Leishmaniases are parasitic diseases caused by obligatory intracellular protozoan flagellates of the genus *Leishmania*. Currently leishmaniasis is considered as the third most important vector-borne disease after malaria and lymphatic filariasis. The sixtieth World Health Assembly, in its resolution, recognized leishmaniasis as one of the most neglected tropical diseases. It is a major public health problem responsible for 57,000 deaths annually and the burden is increasing (Desjeux, 2001; Desjeux, 2004b). In 2002, the World Health Organization (WHO) estimated the number of persons at risk to be around 350 million and the number of new cases to be 23,57,000 per year. Globally the disease is affecting 88 countries: 72 are developing countries and 13 of them are among the least developed. More specifically VL occurs in 65 countries (Desjeux, 1992). The annual estimate for the incidence and prevalence of VL cases worldwide is 0.5 million and 2.5 million, respectively (WHO, 1998). Of these, 90% of the confirmed cases occur in India, Nepal, Bangladesh and Sudan. In India, it is a serious problem in Bihar, West Bengal and eastern Uttar Pradesh where there is under-reporting of VL and PKDL (Post kala-azar dermal leishmaniasis) in women and children 0-9 years of age. Untreated cases of VL are associated with up to 90% mortality, which with treatment reduces to 15% and is 3.4% even in specialized hospitals (Bora, 1999). It is also associated with up to 20% subclinical infection. The situation is specifically crucial in the state of Bihar, India, known as the “heartland of kala-azar”. Here, the burden of disease has increased progressively in the past. A huge amount of research has been conducted on *Leishmania* in numerous scientific fields in the last century. However recent studies have shown the reactivation of several foci such as in Italy, China, Brazil and central Israel (Arias et al., 1996; Nasereddin et al., 2005). New epidemic foci have also emerged in northern and central Israel and Morocco (Jacobson et al., 2003; Al-Jawabreh et al., 2004; Guernaoui et al., 2005; Shani-Adir et al., 2005). Other studies indicate the underevaluation of the severity of these infections and of their socioeconomical impact (Rioux et al., 1990; Desjeux, 2001; Desjeux, 2004b). In addition, co-infection with human immunodeficiency virus is becoming more and more problematic in developing and industrialized countries (southern Europe) (Desjeux, 2001; Desjeux, 2004b; Wolday et al., 2001). Further epidemicity and unresponsiveness to chemotherapy has highlighted the increasing threat of HIV–*Leishmania* co-infections, particularly in India and East Africa (Cruz et al., 2006).
The causative agent of the disease, the *Leishmania* belongs to the family Trypanosomatidae. There are about 21 species of *Leishmania* which are capable of infecting man. They can be classified into two main groups: Old World: *L. major*, *L. tropica*, *L. aethiopica* and the *donovani* complex (*L. donovani*, *L. infantum*) and New World: *L. mexicana*, *L. amazonensis* and Viannia complex (e.g. *L. brasiliensis*, *L. guyanensis*). Old World forms of *Leishmania* are transmitted by sandflies of the genera *Phlebotomus* and are endemic in Africa, Asia, the Middle East, and the Mediterranean. The New World form is transmitted mainly by flies of the genus *Lutzomyia* that are endemic from Texas through South America, Australia and the South Pacific are not considered leishmaniasis-endemic regions. There are about 30 species of phlebotomine sandflies which are capable of transmitting the parasite (Dowlati, 1996; Roberts, 2006).

The parasite has a digenetic life cycle with an extracellular developmental stage in the insect vector and a developmental stage in mammals which is mostly intracellular. In sandflies, development of the parasite occurs in the alimentary canal with the formation of a motile, flagellated and elongated form termed as ‘promastigote’. The promastigote matures in the insect midgut into an infective metacyclic promastigote. Inoculation into the mammalian host occurs when sandflies feed on blood which is a requirement for oviposition (Neuber, 2008). A typical inoculum contains around 100–1000 metacyclic promastigotes which quickly become engulfed by leucocytes, particularly macrophages, neutrophils and dendritic cells. The parasites undergo a further transformation within these cells to form amastigotes. A morphological change occurs as the parasite takes on an ovoid shape with a short flagellum, hence the term ‘amastigote’, and possibly a metabolic change with a switch to anaerobic metabolism under acidic conditions found chiefly in the phagolysosome compartment (Descoteaux and Turco, 1999).

VL was first described in 1903 by Leishman and Donovan. Both of these physicians separately but simultaneously demonstrated parasites in stained smears from the spleen of patients suffering from a malaria-like illness, which is known as visceral leishmaniasis and its causative agent was named *L. donovani*. This is not to say that leishmaniasis did not exist before 1903, on the contrary. Archibadi in 1922 described an epidemic of Kala-azar which occurred in the Garo hills of Assam in Saudi Arabia as far back as 1870. Cunningham recorded a similar disease that occurred in 1885, caused by a parasite which was later named *Leishmania tropica*, the causative agent of cutaneous leishmaniasis. Nicolle in 1908 reported that mammals including dogs could act as
reservoir hosts for the *Leishmania* parasite. Swaminath and cowerkers in 1942, proved using human volunteers that the *Leishmania* parasite could be transmitted by the phlebotomus sandflies (WHO, 1997). Three major clinico-pathological categories are recognized: cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) each caused by distinct species.

2.1 Cutaneous leishmaniasis

The cutaneous leishmaniasis (CL) is also known as "Aleppo boil," "Baghdad boil," "Bay sore," "Biskra button," "Chiclero ulcer," "Delhi boil," "Kandahar sore," "Lahore sore," "Leishmaniasis tropica," "Oriental sore," "Pian bois," and "Uta" (Grevelink and Lerner, 1996). It is the most common form of leishmaniasis with a global annual burden of 1.5 million. About 90% of the CL cases occur in only seven countries (Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia, and Syria), whereas approximately 90% of visceral leishmaniasis (VL) cases occur in rural and suburban areas of five countries (Bangladesh, India, Nepal, Sudan, and Brazil) (Desjeux, 2004b).

Surveillance data indicate that the global number of cases has increased during the past decade, as documented in Afghanistan, (Reithinger et al., 2003) Bolivia, (Davies et al., 2000) Brazil, (Brandao-Filho et al., 1999) Colombia, (Davies et al., 2000; King et al., 2004) Peru, (Davies et al., 2000) and Syria (Tayeh et al., 1997). Such increases can be explained in part by improved diagnosis and case notification (Yadon et al., 2001), but are also a result of inadequate vector or reservoir control, increased detection of cutaneous leishmaniasis associated with opportunistic infections (eg, HIV/AIDS) (Molina et al., 2003) and the emergence of antileishmanial drug resistance (Croft and Engel et al., 2006). However, because many infections are symptomless or misdiagnosed (Escobar et al., 1992), the global burden of cutaneous leishmaniasis is likely to be underestimated. Transmission cycles are adapting to peridomestic environments and are spreading to previously nonendemic areas as a result of urbanisation and deforestation (Desjeux, 2001), with domestic animals as potential reservoirs (Davies et al., 2000; Reithinger et al., 2003). Additionally, economic hardship, (Guthmann et al., 2005) natural disasters (Massoom and Marri, 1993) and tourism (Blum et al., 2004) cause susceptible populations to migrate to areas endemic for cutaneous leishmaniasis, where exposure to infection results in noticeable epidemics. For example, whereas cutaneous leishmaniasis caused by *Leishmania tropica* was rare in Kabul, Afghanistan, before 1990, more than
25,000 mainly autochthonous cases were treated in 2003 with incidence estimated to be up to 67,500 new cases per year (Reithinger et al., 2003). New foci of L. tropica are also reported in Morocco (Chiheb et al., 1999), Israel (Jacobson et al., 2003), Syria (Tayeh et al., 1997), Iran (Motazedian et al., 2002) and Pakistan (Brooker et al., 2004).

In India, CL is reported primarily in some pockets in the Thar Desert of Rajasthan state, located in the western part of the country and bordering Pakistan (Dogra et al., 1990; Bari and Rahman, 2006; Lodha et al., 1971). The first evidence for existence of CL was based on clinico-epidemiologic analysis of cases in 1973 during a large-scale outbreak of the disease in Bikaner (Kumar et al., 2007). More than 2,000 people suffered from this infection; during this time, sporadic cases were detected in villages in the vicinity of the Rajasthan canals. It was reported that Indian desert gerbils, Meriones hurrianae, and dogs were the reservoirs of this infection (Kumar et al., 2007; Sharma et al., 1973). There is no authentic information regarding the vector species transmitting CL infection, but a few studies concluded that in this region one or both species, Phlebotomus papatsi and P. sergenti are the vectors (Sharma et al., 1973).

CL can be differentiated into four types: localized cutaneous leishmaniasis (LCL), leishmaniasis recidiva cutis, disseminated leishmaniasis (DL) and diffuse cutaneous leishmaniasis (DCL). LCL is the most prevalent form of the disease and is most commonly caused by L. major, L. tropica and L. aethiopica, species that are prevalent in the Old World (Scarisbrick et al., 2006). In 86–98% of cases it usually cures spontaneously after a period ranging from 3 months to 2 years. The lesions appear on an exposed area of the body surface, varying in number from one to ten. The established lesion is a round, painless ulcer that is well delimited with a central crust that is sometimes hemorrhagic. It may cure spontaneously, leaving a hypopigmented, smooth, thin scar. Depending on the host–parasite balance and other undefined factors, some cases evolve to other forms of the disease (Akilov et al., 2007). Leishmaniasis recidiva cutis is known in the Old World to be associated with L. (L.) tropica infection, the occurrence of which is rare in the New World. Characteristic papular and vesicular lesions appear after clinical cure in or around the scar of the healed sore after a variable period of time from months to years. DL is characterized by the presence of multiple (10–300) pleomorphic lesions, mainly acneiform and papular, in two or more noncontiguous areas of the body (Reithinger et al., 2007). Diffuse cutaneous leishmaniasis is a true anergic form of tegumentary leishmaniasis and is characterized by the presence of nodular lesions that do
not ulcerate (Barral et al., 1995). It is a rare condition and has been reported in South America, Central America and Ethiopia. The lesions are rich in parasites and the species involved are *L. (L.) mexicana*, and *L. (L.) amazonensis* in the New World and *L. (L.) aethiopica* in the Old World.

### 2.2 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL), or espundia, produces lesions, which can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities. Although mucosal leishmaniasis can be caused by *L. panamensis*, *L. guyanensis*, *L. amazonensis*, *L. major*, *L. tropica*, and *L. infantum*, it is most commonly associated with *L. braziliensis* (Reithinger et al., 2007). The frequency of MCL varies according to the geographical location. In Brazil, it varies from 0.4% in the south (de Castro et al., 2005) to 1.4% in the central region (Gontijo et al., 2002), and to 2.7% in the northeast (Jones et al., 1987). In Andean countries, MCL reaches an average of 7.1% (Davies et al., 2000). Bolivia exhibits a high frequency of 20% (Garcia et al., 2009), Ecuador a medium frequency of 7.7% (Hashiguchi and Gomez Landires, 1991), Colombia a low frequency of 2.3% and Venezuela a very low frequency of 0.4% (Davies et al., 2000).

In most endemic areas, 1–10% of LCL complicates into mucosal leishmaniasis 1–5 years after LCL has healed (Convit et al., 1993), but reports do exist for which mucosal leishmaniasis presented at the same time as LCL (Boaventura et al., 2006), or for which up to 25% of LCL infections resulted in mucosal leishmaniasis (Dimier-David et al., 1993). In MCL parasite metastasises into mucous tissues by lymphatic or haematogenous dissemination. It typically begins with nasal inflammation and stuffiness followed by ulceration of the nasal mucosa and perforation of the septum. In some cases, the lips, cheeks, soft palate, pharynx, or larynx are also involved. Mucosal leishmaniasis never heals spontaneously, is very difficult to treat. Damage may be severe enough to cause death through malnutrition and acute respiratory pneumonia (Lawyer and Perkins, 2004; Grevelink and Lerner, 1996).

### 2.3 Visceral leishmaniasis

Various terms have been used to describe visceral leishmaniasis including Dum-dum fever, Sikari disease, Burdwan fever, Shahib's disease and tropical splenomegaly.
However, the most commonly used term is Kala azar, which in Hindi means black sickness or black fever. The terms originally referred to Indian VL due to its characteristic symptoms, blackening or darkening of the skin of the hands, feet, face and the abdomen (Lainson and Shaw, 1987). Visceral leishmaniasis is caused by the parasites *Leishmania donovani donovani*, *Leishmania donovani infantum* and *Leishmania donovani archibaldi* in the Old World and by *Leishmania donovani chagasi* in the New World. For the past 10 years, epidemics consistently reoccur in Bangladesh, India, Sudan, and Brazil, where more than 90% of the cases occur (Editorial, 2006). Each year, 500,000 new cases are reported of visceral leishmaniasis (VL) and of this, 5,000 have resulted in death (WHO, 1998). Infection with *L. donovani* is particularly rampant in India. It is a serious problem in Bihar, West Bengal and eastern Uttar Pradesh where there is under-reporting of kala-azar and post kala-azar dermal leishmaniasis in women and children 0-9 years of age. Untreated cases of kala-azar are associated with up to 90% mortality, which with treatment reduces to 15% and is 3.4% even in specialized hospitals. It is also associated with up to 20% subclinical infection. Unlike other countries endemic with visceral leishmaniasis, India is unique in that transmission may occur without an animal reservoir. The disease is chronic and onset is gradual. Although people of all ages are susceptible in the Old World, children below the age of 15 are more commonly affected with *L.d infantum* being largely responsible (Rab *et al*, 1995). In sporadic and epidemic cases of VL the disease is usually acute and symptoms appear suddenly with people of all ages being at risk except those who have been conferred immunity due to a past infection.

The symptoms of VL vary between individuals and according to geographical foci. However, some of the common symptoms include high undulating fever often with two or even three peaks in 24 hours and drenching sweats which can easily be misdiagnosed as malaria, chills, rigors, weight loss, fatigue, poor appetite, cough, burning feet, insomnia, abdominal pain, joint pain, anorexia, epistaxis and diarrhoea. Clinical signs include splenomegaly, hepatomegaly and lymphadenopathy (Hashim and Elhassan, 1994). The incubation period is highly variable; the disease can appear anything between ten days to over one year. Even longer incubation periods have been documented (WHO, 1997). The duration of the disease can be 1-20 weeks, in endemic areas of Western Sudan the illness usually lasts about 12-16 weeks with an average of about 6 weeks (Hashim and Elhassan, 1994).
Visceral leishmaniasis can be complicated by serious secondary bacterial infections such as pneumonia, dysentery and pulmonary tuberculosis, which often contribute to the high fatality rate of VL patients. Other complications, though rare include haemolytic anemia, acute renal damage and severe mucosal haemorrhage (WHO expert committee report, 1991).

2.4 Post Kala Azar Dermal Leishmaniasis (PKDL)

PKDL occurs in India and mainly in Sudan and Kenya in Africa (Hashim and Elhassan, 1994). Reports of PKDL in China and Iraq have also been documented (Ramesh and Mukherjee, 1995). In the New World PKDL is extremely rare (WHO expert committee report, 1991). Usually PKDL follows recovery from a kala azar infection, though less commonly, it has been known to occur in patients who have not suffered previously from kala azar (Hashim and Elhassan, 1994). Both the Indian and the African PKDL display similar symptoms. The disease begins with small measel-like lesions (hypopigmented macules, papules or nodules) appearing on the face, and gradually increase in size. Eventually the lesions spread to the upper trunk, arms, forearms, thighs, legs, abdomen, the neck and the back. Multiple lesions can coalesce to form larger lesions and can lead to the gross enlargement of facial features such as the nose and lips, giving an appearance similar to leprosy. The disease is particularly severe if the lesions spread to the mucosal surfaces of the nasal septum, hard and soft palate, oropharynx, larynx or the eye lids and the cornea leading to blindness (Hashim and Elhassan, 1994; Ramesh and Mukherjee, 1995). Potentially the lesions can appear on any part of the body. Hence there is a possibility of PKDL being transmitted sexually. In addition to the disease being confused with leprosy, PKDL can also resemble cutaneous leishmaniasis, secondary syphilis and sarcoidosis (Ramesh and Mukherjee, 1995). The lesions are usually self-limiting, however those that do not heal spontaneously within six months have to be treated (Hashim and Elhassan, 1994).

2.5 Animal models

For in vivo testing of vaccine several animal species have served as experimental hosts for visceral leishmaniasis. Important among them are BALB/c mice and Syrian golden hamsters (primary tests), dogs (secondary tests) and monkeys viz., squirrel, vervet and Indian langur monkeys as tertiary screens.
2.5.1 Models for primary testing: Rodents

Models for primary testing include hamsters (European, Chinese and Syrian); mouse (BALB/c, NMRI, DBA/1, C57BL/6) rat, squirrel, gerbil etc. (Hommel et al., 1995). Of the various animals tried, BALB/c mice and Syrian golden hamsters are the commonest and currently used animal models for drugs and vaccine testing against VL.

2.5.1.1 Mice:

It has been found that outbred mice are generally resistant to infection with *L. donovani*, but inbred strains display marked differences in susceptibility. Most of the mice strains like C57BL/6, CBA/J, C3H or BIOD2 resist the infection with clinical cure within few months (Handman et al., 1979). BALB/c and all T-cell immunodeficient strains manifest a systemic visceral *Leishmania* leading to death (Howard et al., 1980). Resistance and susceptibility are closely related with the development of T-cell responses of Th1 or Th2 type respectively. C57BL/6 mice mount early Th1 immune response and prevent the further growth of the parasite causing self-healing phenotype (Lehmann et al., 2000), whereas susceptible BALB/c strain mounts early Th2 response and results in non healing lesion and exaggeration of disease (Himmelrich et al., 2002). Respective resistance and susceptibility of C57BL/6 and BALB/c strains depend not only on the Th1 and Th2 type of immune response of CD4$^+$ T cells but also on the genetic background of the host.

Though, BALB/c strain of mice infected with *L. donovani* or *L. chagasi* is the most widely studied model of VL, this is considered to be susceptible wherein the infection progresses during the first two weeks and it is then controlled by the host immune response (Murray et al., 1987). The mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response. On the other hand, the better model to study the progressive disease is hamster infected with *L. donovani* or *L. chagasi* that develops a disease similar to human progressive visceral leishmaniasis with hepatosplenomegaly, hypoalbuminaemia, hypergammaglobulinaemia and pancytopenia (Rodrigues-Jr et al., 1992). Therefore, this model is mainly used to study the mechanisms of immunosuppression.

2.5.1.2 Hamster:

The Syrian golden hamster (*Mesocricetus auratus*) is uniquely susceptible to a variety of intracellular pathogens and is an excellent model for a number of human
infectious diseases. The golden hamster was used as one of the early animal models for the study of visceral leishmaniasis. Infection with *L. donovani* leads to visceral disease and death making it a useful tool for the characterization of molecules and mechanisms involved in pathogenesis (Hommel et al., 1995). Hence, they are ideal for most of the experimental studies including vaccine testing because they almost mimic the situation in a kala azar patient (Melby et al., 2001a). A major disadvantage of the visceral models is that, in the prime model of visceral disease (the hamster model), only high dosages of antimony could suppress established lesions. The model has also been used for vaccination studies (Sharma et al., 2003) but the molecular basis for this high level of susceptibility is unknown and immunological studies related to this model have been limited by the lack of available reagents (Sharma et al., 2004).

