2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PARASITE STRAINS AND ISOLATES

L. major 173 wt – *L. major* WHOM/IR/-/173

L. mexicana wt – *L. mexicana* MNYC/BZ/62/M379

L. mexicana::dsred14 cl. 34 – *L. mexicana* MNYC/BZ/62/M379 DsRed integrated into sRNA locus; hygromycin resistant (20 µg/ml)

2.1.2 BACTERIAL STRAINS

XL1-Blue – Genotype*: E. coli recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]

(Genes listed signify mutant alleles. Genes on the F´ episome, however, are wild-type unless indicated otherwise).

BL21-CodonPlus(DE3)-RIPL – Genotype: *E. coli* B F^{-} *ompT hsdS* (r_B^- m_B^-) *dcm*$^{+}$ Tet^{r} *gal* λ(DE3) *endA* Hte [*argU proL* Cam^{r}] [*argU ileY leuW* Strep/Spec^{r}]

2.1.3 MICE STRAINS

All animal experiments were approved by an ethics committee and licensed by the legal authority. BALB/c and C57BL/6 were from Harlan UK (Loughborough, UK).

2.1.4 ANTIBIOTICS

Ampicillin	stock solution 100 mg/ml; working solution 100 µg/ml
Kanamycin	stock solution 50 mg/ml; working solution 50 µg/ml
Penicillin	stock 10000 U/ml; working solution 100 U/ml
Streptomycin	stock 10000 µg/ml; working solution 100 µg/ml
Hygromycin	stock solution 20 mg/ml; working solution 16 – 32 µg/ml
G418	stock solution 100 mg/ml; working solution 16 µg/ml

2.1.5 CHEMICALS

Chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.1.6 BUFFERS

10x PBS

1.36 M	NaCl
27 mM	KCl
17.6 mM	KH_2PO_4
100 mM	Na_2HPO_4

adjust to pH 7.2

10x TBS

500 mM	tris(hydroxymethyl)aminomethane (Tris)
1.5 M	NaCl

adjust to pH 7.5

TBST

1x TBS with 0.5 % Tween 20

10x TBE

890 mM	Tris
890 mM	Boric acid
20mM	0.5M EDTA

10x SDS running buffer

250 mM	Tris
1.92 M	Glycin
1% (w/v)	SDS

adjust to pH 8.8

8% paraformaldehyde solution

16 g paraformaldehyde (PFA) was dissolved in 55 °C warm alkaline water. 4.766 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HEPES were dissolved in 50 ml water and added to dissolved PFA. pH was adjusted to 7 and water was added to a final volume of 200 ml.

Aliquots were stored at -20 °C.

2.1.7 BUFFERS FOR SOUTHERN BLOTTING

Denaturing solution

1.5 M	NaCl
0.5 M	NaOH

Neutralising Solution

1.5 M NaCl

1 M Tris

adjust to pH 7.4 with HCl

20x SSC

3 M NaCl

0.3 M Trisodium citrate

Prehybridisation Buffer

5% dextran sulphate

5x SSC

10% liquid Blocking Reagent (Amersham Bioscience' #RPN3601)

0.1% SDS

Hybridisation Buffer:

Prehybridisation supplemented with appropriate amount of probe.

Detection Buffer

100 mM Tris-HCl pH 9.5

100 mM NaCl

Buffer A :

100 mM Tris pH 9.5

300 mM NaCl

Blocking buffer:

1 in 10 dilution of liquid block in buffer A.

2.1.8 CULTURE MEDIA

Cell culture mediums for *Leishmania* spp.

2 x SDM

	10 l	5 l
MEM medium	140 g	70 g
M199 medium	40 g	20 g
MEM essential amino acid solution	160 ml	80 ml
MEM non-essential amino acid solution	120 ml	60 ml
Glucose	20 g	10 g
HEPES buffer	160 g	80 g
MOPS buffer	100 g	50 g
Sodium pyruvate	2 g	1 g
L-alanine	4 g	2 g
L-glutamine	6 g	3 g
L-arginine	2 g	1 g
L-methionine	1.4 g	0.7 g
L-phenylalanine	1.6 g	0.8 g
L-proline	12 g	6 g
L-serine	1.2 g	0.6 g
L-taurine	3.2 g	1.6 g
L-threonine	7 g	3.5 g
L-tyrosine	2 g	1 g
Adenosine	0.2 g	0.1 g
Guanosine	0.2 g	0.1 g
Glucosamine – HCl	1 g	0.5 g
Folic acid	0.08 g	0.04 g
p-Aminobenzoic acid	0.04 g	0.02 g
Biotin	0.004 g	0.002g

Top up to 9.5 L or 4.5 L with pure water and adjust to pH 7.0. Add 40g and 20g for 10 L and 5 L respectively. Adjust to pH 7.3 and fill up to 10 L and 5 L respectively. Filter sterilise solution (0.22 µm filter) and store at -80 °C.

SDM / 7 % FCS

500 ml 2x SDM

70 ml FCS

10 ml P/S solution

adjust to 1 L with sterile deionised water.

Amastigotes medium / 20 % FCS

500 ml Schneider's Drosophila

140 ml FCS

7 m l P/S

70ml 10 x MES (10 x MES – 3.9 g MES in 100 ml pure water)

Adjust to pH 5.5 with HCl and filter sterilise (0.22 µm filter).

