Chapter 2

Review of Literature
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*Leishmania* are kinetoplastid protozoan parasites exhibiting a digenetic life cycle between the insect vector and vertebrate hosts. In the vertebrate host, these organisms are responsible for causing diseases with diverse severity and wide range of clinical manifestations, collectively termed leishmanises. The disease is spread in all the continents except Australia, and affects nearly 12 million people. It has been estimated that nearly one third of the world’s population lives in the endemic areas and as many as 350 million people are exposed to the risk of infection (WHO report, 1998). Leishmaniasis is not a single disease, but constitutes a variety of syndromes with clinical manifestations ranging from local self-healing skin lesions, to a severe life threatening systemic disease (Pearson and Sousa, 1996) depending upon the species involved and the immune response of the host at the time of infection. Based on the clinical manifestations, the disease has been broadly classified into three types viz. cutaneous leishmaniasis (CL) or the oriental sore, mucocutaneous leishmaniasis, or espundia and visceral leishmaniasis (VL) or kala azar. VL, caused by the members of *L. donovani* complex, is the most severe form of the disease, involving 500,000 new cases annually, 90% of which occur in Bangladesh, Brazil, India, Nepal and Sudan (WHO, 1998).

In the absence of any effective vaccine, the control measures rely mainly on chemotherapy to alleviate the disease and on vector control to reduce transmission. But the emergence of resistance in the parasite against the commonly used drugs poses a major roadblock in the control of leishmaniasis by chemotherapy. Moreover, with one-third of the HIV infected people living in the zones of endemic leishmaniasis, this disease has emerged as an opportunistic infection in AIDS patients (Alvar *et al.*, 1997).

2.1 EPIDEMIOLOGY

The members of the *L. donovani* complex have a wide geographical distribution. *L. infantum*, the causative agent of infantile visceral leishmaniasis, occurs mainly in the Mediterranean basin, including south European countries, extending eastwards through south-west Asia to China and westwards to Central and South America. Visceral leishmaniasis is distributed mainly on the Indian subcontinent, in Bangladesh, north-east
India and Nepal, and in eastern Africa, particularly Sudan and Kenya (Ashford, 2000). *L. chagasi* is prevalent in tropical zones of South- and Central America. Cases of cutaneous leishmaniasis, caused by the members of *L. tropica* and *L. braziliensis* complexes are mainly reported from Afghanistan, Iran, Saudi Arabia, Syria, Brazil and Peru. CL caused by other species like *L. aethiopica* in the highlands of Ethiopia and *L. peruviana* in the western Andes, have focal distribution, which depends upon the presence of reservoir hosts and/or vectors in sufficient numbers and proximity to humans.

### 2.1.1 Leishmaniasis in India

The disease does not find any mention in the ancient medical literature of the country. It first made its appearance in the plains of Bengal in 1824 and reached epidemic proportions by 1862 (Sanyal, 1985). As a consequence of extensive spraying of DDT under the National Malaria Eradication Programme during 1940-1960, the disease was believed to be eradicated from the eastern regions (Mallik and Basu, 1993), but the resurgence of both visceral and cutaneous forms was reported in the late 60s and early 70s (Chowdhury, 1982). It attained epidemic proportions in the early 90s (Thakur, 1993a) along with increasing unresponsiveness to pentavalent antimonials in Bengal (Mallik and Basu, 1993) and Bihar (Thakur, 1993b). From Bihar and Bengal, the disease has spread to other parts of the country and currently kala azar is endemic in the states of Bihar, West Bengal, Assam, Tamil Nadu, Orissa, Gujarat and Uttar Pradesh. It is rapidly spreading to non-endemic areas like Delhi, Meghalaya, Maharashtra and Karnataka (Dhanda et al., 1982; Dhiman and Sen, 1991). The dermal form of the disease, also called post kala azar dermal leishmaniasis (PKDL) is restricted to the eastern parts of India, while cutaneous leishmaniasis caused by *L. tropica* is prevalent in the arid zones of western Rajasthan (Bhattacharya et al., 1993). However, controversy prevails over the coendemic existence of this species with *L. donovani* and its involvement in visceral leishmaniasis (Schnur, 1989; Sacks et al., 1995a; Chatterjee et al., 1995).

The major factors responsible for the spread of leishmaniasis in India include population movement (Chowdhury, 1982), non-availability of drugs (Thakur, 1993b) and emergence of drug resistant strains (Mallik and Basu, 1993). More alarming is the emergence of this disease as an opportunistic infection in immunodeficient patients including AIDS patients.
2.2 THE Leishmania PARASITE

In 1885, *Leishmania* parasites were first observed by Cunningham in the macrophages in sections of "Delhi Boil", and were subsequently described by Leishman and Donovan in 1903 independently in the spleens of patients dying from kala azar in India. These were initially called *Piroplasma donovani* by Laveran and Mesnii and subsequently the name was changed to *Leishmania donovani* in 1903 by Ross (Peters, 1988).

*Leishmania* parasites are dimorphic, unicellular, kinetoplastid, protozoan parasites having a digenetic life cycle. They have been classified as follows:

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*Leishmania* occurs in two distinct morphological forms, the promastigotes and the amastigotes. The promastigotes are motile, flagellated, spindle-shaped, approximately 10-15 μm in length and grow in the gut of the sandfly vector or in culture media. The amastigotes on the other hand are non-motile, aflagellated, spherically-shaped, about 2-3 μm in diameter and grow intracellularly in the vertebrate hosts.

Leishmaniasis is considered to be a zoonotic disease and the humans are generally accidental hosts. But in some cases the infection is anthroponotic, with infected humans serving as reservoirs, as observed for cutaneous leishmaniasis caused by *L. tropica* in Middle East and visceral leishmaniasis caused by *L. donovani* in India (Ashford, 2000). Of the 30 species of *Leishmania* currently known, around 20 have been reported to cause disease in humans (Ashford, 2000). These have been classified into 3 major complexes based on the clinical manifestations resulting from the infection (Tanner, 1996):
i) *donovani* complex includes *L. donovani, L. chagasi* and *L. infantum*. The members of this complex result in visceral leishmaniasis, also termed kala-azar. The parasite replicates in the mononuclear phagocytes of spleen, liver, lymph nodes and bone marrow resulting in chronic infection leading to death if left untreated. The disease is characterized by fever, malaise, weight loss and diarrhoea associated with anemia, skin darkening and hepatosplenomegaly. In some patients treated with chemotherapy, skin becomes the major focus of infection resulting in post kala-azar dermal leishmaniasis (PKDL) (Rees and Karger, 1987).

ii) *tropica* complex includes *L. tropica, L. aethiopica, L. major* and several sub-species of *L. mexicana*. The parasite replicates in the dermal histiocytes resulting in self limiting skin lesions (localized cutaneous leishmaniasis LCL). The disease is characterized by the presence of a granuloma composed of a prominent infiltration of lymphocytes, epitheliod cells and parasites. *L. tropica* has also been suggested to cause visceral leishmaniasis, which was later contradicted (Sacks *et al.*, 1995a; Chatterjee *et al.*, 1995).

iii) *braziliensis* complex includes several sub-species of *L. braziliensis* which are prevalent in the New World. The members of this complex cause local cutaneous and mucocutaneous leishmaniasis. The latter results from the tropism of the parasites towards the macrophages of the oro-naso-pharyngeal area and can cause permanent disfiguring of facial features.