2.5.2 Models for secondary testing: Canines

As all rodent models have the disadvantage of having different metabolisms and kinetics of the drugs, eliciting responses different from those seen in humans, the secondary testing in higher models such as dogs, cats and monkeys, which have responses close to human, would further strengthen the claim of primary screening and would help in picking the most promising molecules/epitopes, which need to be pursued in successive steps in a vaccine development programme (Abranches et al., 1991). The dog is the major reservoir of *L. infantum* in the Middle East and the Mediterranean region and *L. chagasi* in South America. There has been no such reservoir for VL noticed in India; the disease pattern in dogs and humans is similar with a long period of asymptomatic infection followed by wasting, anaemia, enlarged lymph nodes and fever. As in humans, the infection remains asymptomatic in some dogs (Abranches et al., 1991; Pinelli et al., 1995). One of the few differences is the presence of skin lesions in the dogs rarely detected in humans (Hommel et al., 1995). The unpredictable nature of the infection has been a major problem in establishing experimental models for canine VL, but appears to reflect the spectrum of clinical responses seen in natural infections. The dog may be the best animal model for VL in which relevant immunological studies and vaccine development could be performed (Mendonca et al., 1995). With the recent cloning of several dog genes encoding cytokines and immunologically important cell markers, as well as the development of monoclonal antibodies to these molecules, there is a hope for a more sustained exploitation of this excellent animal model (Gradoni, 2001). Dog populations are an important reservoir of visceralizing *Leishmania* in many endemic areas.
and vaccination of these animals would presumably constitute a major step towards control of the logical substitute at least for *L. infantum*, for which the dog is the natural reservoir. *L. donovani* also multiplies within the viscera of mongrel and beagle dogs. German shepherd dogs have been reported to give better results than beagles (Keenan et al., 1984), whereas some researchers claimed highly successful infection rates with mixed breeds (Abranches et al., 1991).

### 2.5.3 Models for tertiary or preclinical testing: Non human primates

A vaccine for man needs to be tested in primates due to their close phylogenetic relation to humans in the evolutionary tree. For VL, the availability of a non human primate model would increase the understanding of various aspects of host parasite interactions. Earlier efforts in establishing VL in New and Old World monkeys demonstrated that *Aotus trivirgatus* (owl monkeys) and *Saimiri sciureus* (Squirrel monkeys) developed fulminating but short lived infection. Antileishmanial screening has been performed in owl monkey (Chapman et al., 1983) and squirrel monkey (Chapman and Hanson, 1981). Old World monkeys such as *Macaca* spp. viz., *M. mulatta*, *M. fascicularis* and *M. nemestrina* and African vervet monkeys develop low and/or inconsistent infections (Dube et al., 2004). Attempts to establish VL in *Presbytis entellus* showed that this species was highly susceptible to single intravenous inoculation of hamster spleen-derived *L. donovani* amastigotes, which invariably produced consistent and progressive acute fatal infection, leading to death between 110 to 150 days post infection. The infected animals presented all the clinicoimmunopathological features as observed in human kala-azar (Anuradha et al., 1990; Dube et al., 1999). The Indian langurs have also been used for preclinical evaluation of potential antileishmanial drugs (Sharma et al., 2004) and vaccine (Dube et al., 1998; Misra et al., 2001).

### 2.6 Treatment

The available treatment options for visceral leishmaniasis (VL) present several problems, including high toxicity and many adverse effects, leading to patients withdrawing from treatment and emergence of resistant strains. In addition to these problems, the high cost of the compounds makes the treatment far from suitable and, regrettably, it has been increasing gradually throughout the years (Yardley et al., 2002; Singh and Sivakumar, 2004). The first line treatment against leishmaniasis includes
pentavalent antimonials, mostly in sodium stibogluconate and N-methylglucamine antimoniate forms, used since the 1940’s (Berman, 1988; Olliaro and Bryceson, 1993; Raht et al., 2003). The other drugs, such as pentamidine, amphotericin B, and paromomycin are used as a second option in resistant cases, despite their great toxicity to the host (Ramos et al., 1990; Kuhlencord et al., 1992; Escobar et al., 2001; Bray et al., 2003; do Socorro S Rosa Mdo et al., 2003). Pentamidine resistance has also been reported in the literature (Bray et al., 2003) as well as difficulties in treating immune-depressed patients (i.e., HIV), in whom conventional drugs are less efficient and higher drug doses and a long treatment period are commonly necessary (Escobar et al., 2001). For patients in resource-rich countries, liposomal amphotericin B appears to be a better treatment option. In South Asia, miltefosine is being used; the combination of single dose liposomal amphotericin B and short course miltefosine looks encouraging but has the problem of potential reproductive toxicities in females. Several researches have been carried out to develop new protocols and chemotherapies for leishmaniasis treatment. The purpose is to reduce the problems related to medicines already in use and increase their efficiency (Ma et al., 2004).

Pentavalent antimonials are the first-line drugs used to treat all types of leishmaniasis. For VL treatment they have been in use since the 1940s (Moore and Lockwood, 2010). It is unfortunate that, treatment failure has been documented in several regions of the world. Currently, there are two formulations in use: sodium stibogluconate (SSG), which contains 100mg antimony/100ml 'Pentostam', (sodium stibogluconate) and meglumine antimoniate, which contains 85 mg antimony/100 ml. Both formulations are comparable in efficacy and toxicity (Chulay et al., 1988). Oral absorption of these drugs is poor, therefore they are injected via intramuscular or intravenous routes. The drug acts by inhibiting the activity of the glycolytic and fatty acid oxidative pathways in amastigotes (Mahajan and Sharma, 2007). The major side effects include arthralgy and myalgia. However, side effects related to cardiotoxicity or renal failure are also found in older patients. The drug is contraindicated during pregnancy. Variations are found in the efficacy of these drugs according to the geographic region, species of Leishmania and clinical presentation (Moore and Lockwood, 2010).

In the New World, the efficacy of antimonials for the treatment of cutaneous leishmaniasis has been variable. In Bolivia, treatment failure was observed in 7% of patients (Bermudez et al., 2006), 16% in Brazil (Oliveira-Neto et al., 1997), and in 39%
of patients in Colombia (Palacios et al., 2001). In the Old World, failure of this drug is approximately 13%, and this drug is considered as satisfactory for the treatment of cutaneous leishmaniasis. In MCL, the cure rate ranges from 30 to 90% with antimonials, depending on the country in which the study was carried out and the dosage used (Franke et al., 1990; Romero et al., 1998; Amato et al., 2009).

Although amphotericin B has shown excellent in vitro leishmanicidal activity, however its use was initially limited by toxicities. In some parts of the world still, it is the drug of choice. This drug reacts with ergosterol present in the Leishmania membrane. It increases the permeability of the cell membrane which in turn promotes an ion influx into the parasite, both promastigotes and amastigotes, leading to their death (Ellis et al., 2009). There are four drug formulations: amphotericin B deoxycholate, liposomal amphotericin B, cholesterol dispersion amphotericin and lipid complex amphotericin. All formulations have a similar efficacy approximately, however, they differ in causing side effects (Trigo et al., 2010). Liposomal Amphotericin B has been observed to have a better tissue penetration and is more effective at lower doses with low toxicities (Adler-Moore and Proffitt, 2002). However the cost for the treatment courses is approximately double for liposomal Amphotericin B as compared to Amphotericin B. It has therefore limited its use in many areas despite better side effect profile (Moore and Lockwood, 2010).

Pentamidine was a preferred drug in cases of antimony resistance, but studies have shown inferior cure rates to amphotericin B (Mishra et al., 1992). This drug acts by interfering with Leishmania DNA synthesis and modifying the morphology of the kinetoplast which promotes fragmentation of the mitochondrial membrane and kills the parasite. The well known toxicities (cardiac, diabetes mellitus, hypotension, gastrointestinal side effects) associated with it have limited its use. However, it has been found to be useful in prevention of relapse in patients with successful initial cure with another agent but have a high risk of relapse secondary to HIV and other immunodeficiencies (Patel and Lockwood, 2009). It has been used with success to treat cutaneous leishmaniasis or MCL in some regions of the New World. Its efficacy has been found to be the same as that of antimonials (Tuon et al., 2008).

Miltefosine was originally used as an anticancer drug. It is a phosphorylcholine ester of hexadecanol, a membrane-active alkylphospholipid. Miltefosine was first shown to be active against L. donovani in vitro (Croft et al., 1987) and rapidly was shown in
small studies to have a favourable therapeutic index for Indian visceral leishmaniasis. It is an orally active drug. It has been suggested that the optimal dose to balance efficacy and tolerance is 100 mg/day for 28 days (Sundar et al., 1998a). A few studies on the treatment of tegumentary leishmaniasis have also been reported. In Colombia, a cure rate of 89–100% was observed for cutaneous leishmaniasis and was dependent on the dose used (Soto et al., 2001), however, in general, the results in the New World are poor (Soto et al., 2004). It is contraindicated in pregnant women because of its teratogenic effects in animal studies (Croft et al., 2006).

Paromomycin (formerly known as aminosidine), an aminoglycoside antibiotic produced by Streptomyces riomosus, was developed in the 1960s as an oral therapy for intestinal protozoa. Later on it was found to be active against gram negative bacteria, some gram positive bacteria, mycobacteria, some cestodes and Leishmania parasites. It is administered by intramuscular injections and the main side effects include ototoxicity, pain at injection site and raised liver enzymes (Moore and Lockwood, 2010). Since August 2006, paromomycin was approved in India as an alternative for visceral leishmaniasis treatment. A suitable monotherapy regimen of paromomycin sulphate is 15 mg/kg/day for 21 days. It can be effectively combined with pentavalent antimonials; the combination is given for 17 days, with the two drugs injected into different buttocks (Davidson et al., 2009). The mechanism of its action is unclear. In Leishmania, it can interfere with RNA synthesis and membrane permeability (Maarouf et al., 1997).

Sitamaquine is an orally active 8-aminoquinoline analogue of Primaquine that showed evidence of efficacy against VL more than 20 years ago. Phase 2 studies have been conducted in a range of VL settings with variable results. In Kenya, used at a dose of 1 mg/kg for 28 days, it had a 50% final cure rate (Sherwood et al., 1994). In Brazil, the dose of 2 mg/kg had a 67% cure rate but unexpected nephrotoxicities in higher doses (Dietze et al., 2001). In India, doses of 2-2.5 mg/kg have reported 80-100% cure rate with common toxicities of vomiting, dyspepsia, nephrotoxicity and cyanosis secondary to methaemoglobinæmia (Jha et al., 2005). Further dose finding studies in Kenya using 1.75-3 mg/kg had 80-90% cure rate; abdominal pain and headache were common but tolerable side effects, and rare but severe nephrotoxicity were seen at the higher doses (Wasunna et al., 2005).
Combination therapy for the treatment of visceral leishmaniasis has increasingly been advocated as a way of preventing the *Leishmania* parasites developing resistance to chemotherapeutic agents (Bryceson, 2001; Jha, 2006). The increase in antimonial resistance in India has fueled this debate. The difficulties in achieving definitive cure in HIV/VL co-infected patients adds to the situation as these patients harbor drug exposed parasites which may be transmitted on to others. There is also a desire to provide the short duration treatment which may well be better achieved by combination treatment. This might avoid excessive costs for patients (especially in South Asia settings) and would be helpful in VL foci unsafe from war (Southern Sudan). However, this approach has not been fully validated and there are risks of augmenting drug toxicities, which in many areas are difficult to monitor for and manage (Moore and Lockwood, 2010). The combination of sodium stibogluconate and paromomycin appears to have been an effective practical approach (Seaman *et al.*, 1993). Good results have been shown with single-dose ambisome followed by 7-14 days of miltefosine (Sundar *et al.*, 2008). In India, although there is no data to support the contention that this regimen will avoid the development of parasite resistance to miltefosine. Trials are ongoing in Africa and India evaluating various drug combinations (Moore and Lockwood, 2010).

### 2.7 Diagnosis

Early and accurate diagnosis remains a key component of leishmaniasis control and is essential for both individual patients and for the community. Leishmaniasis shares many of the clinical and pathological features with other diseases like malaria, tropical splenomegaly syndrome, schistosomiasis, cirrhosis with portal hypertension, African trypanosomiasis, milliary tuberculosis, brucellosis, typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma and leukaemia. Hence other diagnostic methods are required to confirm the clinical suspicion (Singh and Sivakumar, 2003; Singh *et al.*, 2005; Herwaldt, 1999; Lainson and Shaw, 1987).

Diagnosis of VL has typically relied on microscopic examination of bone marrow/splenic aspirate but serology and molecular methods are now better alternatives. Recently several recombinant proteins have been developed to accomplish accurate diagnosis. Recombinant kinesin protein of 39 kDa called rK 39 is the most promising of these molecules. The antigen used in various test formats has proved to be highly...
sensitive and specific for visceral leishmaniasis. It is useful in the diagnosis of HIV-
Leishmania co-infections and as a prognostic marker. Molecular techniques targeting
various genes of the parasite have also been reported, the PCR being the most common
molecular technique successfully used for diagnosis and for differentiation of species
(Singh and Sivakumar, 2003).

2.7.1 Parasitological diagnosis

The visualization of the amastigote form of the parasite by microscopic
examination of aspirates from lymph nodes, bone marrow or spleen is the classical
confirmatory test for VL. This test presents sensitivity values of 53–65%, 53–86 % and
93–99 %, for lymph node, bone marrow and spleen aspirates, respectively (Zijlstra et al.,
1992). Its specificity is 100% and for this reason the direct parasitological examination is
considered to be the gold standard. However, splenic aspiration can be complicated by
life threatening haemorrhages in approximately 0.1% of individuals and therefore
requires considerable technical expertise (Sundar and Rai, 2002), as well as facilities for
nursing surveillance, blood transfusion and surgery. Moreover, the accuracy of
microscopic examination is influenced by the ability of the laboratory technician and the
quality of the reagents used (Reithinger et al., 2007).

The diagnosis of CL and MCL can be performed using light microscopy to
directly examine the biopsy specimens, scraping or impression smears subjected to
giemsa staining. Biopsy and aspirate samples can be further cultured in blood agar base,
formerly known as Novy, McNeal and Nicolle medium, overlaid by liver infusion triptose
or Schneider’s liquid medium, or injected into a susceptible animal such as a hamster for
parasite recovery. The sensitivity of the direct examination is low, at approximately 50–
70% in the Old World (Vega-Lopez, 2003; Al-Hucheimi et al., 2009) and even lower, at
approximately 15–30%, in the New World where chronic cases and MCL are frequent
(Sotto et al., 1989; Schubach et al., 2001). The detection level is higher, reaching 44–58%
by culturing the samples and 38–52% by injection into hamsters (Schubach et al., 2001;
Weigle et al., 1987). Excluding direct microscopic examination, other methods require a
complex laboratory structure and technical skills, as well as longer periods of time to
obtain the results.
2.7.2 Leishmanin Skin Test (LST)

The leishmanin skin test (LST) measures delayed type hypersensitivity reactions to intradermal injection of leishmanin antigen (killed *L. donovani* parasite) in patients' fore arm (Palma and Gutierrez, 1991). After 48-72 hours the induration is measured in mm; a reaction of 5 mm or more is considered positive. No cross-reactions occur with Chagas' disease, but some cross-reactions are found with cases of glandular tuberculosis and lepromatous leprosy. This test is usually used as an indicator of the prevalence of cutaneous and mucocutaneous leishmaniasis in human and animal populations and successful treatment of visceral leishmaniasis (Weigle *et al*., 1987; Singh, 2006).

During active kala-azar disease the LST will be negative (Liarte *et al*., 2001). However, leishmanin antigen is not commercially available and no field study has been carried out in Indian subcontinent. Despite the availability of large number of serological tests, no serological method is helpful for cutaneous and mucocutaneous leishmaniasis because antibodies tend to be undetectable or present in low titre due to poor humoral response (Amaral *et al*., 2000; Ahluwalia *et al*., 2004).

2.7.3 Serological diagnosis

Conventional smear or culture techniques are low in sensitivity particularly in detecting the occult and sub-clinical infections. The serological diagnosis is widely and frequently used and is based on the presence of specific humoral response, as in cases of visceral leishmaniasis or cell mediated immune response, as in cases of cutaneous and mucocutaneous leishmaniasis (Herwaldt, 1999). Commonly used serological methods are described below.

2.7.3.1 Indirect Fluorescent Antibody Test (IFAT)

The Indirect fluorescent antibody test has been widely used for the diagnosis of leishmaniasis (Duxbury and Sadun, 1964) and is one of the most sensitive tests available. The test detects antibodies, which are present in the very early stages of infection and disappear six to nine months after cure. If the antibodies persist in low titers, it is a good indication of probable relapse. Titers above 1:20 are significant and above 1:128 are diagnostic (Davidson, 1998). Due to the phylogenetic similarity between *Leishmania* spp. and *Trypanosoma cruzi*, serological cross-reactions and false-positive results are quite common (Troncarelli *et al*., 2009). However, this problem can be overcome by using amastigote stage of the parasite as the antigen instead of promastigotes (Williams *et al*.,
2006). Although this test is more sensitive (96%) and specific (98%) than soluble antigen ELISA, its application requires a high level of skill and experience and expensive laboratory facilities. Another limitation is the fact that serial dilutions of serum must be made, which makes the test laborious and not practical for screening of large number of samples (Sassi et al., 1999). The direct fluorescent antibody test is more useful in the diagnosis of CL, MCL and PKDL. In place of fluorescence, horse radish peroxidase (HRP) can be used to tag the antibody. This will not require fluorescence microscope and the stained slides can be stored for long time (Singh, 2006).

2.7.3.2 Direct Agglutination Test

The direct agglutination test (DAT) is a highly specific and sensitive test. It is cheap and simple to perform making it ideal for both field and laboratory use (Meredith et al., 1993). The method uses whole, trypsinized, Coomassie stained promastigotes either as a suspension or in a freeze-dried form. Its performance was influenced by neither the region nor by the Leishmania species. Although several researchers defend its use in field conditions (Neogv et al., 1992; Schallig et al., 2002), one of the limitations of DAT is the relatively long incubation time (18 h) and the fact that serial dilutions of blood or serum must be made, which makes the test laborious and not suitable for screening of large number of samples (Harith et al., 1989). Also, DAT has no prognostic value as the test may remain positive for several years after cure. Nevertheless the development of a freeze-dried antigen makes DAT very suitable for use under harsh field conditions since it remains stable at high temperatures (Meredith et al., 1995; Schallig and Oskam, 2002). In various studies it has been shown to be 91 to 100% sensitive and 72 to 100% specific (Liarte et al., 2001; Tavares et al., 2003). In Sudan, well equipped field laboratories, use DAT for diagnosis of VL; patients with high titers receive treatment, and a confirmatory parasitic diagnosis is done in those with low titers (Boelaert et al., 1999). From India, several laboratories reported satisfactory sensitivity and specificity levels for this test (Sundar et al., 1996; Vinayak, et al., 1994).

Although DAT showed a high degree of repeatability within the centers, its reproducibility across the centers was quite weak (Boelaert, 1999). Moreover, difficult field conditions, the fragility of aqueous antigen, the lack of cold chain, and batch to batch variations in the antigen, along with the nonstandardization of test readings, have...
severely limited its widespread applicability in regions of endemicity (Singh and Sivakumar, 2003). The fast agglutination screening test (FAST) combines a higher parasite concentration with a smaller test volume. Furthermore, it requires a single serum dilution and results are read after three hours. The FAST claims advantages over the DAT as it uses freeze-dried antigen which gives more antigen stability, reproducibility, specificity and sensitivity (Singh and Sivakumar, 2003).

