CHAPTER 1

Introduction and Review of Literature
1.0 Overview

Leishmaniasis is caused by a protozoan parasite with a predilection for a specific host cell, the macrophage. Among the parasitic infections, this disease is responsible for the highest number of DALYs (Disability Adjusted Life Years, a measure of health burden) after malaria. The flagellated form of the parasite, the promastigote is introduced into the human host by the blood sucking sandfly, Phlebotomus. The promastigotes attach to mononuclear phagocytes via a receptor-mediated mechanism, are taken up by phagocytosis into a phagosome, which fuses with lysosomes to form the phagolysosome. Once inside the macrophage, the promastigotes undergo significant biochemical and metabolic changes, which results in the obligatory intracellular form of the parasite, the amastigote (Chang and Bray, 1985). In addition to macrophages, dendritic cells and fibroblasts may also harbor the organism. However, Leishmania have evolved mechanisms to subvert these host “seek and destroy” cells for their survival.

Leishmaniasis is a spectrum of diseases of different clinical manifestations depending on a combination of host and parasite genetic factors. Thus, Leishmania donovani tends to home to the liver and spleen causing (usually fatal) visceral leishmaniasis, Leishmania braziliensis homes to the lining of the nose and throat causing the mutilating mucocutaneous disease, and Leishmania major homes to the skin causing the self limiting skin ulcers, called cutaneous leishmaniasis.

Leishmaniasis affects at least 12 million individuals each year, with about 300 million people at risk, both in the developed and developing world. In the last decade, visceral leishmaniasis has surged in epidemic proportions in new areas in the Sudan, Pakistan and China. It has also become a major problem in AIDS patients in Europe and South America.
To date, there is no vaccine against leishmaniasis, and the drugs available are toxic, expensive, and difficult to administer. Moreover, there is evidence of emerging resistance of the parasites to the commonly used antimony drugs (Berman, 1988).

The major aim of my present work is to use state of the art of biochemical and molecular tools in order to understand the biology of the parasite and its interaction with the host. This was done by investigating the molecular mechanism involved in the signal transduction in macrophages by *Leishmania* lipophosphoglycan (LPG), a surface glycoconjugate of *Leishmania* involved in the internalization of the parasite in the macrophages as well as in the protection against microbicidal activities displayed by macrophages. The physiological relevance of the kinases (Protein kinase C and MAP kinase) and transcription factors, nuclear transcription factor-κB (NF-κB), interferon regulatory Factor-1 (IRF-1) and activating protein-1 (AP-1) in the regulation of macrophage effector functions has been worked out. A signal transduction pathway represents an attractive target for an intracellular pathogen, as this would enable the parasite to alter the gene expression and therefore alter the normal cellular processes without even entering the nucleus. Thus selective impairment of signal transduction may therefore represent a new strategy for identifying parasite targets for design of vaccines and new specific drugs for treating *Leishmania* infection.

2.0 Epidemiology

Leishmaniasis has a long history. Design on pre-Columbian pottery and the existence of thousand year old skulls with evidence of leishmaniasis prove that the disease has existed in America for a long time. It has also been present in Africa and India since the mid eighteenth century, atleast. It was Ronald Ross who named the new protozoa the *Leishman Dovovan* body (Cahill et al., 1989). In honor of Major William Leishman (1900)
and Caption James Donovan (1903), who separately described the protozoa now called *Leishmania donovani* found in splenic tissue from patients in India with the life-threatening disease now called visceral leishmaniasis.

Leishmaniasis is a worldwide disease, affecting 88 countries: 72 are developing countries and 13 are among the least developed. Ninety percent of visceral leishmaniasis (VL) cases occur in 5 countries: Bangladesh, India, Nepal, Sudan and Brazil; and 90% of cutaneous leishmaniasis (CL) cases occur in 7 countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. Annual incidence is estimated at 1-1.5 million cases of CL, 500,000 cases of VL. Overall prevalence is 12 million people and the population at risk is 350 million (Fig. 1). The DALY (Disability adjusted life years) burden is 860,000 for men and 1.2 million for women.

In several areas of the world, there is a clear and disturbing increase in the number of cases, e.g. CL in Brazil (1998: 21,800 cases; 1999: 30,550 cases; 2000: 35,000 cases), CL in Kabul, Afghanistan (1994: 14,200 cases, 1999:200,000 cases), and CL in Aleppo, Syria (1998: 3900 cases; 1999: 4700 cases; 2000: 5900 cases).

The geographical distribution of leishmaniasis is limited by the distribution of the sandfly, its susceptibility to cold climates, its tendency to take blood meal from humans or animals and its capacity to support the internal development of specific species of *Leishmania*.

More recently, the overlapping of VL and AIDS has led to an emerging new entity: *Leishmania / HIV* co-infections. Coexistence of leishmaniasis with HIV adds a serious dimension to the problem. Leishmaniasis is spreading in several areas of the world as a result of the rapidly spreading epidemic of AIDS. The immune deficiency has led to increased susceptibility to infections, including leishmaniasis. So far, co-infections have
been reported in 33 countries worldwide (Fig. 2). Co-infection with HIV has led to the spread of leishmaniasis, typically a rural disease, into urban areas. In patients infected with HIV, leishmaniasis accelerates the onset of AIDS by cumulative immunosuppression and by stimulating the replication of the virus. It also may change asymptomatic *Leishmania* infections into symptomatic ones. Sharing of needles by intravenous drug users can spread not only HIV but also leishmaniasis.

3.0 **Leishmaniasis: The disease**

Leishmaniasis is a protozoan parasitic disease. There are three main forms of the disease: Cutaneous, Mucocutaneous and Visceral (Fig. 3). The mucocutaneous form is found mainly in the "New World" (i.e. America) where as the other two forms of the disease are found primarily in the "Old World" (i.e. Europe and Africa). Each of the three forms of the disease is caused by a different strain of *Leishmania*. Mainly *L. tropica* and *L. mexicana* cause the cutaneous form. *L. braziliensis* is responsible for the mucocutaneous form and *L. donovani* causes the visceral disease. *L. tropica* and *L. donovani* are mainly found in Africa, Europe and Asia.

3.1 **Cutaneous Leishmaniasis**

Cutaneous leishmaniasis (Aleppo boil, Aleppo button, Baghdad boil, Baure ulcer, Delhi boil, Oriental sore, Tropical sore) is found in central Asia, India, the Mediterranean coastal plain and West Africa. The single or multiple lesions commonly occur on arms and legs. The lesions appear 2 to 8 weeks after the sandfly bites and begin as small areas of skin elevation and inflammation. The elevated skin areas enlarge slowly, and within a few months, can develop into ulcers that remain for up to 2 years, followed by slow healing (Fig. 3a).
3.2 **Mucocutaneous Leishmaniasis**

Mucocutaneous leishmaniasis (American leishmaniasis, Chiclero ulcer, Espundia, Forest yaws, Uta) is found in Central and South America. The ulcers may spread to the mucous membranes of the nose and mouth and invade the nasal septum resulting in severe facial lesions with extensive deformity (Fig. 3b).

3.3 **Visceral Leishmaniasis**

Visceral leishmaniasis (Dumdum fever, Kala azar) is found in parts of China, the Indian subcontinent, the Mediterranean coastal plain, East Africa and South America. Visceral leishmaniasis (Fig. 3c) is primarily transmitted through the bite of sandflies. The causative parasite invades the spleen, liver, bone marrow, lymph nodes and skin. After an incubation period of 2-6 months, symptoms appear with fever, dizziness, weakness and weight loss. If left untreated, this infection can become chronic and result in complications and infections such as pneumonia and can lead to death. In 5-10% of treated cases in some areas, a disfiguring skin condition called Post-Kala-azar Dermal Leishmaniasis (PKDL) has been reported.

Visceral leishmaniasis (VL) accelerates the onset of AIDS and shortens the life expectancy of HIV-infected people. In addition, HIV infection encourages the spread of VL, increasing VL risk by 100 to 1000 times in endemic areas. Because of HIV infection, however cases of VL are no longer restricted to endemic areas and VL is currently being diagnosed in many Western-European and African countries where it had previously been eradicated.

4.0 **Life cycle and biology of the parasite**

*Leishmania* are obligatory intracellular parasites, transmitted by the bite of an infected female *Phlebotomine* sandfly. The leishmaniasis is a globally widespread disease.
Sandflies are primarily infected by animal reservoir hosts, but humans are also a reservoir for some forms. The parasite exists in two forms: Promastigotes (flagellar form) in the midgut of the vector insect and obligatory intracellular amastigotes that live and multiply inside the cells of the mononuclear phagocytic system, the macrophage cells. A third metacyclic form of the parasite has also been recently observed.

4.1 Promastigote form

The promastigotes are mostly free-swimming forms (14-20 μm) each with a long anterior flagellum. The flagellum is often as long as or longer than the cell body. The surface membrane has binding site molecules like glycoproteins and mannose receptors. These are important in the uptake of the promastigotes by the macrophages. Antibodies in the host serum bind to the promastigotes and facilitate uptake and entry into the macrophage.

No morphological differences can be seen between species. A sexual phase of Leishmania has not been found. They divide by mitosis in a way similar to binary fission of bacteria.

4.2 Amastigote form

Amastigotes are spherical in shape, only about 2.5 to 5 μm in diameter, and are contained within a parasitophorous vacuole within a macrophage. There is a prominent nucleus and kinetoplast, and the cytoplasm is vacuolated and contains lysosomes. The outer membrane has a polysaccharide component but there is no surface coat.

4.3 Metacyclic form

This form of the parasite occurs in the sandfly vector shortly before it is transmitted to the host. Morphologically the parasite is similar to the promastigote form; only it is
shorter and more motile than the latter. The abundant form of lipophosphoglycan (LPG) present at this stage suggests that this is the most virulent form of the parasite.

At the ultrastructural level, most of the organelles present are similar to those present in other *Trypanosomatids*. Three unique features that are present in *Leishmania* are the plasma membrane and the flagellar reservoir, the microtubular systems and the mitochondrion-kinetoplast complex (Chang, Fong and Bray, 1985).

The plasma membrane of the *Leishmania* can be separated into three sections. At the anterior end of the parasite, it is invaginated to form a reservoir, in which the flagellum emerges from the reservoir lining membrane above the basal body. The flagellum extends up to and beyond the opening of the flagellar reservoir for amastigotes and promastigotes respectively. Desmosomes are present at the membrane junction between the flagellum and the opening of the flagellar reservoir. Secretory and endocytic activities are limited to the reservoir lining membrane, which apparently gives rise to or provides the continuity between the plasma membrane of the flagellum and of the cell body.