Other Cell culture medium used

Dulbecco's modified Eagle's medium (DMEM; Invitrogen, # 41966029)

Roswell Park Memorial Institute (RPMI; Invitrogen, # 52400025)

RPMI glucose free (Sigma, # R1383)

Fetal bovine serum (FCS; Biochrom; Berlin, Germany; # S0115, Lot no. 0446K)

2.1.9 CELL CULTURE MEDIUMS FOR *E. COLI*

LB Medium

10 g Tryptone/Peptone

5 g yeast extract

5g NaCl

adjust pH to 7.0

adjust to 1 L with deionised H_2O

autoclave

LB Plates

As LB medium plus 6g bacto agar per 400 ml.

SOB Medium

10 g Tryptone / Peptone

2.5 g Yeast extract

0.25 g NaCl

add 450 ml of deionised H_2O

shake until solutes have dissolved

add 5 ml of a 250 mM KCl solution

adjust to pH 7.0 with NaOH (5N)

adjust to 500 ml with deionied H_2O

autoclave solution

let solution cool down to around 60°C

add 2,5 ml of a sterile solution of 2M MgCl2

SOC Medium

SOB Medium which contains 20mM glucose.

2.1.10 SOLUTION TO GENERATE CHEMICAL COMPETENT *E. COLI*

RF1

10 mM RbCl

50 mM $MnCl_2$

30 mM Potassium Acetate

10 mM $CaCl_2$

10 % v/v Glycerol

adjust to pH 5.0 with 0.2 M acetic acid

RF2

10 mM RbCl

10 mM 3-(N-morpholino)propanesulfonic acid (MOPS)

75 mM $CaCl_2$

15 % v/v Glycerol

adjust to pH 6.8 with NaOH

2.1.11 PRIMERS

TABLE 2. List of primers used for sequencing and cloning of *L. major* and *L. mexicana decr*, respective 5' and 3' flanking regions and selection markers used to generate replacement constructs

Name	sequence in 5' → 3' direction
for general sequencing	
M13/pUC F	GTAAAACGACGGCCAGTG
M13/pUC R	CAGGAAACAGCTATGAC
T7 Promoter F	TAATACGACTCACTATAGGG
T7 Terminator R	TATGCTAGTTATTGCTCAG
pGEX F	CTGGCAAGCCACGTTTGGTG
pGEX R	GGAGCTGCATGTGTCAGAGG
inv F LmjF33.0830	CCCGTGTGCATGCTGG
inv R LmjF33.0830	GTCAAGGCAGTGGATGGG
inv R LmjF33.0830 mex	CATGTTCCAGCGCACCCG
LmjF33.0830 mex	GAAGGCGGGCACATTTTG
LmjF33.0830	GGGTGCGCTGGAACATGG
LmjF33.0830 maj	GTGTCCCACTCATCCAACG
LmjF33.0830 F	CAACCTGAAACACGGCAATCTATG
LmjF33.0830 R	CATGAGAAGAGATAAGCGGCTAAG
5' ir LmjF33.0830 mex	GCTTCAGGTTGTGCGCCTTC
3' ir LmjF33.0830 mex	GGGAGTACATGTGTGTGATGCG
Verifying correct targeting	
LmjF33.0820	CCGAGTCCAACATGAACG
LmjF33.0840	GACGCTGAGAAGTGTAGCC
Hygro inv Rv	GCTTGTATGGAGCAGCAGACG
Hygro inv F	CATATCCACGCCCTCCTACATCG
Neo inv R	CCTGCTTGCCGAATATCATGG
Neo inv F	CAGCCGATTGTCTGTTGTGC
LmjF33.0830 -1344nt	CTATCTCCGTGTGTGTTCGAC
LmjF33.0830 +629nt	ACTTGAGTAGAGGAGCCAAGC
for cloning and partial sequencing	
LmjF33.0830 *Eco*RI	GT**GAATTC**AAGCAGTACGCAAAGATTTTGGAG
LmjF33.0830 *Xho*I	GT**CTCGAG**CAGACGTATCGCCACACTGTG
5ir LmjF33.0830 maj *Hind*III	GCT**AAGCTT**GAGTCTTGTCAGTCCTTCTTG
5ir LmjF33.0830 maj *Spe*I/*Xba*I	GCT**TCTAGACTAGT**TTTTGTGCAATTTCGTTGCCG
3ir LmjF33.0830 maj *Bam*HI	GCT**GGATCC**GGGTCGACTAGGTTGCCGTCAC
3ir LmjF33.0830 maj *Hpa*I/*Kpn*I	GCT**GGTACCGTTAAC**GGCACCAGCACACCACAGCA GAAGATACATGAGAAGAGATAAGCGGCTAAG
5ir Lmex Decr_*Hind*III	CAG**AAGCTT**GTTCATTGGATGTGGACCGAG
5ir Lmex Decr_*Spe*I_*Xba*I	CAG**TCTAGACTAGT**GTTTCTCTATTTCTTTGCCCTGTG
3ir Lmex Decr_*Bam*HI	GTCGGATCCGGGTCGACTTGGTTGCCGTC
3ir Lmex Decr_*Eco*RI	CAGGAATTCGCAATGCTCTGCCGTACCAGCAC
Hyg_F *Spe*I	GCT**ACTAGT**ATGAAAAAGCCTGAACTCACCG
Hyg_R *BamH*I	GCT**GGATCC**CTATTCCTTTGCCCTCGGACG
Neo_F *Spe*I	GCT**ACTAGT**ATGATTGAACAAGATGGATTGCACG
Neo_R *BamH*I	GCT**GGATCC**TCAGAAGAACTCGTCAAGAAGGC

Text in **bold** represents recognition site of restriction enzyme mentioned in primer name.