2.7.3.3 Enzyme Linked Immunosorbent Assay

The Enzyme Linked Immunosorbent Assay (ELISA) is a potential serodiagnostic tool for almost all infectious diseases, including leishmaniasis. The technique is highly sensitive, but its specificity depends upon the antigen used. Several antigens have been tried. The commonly used antigen is a freeze-thawed crude soluble antigen (CSA). In this case sensitivity is high but cross-reactions with sera from patients with trypanosomiasis, tuberculosis, and toxoplasmosis have been recorded (Bray, 1976; Bray, 1985; Choudhry, et al., 1990; Kumar et al., 2001; Singh et al., 1995; Smrkovski and Larson, 1977). On the other hand, when various selective antigenic masses (116 kDa, 72 kDa, and 66 kDa) were used, a specificity of 100% could be achieved, but only at the cost of sensitivity, which went down to as low as 37.5% (De Colmenares et al., 1995; Vinayak, 1994). The use of a 36 kDa glycoprotein, a fucose mannose ligand, in ELISA has been found to result in 100% sensitivity and 96% specificity (Palatnik-de-Sousa, et al., 1995). The sensitivity and specificity of ELISA in diagnosing VL could also be increased by the use of soluble antigens derived from promastigotes cultivated in a protein-free medium. Ten recombinant or purified Leishmania antigens have been compared on the basis of ELISA for the serodiagnosis of Mediterranean visceral leishmaniasis. These included crude soluble Leishmania antigens (SLAs), recombinant (r) antigens (namely: rgp63, rK39, gene B protein, rH2A and r H2B histone proteins, rLACK, rPSA-2, r P20) and purified lipophosphoglycan. Most of the test antigens showed good performance (sensitivity > 85%, specificity > 80%). The best specificity (97%) and sensitivity (92%) was obtained with rK39 based ELISA. The rK39 antigen is a 39-amino acid repeat from a kinesin like protein that is predominant in L. chagasi amastigotes, useful to diagnose VL caused by L. donovani, L. chagasi or L. infantum (Badaro et al., 1996; Bern et al., 2000; Burns et al., 1993). An rK39 based ELISA was then developed into an immunochromatographic test (ICT), or dipstick, format that was more suitable for field use. However, ELISA is technically demanding and has the requirement of test samples to be investigated in
batches while ICT is rapid and is easy to perform. It may also be used in HIV-positive patients. Antibody levels against rK39 decline rapidly after successful treatment (Badaro et al., 1996; Houghton et al., 1998). Studies using samples from endemic area showed that rK39 based ICT strip test is highly sensitive and specific for diagnosis of VL and PKDL in India and Nepal (Kumar et al., 2001; Qu et al., 1994; Singh et al., 1995). However, this test has been shown to be less accurate in East Africa (Boelaert et al., 2008).

Serological tools have also been used in the diagnosis of CL. ELISA using few recombinant antigens viz., gene B protein (GBP) from *L. major*, recombinant major surface glycoprotein, gp63, from *L. major*, and 2 recombinant proteins, T26-U2 and T26-U4, from *Leishmania (Viannia) peruviana* have been tried but have been found less suitable for diagnosing CL (Schoone et al., 2001; Zijlstra and El-Hassan, 2001). Other recombinant antigens such as *L. major* Hsp60 (Rey-Ladino et al., 1997) and *L. braziliensis* Hsp70 (Amorim et al., 1996), were cloned and the products tested using cutaneous leishmaniasis and mucocutaneous samples from Colombia with promising results. *L. (L.) infantum* Hsp83 (Celeste et al., 2004) has also been tested using a limited number of cutaneous and mucocutaneous samples which showed 100% reactivity, interestingly without any cross-reactivity with Chagas’ disease sample.

### 2.7.3.4 Immunoblotting

Immunoblotting is among the most sensitive and specific serological method that provides information about the parasite’s antigenic profile. The band pattern can correlate with disease stages (Ravindran et al., 2004). Most of the work concerning the use of immunoblotting in the diagnosis of leishmaniasis has been done on visceral leishmaniasis. In cutaneous leishmaniasis, anti-*Leishmania* antibodies though detectable, are present in low titres. Hence immunoblotting is not widely adopted for diagnosing CL (Singh, 2006). A 72-74 kDa antigen band, obtained using cytoplasmic, soluble antigens from 5 Indian strains of *L. donovani* and three *L. major* strains from Pakistan separated by SDS-PAGE and electrotransferred on nylon membrane followed by Western blotting with Indian PKDL patients was found to be the most prominent (Singh and Sivakumar, 2003). The commercially available electrochemiluminiscent kit (ECL, Amersham, UK) enhances its sensitivity, several-folds. It also has an added advantage of permanent documentation (Singh, 2006).
2.7.3.5 Antigen Detection

Antigen detection test would, in principle provide better means of diagnosis of active leishmaniasis. Since antigen levels are expected to theoretically correlate with the parasite load this method should be a better alternative to the antibody detection methods, especially in immunocompromised patients, where antibody response is very poor. They can also distinguish active from past infections. However, detection of antigen in the patient’s serum is complicated by the presence of high level of antibodies, circulating immune complexes, serum amyloid, rheumatoid factor and autoantibodies; all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of free antigen. Though a few reports are published, no satisfactory antigen detection system is currently available (Senaldi et al., 2001). A latex agglutination test (KATEX) for the detection of leishmanial antigens in the urine of patients with VL is developed with comparable sensitivity for HIV patients (Singh and Sivakumar, 2003). This is a simple, easy performing, inexpensive, field applicable, and rapid test, which can be performed at patient’s bedside. It detects a 5–20kDa carbohydrate low molecular weight antigen that is released from *Leishmania* parasite into patient’s urine (Sarkari et al., 2002; Salotra and Singh, 2006). The test works well regardless of the geographical origin of samples as evident from the results obtained with KATEX using samples collected from patients of different foci of VL. The test has 100% specificity and sensitivity lies between 68-100% (Singh, 2006). However, in East Africa and the Indian subcontinent studies showed good specificity but only low to moderate (48–87%) sensitivity (Attar et al., 2001). Apart from its low sensitivity, there are two practical limitations: the urine must be boiled to avoid false-positive reactions and it is difficult to distinguish weakly positive from negative results, which affects the reproducibility of the test (Chappuis et al., 2006; Rijal et al., 2004). Boiling declines field applicability of the test (Hatam et al., 2009). There are no antigen detection systems currently available for CL and MCL (Singh and Sivakumar, 2003).

2.7.4 Molecular methods

Due to the limitations inherent in techniques used for detection of parasites, a variety of nucleic acid detection methods targeting DNA and RNA genes have been developed for leishmaniasis (Singh and Sivakumar, 2003). Amongst these, the PCR has proved to be a powerful approach to the application of molecular biology techniques to
the diagnosis of leishmaniasis. It is a highly sensitive and specific technique. Some of the advantages of this method when compared with other parasitological methods are: the possibility of detecting *Leishmania* DNA, even with a low parasite load; specificity; the fast availability of results; the possibility of using different biological materials; and the possibility of detecting the DNA of amastigotes and promastigotes. In addition to diagnosis, PCR may be used in prognosis of disease, strain identification of parasite and molecular epidemiology, detecting HIV-*Leishmania* coinfection and can also be utilized for determining the drug resistance. The PCR-based methods have been used on bone marrow, lymph node, and splenic aspirates (Mathis and Deplazes, 1995; Piarroux *et al*., 1996; Adhya *et al*., 1995; Andresen *et al*., 1997; Osman *et al*., 1997; Piarroux *et al*., 1994), as well as peripheral blood and serum samples (Hu *et al*., 2000).

Different primer sequences specific for different targets in the DNA of *Leishmania* have been used to improve parasite detection. Some are nuclear DNA such as the SSU rRNA gene, multilocus microsatellite DNA, some repetitive sequences, the tubulin gene, the *gp63* gene locus and internal transcribed spacer regions. Others are extrachromosomal DNA such as the repetitive kinetoplast DNA (kDNA). The latter is considered as an attractive target for PCR due to the abundance of minicircles in the parasite (Attar *et al*., 2001; Santos-Gomes *et al*., 2000; El Tai *et al*., 2001; Pizzuto *et al*., 2001; Wortman *et al*., 2001).

A highly sensitive and specific PCR assay based on kDNA sequences for detection of *L. donovani* provides a very reliable, non invasive test for diagnosis of both VL and PKDL in India (Salotra and Singh, 2006). Further, it permits simultaneous species identification of the parasite *L. donovani*. This assay was evaluated in an endemic area for diagnosis of Indian VL as well as the assessment of cure. The results of the study were very promising, showing 99 % sensitivity and 100% specificity and the potential to identify a successful disease outcome. In another study sensitivity was 100% in cutaneous leishmaniasis (Nasereddin *et al*., 2008) and 97.1% in MCL (Oliveira *et al*., 2005).

The PCR-SSCP technique has also been developed for the detection of sequence variation in rRNA genes within the *L. donovani* species (Singh and Sivakumar, 2003). In addition, it can be performed easily and rapidly from clinical samples without prior need of cultivation of the parasite. Real time-PCR (RT-PCR) using primers specific to the *Leishmania* genus or species has been evaluated more recently for the diagnosis of
leishmaniasis. It mainly aims to approach its polymorphism to identify *Leishmania* species but also to measure parasite load in the lesion (de Monbrison *et al.*, 2007; Antinori *et al.*, 2009; Castilho *et al.*, 2008). It is a very promising method and, although it requires the appropriate laboratory structure (expensive equipment and technical skill), the results are obtained much more quickly, with less likelihood of contamination, compared with conventional PCR (Goto and Lindoso, 2010).

### 2.8 Th1/Th2 Paradigm

In susceptible murine models of *L. major* infection, a clear Th1/Th2 polarization with distinct patterns of cytokine production by CD4+ T cells, *i.e.*, IFN-γ/IL-2 associated with resistance and IL-4/IL-10 associated with susceptibility was determined (Heinzel *et al.*, 1989). However, many leishmanial antigens, which stimulate a Th1 immune response during the disease or even after the disease is cured, have been shown to have no protective action. Paradoxically, antigens associated with an early Th2 response have been found to be highly protective if the Th1 response to them is generated before infection (Campos-Neto, 2005). For example, the Ldp23 antigen produces high levels of IFN-γ and undetectable amounts of IL-4, a typical Th1 response (Campos-Neto *et al.*, 1995). However, vaccination with Ldp23 in combination with adjuvants that are well known to induce Th1 responses, such as IL-12 and monophosphoryl lipid A plus squalene (MPL-SE), provides no protection in BALB/c mice, despite stimulating a strong antigen-specific Th1 response in the absence of any detectable Th2 response (Campos-Neto *et al.*, 2002). This lack of reciprocity with protective immune response was also observed in other leishmanial antigens that have been selected on the basis of Th1/Th2 paradigm and using peripheral blood mononuclear cells from cured leishmaniasis patients as immunological readouts (Campos-Neto *et al.*, 2002; Probst *et al.*, 2001). For example, the LACK antigen produces a strong Th2 response that can be observed soon after infection of BALB/c mice with *L. major*. Despite this, LACK induces a significant protective immune response in BALB/c mice if injected in conjunction with adjuvants that stimulate Th1 responses (Mougneau *et al.*, 1995; Julia *et al.*, 1996). Similarly, a *Leishmania mexicana* cysteine protease antigen named CPB2.8 is a potent Th2-inducing molecule during experimental leishmaniasis. It also induces substantial protection if administered with adjuvants producing Th1 type of immune response (Pollock *et al.*, 2003). Another antigen, LmST11 induces mixed Th1/Th2 responses in *L. major* model of BALB/c mice.
The sera of these experimental models contain high titers of IgE, IgG1, and IgG2a anti-LmST11 antibodies (Webb et al., 1996). It also induces excellent protection in BALB/c mice and in monkeys when used in combination with IL-12 (Campos-Neto et al., 2001) or MPL-SE (Skeiky et al., 2002) as adjuvant. No sequence similarities were found between these two antigens (LACK and LmST11) and yet they were found to be equally involved in inducing a Th2 response in L. major model of BALB/c mice. Therefore, it seems that the biased Th2 response is not dependent on a particular molecular characteristic of leishmanial antigens. Therefore, finding disease-associated Th2 antigens and inducing a Th1 immune response to them using defined vaccination protocols is an interesting unorthodox alternative approach to the discovery of a Leishmania vaccine (Campose-Neto, 2005).

2.9 Immunology

Infection with L. donovani and L. infantum results in the establishment of the parasite in the liver, spleen and bone marrow in mice. In most mouse strains the liver is, the site of an acute resolving infection associated with the development of inflammatory granulomas around infected Kupffer cells and resistance to re-infection. In contrast to the liver, parasites persist in the spleen. Persistent parasites are characterized by lack of granuloma formation, splenomegaly, enhanced hematopoietic activity and disruption of lymphoid tissue micro-architecture, the latter postulated to contribute to the immuno-compromised status of the host. Splenic pathology is linked to high levels of both TNF-α and IL-10. TNF-α mediates destruction of marginal zone macrophages and gp38⁺ stromal cells, while IL-10 is responsible for impaired DC migration into T cell areas and defective T cell priming. Furthermore, the altered stromal cell functions can promote development of IL-10 producing DC, with immuno-regulatory properties (Stanley and Engwerda, 2007).

Because Leishmania manipulates the complement system in order to expedite phagocytosis, humoral immunity (B cells and antibodies) is largely ineffective against the organism. This has been proven in many experiments. For example, the course of the infection is unaltered in animals that have been depleted of B-cells. High levels of Leishmania specific antibodies are observed in patients with VL and other severe forms of leishmanial disease and there are sufficient evidences that B cells and antibodies correlate with pathology (Sharma and Singh, 2009).
2.9.1 Neutrophils as Trojan Horses

It has been assumed that Leishmania infection is initiated by direct parasitization of skin resident macrophages (Locksley et al., 1988) whereas uptake by skin DCs has been linked to priming and shaping the T cell responses (Leon et al., 2007). However, in vivo imaging of sandfly transmitted L. major infection revealed that neutrophils are the first cells to be infected by the parasites (Peters et al., 2008) and are most crucial antimicrobial effector cells. They are also the first cells to be mobilized and arrive within hours to the site of tissue damage and parasite entry. Some factors released by the parasites such as IL-8, KC (Gro-α), MIP-2 (Gro-β), IL-17, TNF, Leishmania chemotactic factor (LCF) contribute to the initial neutrophil ingress during sandfly bite (Wozencraft and Blackwell, 1987). Infection of neutrophils is transient and within a week post infection the infected neutrophils gradually undergo apoptosis and are ingested by macrophages (Peters et al., 2008) highlighting another means by which macrophages are parasitized by Leishmania. Thus, parasitized neutrophils were suggested to function as a ‘Trojan horse’, to transfer Leishmania silently to macrophages. However, in human VL neutrophils have been reported to harbor parasites during active disease (Wickramasinghe et al., 1987; Pahwa et al., 2004). It may be noted that human blood is much more neutrophil rich than that of mice (Ganz, 2003). This study was supported by an in vitro study by Laskay and colleagues showing that Leishmania infected apoptotic neutrophils can be taken up by macrophages that allow parasites to thrive (Laskay et al., 2008; van Zandbergen et al., 2004). Apoptotic neutrophils are normally cleared without triggering activation of macrophages. Thus, uptake of infected apoptotic neutrophils could facilitate silent entry and infection of macrophages. Leishmania may also extend the life span of a neutrophil by delaying apoptosis, suggested to give monocytes time to infiltrate the site of infection and become infected by apoptotic neutrophils (van Zandbergen et al., 2004). Further it has also been shown that neutrophils can express a large number of factors including chemokines and cytokines that could influence the microenvironment at the site of infection and thus the subsequent immune response (Nylen and Gautam, 2010).

Nevertheless, when appropriately activated, neutrophils can kill Leishmania and there are several reports suggesting that neutrophils play a role in early protection against leishmanial disease (Lima et al., 1998; McFarlane et al., 2008; Rousseau et al., 2001). For example during cutaneous leishmaniasis, neutrophils extracellular traps (NETs)
facilitate the induction and killing of promastigotes. The same would appear to be the case for amastigotes, albeit not to the same extent as promastigotes. Furthermore, neutrophils were found to kill *L. donovani* amastigotes in a mouse model of infection (Nylen and Gautam, 2010).

The capacity of neutrophils to function as immune evasion target probably depends on the genetic background of the host, the parasite strain and the developmental stage of the parasite used (Ritter *et al.*, 2009). While metacyclic promastigotes may survive in neutrophils, non-metacyclic ones can rapidly be killed. In both human and murine leishmaniasis neutrophils are prominent infiltrates in lesions (Donnelly *et al.*, 1998; Palma and Saravia, 1997). Their presence at the site of infection can cause immune mediated tissue pathology (Smelt *et al.*, 2000; Lopez Kostka *et al.*, 2009).

### 2.9.2 Role of Macrophages

Promastigotes are phagocytosed by macrophages, either directly through receptor mediated endocytosis or after infection of neutrophils initially recruited to the sandfly bite (van Zandbergen *et al.*, 2004). After binding to the macrophage cell surface, promastigotes are endocytosed into a phagosome known as a parasitophorous vacuole, which undergoes a series of fusion events to become a phagolysosome (Lodge *et al.*, 2006). Macrophages possess potent antimicrobial functions and activated macrophages can kill *Leishmania*. To survive, the parasite needs to avoid macrophage activation and recognition by T cells. The survival in macrophages is partly stage specific with metacyclic promastigotes having better capacity to survive in comparison to procyclic promastigotes (Ueno *et al.*, 2009). The parasites use several strategies by which macrophage activation can be prevented. One of these strategies includes silent entry utilizing macrophage receptors such as CR1 and CR3 (Rosenthal *et al.*, 1996; Wanderley *et al.*, 2006) which bind to complement proteins C3b and C3bi respectively. Entry through these receptors provides a survival advantage, since CR1 and CR3 promote phagocytosis without prompting an oxidative burst (Oliviera *et al.*, 2005). Another benefit to using the CR3 receptor is that it inhibits IL-12 induced cell-mediated immunity, thus protecting the promastigote (Dey *et al.*, 2007). *L. major* and *L. donovani* use the CR3 receptor (Antoine *et al.*, 2004). *Leishmania* amastigotes also use other receptors to gain entrance into the macrophage. *L. major* and *L. mexicana* amastigotes have both been shown to use immunoglobulin opsonization as a means of entering the macrophage.
through the Fc receptor. *L. amazonensis* can bind to heparin sulfate and a fibronectin receptor because the surface protein gp63 appears to mimic fibronectin (Oliviera *et al.*, 2005). *L. donovani* can bind to the mannose–fucose receptor. *L. major* can attach a lectin-like receptor that recognizes LPG (McConville *et al.*, 1995). The ability to use a variety of different receptors makes it easier for the promastigotes to enter the macrophages.

Macrophage tries to destroy the parasite by these hydrolytic and acidic enzymes. The attack of these enzymes occurs through phagosome–endosome fusion. The *Leishmania* have adapted a survival strategy which makes it resistant to this acidic attack. It has a proton pump in the surface which allows its intracellular pH to remain close to neutral. Lipophosphoglycan (LPG) repeating units present on the protozoan membrane play an active role to inhibit phagosome–endosome fusion. LPG may act as a degradation barrier because of its highly anionic nature and its unique galactose-β1,4-mannose linkages within the repeating units. These surface glycoproteins are resistant to host lysosomal enzymes and may destroy them (Cunningham, 2002). The gp63 protease, which exhibits optimal activity under the acidic conditions of the phagolysosomes, has been shown to degrade lysosomal enzymes, as well (Cunningham, 2002).

The other strategies utilised by the parasite to survive in macrophages include: more direct inhibition of macrophage function by interfering with NFB transcription and IL-12 production, down regulation of MHC class II and promoting production of regulatory cytokines like IL-10 and TGFβ (McDowell and Sacks, 1999; Kim, 2007). In addition to repressing the microbicidal activities of the host macrophage, *Leishmania* inhibits the ability of the host cell to display parasite antigens to other components of the immune system (Reiner *et al.*, 1987). *L. donovani* inhibits antigen presentation by repressing major histocompatibility complex (MHC) class II gene expression, both basal and particularly following stimulation with IFN-γ (Oliviera *et al.*, 2005).

