Chapter II

Materials and Methods
2.1. Materials and their sources

2.1.1. Tissue culture chemicals:
M-199 and RPMI-1640 media, bovine serum albumin (BSA), penicillin-G, streptomycin, sodium bicarbonate are procured from Sigma Chemical Co., USA., fetal Calf Serum (FCS) from Gibco-BRL Ltd., USA.

2.1.2. General Chemicals:
Ampicillin, sodium dodecyl sulphate (SDS), tris [hydroxymethyl] aminomethane (Tris), dimethyl sulfoxide (DMSO), ammonium persulphate (APS), acrylamide, N-N’- methylene -bis-acrylamide, coomassie brilliant blue R-250, coomassie blue G-500, CaCl₂, formamide, 2-mercaptoethanol, ethidium bromide, formaldehyde, 3-(N-Morpholino) propane sulfonic acid (MOPS), diethylpyrocarbonate (DEPC), phenol, phenyl methyl sulphonyl fluoride (PMSF), N,N,N’,N’-Tetramethylethylenediamine (TEMED), triton-X-100, were all purchased from Sigma Chemical Co., USA.

2.1.3. Analytical grade: Chemicals that were purchased locally are listed below:
Methanol, glycerol, ethanol, chloroform, HCl, isoamyl alcohol, isopropanol, KCl, KH₂PO₄, MgCl₂, acetic acid, NaCl, NaH₂PO₄, Na₂HPO₄, NaOH, boric acid, ethylenediaminetetraacetate (EDTA), hydrogen peroxide (H₂O₂), NEDD (N-1-naphthyl ethylene diamine dihydrochloride), sulphanilamide, orthophosphoric acid, etc.

2.1.4. Molecular biology reagents:
Restriction enzymes, ligases, DNaseI, other modifying enzymes etc. were purchased from MBI Fermentas, Promega, New England Biolabs, USA, salmon sperm DNA, triPure- reagent for RNA isolation and protease inhibitor cocktail were from Roche.

2.1.5. E. coli cells:
DH5α was from Gibco BRL, Ltd., USA.
2.1.6. Molecular weight markers:
Protein molecular marker, 100 bp DNA ladder and Mass ruler were obtained from MBI Fermentas Inc, MD, USA.

2.1.7. Radioactive chemicals:
\(^{55}\)FeCl\(_3\) was purchased from PERKIN ELMER. All other radiochemicals are purchased from BERC.

2.1.8. Kits used:
RT-PCR was performed either with One Tube RT-PCR Kit from Roche or using Superscript III RT from Invitrogen. Mini prep kit for plasmid DNA isolation and purification, and QIA quick Gel Extraction kit was purchased from Qiagen, Germany. Genomic DNA extraction Kit was purchased from Promaga.

2.1.9. Others
Membranes filter papers were purchased from Whatman, USA. PVDF membranes were purchased from MDI and Millipore. Hybond N+ membranes were purchased from Amersham Biosciences, USA. X-ray film: X-ray film, developer and fixer were purchased from Kodak, India Photographic Company Ltd.

2.2. Methods
2.2.1. Culture media
All the media prepared were sterilized before use and handled under aseptic conditions.

2.2.1.1. Growth medium for bacterial culture:-
2.2.1.1.1. Luria-Bertani (LB) broth
For 1L of LB media 20g of LB powder was added and sterilized by autoclaving.
2.2.1.1.2. LB-agar plate
To each liter of LB, 20 g of agar-agar was added and sterilized by autoclaving. The media was cooled to 60°C and the desired antibiotic was added and poured into 90
mm plates (25-30 ml per plate). The plates were kept for at least 30 min under the laminar flow for solidification and drying.

2.2.1.1.3. Antibiotic solutions
Ampicillin solution was made by addition of 100 mg of ampicillin into 1ml sterile water. Kanamycin solution was prepared by adding 50 mg of Kanamycin in 1ml of sterile water.