There are four functional types of microtubules in *Leishmania*. These are subpellicular, flagellar, mitotic and reservoir-associated or cytopharyngeal microtubules. The subpellicular microtubules lie under the cell body section of the plasma membrane and are responsible for providing rigidity and shape to the cytoskeleton of *Leishmania* and other *Trypanosomatids*. The flagellar microtubules are confined to the flagellar region of the parasite and their structure is more or less identical to that in other organisms with flagella as the organelle of cell motility. In line with the observations of other *Trypanosomatids*, mitotic and reservoir-associated microtubules of *Leishmania* probably function in the control of their nuclear division and endocytosis respectively.
The *Leishmaniae* have a mitochondrial-kinetoplast complex. The single mitochondrion in all *Trypanosomatids* branches into many sections, forming a reticulum that often lies below the plasma membrane-subpellicular microtubular system. The ubiquitous kinetoplast is a section of the mitochondrion, located just below the basal body of the flagellum. The mitochondrial DNA in the form of maxicircles and minicircles is condensed into regularly arranged arrays of fine fibrils in the kinetoplast. Amastigotes resemble promastigotes ultrastructurally, except for having less number of certain cell organelles e.g., subpellicular microtubules, shorter flagella (concealed within the flagellar reservoir) and no flagellar paraxial rod. Promastigotes of some *Leishmania* species may also have no paraxial rods.

The life cycle of the parasite (Fig. 4) begins when the *Phlebotomine* sandfly bites an infected host. Some amastigotes get ingested along with the blood meal of the fly. These amastigotes migrate to the midgut of the sandfly and get transformed into promastigotes; which subsequently divide by binary fission and get anchored to the surface of the gut epithelial cells. At this stage, the promastigotes are avirulent. These promastigotes subsequently stop dividing, get detached from the epithelial cells, migrate upwards towards the pharynx of the fly and become virulent. This process is called metacyclogenesis (Sacks, 1989). Thus, when the sandfly bites a new host and regurgitates into the wound, the parasite finds its way into the blood stream of the new host. The promastigotes are taken up by the macrophages of the vertebrate host where they bypass the defense mechanism of the host. They get transformed into amastigotes and survive within the intralysosomal compartment of the macrophages. The parasites multiply by binary fission until the macrophages become full and bursts, releasing these amastigotes into the bloodstream.
These amastigotes further infect more number of macrophages and the infection spreads further to encompass the entire reticuloendothelial system of the host.

5.0 Chemotherapy of Leishmaniasis

5.1 Pentavalent Antimony Compounds:

Pentavalent antimony compounds, derived from the heavy metal antimony (Sb), are the drugs of choice for treating cutaneous and visceral leishmaniasis. Sodium stibogluconate and meglumine antimoniate drugs are administrated parenterally at a dosage of 10-28 mg/Kg body weight per day for 10 to 30 days. Failures and relapses occur in all forms of leishmaniasis and constitute approximately 10-25% of cases. Further complicating the issue is that parasite resistivity seems to be increasing and relapse rates in some areas have been reported as high as 50-70% with mucocutaneous leishmaniasis and 10% with visceral leishmaniasis.

Side effects include: Cardiotoxicity, reversible renal insufficiency, pancreatitis, anemia, leukopenia, rash, headache, abdominal pain, nausea, vomiting, arthralgia, myalgia, thrombocytopenia.

5.2 Amphotericin B:

Amphotericin B has been shown to be more effective than pentavalent antimony in vitro, but has not been used extensively in the past due to extreme toxicity. However, more recent developments have made Amphotericin B less toxic and more useful. It has also become useful in treating strains of visceral leishmaniasis causing organisms that are resistant to pentavalent antimony. Amphotericin B cannot be used in the treatment of mucocutaneous leishmaniasis because very large doses are required.

Side effects include: Fever, nausea, vomiting, malaise, anemia, phlebitis, hypokalemia, hypomagnesemia and nephrotoxicity.
5.3 Pentamidine Isoethionate:

Pentamidine isoethionate is a general anti-parasitic drug that has demonstrated some success with the treatment of visceral leishmaniasis. Unfortunately the cure rate has declined from the early 1980s to the 1990s due to resistance of *Leishmania* to pentamidine. Nonetheless, it can be used as a second-line agent in the event that the pentavalent antimony compounds fail.

**Side effects include:** Nausea, vomiting, abdominal pain and headache.

5.4 Allopurinol, a drug in use for the treatment of gout. It is supposed to function as an alternative substrate for the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRTase), so allowing the incorporation of allopurinol riboside into RNA, which leads to the inhibition of protein synthesis in the parasite. It is also on trial for the treatment of Chagas’ disease and the drug is now widely used for the treatment of leishmaniasis in dogs.

5.5 Ambisome, a formulation of Amphotericin B in liposomes. Owing to the high capacity of macrophages, the host cell of the *Leishmania* parasite, for phagocytosis the drug is specifically targeted and taken up by these cells. This increases the efficacy and reduces toxicity of the drug.

5.6 Aminosidine

Aminosidine or paromomycin is an aminoglycoside. It is used either alone or in combination with antimonials. Pilot studies with the drug have shown that it has no serious side effects, though nephrotoxicity is common (Sundar, 1996). However, one advantage of this drug is that it also has antibacterial and anti amoebic properties.

5.7 Ketaconazole (Janssen Pharmaceutica), an inhibitor of cytochrome P450 and the synthesis of the membrane sterol ergosterol and in use for the tropical and systemic treatment of candidosis and other fungal infections, also inhibits sterol synthesis in
Leishmania and interferes with its growth and division of intracellular amastigotes. It is on trial for the use in dogs and in humans in certain Latin American countries.

5.8 WR 6026

This compound is a primaquine analogue that has been found to be effective against Kala-azar in some animal models. However, clinical trials in Kenya and Brazil have shown that it is not very effective in vivo in humans (Sundar, 1996).

5.9 Fluconazole and other antifungal agents

Fluconazole is an antifungal agent that acts by increasing methylated sterols and decreasing demethylated sterols in leishmanial promastigotes and amastigotes (Berman, Holz and Beach, 1984). Clinical trials with this drug have shown that though it is successful initially in curing visceral leishmaniasis but there is relapse of the disease in all cases, indicating that trials need to be done with either higher doses or to give this drug in combination with other antileishmanial agents in order to achieve a proper chemotherapeutic response (Sundar, 1996). Other antifungal imidazoles that have been tried against the Leishmania are clotrimazole, micorazole, ketoconazole etc. These have also been found to have antileishmanial properties (Berman, 1981; Uraiyo and Zaias, 1982).

Despite the fact that such a large number of antileishmanial agents have been synthesized and used in vitro and in vivo, a need can still be felt for a viable alternative to the currently used antimonial compounds pentostam and glucantime. Thus, efforts are on to develop a rational approach towards leishmanial chemotherapy.

5.10 Other Agents:

The following drugs and therapeutic approaches seem promising and are currently being studied, but have not been approved for general use.
5.10.1 Cytokine therapy:

Cytokines such as interferon gamma and granulocyte macrophage colony-stimulating factors are being used in combination with pentavalent antimony in attempts to enhance response.

5.10.2 Miltefosine:

An oral drug for visceral leishmaniasis is showing progress in studies. Apparently it is less toxic and exhibits higher cure rates than traditional drugs. Furthermore, a cheap, orally administered treatment would be extremely beneficial for the people most affected by leishmaniasis (WHO, 1999).

6.0 Host-parasite interaction: The leishmanial surface molecules that mediate promastigote complement resistance, attachment and uptake by macrophages

6.1 Microbial Glycoconjugates

The surface of all microbes is coated with complex carbohydrates molecules such as lipopolysaccharide (LPS) in gram-negative bacteria, capsular polysaccharides in bacteria, lipoarabinomannas in mycobacteria, lipophosphoglycan (LPG) and proteophosphoglycan (PPG) in Leishmania. The basic structures of these glycoconjugates are known and, in case of pathogens, they can function as virulence determinants (Mengeling and Turco, 1998). The determination of the structure of some of these, especially those that function as virulence determinants, has set the stage for understanding mechanisms related to structure-function. In recent years a lot of emphasis has been made to study the role of LPG and PPG in pathogenicity of Leishmaniasis (Ferguson et al., 1994).
6.2 Phosphoglycans of Leishmania

At all stages, Leishmania has to survive under hostile environment e.g. in digestive system of the insect gut, in the blood stream of the host and inside macrophage, counteracting hydrolytic enzymes and microbicidal oxidative burst. It is believed that the key molecules involved in these activities are the glycoconjugates PPG and LPG (McConville and Ferguson, 1993; Descoteaux and Turco, 1999; Ilg, 2000). Free GPI containing molecules glycoinositolphospholipids (GIPLs) play an important role in pathogenicity (McConville and Ferguson, 1993). All Leishmania species studied express at least two of the three known phosphoglycans. Avirulent strain doesn't express any of these molecules or make truncated molecules.

6.3 Lipophosphoglycan (LPG) of Leishmania

Leishmania is a protozoan parasite and is the etiologic agent of leishmaniasis. Clinical leishmaniasis shows multiple forms namely: cutaneous, mucocutaneous and visceral (or kala-azar). Life cycle is digenetic; the parasite exists as two forms: a) Promastigote: Extracellular in human, flagellated inside the gut of sandfly vector, where it multiplies and undergoes metacyclogenesis to virulent form (in salivary gland) from avirulent form (in gut). b) Amastigote: It is nonflagellated and exists inside the phagolysosome of the macrophage.

At all stages the parasite has to survive under hostile environment e.g. in the digestive system of the insect gut, in the blood stream of the host and inside macrophage, counteracting hydrolytic enzymes and microbicidal oxidative burst. Cell-surface glycoconjugates such as LPG, a heterogeneous lipid containing polysaccharide, play a key role in the survival of Leishmania throughout its existence. LPG was first identified and characterized by Turco et al., in 1984. Subsequently, the complete structure of this
molecule has been determined from a number of different species of *Leishmania*. It is the major glycoconjugate of the promastigote and is localized over its entire surface including flagellum (King et al., 1987). Each parasite contains several million molecules of LPG (McConville and Blackwell, 1991). A large number of observations also suggest a number of different functions of LPG. Current knowledge regarding structure-function relationship of LPG is summarized below.

6.4 Functions of LPG

6.4.1 Inside the Sandfly vector

Soon after the ingestion of amastigotes during a blood meal of an infected host, differentiation and multiplication of promastigotes takes place inside the vector gut. Metacyclogenesis, or development of dividing non-infective forms into resting infective forms, take place as parasite moves from gut to the salivary gland. This process involves major structural modifications of LPG (Sacks et al., 1985; Sacks et al., 1990). These development-regulated structural changes may control attachment and detachment of maturing promastigotes from midgut epithelial cells and their migration towards the mouthparts.

6.4.2 In the blood stream of host

It performs the important function of resisting attack by complement and as ligand for recognition by macrophages.

(a) Complement activation and resistance to complement-mediated lysis

Metacyclic promastigotes show increased resistance to lysis as compared to non-infective promastigotes (from log phase culture) (Franke et al., 1985). The activation of complement is both via classical pathway (in case of metacyclic promastigote) and by alternate pathway (for log phase promastigotes). LPG is thought to be the acceptor of C3 in
all cases (Puentes et al., 1988). Since LPG is a large molecule and covers the whole surface of the parasite, it provides a barrier against anti-leishmanial antibodies. The masking of surface antigens by LPG could be one of the important mechanism by which leishmanial parasite evades humoral immune response. Using LPG-deficient mutants, human immune serum was shown to have strong seropositivity whereas wild type parasite failed to recognize the same sera (Karp et al., 1991).