### 2.2.1 Life cycle of *Leishmania* parasite

*Leishmania*, having a digenetic life cycle, are transmitted to long-lived vertebrate hosts by short-lived phlebotomine sand flies of the genus *Lutzemia* (New World) and *Phlebotomous* (Old World). Both in the vector and in the vertebrate hosts the parasite has a cycle of development and multiply by binary fission. In the insect vector, the parasite survive in the gut as motile, flagellated, extracellular promastigotes, while in the vertebrate host, they live as non-motile, aflagellated, intracellular amastigotes in the mononuclear phagocytes (Fig. 1) (reviewed in Alexander and Russel, 1992).
Intracellular amastigote

Proliferation

Uptake

Phagolysosome

Attachment

Sandfly bite

Macrophage

Proliferation in the midgut

Figure 1: Schematic representation of life cycle of Leishmania
Adapted from Handman, 2001a.
Following ingestion of a blood meal, the parasites initially reside within the peritrophic membrane inside the sand fly's midgut. From the infected macrophages of the blood meal, the amastigotes are released which subsequently differentiate into motile promastigote stage. They synthesize a dense coat of glycocalyx, composed of a variety of glycoconjugates including lipophosphoglycan (LPG), proteophosphoglycans (PPGs) and GPI-anchored proteins. After about 2 days, the peritrophic membrane ruptures and the liberated promastigotes attach to the midgut epithelium through specific binding of LPG. These rapidly dividing promastigotes are termed procyclics and are not infective to vertebrate host. After a period of further 4-7 days, the promastigotes undergo metacyclogenesis wherein they detach from the midgut, cease to divide and mature into infective metacyclic forms, which synthesizes a structurally altered LPG incapable of binding to the midgut wall (Sacks et al., 1995b). The metacyclic promastigotes migrate to the foregut and oesophagus, where they remain suspended in the sand fly's saliva, waiting to be transmitted to the vertebrate host during subsequent blood meal. The sand fly saliva plays an important role in the survival and development of the parasite (Ghosh and Mukhopadhyay, 1998) and enhance the infectivity of these promastigotes by having immunomodulatory effects on the host immune system (Mbow et al., 1998; Lima and Titus, 1996). Immediately after transmission and before its entry into the host cells, the parasite resists the lytic effects of the serum complement factors by spontaneous shedding of C5b-C9 complex from the surface (Puentes et al., 1990) and by producing serine/threonine protein kinases which inactivate C3, C5 and C9 by phosphorylation (Hermoso et al., 1991; Li et al., 1996). Moreover, the parasite appears to harvest the binding of certain complement factors for their uptake by the host cells. Due to the lack of inducible nitric oxide synthase, the epidermal Langerhans cells may function as safe habitat for the promastigotes and can serve as vehicles for transporting the parasite from the infected skin to draining lymph nodes (Blank et al., 1996). The visceralizing strains of Leishmania migrate to internal organs especially to splenic macrophages, Kupffer cells in liver and myelocytes in the bone marrow. The uptake of Leishmania parasite by the macrophages comprises multiple mechanisms involving both parasite ligands and macrophage receptors. There is active uptake of parasite by either 'coiling' phagocytosis (Rittig et al., 1998b) or by conventional 'zipperlike' interactions (Rittig et al., 1998a). The macrophage receptors involved in uptake include mannose-fucose receptor, fibronectin receptor and the receptor for C-reactive protein (reviewed in Bogdan and Röllinghoff, 1998). Although under physiological conditions, complement receptors CR1
and CR3 are most important (Sutterwala et al., 1996). LPG and gp63 of *Leishmania* promastigotes are the two major parasite ligands which are thought to interact with the macrophage surface receptors (Mosser and Rosenthal, 1993). Once inside the macrophages, the parasites can be localized into phagosomes which later fuse with endosomal and lysosomal compartments eventually maturing into parasitophorous vacuole (PV) which contains hydrolytic enzymes (Lang et al., 1994a; 1994b). In PV, the parasites transform into non motile amastigotes in 2-5 days depending upon the species involved, divide by binary fission and exert the clinical manifestations of the disease (Pearson and Wilson, 1989). The amastigotes are released by bursting of the host macrophage and can infect new cells. Recently emerging evidence suggest that the *Leishmania* parasites within the parasitophorous vacuoles are able to influence the trafficking of host cell vacuolar system and the amastigotes have been suggested to recruit the exocytic machinery to gain release from the host cells (Rittig et al., 1998a).

### 2.2.2 *In vivo* and *in vitro* maintenance of *Leishmania* parasite

#### 2.2.2.1 *In vivo* infection model

Animal models of leishmaniasis have been developed in order to mimic the pathological features and immune response observed in humans. The inbred and congenic mouse strains can reproduce the entire spectrum of leishmaniasis as observed in humans. The stationary phase promastigotes are more infective in mice (Sacks and Perkins, 1984). For cutaneous leishmaniasis caused by *L. major* infections, the resistant strains of mice (CBA, C3H/HeJ and C57BL/6) develop self-healing cutaneous lesions, while the susceptible mice (BALB/c and DBA/2) succumb to infection (Leiw, 1989). The susceptibility of the mouse strains for *L. major* infections are controlled by *Scl-1*, *Scl-2* and H11 linked genes (Blackwell et al., 1985a). Moreover, the susceptibility or resistance towards infection has also been shown to be dependent on parasite dose and the cytokine microenvironment (Fowell and Locksley, 1999). *L. major* infection model has been well characterized and has been used to unravel the immunological determinants involved in *Leishmania* infection (Liew and O'Donnel, 1993; Reiner and Locksley, 1995). Unlike cutaneous leishmaniasis, there is no suitable mouse model that mimics progressive visceral leishmaniasis. Though the mouse model is good to study early parasite
replication, followed by immunological control and subclinical infection, it does not mimic the progressive disease observed in active human visceral leishmaniasis. To achieve a detectable visceral infection in mice a relatively large inoculum of parasite is required. However, even this high parasite dose does not lead to the death of the animals. Instead, the parasite burden after a moderate rise gradually falls to low and sometimes undetectable levels. This suggests the development of protective immune response against infection (Tumang et al., 1994; Murray et al., 1995; Leclerq et al., 1996; Honore et al., 1998; Engwerda et al., 1998). Some of the early studies indicated the susceptibility of golden hamsters for the development of *L. donovani* infection (Stauber, 1958; Keithly, 1976). Later several studies employed this model for the evaluation of various immunological parameters and therapeutic regimes (Gifawesen and Farrell, 1989; Rodrigues et al., 1998; Bories et al., 1998; Ghose et al., 1999). Recently, studies using *L. donovani* and *L. infantum* have conclusively demonstrated that hamsters serve as very good models to study human visceral leishmaniasis, exhibiting clinicopathological pattern and cytokine profile similar to that observed in humans (Requena et al., 2000; Melby et al., 2001)

### 2.2.2.2 *In vitro* infection models

*In vitro* infection of macrophages by *Leishmania* has been used to study cellular and molecular mechanisms involved in the infection, efficacy of antileishmanial drugs and microbicidal mechanisms of macrophages. Additionally, the *in vitro* infection system has been used for maintenance and production of amastigotes (Chang et al., 1985). Unlike the *in vivo* infection models, the multiplication of intracellular parasite under *in vitro* condition, is not affected by the source of the host cells (Olivier and Tanner, 1987; Crocker et al., 1987). The parasite shows a similar proliferation pattern in the macrophages isolated from both sensitive and resistant strains of mice. Apart from infecting the cells of monocyte macrophage lineage, under *in vitro* conditions, the *Leishmania* promastigotes have been demonstrated to infect a wide range of other host cells (reviewed in Rittig and Bogden, 2000). The *in vitro* models have helped towards the characterization of the nature of parasite macrophage interactions at the time of infection as well as the mechanisms involved in the uptake of the parasite. These models have also been helpful in understanding the biogenesis of parasitophorous vacuoles (Antonie et al., 1998) and kinetics of promastigote to amastigote transformation (Courret et al., 2001).
Most of our understanding of the macrophage effector mechanisms like nitric oxide mediated parasite killing and cytokine production, is based on the *in vitro* studies.