2.2.1.2. Growth media for Tissue culture:-
2.2.1.2.1. M-199 medium
The M-199 medium was purchased from sigma (Sigma-Aldrich, Inc., MO). To the final 500ml of medium 5ml of penicillin and streptomycin (100 units/ml penicillin, 100mg/ml streptomycin) and 50ml of FBS were added.

2.2.1.2.2. RPMI 1640 medium
The RPMI 1640 medium was purchased from sigma (Sigma-Aldrich, Inc., MO). To the final 500ml of medium 5ml of penicillin and streptomycin (100 units/ml penicillin, 100mg/ml streptomycin) and 50ml of FBS were added.

2.2.2. Cell Lines and Culture Conditions:-
2.2.2.1. Macrophage culture
Mouse macrophage cell line J774A.1 (American Type Culture Collection) were cultured in RPMI1640 (Sigma) supplemented with 10% heat inactivated fetal bovine serum, FBS (Gibco/hyclone), 100 units/ml penicillin, 100 mg/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C (Heraeus BB 15).

2.2.2.2. Animals
BALB/c female mice of 4-12 weeks old (National Centre for Laboratory Animal Sciences, Hyderabad, India) were used for the experiments and for the propagation of the virulent strain of Leishmania donovani. For experimental use of the animals prior approval from the Institutional Animal Ethics Committee was taken (Reg: 19/1999 CPCSEA, 03.10.1999).
2.2.2.3. Parasite culture

*Leishmania donovani* (MHOM\IN\1983\AG83) was used for all the experiments. For *in vitro* culture, promastigotes were maintained in M199 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin at 22°C in BOD incubator. Sub-culturing was done on every fourth or fifth day when the promastigotes reached stationary phase of growth.

Promastigotes from freshly transformed AG83 amastigotes were cultured in M199 medium supplemented with 30% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. A small aliquot of stationary phase culture was added to fresh media for propagation of each subculture.

For *in vivo* culture, the virulent strain of *Leishmania donovani* AG83 was maintained in BALB/c mice by passaging every 3-4 weeks. Infected spleen of BALB/c mice was removed aseptically, homogenized under sterile conditions and suspended in M199 with 30% FBS. This suspension was incubated at 22°C in BOD incubator for 48 to 72 hours. Freshly transformed promastigotes were checked under the microscope and counted, the suspension was centrifuged at 1000 rpm for 10 min at 4°C to remove splenic debris and the promastigotes were pelleted down at 5000 rpm for 15 min at 4°C. The pellet was resuspended in PBS (pH 7.4) at a concentration of 1X10^8 cells/ml. This freshly transformed promastigotes (100 μl) were injected in the tail vein of 2-4 weeks old mice.

2.2.3. Infection of macrophage

J774A.1 macrophage cells were infected with stationary phase *L. donovani* promastigotes at a ratio of 20 parasites per macrophage. After appropriate time of infection, macrophages were washed three times with phosphate buffered saline (PBS), and fresh RPMI medium was added for further experiments.

2.2.4. Amastigotes isolation

Amastigotes were obtained by infection of J774 macrophages. After definite time of infection, cells were washed with PBS once and flush the respective groups using a Pasteur pipettes to dislodge adherent macrophage. Maximum macrophages were removed and suspend in a 1.5ml micro-centrifuge tube (which will resist freeze cycle with liquid nitrogen). The macrophage content was collected and centrifuged at 1000
rpm for 10 min (Rotor radius = 6cm, Biofuge, Heracus). The pellet was resuspended in 2-3 ml of PBS and dispersed well. The tube was placed into liquid nitrogen until it freezes or in -80°C chamber for one hour. Then it was put on 37°C water bath. This step was repeated for four times. A 90-40-20% percoll gradient should be prepared previously in 15 ml centrifuge tube. All three layers should be distinctly visible. The cells were discharged slowly on the top of the gradient and spin at 3000 rpm for one hour. Without disturbing the gradient, there will be two layers separating out. The content of these two entities was checked on a microscope. The appropriate amastigote population was picked out and washed the amastigotes once with PBS to remove percoll.

