 30 min at 37°C. Cell viability was measured spectrophotometrically at 570 nm with Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland). Absorbance values were blanked against acidic isopropanol and the absorbance of cells exposed to medium only (without any treatment) were taken as 100% cell viability (control). The cell viability and IC_{50} were determined from the concentration response curve generated using GraphPad Prism 8.0 Software.

3.24. Intracellular parasite burden analysis

THP-1 monocyte cells were cultured on cover slips, in six well culture plate constituted with PMA and kept for 48 hour for differentiation into THP-1 macrophage cells. Differentiated macrophages further infected with *L. donovani* promastigotes in a ratio of 1:10 (macrophage: parasite) for 24 h to study the anti-leishmanial activity of the IMPA compounds 2 and 12[10]. Unbound and uninternalized parasites were washed twice with 1X PBS and cells were further incubated with the IC_{50} dose of IMPA 2 and IMPA 12. Upon treatment completion, cells were washed with 1X PBS, fixed in methanol and stained with Giemsa. The no. of amastigotes /100 macrophages were counted for determination of parasite load in macrophage cells for checking the effectivity of IMPA 2 and 12 respectively.

3.25. Acridine orange/Ethidium bromide staining

L. donovani promastigotes were seeded in 24-well tissue culture plates (1×10^6 cells/well), treated without or with IC_{50} concentrations of IMPA2 and IMPA 12 accordingly for 24 h, and the cells were washed two times with 1XPBS. Then, the cells were stained with a mixture of acridine orange (3 µg/ml) and ethidium bromide (10 µg/ml) for 10 minutes in dark. After staining immediately place 10 µl of cell suspension onto a microscopic slide, and covered with a glass coverslip, and images were taken using inverted fluorescent microscope (Leica, DMi8, Germany) using 20X objective.

3.26. Measurement of reactive oxygen species (ROS)

2×10^4 promastigotes /well were seeded into 96-well plates, and incubated with DCFDA solution (25 µM, 100 µl/well) diluted in Kreb's Ringer Buffer solution for 45 min at 37°C in dark. After treatment completion, cells were washed with 1X PBS and The Kreb's Ringer Buffer was then removed and serum free M199 medium (150 µl/well) was added with or without IMPA 2 and IMPA 12 for 24 h. Medium was removed from each well and cells were washed with PBS (150 µl/well). The cell lysis buffer (150µl/well) was added to each well and incubated for 10 min. *L. donovani* promastigotes untreated by IMPAs but stained with DCFDA kept as control. Cellular ROS levels was measured in Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland) at Ex/Em = 492/517 nm.

3.27. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

To examine the mitochondrial membrane potential change, JC-1 dye was used, which is a cationic dye that exhibit potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red. *L. donovani* Promastigotes were cultured in 24-well tissue culture plates (1×10^6 cells/well), without or with IC_{50} concentrations of IMPA2 and IMPA 12 accordingly for 24 h at 25°C.Post incubation, parasites were washed two times with 1X PBS, then stained with 5µM JC-1 dye was added for 30 min at room temperature

with parasites in 1X PBS in the dark. Relative change of red to green mean fluorescence intensity was measured to know the change in mitochondrial membrane potential ($\Delta\Psi_m$) of parasites. Flow cytometric data were acquired using a BD FACSLyricTM Flow Cytometry System (BD Biosciences) equipped with three laser configurations (488, 405, and 640 nm) and the BD FACSuiteTM Software for acquisition and analysis. Samples were processed by using excitation wavelength of 488 nm and emission band pass 505–550 nm (green) and a long pass of 575 (red) [11].

3.28. Flow cytometric analysis of cell apoptosis by Annexin V-FITC and PI staining

L. donovani promastigotes apoptosis analysis was performed by using flow cytometric determination of phosphatidylserine externalization by Annexin V-FITC and propidium iodide (PI) double staining (FITC-Annexin V apoptosis detection kit, BD Biosciences) followed by manufacturer's protocol. 2×10^6 *L. donovani* promastigotes were incubated without or with IC_{50} concentrations of IMPA2 and IMPA 12for 24 h. Post treatment parasites were harvested and washed twice in 1XPBS. After that, cell pellet was resuspended in 195 µl of 1X binding buffer supplemented with 5 µl Annexin-FITC and 5 µl PI and incubated for 15 min at room temperature in dark. Samples were then subjected to flow cytometric quantification within 1 hour using BD FACSLyricTM Flow Cytometry System (BD Biosciences) equipped with three laser configurations (488, 405, and 640 nm) and the BD FACSuiteTM Software for acquisition and analysis. Dot-plot graphs were used to illustrate the viable cells (the lower left quadrant), early phase apoptotic cells (the upper left quadrant), late-phase apoptotic (the upper right quadrant), and the dead or necrotic cells (the lower-right quadrant).

