4. Materials and Methods

4.1 Chemicals, reagents, laboratory-wares and services were procured from following sources:

Takara Bio Inc.

TB Green Premix Ex Taq II with Tli RNase H (RR820), Clontech® His60 Ni Superflow Resin (635661), Taq™ DNA Polymerase, with Mg2+ free buffer (R001BM)

New England Biolabs (NEB)

BamHI (R0136), BamHI-HF (R3136), XbaI (R0145), NheI (R0131), EcoRI (R0101), T4 DNA ligase (M0202), Phusion® high fidelity DNA polymerase (M0530)

1st Base Laboratories

Single-pass DNA sequencing (SS1001)

Applied Biosystems™

MicroAmp™ Optical 8-Cap Strips (432032), MicroAmp™ Optical 96-Well Reaction Plate (N8010560), MicroAmp™ Clear Adhesive Film (4306311)

Genaxy Scientific Pvt. Ltd.

0.1 ml 96-Well PCR Plate, fit ABI, Low profile, Natural (GEN-96-010-ES-ABC).

Axygen, Inc.

PCR tubes (PCR-02-C), Microtubes (MCT-175-C, MCT-060-C), Pipette tips (T-1000-B, T-200-Y, RF-300-C), Aerosol-barrier tips (TF-200-R-S, TF-100-R-S, TF-20-R-S, TF-300-R-S)

Invitrogen

Mu-MLV reverse transcriptase buffer (18057-018),Mu-MLV-Reverse transcriptase (28025-021), 0.1M dithiothreitol, 10mM dNTP mix (18427-088), Random Primers (48190-011), 10X PCR buffer (18067-017), 50mM $Mgcl_2$, Penicillin-Streptomycin (15140-122), L-glutamine (250030-081), Sodium Pyruvate (11360-070), Agarose (15510-027)

Milipore

0.22 µm (SLGP033RS) and 0.45 µm (SLHV033RS) syringe filters, Immobilon-P 0.45 µm PVDF membrane (IPVH00010)

Roche Holding AG

cOmplete^{TM}, Mini protease inhibitor cocktail (11836153001) and Streptavidin-POD conjugate (11089153001).

Corning Inc.

Corning® 96-well Clear Polystyrene High Bind Stripwell™ Strips, without Frame or Lid, Nonsterile (2580), Corning® 96-well Stripwell™ Egg Crate Strip Holder (2572), Costar® Spin-X® Centrifuge Tube Filters, 0.22 µm Pore CA Membrane (8160), Cell Scrapers (CLS3010), 25 cm^2 flask ().

Bio-Rad Laboratories

Precision Plus Protein™ Dual Color Standards (1610394), Goat Anti-Mouse IgG (H+L)-HRP Conjugate (1721011), Protein Assay Dye Reagent Concentrate (5000006), Clarity Max^{TM} Western ECL substrate (1705062)

Pierce Biotechnology

Pierce™ BCA Protein Assay Kit (23225), SnakeSkin® pleated dialysis tubing (68035), High Capacity Endotoxin Removal Resin (88270)

Sigma-Aldrich

TRI Reagent® (T9424), Ethidium Bromide (E7637), Sodium dodecyl sulphate (L3771), Ammonium persulphate (A3678), TEMED (T9281), 2-mercaptoethanol (M7522), Bovine Serum Albumin (B6917), Ponceau S (P3504), Bromophenol blue (B8026), Trypan Blue (T8154), Acrylamide (A9099), N,N-methylene bisacrylamide (M7279), Imidazole (I5513), Tris-saturated Phenol (P4557), Lysozyme (L6876), Cesium chloride (C4036), Carestream® Kodak® autoradiography GBX developer/replenisher (P7042), GenElute™ PCR Clean-Up Kit (NA1020), GenElute™ gel extraction kit (NA1111), Freund's adjuvant, incomplete (F5506), Amicon Ultra 0.5 ml centrifugal filter, 3kDa (Z677094), PMA (P 1585)

Fischer Scientific International, Inc. (Thermo Fischer Scientific)

Sodium chloride; NaCl (27605), Glycine (24755), Pottasium dihydrogen orthophosphate; KH_2PO_4 (19465), Sodium hydroxide; NaOH (15895), Formaldehyde (12755), Glycerol (15455), Ethylene diamine tetraacetic acid (EDTA), disodium salt (12635), Tris

(hydroxymethyl) methylamine (15965), Methanol (32406), Propan-2-ol (13825), Glacial acetic acid; CH_3COOH (11005), Hydrochloric acid; HCl (29505), Sulphuric acid; H_2SO_4 (29995), Chloroform (12306)

GIBCO® (Thermo Fischer Scientific)

Fetal Bovine Serum, USA, certified (16000-044), Advanced RPMI-1640 (12633-012), Dulbecco's modified Eagle medium (12430-054), Hank's Balanced Salt Solution; HBSS (14025).