2.2.5. Transfection

*Leishmania* was cultured on M199 medium supplemented with 10% fetal bovine serum up to late log phase. Cells were collected by centrifugation and suspended at a density of 10⁸ cells/ml in HEPES buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄, 6 mM glucose, pH 7.4). For transfection the mode was set on 500V at Low Voltage mode of RESISTANCE & CAPACITANCE. The parameters were set as Capacitance at 1300μF, Resistance at 25 ohms, Chamber Gap 4 mm, Charging Voltage at 450V, Field Strength of E 2.25 kV/cm and a desired Pulse Length of 7 milli Second.

Alternatively, the mode was set on 2500V at high Voltage mode of RESISTANCE & CAPACITANCE. The altered parameters were like Capacitance at 25 μF, Resistance at 25 ohms, the Chamber Gap was 4 mm, and a desired Pulse Length of 2 milli Second.

Procedure: - Cells were collected by centrifugation and suspended at a density of 1x10⁸ cells/ ml in HEPES buffered saline (approximately 400 µl) and was kept on ice for 10 minutes and Plasmid DNA (1mg/ml in 10 mM Tris-1mM EDTA, pH 7.4) was added to it before electroporation. One pulse was provided in case of low voltage mode and two pulses in 10 sec gap in case of high voltage mode. Transfected parasites were kept on ice for 10 minutes and then transferred to 10 ml of drug-free medium. After one day the antibiotic G418 solution was added (50 μg/ml) for selection.
2.2.6. Isolation of genomic DNA from *Leishmania donovani*

Isolation of genomic DNA was done by Promega Wizard genomic DNA purification kit as per manufacture’s protocol.

10 ml of 5 day old (stationary phase) culture of *Leishmania donovani* was pelleted at 3000 rpm for 10 minutes. The pellet was re-suspended in 1ml PBS (1X) and was transferred into 1.5 ml micro-centrifuge tube. Cells were pelleted down again. 300 µl of nucleus lysis solution was added to the pellet and mixed gently by pipette. Then, 100 µl of protein precipitation solution was added and vigorously vortexed for 20 second. After keeping on ice for 5 minutes, the sample was centrifuged at 12000 rpm for 3 minute. The supernatant containing DNA was transferred to a clean 1.5 ml micro centrifuge tube containing 300 µl of isopropanol. Mixing was done by inverting gently until the thread like strand of DNA formed a visible mass. Centrifuge was done at maximum speed for 2 minute. Supernatant was removed and 300 µl of 70% ethanol was added to the pellet. Centrifuge was done at maximum speed for 2 minute. Ethanol was removed and pellet was kept for drying. 50 µl of DNA rehydration solution was added (as per manufacture’s protocol). RNAse treatment was given whenever needed. Genomic DNA was stored at 2-8 °C

2.2.7. Primer sequences and PCR conditions

Both *Leishmania donovani* and *Leishmania major* show a very high homology between DNA and protein sequences (> 95%), so we assume that primers designed from *Leishmania major* will also amplify the sequence from *Leishmania donovani*. We also made longer primers (18 mer) to make annealing easy. Primer sequences were:

FORWARD PRIMER

5’ATA CAT **GGA TCC** atg cgt gca ccg ctc gcg 3’

Bam H1

REVERSE PRIMER

5’ATA CAT **TCT AGA** tca cgc gcg gcg ctc gag 3’

Xba1

By this strategy we amplified the 1.9 kb DNA fragment representing the putative gene for MCO from *Leishmania donovani* genomic DNA by PCR. PCR condition was as follows:
Components

Template 300 ng
Primer For 100 ng
Primer Rev 100 ng
DMSO 2.5%
dNTPs 1 μl (from 10 mM stock)
MgCl₂ 1.5 μl (from 25 mM stock)
10X buffer 5 μl
Taq polymerase 1 unit
DD H₂O As per needed