### 2.9.3 Role of Dendritic cells

Interactions between dendritic cells (DC) and *Leishmania* parasites are complex, and involve paradoxical functions that can stimulate or halt T cell responses and may lead to cure or exacerbation of disease (Soong, 2008). Activation of DC varies in magnitude and profile depending upon developmental stage and the species/strain of *Leishmania* as well as DC cell subset, serum opsonization and exogenous stimuli involved in different studies (Soong, 2008). Vanloubbeeck and Jones revealed that DCs have a unique and
central role in pathogen-specific immunity in that they present antigens to CD4+ cells on MHC class II as well as presenting them to CD8+ cells on MHC class I. This is known as cross-priming and is important because both CD8+ and CD4+ cells are considered to be involved in the immune defense against *Leishmania* (Vanloubbeeck and Jones, 2004). DC play an essential role in conferring resistance or susceptibility to *Leishmania* by driving the differentiation and proliferation of CD4+ cells to either Th1 or Th2 cells. On presentation of *Leishmania* antigens to CD4+ cells, the concomitant secretion of IL-12 drives the proliferation of IFN-γ secreting Th1 cells and NK cells, which activate macrophages and inhibit Th2 responses (Von Stebut et al., 1998). Conversely, the secretion of IL-4 during antigen presentation to CD4+ cells drives Th2 cell development that inhibits Th1 responses and promotes B lymphocyte growth and development. Therefore, it is evident that DC play an essential role in both initiation and regulation of antimicrobial immune responses to *Leishmania* (Schleicher et al., 2007).

There are several subtypes of human and mouse DCs depending upon surface antigen differences. In human beings, they include CD11c+ myeloid DCs (DC1) circulating in the blood, CD11c- plasmocytoid DCs (DC2), which are found in lymphoid organs and Langerhans cells (LCs) in the skin. Several subtypes of murine DCs can be characterised on the basis of the expression of CD8, CD4, CD205 and CD11b surface markers (Shortman and Liu, 2002). Human and mouse myeloid and plasmocytoid DC subsets show partly different functions. Moll first demonstrated that murine epidermal LCs are the important cells for sensing, uptake and transport of *Leishmania* to the lymph nodes (Moll et al., 1995). Further studies have shown that dermal DCs are involved in the early recognition of the parasite. They can efficiently take up and incorporate parasites in vacuoles (Ng et al., 2008) and have been suggested by some to act as principal antigen presenting cells in leishmaniasis (Ritter et al., 2004) while others suggest lymph node resident DC as the initiators of the immune response (Iezzi et al., 2006). DCs are the main source of IL-12 in early *Leishmania* infection. The effect of *Leishmania* infection on IL-12 induction and DCs maturation may vary according to DC subtype and to *Leishmania* species (Nylen and Gautam, 2010).

### 2.9.4 Role of Natural Killer (NK) Cells

Together with phagocytes, NK cells represent sentinel components of the innate immune response to infectious pathogens (French and Yokoyama, 2003; Korbel et al., 2004). They act by two principal mechanisms, cytolytic destruction of host cells infected
with certain viruses, bacteria, or protozoa and secretion of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α) and the maturation of DCs (Moretta, 2002; Degli-Esposti and Smyth, 2005). During early experimental studies in C3H/HeN mice it was indicated that IFN-γ production by NK cells was important for control of leishmaniasis. During subsequent studies in C57BL/6 mice it was demonstrated that NK cells are not required for generation of adequate Th-1 response and protective immunity. Their presence however, may delay onset of disease as BALB/c mice lacking NK cells develop lesions faster and harbor more parasite (Nylen and Gautam, 2010; Laurenti et al., 1999). Thus, NK cells have a direct protective role during early phases of *Leishmania* infection through their ability to rapidly respond with IFN-γ production (Becker et al., 2003).

2.9.5 Role of CD4+ cells

Cell mediated immunity is known to play a central role in the host response to control leishmaniasis. CD4+ Th1 helper cells are a key component of this immunity (Nabors et al., 1995). Naive CD4+ T cells recognise antigen which has been processed and is presented in association with major histocompatibility complex (MHC) class II molecules expressed on specialised APCs (Banchereau et al., 1994). Specialised APCs are primarily dendritic cells, macrophages and B cells. Cell recognition of antigen involves direct cell-cell contact between the antigen specific surface receptor on the CD4+ cell and an MHC/peptide complex at the surface of a antigen presenting cell. After recognising antigen, naive CD4+ cells are activated to release large amounts of cytokines like IFN-γ, IL-2 and TNF-α (Kharazmi et al., 1999). These cytokines further interact with specific receptor molecules on their target cells like macrophages. By releasing cytokines in response to antigenic stimulation, CD4+ T cells are able to orchestrate an appropriate cell mediated immune response to infection. The interaction between antigen specific CD4+ T cells and macrophages forms the basis of the delayed type hypersensitivity response, which is one of the main cell mediated immune effector mechanisms. They are also known to exert cytolytic activity on Natural Killer cells (Pirmez et al., 1993) and sustain functional CD8+ T memory cells (Zwingenberger et al., 1990; Berman, 1998).

2.9.6 Role of CD8+ Cells

CD8+ cells were for a long time thought to play a secondary role as CD8+ cells alone could not induce protective immunity and CD8+ defective mice, were able to control infection (Wang et al., 1993). Belkaid and coworkers also demonstrated that
CD8<sup>+</sup> cells actually were required for healing when C57BL/6 mice were infected with a low and more physiological relevant, dose of parasites and in experimental infection with *L. donovani*. Both CD8<sup>+</sup> and CD4<sup>+</sup> cells can on their own prevent reactivation of disease (Belkaid *et al.*, 2002; Murray *et al.*, 2005).

CD8<sup>+</sup> cells appear to play multiple roles comprising both cytotoxic activity and secretion of cytokines and chemokines during the course of experimental infection. This activity was mediated by both the perforin and the Fas/FasL pathway, as judged from *in vitro* and *in vivo* assays. The CD8<sup>+</sup> cells also up-regulated mRNAs for cytokine (IFN-γ and TNF-α) and C-C chemokines, which have a major role in immunity against the pathogen. These cells have been found to be associated with both cure and pathology in human leishmaniasis. An expanded CD8<sup>+</sup> cell population was observed in the draining lymph node prior to ulcer development, implicating CD8<sup>+</sup> mediated immunity in the early containment of *Leishmania* infection (Bomfim *et al.*, 2007). It was found that CTL were involved in the elimination of *L. major* as well as establishment and maintenance of immunity as the inhibition of CTL with monoclonal antibodies rendered resistant mice susceptible to *L. major*. An increase in responding CD8<sup>+</sup> cells has been associated with cure of *L. braziliensis* (Coutinho *et al.*, 1998; Da-Cruz *et al.*, 1994). Exacerbated CD8<sup>+</sup> activity, in addition to a poor regulatory response, could however, underlie an unfavorable fate with regard to MCL. Basu *et al.*, (2005) demonstrated that CD8<sup>+</sup> T cells are vital in a protective response against *L. donovani* following hybrid cell vaccination, as the depletion of these cells resulted in a higher parasite burden in the spleen and liver. Recruitment of CD8<sup>+</sup> T cells expressing granzyme associated with lesion progression of CL caused by *L. braziliensis* and more CD8<sup>+</sup> cells were found in relapse cases (Faria *et al.*, 2009; Tuon *et al.*, 2008). Accumulation of CD8<sup>+</sup> cells has also been linked to PKDL (Ghosh *et al.*, 1995; Nylen and Gautam, 2010).

### 2.9.7 Role of Cytokines

#### 2.9.7.1 IFN-γ

IFN-γ is released by CD4<sup>+</sup> cells, CD8<sup>+</sup> cells and NK cells (Nylen *et al.*, 2003; Mosmann and Coffman, 1989; Sad *et al.*, 1995). It is a macrophage-activating cytokine and enhances its leishmanicidal activity. Resistant strains of mice with targeted disruption of either the IFN-γ or the IFN-γ-receptor gene are unable to restrict growth of *L. major in vivo* and suffer fatal infection (Belosevic *et al.*, 1990). In a number of studies,
recombinant IFN-γ was capable of activating infected macrophages from both resistant and susceptible mice to clear *L. major* in *vitro* (Reiner and Locksley, 1995). In visceral leishmaniasis also IFN-γ induces microbicidal activity against both the promastigotes and amastigotes of *L. donovani* in monocyte derived human macrophages, in H_{2}O_{2}-dependent (Murray *et al*., 1983) as well as in NO mediated pathway (Vouldoukis *et al*., 1997). During effective chemotherapy it increases uptake of antimony compounds by macrophages. In active human VL or DCL, PBMCs exhibit poor proliferative response to parasite antigens and fail to generate IFN-γ in *vitro*. This lack of IFN-γ production by PBMCs seems to predict progression of the infection into fulminant VL. In contrast, lymphocytes from patients cured of disease demonstrate a vigorous proliferative response and rapidly release IFN-γ, IL-2 and IL-12 on stimulation with parasite antigens *in vitro*. High expression of IFN-γ mRNAs in samples of the lymphoid organs and lesions from CL patients suggest that the immune system is highly activated (Harms *et al*., 1989). It is also produced in higher amounts by PBMC from MCL than from CL patients (Carvalho *et al*., 1985). It has been found to be effective in the chemotherapy of VL or CL patients (Badaro *et al*., 1990; Harms *et al*., 1989).

### 2.9.7.2 IL-2

IL-2 is produced by CD4⁺ T cells in the Th1 response to promastigotes attaching to reticuloendothelial cells (Piscopo and Mallia, 2006). It has been found to be fundamental in the regulation of T-cell dependent immune responses (Smith, 1984; Watson and Mochizuki, 1980; Malek, 2008). It mediates several lymphocyte functions (Farrar *et al*., 1980; Smith, 1984). In experimental visceral leishmaniasis, acquired resistance is T cell-dependent, involves IFN-γ activated macrophages, and is expressed in the tissues by granuloma formation. Resistance is also correlated with antigen-stimulated IL-2 secretion. Continuous administration of IL-2 to *L. donovani*-infected BALB/c mice reduced liver burdens by > 50% and led to marked accumulation of granuloma mononuclear cells. IL-2 increases IFN-γ mRNA expression *in vivo* and is required for IFN-γ secretion *in vitro*, and anti-IFN-γ mAb administration abolishes the antimicrobial effect of exogenous IL-2 (Murray *et al*., 1993). IL-2 was also shown to be associated with protective immunity against the leishmaniasis. (Nagill and Kaur, 2010; Kaur *et al*., 2011). The protective immune response was dominated by CD4⁺ Th1 cells secreting IFN-γ, IL-2 and TNF in resistant strains of mice during cutaneous leishmaniasis (Lonardoni *et al*., 2000).
2.9.7.3 IL-4

IL-4 is a pleiotropic cytokine secreted primarily by CD4+ cells which binds to high affinity receptors expressed on hematopoietic and non-hematopoietic cells (Paul, 1991). The IL-4 receptor is a heterodimeric molecule comprised of IL-4 alpha chain and the common gamma chain, which is shared by receptors for IL-2, IL-7, IL-9 and IL-15 (Beckmann et al., 1992; Keegan et al., 1994; Russell et al., 1993; Kimura et al., 1995; Giri et al., 1994). On T cells, IL-4 promotes the differentiation of naive T cells into IL-4-secreting Th2 cells and suppresses IFN-γ responses (Swain et al., 1990; Seder et al., 1992; Hsieh et al., 1992).

The skewing toward Th2 responses in vivo is thought to be involved in allergic responses and the susceptibility to infectious diseases including tuberculosis, malaria, AIDS, and leishmaniasis. The role of IL-4 in disease progression has been implicated in several studies in which the administration of anti-CD4 and anti-IL-4 antibodies healed Leishmania infection (Awasthi et al., 2004). Furthermore, it was demonstrated that the disruption of the IL-4 gene in susceptible BALB/c mice rendered them resistant to infection with L. major, a finding that clearly reveals the effects of this cytokine on disease progression (Kopf et al., 1996).

Although, IL-4 is reported to be present in the sera, PBMC supernatant (Zwingenberger et al., 1990; Sundar et al., 1997) or as mRNA in human VL (Carvalho et al., 1994; Ghalib et al., 1993; Kenney et al., 1998), there are reports that IL-4 is not always produced in VL patients (Carvalho et al., 1994; Cillari et al., 1988; Kemp et al., 1994) and that it has no immunomodulatory effect in downregulating the Th1 response during disease (Carvalho et al., 1994; Bacellar et al., 2000). However, these inconsistent detections of IL-4 in VL patients might be due to the fact that there are disease specific soluble IL-4 receptors in the serum of VL patients, which can neutralize both the bioactivity and immunologic detection of this cytokine (Sang et al., 1999).

2.9.7.4 IL-10

IL-10 seems to represent the main macrophage-deactivating cytokine in opposition to IFN-γ, being present in many different clinical presentations of human leishmaniasis. It has pleiotropic, primarily down-modulating, effects on innate as well as acquired immune responses. IL-10, however, is not a strict Th2 cytokine, since it can be
produced by macrophages, B cells and mast cells, besides Th2 cells experimental evidences indicate that IL-10 is intimately linked with disease progression of both murine and human *Leishmania* infection (Nylen and Sacks, 2007). Experimental models have clearly demonstrated the central role played by IL-10 in pathology and parasite persistence (Anderson et al., 2007; Kane and Mosser, 2001). In human VL, elevated levels of IL-10/IL-10 mRNA are found systemically as well as in spleen, bone marrow and lymph nodes and the levels decrease after successful chemotherapy (Karp et al., 1993; Ghalib et al., 1993; Kenney et al., 1998). A role for IL-10 in human VL pathology is supported by studies indicating that IL-10 blockade can enhance VL PBMC IFN-γ responses and inhibit VL serum promoted parasite replication in macrophages (Nylen et al., 2003; Carvalho et al., 1994; Ghalib et al., 1993). Recombinant IL-10 suppresses NO mediated killing of *L. infantum*, *L. major* and *L. braziliensis* in human macrophages, derived from the monocytes of healthy individuals (Vouldoukis et al., 1997). It had been shown that only lymphoproliferative response by cultures of PBMCs from VL patients was restored with a combination of exogenous IL-2 and IFN-γ, while both the lymphoproliferative responses and the IFN-γ production of leishmanial antigen were produced in a large amount with a combination of anti-IL-4 and anti IL-10 mAb. This clearly indicates that the inhibition of the *Leishmania* specific Th1 kind of response in the patients is the main reason for the disease susceptibility and this suppression is regulated by IL-10 (Carvalho et al., 1994). Decline in the level of IL-10 mRNA after successful chemotherapy therefore supports the fact that persistence of high levels of IL-10 in the host cells is beneficial for the parasite survival and pathology. In human CL, elevated IL-10 has been demonstrated in lesions (Bourreau et al., 2001; Bourreau et al., 2009; Salhi et al., 2008). A recent genetic analysis of IL10-819C/T polymorphism, in the IL10 promoter, showed that the C allele, which is linked to higher levels of IL-10 production, is associated with increased risk of developing cutaneous lesions in populations exposed to *L. braziliensis* (Salhi et al., 2008; Nylen and Gautam, 2010).

### 2.9.7.5 IL-12

IL-12 is a heterodimeric cytokine composed of two disulfide-linked subunits. IL-12 is produced by monocytes, macrophages, dendritic cells, neutrophils, and to a lesser extent B cells. One major action of IL-12 is its induction of other cytokines, particularly IFN-γ, which coordinate the ensuing immune response. IL-12 initially induces IFN-γ production in NK and T cells (Watford et al., 2003). IFN-γ stimulates the leishmanicidal
activity of phagocytic cells and, therefore, boosts the innate immune response. Moreover, IL-12 acts on DCs to induce further production of IL-12 (Grohmann et al., 1998) and promotes the maturation of skin langerhans cells. IL-12 enhances the cytolytic activity of NK and T cells. Consequently, neutralisation of IL-12 leads to disease exacerbation in L. major and L. donovani infections (Engwerda et al., 1998). A major function of IL-12 is its regulation of the adaptive immune response. Cytokines play a critical role in the developmental regulation of naive CD4+ T cells into either T helper 1 (Th1) or Th2 cells. Appropriate T helper cell development is essential for an effective adaptive immune response. Th1 cells produce IFN-γ and promote cell-mediated immunity, which is essential for the response against intracellular pathogens. IL-12 is the main cytokine that regulates Th1 differentiation and has a number of important actions that serve to promote cell-mediated immunity (Dong and Flavell, 2001; Glimcher, 2001; O'Shea and Paul, 2002; Farrar et al., 2002; Ho and Glimcher, 2002). IL-12 is shown to be a potent inducer of protective immunity in murine L. major infection (Heinzel et al., 1993; Sypek et al., 1993). Murine rIL-12 as an adjuvant with soluble Leishmania Ag has been shown to protect susceptible mice. L. major and L. donovani-susceptible mice are effectively cured by treatment with exogenous IL-12 (Heinzel et al., 1993; Murray and Hariprashad, 1995). In in vitro studies the parasite is able to inhibit IL-12 production by the infected macrophages (Reiner et al., 1994; Carrera et al., 1996). Treatment of susceptible animals with IL-12 renders them resistant (Murray et al., 1997, Heinzel et al., 1993; Sypek et al., 1993; Wang et al., 1994), and has also been used as an effective adjuvant for a killed vaccine for L. major (Afonso et al., 1994). In human leishmaniasis, expression of IL-12 mRNA has been shown in active CL cases (Melby et al., 1996). In VL patients, IL-12 enhances Th1 restoring lymphocyte proliferative responses, IFN-γ production and cytotoxic responses (Bacellar et al., 2000). IL-12 also decreases spontaneous or Ag-induced PBMC apoptosis in VL patients. When it is used in combination with Leishmania antigen it restores proliferation of PBMCs from VL patients more strongly than the use of anti-IL-4 or anti-IL-10 monoclonal antibodies or even of both monoclonals combined (Barral-Neto et al., 1998).

2.9.7.6 TNF-α

TNF was first named in 1975 as an endotoxin-induced factor that affected the necrosis of established tumors (Carswell et al., 1975). Tumor necrosis factor (TNF) is a proinflammatory cytokine that plays a key role in the pathogenesis of autoimmune
diseases and is an important constituent of the human immune response to infection (Tektonidou and Skopouli, 2008). Treatment with recombinant TNF resulted in reduced lesion size and parasitic burden (Titus et al., 1989) whereas the application of neutralizing anti-TNF antibodies caused a transient aggravation of the symptoms (Titus et al., 1989; Liew et al., 1990; Theodos et al., 1991; de Kossodo et al., 1994). Tumor necrosis factor-α (TNF-α) is important in regulation of leishmaniasis through activation of macrophage antimicrobial activity and granuloma formation (Murray et al., 2000). *Leishmania* promastigotes and amastigotes induce the synthesis and release of TNF in macrophages. TNF is essential for maximal expression of cytotoxic levels of nitric oxide which mediates the intracellular killing of *Leishmania* (Green et al., 1990). Anti-TNF-α treatment has resulted in the development of VL as a reactivation process in patients being treated for arthritis (Bassetti et al., 2006; Fabre et al., 2005; Romani-Costa et al., 2004). However, when produced at very high levels, TNF-α might have a disease-enhancing effect; one genetic study has identified a linkage between VL and a polymorphism in an allele associated with elevated serum TNF-α (Karplus et al., 2002). High levels of TNF-α might promote the generation of IL-10 producing T cells as a homeostatic response to excessive inflammation (Ato et al., 2002; Lundqvist et al., 2005). Reoccurrence of clinical symptoms of leishmaniasis occur as a result of blocking TNF which further supports the important role of TNF (De Leonardis et al., 2009; Franklin et al., 2009).

