Studies on Stage Regulated Gene Expression in *Leishmania donovani* Isolated from Indian Kala Azar Patients using Genomic Microarrays

THESIS

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By

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Under the Supervision of

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PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled '**Studies on stage regulated gene expression in *Leishmania donovani* isolated from Indian Kala azar patients using genomic microarrays'** and submitted by Paresh Sharma ID No. 2005PHXF420 for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

Signature in full of the Supervisor _____

Name in capital block letters _____

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Date _____

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December 10, 2009 Paresh Sharma

Content

Abbreviations

A1	Amastigote stage up regulated gene
A_{280}	Absorbance at 280 nm
A_{600}	Absorbance at 600 nm.
Ab	Antibody
Am	Amastigote
Amp	Ampicillin
APS	Ammonium persulfate
ASS	Argininosuccinate synthase
Ax Am	Axenic amastigotes
bp	base pair
BLAST	Basic Local Alignment Search Tool
BMA	Bone marrow aspirates
BSA	Bovine Serum Albumin
^{o}C	Degree Celsius
cDNA	Complementary DNA
Ci	Curie
CL	Cutaneous Leishmaniasis
Cpm	Counts per minute
C terminal	Carboxy terminal
DAB	Diamino Benzidine
dATP	Deoxyadenosine tri phosphate
dCTP	Deoxycytidine tri phosphate
dGTP	Deoxyguanosine tri phosphate
ddH_2O	Double Distilled water
DMSO	Dimethylsulfoxide
ddNTP	Di-Deoxyribose nucleotide tri phosphate
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate

DTT	Dithiothreitol
dTTP	Deoxythymidine tri phosphate
dUTP	Deoxyuridine tri phosphate
dpm	Disintegrations per minute
EDTA	Ethylene diamine tetra-acetic acid
FCS/FBS	Fetal Calf/Bovine Serum
g	Gravitational force
Gms	Grams
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
HEPES	N-2-hydroxyethylpiperazine-N-2 ethane sulfonic acid
hr/hrs	Hour/hours
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSP	Heat shock protein
Hs	Homo sapiens
IFA	Immunofluorescence assay
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-β-D-thio-galactosidase
KA	Kala-azar
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
Ld	*Leishmania donovani*
Lm	*Leishmania major*
M	Molarity
MAP kinase	Mitogen activated protein kinase
mCi	Millicurie
mg/ ml	Milligram/ Milliliter
min/mins	Minute/Minutes
MOPS	3-[N-Morpholino] propanesulfonic acid
NDDH	NAD/FAD dependent dehydrogenase:

$NaHCO_3$	Sodium bicarbonate
N	Normality
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
N terminal	Amino-terminal
O/N	Over night
OD	Optical Density
ORF	Open Reading Frame
P27	Trypanosomatid amastigote specific gene of 27 kDa
PAGE	Poly Acrylamide Gel Electrophoresis
PBS/ PBS-T	Phosphate Buffered Saline/ PBS with Tween 20
PCR	Polymerase chain reaction
PKDL	Post Kala-azar Dermal Leishmaniasis
PMSF	Phenyl Methyl sulfonyl fluoride
PPG	Proteophosphoglycan
Pro	Promastigotes
PSA	Parasite surface antigen
rpm	Revolution per minute
RNase	Ribonuclease
RPMI	Roswell Park memorial Institute
RT-PCR	Reverse transcription- PCR
qRT-PCR	Quantitative real-time PCR
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly acrylamide Gel Electrophoresis
Sec/Secs	Second/Seconds
SJH	Safdarjung Hospital
SSC	Sodium chloride sodium citrate
TAE	Tris acetate EDTA
Tb	*Trypanosoma brucei*
Tc	*Trypanosoma cruzi*
TEMED	N,N,N’,N’ tetramethyl ethylene diamine

Tris	Tris (hydroxymethyl) amino acid
U	Unit
Uba5	Ufm1 activating enzyme 5
Ubls	Ubiquitin-like modifiers
Ufc1	Ufm1 conjugating enzyme 1
Ufm1	Ubiquitin fold modifier 1
UV	Ultra Violet
VL	Visceral Leishmaniasis
WT	Wild type
X g	Times gravity (centrifugal force)
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
β-ME	Beta mercaptoethanol
μg/μl	Microgram/ Microliter
μM/mM	Micromolar/ Millimolar
%	Percentage
~	Approximately

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Introduction

Introduction

Leishmaniasis, a vector-borne disease that is caused by obligate intra-macrophage protozoa, is endemic in large areas of the tropics, subtropics and the Mediterranean basin. This disease is characterized by both diversity and complexity (Herwaldt, 1999): it is caused by more than 20 leishmanial species and is transmitted to humans by ~30 different species of phlebotomine sandflies (Pearson and Sousa, 1996).

The two major clinical forms of leishmaniasis, cutaneous and visceral, are the result of infection by different species of the parasite. However, in addition to the infecting species, the clinical outcome of leishmaniasis also depends on the immune response of the host (Rivas et al., 2004). Epidemiology, immunopathology and outcome are similarly diverse, since infection occurs in multiple endemic regions, in both children and adults (Herwaldt, 1999; Desjeux, 1999). Environmental risk factors such as massive displacement of populations, urbanization, deforestation, new irrigation plans and individual risk factors such as HIV, malnutrition and genetic susceptibility make leishmaniasis an important public health problem (Desjeux, 2004). Though the most significant public health effects of leishmaniasis are concentrated in developing countries, occasional cases occur in developed countries as well. Visceral leishmaniasis, fatal if not treated, is caused by parasites of *L.donovani* complex comprising of *L.donovani, L.infantum,* and *L.chagasi*. More than 90% of the visceral cases in the world are reported from Bangladesh, Brazil, India and Sudan (Desjeux, 1992). In India, the official estimate of 430,000 VL cases in Bihar state over the past 11 years may represent only a fraction of the real numbers. The actual number is believed to be at least 5 times as great (Monograph, 1991-1996).

Current treatment of leishmaniasis is based on chemotherapy, which is difficult to administer, expensive and becoming ineffective due to the emergence of drug resistance. To date, there is no vaccine against

Leishmania in routine use anywhere in the world though several vaccine preparations are in advanced stages of testing. The current techniques for discovery of new genes that determine parasite survival and the possibility of manipulation of the *Leishmania* genome reinforce the potential of a live attenuated parasite vaccine. Current molecular techniques have led to the development of recombinant antigen vaccines, though they have not protected to the level of live attenuated parasites (Rivier et al., 1999). Attenuated parasites, though effective, have been unacceptable for human use due to the absence of a defined genetic profile and danger of reversion (Handman, 2001; Kedzierski et al., 2007).

During their life cycle, the parasites undergo profound morphological changes. The life cycle of *Leishmania* includes two developmental stages: the extracellular promastigote form, transmitted to the mammalian host by the sand-fly vector, and the amastigote form, adapted to resist and replicate within the threatening environment of the phagolysosomes. This adaptation requires a dynamic process implicating morphological and physiological changes within the parasite (MacFarlane et al., 1990; Turco and Sacks, 1991; Zilberstein and Shapira, 1994; Goyard et al., 2003) that are mainly orchestrated by the differential expression of a variety of genes. The process of promastigote-to-amastigote differentiation can be mimicked in axenic culture by shifting promastigotes from an insect-like (26^0C, pH 7.4) to an intralysosomal-like (37^0C, pH 5.5 and 5% CO_2) environment (Saar et al., 1998; Gupta et al., 2001; Somanna et al., 2002; Debrabant et al., 2004; Barak et al., 2005).

Microarray is a very sensitive technology to discover genes that may be expressed transiently at a critical stage of parasite. This kind of analysis produces data that suggest the coordinate regulation of group of genes, and could shed light on how multiple genes and their products integrate into pathways to determine the functioning of cells and organisms and also improve our knowledge of highly complex networks that cross communicate in hitherto unknown ways both in health and disease.

The goal of the present study was to use Genomic microarray chip to investigate the stage regulated gene expression during the two extreme stages, promastigote and amastigote of *L. donovani* to search for genes that may play a role in parasite virulence. There are hardly any study comparing expression profiling of promastigote with the amastigotes of *L. donovani* which we propose to take up using parasites isolated from the bone marrow aspirates of VL patients with minimum *in vitro* passages. The gene expression changes have generally been studied in axenic amastigotes, which may not reflect the exact position operative in the true amastigotes since axenic amastigotes may have different metabolic processes than intracellular amastigotes.Therefore we propose to carry out a comparative analysis of the gene expression in axenic/hamster derived amastigotes/ bone marrow aspirates of VL patients.

The proposed study will lead to identification of stage-regulated genes of *L.donovani* which may have a role in virulence or in the survival of the parasite. Funtional characterization of selected genes will be undertaken to help in deciphering new pathways in *Leishmania* and unravel novel targets for vaccine/drug development against VL.

Chapter 1

Review of Literature

Review of Literature

Leishmania are obligate intracellular protozoan parasite of the order Kinetoplastida and family Trypanosomatidae. They are the causative agents of leishmaniasis,which is endemic in various tropical and subtropical regions(Herwaldt, 1999a). Around 20 species of *Leishmania* infect humans and 30 phlebotomine sand fly species have been identified as vectors (Desjeux, 2004). Leishmaniasis is a complex disease, which may be classified into four clinical forms:

1. Visceral leishmaniasis (VL), also known as kala azar, causes life-threatening systemic infection. Symptoms include fever, severe weight loss, anaemia, hepatosplenomegaly, pancytopenia and hypergammaglobulinaemia. VL is caused by *L.donovani*, particularly in the Indian subcontinent and Eastern Africa. In the Mediterranean and the New World VL is due to *L.infantum* and *L.chagasi*, which are now generally accepted to be one species (Olliaro et al.,2002).

2. Localized cutaneous leishmaniasis (CL) is the most common form and causes 1-200 skin lesions, generally confined to bite site. It is caused mainly by *L.major* , *L.tropica* and *L.aethiopica* in the Old World and by *L.mexicana* complex, *L.guyanensis* and *L.panamensis* in the New World. The lesions usually self heal within a few months, though they can cause disfiguring scars. It can generally be resolved without recourse to drugs(Desjeux, 2004).

3. Diffuse cutaneous leishmaniasis is a chronic progessive form charecterised by disseminated cutaneous lesions, which are non-ulcerative (Herwaldt, 1999a), due to a defective cell-mediated immune reponse (Desjeux, 2004). The lesions are comparable to those of leprosy and, unlike those of localized CL, do not self-heal. It is caused by *L.aethiopica* in the Old World and *L.mexicana* complex species particularly *L.amazonensis*, in the New World.

4. Mucocutaneous leishmaniasis is a metastatic complication of cutaneous leishmaniasis caused by *L.braziliensis* subspecies. Sores develop on the naso-oropharyngeal mucosal membranes with consequent disfigurement (Herwaldt, 1999a).

Leishmaniasis is endemic in 88 countries worldwide, 66 in the Old World and 22 in the New World (Desjeux et al., 2001), as shown in figure 1.1. The disease burden is very high, with an estimated prevalence of 12 million people infected and 350 million people at risk. There is substantial under-reporting of the number of cases, but it is estimated that there are 1-1.5 million new cases of CL and 500,000 of VL annually (www.who.int/entity/leishmaniasis). Over 90% of cases of VL occur in Bangladesh , Brazil ,India, Nepal and Sudan ; 90% of cases of CL occur in Algeria,Afganisthan,Brazil, Iran,Iraq, Peru, Saudi Arabia and Syria (Herwaldt, 1999a).

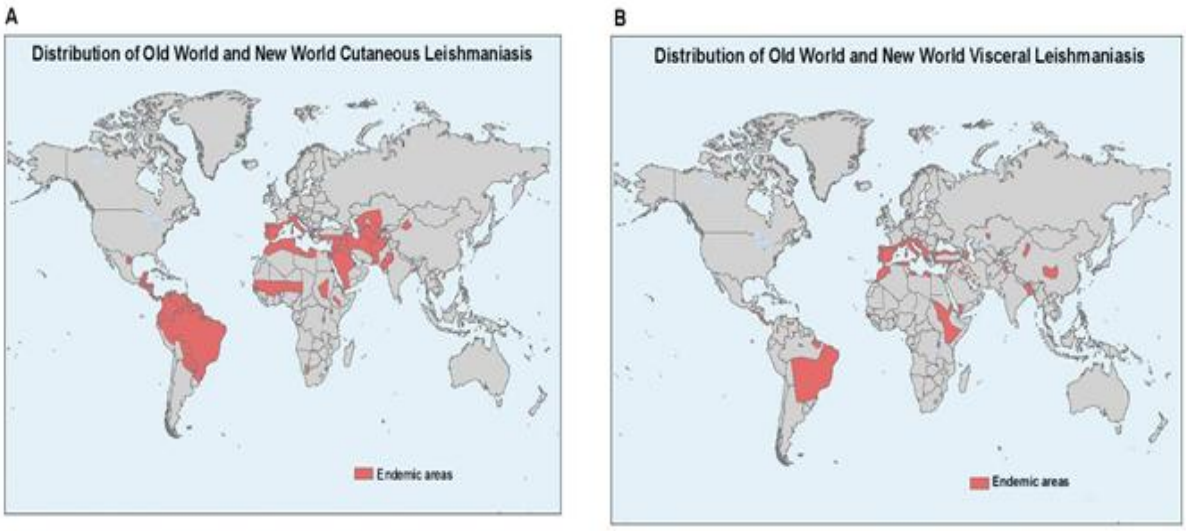


Fig 1.1 Distribution of Cutaneous and Visceral Leishmaniais worldwide.
A= Cutaneous Leishmaniasis, B=Visceral Leishmaniasis
Maps from World Health Organisation (www.who.int/leishmaniasis/leishmaniasis_maps)

Visceral Leishmaniasis

VL or Kala-azar (KA) is endemic in three countries of South East Asia (SEA) Region – Bangladesh, India and Nepal. The disease is now being reported in 45 districts in Bangladesh, 52 in India and 12 in Nepal (Fig. 1.2). The total number of districts reporting Kala-azar exceeds 109. India alone contributes more than 80% of the cases in the SEA Region. In India, states of Bihar and West Bengal are highly endemic foci of KA where periodic epidemics are common (Bora, 1999). The situation is getting worse due to asymptomatic cases, post kala-azar dermal leishmaniasis (PKDL), under nutrition and kala-azar/HIV co-infections. However, the mortality is stable because of improved case management in recent years due to availability of better diagnostic tools (rK39 kits) and oral drug miltefosine. There are inadequacies in reporting since only government agencies are reporting the disease to the programme. The number of cases being reported are increasing and this is probably a reflection of some improvement in the drugs and diagnostic services provided by the government. There is still a large gap between the reported cases and estimated cases.

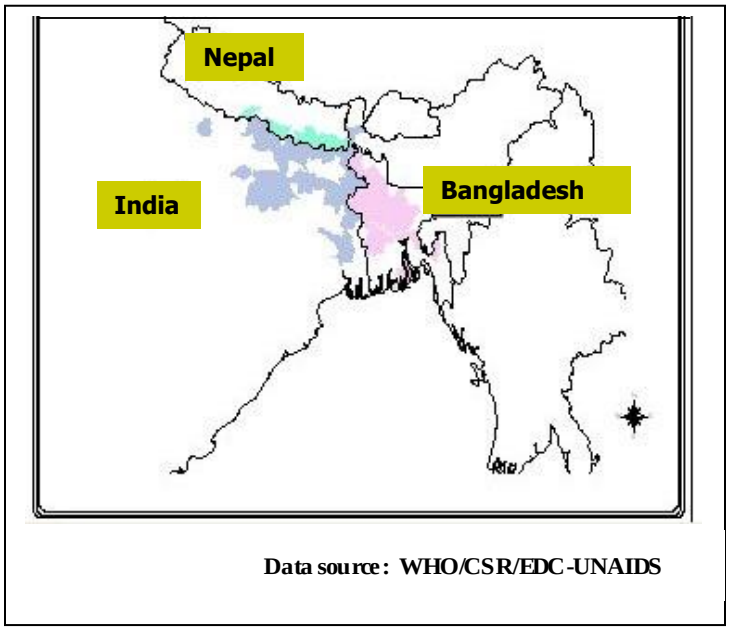


Fig.1.2. Kala-Azar endemic districts of SEA region, 1995-2005

Post kala-azar dermal leishmaniasis (PKDL)

It is a dermatropic form of leishmaniasis developed in 10-15% of apparently cured VL cases, at the same time, cases without any previous known history of VL are also reported (el Hassan et al., 1992). The disease presents as erythema, hypopigmented macules and combination of papules, nodules and plaques, which first appear around the mouth, become denser and slowly spread over the entire body. The interval between the end of treatment of VL and the onset of PKDL is variable (Zijlstra et al., 2003). The disease is relatively common in the Indian subcontinent (India, Nepal, and Bangladesh), East Africa (Sudan, Ethiopia, and Kenya), and China, where the causative agent for VL is *L. donovani* (Leng et al., 1991; Ramesh and Mukherjee, 1995; Mofredj et al., 2002). In the absence of an animal host, cases of PKDL serve as a major reservoir for the leishmanial parasite and play an important part in VL transmission.

Cutaneous Leishmaniasis (CL)

In India, CL is reported primarily in some pockets in the Thar Desert of Rajasthan state, located in the Western part of the country and bordering Pakistan (Lodha et al., 1971; Dogra et al., 1990; Bari and Rahman, 2008). The first evidence for existence of CL was based on clinico-epidemiologic analysis of cases in 1973 during a large-scale out break of the disease in Bikaner (Mohan et al., 1975). More than 2,000 people suffered from this infection; during this time, sporadic cases were detected in villages in the vicinity of the Rajasthan canals (Sharma et al., 1973a; Sharma et al.,1973b). It was reported that Indian desert gerbils, Meriones hurrianae, and dogs were found to be the reservoirs of this infection (Sharma et al.,1973b ; Mohan et al., 1975). There is no authentic information regarding the vector species transmitting CL infection, but a few studies concluded that, in this region, one or both species of the two sand fly, *Phlebotomus*

papatasi and *P. sergenti*, are the vectors. A recent report from Himachal Pradesh, an emerging foci for CL situated in the western Himalayas in northern India, has provided concrete evidence of CL cases in India caused by *L. tropica* and *L. donovani* (Sharma et al., 2005).

Life Cycle of the *Leishmania* parasite

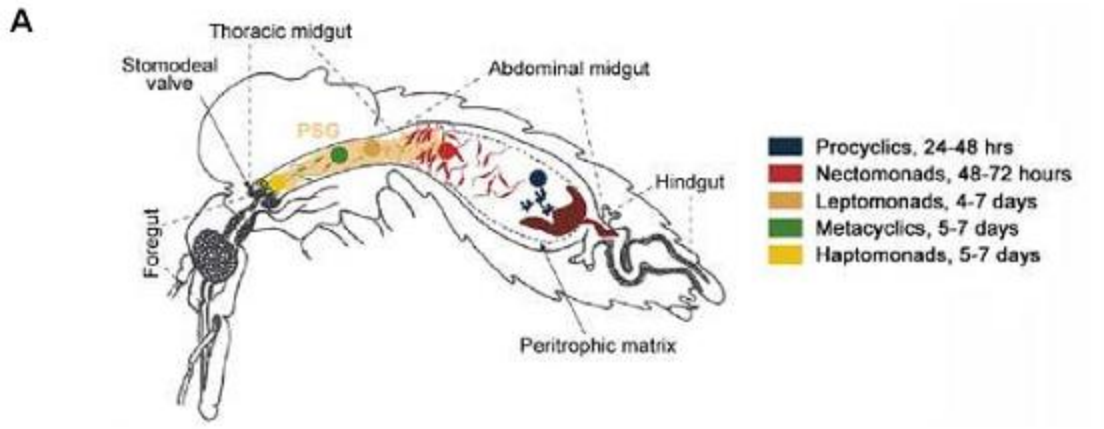
Leishmania alternate between their mammalian host and the digestive tract of female phelbotomine sand flies. Most *Leishmania* species (those of subgenus *Leishmania*) are suprapylarian parasites, restricted to the midgut of the sandfly, but the New World Viannia subgenus parasites, entering the hindgut before moving forward to midgut (Anderson et al., 2006).When a sandfly bites an infected mammal it takes up a bloodmeal containing *Leishmania* amastigotes. These amastigotes must differentiate into promastigotes to survive in the sand fly. Six stages of promastigotes have been described, in which *Leishmania* differentiate sequentially as they move anterially through the midgut (Rogers et al., 2002) . Amastigotes transform into procyclic promastigotes, which then develop into nectomonad promastigotes, then leptomonad promastigotes and finally metacyclic promastigotes, which is the stage infective to mammls. There are also haptomonad promastigotes and paramastigotes, both of them have been found only in low numbers, and none of these stages have definite precursor form (Rogers et al., 2002;Anderson et al., 2006). Only two of the six stages identified have been shown to be actively replicative: i) procyclic promastigotes, in the abdominal midgut, the bloodmeal phase, and ii) leptomonad promastigotes, in the thoracic midgut and foregut, the sugarmeal stage (Gossage et al., 2003). This life cycle is illustarted in Figure 1.3.

The development of *Leishmania* within the sand fly, from amastigote to infective metacyclic promastigotes, has been found to take 6-9 days, depending on the species (Anderson et al., 2006). Metacyclic promastigotes accumulate at the stomodeal valve for transmission. The

thoracic midgut is filled by promastigote secretory gel (PSG) which contains mostly leptomonad and metacyclic promastigotes and which blocks the midgut, forcing open the stomodeal valve (Rogers et al., 2002). The presence of the PSG plug and a heavy infection with *Leishmania* is associated with a reduced ability to take in blood, numerous attempts to feed and thus more chances for transmission. It has been found that around 1000 metacyclic promastigotes are regurgitated into the mammalian host when a sand fly feeds, with most coming from behind the stomodeal valve (Rogers et al., 2004). The PSG plug has also been shown to be regulated into the mammalian host and it has been found that the main component of this, filamentous proteophosphoglycan (fPPG), enhances disease progression (Rogers et al., 2004).

When metacyclic promastigotes are injected into the mammalian host, polymorphonuclear neutrophil granulocytes (PMN) are the first cells recruited to the bite site, appearing within 10-24 hours post infection (Muller et al., 2001). *L.major* promastigotes were found to be chemotactic for neutrophils, attracting them to the bite site(van Zandbergen et al., 2004). *Leishmania* promastigotes are phagocytosed by PMN and inhibit spontaneous apoptosis of the PMN, prolonging the cell lifespan until the point when macrophages are recruited to the bite site, normally within 1-2 days after infection. Macrophages then phagocytose the infected PMN (Aga et al., 2002). This has been suggested to be a "Trojan horse" by which the parasite can infect the macrophage silently, without activating the antimicrobial functions of the macrophage (Laskay et al., 2003). PMN can be infected by promastigote *Leishmania*, but the parasites stay in this form: and do not transform into an amastigote, the mammalian replicative stage of the parasite (van Zandbergen et al., 2004), do not multiply (Laskay et al., 2003), until they are within the parasitophorous vacuole (PV) of macrophage. Amastigotes are aflagellate and round-oval in shape with a diameter of approximately 4µm (Herwaldt, 1999a). Amastigotes

reside within PVs within the macrophage and these vacuoles differ depending on the species of *Leishmania*: they can be tightly or loosely fitted around the amastigotes, and one vacuole can harbor a single amastigote or multiple amastigotes (Rittig and Bogdan, 2000). For example, *L.mexicana* and *L.amazonensis* PVs are large and contain many amastigotes, whilst *L.major*, *L.infantum* and *L.donovani* produce only small vacuoles which fit closely around the amastigotes (Handman and Bullen, 2002), as illustrated in Figure 1.4. The amastigotes multiply within the PV and are released to infect further cells. The mechanism of the release is not fully characterized. The macrophage was presumed to burst due to the physical pressure of large numbers of amastigotes, but recent evidence suggests that the parasite may induce the exocytosis machinery of the macrophage to enable release (Rittig and Bogdan, 2000).



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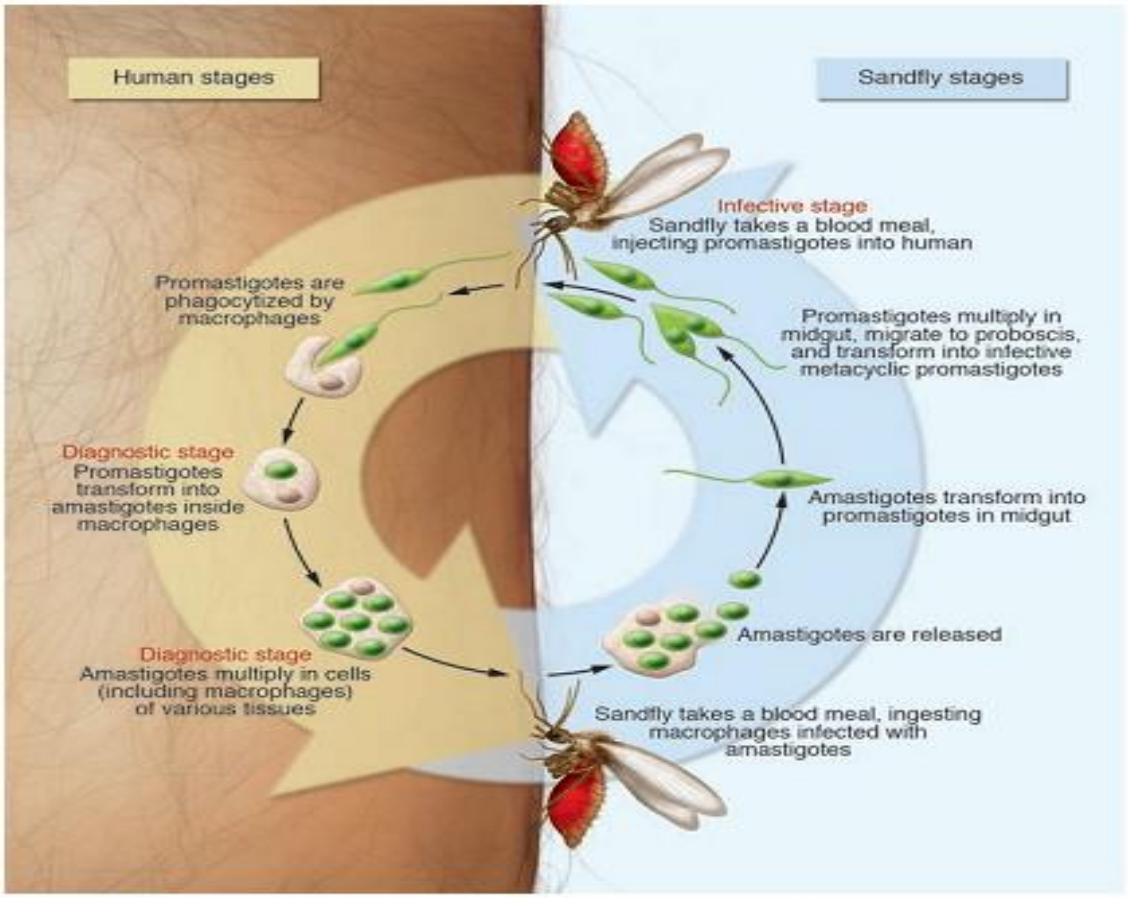

Figure 1.3 Life cycle of *Leishmania*

A= Schematic representation of a *Leishmania* infected phlebotomine sand fly, illustrating time dependent appearance of distinct morphological forms of promastigotes within the midgut. Thoracic midgut is filled with promastigote secretory gel (PSG) and the stomodeal valve is held open by this PSG. (From (Anderson et al.,2006)).
B= Life cycle of *Leishmania* spp. Infection with different species of *Leishmania* causes distinct forms of leishmaniasis. Sandflies are the vectors that transmit the disease-causing protozoan parasites, injecting infective promastigotes when they take a blood meal. The parasites invade mammalian macrophages by receptor-mediated endocytosis, where they transform into amastigotes that multiply by binary fission. Sandflies become infected by ingesting infected cells when they take a meal of parasite-containing blood from an infected human or animal. The amastigotes transform into promastigotes and develop in the gut into metacyclic promastigotes that are infective to humans. (From (Stuart et al, 2008).

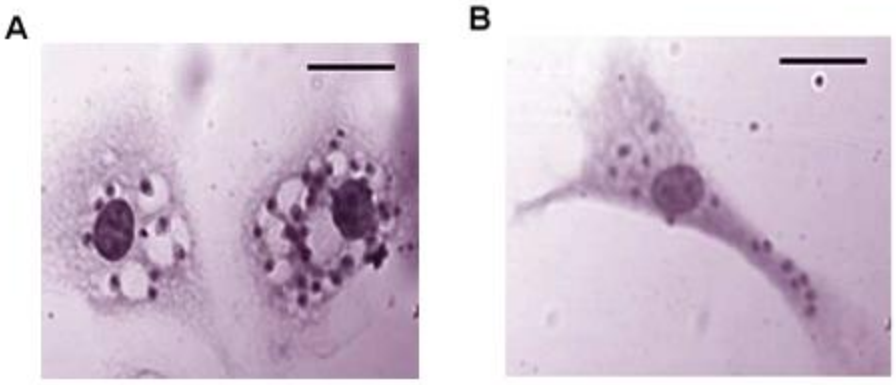

Figure 1.4 Amastigotes within parasitophorous vacuoles in mouse peritoneal exudate macrophages. A= PEM infected with *L.mexicana*, B=PEM infected with *L.infantum*. Infected PEM were stained with Giemsa's stain. False colour image. Scale=10µm.

Ultrastructure of *Leishmania*

The ultrastructure of *Leishmania* differs from that of other kinetoplastids and also shows variation between the different life cycle stages of the parasite (Clayton et al., 1995). The various forms of the *Leishmania* life cycle were initially characterized according to their dimensions; flagellar morphology, kinetoplast (mitochondrial DNA) position and the relative distance from the nucleus to the basal body of the flagellum (Clayton et al., 1995). The characteristic shapes of the stages are maintained by an array of cross-linked subpellicular microtubules underneath the plasma membrane (McConville et al., 2002).

The ultrastructure of the promastigote and amastigote stages of *Leishmania* is illustrated in Figure 1.4. Promastigotes are motile cells with long thin bodies, of size up to 5 µm by 20 µm, with a flagellum up to 20 µm, while amastigotes are non-motile ovoid cells of approximately 4 µm diameter with only a remnant flagellum.

Specialised Organelles

Leishmania contain many of the organelles found in higher eukaryotes, but they also contain a number of special organelles. The surface membrane of *Leishmania* is covered by a distinct glycocalyx made of glycolipids. This glycocalyx is thick on promastigote stages and thin on amastigotes and is thought to enable survival of the promastigote in the sand fly (Turco et al., 2001). The main surface molecule is the hyperglycosylated form of glycoinositol-phospholipid (GPI), lipophosphoglycan (LPG). GPI-linked proteins, including the GPI-linked surface metallopeptidase GP63 and proteophosphoglycans (PPGs) are also present on the surface of *Leishmania* (McConville et al., 2002). The role of LPG in virulence in mammalian hosts is dependent on the species. LPG is essential for the survival of *L.major* and *L.donovani* promastigotes in the sand fly (Sacks et al., 2000) and for *L.major* infection of macrophages and mice (Spath et al., 2000). However, *L.mexicana* parasites lacking LPG are still infective to macrophages and mice (Ilg et al., 2001). The role of all surface molecules containing phosphoglycan (PG) has also been investigated in *L.major*, with a lack of PGs on promastigotes confirmed to lead to a defect in macrophage infectivity. However, there was no effect on the infectivity of amastigotes(Capul et al., 2007). This is probably due to amastigotes having a much reduced glycocalyx, lacking GPI-anchored macromolecules, having instead a surface layer of free GPIs and host –derived glycophospholipids (McConville et al., 2002).

Both promastigotes and amastigotes secrete a large quantity of soluble PPG. They form, individually or after self association, large filamentous structures. PPG structures may lead to promastigote aggregation and the transmission of large clusters of promastigotes in the sand fly bite (McConville et al., 2002), with PPG known to be the main component of the PSG plug in

the sand fly, which, as stated earlier, is regurgitated into the mammalian host and enhances disease progression (Rogers et al., 2004).

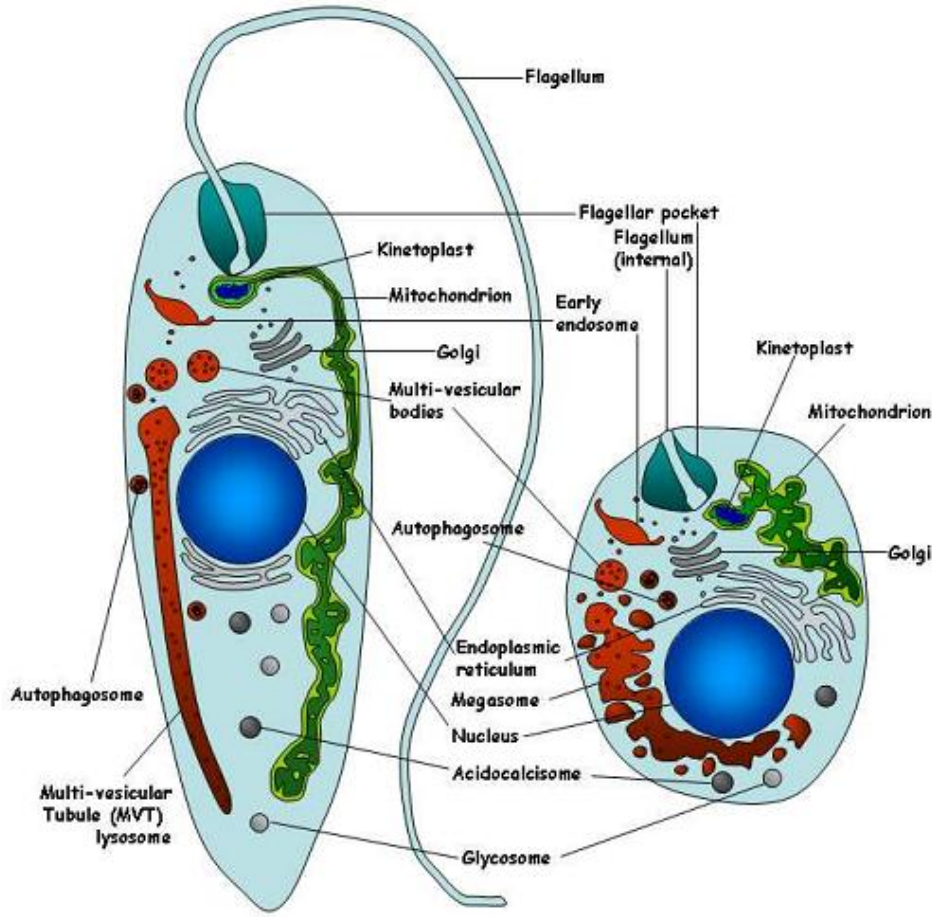

Figure 1.5 Schematic representation of the main organelles of *Leishmania* in both promastigote and amastigote form. Promastigote on the left and amastigote on the right. The flagellar pocket is at the anterior end of the cell. Taken from Besterio et al., 2007.

The subpellicular microtubules below the cell membrane form the cytoskeleton and restrict vesicular transport to the only region of the cell not subtended by the microtubules: the flagellar pocket at the anterior end of the cell (Clayton et al., 1995). The flagellar pocket is an invagination of the plasma membrane, with the junction between the cell membrane and the

flagellum being formed of desmosome-like tight junctions (de Souza, 2002). The flagellar pocket is the single location for endo- and exo-cytosis for integration of membrane proteins into the cell membrane, and is accessible to large macromolecules(Landfear and Ignatushchenko, 2001), for example secreted LPG and PPG, and endocytosed MHC class II molecules(De Souza et al., 1995).

The flagellum contains a classical 9+2 microtubule doublet axoneme as well as the trypanosomatid – specific paraflagellar rod (Bastin et al., 2000). The flagellum is thought to have a number of functions. It is responsible for the motility of *Leishmania* promastigote, and is known to mediate attachment of *Leishmania* to the sand fly gut epithelium (Killick-Kendrick et al., 1974a; Killick-Kendrick et al., 1974b). It is also postulated to act as an environmental sensor and to be involved in signaling (Bastin et al., 2000). The amastigote stage has a much shortened flagellum which does not emerge from the flagellar pocket, making this stage immotile.The flagellum of *Leishmania* is anchored into the cell by a basal body, which is physically linked to the mitochondrion. A second basal body, lying perpendicular to the major one, develops during cell divison. In *T.brucei*, the basal bodies are necessary for kinetoplast division (Ogbadoyi et al., 2003), whilst in *L.donovani* duplication and segregation of kinetoplasts have been observed despite a lack of basal body duplication (Selvapandiyan et al., 2004).

The kinetoplast is a disc shaped structure, comprising the condensed mitochondrial DNA (Liu et al., 2005). The kinetoplast DNA (kDNA) represents 10-20% of the total cell DNA and is composed of a few dozen maxicircles and thousands of minicircles (Stuart, 1983). The maxicircles have some of the functions of higher eukaryote mitochondrial DNA, encoding genes for the respiratory complexes. The minicircles are responsible for the network structure of kDNA (Stuart, 1983) and encode guide RNAs which mediate the editing of the maxicircle transcripts to

form functional mRNA, by insertion of uridylate residues to precise sites (Liu et al., 2005). These filaments constitute the tripartite attachment complex (TAC), which may mediate interactions between the mitochondrion and the cytoskeleton (Ogbadoyi et al., 2003). It has been postulated that the TAC is present in *Leishmania* and the other *Trypanosomatids* (Ogbadoyi et al., 2003), though this has not been confirmed.

In *Leishmania* there is a single mitochondrion which extends throughout the cell. In dividing promastigotes the mitochondrion is a symmetrical organelle, extending from both sides of the kinetoplast region and joined at the posterior end of the cell. In contrast, in non-dividing promastigotes the mitochondrion is asymmetric, extending from one end of the kinetoplast past the nucleus to the cell posterior (Simpson and Kretzer, 1997), as shown in Figure 1.5. In amastigotes the mitochondrion is in the form of a complex network extending throughout the cell (Coombs et al., 1986). In addition to the mitochondrion, *Leishmania* produce energy within specialized organelles called glycosomes, which contain the glycolytic enzymes responsible for converting glucose to 3-phosphoglycerate (De Souza, 2002). Glycosomes are small, spherical organelles, belonging to the peroxisome family (Michels et al., 2006). *Leishmania* also contain acidocalcisomes, spherical organelles preferentially located at the periphery of the cell, which are believed to be storage organelles for a number of elements including phosphorus, calcium, magnesium, sodium and zinc (De Souza, 2002).

As with other eukaryotes, *Leishmania* contain a single Golgi apparatus, located between the nucleus and the flagellar pocket and consisting of a stack of 3 to 10 cisternae and a polymorphic trans-Golgi network. The endoplasmic reticulum (ER) consists of the nuclear envelope and a system of cisternae that are often closely associated with the plasma membrane (McConville et al., 2002). The ER provides some of the membranes necessary for the

biosynthesis of endosomes and lysosomes. In promastigotes the endocytic pathway has been shown to consist of three parts :i) a network of tubular endosomes close to the flagellar pocket, ii) a population of multivesicular bodies also in the flagellar pocket region of the cell, and iii) an unusual multivesicular tubule (MVT) running along the anterior –posterior axis of the cell (Waller and McConville, 2002). The MVT is the terminal compartment of the endocytic pathway, forming the mature lysosome and so is known as the MVT-lysosome (Ghedin et al., 2001; Mullin et al., 2001;McConville et al., 2002).The lytic capacity of the MVT increases as the promastigotes reach the stationary phase of growth (Mullin et al., 2001). In *Leishmania* amastigotes, there is an extended lysosomal compartment comprising one or more large vesicles that have been termed megasomes, which were originally found only in *L.mexicana* group species (Pupkis et al., 1986; Ueda-Nakamura et al., 2001), but have recently been identified in *L.chagasi* amastigotes (Alberio et al., 2004).

The lysosome of *Leishmania* is postulated to have a number of functions. For example, it may be involved in nutrient acquisition, evasion of the immune system and regulating the levels of endogenous proteins (Waller and McConville, 2002). Lesion amastigotes have been found to degrade host macromolecules (Schaible et al., 1999) and to influence the immune system by degrading host MHC II complexes, thus governing the range of peptides which can associate with MHC molecules.

Leishmania genome organization

The *Leishmani*a genome is a relatively small eukaryotic genome with an estimated size of $3.5X10^7$ bp. Old World *Leishmania* (*L. donovani* and *L. major* groups) have 36 chromosome pairs (0.28 to 2.8 Mb)(Wincker et al., 1996), whereas New World species have 34 or 35, with chromosomes 8+29 and 20+36 fused in the *L. mexicana* group and 20+34 in the *L. braziliensis*

group (Britto et al., 1998) and possess repetitive telomeric sequences which do not condense during the mitotic cycle. Gene order and sequence are highly conserved among the ~30 *Leishmania* species (Ravel et al., 1999). The *Leishmania* genome differs from the typical eukaryotic genome. Variations in the relative sizes of homologous chromosomes have been reported in the Old World species of *Leishmania* (Britto et al., 1998). The chromosomal organization of *Leishmania* is similar to many protozoan parasites; a compartmentalization into conserved core domains and polymorphic chromosome ends (Lanzer et al., 1995). In terms of structure and maintenance of chromosomal termini, *Leishmania* conforms to those described in other eukaryotes. Telomerase activity, the activity of the ribonucleoprotein enzyme complex responsible for addition of deoxyribonucleotide triphosphate to the 3' ends of chromosomal strands, has been demonstrated in *Leishmania* with primer recognition and elongation properties similar to those of other eukaryotes (Cano et al., 1999).

Leishmania were found to be more G/C rich (58%) than *T. brucei* (51%) or *T. cruzi* (44%) (Alonso et al., 1992). Further, coding regions had a higher G/C content than non-coding regions (NCR) and 3' NCR were more G/C rich than 5' NCR. It was speculated that the high G/C content of *Leishmania* might be a reflection of the more primitive nature of these organisms. Moreover, *Leishmania* were found to share a similar base-utilization scheme at all three codon positions. Within a codon, there is a strong preference (about 85%) for G or C in the third, or 'wobble', position of *Leishmania* amino-acid codons, a slight A/T bias (about 55%) in codon position 2 and a G/C bias (about 60%) in codon position 1 (Alvarez et al., 1994).

The genome sequence of *L. major* released in July, 2005 were obtained by shotgun sequencing large-insert clones and purified chromosomal DNA (Ivens et al., 2005). A single contiguous sequence was generated for each of the 36 chromosomes although the "right" end of

chromosome 8 lacks a small amount of sub-telomeric sequence and telomeric hexamer repeats. Although the genome is partially aneuploid (Sunkin et al., 2000) and there are three large scale allelic differences, there are very few (<0.1%) sequence polymorphisms.

Analysis of the *L. major* sequence using several algorithms predicts 896 RNA genes, 71 pseudogenes, and 8370 protein coding genes, of which 3083 cluster into 662 putative families of related genes. Most of the smaller (<10 members) gene families appear to have arisen from tandem gene duplication, whereas most members of larger (>10 members) families have multiple loci containing single genes and/or tandem arrays; many of the latter contain *Leishmania*-specific genes. Out of ~8379 genes, 310 are experimentally characterized, the identity of 2610 genes is inferred from homology to other databases, 4674 genes code are conserved hypothetical proteins, 690 sequences are orphan meaning no predicted function can be attributed to them and 71 genes are termed as pseudogenes and have premature stop codons and/or frame shifts. The *L. infantum* genome contains 8184 genes present on 36 chromosomes (last update March 2008) and *L. braziliensis* has 8312 genes present on 35 chromosomes (last update Jan 2007). Full annotations of the genes of both species are under processing. The difference in chromosome number is due to the fusion of chromosomes 20 and 34 to make a single chromosome in *Leishmania braziliensis*.

Comparison of the genomes of *L. major, L. infantum* and *L. braziliensis*

From an evolutionary perspective, phylogenetic analyses have suggested a neotropical origin for the *Leishmania* genus (Stevens et al., 2001) and, while there has been some controversy in this designation (Kerr, 2000), this has been largely resolved in a recent multifactorial genetic study (Lukes et al., 2007). Irrespective of this debate, *L. braziliensis* is the most genetically and biologically divergent of the three sequenced species. Divergence between

the *Leishmania* species complexes is estimated to have occurred 15–50 million years ago (Lukes et al., 2007), within the same range as two potential host species, mouse and human. Given this period of isolation, it was expected that there would be significant differences in both genome architecture and gene repertoire between *L. braziliensis*, *L. infantum* and *L. major*. Indeed, while the genomes have a similar DNA content of around 33 Mb, karyotypic differences had already been identified by linkage group analysis (Britto et al., 1998): *L. major* and *L. infantum*, in common with other Old World species, have a haploid content of 36 chromosomes, while the New World species have either 35 (*L. braziliensis* complex) or 34 (*L. mexicana* complex). Surprisingly, comparison of the respective orthologous chromosomes has revealed remarkable conservation of both gene content and gene order in all three genome species. Despite the differences in gene copy number within some of the major protein-coding families described above, not a single chromosomal re-arrangement has been identified between *L. major* and *L. infantum* across the whole genome, while *L. braziliensis* has only a few possible sequence re-arrangements (Peacock et al., 2007). Equally surprising, from the total content of 8,300 genes in each species, only 200 can be identified as differentially distributed between the three genomes. The most divergent, *L. braziliensis*, possesses 47 genes that are absent from the other two species. In comparison, *L. major* has 27 species-specific genes while *L. infantum* has only five. A number of the other differentially distributed genes are found in two out of the three species. Some of these species-specific sequences have already been analyzed at the molecular level. Examples include the *L. major* A2 gene that encodes an amastigote-specific repeat-containing protein previously characterized in *L. donovani*, the only *Leishmania* sequence to date that confers a change in virulence phenotype when introduced into *L. major* by genetic transfection (Zhang et al., 2003); and the HASP and SHERP genes, expressed from a single locus (absent in

L. braziliensis) in infective stages of *L. major* and *L. infantum*, with their protein products localizing to the plasma membrane and intracellular membranes, respectively, in these species (Denny et al., 2000; Knuepfer et al., 2001). In the Tritryp genome analyses, most genes specific to each of the representative species were found either at the ends of the DGCs or in the sub-telomeric regions of the chromosomes, regions that appear to be more tolerant to genome re-arrangement. However, comparison of the three *Leishmania* genomes has revealed that gene variation is not predominantly restricted to the sub-telomeric regions or even the SSRs but is evenly distributed across the genome (Peacock et al., 2007). *Leishmania* is also distinctive from other eukaryotes in the apparent mechanism by which species-specific gene variation occurs. Whereas insertions/deletions and sequence re-arrangements play major roles in gene diversification in most other eukaryotes characterized to date, degeneration of existing genes (leading to probable loss of function) accounts for 80% of the species differences in *Leishmania*. These degenerate sequences have in-frame stop codons and frame shifts, generating truncated open reading frames that are presumably not translated. One example is the gene encoding cysteine peptidase Pfp1, which is present as an intact gene and translated in *L. major* (Eschenlauer et al., 2006). However, there are five in-frame stop codons and a frame shift in the *L. major* orthologue, while the syntenic region in *L. braziliensis* is even more degenerate. Pfp1 like some of the other species-specific genes appears to be another candidate for lateral gene transfer from bacteria. Of the remaining species-specific sequences not caused by loss of function, many also fall into this category. One example is the cyclopropane fatty acyl synthase (CFAS) gene, present in *L. infantum* and *L. braziliensis* but absent from *L. major*. Acquisition of novel genes in this way may be a mechanism for environmental adaptation to promote survival; similar adaptations to stress or other stimuli may lead to the redundancy of other sequences

clearly identified as pseudogenes in the *Leishmania* genomes (Peacock et al., 2007). In the case of CFAS, acquisition of this gene may have an impact on parasite survival in the host, since the CFAS orthologue in *Mycobacterium tuberculosis* is associated with increased virulence and persistence, functions that apparently require cyclopropanation of a mycolic acid substrate in the bacterial cell wall (Aruna et al., 2005). Despite its chromosomal plasticity (Martinez-Calvillo et al., 2005), the incredible conservation of synteny revealed by comparative genomic analyses of these three species suggests that the *Leishmania* genome is highly stable and has not undergone major genomic re-arrangements during speciation. One contributing factor to this stability could be a lack of mobile DNA elements, as originally demonstrated in the *L. major* genome and verified in *L.infantum* (Bringaud et al., 2006). The comparative sequencing project has revealed some surprising observations, however, one of the most striking being the presence of transposable elements in *L.braziliensis*.