---------------------------------------------

Reaction volume: 50 μl

Conditions:
Cycle = 30

94 °C 5 min
94 °C 1 min
60 °C 1 min 30 sec
72 °C 2 min
72 °C 10 min
4 °C ∞

The amplification of the right product was confirmed by DNA sequencing and by restriction mapping. The sequencing of nucleotides from both the ends revealed that the putative MCO gene from *Leishmania donovani* is 94% homologous to putative MCO gene from *Leishmania major* and 98% homologous to yet annotated sequence of *Leishmania infantum* MCO gene.

2.2.8. Expression vectors for LDMCO expression.

2.2.8.1. pMAL-c2X vector

We cloned the putative LDMCO into pMAL-c2X vector from NEB between the BamH1 and XbaI sites using selection marker as ampicillin. The pMAL-c2X vectors carry an N-terminal maltose binding protein gene sequence so expressed protein will have a N-terminal maltose binding domain of about 40 kD. In this vector after the
cloning, the coding sequence is transcribed by a CMV promoter, which is inducible by IPTG in *E. coli* (any strain). The advantage of MBP tag is that it makes protein more soluble. The expressed protein remains in the cytoplasm mostly in soluble form. MBP-LDMCO can be purified by affinity chromatography by using maltose resin.

## 2.2.8.2. pET-28a (+) vector

We also cloned the putative LDMCO into pET-28a (+) vector from Novagen between the BamH1 and Hind III sites using the selection marker Kanamycin. The pET-28a (+) vector carries an N-terminal His Tag/thrombin/T7 Tag configuration of about 2 kD plus an optional C-terminal His Tag sequence. We did not remove the stop codon from LDMCO ORF so expressed Protein will not have C-terminal His tag sequence. In this vector after the cloning, the coding strand is transcribed by T7 RNA polymerase in *E. coli* strain BL 21. This strain contains T7 RNA polymerase gene inserted into its genome under the control of a promoter, which is inducible by IPTG. IPTG induce the promoter and produce T7 RNA polymerase in high amount that in turn transcribes the cloned sequence. By this approach we may get a very high expression of the cloned gene. The resultant protein will be tagged with His tag and
that can be purified from other proteins of bacteria by affinity chromatography by using Ni-NTA resin.

The pET-28a(-) vectors carry an N-terminal His Tag** polyhistidine/TT-Tag® configuration plus an optional C-terminally His Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 DNA polymerase is shown below. The T7 origin is oriented so that infection with helper phage will produce viruses containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 60337-3).

Map of pMAL-2 Vectors

The E. coli BL 21 containing the cloned MCO in pET 28a (+) when treated with IPTG was found to express an inducible protein close to the calculated molecular weight of 70 kDa. Due to very high expression and because of its being membrane protein, most of the protein fractions was found in the inclusion body. So the same 1.9 kb DNA fragment was cloned into maltose tagged pMALc2X vector. The molecular weight of
protein was about 110 kDa, IPTG inducible band was found mostly into the soluble fraction.

2.2.9. Purification of LDMCO

2.2.9.1. Purification of His tagged LDMCO

The primary culture of E. coli BL 21 containing the cloned LDMCO into pET28a(+) vector was grown in 10 ml LB in presence of Kanamycin (50 µg/µl) overnight at 37°C. 200 ml of LB was inoculated with the 4 ml of primary culture in the presence of Kanamycin (50 µg/µl) and incubated at 37°C until the OD appeared about 6 (approximately three hours). Then the culture was induced by addition of IPTG (100 µM). The culture was now incubated at 28°C for 5 hours. Cells were pelleted down quickly after the incubation on ice for 30 minutes. 10 to 20 ml of PBS was added and cells were re-suspended and pelleted down again. Cell pellet was kept at -20°C overnight after PBS wash. Pellet could be stored at this stage.