(b) **Attachment to target macrophages**

A recent study clearly show that LPG acts as a ligand for recognition of leishmanial parasites by macrophages. The receptor for LPG on macrophage has been identified as “CR1” which is also a receptor for the C3bi, a cleavage product of C3 generated during activation of complement. But the binding site of LPG on CR1 is different from that of C3bi (Kelleher et al., 1992). An important fact is that CR1 mediated internalization in macrophage promotes phagocytosis without triggering oxidative burst (Wright and Silverstein, 1983).

6.5 **Intracellular Functions**

*Leishmania* parasite manages to survive inside the phagolysosomes of macrophages where it encounters degradative enzymes and toxic oxygen products. This is thought to be due to LPG, since phagocytosed a virulent LPG-deficient clone of *L. major* was killed within 18 h inside the macrophages (Handman et al., 1986). The effect of LPG on macrophages may be due to its effect on a number of biochemical pathways such as:

(a) Inhibition of macrophage Protein Kinase C and consequently inhibiting generation of oxidative burst (Buchmuller-Rouiller and Mauel, 1987; Pearson et al., 1982; McNeely and Turco, 1990) and chemotaxis (Frankenburg et al., 1990; Frankenbrug et al., 1992).
(b) Chelation of calcium thereby affecting a host of Ca$^{2+}$-dependent processes (Eilman et al., 1985).

(c) Inhibitor of hydrolytic enzymes and thereby increasing its own survival. *In vitro* studies show that partially purified excreted factor (LPG) strongly inhibited β-galactosidase activity of macrophage after 3 h of incubation. But activity of three other enzymes (acid phosphatase, B-glucoronidase and N-acetyl-B-glycosaminidase) was not affected (El-On et al., 1989). In another experiment the rate of cytolysis of erythrocytes coated with LPG was much less than the uncoated erythrocytes (Pearson, et al., 1983).

(d) Inhibition of c-fos gene expression, which in turn affects cytokine gene expression (Descoteaux and Matlashewski, 1989).

(e) Removal of toxic O$_2$ metabolites, which would increase survival of the parasite (Chan et al., 1989).

(f) Inhibition of IL-1 production and modulation of TNF production leading to parasite survival (Reiner, 1987; Green et al., 1990).

### 6.6 Structure of the Leishmanial LPG

Complete structures of LPG molecules, derived from both promastigote (McConville et al., 1990; Ilg et al., 1992; Thomas et al., 1992) and amastigotes (Moody et al., 1993) of different species of *Leishmania* have been determined using a combination of chemical and biochemical techniques.

All LPG molecules show mainly four domains namely, (a) a phosphatidyl inositol lipid anchor (GPI), (b) a phosphosaccharide core, (c) a repeating phosphorylated saccharide region and, (d) small oligosaccharide caps. Structural analysis of LPG from different species of *Leishmania* shows complete conservation of lipid anchor and extensive
conservation of phosphosaccharide core. Majority of the polymorphism observed resides in the repeating phosphorylated saccharide and polysaccharide units. Detailed structure of the LPG of *L. donovani* is depicted in Fig. 5.

6.6.1 The Lipid anchor of LPG

The polysaccharide portion of LPG is anchored to the membrane through an unusual phospholipid derivative, 1-alkyl-2-lyso-phosphatidyl (myo) inositol. The aliphatic chains consist of either a C24 or C26 saturated, unbranched hydrocarbon in LPGs from different species such as *L. donovani*, *L. major* and *L. mexicana* (Turco and Descoteaux, 1992). GPI-anchored lipid tail can be removed from LPG by hydrolysis with bacterial phosphatidylinositol specific phospholipase C (PI-PLC) similar to the GPI anchored membrane protein (Orlandi and Turco, 1987).

6.6.2 The Phosphosaccharide Core

The phosphosaccharide core region is attached to the lipid anchor via a non-acetylated glucosamine attached to the inositol head of the lipid anchor. In *L. donovani*, *L. major* and *L. mexicana*, the glycan core consists of an unacetylated glycosamine, two mannoses, a galactose-6-phosphate, a galactopyranose and a galactofuranose. The presence of a galactofuranose is extremely unusual in eukaryotic glycoconjugates. As with all other GPI-anchored proteins reported so far (Reiner, 1987), LPG displays Man (α1,4) GlcN (α1,6) myo-inositol-1-PO₄ motif. The polysaccharide core of *L. donovani* and *L. mexicana* has glycosyl-α-1P substituted through phosphodiester linkage to the C6 hydroxyl of the proximal mannose residue. Substitution by glucosyl-1-in the core region has also been observed in LPG derived from *L. major*. Interestingly, Gal (α1,3) Gal- unit in the saccharide core region was shown to be an epitope for circulating antibodies in *Leishmania* patients (Towbin et al., 1987).
6.6.3 The Phosphorylated saccharides repeat Unit

The oligosaccharides repeat units constitute the major portion of the LPG molecule, and differ from species to species and among different stages within a species. All LPG molecules reported so far contain multiple units of backbone structure [-PO₄ 6 Gal (β1,4) Man (α1)]ₙ. The LPG derived from *L. donovani* contains no other substitution, whereas in *L. mexicana* approximately 25% of galactose residues are substituted at C3 hydroxyl with β Glc residues. The repeat units of *L. major* are most complex as 87% of galactose residues are further substituted with small saccharide side chains containing one to four residues of galactose, glucose or arabinose. The detailed structure of LPG of different species is given in Fig. 6. The number of repeat units per LPG molecule directly depends on the growth stage of the promastigote.

6.6.4 Capping Oligosaccharides

LPG is terminated at the non-reducing end with one of several small neutral oligosaccharides containing galactose or mannose. *L. major* has Man (α1,2) Man (α1) disaccharide as the capping unit. *L. mexicana*, on the other hand, has a bigger cap structure—a branched trisaccharide i.e, Gal (β1,4) Man (α1,2) Man (α1).

6.7 The tertiary structure of LPG

The immunochemical structure and surface arrangement of *L. donovani* LPG was determined by Turco et al., (1987) and Tolson et al., (1989) respectively, using monoclonal antibodies. Three-dimensional conformation of mainly repeating units was deduced using physico-chemical techniques. The orientation of LPG on the cell surface and the accessibility of repeating phosphorylated disaccharide and phosphosaccharide core epitopes. The main structural difference between PI-anchored proteins and LPG is the conventional diacylated PI for proteins and a lysoalkyl-PI for LPG.
6.8 Inter-and intra-species polymorphism of LPG

It has been known for sometime that LPGs are highly polymorphic molecules as shown by their reactivity to species- and strain-specific antibodies (IIg et al., 1992). This polymorphism is largely restricted to differences in the branching saccharide moieties of the phosphorylated disaccharide repeat units. Some differences in the range of mannose-containing cap structures are also evident. Intrastrain polymorphism has also been seen on the basis of side chain substitutions in the repeat unit. Leishmanial LPG has been classified into three different classes (McConville et al., 1995). Whereas the type 1 LPGs contain no side chains, type 2 LPGs contain a variety of saccharide side chains all of which are linked to C-3 position of the Gal residue in the disaccharide repeat unit. On the other hand, type 3 LPGs has a side chain on both C-2 position of mannose residue and C-3 position of galactose residue in the disaccharide repeat unit.

6.9 Biosynthesis of LPG

Since LPG is essential for survival of the parasite, detailed knowledge of LPG biosynthesis can provide specific targets in the search for efficacious drugs. The PI anchor region of LPG, which possesses a lyso-1-O-alkyl PI anchor, is synthesized probably via the formation of a 1-O-alkyl-2-alkyl PI precursor called glycoinositol phospholipid (GIPL) (Proudfoot et al., 1990). The suggested pathway of LPG biosynthesis in Leishmania is shown in Fig. 7. It is believed that galactose and mannose residues of LPG repeating units of L. donovani are added by their respective nucleotide sugar donors sequentially and directly to LPG and these are assembled in the golgi (Bates et al., 1990). An analysis of the capping oligosaccharide structures of various leishmanial LPG reveals that all but one contains a man (α1,2) man (α1) at the reducing end. It is therefore, speculated that there exists an enzymes like man (α1,2) mannosyl transferase. The activity of this putative
enzyme would result in the signal for cessation of LPG elongation with the formation of a chain terminating man (α1,2) man-containing cap oligosaccharide (Turco and Descoteaux, 1992).

6.10 Genes directing the biosynthesis of LPG

Nothing much is known about the genes responsible for biosynthesis of LPG except for two genes identified recently. The product of LPG1 has been identified and found to be a glycosyl transferase, using genetic complementation in LPG deficient R2D2 mutant of \textit{Leishmania}. LPG, thus, represents a class of genes (class 1) encoding the biosynthetic enzymes (Ryan et al., 1993). Recently, a second class of LPG mutants C3PO that affect compartmentalization and LPG assembly, have been reported. This C3PO mutant synthesizes a truncated LPG containing only the glycan core and the lipid anchor, which is not seen when LPG expression is restored by transfection with LPG2 (Descoteaux et al., 1995). The LPG2 gene codes for a 341 amino acid putative protein that shares homology with yeast Vrg4/van 2, which are essential for correct protein N-glycosylation and maintenance of golgi structure and function. The LPG2 locus was seen to be deleted in the C3PO mutant.

6.11 GIPLS of \textit{Leishmania}

GIPLs, a class of GPI-anchored glycolipids found in \textit{Leishmania} (McConville and Bacic, 1989), show similarity to PI-containing glycolipid membrane anchors of several eukaryotic proteins and to the lipid moiety of LPG. The overall structure of GIPLs from \textit{Leishmania}. Glycan units of \textit{L. major} GIPLs are composed of 10 to 14-saccharide residue attached to the lipid tail by non-N acetylated glucosamine linked to the 1 myo-inositol group. Heterogeneity in lipid moieties is attributed to the presence of either 1-alkyl-2-acylglycerol composed of saturated unbranched alkyl chains with carbon chain lengths of
18-26 and acyl chains of myristate, palmitate and stearate (in GIPs 1-4), or lyso-alkyl glycerol composed of C24:0 and C26:0 alkyl chains in GIPs 5 and 6 (McConville et al., 1990b). Detailed structure of these GIPs from *L. major* shows that the glycan unit of the GIP-3 has high resemblance to the LPG core, indicating a role of GIPs as precursor in the biosynthesis of LPG (McConville, et al., 1993b).

The GIPs of *L. mexicana*, *L. braziliensis*, *T. cruzi* and *T. rangeli* were identified and characterized. The glycan core can be used to divide them into different classes and human antibody immune responses to these different classes have also been shown (Avila et al., 1991).

There are three different classes of GPI anchors-two of them represent the two types of GIPs- (a) hybrid type containing alkyl acyl-PI linked core with C16:0 and C18:0 as fatty acids and C18:0 alkyl chain, and (b) Type-2 GIPs (precursor of LPG) with alkyl acyl PI or lyso-alkyl PI as lipid moiety. The third class is that of GPI anchors of glycoproteins with alkyl-acyl PI moiety which contains C24:0 or C26:0 as fatty acids. These three types of GPI anchors have been suggested to arise form three distinct biosynthetic pathways (McConville et al., 1993a).

The level of expression of LPG is much less in amastigote as compared to promastigote. However, GIPs are expressed at near constant levels in both developmental stages (Schneider et al., 1993).

