Identification and Characterization of Virulence–Related Genes of *Leishmania donovani* using Microarray Technology

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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Under the Supervision of

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2008

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled '**Identification and Characterization of Virulence–Related Genes of *Leishmania donovani* using Microarray Technology'** and submitted by G Srividya ID No. 2004PHXF425 for award of Ph.D. Degree of the Institute, embodies original work done by her under my supervision.

Signature in full of the Supervisor _____

Name in capital block letters _____

Designation _____

Date _____

Acknowledgement

The thesis work has followed the pattern motivated by the ideas, principally of my mentor Dr. ***Poonam Salotra****. My preparative years with her are studded with myriad take away lessons of enduring value. Her expert guidance and readiness to solve any difficulties instilled a sense of spontaneous zeal during the work. I take this opportunity to thank her for giving me infinite amount of freedom, for lending clarity to my ideas and her phenomenal tolerance to my shortcomings.*

I would not have succeeded in this effort, had it not been for the ideas, discussions and resources at several crucial stages from ***Dr Hira L Nakhasi*** *and* ***Dr Robert C Duncan****, CBER, FDA, USA. They had been instrumental in imparting expert advice and guidance in accomplishment of this work. I would like to express my sincere thanks for all the help they have extended to me.*

The Director of Institute of Pathology (ICMR) deservers special mention because she is the one who helped me through on the conduct of this research work. I sincerely thank ***Dr. Sunita Saxena*** *for the renderance of her help.*

My thanks are due to ***Dr Ravi Prakash****, Professor, BITS,* ***Dr. Dinesh Kumar*** *and* ***Dr. Regalla Srinivas Prakash*** *who have facilitated my work in a great deal. I wish to thank all the members of the Research and Consultancy Division of BITS, Pilani for being instrumental in shaping my career.*

I owe my thanks to ***Dr. B. V. Subba Raju*** *and* ***Paresh*** *for their painstaking and stupendous efforts of assistance in my experimental work. With obligation and praise I sincerely thank* ***Dr. Ruchi Singh, Dhiraj, Rajesh, Dr. Nasim, Sandeep, Arpita, Gajendra, Anand*** *and* ***Vasundhara*** *for making a wonderful working and congenial environment. Their timely help made things easy for me and I profusely thank all of them. My special thanks are due to* ***Dr. G Sreenivas*** *for his advice and valuable suggestions for carrying out this research work. I wish to recall the all the guidance and help I received from* ***Dr. L. C. Singh, V. Ramesh*** *and* ***Dr. N.S. Negi****, which I acknowledge with appreciative gratitude.*

I could always rely on Mrs Saratha for her help in countless instances and express my deep felt thanks to her. I thank ***Mr. Ramchandra Chhetri*** *and* ***Mr. Anish Saxena*** *for their sincere support in everyday lab work.*

Financial assistance granted from Council of Scientific and Industrial Research, India during research work is greatly acknowledged.

I thank my parents ***Mrs. Meera Gurumurthy*** *and* ***Mr*** *K. Gurumurthy for being constant source of encouragement all through my life. I acknowledgements are due to my sister* ***Mrs. Sowmya*** *and Brother-in-law* ***Mr. K, Venkatesh*** *for being helpful and supportive. They provide me the greatest strength.*

*My husband **Mr. S. Ganesh** has been my strongest source of support all through and deserves all the ways of gratitude as he had sacrificed the best part of my marital life. Although my research works he was instrumental in making this work a success. The budding laugh of my daughter **Srinidhi** encouraged me to complete the work in time and join her playful evenings. My heartfelt thanks are due to her for being patient during the final year of my research work.*

*I sincerely acknowledge the patience of my in laws and the support they landed during crucial times. Blessings and strong support of Nani ma was always there whenever I was in trouble to sail through the tough times. I extend my special thanks to my sister-in-law **Mrs. Chitra** and cousins **Karthik** and **Vasudevan** for their help.*

*"Above all of us "It is almighty who traversed me through the darkness and the roughness of this voyage. Hence part all the few mentioned and many unmentioned, I humbly fall at the lotus feet of him because it is **"HE"** who made me one what I am today.*

July 2008 ***Srividya. G***

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Abbreviations

A_{280}	Absorbance at 280 nm
A_{600}	Absorbance at 600 nm.
Amp	Ampicillin
APS	Ammonium persulfate
AxAm	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
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-β-D-thio-galactosidase
KA	Kala-azar
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
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	Protein of 27 kDa size
PA24	Promastigote-to amastigote differentiation at 24hrs time point
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
TEMED	N,N,N',N' tetramethyl ethylene diamine
Tris	Tris (hydroxymethyl) amino acid
U	Unit
UV	Ultra Violet
VL	Visceral Leishmaniasis
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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Review of Literature

Leishmaniasis

Leishmania is a member of the trypanosomatid protozoa belonging to the order kinetoplastida. It is the causative agent of leishmaniasis, diseases transmitted by the blood sucking phlebotomine sandfly. Early accounts of the occurrence of human leishmaniasis were recorded around 1824AD in Asia near the Indian border of Bangladesh. Subsequently epidemics were described in the Gangetic plains where it still has a major presence.

The leishmaniasis afflicts the world's poorest populations. Humans are infected via the bite of phlebotomine sandflies, which breed in forest areas, caves, or the burrows of small rodents. There are four main types of the disease:

- In cutaneous forms, skin ulcers usually form on exposed areas, such as the face, arms and legs. These usually heal within a few months, leaving scars.
- Diffuse cutaneous leishmaniasis produces disseminated and chronic skin lesions resembling those of lepromatous leprosy. It is difficult to treat.
- In mucocutaneous forms, the lesions can partially or totally destroy the mucous membranes of the nose, mouth and throat cavities and surrounding tissues.
- Visceral leishmaniasis, also known as kala azar, is characterized by high fever, substantial weight loss, swelling of the spleen and liver, and anaemia. If left untreated, the disease can have a fatality rate as high as 100% within two years.

Occurring in several forms, the disease is generally recognized for its cutaneous form which causes non-fatal, disfiguring lesions, although epidemics of the potentially fatal visceral form cause thousands of deaths.

Visceral Leishmaniasis (VL)

Visceral leishmaniasis (VL), the most severe in the disease spectrum, being fatal if left untreated, is caused by the members of *Leishmania donovani* complex that includes *L. d donovani* and *L. d. infantum.* In the Indian subcontinent and East Africa, VL is mainly caused by *L. d. donovani*, while in the Mediterranean basin and South America it is *L. d. infantum*. The disease is characterized by irregular fever, weight loss, swelling of the liver and spleen and anemia. The annual incidence and prevalence of VL cases worldwide is 0.5 million and 2.5 million respectively. More than 90% of the VL cases in the world are reported from Bangladesh, Brazil, India, Nepal and Sudan [WHO, 2003].

For many years, the public health impact of the leishmaniasis has been grossly underestimated, mainly due to lack of awareness of its serious impact on health. Over the last 10 years, endemic regions have been spreading further and there has been a sharp increase in the number of recorded cases of the disease. As declaration is compulsory in only 32 of the 88 countries affected by leishmaniasis, a substantial number of cases are never recorded. In fact, 2 million new cases (1.5 million for CL and 500 000 for VL) are considered to occur annually, with an estimated 20 million people presently infected worldwide. Globally leishmaniasis is responsible for approximately 59,000 deaths annually with 350 million at risk. The global burden of leishmaniasis is estimated at 2.4 million disability adjusted life years lost [WHO, 2002]. The two major clinical forms of leishmaniasis, cutaneous and visceral are the result of infection by different species of the parasite.

Widespread in 22 countries in the New World and in 66 nations in the Old World, leishmaniasis is primarily found in South-east Asia, East Africa, South America and Mediterranean region. Human infections are found in 16 countries in Europe, including

France, Italy, Greece, Malta, Spain and Portugal (Fig.1.1). Among the two million new cases each year in the 88 countries where the disease is endemic, it is estimated that 80% earn less than $2 a day [Davies *et al.*, 2003].

Fig.1.1 Distribution of Old World and New World visceral leishmaniasis

Data source: WHO/CSR/EDC-UNAIDS

The problem of VL has become more serious because of co-infection with HIV that is becoming increasingly frequent with cases reported in 34 countries [Pintado and Lopez-Velez, 2001; Desjeux, 2003]. Control of leishmaniasis is complicated by the multiplicity of *Leishmania* species and their diverse clinical manifestations [Amaral *et al.*., 2002].

VL in South East Asia (SEA) Region

VL or kala-azar (KA) as it is known in India is a significant infectious disease in the developing world and of late in the developed world because of increased international travel

and HIV infection. The disease is endemic in three countries of WHO's South East Asia Region –Bangladesh, India and Nepal. Approximately 200 million people in the region are "at risk" from the disease. 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. Of the estimated 500,000 people in the world infected each year, nearly 100,000 are estimated to occur in the region. In the endemic countries, kala-azar affects the poorest among the poor. The very poor have little knowledge about the disease and hence they are unlikely to seek early treatment, and most of those who start treatment cannot afford to complete it. The occurrence of the disease drags then further into the downward spiral of poverty from which they are unable to recover. Kala azar worsens the poverty amongst the people. It contributes to poor development of the area and stresses the overstretched health system.