The cell pellet was put on ice from -20°C for 30 min and then suspended in 2ml of PBS containing Lysozyme 20 µg/µl and protease inhibitor cocktail. The solution was incubated on ice for 1 hour or until the cells lyse.

After cells were lysed, sonication was done at amplitude of 4 for 30 seconds in ice. This step of sonication was repeated for four times or until the solution loses its viscosity with an interval of one minute on ice. The solution was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 4°C for 20 min at maximum speed in a microfuge. Most of the protein was found within the inclusion bodies so the supernatant was discarded and pellet was re suspended again into 1ml PBS containing 0.3 % sarcocine and protease inhibitor cocktail and was kept for one hour in ice. The solution was centrifuged again at 4°C for 20 min at 13000 rpm speed. Now the supernatant was taken and mixed with the active Ni-NTA beads slowly and the mixture was incubated at 4°C rotating slowly for 1 hour. To make beads active they were incubated with nickel sulfate solution for 10 min on ice and washed twice with PBS at 1500 rpm for 30 second. After 1 hour of binding step, the mixture was centrifuged at 1500 rpm for 30 second. The supernatant was discarded and the pellet was washed 5-6 times with PBS containing 20µM of imidazol at 1500 rpm for 30 second each. The protein was eluted with PBS containing 200 µM of imidazole.
2.2.9.2. Purification of MBP tagged LDMCO

The primary culture of *E. coli* containing the cloned LDMCO into pMALc2X vector were grown in 10 ml rich media in the presence of ampicilin (100 μg/μl) overnight at 37°C. 200 ml of rich media was inoculated with the 4 ml of primary culture in the presence of ampicilin (100μg/μl). Glucose was added to the media with the final concentration of 0.2 % and incubated at 37°C until the OD appeared about 6 (approximately three hours). Then the culture was induced by adding IPTG (200 μM). The culture was now incubated at 28°C for 5 hours. Cells were pelleted down quickly after the incubation on ice for 30 minutes. 10 to 20 ml of PBS was added and cells were re-suspended and pelleted down again. Cell pellet was kept in -20°C overnight after PBS wash.

The cell pellet was put on ice from -20°C for 30 min and then suspended in 2ml of lysis buffer containing Lysozyme 20 μg/μl and protease inhibitor cocktail. The solution was incubated on ice for 1 hour or until the cells were lysed.

After the lyses of cells, sonication was done at amplitude of 4 for 30 seconds on ice. This step of sonication was repeated for four times or until the solution loses its viscosity with an interval of one minute on ice. The solution was transferred into 1.5 ml micro centrifuge tubes and centrifuge at 4°C for 20 min at 13000 rpm in a microfuge. The supernatant was taken and mixed with the maltose beads slurry slowly and the mixture was incubated at 4°C rotating slowly for 1 hour. To make slurry, beads were washed twice with PBS at maximum speed for 30 second. 200 μl of PBS was added to the pellet to make slurry. After 1 hour of binding step, the mixture was centrifuged at maximum for 30 second. The supernatant was discarded and the pellet was washed 5-6 times with PBS at maximum for 30 second each. The protein was eluted with PBS containing 250 μM of maltose.

2.2.10. Ferroxidase assay.

Ferroxidase activity was performed at 25°C by using ferrous sulfate as the electron donor and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a chelator to specifically detect the ferrous iron remaining at the end of the reaction. Each assay mixture (1 ml) contained 100 mM Na-acetate buffer, pH 5.2, and the reaction was started by addition of FeSO₄ to 0.03 mM. Samples were quenched at time intervals by adding ferrozine to 0.3 mM, and the rate of Fe(II) oxidation was
determined by measuring the absorbance of residual Fe(II)-ferrozine 
\((E_{570} = 7.26 \text{ mM}^{-1} \text{ cm}^{-1})\).