6.12 Lipophosphoglycan in other organisms

GPI-anchored glycoconjugates have been isolated and characterized in developing neurons (Nedelec et al., 1992). These consist of two types of LPG-like molecules of average molecular mass 28-33 kDa and 50-70 kDa. These also show striking similarity to leishmanial LPG in terms of binding to DEAE cellulose, sensitivity to endoglycosidases,
liability to mild acid hydrolysis, sensitivity to PI-PLC and presence of multiple phosphate
groups.

LPG like glycoconjugate bearing a striking similarity to leishmanial LPG has been reported in two Trichomonad species- T. vaginalis and T. foetus (Singh, 1993a), migrating as a single polydisperse in both the species. LPG like glycoconjugates has also been reported in Trypanosoma cruzi epimastigote (Singh et al., 1993b). Though complete structure has not been determined but studies show structural features similar to that of leishmanial LPG. It migrates as a homogenous broad band (42 kDa) on SDS-PAGE. Mass spectrometry indicated that actually there are two major components whose molecular masses are about 18.4 kDa and 22.5 kDa. Treatment with PI-PLC released an alkylglycerol lipid moiety with a C16:00 at both alkyl and acyl positions (Singh et al., 1993a). On the other hand, the lipid moiety of T. vaginalis and T. foetus LPG revealed that these are anchored to the membrane via an inositol-phosphate-ceramide. Presence of ceramide as the lipid moiety in LPG molecule has been reported for the first time (Singh et al., 1994).

LPG-like glycoconjugate was reported in E. histolytica by (Bhattacharya et al., 1992b and Stanley et al., 1992). It was shown to possess gross structural similarities to leishmanial LPG like presence of sugar unit attached to a lipid tail by a GPI anchor, which is sensitive to PI-PLC and nitrous acid treatment. Just like Leishmanial LPG, amoebic LPG also has sugar phosphate repeating groups. In the strain HM-1:IMSS one of the repeating groups in Gal β-1-4 Man is similar to that present in L. donovani. On SDS-PAGE E. histolytica LPG migrates as two poly-disperse bands of relative molecular mass in the range of 110 kDa and 45 kDa.
7.0 GP63

In the promastigote stage, the surface expression of an abundant glycosylphosphatidyl inositol (GPI)-anchored metalloproteinase (Bouvier et al., 1985; Bordier et al., 1986; Schneider et al., 1990) called glycoprotein 63 (gp63) or promastigote surface proteinase, has been shown to be important in the parasite-host interaction of all Leishmania species (Medina-Acosta et al., 1993). Gp63 is a zinc-dependent metalloprotease found on the surface of the parasite that facilitates complement inactivation in serum and expressed in 500,000 copies on each promastigote, as a parasite virulence factor.

The importance of the major surface glycoprotein of Leishmania promastigotes, gp63 in the binding of promastigotes to macrophages has been inferred largely due to its abundance, surface location, and proteolytic activity (Bouvier et al., 1985; Etges et al., 1986; Chang et al., 1986). Gp63 interact directly with macrophage receptors, including the macrophage complement receptor type 3, Mac-1 (CD11b/CD18) (Russell et al., 1988) and the fibronectin receptor (Rizvi et al., 1988).

8.0 Receptor on macrophage, which contribute for attachment and internalization in the parasite

The concept of receptor originated at the end of the 19th century and the beginning of the 20th century through the studies of John Langley on atropine and of Paul Ehrlich on antibody. Receptors are specific cellular components that interact with specific ligands, which include hormones, neurotransmitters, drugs, antibody, lectins and growth factor.

A receptor is an entity that recognizes and is receptive to a specific ligand. Similar to enzymes and hemoglobin, receptors are protein molecules that display a high degree of specificity and selectivity towards the natural ligands designed to interact with the receptor.
The macrophage is the only cell *Leishmania* species inhabit, implying a high degree of specificity for this cell by the parasite. Entry into macrophages involves recognition of specific parasite ligands by receptors on the macrophage surface and eventual internalization of the parasite by the macrophage. Macrophage receptors involved in the entry of *Leishmania* promastigotes include the following:

### 8.1 Mannose-fucosyl receptor

This receptor binds specifically to ligands having terminal L-fucose or L-mannose groups. This interaction is a serum independent interaction that follows saturation kinetics and partially inhibited by these sugars and requires calcium (Lehrman, Haltiwanger and Hill, 1986). LPG and GP63 are the ligands that have been suspected to be involved in these interactions. Wilson and Pearson (1986) have demonstrated the binding of LPG to the mannose receptor of macrophages.

### 8.2 The C3b receptor

This receptor is supposed to be dependent on the amount of serum present. This macrophage-parasite interaction occurs when the complement factors get adsorbed on the parasite surface and the macrophages bind via their C3b receptors to the parasite (Wilson and Pearson, 1988). The protease activity of GP63 has been implicated in cleaving C3 into C3b and C3a (Etges, Bouvier and Bordier, 1986; Wright and El Amin, 1989) and it may also be involved in direct binding to the macrophage through its mannose moieties.

### 8.3 The fibronectin receptor

The fibronectin receptor has also been thought to play a role in leishmanial binding and uptake. However, this receptor is of lesser importance than the C3b or mannose-fucosyl receptor (MFR) since it is not specific to the macrophages and promotes rather than actively mediates phagocytosis (Chang, Chaudhari and Fong, 1990).
8.4 Toll-like receptor

Toll receptors were first discovered in the fruit fly, *Drosophila*. The Toll like receptor (TLR) family consists of phylogenetically conserved transmembrane proteins that are essential for innate immunity. *Drosophila* Toll is involved in dorsoventral patterning and also in host defense against fungal infection (Anderson, 2000). The *Drosophila* Toll system is structurally conserved and homologous to the mammalian TLR family. Microbial ligands, including lipopolysaccharide (LPS) and bacterial lipoproteins, have been shown to activate mammalian TLRs, facilitating transcription of genes that regulate the adaptive response, including cytokines and costimulatory molecules. Human TLR4, the mammalian homolog of Toll, was first identified by Janeway’s group (Medzhitov et al., 1997). To date, more than ten members have been reported to belong to the TLR family in mammals (Medzhitov et al., 1997; Rock et al., 1998; Takeuchi et al., 1999 and the EMBL/GenBank/DDBJ databases). Accumulating evidence suggests that mammalian TLRs are involved in recognizing pathogen-associated molecular patterns (PAMPs). Similar to *Drosophila* Toll, TLRs carry extracellular leucine-rich repeat (LRR) domain, a single transmembrane domain and an intracytoplasmic domain, which is homologous to that of the IL-1 receptor (IL-1R) family, including IL-1R and IL-18R (O’Neill and Dinarello, 2000). The intracytoplasmic domain is crucial for signal transduction and is referred to as the Toll/IL-1 receptor homology (TIR) domain (Michael et al., 2003).

8.5 Lipid Rafts

Rafts are dynamic small platform, composed of sphingolipids and cholesterol in the outer exoplasmic leaflet, connected to phospholipids and cholesterol in the inner cytoplasmic leaflet of the lipid bilayer (Simons et al., 2002). Lipid rafts form distinct
liquid-ordered phases in the lipid bilayer, dispersed in a liquid-disordered matrix of unsaturated glycerolipids (Brown et al., 1998; Schroeder et al., 1994).

The most important properties of lipid rafts are that they can include or exclude proteins to variable extents. Proteins with rafts affinity include glycosylphosphatidylinositol (GPI) anchored protein (Hooper, 1999), doubly acylated proteins, such as Src-family kinases or the α-sub-units of heterotrimeric G proteins (Resh, 1999), cholesterol-linked and palmitoylated proteins such as Hedgehog (Rietveld et al., 1999) and transmembrane proteins, particularly palmitoylated ones (Brown et al., 1998).

Lipid rafts play a central role in many cellular processes, including membrane sorting and trafficking, cell polarization and signal transduction processes that have been best studied in T cells (Janes et al., 2000). B cells (Cherukuri et al., 2001) and in the allergic response (Sheets et al., 1999; Holowka et al., 2001). Ceramide/sphingomyelin signaling, which regulates cell growth, survival and death, also involves raft clustering (Kolesnick, 2002). Several groups of pathogens—bacteria, prions, viruses and parasites hijack lipid rafts for their purposes (Van der Goot et al., 2001) and also involved in the generation of pathological forms of proteins associated with Alzheimer’s and prion diseases (Thomas et al., 2004).

8.6 Other protein mediating macrophage-parasite interactions

Another protein has been recently implicated in binding to *Leishmania donovani* lipophosphoglycan and increasing the uptake of the parasite into human macrophages (Culley et al., 1996). This is the C-reactive protein (CRP), a major acute phase protein that binds to promastigotes at the infectious, metacyclic stage of development, at concentrations found in normal human serum. The phosphosaccharide repeats of the lipophosphoglycan backbone are probably involved in binding to CRP.
In spite of the fact that certain mechanisms involved in macrophage-parasite interaction have been elucidated, the exact molecular interactions involved are not absolutely clear.

9.0 Evasion strategies of *Leishmania* parasite for intracellular survival inside the hostile environment of host

9.1 Alteration of the host complement system

The activation of the complement system through either the classical, lectin, or alternative pathways converges on the deposition of C3b on the parasite surface. The extracellular promastigote stage of *Leishmania* spp., found in the sandfly vector, activate the alternative and lectin-mediated pathways of complement (Green et al., 1994; Mosser and Edelson, 1984). Non-infective (procyclic) *Leishmania* promastigotes are sensitive to direct lysis by the serum complement system, whereas, metacyclic promastigote are relatively resistant to direct serum killing, by the spontaneous release of C5b-C9 complexes from the parasite surface (Puntes et al., 1990), which linked to the elongation of the phosphoglycan chain of the surface LPG (Sacks et al., 1995). In addition, leishmanial protein kinases have been reported to phosphorylate several components of the complement system (C3, C5 and C9) with subsequent inhibition of the classical and alternative complement pathway (Hermoso et al., 1991; Sacedoti-Sierra et al., 1997). The 63-kDa surface metalloproteinase (gp63); abundant in the metacyclic stage of the parasite also resist the complement-mediated lysis. The surface proteinase gp63 (Kweider et al., 1989) can cleave C3b to the inactive iC3b form and so prevent deposition of the C5b-C9 complex (Mosser and Brittingham, 1997). IC3b will opsonize the parasites for phagocytosis through complement receptors such as Mac-1 (Blackwell et al., 1985; Mosser and Edelson, 1985). This process of complement-mediated phagocytosis is important because it targets the
parasites to the macrophage, their host cell of choice and is also important for the intracellular survival of the parasite (Mosser and Edelson, 1987).

9.2 Protection against antileishmanial effector mechanisms

The effector mechanisms required for protection against any given infection are quite varied and will depend on specific characteristics of the parasite, such as the location of the parasites within the host, the number of life cycle stages within the host, and the evasion strategies developed by the parasite.