Qualigens fine chemicals (Formerly a part of Glaxo India Pvt. Ltd.)

Calcium chloride (22185), Magnesium chloride (25165)

HiMedia Laboratories Pvt. Ltd.

Potassium acetate; CH_3COOK (P1190), LB broth (M1245), LB Agar (modified) (M1151F), HEPES (RM380), MOPS (RM660), Manganese chloride; $MnCl_2$ (RM3925), Ionomycin, calcium salt (RM9641).

MP Biomedicals

Triton-X-100 (219485483), Boric acid (194810), N, N'-methylene bis(acrylamide) (195316), IPTG (MP114064102).

USB Chemicals

Sodium phosphate, monobasic, monohydrate; $NaH_2PO_4.H_2O$ (20233), Sodium phosphate, dibasic, heptahydrate; $Na_2HPO_4.7H_2O$ (20232)

Affymetrix Inc.

Tween-20 (AAJ20605AP)

Difco™

Fluid Thioglycollate Medium (DF0256)

Merck™

Novagen™ Benzonase™ nuclease (10349963), Dextrose (17809)

Beckman Coulter Inc.

Quick-Seal® Polypropylene Tubes, 16 X 57 mm (344621)

Qiagen

Effectene® Transfection reagent (301425)

BD biosciences

FITC Rat anti mouse-CD3 (555274), PB Rat anti mouse-CD4 (558107), PerCPCy5.5 Rat anti-mouse CD44 (560570), APCCy7 Rat anti mouse-CD62L (560514), APCCy7 Rat anti mouse-CD25 (557658), APC Rat anti mouse-FOXP3 (560402), PE Rat anti mouse-IFNγ (554412), PE Rat anti mouse-IL4 (554435), and PE Rat anti mouse-IL10 (554467), FITC Rat anti mouse-B220 (553087), PE Rat anti mouse-CD19 (557399), Horizon™ V450 Streptavidin (560797), FITC mouse IgG1, κ isotype control (550616), PE Rat IgG2a, κ

isotype control (554689), PerCP-Cy5.5 mouse IgG1, κ isotype control (550795), PE-Cy7 Hamster IgG1, κ isotype control (552811), APC Rat IgG2b, κ isotype control (556924), APC-Cy7 Rat IgG1, λ isotype control (557663), Pacific Blue Rat IgG2a, κ isotype control (558109), Cytofix/Cytoperm-plus kit with Golgi Plug (555028), TMB substrate reagent set (555214), recombinant anti mouse IFN-γ (551216), Biotin Rat anti mouse IgG2a (553388), Biotin Rat anti mouse IgG1(553441), Biotin Rat anti mouse IgM (553406), Purified Rat anti-mouse IL-4 (554387), Biotin Rat anti-mouse IL-4 (554390), Purified Rat anti-mouse IL-10 (551215), Biotin Rat anti-mouse IL-10 (554423), Purified Rat anti-mouse IL-12 (551219), Biotin Rat anti-mouse IL-12 (554476), Purified Rat anti-mouse IFN-γ (551216), Biotin Rat anti-mouse IFN-γ (554410), Falcon® 6-well Clear Flat Bottom TC-treated (353046), Falcon® 24-well Clear Flat Bottom TC-treated (353047), Falcon® 50 ml conical centrifuge tubes (14-432-22), Falcon® 15 ml conical centrifuge tubes (14-959-49B),

Biolegend

PECy7 Rat anti-mouse/human CD44 (103030), PerCPCy5.5 Rat anti mouse-CD62L (104432), APC Rat anti mouse-Tbet (644804), APC Rat anti mouse-IL17 (506916)

eBioscience, Inc.

APC Rat anti mouse-GATA3 (50-9966-42)**,** PE Rat anti mouse-RORγT (12-6981-82)

Santa Cruz® Biotechnology, Inc.

Anti-his tag antibody, monoclonal IgG1 (sc-8036)

HMD Healthcare Ltd.

DispoVan® 1 ml (31G) and DispoVan® 2, 5, 10 and 20 ml sterile syringes (Luer Mount).

Fujifilm

X-ray films

Indo-Chem Laboratories

Hi-Fix® high speed liquid fixer and Hardener

IDT, Inc.