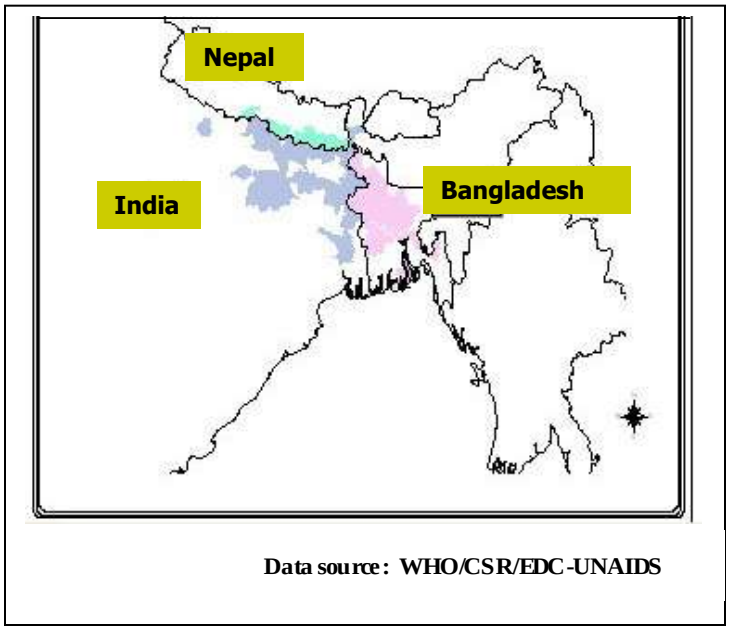
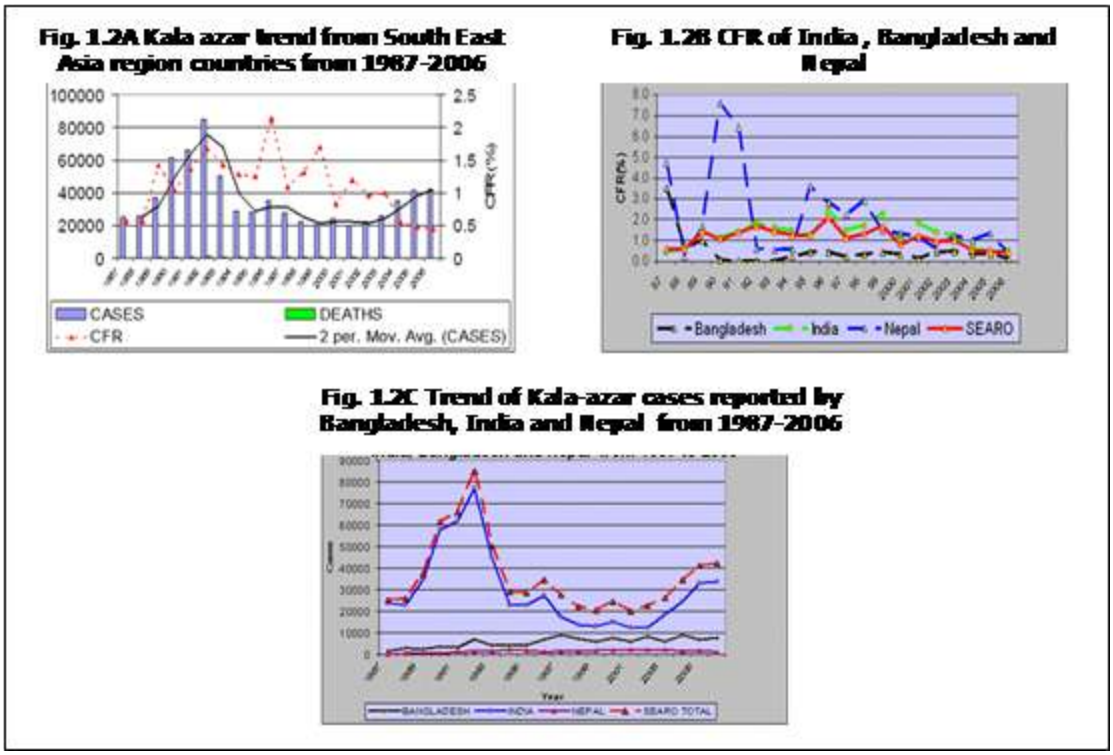


Fig.1.2. Kala-Azar endemic districts of SEA region, 1995-2005

The situation is worsening due to asymptomatic cases, PKDL, undernutrition and kala-azar/HIV co-infections (Fig. 1.3 A). However, the mortality is stable because of improved case management (Fig. 1.3 B) in the recent years due to availability of better diagnostic tools (rK39 kits) and oral drug miltefosine. India alone contributes more than 80% of the cases in the South East Asia (SEA) Region (Fig. 1.3 C). There are inadequacies in reporting since only government agencies are reporting the disease to the programme. The number of cases reported is 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**.**

After recovery from VL nearly 10-20% of the patients develop chronic Post Kala-azar Dermal Leishmaniasis (PKDL) in India that requires long and expensive treatment. In the absence of any animal hosts PKDL patients are deemed singular source of *L donovani* in India [Thakur and Kumar, 1992]. PKDL is an unusual dermatosis that develops as a sequel of KA, producing gross cutaneous lesions in the form of hypopigmented macules, erythema and nodules. The disease is relatively common in the Indian subcontinent and in East Africa, but less frequent in the American and European continents [Ramesh and Mukherjee, 1995]. The number of reported cases of PKDL is not clear. India estimates the PKDL to be about 10-20% while Nepal estimates it at 10%. There are difficulties in recognizing cases of PKDL.



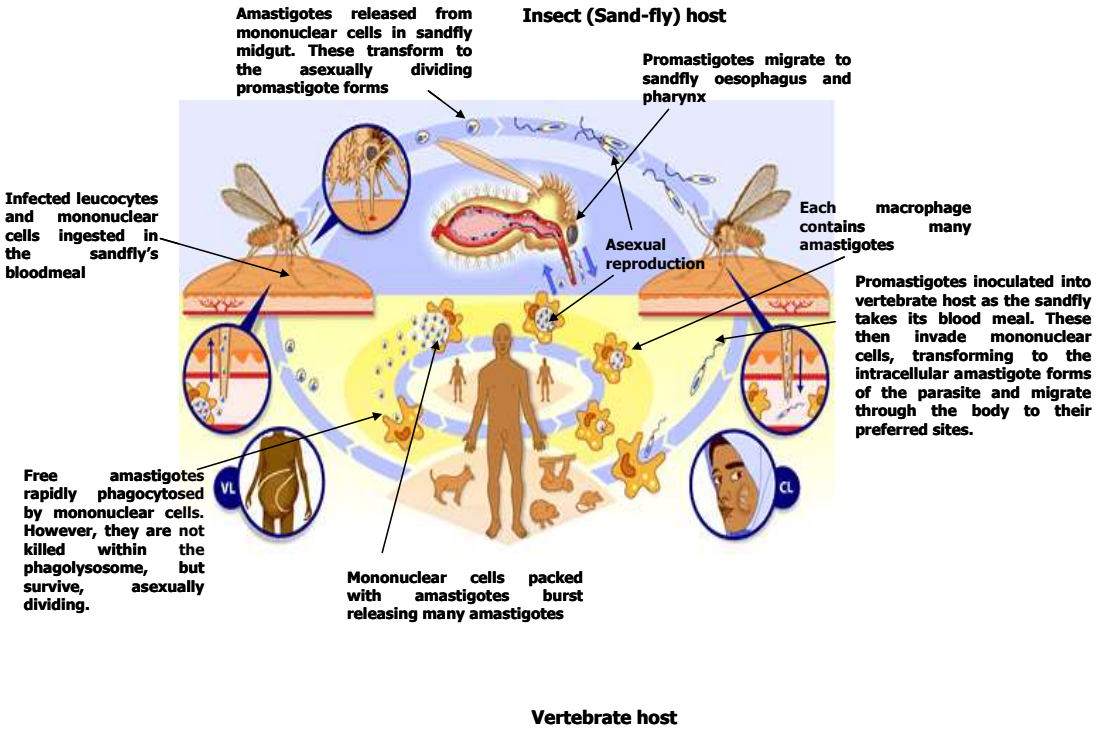
Leishmania transmission

Leishmania are transmitted between long-lived vertebrate hosts by short lived phlebotomine sand flies (*Phlebotomus spp., Lutzomyia spp. and Psychodopygus spp.)* and have a cycle of development in each host. In the sand fly the parasites are in the promastigote form (1.5-3μm x 10-20 μm) with an anterior flagellum and in the vertebrate host they reside intra- and extracellularly as oval, non-motile cells with only a very short flagellum and a maximum diameter of 2.5 x 6.8 μm, which are called amastigotes. Multiplication of each form is by binary fission [Bryceson, 1996].

Life cycle of the *Leishmania* parasite

Leishmania parasites are dimorphic and reside in two hosts, the sand-fly vector and the mamamalian host. A schematic diagram of *Leishmania* life cycle is shown in Fig. 1.4.

Fig. 1.4. Schematic diagram of the *Leishmania* digenic life cycle.



The sand fly vector of genus *Phlebotomus* (old world) or *Lutzomyia* (new world) becomes infected when feeding on the blood of an infected individual or an animal reservoir (Fig 1.4). The *Leishmania* parasites live in the macrophages as round, non-motile amastigotes. The fly ingests the macrophages during the blood meal and the amastigotes are released into the stomach of insect [Killick-Kendrick, 1990]. Almost immediately the amastigotes transform into the motile, elongated, flagellate promastigote form. The

promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission [Guevara *et al*., 2001]. Sand fly saliva selectively inhibits parasite killing by macrophages and nitric oxide production [Hall & Titus, 1995]. The major surface glycoconjugate lipophosphoglycan (LPG) constitutes a dense glycocalyx that covers the entire surface of the parasite including the flagellum. Immature organisms, termed procyclics, express shorter LPG molecules but mature metacyclics bear the capping at the terminal β- galactose residues with α-arabinose and elongation by increasing the numbers of repeating disachharides unit by two to three folds [Pimenta *et al*., 1991, 1994]. This mature metacylic form of the organism is released from the midgut and migrates to the proboscis. PpGalec, a tandem repeat galectin expressed in the midgut of the sandfly *P. papatasi,* is a LPG receptor, used by *L. major* for mediating specific binding to insect midgut via LPG. The presence of species specific receptor in the sandfly midgut demonstrates the feasibility of using midgut receptors for parasite ligands as target antigens for transmission – blocking vaccines [Kamhawi *et al*., 2004].