3.29. Cell cycle analysis by flow cytometry

Briefly, 2×10^6 *L. donovani* promastigotes were treated with or without IMPA 2 and IMPA 12 following IC_{50} dose for 24 h. Post incubation parasites were harvested, washed twice in 1X PBS and fixed with 70% ethanol at 4 °C for 24 h. Pellet was

further washed with 1X PBS and further supplemented with 500 µl DNAs free RNaseA(100 µg/ml). Cell Pellet was further stained with propidium iodide (PI) dye (10 µg/ml) in the dark for 20 min. The percentage of cell counts in different phases of cell cycle was analyzed by using BD FACSLyricTM Flow Cytometry System (BD Biosciences). Data acquisition and analysis were performed by using BD FACSuiteTM Software.

3.30. Site directed mutagenesis

A pcDNA3-TLR4 plasmid was used as a template for the generation of TLR4-LRR mutants using a QuickChange Lightning Multi Site-Directed Mutagenesis kit following the manufacture's protocol. Primers used to mutate the specific LRRs were designed with the help of QuickChange Primer Design Program available online at www.agilent.com/genomics/qcpd. Forward and reverse primer sequences used for mutated LRRs are listed below in Table 3.2.

Table 3.2: List of primers used for mutation of LRRs in this study

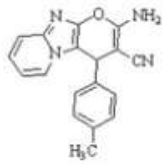
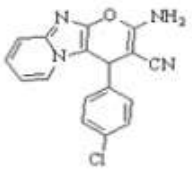
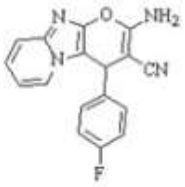
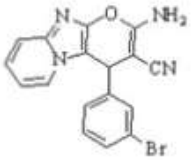
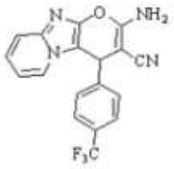
LRR Region	Region of Deletion	Sequence (5'-3')
LRR1-hTLR4	del 361-518 (F)	CAACCTCCCCTTCTCAACCATGAACTTTATCCAACCAG
	del 361-518 (R)	CTGGTTGGATAAAGTTCATGGTTGAGAAGGGGAGGTTG
LRR2-hTLR4	del 533-541 (F)	CCAGGTGCATTTAAAGAAATTAGGCTTGATAGTTTAAATGTAATGAAAACTTGTA
	del 533-541 (R)	TACAAGTTTTCATTACATTTAAACTATCAAGCCTAATTTCTTTAAATGCACCTGG
LRR3-hTLR4	del 637-645 (F)	GACTTTTCTTATAATTTCGGATGGTTTGGACAGTTTCCCACATTGAAA
	del 637-645 (R)	TTTCAATGTGGGAAACTGTCCAAACCATCCGAAATTATAAGAAAAGTC
LRR4-hTLR4	del 658-666 (F)	CACATTGAAACTCAAATCTCTCGGTGGGAATGCTTTTTCAGAAG
	del658-666 (R)	CTTCTGAAAAAGCATTCCCACCGAGAGATTTGAGTTTCAATGTG

LRR5-hTLR4	del 680-688 (F)	GAAGTTGATCTACCAAGCCTTTTGAGTTTCAAAGGTTGCTG
	del680-688 (R)	CAGCAACCTTTGAAACTCAAAAGGCTTGGTAGATCAACTTC
LRR6-hTLR4	del 706-714 (F)	GATTTTGGGACAACCAGCCTAGTTATTACCATGAGTTCAAAC
	del 706-714 (R)	GTTTGAACTCATGGTAATAACTAGGCTGGTTGTCCCAAAATC
LRR7-hTLR4	del 729-737 (F)	TTCAAACTTCTTGGGCTTAGAACAACTATTGAAACAAATGAGTGAGTTT
	del 729-737 (R)	AAACTCACTCATTTGTTTCAATAGTTGTTCTAAGCCCAAGAAGTTTGAA
LRR8-hTLR4	del 754-762 (F)	CCTATCACTCAGAAACCTCACCAGAGTTGCTTTCAATG
	del 754-762 (R)	CATTGAAAGCAACTCTGGTGAGGTTTCTGAGTGATAGG
LRR9-hTLR4	del 533-762 (F)	ACCAGGTGCATTTAAAGAAATTAGGCTTACCAGAGTTGCTTTCAA
	del 533-762 (R)	TTGAAAGCAACTCTGGTAAGCCTAATTTCTTTAAATGCACCTGGT
LRR10-hTLR4	del 778-884 (F)	CAATGGCTTGTCCAGTCTCTTTGCTTGTACTTGTGAAC
	del 778-884 (R)	TTGAAAGCAACTCTGGTAAGCCTAATTTCTTTAAATGCACCTGGT

3.31. Details of IMPA derivatives

The IMPA derivatives were synthesized by Dr. Rambabu Gundla and his group and kindly gifted to examine their anti-leishmanial activity against *Leishmania donovani* promastigotes. The synthesis and characterization of the IMPA derivatives were described in their previous publication [9]. These Five IMPA compound have shown potent anti-cancer activity and cytotoxicity in non-small cell lung cancer (NSCLC) as previously reported [9]. Structural details of these five IMPA derivatives were mentioned below.