### 2.2.2.3 Promastigote culture

The amastigotes isolated from the infected animals can be transformed to promastigotes, which can be maintained under *in vitro* conditions (Chang and Hendricks, 1985; Evans, 1987). Freshly transformed promastigotes have been shown to resemble the infective metacyclic promastigotes (Sacks and da Silva, 1987; Kweider *et al*., 1987) and have been used to initiate *in vivo* and *in vitro* infection. The cultured promastigotes have been used for delineating various metabolic pathways and studying several biochemical and genetic aspects of the *Leishmania* parasite (Mukkada, 1985; Marr and Berens, 1985; Chance, 1985). Promastigotes have also been used to study the mechanism of action of various anti-leishmanial drugs and identification of new anti-leishmanial compounds (Hart *et al*., 1981).

### 2.2.2.4 Axenic Amastigote Culture

The clinical manifestations of all forms of leishmaniasis are due to amastigotes. Therefore, the understanding of biochemical machinery and antigenic profile of this stage of parasite is important for chemotherapeutic interventions and vaccine development. However, much of the information on biochemical and molecular aspects of *Leishmania* are based on the studies carried out on promastigotes owing to the ease of culturing them (Bates, 1993). Initially, amastigotes were obtained either from infected tissues, or cultured in mouse peritoneal macrophages and cell lines, where the supply was limited. In addition to the doubts about their purity, these preparations were often contaminated with the adsorbed host components (Bates, 1993; Gupta *et al*., 2001). To overcome all these limitations, various groups have attempted to culture amastigotes of different species axenically. Axenic cultures of amastigotes have been established using both promastigotes and amastigotes isolated from infected tissues by culturing them at relatively higher temperature and acidic pH (Shapira *et al*., 1988; Doyle, *et al*., 1991). However, these amastigotes should meet certain morphological, biochemical and immunological parameters if they are to be compared to the true amastigotes (reviewed Gupta *et al*., 2001). Axenic amastigotes have been used for the screening of anti-leishmanial agents (Callahan, 1997) and studying the mechanisms of action of anti-
leishmanial drugs. These amastigotes have also been helpful in identification immunological, molecular and biochemical differences between the two stages of the parasite (Saar et al., 1998)

2.3 Leishmania-MACROPHAGE INTERACTIONS.

2.3.1 Uptake of Leishmania parasite

The primary step in establishment of Leishmania infection in the host cells is the attachment of the parasite to the cell surface and subsequent uptake by phagocytosis. This early event is thought to involve numerous host cell receptors like mannose-fucose receptor, the fibronectin receptor, the receptor for C reactive protein (reviewed in Bogdan and Röllinghoff, 1998) and complement receptor type I (Sutterwala et al., 1996). These receptors interact with a variety of parasite ligands like LPG and gp63. The initial attachment triggers phagocytosis, which can occur in conventional “zipper-type” fashion, targeting the parasite to phagosome. Alternatively, phagocytosis may proceed in coiling fashion where, asymmetrical pseudopodia coil and stack, which occasionally targets the parasite to the cytoplasmic compartment. In both the cases the complement receptors CR1 and CR3 appear to play major role (Rosenthal et al., 1996). Recently, a new mechanism for the uptake of the parasite has been demonstrated which utilizes the alternative pathway of complement activation. Using an in vitro system, the promastigotes were shown to bind natural IgM antibodies present in the human blood and attach to erythrocyte surface through the complement receptor CR1. These promastigotes then invade neutrophils, where they are destroyed, and also macrophages, where they survive (Dominguez and Torano, 1999).

The uptake of amastigotes by macrophages appears to involve the interaction between both common and distinct set of host and parasite molecules. Like the promastigotes, the major ligand interacting with macrophages is LPG in L. major amastigotes. But in the amastigotes of certain species which do not express LPG at this stage, proteophosphoglycans might play an important role. For L. amazonensis amastigotes, an undefined heparin binding molecule (Love et al., 1993) and amastigote specific glycosphingolipids (Straus et al., 1993) have been implicated in the attachment to the macrophages. The macrophage receptors involved in amastigote uptake include the Fc receptor, CR3 receptor and mannose receptor (Peters et al., 1995). Langerhans cells
and dendritic cells have been reported to carry the amastigotes from the initial site of infection in skin to the draining lymph nodes. Also, in the dendritic cells the parasite may persist for a long time as silent infection. The host and the parasite molecules responsible for the interactions of amastigotes with these cell populations are not known (Rittig and Bogdon, 2000).

2.3.2 Development of Parasitophorous vacuole

Following phagocytosis, the parasites are localized in the resulting membrane bound phagosome, which becomes modified after fusing with secondary lysosomes and matures into a parasitophorous vacuole (PV) (Chang, 1983). PVs induced by all Leishmania species are acidic compartments rich in microbicidal peptides and hydrolytic enzymes and have lysosomal membrane markers LAMP-1 and LAMP-2 (Antoine et al., 1998). The acidic pH is maintained by a vacuolar H⁺-ATPase of macrophage origin and expressed in the PV membrane. Despite these common features the PVs induced by various Leishmania species show marked morphological differences. The New world species induce large PVs with amastigotes arranged around the periphery while L. major and L. donovani produce small PVs with little space around the amastigotes (Antoine et al., 1998). The large size of the PVs in case of L. mexicana has been attributed to amastigote specific proteophosphoglycan (aPPG) which is secreted in the parasitophorous vacuole (Peters et al., 1997). In the PV the promastigotes transform into amastigotes. This transformation is characterized by the loss of flagellum, closing of flagellar pocket, reduction in size and major changes in gene expression. The acidic pH of the PV and the higher temperature of the mammalian host, in addition to some unknown factors play major role in this transformation (Alexander and Vickerman, 1975; Chang and Dwyer, 1976; Antoine et al., 1990). The role of parasite in the development of PV is poorly understood though some secretory factors of amastigotes have been speculated to play a role in PV biogenesis (Ilg et al., 1995, Peters et al., 1997). The presence of MHC II molecules has been demonstrated in the PVs harboring Leishmania amastigotes. The capability of these molecules to present amastigote antigens is highly debated. Also, the amastigotes have been shown to selectively internalize and degrade MHC II molecules (Lang et al., 1994 a; 1994 b; Antoine et al., 1998).
2.3.3. Modulation of Macrophage Effector Functions

2.3.3.1 Leishmanicidal mechanisms

The two main effector mechanisms by which the macrophages destroy *Leishmania* and other intracellular pathogens are the release of superoxide by neutrophils and macrophages by NADPH oxidase complex, and cytokine induced nitric oxide (NO) production in macrophages. *Leishmania* parasite and/or their components have been demonstrated to interfere in both the pathways. \( \cdot o^\cdot \) and \( H_2O_2 \) production in response to phorbol esters is drastically reduced in *Leishmania* infected macrophages (Buchmuller-Rouiller and Mauel 1987; Olivier et al., 1992; Passwell et al., 1994). LPG has been demonstrated to be responsible for this effect by inhibiting protein kinase C (PKC). Additionally, gp63 and other glycoconjugates have also been associated with the inhibition of oxidative burst (Sorensen et al., 1994). Moreover some of the *Leishmania* molecules scavenge the reactive oxygen radicals (LPG). Recently, a novel hydrogen peroxide catabolizing pathway, mediated by tryptaredoxin peroxidase, was described for the protection of parasite against oxidative damage (Levick et al., 1998). *Leishmania* infection also inhibits NO production by the macrophages in response to IFN-\( \gamma \) activation. This is due to the strong inhibitory effects of *Leishmania* GPILs on the induction of nitric oxide synthase-2 (NOS-2) (Proudfoot et al., 1996).