TABLE 3. Primers used in qRT-PCR

Name	Sequence 5' → 3' direction
Abca1 F	TCGAAGGAGACAAACATGTCAGCT
Abca1 R	CTCTGAGCGTCTGAGAACAGGCGA
adrp F	ATGAGAGTGGCCAACCTGCCCT
adrp R	CAACAATCTCGGACGTTGGCTGGT
alox15 F	TTGTAGTCAGCAGTACCTGG
alox15 R	TCAGAAGATGAGCCTGTAGC
apoe F	TTGCTGACAGGATGCCTAGCCGA
apoe R	AGATCCTCCATGTCGGCTCCGAG
CD36 F	GTAGAGATGGCCTTACTTGG
CD36 R	AGAGAGAGAGAGCACACACC
fabp4 F	TCACCATCCGGTCAGAGAGT
fabp4 R	CTCTTGTGGAAGTCACGCCT
fabp5 F	ACCGAGAGCACAGTGAAGAC
fabp5 R	CACTCTGGCAGCTAACTCCT
fabp7 F	TCTGCGCAACCTGGAAGCTGAC
fabp7 R	TCCCCAAAGGTAAGAGTCACGACC
fdps F	GGGATGCTATTGCCCGGCTCAA
fdps R	GCTTCCAGAAGCAGAGCGTCGT
gamma actin F	GAAGATGACGCAGATAATGTTTGAA
gamma actin R	CCAGGTCCAGACGCAAGAT
hmgcr F	AATGTTGTCAAGACTTTTCCGGA
hmgcr R	GTACTTGGACCCAAGCTGCCGTA
ldlr F	GTGTGATGGAGACCGAGATT
ldlr R	CTGCGATGGATACACTCACT
lipa F	GATGCAACTCCAGGGCCTGGT
lipa R	TACTAGAATCTGCCAGCAAGCCGT
lipe F	CATCACTGAGATTGAGGTGC
lipe R	TTGAGTACCTTGCTGTCCTG
Mhprt F1	GGACCTCTCGAAGTGTTGGA
Mhprt R1	GGCCACAGGACTAGAACACC
Mhprt F2	TGCTGACCTGCTGGATTACA
Mhprt R2	TCCAACACTTCGAGAGGTCC
npc1 F	GGCTATGACTTAGTGCAGGA
npc1 R	CCTCAGTTGCTGTCACATTC
npc2 F	GAATGTGAGCCCATGTCCCACCGA
npc2 R	CTGAACTGGGATCTCCCAGCAG
Nr1h3 F	AACGGAGTTGTGGAAGACAG
Nr1h3 R	GTTGTAATGGAAGCCAGAGG
plin F	GACGACGAGGAGGAGGAAGA
plin R	GCTGGTGTGAGGTGCAGGAT
scd1 F	CGCCCAAGCTGGAGTACGTCTG
scd1 R	CACAAGCAGCCAACCCACGTG
scp2 F	CCTTACTCCGCAGTGGAACAGGCA
scp2 R	TCAGTTGGAGTGGTCCGATCTGAG
sgpl F	CACCAAATATGAGCCCTGGCAGCT
sgpl R	AGCTGTGCCCATACCCTGAGCA
soat1 F	ACATCCTAGTCTCCGACCGT
soat1 R	ATAGTGGCTTCAGCTCCTCC
sqle F	CATCGTGGGATCTGGTGTGCTTGG
sqle R	GCCATAGCTGCTTTCCGGAGACTC

2.1.12 GEL ELECTROPHORESIS

TABLE 4. Pipetting scheme SDS-mini-gels

	separation gel				stacking gel
	8%	10%	12.50%	15%	5%
Rotiphorese Gel 30	5.4	6.65	8.2	10	1.34
1 M Tris/HCl pH 8.8	7.5	7.5	7.5	7.5	
1 M Tris/HCl pH 6.8					1
10% SDS	0.2	0.2	0.2	0.2	0.08
H_2O	7	5.75	3.7	2.1	5.4
APS	0.17	0.17	0.17	0.17	0.08
Temed	0.01	0.01	0.01	0.01	0.008

volume in ml, Rotiphorese (Roth, #3029.1)

4x protein sample buffer

8 ml 1 M Tris/HCl, pH6.8

4 ml 2-Mercaptoethanol (Roth, #4227.1)

16 ml glycerol

12 ml 20% (v/v) SDS)

8 mg Bromophenolblue

2.2 METHODS

2.2.1 CULTURING OF *LEISHMANIA* SPP. PARASITES

Passage of promastigotes

Promastigotes were cultured axenically in SDM / 7% FCS at 27 °C. Cultures were maintained in volume of 10 ml in T25 tissue culture flask without CO_2 and passaged weekly by inoculating 30 -50 µl of stationary phase culture. Parasites were kept in culture for not more than 15 passages, unless stated otherwise.

Parasites were passaged through BALB/c mice to maintain virulence. Stationary phase promastigotes were washed three times with PBS and resuspendend therein in appropriate density. Mice were infected with 2×10^7 parasites in 30 µl at the base of the tail by sub-cutaneous injection. Mice were sacrificed when a lesion at the site of infection developed and the popliteal lymph nodes were excised. Lymph nodes were cultured in 10 ml SDM / 7% FCS 27 °C.

Generation of axenic amastigotes

Leishmania mexicana amastigotes were cultured axenically as decribed by Bates *et al.* (Bates, P. A. 1994).