Table 3.3: Structure of IMPA Compounds

	**IMPA 2:** 2-Amino-4-(*p*-tolyl)-4*H*-pyrano[2',3':4,5]imidazo[1,2-*a*]pyridine-3-carbonitrile
	**IMPA 5:** 2-Amino-4-(4-chlorophenyl)-4H-pyrano[2',3':4,5]imidazo[1,2-*a*]pyridine-3-carbonitrile
	**IMPA 6:** 2-Amino-4-(4-fluorophenyl)-4*H*-pyrano[2',3':4,5]imidazo[1,2-*a*]pyridine-3-carbonitrile
	**IMPA 8:** 2-Amino-4-(3-bromophenyl)-4*H*-pyrano[2',3':4,5]imidazo[1,2-*a*]pyridine-3-carbonitrile
	**IMPA 12:** 2-Amino-4-(4-(trifluoromethyl)phenyl)-4*H*-pyrano[2',3':4,5]imidazo[1,2-*a*]pyridine-3-carbonitrile

3.32. Statistical analysis

All data were derived from at least three independent experiments and statistical analyses were conducted using Sigma Plot 10.0 software. Densitometric analysis of RT-PCR and Western blot data was performed using ImageJ software (1.48v, NIH,

USA). Data were analyzed by unpaired Student's ttest, where the p value indicated significance. All values were means ± SEM. A level of $p < 0.05$ was considered significant.

3.33. Bibliography

[1] Mallick, S., Halder, S., Dutta, A., Dey, S., Paul, K., Maiti, S.,& Pal, C.. Chromone linked nitrone derivative induces the expression of iNOS2 and Th1 cytokines but reduces the Th2 response in experimental visceral leishmaniasis. *International Immunopharmacology*, 15(4), 772-779, 2013.

[2] Banerjee, D., Sinha, A., Saikia, S., Gogoi, B., Rathore, A. K., Das, A. S., Pal, D., Buragohain, A.K. & Dasgupta, S. Inflammation-induced mTORC2-Akt-mTORC1 signaling promotes macrophage foam cell formation. *Biochimie*, 151, 139-149, 2018.

[3] Kumar, G. A., Karmakar, J., Mandal, C., & Chattopadhyay, A.. Leishmania donovani internalizes into host cells via caveolin-mediated endocytosis. *Scientific Reports*, 9(1), 12636, 2019.

[4] Loike, J. D., & Silverstein, S. C. (1983). A fluorescence quenching technique using trypan blue to differentiate between attached and ingested glutaraldehyde-fixed red blood cells in phagocytosing murine macrophages. *Journal of immunological methods*, 57(1-3), 373-379, 1983.

[5] Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-75, 1951.

[6] Dey, S., Mukherjee, D., Sultana, S.S., Mallick, S., Dutta, A., Ghosh, J., Hussain, A., Sarkar, B., Mandal, S., Patra, P.,Saha, B., Pal, C.. Combination of Mycobacterium indicus pranii and heat-induced promastigotes cures drug-resistant Leishmania infection: critical role of interleukin-6-producing classical dendritic cells. Infect. Immun. 88(6), 1-20, 2020.

[7] Hussain, A., Ghosh, S., Roy, K., Nath, S., Sarkar, B., Dutta, A., A., Maji, P., Basu, S., Paul, S., Dey, S. and Chakraborty, K.& Pal, C.. A mushroom derived 'carbohydrate-fraction'reinstateshost-immunity and protects from Leishmania donovani infection. *Parasite Immunology*, 43(3), e12806, 2021.

[8] Su, H., & Xu, Y.. Application of ITC-based characterization of thermodynamic and kinetic association of ligands with proteins in drug design. *Frontiers in pharmacology*, 1133, 2018.

[9] Bhavya, K., Mantipally, M., Roy, S., Arora, L., Badavath, V. N., Gangireddy, M., Dasgupta, S., Gundla, R. & Pal, D..Novel imidazo [1, 2-a] pyridine derivatives induce apoptosis and cell cycle arrest in non-small cell lung cancer by activating NADPH oxidase mediated oxidative stress. *Life Sciences*, 294, 120334, 2022.

[10] Ali, R., Islamuddin, M., Tabrez, S., Alsaweed, M., Alaidarous, M. A., Alshehri, B. M., Banawas, S., Dukhyil, A.A.B.& Rub, A.. Embilica officinalis L. inhibits the growth and proliferation of Leishmania donovani through the induction of ultrastructural changes, mitochondrial dysfunction, oxidative stress and apoptosis-like cell death. *Biomedicine & Pharmacotherapy*, 143, 112156, 2021.

[11] Shadab, M., Jha, B., Asad, M., Deepthi, M., Kamran, M., & Ali, N.. Apoptosis-like cell death in Leishmania donovani treated with KalsomeTM10, a new liposomal amphotericin B. *PloS one*, 12(2), e0171306, 2017.

[12] Murray HW, Miralles GD, Stoeckle MY et al. Role and effect of IL-2 in experimental visceral leishmaniasis. J Immunol; 151: 929–38, 1993.