Following table enlists the primer pair procured :

Gene	**Sense**	**Anti-sense**
LmjF_36_3850 (cloning)	5'-TATCGCGGATCCTGATGTTCAATCGTCTC-3'	5'-TATCTGCTCTAGAGTTACGCAAGCTCCGC-3'
LmjF_36_3850 (RT-PCR)	5'-ATGTTCAATCGTCTCTTTGGCAG-3'	5'-TTACGCAAGCTCCGCCTC-3'
LmpcDNA (cloning)	5'-TATACCGGAATTCTGCCACTGTTTATCAT-3'	5'-TATCTGCTCTAGAGCTTTGCCAG-3'

LmAdeK (RT-PCR)	5’-AAGGGGACAGTGAGCCATC-3’	5’-TGTCAGAGTAATGGGCGATGA-3’
Lmpet28a+ (cloning)	5’-CTAGCTAGCATGCCACTGTTTATCATTC-3’	5’-CGGGATCCCAGCTTTGCCAGAGGGAC-3’
β-tubulin	5’-AGCAGTTCACGGGTATGTTC-3’;	5’-GAGACGAGGTCGTTCATGTT-3’;
LmjF_36_3850	5’-GGATGAGCTGAAGCGTTCTAA-3’;	5’-CTTGTCCATCTCCTCCATCAAG-3’;
n-nitro phenyl phosphatase	5’-CCTTGTACATCCAGGAGAAGTT-3’	5’-GACGGACTGCACCTCATTAT-3’
gp63 (Leishmanolysin)	5’-TACGAGGAAAGCCGCATAAC-3’	5’-ATCCTCCATCTCCAGATACTCC-3’

NAD+ synthase	5’-CTTCCCGTACGACTTTGTAGAG-3’	5’-GTTTCGGGCCTCATCACATA-3’
Adenylate kinase	5’-AGGAAGCGTCAGGATGAATAC-3’	5’-CGAGCAACTTCGGCAAATAC-3’
DNA polymerase ε subunit B	5’-GTGCGGGATGACGAAGTATT-3’	5’-CCACAGTCAGGAACCCATTT-3’
Phosphatidic acid phosphatase	5’-GCATCTTCACGGGTCTTCTT-3’	5’-GTCGAAGTAGTGACGGTTGTC-3’
Thiol dependent reductase-1	5’-CGATCTCTTCGCCCACTTT-3’	5’-CGGATGTGCTCCTTGTACTC-3’
Pyruvate carboxylase	5’-CACAAGATCGACCCTCTCAAA-3’	5’-ACTTCTTCGACCGCCTTTAC-3’
HGPRT	5’-CCGTGGAGAATCGCCATATT-3’	5’-GAGCATGAACCGCATCAGATA

		-3’
LmjF_33_2620	5’-CCGTGCGTTTAAGCACTTTC-3’	5’-CCCAAACACCAAGAACACAAC-3’
ARP2/3 complex subunit	5’-TATTGAGGCATCGTGTGACAG-3’	5’-GAGCAGAAGAACGCCATGTA-3’
IFN-γ	5’-TGGATATCTGGAGGAACTGG-3’	5’-TCGGCTTGCTGTTGCTGA-3’
IL-4	5’-GGTGTTCTTCGTTGCTGTGA-3’;	5’-TCTCGAATGTACCACGAGCC-3’;
IL-10	5’-AACATACTGCTAACCGACTCC-3’	5'-TCCTTGATTTCTGGGCCATG-3’
IL-17	5’-CTCCACCGCAATGAAGACC-3’	5’-CGGTCTTCATTGCGGTGGAG-3’
FOXP3	5’-GGCCCTTCTCCAGGACAGA-3’	5’-GTCTGTCCTGGAGAAGGGCC-3’
CTLA4	5’-TACCCACCGCCATACTTTGT-3’	5’-AGAAACAGCAGTGACCAGGA-

		3’
GAPDH	5’- ATTGTCAGCAATGCATCCTG-3’	5’- ATGGACTGTGGTCATGAGCC-3’

4.2 Buffers and solutions

4.2.1 Phosphate-buffered saline (PBS), pH 7.4

NaCl	4 g
$Na_2HPO_4.7H_2O$	0.72 g
KH_2PO_4	0.12 g
KCl	0.1 g
Deionized water	q.s. to 500 ml

Contents were dissolved in 350 ml of distilled water and volume was made up to 500 ml. pH of solution was adjusted by 1N NaOH. Solution was autoclaved and stored at 4°C. ELISA washing buffer was made by mixing 0.05% tween-20 in PBS.

4.2.2 Tris-borate-EDTA (5X)

Tris	27 g
Boric acid	13.75 g
0.5M EDTA	10 ml
Deionized water	q.s. to 500 ml

Contents were mixed and dissolved in deionized water and volume was adjusted to 500 ml

4.2.3 Lysis buffer

Tris-Cl (pH 7.5)	20mM
NaCl	150 mM
EGTA	1 mM
EDTA	1 mM
Glycerol	10%
NP-40	1%
Protease inhibitor cocktail	1 tablet/10 ml of lysis buffer (fresh addition)

Contents are diluted from their stock solutions in deionized water and volume was adjusted accordingly. Aliquots were prepared and stored at -20°C until further use.