Stationary promastigotes were diluted 1:10 in amastigotes medium and cultured at 34 °C and 5% CO_2. Amastigotes were passaged weekly 1:5 -1:10. Amastigote clumps were dispersed by passing them five times through a 23 gauge (G) needle, attached to 1 ml syringe, before inoculation.

2.2.2 FREEZING OF *LEISHMANIA* SPP.

Promastigotes ($1x10^7$) of a late logarithmic growth phase were centrifuged at 1200 ×g for 10 min at 4 ºC. Supernatant was discarded and the pellet resuspended in 500 µl cold SDM / 7% FCS. Then 500 µl of 15% sterile glycerol in PBS was added, mixed and transferred into a cryo vial. Tube was placed in a RT cryo box and incubated for 24 at -80 ºC. Tubes were either stored at -80 °C or in a liquid nitrogen tank for long term storage.

2.2.3 THAWING OF *LEISHMANIA* SPP.

Cryovial was transferred from either -80 °C or liquid nitrogen tank to a 37 °C water bath and incubated until ice was almost thawed. Cells were transferred into a 15 ml tube prepared with 10 ml pre-warmed SDM / 7% FCS, centrifuged at 1200

×g for 10 min at RT. Supernatant was discarded and cells resuspended in 1 ml SDM / 7% FCS and transferred into a non vented tissue culture flask prepared with 9 ml SDM / 7% FCS with or without appropriate antibiotic.

2.2.4 OBTAINING BONE MARROW

Femuri, humeri and tibiae were freed from remaining tissue. Ends of bones were cut off and bone marrow was flushed out by using a 5 ml syringe and a 26 gauge (G) hypodermic needle with either DMEM or RPMI 1640 supplemented with 10 % FCS. Bone marrow was transferd into a 70 µm cell strainer and forced through with the pistil of 1 ml syringe. Cell strainer was rinsed with medium /10% FCS. Cells were centrifuge 10 min 300 ×g at RT, and supernatant discarded. Erythrocytes were lysed with erythrocytes lysis buffer (approximately 1 ml per bones of 2 mice) and incubated at RT for 1 – 2 min. This was then toped up to 30 – 40 ml with medium and centrifuged. Supernatant was discarded and cells were washed twice more.

2.2.5 DIFFERENTIATION OF BONE MARROW TO MACROPHAGES

Bone marrow (3.5×10^6 cells) was placed in DMEM complemented with 10 % FCS and 20% L929 conditioned medium per Ø 10 cm and 1.2×10^7 per Ø 14.1 cm bacterial grade petri dish. Petri dishes were incubated for 6-7 d at 37 °C at 5% CO_2.

Medium was removed and bone marrow derived macrophages (bmdm) were washed once with cold PBS. Bmdm were rinsed from the petri dish with pre-warmed PBS. Cells were transferred into a 50 ml tube containing 2 ml FCS, centrifuged at 300 ×g for 10 min at RT. Supernatant was discarded and bmdm were resuspended in DMEM/ 10% FCS/ 4% L929 conditioned medium. Cells were plated out at density stated and incubated at 37 °C, 5% CO_2.

2.2.6 INFECTION OF MACROPHAGES WITH *LEISHMANIA* AMASTIGOTES OR BEADS

Leishmania mexicana axenic amastigotes were washed in DMEM / 10%FCS / 4% L929 conditioned medium. Centrifugation steps were carried out at 1200 ×g. Axenic amastigotes were singularised in 1 ml medium with a 1 ml syringe and a blunt end 23 G hypodermic needle. Bmdm were infected, at a multiplicity of infection (MOI) stated, as follows. Parasites were given to cells and the tissue

culture well plate was centrifuged for 5 min at 1200 ×g. In the case of the time course a 0 h timepoint was taken immediately after this centrifugation step. Medium was changed every 24 h, and infected and control cells kept at 34 °C and 5% CO_2.

Immunoglobulin G (IgG) from mouse serum was coupled to Polybeads-carboxylated 4.5 micron micropsheres (Polysciences, #9003-53-6) with PolyLink-protein coupling KIT for COOH microspheres (Polysciences, #24350-1) according to manufacturer's instructions. IgG coated beads were washed in the same way as the parasites and sonicated for 5 min in a water bath prior to exposure to cells (RK100H Sonorex, Bandelin, Berlin, Germany). Density of beads was adjusted as stated.

2.2.7 DNA EXTRACTION

Minipreps were carried out with QIAprep Spin Miniprep Kit (Qiagen; Crawley, UK, #27104) according to manufacturer's instructions. DNA was extracted from Gel with QIAquick Gel Extraction Kit (Qiagen; Crawley, UK, #28704) and PCRs were either applied to a gel and then extracted or purified with QIAquick PCR Purification Kit (Qiagen; Crawley, UK, #28104) according to manufacturer's instructions.

2.2.8 ISOLATION OF GENOMIC DNA FROM *LEISHMANIA*

Leishmania parasites were taken and transferred, dependent on the scale of the preparation, either into a 2 ml or a 15 ml tube, centrifuged at 1200 ×g for 10 min at RT. Parasites were washed with PBS and the pellet either frozen and stored until needed at -20 °C or resuspended in a appropriate volume (1 ml or 3 ml dependent on scale) of buffer P1 of Qiagen Miniprep Kit (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 100 µg/ml RNAse A) supplemented with 100 µg/ml Proteinase K and 10% SDS. This was incubated for 3 h or over night at 50 °C. The DNA was extracted by adding an equal volume of Phenol:Chloroform:Isoamyl alcohol (# P2069, Sigma-Aldrich, Irvine, UK) and incubated on rotator for 15 min. To separate phases the solution was centrifuged at 4500 ×g. Top phase was transferred into a new tube and extraction was repeated. To remove traces of phenol an equal volume of chloroform is added and incubated upside down for 15 min, followed by centrifugation as above. The top phase is again transferred into a

new tube and the chloroform extraction in repeated. Subsequently the DNA is precipitated.