For establishment of an infection in the mammalian host, *Leishmania* must survive the process of phagocytosis and later resist the acidic and protease-rich milieu in the phagolysosome. Diverse families of molecules (e.g., LPG, 63 kDa glycoprotein (gp63), glycoinositolphospholipids (GIPLs), non-inositol containing glycosphingolipids (GSLs) are differentially expressed on the surface of pro- and amastigote *Leishmania*, which contribute to intraphagosomal and intraphagolysosomal survival. LPG and GIPLs of *Leishmania* have a number of downmodulatory effects on macrophage function, including inhibition of IL-12 production.

LPG and GIPLs of *Leishmania* have a number of downmodulatory effects on macrophage function, including inhibition of phagosome-endosome fusion (Desjardins and Descoteaux, 1997), efficiently scavenges hydroxyl radicals and superoxide anions (Chari et al., 1989), which are rapidly released after activation of NADPH oxidase during phagocytosis and also inhibit IL-12 production as well as reduced expression of inducible nitric oxide synthase and TNF-R (Carrera et al., 1996; Proudfoot et al., 1995). LPG also can interfere with signaling in infected cells and is unable to activate protein kinase C (Descoteaux et al., 1991; Feng et al., 1999).
9.3 Suppression of the synthesis of anti-leishmanial molecules

The two main antimicrobial effector mechanisms active against *Leishmania* parasites are the release of superoxide (O$_2^-$) by neutrophils and macrophages via the NADPH oxidase complex, and the molecular oxygen catalysed by nitric oxide synthase-2 [NOS-2] (iNOS), which occurs in macrophages. Infection of mouse or human macrophages with *L. major* or *L. donovani* drastically reduce the production of O$_2^-$ or H$_2$O$_2$ in response to phorbol esters (Buchmuller-Rouiller et al., 1987, Olivier et al., 1992; Passwell et al., 1994). *L. donovani* LPG also mimics the effect of complete parasites and to inhibit the activity of protein kinase C (PKC). Activation of PKC with translocation of the enzyme from the cytosol to the inner layer of the plasma membrane is a key requirement for the oxidative burst.

9.4 Modulation of cytokine production

The ability of certain parasites to invade and replicate within host cells allows them to evade the humoral immune response. Therefore, a recognition process is required to alert the immune system to the presence of infected cells, along with a series of effector mechanisms to control parasite replication. Infected macrophages are triggered to secrete certain cytokines (TGF-β, IL-10). The effect of TGF-β *in vivo* is likely to be due to its ability to suppress Natural killer (NK) cell and macrophage effector functions (parasite killing via production of NO and superoxide), an activity, which is also shared by IL-10. Infection of macrophages with *Leishmania* also blunts their ability to release T cell-stimulatory cytokines such as IL-1 and TNF-α. One of the most important cytokines in the initiation of cell-mediated immunity is interleukin-12 (IL-12), which as an active dimer (p70) and composed of p35 and p40 subunit. IL-12 suppression is one reason for profound immunosuppression accompanying Leishmaniasis (Christian Bogdan et al., 1998; Cristan
Bogdan and Andre Gessner, 1996; Alexander and Abhay, 1999). The production of IL-12 in response to infection plays a critical role in the activation of NK cells to produce gamma interferon (IFN-γ), which in turn is responsible for the activation of effector mechanisms required to control replication of intracellular pathogens. Although IL-12 is important for the development of protective immunity to leishmaniasis, early studies indicated that promastigotes of *Leishmania major* do not activate macrophages to produce IL-12 (Carrera et al., 1996; Reiner et al., 1994) and that infected macrophages have an impaired ability to produce this cytokine (Belkaid et al., 1998). It is now recognized that murine dendritic cells have the capacity to make IL-12 in response to *Leishmania* amastigotes and promastigotes (Gorak et al., 1998; Von Stebut et al., 1998) and thus may provide the initial source of the cytokine during *Leishmania* infection.

9.5 Inhibition of antigen-presentation and T cell stimulation

In the context of the antigen presentation process, parasitophorous vacuoles (PVs) might be described as potential compartments for parasite antigen processing. PVs are located on the intracellular endocytic pathway used by MHC class II molecules, thus location of *Leishmania* in an endocytic compartment of macrophages can initiate the host activation cascade by presenting antigens and co-stimulatory molecules. This has to be prevented and the parasite has specific mechanisms to modulate the build up of host immune response in its own favour (Jean-Claude and Eric Prina, 1998; Thierry and Raymond, 1994). *Leishmania* is known to avoid the induction of antigen presentation. Analysis of the intracellular localization of MHC-II molecules shows that there is a redistribution and sequestration of intracellular MHC-II molecules in the parasitophorous vacuoles and that they are polarized at the attachment site between amastigotes and the PV in macrophages. This suggests specific interaction between parasites and MHC-II
molecules in PV. Apart from this *Leishmania* infection interferes with the intracellular loading of MHC-II molecules with antigenic peptides. Even if productive peptide loaded MHC-II are generated they are prevented from reaching the macrophage surface for interaction with T cells. It has also been reported that the sequestered MHC-II molecules are being internalized and degraded by the parasite causing a marked downregulation of MHC-II molecules. All these events in combination lead to ineffective CD4⁺ T cell activation (Christan and Martin, 1998; Jean-Claude and Eric Prina, 1998; Ulrich and Nathaly Solioz, 1993; Selma and Thierry Lang, 1995).

In addition to the antigen-specific interaction between antigen presenting cells (APC) and T cells, the latter require costimulatory molecules for activation. Modulation of costimulatory signals leads to significant modification of antileishmanial T cell responses. The two sets of costimulatory molecules on macrophages (APC) modulated by parasite are:

(i) **Role of B7-1/B7-2:** Relative B7-1/B7-2 expression has a direct bearing on establishment of Th1 or Th2 type of CD4⁺ T cell activation. These costimulatory molecules interact with CD28 molecule on T cells to activate Th1 (B7-1) or Th2 (B7-2) by providing a costimulatory signal. *Leishmania* causes decreased B7-1 expression in comparison to B7-2 and thus Th1 subset of CD4⁺ T cells are not activated. A decrease in B7-2 expression is also seen. Relevance of relative expression of these molecules varies with the courses of infection.

(ii) **Role of CTLA-4:** CTLA-4 is a structural homologue of CD28 on T cells, which also interacts with B7-1/B7-2 on APC. However on binding to B7 molecules CD28 and CTLA-1 deliver opposing signals to T cells, the former being positive and latter negative. These antagonistic signals appear to be integrated by the T cell in determining the response to activation. Also the binding affinity of CTLA-4 to B7
molecules is significantly higher than that of the CD28 molecule. A decreased expression of B7 molecules thus affects both positive as well as negative signal, but on a comparative scale, positive signal is decreased to a greater extent than the negative signal resulting in an overall negative stimulus for the T cells (Mathew et al., 1995). Thus CTLA-4 engagement by this ligand B7 causes *Leishmania* induced unresponsiveness in CD4+ T cells.

### 9.6 Alteration of T cell differentiation/function

T-helper response basically comprises of 2 subsets- the Th1 and Th2 type of responses. The outcome is determined by the cytokine milieu at the site where T cells interact with antigen presenting cells. An experimental study using murine models has shown that there is a biased Th2 type of response in Leishmaniasis. This type of response allows the parasite to propagate since it exists as intracellular form. The Th1 type of response, which confers cell-mediated immunity, is antiparasitic, thus the parasite successfully skews the system towards Th2 type and happily survives within host’s cells even though massive humoral response is generated against it. The mechanism behind the regulation of cytokine milieu in favour of the parasite is not very clear, but parasite does play a role by differential up regulation and down regulation of Th1 and Th2 specific cytokines (Christan and Martin, 1998; Cristan and Andre, 1996 and Alexander et al., 1999).

The *L. major*-mouse model of human cutaneous leishmaniasis provides a valuable model for examining the roles of Th1 and Th2 cells *in vivo*; Th1 cells are host protective whereas Th2 responses exacerbate disease and allow uncontrolled infection (Reiner and Locksley, 1995). In humans, disease develops as a result of poorly controlled parasite replication leading to the development of skin or mucosal lesions (*L. major, L. amazonensis, L. braziliensis, and L. chagasi*), visceralization and major-organ involvement
(L. donovani and L. braziliensis) or excessive and damaging immune responses to the parasite (mucocutaneous leishmaniasis due to L. amazonensis). Human with visceral leishmaniasis have high circulating levels of IL-10, which may partly explain their inability to control the infection (Holaday et al., 1993; Karp et al., 1993).

10.0 Modulation of host signal transduction cascades by Leishmania

10.1 General preview on how signal transduction involved in different Intracellular protozoan parasites: diverse strategies to modulate parasite-host interaction

Protozoan pathogens such as Plasmodium, Leishmania, Trypanosoma are responsible for several of the most widespread and lethal human diseases. Their successful survival depends mainly on evading the host immune system by, for example, penetrating and multiplying within cells, varying their surface antigens, eliminating their protein coat, and modulating the host immune response. Immunosuppression is sometimes caused directly by parasite products and sometimes involves antigenic mimicry, which often appears in association with parasitic diseases (Zambrano-Villa et al., 2002). However, one of the most sophisticated mechanisms of evasion is the selective impairment of pathway by infectious pathogens.

Finally, parasitic protozoa have evolved strategies to down-modulate signaling pathways leading to host cell apoptosis, thereby prolonging the life of the host cell and their own intracellular survival (Luder et al., 2001). Induced apoptotic pathways in macrophages infected with either Leishmania donovani promastigotes (Moore et al., 1994) or T. gondii tachyzoites (Nash et al., 1998) were strongly inhibited, perhaps by parasite induced up-regulation of Bcl-2 homologs. Interestingly, the pro-apoptotic effects that T. cruzi infection has on both CD4⁺ and CD8⁺ T cells indirectly enhanced parasite growth, as the binding of
apoptotic lymphocytes to vitronectin receptors on macrophages triggered TNF-β production and a burst of parasite replication in infected cells (Freire-de et al., 2000).

10.1.1 Plasmodium:

In humans, malaria is caused by *Plasmodium vivax, Plasmodium falciparum, Plasmodium ovale* and *Plasmodium malariae*. Once the plasmodium parasite is established in the host, it evades the immune response (IR) by changing its surface antigen as it passes through the stages of its lifecycle. The development of an effective immune response (IR) is hampered by following evasion strategies like antigenic variation and/or polymorphisms, induction of blocking antibodies, molecular mimicry and by altering peptide ligand (Zambrano-Villa et al., 2002).

10.1.2 Trypanosomes:

African trypanosomiasis (sleeping sickness) is produced in humans by two African trypanosomes (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*) and in cattle by four (*Trypanosoma brucei brucei, Trypanosoma vivax, Trypanosoma evansi* and *Trypanosoma congolense*). These *trypanosomes* live in the blood and lack intracellular stages, so the parasite is a target for antibody-mediated destruction. Parasites survive and establish the infection by antigenic variation of the so-called variant surface glycoprotein (VSG). During parasitemia, several populations of B and T cells are altered. *Trypanosome* products also activate macrophage and CD8+ T cells in a particular way that causes changes in the pattern of cytokines release (Zambrano-Villa et al., 2002). Interestingly, *Trypanosoma cruzi* is unable to invade cells lacking transforming growth factor-β (TGF-β) receptors I or II (Ming et al., 1995) that suggests triggering this signaling pathway, perhaps by some parasite TNF-β homolog, might be needed to deactivate host cells during the early stages of infection.
10.1.3 *Toxoplasma*:

*Toxoplasma gondii* resides in a phagosome that restricts its fusion with host endosomes and lysosomes. *Toxoplasma* actively penetrates both phagocytic and nonphagocytic cells, propelled by an actin-myosin-dependent gliding motility (Sibley et al., 2000). In the process, it establishes a non-fusigenic compartment, called the parasitophorous vacuole (PV), which lacks integral membrane proteins of host cell origin, but is extensively modified by secreted parasite proteins (Mordue et al., 1999 and Lingelbach et al., 1998). This remodeling seems to be crucial to the inhibition of PV acidification and lysosome fusion, as these events proceed normally after uptake of dead or opsonized parasites, which are internalized via classical receptor-mediated phagocytosis.