2.3.3.2 Impaired antigen presentation

Macrophages, the primary hosts of *Leishmania* parasite, are classical antigen presenting cells and present the antigens in association of the MHC class I (MHC I) or class II (MHC II) molecules, to CD4\(^+\) or CD8\(^+\)-T cells respectively. However, the capability of *Leishmania*-infected macrophages to present antigens to T-cells has been debated since long. *Leishmania* infection does not affect the IFN-\( \gamma \) induced expression of MHC II neither does it affect the quantitative expression of these molecules in the macrophages. The surface expression of MHC II is also unaffected in the early stages of infection, which however undergoes dramatic redistribution in the cells at the later stages of infection. Most of the MHC II molecules are distributed in the PV and are localized at the site of attachment of amastigotes in the PV membrane. The amastigotes have been demonstrated to internalize and possibly degrade MHC II molecules (Lang et al., 1994a; 1994 b; Antoine et al., 1998). However, *Leishmania*-infected macrophages have been
found to present antigens to CD4+ T cells. Wolform et al. (1995; 1996) demonstrated that antigen could be processed in the parasitophorous vacuole to be presented at the macrophages surface to activate T cells by macrophages infected with *L. mexicana* that overexpressed membrane bound acid phosphatase at the parasite surface as a secreted product. But the failure of macrophages infected with wild type parasite to stimulate the T cells suggests that the intracellular antigens of intact *Leishmania* are not normally available for presentation (Wolform et al., 1996). However, the abundant antigens like cysteine proteinases from the dead parasite can be presented to T cells (Wolform et al., 1995). For effective T cell activation, in addition to the presentation of antigens by MHC II, co-stimulatory molecules are also required to provide the secondary signal. This is provided by the ligation of B7-1/B7-2 and CD40, present on the macrophages, with CD28 and CD40L on T cells. *L. donovani* infected macrophages fail to upregulate B7-1 expression upon exposure to inflammatory cytokines (Kaye et al., 1994; Saha et al., 1995).

### 2.3.3.3 Impaired cytokine production

IL-12 produced by the macrophages plays an important role in the induction of protective immunity against the intracellular pathogens (both innate and acquired Th1). Both infective forms of *Leishmania* metacyclic promastigotes and amastigotes inhibit the macrophage production of this cytokine *in vivo* as well as *in vitro*. LPG of the parasite has been shown to suppress IL-12 production at transcriptional level (Piedrafita et al., 1999). TGF-β and IL-10 are macrophage derived cytokines which inhibit the killing of *Leishmania* by macrophages by counteracting the development of Th1 response. Both *in vitro* and *in vivo* infection with *Leishmania* induce the production TGF-β. *L. major* promastigotes have also been demonstrated to induce the production of IL-10 β. Infection of macrophages *in vitro* with *L. major* promastigotes has also been shown induce IL-10 production. Additionally, *L. donovani*-infected macrophages fail to release T cell stimulatory cytokines IL-1 and TNF-α in response to LPS stimulation (reviewed in Bogdan and Röllinghoff, 1998).

### 2.3.3.4 Inhibition of macrophage apoptosis

By extending the life of the host cell the parasite ensures its persistence in the host. This is primarily achieved by inhibition of apoptosis of macrophages through the
induction of pro-survival cytokines like macrophage colony stimulating factor (M-CSF), TNF-α, and IL-6 (Moore and Metlashewski, 1994; Antoine et al., 1998).

2.3.3.5 Impaired signal transduction

Many of the macrophage dysfunctions resulting from Leishmania infection have been speculated and demonstrated to result from the interference of various cell signaling pathways. Leishmania donovani infected macrophages exhibit altered activation and translocation of protein kinase C (PKC). As a result the infected macrophages had defective oxidative burst in response to phorbol myristate acetate (PMA) (Olivier et al. 1992a; 1992b). It was also demonstrated that LPG inhibits purified PKC activity (McNeely and Turco, 1987), and incubation of macrophages in vitro with LPG renders the cells refractory to PKC-dependent c-fos gene expression (Moore et al., 1993). PKC isolated from macrophages harboring Leishmania amastigotes required two-fold increase in the concentration of allosteric activator 1,2-dioleoyl-rac-glycerol to achieve half-maximal PKC activation. Another mechanism employed by Leishmania to downregulate PKC signaling is by degradation of PKC substrate. Recently Corradin et al., (1999), demonstrated that the purified gp63 from Leishmania is capable of degrading MRP (Myristoylated Alanine Rich Protein kinase C substrate (MARCKS)- Related Protein) which is a major intracellular substrate of PKC. Ca^{2+}-regulated signaling has also been found to be defective in Leishmania infected macrophages. Although, the level of intracellular Ca^{2+} is higher in infected cells, the rapid release of Ca^{2+} from intracellular stores in response to fMLP is diminished. This correlated with diminished generation of endogenous inositol phosphate, an endogenous Ca^{2+} mobilizing agent (Olivier et al., 1992a; 1992b). Some effector mechanisms of macrophages, like phagocytosis, involve protein tyrosine phosphorylation. Attachment of promastigotes and fixed amastigotes triggers tyrosine phosphorylation in macrophages. However, Leishmania amastigotes were demonstrated to alter tyrosine phosphorylation of extracellular signal-regulated protein kinase (ERK1 MAP kinase) and some other macrophage molecules. The decreased tyrosine phosphorylation was attributed to an unidentified ecto-protein phosphatase activity on parasite surface (Martiny et al., 1999).
2.3.3.6 Down regulation of gene expression

*Leishmania* mediated modulation of macrophage functions has been studied in terms of specific gene expression for example, IL-12 and other cytokine genes. Recently, Buates and Metlashewski (2001) demonstrated that *L. donovani* infection results in general suppression of gene expression in infected macrophages. Though the expression of housekeeping genes remains unaffected, nearly 40% of other genes examined were downregulated in the infected macrophages. However, infection resulted in up-regulation of few genes, including genes of macrophage inflammatory proteins (MIP) 1α and 1β which might be helpful in the recruitment of new macrophages at the site of infection. *Leishmania* infection has also been demonstrated to alter the expression of small interspersed repetitive elements (SINEs) in the macrophages. In contrast to uninfected cells, there is down-regulation of pool size of small B1-RNAs and an increase in the amount of pol II directed large B1 transcripts (Ueda and Chaudhuri, 2000).