2.2.9 RESTRICTION DIGESTION

For restriction digestion enzymes were from New England Biolabs (Hitchin, UK) and were used in single, double and triple digestion/s according to manufacturer's instructions. *Pvu*II was from Promega (Southampton, UK).

2.2.10 DNA PRECIPITATION

1/10 Vol. of 3 M sodium acetate pH 5.2 and 2.5 Vol. 100% Ethanol p.a. was added to the DNA solution. Precipitation was carried out either at -80 °C for 1 h or at -20 °C over night. DNA was centrifuged for 20 min at 16,000 ×g at 4 °C. Supernatant was carefully removed and pellet was washed with 1 ml of 70% Ethanol. Pellet was air dried and resuspended in water in an appropriate volume.

2.2.11 DNA SEQUENCING

Sequencing of DNA was carried out by The Gene Pool, the sequencing service of the School of Biological Sciences at the University of Edinburgh. Clean DNA and sequencing primer were delivered and a BigDye (Applied Biosystems) sequencing, clean-up and capillary analysis on the ABI3730 was performed.

2.2.12 POLYMERASE CHAIN REACTION

1x PCR buffer

1.5 mM $MgCl_2$

10 mM dNTPs

10 µM primer I

10 µM primer II

1-5 U polymerase

20 – 400 ng template

H_2O

Mango Taq DNA polymerase (Bioline, #BIO-21083) was used in general for Polymerase chain reactions (PCR). Platinum Pfx DNA Polymerase (Invitrogen, #11708021) was used for cloning purposes.

2.2.13 PURIFICATION OF RNA AND GENERATION OF COMPLEMENTARY DNA

RNA was purified from 1.1×10^6 infected or uninfected MΦ. Infections were routinely done in 6 well tissue culture plates. Cells were washed three times with PBS and 1 ml TRIzol (Invitrogen, #15596026) was added to well. Purification of RNA was performed according to manufacturer's instructions.

RNA was resuspended in a small volume of 17 µl. RNA was treated with DNA-free (Ambion – Applied Biosystems, # AM1906) according to manufacturer's instructions.

RNA yield was determined with NanoDrop 1000 (Thermo scientific).

RNA was transcribed with ThermoScript™ RT-PCR System (Invitrogen, #11146016) according to manufacturer's instructions, briefly: Whole time course was transcribed at once. Samples were adjusted with water to the respective amount of 9 µl RNA solution of sample with lowest RNA concentration. Oligo $(dT)_{20}$ was used as primer. Transcription was performed for 60 min at 50º C. After transcription RNase H step was performed. cDNA was diluted with sterile water to appropriate concentration.

2.2.14 QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Reactions were performed in fast optical 96 well plates (Applied Biosystems, #4346906). Reaction mixture was as follows: 10 µl of diluted cDNA, 5 µl primer mix (2 µl of each primer (10 mM)) and 15 µl Power SYBR Green PCR Master Mix (Applied Biosystems, #4367659). Plates were sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, #4311971). Quantitative Reverse Transcription Polymerase chain reaction (qRT-PCR) was performed with StepOnePlus real time PCR machine. The amplification program was as follows (50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec followed by 60 °C for 1 min) After 40 cycles a dissociation protocol was performed. Data were analysed with StepOne software version 2.0.

2.2.15 PROBE GENERATION

Probes were labelled with 'DIG DNA Labeling KIT' (#11 175 033 910; Roche, Penzberg, Germany) according manufacturer's instructions and using 3000 ng of purified PCR product as the template.

2.2.16 PROBE DETECTION

Probes were detected with 'Anti-Digoxigenin-AP' (#11 093 274 910; Roche, Penzberg, Germany) in a 1:25000 dilution in Buffer A.

2.2.17 SOUTHERN BLOTTING

Genomic DNA (gDNA; 2.4 µg) was digested in 400 µl of appropriate buffer supplemented with BSA. 1 U of enzyme was added to per 100 ng gDNA and incubated over night at 37 °C on a rotating wheel.

Digest was precipitated over night as mentioned in section 2.2.10. Pellet was resuspended in 15 µl sterile water, loading buffer was added and sample was applied to a 0.8% agarose gel. After the digest was separated on the gel a picture with ruler was taken.

Genomic DNA was depurinated by incubating the gel for 20 min in 0.25 M HCl solution with gentle agitation. Genomic DNA was denatured by gentle agitation of the gel for 30 min in denaturing solution. Gel was neutralised for 30 min in neutralising solution with gentle agitation.

Lunchbox was placed upside down in a larger box and a gel tray placed upside down on top of the lunchbox. A wick was formed with MME paper which covered the gel tray and 20x SSC was added in the reservoir. Gel was placed on MME paper and everything was sealed with Saran film, leaving only the gel uncovered. Nylon membrane (Hybbond-N^+, #RPN203B Amersham Pharmacia Biotech) was pre-wetted with water and placed on gel. Two pieces of MME paper, pre-wetted in 2x SSC were placed on top of the gel, air bubbles removed and 5 cm of tissue placed on top. A weight was placed on top of the tissue pile and the transfer performed over night (Southern, E. M. 1975).