When the sand fly next feeds on a mammalian host, it transfers the metacyclic *Leishmania* promastigotes to the host along with the saliva [Sacks, 2001]. The sand fly rips up the epidermis and eventually gains access to dermal capillaries. During this process, parasites are regurgitated into the bite wound. Under natural conditions, sand flies transmit very low numbers of promastigotes, which are able to induce the disease. Several changes, individually and collectively, allow the metacyclic promastigotes to withstand complement activation and to infect macrophages successfully. The lipophosphoglycan binds to serum-mannan binding protein, which has a complement activating C1q domain [Green *et al*., 1994]. This results in the lysis of procyclic but not metacyclic promastigotes. *L. donovani*

and *L. major* metacyclics are protected by a thick glycocalyx augmented by elongation of lipophosphoglycan through an increase in the number of phosphorylated disaccharide repeat units [Sacks *et al*., 1995]. Gp63, which is upregulated in metacyclic, also inhibits complement-mediated lysis and promotes parasite uptake by cleaving C3b to C3bi [Brittingham & Mosser, 1996]. Opsonisation of parasites with C3b and more particularly with C3bi, which bind to the macrophage receptors CR1 and CR3 respectively, provides the predominant means by which metacyclics bind to and access the host macrophages. Other receptors for uptake of promastigotes by macrophages that have been identified include mannose-fucose receptor, CR4, the fibronectin receptor, the receptor for advanced glycosylation end products, the Fc receptor and the C-reactive protein receptor [Alexander *et al*., 1999].

Presence of multiple receptors allows the parasite an easy access into macrophages and langerhans cells where the parasites transform into amastigotes. Langerhans cells are thought to provide a safe haven for the parasite by their failure to produce inducible nitric-oxide synthase. More significantly, although parasites fail to replicate in langerhans cells, they are not rapidly killed and might save the host cells from apoptosis [Moore & Matlashewski, 1994]. Once in the host, the promastigotes are taken up by the macrophages where they rapidly revert to the amastigotes form [Pulvertaft & Hoyle, 1960], survive and multiply within the phagolysosome of the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues. Infected macrophages either remain in the skin and cause cutaneous disease or disseminate throughout the reticuloendothelial system producing disseminated disease.

Occasionally, sandflies are not involved in transmission. VL can be directly initiated by amastigotes via blood (shared needles, transfusion, transplacental spread) or organ transplantation; cutaneous infection can develop after inadvertent needle stick if the needle or syringe contains infected material [Cruz *et al*., 2002; Basset *et al*., 2005]. Risk of acquiring infection is determined by local sandfly behaviour and by the presence of an infected animal or human reservoir.

***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 for 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* 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 *L. major, L. infantum* and *L. braziliensis* genomes

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). These differences were shown to be due to fusion of pairs of chromosomes (chromosomes 20 +34 in *L. braziliensis*; chromosomes 8 +29 and 20 +36 in *L. mexicana*), with the former observation now confirmed in the sequencing project. 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 analysed 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. major* 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 [Rao *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. major* [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 [Dwyer *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 [Weise, 2007]. A surprising number of these kinases are also involved in modulating flagellum length (LmMPK9, LmMPK13, LmMPK3), which varies enormously in different promastigote stages and amastigote. 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. Belesterio *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 overlapping or complementary roles in the amastigotes.

***Leishmania* survival in macrophages**

Leishmania promastigotes preferentially utilize glucose as their primary carbon source and are thought to have acquired a number of enzymes involved in carbohydrate scavenging/metabolism to exploit the sugar-rich environment of the sandfly digestive tract [Opperdoes and Coombs, 2007]. By contrast, recent studies suggest that the *Leishmania*-occupied phagolysosome (PV) contains low levels of sugars and that amastigotes may dependent on amino acids as their major carbon source [Naderer *et al.*, 2006]. Consistent with this notion, the genomes of *Leishmania* spp. contain many amino acid permeases that would allow amastigotes to scavenge amino acids from the lumen of the PV in competition with the host PV transporters. Amino acid uptake is also required to satisfy the complex amino acid requirements of these parasites, which are auxotrophic for at least 10 amino acids [McConville *et al.,* 2007]. Intracellular amastigotes can also

endocytose host proteins that are continuously delivered in the parasite-occupied phagolysosome. The degradation of host proteins in the amastigote lysosome would provide another source of amino acids and other essential metabolites (Fe, heme). Levels of lysosomal proteinases are increased in the amastigotes of many species, and *L. mexicana* mutants lacking some of the proteinases exhibit a severe loss of virulence [Besterio *et al.,* 2007]. While both amino acid scavenging and lysosomal degradation pathways are likely to be important for parasite growth in macrophages, it is notable that amastigote growth can be stimulated by supplementation of the culture media of infected macrophages with essential free amino acids [Iniesta *et al.,* 2005; Wanasen *et al.,* 2007], indicating that some amino acids may still be limiting for intracellular growth. The up-regulation of cysteine proteinase expression in amastigotes represents one of the few examples of stage-specific transcriptional regulation of metabolic enzymes in these parasites. Cohen-Freue *et al.* [2007] have reviewed recent genome-wide transcript profiling studies that reveal that the vast majority (>95%) of the genes in the *L. major* and *L. mexicana* genomes are constitutively expressed. Of the mRNAs that did change in a stage-specific manner, only a minority did so in both species, further emphasizing the lack of stage-specific changes in mRNA levels. These findings suggest that stage-specific differences in metabolism and ultrastructure are likely to be mediated by a combination of changes in protein translation, turnover and/ or post-translational modification. This is supported by proteomic analyses of promastigotes and amastigotes which have revealed significant changes in the levels of many proteins [Cohen-Freue *et al.,* 2007]. However, analysis of the proteome has not revealed the concerted changes in metabolic pathways observed in many other prokaryotic and eukaryotic pathogens. It is possible that the

constitutive expression of enzymes involved in major pathways of central carbon metabolism confers a selective advantage, by allowing these parasites to exploit variable nutrient conditions in the sandfly or mammalian host. Interestingly, the constitutive expression of genes and proteins essential for pathogenicity has also been reported in another obligatory intracellular pathogen, *Mycobacterium tuberculosis* [Rengarajan *et al.*, 2005]. The absence of a conventional network of transcription factors in *Leishmania* and the constitutive expression of (most of) the parasite genome presents a particular challenge to scientists studying the control and regulation of metabolic pathways. It is possible that studies on metabolic regulation in *Leishmania* will reveal mechanisms that exist in other eukaryotes but have been largely overlooked because of overlying transcriptional/translational regulatory mechanisms. Alternatively, these organisms may have evolved new regulatory mechanisms. Regardless, exploration of metabolic regulation in *Leishmania* should provide interesting new information.

Virulence factors in *Leishmania*

A number of *Leishmania* antigens found to elicit antibodies often at high titers in kala-azar patients [Requena *et al.*, 2000]. Fig. 1.5 summarizes few pathoantigenic determinants identified till now.

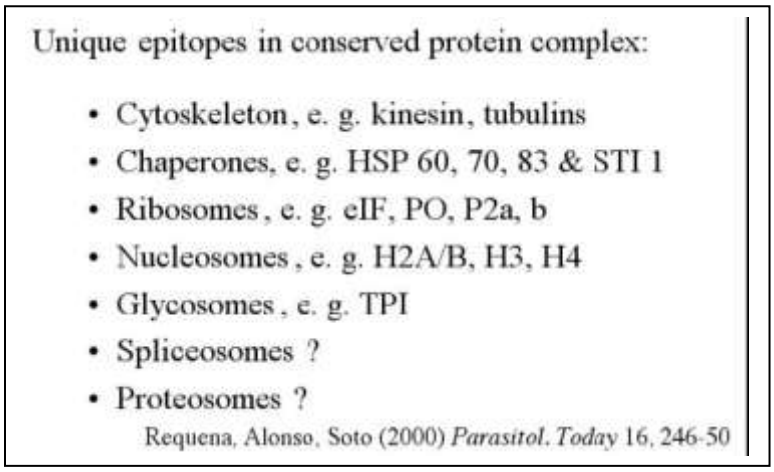


Fig.1.5
Some *Leishmania* pathoantigenic determinants proposed to cause immunopathology manifested as the clinical symptoms in leishmaniasis. The molecules listed have been found to contain immunogenic B-cell epitopes.

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 worthy of mention 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 immunopathology, 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. 1.6

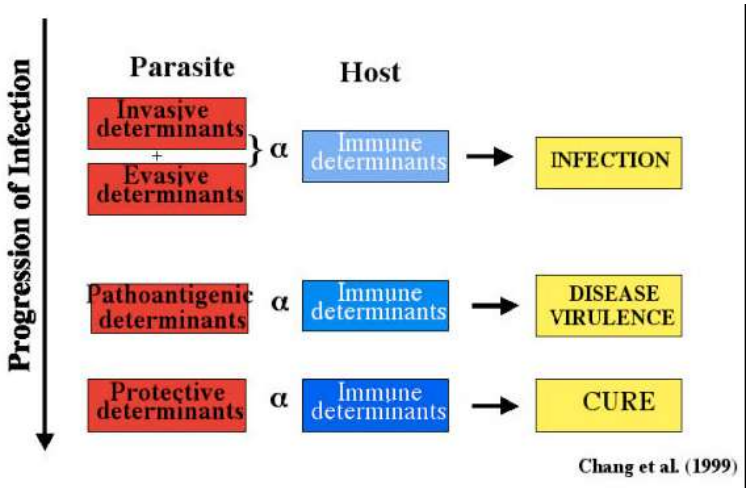


Fig.1. 6
A hypothetical model to explain virulent phenotype in leishmaniasis. The three groups of determinants are thought to interact with host immune system independently, but may progress sequentially to produce the spectrum of sub clinical and clinical manifestations as the basis of virulent phenotypes 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 for the transition from asymptomatic phase to symptomatic phase, especially when host immunity becomes downregulated. 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 due, perhaps, 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 virulenceref.