10.1.4 Signal transduction in reference with *Leishmania*

10.1.4a Inhibition of host cell signaling pathways- by *Leishmania*

*Leishmania* parasites lack the machinery necessary for active invasion and are confined instead to professional phagocytes, mainly macrophages, with some exceptions (for example, fibroblast, dendritic cells and neutrophils) (Rittig et al., 2000). The uptake of *Leishmania* by macrophages proceeds via conventional receptor-mediated phagocytosis that involves a diversity of opsonic or pattern-recognition receptors (for example, CR3, CR1 and mannose fucose receptors) (Alexander et al., 1992) that are used depending on the species and stage of parasite and the presence or absence of fresh serum. Macrophages have to become activated in order to kill infectious organisms indicating the need for signal transduction pathways that can transmit the information from the cell membrane to the nucleus. Since *L. donovani* is known to survive within the macrophages, this could be due to the impairment of several macrophage accessory functions responsible for microbicidal action. For instance *L. donovani* infected macrophages are known to have an impaired
oxidative burst, which is a primary defense mechanism of the cell during *L. donovani* invasion (Buchmuller-Roiller and MaueI, 1987). Infected macrophages are impaired in their ability to produce gene products like IL-1β or MHC, which are essential for induction of T cell dependent immune responses (Reiner, 1987; Reiner, Ng and Mc Master, 1987; Reiner et al., 1988). Impairment of signal transduction pathways in *L. donovani* infected macrophages is yet another mechanism by which parasite evades intracellular killing (Descoteaux et al., 1991; Moore, Labrecque and Matlashewski, 1993). Thus, the signal transduction pathway represents an attractive target for the parasite to result in inactivation of macrophage functions. Several early genes like c-fos, c-jun and c-myc protooncogenes are involved in the general process of cell activation (Hamilton and Adams, 1987).

A major cell surface molecule of the *Leishmania* species is the glycoconjugate, lipophosphoglycan (LPG). LPG is involved in macrophage-parasite interaction (Turco and Descoteaux, 1992) especially in the attachment and internalization of the promastigotes (Descoteaux et al., 1991) as well as in the protection against microbicidal activities displayed by macrophages. One of the most important features of LPG may be its ability to inhibit the activation of PKC as shown earlier in *in vitro* systems (McNeely and Turco, 1987). Since PKC is the key enzyme of the cell machinery, which conveys signals from the surface of the cell to the nucleus (Nishizuka, 1986), inhibition of PKC is indeed a crucial breach in the defense mechanism of the cell.

### 10.1.4b *Leishmania* induced macrophage-signaling alteration

As *Leishmania* parasites resides inside the host macrophage cells during their life cycle. Development of effective cell-mediated immune responses to these organisms requires that infected macrophages induce significant Th1 cell activation against leishmanial antigens (Locksley et al., 1987, 1991, and 1995; Heinzel et al., 1988, 1989;
Scott et al., 1989; Scott, 1990, 1991; Locksley and Scott, 1991; Reiner and Locksley, 1995; Scharton-Kersten and Scott, 1995; Fearon and Locksley, 1996). The subsequent type 1 cytokine response activates macrophages to become microbicidal effector cells (Belosevic et al., 1990; Mauel, 1990; Olivier et al., 1989a, 1989b; Murray et al., 1982, 1987; Olivier and Tanner, 1989). IFN-γ has been recognized as the most potent activator of macrophage functions important for parasite control; IFN-γ ligation with the IFN-γ receptor leads to rapid activation of the JAK2-STAT1α signaling pathway (Leonard and O'Shea, 1998), which regulates the expression of key macrophage genes and proteins. The integrity of this type of signaling pathway is of paramount importance for host protection against Leishmania.

However, infection of macrophage with Leishmania donovani leads to the inhibition of many cellular functions including phagocytosis, IFN-γ-inducible MHC class II expression, IL-1 production, lipopolysacchride (LPS) and phorbol ester- mediated c-fos gene expression, and generation of oxygen radicals in response to the chemotactic peptide fMet-Leu-Phe (fMLP) or phorbol myristate acetate (PMA) (Olivier, 1996).

10.1.4c Ca²⁺ Mobilization

L. donovani infection causes the induction of abnormal macrophage plasma membrane permeability to Ca²⁺, leading to rapid and sustained elevation of the intracellular Ca²⁺ concentration ([Ca²⁺]) (Olivier et al., 1992a), possibly involving a capacitative mechanism maintained by persistent depletion of intracellular Ca²⁺ stores. Ca²⁺ mobilization may also led to the activation of an inositol triphosphate (IP₃) phosphatase that can dephosphorylate and degrade the inositol phosphates (Kukita et al., 1986), which results in the low fMLP-stimulated IP₃ activity measured in L. donovani infected macrophages (Olivier et al., 1992a). On the other hand, such [Ca²⁺] elevation may have
promoted the activation of Ca\textsuperscript{2+}-sensitive phosphoprotein phosphatases, such as the Ser/Thr phosphatase calcineurin (Klee et al., 1979), leading to the dephosphorylation and inactivation of several critical cellular proteins. In addition the other phosphatases, like phosphotyrosin phosphatases (PTP), endogenously produced by host cells in response to \textit{Leishmania} infection (Blanchetter et al., 1999) or produced by the pathogen, as reported for \textit{Yersinia} infection, in which the PTP YopH is inducible by [Ca\textsuperscript{2+}]\textsubscript{i} present in infected cells, may cause dephosphorylation of tyrosyl residues of host cell proteins and interfere with signaling cascades (Blanchette et al., 1999; Bliska et al., 1991, 1993).

\textbf{10.1.4d Protein phosphorylation}

Phosphorylation of tyrosyl residues by phospho tyrosine kinases (PTK) is a primordial step in the regulation of many cellular events. Tyrosine phosphorylation is a common event in the initiation of cell proliferation, but its role in signal transduction regulating the cellular functions of nonproliferative hematopoietic cells is also well documented (Dong et al., 1993a, 1993b). Activation of macrophage functions such as generation of a respiratory burst in response to zymosan (Green et al., 1992), phagocytosis via Fc receptors (Greenberg et al., 1993), tumoricidal activity induced by LPS and IFN-\gamma (Dong et al., 1993a), nitric oxide (NO) production in response to LPS and IFN-\gamma (Dong et al., 1993b; Olivier et al., 1998), regulation of eicosanoid biosynthesis (Glaser et al., 1993), and MHC class II expression following IFN-\gamma stimulation of 2C4 cells in somatic-cell genetic experiments (Watling et al., 1993) has proved the involvement of tyrosine phosphorylation-mediated signaling events.

IFN-\gamma induces tyrosine phosphorylation-mediated cellular functions (Dong et al., 1993a, 1993b; Olivier et al., 1998), and there is strong evidence that this cytokine can specifically activate JAK2 kinase, a PTK, to achieve its effect (Hunter, 1993; Muller et al.,
1993; Shuai et al., 1993; Silvennoinen et al., 1993 and Watling et al., 1993). JAK2 kinase becomes rapidly phosphorylated and subsequently induces tyrosine phosphorylation of the latent cytoplasmic transcriptional activator STAT proteins (Shuai et al., 1993). This signaling pathway is altered in L. donovani-infected macrophages due to immediate PTP SHP-1 activation following the initial parasite-macrophage interaction. This also explains how IFN-γ-inducible phagocyte functions (e.g., MHC class II expression and IL-12 generation) are inhibited by Leishmania. It could be possible that the IFN-γ receptor may be differentially expressed in Leishmania-infected macrophages, and this might explain the cellular unresponsiveness to IFN-γ stimulation. IFN-γ-induced IL-12, inducible NO synthase (iNOS), an MHC class II expression are affected by Leishmania infection, the induction of MHC class I antigen presentation (Kima et al., 1987) and the expression of immunoproteasome subunits LMP-2, LMP-7, and MECL-1 mRNA in response to this cytokine are normal (unpublished data). This shows that IFN-γ-receptor generally is not affected in Leishmania-infected cells and further reinforce the notion that other signaling pathways are triggered in response to IFN-γ stimulation.

10.1.4e Proteasomes

Proteasomes are known to modulate several transcriptional regulators, including Iκ/NF-κB activation (Chen et al., 1995), as well as STAT proteins (Yu and Burakoff, 1997). STAT1α inactivation following Leishmania infection could involve the participation of proteasome (26S [20S+PA700, 11S]; responsible for ATP-dependent proteolysis) and/or other proteolytic molecules. Whereas the 26S proteasome is involved in the degradation of regulatory proteins controlling various biological processes, the IFN-γ-inducible immunoproteasome formed by LMP-2, LMP-7, and MECL-1 subunits and activation of the 20S regulatory subunits PA28α,β is essential for appropriate peptide generation in the
context of MHC class I antigen presentation (Rivett, 1998). Presentation of antigen by the MHC class I-processing pathway occurs in *Leishmania*-infected cells (Kima et al., 1987).

Immunoproteasome modulation by IFN-γ is reflected in enhanced mRNA expression of the different subunits (Brown et al., 1991; Rivett, 1998; Stohwasser et al., 1997) in *Leishmania*-infected macrophages. *Leishmania* infection, affect the expression of some IFN-γ-inducible immunoproteosome subunits (i.e., LMP-2 and PA28α).