2.2.11. DNA synthesis assay
DNA synthesis was measured by incorporation of \(^3\)H thymidine into trichloroacetic acid (TCA) precipitable material. 1 x 10^7 *Leishmania* cells were incubated at 22°C for 10 h. \(^3\)H thymidine was added to culture as 0.4 µCi/ml concentration and cells were incubated for 4 h at 22°C. After the incubation cells were pelleted down and washed with 5% TCA three times. 0.5 ml of 0.25 M NaOH was added to pellet and pellet was mixed slowly on shaker platform for 10 min at room temperature. 50µl of 6 M HCl was added to neutralize the solution to minimized artifact due to chemiluminesence in the scintillation counter. Equal number of cells was taken for scintillation counting.

2.2.12. \(^{55}\)Fe Iron uptake assay in intracellular amastigotes
\(^{55}\)Fe iron uptake assay was done by using \(^{55}\)Fe-NTA. \(^{55}\)Fe-NTA was prepared by adding about 3µg of \(^{55}\)Fe in 100µl of NTA solution (600µg of NTA in 100µl of carbonate buffer) for 10 min at room temperature. 1X10^6 J774A.1 macrophage cells were plated in 100mm dishes in RPMI media with 10% serum and incubated for 24 h in the CO₂ incubator. After the incubation cells were kept in serum free RPMI media for 16 h at 37°C in 5% CO₂. About 10µl of \(^{55}\)Fe-NTA solution was added to each plate and kept for 6 h in above conditions. After washing the macrophages thoroughly to wash out extra-cellular \(^{55}\)Fe, cells were infected with stationary phase LD promastigotes at a ratio of 20 parasites per macrophage. After indicated time points of infection, macrophages were washed 3 times with 1 ml phosphate buffered saline (PBS) and suspended in a falcon tube (to resist freeze cycle with liquid nitrogen). Cells were dispersed well. The tube was put into liquid nitrogen until it freezes or in -80°C for one hour. Then it was put on 37°C water bath. This step was repeated for four times. To isolate and purify intracellular parasites a 90-40-20% percoll gradient, which should be prepared previously was used (Chang. 1980). The cells were discharged slowly on the top of the gradient and spin them at 3000 rpm for one hour. Then two layers were separated out according to the gradient and they were collected individually without disturbing the gradient. The content of these two entities was checked under microscope to pick out the appropriate amastigote population. The
amastigotes was washed once with 10% DMEM or PBS to remove percoll. Equal number of amastigotes was taken for scintillation counting.

2.2.13. Counting of LD
The LD promastigotes and amastigotes were count on a neobar chamber in light microscope at 40X magnification. LD was counted from all four 16-big squares and an average was considered for further calculations. The formula used is:

\[
\text{No of cells} = \text{Average counting by neobar chamber} \times \text{dilution factor} \times 10^4 \text{cells/ml}
\]

The generation of knockout constructs was based on the pXG-HYG and pXG-NEO vectors (kind gifts from Dr. S. Beverly, Washington University) that consist of backbone of a pSP6-T3 (Life Technologies Inc.) and a cassette comprising a gene encoding resistance to hygromycin B (hyg) or G418 (neo) and flanking dihydrofolate reductase-thymidylate synthase (DHFR) sequences that are required for proper gene expression. To disrupt the LDMCO locus, the flanking regions of LDMCO DNA sequences were cloned into pX63-HYG and pX63-NEO such that the hyg expression cassette (2,837bp) and neo expression cassette (2,687bp) were flanked at both ends by these sequences. This strategy permitted subsequent excision of the entire insert, excluding vector DNA, for transfection into the parasites to promote homologous recombination with chromosomal LDMCO sequence. The 5' LDMCO plus a portion of LDMCO ORF were PCR amplified, yielding a 1044-bp fragment (nucleotides -549 to 495) that was cloned into the Hind III/Sal I sites in both vectors. The unique 3' LDMCO homologous-region insert was PCR amplified, yielding a 1187-bp fragment that was cloned into the Smal/BglIII sites of both vectors, resulting in the pXGHYG-LDMCO-KO and pXGNEO-LDMCO-KO vectors. The vectors were digested with Hind III and Bgl II, and the knockout fragment containing the selectable gene marker was purified. The procedures used in the transfection of LD promastigotes were described before. Wild-type parasites were transfected with 10 μg of the linearized knockout constructs by electroporation, and transformants were selected at 25 μg of hygromycin B/ml or 50 μg/ml of G418/ml. By this strategy we generated half knock out of LDMCO, one with selection marker of hygromycin B and other with selection marker G418. It is known that continuous subpassage of parasites may result in a decrease in virulence; therefore, the half knock outs and wild-type strains were
electroporated and subpassaged in parallel the same number of times under the same culture conditions.