***In vitro* differentiation of promastigotes-to-amastigotes**

To study these parasites, promastigotes and amastigotes have been cultured under different *in vitro* laboratory conditions and have been the subject of numerous biological and biochemical studies. Studies undertaken on *in vitro* cultured stages of *Leishmania* suggest that two environmental factors are sufficient to induce differentiation of promastigotes to amastigote-like forms (axenic amastigotes); a mild rise in temperature to 33–37^{o}C and decrease in pH to 5.5, conditions that mimic the environment in the macrophage phagolysosome (Porter-Kelley et al., 2004). However, not all species of *Leishmania* can be induced to differentiate with these stimuli, and other factors (such as opsonization with host serum components (Bee et al., 2001) may be required for differentiation *in vivo*. Very little is known about how these external signals are perceived by *Leishmania*, or how they are transmitted to down-stream targets responsible for

differentiation. In fact, no signal transduction pathways have been fully elucidated in any of the trypanosomatid parasites. While it is possible that *Leishmania* differentiation is triggered by the activation of specific cell surface receptors/channels or kinases, analysis of the *L. major* genome have not revealed any candidate proteins (i.e. G-protein coupled receptors, receptor tyrosine kinases). Moreover, pharmacological agents that induce protein misfolding and/or the promastigote heat shock response can trigger promastigote-amastigote differentiation *in vitro*, indicating that differentiation signals could originate in the cytosol (Wiesgigl and Clos, 2001; Barak et al., 2005). The next challenge is to identify how these stress responses are transmitted to other down-stream targets that regulate the biogenesis of different organelles and cellular metabolism. In other eukaryotes, the mitogen-activated protein (MAP) kinase pathway plays a key role in regulating cellular responses to various stresses and nutrient signals. Several of the *L. mexicana* MAP kinases have been shown to be important for parasite growth in rich medium (i.e. LmMPK4, LmMPK2) or lesion development in animal models (LmMPK1 and LmMPK5) indicating critical roles in normal growth and stress responses (Wiese, 2007). A surprising number of these kinases are also involved in modulating flagellum length (LmMPK9, LmMPK13, LmMPK3), which varies enormously in promastigote and amastigote stages. Whether these kinases are directly regulating the intra-flagellum transport machinery or modulating other cellular processes that impact on flagellum length remains to be defined. A major challenge now is to define further up-stream kinases and signals, as well as down-stream targets of these signalling cascades. Increased protein turnover and degradation is likely to be a particularly important process in differentiation, given the dramatic remodelling of the endomembrane system and contraction in cell size that accompanies promastigote-amastigote differentiation. Besteiro et al. (2007) describe the major proteolytic systems in *Leishmania*, and

highlight recent studies on the possible role of autophagy in parasite differentiation and nutrition. This group has recently shown that autophagy is markedly increased in stationary phase promastigotes and is required for metacyclogenesis (the transition to a mammalian-infective promastigote stage) and subsequent differentiation to amastigotes. It will be of interest to determine whether autophagy is also essential for ongoing survival of amastigotes in the macrophage, and the extent to which protein turnover via the proteosome and autophagy have so overlapping or complementary roles in the amastigotes.

Interaction between *Leishmania* and Host Cells

Invasion of macrophages

The entry of *Leishmania* into macrophages is thought to involve both silent entry through the "Trojan Horse" of infected neutrophils, and direct entry into macrophages. The binding of *Leishmania* promastigotes to macrophages is thought to be primarily through the complement receptors type 1 (CR1) and type 3 (CR3), which bind to complement components attached to molecules on the parasite plasma membrane (Antoine et al., 1998). The CR1 binds the C3b fragment of complement and CR3 binds iC3b, with the latter thought to be the more important (Mosser and Rosenthal, 1993). Bound iC3b on the parasite surface prevents complement lysis, with GP63 on the parasite surface being responsible for rapid conversion of C3b to iC3b (Cunningham, 2002; Denkers and Butcher, 2005). Amastigote binding to macrophages is not dependant on serum and involves different receptors than that of promastigotes (Mosser and Rosenthal, 1993).

Leishmania are thought to be taken up by conventional phagocytosis, with elongated pseudopodia advancing tunnel-like along the parasites(Rittig and Bogdan, 2000), into a phagosome (Bogdan and Rollinghoff, 1999). When promastigotes are phagocytosed, LPG on the

parasite surface inhibits phagosome endosome fusion (Desjardins and Descoteaux, 1997;Dermine et al., 2000). Since promastigotes are acid labile but amastigotes are not (Bogdan and Rollinghoff, 1999), delaying the development of this organelle promotes survival, allowing promastigote-amastigote differentiation to be completed (Bogdan and Rollinghoff, 1999). Amastigote survival in the acidic PV is thought to be due to the protective effects of the abundance of cell surface and secreted glycoconjugates produced by this stage (Sacks and Sher, 2002).

The phagolysosome has features of lysosomal compartments, being positive for the lysosomal–associated membrane proteins LAMP-1 and LAMP-2, having acidic pH and containing acidic hydrolases normally found in lysosomes (Russell et al., 1992). They are seen as being a mixed organelle, also having features of late endosomes, since they contain the late – endosome associated proteins rab7p and macrosialin (Lang et al., 1994; Courret et al., 2002). Fusion with endocytic organelles is believed to occur in a sequential manner, with endosomes fusing prior to lysosomes (Duclos and Desjardins, 2000). A kiss and run" fusion hypothesis has been proposed for the biogenesis of phagosomes, with organelles joining their plasma membranes to form a fusion pore, which allows exchange of molecules between organelles; the pore then closes and the organelles separate (Duclos and Desjardins, 2000). This process would allow gradual acquisition of molecules and size selection based on the size of the pore.

Survival of *Leishmania*

In order to survive within macrophages *Leishmania* must achieve silent infection, avoiding activating the anti-microbial respiratory burst and evading immune responses (Bogdan and Rollinghoff, 1999). Induction of a Th1 immune response is associated with clearance of *Leishmania* infection, whilst a Th2 response leads to persistence. The regulatory cytokines TGF-

β and IL-10 have also been suggested as important as immunosuppressive signaling molecules in *Leishmania* infections (Olivier et al., 2005). The release of both cytokines has been found to be induced by infection of macrophages by *Leishmania* and however in mice constitutive expression of IL-10 cannot control *Leishmania* infection (Olivier et al., 2005). *Leishmania* have also been shown to inhibit production of the Th1 response promoting cytokines IL-1, IL-12 and TNF-α (Bogdan and Rollinghoff, 1999; Olivier et al., 2005). Recent *in vivo* studies using *L.major* and *L.donovani* have found that pro-inflammatory cytokines were actually induced in early infection, with the induction being higher in *L.major* infections, suggesting one possible reason for the differences in pathology associated with the two species (Olivier et al., 2005). An analysis of the genes expressed by macrophages following infection by *L.chagasi* has also revealed a general trend for genes encoding Th1-type responses to be down-modulated, whilst genes involved in an anti-inflammatory, Th2-type response were up regulated (Rodriguez et al., 2004). *Leishmania* thus modulate the immune system by down-regulating Th1 response and up-regulating Th2 responses.

The primary receptor used for uptake of *Leishmania* into macrophages is thought to be protective for the parasite, as uptake of complement opsonised *L.major* via CR3 leads to inhibition of IL-12 release, while uptake via other receptors induces IL-12 release (Schonlau et al., 2000). Uptake of *L.major* or *L.mexicana* amastigotes also suppresses IL-12 production, though this is not dependent on opsonisation by complement (Weinheber et al., 1998). IL-12 is a Th1 response initiating cytokine which activates natural killer (NK) cells, which in turn produce interferon (IFN)-γ which activates macrophages and is important for early control of infection (McMahon-Pratt and Alexander, 2004).

Binding of complement by *L.major* and *L.donovani* has also been found to increase parasite survival by leading to a reduction of the respiratory burst of macrophages when complement-opsonised promastigotes were phagocytosed (Mosser and Rosenthal, 1993). IFN-γ induces macrophages to produce inducible nitrogen oxide synthase (iNOS) and nitric oxide (NO) production, which are known to be effective at killing *Leishmania*(Olivier et al., 2005). *L.major* has been found to interfere with the induction of iNOS, and purified LPG alone is able to suppress iNOS expression and NO production (Proudfoot et al., 1996). The inhibition of iNOS has been shown to involve abnormal protein kinase C activity (Olivier et al., 2005).

Nutrient acquisition by *Leishmania*

To survive within their host cell, *Leishmania* must acquire nutrient sources from the macrophage. A number of ligands have been shown to be taken up by endocytosis and delivered to the parasitophorous vacuole (PV) through the endosomal system, with increased efficiency of uptake with increasing age of infection (Russell et al., 1992). The ligands were also detected within the flagellar pocket and megasomes of amastigotes, illustrating that *Leishmania* can take up molecules delivered from the endosomal system.

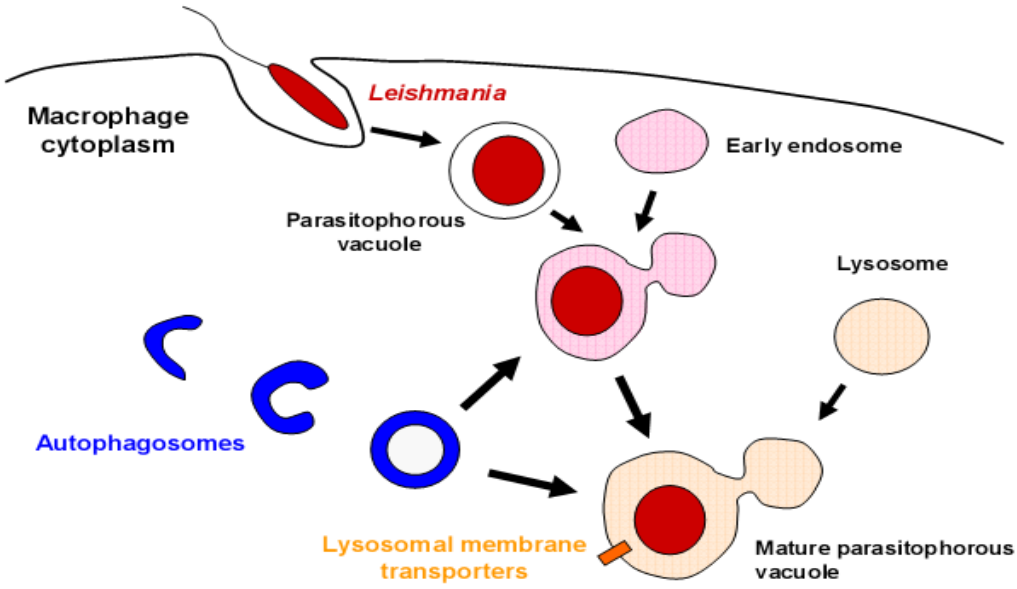

Fig 1.6 Nutrient acquisition by *Leishmania* via interaction with the endosomal and autophagosomal systems of the macrophage and lysosomal membrane transporters. (Adapted from Duclos and Desjardins, 2000).

As well as molecules delivered to the PV via endocytosis, molecules from the host cell cytosol are delivered to the PV. Two mechanisms have been demonstrated whereby molecules can be delivered from the cytosol firstly via an organic anion transporter on the vacuole membrane, and secondly via the autophagic pathway (Schaible et al., 1999). Autophagy is a self-digestion process whereby cells degrade proteins and organelles in the cytoplasm, by sequestration into a double-membrane vesicle, which normally fuses with the lysosome to allow the degradation of the cargo by hydrolases (Reggiori and Klionsky, 2005). Delivery of molecules to the PV via autophagy may provide nutrients to *Leishmania* in addition to those delivered via fusion with endosomes (Schaible et al., 1999), particularly purines which *Leishmania* are unable to synthesize (Hassan and Coombs, 1985). Although there is no evidence of other transporters in the PV membrane, a number of other transporters have been identified on lysosomal membranes (Pisoni and Thoene, 1991) and it is likely that these would also be found on the PV membrane. If present, these would allow the movement of monosaccharide's, nucleosides, amino acids, sulphate and phosphate from the cytosol into the PV(Burchmore and Barrett, 2001). The various methods of nutrient acquisition by the PV are illustrated in Figure 1.6.The hydrolases present in the PV ensure that most macromolecules delivered to this organelle are rapidly degraded to low molecular weight solutes (Burchmore and Barrett, 2001). These solutes ought to be ideal nutrient sources for *Leishmania*, with promastigotes being able to use fatty acids, amino acids and glucose as carbon sources (Hart and Coombs, 1982) and amastigotes taking up glucose, proline and polyamines through transporters with acidic pH optima (Burchmore and Barrett, 2001). A number of proteins characterized as peptidases or involved in proteolysis were detected in an analysis of the *L.donovani* of host proteins for the

nutrient needs of the parasite. Several *Leishmania* peptidases have been identified as potential virulence factors for survival in the host and this secretion into the PV may be an additional survival mechanism (Silverman et al., 2008).

Virulence factors in *Leishmania*

Virulence (defined as the capacity of a pathogen to proliferate and induce disease) is illustrated by the severity of clinical manifestations, which vary from localized, self-healing cutaneous lesions to diffuse cutaneous diseases (e.g. disease caused by *L. amazonensis, L. braziliensis, L. major, L. mexicana* and *L. tropica*) and from asymptomatic infection to fatal visceral dissemination (e.g. disease caused by *L. donovani* and *L. infantum*). Both host and parasite-specific factors contribute to virulence. A molecule is classified as a virulence factor if its absence results in an avirulent or attenuated phenotype and if its re-expression restores virulence (Turco et al., 2001). In these aspects, *Leishmania* determinants are considered as the driving force of virulent phenotype. Host and vector determinants are undoubtedly involved, but they play a secondary or passive role in natural conditions.A number of *Leishmania* antigens elicit antibody response, often at high titers, in kala-azar patients (Requena et al., 2000). Fig. 1.7 summarizes a few pathoantigenic determinants identified till now.

Unique epitopes in conserved protein complex:

- Cytoskeleton, e. g. kinesin, tubulins
- Chaperones, e. g. HSP 60, 70, 83 & STI 1
- Ribosomes, e. g. eIF, PO, P2a, b
- Nucleosomes, e. g. H2A/B, H3, H4
- Glycosomes, e. g. TPI
- Spliceosomes ?
- Proteosomes ?

Requena, Alonso, Soto (2000) *Parasitol. Today* 16, 246-50

Fig 1.7 Some *Leishmania* pathoantigenic determinants proposed to cause immunopathology manifested as the clinical symptoms in leishmaniasis. The molecules listed have been found to contain immunogenic B-cell epitopes.

These *Leishmania* antigens are identified by Western blot analysis and/or by immunoscreening of *Leishmania* expression libraries with patients' sera. Another striking feature of those listed in above figure is that they are all conserved structural or soluble cytoplasmic proteins, which are often complexed with other molecules to form subcellular particles. Although some of them, e.g. histones and heat shock proteins, are seemingly shared with those found in autoimmune diseases, they are not cross-reactive. Epitope-mapping reveals unique *Leishmania* sequences, which are recognized only by sera from patients with kala-azar (Requena et al., 2000). One example worth mentioning is the unique 117 bp repeats in the *Leishmania* kinesin-like gene (Burns et al., 1993). It is expressed by the amastigotes of visceralizing *Leishmania*, but not by cutaneous species. Some *Leishmania*-specific T-cell epitopes may also exist and cause additional immune-pathology, although these epitopes have not been extensively studied in human leishmaniasis. Work in the direction of elucidating protective immunity has identified T-cell epitopes, which exist also in *Leishmania* cytoplasmic molecules (Probst et al., 2001). Little is known about the protein components of the putative trans-spliceosome, although a number of small nuclear RNAs are known to participate in the process. Because cis-splicing of introns in yeast and mammals and trans-splicing of the spliced leader in *Leishmania* and *Trypanosomes* are mechanistically similar, however, it seems likely that similar proteins participate in these two processes.

Another group of parasite molecules is hypothetically perceived as vaccine determinants. Their interactions with the host immune system lead to the elimination or reduction of parasites

to affect a clinical cure. Differential expression of these determinants alone by parasites may alter their interactions with the hosts. Virulent phenotype is consequently presented as a spectrum of manifestations from asymptomatic infection to fatality. A secondary level of regulation lies in host genetic and environmental factors. A hypothetical model to explain virulent phenotype in leishmaniasis in shown in Fig. 1.8

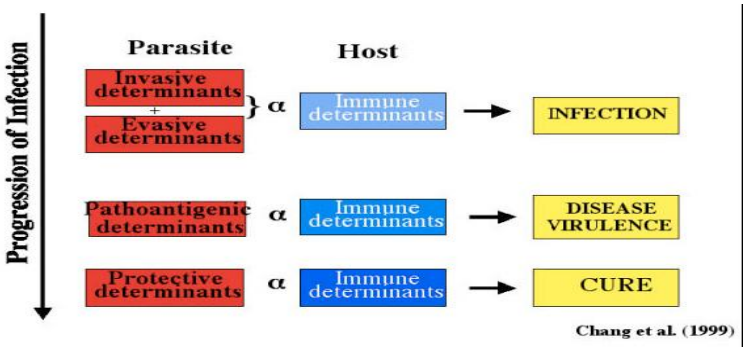

Figure 1.8 A hypothetical model to explain virulent phenotype in leishmaniasis. The three groups of determinants are thought to interact with host immune system independently, but may progress sequentially to produce the spectrum of sub clinical and clinical manifestations as the basis of virulent phenotypes seen.

The invasive/evasive determinants of *Leishmania* help to overcome the host immune and non-immune barriers to establish intracellular infection of macrophages. Infection must be maintained in order to transit from asymptomatic phase to symptomatic phase, especially when host immunity is down regulated. The latter event alone produces no leishmaniasis without persistence of the infection. During the subsequent chronic course of infection, it appears that some intracellular amastigotes are killed or lysed inadvertently perhaps due to the incomplete protection by their invasive/evasive determinants. As a result of this, some cytoplasm molecules of amastigotes are exposed to the host immune system. The resulting immune response to these unique epitopes does not contribute to the anti-*Leishmania* immunity, but to the clinical symptoms observed in leishmaniasis. Thus, *Leishmania* determinants of infection and

immunopathology are considered here as different, but sequentially necessary components for the expression of virulence.

Stage regulated gene expression in *Leishmania*

Several techniques for the quantitative analysis of gene expression at mRNA level are available, such as Northern blotting, polymerase chain reaction after reverse transcription of RNA (RT-PCR), nuclease protection, cDNA sequencing, clone hybridization, differential display, subtractive hybridization, cDNA fingerprinting and serial analysis of gene expression (SAGE). However, these methods each have their limitations, which render them unsuitable if large number of expression products have to be studied at the same time.

The nature of an organism is defined by the genes that it expresses. Genome and expressed-sequence-tag (EST) sequencing projects are underway for many of the major parasites of humans and animals. These provide essential data sets that delineate the genes present in an organism and, in the case of ESTs, some quantitative information on gene expression. The temporal and quantitative analysis of gene expression is essential to fully exploit these datasets and define the biology of the parasite at the molecular level. SAGE is a technique that allows the rapid, quantitative analysis of thousands of transcripts. It complements microarray analysis with the advantage that it is affordable for standard laboratories. It provides a platform to define complete metabolic pathways and has been applied to study responses to drug treatment and the molecular events that are associated with arrested larval development (Knox and Skuce, 2005).

Complex phenotypes are likely to be the summation of the effect of multiple genes. Techniques described earlier that have been used for screening such genes in the past have either measured small groups of genes at a time or measured differential RNA levels that were not reproducible. In recent times, substantial improvement in sensitivity and throughput of

expression screening has been obtained by the introduction of DNA microarray technology. The study of gene expression by DNA microarray technology is based on hybridization of mRNA to a high-density array of immobilized target sequences, each corresponding to a specific gene. In parasitological studies, microarray technology has been used successfully to identify critical genes expressed during development of *Trypanosoma* (Imai et al., 2005), *Plasmodium* (Young et al., 2005) and *Leishmania* (Saxena et al., 2003; Akopyants et al., 2004; Almeida et al., 2004; Holzer et al., 2006). However, results to date for the Kinetoplastid parasites *Trypanosoma* and *Leishmania* have not advanced substantially beyond the stage of validation and new gene discovery (Duncan et al., 2004). Microarray based studies in *Leishmania* have been carried out mainly with *L major* and *L mexicana* and are warranted for *L. donovani*, which causes fatal visceral leishmaniasis. Transcript profiling has been used frequently in expression studies of several model organisms including selected protozoan parasites (Duncan, 2004; Duncan et al., 2004). Expression profiling in *Leishmania* has been performed for *L. major* using array probes amplified from either cDNA or randomly sheared genomic DNA (Akopyants et al., 2001; Almeida et al., 2002; Saxena et al., 2003; Akopyants et al., 2004). Leifso et al. [2007] used DNA oligo-nucleotide genome microarrays representing 8160 genes to analyze the mRNA expression profiles of *L. major* promastigotes and lesion derived amastigotes. Over 94% of the genes were expressed in both life stages and a low degree of differential mRNA expression was observed: 1.4% genes in amastigotes and 1.5% in promastigotes. Comparisons between replicating *L. major* promastigotes and lesion derived amastigotes resulted in a wide range of estimates of the number of transcripts that were regulated (0.6–35%) (Almeida et al., 2004; Holzer et al., 2006). Expression profiling of promastigote vs axenic amastigotes with the parasites isolated from the

field isolates and comparative analysis between axenic and hamster derived/ true amastigotes had not been carried out before, also there are very few studies with *L.donovani.*

Treatment of Leishmaniasis

The drugs currently available for treating leishmaniasis are the pentavalent antimonials, i.e. sodium stibogluconate (Pentostam) and meglumine antimonite (Gluantime); amphotericin B and its lipid formulations ; pentamidine ; miltefosine and paromomycin (Croft and Coombs, 2003). All current drugs have their limitations, with all except miltefosine requiring parenteral administration, necessitating long hospitalization. In addition, the drugs available are not equally effective against all types of leishmaniasis. For visceral leishmaniasis, the pentavalent antimonials have been the first line treatment for over 60 years (Croft and Coombs, 2003). Antimonials are toxic drugs, giving rise to musculoskeletal pain and gastrointestinal side effects, with potential for serious adverse effects including cardiac arrhythmia and pancreatitis. High levels of resistance to these drugs have been reported in endemic regions, particularly around the focus of infection in Bihar, India (Sundar and Rai, 2002b). Amphotericin B is highly effective at treating antimony –resistant cases, but is also toxic, with infusion related fever and chills, nephrotoxicity and first-dose anaphylaxis reported. It has, however replaced antimonials as a first line treatment in some areas of Bihar (Chappuis et al., 2007). The lipid formulation, Ambisome, has much reduced toxicity, but its high cost has limited its use in developing countries until recently (Croft and Coombs, 2003). The WHO announced a price reduction in May 2007 for Ambisome in VL-endemic countries, which reduced treatment cost from around $2,800 to $200 (Chappuis et al., 2007).

Miltefosine was licensed for use in India in 2002 (Davies et al., 2003). It is an alkyl phosphocholine which was originally designed as an anti-cancer drug and was found to have

anti-leishmanial activity against *L.donovani* and *L.infantum* in a mouse model (Croft et al., 1987; Kuhlencord et al., 1992; Croft et al., 1996). Miltefosine has been shown to be safe for both adults and children in India and although gastrointestinal side effects are common, these do not generally prevent completion of treatment (Sundar, 2003). However, it was found to be a possible teratogen in animal studies (Herwaldt, 1999b), so its use in women of child –bearing age has to be carefully monitored (Sundar and Rai, 2002a). Miltefosine has the advantage of being orally administered, but it has a long half life in the body, which may influence the development of resistance (Guerin et al., 2002), and such resistance has been generated in a laboratory setting (Perez-Victoria et al., 2006; Seifert et al., 2003; Seifert et al., 2007). In addition, it has not been established whether miltefosine will be as effective against *L.infantum/Chagasi* as it is against *L.donvani* (Croft and Coombs, 2003).

Increased levels of HIV prevalence in *Leishmania*-endemic countries has lead to increasing numbers of co-infected patients, with 34 countries currently reporting co-infection (www.who.int/leishmaniasis). HIV –*Leishmania* co-infection leads to worse outcomes for the patient for both diseases. Both HIV-coinfected patients and immune-suppressed patients respond slowly to treatment and in most cases relapses occur (Olliaro et al., 2002). Co-infected or immune-suppressed patients are also believed to be likely to develop resistance to anti-lesishmanial drugs if given in monotherapy, resistance which could then spread throughout the community, meaning combination therapy is desirable (Bryceson, 2001).

Future treatment options

New treatments for leishmanisis are needed, however development is hampered by the lack of funds available for drug development (Guerin et al., 2002). A number of potential anti-leishmanial drugs have been identified and are at various stages of development. Those furthest

through development are sitamaquine and the azoles. Research into potential drugs continues, with a more rational approach to drug design being taken (Davis et al., 2004). Fundamental research into *Leishmaina* – specific drug targets is one of the most promising areas. Through a rational approach to identifying drug targets, several have already been proposed and investigated, including cysteine peptidases, the metabolism of trypanothione and sterol biosynthesis enzymes and protein kinases (Croft and Coombs, 2003). However, the recent availability of the genomes of three *Leishmania* species and powerful bioinformatic tools has identified *Leishmania* – specific genes, making them attractive potential drug targets (Peacock et al., 2007), and will hopefully lead to identification of the parasite factors responsible for disease tropism and pathology of the disease which may be good potential targets (Ivens et al., 2005). As early branching eukaryotes, the organization of their cells show extensive differences from that of mammalian cells, suggesting that there will be ample basis for identifying novel proteins, structures and processes that may exploitable as drug targets (Barrett et al., 1999).

Vaccine development

Most of the infected people do not develop clinical symptoms and past episode of Leishmaniasis leads to life long immunity against reinfection with the same subspecies, once the infection is healed (Von Stebut, 2007). This makes the development of vaccine a realistic goal. The extensive knowledge that has been gathered from animal models has shown that protection against live challenge could be achieved using parasite-specific proteins, DNA(Kedzierski et al., 2006) or genetically attenuated parasites (Amaral et al., 2002 ; Uzonna et al.,2004 ; Alexander et al., 1998) advances in our understanding of *Leishmania*–host interactions, *Leishmania* pathogenesis, protective immunity (Saha et al., 2006) and the availability of the complete *Leishmania major* genome sequence (Ivens et al., 2005), could take this a step further. So far,

however, progress in developing a protective vaccine against the different human leishmaniasis has been limited. 'Leishmanization', which simulates live infection by inoculation of live *L. major* as a vaccine for protection against Cutaneous leishmaniasis, has been carried out in Uzbekistan, Iran and Israel (Nadim et al., 1983; Greenblatt, 1988). However, this technique is not suitable for large-scale use or for use in HIV-endemic areas, and has been discontinued. Although studies in a high-prevalence endemic site in Eastern Sudan showed that previous natural *L. major* cutaneous disease or exposure to it can protect from VL (Zijlstra et al.,1994), a trial of a killed *L. major* vaccine that was administered with the tuberculosis vaccine bacille Calmette–Guérin (BCG) failed(Khalil et al., 2000). In fact, none of the preparations of killed parasite (which are crude preparations and difficult to define and standardize) with or without adjuvants has shown significant prophylactic efficacy (Khamesipour et al., 2005; Velez et al., 2005). A second- generation vaccine is now in clinical development as a therapeutic or prophylactic vaccine against VL; it contains a recombinant protein comprising three leishmanial antigens (Leish-111f) (Skeiky et al., 2002) and a defined adjuvant (MPL-SE) (Reed et al., 2003).

Interest in developing immunochemotherapeutic vaccines, which combine a vaccine with drug treatment and which have the potential to become a practical and affordable treatment for PKDL and other persistent forms of Leishmaniasis, is gaining momentum. Encouraging results have been obtained using alum-precipitated autoclaved *L. major* plus BCG together with pentavalent antimonials to treat persistent PKDL in Sudan (Musa et al., 2005). The use of such a therapeutic vaccine could reduce the dose and duration of chemotherapy and be an important control tool. Further studies should focus on exploring the *L. major* genome sequence for candidate protective antigens, testing these candidates in animal models and translating these

studies to humans using novel antigen and adjuvant combinations. Clinical trials should be performed in well-defined sites where there is a high prevalence of the target leishmaniasis.

Gaps in Research

Little is known about the molecular processes that mediate promastigote-to-amastigote differentiation, but it is likely that exposure to the higher temperature and lower pH of the intralysosomal environment initiates a series of changes in gene expression that lead to the morphological changes associated with amastigotes. The different parasite stages therefore, must accomplish an adaptation of the metabolic pathways. Further gene expression profiling between lesion-derived amastigotes and axenic amastigotes in *L.mexicana* have found many genes that show significant differences, such studies are lacking in *L.donovani.* Traditionally these differentially expressed genes have been identified and studied individually or in families, however microarray hybridization techniques are now available that enable the study of gene expression on a genome-wide scale.

The goal of the study was to explore the utility of *L.donovani* genomic microarrays to examine the changes in gene expression as promastigote differentiate into amastigotes. Since DNA microarrays allow examination of gene expression on a genome-wide scale, the study would reveal substantial new information about the dynamics of transcript abundance during this differentiation process. We next expanded the scope of expression profiling from *in vitro*(axenic Am) to *in vivo* (true/hamster derived amastigotes and in tissue lesions of VL patients) and compared the expression of a few important genes which were up regulated in the axenic amastigote stage in the microarray. The study will contribute towards understanding the mechanism of *Leishmania* pathogenicity and virulence.

Chapter 2

Aims and Objectives

Aims and Objectives

The aim of the study is to identify and characterize virulence-related genes in *Leishmania donovani* parasites isolated from Indian kala-azar patients exploiting the massively parallel screening by microarray technology. The study will contribute towards understanding the mechanism of *Leishmania* pathogenicity and virulence. The specific objectives of the present study have been defined as under.

1. **To set up cultures of *L. donovani* from Kala-azar patient samples**

Leishmania have a tendency to spontaneously lose virulence during *in vitro* culture by processes not clearly understood. To ensure the representation of critical virulence genes, it is planned to use parasites isolated from Indian KA patients instead of standard WHO isolates that have been cultured *in vitro* for the past several years. Since it is difficult to obtain enough parasites from a clinical sample to perform biochemical and molecular analysis, it is necessary to culture the parasites for a few passages to obtain parasite material in sufficient quantity. Cultures of Indian isolates of *L. donovani* will be prepared from bone marrow aspirates of VL patients. The parasite isolates will be characterized using species specific PCR.

2. To generate axenic amastigotes in culture

In vitro transformation of promastigotes into amastigotes will be done in order to obtain a continuous and developmentally staged parasite cultures. Homogeneous source of axenic amastigotes that are free from host contaminations will be obtained.

3. **Microarray hybridization to study stage regulated gene expression in Promastigote and axenic amastigote stages of *L.donovani***

Total RNA will be isolated from three biological preparations of promastigote and axenic amastigote stages of *L. donovani*. Fluorescently labeled cDNA probes will be prepared from

promastigotes (Pro) and axenic amastigotes (Axe Am) that represent all the genes expressed in parasites at the particular life stage of interest. A cDNA probe from a Pro sample labeled with one flourochrome (Cy3/Cy5) will be mixed with a probe from a Axe Am sample labeled with a contrasting flourochrome (Cy5/Cy3) and hybridized to the genomic microarray. Multiple microarray hybridizations using three different biological preparations as well as reverse labeling experiments will be carried out. Scanning the microarray with Axon 4100A scanner will measure the intensity of Cy3 and Cy5 signals at each spot and transfer the data directly to a computer for analysis. For each spot on the array, the ratio of intensities of the two signals will identify clones specifically increased or decreased in expression in the test sample. Analysis of microarray data will be carried out using Acuity 3.1 software and MS-Excel and clones showing consistent higher expression at amastigote stage will be selected. Selected clones showing significantly higher and consistent expression at a particular stage will be sequenced. The sequences will be searched against Genbank to look for previous identification or homology to known proteins.

4. Validation of microarray data

Genes that are up regulated in the axenic amastigote stage will be selected for further validation and analysis. Individual DNA clones selected by microarray experiments will be used as probes in Northern analysis with total RNA isolated from promastigotes and axenic amastigotes. Alternatively, RT-PCR of such clones will be carried out to validate the differential expression. Molecular characterization of such genes will be undertaken.

5. Comparison of genes up regulated at the axenic amastigote with hamster derived amastigotes

Comparative analysis of differentially expressed genes which were found significantly up regulated at the axenic amastigote stage was verified in hamster derived amastigotes using Real time PCR.

6. Functional analysis of selected genes.

Full-length genes with potential for functional analysis will be expressed as recombinant proteins and antibodies will be raised. The differential expression of these genes will be verified at protein level. The gene (s) will be over expressed by transfection in parasites and genetically altered parasites will be assessed for growth and differentiation as evidence of the gene's function.

Scopes and Limitation of the study

Due to a large spectrum of drug resistance and emergence of kala-azar as an opportunistic infection in HIV infected persons, the incidence of the disease is increasing at an alarming proportion throughout the world. Chemotherapy and vector control are the only effective measures for combating the disease, which is nearly always fatal, if left untreated. Past efforts for development of vaccines have clearly shown the need for a better understanding of the mechanism of *Leishmania* pathogenesis. Studies outlined here would contribute toward our understanding of virulence related genes and pathways playing a role in parasite biology, which is of paramount importance towards identifying new drug targets and development of vaccine candidates to control the infection.

Identification of Amastigote up regulated genes

Introduction

Intracellular parasitism is a process by which micro-organisms cycle between vector and host as a result of which the parasites encounter extreme environmental changes during their life cycle, to which they respond by differentiating into highly adapted forms that enable them to invade and proliferate inside their hosts. *Leishmania donovani*, the causative agent of visceral leishmaniasis (known as kala-azar in India), is a parasitic protozoan that cycles between the alimentary tract of sand flies and mammalian macrophages. In the insect vector, the parasites grow as extracellular flagellated promastigotes, which differentiate into intracellular aflagellate amastigotes upon entering the phagolysosome of the host macrophages (Chang and Dwyer, 1976; Killick-Kendrick, 1999). The amastigotes are adapted to grow and proliferate in the hydrolytic environment inside phagolysosomes (Zilberstein and Shapira, 1994; Burchmore and Barrett, 2001). Promastigote (Pro) -to- amastigote (Am) differentiation is a complex process that is accompanied by a number of morphological and biochemical changes. Parasites change shape from elongated to spherical and lose most of their flagellum. They undergo a major shift in metabolism, especially in the rate and pH optima for several processes, including DNA synthesis (Mukkada et al., 1985) and nutrient uptake (Mazareb et al., 1999). A number of amastigote-specific genes have been identified, including a 3'- nucleotidase (Bates, 1993), the A2 gene family (Charest et al., 1996; Zhang and Matlashewski, 1997), HSP100 (Hubel et al., 1997), and a MAP kinase, LMPMK (Wiese and Gorcke, 2001). In addition, certain members of the GP63 and PSA-2 gene families are differentially expressed in amastigotes, and there are differences in the GPI anchor of the latter (Handman, 2001). In contrast, some processes are down-regulated in amastigotes (McConville and Blackwell, 1991; Turco and Sacks, 1991) most notably lipophosphglycan (LPG) biosynthesis, resulting in its replacement by glycoinositol phosholipid

(GIPL) as the major component of the parasite surface coat (McConville and Blackwell, 1991). Regulation of gene expression in *Leishmania* is unusual because their protein-coding genes are transcribed as polycistronic RNAs with tens-to-hundreds of adjacent genes on the same DNA strand (Myler et al., 1999; Martinez et al., 2003; Worthey et al., 2003; Martinez-Calvillo et al., 2004; Ivens et al., 2005). Mature mRNAs are subsequently obtained from coordinated polyadenylation and trans-splicing, which adds a 39-nt spliced leader (SL) sequence to the 5' end of all mRNAs (Perry and Agabian, 1991; LeBowitz et al., 1993). As a consequence of this unusual gene organization, *Leishmania* gene expression appears not to be regulated at the level of transcription (Clayton, 1999), but stage-specific expression of a number of genes has been shown to be regulated via mRNA stability (Aly et al., 1994; Charest et al., 1996; Beetham et al., 1997; Burchmore and Landfear, 1998; Wu et al., 2000; Brittingham et al., 2001; Ouellette et al., 2003). The study of the relative changes in transcript abundance during the life cycle may aid in the identification of genes that are essential in the cytodifferentiation process and thus are potential candidates for vaccine or drug targets for control of infection (Almeida et al., 2002; Duncan et al., 2004). A number of studies have been conducted searching for changes in gene expression amongst *Leishmania* stages, using methods such as differential or subtractive hybridization (Diatchenko et al., 1999), AP-PCR (Pogue et al., 1995), SAGE (Velculescu et al., 1995). Traditionally the differentially expressed genes like Amastin, A2 and gp63 (Wilson et al., 1993; Wu et al., 2000; Zhang and Matlashewski, 2001) identified through these techniques are studied individually or in families. However microarray hybridization techniques are now available that enable the study of gene expression on a genome-wide scale.

In parasitological studies, microarray technology has been used successfully to identify critical genes expressed during development of *Plasmodium* (Young et al., 2005), *Trypanosoma*

(El-Sayed et al., 2000; Imai et al., 2005) and *Leishmania* (Almeida et al., 2002; Saxena et al., 2003; Akopyants et al., 2004; Duncan, 2004; Duncan et al., 2004; Goyal et al., 2006; Holzer et al., 2006). These array platforms support thousands of DNA elements representing up to two-thirds of the genome. The differentiation process can be mimicked in axenic culture by shifting promastigotes from an insect-like (24°C, pH 7) to an intralysosomal-like (37°C, pH 5.5 and 5% CO2) environment (Saar et al., 1998; Gupta et al., 2001; Somanna et al., 2002 ; Debrabant et al., 2004; Barak et al., 2005). However, because of the difficulties in obtaining adequate amounts of viable amastigotes that are free of host cell contamination as well as the limited availability of effective and sensitive methods (Coulson and Smith, 1990; Brodin et al., 1992), molecular biology studies on the parasite, particularly at the amastigote stage have been hindered greatly. Whereas, there are studies on stage-specific expression of genes in promastigotes and terminally differentiated amastigotes (Coulson and Smith, 1990; Joshi et al., 1993; Zhang and Matlashewski, 1997; Krobitsch et al., 1998; Wu et al., 2000), little is known about the molecular processes that mediate promastigote-to-amastigote differentiation, but it is likely that exposure to the higher temperature and lower pH of the intralysosomal environment initiates a series of changes in gene expression that lead to the morphological changes associated with amastigotes. Studies suggest that axenic amastigotes from *Leishmania* strains are morphologically and biochemically similar to those grown in macrophage cell lines or to lesion-derived amastigotes (Pan et al., 1993 ; Hodgkinson et al., 1996 ; Balanco et al., 1998; Gupta et al., 2001). Nevertheless, recent reports with *L.mexicana* have found many genes that show significant differences between lesion-derived amastigotes and axenic amastigotes (Holzer et al., 2006). Comparisons between replicating *L. major* promastigotes and lesion-derived amastigotes resulted in a wide range of estimates of the number of transcripts that were regulated (0.6–35%)

(Akopyants et al., 2004). The gene expression changes have been studied in axenic amastigotes. However by removing the parasite from its intracellular niche, the genes required for penetration of the parasite into the host cell and its survival in the peculiar environment of the macrophage phagolysosome may not be screened efficiently. In addition, axenic amastigotes may have different metabolic processes than intracellular/true amastigotes.

The goal of the present study was to use DNA microarray technology using custom made prototype chip to investigate the stage regulated genes during promastigote and amastigote stage of *L.donovani,* to search for genes that may contribute to play a role in virulence. The *L. donovani* parasite used for microarray construction and hybridization was well characterized, predominant isotype obtained from an Indian KA patient (Sreenivas et al., 2004). There are hardly any studies comparing expression profiling of promastigotes with the amastigotes of *L. donovani* which we propose to take up using parasites isolated from the bone marrow aspirates of VL patients with minimum *in vitro* passages. Comparative analysis of differentially expressed genes which were found significantly up regulated at the axenic amastigote (Axe Am) stage was verified in hamster derived amastigotes using Real time PCR.

Materials and Methods

Reagents and Chemicals

Agarose, Bovine Serum Albumin, Glycine, Glutamine, HEPES, MES, Penicillin, Streptomycin, Gentamycin sulphate, Vitamin solution, Amino acid solution, Adenosine, Tris base, Medium-199, RPMI-1640, MOPS, Kanamycin sulphate and other fine chemicals for microarray experiments i.e. yeast tRNA, aminoallyl-dUTP and Poly A were from Sigma Chemicals, USA. Trizol, *Taq* polymerase, dNTPs, $MgCl_2$, DTT, Superscript II reverse transcriptase, oligo dT_{20}, Cot-1 DNA, RNasin were from Invitrogen, USA. Fetal bovine serum

was obtained from Biological Industries, Israel and Gibco BRL, USA. Cy3-monofunctional dye or Cy5-monofunctional dye for labeling in microarray experiments were from Amersham Biosciences, USA. SpotReport® Alien cDNA array validation system (alien mRNA spikes 1-3) was from Stratagene. pGEM®-T Easy cloning kit was from Promega corporation USA and kits for gel extraction, PCR purification and RNA cleanup (RNeasy kit) were obtained from Qiagen. RNA marker and restriction enzymes were from MBI Fermentas & New England Biolabs, USA. SYBR Green I PCR Master mix was provided by Applied Biosystems, USA. Gene specific oligonucleotides were synthesized from Sigma, USA. Glycerol, Disodium hydrogen phosphate, Dipotassium hydrogen phosphate, Isopropanol, Potassium dihydrogen phosphate, Potassium chloride, Potassium acetate, SDS, Sodium acetate, Sodium chloride, sodium dihydrogen phosphate, Sodium hydroxide, Sodium bicarbonate were purchased from SRL, India Magnesium sulphate, Glucose were from Qualigens, India. Charged nylon membrane was from Amersham Biotech, USA. Radioactive [α-^{32}P] dATP was purchased from BRIT, Hyderabad. Cell culture plasticware were obtained from Corning, USA. Diff-Quik solutions were from Dade Behring Ag, Dӧdingen, Switzerland. Hamsters were obtained from the National Institute of Nutrition, Hyderabad.

Experimental methods

1. Parasite culture

A. Isolation and propagation of parasites

Parasite isolates of *L. donovani* were prepared from bone marrow aspirates of KA patients originating from Bihar and reporting to Safdarjung Hospital (SJH), New Delhi using NM+30% FCS, as described before (Salotra et al., 2001; Sreenivas et al., 2004). The diagnosis of KA was confirmed by demonstrating the presence of LD bodies in the bone marrow. Informed

consent was obtained from patients before collecting the bone marrow samples, according to the guidelines of the Ethical Committee, SJH. The Promastigotes (Pro) were cultured in Medium 199, 25mM HEPES N-[2-hydroxyethyl]piperazine-N^{-1}-[2-ethanesulfonic acid], 100 IU and 100µg/ml each of penicillin G and streptomycin sulphate, respectively from 10,000IU and 10,000µg/ml combined stock, supplemented with 10% heat- inactivated FBS (fetal bovine serum) at 24^0C and 7.4 pH. Promastigote forms of these parasites were routinely maintained in 15 ml sterile conical centrifuge tubes and transferred into fresh medium every 4-5 days, as necessary.

B. Generation of axenic amastigotes

Amastigotes were generated and grown *in vitro* by serially adapting them to grow at elevated temperatures and reduced pH conditions according to the method described by Debrabant *et al.,* [2004]. Initially, the promastigote were adapted to grow in Medium 199 + 25mM HEPES + 10% FBS supplemented with a final concentration of 2mM L- Glutamine, 100µM adenosine, 23µM folic acid, 100 IU and 100µg/ml each of penicillin G and streptomycin sulphate, respectively from 10,000 IU and 10,000µg/ml combined stock, 1X RPMI vitamin mix, 4.2mM $NaHCO_3$. The final medium was adjusted with 2N HCl (drop wise, while stirring) to pH 6.8 at 26^0C, sterilized by filtration (0.22µM, Millipore) and stored at 4^0C prior to use. Promastigote forms of these parasites were transferred into fresh medium every 4-5 days which is an essential need for amastigote development.

The parasites were gradually adapted to grow at 26^0C in potassium buffered (~140mM) RPMI based medium. This medium was formulated to contain the following salts at a final concentration of: KCl (15mM); KH_2PO_4 (114.6mM); $K_2HPO_4.3H_2O$ (10.38mM); $MgSO_4.7H_2O$ (0.5mM) and $NaHCO_3$ (24mM). Other constituents of the medium were 1X RPMI vitamin mix

solution, 1X RPMI amino acid mix, 4mM L- glutamine, 100μM adenosine, 23μM folic acid, 100IU and 100μg/ml each of penicillin G and streptomycin sulphate, 1X phenol red, 22mM D-Glucose and 25mM 2-(N-morpholino) ethane sulfonic acid. To this, 1L of potassium based–basal medium, 256 ml of heat-inactivated FBS (25.6% v/v final serum concentration), was added. The pH of the medium was adjusted to 5.5 with 2N HCl (dropwise, while stirring). The medium was sterilized by filtration using 0.22μM filter and stored at 4^0C prior to use. These parasites were grown in 25cm^2 tissue culture flasks at 26^0C and the ratio of culture fluid volume to the total surface area (cm^2) of the culture flask (i.e. 1:5) was stringently maintained at all subsequent culture conditions. The temperature was gradually increased to adapt the parasites to grow at 37^0C. Subsequent to several passages under these conditions, the parasites transformed and grew as intermediate forms. As a final step towards the generation of axenic amastigotes, these intermediate form parasites were subsequently grown in potassium based RPMI-1640+MES/pH5.5 at 37^0C in a humidified atmosphere containing 5% CO_2. Once adapted, these parasites were able to grow as axenic amastigotes and were routinely shuttled as promastigotes and axenic amastigotes.