### 2.9.8 Role of T Regulatory (Treg) cells

T Regulatory cells have emerged as a dominant T-cell population capable of mediating peripheral tolerance to auto- and alloantigens, tumour antigens and pathogen-derived antigens. Persistent pathogens are known to establish chronic infections by engaging Treg cells that suppress host immunity and control excessive effector or immune responses (Belkaid et al., 2002; Campanelli et al., 2006; McKee and Pearce, 2004). They are a unique subset of thymus-derived T cells that constitute 5–10% of peripheral CD4+ T cells in normal animals and humans. They constitutively express surface CD25, the α-subunit of the interleukin-2 receptor (IL-2R), before activation (Sakaguchi et al., 1995) and the transcriptional regulator *Foxp3* (Brunkow et al., 2001; Hori et al., 2002; Khattri et al., 2003). Other molecules with constitutive expression on Treg cells are glucocorticoid-induced tumor-necrosis factor receptor (GITR) a family-related gene (Shimizu et al., 2002), cytotoxic T-lymphocyte antigen 4 (CTLA-4) (Read et al., 2000) and a chain...
(CD103) of the αEb7 integrin (McHugh et al., 2002). Treg cells require activation via T cell receptor to exert regulatory function and their suppressive reactivity is related to their ability to inhibit IL-2 production via a mechanism requiring direct cell contact (Thornton and Shevach, 2000) and possibly linked to CTLA-4 expression. Controversy still exists regarding the molecular mechanisms underlying Treg in vivo and in vitro function, although several studies have suggested the involvement of immunoregulatory cytokines such as transforming growth factor (TGF)-b1 (Nakamura et al., 2004) and IL-10 (Annacker et al., 2001; Asseman et al., 1999) in Treg suppressive activity in vivo. These cells play an important role in the regulation of immune responses and are also responsible for immunologic tolerance. It has been shown that Treg cells can control a large number of infections by modulating the intensity of the effector immune response (Belkaid and Tarbell, 2009), which also prevents the expression of immune mediated lesions. In a murine model of L. major infection, natural Treg cells favour parasite survival and expansion in a genetically resistant strain; these cells accumulate at sites of infection and locally control the expression of effector T cell functions. In susceptible mice, Treg cells prevent the early expression of lesions but bring about better control of the parasite in the long term (Belkaid et al., 2002). It has been postulated that Th2 cells are more susceptible to the inhibitory effect of CD4⁺CD25⁺ cells than the Th1 cells (Xu et al., 2003). This information strongly indicates that CD4⁺CD25⁺ T cells may have similar immunomodulatory role in human VL, which needs to be investigated (Saha et al., 2006).

2.9.9 Role of Chemokines

Chemokines and chemokine receptors have been shown to play a crucial role in determining the outcome of leishmaniasis. Chemokines are chemotactic cytokines that coordinate recruitment of leukocytes involved in homeostasis as well as in innate and adaptive immune responses. They are single polypeptides of about 67 to 127 amino acid residues in length (Moser and Willimann, 2004). Chemokines mediate their actions through binding of chemokine receptors, which are cell surface G-protein coupled receptors with seven transmembrane domains. Chemokine receptor engagement leads to numerous distinct signal transduction pathways ultimately resulting in a variety of biological functions including integrin activation and cell migration along a chemokine gradient (Viola and Luster, 2008). Some chemokines have been shown to regulate cell differentiation (Gu et al., 2000) and distinct patterns of chemokine secretion has been
observed in differentiated cells (Muller et al., 2003). Approximately 50 human chemokines and 20 chemokine receptors have been identified to date (Viola and Luster, 2008). These biological molecules have been shown to play a vital role in the regulation of immunity to various diseases (Del Rio et al., 2001; Jones et al., 2003; Rodriguez-Sosa et al., 2003). Indeed distinct chemokines and chemokine receptor-integrin combinations are associated with particular diseases and control effector cell migration to their respective infected tissue sites (Rot and von Andrian, 2004). Several studies have shown that chemokines play an important role in the generation of innate and acquired immunity against CL (Vester et al., 1999; Ritter and Moll, 2000; Rosas et al., 2005; Vasquez et al., 2008). The chemokines produced at the site of an infection are critical in determining the composition of infiltrating cells and defining the eventual outcome of the disease (Teixeira et al., 2006). This is evident in cases of localized versus diffuse American cutaneous leishmaniasis in humans caused by L. mexicana (Ritter and Korner, 2002). In localized cutaneous leishmaniasis (LCL) which is self healing, a Th1 chemokine profile is observed in the lesions consisting of CCL2, CXCL9 and CXCL10 and is associated with a concentrated dermal infiltrate comprising of macrophages and large numbers of CD4 positive cells. In contrast, the chemokine profile of lesions of chronic diffuse cutaneous leishmaniasis (DCL) is Th2 associated dominated by the expression of CCL3, and the dermal infiltrate is more diffuse with fewer CD4 positive cells (Ritter and Korner, 2002). In VL, some chemokines and chemokine receptors have a role in the development of the Th1 response because their deletion influences IFN-γ production by T cells. L. donovani infected mice undergo a rapid hepatic accumulation of MIP-1α, CCL2 and CXCL10 after infection (Cotterell et al., 1999). However, only CXCL10 expression, amplified by T cells, remains high during the late phase, and this is essential to enable liver granuloma formation and the inflammatory response (Hailu et al., 2004).

2.10 Vaccines

Current treatment of leishmaniasis primarily relies on chemotherapy, with some attempts at using immunotherapy (Ghosh et al., 2003; Santos et al., 2003; Borja-Cabrera et al., 2004). The drugs based on pentavalent antimonials are the first line of treatment in most of the endemic areas. However, its use is related to the emergence of resistance. The drugs such as amphotericin B and pentamidine are the second line of treatment which are characterized by high efficacy, but are relatively expensive and have severe side effects
The other drugs, such as the lipid formulations of amphotericin B are effective in the treatment of visceral leishmaniasis, but they are also very expensive (Berman, et al., 1998; Murray, 2004). A breakthrough has been the introduction of orally administered miltefosine for the treatment of visceral, cutaneous, and mucocutaneous leishmaniasis, but the relative high cost and concerns about teratogenicity and potential development of resistance have limited the use of this drug (Sundar et al., 2002; Croft and Engel, 2006). On the other hand the spread of HIV infections (AIDS), the widespread use of immunosuppressive drugs (in the case of organ transplantation) and cancer chemotherapy that compromises the immune system have lead to the sudden outburst of leishmaniasis infections. Thus, an effective and affordable vaccine remains the only realistic hope of controlling such a parasitic disease. Considering the severity of human kala-azar, the spreading of the epidemic and the limitations of chemotherapy, the development of immunoprophylactic vaccine against VL has been strongly encouraged by WHO (Courtenay et al., 1996).

The relatively uncomplicated leishmanial life cycle and the fact that recovery from a primary infection renders the host resistant to subsequent infections indicate that a successful vaccine is feasible. An ideal anti-leishmanial vaccine would need to possess several attributes, but not all of them may be easily achievable. These include; (1) Safety; (2) Affordability to the populations in need; (3) Induction of CD4⁺ and CD8⁺ T cell responses and long-term immunological memory that can be boosted by natural infections, thus minimising the number of immunizations; (4) Effectiveness against species causing CL and VL; (5) Stability at room temperature eliminating the need for a cold chain to preserve potency and; (6) Effectiveness as a Leishmania vaccine, prophylactic as well as a therapeutic vaccine. While the cost-effectiveness and safety issues can be relatively straightforward to resolve, the induction and maintenance of the required immune responses are much more difficult to solve and cross-species protection may not be achieved by the same vaccine. Despite the wealth of information regarding the genetics of the parasite and the experimental immunology of the disease, there is currently no vaccine against leishmaniasis. However extensive efforts have been made to search for an effective Leishmania vaccine during the past several decades.

In general, the development of Leishmania vaccines may be classified into: live Leishmania; including new genetically modified constructs, first generation vaccines
consisting fractions of the parasite or whole killed *Leishmania* with or without adjuvants and second generation vaccines (SGV) including all defined vaccines, *i.e.*, recombinant proteins, DNA vaccines and combinations thereof.

First generation vaccines include trials of using whole living virulent organisms, crude parasite preparations or killed *Leishmania* with or without adjuvants (Convit *et al.*, 1987; Castes *et al.*, 1994; Nascimento *et al.*, 1990; Antunes *et al.*, 1986; Santos *et al.*, 1999; Maingon *et al.*, 1995; Khalil *et al.*, 2000). These vaccines were developed against cutaneous leishmaniasis and resulted in variable protection ranging from good to no protection at all (Antunes *et al.*, 1986; Mayrink *et al.*, 1985; Genaro *et al.*, 1996; Sharifi *et al.*, 1998; De Luca *et al.*, 2001; Khalil *et al.*, 2000). These vaccines have been the subject of many investigations over several decades and are the only leishmaniasis vaccine candidates which have undergone phase III trials, even against human kala-azar, due to the ease of large-scale production and absence of toxicity. The most noticeable feature of the first-generation human vaccines is that a leishmanin skin test (LST) is used for candidate selection and for evidence of immunogenicity (Bettini *et al.*, 1983). Whenever the LST is performed, average vaccine efficiency is obtained among the individuals whose skin tested positive (Sharifi *et al.*, 1998; Khalil *et al.*, 2000), whereas no efficacy is detected in assays that did not include an LST (Velez *et al.*, 2005).

The thinking behind the second generation vaccines is that an effective antileishmanial vaccine needs to be consistent, safe, cost effective and it should mimic as many aspects as possible of the types of antigens and the microenvironment from which the antigens are delivered during natural infection. The vaccine should also consider some practical aspects. For example, vaccine should be delivered as a single, defined molecule to facilitate compliance with regulatory and manufacturing standards and to lower the overall production costs. Ideally, the vaccine should protect against multiple forms of leishmaniasis (Kedzierski *et al.*, 2006).

With the disease having 14 million clinical cases on a worldwide scale and 350 million people at risk of infection (WHO, 2006) urgent control measures are needed to stop the over 2 million infections reported each year. Cases of self cure in cutaneous leishmaniasis (CL), accompanied by immunity to reinfection, make vaccine development a feasible leishmaniasis control method. Over the years, some of the antigens tested
include live vaccines, killed vaccines, live-attenuated vaccines, recombinant and synthetic vaccines, naked DNA vaccines and vector-derived vaccines. However, so far, there is no vaccine against leishmaniasis in routine use anywhere in the world.

2.10.1 Historical Background

From ancient times it was known in western and south-western Asia, that natural infection with cutaneous leishmaniasis (CL) due to *L. major* is followed by a strong immunity to the disease (Bray and Modabber, 2000). Bedouin or some Kurdistani tribal societies traditionally expose their babies’ bottoms to sandfly bites in order to protect them from facial lesions. Another ancient technique practised in the Middle East has been the use of a thorn to transfer infectious material from lesions to uninfected individuals. With the establishment by Nicolle and Manceau in 1908 (Nicolle, 1908) of culture conditions able to support the growth of promastigotes, live organisms started to be used for vaccination. This approach later became known as “leishmanization” (LZ). The success of this strategy depended critically on the viability and infectivity of the injected organisms. Organisms which had lost virulence were shown to induce delayed-type hypersensitivity but did not protect from subsequent natural infection (Kellina, 1965). Leishmanization as a prophylactic vaccine was used in Israel in 1970s and in Iran in 1980s (Jaffe and Greenblat, 1991; Khamesipour et al., 2006). In Israel the programme was stopped mainly due to loss of infectivity of the parasite as a result of continuous subculturing as well as immunosuppression as seen by reduced responsiveness to diphtheria, pertussis, and tetanus (DPT) vaccine in children following LZ. Some individuals vaccinated with the virulent parasites developed small numbers of aberrant and intractible lesions that required long term medical treatment. Therefore this practice has also been abandoned in Iran mainly due to safety issues (Khamesipour et al., 2006). At present, Uzbekistan is the only country where this practice is in current use. This prophylactic vaccine is a mixture of live virulent *L. major* promastigotes and killed *Leishmania* promastigotes on the basis that such a mixture will be less virulent. The parasite is isolated from an active lesion to produce the vaccine each year to overwhelm the problem of loss of infectivity (Gafurov, 1999; Thakur and Kumar, 1992). Efforts have been made to improve safety of leishmanisation by the inclusion of drug-sensitive *Leishmania* mutants with suicide genes for controlled infection (Muyombwe et al., 1997; Davoudi et al., 2005), inclusion of genetically modified parasites that do not produce the pathology,
but induce protection against wild type parasites, inclusion of killed parasites to reduce the size and duration of lesions, or by using adjuvants that promote more rapid onset of anti-leishmanial immunity and swift cure of disease (Khamesipour et al., 2005; Tabbara et al., 2005). These mutant parasites have been developed by mutagenesis and selection (Daneshvar et al., 2003), gene targeting methods (either to introduce foreign genes into the genome or to derive deficient strains, known as knock-out mutants) to assess the function of individual genes or gene families (Cruz et al., 1991; Webb and McMaster, 1994; Joshi et al., 1998; Joshi et al., 2002).

2.10.2 Killed Leishmania vaccines

The early trials with killed Leishmania as a vaccine were conducted in Brazil in 1940s by Sales-Gomes followed by Pessoa and colleagues. They evaluated 3 doses of a polyvalent vaccine of 18 strains of Leishmania without any adjuvant and observed 80% efficacy (Genaro et al., 1996; Pessoa and Pestana, 1941; Sales Gomes, 1939). Beginning in the 1970s and 1980s, Mayrink and colleagues in Brazil and Convit evaluated two main vaccines, the pentavalent preparation known as Leishvacin® and the simplified monovalent L. amazonensis vaccine. These vaccines induced significant protection from natural infection (Mayrink et al., 1978; Mayrink et al., 1979; Modabber, 1995; Armijos et al., 1998; Genaro et al., 1996; De Luca et al., 1999). These studies also indicated that delayed type hypersensitivity skin test conversion can be used as a surrogate marker for protective immunity. Despite promising results, due to the problems in its production and standardization, the 5-strain Leishvacin® presented difficulties in preparation and registration. Additionally, subsequent studies demonstrated that 2 doses of L. amazonensis promastigotes killed by merthiolate and sonications have similar immunogenicity in mice as the 5-strain Leishvacin (Mayrink et al., 2002). Mayrink et al., in 1996 also demonstrated enhanced lymphocyte proliferation and significant protection against infection by Leishmania in Brazilian dogs that had received merthiolated, ultrasound-disrupted promastigotes of L. braziliensis together with Bacillus Calmette Guerin (BCG) in phase I and II clinical trials. Strong cellular proliferation to soluble Leishmania antigens (SLA) has also been reported in dogs vaccinated with autoclaved L. major promastigotes (ALM) plus BCG (Lasri et al., 1999).

In Colombia a monovalent vaccine of L. amazonensis along with BCG was found to be safe and immunogenic in clinical trials. This vaccine has undergone phase 3 trials
without BCG in army volunteers but was found to give no protection against natural infection with *L. panamensis* (Velez *et al*., 2005). In Ecuador a clinical trial was done to assess the safety, immunogenicity and efficacy of two intradermal doses of a locally prepared trivalent vaccine against two doses of BCG alone. The vaccine consisted of *L. braziliensis*, *L. guyanensis* and *L. amazonensis* promastigotes originally obtained from the lesions of patients plus BCG providing 73% efficacy (Armijos *et al*., 1998). In Iran a single dose of a formulation of autoclaved promastigotes of *L. major* plus low dose of BCG was tested in Iran against two different forms of cutaneous leishmaniasis without showing efficacy, except may be in those who converted their skin reaction (Momeni *et al*., 1999) to leishmanial antigens (leishmanin) (Sharifi *et al*., 1998). Same vaccine was tested in Sudan against visceral leishmaniasis in a clinical trial showing no significant protection against visceral leishmaniasis (Khalil *et al*., 2000). Convit and his group in Venezuela introduced their autoclaved *L. mexicana* + BCG for immunotherapy and/or immunochemotherapy (Convit *et al*., 1987). As in Venezuela with Convits vaccine, Mayrinks vaccine was also effective in reducing the dose of antimony required to achieve cure (Jackson *et al*., 2002). Based on these trials the vaccine was registered as an adjunct to antimony therapy in Brazil but not for prophylactic use. In Venezuela, autoclaved killed *L. mexicana* is now used to treat patients with CL. If the patient does not respond after three injections (2 months), then antimony treatment is initiated (Convit *et al*., 2003). In Ecuador, two doses of a vaccine composed of *L. amazonensis* and *L. mexicana* mixed with BCG induced 73 per cent protection (Armijos *et al*., 1998). In a recent study, immunization of either susceptible or resistant mice with either autoclaved *L. major* (ALM) or freeze–thawed-killed *L. major* (KLM) mixed with low dose of *Mycobacterium vaccae* increased protection defined by significantly smaller ulcer size in immunized mice compared with the PBS-injected control group (Keshavarz Valian *et al*., 2008). ALM mixed with BCG was tested in zoonotic (Momeni *et al*., 1999) as well as anthroponotic (Sharifi *et al*., 1998) foci of Iran. The results showed that vaccine was safe and acceptable but efficacy was only 38% with measureable IFN-γ production. In order to enhance the immunogenicity of the vaccine (ALM+BCG), ALM was adsorbed to alum (aluminum hydroxide), and the resulting alum-ALM was mixed with BCG just prior to injection. The vaccine alum-ALM + BCG protected Langur monkeys against visceral leishmaniasis (Misra *et al*., 2001). A single injection of alum-ALM mixed with BCG showed 70 per cent protection in canine leishmaniasis in Iran (Mohebali *et al*., 2004). In Sudan this safety-immunogenicity trial was repeated and essentially all participants responded
strongly with a single dose. An immunochemotherapy trial of 4 injections of 100 mg of alum-ALM+BCG combined with sodium stibogluconate (Pentostam) in chronic PKDL patients in Sudan was completed and encouraging results were obtained (Khalil et al., 2005).

Surprisingly, in view of the prophylactic vaccine studies discussed above, therapeutic trials of first generation leishmaniasis vaccine have shown very encouraging results. These trials made a significant contribution to improving the overall quality of vaccine investigation in the endemic countries where they were conducted, training personnel and identifying particular issues related to vaccine development in general and vaccines against leishmaniasis in particular (Convit et al., 1987; Convit et al., 2003; Machado-Pinto et al., 2002).