2.2.15. Preparation of vector for knock down and over expression
For gene knock down studies, the entire coding region of the LDMCO gene was cloned into the BamHI sites of the pXG-Neo expression vector in reverse direction to generate the antisense LDMCO vector, and for gene complementation studies, the entire coding region of the LDMCO gene was cloned into the BamHI sites of the pXG-Neo expression vector to generate the pXGNEOLdmco vector. Both vectors were transfected to LD by electroporation and transformants were selected at 50µg of G418/ml.

2.2.16. Preparation of GFP-LDMCO vector
For localization studies, the entire coding region of LDMCO gene without stop codon was cloned into the Sma1/BamH1 sites of pXG-‘GFP+ expression vector to generate pXG-LDMCO-GFP vector.

2.2.17. Immunostaining for localization of LDMCO protein in LD
A smear of LD was made on cover slip and let it dry for 30 min. Cover slip was kept in a 30mm dish and 2 ml of chilled methanol was added slowly over the cover slip. After 10 min of incubation methanol was pipette out and cover slip was dried again. After this the cover slip was put in 3 ml of PBS containing 1% BSA for 3h. After this incubation the cover slip was washed twice with PBS for 5 min without shaking. Then the cover slip was put in anti-LDMCO primary antibody (1:5000 in PBS + 1% BSA) for 1 h at RT. Three washes with PBS were given for 5 min each without shaking. Then the cover slip was put in the secondary antibody solution (1:1000 Cy3 anti rabbit +1:1000 DAPI in PBS + 1% BSA) for 1h. Five washes with PBS were given for 5 min each without shaking. The cover slip was mounted in 10% glycerol on slide for microscopic examination.

2.2.18. Immunoblot analysis:
Proteins were denatured at 95°C by mixing with appropriate volume of 2X SDS-PAGE gel loading buffer and loaded onto SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (percentage of the gel was determined according to the protein size
analyzed) under reducing condition. After resolving, proteins were transferred to the PVDF membrane (Millipore), blocked with 5% non-fat milk in TBST buffer and incubated with specific anti-LDMCO antibody solution (1:5000 anti LDMCO in TBST + 1% BSA) for 1h. 5 washes were given with TBST buffer for 5 min each. After the washes blot was incubated with horseradish peroxidase-conjugated solution (1:2500 secondary antibody in TBST + 1% BSA) for 1h. After the incubation the blots were washed for 5 times with TBST buffer and 3 times with TBS buffer. Finally, proteins were visualized by enhanced chemiluminescence (ECL) following manufacturer's protocol (Amshesam Biosciences).

2.2.19. RNA Isolation and semi-quantitative Reverse Transcriptase-PCR (RT-PCR) from LD
Total RNA was isolated from LD, using TriPure reagent (Roche), according to the manufacturer's protocol. In the case of intracellular amastigotes, RNA was obtained directly from the infected macrophages. RNA (4μg) was reverse transcribed using the first Strand cDNA synthesis kit for RT-PCR (Invitrogen) with specific reverse primer in a 25μl reaction. 10% of the cDNA solution was taken for doing PCR to quantify the RNA.