10.1.4f Factors mainly affected by *Leishmania* infection

i) Protein kinase C

*Leishmania* adversely affect the signal transduction pathways of macrophages. One particular signaling mechanism modulated by *Leishmania* infection is protein kinase C (PKC)-mediated events. PKC is a family of Ca$^{2+}$-dependent and -independent and phosphatidylserine-dependent protein kinases that play pivotal roles in the transmission of extracellular signals from the cell surface to the nucleus. Signals that act through inositol phospholipid hydrolysis stimulate a rapid increase in intracellular Ca$^{2+}$ levels and diacylglycerol (DAG) formation. Cytoplasmic PKC is inactive and in the presence of increased Ca$^{2+}$ levels, will translocate to the inner leaflet of the macrophage plasma membrane. The translocated PKC, while loosely associated with the plasma membrane, is still inactive. However, newly formed DAG destabilizes the bilayer, enabling PKC to partition into the bilayer and undergo a conformational change to become an active enzyme. This results in ATP-dependent phosphorylation of target proteins, activating events such as the respiratory burst, c-fos gene expression, IL-1 production, and chemotaxis (Adams and Hamilton 1987, Kiyotaki and Bloom 1984, Smith et al. 1992). PKC represents an ideal target for *Leishmania* to impair macrophage responsiveness to activating signals.
Ornithine decarboxylase (ODC) activity appeared to be coupled to the activation of protein kinase C (PKC) in macrophages, as activators of PKC caused a rapid increase in the ODC activity. ODC is the first and the rate-limiting enzyme in the biosynthesis of polyamines, which is implicated to have a role in a variety of metabolic processes (Pegg and McCann, 1982; Tabor and Tabor, 1984). The accumulation of ODC mRNA in mammalian cells is associated with the stimulation of a variety of physiological responses, indicating that the ODC gene belongs to a group of early genes, e.g., c-fos, c-myc, and c-jun protooncogenes (Kaczmarek and Kaminska, 1989). A major cell surface molecule of the *Leishmania* species is the glycoconjugate lipophosphoglycan (LPG), which inhibits the activation of protein kinase C (PKC), as shown earlier in *in vitro* systems (McNeely and Turco, 1987). Because PKC is the key enzyme of the cell machinery that conveys signals from the surface of the cell to the nucleus (Nishizuka, 1986), inhibition of PKC is indeed a crucial breach in the defense mechanism of the cell.

ii) *Mitogen-activated protein kinases (MAP kinases)*

The MAP kinases are an important group of serine/threonine signaling kinases that, by modulating the phosphorylation, and hence activation status of transcription factors, link transmembrane signaling with gene induction events in the nucleus (Karin, 1992 and Radler-Pohl et al., 1993). In mammalian cells, three major subgroups of MAP kinases have been identified, and these comprise the extracellular signal-regulated kinases (ERKs), the c-jun amino-terminal kinases (JNKs), and the p38 MAP kinases (Cobb et al., 1995). These MAP kinases are all activated by phosphorylation of a common threonine-X-tyrosine regulatory motif by their distinct upstream dual-specificity (Thr/Tyr) MAP kinase kinases (Her et al., 1993; Ward et al., 1994 and Canagarajah et al., 1997). Although LPS has previously been reported to activate all three types of MAP kinases, the physiological
relevance of such MAP kinase signaling to macrophage function remains unclear. Also Mitogen-activated protein (MAP) kinases are downstream targets of PKC (Seger et al., 1992), suggesting the possibility of impaired signaling through these enzymes in *leishmania*-infected cells. Two closely related MAP kinases like extracellular signal regulated-protein kinases 1 and 2, function as essential relays in many signal transduction processes. These kinases are in part responsible for regulating gene expression in response to diverse extracellular stimuli such as growth factors, cytokines, and hormones that influence cell proliferation, differentiation, and other functions (Blenls, 1993 and Pelech et al., 1992). MAP kinases have pleiotropic effects on processes in the cytoplasm, the nucleus, the cytoskeleton, and the plasma membrane (Blenls, 1993 and Pelech and Sanghera, 1992). Phosphorylation of MAP kinases on tyrosine and threonine is essential for their activation (Payne et al., 1991). Because of the broad range of effects of MAP kinases, their activities are tightly controlled by a family of dual specificity enzymes known as MAP kinase phosphatases that dephosphorylate MAP kinases on both threonine and tyrosine thereby rendering them inactive (Hunter, 1995). It has also been reported that the Src homology 2 domain containing tyrosine phosphatase (SHP-1) is involved in deactivating MAP kinases to dephosphorylation of tyrosine residues.

**iii) Transcription factor: NF-kappa B**

NF-κB (Nuclear transcription factor-κB) was first identified by Sen and Baltimore (Sen and Baltimore, 1986) as a constitutively active transcription factor binding the kappa light chain immunoglobulin enhancer in B cells and also NF-κB present in other cell types in an inactive cytoplasmic form which upon cellular stimulation could be induced to translocate to the nucleus and bind DNA (Baldwin, 1996; Pahl, 1999).
The transcription factor NF-κB consists of 2 subunits: a 50-kilodalton subunit (p50) and a 65-kilodalton subunit (p65, also known as RelA), it has attracted widespread attention among researchers in many fields based on the following: its unusual and rapid regulation, the wide range of genes that it controls, its central role in immunology processes, the complexity of its subunits, and its apparent involvement in several diseases. A primary level of control for NF-κB through interactions with an inhibitor protein called IκB. NF-κB can be activated by exposure of cells to LPS or inflammatory cytokines such as TNF or IL-1, viral infection or expression of certain viral gene products, UV irradiation or T cell activation, and by other physiological and non-physiological stimuli (Barnes and Adcock, 1997; Chen et al., 1999; Ghosh et al., 1998 and Thurberg and Collins, 1998). Activation of NF-κB to move into the nucleus is controlled by the targeted phosphorylation and subsequent degradation of I-κB dimers bind to target DNA elements and activate transcription of genes encoding proteins involved with immune or inflammation responses and with cell growth control.

In contrast to many pathogens, which augment the immune activation as in the case of T. cruzi, L. donovani induces an immune “silencing” mechanism for its intracellular survival. A recent study has reported the ability of L. donovani promastigotes to evade the induction of NF-κB in naïve bone marrow macrophages (Prive and Descoteaux, 2000). However, the signal transduction pathway leading to the deactivation of the NF-κB remains unclear.

iv) Interferon regulatory factor-1 (IRF-1)

Interferons (IFNs) are a group of multifunctional, secretory glycoproteins of about 20 Kda that function as important cytokines and regulate a variety of functions in mammalian cells (reviewed in Stark et al., 1998). IFNs are mainly of two types: type I
(IFN-α and IFN-β) and type II (IFN-γ). IFN-α molecules are coded by multiple (at least 18) genes but IFN-β and IFN-γ, each coded by a single gene. Broadly, IFNs have antiviral, antiproliferative and immunomodulatory properties (Weissmann and Weber, 1986; DeMaeyer and DeMaeyer-Guignard, 1988). Type I IFNs are recognized by a common receptor, IFNAR but type II or IFN-γ binds to a distinct receptor, IFNGR (Pestka et al., 1987). Both receptors are located on surface of most mammalian cells. IFN-α is mostly secreted by lymphoid cells and is often referred as lymphocyte IFN. IFN-β is mostly secreted by fibroblasts and is referred as fibroblast IFN. IFN-γ is known as immune interferon and is secreted by cells of the immune system and some other cell types.

The Interferon regulatory factor (IRF) family of transcription factors are cellular DNA binding proteins that act as activators or repressors of promoters containing variations on the IRF binding sequence. Family members include IRF-1 (ISGF-2), IRF-2, IRF-3, IRF-4 (lymphocyte-specific IRF), ICSBP, and ISGF-3γ(p48) (Nguyen et al., 1997; Sato et al., 2001). IRF-1 is a positive transcription factor for many mammalian genes. IRF-1 is an IFN-inducible transcription factor that was originally identified by its ability to positively regulate the IFN-β promoter through the PRD I motif (Fujita et al., 1998). Deletion analysis of IRF-1 reveals that the N terminus contains a DNA binding domain, while the C terminus has the ability to transactivate gene expression (Lin et al., 1994). IRF-1 is generally present only at low levels within resting cells; but after treatment with either type I or type II IFN, transcription of the IRF-1 gene is increased, and levels of IRF-1 protein rise dramatically (Miyamoto et al., 1988; Fujita et al., 1989). In addition, IRF-1 production is induced by other cytokines, such as IL-1, IL-6 and TNF-α (Fujita et al., 1989), viral infection (Miyamoto et al., 1988), and prolactin (Yu Lee et al., 1990). IRF-1 enters the nucleus
rapidly after synthesis, where it binds to a DNA motif with the sequence G
(A)AAA(G/C)(T/C)-GAAA(G/C)(T/C) (Tanaka et al., 1993). This motif has been found in
the upstream promoter elements of a number of IFN-inducible genes such as IFN-β (Ohno
and Taniguchi, 1983), major histocompatibility complex class 1 (Johnson and Pober, 1994),
and 2-5-oligo (A) synthetase (Benech et al., 1987). The IRF gene family does not have any
sequence similarity to those of other known transcription factors. Thus, IRF-1 binding to
DNA and other proteins is central to the formation of transcription factor complexes, or
enhanceosomes, which mediate the transcriptional regulation of a number of IFN-regulated
genes. A number of studies have shown the importance of IRF-1 in mediating
transcriptional upregulation of these and other IFN-induced genes (Tanaka et al., 1993).
Earlier studies have shown that the gene for inducible nitric-oxide synthase (iNOS) is
regulated by IRF-1 (Martin et al., 1994; Kamijo et al., 1994). Within the mouse iNOS
promoter sequence there are four copies of an IFN-γ response element, two copies of γ-
activated site, and two copies of the IFN-stimulated response element (ISRE). The
complimentary nucleotide sequence of one of these ISRE core closely matches a consensus
sequence termed IFN-regulatory factor element (IRF-E) (Feng et al., 1999). IRF-1 is an
IRF-E binding protein (Feng et al., 1999).

v) Activating Protein-1 (AP-1)

The transcription factor AP-1 (Activating Protein-1) was first defined as a DNA-
binding activity specific for positive regulatory elements in SV40 early promoter. The AP-1
is a sequence-specific transcriptional activator composed of homodimers and heterodimers
of Jun (v-Jun, C-Jun, Jun B, Jun D), Fos (V-Fos, C-Fos, FosB, Fra1, Fra2) or activating
transcription factor (ATF2, ATF3/LRF1, B-ATF) (Vogt and Bos 1990; Angel and Karin
1991). These proteins, which belong to the bZIP (basic region leucine zipper) group of
DNA binding proteins that bind to a common DNA site, the AP-1 binding site. Although Jun proteins form very stable heterodimers with Fos- and ATF (activating transcription factor) family members, they can also homodimerize among themselves (Ziff, 1990; Kouzarides and Ziff 1988 and Dorsey et al., 1995). Jun-Jun and Jun-Fos dimers preferentially bind to the phorbol 12-O-tetradecanoate-13-actetate (TPA)-responsive element (TRE: this element has the base sequence (TGACTCA), whereas Jun-ATF dimers or ATF homodimers prefer to bind to the cAMP-responsive element (CRE: this element has the base sequence (TGACGTCA) (Hai and Curran, 1991). Both elements are palindromic and contain the same AP-1 half-site.