2.4 TARGET MOLECULES OF *Leishmania* PARASITE

During its life cycle, the *Leishmania* parasite encounters three different hostile environments; (i) the gut of the sandfly where the parasite is susceptible to degradation mediated by hydrolytic digestive enzymes, (ii) the bloodstream of the vertebrate host, where the parasite is exposed to lytic complement pathway and, (iii) the phagolysosome of host macrophages where it is prone to the action of hydrolytic enzymes and macrophage microbicidal mechanism like oxidative burst. The parasite has developed its own arsenal to counter these destructive mechanisms, which serve as virulence factors and allow the parasite to grow in these hostile environments. Majority of these factors are membrane bound or secretory glycoconjugaes and glycoproteins. Some of the important virulence factors of *Leishmania* include; lipophosphoglycan (LPG), proteophosphoglycans (PPGs), acid phosphatases (APs), cysteine proteinases and a surface metalloprotease-gp63.
2.4.1 Lipophosphoglycan

Promastigotes of all the *Leishmania* species analyzed to date have been demonstrated to produce lipophosphoglycan (LPG). Several million copies of this immunodominant glycoconjugate are present per cell, which cover the entire promastigotes surface including the flagellum, forming a dense filamentous glycocalyx.

2.4.1.1 Structure

*Leishmania* LPG is organized into four domains; a 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol membrane anchor, a diphosphoheptasaccharide core, a backbone comprising of repeating phosphodisaccharide (PO₄-6Gal(β1,4)Man(α1) and mannose-rich cap structures. While the lipid anchor and the core structure are conserved, the backbone and the cap exhibit high degree of stage/species variation. The repeating units of the phosphosaccharide backbone are unsubstituted in *L. donovani* LPG, while in *L. mexicana* LPG 25% of Gal residues are substituted with βGlc. *L. major* LPG exhibits most complex repeating units wherein nearly 87% of the galactose residues are further substituted with small saccharide side chains consisting of one to four residues of galactose, glucose or pentose arabinose. Despite the complexity in the composition of repeating units, the cap of *L. major* LPG is simplest and consists of the disaccharide Man(α1,2)Man(α1), while the most abundant terminal oligosaccharide in *L. donovani* and *L. mexicana* LPG is a branched trisaccharide Gal(β1,4)Man(α1,2)Man(α1) (Turco and Descautex, 1992).

2.4.1.2 Functions

LPG has been regarded as a multifunctional virulence factor. It has been demonstrated to serve important functions for the parasite in both insect and the mammalian hosts.

In the sandfly vector, LPG has been long thought and recently demonstrated to aid in the protection of parasite against the hydrolytic digestive enzymes of the sandfly gut (Turco and Descautex, 1992). Recently, Sacks *et al.* (2000) demonstrated that LPG is not essential for the survival of the parasite in the early blood-fed sandfly midgut, but along with other secreted phosphoglycans, can protect the parasite from the digestive
enzymes. Importantly, LPG is involved in the attachment of parasite to midgut of insect, thereby preventing the parasite from being excreted along with the excreted bloodmeal. The interaction of LPG with the sandfly vector midgut is one of the key factors governing the vectorial competence of the given *Leishmania* species. The subtle variations in the composition of the LPG amongst various species determine its capability to be retained or excreted out along with the bloodmeal. LPG undergoes modification in the side chains during metacyclogenesis. It has been proposed that this modification helps in the detachment of the infective metacyclic promastigotes from the gut of the sandfly (Sacks *et al.*, 1995b).

The bloodstream of the mammalian host is the next frontier where LPG contributes significantly towards parasite survival (Turco and Descoteaux, 1992). In contrast to log phase or procyclic promastigotes, the stationary phase parasites, where the population of metacyclics is more, are more resistant to complement mediated lysis (Puentes *et al.*, 1988). This has been attributed to the thickening of the glycocalyx resulting from the metacyclogenesis associated elongation of LPG backbone (Turco and Descoteaux, 1992). Also this thick coat is thought to protect the parasite from the lytic antibodies present in the kala azar patients, by masking the other functionally important molecules. The finding that there is enhancement in the recognition of antigens by kala azar patient sera in LPG deficient mutant R2D2 substantiates this view (Karp *et al.*, 1991).

The *Leishmania* LPG has been implicated in the attachment of the parasite to the host cells during infection. As discussed earlier (2.3.1), the interaction and uptake of *Leishmania* is facilitated by multiple ligand-receptor interactions. Several lines of evidence suggest the involvement of this abundant surface glycoconjugate as a parasite ligand/receptor for the macrophages. The Fab fragment of anti- *L. major* LPG antibody inhibits the binding of *L. major* promastigotes to the macrophages *in vitro* (Handman and Goding, 1985). Purified LPG from *L. major* and *L. donovani* bind to macrophages and other cell types (Tolson *et al.*, 1990). Moreover due to the ability of LPG to bind to the C3 complement protein, the molecule may further exploit the C3 receptor on the macrophages for the entry of the parasite (Talamas-Rohana *et al.*, 1990).

The final frontier for *Leishmania* parasite is the phagolysosome of the host cells. In the host cells LPG has been demonstrated to carry out various functions, which might

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help the parasite to combat the hostile macrophage microbicidal effector mechanism and inhibit the maturation of the protective host immune response against *Leishmania*. LPG inhibits phagosome lysosome fusion, thereby interfering with the maturation of phagolysosome, which is otherwise rich in hydrolytic enzymes. Still some hydrolytic enzymes are present in *Leishmania* harboring parasitophorous vacuoles and LPG has been demonstrated to inhibit some of these enzymes, like β-galactosidase. Reactive oxygen species produced as a result of oxidative burst is one of the key macrophage microbicidal mechanisms. The LPG protects the parasite against destruction by this pathway at two levels. The repeating unit sugars are rich in hydroxyl groups, which can be oxidized to ketones, thereby helping the LPG molecule to serve as a scavenger of reactive superoxide anions and hydroxyl radicals. Secondly, by inhibiting the PKC translocation and activation of this pathway the initiation of oxidative burst is inhibited. Being rich in phosphate groups, the LPG molecule is highly negatively charged. Thus, the molecule serves as a chelator of Ca\(^{2+}\) ions and might affect the signaling pathways involving Ca\(^{2+}\). The purified *Leishmania* LPG has also been demonstrated to alter the production of various cytokines from the macrophages. It has been shown to inhibit the production of IL-12, TNF-α and IL-1β by the macrophages (Proudfoot *et al.*, 1996; Hatzgeorgiou *et al.*, 1996). Recently, purified LPG was shown to inhibit the expression of inducible nitric oxide synthase in macrophages (Piedrafita *et al.*, 1999). Additionally, LPG inhibits endothelial adhesion and trans-endothelial migration of monocytes thereby helping the parasite survival and spreading (Ho *et al.*, 1996; Lo *et al.*, 1998).

In light of all these observations related to its function, LPG has been widely regarded as a multifunctional virulence factor. On the expected lines were the results obtained with the mutant parasites deficient in LPG biosynthesis. Several LPG deficient mutants of *L. donovani* were obtained after heavy mutagenesis and subsequently selected for their failure to be agglutinated by ricin (King and Turco, 1988). These mutants were found to be avirulent. In one of the mutants, R2D2, a gene for LPG biosynthesis (*lpg1*) was identified and null mutant in *L. major* background was created (Spath *et al.*, 2000). The uptake of this mutant by macrophages was unaffected, but intracellular survival of the parasite was compromised and the parasites were destroyed after 2 days of infection. However, some recent studies have raised doubts over this belief. At least in *L. mexicana*, LPG is not required for the infection of macrophages or mice (Ilg, 2000). It was demonstrated that the parasites lacking *lpg1* gene, which is involved in LPG biosynthesis,
do not show difference with the wild type in respect to attachment, uptake and survival of parasite inside the macrophages. Moreover, the parasite lacking the phosphoglycan repeats, which in addition to LPG are important components of PPGs, are also infectious to the macrophages and mice (Ilg et al., 2001). These results have raised serious doubts over the role of LPG as a universal virulence factor in all the species of *Leishmania*. This possibly points towards some degree of redundancy built in the parasite surface molecules thereby enabling the parasite to interact with the macrophage receptors in more than one ways (Handman, 2001b). This issue has been extensively discussed in a recent review (Turco et al., 2001).