2.10.3 Genetically modified *Leishmania* vaccines

Genetically modified *Leishmania* are “knock-out” *Leishmania* species, which lack genes essential for long term survival in mammalian host. These parasites can undergo a limited number of replications in macrophages during a short life cycle, enough to generate a specific immune response causing abortive infection and no disease in man (Cruz et al., 1991). Permanent alteration of the genetic makeup of these parasites can be achieved using gene replacement through homologous recombination, a powerful method for altering and testing gene function (Capecchi, 1989; Cruz et al., 1991). *L. major* lacking dihydrofolate reductase - thymidylate synthase (*dhfr-ts*) induced protection against infection with either *L.major* or *L.amazonensis* in mouse model (Amaral et al., 2002; Titus et al., 1995; Cruz et al., 1991). But *dhfr-ts* mutants could not protect rhesus monkeys on challenge with virulent parasite. Vaccination with a genetically altered mutant of *L. donovani* reduces the growth of the parasite *in vitro* as well as in the macrophage due to overexpression of the gene *centrin* (*Ldcen*). The mutant phenotype has defects in cytokinesis in the amastigote form (Selvapandian et al., 2004). *L. major* mutants deficient for leishmanolysin genes showed normal development in macrophages, *in vitro*, they showed delayed lesion formation in susceptible BALB/c mice (Joshi et al., 1998; Joshi et al., 2002). *L. major* mutants that lack LPG1 (the gene encoding a galactofuranosyl transferase) showed attenuated virulence in mice (Spath et al., 2000) whereas the parasites that lack LPG2 (the gene encoding a Golgi GDP-Mannose transporter) persisted indefinitely at a low level in mice without displaying disease and
provided protection from virulent *L. major* challenge (Uzonna *et al.*, 2004). Such protection from virulent challenges in mice was also achieved after gene knockout for other genes: cysteine protease in *L. mexicana* (Alexander *et al.*, 1998; Souza *et al.*, 1994; Mottram *et al.*, 1996) and biopterin transporter in *L. donovani* (Papadopoulou *et al.*, 2002).

The use of genetically altered parasites is very attractive because they closely mimic the natural course of infection and may therefore lead to similar immune responses because many other parasite antigens are also delivered by the transient infection in comparison to the limited number possible with subunit or recombinant antigens (Handman, 2001). Moreover, the immune responses may be skewed even more towards a Th1 protective response than in natural infection because of the small load of antigen. The disadvantages of such vaccines are the logistics of their large-scale production and distribution in the field (Constant *et al.*, 1995; Metz and Bottomly 1999).

### 2.10.4 Purified *Leishmania* fractions

The antigens are made from purified/enriched components of whole cultured organisms, or from secretory/excretory macromolecules. Different antigens studied for vaccination are described below.

#### 2.10.4.1 FML

The fucose-mannose ligand (FML), a glycoproteic complex of *L. donovani* has been characterized as a major antigenic complex of this parasite species (Palatnik-de-Sousa *et al.*, 1994; Santos *et al.*, 1999; Santos *et al.*, 2002), which is present on the surface of the parasite throughout the life cycle (Palatnik-de-Sousa *et al.*, 1993). Its most immunogenic fraction, a glycoprotein of 36 kDa strongly protected BALB/c mice from *L. donovani* infection (Paraguai de Souza *et al.*, 2001). It strongly inhibits the *in vitro* infection of murine macrophages by promastigotes and amastigotes of *L. donovani* (Palatnik-de-Sousa *et al.*, 1989; Palatnik-de-Sousa *et al.*, 1993). This inhibition was found to be species-specific for the genus *Leishmania* (Palatnik-De-Sousa *et al.*, 1990) and was also found to be a potent immunogen in rabbits and mice (Palatnik-de-Sousa *et al.*, 1993; Palatnik-de-Sousa *et al.*, 1990; Santos *et al.*, 1999). Its proteic moiety (36 kDa) was cloned and expressed in *Escherichia coli* system as the nucleoside hydrolase of *L.*
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*donovani*, being strongly antigenic for *L.chagasi* infected dogs’s sera (Santana et al., 2002).

FML is also a sensitive, predictive and specific antigen used in serodiagnosis of human (Palatnik-de-Sousa et al., 1995) and canine visceral leishmaniasis (Borja-Cabrera et al., 1999). The kala azar patients sera specifically reacted with the 36 kDa glycoprotein (Palatnik-de-Sousa et al., 1996).

### 2.10.4.2 Leishmune

The FML-vaccine containing FML antigen and saponin was assayed in phase I–IIa trials in the isogenic CB hamster, Swiss albino mice and BALB/c mouse models of visceral leishmaniasis. An average 87.7% (P < 0.01), 84% (P < 0.001) and 85% (P < 0.01) protection was achieved in the BALB/c, Swiss Albino outbred and CB hamster models, respectively (Palatnik-de-Sousa et al., 1994; Santos et al., 1999). Later on this vaccine was licensed for prophylaxis against canine visceral leishmaniasis (CVL) in Brazil and became commercially available (Borja-Cabrera et al., 2008; Nogueira et al., 2005, Palatnik-de-Sousa et al., 2009). It is the first registered vaccine against CVL composed of purified fraction named fucose mannose ligand (FML), isolated from *L. donovani* promastigotes and uses saponin as adjuvant (Palatnik-de-Sousa et al., 1989; Palatnik-de-Sousa et al., 1993).

Phase III trial reported the efficacy of the Leishmune vaccine in Brazil, an endemic area both, for human and canine kala-azar (da Silva et al., 2001; Palatnik-de-Sousa et al., 1995; Borja-Cabrera et al., 1999). The vaccine induced 92–95% specific protection against natural visceral leishmaniasis highlighting the long lasting and strong immunoprophylactic effect. Vaccination reduced both morbidity and incidence of the canine disease. This effect lasted for at least 3.5 years and was concomitant with the reduction of the human incidence of the disease in the area (da Silva et al., 2001; Borja-Cabrera et al., 2002). In another study the FML antigen of *L. donovani* was administered in combination with either Riedel de Haen (R), QuilA, QS21 saponins, IL12 or BCG in an outbred murine model against visceral leishmaniasis (VL) (Santos et al., 2002). The QS21-FML and QuilA-FML groups achieved the highest IgG2a response. QuilA-FML developed the strongest DTH and QS21-FML animals showed the highest serum IFN-γ concentrations. The reduction of parasitic load in the liver in response to each FML-vaccine formulation was: 52% for BCG-FML, 73% for R-FML, 93% for QuilA-FML and

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79.2% for QS21-FML treated animals, respectively. Significant, specific and strong protective responses were achieved by FML-saponin vaccines against murine visceral leishmaniasis. Minor and non-specific protection was induced by BCG-FML. Although IL12-FML enhanced the specific antibody response and delayed type of hypersensitivity response (DTH) to promastigote lysate it failed to reduce the parasitic load of infected animals (Santos et al., 2002).

It has also been shown that the Leishmune has an immunotherapeutic effect when administered to L. donovani or L. chagasi infected dogs while they were still asymptomatic (Borja-Cabrera et al., 2004). Recently the immunotherapeutic effect of Leishmune was assessed in combination with allopurinol or amphotericin B/allopurinol in infected dogs. The immunotherapy promoted the control of the clinical and parasitological signs of canine visceral leishmaniasis thereby reducing the death rate (Borja-Cabrera et al., 2010).

2.10.4.3 LiESAp

Excreted/secreted antigen purified from defined-medium culture supernatant of L. infantum promastigotes (LiESAp) was employed to protect dogs against the experimental infection with intravenous metacyclic promastigotes of this species (Lemesre et al., 2005). Different dosages of the antigen in formulation with muramyl dipeptide were assayed in groups of 3–6 beagle dogs. None of the 9 dogs receiving 2 subcutaneous injections of 100 or 200 µg of antigen showed evidence of infection at a 14-month follow up, in contrast with 6/6 placebo-treated dogs (Lemesre et al., 2005).

2.10.5 Vaccination with Recombinant DNA-Derived Proteins.

2.11.5.1 Gp63-leishmanolysin

The most comprehensively studied anti Leishmania vaccine candidate is the surface-expressed glycoprotein gp63, or leishmanolysin. Gp63 is a conserved glycoprotein and is demonstrated to be the major surface antigen on all species of promastigotes analysed (Lepay et al., 1983; Colomer-Gould et al., 1985). Biochemical and molecular analysis have identified that gp63 is a zinc metalloprotease. It binds to the macrophage complement receptor 3 (CR3) mediating internalization of promastigotes (Russell and Wilhelm, 1986; Handman, 1999). Surface iodination, immunostaining and
biochemical analysis have demonstrated not only that gp63 is the most abundant protein on the surface of all species of *Leishmania* studied, but that its expression increases as promastigotes become more infectious (Kweider *et al.*, 1987; Grogl *et al.*, 1987; Kweider *et al.*, 1989; Ramamoorthy *et al.*, 1992; Brittingham *et al.*, 1995). It is an attractive vaccine candidate because of its expression in both promastigotes and amastigotes. Due to the great abundance, surface location, and proteolytic activity of gp63, much work has been done to define a role for gp63 in *Leishmania* virulence. These include studies implicating a role for gp63 in the attachment of promastigotes to macrophages (Russell and Wilhelm, 1986), as well as a role in the survival of phagocytosed promastigotes (Chaudhuri *et al.*, 1989).

The gp63 gene from *L. major* was cloned (Button and McMaster, 1988). Genomic organization of this gene was studied and it was revealed that it belonged to a family of tandemly linked genes. In *L. major*, there are 6 copies of the gene, 5 tandemly arranged and one additional copy 8 kb downstream of the tandem array (Button *et al.*, 1991). *L. donovani* has 7 copies of the gp63 gene, 5 tandemly arranged, and 2 downstream (Webb *et al.*, 1991). *L. amazonensis* possesses a more complex array of gp63 genes, with at least 10 copies of the gene, split into 3 distinct complexes, all occurring on the same chromosome (Medina-Acosta *et al.*, 1989). Soon after cloning a recombinant protein (rgp63) was developed and tested for immunogenic properties in several laboratories using a number of expression systems such as BCG, *Vaccinia* virus and *S. enterica* serovar *typhimurium* (Russel and Alexander, 1988; Olobo *et al.*, 1995). The results are conflicting, but the main evidence suggests a recombinant protein expressed in *E. coli*, gp63 failed to protect mice against *L. major* infection (Handman *et al.*, 1990). In contrast, immunization with the native protein purified form *L. major* led to protection of mice against challenge with either *L. mexicana* or *L. major* (Russell and Alexander, 1988; Rivier *et al.*, 1999). More over a Th2-type response was demonstrated in PBMC cultured from subjects with subclinical or cured VL and stimulated with rgp63 (Kurtzhals *et al.*, 1994; Vinhas *et al.*, 1994). A small-scale phase I and II vaccine trials were performed with rgp63 against *L. major* infection in a vervet monkey model (Olobo *et al.*, 1995). Three doses of the recombinant antigen were administered with BCG as adjuvant. After vaccination, PBMC from these animals neither proliferated nor produced IFN-γ following stimulation with antigen, and only partial protection was achieved after challenge with virulent *L. major* promastigotes. Successful vaccination was achieved using gp63
expressed in BCG (Connell et al., 1993; Abdelhak et al., 1995) and attenuated Salmonella (Yang et al., 1990; Xu et al., 1995; McSorley et al., 1997; Gonzalez et al., 1998). An oral vaccine against leishmaniasis was constructed by using *S. typhimurium* that expresses the *L. major* promastigote surface protease gp63. The vaccine was shown to be stable, capable of expressing the protein and to induce a protective Th1 type response in mice (Yang et al., 1990). These promising findings were overshadowed by variable (mostly negative) T cell responses in humans (Jaffe et al., 1990; Kemp et al., 1991; Mendonca et al., 1991; Russo et al., 1991) and the inability of T cells from mice immunized with gp63 to elicit protective responses in macrophages (Rivier et al., 1999). Nonetheless, researchers focused their efforts on gp63 DNA-based vaccines (described below) and gp63-derived synthetic peptides. The latter were successfully tested in an animal model of cutaneous leishmaniasis and triggered long-lasting T cell responses (Jardim et al., 1990; Spitzer et al., 1999). In another study in BALB/c mice, the intramuscular injection of a *L. major* gp63 plasmid DNA construct resulted in partial protection against *Leishmania* infection, where IFN-γ but not IL-4 was produced by immune CD4+ cells upon restimulation with parasite antigens, suggesting a bias towards a Th1 response (Walker et al., 1998; Xu and Liew, 1995). Gp63 was also used to pulse dendritic cells (DC) for investigating the potential of a DC-based subunit vaccine (Berberich et al., 2003; Tsagozis et al., 2004). Gp63-pulsed DCs were able to give protection to mice against infection, through a Th1 response (Flohe et al., 1998). It has been shown that mice immunized with DCs loaded with leishmanolysin were capable of controlling cutaneous leishmaniasis by *L. major* (Berberich et al., 2003). However, the selection of antigen used for pulsing the DCs is cyanical, since some gp63-derived synthetic peptides triggered protection while others resulted in exacerbation of disease accompanied by a switch to a Th2 profile (Tsagozis et al., 2004). A gp63 co-expressed with human CD40L has been found to be a potent inducer of IL-12 production (Chen et al., 2001). Vaccination with cells expressing both molecules partially protected C57BL/6 mice against *L. amazonensis* infection. Gp63 has been reported to be a major component of the Leishvacin vaccine tested in healthy army conscripts in Brazil (Nascimento et al., 1990). Protective efficacy of the Leishvacin components was tested in mice using a variety of antigenic preparations along with gp63 against cutaneous leishmaniasis by *L. (L.) amazonensis* (Mora et al., 1999). Gp63 was also the immunodominant component of the *L. donovani* mixture of promastigote proteins encapsulated in liposomes and tested for protection against experimental visceral leishmaniasis (Afrin et al., 2002). Recently it has been shown that
cationic liposomes, when used as vaccine adjuvant with the immunodominant 63-kDa glycoprotein (gp63) of *L. donovani* promastigotes, induced significant protection against progressive visceral leishmaniasis in susceptible BALB/c mice (Bhowmick et al., 2007). Cocktail of gp63 and Hsp70 has been shown to induce protection against *Leishmania donovani* in BALB/c mice (Kaur et al., 2011).

### 2.10.5.2 LACK

The LACK (*Leishmania* homologue for receptors for activated C kinase) antigen is a 36 kDa protein highly conserved among related *Leishmania* species expressed in both leishmanial life cycle stages (Mougneau et al., 1995). The protein belongs to the family of WD 40 repeat proteins restricted to eukaryotes and is involved in several regulatory functions. LACK is localised in the cytoplasm, bound to multiprotein complexes involved in DNA replication and RNA synthesis (Gonzalez-Aseguinolaza et al., 1999). The antigen is of interest as a vaccine candidate for the leishmaniasis because of its immunopathogenic role in murine *L. major* infection. LACK is a preferential target for the early anti-parasite immune response, driving the expansion of IL-4 secreting, disease-promoting T cells (Launois et al., 1997; Julia and Glaichenhaus, 1999). The early Th2 responses were diminished in susceptible BALB/c mice that were made tolerant to LACK leading to a healing Th1 response and control of *L. major* infection (Julia et al., 1996). LACK protein was cloned from an *L. major* expression library screened through recognition by a protective CD4+ T-cell clone (Mougneau et al., 1995). The LACK homologue in *L. infantum* has also been cloned (Gonzalez-Aseguinolaza et al., 1999). Despite its propensity to induce Th2 type immune responses, immunization with a 24-kilodalton portion of this antigen protected BALB/c mice against *L. major* when administered as a vaccine with IL-12 before infection (Mougneau et al., 1995) and recombinant LACK and IL-12 triggered short term protective responses, but failed to elicit long-term immunity (Gurunathan et al., 1998). A heterologous prime/boost vaccination with vaccinia virus expressing LACK was shown to confer protective immune responses and partial protection in homologous (Gonzalo et al., 2001) and heterologous (Gonzalo et al., 2002) challenge systems. Since coadministration of IL-12 appeared to be an essential part of experimental vaccination of mice with LACK, a vaccine trial was conducted using LACK expressing *Listeria monocytogenes* as a live recombinant vector. *L. monocytogenes* is a well-known inducer of IL-12 secretion by
macrophages and vaccination with recombinant bacteria induced the development of potentially protective IFN-γ secreting Th1 CD4+ T lymphocytes and partial protection against cutaneous leishmaniasis by *L. major* (Soussi et al., 2000).

2.10.5.3 **gp46/M2/Parasite Surface Antigen 2 (PSA-2)**

Another vaccine candidate tested in animal models is a glycoinositol phospholipids anchored membrane protein, gp46/M2 or parasite surface antigen 2 (PSA-2), that belongs to a multicity gene family present in all *Leishmania* species bar *L. braziliensis* (Jimenez-Ruiz et al., 1998; Murray et al., 1989; Lohman et al., 1990; McMahon-Pratt et al., 1992). PSA-2 consists of several leucine-rich repeat (LRRs) motifs that are shared with an unrelated surface proteophosphoglycan (PPG). LRRs function in protein-protein interaction (Montgomery et al., 2000). PSA-2 is involved in macrophage invasion through the interaction of its LRRs with CR3 (Kedzierski et al., 2004). *L. major* PSA-2 comprises three polypeptides with approximate molecular weights of 96,000, 80,000 and 50,000 (Handman, 1995a). The amastigote form expresses a distinct but closely related PSA-2 with molecular weight of 50,000 kDa. There is a great deal of similarity between PSA-2 proteins from different species, but there is a high degree of heterogeneity between different members of the family (Murray and Spithill, 1991; Symons et al., 1994). One of the PSA-2 genes was cloned and expressed in both *E. coli* and *L. mexicana* promastigotes. Vaccination with recombinant PSA-2 purified from *E. coli*, using *Corynebacterium parvum* as adjuvant, did not confer protection to C3H mice challenged with virulent *L. major*, in contrast with PSA-2 expressed in *L. mexicana*, which provided good protection. This study indicates that correct protein folding in recombinant antigens may be required to induce protective immunity. Immunization with *L. major* PSA-2 expressed episomally in *L. mexicana* promastigotes conferred protection against cutaneous leishmaniasis in mice (Handman et al., 1995b). It has also been shown that vaccination with native PSA-2 with *Corynebacterium parvum* as adjuvant protects mice from *Leishmania* through a Th1 mediated response, but the recombinant PSA-2 purified from *Escherichia coli* and administered in immuno stimulating complexes (ISCOMs) or mixed with *C. parvum* as an adjuvant, does not induce protective immunity despite the induction of Th1 responses. Both C3H and BALB/c mice showed good protection against *L. major* challenge when the DNA was administered as a prophylactic vaccine, but also significant healing from established *L. major* infection was seen when
the plasmid was given as an immunotherapeutic agent (Sjolander et al., 1998a). Protective vaccination was also achieved against *L. amazonensis* (Champsi and McMahon-Pratt, 1988; McMahon-Pratt et al., 1993). In another study, protection of susceptible BALB/c mice by vaccination with secreted/excreted *L. major* antigens was attributed to a combination of PSA-2 and LPG contained in the preparation (Tonui et al., 2004). However, the synergistic or additive effect of other molecules in the mixture could have enhanced the protective efficacy of PSA-2.