AP-1 activity was found to be induced by many other stimuli, including growth factors, cytokines, T cell activators, neurotransmitters and UV irradiation (Angel and Karin 1991). Most of the genes that encode AP-1 components behave as “immediate-early” gene, i.e. genes whose transcription is rapidly induced, independently of de novo protein synthesis following cell stimulation. A cAMP response element mediates c-fos induction in response to neurotransmitters and polypeptide hormones, which by using either cAMP or Ca^{2+} as second messengers activates either protein kinase A or calmodulin-dependent protein kinases. A serum response element (SRE) mediates c-fos induction by growth factors, cytokines, and other stimuli that activate MAPKs (Treisman, 1992), and a cis-inducible enhancer mediates induction by stimuli that activate the JAK (Janus kinase) group of protein kinase (Darenell, Kerr and Stark, 1994). Since SRE (serum response element) mediates c-fos induction in response to external stimuli, which results in ERK (extracellular stimulus responsive kinase) activation leads to elevated AP-1 activity via c-fos induction. This results in increased synthesis of c-fos, which upon translocation to the nucleus combines with pre-existing Jun proteins to form AP-1 dimers that are more stable
than those formed by Jun proteins alone (Smeal et al., 1989). Increased stability results in higher levels of AP-1 DNA binding activity because it shifts to equilibrium toward dimer formation, which is essential for DNA binding. The AP-1 protein function is regulated primarily by phosphoregulation (Cohen, et al., 1989; Cook et al., 1999; Derijard et al., 1994). The AP-1 family members differ in their ability to repress transcription (Ryseck and Bravo, 1991 and Vandel et al., 1995).

vi) **Inducible nitric oxide synthase (iNOS)**

Nitric oxide (NO), a reactive free-radical gas, was found to be generated enzymatically from L-arginine and molecular oxygen by constitutive or inducible NO synthase (NOS) in a variety of cells, such as neuronal cells, endothelium, adrenal glands, neutrophils, kupffer cells, and activated macrophages (Moncada et al., 1991 and Nathan, 1992). NO plays several physiological roles in mammalian systems: (i) mediator of endothelial-derived relaxation of smooth-muscles in the artery; (ii) synaptic neuronal messenger; (iii) cytotoxic agent released by macrophages; and (iv) signaling molecule that acts by binding the haem iron at the active site of soluble guanylate cyclase, stimulating the enzyme to generate cyclic GMP (Moncada et al., 1991 and Nathan, 1992). In macrophages, NO is generated by inducible NOS (iNOS), and interferon-γ (IFN-γ) is an important factor for the priming of macrophages, and tumor necrosis factor α (TNF-α) or some other cytokines or lipopolysaccharide (LPS) is necessary for full induction of NO from activated macrophage (Lanier et al., 1992; Phillips et al., 1992; Farias-Eisner et al., 1994; Xie et al., 1995). Also macrophage plays a key role in directing the host immune response to infection. Recruitment and stimulation of macrophages by cytokines and/ or microbial products such as LPS results in the induction and release of several key immune effector molecules such as NO and IL-12, a number of intracellular pathogens, including
Leishmania major, Mycobacterium tuberculosis, Listeria monocytogenes, and Toxoplasma gondii (Green et al., 1990; Liew et al., 1991; Hsieh et al., 1993; Heinzel et al., 1993; Sypek et al., 1993; Chan et al., 1995 and Nathan et al., 1994). Indeed, survival of the Leishmania parasite has been shown to be associated with the ability of lipophosphoglycan glycoconjugates expressed on the cell surface of Leishmania promastigotes to regulate production of both NO (Proudfoot et al., 1996 and IL-12 (Piedrafita et al., 1999) in macrophages. NO, which mediates many of the nonspecific cytotoxic and inflammatory responses of macrophages following infection by pathogens, is generated following the upregulation of expression of the inducible form of NO synthase (iNOS) (Hsieh et al., 1993).

vii) Macrophage-associated cytokine production on L. donovani infection

Induction of protective immunity against leishmaniasis is also generally thought to depend on the production of interleukin (IL)-12 (Afonso et al., 1993; Heinzel et al., 1993; Liew and O'Donnell, 1993). This macrophage-associated cytokine drives a CD4+ Th1 response and induces IFN-γ production from both natural killer (NK) cells and T cells. IFN-γ in turn mediates protection by inducing NOS2 expression and NO production. Consequently, neutralization of IL-12 leads to disease exacerbation in L. donovani and L. major infections (Heinzal et al., 1995; Engwerde et al., 1998).

It is hardly surprising, therefore, to find that Leishmania metacyclic promastigotes are potent inhibitors of macrophage IL-12 production both in vitro (Carrea et al., 1996; Sartori et al., 1997; Piedrafita et al., 1999) and in vivo (Belakaird et al., 1998; Reiner et al., 1994). Metacyclogenesis modulates the ability of promastigotes to induce IL-12 production and procyclins fail to inhibit IL-12 production in human peripheral blood monocytes (Sartori et al., 1997). Piedrafita et al. (1999) have recently shown that the phosphoglycan moiety of L. major LPG regulates IL-12 synthesis in J774 cells at the transcriptional level,
although this pathway is not mediated through the nuclear factor NF-κB. Whether amastigotes can suppress the induction of this cytokine remains controversial. Studies using lesion-derived *L. major* amastigotes have shown that they stimulate IL-12 production in bone-marrow-derived macrophages (Reiner et al., 1994) and in J7774 cells (Piedrafita et al., 1999). However, in further studies using inflammatory macrophages obtained from non-immune granulomas from resistant and susceptible mice, *L. major* amastigotes failed to induce IL-12 production (Belakaird et al., 1998), and infected host cells lost the ability to produce IL-12 following stimulation with LPS plus IFN-γ. Similarly, infecting macrophages with *L. mexicana* amastigotes resulted in sustained suppression of IL-12 production (Weinheber et al., 1996). This was not dependent on opsonisation of the parasites with serum components and was not associated with production of IL-10 or transforming growth factor-β (TGF-β). It also appeared to be a post-transcriptional event. Immunohistochemical studies on *L. donovani* in BALB/c mice suggest, with this species, that dendritic cell, but not macrophages, produce IL-12 during the early stage of infection (Gorak et al., 1998).

Macrophages secrete other cytokines, in addition to IL-12, that not only regulate macrophage function in an autocrine manner but also play an important role in the modulation of acquired immune responses. For instance, *in vitro* studies have demonstrated that TNF-α, and monocyte chemotactic and activating factor (MCAF), can enhance the leishmanicidal activity of macrophages (Mannheimer et al., 1996). Similarly, migration inhibitory factor (MIF) also plays a protective role during *L. major* infection *in vivo* and *in vitro*, possibly by inducing NO production (Juttner et al., 1998; Xu et al., 1998). Additionally, a recent study has indicated that type I interferon (IFN-α/β) and NOS2 are critical regulators of the innate response to *L. major* (Diefenbach et al., 1998).
macrophage-derived cytokines (IL-6, IL-10 and TGF-β) downmodulate macrophage leishmanicidal activity and are believed to play a role in pathogenesis of leishmaniasis (Hatzigeorgiou et al., 1993; Karp et al., 1993; Barral et al., 1993, 1995). The roles for IL-1β and granulocyte-macrophage colony stimulating factor (GM-CSF) in leishmaniasis vary depending on the study. Whereas some in vitro and in vivo studies indicated that IL-1β and GM-CSF enhance macrophage leishmanicidal activity and play a protective role in leishmaniasis (Hatzigeorgiou et al., 1993), others reported that these cytokines play a deleterious role. Significantly for parasite survival, during L. major infection IL-1 is downregulated and during L. donovani infection IL-1 and TNF-α are downregulated by an LPG-associated activity. Conversely, macrophage-derived regulatory cytokines, such as TGF-β and IL-10, which downregulate cytokines, such as TGF-β and consequently exacerbate disease, are unregulated during infection.

11.0 Rationale of the present study

During the course of evolution protozoan parasites have evolved strategies to subvert the immune responses of their host in order to multiply, reproduce and survive. One of these strategies is to modulate the host signal transduction mechanism. Leishmania, an intracellular pathogen is capable of promoting mononuclear phagocyte dysfunctions.

Infection of mononuclear phagocytes with Leishmania leads to the inhibition of many of their functions, including phagocytosis (Buchmuller-Rouiller and Mauel, 1987); the production of interleukin 1 (Oliver, 1996), expression of c-fos gene in response to lipopolysaccharide and phorbol esters (Oliver, 1996), the expression of major histocompatibility complex (MHC) class II molecule in response to interferon gamma (Oliver, 1996) and generation of oxygen radicals (Oliver, 1996). Leishmania donovani has evolved to selectively impair host cell biochemical processes involved in transmitting
information to the nucleus and such activity enhances the level of infection with this protozoan. Selective impairment of signal transduction may therefore represent a new strategy for identifying targets for treating Leishmania infection.

Major surface molecule of Leishmania promastigotes, lipophosphoglycan can promote as well as inhibit NO synthesis by the murine macrophages, thereby playing an important role in the host-parasite relationship. An increased understanding of the molecular mechanisms by which parasite surface molecule, LPG, signals to the host cells, leading to the expression of iNOS may allow the development of novel therapies for disease using signaling agonists or antagonists to modify the pathophysiological responses to this infection.

Leishmaniasis is a major public health problem in parts of India and the development of resistance of the parasite to existing drugs has created a need to understand the host-parasite relationship and to develop new combating strategies. Impairment of signal transduction pathways in L. donovani infected macrophages is one of the mechanism by which parasite evades intracellular killing. Thus, the signal transduction pathway represents an attractive target for the parasite to result in inactivation of macrophage functions. Selective impairment of signal transduction may therefore represent a new strategy for identifying targets for treating Leishmania infection.

Differential sensitivity of the macrophage effector responses to pharmacological modulators would suggest that such reagents could be used as potential immunomodulators to generate the required immunity to combat a particular pathogen or alternatively reduce inflammation/pathology associated with certain disease status.

In order to resolve the critical strategy through which the parasite establishes a tryst with macrophages so that it can reside, mature and multiply within macrophages it is vital
to study how parasite down-regulates macrophage activation. The main objective of the present study is to investigate the molecular mechanism involved in the signal transduction in macrophages by *Leishmania* surface glycoconjugate, lipophosphoglycan (LPG).

**Main Objective:**

The aim of the present work is to investigate the molecular mechanism involved in the signal transduction in macrophages by *Leishmania* surface glycoconjugate, lipophosphoglycan (LPG).

**Specific Objectives:**

(a) Role of LPG in regulating signal transduction through protein kinase C and mitogen activated protein (MAP) kinase in murine macrophages.

(b) Activation/down regulation of transcription factors by LPG in murine macrophages: NF-κB, IRF-1 and AP-1

(c) Regulation and expression of macrophage effector molecule, nitric oxide (NO).
Fig. 1: Global distribution of leishmaniasis
Distribution of leishmaniasis/HIV co-infection

Fig. 2: Countries reporting leishmaniasis/HIV co-infection (Source WHO/CTD, 1997)
Fig. 3: - Pathology of Leishmaniasis (Adapted from pathology of Tropical and Extraordinary Diseases, an Atlas, Vol.1)
a) Diffuse anergic cutaneous leishmaniasis.
b) Mucocutaneous leishmaniasis showing deformed nose.
c) Visceral leishmaniasis showing enlarged liver and spleen.
Fig. 4: Life cycle of *Leishmania*
Fig. 5: Structure of LPG from Leishmania donovani
Fig. 6: Structure of LPGs from the three species of *Leishmania* promastigotes grown in log to late-log phase of growth (Turco and Descoteaux, 1992)
Fig. 7: Proposed pathway of assembly of the repeating units and capping oligosaccharides of *L. donovani* LPG. The core structure is Gal (α1,6) Gal (α1,6) Gal(β1,3) Man (α1,3) Man(α1,4) GlcN(α1,6) and PI is lyso-1-O-alkyolphosphatidylinositol. The sequence of monosaccharide addition is not yet known (Turco and Descoteaux, 1992).