C. Isolation of hamster derived amastigotes

Golden Syrian Hamsters (6 to 8 weeks old, male) were intracardially inoculated with $1X10^8$ cells (promastigotes) of *L. donovani* per animal. Spleens from 8 week infected hamsters (n-12) were excised and diced and amastigotes were purified. Three biological preparations of amastigotes were prepared pooling spleen from 4 hamsters. The excised splenic material was transferred to a glass dounce homogenizer in 30 ml PSGEMKA buffer (20mM **S**odium-Phosphate, 104mM **S**odium Chloride, 0.5mM **G**lucose, 10mM **E**DTA, 10mM **M**agnesium Chloride, 10mM Potassium chloride (**K**Cl), 0.02% Bovine Serum **A**lbumin buffer), and parasites

released from macrophages with seven thrusts. Centrifugation at 80s for 5 min at 4^{o}C pelleted large cellular debris. The supernatant was then centrifuged at 1300g for 10 min at 4^{o}C to pellet amastigotes After the centrifugation steps, red cells were lysed by incubating for 7 min in 0.05% saponin in PSGEMKA at 4^{o}C After one wash, amastigotes were resuspended in buffer (10ml per spleen) and were filtered through polycarbonate filters of pore size 5µm using a gentle vacuum. Host cells and debris were retained on the filters and parasites were collected in the filtrate. Amastigotes were washed twice with PSGEMKA before use. The viability of isolated amastigotes were checked by staining with solution comprising of acridine orange and ethidium bromide and observed under fluorescent microscope.

D. Patients' samples

Six patients each of confirmed VL, PKDL and CL, hailing from the endemic area were included in this study. Diagnosis was confirmed by either microscopic demonstration of the Ld bodies and/or by PCR bone marrow aspirate (BMA) samples for VL or skin biopsy for PKDL and CL lesions. The causative parasites were characterized by genotyping with ITS1-RFLP assay as *L.donovani* in VL and PKDL and as *L. tropica* in CL . The study was approved by and carried out under the guidelines of the Ethical Committee of Safdarjung Hospital, New Delhi, with informed consent was obtained from all patients or their guardians. Two normal skin biopsy samples were collected as controls from healthy volunteers.

2. Isolation of RNA

The cells were collected at identical growth points (log phase promastigotes and axenic amastigotes) and grown under identical conditions to contain variations in the quality of RNA. Total RNA was isolated from promastigotes, axenic amastigotes and hamster derived amastigotes using Trizol reagent. Briefly, the cells (1-1.5X10^{9}) were lysed in Trizol, at room

temperature and to the homogenized samples, 0.2 volumes of chloroform was added followed by centrifugation at 12,000 x g for 15 min. The aqueous phase was collected and 0.5 volumes of isopropyl alcohol were added to precipitate total RNA. The total RNA was pelleted by centrifugation and washed in 70% ethanol (in autoclaved DEPC treated water) and stored at –70°C till further use. Approximately 130-140µg of the total RNA was cleaned up to eliminate salts and contaminating genomic DNA by preferential binding of RNA to the RNeasy columns before the labeling reaction, using RNeasy mini kit. The purified RNA was quantified spectrophotometrically by checking the absorbance at 260nm. The quality and integrity of RNA was checked using agarose/formaldehyde gel electrophoresis. To 1µg RNA, 0.5µl 10X MOPS buffer, 4µl 12.3 M formaldehyde, 4µl formamide and 0.5µl ethidium bromide were added. The samples were heat denatured at 66^{o}C for 10 mins, mixed with 2X RNA loading buffer and loaded onto the gel. Electrophoresis was carried out at 65V for 3hrs and products visualized under UV transilluminator on 0.6% Formaldehyde Agarose Gel.

3. Microarray hybridization, Scanning and Analysis

A. Description of *L. donovani* Genomic Microarray

The *L. donovani* genomic microarray used in this study was constructed as a part of recently completed collaborative project with Dr. H.L. Nakhasi and Dr. R. Duncan at CBER, FDA, USA as described by Duncan et al., [2004]. Briefly, a library of 1-1.5 kb randomly sheared fragments of genomic DNA from a fresh isolate of *L. donovani* prepared from an Indian KA patient was ligated into the pZERO-2 vector. Various steps of assembling of genomic clones and microarray constructions are shown in a schematic flow diagram in Fig. 3.1. A total of 4224 spots, representing ~37% of the expressed genes (Akopyants et al., 2001), comprised of 4188 PCR amplified inserts from library clones along with 24 known *Leishmania* genes viz.

28SrRNAζε, *Leishmania* protein kinase 2 (c-lpk2), *Leishmania* stress inducible protein (LSIP), LdS-6-1, Calreticulin, Centrin, protein disulfide isomerase (PDI), Protein kinase A (PKA) etc. and 12 negative controls inclusive of human genes, bacterial and salmon genomic DNA, hamster genes, empty vector, poly A, cot-1 etc, along with SpotReport® Alien cDNA array validation system, were printed in duplicate arrays on polylysine slides.

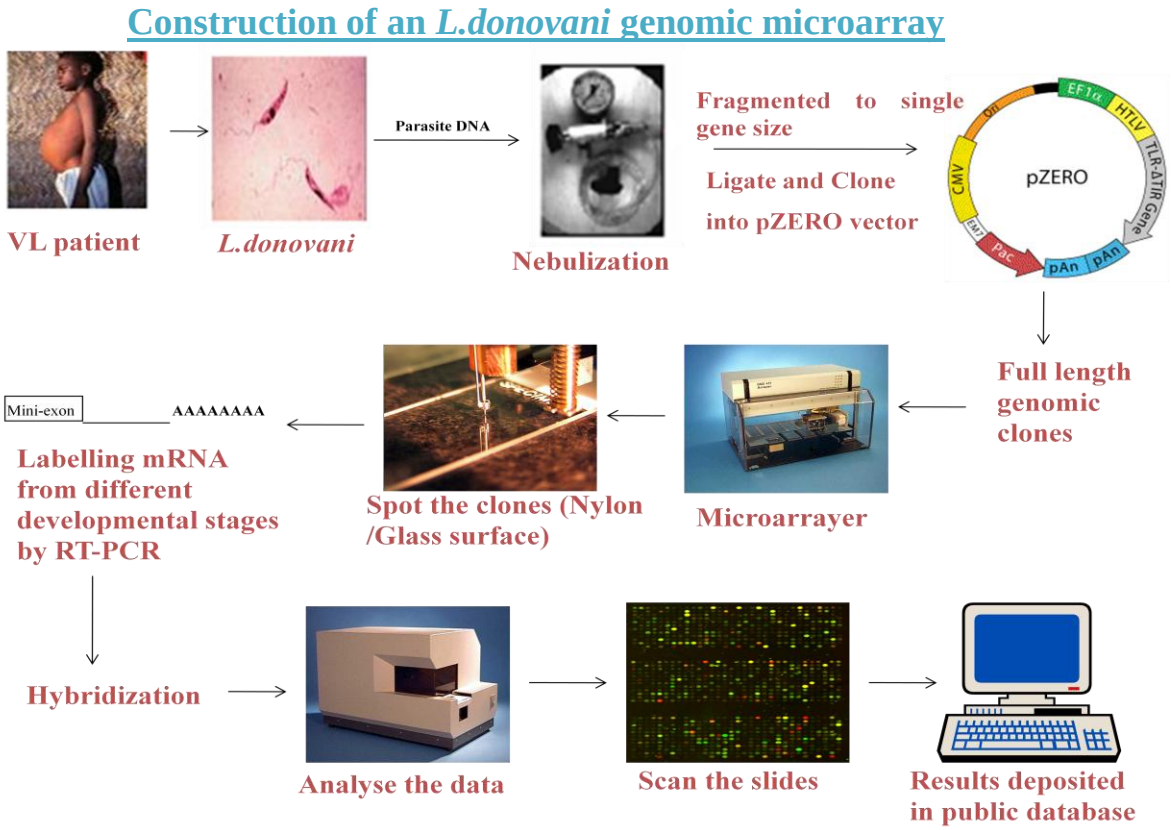

Fig 3.1. Flow diagram of the process of constructing a genomic microarray for *Leishmania donovani*. 1) Parasites collected from patient expanded in culture. 2) DNA extracted. 3) DNA randomly fragmented and size selected 4) DNA fragments ligated into a pZErO-2 plasmid vector, bacteria transformed, individual colonies picked, cultured and plasmids extracted. 5) Common flanking primers used to synthesize sufficient insert DNA by PCR. 6) PCR products transferred with a robotic pin spotter onto glass slides, fixed and blocked. 7) Fluorescently

labeled oligo dT primed cDNA synthesized from total RNAs of interest, hybridized to the microarray and image captured with a two-color laser scanner.

B. Microarray Hybridization and analysis Different steps for the microarray experiments for comparing the gene expression profile of parasites at different stages are shown in the Fig 3.2.

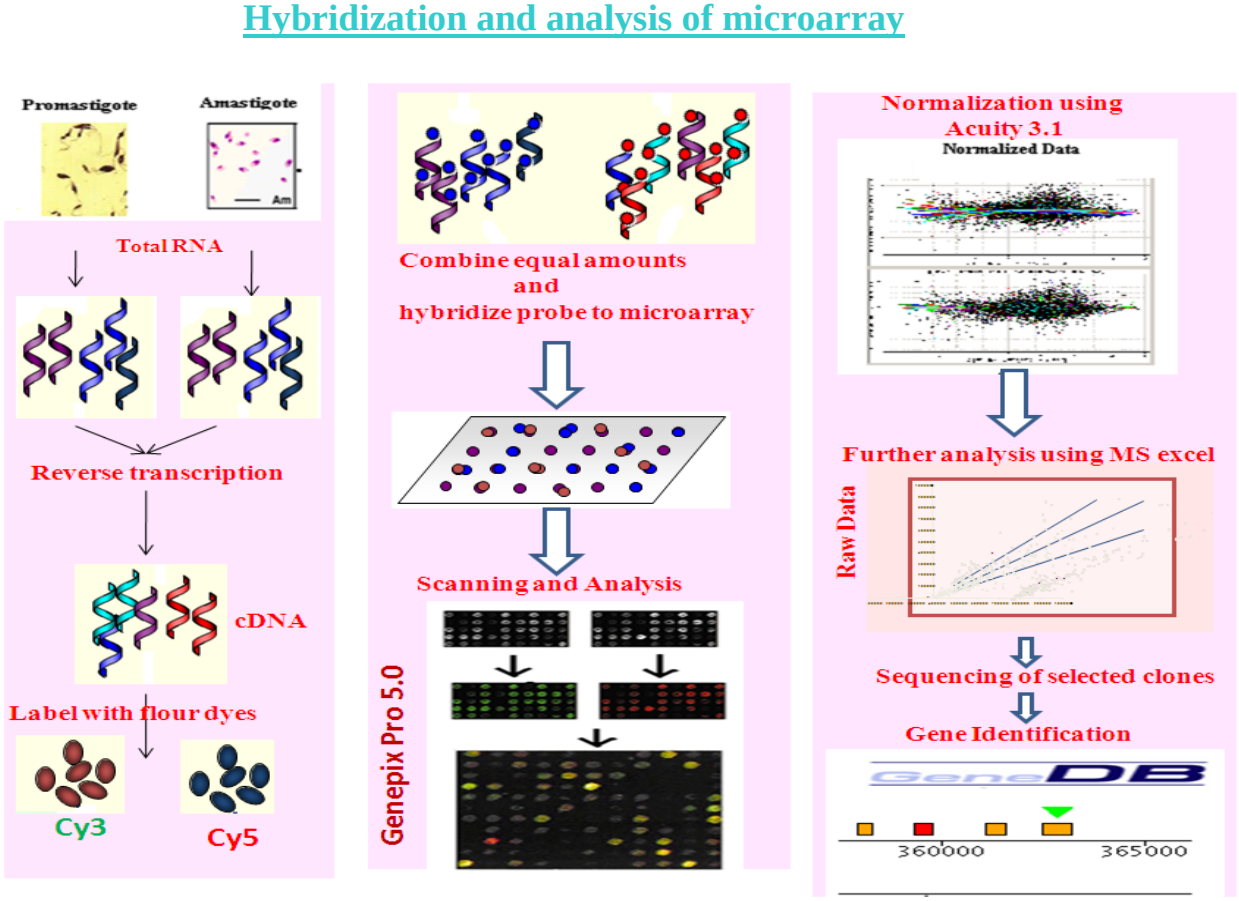

Fig 3.2 Flow diagram showing steps for microarray based gene expression profiling of Pro and Am stages. *Leishmania* parasites were grown in large quantity and total RNA was isolated from the parasites. cDNA was prepared and labeled with fluorochrome Cy5 or Cy 3 containing amino-allyl dUTP by indirect incorporation. After unbound fluorochrome removal, both the labeled probes were pooled and hybridized with *L.donovani* genomic microarray. The image was visualized using GenePixPro 5.0 software and normalized and analyzed using Acuity 3.1. The

clones showing consistent and significant higher expression were sequenced and BLAST analyzed in *Leishmania* geneDB and the corresponding gene identity was determined.

i. Production of differential probes

Fluorescently labeled cDNA copies of the total RNA isolated from log phase promastigotes and axenic amastigotes were prepared that represent all the genes expressed at that stage. Alien RNAs that are complementary to the Alien external control DNAs printed on the slide were also included in the labeling reaction to facilitate validation and optimization of hybridization reactions. cDNA was prepared by using 20μg of total RNA spiked with alien RNAs. 1μg oligo $(dT)_{20}$ primer, 10mM each of dATP, dCTP and dGTP, 6mM of dTTP, 4mM of amino-allyl-dUTP, 9mM DTT and 400 units Superscript II reverse transcriptase in reaction buffer provided. The RNA and primer were incubated at 70^0C for 5 mins and snap-chilled on ice, before other components of the reaction were assembled. The reaction was incubated at 42^0C for 1hr. The residual RNA in the reaction tube was then degraded and reaction neutralized by keeping the tube at 65 degree Celsius for 5 min. The cDNA products were purified using Qiagen MiniElute PCR purification kit and concentrated using DNA plus vacuum concentrators. A cDNA probe from a reference sample (Pro) labeled with one fluorochrome was mixed with a probe from a differentiating sample (Axe Am) labeled with a contrasting fluorochrome and hybridized to the microarray, using hybridization chambers.

ii. **Promastigotes vs Axenic Amastigote Hybridization**

The two probes, Pro and Axe Am labeled with respective fluorochrome were mixed and hybridized with the genomic microarray chip containing 4224 clones in a solution containing 3.5X SSC, 0.3% SDS, 10μg COT-1 DNA, 4μg yeast t-RNA, 10μg poly A in a hybridization chamber. The hybridization reaction was allowed to proceed for 16hrs at 65^0C.The hybridized

microarrays were washed at room temperature for two minutes in 2X SSC/0.1% SDS, and 1X SSC for 10 mins, 0.2X SSC for 10 mins and finally 0.05X SSC for 10mins. All the hybridizations were carried out with three biological preparations of *L. donovani*.

iii. Scanning and Analysis of microarray data

After hybridization, scanning and analysis of the two chips were carried likewise. The arrays were spun dried in a centrifuge and scanned in a microarray scanner (Axon 4100A). The intensity of the two signals at each spot was transferred directly to a computer for visualization and analysis. The images were visualized using GenePix Pro5.0 software. The slide was initially pre scanned at pixel size 40μm. The fluorescence intensities were calculated for control features and the PMT voltages were adjusted to yield an intensity ratio of 1.0. Data scan was carried out at a resolution of 5μm, after adjusting the PMT voltages. The individual features on the microarray were manually examined to assess their quality and those exhibiting a sum of Cy3 and Cy5 intensity value lower than the control features included on the array (< 1500) or those having saturation intensity or poor quality were flagged and discarded from further analysis. Local background was subtracted from the intensity value of each feature on the array. The relative intensity of the two signals identified clones expressed differentially at Axe Am stage in comparison with Pro stages. Analysis was carried out using statistical analysis software package Acuity 3.1 and MS Excel. The microarray data were normalized based on medians of ratios as well as by Z score transformation as described by Cheadle *et al.,* [2003]. In order to compare expression levels between RNAs, the medians of Cy5/Cy3 ratios were calculated from the normalized values of replicate arrays. The ratios from reverse-labeled experiments were reciprocated before analysis. Clones showing consistent ratios in replicate experiments were selected for further analysis. These fold changes were relative to the amount of RNA in the cell.

For calculation of Z ratios of dye flip experiments, the ratios were inversed before analysis. Z-scores were calculated by subtracting the average gene intensity within one array replicate from the trimmed mean scaled intensity and then dividing the result by the standard deviation of all intensities. This step corrects the data internally in each hybridization and expresses the intensity values as units of standard deviation away from the mean of the array, which is set to zero, while still reflecting the quantitative integrity of the data. Z-ratios were calculated by taking the difference of the average of the replicate Z-scores between two samples and then dividing by the standard deviation of all the averaged Z-score differences for that particular comparison. Average *Z*-scores were also subjected to sample comparisons by grouping the replicates by sample and then applying the *t*-test comparison measure. Only two samples were compared in any one analysis (Pro versus Axe Am). Each comparison produced a single *p*-value for each gene, which were corrected for multiple testing by Bonferroni's correction an error rate of 0.05.

Fourteen genes which were up regulated in the amastigote stage were checked for the presence of 450 nucleotides regulatory sequence that was observed in the 3'UTR of the amastigote specific genes (Wu et al., 2000). The 3'UTR of the amastigote specific genes and the standard 450 nucleotides sequence were aligned in the GCG software, a multiple alignment program to find percentage similarity between the sequences.

4. Northern hybridization

Northern hybridization was carried out essentially as described by Sambrook *et al.*, [1989] as per the following details.

Sample preparation

The total RNA prepared from either promastigotes or axenic amastigotes stored in 70% ethanol were centrifuged at 3000 rpm for 60 secs to completely remove traces of ethanol. After

briefly drying under vacuum the samples were rehydrated by addition of 30-40μl RNAse free water and incubated in a dry bath at 65°C for 15mins and immediately shifted to ice bath. The concentration was determined by measuring A_{260}.

10X MOPS Electrophoresis buffer

10X MOPS	:	50ml
35% Formaldehyde	:	50ml

Made up to 500ml with DEPC treated water

Sample Mixture

Total RNA	:	10μg (1-3μl)
10XMOPS	:	2.0μl
Formamide	:	20μl
Formaldehyde	:	7μl

The mixture was incubated at 65°C in dry bath for 15mins and stored on ice till loading after a brief spin. To the denatured RNA samples, 2μl of 10X RNA gel loading buffer (50% glycerol, 10mM EDTA, 0.25% w/v Bromophenol blue 0.25% w/v Xylene cyanol) and 1μl of ethidium bromide solution (10μg/ml) were added before loading samples on formaldehyde agarose gel. RNA marker was run along with the samples.

Electrophoresis on formaldehyde gels

Gel boats and electrophoresis tank were initially treated with 3% solution of H_20_2 for 10 mins and rinsed thoroughly with DEPC treated water to remove contaminating RNases. 0.6g of agarose was dissolved in 43ml of DEPC treated water and boiled in microwave oven. After letting it cool to 55°C, 6ml of 10X MOPS buffer and 10.8ml of formaldehyde were added. The gel was cast and allowed to polymerize for 30 mins and electrophoresed at 65V for 30 mins

before loading the RNA samples. The denatured RNA samples were electrophoresed for 3 hrs at 65V. After the electrophoresis, photograph was taken and the position of the markers noted.

Northern blot

The formaldehyde gel was rinsed briefly with double distilled water followed by soaking in 5 gel volumes of alkaline transfer buffer (0.01N NaOH/3M NaCl) for 20 mins. Nylon^{+} membrane of appropriate size was floated in DEPC water for soaking and then in alkaline transfer buffer for 5 mins. The gel containing fractionated RNA was placed on the transfer set up. Nylon membrane was placed on top of the gel and the RNA was allowed to blot by capillary transfer using alkaline transfer buffer. After overnight transfer, the membrane was marked for origin and wells. The side of membrane carrying RNA was cross linked by UV irradiation in a cross-linker for 5 mins (1.5J/cm^2 for damp membranes).

Hybridization and autoradiography

For validation, microarray selected clones were grown and plasmid prepared followed by digestion with *XbaI* and *HindIII* (pZErO-2 vector) released *Ld* inserts of 1-1.5kb that were labeled with α-P^{32} and used as probe in Northern analysis.

Probe Preparation

Random priming reaction*:*

50ng of the template DNA in nuclease free water was denatured in dry bath for 10 mins at 95°C and quickly placed on ice. The tube was centrifuged briefly in cold and the reaction was set up as follows

Template DNA	-	50ng
10x labeling buffer including random oligomers	-	5μl
dNTP mixture (dCTP, dGTP, dTTP) each	-	2μl

[α-^{32}P] dATP (3000ci/mM, 50μci)	-	5μl
Klenow fragment	-	1μl
Nuclease free water, to	-	50μl

The reaction was incubated at 37°C for 6hr and terminated by addition of 5μl of 0.2M EDTA. The unincorporated [α-^{32}P] dATP was removed by cleaning the probe with Qiagen Nucleotide removal kit as per instructions supplied.

Hybridization protocol

Formamide pre-hybridization solution

	Final conc.	Stock	10ml
Formamide	50%	100%	5 ml
20X SSC	5X	20X	2 ml
Sodium phosphate (pH 6.5)	0.5M	20mM	0.4 ml
Denhardt's solution	5X	50X	1ml
Glycine	1%	10%	1ml
SDS	1%	10%	1ml

Sonicated salmon sperm DNA (Sigma, USA) was denatured and added at a final concentration of 10μg/ml prior to use.

The probe was labeled to a specific activity of $>10^8$ dpm/μg. The membrane carrying immobilized RNA was soaked in 5XSSC. The membrane was placed with RNA side up in hybridization bottle and approximately 1ml of formamide pre-hybridization solution was added per 10cm^2 of membrane. The pre-hybridization was carried out in hybridization oven by incubating at 42°C for 3hrs. The radiolabeled probe ($1x10^7$cpm) was denatured by heating at 100°C for 10 mins and quickly transferred to ice. The denatured probe was added and the

hybridization reaction was allowed to continue for 16 hrs. The hybridization solution was collected into 15 ml tube and the membrane was washed by the following steps.

Washing and Autoradiography

The membrane was washed with 5XSSC twice at room temperature for 30 secs followed by washing with 2XSSC twice at 42°C for 15 mins each. Finally, the membrane was washed in 0.2XSSC once at 65°C for 30 mins. After washings, membrane was damped in 2XSSC, exposed to X-ray film (Biomax, Kodak) and kept at -70°C. After 72-96 hrs exposure, X-ray film was developed. The intensity of the hybridized bands was quantified from autoradiograms using AlphaEase software (AlphaImager). Normalization of the RNA added in each lane was done by the ribosomal RNA quantity.

5. RT-PCR (Reverse transcriptase) analysis

Primers for RT-PCR to amplify the ORF regions of selected genes were designed using Clone 2 or Primer 3 softwares and synthesized from Microsynth Inc. or Bioserve Ltd (Table 3.1). First strand cDNA was prepared for RT- PCR using 5 µg total RNA and 1µg oligo $(dT)_{20}$ primer. The RNA and primer were incubated at 70^0C for 5 mins and snap-chilled on ice, before other components of the reaction were assembled. 10mM each of dATP, dCTP, dTTP and dGTP, 10mM DTT and 200 units Superscript II reverse transcriptase were added in the provided reaction buffer. The reaction was incubated at 42^0C for 1hr. Amplification reactions were conducted with primers for constitutively expressed housekeeping gene α-tubulin for normalization of amounts of RNA used in our reactions. The reaction mixture (20 µL) for PCR contained normalized cDNA, 200 mmol/L each dNTP, 1.5 mmol/L MgCl2, 25 pmol of each primer, and 0.5 U of Thermus aquaticus (Taq) DNA polymerase in PCR buffer (Invitrogen, USA). PCR was performed in a DNA thermal cycler (Applied Biosystem, USA). After the PCR

amplification, 2 µL of tracking dye was added to the sample, and 10µL of amplification products were run on 1.5% agarose gel containing ethidium bromide (1 mg/mL) in TAE buffer (0.04 mol/L Tris acetate, 0.001 mol/L EDTA). A 1-kb DNA ladder was used as a molecular marker. Control experiments were performed with amplification using total RNA but no reverse transcriptase to check for DNA contamination.

Table 3.1: List of genes and primer sequences used for RT-PCR study

Gene	Primer (F)	Primer (R)	T_A	Product size
LdUba5	5' ATG CCC TTA TCC TCC TCC CC 3'	5' CTA CTG GAT GGC CTT CAT GCG 3'	61^0C	1.2kb
Trypanosomatid specific gene (p27)	5' ATG TCT CGC TGC ACG ACC AAG 3'	5' TTA CAC ACC GTG GCC GGA CAT 3'	63^0C	261bp
Calpain-like cysteine proteinase (35C8)	5' GTT AAC GGC AGA CGG CAA AGG G 3'	5' CGG TAA GTC CTC CAG CGC CAT 3'	62^0C	1.2kb
Phosphomanose mutase (40B11)	5' GCC TCG CAA TCC GGA AAC CCT 3'	5' AAT CGT TGC CGC CCT CTG ACG 3'	57^0C	747bp
MAPkinase homolog (28F11)	5' ATG ACC TCC TAT GGC ATC GAC 3'	5' CAA CTT CAG TCT GTC GCT AAT AAC 3'	59^0C	927bp
Hypothetical protein (45E11)	5' ATG ATC ACC ACG AGC GGC CG 3'	5' GCG CAA AGC GAG AAC AAA CG 3'	57^0C	1.6kb

Hypothetical protein (36G8)	5' ATG CTC GCT CGT CTC AAC CTG AAC 3'	5' CTG CGT GTG AGG AAG CGG GAC 3'	64^0C	599bp
28F12(CPN10)	5' ATG ACC TCC TAT GGC ATC GAC 3'	5' CAA CTT CAG TCT GTC GCT AAT AAC 3'	59^0C	627bp
A2	5' ATG GGA TCC CCG CAC AAG GCG GCC GTT GAC 3'	5' CAT GCC TCA TGG CAT ATA CTT TAC GC.3'	63^0C	1.1kb
Parasite surface antigen 2 (PSA2)	5' CGT GCG ATC CCT GAG CTT 3'	5'CCG GCA TAC TTT GGC TGA AA 3'	60^0C	100bp
V-type ATPase	5' AGT GGT GCA TXG ATC AGC GGT 3'	5' CCT TTT TGC GCC ATC AGA ATG C 3'	52^0C	394bp
A1	5' ATG GAC GCC GCC AGG AAA C 3'	5' CAC GCT TCC TCG G3'	54^0C	120bp
Argininosuccinate synthase (ASS)	5' CGA CAC TGA AGC TGT ACA AAG GTA 3'	5' GTC GTG GTG ATT CTA TAG GTG TTG3'	54^0C	123bP
Leishmania specific GAPDH	5'GAA GTA CAC GGT GGA GGC TG 3'	5'CGC TGA TCA CGA CCT TCT TC 3'	54^0C	206bp
Leishmania specific α-tubulin	5'TTT TTT TTC GCC CTC GTC G '3	5'GCC CCC CTC CAC AGA TAC AC 3'	55^0C	207bp
Human HPRT	5' CGA GAT GTG ATG AAG GAG ATG GG 3'	5' CCT GAC CAA GGA AAG CAA AGT CTG 3'	66^0C	303bp

6. Quantitative Real-time PCR analysis

cDNA was generated from the cleaned up total RNA samples using the Superscript II reverse transcriptase (Invitrogen) using 1μg oligo $(dT)_{20}$ primer following the manufacturer's instructions. SYBR Green real-time PCR gene expression assays were purchased from Applied Biosystems, Inc. (ABI) and were performed according to the manufacturer's instructions. For all qRT-PCR experiments, 20 μl reactions were performed in 96-well plates and contained 1XSYBR

Green Master Mix (Applied Biosystems), 200 nM gene-specific forward and reverse DNA oligo primers, and 0.01–1 ng of cDNA template. Reactions were cycled on an ABI Prism 7000 Sequence Detection System. The thermal profile consisted of three stages: (1) incubation at 50^{o}C for 2 min, (2) incubation at 95^{o}C for 10 min, and (3) incubation at 95^{o}C for 0.15 min, then at 60^{o}C for 1 min. The third stage was repeated for 40 cycles. A dissociation protocol was performed with 60^{o}C set as the starting temperature. Relative quantification was carried out using two *Leishmania* specific internal standards α-tubulin and GAPDH. Gene-specific oligonucleotide primers for qRT-PCR were designed with Primer Express Software (Applied Biosystems). Primer efficiency of each primer was determined by serial dilution of cDNA template in a series of qRT-PCR reactions and standard curve was plotted. A *C*t was determined for every reaction on the plate and relative fold change determined with respect to either α-tubulin or GAPDH. Every reported reading for a gene was the average of experiments run on at least three biological cDNA preparations with two replicates on each plate. A list of primer sequences is given below (Table 3.2). PCR efficiency was measured using a standard curve generated by serial dilutions of cDNA, starting with the initial cDNA concentration of 100 ng/µl which was serially diluted 10 fold (100ng, 10ng, 1ng, 0.1ng, and 0.01 ng) for the real time PCR assay for generating relative standard curve for quantitation. The PCR efficiency (E) was calculated by the formula: $E=10^{(1/-slope)}-1$, and ranged from 90-100% in the different assays. We used the $2^{-\Delta\Delta Ct}$ method to calculate relative changes in gene expression determined from real time quantitative PCR experiments as described earlier (ABI., 1997).

Table 3.2 List of genes and primer sequences used for Real time-PCR

Gene	Primer Sequence (5'→ 3')	Primer Sequence (3'→ 5')	Amplicon size (bp)
P27	CGGTCTCGGTGTTGGTGACG	CCAGATACGGCTCGTTCGC	261 bp
A1	ATGGACGCCGCCAGGAAAC	CACGCTTCCTCGG	120 bp
PSA-2	GCGATAGCCGAGGTGCAA	CGGGCTGTCGGTCCAAAG	106 bp
Ld Uba5	GGGCATCGTTGCTGGCTTCCT	CATGGCGTCGTAGCCGACATACTC	105 bp
MAPK	ATGACCTCCTATGGCATCGAC GGTGAGGTT	ATACACGTCTGTTATGTGATGGCG GTAGCGCA	215 bp
A2	TGCAGGAAGATCATTGCTTTGCGG	CGCTCGCCTGATGCACATATTCTT	162 bp
V type ATPase	AGTGGTGCATXGATCAGCGGT	CCTTTTTGCGCCATCAGAATGC	394 bp
α-Tubulin	TTTTTTTTCGCCCTCGTCG	GCCCCCCTCCACAGATACAC	207 bp
HPRT	CGAGATGTGATGAAGGAGATGGG	CCTGACCAAGGAAAGCAAAGTCTG	303 bp

Results

Comparison of gene expression profiles between promastigote and amastigote stage of *L. donovani*

Changes in mRNA abundance during differentiation of promastigotes into amastigotes were examined by genome-wide expression profiling using genomic DNA microarrays. The hybridized scanned array was subjected to analysis as described in Materials and Methods (Fig 3.2). Multiple replicates of all hybridizations were performed to account for sample heterogeneity and possible variation due to hybridization. A series of replicate experiments using the same RNA sample were done initially to obtain an estimate of the accuracy and precision of the system. Microarrays were hybridized with fluorescently labeled Cy3 and Cy5 cDNA, both prepared from promastigote RNA. This theoretically should give a log expression ratio (Cy5/Cy3) of 1 for all the elements arrayed on to the slide. A log10 plot of Cy3 (532nm) versus Cy5 (635nm) calibrated fluorescent response from a representative hybridization is shown in Fig. 3.3A. The plot shows tightly packed distribution of most genes along the line of best fit, with a regression correlation coefficient (r) of 0.99. When a similar experiment was conducted with

Cy3-labeled promastigote cDNA and Cy5-labeled amastigote cDNA, the distribution was not as tightly packed with a fraction of the points deviating from the line of best fit (Fig. 3.3B), indicating differential expression.

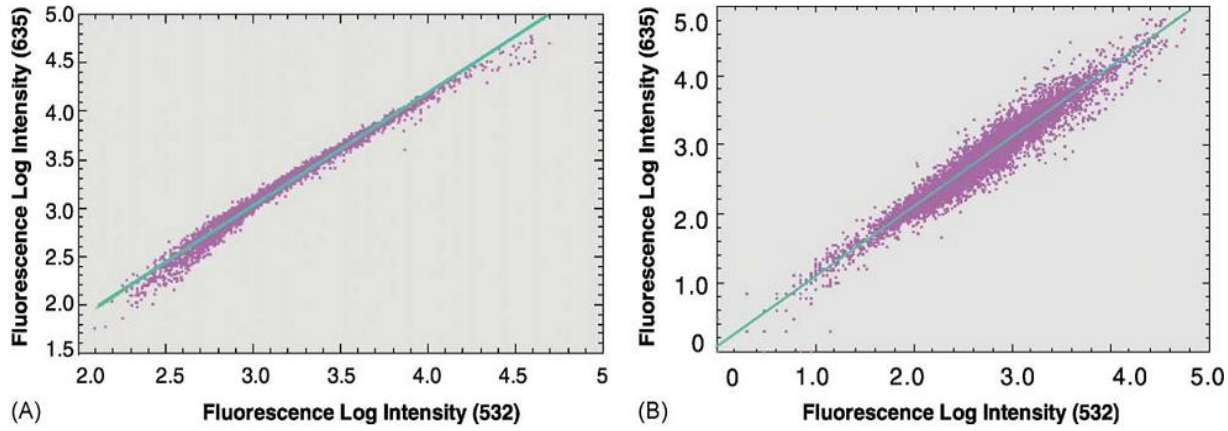

Fig. 3.3. Reproducibility of microarrays. A log10 plot of Cy3 (532nm) vs. Cy5 (635nm) calibrated fluorescent response from representative hybridizations for (A) Cy3-labeled promastigote RNA vs. Cy5-labeled promastigote RNA, and (B) Cy3-labeled promastigote RNA vs. Cy5-labeled Axenic Amastigote RNA. The line of best fit is shown in both panels.

In order to compare the relative contributions of experimental and biological variations, we compared the results above with those obtained from hybridization of cDNAs from RNA extracted with the same day Pro and Axe Am cultures (Fig. 3.4A) with those from different day pro and Axe Am cultures (Fig. 3.4B). The correlation coefficient (r) for the two duplicate experiments conducted with cDNAs from the same RNAs was 0.794 (Fig.4.4A), while RNA extracted from different cultures showed r of 0.787 (Fig. 3.4B). Thus, the experimental variation was similar to the biological variation.

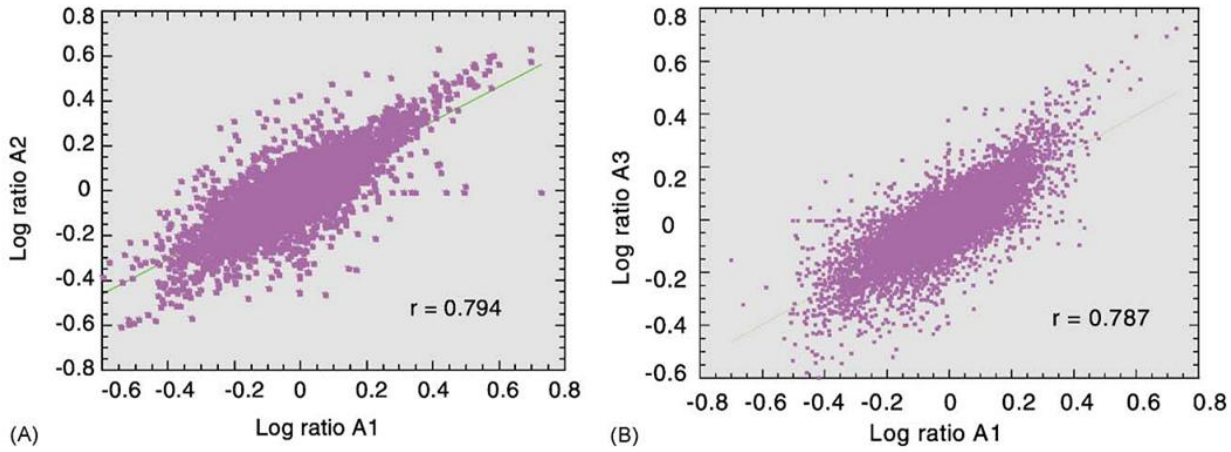

Fig. 3.4. Comparison of expression ratios in duplicate experiments. Log10 expression ratios (Cy5:Cy3) are plotted for arrays probed with Cy5-labeled Axe Am RNA vs. Cy3-labeled promastigote RNA. (A) Duplicate hybridizations (A1 and A2), using cDNA from the same pair of promastigote and Axe Am RNA samples. (B) Hybridizations (A1 and A3), using cDNAs from different pairs of RNA samples.

Replicate experiments with three biological preparations were performed comparing Axe Am with Promastigotes. A representative image of a hybridized microarray comparing Axe Am with Pro stage is shown in Fig. 3.5. Panel A shows the image of a hybridized array captured with laser scanner. Panel B represents enlarged view of the triplicate blocks from the array. The array quality control report was generated before going for further analysis; various criteria for quality control as defined by the software (GenePix Pro 5.0) were passed. The quality control report is shown in Table 3.3.

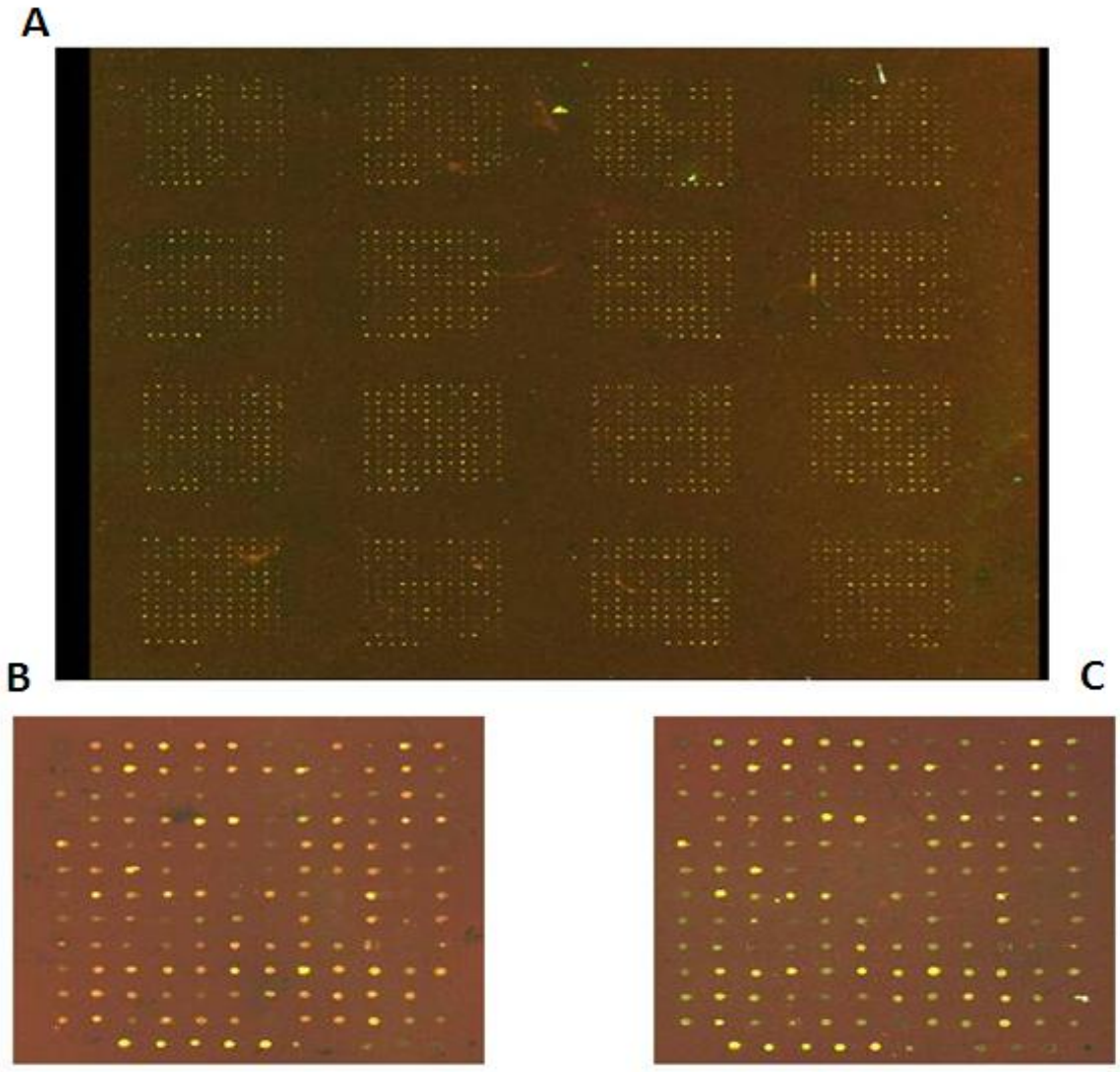

Fig 3.5 Microarray image using Pro vs. Axe Am RNA samples. Panel A shows the image of a portion of microarray with 16 blocks. Panel B and C show image of a single block from two dye-swap hybridizations, image captured with laser scanner. Panel B shows Axe Am labeled with Cy5 and Pro with Cy3, panel C shows the reverse. Spike-in controls are in the row at the bottom of each panel, position 1 to 5 from the left. The remaining spots in the control row are negative controls: 3XSSC, 3XSSC, Salmon DNA, tRNA, poly Adenosine, human cot-1 DNA

from left to right. Total RNA was used to synthesize Cy3 or Cy5 labeled cDNA and hybridized to the DNA chip under stringent conditions.)

Table 3.3: Array Quality Control Report

Scanned by:	GenePix 4100A 01 [100423]	**Analyzed by:**	GenePix Pro 5.0.1.24
Scanned on:	2/25/2006 12:13:16 PM	**GPS file:**	**PL052.gps**
Image wavelengths:	532, 635	**GAL file:**	4224CombinedPL3-1.txt
PMT:	417, 526 V	**Temperature:**	26.4 V
Laser Power:	2.2, 2.1 V	**Laser On-time:**	0, 0
Scan Power:	100, 100 %	**Barcode:**	None
Normalization Factors:	1, 1	**Normalization Method:**	None
Wavelength Image Files:	Not Saved		
Comment:		None	

Vital Statistics

	532	**635**	**Threshold**	**Results**	
				532	**635**
Median signal-to-background	2.9	2.8	> 2	**Pass**	**Pass**
Mean of median background	240.382	197.982	< 500	**Pass**	**Pass**
Median signal-to-noise	4.4	2.1	> 2	**Pass**	**Pass**
Median % > B+1SD	97	95	> 90	**Pass**	**Pass**
Feature variation	0.379	0.489	< 0.5	**Pass**	**Pass**
Background variation	0.454	0.433	< 0.5	**Pass**	**Pass**
Features with saturated pixels	0.28 %	0.37 %	< 0.5 %	**Pass**	**Pass**
Not Found features	930/13584 (6.8%).		< 7 %	**Pass**	
Bad features	253/13584 (1.9%).		< 7 %	**Pass**	

To adjust for unequal fluorescence intensities of the two RNA samples and to allow comparison from experiment to experiment, the data was normalized using Acuity 3.1 software. Normalization was carried out based on the premise that most genes on the array are not differentially expressed; therefore, the arithmetic mean of the ratios from every feature on the array is equal to 1. To produce a continuous distribution of up and down regulated spots, the ratios were transformed to the $\log_2$ scale. Further normalization to account for the systematic dependence of ratio on intensity was performed by locally weighted linear regression (LOWESS). Scatter plot of a representative hybridization comparing the expression in Axe Am and Pro following LOWESS normalization is shown in Fig. 3.6. From the scatter plot, it is seen that the majority of the log ratio values are clustered close to zero reflecting no change in expression as expected, with outliers representing the differentially expressed genes. Similar plots were obtained in all the hybridizations comparing promastigote with Axe Am RNA expression (Data not shown).

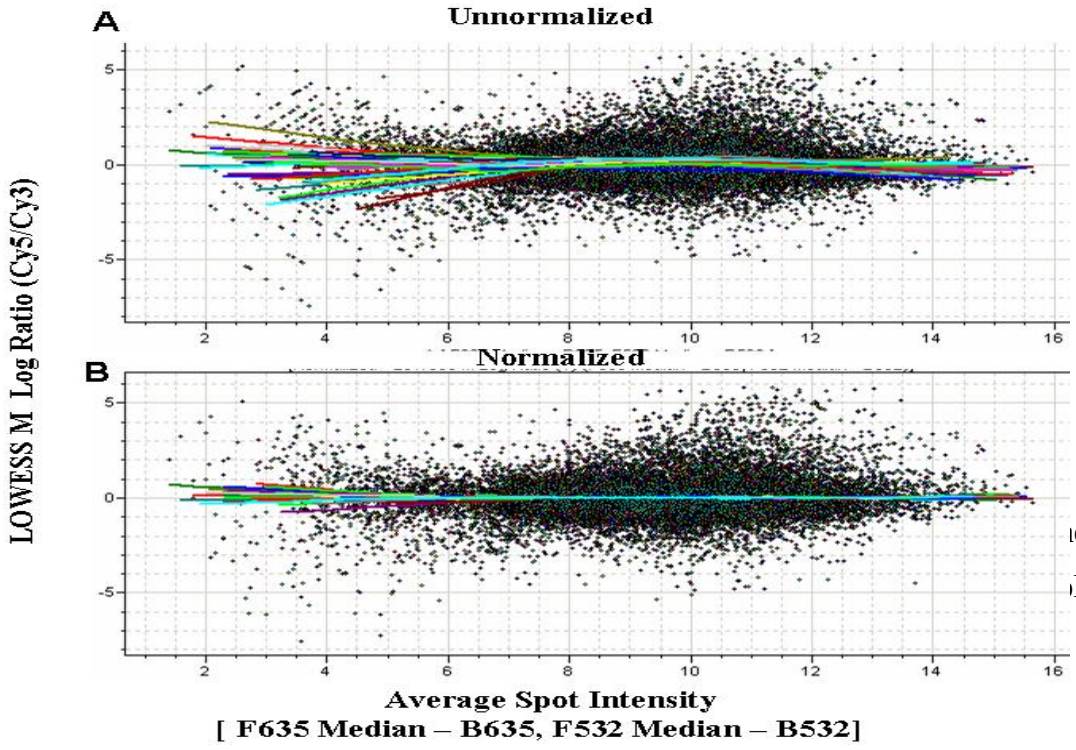

Fig. 3
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Analysis of microarray experiments revealed a number of DNA clones showing differential expression in Am/Pro microarrays (Table 3.4). Of the 4224 genomic DNA clones, those showing ≥ 1.7 fold differential expressions in either of *Leishmania* life cycle stages were considered for further analysis. Initially when a cut off of ≥ 2.5 fold was used, 0.59% (25/4224) clones showed differential expression in Am/Pro microarrays. However, by reducing the cut off value to ≥ 2 fold, there were 5.04% (213/4224) clones in Am vs. Pro microarrays showing differential expression. Of these, the clones showing significant and consistently higher expression with ratio ≥ 1.7, Z ratio >1.9 in three microarray hybridizations and reproducibility in dye flip microarray experiments were chosen for further analysis. Z-ratios are a direct measure of the likelihood that an observed change is an outlier in an otherwise normal distribution and are independent from their underlying intensity values. All the raw data for differentially expressed genes in Pro vs axe amastigote have been submitted to ArrayExpress (accession no. E-MEXP-866). Thirty eight clones ranking top in the fluorescence intensities with ratio ≥ 1.7 and reproducibility in replicate experiments were selected and sequenced for further analysis.