The following day nylon membrane was removed and the lanes were marked with a pencil. DNA was UV cross linked (Stratalinker; on autolinker start counts from 1200).

Membrane was placed in hybridisation bottle containing 10 ml of pre-warmed pre-hybridisation buffer and incubated for 1 hour at 65 °C.

Next an appropriate amount of labelled DIG-labelled DNA probe (usually 1 µl) was placed in 50 µl water and boiled for 10 min at 100 °C. After boiling the probe was immediately transferred into 10 ml pre-warmed prehybridisation buffer.

Subsequently pre-hybridisation buffer without probe was replaced with pre-hybridisation containing probe and hybridisation was performed over night at 65 °C

Membrane was washed twice with 50-100 ml of pre-warmed 1x SSC, 0.1 % SDS for 30 min at 65 C, and then twice with 50-100 ml of pre-warmed 0.1x SSC, 0.1 % SDS for 30 min at 65 °C.

Membrane was transferred to lunch box and incubated for 1 h in 50 ml blocking buffer. Next it was incubated for 1 h with gentle agitation in 50 ml of Buffer A containing Anti-Digoxigenin-AP antibody 1:25,000. Membrane was washed three times for 15 min in 100 ml Buffer A / 0.3% Tween 20. Membrane was incubated for 2 min in detection buffer. Excess detection buffer was removed. Membrane was placed in bag W and 1 ml of CDP-Star (#11 685 627 001; Roche, Penzberg, Germany) was applied, diluted 1 in 100 in detection buffer. Incubated for 2 min and excess CDP-Star solution was removed but membrane was kept moist. Membrane was sealed into bag W with a heat sealer. Membrane was developed with Amersham Hyperfilm ECL (GE Healthcare UK, #28-9068-37).

2.2.18 PROTEIN PURIFICATION

Expression induction

Single cell colony from the plate was inoculated into 20 ml of liquid LB medium with appropriate antibiotics and cultures were grown at 37 °C with 200 rpm shaking. The next day culture was inoculated to expression culture (1/100 dilution; 100-500 ml). Cells were grown at 37 °C with 200 rpm until an OD_{600} = 0.6-0.8. A 500 µl aliquot was withdrawn (centrifuged 3 min at top speed in table top Eppendorf centrifuge and, supernatant discarded and pellet resuspended in 1x SDS sample buffer and boiled for 5 min at 95 °C). Cells were induced with stated amount of Imidazole and 0.13 mM riboflavin was added. Cells were grown at 37 °C for 5 h and then a 500 ml aliquot was withdrawn. Cells were centrifuged at 4500 ×g for 30 min at 4 °C, supernatant discarded and the pellet lysed immediately or stored at -80 °C until needed.

Lysis

Pellet was resuspended in 5 ml lysis buffer (20 mM Tris, 250 mM NaCl, 1% Triton X-100, 1 mM 2-mercapthoethanol, 5 mM Imidazole, pH 7.5). Solution was sonicated in 50 ml tube, placed in a bucket containing ice, six times for 30 sec with 30 sec intervals with MSE soniprep 150. Lysate was centrifuged at 20000 ×g for 20 min at 4 °C and applied onto column.

Purification

A HiTrap Chelating HP (GE Healthcare, #17-0408-01) column was used.

Column was washed with 5 column volumes (Vol.) distilled water and charged with 0.6 ml 10 mM $NiSO_4$. After column was charged, column washed again with 5 column Vol. distilled water and 2 column Vol. (20mM HEPES pH7.3, 500 mM NaCl, 5 mM Imidazole, pH 7.6). Lysate was applied onto column and flow through was collected and reapplied. Column was washed with 5 column Vol. of each of the following buffers with increasing Imidazole concentration and fraction of 1 ml portions were collected.

Wash 1: 20mM HEPES pH7.3, 500 mM NaCl, 5 mM Imidazole, pH 7.6

Wash 2: 20mM HEPES pH7.3, 500 mM NaCl, 50 mM Imidazole, pH 7.6

Elution 1: 20mM HEPES pH7.3, 500 mM NaCl, 100 mM Imidazole, pH 7.6

Elution 2: 20mM HEPES pH7.3, 500 mM NaCl, 200 mM Imidazole, pH 7.6

Elution 3: 20mM HEPES pH7.3, 500 mM NaCl, 300 mM Imidazole, pH 7.6

Elution 4: 20mM HEPES pH7.3, 500 mM NaCl, 500 mM Imidazole, pH 7.6

Elution 5: 20mM HEPES pH7.3, 500 mM NaCl, 1 M Imidazole, pH 7.6

2.2.19 LOW DENSITY LIPOPROTEIN LABELLING

For labelling of LDL the lipid dispersion technique was done according to Groener *et al.* (Groener, J. E. *et. al.*, 1986). Briefly, five µCi of cholesteryl linoleate [cholesteryl-1 2 6 7-3H(N)] (#ART1203, American Radiolabeled Chemicals, St. Louis, Missouri, USA) was mixed with 1 µmol phosphatidylcholine (Sigma, #P3556) and 20 nmol of butylated hydroxytoluene (Sigma, #W218405) in chloroform. Chloroform was evaporated in a stream of nitrogen. Ester and phosphatidylcholine were dissolved in 50 mM Tris HC1, pH 7.4, containing 0.1 g/l EDTA and the tube was flushed with nitrogen. The suspension was sonicated twice (RK100H Sonorex,

Bandelin, Berlin, Germany), for 5 min at RT. Sonicated mixture was added to 5 mg LDL (Sigma, #L8292) and 5 ml lipoprotein deficient serum (LPDS; Sigma #S5394), 0.6 ml of a 10 mmol/l 5,5-dithiobis(2-nitrobenzoic acid) (#D8130) solution and 80 µl of a 100 g/L EDTA solution were added. Tube was flushed with nitrogen and incubated for 24 h at 37 °C.