Regulation of virulence

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 classed 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.

Modulation of host macrophages by invading parasites

Many studies have shown that *Leishmania* promastigotes can engage a variety of macrophage receptors and that the utilization of di .erent receptors can elicit different responses from the host cells. However, the physiological relevance of these interactions has recently been questioned with the finding that promastigotes may initially invade neutrophils (the first population of host cells to arrive at the site of infection) rather than macrophages [van Zandbergen *et al*., 2004]. Infected neutrophils subsequently undergo apoptosis and are then engulfed together with their parasite cargo by macrophages, where they transform into amastigotes and establish infection. While the extent to which promastigotes interact with and survive within neutrophils *in vivo* might be variable (and is still controversial [Lima *et al*.,1998]), it is clear that the interactions between amastigotes and macrophages are of paramount importance in establishing and maintaining infection in the animal host. As described by Kima [2007] it is clear that *Leishmania* amastigotes, like the promastigote stages, may engage a variety of different classes of macrophage phagocytosis receptors, including the Fc Receptor, the Complement Receptors, phosphatidylserine (receptor for apoptotic cells) and DC-SIGN (in the case of dendritic cells), as well as membrane domains not normally associated with phagocytosis (i.e.caveolae) [Kima, 2007]. The diversity of receptor combinations used by these parasites to invade macro- phages and dendritic cells may contribute to the remarkable capacity of most *Leishmania* species to invade a wide variety of animal hosts. While the mode of parasite internalisation may strongly influence initial macrophage

responses, there is growing evidence that *Leishmania* amastigotes actively modulate signaling pathways in the host cell once they are established in the phagolysosome. Notably, *Leishmania* invasion (by either promastigotes or amastigotes) results in the suppression of superoxide and nitric oxide production and inhibition of IL-12 secretion (required for a host protective TH 1 response). On the other hand, secretion of cytokines such as IL-10 and TGF-β is increased. While the degree to which host macrophages become activated may reflect the initial mode of entry, the long term anergy of infected macrophages indicates the active suppression of host signalling pathways by resident amastigotes. Much of the information on the interaction of the parasite and host macrophage derives from studies in mouse models of disease and an important challenge is to relate these findings to the situation in humans. As the contribution of host genetics to these processes is only now starting to be elucidated [Blackwell *et al.*, 2004] the delineation of host-parasite interactions at this level is likely to be complex. The other challenge is to identify parasite molecules that might be involved in orchestrating macrophage functions. While there is evidence that components of the prominent promastigote surface glycocalyx suppress IL-12 secretion and superoxide production, many of these components are not expressed on amastigotes. Moreover, *Leishmania* mutants lacking the enzymes needed for their synthesis are still able to inhibit macrophage signalling and cytokine secretion. There is increasing evidence that some parasite proteins, including members of the cysteine proteinase B family of lysosomal proteins and EL-1 a are transported to the cytosol of the macrophage and interact directly with host signalling pathways [Kima, 2007]. It remains to be determined how transport of parasite proteins from the phagolysosome lumen to the host cytosol occurs. It is

conceivable that transport occurs directly across the phagolysosome membrane via endogenous (or parasite-encoded) transporters, or that some parasite proteins are transported from the phagolysosome to the endoplasmic reticulum via retrograde membrane transport pathways, and retro-translocated to the cytosol. Finally, while the focus of these studies has been on identifying processes that modulate host cell microbicidal processes, it is likely that *Leishmania* may also manipulate the metabolism of macrophages to facilitate access to nutrients. A number of recent studies have shown that alterations in the metabolic state of macrophages can influence the growth rate of intracellular amastigotes. For example, activation of infected macrophages with IL-4 (or in some cases with INF-γ can increase arginine and polyamine levels in the host cell and promote parasite growth, presumably reflecting the transport of low molecular weight nutrients from the host cytosol to the phagolysosome lumen [Iniesta *et al*.,2005; Wanasen *et al*.,2007]. As there are many precedents for other pathogens highjacking the signalling or metabolic machinery of their host cell, this promises to be a rich area of investigation for understanding the amastigote-macrophage biology, and an essential step toward the development of novel drugs and vaccines.

Host responses

Leishmania are susceptible to complement-mediated lysis, and it is to the parasites' advantage to enter the macrophage in an expedient manner. It is known that increased production of TGF-β and IL-10 are closely associated with disease susceptibility in humans and in animal models [Heinzel *et al*., 1991; Barral-Netto *et al*., 1992; Ghalib *et al*., 1993; Reed & Scott, 1993]. *Leishmania* are obligate intracellular parasites of macrophages, and each of these above cytokines is associated with increasing

virulence of this and other pathogens. It is possible that induction of one or both of these molecules by vector saliva components could increase macrophage infection. Conversely, IL-12 and IFN-γ are cytokines associated with resistance to leishmaniasis [Scott 1991; Scharton-Kersten *et al*., 1995]. Kamhawi *et al.* [2000] demonstrated that the delayed-type hypersensitivity (DTH) response was induced in mice by prior exposure to sand fly bites.

The DTH response was characterized by a massive cellular infiltrate; with T cells that produced IFN-γ, the effector cytokine most closely associated with limiting parasite replication in macrophage, and was correlated with reduced infections when the mice were subsequently bitten by infected sand flies [Behin *et al.*, 1997]. At sites of infection, complex innate responses include multiple factors: cells (neutrophils, monocytes, natural killer cells, macrophages, dendritic cells); recognition receptor mechanisms eg, toll-like receptors [Brandonisio *et al*., 2004] and soluble products like complement, released cytokines including IL-1α, IL-12, TNFα [Sacks & Sher, 2002]. Three hypothesis have been proposed for explaining the Th1/Th2 imbalance in experimental leishmaniasis (Reiner & Locksley, 1995): (1) different peptides stimulate distinct groups of Th1 or Th2 clones, (2) a particular pattern of cytokines and cofactors, produced by innate immune system accessory cells, would be the reason for the divergence, (3) under stimulation, T cells of different mice strains would have an innate tendency for the development of one of the two poles of the response (clones of T cells derived from C57BL/6 mice for example, would take the Th1 direction while cells from BALB/c would go for the Th2 pole).

Leishmania infection in humans induces life long protection in a majority of individuals. Several studies have shown that the parasites persist at the lesion site

[Schubach *et al.*, 1998; Engwerda *et al*., 2004; Mendonca *et al*., 2004] and that a generalized infection can recur in cases of immunosuppression [Desjeux, 1999]. The central memory and effector memory T-cell compartments are maintained in people living in endemic areas [DaCruz *et al*., 2002]; they are maintained by persistent parasites but might also be boosted by frequent re-infection if the individual remains in an endemic area, as might be the case for asymptomatic individuals living in such areas [Follador *et al*., 2002].

Gene expression analysis

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 datasets 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 & Skuce, 2005].

Complex phenotypes are likely to be the summation of the effect of multiple genes. Screening techniques described earlier that have looked for such genes in the past have either measured small groups of genes a few 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. This technique has been successfully applied to a range of biological questions in pertaining to *Leishmania* biology including differentiation, drug resistance and PKDL biology [Goyal *et al*., 2006; Salotra *et al.*, 2006; Saxena et al., 2003; Saxena *et al.*, 2007; Srividya *et al.*, 2007].

Differential gene expression in the procyclic and metacyclics promastigotes of *L. major* was compared and confirmed the stage-specific expression of several known genes, as well as identified a number of novel genes that were up-regulated in either procyclics or metacyclics [Saxena *et al*., 2003]. Expression profiling in parasite *L. major* has been carried out using random genomic DNA microarrays, to identify differentially expressed genes associated with the three major developmental stages of the protozoan, replicating promastigotes, infective non-replicating metacyclics, which occur in the sand fly vector, and in the amastigote stage residing within macrophage phagolysosomes in mammals [Akopyants *et al.*, 2004]. Studies with cDNA microarray comparing stage-regulated gene expression in *L. major* revealed ~35% genes upregulated in amastigotes

compared to ~12% in metacyclic promastigotes [Almeida *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 low degree of differential mRNA expression was observed: 1.4% genes in amastigotes and 1.5% in promastigotes. Genomic microarray technology was employed to identify genes that are expressed during early stages of *L. donovani* promastigote to amastigotes differentiation in *in vitro* systems [Srividya *et al.*, 2007]. Approximately, 3.7% and 9.1% differentially expressed clones were identified at an intermediate stage of differentiation and terminally differentiated amastigotes respectively.

In conclusion, the analysis of *Leishmania* promastigote and amastigote life stages by genome microarrays demonstrated that the vast majority of *Leishmania* mRNAs are constitutively expressed. Differentiation, virulence and pathogenesis may, therefore, not be dependent on the induction or regulation of gene or protein expression,. Rather, given the abundance of nutrients available to both the promastigote and the amastigote, *Leishmania* may be largely constitutively adapted for survival and replication in either the sand fly vector or macrophage host utilizing an appropriate set of genes/proteins for each vastly different environment.

Treatment for VL

Chemotherapy offers several advantages in the control of Leishmaniasis. Conventionally, long parenteral courses of pentavalent antimonials (SbV) drugs have been used for both visceral and cutaneous leishmaniasis. They include sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), the branded drugs which are quite

expensive, and the generic drug Sodium antimony gluconate (SAG) from Albert David Ltd, Kolkata, India. Both the branded and generic antimonial drugs were equally effective in a randomized trial under field conditions [Guerin *et al.*, 2002]. The second line drugs Amphotericin B and the less frequently used pentamidine are quite toxic. The difficulties of treatment are exacerbated by the spread of resistance to antimony in India [Sundar *et al.*, 2000] and the intractability of the disease to all drugs in patients co-infected with HIV. In most endemic areas the use of some excellent treatments, notably liposomal Amphotericin B for VL, is limited by the cost [Sundar *et al*., 2001a].