2.4.2 Glycoprotein 63 (gp63)

GP63 is the major surface glycoprotein of *Leishmania* promastigotes. Often referred as leishmanolysin, in promastigotes this GPI anchored zinc metalloprotease might account for as much as 1% of the total cellular proteins and with $5 \times 10^5$ copies per cell covering the entire cell surface. In amastigotes it is expressed at lower levels, lacks GPI anchor and is localized in the flagellar pocket (Medina-Acosta et al., 1989). Recently the crystal structure of this molecule was solved and it revealed an active site structural motif similar to that found in other zinc metalloproteases (Schlagenhauf et al., 1998). A multigene family organized in tandem repeats, that vary in size and copy number in a species-dependent manner, encodes different forms of this protein in *Leishmania* (Button et al., 1989; Medina-Acosta et al., 1993). There is high degree of polymorphism exhibited in the gp63-coding regions than that observed in gp63 intergenic regions at this locus (Guerbouj et al., 2001). The expression of various gp63 genes is developmentally regulated. The expression of gp63 is upregulated in metacyclic promastigotes and this is thought to result in the enhancement of infectivity (Sacks and Perkins, 1984; Russel and Wright, 1988; Medina-Acosta et al., 1989). Gp63 helps the parasite avoid complement-mediated lysis by proteolytically converting the deposited C3b into inactive iC3b. By acting as a ligand for macrophage surface β1-integrins, GP63 can directly augment parasite internalization (Brittingham et al., 1995; Brittingham et al., 1999). Recently, Corradin et al. (1999) demonstrated the degradation of MRP, a PKC substrate in macrophages by gp63, indicating that gp63 might affect MRP mediated events in the macrophages. The genetic manipulations have confirmed the role of gp63 in complement resistance. However, the general function of gp63 in the survival of the
parasite is not defined due to the difficulty in complete elimination of gp63 genes. Targeted deletion of 6 out of seven gp63 genes in *L. major* did not affect parasite growth *in vitro* or prevent formation of disease in mice (Joshi et al., 1998). Recently Hilley *et al.* generated a knockout of GPI8, GPI: protein transaminidase, that eliminated the expression of GPI-anchored proteins, including gp63. The growth of the parasite in culture and its ability to infect macrophages *in vitro* was unaffected. Importantly, the mutant was able to establish infection in mice thus suggesting that GP63 is not important for growth or infectivity in mammals.

### 2.4.3 Proteophosphoglycans

As discussed in the previous section (2.4.1), LPG is an important multifunctional virulence factor in *Leishmania*. Phosphoglycan repeats account for most of the biological activity of *Leishmania* LPG. However these structures are not restricted to LPG alone, but also occur on a family of *Leishmania* glycoproteins termed proteophosphoglycans (PPGs). These proteins exhibit a novel protein-glycan linkage, phosphoglycosylation, wherein the phosphosugars are attached to the serine/threonine residues of protein backbone through a phosphodiester linkage (Ilg, 2000). Due to heavy glycosylation and high molecular weights, these glycoproteins have been compared to the mucins of the higher organisms (Ilg *et al.*, 1999). Few of these have been well studied and characterized. These include secreted acid phosphatases (sAPs), filamentous proteophosphoglycan (fPPG), membrane bound proteophosphoglycan (mPPG) and amastigote proteophosphoglycan (aPPG) (Ilg *et al.*, 1994; Ilg *et al.*, 1995; Ilg, *et al.*, 1996).

**SAPs** are secreted, via flagellar pockets, by the promastigotes of most *Leishmania* species, in the surrounding medium (Gottlieb and Dwyer, 1982; Ilg, *et al.*, 1994). The sAPs of *L. donovani*, *L. tropica*, and *L. mexicana* were found to share some structural elements with LPG. sAPs of *L. mexicana* have been characterized extensively. These are encoded by two genes, *lmsapl* and *lmsap2*, which are expressed in the promastigotes. They differ only in length of a region coding for Ser/Thr-rich repeats at 3'end. The gene products of these genes, sAP1 and sAP2 form linear heteropolymers via their globular, enzymatically active, N-terminal domains (Weise *et al.*, 1995). The C-terminal Ser/Thr rich domains are subjected to extensive phosphoglycosylation and project from the central polymer conferring upon the molecule a bottlebrush structure.
phosphatase activity associated with these molecules, the function of these molecules has been elusive. The *L. major* promastigotes lack sAPs, while the gene knockout of *L. mexicana* sAP indicated that the enzyme is neither required for the *in vitro* promastigotes growth nor for parasite infectivity (Ilg, 2000).

**fPPG** like sAPs is secreted through the flagellar pocket by the promastigotes. These form a gel-like network of fibrous filaments and results in the aggregation of promastigotes in this network (Stierhof *et al*., 1994). This molecule recently been implicated in the transmission of parasites by the insect vector by forming a plug which blocks the lumen of the stomodeal valve and may result in the deposition of the parasites into the wound due to reflux during the bloodmeal (Ilg, *et al*., 1999; Ilg, 2000). fPPG has also been demonstrated to have immunomodulatory effects. On one hand they inhibit the LPS induced production of TNF-α, which is beneficial to the parasite while on the other, they synergise the production of nitric oxide in combination with IFN-γ, which had parasiticidal effects (Piani *et al*., 1999).

**mPPG** is a membrane bound PPG molecule which is present in primarily in the promastigotes and to a lower extent in the amastigotes (Ilg *et al*., 1999). It is a large polypeptide of ~2300 amino acids, encoded by *ppgl* a member of *L. major* PPG multigene family, and like other *Leishmania* molecules, the exact function of this molecule is not known but it is speculated that the carbohydrate rich central domains displays potential ligands for sandfly and macrophage receptors, and may also serve as complement acceptors (Ilg *et al*., 1999; Ilg, 2000).

Amastigotes of most of *Leishmania* species do not synthesize LPG or sAPs, but secrete aPPG in the parasitophorous vacuoles of the host cells. It contains all the glycans identified previously to e present in LPG or sAPs, but additionally contains some amastigote specific or novel structures including some mono- and multiphosphorylated glycans (Ilg *et al*., 1995). The concentration of secreted aPPG in the parasitophorous vacuole may reach in the range of mg/ml. Due to its high concentration and polyanionic nature owing to the phosphoglycans, this molecule has been implicated in the biogenesis of parasitophorous vacuole (Peters *et al*., 1997).
2.4.4 Cysteine Proteinases

*Leishmania* produce cathepsin L-like cysteine proteinases (CPs), which are predominantly expressed in amastigotes and to a lesser extent in the metacyclic promastigotes (Sakanari *et al.*, 1997; Mottram *et al.*, 1998). This observation together with the failure of the parasite to grow within macrophages in presence of CP inhibitors, suggest an important role of these proteins in the intracellular survival and parasitism (Mottram *et al.*, 1996; Alexander *et al.*, 1998). Another class of CPs, cathepsin B-like cysteine proteinases is also expressed in this parasite. Cathepsin L-like CPs are encoded by a single copy gene *cpa*, while a multicopy gene family, *cpb* encodes cathepsin B-like CPs (Coombs and Mottram, 1997a). Mutants lacking *cpa* retained the virulence indicating that this particular CP is not essential for the interaction of the parasite with the mammalian host. However disruption of 19 copies of *cpb* genes led to the decrease in the virulence of the parasites. The *in vitro* infectivity to macrophages was reduced by 80% while in mice the development of subcutaneous lesions was slower than the wild type. The pathogenicity was completely restored by transfecting a single copy of *cpb* gene in the null mutant (Mottram *et al.*, 1996; Descautex, 1998). However different *cpb* genes vary in their ability to restore full infectivity in *cpb* null mutants. Additionally, expression of metacyclic specific CPBI failed to restore the virulence in *cpb* null mutants suggesting that this form of the enzyme might have a role in the interaction of the parasite with the sandfly or during the initial contact with the mammalian host (Mottram *et al.*, 1997). However, different CPs expressed in the amastigotes have been demonstrated to vary in their substrate specificity when analyzed with the artificial substrates Mottram 1996; 1997). Clear identification of natural substrates for these CPs will provide important clues to their role in the intracellular survival and pathogenesis of *Leishmania*. Some speculated functions of amastigote CPs include degradation of MHC II and hydrolytic enzymes present in the parasitophorous vacuoles.