Table 3.4: Results of Microarray Experiments

Hybridization	Stage showing over expression	Clones with ratio >1.7	Clones with ratio > 2.0	Clones with ratio > 2.5
Am vs. Pro	Am	239 (5.65%)	130 (3.07%)	46 (1.09%)
Am vs. Pro	Pro	144 (3.41%)	83 (1.96%)	31 (0.73%)

Of the 38 clones, 28 were up regulated at the Ama stage (Table 3.5) while 10 were found to be down regulated at the Ama stage (Table 3.6). The identities of these genomic clones were assigned by homology to regions in the ORF, 5'UTR (upto ~ 500 bp) or in 3'UTR (within1.5kb) as listed in GeneDB/NCBI. The identities of these selected clones, their NCBI-GSS accession

numbers, mean expression ratios from three replicate arrays along with standard deviation, Z-ratios and the gene ontology category to which they belong are given in Tables 4.5 and 4.6. In addition to identifying homologues in *L. major*, the clone sequences were also searched by BLAST in the *L. infantum* genome. Based on their identities, putative functions were assigned to proteins of known function. The genes coding for proteins involved in basic cell metabolism for maintenance of life were grouped as maintenance genes whereas those coding for surface proteins were classified as surface genes. Kinases / phosphatases form the category of genes involved in the signaling cascasde and HSP/chaperonins represent the chaperone category. The category, intergenic region, consists of the clones that are homologous to a region in *L. major* genome which does not code for any protein as they are located outside the coding or untranslated region (5' or 3' UTR) of mRNA.

Table 3.5: Clones up regulated at Axe Am Stage

Gene ontology category	Clone ID & NCBI No.	Accession No. *L. major* Gene DB[a]	Am/Pro Median of ratios ± SD	Am/Pro Z ratio (p-value)	Gene identity
Signaling cascade	15B2* **CL844754**	**LmjF23.1665**	2.19±0.07	2.13 (0.01)	PA phosphatase, putative
	28F11 **ED004281**	**LmjF36.6470**	1.48±0.17	1.05 (0.00006)	MAP Kinase
	31C2 **ED004285**	**LmjF31.1530**	1.97±0.17	1.53 (0.002)	Protein kinase
	45F10 **ED004304**	**LmjF36.6470**	1.98±0.35	1.29 (0.03)	MAP Kinase
	65F10 **ED004318**	**LmjF04.1210**	2.98 ±0.51	2.08 (0.04)	Protein kinase, putative
Chaperone	28F12 **ED004282**	**LmjF26.0620**	2.44±0.20	2.07 (0.02)	HSP 10, putative
	16C11 **ED004268**	**LmjF33.0314**	2.36±0.13	2.71 (0.03)	HSP 83
Surface protein	65G7 **ED004319**	**LmjF36.0430**	2.27±0.16	2.00 (0.03)	ABC1-like protein
	18D8 **ED481185**	**LmjF35.0520**	4.83±0.63	3.30 (0.005)	Proteophosphoglycan 4
	42A11# **ED004298**	**LmjF12.0910**	3.27 ± 0.07	1.87 (0.001)	PSA-2
	14D7 **CL844745**	**LmjF08.0670**	3.10±0.53	3.64 (0.04)	Amastin-like protein
	29B8 **ED004283**	**LmjF31.0450**	2.95±0.38	3.24 (0.02)	Amastin, putative
Maintenance Protein	40B11 **ED004292**	**LmjF36.1960**	2.25±0.33	1.31 (0.007)	Phosphomannose mutase, putative
	22C10 **ED004275**	**LmjF35.0980**	3.1±0.29	3.38 (0.01)	Aldose-1 epimerase like protein
	35C8 **ED004287**	**LmjF31.0410**	3.34± 0.34	2.38 (0.004)	Calpain-like cysteine Proteinase, putative
	46F7 **ED004306**	**LmjF35.SnoRNA.0053**	2.73 ±0.48	3.00 (0.002)	Spliced leader RNA
	29C8 **CL844612**	**LmjF15.0970**	3.49±0.24	5.79 (0.002)	NAD/FAD dependent dehydrogenase
	41B10 **ED004295**	**LmjF18.0450**	2.48 ±0.39	2.03 (0.005)	Serine carboxypeptidase CBP-1, putative
Hypothetical protein	46G8 **ED004307**	**LmjF28.0980**	3.61±0.26	3.94 (0.003)	Hypothetical protein
	14C7 **ED004266**	**LmjF22.0640**	2.53±0.34	2.00 (0.01)	Hypothetical protein
	45E11 **ED004302**	**LmjF29.2760**	2.89±0.20	3.42 (0.02)	Hypothetical protein
	36G8 **ED004289**	**LmjF19.0490**	1.90±0.12	1.26 (0.002)	Hypothetical protein
	16D8 **ED004269**	**LmjF19.0980**	4.11±0.46	2.64 (0.005)	Hypothetical protein
	63C5* **ED004316**	**LmjF09.0640**	2.80±0.56	2.15 (0.05)	Hypothetical protein
Intergenic region#	34E12 **ED004286**	**LmjF33**	2.18±0.11	1.98 (0.0005)	Intergenic region
	61B6 **ED004313**	**LmjF31**	1.70±0.20	1.15 (0.01)	Intergenic region
	43E10 **ED004299**	**LmjF36.0010** **LmjF35.0010** **LmjF31.3190** **LmjF25.2450**	2.24±0.11	2.07 (0.005)	Intergenic region b/w histone H4 & Phosphoglycan β-1,3-galactosyltransferase
	53H4 **ED004312**	**LmjF34**	7.27±0.32	8.08 (0.002)	Intergenic region

[a] The sequences of clones were BLAST analyzed in *L.major* and *L.infantum* gene databases. All clones aligned with the respective *L. major* sequences with an E-value of 10^{-40} or smaller.

∗ Clones showing different alignment in *L. infantum* database - 15B2 - calpain-like protein, 63C5 - Zn finger domain-like protein.

Classified as intergenic region as the distance between two ORFS is large and therefore no ORF could be assigned to these DNA clones.

Table 3.6: Clones down regulated at Axe Am Stage

Gene ontology category	Clone ID/ NCBI No.	Accession No. *L.major* Gene DB[a]	Am/Pro Median of ratios ± SD	Am/Pro Z-ratio (p-value)	Gene Identity
Maintenance Protein	23G11 **ED004279**	**LmjF09.0120**	0.38±0.57	1.78 (0.002)	Kinesin, putative
	13H8 **ED004265**	**LmjF05.1040**	0.35±0.27	1.32 (0.0002)	Stomatin-like protein
	44D6 **ED004300**	**LmjF30.3660**	0.4±0.98	2.72 (0.0002)	V-type ATPase, C subunit, putative
	67B10 **ED004320**	**LmjF35.0420**	0.48±0.47	2.06 (0.0009)	40S ribosomal proteinS3a, putative
	41D6 **ED004296**	**LmjF36.3880**	0.34±0.27	1.88 (0.005)	Eukaryotic translation initiation factor 3 subunit, putative
	68D7 **ED004322**	**LmjF22.0090**	0.43±0.92	1.85 (0.03)	Methyltransferase, putative
Hypothetical protein	23G7 **ED004280**	**LmjF27.0600**	0.22±0.32	2.70 (0.003)	Hypothetical protein, conserved
	23A3 **ED004275**	**LmjF34.1390**	0.39±0.43	2.06 (0.002)	Conserved hypothetical protein
	23A11 **ED004276**	**LmjF34.4110**	0.28±0.20	2.51 (0.003)	Conserved hypothetical protein
	23B8* **ED004278**	**LmjF25.1445**	0.36±0.63	1.75 (0.01)	Conserved Hypothetical protein

∗ Clone 23B8 aligns to peptide chain release like protein in *L.infantum* database.

Validation of expression pattern of Ama up regulated genes with Reverse transcriptase PCR and Northern blots:-

The expression changes in selected representative clones were verified by RT-PCR and Northern hybridizations. Of the genes differentially expressed in amastigote stage, total 11 genes were selected on the basis of the homology search (NCBI and Gene DB) out of which 5 clones were tested with Northern hybridizations in two patient isolates (Fig. 3.7) and 6 other genes were verified using Reverse transcriptase PCR in three different patient isolates of *L. donovani* (Fig. 3.8). Two amastigote stage up regulated genes A1,amastigote up regulated gene (Sreenivas et al., 2004) and ASS, Argininosuccinate synthase, identified in previous gene differential expression studies from our lab in *L.donovani*, were also included in the study. The genes taken for validation are 29C8: Ubiquitin activating E1 like Uba5 (LdUba5), ASS, 46G8: mitochondrial membrane protein of 27 kDa (P27), 49A11: Parasite surface antigen 2 (PSA2), 28F11: MAP kinase (MAPK), 35C8 (Calpain-like cysteine peptidase), 15B2 (Conserved hypothetical protein), 36G8(both are hypothetical proteins), 28F12 (heat shock protein 10), 45E11 (Conserved hypothetical protein) and 40B11 (Phosphomannose

mutase). Northern blots and Reverse transcriptase-PCR with different parasite lines gave similar expression levels. Though the fold changes observed by Northerns and Reverse transcriptase-PCR were different from those seen in microarray results, the expression patterns were found to be similar to microarray results. PCR reactions were also carried out with parasite RNA without a reverse transcriptase step for all of the above mentioned primer sets, but no amplification was seen with RNA indicating the absence of DNA contamination in the RNA samples.

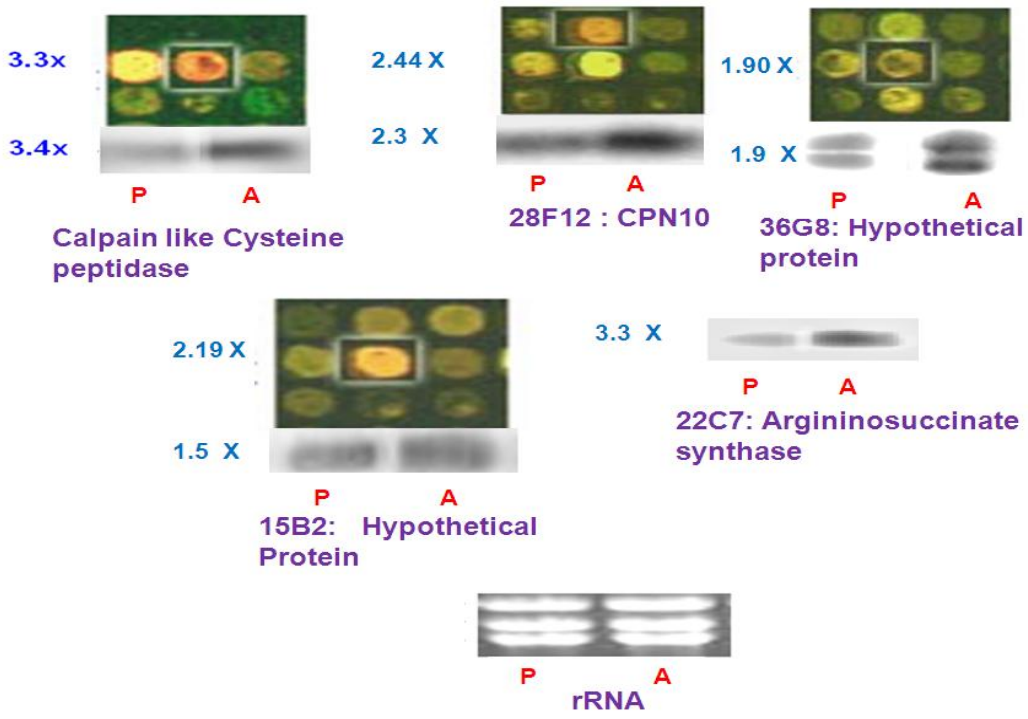


Fig 3.7 Northern blot analysis of expression in Pro and Ama stages. The results are shown from representative microarrays (top panels) and Northern blots (bottom panels) for DNA elements which were found to be up regulated in the Axe Am stage. The DNA element is indicated by the boxed spot in the top panels and the name below the bottom panels. In the bottom panels P denotes Promastigote RNA and A denotes Axe Am RNA.

The ratio of mRNA level in Axe Ama to Pro RNA as determined by both methods is indicated to the left of each panel.

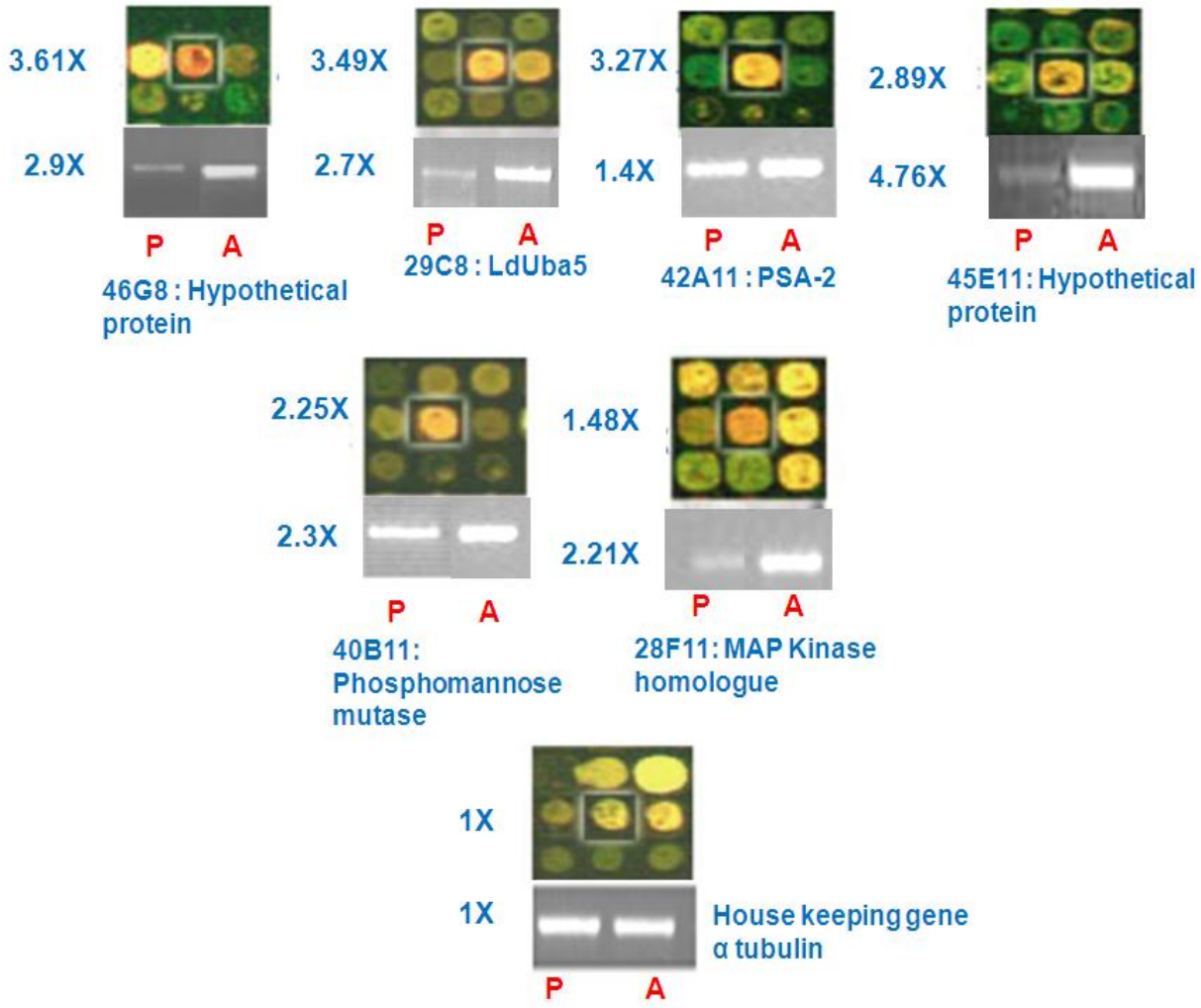


Fig 3.8 Reverse transcriptase (RT) PCR confirmation of microarray results. The results are shown from representative microarrays (top panels) and RT PCR (bottom panels) . The element is indicated by the boxed spot in the top panels and the name below the bottom panels. In the bottom panels P denotes Promastigote RNA and A denotes Axe Am RNA. Expression pattern was verified in three different field isolates of *L. donovani* and representative picture with the RNA of the parasite used in the microarray is shown. The RNAs from the two stages Promastigote (Pro) and Axenic

Amastigote (Axe Am) were reverse transcribed and amplified by gene-specific primers using α- tubulin for normalization . Fold changes at the two stages are indicated to the left of each panel.

Quantitative Real Time PCR for Transcript demonstration in hamster derived amastigotes

Our microarray study had been carried out with axenic amastigotes. As they are not the true amastigotes we next investigated the expression of selected genes in the true/hamster derived amastigotes. Hamsters were infected with *L.donovani* K59 strain and after two months amastigotes were isolated from the spleen of the infected hamsters and RNA is prepared as discussed in earlier section. Out of 12 Axe Am stage up regulated genes, 6 genes (*Ld*Uba5, ASS, P27, PSA2, MAPK and A1) were further selected through bioinformatic analysis which may include novel virulence factors. The expression was then further verified using relative quantification study between the three stages Pro, axenic Am and hamster derived amastigotes. The relative fold change in expression at different stages was determined with respect to promastigotes using either *Leishmania* specific α-tubulin or *Leishmania* specific GAPDH as internal controls. Initially, primer efficiency of all the primer pairs were determined using absolute quantification method and slope of each primer pair was observed to be ≥ -3. A representative slope with one of the primers is shown in Fig. 3.9.

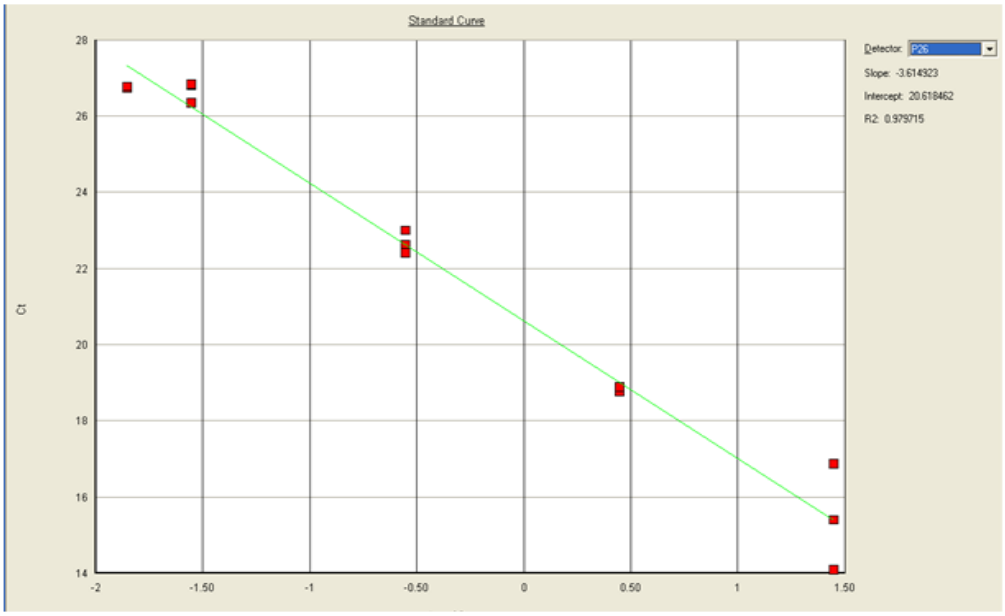


Fig 3.9 Standard curve for determining primer efficiency of *Ld*Uba5. The cDNA concentration is plotted against Ct values.

After determining the efficiency of the primers, the relative fold changes of various genes were determined by relative quantification study using ΔΔCt method. A representative amplification plot is shown in Fig. 3.10. It is seen from the figure that the endogenous controls have similar Ct values at various stages suggesting equal amounts of RNA in the samples. The relative fold change of each gene at different life cycle stages were determined in three different biological preparations and duplicates were included in each plate. Table 3.7 summarizes the relative fold change of each gene at lesion derived and axenic amastigote stages with respect to promastigotes using GAPDH and α-tubulin as endogenous controls. The expression pattern observed by Real time PCR was found to be similar to microarray results.

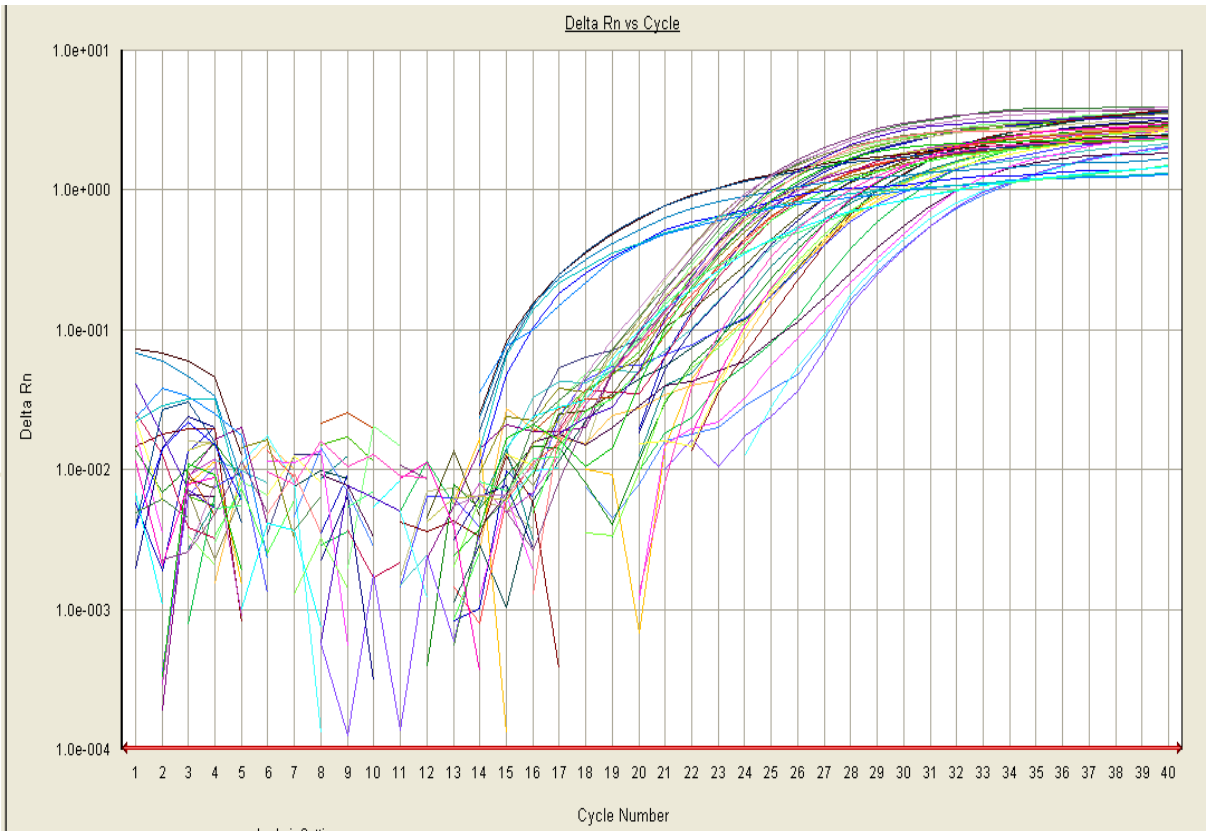


Fig 3.10 Amplification plot showing Delta Rn is plotted against cycle number.

Table 3.7 Relative quantification using either GAPDH or α- tubulin as endogenous control

S. No.	Gene	Endogenous control	Fold Change		
			Promastigotes	Axenic amastigotes	Hamster-derived amastigotes
1.	P27	GAPDH	1	12.1 ± 4	3.075 ± 0.38
		α- tubulin	1	16.2 ± 2.7	2. 08 ± 0.73
2.	*Ld* UBA5	GAPDH	1	12.31 ± 3.1	2.35 ± 0. 35
		α- tubulin	1	14.98 ± 3.6	2.24 ± 0.49
3.	ASS	GAPDH	1	1.88 ± 0.2	2.11± 0. 13
		α- tubulin	1	1.61 ± 0.2	2.98 ± 0.18
4.	PSA2	GAPDH	1	1.6 ± 0.98	3.15± 0.01
		α- tubulin	1	2.5 ± 0.82	3.99 ± 0.01

5.	MAPK	GAPDH	1	2.6 ± 0.98	3.15± 0.01
		α- tubulin	1	3.5 ± 0.82	2.99 ± 0.01
6.	A1	GAPDH	1	4.3 ± 2.10	2.34 ± 0.38
		α- tubulin	1	3.35 ± 0.57	1.98 ± 1.67

Gene Transcripts in VL lesion tissues

We next sought to check if gene transcripts of selected *Leishmania* genes up regulated in Axe Am and hamster derived Ama is detectable in bone marrow of VL patients. The genes selected for the study were *Ld*Uba5, ASS, *Ld*p27, PSA2, MAPK, A1 & using A2 (amastigote-specific protein) (Ghedin et al., 1997) gene as a positive control. The presence of *Leishmania* RNA in the lesion derived RNA samples was confirmed by using *Leishmania* specific α-tubulin primers. Transcripts were demonstrated in human bone marrow samples of VL patients (n=6) by RT-PCR, representative data is shown in Fig. 3.11. RNA for *Leishmania* specific α-tubulin was detectable in all the VL^+ samples but absent in VL^- control (n=2). The transcripts of P27, Ld Uba5, ASS, PSA-2, MAPK and A2 were detected in 6/6 (100%) while A1 transcripts were detected in 5/6 (75%) of the VL^+ bone marrow samples tested. The gene transcripts of promastigote specific gene V-type ATPase (Ref) were not detectable in the human bone marrow samples (Fig 3.11).

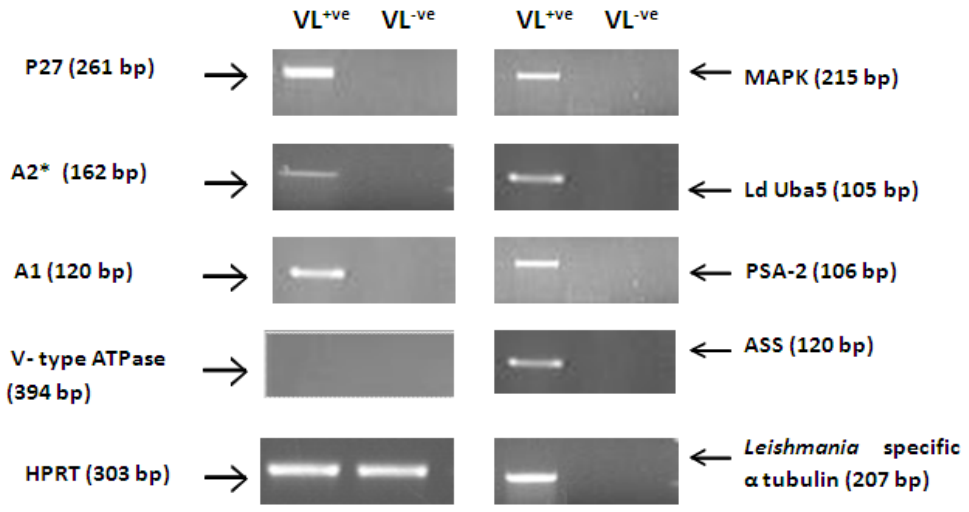


Fig 3.11: RT-PCR of selected genes with human bone marrow samples. Bone marrow RNA from 4 VL^+ and 1 VL^- were used for the study.
* Known amastigote specific gene A2 was included as a positive control.
#RNA samples were normalized using human HPRT gene.

Expression of selected genes in VL, PKDL and CL infection

The relative abundance of these gene transcripts was assessed in VL bone marrow samples, skin lesions of PKDL as well as CL patients by real-time RT-PCR, using human HPRT as endogenous control. The relative fold change in expression of different genes was determined with respect to *Leishmania* specific α-tubulin. Initially, primer efficiency of all the primer pairs were determined using the absolute quantification method and slope with each primer pair was observed to be ≥ -3 (Data not shown). Analysis of the real- time PCR experiments revealed P27 to be 7081.7 ± 4266 folds higher than α-tubulin in the bone marrow of VL patients. The expression of other genes were in the order MAPK (4758.04 ± 2396.29) > A1 (2623.10 ± 1015.07) > PSA-2 (1599.33 ± 550.85) > ASS (1539.33 ± 445.36) > Uba5 (441.85± 195.36) > A2 (376.24 ± 97.56) (Fig.3.12).

The expression of these genes were compared in bone marrow samples of VL patients and in the skin lesions of PKDL patients. Our results showed PSA-2 transcripts

to be most abundant in the PKDL tissues followed by P27 and A1. Approximately 8 fold higher abundance of PSA-2 transcripts was found in PKDL tissues compared to VL tissues. The expression of Uba5, ASS and A2 was found to be less than α-tubulin in PKDL tissues, though these were abundant in bone marrow samples in VL patients. Evaluation of gene expression in the skin tissues of CL patients depicted that the expression of MAPK and A1 were significantly higher in CL lesions compared to PKDL though it was significantly lower than that in VL lesions. Transcripts of P27 were abundant in the tissues of all patients irrespective of disease pathology. The expressions of Uba5, ASS and A2 were low in CL similar to that observed in PKDL. PSA-2 gene transcripts were not detectable in CL patient samples.

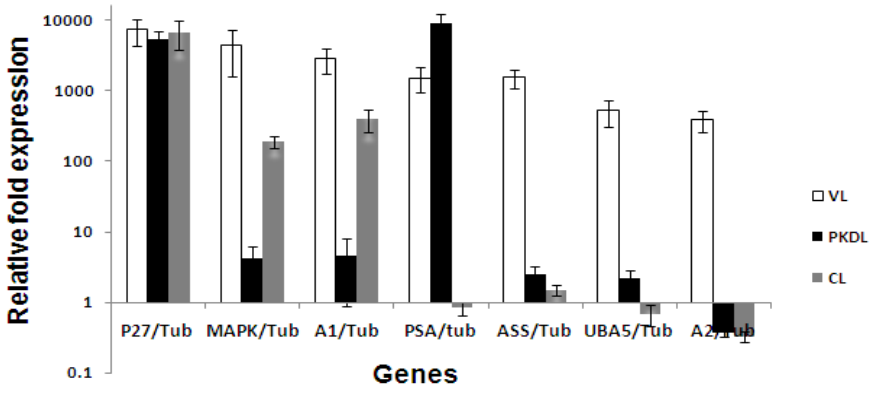


Fig 3.12 Transcription levels of p27,MAPK,A1,PSA-2, ASS, Uba5 and A2 across VL, PKDL and CL. The samples were normalized using human HPRT gene. The results were expressed in terms of mean of expression +SE. *In case of VL, mean of expression in 3 samples were taken for the A1 gene.

Discussion

Promastigote-to-amastigote differentiation in *Leishmania* is a complex process, but the morphological changes that take place appear to be well-coordinated and regulated (Barak et al, 2005). DNA microarray analysis has become a popular and powerful method for examining changes in gene expression during differentiation and/or

adaptation to new growth conditions (DeRisi et al., 1997; Jiang et al., 2001; Bozdech et al., 2003; Maeda et al., 2003; Smith and Greenfield, 2003; Wagner et al., 2005). This approach has been used previously to compare the different life cycle stages (procyclics, metacyclics and axenic amastigotes) of *L. major* (Saxena et al., 2003; Akopyants et al., 2004; Almeida et al., 2004), *L. donovani* (Duncan et al,2004; Saxena et al., 2007), *L. infantum* (Mcnicoll et al,2006), and *L. mexicana* (Holzer et al.,2006). There are limited studies in case of *L. donovani* and work presented here represents the first comparative gene expression profiling of promastigote with axenic amastigotes, followed by validation of expression with hamster derived amastigotes and directly in tissue lesions of VL patients. Further the comparative assessment of the parasite gene expression in infected tissues of VL, PKDL and CL was carried out, which we believe is particularly relevant and important since the genes involved in the different pathologies would be expected to include a prospective set of genes required for persistence of parasites in infected hosts.

The present study is focused on differences in gene expression that can be seen as the visceral parasite, *L. donovani*, differentiates from the life cycle stage found in the insect vector (Pro) into the human infectious stage (Am). DNA microarrays either in the form of random genomic fragments or cDNA have proven their utility in identifying differentially expressed genes in *Leishmania* (Saxena et al., 2003; Holzer et al., 2006). Genomic arrays have the advantage over cDNA arrays that the genes represented on the array are not biased by the expression levels in the RNAs used for microarray construction. Therefore, to screen for expressed genes in an unbiased way we utilized a microarray constructed of random genomic fragments. Furthermore, to ensure representation of important virulence associated genes, we prepared the genomic clones

from parasite DNA obtained from a strain derived from a kala-azar patient, after minimum number of *in vitro* passages. Use of microarray technique allowed us to measure differential RNA levels over a large portion of the uncharacterized genome. Microarray analysis with Pro vs Axe Am revealed a modest number of transcripts showing up- or down regulation, which is in line with the small-scale changes found throughout the infectious cycle in *Leishmania* (Akopyants et al., 2004). This is as expected as *Leishmania* relies more heavily upon regulatory mechanisms at the protein level than upon changes in mRNA abundance to control gene regulation (Beverley et al., 2002). Changes in mRNA abundance revealed by the microarray, though moderate, are likely to point toward changes that, when further amplified by differences in translation and post-translational modification, will result in the ultimate phenotypic changes that determine the traits of interest. In kinetoplastids, most protein coding genes are transcribed as large polycistronic molecules, initiated without defined promoters, and subsequently processed into mature mRNA (Myler et al., 2000; Clayton, 1999). The abundance of mRNA species is controlled largely by its stability, which has been shown to be regulated by sequences in the 3' untranslated region (Furger et al., 1997; Wu *et al.*, 2000; D'Orso et al., 2003). Consequently, we checked for the presence of 450 nucleotides regulatory sequence in our amastigote up regulated genes. Our analysis revealed that except for amastin which showed 66% homology with this sequence, all other amastigote up regulated genes did not contain this sequence suggesting that the presence of this regulatory sequence may not be a pre-requisite for stage-specific expression.

The expression of a variety of genes was found to be modulated, the prominent ones being the surface molecules, kinases and maintenance genes. Of the axenic

amastigote stage over expressed genes, the putative signal-transducing molecules such as protein kinases (clones 28F11, 31C2, 45F10) showed higher expression. Protein kinases and phosphatases, and their regulation, are likely to be critical for differentiation and proliferation of *Leishmania*. In eukaryotes, the signal transducers mediate phosphorylation and dephosphorylation of serine, threonine and tyrosine residues in proteins and function as control switches in cellular networks. The increase in the expression of these protein phosphorylation molecules suggest an important role of these enzymes in the amastigote stage that may contribute to virulence.

Apart from protein phosphorylation, another important phenomenon that occurs during the temperature increase as the parasite travels from the insect to the mammalian host is the production of various heat shock proteins (HSPs) that may protect the parasite against the adverse effects of elevated temperature by chaperoning proteins. Earlier studies have reported *L. donovani* CPN10/HSP10 to be expressed preferentially under heat stress and in axenic amastigotes unlike HSP70 and HSP90 that show high constitutive abundance and only a marginal increase in the amastigote stage (Brandau et al., 1995; Zamora-Veyl et al., 2005). In our array data, CPN10 (clone 28F12) and HSP83 (clone 16C11) were both up regulated in axenic Am stage, indicating their significant role during Am stage.

The expression of surface genes amastin (clones 29B8 and 14D7), Promastigote surface antigen (PSA 2) (clone 42A11 and 49A11) and proteophosphoglycan (PPG, clone 18D8) was found up regulated during the amastigote stage. These genes are known to be expressed specifically at the amastigote stage in *L. d. infantum* (Piani et al., 1999; Boucher et al., 2002) and are thought to play a role in the intracellular survival and pathogenesis in the mammalian host. The PSA-2 cell surface protein gene which showed

1.8 – 3.2 fold higher expression at amastigotes in comparison with promastigotes suggest an increase in complexity of the cell surface that is at the interface with the harsh environment of the phagolysosome. PSA-2 of *Leishmania* has previously been shown to increase about 30-fold as *in vitro* cultured parasites progress from logarithmic to stationary phase, growth phases that are, respectively associated with parasites having low and high infectivity to mammals (Lincoln et al., 1999; Beetham et al., 2003). These antigenic proteins might have a role in immune evasion during insect to mammal transition.

The membrane bound kinesin (clone 23G11) and V-type ATPases (clone 44D6) were found to be over expressed in Pro in our microarray analysis. Kinesins perform a variety of cellular functions that include cell division, signal transduction, microtubule dynamics and trafficking of macromolecular complexes and organelles including mitochondria, lysosomes and synaptic vesicles (Rodrigues et al., 1999). V-type ATPases are present in acidocalcisomes of *Leishmania* and play a role in pH homeostasis. This molecule is believed to protect the parasites from osmotic shock inside the sandfly. The expression of these genes correlated with the known functions of these genes at the two stages (Dutoya et al., 2001).

From our observation, among the 38 identified genes, 12 genes were maintenance genes. The maintenance category included some of the enzymes required for the cell's metabolism and maintenance. In amastigotes, a substantial number of maintenance genes were down regulated while other genes were up regulated. As the parasite differentiates from Pro to Am, there is a shift in the metabolic pathway due to differences in the environment and consequent change in nutrient availability at different stages. Thus, a large turnover in the expression of these enzymes during the process of differentiation is

not unexpected. However, the increased expression of the enzyme *Ld*Uba5 (clone 29C8) in Axe Am stage and relatively very low expression in Pro suggests this molecule to be an extremely important molecule involved in parasite's survival in the mammalian host. Homology search (NCBI) had shown this clone to be homologous to human Uba5 (E1-like ubiquitin-fold modifier) which is involved in an ubiquitin-fold modifier 1 (Ufm1) linked pathway. The Ufm1, one of a variety of ubiquitin-like modifiers, covalently attaches to target proteins via Uba5 and Ufm1-conjugating enzyme 1 (Ufc1), which are analogous to the E1 and E2 ubiquitylation enzymes, reported in humans. Ufm1-related proteins are conserved in metazoa and plants but not in yeast. (Komatsu et al, 2004)

From our array work, 8/38 identified genes were identified as hypothetical genes that showed differential expression at either of *Leishmania* life cycle stages. This can be explained by the fact that a large proportion of the differentially expressed genes comprised of hypothetical proteins indicating the extent of genetic difference between this parasite and the other well studied organisms. In the recently completed *L. major* genome, 4673 (>50%) out of 8370 coding sequences are conserved hypothetical proteins [www.geneDB.org; Ivens *et al.,* 2005] meaning no homologue that is functionally characterized has been found in other organisms. Characterization of some of these genes may help in studying the processes involved in *Leishmania* pathogenesis.

Total 12 genes, 10 genes from the microarray (pro vs. axe) *Ld*Uba5, P27, PSA2, MAPK, 35C8, 15B2, 36G8, 28F12, 45E11 and 40B11 and two genes A1 (Sreenivas et al., 2004) and Argininosuccinate synthase (ASS) form previous differential expression studies of our lab were selected for validation and further studies because they showed higher expression in the axenic amastigote stage than the promastigote stage in *L.donovani*. On the basis of homology search (NCBI and GeneDB) 6 out of 12 genes,

ASS, *Ld*Uba5, P27, PSA-2, MAPK, and A1 were taken further for verification in hamster derived amastigotes and in tissue lesions of patients suffering from VL, PKDL caused by *L.donovani* and CL by *L.tropica*. These genes may be required for survival in the amastigote stage and some of them may be directly responsible for disease pathogenesis.

Species-specific differential gene expression may be attributed to a dynamic mode of regulation, which is suitable to adaptations to different insect vectors and life cycle features, different target tissues, and distinct disease pathogenesis. Commonly, genes up regulated *in vivo* may fulfill essential features of the parasite such as metabolism, motility, infectivity and interaction with the host. A recent study had shown that only 10–12% of the life stage regulated genes were common to both *L. major* and *L. infantum* (Rochette et al., 2008). A central question in the study of *Leishmania* pathogenesis is why some species cause cutaneous pathology while others migrate away from the site of infection and cause fatal visceral infections. Another question which needs to be resolved is why same species causing VL (*L.donovani*) in the visceral organs migrate to dermal region in the sequel of VL i.e. PKDL. Different virulence factors have been identified for distinct *Leishmania* species (McMahon and Alexander., 2004), and there are profound differences in the immune mechanisms that mediate susceptibility/resistance to infection and in the pathology associated with disease. These variations not only point to interesting features of the host–pathogen interaction and immunobiology of this genus of parasitic protozoa, but also have important implications for immunotherapy and vaccine development (McMahon and Alexander.,2004). We believe that the comparison of parasite gene expression in infected tissues of VL, PKDL and CL is particularly relevant and important since the genes involved in the different

pathologies would be expected to include a prospective set of genes required for persistence of parasites in infected hosts.

Our finding that P27, a trypanosomatid amastigote specific protein of M_w of 27 KDa is abundantly expressed in VL, PKDL and CL suggests that the gene is active in all the three forms of the disease and may have some essential metabolic role in the intracellular form of *Leishmania* that is required for the survival of the pathogen inside the host cell, irrespective of the phenotype of the disease. Further studies of this gene product should shed light on its role in the pathogenesis of all the three forms of *Leishmaniasis*.

Expression of MAPK was substantially higher in VL when compared with PKDL, while it was of intermediate level in CL. Protein kinases and phosphatases, and their regulation, are likely to be critical for differentiation and proliferation of *Leishmania*. A previous study had shown MAPK to be essential for the survival of *L .mexicana* in the infected host by affecting the cell division of the amastigotes (Wiese, 1998). Low expression of MAPK kinase in PKDL may be attributed to the unique feature of the causative parasites since they donot differentiate from promastigotes, but are visceral amastigotes that transition to cutaneous amastigotes.

PSA-2 gene showed higher expression in PKDL as compared to VL, extending our earlier results which showed up regulation of PSA-2 in parasites isolated from PKDL lesions as compared to those from VL bone marrow lesions, at transcript as well as protein levels (Salotra et al., 2006), while in CL the expression of PSA-2 was very low as compared to VL and PKDL. Glycoproteins on the surface of the *Leishmania* play important roles in parasite survival in both the amastigote and promastigote life stages. Among the glycoproteins, PSA-2 is one of the major classes of membrane

proteins present at the surface of the parasitic protozoan *Leishmania*. While it harbours leucine rich repeats, which are suggestive of its involvement in parasite-to-host physical interactions, its exact role is largely unknown (Devault and Banuls., 2008). Further, the role of PSA-2 in parasite attachment and invasion of macrophages and its association in resisting complement-mediated lysis was also demonstrated (Kedzierski et al., 2004; Lincoln et al., 2004). High expression of PSA-2 in PKDL lesions may promote degradation of extracellular matrix proteins as well as resistance to complement mediated lysis which may allow the sequestered parasite to move to and proliferate at the skin surface (Salotra et al., 2006). An understanding of this persistence is particularly relevant for Indian patients where PKDL is known to develop even after 20 years of cure from VL.

Uba5 which belongs to the family of ubiquitin activating enzyme E1 (Komatsu et al., 2004) had abundant expression in VL lesions where as the expression was low in PKDL lesions. The pattern of expression of Uba5 was similar to that previously observed by us in parasite isolates from VL and PKDL patients (Salotra et al., 2006). The expression of Uba5 gene was barely evident in dermal lesions of CL patients being even lower than the expression in dermal lesions in PKDL. The ubiquitin fold modifier 1(Ufm1) linked pathway, one of a variety of ubiquitin like modifiers (Ubls) is recently discovered in humans (Komatsu et al.,2004). Homologs of 6 out of 9 major UBL families, including ubiquitin, Nedd8 (neural precursor cell-expressed developmentally down regulated 8), small ubiquitin related modifier (SUMO), Hub1, ubiquitin related modifier-1 (Urm 1) and autophagy-8 (Atg 8) are identified in parasitic protozoa (Ponder and Bogyo., 2007). Of the identified families of Ubls, only ubiquitin and Atg8 have been characterized in parasitic protozoa including *Leishmania* (Ponder and Bogyo., 2007). As

there are many types of E1 like enzymes involved in as many different Ubiquitin dependent pathways in protozoa and other eukaryotic cells, the data indicates that the Ufm1 pathway may have a role in VL. Protein modifications mediated by UFM1 and the consequent physiological alterations are currently unknown. Presumably such conjugation of UFM-1 might result in a wide variety of changes including variations in protein turnover or sub cellular localization of the target proteins. Identification of proteins that are targets for modification by UFM-1 should illuminate further the role of UBA5 in VL.

Expression of ASS gene was highest in VL, while in CL and PKDL it was low. Argininosuccinate synthetase (ASS) catalyzes the synthesis of argininosuccinate from citrulline and aspartate which is then cleaved by argininosuccinate lyase (ASL) to produce L arginine and fumarate. L-arginine has long been identified as an essential amino acid for *Leishmania* growth and activities of iNOS and arginase can be greatly influenced by its availability. Therefore, L-arginine transport from the extracellular milieu is critically important for parasite growth and killing within an infected cell (Wanasen and Soong.,2008).

Expression of the A1 gene was highest in VL, while in CL it was low but higher than in PKDL. Our results are consistent with the previous results which have shown the presence of A1 gene in different species of *Leishmania* including *L. tropica* (Sreenivas et al., 2004). Earlier *in vitro* studies had shown significantly higher abundance of the A1 mRNA in amastigote as compared with the promastigote stage suggesting its role in amastigote physiology, the present studies demonstrating its transcripts in VL lesions further point to its role *in vivo*, although the exact function of A1 remains to be investigated.

A2 corresponds to the amastigote-specific protein (A2) which was found to be expressed in the VL and negligible in the CL and PKDL cases. Our results are consistent with the earlier findings which revealed that *L. donovani* A2 protein coding sequences are not present in the genome of all *Leishmania* species and notably appeared to be absent in *L. major* and *L. tropica* (Ghedin et al., 1997). Interestingly, the *L. donovani* A2 gene (pseudogenes in *L. major*), is the only gene implicated so far in disease tropism (Zhang et al., 2003). Earlier studies with BALB/c mice had shown that A2 plays a role in the visceralization of infection associated with *L. donovani* (Zhang and Matlashewski., 2001). Current study confirms the role of A2 in human VL. Role of A2 in visceralization is further emphasized by the striking difference in its expression between VL and PKDL where the causative species is the same. Loss of expression of A2 could be an important factor facilitating the change to PKDL phenotype where the parasite does not visceralize and resides in the dermis.

The six genes evaluated in this study were selected because they showed higher expression in the amastigote stage than the promastigote stage in *L. donovani*. The confirmation of a high level of expression in VL tissue lesions demonstrates the relevance of the in vitro studies. These genes may be required for survival in the amastigote stage and some of them may be directly responsible for disease pathogenesis. It is significant to understand that the same species in a different disease presentation show a different pattern of gene expression as seen with *L. donovani* in VL with a high level of *Ld*Uba5 and A2, yet these genes are barely expressed in *L. donovani* in PKDL. Similarly in the same tissue tropism of CL and PKDL, different species show a different pattern of expression. For example, PSA2 is high in *L. donovani* in the skin, but low in *L. tropica* or A1 is much higher in *L. tropica*, but low in *L. donovani* in skin lesions. This

study opens the way for further studies of the correspondence between parasite gene expression and disease pathogenesis.

In summary, we have demonstrated the utility of *L.donovani* genomic microarrays to examine the changes in gene expression as promastigote differentiate into amastigotes. Since DNA microarrays allow examination of gene expression on a genome-wide scale, the study revealed substantial new information about the dynamics of transcript abundance during this differentiation process. However, a complete view of gene expression was not possible, since the *Leishmania* genome is not yet completely sequenced and annotated, and the arrays used do not represent every gene present in the *Leishmania* genome. Also there is a possibility that axenic amastigotes may have different metabolic processes than intracellular amastigotes therefore, we expanded the scope of expression profiling in axenic stage to true/hamster derived amastigote stage, and compared the expression of a few important genes which were up regulated in the axenic amastigote stage in the microarray. Further the assessment of selected gene transcripts *in vivo* in tissue lesions highlighted substantial differences in gene expression patterns between the three different forms of leishmaniasis VL, PKDL and CL. Our array identified several differentially expressed molecules that might enable the parasite to survive within the phagolysosome of vertebrate macrophages and its transmittance by sand fly vectors. The functional study of some of the identified genes will provide useful insights in *Leishmania* survival and pathogenesis. The proposed study lead to identification of some important stage-regulated genes which may have a role in virulence or in the survival of the parasite. Such genes may be used as targets for preparing attenuated parasites with potential as live attenuated vaccines or as drug targets against VL.