The problem of treatment failure for VL is exacerbated by geographical variations in antimonial treatment regimens, severity of disease, and sensitivity of *Leishmania* species. In North Bihar in India, there is clear evidence of acquired resistance of *L.donovani* for VL to antimonial drugs with up to 60% failure rate with treatment. Amphotericin B has been a standby treatment during this developing crisis; the drugs can be used in short course and gives >90% cure rate [Sundar *et al*., 2001b]. Pentamidine proved to be an unsatisfactory substitute for antimony in India. However, conventional amphotericin B is highly effective, albeit an arduous treatment because of infusions lengthy administration (20–30 days), and adverse reactions [Sundar *et al.*, 2004]. Lipid formulations of amphotericin B, representing macrophage-targeted treatment, induce side effects much less frequently than the free drug and are very active in 5–10 day regimens. Indian kala azar is especially responsive to low total doses and even a single infusion of liposomal amphotericin B cures 90% or more of patients [Sundar *et al*., 2001]. Paromomycin, an aminoglycoside identical to aminosidine [Jha *et al*., 1998], has completed phase III testing in India and is being tested in East Africa. Once

commercially available, paramomycin's anticipated high-level effcacy, minimum toxicity, and low cost for the 21-day course [Guerin *et al*., 2002] should provide an injectable alternative to amphotericin B in India and a potential substitute for antimony worldwide.

However, it is the alkylphosphocholine Miltefosine, first developed as an anticancer drug that offers the most hope as an oral drug against KA. In a series of trials this drug achieved a 94% cure rate at doses of about 2.5 mg/kg (100 mg/day for four weeks) even among patients with antimony resistant disease [Jha *et al*., 1999]. Miltefosine was registered for treating VL in India in March 2002. Subsequent trials in children have yielded similar results. However, teratogenic potential of this drug requires that it should be used with caution in women of childbearing age. Another potential oral drug sitmaquine, lacked a linear correlation between dose and cure rate and had an unsatisfactory safety and efficacy profile [Davies *et al.*, 2003]; a dose ranging study of sitamaquine for the VL treatment has shown good response [Jha *et al*., 2005]. HIV co-infections with *L infantum* have proved difficult to treat, with over 60% failure rate with most antileishmanial drugs used either alone or in combination. HAART (highly active antiretroviral therapy) has some effect on the relapse rate [de La Rosa *et al*., 2002].

VACCINATION AGAINST VISCERAL LEISHMANIASIS

To date no vaccine is available despite substantial efforts by many laboratories. Advances in our understanding of *Leishmania* pathogenesis and generation of host protective immunity, together with the completed *Leishmania* genome sequence, open new avenues for vaccine research. The major challenges are the translation of data from animal models to human disease and the transition from the laboratory to the field.

The demands from a VL vaccine are more complex than for a CL vaccine and it is believed that human VL trials will follow any successful CL immunization programme. Whether the same vaccine will work against both forms of the disease remains to be seen. Similar to the situation in CL, protection against VL correlates with production of IFN-γ by Th1-type cells. However, co-existence of Th1 and Th2 responses has been reported in VL patients as well as experimental animals. In contrast to CL, Th2 responses do not hinder Th1 responses and early Th2 cytokines may in fact enhance IL-12 and IFN- γ production later on. Moreover, humoral immune responses seem to play a role in anti-VL immunity. It appears that a vaccine against VL may need to generate both cellular and humoral immune responses [Ravindran and Ali, 2004]. VL vaccination studies have been hampered by the lack of a suitable animal model of disease. The best animal models are the natural combination of dogs and *L. infantum* or *L. chagasi* [Hommel *et al.*, 1995] and *L. donovani* in golden hamsters [Requena *et al.*, 2000]. Several clinical symptoms and pathogenic features of infection in both models are similar to the human disease. The canine model is particularly useful in evaluating vaccine candidates since successful vaccination of dogs is thought, at least to some extent, to control the spread of disease to humans in endemic areas where the dog is the reservoir of infection [Tesh, 1991]. However, both models which use outbred animals also suffer from lack of immunological reagents and assays needed for the dissection of immune responses. The mouse model of VL has been the most widely used system. It has the advantage that there are many different knockout mice with specific lesions in the immune system and there are good immunological reagents. The Th1 and Th2 polarisation has not been observed for *L.*

donovani and often the mice have to be injected intravenously with large numbers of amastigotes in order to achieve visceral disease [Ravindran and Ali, 2004].

Single antigen vaccines

The protective efficacy of several antigens delivered either as DNA vaccines or subunit vaccines has been tested in the canine model of visceral *Leishmania*sis. Early studies showed that dp72 protected mice against *L. donovani* infection [Jaffe *et al.,* 1990; Rachamim and Jaffe, 1993]. Despite these early successes, there has been no progress on the use of this antigen for the development of vaccines. A handful of other recombinant proteins have been tested against visceral Leishmaniasis in murine models. The LACK DNA vaccine was tested in dogs and mice with variable outcomes [Melby *et al.,* 2001]. The *L. donovani* amastigote LCR1 protein containing 67-amino acid repeats homologous to repeats in a *Trypanosoma cruzi* flagellar polypeptide, was administered as recombinant protein or expressed in BCG and tested for protection in mice. The recombinant protein induced partial protection against *L. chagasi* challenge [Wilson *et al*., 1995]. Immunisation with BCG-LCR1 elicited better protection than the protein alone, but protection depended on the site of immunisation, subcutaneous delivery being better than intra-peritoneal [Streit *et al.,* 2000]. Immunisation with the A2 cysteine proteinase delivered as recombinant protein or as DNA offered protection against invasion of macrophages and disease progression [Ghosh *et al*., 2001a, b]. Recombinant hydrophilic acylated surface protein B1 (HASPB1), a member of a family of proteins expressed only in metacyclic and amastigote stages of development of several *Leishmania* species, was protective in the mouse model of VL and interestingly, protection did not require any adjuvants and seemed to be generated via mechanisms reminiscent of DNA vaccination

[Stager *et al.,* 2000]. The PapLe22 antigen, a protein of unknown function, which localises to the promastigote nucleus is recognised by T cells from visceral Leishmaniasis patients [Suffia *et al.,* 2000]. Although PapLe22DNA vaccination led to a marked decrease in parasite burden in immunised hamsters [Fragaki *et al*., 2001], it induced IL-10 production in peripheral blood mononuclear cells from visceral Leishmaniasis patients indicating that in humans it might actually contribute to pathogenesis. Therefore, its use as a vaccine would need to consider the possibility that it may exacerbate disease. PapLe22 vaccine may be able to protect if the vaccine formulation would redirect T cell responses towards Th1 type responses. The *Leishmania* antigen ORFF, also a protein of unknown function [Ghosh *et al.,* 1999], was able to induce protective immunity against *L. donovani* challenge when administered with CpGs oligonucleotides [Tewary *et al.,* 2004].

Poly-protein vaccines

Apart from defined single molecules, multicomponent vaccines have been demonstrated to afford protection against VL in experimental animals. Recombinant Q protein formed by fusion of antigenic determinants from four cytoplasmic proteins from *L. infantum* (Lip2a, Lip2b, P0 and histone H2A) co-administered with live BCG protected 90% of immunised dogs by enhancing parasite clearance [Molano *et al*., 2003].

DNA vaccines

In 1995, DNA vaccination was proposed to be the way of the future [Waine and McManus, 1995]. DNA vaccines are relatively simple to produce and administer, they are often very immunogenic and offer a protein that is usually correctly folded and may

be post-translationally modified in a fashion similar to the native protein. Such vaccines are able to elicit humoral, CD4+ and CD8+ T cell immune responses, which can be further modulated by the addition of cytokines and/or CpG oligonucleotides [Alarcon *et al*., 1999]. They can also be modulated by prime-boost strategies that involve priming with DNA and boosting with protein [McShane, 2002]. Most nucleic acid vaccination efforts have been directed against viral infections, which require induction of CTL responses, a major feature of DNA vaccines. This method of immunisation is also attractive for Leishmaniasis since the induction of Th1 responses is also a general property of DNA vaccines [Gurunathan *et al.,* 2000]. In addition, a growing body of evidence implicates CD8+ Tcells in anti-Leishmanial immunity [Rodrigues *et al.,* 2003]. Most of the antigens described in the previous sections and delivered as recombinant proteins or expressed in live, microbial delivery systems have also been tested as DNA vaccines. The gene encoding gp63 was the first to be used as a DNA vaccine, and immunised mice developed strong Th1 responses as well as significant resistance to infection with *L. major* [Xu and Liew, 1994]. In another study, 30% protection was reported in immunised mice, with indications of strong Th1 responses being elicited by vaccination [Walker *et al*., 1998]. More recently, a comparative study evaluating *Leishmania* vaccines S97 different DNA vaccine candidates including gp63 showed that protection was transient, and eventually the immunised mice developed lesions similar to those observed in controls [Ahmed *et al*., 2004]. The same study also included PSA-2, which did not confer protection. This is in contrast with previous studies using PSA-2 DNA immunisation as either prophylactic [Sjolander *et al*., 1998] or therapeutic vaccines [Handman *et al.,* 2000], which showed protection associated with strong Th1 responses.