2.4.5 A-2 proteins

*A2* gene family was first identified in *L. donovani*, as the transcripts were highly abundant in the amastigotes but hardly detectable in promastigotes (Charest and Matlashewski, 1994). Recently *A2rel* genes were also discovered, which are expressed at equal levels in the promastigotes and amastigotes. Multicopies of *A2* genes are clustered on the 850 kb chromosome of *L. donovani*, alternating with a *A2rel* genes (Ghedin *et al.*, 1998).
1997). *A2rel* gene encodes a putative 44 kDa, hydrophobic protein with an unknown biological function (Ghedin *et al.*, 1998), while the *A2* genes are composed predominantly of a 10 amino acid encoding sequence that is repeated from 40 to over 90 times depending upon the species of *A2* gene. The *A2* protein is strongly homologous to a developmentally expressed S-antigen of *Plasmodium falciparum*. The family of *A2* proteins ranges from 40-100 kDa and is present only in the cytoplasm of the amastigotes (Charest and Matlashewski, 1994; Zhang *et al.*, 1996). By expressing the *A2* antisense RNA in *L. donovani*, Zhang and Matlashewski (1997) established that *A2* proteins are not required for parasite proliferation *in vitro* but is essential for the survival of the amastigotes in the mammalian host. Recently, *A2* proteins have been demonstrated to be involved in the visceralization of the parasites in the mammalian hosts (Zhang and Matlashewski, 2001). Partial *A2-A2rel* gene cluster knockouts of *L. donovani* had reduced proliferation in culture and reduced virulence in mice. Transfection of *A2* gene in *L. major*, which otherwise lacks this genes and causes cutaneous leishmaniasis, increased its ability to survive in the spleen of mice. Thus it appears that the *A2* gene is involved in the visceralization of VL causing species (Zhang and Matlashewski, 2001)

### 2.5 *Leishmania* Antigens on Infected Macrophages

In the mammalian hosts, the *Leishmania* parasite survives intracellularly as amastigotes in the cells of monocyte-macrophage lineage. Several groups have demonstrated the presence of parasite antigens on the surface of infected macrophages. Farah *et al.*, (1975) and Handman *et al.* (1979) demonstrated the presence of promastigotes antigens on the surface of *in vitro* infected mouse peritoneal macrophages. Due to the presence of the antigen on the uninfected cells also, it was proposed that these antigens could be secreted in the culture medium and then absorbed alone, or as part of immune complexes (Handman *et al.*, 1979). Dwyer (1979) demonstrated the recognition of the surface of *L. donovani* infected bone marrow macrophages, explanted from *in vivo* infected hamsters, by anti-*L. donovani* amastigote antibodies. These amastigote antigens were thought to be incorporated into the parasitophorous vacuole membrane and appeared on the surface following the fusion of PV membrane with the macrophage membrane. The presence of *Leishmania* antigens was also demonstrated on the surface of *in vitro* *L. donovani* and *L. tropica* infected human monocyte derived macrophages (Berman and Dwyer, 1981). It was shown that the infected macrophages express certain amastigote
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antigens on their surface and these antigens might not be present in large quantities in the promastigotes. The latter was supported by the following observations: (i) 3-6 days post-infection, the infected cells were labeled specifically with the antisera generated against amastigotes of homologous Leishmania spp.; (ii) failure of anti-L. donovani promastigotes antiserum to detect macrophages infected with L. donovani promastigotes; and (iii) the failure of any antiserum to recognize the surface of uninfected sera cultured in L. donovani conditioned medium. Handman and Hocking (1985) reported the recognition of infected macrophages with 4 MAbs. Of all the antigens present on the infected macrophage surface the identity of LPG has been established most conclusively. Handman and Goding (1985) demonstrated the binding of hydrophilic extracellular form of L. major LPG, which is generated by the activity of endogenous phospholipase, to the macrophages. The binding of this glycoconjugate and promastigotes to macrophages was inhibited by the Fab fragments of MAb WIC79.3 (which recognizes L. major LPG), thereby suggesting that this is an important parasite ligand for macrophages receptors. The LPG epitopes present on the L. major infected macrophage surface were partially defined using various anti-LPG MAbs (Handman, 1990). It was demonstrated that the expression of LPG was time dependent and the epitopes displayed on LPG were present in the amastigote LPG. It was suggested that the attachment of LPG to the infected cell surface was most likely either through GPI anchor and/or as water-soluble phosphoglycan form bound to macrophage integrins (Handman, 1990). In L. donovani promastigote infected macrophages, appearance of repeating phosphorylated disaccharide epitope of LPG was demonstrated as early as 5-10 min post infection (Tolson et al., 1990). These epitopes were initially localized at the point of attachment/internalization of promastigotes and were later evenly distributed over the entire macrophage surface by 25 min post infection. The phagolysosomal degradation was not involved in the expression of this epitope. However, the there was drastic reduction or complete abolition of LPG expression upon treatment of macrophages with proteinase K. This might be because of the effect of proteinase K on macrophage membrane flow and therefore phagocytosis. It was proposed that these epitopes were either sloughed off from promastigotes and directly transferred onto host cells during internalization. Failure of the antibodies which recognize the core of LPG, to detect the LPG and PG molecules expressed on the macrophage surface led the authors to suggest that the orientation of these molecules on macrophage surface is similar to that found in the promastigotes. With the help of a monoclonal antibody raised against a dominant surface antigen of L. panamensis
promastigotes, William et al. (1986) demonstrated the expression of 15 kDa glycoconjugate on macrophage surface. This antigen appeared after 6 hr post infection and was also detected in the amastigotes but not on the surface of the uninfected cells co-cultured with the infected cells. Recently a 51 kDa antigen has also been demonstrated in the membrane of mouse peritoneal macrophages and human monocyte-derived macrophages infected with *L. donovani* (Basu et al., 1994). This antigen was present in both promastigotes and amastigotes and was also detected with the kala azar patient sera and F(\(ab\))\(_2\) fragment of polyclonal antibodies raised against the soluble antigen of *Leishmania*. The time-kinetics study of the expression of this antigen revealed that this antigen is not expressed until 12 hr post infection, attained maximum level by 24 hr and a steady state level was maintained till 96 hr post-infection. Trypsin leeching of the macrophages confirmed the surface expression of this molecule and degraded it into 26, 11 and 10 kDa fragments all of which were recognized by the polyclonal antiserum. This points towards the possibility of three different epitopes or a shared common antigenic determinant.