Chapter 4

Cloning and Characterization of ASS

Introduction

Leishmania parasites alternate between two distinct life stages, the promastigote and the amastigote. The extracellular, motile promastigotes are rapidly engulfed by phagocytic cells at the site of infection, and differentiate into the obligate intracellular amastigote in response to the acidic environment of the macrophage phagolysosome and the elevated temperature. Very little is known about how these external signals are perceived by *Leishmania*, or how they are transmitted to down-stream targets responsible for differentiation. In fact, no signal transduction pathways have been fully elucidated in any of the Trypanosomatid parasites. While it is possible that *Leishmania* differentiation is triggered by the activation of specific cell surface receptors/channels or kinases, analysis of the *L. major* genome have not revealed any candidate proteins (i.e. G-protein coupled receptors, receptor tyrosine kinases). Pharmacological agents that induce protein misfolding and/or the promastigote heat shock response can trigger promastigote-amastigote differentiation *in vitro*, indicating that differentiation signals could originate in the cytosol (Barak et al., 2005; Wiesgigl and Clos, 2001). However, the exact molecular mechanism is yet to be discovered. Understanding of the amastigote stage of *Leishmania* and its interaction with host macrophages is needed to better control leishmaniasis. Many house keeping genes are expressed constitutively while the morphological and biochemical differences between the two life stages should, in large part, result from the stage-specific expression of a discrete number of regulated genes (Bellatin et al., 2002). These amastigote-specific proteins are responsible for intracellular parasite survival and, furthermore, the interactions between such gene products and the host cell components likely determine *Leishmania* pathogenesis (Chang and McGwire, 2002).

Although differentiation from promastigotes to amastigotes is a central biological process required for survival during the life cycle, very little is known about this process. Surprisingly, few stage-specific molecules have been described and the functional role defined in still less number of cases. Amastigote-specific molecules are likely to play central roles in survival of parasite in the mammalian host and can therefore be considered virulence factors (Matlashewski, 2001). A number of molecules from *Leishmania* have been described that can be considered virulence factors such as secreted acid phosphatases (Ilg et al., 1991), cysteine proteinase (Mottram et al., 1996), A2 family (Zhang and Matlashewski, 1997), major surface glycoprotein gp63 (Chen et al., 2000; Joshi et al., 1998), lipophosphoglycan (Spath et al., 2000), GDP-mannose pyrophosphorylase (Garami and Ilg, 2001). More such virulence molecules need to be identified to fully understand the *Leishmania* pathogenesis.

Previous differential gene expression studies from our lab in *L.donovani* have found a few important genes, out of which Argininosuccinate synthase (ASS) found to be over expressed in the axenic amastigote stage was selected for further studies. The present study is aimed at characterizing the ASS gene that showed abundant expression in amastigote stage.

Materials and Methods

Reagents and Chemicals

Acrylamide, Ampicillin, Chloramphenicol, Bovine Serum Albumin, Coomassie brilliant blue R-250, Calcium chloride, Glycine, Glutamine, Glycerol, PMSF, SDS, Imidazole, Tris, Tween-20, Isopropyl-thio-β-D-galactopyranoside (IPTG) and other fine chemicals were purchased from Sigma Chemicals, USA. Nitrocellulose membrane was from Millipore, USA. Plasmid purification, gel extraction, PCR purification kits, Ni-NTA agarose columns were purchased from Qiagen, Germany. The anti-6xHis-tag antibodies were from Qiagen, Germany.

Anti α–tubulin, Anti-mouse IgG-HRP conjugate, Anti-rabbit IgG-HRP conjugate, Anti-rabbit IgG-FITC conjugate and anti human IgG HRP conjugate were from Sigma. The enzymes and chemicals used for DNA manipulation were purchased primarily from Invitrogen, USA. Restriction enzymes used were from New England Biolabs, USA and MBI Fermentas, USA. The vector pCR2.1 and pCRT7/CT-TOPO, Superscript II reverse transcriptase enzyme, dNTPs, DTT, oligo dT_{20}, RNasin and *Taq* polymerase were obtained from Invitrogen, USA. RNA marker, DNA marker and restriction enzymes were from MBI Fermentas & New England Biolabs, USA.

Bacterial strains and animals

E. coli strains Top10F' and BL21 (DE3) pLysS were purchased from Invitrogen, USA.

Experimental methods

1. RNA Preparation

RNA isolation and cDNA preparation from *L. donovani* promastigote, axenic amastigote, hamster derived true amastigote and bone marrow samples of VL patients was done as described previously in chapter 4.

2. RT-PCR and quantitative Real-time PCR

Reverse Transcriptase (RT) PCR and Real time PCR analysis was carried out as described previously in chapter 4.

3. Cloning full length ASS gene from *L. donovani*

a. Isolation of DNA

Genomic DNA was prepared from minimally passaged parasites from kala-azar patient. Mid log phase parasites ($1\text{-}2X10^9$) were washed twice with cold PBS and used for DNA isolation by Wizard Genomic DNA kit. The experimental outline of cloning ASS is shown in Fig. 4.1. The nucleotide sequence of the amplified product was confirmed by cloning into pCR2.1-TOPO

before bacterial expression was attempted. The vector and sequence map of pCR2.1-TOPO TA cloning vector is shown in Fig. 4.2. The sequencing reactions were carried out on Automated sequencing machine 3730 Version 3.0 (ABI PRISM) at Sequencing facility department, Delhi University South Campus, New Delhi.

b. Expression of ASS gene from L. donovani in E. coli BL21 (DE3)pLysS Cells

The vector pCRT7/CT-TOPO is a T7 based expression plasmid. The T7 expression system uses the T7 promoter and T7 RNA polymerase for high-level transcription of the gene of interest. High level expression is achieved due to the higher processivity of T7 RNA polymerase compared to *E. coli* RNA polymerase. Expression of the target gene is induced by providing a source of T7 RNA polymerase in the host cell. This is accomplished by using BL21 *E. coli* host, which contains a chromosomal copy of the T7 RNA polymerase gene. This gene is under the control of *lacUV5* promoter that can be induced by IPTG.

Cloning into pCRT7/CT-TOPO takes advantage of the religating activity of topoisomerase I enzyme and enables faster ligation reactions which yields ≥ 95% recombinants. The ligation of the linearized vector that has single 3'-T overhangs and the PCR products that possess 3'-A overhangs at each end because of the terminal transferase activity of *Taq* polymerase, is achieved in a very short interval of time.

The vector pCRT7/CT-TOPO contains all the elements essential for protein expression from the cloned gene of interest and subsequent purification that include a strong T7 promoter, a 6X-histidine tag, gene coding for ampicillin resistance, and a synthetic ribosome binding site (RBS) along with translational stop codon and transcriptional terminator. Additionally, it also has V5 epitope region as a fusion tag for detection of recombinant polypeptides. This plasmid

can be transformed into BL21 (DE3) pLysS *E. coli* host strain for efficient expression. Schematic diagram with the multicloning site and the location of the affinity tag is shown (Fig 4.3).

Polyhistidine tracts bind tightly to a number of transition metals and transition metal chelate complexes such that a protein carrying an exposed His-6 region will bind to a resin charged with divalent nickel ions. Few natural proteins bind with significant affinities to such matrices, His-6 labeled proteins generated by recombinant methodologies can be purified substantially in a single step by affinity chromatography. Moreover, His-6 tag is much smaller than most other affinity tags and is uncharged at physiological pH. It rarely alters or contributes to protein immunogenicity, making it widely used affinity tag. In addition, His-tag allows for easy detection of the recombinant protein on Western blot.

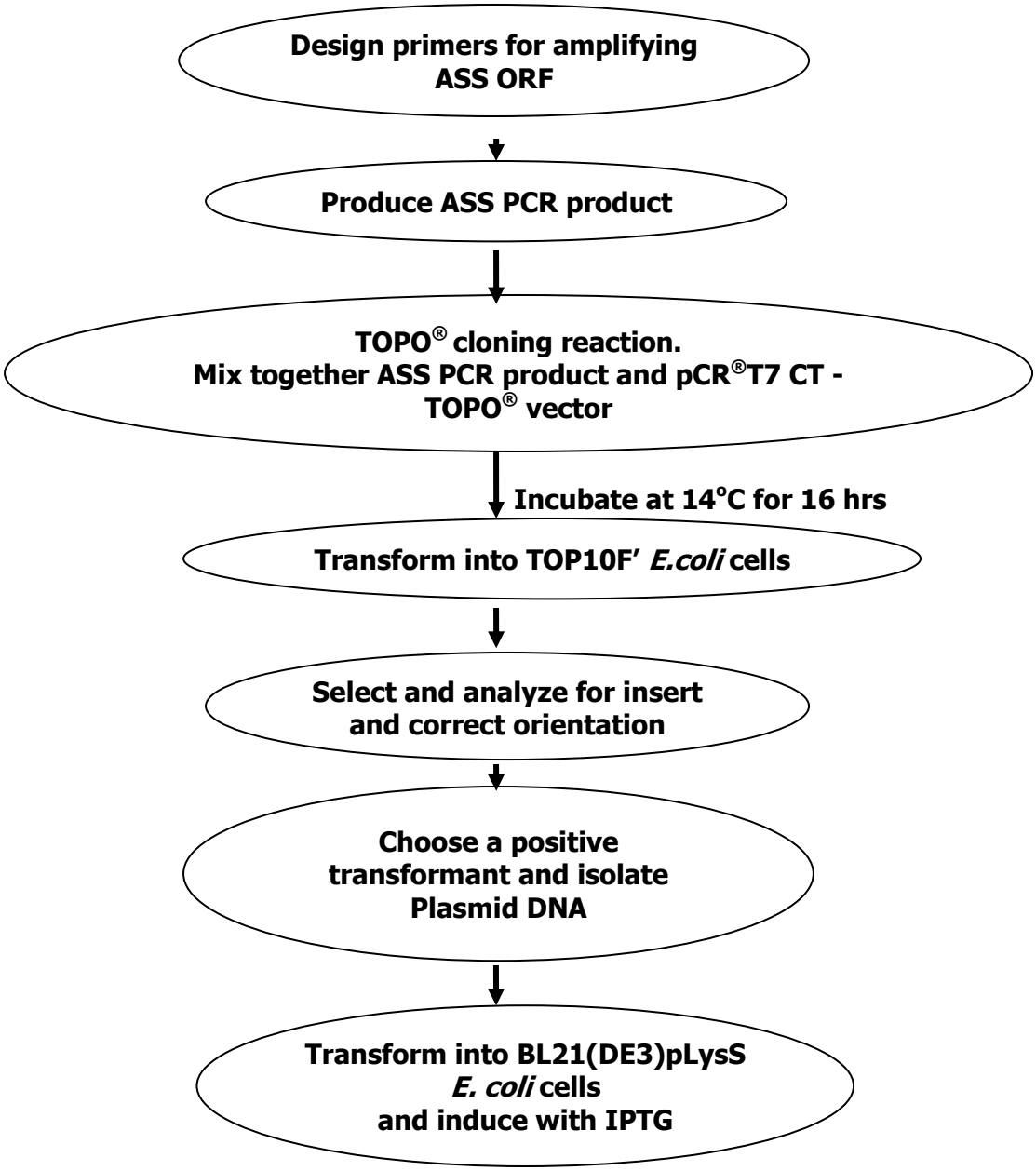
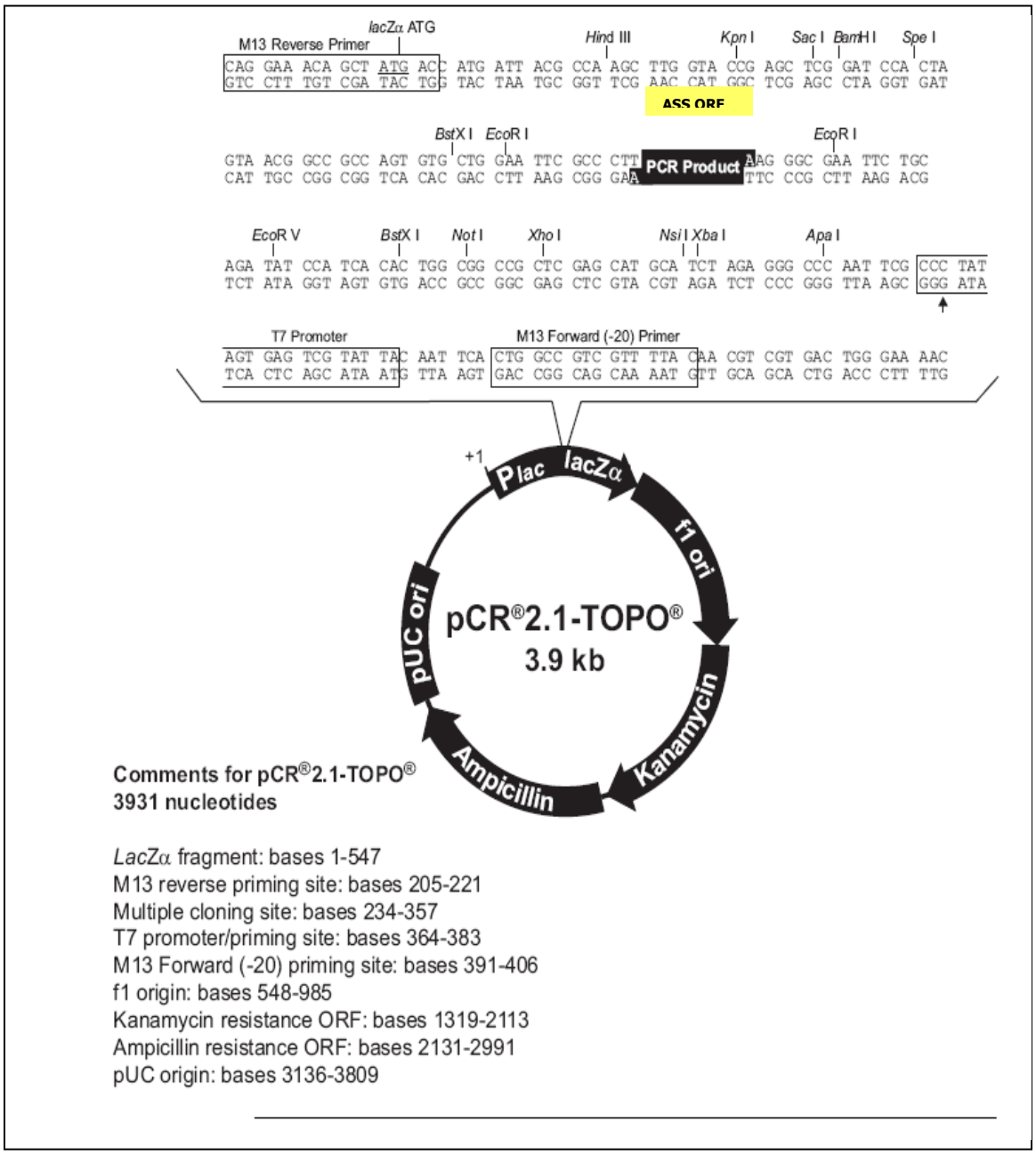


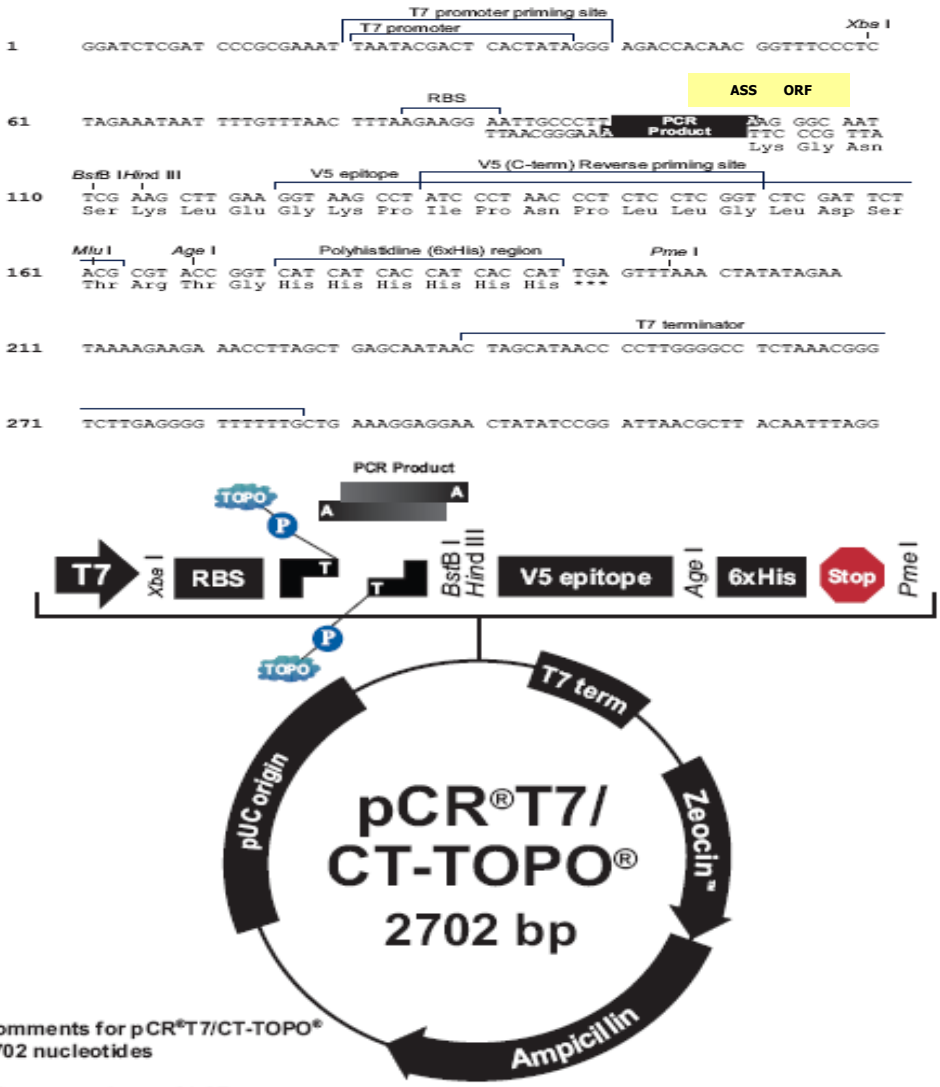
Fig 4.1 Experimental outline for cloning ASS.



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Fig 4.2 Sequence and map of pCR® 2.1 TOPO cloning vector.



Comments for pCR®T7/CT-TOPO®
2702 nucleotides

T7 promoter: bases 21-37
T7 promoter priming site: bases 21-40
Ribosome binding site: bases 85-91
TOPO® Cloning site: bases 100-101
V5 epitope: bases 122-163
V5 (C-term) Reverse priming site: bases 131-151
Polyhistidine (6xHis) region: bases 173-190
T7 transcription terminator: bases 240-287
Zeocin™ resistance gene: bases 367-830
ORF: bases 456-830
Ampicillin resistance gene: bases 834-1711
ORF: bases 851-1711
pUC origin: bases 1856-2529

Fig 4.3 Sequence and map of pCR® T7/CT-TOPO expression vector

Scheme for recombinant ASS expression

The ASS gene was expressed as a fusion protein with 6x Histidine affinity tag using the vector pCRT7/CT-TOPO. The ASS gene was amplified by PCR and the amplified products were separated on 1% agarose gel. The amplified fragment of ~1254 bp was excised and the DNA was eluted using the Gel extraction kit as described below. The PCR product and the vector were ligated overnight at 14°C and transformed into *E. coli* BL21(DE3)pLysS competent cells. Preparation and transformation of competent *E. coli* bacteria were performed according to procedures described by Sambrook *et al.,* [1989]. The transformation mixture was plated on Luria agar plates containing 100µg/ml of ampicillin. The plates were incubated for 16hr at 37°C. Colonies from the plate were screened for the recombinant plasmid by mini-preparations of plasmid DNA [Sambrook *et al*., 1989]. The desired recombinant plasmid was confirmed by restriction digestion with *Xba*I and *PstI*. The recombinant ASS was expressed by induction with IPTG and the protein was purified using Nickel agarose affinity chromatography.

The experimental details are discussed below.

I. PCR amplification

PCR was performed in 50µl reaction volume using DNA thermal cycler in 0.5ml PCR tubes. The reaction mix consisted of 50ng of genomic DNA from *Ld*K59, 0.2mM of each dNTP, 0.2nM of oligonucleotide, 5µl of PCR buffer (10X) and 1unit of Taq DNA polymerase. The genomic DNA was initially denatured at 95^{o}C for 5 min, followed by 30 cycles of 95^{o}C for 30 sec, primer annealing at 61^{o}C for 30 sec and extension at 72^{o}C for 30 sec, final extension was carried out at 72^{o}C for 7 min to obtain a product of 1.2 Kb. PCR products were stored at -20°C until further use.

Sequences of the Primers were:

Forward: 5’ ATG CCT GCA ACG GCG ACG GA 3’

Reverse : 5’ CAA GCT GCT CGG CAT CTC CTT CGC 3’

II. Extraction of DNA from agarose gel

The PCR reaction mix was fractionated by agarose gel electrophoresis. DNA fragments of right size were excised from agarose gel and were eluted from the gel slice using gel extraction kit as previously described.

III. Ligation

The amplified DNA fragment resolved on agarose gel and extracted from gel was cloned in pCRT7/CT-TOPO. Ligation was set up as follows.

Water	2.0 µl
ASS DNA (64.5 ng)	2.0 µl
Salt	1.0 µl
pCRT7/CT-TOPO vector DNA (5-10ng)	1.0 µl
Total	6.0 µl

The ligation reaction mixture was incubated at room temperature for 10-15 minutes for 16 hrs. An aliquot of the ligation mix was transfered (10 µl or less) into a cold sterile 1.5 ml microcentrifuge tube, and kept on ice. Aliquot of frozen competent *E. coli* BL21 (DE3) pLysS competent cells was thawed. Cells were resuspend and 100 µl of cell suspension was transferred into microcentrifuge tube with the ligation mix, mixed carefully, and kept on ice for 20 min. The

tube was transferred to a 42°C water bath or heating block for 90 sec. Added 500 µl of Super broth to the cells and incubated for 60–90 min at 37°C. Shaking increases transformation efficiency. Plated out 50, 100, and 200 µl aliquots on LB-agar plates containing ampicillin (100µg/ml) and chloroamphenicol (34µg/ml). Incubated the plates at 37°C overnight. A few recombinant transformant clones were grown in LB broth for plasmid preparation, which were further checked for right size of insert by restriction digestion. Single pass automated sequencing of the plasmid DNA was carried out on an Automated sequencing machine 3730 Version 3.0 (ABI PRISM) at Sequencing facility department, Delhi University South Campus, New Delhi.

IV. Screening of the transformants and expression of recombinant proteins

The presence of the ligated plasmid product was confirmed by mini-preparations of plasmid DNA. The DNA of the construct thus obtained was digested with *Xba*I and *PstI* and run on agarose gel to check for the size of insert for ASS gene. The orientations of positive colonies were determined by restriction analysis and the positive colonies were sequenced.

To ensure the presence of pLysS chloramphenicol was added to the medium for expression. The *E. coli* BL21 cells carrying the recombinant plasmid were grown at 37°C in 100ml Luria broth with ampicillin (100µg/ml) and chloramphenicol (34µg/ml). To establish the expression of the recombinant protein, cells were grown and induced by addition of IPTG (1mM final concentration). After 5 hrs of induction, cells were harvested by centrifugation at 4000g for 5 mins. The purification of recombinant proteins was executed using Ni NTA column according to manufacturer's protocol (Qiagen, Germany) (Fig 5.4). Purified elutes were quantified by Bradford's method and subjected to western blot using anti -His antibody.

The expressed protein was loaded on to nickel agarose column and eluted by 250mM imidazole in elution buffer (20mM Sodium phosphate, 500mM Sodium chloride pH7.0). SDS-

PAGE of the purified recombinant protein followed by the Western blotting analysis determined the presence of recombinant protein.

V. Polyacrylamide gel electrophoresis

A 12% separating gel was used for the electrophoretic analysis of proteins. For stacking of proteins 5% gel was used. Protein samples for SDS-PAGE were prepared by the addition of the 6X loading buffer to the final concentration of 1X, boiled for 5 minutes followed by centrifugation at 12,000g for 5 minutes at room temperature. Proteins were analyzed by SDS-PAGE according to method of Laemmli [1970] at a constant voltage of 85V in a Bio-Rad mini gel apparatus. The resolved proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250 for 30 minutes followed by destaining the gel to remove excess stain.

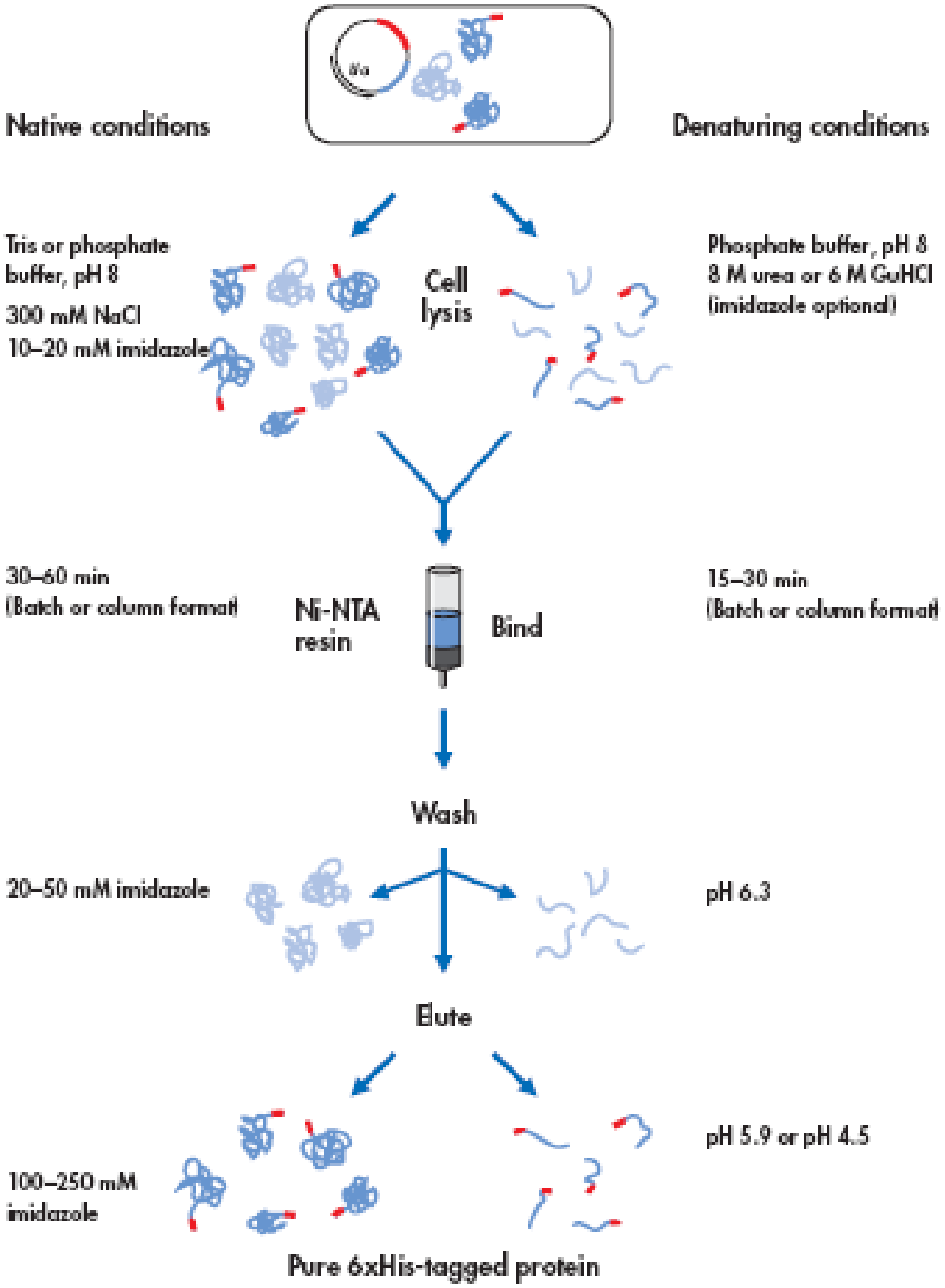


Fig 4.4 Purification of ASS (6X His-tagged protein) using QIAexpress system (Qiagen)

4. Raising antisera to r-ASS

Antisera were raised against recombinant ASS. For raising antisera to r-ASS protein, 6 months -1 year old rabbits were purchased and adapted to the lab conditions a week prior to actual experiments. 2 ml blood was drawn from the rabbits a day prior to injection of recombinant proteins and serum was separated from it to be used as control in western blot experiments. For immunization, an emulsion was made by taking 200µg purified r-ASS protein and mixing it with an equal volume of Freund's complete adjuvant. The emulsion was injected into the rabbit intra muscularly and the rabbit was observed for 15 days. On the 15^{th} day, 2 ml blood was drawn from ear vein to obtain serum. Two booster doses were given with 200µg purified protein emulsified in equal volume of Freund's incomplete adjuvant at 21 days interval each after drawing 2-3ml blood before each immunization. After third booster dose, 3 ml blood was drawn from the rabbit ear vein and serum was separated from it.

5. Preparation of protein lysates

Total protein lysate was prepared from promastigotes, axenic and hamster derived amastigotes. Parasites ($1\text{-}2X10^9$) at different stages were washed thrice with ice cold PBS and lysed with 4X SDS lysis buffer at 100^oC for 10min. The lysate was snap chilled on ice and stored at -70^oC until further use.

6. Western Blot analysis

Western blot analysis was performed following the method as described by Towbin *et al*. (1979). 10ng of purified r-ASS protein/50µg of crude parasite lysate was resolved on SDS-PAGE and transferred to nitrocellulose membrane at a constant current of 100 ampere for 2hr. Blocking was done by using 2% BSA in PBST for 3hrs. For probing the blot with antibody, the membrane was incubated for 2hr at room temperature with anti- His monoclonal antibody (1:5000)/ polyclonal anti-ASS antibody (1:1000) in PBST buffer (pH7.4) containing 1% BSA.

The membrane was washed and then incubated for 1hr in anti-mouse IgG-HRP-conjugated antibody (1:5000)/ anti-rabbit IgG-HRP-conjugated antibody (1:2500). The protein bands were visualized using the Chemiluminescent HRP substrate Bound ECL Western blotting detection system (Immobilon^{TM} from Millipore, U.S.A.). For evaluating immunogenic potential of r-ASS, the 10ng recombinant protein was blotted on nitrocellulose membrane, blocked as previously and allowed to react with patient's sera (1:100) dilution. Anti-human IgG-HRP conjugated antibody (1:2500) was used as secondary antibody. The protein bands were visualized using the peroxidase substrate 3, 3 '- diaminobenzidine and H_2O_2.

7. pSORTII analysis for protein localization

For prediction of protein localization in *Leishmania,* the DNA sequence was fed into web based protein localization software WoLF PSORT. WoLF PSORT is a new program for predicting protein subcellular localization from amino acid sequence. This program combines signal based features of PSORTII and iPSORT with amino acid content. The prediction accuracy is increased by using feature selection [Horton *et al.,* 2006].

8. Immunofluorescence assay

L. donovani promastigotes were fixed in suspension of 4% (w/v) paraformaldehyde in PBS (50 mM Na_2HPO_4, 150mM NaCl, pH 7.4) for 20min at room temperature, washed three times in PBS, and allowed to attach to poly-L- lysine coated glass slides. After air drying, the slides were immersed in ice-cold methanol for 5 min, blocked for 2hrs in 1% (w/v) bovine serum albumin in PBS, and incubated for 1hr with the anti-ASS diluted (1:50) in 1% BSA in PBS. After three washes in cold PBS, cells were incubated for 1hr with fluorescein-conjugated anti-rabbit IgG antibody (Sigma). These secondary antibodies were diluted 1:200-fold in PBS containing 1% BSA. Cells were subsequently washed three times with cold PBS and mounted in

10% glycerol in PBS. Rabbit non reactive serum (NRS) was used as control. Cells were examined for fluorescence under a confocal laser microscope (Zeiss), with epi-fluorescence. The focal plane chosen in all the cells was in the middle of the cells.

Results

Differential expression of ASS in Amastigote stage

ASS exhibited 2.7 (± 0.26) fold higher expression at axenic Am stage, compared to promastigote with Reverse transcriptase PCR . Relative Quantification also displayed ~ 3 fold higher abundance of ASS in axenic as well as hamster derived amastigotes (data shown in chapter 4).

Detection of ASS transcripts in bone marrow of VL patients

The expression of ASS was determined directly in the human system in bone marrow samples from VL patients. The presence of ASS gene transcripts in KA patient's bone marrow emphasizes the importance of this gene in disease pathogenesis.

Cloning and expression of ASS

The ASS gene sequence aligned within the ORF region of the gene *Lm*JF23.0260 (ASS) in *L.major* genome, which codes for a protein of ~46.2kDa(Fig 4.5).

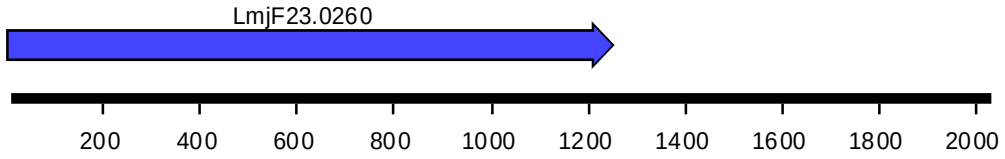


Fig 4.5 Alignment of the *L. donovani* ASS clone sequence (blue) with a segment of the *L. major* genome. Blue arrows indicate the presence of open reading frames with the gene annotation from www.genedb.org, version 1.0.

PCR was carried out with gene specific primers for the amplification of ASS ORF from *L. donovani* genome. The reaction yielded a ~1254 bp product with *L. donovani* DNA (Fig 4.6).

The PCR product was cloned into pCR2.1®-TA cloning vector and its nucleic acid sequence determined. The orientation of the gene was checked by digestion with SphI and Hind III digestion. Further the confirmed colonies were sequenced in an automated sequencer (ABI3770) using M13 forward and reverse sequence for correct orientation and fidelity of the PCR product.

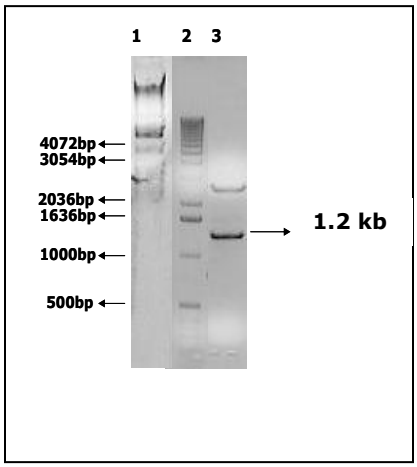


Fig 4.6: Agarose gel picture of recombinant pCR 2.1TOPO vector and restriction analysis
Lane 1: Recombinant plasmid;
Lane 2: Molecular weight marker
Lane 3: Digestion with EcoR1 enzyme

Alignment of nucleic acid sequence of ASS ORF cloned from *L. donovani* with the ASS from *L.infantum* and *Homo sapiens* using Clustal W algorithm showed high level of conservation from parasite to human, while interestingly it was found absent in trypanosomes like *T.brucei and T.cruzi* suggesting some specific role of this protein in *Leishmania* (Fig 4.7). The ORF size in *L. donovani* was found to be ~1.2 Kb similar to *L. major, L. infantum and L. braziliensis* the sequences of which were obtained from geneDB. Sequence analysis indicated that the ASS gene contained a 1.2Kb ORF of 419 amino acids, coding for a ~46.2kDa protein and showed 100% homology to corresponding gene LinJ23_V3.0300 in *L. infantum*.

Fig 4.7 Multiple alignment of ASS ORF sequences: Hs,H.sapiens (ACE87601);*Ld*, *L.donovani* and *Li*, *L.infantum*(*Lin*J23_V3.0300) . The accession numbers for the *Leishmania* gene is available from GeneDB while the Human sequence is available from GenBanK data bank. The nucleotide sequence of ASS was compared by Clustal W program. Asterisks denote identical nucleic acids; single and double dots denote weakly and strongly similar nucleic acids, respectively, determined by the criteria of ClustalW program.

Bioinformatic analysis revealed that the single copy of the gene was present in *L. infantum, L. major, L.braziliensis*. The amino acid sequence predicted by the *L. donovani* coding sequence

with those of *L. infantum* (LinJ23_V3.0300) and other *Leishmania* species was aligned using Clustal W algorithm. Table 4.1 shows the high level percentage homology of *ASS* ORF from *L. donovani* with other *Leishmania* species. The analysis revealed 100%, 94% and 64% homology respectively with *L. infantum, L. major and L.braziliensis* . Further the amino acid sequence of the protein was submitted to WoLF pSORT for prediction of subcellular localization. The results of WoLF pSort analysis reveals the protein to be localized in cytoplasm.

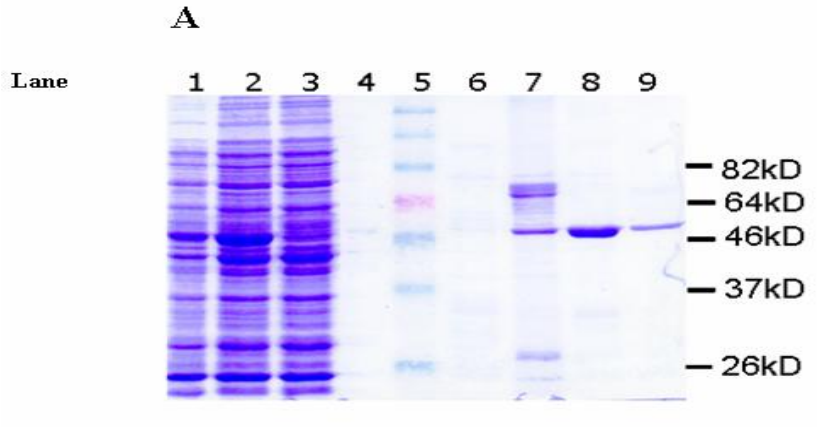
	Ld	***Li***	***Lm***	***Lb***
L. donovani	**1257**	**100%**	**94%**	**64%**
L. infantum	**1257**	**1257**	**94%**	**64%**
L. major	**1184**	**1184**	**1184**	**63%**
L. braziliensis	**793**	**793**	**793**	**793**

Table 4.1 Homologues of *L.d.*ASS found in *Leishmania infantum* (LinJ23_V3.0300), *L. major* (LmjF23.0260) and *L.braziliensis* (LbrM23_V2.0290). Percent similarity is shown in blue and number of bases showing match is shown in red. The ORF size is shown in shaded box.

Expression of recombinant ASS and western blotting

To demonstrate that the ASS putative protein is expressed in *Leishmania*, the ORF was cloned into an expression vector and recombinant protein purified from *E. coli* utilizing a 6x-His tag. Further, expression of the ORF was considered necessary to confirm its deduced coding capacity. The ASS ORF (~1254 bp) was PCR amplified from genomic DNA of *Ld*k59 and was ligated into pCR®T7/CT-TOPO bacterial expression vector in frame with a 3' histidine tag. The

protein expression was induced by IPTG (Fig 4.8 A). The recombinant ASS protein was purified using nickel agarose affinity chromatography (Qiagen). Western blot analysis of the recombinant ASS protein with anti-6Xhis antibodies showed that the recombinant product was a ~46 kDa protein (Fig 4.8 B). The acrylamide gel showed that primarily one protein was purified from the lysate and Western blot with anti-His Ab showed the major polypeptide eluted was the recombinant protein. A sufficient quantity of the recombinant ASS was produced to inject into a rabbit for the production of antibodies to *Ld*ASS as per details given in the method section. The antibodies raised in rabbit were tested by reacting the sera with recombinant protein. A dark band corresponding to ~46kDa ensured the presence of anti- ASS protein antibody in the rabbit serum (Fig 4.9).



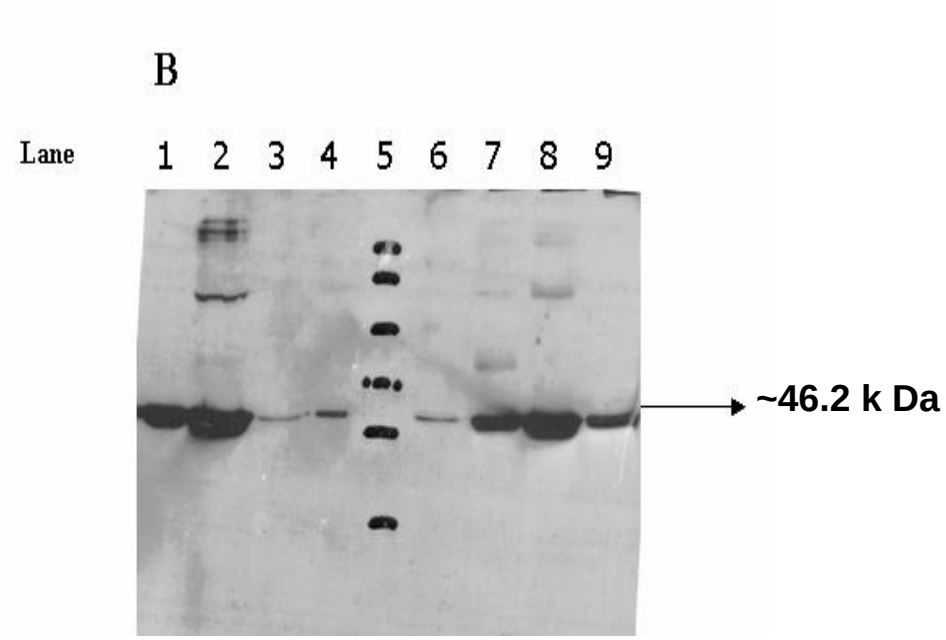


Fig 4.8 : (A)SDS PAGE and (B)western blot analysis with anti-His Ab. Lane 1 : Crude SDS lysate, Lane 2: Nondenaturing lysate, Lane 3 : ND lysate, post-Ni, Lane 4 : Post elution Ni-beads, Lane 5 : Marker proteins, Lane 6 : Elution #1, Lane 7 : Elution #2, Lane 8 : Elution #5, Lane 9 : Elution #7.

Differential expression of ASS at protein stage

The antibodies raised against recombinant ASS bind a single band of approximately 46 kDa. The amastigote-up regulated pattern of expression, originally identified by RNA levels, was observed to manifest at the level of protein as well. Western blot results showed significant expression of ASS protein at axenic as well as hamster amastigote stage, when compared with the promastigotes (Fig 4.9).

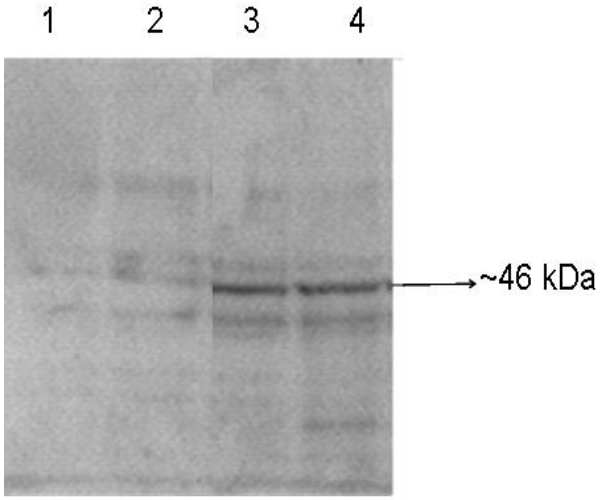


Fig 4.9. Western blot showing differential expression of argininosuccinate synthase at various life cycle stages of *Leishmania* Lane 1: Log promastigotes; Lane 2: Stationary promastigotes; Lane 3: Axe Am; Lane 4: Hamster derived Am.

Immunogenic potential of recombinant ASS

Serum from 10 kala-azar patients and 5 control individuals were reacted against recombinant ASS protein. No interaction was seen with any of the sera with ASS protein indicating that the protein does not elicits a humoral response in VL patients (data not shown).

Immunofluroscence assay(IFA)

*Ld*ASS promastigote cells were subjected to IFA using polyclonal α-ASS antisera. Immunofluorescence revealed *Ld*ASS to be localized in cytoplasm in promastigotes of *L.donovani*. Rabbit non reactive serum (NRS) was used as control (Fig 4.10).

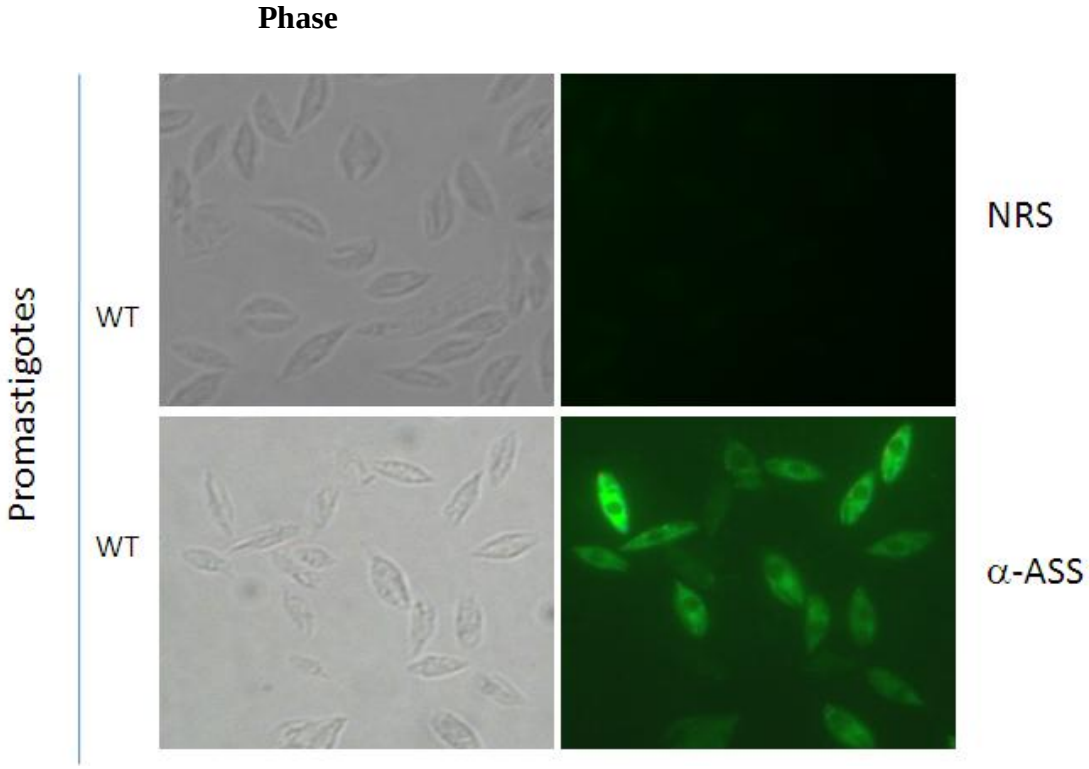


Fig 4.10: Immunofluroscence assay of *Ld* ASS. Native anti-ASS antibody conjugated with FITC were used for the immunofluorescence assay. DAPI was used to stain the nucleus.