4.2.4 30% acrylamide solution

Acrylamide	29.2 g
N, N'-methylene-(bisacrylamide)	0.8 g
Deionized water	q.s. to 100 ml

Contents are mixed and volume was adjusted accordingly. Solution was kept at 4°C in an amber coloured bottle.

4.2.5 Tris buffer (pH 8.8) for resolving gel

Tris base	18.17 g
Deionized water	q.s. to 100 ml

pH was adjusted by drop-wise addition of 1N HCl and solution was kept at 4°C

4.2.6 Tris buffer (pH 6.8) for stacking gel

Tris base	12.11 g
Deionized water	q.s. to 100 ml

pH was adjusted by 1N HCl and solution was kept at 4°C

4.2.7 10% (w/v) Sodium dodecyl sulphate (SDS)

SDS	5 g
Deionized water	q.s. to 50 ml

Dissolved by light stirring in a beaker, volume was adjusted accordingly and stored at room temperature

4.2.8 10% (w/v) Ammonim persulphate (APS)

1 g APS was dissolved in deionized water and volume was adjusted to 10 ml. Solution was stored, protected from light at 4°C.

4.2.9 10X SDS-electrophoresis buffer (pH 8.3)

Tris-base (250 mM)	15.1 g
Glycine (2.5 M)	94 g
SDS (1%)	5 g
Deionized water	q.s. to 500 ml

Glycine was added first and allowed to dissolve by vigorous stirring. Remaining contents are added and volume was adjusted accordingly. Solution was diluted in deionized water before use.

4.2.10 Recipe for resolving and stacking gels

Component	12%	15%
1.5 M Tris-HCl (pH 8.8)	2.5 ml	
Deionized water	3.3 ml	
30% acrylamide	4 ml	
10% APS	100 µl	
10% SDS	100 µl	
TEMED	4 µl	

500 µl isopropanol was added onto top of resolving gel after pouring between plates

Component	5% (Stacking gel)
1.5 M Tris-HCl (pH 6.8)	1 ml
Deionized water	5.5 ml
30% acrylamide	1.3 ml
10% APS	80 µl
10% SDS	80 µl
TEMED	8 µl

4.2.11 4X Lamelli's sample buffer

Tris pH 6.8 (200mM)	2.42 g
2-mercaptoethanol (20%)	20 ml
SDS (8%)	8 g
Bromophenol blue (0.4%)	0.4 g

Glycerol (40%)	40 ml
Deionized water	q.s.to 100 ml

Tris was dissolved in 30 ml deionized water and pH was adjusted using 1N HCl. SDS, Bromophenol blue and glycerol were dissolved by gentle heating at 37°C and 20 ml 2-mercaptoethanol was added. Volume was adjusted accordingly, aliquots were stored at -20°C

4.2.12 Western transfer buffer

Tris	2.9 g
Glycine	14.5 g
Methanol	200 ml
Deionized water	1000 ml

Components were dissolved and volume was adjusted accordingly. Buffer can be reused and kept at -20°C

4.2.13 Ponceau S

Ponceau S	0.1 g
Acetic acid	5 g
Deionized water	q.s. to 100 ml

4.2.14 10X Tris buffered saline (TBS) pH 7.6

Tris	24.2 g
NaCl	80 g

Deionized water	q.s. to 1000 ml

Components were dissolved in 800 ml solvent and pH was adjusted by 1N HCl. Volume was adjusted accordingly. Solution was diluted 10 times and 1 ml tween-20/1000 ml solution was mixed to make PVDF membrane washing buffer (TBS-T).

4.2.15 Stripping solution

PVDF membrane was boiled for 5 minutes in 5mM EDTA solution and incubated for 2 minutes and kept in blocking solution.

4.2.16 Competent cells preparation buffers

Component	**TFB-I (pH 5.8)**	**TFB-II (pH 7.0)**
KCl	100 mM	10 mM
$MnCl_2$	50 mM	-
CH_3COOK	30 mM	-
$CaCl_2$	10 mM	75 mM
Glycerol	10%	10%
MOPS	-	10 mM

TFB-I pH was adjusted by 0.2 M CH_3COOH and TFB-II pH was adjusted by 1N NaOH

4.2.17 Solutions for plasmid extraction

Solution I (Resuspension)	**Solution II (Lysis)**	**Solution III (Neutralization)**
50 mM Dextrose	1% SDS	2 M Glacial CH_3COOH
10 mM EDTA	0.2 % NaOH	3 M CH_3COOK

25 mM Tris	-	-

Solutions I and III were stored at 4°C, solution II was freshly prepared.