### 2.10.5.4 Cysteine Proteases (CPs)

Cysteine proteases are enzymes that belong to one of the four major classes of proteolytic enzymes and are produced by a variety of organisms including viruses (Allaire et al., 1994), bacteria, yeast, plants, helminths, insects, mammals and protozoa including *Leishmania* (Baker and Drenth, 1987; Shaw, 1990; Berti and Storer, 1995). In *Leishmania* the cathepsin L-like cysteine proteinases (CPs) belonging to the papain superfamily are thought to be good vaccine candidates due to their high immunogenicity and important role in host-parasite interaction (Wolfram et al., 1995; Rafati et al., 2003). Three classes of CPs have been identified; Type I (CPB), Type II (CPA) and Type III (CPC) (Robertson and Coombs, 1993). CPB is encoded by genes that occur in multicopy tandem arrays, both in *L. major* and *L. mexicana* (Rafati et al., 2001; Mottram et al., 2004). CPA is encoded by a single copy gene. CPA has been described in *L. major*, *L. mexicana*, and *L. infantum* (Rafati et al., 2001; Denise et al., 2006; Williams et al., 2006). Finally CPC is reported in *L. mexicana*, *L. major*, *Leishmania* (*L.*) *donovani*, and *L. chagasi*, which is also encoded by a single copy gene (Somanna et al., 2002; Mottram et al., 2004) and present in all life cycle stages of *Leishmania* (Rafati et al., 2003). Furthermore, an unusual 100 amino acid C-terminal extension that is frequently glycosylated is a distinctive feature of these CPs (Souza et al., 1992). CPs are predominantly expressed and active in the amastigote form and to a lesser extent in metacyclic promastigotes. Furthermore, *Leishmania* cannot grow within macrophages in the presence of CP inhibitors. These observations suggest that CPs are necessary for successful intracellular parasitism (Mottram et al., 1998). The parasite’s enzymes might also modulate cytokine production in a way that is beneficial to the parasite itself. They are reported to stimulate an increase in the expression of IL-4 and IL-1 (Finkelman and Urban, 1992), driving the differentiation of the CD4 precursors towards the Th2 phenotype and thus favoring the proliferation of the parasite. Amastigote replication may also be promoted through the
decreased production of nitric oxide that results from the down-regulation of nitric oxide synthase by the transforming growth factor-β (TGF-β) that is secreted in large amounts by *Leishmania*-infected macrophages (Barral et al., 1993). It is possible that leishmanial CPs are involved in this production of TGF-β. CPA and CPB have been administrated in experimental vaccination in both mouse and dog model and have shown acceptable level of protection (Rafati et al., 2005; Rafati et al., 2006; Pinto et al., 2000; Ferreia et al., 2008). Immunization of mice with recombinant cysteine proteinase induced high production of IFN-γ and offered partial protection against *L. major* challenge (Rafati et al., 2000). More detailed vaccination studies with CPB and CPA demonstrated that only recombinant CPB, but not CPA was able to trigger immune responses that partially protected experimental animals against challenge, and protection depended on IFN-γ producing CD8+ T cells (Rafati et al., 2002). *L. mexicana* CP is a T-cell immunogen, resulting in the development of potentially protective Th1 cell lines (Wolfram et al., 1995). This finding suggests that the CP itself is a vaccine candidate and that homologous enzymes in other species may also be so. A similar *L. amazonensis* CP provided some protection against subsequent challenge, apparently through inducing a Th1-associated response (Beyrodt et al., 1997). A hybrid fusion protein composed of CPA and CPB was used to immunize mice and partial protection against *L. major* infection was obtained (Zadeh-Vakili et al., 2004). Protection was similar to that obtained by immunization with CPB alone (Rafati et al., 2002), but much higher levels of IFN-γ were detected upon immunization with the hybrid protein, pointing towards a dominant Th1 response. In other leishmanial species, a homologue of cysteine proteinase (A2) from *L. pifanoi* partially protected mice against homologous challenge. A CP of *L. pifanoi*, however, provided rather little protection for the host against infection with the parasite (Soong et al., 1995) and inclusion of recombinant *L. mexicana* cysteine proteinase 5 in a cocktail with gp63 and acid phosphatase triggered protective responses in C57BL/6 mice, but not BALB/c or CBA/J mice (Aebischer et al., 2000). The protective effect of CPs has also been assessed in visceral leishmaniasis (VL) in the canine model (Rafati et al., 2003). Immunization with CPB and CPA from *L. infantum* delivered as a combination of DNA and protein showed good efficacy (Rafati et al., 2005). A recombinant cysteine proteinase from *L. chagasi*, rLdccys1, is found to be a suitable immunological marker for different stages of VL in humans and dogs and provided high sensitivity and specificity for serodiagnosis of human VL by ELISA assay (de Souza Dias et al., 2005; da Costa Pinheiro et al., 2005). In 2009 the rLdccys1 antigen was assessed in DTH and ELISA assays to evaluate its ability in the discrimination of the clinical and subclinical forms of
VL and serodiagnosis in naturally infected dogs living in an endemic region in Brazil and was found to be potentially useful for diagnosis of canine leishmaniasis (Pinheiro et al., 2009).

2.10.5.5 LCR1

LCR1 is another recombinant protein containing 67-amino acid repeats homologous to repeats in a *Trypanosoma cruzi* flagellar polypeptide. It has originated from an *L. chagasi* amastigote cDNA library screened by a double-antibody T-cell technique (Wilson et al., 1995). The antigen stimulated proliferation of splenic T-lymphocytes from *L. infantum*-infected C3H and BALB/c mice, and induced IFN-γ but not IL-4, IL-5, or IL-10 secretion. Immunization with LCR1 partially protected BALB/c mice against challenge with *L. infantum*. Immunization with BCG-LCR1 elicited better protection than the protein alone, but protection depended on the site of immunization, subcutaneous delivery being better than intra-peritoneal (Streit et al., 2000).

2.10.5.6 HASPB1

Recombinant hydrophilic acylated surface protein B1 (HASPB1) is a member of a family of proteins expressed only in metacyclic and amastigote stages of development of several *Leishmania* species. rHASPB1 is able to confer protection in the mouse model against experimental challenge with *L. donovani*. Interestingly, protection did not require any adjuvants and seemed to be correlated with the presence of rHASPB1 specific IFN-γ producing CD8+ Th cells (Stager et al., 2000). The protective efficacy of *Leishmania* recombinant proteins histone 1 (H1) and hydrophilic acylated surface protein B1 (HASPB1) was assessed in beagle dogs against infection challenge with intravenous *L. infantum* promastigotes. Dogs were infected 45 days after immunization (Moreno et al., 2005). At a 16-month follow up, 6/8 untreated control dogs showed symptomatic leishmaniasis, in contrast with 4/8 dogs immunized with either HASPB1 or the combination of HASPB1 plus H1, and 3/8 dogs immunized with H1 antigen (Moreno et al., 2005).

2.10.5.7 A2

A2 proteins are predominantly expressed in the amastigote stage of *Leishmania* parasites, first described in *L. donovani*. The A2 genes are present within a multigene family and the corresponding A2 proteins are composed of predominantly multiple copies.
of a 10 amino acid repeat sequence. The A2 proteins are abundant in amastigotes and range in molecular weight from 42 to 100 kDa depending on the number of repeats within each protein species. These proteins are required for *Leishmania* survival in mammalian hosts (Charest and Matlashewski, *et al.*, 1994; Zhang and Matlashewski, 1997; Zhang and Matlashewski, 2001). It was also demonstrated that A2, in DNA or protein formulations, can protect mice against *L. donovani*, *L. amazonensis* and *L. chagasi* (Ghosh *et al.*, 2001; Coelho *et al.*, 2003; Zanin *et al.*, 2007). A2-specific antibodies have been detected in sera from patients with active VL, confirming that A2 proteins are expressed during active infection (Ghedin *et al.*, 1997; Carvalho *et al.*, 2002). It has been shown that generation of A2 deficient *L. donovani* resulted in avirulent amastigotes, which could not survive in a mouse model but were able to multiply in axenic culture as promastigotes (Zhang and Matlashewski, 1997). Antibody responses to A2 are also observed in sera of infected dogs (Carvalho *et al.*, 2002), including symptomatic and asymptomatic animals, though higher numbers of asymptomatic animals are reactive as compared with the symptomatic dogs (Porrozzi *et al.*, 2007). In addition, A2 was identified as capable of stimulating *in vitro* CD4⁺ and CD8⁺ cells from infected C3H/HeJ mice, during a systematic screening of *L. chagasi* amastigote antigens (Martins *et al.*, 2006). Moreover, in a recent study, comparing different proteins for diagnosis of leishmaniasis in dogs, A2 showed the best results to identify asymptomatic dogs, suggesting that it is associated with protective immunity (Porrozzi *et al.*, 2007).

### 2.10.5.8 P4 and P8

P4 and P8 are amastigote-specific membrane proteins which have been used to induce protection against *L. pifanoi* challenge (Soong *et al.*, 1995). P4 is a membrane-associated, single strand-specific nuclease present in all *Leishmania* species (Sujata *et al.*, 2000). P4 selectively elicited Th1-like responses in peripheral blood mononuclear cells from patients infected with *L. braziliensis* (Haberer *et al.*, 1998). CD4⁺ T cells producing IFN-γ, lymphotoxin and macrophage migration inhibitory factor played a dominant role in protection against *L. pifanoi* in animals vaccinated with P4 (Kar *et al.*, 2005). The function of P8 is not known, but the *L. pifanoi* polypeptide conferred complete protection in a heterologous challenge system against *L. amazonensis* in CBA/J mice and partial protection in BALB/c mice (Soong *et al.*, 1995). T cell recall responses were also triggered in patients with *L. braziliensis* and *L. amazonensis* infections by P8 antigen (Silveira *et al.*, 1998).
2.10.5.9 P0

*Leishmania* infantum* acidic ribosomal P0 protein (rLiP0), a structural component of the large ribosome subunit has been described as an immunodominant antigen recognized by sera from both patients and dogs infected with *L. chagasi-L. infantum* (Skeiky *et al.*, 1994; Soto *et al.*, 1995b). It has been shown that genetic vaccination with this antigen induces protective immunological effector mechanisms against *L. major* infection in the BALB/c mouse model. Protection was associated with priming of both CD4+ and CD8+ T cells, which, with the *L. major* challenge, were boosted to produce significant levels of IL-12-dependent, antigen-specific IFN-γ (Iborra *et al.*, 2003). In addition, the C-terminal region of the LiP0 is present in a multicomponent protein that when administered to dogs in combination with BCG as adjuvant, confers protection against *L. infantum* infection (Molano *et al.*, 2003). In another study a vaccine consisting of *Leishmania* ribosomal P0 protein plus MPL® as adjuvant achieved partial protection against *L. donovani* challenge in hamsters that correlated with a decrease in IL-10 expression (Bhardwaj *et al.*, 2009).

2.10.5.10 Leishmanial Histones

Histones are evolutionarily conserved proteins which associate with DNA to form the chromatin structural unit in eukaryotes, the nucleosome. *Leishmania* histones are intracellular components that behave as pathoantigens related to the virulent *Leishmania* phenotype and pathological stage of disease (Chang and McGwire *et al.*, 2002). The first report of the elicitation of a humoral immune response against parasite histones during infection was made in 1995, in which a response against *Leishmania infantum* H2A during canine visceral leishmaniasis (CVL) was described (Soto *et al.*, 1995b). *Leishmania* histone H2B was able to stimulate the production of IFN-γ in a T cell clone established from an immune donor (Probst *et al.*, 2001) and parasite histones H2B, H2A and H3 induced proliferation and IFN-γ production by peripheral blood mononuclear cells (PBMCs) from CL patients (de Carvalho *et al.*, 2003). Histones H2A and H3 have been shown to be more antigenic and prevalent immunogens than histones H2B and H4 since they are highly recognized by the sera from *Leishmania* infected dogs (Soto *et al.*, 1995b; Soto *et al.*, 1996; Soto *et al.*, 1997; Soto *et al.*, 1999). Genetic immunization of BALB/c mice with any one of the plasmids encoding a pair of histones (H2AH3, H2BH4, H2AH4 or H2BH3) the individual histones (H2A, H2B, H3 and H4) only resulted in a delay in lesion development at the site of infection but it could not induce protection.
The protection was only associated with a significant reduction in the cytokine levels of IL-4. In contrast, immunized mice with cocktails of plasmids encoding the four histones developed a specific Th1 immune response, which was associated with an antigen specific production of IFN-γ and a limited humoral response against histones. Both CD4+ and CD8+ T cells contributed to the resistance of vaccinated mice to CL in these experiments (Iborra et al., 2004). These results provide direct evidence that all four nucleosomal histones of *Leishmania* are necessary to maintain complete protection against *L. major* reinfection. Recombinant histone H1 antigen or a long synthetic peptide representing the complete *L. major* histone H1 sequence, each formulated with Montanide ISA 721 as adjuvant, did not produce significant protection in African green monkeys against CL (Masina et al., 2003). Very recently *Leishmania* histone H2B has been reported to be a promising candidate for both vaccination and serodiagnosis. Cellular immune responses were induced by H2B and its divergent amino-terminal (H2B-N) and conserved carboxy-terminal (H2B-C) regions in individuals with a history of Localized Cutaneous Leishmaniasis (LCL) due to *L. major*. H2B induced significantly high PBMC proliferation and IFN-γ levels in LCL individuals whereas significantly lower proliferation and IFN-γ levels were observed with the divergent part of the protein. All proteins induced IL10 in LCL and healthy individuals. Humoral responses were also induced by these proteins in patients with Mediterranean Visceral Leishmaniasis (MVL) due to *L. infantum*. H2B and H2B-N were highly recognized by MVL sera. These results show that the entire H2B protein is more efficient than its amino- and carboxy-terminal regions in inducing a dominant Th1 profile in cured LCL subjects and suggest that this protein may constitute a potential vaccine against leishmaniasis. Furthermore, H2B and H2B-N are shown to be interesting antigens for serodiagnosis of MVL (Meddeb-Garnaoui et al., 2010).

### 2.10.5.11 PapLe22

The PapLe22 (22-kDa potentially aggravating protein of *Leishmania*) antigen is a protein of unknown function, which localises to the promastigote nucleus. It is recognised by T cells from visceral leishmaniasis patients (Suffia et al., 2000). Although PapLe22 DNA immunization led to a marked decrease in parasite burden in immunized outbred golden hamsters (Fragaki et al., 2001), it induced IL-10 production in peripheral blood mononuclear cells from visceral leishmaniasis patients indicating that in humans it might...
actually contribute to pathogenesis (Suffia et al., 2000). Therefore, its use as a vaccine would need to consider the possibility that it may exacerbate disease. PapLe22 vaccine may be able to protect if the vaccine formulation would redirect T cell responses towards Th1 type responses.

2.10.5.12 ORFF

The leishmanial antigen ORFF (The open reading frame F) appears to be a novel and a Leishmania specific protein since its amino acid sequence has no sequence homology to any known protein in the database (Sunkin et al., 2001). It is differentially expressed in the promastigote and amastigote stages of the digenetic parasite (Ghosh et al., 1999). It is encoded by a single copy gene located on the chromosome 35 as a part of the multigene LD1 locus. ORFF has an important role for the parasite survival. Recombinant ORFF protein (rORFF) has been found to be a differential diagnostic and a promising vaccine candidate (Raj et al., 1999; Dole et al., 2001; Sukumaran et al., 2003; Tewary et al., 2004a; Tewary et al., 2004b; Tewary et al., 2005).

2.10.5.13 KMP

Kinetoplastid membrane protein-11 (KMP-11) is a highly conserved surface membrane protein present in all members of the family Kinetoplastidae, and is differentially expressed both in amastigote and promastigote forms of Leishmania (Jardim et al., 1995; Berberich et al., 1998). It was discovered as a protein complex tightly associated with lipophosphoglycan, the major cell surface glycoconjugate of Leishmania promastigotes (King et al., 1987). Its surface expression is higher in amastigotes than in promastigotes and increases during metacyclogenesis in L. amazonensis. The increased expression in metacyclic promastigotes and especially in amastigotes indicate a role for this molecule in parasite survival in the mammalian host (Matos et al., 2010). Interestingly, KMP-11 expression has been associated with virulence of L. donovani (Mukhopadhyay et al., 1998). Peripheral blood mononuclear cells from American tegumentary leishmaniasis patients stimulated with KMP-11 produce high levels of IL-10 (de Carvalho et al., 2003, Carvalho et al., 2005), a cytokine responsible for pathogenesis and parasite persistence in leishmaniasis (Belkaid et al., 2001; Nylen and Sacks, 2007). In contrast this protein has been shown to be highly antigenic for murine and human T cells (Jardim et al., 1991, Russo et al., 1992). Patients cured from visceral leishmaniasis
caused by *L. donovani* showed cellular proliferation against KMP-11 from *L. donovani* and also production of IFN-γ and IL-4 (Kemp *et al.*, 1993, Kurtzhals *et al.*, 1994). It has also been described that the DNA vaccine of KMP-11 protected hamsters experimentally infected with *L. donovani* (Basu *et al.*, 2005).

### 2.10.6 Multi-component vaccines

An ideal vaccine against leishmaniasis is unlikely to consist of a single antigen due to the genetic predisposition of the immune response amongst different individuals and populations. Therefore the idea of multi-component or polyprotein vaccines using more than a single antigen has been promoted. Such a multi-component vaccine, recombinant Q protein formed by the genetic fusion of antigenic determinants from four cytoplasmic proteins from *L. infantum* (Lip2a, Lip2b, P0 and histone H2A) when co-administered with live BCG protected 90% of immunized dogs by enhancing parasite clearance (Molano *et al.*, 2003). In another study it was observed that the Q protein when mixed with CpG-ODN motifs is able to significantly reduce the parasite burden in liver and spleen of BALB/c infected with *L. infantum* and induces long-term protection (Parody *et al.*, 2004). In a recent study, the Q protein from *Leishmania* in the absence of adjuvant protected dogs against an experimental *L. infantum* infection (Carcelen *et al.*, 2009). It has been shown that the Q protein and its components are highly specific molecules and may be used for the diagnosis of the *Leishmania*-induced pathology in dogs and humans (Soto *et al.*, 1995c; Soto *et al.*, 1995a; Soto *et al.*, 1998; Requena *et al.*, 2000a) as well as in hamsters experimentally infected with *L. infantum* (Requena *et al.*, 2000b).

Leish-111f is one of the best examples of polyprotein vaccines and it is the first defined vaccine for leishmaniasis in human clinical trials and has completed phase 1 and 2 safety and immunogenicity testing in normal, healthy human subjects in Brazil, Peru, Columbia, and India (Velez *et al.*, 2009). It is a single polyprotein composed of three molecules fused in tandem; the *L. major* homologue of eukaryotic thiol-specific antioxidant (TSA) (Webb *et al.*, 1998), the *L. major* stress-inducible protein-1 (LmSTI1) (Webb *et al.*, 1997) and the *L. braziliensis* elongation and initiation factor (LeIF) (Skeiky *et al.*, 1998). The selected proteins are expressed in promastigotes and amastigotes across the *Leishmania* genus and have been shown to afford protection in the mouse model when administered as single antigens or as a combination. These three proteins are highly
conserved among *Leishmania* sp. (Campos-Neto et al., 2001; Campos-Neto et al., 2002; Coler et al., 2002; Mendez et al., 2001; Rhee et al., 2002; Skeiky et al., 2002; Webb et al., 1996; Webb et al., 1998). The *L. major* homologue of eukaryotic TSA was discovered by screening expression libraries (Webb et al., 1998) to characterize the immune responses elicited by proteins isolated from filtrates of *L. major* promastigote cultures. Immunizing BALB/c mice with recombinant TSA protein formulated with either IL-12 or TSA DNA results in the development of strong cellular immune responses and confers protective immune responses against infection with *L. major* (Webb et al., 1998; Campos-Neto et al., 2001). LmSTII was identified by screening an *L. major* amastigote cDNA library with sera from BALB/c mice infected with *L. major* (Webb et al., 1996). Vaccination experiments with recombinant LmSTII protein plus either IL-12 or LmSTII DNA elicit a mixed cellular response that is skewed towards a Th1 phenotype, and protects BALB/c mice (Webb et al., 1996; Webb et al., 1997, Campos-Neto et al., 2001; Campos-Neto et al., 2002). LeIF was identified by screening a *Leishmania braziliensis* genomic library with sera from a patient with mucosal leishmaniasis (ML). LeIF stimulates the innate immune system to produce IL-12, IL-18 and IFN-γ and therefore, is a Th1 inducer. LeIF has immuno-therapeutic properties in mice (Borges et al., 2001; Skeiky et al., 1995; Skeiky et al., 1998; Probst et al., 1997). The protective efficacy of LmSTII and TSA has also been tested in rhesus monkeys (Campos-Neto et al., 2001). Monkeys immunized with a preparation containing LmSTII and TSA with the recombinant human IL-12 and alum as adjuvant mount excellent protection against challenge with *L. major* (Campos-Neto et al., 2001). Initial immunization trials in mice demonstrated that Leish-111f was able to provide a long term protection (over 14 weeks) to mice against *L. major* and *L. amazonensis* infection which is unusual for protein antigen (Coler et al., 2002; Skeiky et al., 2002). Upon vaccination with Leish-111f responses to its individual antigens were maintained and the protection was equal or better than that obtained by immunization with a mix of individual antigens. A crucial component of the Leish-111f vaccination programme is the adjuvant. The vaccine is effective when administered with IL-12, as well as MPL-SE (Reed et al., 2003). MPL-SE serves as an efficacious adjuvant to induce protective Th1 responses and is more affordable than rIL-12 (Reed et al., 2003). Leish-111f is to be initially tested as a therapeutic vaccine against cutaneous leishmaniasis. There is some evidence that the recombinant Leish-111f vaccine can also induce significant protection against *L. infantum* infection in mice and hamsters by eliciting CD4+ T cells. Although a report has suggested
that the mixture of the recombinant components of Leish-111f was highly immunogenic in dogs (Fujiwara et al., 2005). Leish-111f failed to protect dogs against infection and did not prevent disease development in a recent Phase III vaccine trial in dogs (Gradoni et al., 2005). A high percentage (95%) of the vaccinated dogs showed evidence of L. infantum infection. In a separate study in Brazil, the curative effect of this vaccine was limited (Miret et al., 2008). In a very recent study intranasal immunization with Leish-111f induced serum Leish-111f-specific IgG2a production, biased IFN-γ production from spleen cells and protective immunity against L. major infection (Sakai et al., 2010).