Discussion:-

Leishmania occupy extracellular niches in the sandfly vector, but are obligate intracellular parasites in the mammalian host, primarily invading and proliferating within the phagolysosome of macrophages and some other phagocytic host cells. The differentiation of (metacyclic) promastigotes to amastigotes is one of the major developmental transitions in the *Leishmania* life cycle and a key event in establishing an infection in the mammalian host. It is conceivable to catalog the genetic determinants of *Leishmania* into those that help the parasite invade the macrophages and those that cause persistent infection and pathology. These virulence factors help *Leishmania* successfully establish infection in macrophages by evading the humoral lytic factors, attachment of parasites to macrophages followed by entry via phagocytosis, intracellular survival, promastigote to amastigote differentiation and replication as amastigotes. However, the spreading of amastigotes to infect additional cells must be considered as crucial for the development of leishmaniasis since infection by itself does not cause the disease, though it is a prerequisite for this state. Infection must be maintained in order for the transition from asymptomatic phase to symptomatic phase, especially when host immunity becomes down regulated. The parasite molecules involved in promastigote-to-amastigote differentiation and replication of the amastigotes may have additional functions beyond infection, especially in the latter case. This dramatic change is accompanied, and is most likely governed, by the modulation of gene expression.

Our study undertaken on comparison of gene expression of axenic and hamster derived amastigotes was to identify genes that showed high expression in both hamster derived

amastigotes and axenic amastigotes as recent reports with *L.mexicana* have found many genes that show significant differences between lesion amastigotes and axenic amastigotes (Holzer et al., 2006). Such genes may include novel virulence factors that affected the growth of the parasite. However, because of the difficulties in obtaining adequate amounts of viable amastigotes that are free of host cell contamination as well as the limited availability of effective and sensitive methods (Brodin et al., 1992; Coulson and Smith, 1990), molecular biology studies on the parasite at the amastigote stage have been hindered greatly. Very few genes involved in the stage transitions have been characterized and the underlying mechanism of differential gene expression not fully understood (Liu et al., 2000). The A-2 gene family was first identified as an amastigote-specific in *L. donovani* since the A2 transcripts are abundant in the amastigotes but hardly detectable in the promastigotes (Charest and Matlashewski, 1994). The A2 genes of *L. donovani* are composed predominantly of a 10-amino acid encoding sequence that is present in multiple copies. The predominant expression of A-2 in amastigote stage was shown to be important for the virulence when the expression of A-2 was inhibited by antisense approach (Zhang and Matlashewski, 1997).

Another class of molecules that display higher abundance in amastigote stage are the heat shock proteins. The major heat shock proteins of *Leishmania*, HSP70 and HSP83, are already extremely abundant under standard culture conditions (Brandau et al., 1995). In contrast to that of HSP70 and HSP83, the expression of the HSP104 homolog in *Leishmania*, HSP100, is chiefly restricted to conditions of heat stress (Hubel et al., 1995). This protein is barely detectable in unstressed insect stages of the parasite, but its intracellular level is increased by 1 order of magnitude by temperatures equivalent to those in the mammalian target tissues. Smejkal *et al.* (1988) had shown that heat treatment increased the virulence of *Leishmania braziliensis panamensis* in the hamster model. The expression pattern of HSP100 (Hubel et al., 1995)

suggested that this protein was the effector molecule which mediated the heat inducibility of virulence (Smejkal et al., 1988). Recently, stage specific expression of HSP 10 has also been shown to be expressed preferentially under heat stress and specifically in the amastigotes stage in *L. donovani* (Zamora-Veyl et al., 2005). Apart from the aforementioned classes of molecules, several genes showing higher expression in amastigote stage were identified which include *L. donovani* proton motive P-type *ATPase 1b* (Meade et al., 1989), *L. mexicana CPB2.8* (Mottram et al., 1997; Souza et al., 1992); *L. mexicana* AF032464 (Liu et al., 2000), *L. donovani infantum* triose phosphate isomerase TIM (El Fakhry et al., 2002), *L. pifanoi CYS2* cysteine proteinase (Traub-Cseko et al., 1993) and *L. major HASPs* (McKean et al., 1997).

Previous studies from our lab on differential expression in *Leishmania* had identified a few amastigote up regulated genes, Argininosuccinate synthase (ASS) was one among such genes showing 2-3 fold up regulation at the axenic amastigote stage. Argininosuccinate synthase (ASS), the rate-limiting step in the regeneration of arginine from citrulline, catalyzes the synthesis of argininosuccinate, AMP, and inorganic pyrophosphate from citrulline, ATP, and aspartate (Xie and Gross, 1997). Argininosuccinate is then cleaved by argininosuccinate lyase (ASL) to produce L arginine and fumarate. L-arginine is involved in many metabolic pathways, including those involved in the synthesis of NO, agmatine, creatine, and urea (Morris, 2004). Two major L-arginine metabolic pathways are particularly relevant to *Leishmania* infection due to their roles in regulating MΦ effector functions. L-arginine in MΦs can either be catabolized by inducible nitric oxide synthase (iNOS) to produce NO, or by arginase for polyamine synthesis, depending on the type of extracellular stimuli. When MΦs are exposed to Th1 cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), together with toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS), the expression of iNOS enzyme is upregulated, driving L-arginine metabolism toward NO production (Green et

al., 1990; Liew et al., 1990). In addition, several Th1–favored chemokines, including MΦ inflammatory protein-1α (MIP-1α/CCL3), MΦ chemoattractant protein-1 (MCP-1/CCL2) (Bhattacharyya et al., 2002; Brandonisio et al., 2002), and gamma interferon-inducible protein-10 (IP-10/CXCL10) (Vasquez and Soong, 2006) can also trigger iNOS activity and promote parasite killing in MΦs. In contrast, Th2 cytokines, such as interleukin (IL)-4, IL-10, IL-13, and transforming growth factor-β (TGF-β), preferentially induce L-arginine utilization towards the production of polyamines (Iniesta et al., 2002 ; Barksdale et al., 2004). These Th2 cytokines induce expression and activity of arginase, which converts L-arginine to L-ornithine, a substrate for ornithine decarboxylase (ODC) that converts it to putrescine (Raina and Janne, 1968). Since polyamines (e.g., putrescine, spermidine, and spermine) are important nutrients being directly utilized by *Leishmania* spp., the exposure to Th2 cytokines leads to the promotion of parasite growth (Kane and Mosser, 2001; Iniesta et al., 2002).

As found in higher eukaryotes, some lower eukaryotes like *Leishmania* and *Trypanosoma* also have their own L-arginine metabolism pathways (Camargo et al., 1978). L-arginine has long been identified as an essential amino acid for *Leishmania* growth. As early as the 1970s, Krassner and coworkers demonstrated that *Leishmania* promastigotes could not be maintained in L-arginine-free media (Krassner and Flory, 1971; Steiger and Steiger, 1977), suggesting mechanisms of L-arginine uptake and utilization in *Leishmania* parasites. Due to the critical roles of L-arginine pathways in determining parasite killing and proliferation, it is anticipated that these pathways are tightly regulated to permit them to compete for available intracellular L-arginine. Several counter-regulatory mechanisms between the iNOS and arginase pathways have been documented. For example, Nω-hydroxy-L-arginine (LOHA), an intermediate product of the iNOS pathway, is known to strongly inhibit arginase function (Iniesta et al., 2001). Conversely, arginase can reduce iNOS activity by competing for L-arginine (Gotoh and Mori, 1999). Thus,

activities of iNOS and arginase can be greatly influenced by L-arginine availability. Although, L-arginine has been described as a non-essential dietary amino acid, because it can be synthesized in virtually all cell types from L-ornithine or L-citrulline, urea cycle intermediates (Peck et al., 1995), the endogenous synthesis of L-arginine is insufficient for certain cellular activities, including the production of NO via iNOS and polyamine synthesis (Yeramian et al., 2006). In MΦs lacking an L-arginine transporter, NO and polyamine synthesis was reduced when these cells were stimulated with Th1 and Th2 cytokines, respectively (Stevens et al., 1996; Yeramian et al., 2006), suggesting that L-arginine availability is the bottle-neck control step for both iNOS and arginase. Therefore, L-arginine transport from the extracellular milieu is critically important for parasite growth and killing within an infected cell.

Our studies with ASS gene from *L.donovani* showed 100% homology to ASS from *L. infantum* (LinJ23_V3.0300). The gene was found to be present in a single copy in *Leishmania* [www.geneDB.org]. ASS was found to be present in almost all species of *Leishmania* while it is absent in *Trypanosoma*. Apart from axenic cultures, hamster derived amastigote stage also showed significantly higher expression confirming that this gene is transcribed at amastigote stage. Further, ASS transcripts were also detected in the RNA isolated from human bone marrow samples suggesting an important role of this gene in the mammalian hosts. We had cloned and characterized this gene from *L. donovani*. A sufficient quantity of the recombinant ASS was produced to inject into a rabbit for the production of antibodies to ASS. The expression of ASS was validated at RNA / protein level, the amastigote-specific pattern of expression, originally identified by RNA levels, is manifest at the level of protein as well. The protein pattern of expression is very significant because the functional properties in living cells are determined by protein activity, thus ASS must perform a function particularly important in the amastigote stage. The gene A1 which had been characterized as amastigote specific gene in our lab earlier was

used as a marker of amastigote stage (Sreenivas et al., 2004). Bioinformatics analysis by PredictProtein and WoLF pSORT (Horton et al., 2007) revealed that the protein contained several phosphorylation sites and suggested ASS to be localized in cytoplasm [Rost et al., 1996, 1998, 2004; Bairoch et al., 1997; Ceroni et al., 2004]. The cytoplasmic localization of ASS was experimentally proven by immunofluorescence assay.

In brief, we have identified a *Leishmania* specific protein ASS absent in *Tyrpanosoma* which was expressed abundantly in the axenic as well as hamster derived amastigotes. Previous studies indicate that ASS acts as a rate limiting enzyme in the L-arginine synthesis suggesting an important role of ASS in *Leishmania*. The higher expression of ASS gene at RNA and protein level in amastigote stage and the presence of gene transcripts in infected bone marrow samples in VL patients suggest a role in the disease pathogenesis in the human host.

Chapter 5

Identification and functional characterization of Ubiquitin like system in the protozoan parasite Leishmania donovani

Introduction

Protein modification by ubiquitin and ubiquitin-like proteins is one of the most complex and intensely studied mechanisms of post-translational protein regulation in eukaryotes. Conjugation of the 76-amino-acid protein ubiquitin is first and foremost a signal for targeting proteins to the proteasome for degradation, but evidence that ubiquitin also plays diverse roles in the regulation of numerous biological pathways is building. In addition, there are many structurally related ubiquitin-like modifiers (Ubls) that utilize mechanistic pathways similar to those utilized by ubiquitin for conjugation to protein substrates and deconjugation. Despite similarities in structure between ubiquitin and other Ubls, modification by Ubls regulates diverse cellular processes such as transcriptional regulation, cell cycle control, and autophagy (Hochstrasser, 1996). Ubiquitin has been identified in the majority of parasitic protozoa, but most Ubls in these organisms have not been characterized (Table 6.1). Even less attention has been paid to the enzymes that regulate protein modification by ubiquitin or Ubls. The complex life cycles and multiple disease-causing states of parasitic protozoa offer a unique context in which to study ubiquitin and Ubl modification pathways. The life cycles of most protozoan parasites within single or multiple hosts rely on strict timing of protein regulation and gene expression for both survival and virulence. While the regulation of gene expression and protein turnover is clearly critical for both life cycle and disease progression in medically important protozoa, the mechanisms regulating these processes are not well understood. Given the known functions of ubiquitin and Ubls in other organisms, a better understanding of these posttranslational modifiers is likely to be critical to understanding how parasites control many basic biological processes (Ponder

and Bogyo, 2007). UBLs (for example SUMO, RUB1/NEDD8, APG12) do not seem to promote proteasomal degradation, but regulate a variety of cellular functions as critical regulators of many cellular processes, such as transcription, DNA repair, signal transduction, autophagy and cell cycle (Jentsch and Pyrowolakis, 2000; Welchman et al , 2005). Like ubiquitin, some UBLs are expressed as inactive precursors; that is, as proteins with C-terminal extensions, which prevent conjugation. These tails, which can either be single amino acids or short peptides, are clipped off by the activity of specific proteases, thereby releasing the active UBL with a C- terminal glycine residue. Other UBLs are already expressed as mature proteins bearing an exposed C-terminal glycine residue (Furukawa et al., 2000). This glycine residue, often part of a di- glycine motif, appears to be crucial for conjugation and de- conjugation from substrates (Wilkinson & Audhya, 1981; Jentsch & Pyrowolakis, 2000). The conjugation pathways for UBLs resemble that of ubiquitin and utilize the same conserved mechanism. The first step involves an activating enzyme (E1), which hydrolyses ATP and forms an E1-UBL thiolester. UBLs are then transferred to conjugating enzymes (E2s), which results in a similar thiolester-linked complex. Finally, the UBLs are covalently attached to the substrates through the formation of an amide bond between their C-termini and a lysine side chain of the substrate protein. An exception is the UBL APG8, which is not attached to proteins but forms an amide bond with an amino group of a lipid (Ichimura et al., 2000).

A new ubiquitin-like protein, Ufm1 (ubiquitin-fold modifier 1), has recently been identified in humans (Komatsu et al, 2004). It shares only 16% sequence identity with Ub but displays a striking similarity in its tertiary structure to Ub (Sasakawa et al, 2006). It has a single Gly at its C terminus, unlike Ub and most other Ubls that have a conserved

C-terminal diglycine motif. Ufm1 in mouse and human is expressed as a precursor with a C-terminal Ser-Cys dipeptide extension that needs to be processed prior to conjugation to target proteins. The matured Ufm1 is specifically activated by an E1-like enzyme, Uba5, and then transferred to its cognate E2-like enzyme, Ufc1. The Ufm1 system is conserved in metazoa and plants but not in yeast. Recently Ufm1-specific proteases named UfSP1and UfSP2, enzymes responsible for the C–terminal processing of the Ufm1 precursor as well as for the reversal of protein conjugation by matured Ufm1 have been identified (Kang et al,2007). E3-like enzymes for the ligation of Ufm1 to target proteins have not been identified (Fig 5.1).

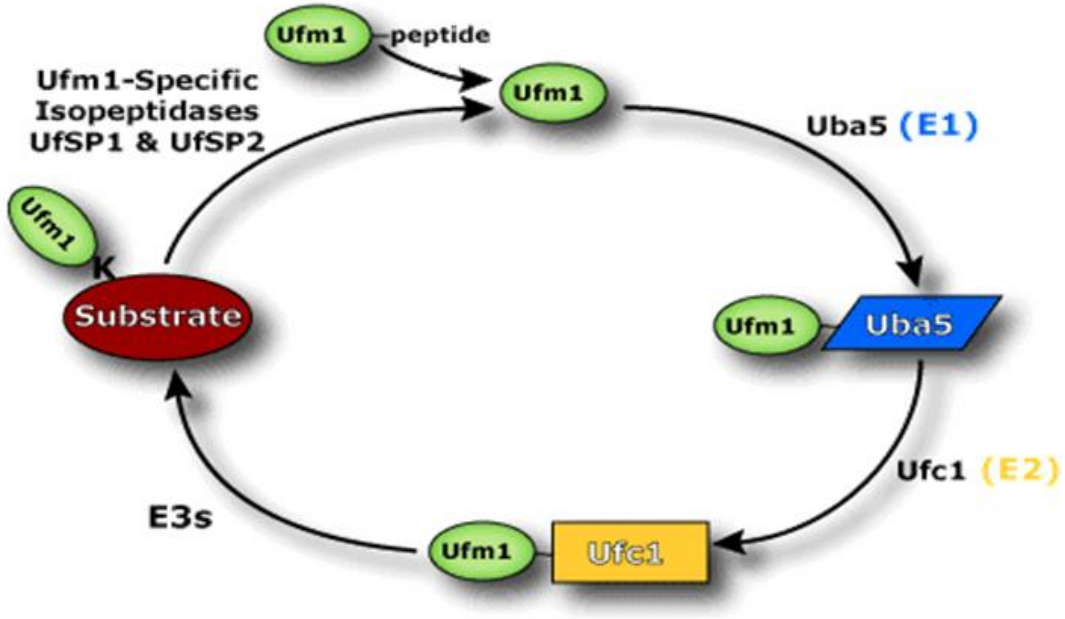


Figure 5.1: Ufm1 conjugation and deconjugation pathway in humans

In the present work, a highly sensitive genomic microarray technology has led to the identification of a gene that is up regulated in the amastigote stage of *L.donovani* and showed homology with human Uba5, thus was termed as *Ld*Uba5. We have attempted to functionally characterize *Ld*Uba5 linked pathway in *L.donovani* and studying its effect on

growth and differentiation of the parasite, this pathway is identified only in humans and in a few multicellular organisms while it is undiscovered in protozoans.

Table 5.1 : Common Ubiquitin like modifiers(Ubls)

S. No.	Ubl	Known function(s)	Protozoa with predicted homologs	Protozoa for which characterization of Ubl has been published
1	Ubiquitin	Protein degradation, internalization,histone regulation	*Plasmodium,Toxoplasma, Leishmania, Trypanosoma, Entameoba, Giardia, Cryptosporidium* and *Theileria* spp.	*Plasmodium, Leishmania, Trypanosoma, Entameoba* and *Giardia spp.*
2	Nedd8	Ubiquitin conjugation	*Plasmodium* spp.	None
3	ISG15	Interferon response	None	None
4	SUMO	Transcriptional Regulation, Protein localization	*Plasmodium,Toxoplasma, Leishmania, Trypanosoma, Entameoba, Cryptosporidium* and *Theileria* spp.	None
5	FAT10	Ubiquitin independent degradation	None	None
6	Hub1	Pre-mRNA splicing	*Plasmodium,Toxoplasma, Entameoba, Cryptosporidium* and *Theileria* spp.	None
7	Urm1	Starvation response	*Plasmodium,Toxoplasma, Leishmania, Trypanosoma, Entameoba, Giardia* and *Cryptosporidium* spp.	None
8	Atg8	Autophagy	*Plasmodium,Toxoplasma, Leishmania, Trypanosoma,* and *Theileria spp.*	*Leishmania and Trypanosoma spp.*

9	Atg12	Autophagy	None	None

Materials and Methods

Reagents and Chemicals

Acrylamide, Ampicillin, Chloramphenicol, Bovine Serum Albumin, Coomassie brilliant blue R-250, Calcium chloride, Glycine, Glutamine, Glycerol, PMSF, SDS, Imidazole, Tris, Tween-20, Isopropyl-thio-β-D-galactopyranoside (IPTG), Anti α–tubulin, Anti α–flag, Anti-mouse IgG-HRP conjugate, Anti-rabbit IgG-HRP conjugate, Anti-rabbit IgG-FITC conjugate, Anti human IgG HRP conjugate and other fine chemicals were purchased from Sigma Chemicals, USA. Nitrocellulose membrane was from Millipore, USA. Plasmid purification, Gel extraction and PCR purification kits, Ni-NTA agarose columns, Anti-6xHis-tag antibodies were purchased from Qiagen, Germany. DNA isolation kit was from Promega, USA. The enzymes and chemicals used for DNA manipulation, vector pCR®2.1 and pCR®T7/CT-TOPO, Superscript II reverse transcriptase enzyme, dNTPs, DTT, oligo dT_{20}, RNasin and *Taq* polymerase were purchased primarily from Invitrogen, USA. The oligonucleotides were obtained from Microsynth, Switzerland or Bioserve Biotechnologies (India) Pvt. Ltd., India. RNA marker, DNA marker and restriction enzymes were from MBI Fermentas & New England Biolabs, USA.

Experimental methods

Isolation of Genomic DNA and RNA

Genomic DNA was isolated from promastigotes according to the methods described in the manual for GENOMIC DNA isolation kit from promega. Total RNA was isolated from promastigote, axenic and hamster derived amastigote of *L. donovani* and bone marrow aspirates (BMA) from VL patients using Trizol reagent (Invitrogen) according to the manufacturer's instructions and stored at -70C (as described in chapter 4).

Quantitative real-time PCR (qRT-PCR)

Real time PCR analysis was carried out as described previously in chapter 4.

Expression of recombinant proteins in *E. coli*

The *Leishmania* gene was amplified by PCR based on primers of the respective genes using *L.donovani* genomic DNA. Subsequently the amplified fragment was cloned into the *E. coli* expression plasmid pCRT7CT-Topo that contains a built-in His_6 sequence (Invitrogen). *E. coli* BL21/pLys host cells (Invitrogen) were transformed with this plasmid, and the expressed, His-tagged protein was affinity purified under denaturing conditions using Ni-nitrilotriacetic acid-agarose (Ni-NTA) beads (QIAGEN) according to the manufacturer's protocol. The primer sequence used to amplify recombinant genes are listed in table 5.1.

Raising antisera to r-*Ld*Uba5

The ~43.2-kDa His-tagged *Ld*Uba5 protein was used to immunize a rabbit according to company protocol (Spring Valley Laboratories) as described in chapter 5. The resulting antiserum (anti-*Ld*Uba5) was analyzed by Western blotting with r-*Ld* Uba5 protein lysates.

Over expression of full length genes in *L. donovani*

The *Leishmania* expression plasmid pKSNeo (Zhang et al., 1996) was used to express full-length genes of *L.donovani*. The nucleotide sequence of the full length gene was confirmed by cloning into pCR®2.1-TOPO. Full length insert was used as template using respective gene primers with *SpeI* site and HA tag for sub cloning into pKSNeo, followed by transfection in *L.donovani* cells.

Following steps are involved for over expression of genes in *Leishmania*:-

I. DNA construction

The DNA encoding full length gene was obtained by PCR from *L.donovani* DNA isolated from the cloned line designated by the World Health Organization as MHOM/SD/62/1S-C12D (LDS 1). It was then sub cloned into pCR®2.1-TOPO TA cloning vector. Ligation was set up as follows.

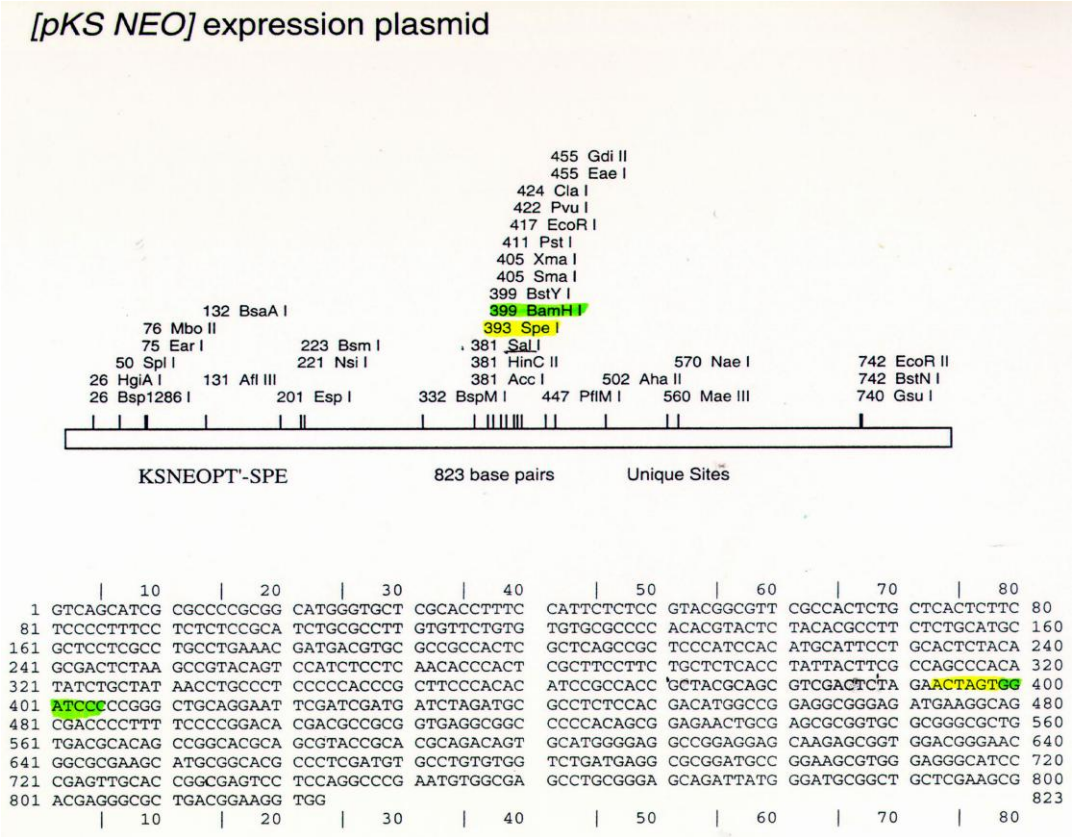
pCR®2.1-TOPO TA cloning vector (50ng)	1.0 µl
DNA (60 ng)	3.0 µl
Ligation buffer (2X)	5.0 µl
T4 DNA ligase (1U)	1.0 µl
Total	10.0 µl

The ligation reaction mixture was incubated at 14ºC for 16 hrs. *E. coli* TOP 10F' competent cells were prepared and the ligated product was transformed and subjected blue/white + Amp^r selection. The recombinant white Amp^r colonies were screened for the presence of gene of interest by digestion with *EcoRI* enzyme. Further the confirmed

colonies were sequenced in an automated sequencer (ABI3730) using M13 forward and reverse sequence for correct orientation and fidelity of the PCR product.

II. **Insertion of full length gene in pKSNeo vector**

Sequence confirmed plasmid DNA of pCR®2.1-TOPO TA -fused with full length insert was used as template using respective gene primers with *SpeI* site and HA tag for sub cloning into the *Leishmania* expression plasmid pKSNeo (Zhang et al., 1996). The vector pKSNeo consists of a 1.6-kb 5' upstream flanking region of the A2 gene, the entire Neo gene and a 1.8-kb fragment containing the A2 3' UTR and a synthetic pyrimidine tract [Zhang *et al.*, 1996]. The sequence and multiple cloning site of the pKSNeo vector is shown in Fig 5.2.



These oligos introduced a hemagglutinin tag at the C-terminus of the fusion protein and contained SpeI restriction sites on either end. The SpeI insert was sub cloned into the SpeI site of pKSNeo, resulting in plasmid constructs that were used to over express wild-type or mutant constructs in *Leishmania* transfectants. The primer sequence used to amplify were as follows and in the same way, SpeI site and HA tag are introduced in other plasmids / mutants also (sequences not shown).

_Ld_Uba5

SpeI site

Forward: 5' GG**A CTA GT** ATGCCCTTATCCTCCTCCCC 3'

HA Tag

Reverse: 5' CC**ACTAGT**CTA**CGCGTAGTCCGGCACGTCGTACGGGTA** CTGGATGGCCTTCATGCG 3'

_Ld_Ufc1

SpeI site

Forward: 5' GG**A CTA GT** ATGGAGCCGTCAGTGAAGGAGAGC 3'

HA Tag

Reverse:5'CC**ACTAGT**CTA**CGCGTAGTCCGGCACGTCGTACGGGTA**CTCAGCAGTCGTGGG CACCGTTG 3'

_Ld_Ufm1

SpeI site

Forward: 5' GG**A CTA GT** ATGAGCGCTG CGAACGACTC CG **3'**

Reverse: HA Tag

5'CC**ACTAGT**CTA**CGCGTAGTCCGGCACGTCGTACGGGTA**CCCCCTCCACCACCGCCACCG

G 3'

The amplified products were ligated using T4 DNA ligase using pKSNeo vector and transormed in *E. coli* TOP 10F' cells as described in chapter 5. The recombinant Amp^r colonies were screened for the presence of gene of interest by digestion with *SpeI* enzyme. The orientation of the gene of insert was checked by digestion with *HindIII* and *XhoI*.

III. Restriction digestion

Plasmids were prepared from bacterial colonies containing the respective constructs along with pKSNeo vector and digested with *SpeI* enzyme to release the insert.

Plasmid (pKSNeo + Insert)	5.0 µl
SpeI enzyme (1U)	1.0 µl
NEB buffer 2	1.0 µl
H_2O	3.0 µl
Total	10.0 µl

E. coli cells containing plasmids with correct inserts were grown in bulk and plasmids were isolated using Endofree plasmid isolation kit (Qiagen) following manufacturer's instructions.

IV. Transfection of construct in *L.donovani*

a) Preparation of cold DNA

20µg plasmid DNA was re-precipitated in 3M sodium acetate and 2 volumes of isopropanol, washed with 80% ethanol, dried and dissolved in 20µl sterile TE buffer and the plasmid was kept in ice until transfection.

b) Electroporation

$2X10^8$ mid log phase *L.donovani* cells were harvested, washed once with electroporation buffer and resuspended in ice-cold electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mMNa_2HPO_4,6mM glucose, pH7.4). 0.5 ml cells were taken in 2mm cuvette and 20µl cold plasmid DNA was added to it for transfection. The DNA was electroporated into *Leishmania* cells at 450 V, 500µF, 800π resistance. Following electroporation, cells were recovered in M199+10%FCS for 24hrs. After 24hrs, cells were washed once with M199 and resuspended in M199 + 10% FCS + 20µg/ml G418 (selectable marker). The drug concentration was gradually increased to a final concentration of 200µg/ml for the selection of mutant cell lines.

Site directed mutagenesis

A point mutation in *Ld*Uba5 for Cys at position 217 to Ser or Ala was generated by PCR-based site-directed mutagenesis. In two separate PCR reactions fragments of *Ld*Uba5 were amplified with primers that introduce point mutations by using pCR®2.1-*Ld*Uba5 as template. Gels purify the two PCR products and add equal amounts of each product in a second round of PCR using the terminal primers (primers which were used to amplify the full length *Ld*Uba5 gene). Ligate into pCR2.1 Topo, pick up colonies and confirm sequence by sequencing. This recombinant DNA construct was termed pCR®2.1- *Ld*Uba5^{C217S} or pCR®2.1- *Ld*Uba5^{C217A}.

Similarly, DNA encoding *Ld*Ufm1 was amplified by PCR from *L. donovani* DNA (LDS 1) and sub cloned into pCR®2.1-TOPO TA cloning vector, rest ligation transformation and sequencing is performed as for pCR®2.1- *Ld*Uba5. The HA 6XHis tag (CAC – Nucleotide codon for His) was introduced at the N-terminus of Ufm1. The C-terminal deletion mutants of *Ld*Ufm1 named *Ld*Ufm1ΔC i.e., deletion of 18 residues from precursor $Ufm1^{1–115}$ protein encoding amino acids 1–97, were generated by PCR. A point mutation for Gly at position 98 to Ala of *Ld*Ufm1 named *Ld*$Ufm1^{G98A}$ was generated by PCR based site-directed mutagenesis. Both the mutants as well as HA6xHis *Ld*Ufm1 were subcloned in pCR®2.1-TOPO TA cloning vector, ligation, transformation and sequencing is performed.

The DNA encoding *Ld*Ufc1 was obtained by PCR from *L. donovani* DNA (LDS 1). The Flag tag was introduced at the C-terminus of Ufc1. The FLAG-tag or FLAG octapeptide, is a polypeptide protein tag that can be added to a protein using Recombinant DNA technology and helps to separate recombinant, over expressed protein by the host organism. The peptide sequence of the FLAG-tag is as follows: N-DYKDDDDK-C (1012 Da).The Flag *Ld*Ufc1 was then sub cloned in the pCR®2.1-TOPO TA. All the mutations were confirmed by sequencing and primers used for site directed mutagenesis or other modifications are listed in table 5.2.

Table 5.2: List of genes and primer sequences

Gene	**Primer (F)** (5'--- 3')	**Primer (R)** (5'--- 3')
***Ld*Uba5**	ATG CCC TTA TCC TCC TCC CC	CTA CTG GAT GGC CTT CAT GCG
***Ld*Ufm1**	ATGAGCGCTGCGAACGACTCCG	CCCCCTCCACCACCGCCACCGG
***Ld*Ufc1**	ATGGAGCCGTCAGTGAAGGAGAG C	CTCAGCAGTCGTGGG CACCGTTG

***Ld* Uba5^{C217S}**	AAGCGGGAGGGCGTCTCCGCGGC GTCGCTGCCG	CGGCAGCGAGGCCGCGGAGACGCCCTCCCGCTT
***Ld* Uba5^{C217A}**	AAGCGGGAGGGCGTCGCCGCGGC CTCGCTGCCG	CGGCAGCGAGGCCGCGGCGACGCCCTCCCGCTT
***Ld* Ufm1 6XHis**	CACCACCACCACCACCACATGAGC GCTGCGAACGACTCCG	CCCCCTCCACCACCGCCACCGG
***Ld* Ufm1∆C**	ATGAGCGCTGCGAACGACTCCG	ACCGACGCGATCACGCGGAATCAG
***Ld*Ufm1^{G98A}**	ATGAGCGCTGCGAACGACTCCG	TCACCCCCTCCACCACCGCCACCGGCTCCTCAGA AGGCGGACGCAGGGGGCGGCAGC
***Ld*Ufc1 Flag**	ATGGAGCCGTCAGTGAAGGAGAG C	TTTATCATCATCATCTTTATAATCCTCAGCAGTC GTGGGCACCGTTG

***In vitro Ld*Uba5 – *Ld*Ufm1 activation assay**

Cell lysates of HA-tagged *Ld*Uba5, *Ld*Uba5^{C217S}, mature Ufm1 with exposed C-terminal Gly^{98} residue (Ufm1) and *Ld*Ufm1^{G98A} constructs over expressed in *L.donovani* were Immuno-precipitated using Anti -HA Ab followed by purification with HA beads. The Transcreener AMP Assay (Bellbrook labs, USA) is a universal biochemical assay designed for enzymes that produce AMP. In the one-step detection protocol, 10µl AMP Detection Mixture (20µg/ml AMP/GMP Antibody and 2nM AMP/GMP Alexa633 Tracer in 0.5X Stop & Detect Buffer B (10X consists of 200mM HEPES (pH 7.5), 400mM EDTA, and 0.2% Brij-35) is added to the 10µl enzyme reaction (4 µg mature Ufm1 or *Ld*Ufm1^{G98A} and 10µM ATP, 2 or 0.2 µg *Ld*Uba5 or *Ld*Uba5^{C217S}), mixed and incubated for 1 hour. Total 20µl of reaction was set up in entirely black with a non-binding surface 96 well plate (Corning) and fluorescence polarization (mP) was measured with SpectraMax M5 spectrofluorimeter. Human Uba5 and Ufm1 recombinant proteins, purchased from BostonBiochem, USA) were used as positive control.

Growth kinetics

Stationary phase parasites were diluted to 0.5 $X10^5$ cells/ml in 10 ml cultures to allow cells to enter logarithmic phase. For each time point, the parasites were pelleted by centrifugation, washed with PBS, and counted by light microscopy. The growth kinetics of all the cell lines was studied at the promastigote stage.

Immunofluorescence assay (IFA)

L.donovani promastigotes/amastigotes were fixed in suspension in 4% (w/v) paraformaldehyde in PBS (50 mM Na2HPO4, 150 mM NaCl, pH 7.4) for 20 min at room temperature, washed three times in PBS, and allowed to attach to glass slides. After air drying, the slides were first immersed in ice-cold methanol (-20°C) for 5 min, blocked for 30 min in 1% (w/v) bovine serum albumin (United States Biochemical Co., Cleveland, OH) in PBS, and incubated 1 h with either the anti-*Ld*Uba5 serum (1:200 dilution) or the anti-HA serum (1:30 dilution) diluted in 1% bovine serum albumin in PBS. After three washes in PBS, cells were incubated for 1 h with affinity-purified fluorescein-conjugated anti-rabbit IgG (H+L) antibody when probed with anti-*Ld*Uba5 Ab and Texas red anti-mouse IgG (H+L) antibody when probed with anti-HA serum antibody. These secondary antibodies (Vector Laboratories Inc., Burlingame, CA), were diluted 1:200-fold in PBS containing 1% BSA. Cells were subsequently washed three times with PBS and mounted in Vectashield containing 4’6-diamidino-2-phenylindole (DAPI) (Vector, Vector Lab. Inc.) to stain both nucleus and kinetoplast. Cells were examined for fluorescence under the microscope (Nikon (DIAPHOT-200), Tokoyo, Japan), with epi-fluorescence and images captured with Pixera (120ES) color digital camera. Confocal studies were conducted under 100 x objective lens of Leica-DM IRBE (Leica Microsystem, Heidelberg, Germany) using krypton and argon/UV lasers. The focal plane chosen in all

the cells was in the middle of the cells. The images were processed using Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA).

Cell fractionation

For Cell fractionation, *Leishmania* cells were washed three times in 15 ml MES buffer (20 mM MOPS, pH 7.0, 250 mM sucrose, 3 mM EDTA). The cell pellet was resuspended in 0.2 ml MES buffer containing 1 mg/ml digitonin and protease inhibitor cocktail (Roche Applied Science). The suspension was incubated at room temperature for 5 minutes and centrifuged at 10,000 ***g*** for 5 minutes. The resulting supernatant was collected as a cytosolic fraction, and the heavy membrane pellet enriched for mitochondria was resuspended in phosphate buffer (20 mM sodium phosphate, pH 7.0, 3 mM EDTA).

Immunological analysis

For immunoblot analysis, cells were lysed with ice-cold 4X SDS lysis buffer(125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 100 mM dithiothreitol, 0.005% bromphenol blue and protease inhibitors) and the lysates were separated by SDS–PAGE (12% gel) and transferred to a Nitrocellulose membrane. Mouse monoclonal anti-Flag antibody (M2; Sigma Chemical Co., St Louis, MO), anti-HA antibody (F7; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal anti-Uba5 antibody were used for immunodetection. Development was performed by the Chemiluminescent HRP substrate Bound ECL Western blotting detection system (Immobilon^{TM} from Millipore, U.S.A.). For immunoprecipitation analysis, cells were lysed by 200 ml of 4x SDS lysis buffer, and the lysate was then centrifuged at 10,000 g for 10 min at 4ºC to remove debris. In the next step, 800 ml of 4x SDS lysis buffer and 30 ml of M2-agarose (Sigma) were added to the lysate, and the mixture was mixed under constant rotation for 12 h at 4ºC. The

immunoprecipitates were washed five times with ice-cold 4x SDS lysis buffer. The complex was boiled for 10 min in SDS sample buffer in the presence of β-mercaptoethanol to elute proteins and centrifuged at 10 000 g for 10 min at 4ºC. The supernatant was subjected to SDS–PAGE, transferred to Nitrocellulose membrane, and analyzed by immunoblots with anti-Flag (M2) or anti-HA or anti-*Ld*Uba5 antibody. For purification of 6xHis-tagged proteins under denaturing conditions, cells were lysed by 1 ml of denaturing lysis buffer (8M urea, 0.1M NaH2PO4, and 0.01M Tris–HCl, pH 8.0) in the presence of 20mM N-ethylmaleimide as an inhibitor of isopeptidases, and the lysate was sonicated briefly and then centrifuged at 10 000 g for 10 min at room temperature to remove debris. Then, 10 ml of Ni-NTA Superflow (QIAGEN) was added to the lysate, and the mixture was shaken under constant rotation for 30 min at room temperature. The precipitates were washed five times with denaturing wash buffer (8M urea, 0.1M NaH2PO4, and 0.01M Tris–HCl, pH 5.9). To elute proteins, elution buffer (8M urea, 0.1M NaH2PO4, and 0.01M Tris–HCl, pH 4.5) was added to the complex, and the mixture was centrifuged at 10 000 g for 10 min at room temperature. The resulting supernatant was subjected to SDS–PAGE, transferred to Nitrocellulose membrane, and analyzed by immunoblots with respective antibodies.

In Vitro Culture of *T. brucei* Parasites for RNAi

T. brucei procyclic form strain 29-13 that harbors integrated genes for T7 RNA polymerase and tetracycline repressor (Wirtz *et al.*, 1999) was used. The parasites were grown in SDM-79 medium supplemented with 10% fetal bovine serum during normal growth and 15% during transfection and clonal selection. G418 (15 µg/ml) and hygromycin B (50 µg/ml) were added in the medium to preserve T7 RNA polymerase

and tetracycline repressor gene constructs within the cells. The parasites were grown and harvested as described previously (Morris *et al.,* 2004).

Transfection of Parasites for RNAi

Gene characterization via cognate mRNA degradation (RNAi) and arrest of translation is a routinely followed molecular technique as described in certain eukaryotes (Ullu *et al.,* 2004). This is achieved through plasmid construct carrying a unique portion of the gene, which after transfection in cell generates double stranded RNA of the gene upon induction (currently with tetracycline), which then targets the cognate native mRNA for degradation. Specifically in this study to amplify PCR fragment of TbUba5 gene for developing RNAi construct, gene specific forward and reverse primers were designed utilizing the putative Uba5 sequence from the *T. brucei* genome sequence databank (Berriman *et al.,* 2005). Oligos were designed (TbUba5 P1 and TbUba5 P2) in which the amplicon constitutes a portion from the 5' untranslated region (from -69 base pairs) of the genes into approximately the middle of the open reading frame (ORF; +364 base pairs) with HindIII and XhoI restriction sites added to the termini of the PCR fragments. The PCR amplified fragment was a 364-base pair unique sequence that had no significant sequence identity with the rest of the *T. brucei* genome sequences. The fragment was subcloned into the HindIII and XhoI sites of the pZJM vector (Wang *et al.,* 2000). The recombinant plasmid was linearized with NotI and DNA transfection carried out on the procyclic form of *T. brucei* using a published procedure (Morris *et al.,* 2004) using a BTX ECM 630 Electroporator (BTX, San Diego, CA, 500 V and 275 µF). Transfectants were selected in the presence of 2.5 µg/ml phleomycin and a clonal cell line was obtained by limiting dilutions. For induction of RNAi, the cloned stable transfectant that reached a constant growth rate after at least three regular subpassages was cultured in the presence

of 1.0µg/ml tetracycline. For monitoring cell growth, the cells were counted at different time intervals using a Coulter Z1 particle counter (Beckman Coulter, Fullerton, CA) with a starting culture of 1 x 10^5cells/ml. To examine the specific reduction in Uba5 transcription during RNAi induction, the cloned stable transfectant, either uninduced or induced with tetracycline for 3 d, was analyzed for TbUba5 mRNA level using Northern blot analysis (as given in chapter 4). The signal intensity was quantitated using a Phosphor Imager system (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ) as described previously (Selvapandiyan *et al.*, 2001).

Primers used for RNAi study

TbUba5 P1	CGC CTC GAG CTC ATG GCA CTT CAG CGG ATG G	364 bp
TbUba5 P2	CGC AAG CTT CTA CCG GAG TGC TGG GCT TTA CAC	
Tb Uba5 P3	CTG CGT CTC TTC CAA CCA CGA TG	460 bp
Tb Uba5 P4	GGC AAC AAA CTC GTC TTC CCT TGG	

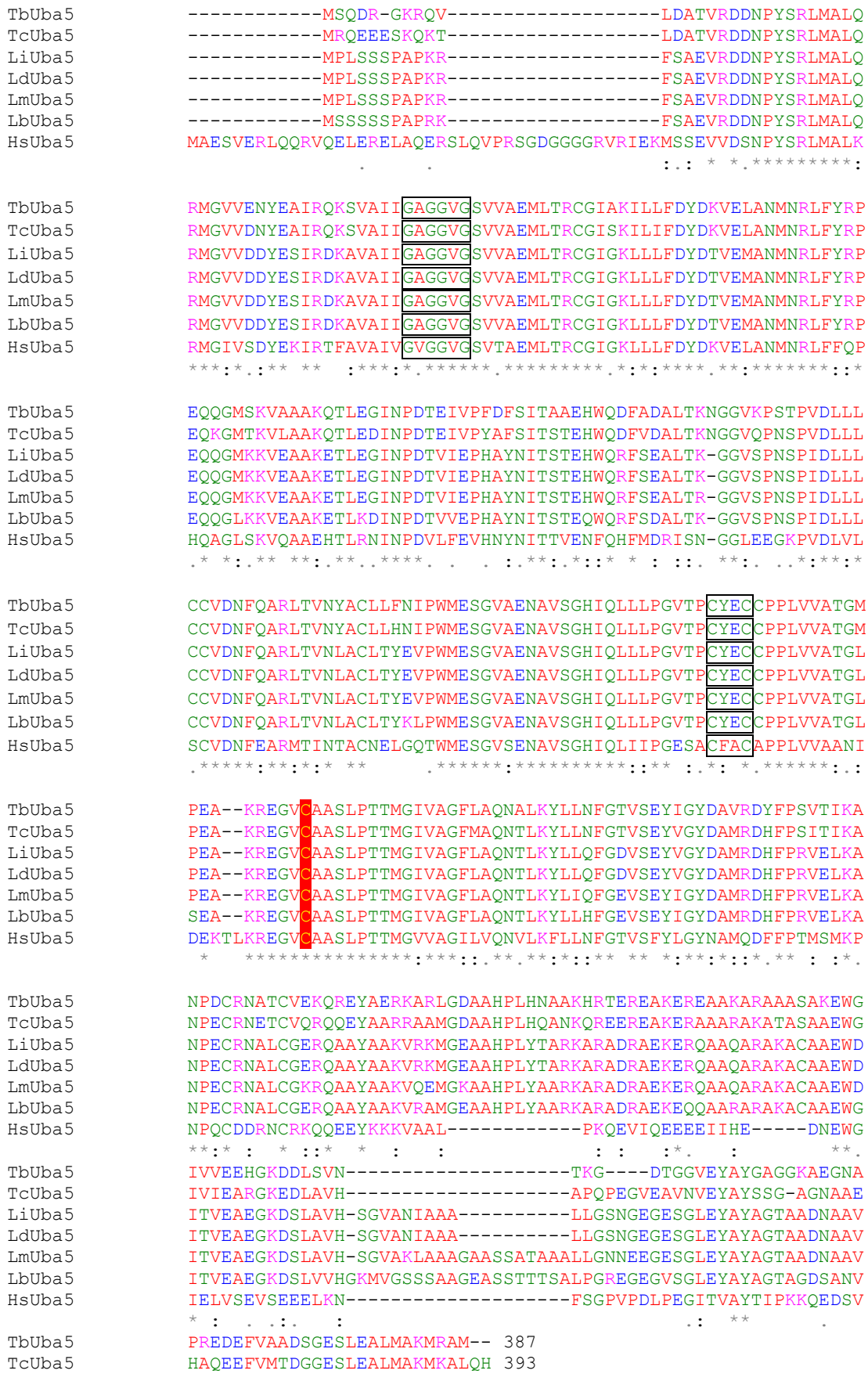
Results:-

Detection of *Ld*Uba5 transcripts in amastigote stage and in bone marrow of VL patients

Stage regulated transcriptome profiling using *L. donovani* genomic microarray led to the identification of a gene that showed 3.49 (±0.24) fold higher expression at axenic Am stage of *L.donovani* and showed homology with human Uba5, thus was termed as *Ld*Uba5. Up regulation of *Ld*Uba5 was validated by Real time PCR in both axenic and hamster derived amastigotes, further *Ld*Uba5 gene transcripts were also shown in bone marrow samples from VL patients (Data shown in chapter 4).