4.2.18 Recipe for cDNA master mix preparation (25 µl reaction mix per sample)

5X first strand buffer	5 µl
dTT	1.5 µl
dNTP	1 µl
Mu-MLV	0.5 µl
Sterile, deionized water	2 µl

It was added to 15 µl mRNA+Random primer mix

4.2.19 Lysis buffer- recombinant protein purification (25 mM Sorenson's phosphate buffer)

$NaH_2PO_4.H_2O$	6.1 mM
$Na_2HPO_4.7H_2O$	19.1 mM
NaCl	300 mM
Glycerol	10%
Triton-X-100	0.1%
2-mercaptoethanol	12 mM
Lysozyme	1 mg/ml
Nuclease (Benzonase®)	250 U
PMSF	1 mM

pH was adjusted to 7.4 before adding detergent, 2-mercaptoetrhanol and enzymes.

4.3 Methods

4.3.1 Mice

Female BALB/c mice were procured from Jackson Laboratories (Bar Harbor, ME) and were bred at the Institute's animal facility. 6-8 weeks old mice were used for experiments following Institutional Animal Care and Use Committee approved protocols.

4.3.2 Peritoneal macrophages collection and culture

2ml of 3% Thioglycollate (DIFCO, Henry St, Detroit, MI) was injected i.p. in female BALB/c mice. On 5^{th} day post-injection, peritoneal cavity was flushed with 10 ml sterile PBS (pH 7.4) and solution was collected. It was centrifuged at 1200 rpm for 8 minutes and resuspended in RPMI-1640 containing 6% FBS supplemented with 1 mM Sodium pyruvate, 2 mM L-glutamine and 1000 U Penicillin-streptomycin at 2x10^6 cells/well in a 6-well plate. It was allowed to adhere for 6h. It was washed twice with 1X PBS, supplemented with fresh media and kept at 37°C, 5% CO_2 for 48h.

4.3.3 *In vitro* and *in vivo* maintenance of *L. major*

L. major strain (MHOM/Su73/5ASKH) was maintained in RPMI-1640 media containing 10% Foetal bovine serum, 1 mM Sodium pyruvate, 1000 U Penicllin-streptomycin and 50 nM 2-mercaptoethanol for *in vitro* infection studies. After *in vitro* serial passaging for approximately 10 generations, parasite was washed twice with 1X sterile HBSS and injected 50 µl of HBSS-suspension containing 2x10^6 stationary-phase metacyclic promastigotes were injected in left hind footpad to retain virulence. After development of footpad lesion, draining lymph node was isolated and crushed with frosted-end slides.

Tissue lysate was centrifuged at 500 rpm for 8 min and supernatant was transferred to fresh RPMI 1640 media in a non-vented 25 cm^2 flask and kept at 25°C for amastigote to promastigote transition and passaged as indicated above. Avirulent (HP) strain was obtained by continuous *in vitro* culture of *L. major* 5ASKH for atleast 600 generations.

4.3.4 *In vitro* infection of macrophages and IFN-γ stimulation

Post 48h, macrophages were infected with *L. major* strain (MHOM/Su73/5ASKH; LP or HP) promastigotes at a 1:10 macrophage:parasite ratio. Macrophages were kept at 33°C, 5% CO_2 for 6h and extracellular parasites were washed with PBS. Macrophages were again kept at 37°C, 5% CO_2 for 8h with or without recombinant IFN-γ stimulation (20 ng/ml).

4.3.5 RNA isolation and first-strand cDNA synthesis

Cells were washed with sterile PBS and lysed with 1ml TRI® reagent. Total RNA was extracted following manufacturer's instructions. 15µl solution containing 2µg RNA and 2µl random primers (RNA mix) was made for each sample. This solution was kept at 65°C for 5min. Meanwhile, the master mix containing first strand buffer, dTT, dNTP, Mu-MLV was prepared and 10 µl of this mixture was added to RNA mix of each sample. It was kept at 37°C for 1h and inactivated at 65°C for 15 min.

4.3.6 Quantitative real-time PCR (qPCR)

qPCR was performed using SYBR premix containing Tli RNaseH and gene-specific primers on a StepOnePlus (Applied Biosystems Inc., Foster City, CA) under following conditions: 95°C (30 sec), 45 cycles of 95°C (5 sec) and 60°C (34 sec), followed by melt

curve cycle. 10µl reactions, each containing 5µl SYBR premix, 0.2µM forward and reverse primer and 15ng cDNA, were performed in duplicates and expression levels (Ct) were normalized against β-tubulin of *L. major* or mouse GAPDH and relative fold change was analyzed by delta-delta Ct method ($2^{-\Delta\Delta Ct}$) method. The gene-specific primers used for amplification are listed in section 5.1.