The difference in outcome between the two studies could be due to the use of susceptible BALB/c mice in the first, and resistant C3H/He mice in the second. Another comparative study demonstrated that gp63 DNA immunisation was able to reduce lesion size as well as parasite burden, while gp46/PSA-2 DNA vaccination led only to a reduction in lesion size without reduction of parasite burden [Dumonteil *et al.*, 2003]. LACK is the most extensively studied DNA vaccine against both CL and VL. DNA vaccination with a plasmid harbouring the LACK gene with, or without co-administration of IL-12 induced robust, long-lasting protection against *L. major* challenge in mice, dependent on the immunoregulatory role of CD8+ T cells [Gurunathan *et al.*, 2000]. In a heterologous challenge system, priming with *L. infantum* LACK followed by a booster with vaccinia virus expressing LACK afforded protection against L. major infection [Gonzalo *et al*., 2002]. The protection was further enhanced by co-administration of plasmids expressing IL-12 and IL-18 cytokines [Tapia *et al*., 2003]. Since previous studies showed that LACK-induced immunity was dependent on CD8+Tcells, boosting with vaccinia virus probably enhanced this immunity by expanding the CD8+ T cells population [Zavala *et al.*, 2001]. Boosting with recombinant *Salmonella* expressing LACK following a priming injection with DNA also conferred protection against infection and skewed responses towards Th1, thus enhancing the protection observed upon immunisation with DNA or *Salmonella* alone [Lange *et al*., 2004]. The prime-boost regimen was also employed to immunise dogs against VL and elicited protective responses in 60% of vaccinated animals [Ramiro *et al*., 2003]. Protective vaccination against *L. major* was also achieved following delivery of LACK in a minimalistic, immunogenically defined gene expression (MIDGE) vector [Lopez-Fuertes *et al*., 2002] with lower doses of plasmids required for

protection. The intranasal delivery of LACK DNA also protected mice against *L. amazonensis* challenge [Pinto *et al*., 2004]. These positive outcomes are overshadowed by several studies where immunisation with LACK offered no protection. These reports are mainly restricted to VL, but there are also reports in the *L. major* [Ahmed *et al*., 2004] and *L. mexicana* models of disease [Dumonteil *et al.*, 2003]. Melby and colleagues [2001] reported that despite triggering strong Th1 responses the LACK DNA vaccine did not induce protection in mice against *L. donovani* challenge. Moreover, the co-administration of IL-12 did not improve the protective outcome. A recent study in the *L. chagasi* model, confirmed that LACK DNA vaccination does not confer protection against VL despite the presence of Th1 responses [Marques-da-Silva *et al*., 2005].

Several other antigens have been successfully tested as DNA vaccines against cutaneous or visceral infection. The former group include acidic ribosomal protein P0 [Iborra *et al.*, 2003], P4 nuclease [Campbell *et al.*, 2003] and paraflagellar rod protein 2 (PRP-2), whereas the latter contains ORFF [Sukumaran *et al*., 2003], kinetoplastid membrane protein-11 (KMP-11) [Basu *et al*., 2005], CPA and CPB [Rafati *et al.*, 2005] and NH36, a main component of the fucose-mannose ligand [Aguilar-Be *et al.*, 2005]. DNA vaccination against *Leishmania* is considered a promising technology, but no development of such a vaccine for use in humans has been reported so far. Conflicting reports as to the protective efficacy of the antigens delivered in this mode add to the confusion in the field. To complicate issues further, protective outcomes seem to be influenced by many factors including plasmid backbone, number of injections, challenge dose and virulence of the *Leishmanial* strain, developmental stage of the parasite (promastigote vs amastigote), experimental protocol employed, immunomodulators and

type of animal model. Therefore, it is not surprising that the initial enthusiasm has been tampered by the complexities and difficulties that have surfaced.

Identification and Characterization of Virulence–Related Genes of *Leishmania donovani* Using Microarray Technology

SYNOPSIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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2008

Introduction

Leishmaniasis is widespread in 22 countries in the New World and in 66 nations in the Old World and afflicts the world's poorest populations. It is primarily found in tropical and sub-tropical regions including South-east Asia, East Africa, South America and Mediterranean region. Occurring in several forms, the disease is generally recognized for its cutaneous form which causes non-fatal, disfiguring lesions, although epidemics of the potentially fatal visceral form cause thousands of deaths.

Visceral leishmaniasis (VL) or Kala-azar (KA) is caused by the protozoan parasites of the *Leishmania donovani* complex, comprising of *L. d. donovani, L. d. infantum* and *L. d. chagasi.* More than 90% of the VL cases in the world are reported from Bangladesh, Brazil, India and Sudan [Desjeux, 2001]. In India, *L. d. donovani* is the primary causative agent of VL and states of Bihar, Eastern Uttar Pradesh and West Bengal are highly endemic foci of KA where periodic epidemics are common [Sundar and Rai, 2002].

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]. These axenic amastigotes resemble animal-derived amastigotes and have been widely used for investigating parasite activities without the complication of host cell material [Mengeling *et al.,* 1997; Shaked-Mishan *et al.,* 2001; Bente *et al.,* 2003; Goyard *et al.,* 2003]. This approach has been used previously to compare the different life cycle stages of *L. major* [Saxena *et al.,* 2003; Akopyants *et al.,* 2004; Almeida *et al.,* 2004], *L. donovani* [Duncan

et al., 2004], *L. infantum* [McNicoll *et al.*, 2006], and *L. mexicana* [Holzer *et al.*, 2006]. Host-free cell differentiation systems allow one to examine the changes in gene expression during the transition from promastigotes to amastigotes, especially the intermediate stage of differentiation, which would be impossible to carry out using animal-derived parasites.

A number of studies have been conducted searching for changes in gene expression amongst *Leishmania* stages, using methods such as differential or subtractive hybridization, AP-PCR, differential display, cDNA and genomic microarrays [Coulson and Smith, 1990; Charest and Matlashewski, 1994; Pogue *et al.*, 1995; Heard *et al.*, 1996; Liu *et al.*, 2000; Wu *et al*., 2000; Almeida *et al.*, 2002; Bellatin *et al.*, 2002; Saxena *et al.*, 2003, 2007; Akopyants *et al.*, 2004; Holzer *et al*., 2006; Leifso *et al.*, 2007]. Microarray analysis holds the promise of tracking the expression pattern of a large collection of genes simultaneously. Expression patterns might change over time or in response to different environmental conditions, or with stages of internal physiological change, or between strains or cell types. This is a very sensitive technology to discover genes that may be expressed transiently at a critical point of differentiation.

Historically, cutaneous leishmaniasis has been the focus of vaccination attempts, probably because it has been known since antiquity that individuals who had healed their skin lesions were protected from further infections. Bedouin or some Kurdistani tribal societies traditionally exposed their babies' bottoms to sand-fly bites in order to protect them from facial lesions. Another ancient technique practiced in the Middle East has been the use of a thorn to transfer infectious material from lesions to uninfected individuals. 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.*, 2006]. 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.

The study aimed at identifying genes that may be expressed at various time points during differentiation of promastigotes into amastigotes. Whereas there are several

studies on stage-specific expression of genes in promastigotes and terminally differentiated amastigotes [Coulson and Smith, 1990; Zhang and Matlashewski, 1997; Krobitsch *et al*., 1998; Boucher *et al*., 2002], the knowledge on genes that are expressed early during the differentiation is limited. Significant changes were observed in a few genes during early stages of differentiation [Duncan *et al*., 2001]. Hence, it is planned to find genes that may be expressed transiently during the intermediate stage of differentiation and might play a significant role in the differentiation process and disease pathogenesis. The transiently modulated genes may be undetected in studies with the two extreme life cycle stages. Genes showing differential expression early during the differentiation event will be identified using microarray technology. Selected gene(s) will be cloned and characterized to elucidate their role in *Leishmania* differentiation and hence, in disease pathogenesis. Such genes products that regulate differentiation into the intracellular amastigote form have great potential as targets to block the infection process.

Specific objectives

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 was planned to use parasites isolated from Indian KA patients instead of standard WHO isolates that had 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. Use of *in vitro* culture system will facilitate obtaining parasites at various stages during the differentiation process.

3. Microarray hybridization to study the events of *Leishmania* differentiation

Total RNA will be isolated from three biological preparations of developmentally staged cultures of *L. donovani*. Fluorescently labeled cDNA probes will be prepared from Promastigotes, an intermediate differentiation stage (PA24) and fully differentiated amastigotes that represent all the genes expressed in parasites at the particular life stage of interest. A cDNA probe from a reference sample labeled with one flourochrome (Cy3/Cy5) will be mixed with a probe from a differentiating 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 will be selected.

4. Characterization of identified clones.

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.

5. Validation of microarray data

Individual DNA clones selected by microarray experiments will be used as probes in Northern analysis with total RNA isolated from promastigotes, intermediate stage parasites and axenic amastigotes. Alternatively, RT-PCR of such clones will be carried out to validate the differential expression. Genes that are expressed at a higher level in any of the stages will

be selected for further analysis. Molecular characterization of selected genes will be undertaken.

6. Functional analysis of selected genes.

Full-length genes with potential for functional analysis will be expressed as recombinant proteins and antibodies will be synthesized. The differential expression of these genes will be verified at protein level. Transcript levels of these genes will be evaluated in human bone marrow samples. 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.

Results and Discussion

Morphological changes during differentiation

To study the structural and biochemical changes that are occurring during the process of differentiation, time course experiments comparing the promastigotes with an intermediate differentiation stage (PA24-promastigote-to-amastigote at 24hrs) were performed. Initially promastigotes were harvested at log phase (1.4×10^8 cells and 8.4×10^7 cells), resuspended in amastigote medium and placed in an incubator at 37^0C, and 5% CO_2 for 24 hrs and 96 hrs respectively. At these times the differentiating parasites (PA24) and the axenic amastigotes were harvested.