Sequence analysis, Cloning and Expression of rLdUba5 in *L.donovani*

The *Ld*Uba5 sequence aligned within the ORF region of the gene LinjF15.1020 (NAD/FAD dependent dehydrogenase) in the *L.infantum* genome, which codes for a protein of ~43.2kDa. Primers were designed for ORF region of this *L.infantum* gene and amplified with the *L. donovani* DNA and sequenced. The *L.donovani* sequence showed 100% homology with the gene from *L.infantum.* Homology search of the open reading frame of *Ld*Uba5 revealed its similarity with a 404-amino-acid protein (Uba5), highly conserved in various multicellular organisms, including *Homo sapiens*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana*, but absent in yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and a member of the E1-like enzyme family. We named this protein *Ld*Uba5, based on its homology to human Uba5. Clustal W analysis of *Ld*Uba5 showed conserved cysteine residue similar to human Uba5 in *Leishmania* and *Tyrpanosoma* (Fig 5.3). *Ld*Uba5 also has an ATP-binding motif (GXGXXG) and a metal-binding motif conserved in other E1-like enzymes such as Uba2, Uba3, Uba4, and Atg7. Most of E1-like enzymes have an active Cys residue within the conserved 10–20 amino-acid (AA) residues downstream from the metal-binding motif (Komatsu et al, 2004). For the production of recombinant *Ld*Uba5 protein, the gene was amplified using DNA isolated from *L.donovani* and cloned into pCR-T7 CT TOPO TA expression vector. A sufficient quantity of the recombinant *Ld*Uba5 protein was produced to inject into a rabbit for the production of antibodies to *Ld*Uba5.


TbUba5 ----------MSQDR-GKRQV-------------------LDATVRDDNPYSRLMALQ
TcUba5 ----------MRQEEESKQKT-------------------LDATVRDDNPYSRLMALQ
LiUba5 ----------MPLSSSPAPKR-------------------FSAEVRDDNPYSRLMALQ
LdUba5 ----------MPLSSSPAPKR-------------------FSAEVRDDNPYSRLMALQ
LmUba5 ----------MPLSSSPAPKR-------------------FSAEVRDDNPYSRLMALQ
LbUba5 ----------MSSSSSPAPRK-------------------FSAEVRDDNPYSRLMALQ
HsUba5 MAESVERLQQRVQELERELAQERSLQVPRSGDGGGGRVRIEKMSSEVVDSNPYSRLMALK
TbUba5 RMGVVENYEAIRQKSVAIIGAGGVGSVVAEMLTRCGIAKILLFDYDKVELANMNRLFYRP
TcUba5 RMGVVDNYEAIRQKSVAIIGAGGVGSVVAEMLTRCGISKILIFDYDKVELANMNRLFYRP
LiUba5 RMGVVDDYESIRDKAVAIIGAGGVGSVVAEMLTRCGIGKLLLFDYDTVEMANMNRLFYRP
LdUba5 RMGVVDDYESIRDKAVAIIGAGGVGSVVAEMLTRCGIGKLLLFDYDTVEMANMNRLFYRP
LmUba5 RMGVVDDYESIRDKAVAIIGAGGVGSVVAEMLTRCGIGKLLLFDYDTVEMANMNRLFYRP
LbUba5 RMGVVDDYESIRDKAVAIIGAGGVGSVVAEMLTRCGIGKLLLFDYDTVEMANMNRLFYRP
HsUba5 RMGIVSDYEKIRTFAVAIVGVGGVGSVTAEMLTRCGIGKLLLFDYDKVELANMNRLFFQP
TbUba5 EQQGMSKVAAAKQTLEGINPDTEIVPFDFSITAAEHWQDFADALTKNGGVKPSTPVDLLL
TcUba5 EQKGMTKVLAAKQTLEDINPDTEIVPYAFSITSTEHWQDFVDALTKNGGVQPNSPVDLLL
LiUba5 EQQGMKKVEAAKETLEGINPDTVIEPHAYNITSTEHWQRFSEALTK-GGVSPNSPIDLLL
LdUba5 EQQGMKKVEAAKETLEGINPDTVIEPHAYNITSTEHWQRFSEALTK-GGVSPNSPIDLLL
LmUba5 EQQGMKKVEAAKETLEGINPDTVIEPHAYNITSTEHWQRFSEALTR-GGVSPNSPIDLLL
LbUba5 EQQGLKKVEAAKETLKDINPDTVVEPHAYNITSTEQWQRFSDALTK-GGVSPNSPIDLLL
HsUba5 HQAGLSKVQAAEHTLRNINPDVLFEVHNYNITTVENFQHFMDRISN-GGLEEGKPVDLVL
TbUba5 CCVDNFQARLTVNYACLLFNIPWMESGVAENAVSGHIQLLLPGVTPCYECCPPLVVATGM
TcUba5 CCVDNFQARLTVNYACLLHNIPWMESGVAENAVSGHIQLLLPGVTPCYECCPPLVVATGM
LiUba5 CCVDNFQARLTVNLACLTYEVPWMESGVAENAVSGHIQLLLPGVTPCYECCPPLVVATGL
LdUba5 CCVDNFQARLTVNLACLTYEVPWMESGVAENAVSGHIQLLLPGVTPCYECCPPLVVATGL
LmUba5 CCVDNFQARLTVNLACLTYEVPWMESGVAENAVSGHIQLLLPGVTPCYECCPPLVVATGL
LbUba5 CCVDNFQARLTVNLACLTYKLPWMESGVAENAVSGHIQLLLPGVTPCYECCPPLVVATGL
HsUba5 SCVDNFEARMTINTACNELGQTWMESGVSENAVSGHIQLIIPGESACFACAPPLVVAANI
TbUba5 PEA--KREGVCAASLPTTMGIVAGFLAQNALKYLLNFGTVSEYIGYDAVRDYFPSVTIKA
TcUba5 PEA--KREGVCAASLPTTMGIVAGFMAQNTLKYLLNFGTVSEYVGYDAMRDHFPSITIKA
LiUba5 PEA--KREGVCAASLPTTMGIVAGFLAQNTLKYLLQFGDVSEYVGYDAMRDHFPRVELKA
LdUba5 PEA--KREGVCAASLPTTMGIVAGFLAQNTLKYLLQFGDVSEYVGYDAMRDHFPRVELKA
LmUba5 PEA--KREGVCAASLPTTMGIVAGFLAQNTLKYLIQFGEVSEYIGYDAMRDHFPRVELKA
LbUba5 SEA--KREGVCAASLPTTMGIVAGFLAQNTLKYLLHFGEVSEYIGYDAMRDHFPRVELKA
HsUba5 DEKTLKREGVCAASLPTTMGVVAGILVQNVLKFLLNFGTVSFYLGYNAMQDFFPTMSMKP
TbUba5 NPDCRNATCVEKQREYAERKARLGDAAHPLHNAAKHRTEREAKEREAAKARAAASAKEWG
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LmUba5 NPECRNALCGKRQAAYAAKVQEMGKAAHPLYAARKARADRAEKERQAAQARAKACAAEWD
LbUba5 NPECRNALCGERQAAYAAKVRAMGEAAHPLYAARKARADRAEKEQQAARARAKACAAEWG
HsUba5 NPQCDDRNCRKQQEEYKKKVAAL----------PKQEVIQEEEEIIHE-----DNEWG
TbUba5 IVVEEHGKDDLSVN--------------------TKG----DTGGVEYAYGAGGKAEGNA
TcUba5 IVIEARGKEDLAVH--------------------APQPEGVEAVNVEYAYSSG-AGNAAE
LiUba5 ITVEAEGKDSLAVH-SGVANIAAA----------LLGSNGEGESGLEYAYAGTAADNAAV
LdUba5 ITVEAEGKDSLAVH-SGVANIAAA----------LLGSNGEGESGLEYAYAGTAADNAAV
LmUba5 ITVEAEGKDSLAVH-SGVAKLAAAGAASSATAAALLGNNEEGESGLEYAYAGTAADNAAV
LbUba5 ITVEAEGKDSLVVHGKMVGSSSAAGEASSTTTSALPGREGEGVSGLEYAYAGTAGDSANV
HsUba5 IELVSEVSEEELKN--------------------FSGPVPDLPEGITVAYTIPKKQEDSV
TbUba5 PREDEFVAADSGESLEALMAKMRAM-- 387
TcUba5 HAQEEFVMTDGGESLEALMAKMKALQH 393

FIG 5.3 Clustal W analysis of Uba5

Multiple alignment of various Uba5 sequences: Hs,H.sapiens (AK026904);*Ld*, *L.donovani*;Lm,*L.major*(LmjF15.0970);Tb,*T.brucei*(Tb09.160.4430);Tc,*T.cruzi*(Tc00.10 47053505843.40). The accession numbers for the *Leishmania* and *Trypanosoma* genes are available from GeneDB while the Human Uba5 is available from GenBank data bank. The amino-acid sequence of Uba5 is compared by Clustal W program. Asterisks denote identical amino acids; single and double dots denote weakly and strongly similar AAs, respectively, determined by the criteria of ClustalW program. AAs are listed in the standard one letter code, and residues identical to Human Uba5 are indicated by dark boxes. The putative active site Cys residue is boxed in red. Open box indicates an ATP-binding motif (GXGXXG). The metal binding motif is underlined.

Expression of *Ld*Uba5 at protein level in Pro and Ama stages of *L.donovani*

The antibodies raised against recombinant *Ld*Uba5 bind to a single band of approximately ~ 45 kDa. Immunoblotting with the *Ld*Uba5 Ab revealed that this protein is expressed equally in both Pro as well as Ama stages of *L.donovani* (Fig 5.4). Normalization of parasite lysates was carried out using anti-α-tubulin antibody. The expression at protein level was tested in two isolates of *L. donovani* and in two biological preparations each.

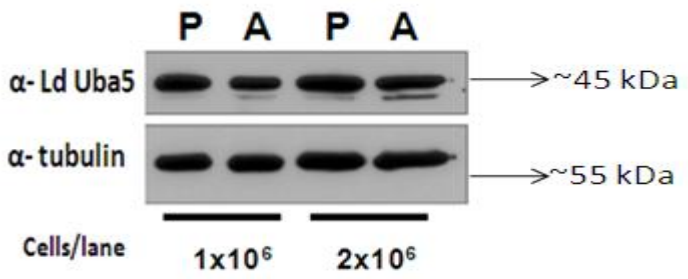


Fig 5.4 Western blot analysis for protein expression of *Ld*Uba5.

Cell lysates (25µg of protein) from promastigote (P) as well as amastigote (A) stage were run on a 12% SDS-PAGE and analyzed by Western blot using anti *Ld*Uba5 Ab. The blots were normalized using α tubulin Ab.

RNAi-induced Reduction of Specific TbUba5 mRNA

The putative *T. brucei* Uba5 ORF sequences obtained from GeneDB were compared with the *Leishmania* and human Uba5 genes by ClustalW (Figure 5.3). Based on the sequence similarity and clustal analysis, the Uba5 from *T.brucei* (Tb09.160.4430) and *L. donovani* share 68% sequence similarity at the amino acid level and was designated as TbUba5.

To characterize *Tb*Uba5 function, we knocked down the mRNA level of this *Tb*Uba5 using RNAi methodology in the procyclic stage of the parasite. Northern blot analysis of RNA obtained from the tetracycline-induced culture on day three revealed the reduction of the cognate mRNA level (Figure 5.5). For normalization, the membrane was reprobed with α-tubulin gene fragment, and its mRNA level was quantitated, the reduction in the amount of Uba5 mRNAs in the tetracycline-induced culture as compared with the uninduced culture was ~92%.

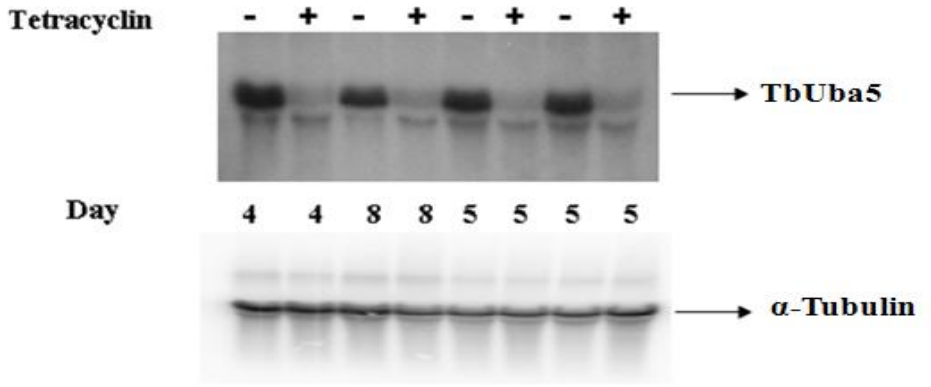


Fig 5.5 Northern blot showing the effect of RNAi: Northern blots of RNA from induced and uninduced TbUba5 culture. Membrane containing 12 µg total RNA from uninduced (Un) and induced (I) cells was used. Membrane was hybridized with specific Uba5 DNA and further reprobed with α-tubulin for normalization.

***Tb*Uba5 is not essential for the Growth of the *T.brucei* Parasite**

The effect of reduction in TbUba5 level on the growth of the TbUba5 RNAi cells was monitored by counting cell number daily for 6 day after RNAi induction (Figure 5.6). The tetracycline- induced parasites showed no significant growth defect as compared with uninduced control cells.

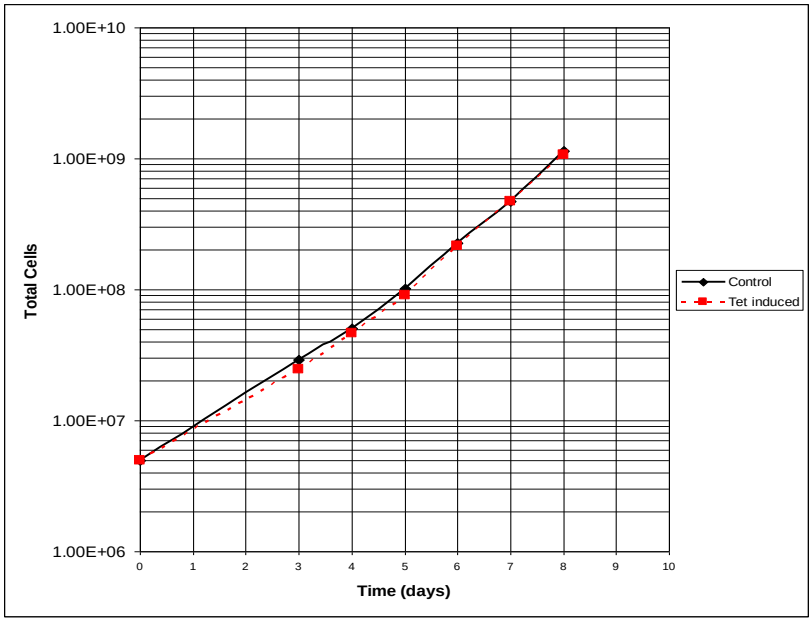
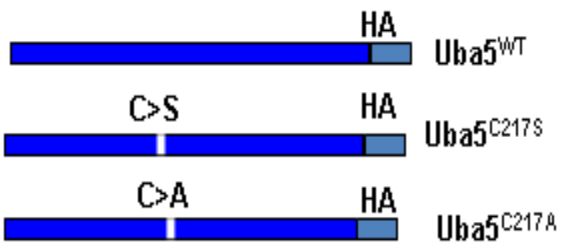


Fig 5.6: **The effect of TbUba5 knockdown on the in vitro growth of *T. brucei* procyclics**. The cells were grown with (I) or without (Un) tetracycline. The data represent the means of ± SD of three independent experiments.

Cysteine^{217} is the active site residue of *Ld*Uba5

If an active site Cys residue within an E1 or E1-like enzymes is changed to Ser, an O-ester bond instead of a thioester bond is formed with its respective modifier protein and the intermediates become stable even under reducing conditions (Komatsu et al, 2004). In human Uba5, Cys ^{250} is the active site residue, multiple alignment of *Ld*Uba5 along with human Uba5 showed Cys^{217} to be the most possible active site residue in *Ld*Uba5 (Fig 5.3). Therefore we mutated Cys within *Ld*Uba5 to Ser or Ala by site directed mutagenesis and prepared constructs Δ*Ld*Uba5^{C217S}or Δ*Ld*Uba5^{C217A}(Fig 5.7A). WT*Ld*Uba5 and mutated plasmid constructs were transfected into *L.donovani* promastigotes. Cell lysates were prepared from each of the transfected parasites at mid-log stage and analyzed by Western blot using anti-*Ld*Uba5 Ab, subsequent to confirmation of transfection with anti HA antibodies (Fig 5.7 B). Parasites transfected with *Ld*Uba5 or Δ*Ld*Uba5^{C217A} gave a single band of ~45kDa (Fig 5.7 B, lane 2, 4) while those transfected with Δ*Ld*Uba5^{C217S} showed an additional band of ~57kDa in addition to 45kDa, indicating that Δ*Ld*Uba5^{C217S} forms a stable intermediate complex with an endogenous protein(Fig 5.7 B, lane 3). Cell lysates from wild type (WT) *Leishmania* cells used as control showed a band of ~45kDa with anti-*Ld*Uba5 Ab(Fig 5.7 B, lane 1).

(A)



(B)

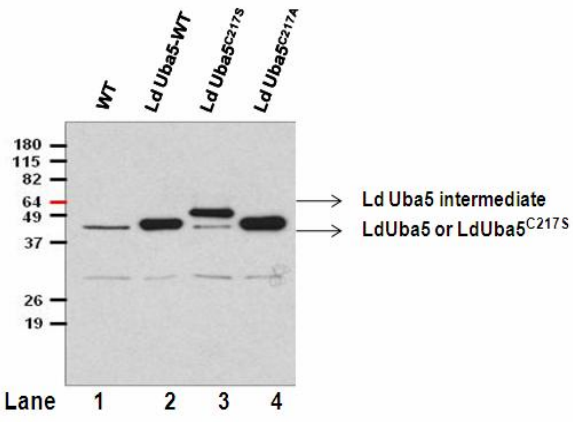


Figure 5.7 Demonstration of intermediate complex linked to *Ld*Uba5 in *Leishmania* cells.

A. Schematic representation of *Leishmania* expression plasmids for *Ld*Uba5 and the derivative mutants. *Ld*Uba5 was constructed with HA tagged at the C-terminus (*Ld*Uba5^{WT}). To construct *Ld*Uba5^{C217S} and *Ld*Uba5^{C217A}, Cys^{217} of HA-*Ld*Ufm1 was mutated to Ser/Ala by site-directed mutagenesis. The *Ld*Uba5^{C217S} and *Ld*Uba5^{C217A} mutants were tagged with HA epitope at C-terminus.

B. Cell lysates (25µg) from parasites transfected with *Ld*Uba5 (WT), Δ*Ld*Uba5^{C217S}(C>S) or Δ*Ld*Uba5^{C217A}(C>A) were subjected to SDS–PAGE and analyzed by immunoblotting with α-*Ld*Uba5 purified IgG. Cell lysate from wild type *L.donovani* cells were used as control.

Identification and Sequence analysis of a novel Ubiquitin-fold molecule, Ufm1

We next sought to determine the identity of the protein of ~12kDa which is forming an intermediate complex with Δ*Ld*Uba5^{C217S} leading to an additional band of ~57kDa in Western blot (Fig 5.7 B). Since human Uba5 has been known to interact with Ufm1 (Komatsu et al, 2004), its homolog was searched in the *L. infantum* GeneDB. An ~115 amino acid protein with a predicted molecular mass of ~12.4kDa showed high percentage of similarity with accession no. Linj16.V31100 (Conserved hypothetical protein). Primers were designed for ORF region of this *L.infantum* gene and amplified with *L. donovani* DNA, sequencing of which showed 100% homology with the *L.infantum* gene. Due to high degree (54%) of similarity with the human Ufm1, we named it as *Ld*Ufm1(Fig 5.8 A). Human Ufm1 protein is conserved in multicellular organisms, but not in yeasts, and although the protein shows no clear overall sequence identity to ubiquitin or other modifiers, the tertiary structure displays a striking resemblance to human ubiquitin. Ufm1 has a single Gly residue conserved across species at the C-terminal region, although the

length and sequences of amino acids extending from this Gly residue vary among species. In case of *Leishmania* (*L.donovani, L.major and L.infantum*) after C terminal Glycine residue 17 AA are there while in humans and *Trypanosoma* only two AA are present (Fig 5.8A). An interesting difference in the size of Ufm1 had been found in different *Leishmania* species; *L.donovani* and *L.infantum* contains 115 AA while in *L.major* 249 AA are there. Clustal W analysis of Ufm1 showed conserved glycine residue similar to human Ufm1 and Ubiquitin in *Leishmania* and *Tyrpanosoma* (Fig 5.8A, B).

(A)

```
LdUfm1  ------------------------------------------------------------
LiUfm1  ------------------------------------------------------------
LmUfm1  MTLCRYFVFAPLSRCASFRYLPVCCAQVPAPLLPSLTTYTHTHTPTHTHASTVLPAVIPS
TcUfm1  ------------------------------------------------------------
TbUfm1  ------------------------------------------------------------
HsUfm1  ------------------------------------------------------------

LdUfm1  ------------------------------------------------------------
LiUfm1  ------------------------------------------------------------
LmUfm1  AFRWHSFSQHFYFFRHSTSLSPRARTIDTDRPGRTDGHLVQNLFDRRSGDEWRERGEGEP
TcUfm1  ------------------------------------------------------------
TbUfm1  ------------------------------------------------------------
HsUfm1  ------------------------------------------------------------

LdUfm1  ---------------MSAANDSGAPAAPSGNKVTFRVILTSERSQPFRVISIAEEAPLTA
LiUfm1  ---------------MSAANDSGAPAAPSGNKVTFRVILTSERSQPFRVISIAEEAPLTA
LmUfm1  QRKQKKTFTSVVRRTMSAANQSGAPAAPSGNKVTFRVILTSERSQPFRVISIAEEAPLTA
TcUfm1  ---------------MSQEEQT-APAA-SGGKVTFRVILTSERTQPFRVISIAEEAPLTA
TbUfm1  ---------------MSQ--NEEAPAR-SGGKVTFRIILTSERSQPFRVISIAEEAPLTA
HsUfm1  ------------------------------MSKVSFKITLTSDPRLPYKVLSVPESTPFTA
                                       .**:*:: ***:   *::*:*:.*.:*:**
LdUfm1  VLRFAAEEFGVASVDSMAATTKDGTGINPAQTAGTVFMKYGQEIRLIPRDRVGAAPCVRL
LiUfm1  VLRFAAEEFGVASVDSMAATTKDGTGINPAQTAGTVFMKYGQEIRLIPRDRVGAAPCVRL
LmUfm1  VLRFAAEEFGIASVDSMAATTKDGTGINPAQTAGTVFMKYGQEIRLIPRDRVGAAPCVRL
TcUfm1  VLRFAAEEFGIASVDSMAATTKDGTGINPAQTAGSVFMKYGQEIRLIPRDRVGAA-----
TbUfm1  ALRFAAEEFGIASVDSMAATTKDGTGINPAQTAGNVFMKYGQEIRLIPRDRVGRV-----
HsUfm1  VLKFAAEEFKVPAATS-AIITNDGIGINPAQTAGNVFLKHGSELRIIPRDRVGSC-----
        .*:****** :.:. * *  *:** *********.**:*:*.*:*:*******
LdUfm1  LRSRWRWWRG
LiUfm1  LRSRWRWWRG
LmUfm1  LRSRW-WCRG
TcUfm1  ----------
TbUfm1  ----------
HsUfm1  ----------
```

(B)

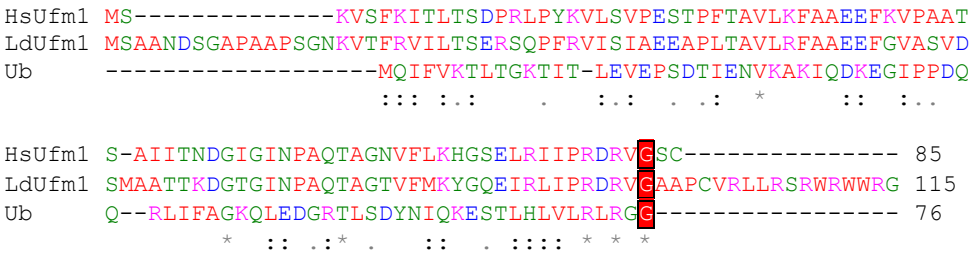


Figure 5.8 Clustal W analysis of Ufm1, a Ubiquitin-fold molecule.

(A) Sequence alignment of *Ld*Ufm1 with its homologs in *Homo sapiens*, *L.major* and trypanosomes. The sequence of hsUfm1 is available from GenBanK under the accession number BC005193. The sequence of *L.donovani* Ufm1; *L. major* (LmjF16.1065); *T.brucei* (Tb927.8.5380); *T.cruzi* (Tc00.1047053507491.59); are from GeneDB . The C-terminal conserved Gly residue is boxed in red.

(B) Sequence alignment of HsUbiquitin with hsUfm1 and *Ld*Ufm1. The C-terminal conserved Gly residue is boxed in red.

***Ld*Ufm1 is post translationally cleaved at C-terminus conserved Glycine residue**

To test whether the C-terminus of *Ld*Ufm1 is post-translationally cleaved, we constructed an expression vector for Ufm1 tagged with HA epitope at the N-terminus (HA- *Ld*Ufm1). It is known that the replacement of C-terminal Gly residue of Ub and other UBLs with an Ala residue inhibits the C-terminal processing (Kabeya et al, 2000; Tanida et al, 2003). Two constructs HA-*Ld*Ufm1^{G98A} (Gly^{98} of HA-*Ld*Ufm1 was mutated to Ala) and HA-*Ld*Ufm1ΔC (equivalent to mature Ufm1$^{1-98}$ protein lacking the C-terminal residues after Gly of proUfm1) were constructed and expressed in *L.donovani* cells to examine whether Gly^{98} of *Ld*Ufm1 is essential for the cleavage (Figure 5.9 A). After transfection of HA-*Ld*Ufm1, HA-*Ld*Ufm1ΔC and HA-*Ld*Ufm1^{G98A} into *L.donovani* cells, the cell lysate were subjected to SDS–PAGE, protein corresponding to each construct was recognized by immunoblotting with anti-HA antibody (Fig 5.9 B). The mobility on SDS–PAGE of HA-

*Ld*Ufm1was similar to that of HA-*Ld*Ufm1ΔC (equivalent to mature Ufm1$^{1-98}$ protein) lacking the C-terminal residues after Gly of proUfm1 (Fig 5.9 B). These results suggested that the C-terminus of Ufm1 is post-translationally cleaved in the cells, producing mature Ufm1 with the C-terminal Gly residue. The mobility of most HA-*Ld*Ufm1^{G98A} on SDS–PAGE was apparently slower than that of HA-*Ld*Ufm1 suggesting that mutation Gly^{98} to Ala confers resistance to its C-terminal cleavage (Fig 5.9B). Results indicate that similar to human Ufm1, *Leishmania* Ufm1 is enzymatically processed as indicated by the G>A mutant which remained as pro-Ufm1 and therefore migrated as an high molecular weight band.

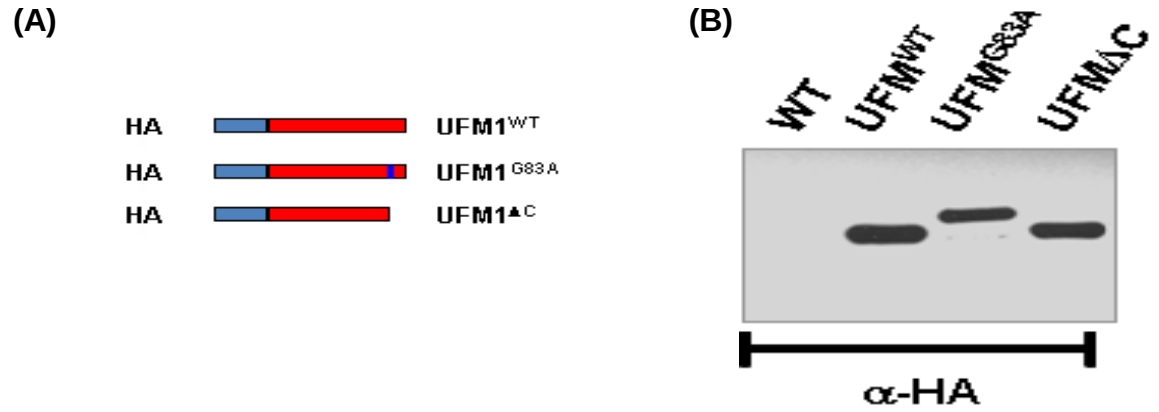


Figure 5.9 Post translational processing of *Ld*Ufm1

(A) Schematic representation of *Leishmania* expression plasmids for Ufm1 and the derivative mutants. *Ld*Ufm1 was constructed with HA tagged at the N-terminus (*Ld*Ufm1WT). To construct *Ld*Ufm1ΔC, residues from C-terminal Gly^{98} of mature Ufm1 were deleted by PCR. To construct *Ld*Ufm1^{G98A}, Gly^{98} of HA-*Ld*Ufm1 was mutated to Ala by site-directed mutagenesis. The *Ld*Ufm1ΔC and *Ld*Ufm1^{G98A} mutants were tagged with HA at the N-terminus.

(B) *L.donovani* cells were transfected with *Ld*Ufm1WT, *Ld*Ufm1ΔC and *Ld*Ufm1^{G98A} using wild type cells as control. The cell lysates were subjected to SDS–PAGE and analyzed by immunoblots with anti-HA antibodies. Cell lysates from wild type (WT)

Leishmania cells are used as control which showed no band with anti-HA Ab(Fig 4B, lane 1).

Uba5 is an Ufm1-activating enzyme

We next investigated whether *Ld*Uba5 forms an intermediate complex with *Ld*Ufm1. We expressed *Ld*Uba5 or Δ*Ld*Uba5^{C217S} with HA & 6XHis-tagged Ufm1 (HA6XHis-*Ld*Ufm1) in *L.donovani* cells. Wild type (WT) *L.donovani* cells were used as control. Each cell lysates were prepared and analyzed by immunoblotting with anti-His antibody. When Δ*Ld*Uba5^{C217S} was co expressed with HA6XHis-Ufm1, a band corresponding to ~ 60kDa (Fig: 5.10, lane 2) was observed while the higher band was not detected when HA6XHis-Ufm1 was co expressed with *Ld*Uba5(Fig: 5.10, lane 3) or in control cells. These results indicate that *Ld*Uba5 forms an intermediate with *Ld*Ufm1and further indicates that like human Uba5 *Leishmania* Uba5 also acts as an Ufm1 activating enzyme.

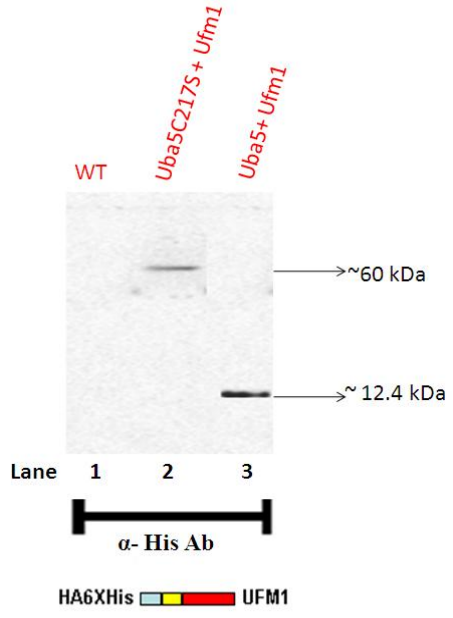


Figure 5.10 Demonstration that Uba5 is an Ufm1-activating enzyme.

Each *Ld*Uba5/*Ld*Uba5^{C217S} and *Ld*Ufm1 were co expressed (lanes 2 and 3, respectively) in *Leishmania* cells using wild type (WT) cells as control (lane 1). The cell lysates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-His antibody. The bands corresponding to *Ld*Uba5 and *Ld*Uba5–*Ld*Ufm1 intermediates are indicated on the right.

In vitro activation of *Ld*Ufm1 by *Ld*Uba5

We subsequently tested whether Uba5 can activate Ufm1 *in vitro.* The Transcreener™ AMP assay was performed using recombinant proteins expressed in *Leishmania*. The Transcreener™ AMP Assay is a far-red, competitive fluorescence polarization assay based on the detection of AMP, and is therefore compatible with any enzyme class that produces AMP, including ubiquitin, SUMO, nucleic acid and protein ligases, phosphodiesterases (PDEs), and synthetases. Human Uba5(Ufm1 activating enzyme) utilizes ATP to adenylate the C-terminal glycine residue of Ufm1, forming a high-energy thiolester bond with the active site cysteine residue and the release of AMP (Komatsu et al., 2004).

Ufm1 Activating Enzyme (Uba5) catalyzed the reaction:

ATP + Uba5 + Ufm1→ AMP + Uba5-Ufm1 + PPi

The Transcreener AMP Assay is a single step, homogenous detection assay enabling the use of unmodified native substrate concentrations of 1 - 1000 μM. The Transcreener AMP Detection Mixture comprises of an AMP Alexa633 Tracer bound to an AMP Antibody. The tracer is displaced by AMP, the invariant product generated during the enzyme reaction. The assay provides excellent signal at low substrate

conversion, with a Z' ≥0.7 and polarization shift (mP) ≥85 at 10% substrate conversion using 10μM donor substrate. Fluorescence polarization measures the parallel and perpendicular components of fluorescence emission using plane polarized excitation. Polarization values (measured in mP units) for any fluorophore-labelled complex are inversely related to the speed of molecular rotation of that complex. During the assay, AMP, produced by stimulating cells or membrane receptors, displaces Fluo-AMP from the antibody. The newly unbound Fluo-AMP rotates faster, decreasing the polarization value. The magnitude of this change in polarization is used to quantitatively measure the level of AMP in a simple, homogenous technique.

When *Ld*Uba5 and mature *Ld*Ufm1 were co-incubated with ATP, the level of AMP was found to be significantly higher as compared when *Ld*Uba5 or *Ld*Uba5^{C217S} or *Ld*Ufm1 or *Ld*Ufm1^{G98A} were incubated alone with ATP or in combination with each other, suggesting that thioester bond formation is taking place only between mature *Ld*Ufm1 and *Ld*Uba5 resulting in the production of AMP. As positive controls incubation of human Uba5 and human Ufm1 along with ATP resulted in significantly higher concentration of AMP as compared when ATP was absent and when Uba5 / Ufm1 were incubated alone with ATP (Fig 5.11 A). In order to prove that *Leishmania* Uba5 is behaving same as human Uba5, human Ufm1 was incubated with *Ld*Uba5 in the presence of ATP which showed significantly higher concentration of AMP as compared when human Ufm1 was incubated with *Ld*Uba5 mutant or alone .

Results indicate that similar to human Ufm1, *Leishmania* Ufm1 is first cleaved at the C-terminus to expose its conserved Gly residue. This Gly residue is essential for its subsequent conjugating reactions. The C-terminally processed Ufm1 is activated by a

novel E1-like enzyme, Uba5, by forming a high-energy thioester bond. Taken together, we concluded that Uba5 is an Ufm1-activating enzyme and has the active site in Cys^{217}.

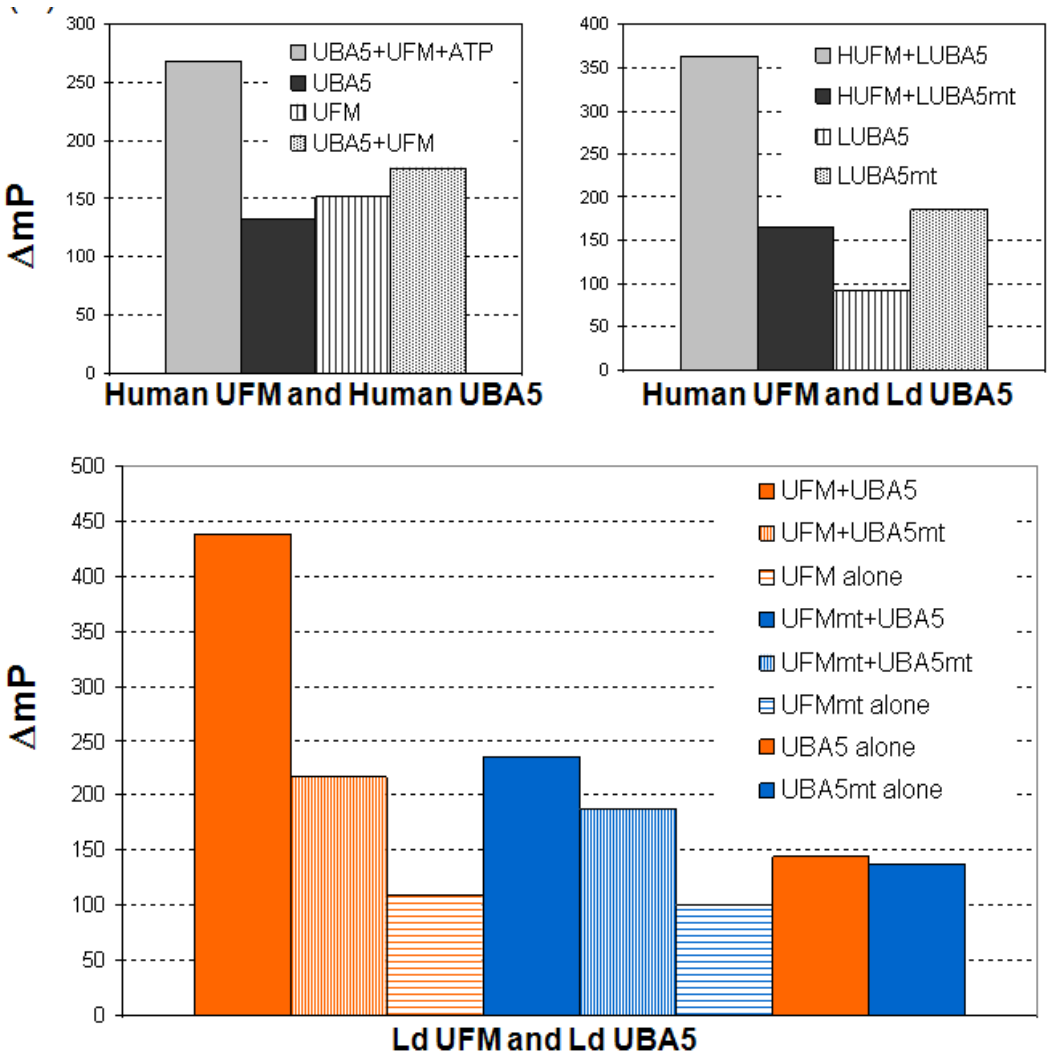


Figure 5.11 *In vitro* activating assay of Ufm1 by Uba5.

Enzyme dependent AMP production was evaluated with Uba5 (human), Ufm1 (human), and *Leishmania* Uba5, Uba5^{C217S}, mature Ufm1, Ufm1^{G98A}. Expected substrate specificity was observed as AMP was preferentially hydrolyzed by human and *Leishmania* Uba5 and Ufm1, respectively (compare figure A, B and C). 20 µl reactions

were performed in standard assay conditions. Polarization (mP) was measured with a SpectraMax M5 spectrofluorimeter. Quantitative measurement of AMP was done in µM.

Identification, sequence analysis of a novel protein-conjugating enzyme, Ufc1

We next investigated in *Leishmania*, Ufc-1 which acts as an E2-like conjugating enzyme in the human Ufm1 pathway. This protein is also conserved in multicellular organisms, like Uba5 and Ufm1. A homolog of human Ufc-1 was searched in the *L. infantum* GeneDB, an ~171 amino acid protein with a predicted molecular mass of ~19.4kDa that showed a high percentage of similarity with a conserved hypothetical protein of accession no. Linj15_V3.1270. Primers were designed for ORF region of this *L. infantum* gene and amplified with *L. donovani* DNA, sequencing of which showed 100% homology with the *L.infantum* gene and it showed a high degree (57%) of similarity with the human Ufc1. *Ld*Ufc-1 also has an active site Cys residue conserved in Ufc-1-like enzymes of other multicellular organisms (Fig: 5.12). We assumed that this protein may be an E2-like conjugating enzyme for *Ld*Ufm1 and thus named it *Ld*Ufm1-conjugating enzyme 1 (*Ld*Ufc1).

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LiUfc1 -MEPSVKESVSRIPLLKTKAGPRDGDKWTARLKEEYASLITYVEHNKASDSHWFHLESNP
LdUfc1 -MEPSVKESVSRIPLLKTKAGPRDGDKWTARLKEEYASLITYVEHNKASDSHWFHLESNP
LmUfc1 -MEPSVKESVSRIPLLKTKAGPRDGDKWTARLKEEYASLITYVEHNKASDSHWFHLESNP
TbUfc1 -MDPAVRESVSRIPLLKTKAGPRDGEQWTQRLKEEYTSLIQFVENNKASDNHWFKLESNE
HsUfc1 MADEATRRVVSEIPVLKTNAGPRDRELWVQRLKEEYQSLIRYVENNKNADNDWFRLESNK
         : :.:. **.**:***:***** : *. ****** *** :**:** :*..**:****

LiUfc1 QGTRWYGTCWTYYKNEKYEFEMNFDIPVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
LdUfc1 QGTRWYGTCWTYYKNEKYEFEMNFDIPVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
LmUfc1 QGTRWYGTCWTYYKNEKYEFEMNFDIPVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
TbUfc1 AGTRWYGTCWTYYKNERYEFNMNFDLAVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
HsUfc1 EGTRWFGKCWYIHDLLKYEFDIEFDIPITYPTTAPEIAVPELDGKTAKMYRGGKICLTDH
        ****:*.**  :.  :***:::**:.:*** :.****:***:***.*********:* *

LiUfc1 FFPLWARNVPYFGISHVLALGLGPWLSIEVPAIVEEGYLKPASAATVPTTAE
LdUfc1 FFPLWARNVPYFGISHVLALGLGPWLSIEVPAIVEEGYLKPASAATVPTTAE
LmUfc1 FFPLWARNVPYFGISHVLALGLGPWLSIEVPAIVEEGYLKPASAATVPTTGE
TbUfc1 FFPLWARNVPYFGISHALALGLGPWLSIEVPAMVEDGVLKPKKVES------
HsUfc1 FKPLWARNVPKFGLAHLMALGLGPWLAVEIPDLIQKGVIQHKEKCNQ-----
       * ******** **::* :********::*:* :::.* ::  .  .
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Fig 5.12 Clustal W analysis of Ufc1, a Ufm1 conjugating enzyme.

Sequence alignment of *Ld*Ufc1 with its homologs in *Homo sapiens*, *L.major* and trypanosomes. The sequence of hsUfm1 is available from GenBanK under the accession number BC005187. The sequence of *L. donovani* Ufc1 (No. to be obtained); *L.major*(LmjF15.1250); *T.brucei*(Tb09.160.4150); *T.cruzi* (Tc00.1047053506445.100); are from GeneDB . The putative active site Cys residue is boxed in red.

_Ld_Ufc1 is an _Ld_Ufm1 conjugating enzyme and interacts with _Ld_Uba5 and _Ld_Ufm1

If *Ld*Ufc1 is an authentic E2 enzyme for *Ld*Ufm1, it is expected to form an intermediate complex with *Ld*Ufm1 via a thioester linkage. To test this possibility we expressed Flag-*Ld*Ufc1, HA-*Ld*Ufm1 and Flag-*Ld*Ufc-1 in combination with HA-*Ld*Ufm1 in *L. donovani* cells (Fig: 5.13 A). Cell lysates from wild type (WT) *L.donovani* were used as control. Each cell lysate was immuno-precipitated with anti-HA antibody, the resulting precipitates were then analyzed by immunoblotting with anti-flag antibody. When Flag-*Ld*Ufc1, HA-*Ld*Ufm1 were expressed alone no band was seen as in the control. A band corresponding to *Ld*Ufc-1 (~19.4 kDa) was seen in cell lysates when both Flag-*Ld*Ufc1 and HA-*Ld*Ufm1 were co expressed (Fig: 5.13 B). These results indicate that *Ld*Ufc1 forms an intermediate with *Ld*Ufm1 in vivo.

To further confirm that *Ld*Ufc1 is an authentic E2 enzyme for *Ld*Ufm1 we next investigated interaction between *Ld*Uba5 and *Ld*Ufc1. Cell lysates from WT, Flag-*Ld* Ufc1, HA-*Ld*Ufm1 and Flag-*Ld*Ufc-1 in combination with HA-*Ld*Ufm1 were immuno-precipitated with anti-Uba5 antibody, the resulting precipitates were then analyzed by immunoblotting with anti-flag antibody. A band corresponding to *Ld*Ufc-1 (~19.4 kDa) was seen in cell lysates where either both Flag-*Ld*Ufc1 and HA-*Ld*Ufm1 were co-expressed and when Flag- *Ld*Ufc-1 alone was expressed, while there was no band when HA-*Ld*Ufm1 was expressed alone or in the control (Fig:5.12C).

To ascertain *Ld*Uba5 and *Ld*Ufc-1 interaction, cell lysate from WT, Flag-*Ld*Ufc1, HA-*Ld*Ufm1 and Flag-*Ld*Ufc-1 in combination with HA-*Ld*Ufm1 was immuno-precipitated with anti-flag antibody, the resulting precipitates were then analyzed by immunoblotting with anti-Uba5 antibody. A band corresponding to *Ld*Uba-5 (~43.2 kDa) was seen in cell lysates when both Flag-*Ld* Ufc1 and HA-*Ld*Ufm1 was co expressed and when Flag- *Ld*Ufc-1 alone was expressed. HA-*Ld*Ufm1 was again unable to form any band, as in the case with control (Fig:5.12D). Taken together, we concluded that similar to human Ufc-1, *Ld* Ufc1 interacts with *Ld*Uba5 and *Ld*Ufm1, the necessary steps to function as an Ufm1-conjugating enzyme.

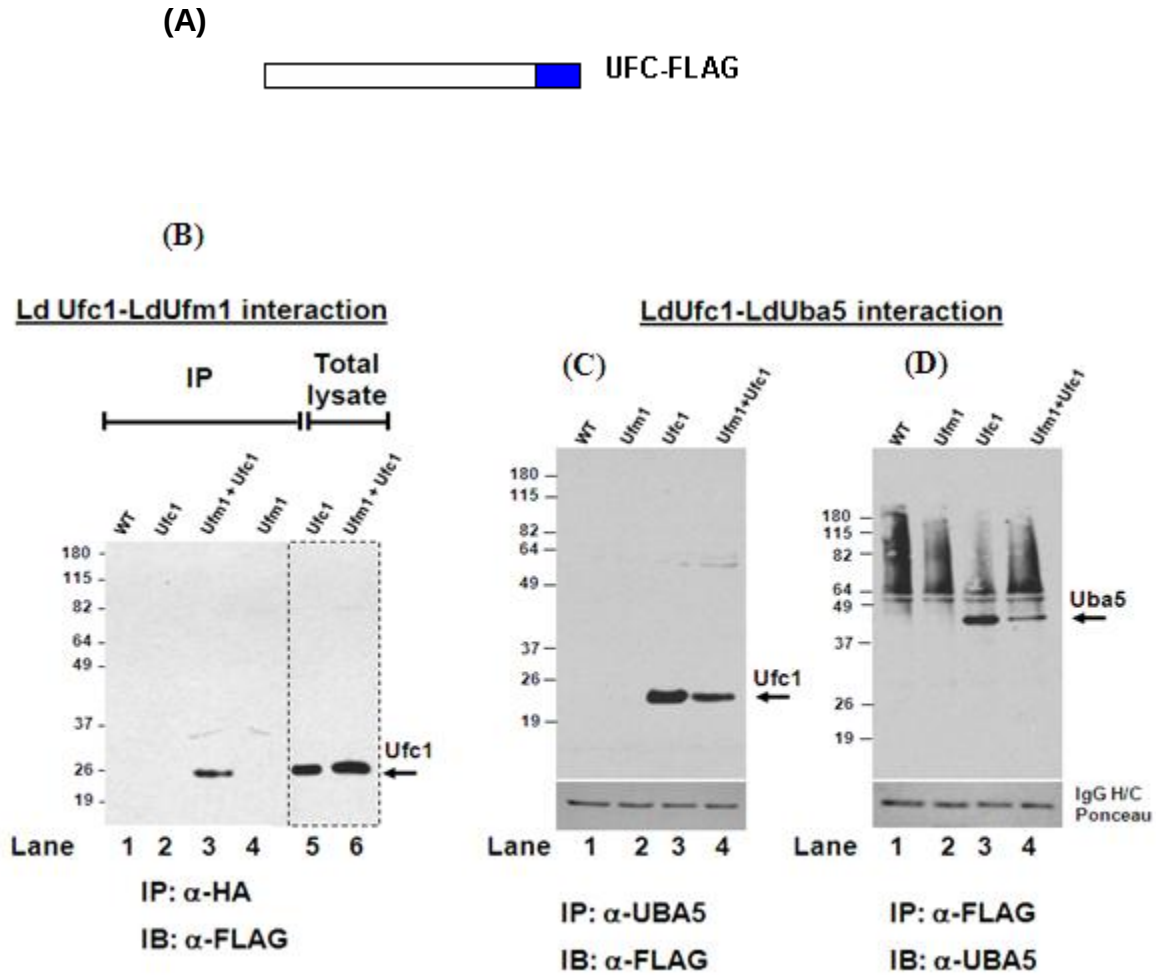


Figure 5.13 Characterization of Ufc1, a novel E2-like enzyme.