4.3.7 Preparation of competent cells

E. coli DH5α and BL-21 (RIL) chemi-competent cells were made by incubating a 100ml (OD_{600}= 0.5) culture pellet grown at 37°C in TFB-I buffer for 90min with occasional mixing. Cells were centrifuged at 4000 rpm for 10 min and resuspended in TFB-II buffer. 80µl aliquots were prepared and stored at -80°C until further use.

4.3.8 Plasmid purification

Plasmid was amplified in *E. coli* DH5α by growing at 37°C for 16h. Cells were resuspended in 200µl, lysed in 400µl and neutralized in 300µl (5min) of solution I, II and III as described in section 5.2. Lysate was centrifuged at 12000 rpm for 10min. Supernatant was collected and incubated with RNase A at 37°C for 30 minutes. 500µl phenol chloroform (1:1) mix was added. It was shaken vigorously for 1 min and centrifuged at 12000 rpm for 10min. Upper layer was collected, equal volume of isopropanol added, incubated at -20°C for 30min, and centrifuged at 12000 rpm for 15min. Pellet was washed with 1ml of 70% ethanol, air-dried and dissolved in sterile water.

4.3.9 PCR-based cloning of *L. major* genes

L. major LmjF_36_3850 was amplified by PCR using cDNA synthesized from *L. major* 5ASKH as a template according to manufacturer instructions. Primers used for amplification contained BamHI and XbaI digestion-susceptible sites. PCR product was purified using GenElute™ PCR purification kit as per provided protocol. Restriction digestion reaction at 37°C for 4 hours produced sticky overhangs in amplified LmjF_36_3850 gene and pcDNA6/HisA. Digested insert and vector were run on a 0.8% agarose gel for 90 minutes and gel portion containing digested insert and vector were excised and purified using GenElute™ Gel extraction kit as per provided protocol. Ligation reaction was set up using NEB T4 DNA ligase at 16°C for 14 h. Enzyme was inactivated at 65°C for 10 min. Inactivated ligation mixture was transformed into E. coli DH5α competent cells and colonies were checked for successful ligation by double-digestion with same enzymes and positive clones were sent for sanger sequencing service. *L. major* adenylate kinase (LmAdeK) was cloned in pcDNA6/HisA and pet28a+ by using EcoRI and XbaI, NheI and BamHI-HF respectively using same protocol.

4.3.10 Preparation of endotoxin free DNA

Cesium chloride density gradient centrifugation method was used for plasmid purification. Plasmid transformed, 500 ml confluent bacterial culture was resuspended in 25ml solution I, lysed with 50ml solution II, neutralized with 37.5 ml solution III. Lysate was centrifuged at 4000 rpm for 15 min in Sorvall™ centrifuge, filtered and 70 ml isopropanol was added to supernatant. It was mixed and incubated at room temperature for 15 min and centrifuged at 5000 rpm for 30 min. Pellet was washed with 70% ethanol, air-dried and dissolved in 5ml TE buffer (pH 8.0). Volume of plasmid solution was adjusted to 5.2 ml and 5.72 gms of CsCl was dissolved and transferred to ultracentrifuge tubes. Remaining volume was

adjusted with 1.1 gms/ml CsCl solution, 300 µl of 10mg/ml ethidium bromide solution and sealed. It was centrifuged at 65000 rpm for 14 hours at 25°C. Plasmid band was eluted, ethidium bromide was removed by NaCl saturated n-butanol. Plasmid was precipitated using isopropanol and 3M sodium acetate. Pellet was washed with 70% ethanol and dissolved in sterile water.

4.3.11 Purification of recombinant *L. major* adenylate kinase (rAdeK)

LmAdeK-pet28a+ (Lmpet28a+) was transformed into *E. coli* BL-21 (RIL) and protein induction was done by 0.1 mM IPTG at OD_{600} = 0.5 and induced culture was grown at 18°C for 15 hours. Cell pellet was resuspended in 25 ml lysis buffer and kept at 4°C for 45 min, sonicated at 4 sec ON cycle and 6 sec OFF cycle for 10 minutes. Lsyate was centrifuged at 12000 rpm for 30 min and supernatant was incubated with 400 µl Ni-NTA beads at 16°C for 90 min at 5 rpm. Beads were washed successively with lysis buffer (without 2-mercaptoethanol, lysozyme, and nuclease) containing 25 mM, 50 mM and 75 mM imidazole for 5 min at 6 rpm. Protein was eluted at 300 mM imidazole (without Triton-X-100). Eluted fractions were dialyzed for 3 cycles (4h, 8h, 12h) and concentrated using columns and endotoxin removal was done by resin as per manufacturer's protocol.