After labelling the LDL was obtained by density-gradient ultracentrifugation as described by Redgrave *et al.* (Redgrave, T. G. *et. al.*, 1975). Briefly, the density of mixture was adjusted with KBr (Sigma, #60093) to 1.063 g/ml and centrifuged for 24 h at ~286,000 ×g at 4 °C. LDL was floating on top of gradient and transferred into 1 ml of LPDS. To stabilize the LDL, porcine albumin was added to obtain a final concentration of 80 g/l.

2.2.20 FLUORESCENCE MICROSCOPY

For microscopy acetone treated 13 mm round cover slips (VWR, #0150) were used and cells were seeded at a density of 1.2 – 1.5×10^5 cells per cover slip.

Flourescence microscopy- filipin staining

Cells were washed three times with cold PBS and fixed for 1 h at RT with 3% paraformaldehyde. After fixation cells were washed three times with cold PBS. Cells were incubated with 1.5 g glycine/PBS 10 min at RT. Staining was performed with 1 ml of PBS/10% FCS containing 0.05mg/ml filipin (25 mg/ml in DMSO; Sigma, #F9765) for 1 h at RT. After staining cells were washed three times and the cover slips were mounted in Mowiol (7g glycerine, 6ml H_2O and 2.4 g Mowiol were stirred for 2 h at RT, 12 ml 0.2 M Tris/HCl (pH 8.5) was added and the solution was stirred until Mowiol was completely dissolved).

Images were recorded with a Leica DMI6000B and Leica DFC360FX camera, 100x magnification, 1.4 numerical aperture, oil immersion. Filipin was excited at 340 – 380 nm. Analysis of images was performed with Volocity (ver. 5.2.0, Improvision, Coventry, UK).

Confocal microscopy – FABP4, 5 and 7

Cells were washed three times with cold PBS and fixed in PBS/3.7% formaldehyde/0.025% glutaraldehyde for 30 min at RT. Cover slips were washed three times with PBS. Cells were permeabilised in permeabilisation buffer

(PBS/0.05% saponin/ 5% FCS/ 0.5 % BSA / 0.1 % NaN_3) for 30 min at RT. Cover slips were stained up side down on 80 µl permeabilisation buffer containing appropriate amount of primary antibody, for 1 h at RT. Cells were washed and stained as above but with secondary antibody. Cover slips were washed three times with PBS and mounted as mentioned above in Mowiol containing 1 µg/µl DAPI.

Anti bodies used: anti-mouse FABP4 (R&D systems, #AF1443) at 10 µg/ml concentration; anti mouse FABP5 (R&D systems, #AF1476) at 15 µg/ml concentration; anti mouse FABP7 (Abcam, #ab32423) at 5 µg/ml concentration; anti-goat IgG-Alexa647 (Molecular Probes; #A21469) at 2 µg/ml concentration; anti-mouse-Cy5 (Stratech, # 715-175-150), 1:100 dilution.

Images were recorded with a Leica SP5 confocal laser scanning microscope at 63x magnification, 1.4 numerical aperture, oil immersion, and analysed with Volocity (ver. 5.2.0, Improvision, Coventry, UK).

2.2.21 PROTEOMICS

Proteome analysis of *Leishmania mexicana* promastigotes and amastigotes[2]

1D and 2D gel electrophoresis as well as processing thereof for mass spectrometry, was performed as mentioned in Paape *et al.* (Paape, D. *et. al.*, 2008). Data acquisition and criteria for protein identification was also done as mentioned in Paape *et al.*

Proteome analysis of potentially secreted proteins of *Leishmania mexicana* amastigotes[3]

Mascot Generic Format (MGF) input files were generated with the Extract_MSN tool (Bioworks 3.3, ThermoQuest Corp, San Jose, CA), and merged with the precursor grouping option disabled. Spectra containing less than 10 fragment data points were discarded. MS/MS data were searched using Mascot version 2.2.03 (Matrix Science Ltd, UK) against a compilation of NCBI (RefSeq database) containing 70303 protein sequences. This database consisted of Mus musculus (35

[2] Core Facilities, Max Planck Institute for Infection Biology, Charitéplatz 1, 10117 Berlin, Germany

[3] Sample processing, mass spectromic analysis as well as calculation of the emPAI was done by M. E. Barrios-Llerena. Stated for completeness.

275 sequences, released in July 2008), Bos taurus (25 279 sequences, released in July 2008), Leishmania major (9 436 sequences, released in March 2008), *L. infantum* and *L. braziliensis* (27 and 47 sequences respectively, released in March 2008). This database was complemented with sequences from *L. mexicana*, frequently observed contaminants (human keratins and trypsin) and the sequence encoding DsRed (239 sequences, released in July 2008).