2.2.20. Northern blot analysis:
Leishmanial genomic DNA was isolated from the cells and 20μg of DNA was used for each sample. After adding 6X loading dye samples were loaded onto 1% agarose gel. After running, the gel was trimmed and washed with 20X SSC at room temperature (25°C) for 45 minutes with constant and gentle shaking. DNA was transferred to the presoaked nylon membrane using 20X SSC overnight by capillary transfer method. DNA was cross-linked to the membrane by UV (strata linker1800, stratagene) and the membrane was incubated in the hybridization buffer (300mM Phosphate buffer, 7% SDS, 1mM EDTA) for pre-hybridization for 6h at 65°C. Radiolabeled probe for LDMCO was prepared using by random priming kit (New England Biolab) with [α-32P]dCTP and purified by sephadex G50 column. The probe was heated at 95°C for 5 minutes, quick-chilled on ice for 2 minutes and added to the membrane with 5ml of hybridization buffer. After 16h of hybridization at 60°C the
membrane was washed sequentially once with each of 2X SSC, 2% SDS and 1X SSC, 1% SDS and bands were visualized by autoradiography.

2.2.21. In gel and In membrane ferroxidase assay

For in gel ferroxidase assay zymograms were obtained by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Samples were applied in nonreducing denaturing loading buffer without boiling and were electrophoresed at 4°C. Gels were equilibrate in 10% glycerol for 40 min and then incubated at room temperature in 100mM sodium acetate buffer (pH 5.2) containing 0.08% ferrous sulfate for 20 min. The ferrous sulfate solution was dumped and gel was rinsed 5 times with Milli-Q water on shaker for 5 min each. Potassium ferrocyanide solution (1g of Potassium ferrocyanide in 50 ml of Milli-Q water + 50 ml of 0.2 N HCl) was made and gel was put in it for one minute. Then gel was rinsed 5 times with Milli-Q water on shaker for 5 min each.

For in membrane assay one gram agarose was taken and added to 100 ml of cold TB buffer in a 250 ml flask. It was placed in a microwave to melt the agarose (making sure that it does not boil over). 25 ml of this melt agarose solution was put to a gel casting tray after cooling for 30 minutes for solidification Samples were prepare by using Ficoll sample Loading buffer. After loading samples the gel was run at 100V (constant voltage) for 2 hours. To transfer protein, required sized pieces of PVDF were cut. Membrane was activated by making wet it in methanol and by washing to remove extra methanol and it was put in TB buffer until ready to use it. Gel blot papers were also put in TB buffer. Two pieces of the wet gel blot paper were placed on the base of semi-dry electroblotter. Then the membrane was placed on the gel blot paper. Next the gel was placed and then the other gel blot papers. Transfer was run up to 2 h at 100 mA (constant current). The membrane was taken out and was put in a Pyrex dish with Milli-Q water in it and the ferrous sulfate solution (0.08 g of ferrous sulfate in 100 ml of the acetate buffer) was made. This solution was put on membrane and incubated at 37°C for 30 minutes on shaker. The ferrous sulfate solution was dumped and membrane was rinsed 3 times with Milli-Q water. Potassium ferrocyanide solution (1g of Potassium ferrocyanide in 50 ml of Milli-Q water + 50 ml of 0.2 N HCl) was made and membrane was put in it for one minute. The membranes were washed in Milli-Q water and scan the membrane while it is still wet.
2.2.22. Apo-transferrin loading assay.
Incorporation of Fe(II) into apo-transferrin by LDMCO was measured \textit{in vitro}. Reactions were carried out in 200 µl volumes and contained 200 µM apotransferrin, 200 µM ferrous ammonium sulfate, 100 µM ascorbate and 50 µg of LDMCO in 0.1 M sodium acetate, pH 5.2. Formation of Fe(III)-transferrin was monitored at 460 nm. The amount of Fe(III)-transferrin formed was calculated using the molar absorption constant of $\varepsilon_{460\text{ nm}} = 2500 \text{ M}^{-1} \text{ cm}^{-1}$/Fe(III)-transferrin formed.