4.3.12 *In vitro* expression of construct

Cloned vector was transfected into HEK293T cells by Effectene® transfection reagent as per manufacturer's instructions and LmjF_36_3850, LmAdeK expression was determined after 48h in transfected and untransfected cells by semi-quantitative PCR at 95°C (3min), 36 cycles of 94°C (1min), 57°C (1min) and 72°C (1min) followed by 72°C (10min) in a thermal cycler.

4.3.13 *In vivo* expression of construct

LmjF_36_3850, LmAdeK expression was determined after 4 days in cloned and control vector injected (im) mice by semi-quantitative PCR as described in 5.2.11.

4.3.14 Immunization regimen and *L. major* challenge infection

For DNA vaccination, 100µg of control or cloned vector (LmjF_36_3850 or LmAdeK) in 100µl HBSS was injected i.m. in left thigh muscle at 0, 15th and 30th day. For recombinant protein vaccination, 1XrPrt regimen includes an i.m. injection of 15 µg rAdeK in 50 µl 1:1 o/w emulsion with IFA. 3XrPrt regimen includes three consecutive doses of 15 µg rAdeK on 0, 15th and 30th day. HPB regimen includes 100 µg DNA injection at 0 and 15th day and 15 µg rAdeK on 30th day. A schematic of LmjF_36_3850 and LmAdeK immunization is shown in Figure (a) and (b) respectively.

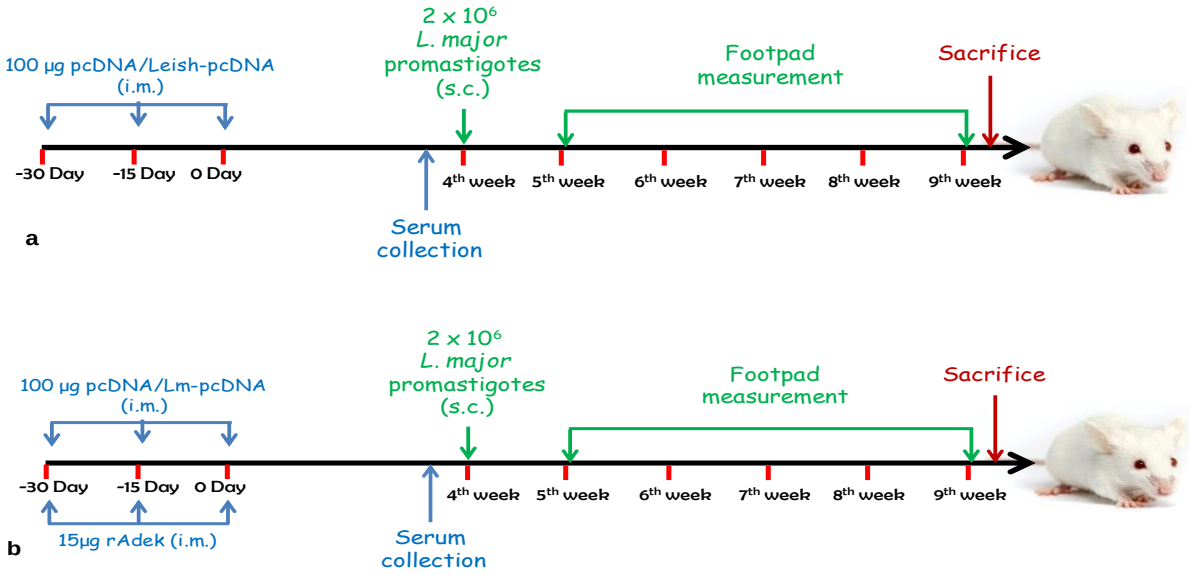

Figure 6. Immunization protocol for vaccination experiments

Table 5. Vaccination/injection regimen in different study groups during LmAdeK vaccination studies

Group I	**Sterile HBSS injection on 0, 15th and 30th day/Naive**
Group II	*L. major* infection(Lm) along with vaccinated groups
Group III	pcDNA injection on 0, 15th and 30th day
Group IV	LmAdek-pcDNA(Lm-pcDNA) on 0, 15th and 30th day
Group V	rAdeK on 0 day and pcDNA on 15th and 30th day
Group VI	rAdeK on 0, 15th and 30th day
Group VII	Lm-pcDNA on 0 and 15th day and rAdeK on 30th day

4 weeks after last immunization, 2x10^6 *L. major* stationary phase promastigotes were injected in left hind footpad.