Protein abundance was estimated by calculating the protein abundance index (PAI) (Rappsilber, J. *et. al.*, 2002) and the exponentially modified protein abundance index (emPAI) (Ishihama, Y. *et. al.*, 2005). The emPAI values were given by the exponential relationship with PAI, $10^{PAI}-1$, where PAI was defined as the number of observable unique peptides per protein normalised by the number of peptides obtained via *in silico* digestion. Proteogest software was used to generate lists of *in silico* digested peptides (Cagney, G. *et. al.*, 2003).

2.2.22 BIOINFORMATICS

Identification of Sequence motifs in the 3'-Untranslated mRNA regions

3'-Untranslated mRNA regions (3'-UTR) analysis was performed using the oligo-analysis tool (van Helden, J. *et. al.*, 2000). The tool identifies oligomers ranging in length from 4 to 8 nt that are more frequent within the UTR sequence of a group of co-regulated genes compared with a set of non-regulated genes. In our case, 51 intergenic sequences from the *L. major* genome were chosen for analysis of regulated loci. This test set was compared against a random selection of intergenic sequences from annotated protein-coding genes on *L. major* chromosomes 4, 7, 12, 18, 21, 25, 28, 30, and 36, totaling 2677 sequences. Statistics and significance values were determined according to van Helden *et al.* (van Helden, J. *et. al.*, 1998).

The 51 regulated test loci (see suppl. TABLE 2) represented the 39-intergenic regions of 35 ORFs for which the respective proteins were solely identified in amastigote samples by the present proteome analysis, and 16 additional 3'-intergenic regions of ORFs encoding proteins found to be more abundant in amastigotes by isotope-coded protein labelling. The cysteine proteinase B ORF array was excluded as synthesis of the homologous *L. major* ORFs appears not to be stage-specifically regulated (Sakanari, J. A. *et. al.*, 1997). Leishmanolysin

(gp63)-encoding loci were excluded for the same reason because the respective genes are not expressed in *L. major* amastigotes (Schneider, P. *et. al.*, 1992). For *L infantum*, 3'-UTRs of 45 corresponding orthologs were searched for the presence of the identified oligonucleotide motifs. To test whether a motif lay within the boundaries of a presumptive 3'-UTR, we predicted putative splice sites of the downstream ORFs that define the 5'-UTR boundary. The longest predicted 5'-UTR was chosen, and the putative end of the 39-UTR of the upstream gene was arbitrarily set 250 nt further upstream, effectively representing a conservative (short) estimate of 39-UTR length. Splice sites of downstream ORFs were predicted with the algorithm developed by Benz *et al.* (Benz, C. *et. al.*, 2005) in the form adapted by Gopal *et al.* (Gopal, S. *et. al.*, 2005) using the following parameters: a minimum length of polypyrimidine tract of 8 with up to two mismatches but at least six consecutive pyrimidines and a splice site predicted within the 400 nt upstream of the ATG (Gopal, S. *et. al.*, 2005).

Other bioinformatics tools used

Codon adaptation indices (CAIs) (Sharp, P. M. and Li, W. H. 1987) were calculated as described by Carbone *et al.* (Carbone, A. *et. al.*, 2003). Subcellular localization was predicted according to Szafron *et al.* (Szafron, D. *et. al.*, 2004). Virtual 2-DE Gel images were generated with JVirGel (Hiller, K. *et. al.*, 2003). Functional classification was performed according to the Sanger database and KEGG2 (http://www.genome.jp/kegg/kegg2.html).

Information on enzymes identified was embedded in an overview chart of the predicted metabolism of L. major produced via the LeishCyc website (http://leishcyc.bio21.unimelb.edu.au/; Doyle, M. A. *et. al.*, 2009). This visualised all enzymes identified and their abundance by a colour code referring to their respective emPAI values.

Prediction of secretion

The *L. major* reference proteome was downloaded from ftp://ftp.sanger.ac.uk/pub/databases/L.major_sequences/DATASETS/ (LmjFwholegenome_20070731_V5.2, 8335 entries) and for *L. infantum*

fromftp://ftp.sanger.ac.uk/pub/pathogens/L_infantum/DATASETS/
(LinJwholegenome_20080508.v3.0a, 8173 entries).

To predict potentially secreted proteins, sequences were first analysed with TargetP 1.1 with the specificity parameter set at >0.95 (Emanuelsson, O. *et. al.*, 2000; Emanuelsson, O. *et. al.*, 2007). Proteins predicted by TargetP as mitochondrial were excluded for further analyses.

Proteins predicted as secreted, prediction of localisation was not possible as well as proteins with a reliability class value >3 were reanalysed with SignalP 3.0 (Bendtsen, J. D. *et. al.*, 2004; Nielsen, H. and Krogh, A. 1998). The set of proteins for which SignalP predicted a signal peptide was finally purged of those in which transmembrane domains were predicted with TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/; Krogh, A. *et. al.*, 2001) and of those with an annotation in the Sanger database indicating a GPI-anchor. The latter is not available for *L. infantum*, hence the annotated *L. major* orthologues were purged from the list. However, only 21 proteins of 653 *L. major* proteins predicted to have a signal peptide were also predicted to be GPI anchored. By analogy only up to 3 % would be expected to be falsely considered secreted/not secreted in *L. infantum* by not being able to purge the data set of putatively GPI anchored *L. infantum* proteins.

2.2.23 STATISTICAL ANALYSIS

Statistical analysis of general linear model was conducted in minitab version 15. When residual did not conform to normal distribution they were $\log_{10}$ tranformed. Residuals showed homogeneity of variance. P values less than 0.05 were considered statistically significant.

Other statistical analyses were performed with GraphPad Prism 4.0.