(A) Schematic representation of *Leishmania* expression plasmids for *Ld*Ufc1. *Ld*Ufc1 was constructed with flag tagged at the C-terminus (Flag-*Ld*Ufc1).

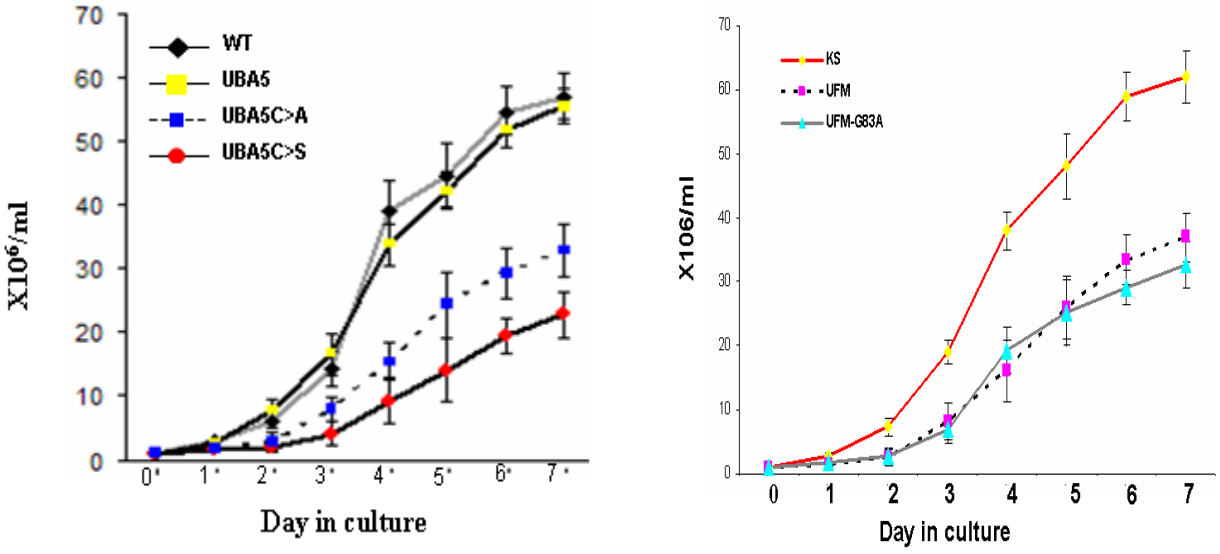
(B) Immunoblotting analysis after immunoprecipitation showing *Ld*Ufc-1 and *Ld*Ufm-1 interaction. Cell lysates of WT (lane 1), Flag-*Ld*Ufc1(lane 2) Flag-*Ld*Ufc1 + HA *Ld*Ufm1(lane 3) and HA-*Ld*Ufm1(lane 4) were immunoprecipitated with anti-HA antibody, resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibodies. The bands corresponding to *Ld*Ufc-1 are indicated.

(C) Immunoblotting analysis after immunoprecipitation showing *Ld*Uba5 and *Ld*Ufc-1 interaction. Cell lysates of WT (lane 1), HA*Ld*Ufm1(lane 2) Flag-*Ld*Ufc1 + HA *Ld*Ufm1(lane 3) and Flag*Ld*Ufc1(lane 4) were immunoprecipitated with anti-*Ld*Uba5 antibody, resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibodies. The bands corresponding to *Ld*Ufc-1 are indicated.

(D) Immunoblotting analysis after immunoprecipitation showing *Ld*Ufc-1 and *Ld*Uba5 interaction. Cell lysates of WT (lane 1), HA-*Ld*Ufm1 (lane 2), Flag*Ld*Ufc1 (lane 3) and Flag-*Ld*Ufc1 + HA *Ld*Ufm1 (lane 4) were immunoprecipitated with anti-Flag antibodies, resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Uba5 antibody. The bands corresponding to *Ld*Uba5 are indicated.

_Ld_Uba5 & _Ld_Ufm1 mutants have a Dominant negative Effect on the Growth of the Parasite.

As a first step towards understanding the role of *Ld*Uba5 & *Ld*Ufm1, growth of parasites was analyzed in culture. The *Ld*Uba5++ parasites showed growth comparable with the control cells. On the other hand, parasites expressing Δ*Ld*Uba5^{C217S}++ $^{\text{or C217A}}$++ displayed a marked (10-fold) reduction in the growth rate compared with the controls (Fig5.14 A). Over expression of *Ld*Ufm1 or *Ld*Ufm1^{G83A} resulted in a significant growth reduction as compared with the control cells (Fig 5.14 B).



(A) **(B)**

FIG 5.14 Effect of the over expression of *Ld*Uba5 and *Ld*Ufm1 and their mutants on the growth of the parasite.

(A) Growth of *L.donovani* promastigote cultures-plasmid control pKSNeo (WT), wild type *Ld*Uba5 and mutant *Ld*Uba5 (C>S and C>A) was monitored. *L. donovani* promastigotes were cultured in vitro at an initial concentration of $1x10^6$ cells/ml. The cells were kept in culture for 7 days and counted at indicated days. Results are the mean of three independent experiments. Error bars indicate the standard deviation.

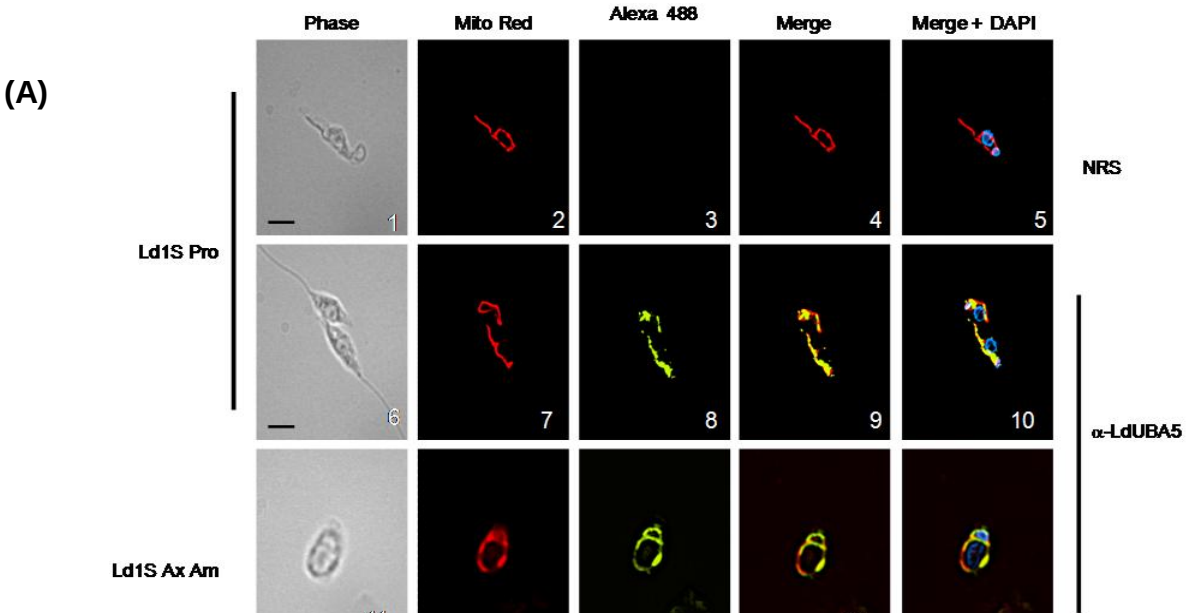
(B) Growth of *L.donovani* promastigote cultures-plasmid control pKSNeo (KS), wild type *Ld*Ufm1 or mutant *Ld*Ufm1^{G83A}(G>A) was monitored.

Sub cellular localization of *Ld*Uba5, *Ld*Ufc1 & *Ld*Ufm1 in *Leishmania* cells

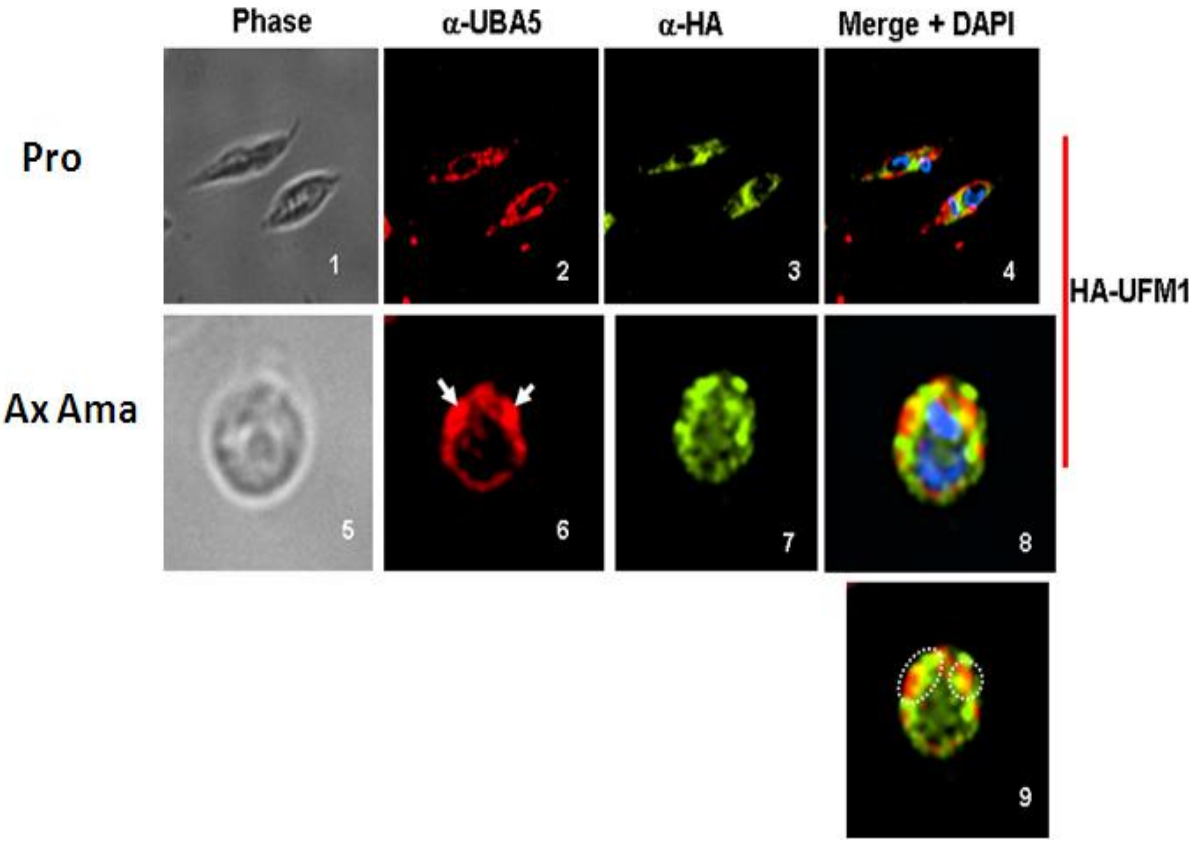
To determine the cellular localization of *Ld*Uba5 in *Leishmania*, antibodies against r*Ld*Uba5 were used in IFA studies. Immunofluorescence assay with these antibodies revealed that endogenous *Ld*Uba5 is localized in mitochondria in promastigotes as well as axenic amastigotes of *L.donovani*, as indicated by its co localization with Mitotracker Red, a mitochondrial marker (Fig. 5.15 A, panel 10,15).

Promastigotes and axenic amastigotes over expressing *LdUfm1* labeled with antibodies against the HA tag (α-HA) to determine the cellular localization of *Ld*Ufm1, revealed it to be localized in mitochondria(Fig. 5.15, panel 7). Further co-localization of *Ld* Ufm1- *Ld*Uba5 using α-HA and α-*Ld*Uba5 Ab respectively revealed strong fluorescence at the mitochondrial region suggesting that *Ld*Ufm1 and *Ld*Uba5 both are localized in mitochondria(Fig. 5.15 B, panel 9). Rabbit non reactive serum (NRS) was used as control.

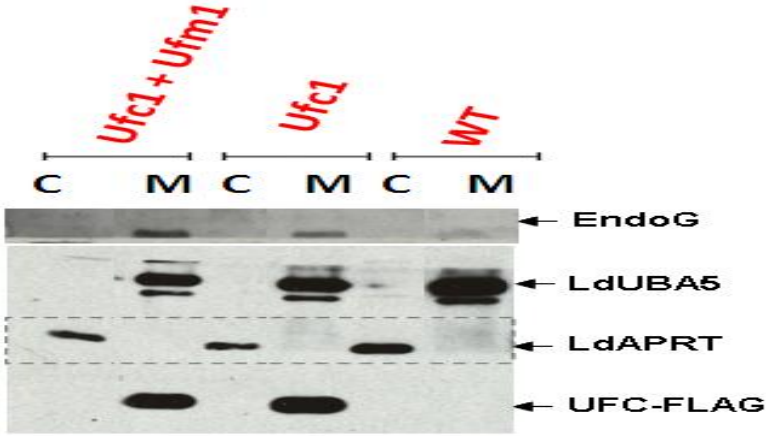
Fractionation experiments with cytosolic, intermediate and mitochondrial extracts from Flag-*Ld*Ufc1++, Flag-*Ld*Ufc1 & HA-*Ld*Ufm1++ (co expressed) and Wild type(WT) *Leishmania* cells as control were performed. The cytoplasmic, intermediate and mitochondrial fractions from these extracts were resolved by SDS-PAGE and immunoblotted with antibodies against anti-Flag Ab. *Ld*APRT, Adenine phosphoribosyltransferase (Zarella-Boitz et al., 2004) present in cytoplasm, Endo G, Endonuclease G (Sreenivas et al., 2007) and *Ld*Uba5 which are present in mitochondria respectively are taken as control genes. Immunoblot showed the mitochondrial localization of *Ld*Ufc-1 in both Flag-*Ld*Ufc1++, Flag-*Ld*Ufc1 & HA-*Ld*Ufm1++ and its absence in the cytosolic and intermediate cell fractions as well in all the WT fractions (Fig. 5.15 C).



(B)



(C)



C - Cytosolic
M - Mitochondrial

FIG.5.15 Localization of *Ld*Uba5, *Ld*Ufm1 and *Ld*Ufc1

(A) IFA of *Ld*Uba5 in promastigotes (pro) and axenic amastigotes (Am) using rabbit anti-*Ld*Uba5 Ab and Mitotracker red revealed it to be localized in Mitochondria. Nucleus (N) and kinetoplast (K) were stained with DAPI. Images were viewed under the confocal microscope. The stained images and phase were merged and shown.

(B) IFA of *Ld*Ufm1 promastigotes (pro) and axenic amastigotes (Am) using anti-HA Ab revealed it to be localized in Mitochondria. Colocalization of *Ld*Ufm1 and *Ld*Uba5 using anti *Ld*Uba5 and anti-HA Ab was carried out by merging the two images, showed both of them to be co localized in mitochondria (indicated by dotted circle in Fig 6.15 B, panel 9). Nucleus (N) and kinetoplast (K) were stained with DAPI. Images were viewed under the confocal microscope. The stained images and phase were merged and shown.

(C) Localization of *Ld*Ufc1: The cytosolic, intermediate and mitochondrial extracts from Flag-*Ld*Ufc1++, Flag-*Ld*Ufc1 & HA-*Ld*Ufm1++ (co expressed) and Wild type(WT) parasites were subjected to SDS–PAGE and analyzed by immunoblots with respective antibodies. The bands corresponding to *Ld*Ufc1 and controls are indicated on the right.

Discussion

The essential roles of Ubiquitin and Ubls in both protein turnover and transcriptional regulation in other organisms suggest that Ubiquitin and Ubl pathway should be explored to better understand basic parasite biology. Homologs of 6 out of 9 major Ubl families, including Ubiquitin, Nedd8 (neural precursor cell-expressed developmentally down regulated 8), small ubiquitin related modifier (SUMO), Hub1, ubiquitin related modifier-1 (Urm1) and authophagy-8(Atg8) are identified in parasitic protozoa. Of the identified families of Ubiquitin like modifiers (Ubls), only Ubiquitin and

Atg8 have been characterized in parasitic protozoa (Ponder and Bogyo, 2007). The molecular apparatus that regulates UFM1-UBA5-UFC1 ubiquination pathway in humans has been widely investigated although the function of this pathway is still not known (Komatsu et al, 2004, Liu et al, 2008).

In the present study, we have shown for the first time in protozoa, a unique UBL called ubiquitin-fold modifier 1, or Ufm1in *Leishmania* which was recently reported in humans. The experimental evidence that Ufm1 acts as a new post-translational UBL modifier in *L. donovani*, includes the following: (1) It is a small protein of ~12.4kDa showing homology with a human Ufm1. (2) Similar to human Ufm1, *Leishmania* Ufm1 is enzymatically processed as indicated by the *Ld*Ufm1^{G98A} mutant which remained as pro-Ufm-1, migrating as a high molecular weight band. (3) The C-terminal processing and exposure of glycine residue is essential for the formation of *Ld*Ufm1 conjugates. Ufm1 is first cleaved at the C-terminus to expose its conserved Gly residue. This Gly residue is essential for its subsequent conjugating reactions. The C-terminally processed Ufm1 is activated by a novel E1-like enzyme, Uba5, by forming a high-energy thioester bond. Activated Ufm1 is then transferred to its cognate E2-like enzyme, Ufc1, in a similar thioester linkage. Ufm1, Uba5, and Ufc1 are conserved in various multicellular organisms as well as in *Trypanosoma* and *Leishmania*, but not in budding and fission yeasts, suggesting that they all have been generated by co-evolution. Functional role of Ufm1 activity in *Leishmania* growth was evident since over expression of *Ld*Ufm1 or *Ld*Ufm1^{G98A} resulted in reduction of the parasite growth. Functional role of human Ufm1 is yet to be identified.

We identified *Ld*Uba5 as an Ubiquitin activating E1 like enzyme for *Ld*Ufm1 with Cys^{217} as the active cysteine residue. The *Ld*Uba5 gene transcripts were detected in

infected bone marrow samples from leishmaniasis patients suggesting a role in the disease pathogenesis in the human host. It was recently reported that Uba5 is induced by certain reagents that induce stress in the endoplasmic reticulum(ER), a so-called 'unfolded protein response' (Harding et al, 2003). However, induction of human Uba5, Ufc1, and Ufm1 by treatment with various compounds known to induce ER stress in mammalian cells did not show any induction. In addition, exposure to other stresses including high temperature or heavy metals also did not induce the appearance of obvious new conjugation band(s) of Ufm1 (Komatsu et al, 2004). During transformation from promastigote to amastigote stage a stress is induced due to temperature and pH shift which initiates unfolded protein response to repair the damage due to this shift, however the expression of *Ld*Uba5 at protein level was found to be the same at the two parasite stages. We attempted to investigate the functional significance of *Ld*Uba5 by RNAi in *T.brucei* / over expressing either with wild type Uba5 or C217S/C217A mutants in *L. donovani*. TbUba5 RNAi did not show any effect on growth of *T.brucei*, though the mRNA level was depleted as confirmed by northern blot. However mutations in human Uba5 have been shown to result in the loss of activity of ubiquitin activating E1 enzyme (Uba5) in mammalian cells (Komatsu et al, 2004). Over expression of either *Ld*Uba5^{C217S} / *Ld*Uba5^{C217A} mutant resulted in over 10 fold reduction in the parasite growth indicating the importance of Uba5 activity in *Leishmania* growth. Similarly we have identified *Ld* Ufc1 as an Ubiquitin conjugating E2 like enzyme for *Ld* Ufm1 which is homologus to human Ufc1 that participates in the recently discovered Ufm1-Uba5-Ufc1 ubiquination pathway in metazoan organisms. Ufc1 is a unique E2-like enzyme with no obvious sequence homology with other E2s, except approximately 10 amino-acid residues encompassing the active site Cys residue. Our studies on subcellular distribution of

*Ld*Uba5, *Ld*Ufc1 and *Ld*Ufm1 in *L.donovani* cells demonstrated their localization in the mitochondria, in contrast with the human Ufm1 which was predominantly localized in the nucleus and diffusely in the cytoplasm suggesting a unique role of this pathway in *Leishmania*.

As UBLs display tertiary structure similar to that of Ub, along with E1 (activating) and E2 (Conjugating) they also require an E3 ligase for target recognition and Ub transfer in the Ub conjugation pathway (Pickart, 2004; Callis and Vierstra, 2000). E3 like enzymes for ligation of *Ld*Ufm1 to target proteins were searched through bioinformatic analysis, however no suitable gene was found in *Leishmania*. Although ubiquitin ligases (E3 like enzymes) have not been identified in humans, in other eukaryotes they recognize the substrate's degradation signal (degron) i.e. N-terminal Arg of a substrate and target the ubiquitylated substrate to the ubiquitin-proteasome system known as N-end rule pathway (Hershko et al, 2000; Pickart, 2004; Petroski and Deshaies, 2005; Varshavsky, 2005). While in prokaryotes ubiquitin–proteaosome system is absent and rather than an E3 ligase, an adaptor protein called ClpS binds to the N- terminal region of substrates and targets the substrate to the ATP dependent Clp protease complex (ClpAP) (Ravid and Hochstrasser, 2008). Likewise, E3 ligases may not be required for *Leishmania* Ufm1 to target proteins.

Overall, in this study, we describe a unique UBL-type modifier named ubiquitin-fold modifier 1 (*Ld* Ufm1) in the trypanosomatid protozoan *L. donovani* that is synthesized in a precursor form consisting of 115 amino-acid residues with amino acid sequence highly similar to that of human Ufm1. We also identified the *L.donovani* specific activating (*Ld*Uba5) and conjugating (*Ld*Ufc1) enzymes for *Ld*Ufm1. Prior to activation by *Ld*Uba5, the extra amino acids at the C-terminal region of the pro-*Ld*Ufm1

protein are removed to expose Gly which is necessary for conjugation to target molecule(s). Functional role of *Ld*Uba5/*Ld*Ufm1 activity in *Leishmania* growth was evident since over expression of either mutants of *Ld*Ufm1 or *Ld*Uba5 resulted in reduction of the parasite growth, indicating the importance of *Ld*Ufm1/*Ld*Uba5 activity in *Leishmania* growth.

This is the first demonstration of an Ufm1-Uba5-Ufc1 ubiquination like pathway in trypanosomatids. The unique feature of *Leishmania* Ufm1 pathway was its localization in the mitochondria as most of the Ubls including human Ufm1 are found in cytoplasm. As *Ld*Uba5/*Ld*Ufm1 are demonstrated as functionally important for the growth of *Leishmania*, it is possible this pathway may be involved in protein turnover and / or transcriptional regulation like other Ubiquitin and Ubls.

Conclusions and future scope of work

In the present study, *in vitro* culture systems were set up for growing and differentiating *L. donovani* promastigotes into axenic amastigotes. Microarray experiments were carried out to study the stage regulated gene expression during transformation from promastigote to amastigote stage of *L.donovani*. Further the expression of amastigote up regulated genes was verified in the hamster derived amastigotes and in the bone marrow samples of the VL patients. Two genes that showed significantly higher expression at the amastigote stage were selected for further characterization.

1. Argininosuccinate synthase (ASS) which was found to be unique to *Leishmania* and absent in *Trypanosomes* was cloned and expressed as a recombinant protein.
2. *Ld*Uba5 which is homologous to human Uba5 was characterized in *L.donovani* which paved the way for discovery of a unique UBL called ubiquitin-fold modifier 1, or Ufm1 linked pathway, as yet undiscovered in protozoans.

Parasites were isolated and promastigote cultures were set up from bone marrow aspirates of KA patients. The parasites, characterized as *L. donovani* by species specific PCR, were serially adapted to grow as axenic amastigotes. In search of stage regulated genes that show differential expression at Pro and Ama stages, the gene expression profiling was undertaken using highly sensitive microarray technology. Analysis of Gene expression at promastigote stage with axenic amastigotes stage using a genomic microarray comprising of 4224 randomly sheared PCR amplified *L. donovani* inserts revealed a number of DNA clones showing stage regulated expression. There were 9.06% (383/4224) differentially expressed clones with ratios ≥ 1.7 in Am vs. Pro parasite stage.

Of these, 38 clones showing significantly higher and consistent expression with ratio ≥ 1.7 in at least 8/9 spots (SD < 1), Z ratio > 1.9 (p value < 0.05) in three microarray hybridizations and reproducibility in dye flip microarray experiments were chosen for further analysis. Of the 38 clones, 28 were up regulated while 10 were found to be down regulated at the amastigote stage. The over expression at amastigote stage was verified by RT-PCR and Northern hybridizations for 12 selected genes. The relative transcript abundance of six genes [Ubiquitin activating E1 like Uba5 (*Ld*Uba5), Argininosuccinate synthase (ASS), trypanosomatid specific gene of 27 kDa (P27), Parasite surface antigen 2 (PSA2), MAP kinase (MAPK) and amastigote up regulated gene (A1)] upregulated in axenic amastigotes was assessed in hamster derived amastigotes and VL bone marrow samples. Quantitative expression of selected genes was compared in lesion tissues of VL, PKDL and CL patients. Assessment of *in vivo* mRNA level highlighted substantial differences in gene expression patterns, in the three different forms of Leishmaniasis. Such differentially expressed genes hold the key to understanding of the disease pathogenesis.

Argininosuccinate synthase (ASS)

Another gene identified as ASS that showed up regulated at the amastigote stage was chosen for characterization. The protein coding gene ASS was cloned and sequenced The nucleotide sequence analysis showed that the gene comprised of a 1.2kb ORF encoding a ~ 45kDa protein. Quantitative real-time PCR experiments revealed that the gene was 2-3 fold up regulated in both axenic and hamster-derived amastigotes. The gene transcripts were demonstrated in human bone marrow samples of VL patients. The recombinant ASS was expressed in *E. coli* as a ~45kDa protein and polyclonal antiserum to this protein was raised in rabbit. Amino Acid sequence analysis revealed a number of

protein phosphorylation sites and a cytoplasmic targeting signal sequence. The cytoplasmic localization was experimentally proved by immunofluroscence assay using anti ASS antibody. The over expression shown at transcript level was further validated at protein level at the amastigote stage.

Ufm1 linked pathway in *Leishmania donovani*

The cloning and characterization of *Ld*Uba5 (Ufm1 activating enzyme 5) that showed consistently up regulated expression in Am stage in microarray experiments was undertaken. The gene transcripts were demonstrated in human bone marrow tissue samples of kala-azar patients. The protein coding gene *Ld*Uba5 was cloned, sequenced and expressed. Abs were raised against the rLdUba5 and the protein expression was demonstrated in pro and ama stages. Clustal W analysis of *Ld*Uba5 showed 48% homology and a conserved cysteine residue similar to human Uba5. In the case of *Ld*Uba5, the Cys^{217} was demonstrated as the active site Cys residue by over expressing mutated Uba5 (Cys^{217} to Ser/Ala) in *L.donovani* cells. Parasites transfected with *Ld*Uba5 or Δ*Ld*$Uba5^{C217A}$ gave a single band of ~45kDa while when *Ld*$Uba5^{C217S}$ was expressed, an additional band with a higher molecular mass of ~57 kDa was observed, indicating that *Ld*$Uba5^{C217S}$ forms a stable complex with an endogenous protein. Since human Uba5 has been known to interact with Ufm1, its homolog was searched in the *L. infantum* GeneDB which led to the identification of a *Leishmania* ubiquitin-fold modifier 1 (*Ld*Ufm1) with a molecular mass of ~12.4 kDa. *Ld*Ufm1 is synthesized as inactive precursor (pro-Ufm1) which has 18 additional amino acids in comparison to human Ufm1 which had two residues beyond the conserved glycine residue. Our studies on *in vitro* activation of *Ld*Ufm1 by *Ld*Uba5 showed that mature *Ld*Ufm1 has an exposed C-terminal glycine which is essential for subsequent activation by its cognate E1 protein

(*Ld*Uba5). This activation step results in the formation of a high-energy thiolester bond between LdUba5 and Ufm1 in the presence of ATP. We next investigated in *Leishmania*, Ufm1 conjugating enzyme (Ufc-1) which acts as an E2-like conjugating enzyme in the human Ufm1 pathway. Immuno-precipitation followed by immuno-blotting analysis showed *Ld*Ufm1 is subsequently transferred from *Ld*Uba5 to its cognate E2-like enzyme (*Ld*Ufc1) via a similar thiolester linkage with a conserved cysteine at the E2 active site. Localization studies revealed that *Ld*Uba5/*Ld*Ufm1/LdUfc1 were localized in mitochondria. Functional role of *Ld*Uba5/*Ld*Ufm1 activity in *Leishmania* growth was evident since over expression of either mutants of *Ld*Ufm1 or *Ld*Uba5 resulted in reduction of the parasite growth, indicating the importance of *Ld*Ufm1/*Ld*Uba5 activity in *Leishmania* growth. Functional role of human Ufm1 is yet to be identified. This is the first demonstration of an Ufm1-Uba5-Ufc1 ubiquination like pathway in Trypanosomatids. The unique feature of *Leishmania* Ufm1 pathway was its localization in the mitochondria as most of the Ubls including human Ufm1 are found in cytoplasm. As *Ld*Uba5/*Ld*Ufm1 are functionally important for the growth of *Leishmania* it is possible this pathway may be involved in both protein turnover and /or transcriptional regulation like Ubiquitin and other Ubls.

Future scope of work

In our study, we have identified a few amastigote up regulated genes of which characterization of two genes has been carried out. Other differentially expressed genes identified such as trypanosomatid specific gene of 27 kDa(P27), Parasite surface antigen 2 (PSA2), MAP kinase (MAPK) and amastigote up regulated gene (A1) need to be investigated and characterized to determine their role in the differentiation process.

Ufm1 linked pathway in *Leishmania donovani*

In this study, UFM1-UBA5-UFC1 ubiquination pathway was discovered and shown to be functionally important in *L.donovani*. This pathway needs to be further explored to better understand basic parasite biology. Ufm1 is conjugated to a variety of target proteins and forms complexes with as yet unidentified proteins. Studies on the biological roles of the Ufm1 conjugation pathway and whether Ufm1 is conjugated to several distinct proteins or to a single target are under investigation in our laboratory. Recently, two novel thiol proteases have been identified (UfSP1 and UfSP2) in humans which cleave Ufm1-peptide C-terminal fusions and also remove Ufm1 from native intracellular conjugates. Both UfSP1 and UfSP2 possess highly conserved Cys and His residues constituting a new subfamily of cysteine proteases Functional characterization of *Leishmania* Ufm1-specific proteases is warranted in order to dissect the UFM1 pathway.

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Appendix

Preparation of reagents

10X MOPS

0.2M MOPS pH 7.0, 20mM Sodium acetate, 10mM EDTA pH 8.0

Filter sterilized with 0.45μm filter.

DEPC water

0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37°C it was autoclaved.

100X Denhardt's solution

Ficoll400	10g
Polyvinylpyrrolidone	10g
Bovine serum albumin Fraction V	10g
dH_2O	500ml

Filter sterilized and stored at 4℃.

20X SSC

175.3gm of NaCl, 88.2gm of Sodium citrate were dissolved in 800ml distilled water. pH was set to 7.0 with HCl. Volume was adjusted to 1ltr, aliquots prepared and the solution was sterilized by autoclaving.

Preparation of bacterial culture media

LB medium (Luria Broth)

10 gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride (Hi Media) were dissolved in 1liter of dw. Media were sterilized by autoclaving for 20 minutes at 15 lb/sq.in.

LB Agar

15gm of agar powder, 10gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride, (Hi media) were dissolved in dw. Media were sterilized by autoclaving for 20 minutes at 15lb/sq.in. LB agar was allowed to cool to 45°C and poured (~30 ml per plate) in 90 mm

disposable petri plates (Tarsons) along with appropriate antibiotics and allowed to solidify.

Antibiotics

Ampicillin

100mg/ml ampicillin stock was prepared in autoclaved dw and sterilized by filtration through 0.22 μm filter. 100μl aliquots were stored by freezing at –20°C.

Kanamycin

50mg/ml kanamycin stock was prepared in autoclaved dw and sterilized by filtration through 0.22 μm filter. 100μl aliquots were stored by freezing at –20°C.

Chloramphenicol

34 mg/ml chloramphenicol stock was prepared in ethanol and stored at –20°C. Chloramphenicol is required to ensure the presence of pLysS.

Stock solution of commonly used reagents

1M Tris

121.1gm of Tris base was dissolved in 800ml of dw and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA

186.1gm of disodium EDTA.-$2H_2O$ was added in 800ml of dw, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

3M sodium acetate

204.5gm of $C_2H_3O_2Na$. $3H_2O$ was dissolved in 400ml of dw, pH set to 5.3 with glacial acetic acid, volume made up to 500 ml and autoclaved.

10% SDS

10gm of electrophoresis grade SDS was dissolved in 70ml of dw, heated at 60°C to dissolve and the volume made up to 100ml.

Ethidium Bromide (10 mg/ml)

10mg of ethidium bromide was dissolved in 1ml dw, stored in a opaque bottle.

30% Acrylamide Stock

29.2gm of acrylamide and 0.8gm of bis-acrylamide were dissolved in 50ml of ddH_2O. Volume was made up to 100ml, the solution filtered through Whatman no. 1 paper, degassed and stored in an opaque bottle.

Calcium Chloride (0.1 M)

1.47gm of $CaCl_2.2H_2O$ was dissolved in 100ml of ddH_2O and sterilized by autoclaving.

IPTG (1M)

238mg of IPTG was dissolved in 1ml of dw, filter sterilized and stored at -20°C in 50µl aliquots.

Coupling Buffer (0.2 M)

0.42 gm of $NaHCO_3$ was dissolved in 40 ml, pH was set to 9.0 and volume made upto 50 ml. Sterilized using 0. 45µm filter

Sodium Phosphate (1M)

Monobasic

138gm of $NaH_2HPO_4.H_2O$ was dissolved in 800ml of dw and volume made up to 1liter.

Dibasic

268gm of $Na_2HPO_4.7H_2O$ was dissolved in 700ml of dw and volume made up to 1liter.

Ammonium persulfate (10%)

To 1gm of ammonium persulfate, 10ml of dw was added and the solution stored for several weeks at 4°C.

10 X TAE buffer (Tris acetate, EDTA)

4.84gm of Tris base in 80ml of dw was dissolved and 1.2ml of glacial acetic acid and 2ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100ml.

10X TBE buffers (Tris borate, EDTA)

8gm of Tris base, 55 gm of boric acid and 9.3gm $Na_2EDTA.H_2O$ were dissolved in 700ml dw and the final volume made up to 1 liter.

Phosphate Buffer Saline (PBS)

8gm of NaCl, 2gm of KCl, 1.44gm of Na_2HPO_4 and 0.2gm of KH_2PO_4 were dissolved in 800ml of dw. pH was set to 7.4 with HCl. Final volume was made up to 1

liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

SDS-PAGE electrophoresis buffer

3gm of Tris base, 14.4gm of glycine and 1gm of SDS were dissolved in 1 liter of dw.

Protein transfer buffer

5.8gm of Tris base, 2.9gm of glycine and 0.33gm of SDS were dissolved in 500ml of dw. 200 ml of ethanol was added and the final volume was made up to 1 liter.

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

Tris-Cl (pH6.8)	100mM
DTT	200mM
SDS	4%
Bromophenol blue	0.2%
Glycerol	20%
β-mercaptoethanol	10%

10X Ligation buffer

Tris.Cl pH 7.8	500mM
$MgCl_2$	100mM
DTT	100mM
ATP	10mM

10 X Amplification buffer

Tris.Cl pH 8.3	100mM
$MgCl_2$	15mM
KCl	500mM
Gelatin	0.1%

DNA loading dye (6X)

0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved dw.

SDS-PAGE reagents

Composition of resolving gel (12%) 10 ml

30% acrlyamide solution	4.0 ml
1.5M Tris-Cl pH 8.8	2.5 ml
dw	3.3ml
10% SDS	100µl
10% APS	100µl
TEMED	10µl

Composition of stacking gel (5%) (5.0 ml)

30% acrylamide solution	0.83ml
1.0M Tris.Cl pH 6.8	0.68 ml
dw	3.4ml
10% SDS	50µl
10% APS	50µl
TEMED	5µl

Staining solution

1gm of coomassie blue was dissolved in 450ml of methanol. 100ml of glacial acetic acid was added and the volume made up to 1liter by double distilled water, filtered through Whatman no.1 filter and stored at room temperature.

Destaining solution

Methanol: water: acetic Acid were mixed in the ratio of 45:45:10 and stored at room temperature.

PSGEMKA Buffer

20µl of 1M Na-Phosphate, 20.8 ml of 1M NaCl, 400µl of 250mM Glucose, 10µl of 0.5M EDTA, 20 ml of 10mM $MgCl_2$, 20 ml of 100mM KCl, 0.02% BSA buffer added to 138ml of autoclaved distilled water.

Electroporation Buffer

0. 25 gms HEPES, 0.403 gms NaCl, 186mg KCl, 6.2mg Na_2HPO_4, 54 mg glucose, pH7.4 added to 100 ml dw and sterilized by filtration.

Publications and Presentations

Publications

1. Identification and role of a novel Ubiquitin-like system in the protozoan parasite *Leishmania donovani*. **Paresh Sharma**, Gannavaram Sreenivas, Robert Duncan, Srividya Gurumurthy, Hira L.Nakhasi and Poonam Salotra (Manuscript Under preparation).
2. Comparative *in vivo* expression of amastigote up regulated *Leishmania* genes in three different forms of Leishmaniasis. **Paresh Sharma**, Srividya Gurumurthy, Robert Duncan, Hira L.Nakhasi and Poonam Salotra (Accepted Nov 2009, Parasitology International).
3. Transcriptome analysis during the process of in vitro differentiation of *Leishmania donovani* using genomic microarrays. Srividya G, Duncan R, **Sharma P**, Raju BV, Nakhasi HL, Salotra P. Parasitology. 2007; 134:1527-39.
4. Circulating nitric oxide and C-reactive protein levels in Indian kala azar patients: correlation with clinical outcome. Ansari NA, **Sharma P**, Salotra P. Clin Immunol. 2007; 122(3):343-8.

Presentation in National/International conferences

1. **Paresh Sharma**, Gannavaram Sreenivas, Robert Duncan, Srividya Gurumurthy, Hira L.Nakhasi and Poonam Salotra . Identification and role of a novel Ubiquitin-like system in the protozoa parasite *Leishmania donovani.* In 4th World Congress on Leishmaniasis (WL4) held at Lucknow, India, 03-07 Feb'09. P 231.
2. **Paresh Sharma**, Gannavaram Sreenivas, Robert Duncan, Srividya Gurumurthy, Hira L.Nakhasi and Poonam Salotra. A novel Ubiquitin-like system in the protozoa parasite *Leishmania donovani*. In the National Conference on Emerging Trends in Life Sciences Research held at BITS, Pilani, Rajasthan, India on 6 -7 Mar'09.P 73.
3. **Paresh Sharma**, Gannavaram Sreenivas, Robert Duncan, Srividya Gurumurthy, Hira L.Nakhasi and Poonam Salotra Identification and role of a novel Ubiquitin-like system in

the protozoa parasite *Leishmania donovani*. In Keystone symposia on The Many Faces of Ubiquitin held at Colorado, USA on January 11 - 16, 2009.

4. Robert Duncan, Ranadhir Dey, Joseph Milone, **Paresh Sharma**, Poonam Salotra, Hira Nakhasi Characterization of *Leishmania donovani* amastigote specific 27kDa protein: a candidate for making a live attenuated vaccine. In Gordon Conference , USA June 2008.
5. Nasim Akhtar Ansari, **Paresh Sharma**, Venkatesh Ramesh, Poonam Salotra. Immuno-determinants in Indian Leishmaniasis: Correlation with disease outcome. In 33rd Indian Immunology society conference held at AIIMS, New Delhi, India on 28th -31th January 2007.
6. Salotra P, Srividya G, **Sharma P**, Subba Raju B.V, Duncan R, Nakhasi H.L Identification of novel vaccine targets for Visceral Leishmaniasis using genomic microarray. In 32nd annual conference of Assoc Clin Biochem , Patna, India in Dec 2005.
7. **Paresh Sharma**. Development of Informative STR Markers and Establishment of DNA Repository from a Linkage Mapping Panel on Buffalo. In 8th ADNAT conference at Center for Cellular & Molecular Biology(CCMB),Hyderabad, India on 23rd & 24th Feb. 2004.

Biography of the Candidate

Name Paresh Sharma

Date of Birth 24 Sep' 1980

Educational qualification

Examination Passed	Board/University	Percentage	Year of passing
Secondary school Examination	CBSE	58	1996
Intermediate	CBSE	56	1998
B.Sc Biotechnology	Jiwaji University, Gwalior	64	2001
M.Sc Biotechnology	Jiwaji University, Gwalior	72	2003

Academic Achievements

1. Won **Indo-US Vaccine Action Program** travel fellowship for a duration of 3 months (Nov' 07 to Jan 08), on the subject of "Discovery of Virulence-related Genes in *Leishmania donovani* using a Genomic Microarray" at the Center for Biologics Evaluation and Research, Bethesda, Maryland, United States of America, in lab of Dr. Hira L Nakhasi (Director, Division of Emerging and Transfusion Transmitted Diseases).
2. Awarded ICMR Senior research fellowship (SRF) for the project entitled "Molecular and Functional Characterization of a Stage regulated gene in *Leishmania donovani*" from Feb'08 –till now.
3. Best Poster Presentation at National Conference on Emerging Trends in Life Sciences Research held at BITS, Pilani, Rajasthan, India on 6 -7 Mar'09.

Conferences Attended

- Attended 4th World Congress on Leishmaniasis (WL4) held during 03-07 Feb'09 at Lucknow, India.

- Attended National Conference on Emerging Trends in Life Sciences Research held at BITS, Pilani, Rajasthan, India from 6 -7 Mar'09.
- Attended 33rd Indian Immunology society conference held at AIIMS, New Delhi, India from 28th -31th January 2007.
- Attended 21st annual conference of Indian Association of Pathologists and Microbiologists, Delhi Chapter organized by Institute of Pathology (ICMR) and Safdarjung Hospital and V.M.M. College held on 16 April 2006.
- Attended 7 ADNAT symposium on proteomics held in CCMB from Feb. 23 – 24, 2003.
- Attended 8 ADNAT symposium on Comparative & Functional Genomics held in CCMB from Feb. 23-24.2004.

Work Experience

- Junior research fellowship (JRF) from Department of Biotechnology (DBT), at Transgenic and Gene Knock out lab, centre for Cellular and Molecular Biology, Hyderabad,India,fromNov'03 to may'04.
- Junior research fellowship (JRF) from Department of Biotechnology (DBT), New Delhi, from May '04 to May '06.
- Senior research fellowship (SRF) from Department of Biotechnology (DBT), New Delhi, from June '06 to Feb'08.
- ICMR Senior research fellowship (SRF) for the project entitled "Molecular and Functional Characterization of a Stage regulated gene in *Leishmania donovani*" from Feb'08 –till now.

Brief Biography of the Supervisor

Personal Particulars

- Name Poonam Salotra
- Designation : Deputy Director
- Place of work : Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi – 110029, India.
- Tel. No: 26198402, 26166124
- Fax No: 26166124
- E. mail: salotra@vsnl.com, salotrap@icmr.org.in

Academic Qualifications

- B.Sc. Hons (Chemistry) from Delhi University in 1974.
- M.Sc. (Biochemistry) from P.G.I., Chandigarh in 1976.
- Ph.D. from V.P. Chest Institute, Delhi University in 1980.

Professional Appointments:

- Postdoctoral Fellow at Roche Institute of Molecular Biology, Nutley, New Jersey, U.S.A. (1980).
- Research Officer at Tuberculosis Research Centre, Madras (1982).
- Principal Investigator under Young Scientists scheme of Department of Science & Technology at Centre for Biotechnology, J NU, New Delhi (1991).
- Pool Research Officer (CSIR) at Centre for Biotechnology, JNU, New Delhi (1993).
- Senior Research Officer at Institute of Pathology (ICMR), New Delhi (1996).
- Assistant Director at Institute of Pathology (ICMR), New Delhi (2000).
- Deputy Director at Institute of Pathology (ICMR), New Delhi (2005 till date)

Research Interests:

- Worked mainly on the molecular basis of pathogenesis of infectious diseases such as Kala-azar, Anthrax, Tuberculosis and Cholera. Currently working on development of diagnostic tests and attenuated vaccines for kala-azar, mechanism of drug resistance in Indian kala-azar, and characterization of immune responses in patients of kala-azar and post kala-azar dermal leishmaniasis.

Awards/Honours

1. Basanti Devi Amir Chand Award conferred by ICMR in 2007.
2. Prof. BK Aikat Award conferred by Indian Council of Medical Research conferred by ICMR in 2007.
3. ICMR International fellowship for Senior Biomedical Scientists for the year 2006.
4. Awarded Courtesy Fellowship by CBER, FDA, USA in Sep 2005.
5. Awarded fellowship by National Foundation of Infectious Diseases, USA in 2005.
6. Granted ICMR Award for Excellent Research output in July 2004.
7. Silver Jubilee award by Indian Association of Medical Microbiology in 2003.
8. Awarded Courtesy Fellowship by CBER, FDA, USA in Dec 2003
9. Kshanika Oration Award, a National award for Eminent Woman Scientist, conferred by Indian Council of Medical Research in 2002.
10. National Science Talent Scholarship awarded by N.C.E.R.T. , New Delhi

Patents

1. Awarded US Patent No. 6,855,522, in 2005, for "Species-specific PCR assay for detection of Leishmania donovani in clinical samples of kala-azar and post kala-azar dermal leishmaniasis".
2. US patent no. 20060240046 for "Live attenuated *Leishmania* vaccines"

MEMBERSHIP OF PROFESSIONAL ASSOCIATIONS

1. Society for Parasitology, India (Life member).
2. Society of Biological Chemists, India (Life member).
3. Association of Clinical Biochemists of India (Life member)
4. Indian Association of Medical Microbiologists, India (Life member
5. Indian Immunology Society, India (Life member).

Publications

PUBLICATIONS IN INDEXED FOREIGN JOURNALS	56
Publications in indexed Indian journals	05
Publications in Proceedings	41
Chapter in Books	03
Total	105