The extent of morphological change as evidence of differentiation was examined by light microscopy of stained cells from these samples. Promastigotes were distinguished by their elongated ellipsoidal shape, centrally located nuclei and prominent flagella. PA24 parasites were shorter and stouter than Pro with flagella present in few cells. Amastigotes were present in clusters and were round to ovoid in shape with no apparent flagella. Complete *in vitro* differentiation of Pro into Am was observed in 72-96 hrs. To quantify the morphological change, a shape factor (width/ length) was calculated for 15 parasite images at each stage.

With a shape factor of 0.897, the axenic amastigotes were about 3.3 times shorter than the promastigotes. Promastigotes at 0.274 were distinctively long and narrow. The

PA24 stage parasites had a mean shape factor of 0.517 and were 1.8 times shorter than promastigotes and 1.7 times longer than the amastigotes.

Transcript profiling of L. donovani differentiation

Changes in mRNA abundance during differentiation of promastigotes into amastigotes were examined by genome-wide expression profiling using genomic DNA microarrays. To incur an estimate of accuracy and precision of the system, microarray hybridization was carried out comparing Cy3-labeled promastigote RNA to itself labeled with Cy5, which showed comparable intensities at both wavelengths (532 nm and 635 nm) at all spots on the array indicating little or no dye bias (data not shown). Replicate experiments with three biological preparations were performed comparing PA24 or Am with promastigotes.

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).

Analysis of microarray experiments revealed a number of DNA clones showing differential expression in PA24/Pro and Am/Pro Parasite stages. 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) and 1.82% (77/4224) clones showed differential expression in PA24/Pro and Am/Pro microarrays respectively. However, by reducing the cut off value to ≥ 2 fold, there were 1.82% (77/4224) clones in PA24 vs. Pro and 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 in at least 8/9 spots (SD < 1), Z ratio >1.9 and p value < 0.05 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 have been submitted to ArrayExpress (accession no. E-MEXP-866).

Fifty-seven clones ranking top in the fluorescence intensities with ratio ≥ 1.7 and reproducibility in replicate experiments were selected and sequenced for further analysis. Of the 57 clones, 30 were upregulated at the intermediate PA24 stage while 27 were found to be downregulated at the PA24 stage. The identities of these genomic clones were assigned by homology to regions in the ORF, 5'UTR (upto ~ 500 bp) or in 3'UTR (within 1.5kb) of known *Leishmania* genes. Among the 30 clones overexpressed at PA24 stage, 16 clones showed further increase in their expression during the differentiation into Am. The expressions of 14 clones showed a transient increase at PA24 while their expression declined in Am though the expressions in Am stage remained significantly higher compared to Pro. Protein kinases and HSP10 were found to follow this pattern.

Among the 27 clones showing two-fold down regulation in PA24 stage compared to Pro stage, 10 clones were under-expressed at PA24 and Am stages. In 13 clones though the expression was low in PA24, their expression was regained as the parasite fully differentiated into Am. Of particular interest were 4 other clones which showed transient decrease at PA24 stage while their expression level increased by > 1.5 fold in Am in comparison to Pro. The identity of these clones revealed them to be surface molecules such as antigenic proteins and amino acid transporters.

Analysis of the differentially expressed clones in PA24 vs. Pro and Am vs. Pro microarray revealed five different patterns of gene expression. The expression of 14 clones increased transiently at PA24 stage when compared to Pro and Am. The clones that followed this expression pattern included PA phosphatase, two MAP kinases, two protein kinases, HSP10, tetratricopeptide repeat protein, ABC-1 transporter, phosphomannose mutase, two intergenic regions and three hypothetical proteins. Of the clones with increased expression at the PA24 stage, 5 maintained the same level of expression as they further differentiated to Am. Among these were HSP83 and a trypanosomatid specific protein of 27kDa (termed as P27) besides several hypothetical proteins. 11 clones showed continuous increase in their expression levels as the parasites

fully transformed into Am. The amastins, proteophosphoglycan, aldose-1 epimerase, calpain-like cysteine proteinase, serine carboxypeptidase, NAD/ FAD dependent dehydrogenase Spliced leader RNA, intergenic region and two hypothetical proteins were observed to follow this pattern. The expression pattern of 17 clones showing transient down regulation at PA24 stage. These were universal mini-circle binding protein, SnRNA, glutamate dehydrogenase, T-complex protein, regulatory subunit of protein kinase A like protein, amino acid transporter, Histone H_2A, short-chain 3-hydroxyacyl coA dehydrogenase, splice leader associated RNA and few hypothetical proteins. Other 4 clones in this category included surface molecules like PSA-2, surface antigen protein 2, amino acid permeases and a hypothetical protein were under expressed at PA24 by -4 fold were significantly overexpressed in Am compared to Pro. The fifth pattern of gene expression consisted of 10 clones showing down regulation at both PA24 and Am stages. Methyltransferase, eukaryotic initiation factor 3 subunit, kinesin, stomatin-like protein, V-type ATPase, 40s ribosomal S3a protein and 4 hypothetical proteins followed this expression pattern. The clones are grouped by gene ontology to facilitate interpretations about the correspondence between the pattern of gene expression changes with differentiation and the gene functions which follow that pattern.

The 3'UTR of 14 amastigote upregulated genes were aligned with standard 450 nucleotides sequence in GCG software, a multiple alignment program to check whether this regulatory sequence is present in the amastigote stage-specific expression. Except for 29B8 (amastin specific) which showed 66% homology, the other amastigote upregulated gene sequences do not seem to contain this 450 nucleotides sequence

The expression changes in representative clones from various categories were verified by RT-PCR and Northern hybridizations. The gene expression changes in 5 clones representing different categories were validated by RT-PCR in three different patient isolates of *L. donovani* and 5 other clones were tested in Northerns in two patient isolates. The clones tested on northern blots were: 15B2, 36G8 and 46G8 (all of which are hypothetical proteins); 39B11 (Parasite Surface Antigen) and 28F12 (heat shock protein 10). The clones 29C8 (NAD/FAD dependent dehydrogenase), 40B11 (Phosphomannomutase), 45E11, 46G8 (Hypothetical protein) and MAP kinase (28F11) were testedby RT-PCR. Northern blots and RT-PCR with different parasite lines gave

similar expression levels. Though the fold changes observed by Northerns and RT-PCR were different from those seen in microarray results, the expression patterns were found to be similar to microarray results.

Characterization of a novel trypanosomatids gene P27

The genomic clone 46G8 representing a transcribed sequence that exhibited 3.53 (± 0.40) fold higher expression at PA24 and 3.61 (± 0.26) fold higher expression at Am stage was selected for cloning of full-length gene. The nucleotide sequence of the clone 46G8 was submitted to GenBank and granted the accession number **ED004307**. The clone sequence aligns with 3'UTR region of the gene LmjF28.0980. The nucleotide sequence of the 46G8 clone revealed it to be a part of 3' UTR an ORF that coded for a protein of ~27kDa.

Transcript demonstration in hamster derived amastigotes

Our microarray study had been carried out with axenic amastigote cultures and we sought to check if P27 gene transcripts can be demonstrated in the hamster derived amastigotes as well. The expression of differentially expressed genes was validated using Relative Quantification study. 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.

Apart from axenic cultures, three folds higher expression was also observed in hamster spleen derived amastigotes confirming that this gene is transcribed in true amastigotes as well

Transcript demonstration in human bone marrow samples

We chose three genes (P27, amastigote specific gene A2 and a promastigotes upregulated gene V-type ATPase) to investigate the presence of transcripts in the cDNA samples of KA patients by semi-quantitative RT-PCR. Human HPRT gene was used for the normalization of bone marrow RNA. As an internal control, bone marrow cDNA was amplified with *Leishmania* specific α-tubulin primers in order to demonstrate the

presence of *Leishmania* RNA in the bone marrow RNA sample. We analyzed bone marrow samples from 4 KA positive patients and a KA negative sample.

The trypanosomatid specific gene transcripts were present in all the 4 samples tested. However, the amastigote specific A2 gene transcript could be detected in 3 of the 4 KA^+ bone marrow samples tested. Another gene transcript V-type ATPase which was upregulated in the promastigote stage was not detectable in the infected patient's sample. Quantitative analysis revealed that the gene expression was 4-39 folds higher compared to α-tubulin gene in human tissue samples which indicated that P27 gene is abundantly expressed in amastigotes in infected tissues of KA patients. The presence of P27 gene transcripts in KA patient's bone marrow emphasizes the importance of this gene in disease pathogenesis.

Cloning and Expression of P27

The gene P27 identified for the first time is specific to the family Trypanosomatidae and coded for a protein whose function is undetermined. A search of GenBank database (NCBI) with BLAST revealed that the P27 gene was unique to trypanosomatids suggesting some specific role of this protein in trypanosomaids. P27 ORF from *L. donovani* genome was cloned in pCR® CT-TOPO expression vector and its nucleic acid sequence determined. The gene was found to be present in a single copy in *Leishmania* whereas *T. brucei* had two copies of the gene. The gene showed 100% homology to P27 from *L. infantum* (LinJ28.1020) and contained homologues in *T. brucei.* The P27 ORF was successfully expressed in a bacterial expression system confirming its coding capacity. The protein band of 27kDa could be readily detected on SDS-polyacrylamide gels after purification with Nickel agarose. A sufficient quantity of the recombinant P27 was produced to inject into a rabbit for the production of antibodies to P27. Subsequently polyclonal anti-sera to recombinant P27 were raised in rabbit for subsequent studies such as immuno-localization in *Leishmania* and detection of the gene product in different life cycle stages of *Leishmania*.