In recent study from this lab, different polyclonal and monoclonal antibodies (MAbs) were generated using *L. donovani* promastigotes and homologous infected macrophage membrane (Goel, 1997). These antibodies exhibited differential recognition of *in vitro* cultured promastigotes and infected macrophages, tested at an early post infection time of 4 hr. MAbs 44G3, 31D6, 33E8, 8G2 and 38C10 strongly reacted with promastigotes in ELISA. Among those, MAbs 31D6 and 44G3 recognized 44 kDa and <29 kDa antigens, respectively in immunoblotting. These antibodies also reacted well with the infected macrophages kept for post infection for 4 hr and an antigen of <29 kDa was identified by 44G3 in the crude membrane fraction of infected cells at early post infection time. Studies also indicated the recognition of the amastigote like forms by some MAbs. While all MAbs reacted with the promastigotes of different *L. donovani* strains (Goel, 1997).

2.6 STAGE SPECIFIC ANTIGENS IN *Leishmania*

The life cycle of *Leishmania* involves two morphologically and biochemically distinct stages, the promastigotes and amastigotes, which are adapted to survive in two distinct but equally hostile environments. The presence of both promastigotes and amastigote specific antigens have been reported in *Leishmania*. Various promastigotes-
specific antigens include: 50 kDa antigen of *L. tropica* (Handman *et al.*, 1981); a 68 kDa triplet and a 90 kDa protein of *L. mexicana* (Fong and Chang, 1982); 40 and 92 kDa proteins of *L. amazonensis* (McMahon-Pratt and David, 1982); six proteins of 24-68 kDa of *L. mexicana* (Chang and Fong, 1982); 50,100 and 200 kDa proteins of *L. tropica* (Jaffe and McMahon-Pratt, 1983); and 46 kDa protein of *L. amazonensis* (Kahl and McMahon-Pratt, 1986). The process of metacyclogenesis has been associated with the increased expression of 68 kDa proteins of *L. amazonensis* (Fong and Chang, 1982); 116 kDa protein of *L. major* (Sacks *et al.*, 1985); 65 kDa protein of *L. braziliensis* (Kweider *et al.*, 1987) and certain glycolipids of *L. major* (Sacks and daSelva, 1987). Recently Cuviller *et al.* (2000) described a promastigotes specific protein LdARL-3A, an ADP-ribosylation factor-like protein, which is important for flagellar integrity. The LPG molecule has also been demonstrated to undergo modifications during metacyclogenesis (Sacks *et al.*, 1990; McConville *et al.*, 1992). Flinn *et al.* (1994) demonstrated the expression of a hydrophilic surface protein (gene B product) in the infective metacyclic promastigotes and amastigotes.

Various amastigote specific proteins have also been described. These include cysteine proteases in various *Leishmania* species (Coombs and Mottram, 1997); A-2 proteins of *L. donovani* (Charest and Matlashewski, 1994); cysteine proteinases (Mottram *et al.*, 1998); aPPG (Ilg *et al.*, 1995) and P-2, P-4, P-8 antigens of *L. pifanoi* (Soong *et al.*, 1995). Amastigotes of *L. donovani* have also been demonstrated to express stage-specific adenosine transporter T2 (Ghosh and Mukherjee, 2000) and genes of amastin gene family, which code for a highly hydrophobic surface protein (Wu *et al.*, 2000). Mensa-Wilmot *et al.* (1999), recently identified two amastigote specific free GPIs, AmGPI-Y and AmGPI-Z, which were demonstrated to be important for the viability, replication and virulence of the amastigotes. Additionally, glycosphingolipids (Straus *et al.*, 1993) and some antigenically distinct forms of LPG are expressed only in the amastigotes (Glaser *et al.*, 1991; Turco and Sacks, 1991).

### 2.7 SPECIES-SPECIFIC ANTIGENS IN *Leishmania*

Several reports have demonstrated the occurrence of species-specific antigens of *Leishmania* promastigotes. These include: 70, 100 and 200 kDa proteins of *L. tropica* (Jaffe and McMahon-Pratt, 1983); 11 and 50 kDa proteins of *L. mexicana* (McMahon-Pratt *et al.*, 1985); and several proteins between 12-140 kDa of *L. tropica* (Jaffe and
Safrstein, 1987). LPG of various species displays species-specific variations in the nature of glycans attached to repeat units (McConville et al., 1990a; Sacks, 1992). Here is evidence of occurrence of sub-species specific antigens in the members of L. donovani (Lemerse et al., 1985), L. braziliensis (McMahon-Pratt, et al., 1982) and L. mexicana complexes (McMahon-Pratt et al., 1985). Handman and Hocking (1982) demonstrated the occurrence of strain specific antigens in L. tropica promastigotes. Using L. donovani strains RMRI-68, AG83 and DD8, it was recently demonstrated from the lab that unlike anti-promastigote antisera, homologous anti-infected macrophage membrane antisera exhibited strain specific recognition of promastigotes. This indicated the existence of strain specific antigens also in L. donovani promastigotes (Goel et al., 1998).

2.8 VACCINATION AGAINST LEISHMANIASIS

To date there is no vaccine against Leishmania in routine use anywhere in the world. Several vaccine preparations are in more or less advanced stages of testing.

Extensive vaccinations with a cocktail of five killed Leishmania stocks or a single strain of L. amazonensis have been demonstrated to induce significant protection against natural infection in the trials in Brazil and Ecuador. A combination of killed L. mexicana or L. braziliensis and M. bovis BCG, when given therapeutically, has been demonstrated to have a high cure rate in severe cases. In both the cases the protection or cure was associated with the development of Th1 response in the recipients. In Iran, a mixed BCG-L. major vaccine has undergone clinical trials (reviewed in Handman, 2001a).

The relative merits of live-attenuated vaccines versus killed vaccines have long been debated with concerns towards the immunogenicity, efficacy, safety, distribution and storage. In recent years, using homologous recombination, stable deletion mutants of several genes have been developed. These live attenuated mutants could cause abortive infection when in man while generating protective responses. Knock-outs of cysteine proteinases and dihydrofolate reductase/thymidylate synthase were observed to render resistance in mice (Souza et al., 1994; Veras et al., 1999). The use of attenuated organisms is very attractive because they are the closest mimic to the natural infection.

Recombinant antigens can be delivered as purified proteins, as the naked DNA coding them or as bacteria or viruses manufacturing them. All these strategies have been
In the earlier days the most abundant glycoconjugate of *Leishmania* surface, LPG was viewed as an important virulence factor and vaccine candidate. LPG is highly immunogenic and immunization of susceptible mice with purified LPG of *L. major* (Handman and Mitchell, 1985) or resistant and susceptible mice with *L. mexicana* LPG (Russel and Alexander, 1988) resulted in complete or partial protection. It was argued that LPG itself does not contain any T cell epitopes but these are expressed on a tightly associated protein component (Mitchell and Handman, 1986; Jardim *et al.*, 1991; Russo *et al.*, 1992). But recently, many novel and interesting microbial antigens including mycobacterial glycolipids have been demonstrated to be recognized by T cells. These antigens are presented to T cells by a special subset of MHC I proteins known as CD1.

Thus, for designing an efficient vaccine against leishmaniasis, it is important to search for *Leishmania* antigens which are presented on the surface of infected macrophages, at various stages harboring either live or growing parasites. These macrophage-surface associated antigens can be either promastigotes left-overs during invasion, or expressed on amastigotes plasma membrane, or secreted by amastigotes in the parasitophorous vacuoles and subsequently displayed on macrophage surface. Besides, such *Leishmania* antigens might serve as an aid in understanding the basic mechanism involved during the pathogenesis of disease.