4.3.15 Western transfer and blotting

L. major lysate or rAdeK was run on 12% SDS-PAGE. Proteins were transferred onto PVDF membrane at 180mA for 210 min. Membrane was blocked and incubated with

1:5000, 1:10000, 1:15000 dilutions of sera from vaccinated or control mice, 1:3000 of anti-His tag antibody overnight at 4°C. It was washed with 1X TBS-T and probed with 1:5000 dilution of Goat anti mouse IgG HRP conjugate for 90 min at 25°C. It was washed with 1X TBS-T and detected by ClarityMax^{TM} ECL substrate.

4.3.16 Preparation of *L. major* crude soluble antigen (CSA)

30 ml *L. major* stationary phase culture from virulent strain was centrifuged and washed with PBS. It was resuspended in 1 ml HBSS containing protease inhibitor cocktail solution. Solution was sonicated for 30 cycles of "2 sec ON and 3 sec OFF" pulse and centrifuged for 20,000xg for 30 minutes. Supernatant was filtered by 0.22 µM centrifuge tube filter, estimated by BCA reagent and kept at -80°C until further use.

4.3.17 Antibody ELISA

Wells were coated overnight with 100 µl of either 30 µg/ml of crude soluble antigen (CSA) or 10 mg/ml rAdeK solution at 4°C. Blocking was done by 1% BSA for 4 hours at 4°C. Different dilutions of sera were prepared in blocking solution and 100 µl/ well were added in duplicates and incubated at 4°C for 16 hours. It was washed with 1X PBS-T and 100 µl/well of biotinylated anti mouse IgG2a (0.25 µg/ml), IgG1 (0.25 µg/ml) and IgM (1 µg/ml) were added and incubated for 90 min at 25°C. Wells were washed with PBS-T and 100 µl/well of Streptavidin POD conjugate dilution (1:20000) was added and incubated for 30 min at 25°C. Wells were washed and developed with 100µl of 1:1 solution of TMB reagent solution/well. Reaction was stopped by 50 µl of 1N H_2SO_4 and absorbance was measured at 450 nm.

4.3.18 Footpad measurement and in vivo parasite load

Lesion progression was measured by the difference in the thickness of left hind footpad and contra-lateral footpad, taken successively for 5 weeks. Draining lymph node was crushed and supernatant was added to RPMI 1640 media in 25 cm^2 flasks, supplemented for parasite growth as described above. Parasite number was counted after 5 days of culture at 25°C.

4.3.19 Cytokine ELISA

0.5 µg/ml capture antibody and 2 µg/ml capture antibody in 50 µl PBS was coated for IL-4 and IL-10, IL-12 and IFN-γ respectively, overnight at 4°C. Blocking of plates was done for 4 h, 4°C. Plates were washed with PBS-T, 50 µl samples were added in duplicates and incubated at 4°C for 16h. Plates were washed and 50 µl/well biotinylated antibody for IL-4 (1 µg/ml), IL-10 (2 µg/ml), IL-12 (2 µg/ml) and IFN-γ (2 µg/ml) were added and incubated at 25°C for 90 min. Post-washing, 100 µl of Streptavidin-POD conjugate was added per well and incubated at 25°C for 30 min. Plates were developed using 100 µl of TMB substrate (A+B) per well, reaction was stopped by 1N H_2SO_4 (50 µl/well) and absorbance was read at 450 nm.

4.3.20 Flow cytometry

Lymphocytes were blocked in 50% FBS for 30 min at 4°C. For surface staining, cells were stained with 0.5 µl of anti-CD3, anti-CD4, anti-CD44, anti-CD62L, anti-CD25 fluorophore-tagged antibodies at 4°C for 1h. Post-staining, cells were washed and permeabilized with 180 µl of BD Cytofix/Cytoperm^{TM} for 20 min. For intracellular staining of effector cytokines, lymphocytes were stimulated with 20 ng/ml PMA and 1 µg/ml ionomycin for 6h and 4 µl Golgi-plug/6 ml of culture medium was added in last 2h.

Post-washing, intracellular staining was done with 0.5 µl of anti-Tbet, anti-IFN-γ, anti-GATA-3, anti-IL-4, anti-RORγT, anti-FOXP3, anti-IL-10 fluorophore-tagged antibodies at 4°C for 75 min. Cells were washed, fixed in 1% PFA and data was recorded with BD FACS CantoII™. Population analysis was performed by FACSDiva software.

For B-cell analysis, lymphocytes were surface stained with anti-B220 and anti-CD19 fluorochrome-conjugated antibodies. Post-washing, surface staining for IgG receptors was done by incubating with biotinylated anti-IgG2a, anti-IgG1 and IgM antibodies. Post-washing, cells were stained with fluorophore conjugated streptavidin antibody, following similar protocols as described above.