Secondary structure prediction and localization of P27

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 to mitochondria. Gavel: prediction of cleavage sites for mitochondrial presequence is predicted to be in the R-2 motif at position 90 GRY|VH. The amino acid sequence was also submitted to Predict Protein Web based server. The output of these predictions revealed the protein to be a part of inner mitochondrial membrane with a transmembrane helix in the center of the protein (residues 103-121). Prosite motif search of the protein revealed that it contains six protein kinase C phosphorylation site at amino acid 5, 12, 48, 120, 139 and 217, two casein kinase II phosphorylation sites at aa 88 and 170, two tyrosine kinase phosphorylation sites at aa 151 and 198, one glycosaminoglycan attachment site at residue 236 and 4 N-myristoylation sites at aa 11, 39, 67 and 136. Disulfide analysis revealed the probability of cysteine linkages to be low and GLOBE predicted the protein to be compact as a globular protein. Further the predictions from PHDhtm, revealed the presence of a transmembrane helix at the centre of the protein. These analyses predict the protein to be an intergrated protein of inner mitochondrial membrane which was also proven experimentally.

Differential expression at various life cycle stages of Leishmania

The antibodies raised against recombinant P27 bind a single band of approximately 27kDa. The amastigote-specific pattern of expression, originally identified at RNA levels on the microarray, was observed to manifest at the level of protein as well. Western blot results showed that P27 protein expression starts immediately after the external stimulus for differentiation is provided and increases until full differentiation into amastigotes had taken place. The protein pattern of expression is very significant because the functional properties in living cells are determined by protein activity, thus P27 must perform a function particularly important in the amastigote stage.

Immunogenic potential of recombinant P27

Serum from 30 kala-azar patients and 20 control individuals were reacted against recombinant P27 protein. All kala-azar patients' sera reacted with P27 protein indicating that the protein elicits an immune response in humans.

Cloning and characterization of PSA-2

The genomic clone 42A11 representing a transcribed sequence that exhibited 4 (± 0.54) fold lower expression at PA24 and 3.27 (± 0.07) fold higher expression at Am stage was selected for cloning of full-length gene. The nucleotide sequence of the clone 42A11 was submitted to GenBank and granted the accession number **ED004298.**

Validation of expression at protein level

Multiple transcripts of PSA-2 were found to be differentially expressed in our microarray experiments and expressions of two PSA-2 genes were validated by Northern blot or RT-PCR. We tested the expression of PSA-2 protein at various life cycle stages of *Leishmania* on a western blot using anti PSA-2 antibodies. These antibodies were a kind gift from Dr. Handman and were raised against *L. infantum* native PSA-2 protein or monoclonal PSA-2 antibody raised against recombinant PSA-2 fusion protein. Polyclonal antibodies to native *L. infantum* PSA-2 detected the protein ~55kDa in all life cycle stages of *L. donovani* with uniform expression. However, PSA-2 ~46kDa was detected only in stationary promastigotes and terminally differentiated amastigotes when monoclonal anti-PSA-2 antibody was used suggesting this PSA-2 to have a role in parasite differentiation and virulence.

Over-expression of PSA-2 in *Leishmania*

Towards understanding the role of this surface protein in parasite differentiation and virulence, PSA-2 was over-expressed in *Leishmania donovani* and its effect in parasite differentiation, virulence and resistance to complement mediated cell lysis was determined. The growth pattern of the promastigotes of *Ld*PSA-2++ and *Ld*pKS Neo were studied and compared with wild type *L. donovani*. The growth pattern of all the three strains was found to be comparable.

***In vitro* differentiation into axenic amastigotes**

The ability of PSA2++ parasites to differentiate into axenic amastigotes *in vitro* was tested by growing the cells at different stages, i.e., promastigotes, PA24 and amastigotes and counting the number of cells with different morphologies at various time

points. At 24 hrs after the differentiation signals were provided, the number of PA24 stage cells were higher in *Ld*PSA-2++ as compared to wild type *L. donovani* or *Ld*pKS Neo. The axenic amastigotes started to appear with 24hrs of the differentiation process in *Ld*PSA-2++ which was not seen in wild type *L. donovani* or *Ld*pKS Neo. This suggested that *Ld*PSA-2++ cells were committed to amastigote stage within first 24 hrs of exposing them to amastigote culture conditions, indicating that the molecule plays a pivotal role in differentiation.

Complement lysis assay

Lysis by complement is one of the first immune mechanisms encountered by metacyclic promastigotes upon inoculation by fly bite into the vertebrate host. PSA has been shown earlier to mediate resistance to complement lysis. We had compared the wild type *L. donovani*, *Ld* pKS Neo and *Ld*PSA-2++ to analyze if over-expressing PSA helps to increase the resistance to complement lysis. Our results showed that over-expression of PSA-2 results in 5 fold increase in resistance to complement mediated cell lysis as compared to wild type *L. donovani*, thus providing the direct evidence that PSA-2 is indeed responsible for increased resistance to complement mediated cell lysis.

Immunofluroscence assay

*Ld*PSA-2++ cells were subjected to immunofluroscence assay using polyclonal anti-PSA-2 antisera. At PA24 stage, the *Ld*PSA-2++ cells were larger in size than wild type *L. donovani* cells at the same stage suggesting some role of PSA-2 in cell differentiation.

Summary

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 pattern of gene expression during the differentiation process of *L. donovani* promastigotes to amastigotes. The gene expression in promastigotes was compared with an intermediate stage of differentiation (PA24) and terminally differentiated amastigotes (Am) using genomic microarrays. Fifty-seven

differentially expressed clones were selected for sequencing. Among the upregulated clones three patterns of expression were observed: those that were transiently upregulated at PA24 stage which included several protein kinases; those that showed higher expression at PA24 stage but their expression were maintained at the same level until full differentiation (HSP83, a trypanosomatid specific protein P27 and several hypothetical proteins) and those that showed continuous increase in expression levels as the parasites fully transformed into Am which included surface proteins like Amastins, PPG and several metabolic enzymes. Two patterns of gene expression were observed among the downregulated clones. First expression pattern consisted of 17 clones showing transient down regulation at PA24 stage. Of these, 4 clones which included surface molecules like PSA-2 and amino acid permeases, were underexpressed at PA24 by ~4 fold and were significantly overexpressed in Am compared to Pro. The second pattern of gene expression consisted of 10 clones showing down regulation at both PA24 and Am stages. The microarray results were confirmed by northern blot analysis and RT-PCR.

Two clones that showed altered expression during the differentiation process were selected for further characterization. A trypanosomatid specific protein that showed overexpression soon after differentiation signals were provided was cloned for the production of recombinant protein. The nucleotide sequence analysis showed that the gene comprised of a 723bp ORF encoding a ~27kDa protein. The gene transcripts were demonstrated in human bone marrow tissue samples of kala-azar patients. Quantitative real- time PCR experiments revealed that the gene was 2-3 fold upregulated in hamster-derived amastigotes and 4-39 folds higher than *Leishmania* specific α-tubulin in human bone marrow tissue samples of kala-azar patients. The recombinant P27 was expressed in *E. coli* as a ~27kDa protein and polyclonal antiserum to this protein was raised in rabbit. Bioinformatic analysis revealed the protein to be a part of inner mitochondrial membrane. The expression of P27 was validated at protein level as well. Antibodies to r-P27 were detected in sera from kala-azar patients but absent in healthy controls suggesting that the protein elicits humoral immune response.

Another clone identified as PSA-2 that showed transient decrease in expression at an early stage of differentiation but was upregulated in terminally differentiated amastigotes was chosen for characterization. Western blot with antibodies against native *L. infantum* PSA-2 detected the protein at all life cycle stages of *Leishmania* whereas

monoclonal PSA-2 antibody raised against 46kDa PSA-2 fusion protein detected the protein in promastigotes and terminally differentiated amastigotes but not in the intermediate stages suggesting that PSA-2 indeed has a role in differentiation. The ORF region of PSA-2 was cloned in *Leishmania* expression vector and electroporated in *L. donovani.* Subjecting the mutant parasites to *in vitro* differentiation process revealed that the PSA-2 overexpressing parasites differentiate into axenic amastigotes sooner than the wild type parasites. Growth kinetics of the transfected parasites was studied and was found to be comparable to wild type *L. donovani*. The PSA-2 overexpressing cells showed a higher resistance to complement proteins when tested with graded concentrations of fresh human serum. These results suggest that PSA-2 protein has a role in promastigote to amastigotes differentiation and may perhaps play a role in immune evasion of *Leishmania* parasite inside the mammalian host.

The study establishes the increased expression of certain stage regulated genes that may be candidates for establishing infection and facilitating parasite survival inside the macrophages. Such differentially expressed genes hold the key to understanding of the parasite pathogenesis and may have a potential to be vaccine candidates.

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Publications and Presentations

1. **Srividya, G.**, Duncan, R., Sharma, P., Subba Raju, B.V., Nakhasi, H.L. and Salotra, P. (2007). Transcriptome analysis during the process of *in vitro* differentiation of *Leishmania donovani* using genomic microarrays. *Parasitology* **134**, 1527-1539.
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3. Raju, B.V.S., **Srividya, G**., Kuhls, K., Schonian, G. and Salotra, P. Genetic typing reveals monomorphism among the SAG sensitive and resistant *L. donovani* isolates causing Kala-azar or post kala-azar dermal leishmaniasis. Manuscript under preparation.
4. **Srividya, G.,** Singh, R. and Salotra, P. Advances in the diagnosis of Visceral Leishmaniasis: a comprehensive review over the last decade. Manuscript under preparation

Presentation in National/International conferences

1. Salotra, P., **Srividya, G.**, Subba Raju, B.V., Sharma, P., Duncan, R. and Nakhasi H.L.. Genomic microarrays for vaccine development against Kala-Azar. (Abstract) *Proceedings of the Annual Association of Clinical Biochemists in India*, Patna, Dec 2005, p35.
2. **Srividya G.** Identification of differentially expressed genes from three life cycle stages of *Leishmania donovani* using genomic microarrays. (Oral presentation) International Conference on Molecular Biology of *Leishmania* held on 26-28 Oct 2006 at ICGEB, Trieste, Italy.