2.10.7 Genetic vaccination with Leishmania DNA

The concept of DNA vaccination is relatively new and was established by Wolff when direct intramuscular injection of plasmid DNA, encoding reporter genes, resulted in expression of the proteins in myocytes (Wolff et al., 1990; Ivory and Chadee, 2004; Garmory et al., 2003). Their study demonstrated that purified recombinant nucleic acids could be delivered to cells in vivo to direct protein expression endogenously (Roberts, 2006). DNA vaccines consist of antigenic proteins encoded on naked plasmid DNA vectors that allow their expression in eukaryotic cells (Dumonteil et al., 2003; Murray et al., 2005). Compared to recombinant protein vaccines, DNA vaccines are much more stable and have the advantage of their low cost of production, no need of cold chain for distribution, and flexibility of combining multiple genes in a simple construct. They are often very immunogenic and offer a protein that is usually correctly folded and may be post-translationally modified in a fashion similar to the native protein. Such vaccines are able to elicit humoral, CD4+ and CD8+ T cell immune responses, which can be further modulated by the addition of cytokines and/or CpG oligonucleotides (Alarcon et al., 1999; Restifo et al., 2000). In addition, genetic vaccines effectively engage both major histocompatibility class I and class II MHC pathways, thereby allowing the induction of both CD8+ and CD4+ T cells. This feature is particularly attractive for leishmaniasis, in which both cell types are involved in protection (Gurunathan et al., 2000). Other unique features that make DNA vaccination particularly attractive are the long lived production of the antigen, which is similar to the situation in natural Leishmania infection, together with improved immunological memory (Scott et al., 2004). They can also be modulated by prime-boost strategies that involve priming with DNA and boosting with protein (McShane, 2002).
Most-studied antigens were those previously assayed as recombinant proteins. Some of them were tested as single antigen vaccines (Aguilar-Be et al., 2005; Ahmed et al., 2004; Campos-Neto et al., 2002; Sukumaran et al., 2003; Basu et al., 2005; Gamboa-Leon et al., 2006) and others as multiple antigen vaccines (Ahmed et al., 2004; Campos-Neto et al., 2002) or as heterologous prime-boost (HPB), which involves an injection of the DNA vaccine followed by an injection of the recombinant protein (Rafati et al., 2005). A Vaccinia virus expressing the recombinant protein has also been tested (Ramiro et al., 2003; Gonzalo et al., 2002).

The encoding for the protein portion of the *Leishmania* surface glycoprotein (gp63) was the first *Leishmania* vaccine delivered as a plasmid. In this study, the level of IFN-γ but not IL-4 was high in spleen cells from immunized mice when stimulated by freeze/thawed antigen. These mice were partially protected against challenge with infectious *L. major* (Xu and Liew, 1994; Xu and Liew, 1995). In another study, 30% protection was reported in immunized mice, with indications of strong Th1 responses being elicited by vaccination (Walker et al., 1998). Further, a comparative study evaluating different DNA vaccine candidates including gp63 showed that protection was partial and transient (Ahmed et al., 2004).

Problems encountered with incorrect protein folding of recombinant PSA-2 were not apparently seen when the corresponding gene was delivered through a mammalian expression vector in mice by intramuscular injection. The antigen was properly expressed as revealed by immunofluorescence assays. Both C3H and BALB/c mice showed good protection from *L. major* challenge when the DNA was administered as a prophylactic vaccine, but also significant healing from established *L. major* infection when the plasmid was given as an immunotherapeutic agent (Handman et al., 2000). However, in a comparative study it was demonstrated that gp63 DNA immunization was able to reduce lesion size as well as parasite burden, while gp46/PSA-2 DNA vaccination led only to a reduction in lesion size without reduction of parasite burden (Dumonteil et al., 2003). LACK is the most extensively studied DNA vaccine against both cutaneous and visceral leishmaniasis. The protective efficacy of LACK DNA was compared with that of LACK protein and IL-12. It was shown that the LACK gene construct induced a strong protective response comparable to that achieved when LACK protein plus recombinant IL-12 was administered, and was better than protection seen with LACK protein alone (Gurunathan et al., 1997). High protective immune responses were seen against cutaneous...
leishmaniasis by co-administration of plasmids expressing IL-12 and IL-18 cytokines along with DNA-p36/LACK. It was followed by a booster with a vaccinia virus recombinant expressing p36/LACK which further enhanced this immunity by expanding the CD8+ T cells population (Zavala et al., 2001; Tapia et al., 2003). It has been shown that DNA-*Salmonella enterica* serovar *typhimurium* primer-booster vaccination strongly induced Th1 responses against *L. major* infection in susceptible BALB/c mice and biased responses towards Th1, thus enhancing the protection observed upon immunization with DNA or *Salmonella* alone (Lange et al., 2004). In a heterologous challenge system, priming with *L. infantum* LACK followed by a booster with vaccinia virus expressing LACK afforded protection against *L. major* infection (Gonzalo et al., 2002). A similar study in a dog model was shown to confer 60 per cent protection against *L. infantum* infection (Ramiro et al., 2003). The intranasal vaccination with LACK DNA also conferred protection in mice against *L. amazonensis* challenge (Pinto et al., 2004). To date, the protective efficacy of LACK has been mainly reported in cutaneous leishmaniasis and LACK failed to protect against visceral leishmaniasis. For example, Melby and colleagues reported that despite triggering strong Th1 responses the LACK DNA vaccine did not induce protection in mice against *L. donovani* challenge. Moreover, the co-administration of IL-12 did not improve the protective outcome (Melby et al., 2001b). Intramuscular immunization with p36 (LACK) DNA vaccine in the *L. chagasi* model, confirmed that this vaccination does not confer protection against VL despite the production of IFN-γ (Marques-da-Silva et al., 2005).

Other antigens which have been successfully tested as DNA vaccines against cutaneous or visceral infection include acidic ribosomal protein P0 (Iborra et al., 2003), P4 nuclease (Campbell et al., 2003) and paraflagellar rod protein 2 (PRP-2) (Saravia et al., 2005), whereas the latter contains ORFF (Sukumaran et al., 2003; Tewary et al., 2005), kinetoplastid membrane protein-11 (KMP-11) (Basu et al., 2005), CPA and CPB (Rafati et al., 2005) and NH36, a main component of the fucose-mannose ligand (Aguilar-Be et al., 2005). Immunization with plasmid DNA encoding *Leishmania* antigens represents a promising approach to vaccination against leishmaniasis in that it has intrinsic adjuvant properties, induces both humoral and cell-mediated immune responses and results in long lasting immunity but no development of such a vaccine for use in humans has been reported so far. Conflicting reports related to the protective efficacy of the antigens delivered as DNA vaccines resulted in confusion in this field. To
complicate issues further, protective outcomes seem to be influenced by many factors including plasmid backbone, number of injections, challenge dose and virulence of the leishmanial strain, developmental stage of the parasite (promastigote vs amastigote), experimental protocol employed, immunomodulators and type of animal model (Bhowmick and Ali, 2008).

2.11 Adjuvants

The concept of adjuvants arose in the 1920s from observations such as those of Ramon who noted that horses that developed an abscess at the inoculation site of diphtheria toxoid generated higher specific antibody titres (Ramon, 1959). The term adjuvant has been used for any material that can augment the specific immune response to an antigen. Adjuvants reduce the amount of antigen or the number of immunizations needed for protective immunity. An ideal adjuvant should be stable with long shelf life, biodegradable, cheap to produce, without side effects, not induce immune responses against themselves and promote an appropriate immune response (Edelman, 1980). The mode of action of adjuvants involves the formation of a depot of antigen at the site of inoculation, with slow, even, and sustained release of small quantities of antigen over a longer period of time while minimizing suppressive effects; the presentation of antigen to immunocompetent cells so that cell to cell interactions could take place to generate plasma cells; and the production of various and different lymphokines by activating the various cells involved in the immune response, either directly or indirectly (Chedid, 1985). There are many evidences which indicate that presentation of antigen via macrophages preferentially stimulates Th1 responses, delayed type hypersensitivity and IgG2a production and B cell presentation stimulates Th2 expansion, IgG1 and IgE production (Gajewski et al., 1991; Abbas et al., 1991; Brewer et al., 1994). It is widely recognized that many vaccines will require the simultaneous administration of adjuvants to enhance immunogenicity and efficacy. In addition, immunity induced by vaccines often necessitates specific enhancement of a polarized immune response, e.g. Th1 versus Th2, and this would require adjuvants possessing specialized mode of actions such as TLR ligation (Pashine et al., 2005). Successful vaccine development requires knowing which adjuvants to use and knowing how to formulate them. Th1 and Th2 paradigm has been used as a strategy for the selection of an antigen in the preliminary vaccine development against leishmaniasis (Handman, 2001). Thus, leishmanial antigens that
primarily stimulate Th1 responses in parasite infected cells have been accepted as "potential protective antigens" and therefore encouraging vaccine candidates. Thus, in recent years there has been a great deal of interest in developing novel, cheap, effective and safe adjuvants which stimulate protective immune response, to be used with vaccines. In addition, the recent unravelling of numerous immunological pathways has facilitated the rational development of new adjuvants and allowed a better understanding of the modes of action of traditional adjuvants (Handman, 2001).

Modern biochemical, biosynthetic, and recombinant DNA techniques have created increasingly pure antigens (Ag). Many of these newer antigens are small or generally weak immunogens and they will require the use of suitable adjuvants to enhance immunogenicity and efficacy. In addition, the trend towards the use of peptides and subunit proteins in modern vaccine design has demanded the use of immunological adjuvants to achieve a high quality/high quantity and memory-enhanced immune response. Studies carried out in animal models for leishmaniasis have demonstrated the requirements for an adjuvant in Leishmania vaccines (Kaeberle, 1986).

In the past few years a number of new adjuvants have become available for use in laboratory animals. Adjuvants that have been tested in Leishmania vaccines are described below.

2.11.1 Interleukin-12 (IL-12)

IL-12 has emerged as a potent adjuvant for vaccination against leishmaniasis in the murine model. IL-12 is produced by macrophages, dendritic cells (DCs), monocytes and neutrophils and to a lesser extent by B cells. IL-12 further stimulates IFN-γ production by NK and T cells. A major function of IL-12 is its regulation of the adaptive immune response. It induces the polarization of CD4+ T cells into Th helper 1 (Th1) cells that produce IFN-γ and help skew the immune response towards a Th1 type, which is important for protective immunity in leishmaniasis (Reiner and Locksley, 1995; Mattner et al., 1997). Moreover, IL-12 also acts on DCs to induce further production of IL-12 and it also enhances the cytolytic activity of NK and T cells (Grohmann et al., 1998).

The first experiments examining the effects of IL-12 as a vaccine adjuvant were performed in the L. major experiment model which reported that mice immunized with soluble Leishmania antigen (SLA) + IL-12 were protected against disease. Mice immunized with SLA + IL-12 produced little IL-4 but large amounts of IFN-γ that were
comparable to those observed in *L. major*-resistant mouse strains which is associated with protective Th1 immune response. Lesions from unimmunized BALB/c mice or mice that had been immunized with SLA alone contained high levels of parasites, as compared to BALB/c mice immunized with SLA+IL-12 formulation (Afonso *et al.*, 1994). Gicheru *et al.*, in 2001 assessed adjuvant potential of two doses of IL-12 along with a killed *L. major* vaccine in vervet monkeys. Low doses of IL-12 induced a small increase in the parameters of cell-mediated immunity, relative to animals that received antigen without IL-12. However higher doses of IL-12 induced a substantial increase in *Leishmania*-specific immune response. Nevertheless, despite the presence of correlates of protection, the animals were not able to protect themselves against challenge infection. Addition of IL-12 to recombinant polyprotein Leish-111f resulted in immunogenic and protective immune response against *L. major* infection in BALB/c mice, whereas no protection was observed in the absence of adjuvant. A limiting factor for use of IL-12 as an adjuvant is that it is costly and difficult to manufacture. Moreover its safety as an adjuvant for human use is questionable (Skeiky *et al.*, 2002). It was also found that rIL-12 along with 78 kDa antigen of *L. donovani* resulted in the generation of protective Th1 type of immune responses (Nagill and Kaur, 2010).

### 2.11.2 Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

Recombinant GM-CSF has been documented to have a potent cytokine adjuvant effect based on its activity of inducing activation, maturation and migration of dendritic cells (Romani *et al.*, 1994). This cytokine is known to yield a dominant Th 1 type of response (Badaro *et al.*, 2001). It has been shown to be effective in doses of 25 mg to 50 mg as an adjuvant (Lin and Jones, 1997), and it has been shown to be well tolerated when used in patients with visceral leishmaniasis (Badaro *et al.*, 1994). A cocktail vaccine for immunotherapy of mucosal leishmaniasis patient containing 5 mg of each of the antigens, TSA, LmSTII, rLbhsps83 and 10 mg of LeIF in combination with 50 mg of GM-CSF (Leukine®) added as an adjuvant showed no adverse reaction, except in one healthy subject who had erythema and induration at the injection site that was considered to be a secondary infection, but possibly due to an intense delayed type hypersensitivity (DTH) response. The cytokine was found to promote wound healing in vaccinated patients suffering from mucosal leishmaniasis (Badaro *et al.*, 2001). When used at high doses and injected at the site of the lesion and in combination with pentavalent antimony, GM-CSF shortens the healing time (Herwaldt, 1999).
2.11.3 BCG

BCG is an attenuated version of the bacterium *Mycobacterium bovis*, which is closely related to *Mycobacterium tuberculosis*, the agent responsible for tuberculosis. Vaccines composed of promastigote antigens and BCG have been clearly associated with the induction of strong cellular response evidenced by a high index of conversion of the intradermal skin reaction to leishmanial antigen (Castes *et al.*, 1994) and is probably the most acceptable Th1 immune response inducing adjuvant presently available for use in humans. In some of the early studies on *Leishmania* killed vaccines, a combination of killed promastigotes and *Mycobacterium bovis* BCG was used by Convit and colleagues in 1987 for immunotherapy of patients in Venezuela against South American leishmaniasis. In the therapeutic mode, vaccination appeared to induce a high cure rate even in patients with severe cases without side effects. Vaccination with BCG plus killed *Leishmania* promastigotes resulted in reduction of acute infection by *T. cruzi*, prolonged survival time, reduced blood parasitaemia and promoted IFN-γ production in mice (Araujo *et al.*, 1999). BCG vectors carrying gp 63 has also been used successfully to induce protection in *L. major* infected animals (Abdelhak *et al.*, 1995; Aebischer *et al.*, 2000). It has also been found that liposomes containing autoclaved *L. major* antigens mixed with BCG could be used to induce a Th1 response in C57BL/6 mice (Sohrabi *et al.*, 2005). Complete protection of Indian langurs was obtained by vaccination with alum-precipitated ALM combined with BCG at a dose of 1 mg per animal against *Leishmania donovani* challenge infection (Misra *et al.*, 2001). Musa *et al.*, in 2008 used alum-autoclaved *L. major* plus BCG in a preliminary trial in refractory PKDL patients, and showed immunochemotherapy to be superior to chemotherapy. A single dose vaccination with killed promastigotes of *L. major* plus BCG was found to be safe in Iranian volunteers with cutaneous leishmaniasis but was not effective in a controlled trial when compared to the BCG alone (Momeni *et al.*, 1999). Hence multiple doses or other adjuvants were suggested as ways of increasing immunogenicity of this antigen. The application of BCG as an adjuvant in vaccines has not always been without problems. A few reports of complications have been reported with the use of BCG, including inflammatory and autoimmune reactions (Misra *et al.*, 2001; Shoenfeld and Isenberg, 1988; Simila *et al.*, 1988; Milstien and Gibson, 1990). Thus, these effects of BCG make it quite undesirable for a safe vaccine.
2.11.4 Montanide ISA 720

Montanide Incomplete Seppic adjuvants (Montanide ISA or MISA) is a family of oil/surfactant based adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two oils. Montanide ISA 720 (MISA 720), a squalene based water-in-oil adjuvant formulation has shown promising results in previous malaria vaccine trials (Lawrence et al., 2000; Genton et al., 2002) possibly due to the slow-release capacity of the inert water-in-oil emulsion and immune stimulating effects of its components (Aucouturier et al., 2002). MISA 720 have also been used in experimental vaccines in malaria, HIV and cancer. It has been recommended for clinical trials in humans as an alternative adjuvant to aluminium hydroxide (Masina et al., 2003; Toledo et al., 2001; Oliveira et al., 2005). It has been shown to be immunogenic, inducing both Th 1-type cellular and humoral immune responses in humans (Masina et al., 2003). MISA 720 has also shown good results in non-human primate vaccination studies (Masina et al., 2003; Collins et al., 2006).

In leishmaniasis vaccine studies, Montanide ISA 720 adjuvant combined with recombinant glutathione-S-transferase-Histone-1 (Leishmania antigen) were able to generate a durable cellular response that was sufficient to control infection in the majority of the vervet monkeys (Masina et al., 2003). Studies on this adjuvant in Leishmania vaccines are limited. In another vaccination study immunization with L. major exogenous antigens along with MISA 720 in mice was not able to show an increase in protection by the adjuvant. Alum is currently the only Food and Drug Administration-approved adjuvant in clinical use in humans. The other adjuvants, rmIL-12 and Montanide ISA 720, have shown good results in several primate and human vaccination studies (Tonui et al., 2004).

2.11.5 Aluminium salts

In 1926, Glenny and associates first demonstrated the adjuvant activity of aluminium compounds and discovered that a suspension of alum adsorbed diphtheria toxoid had a much higher immunogenicity than the fluid toxoid. Since then, alum has been the most widely used adjuvant in clinical trials in vaccines because of their generally mild inflammatory reactions, safety and efficacy for generating memory. The large surface area of aluminum salts, particularly the hydroxide and phosphate derivatives,
provide a high adsorptive capacity for antigens (Edsall, 1966). Alum adjuvants primarily function to increase antibody responses to an immunogen.

Alum salts work by forming a repository of antigen at the inoculation site from where antigen is released slowly to produce particulate antigen for presentation to immune cells. The trapping of soluble antigen in the alum gel may also increase the duration of antigen interaction with the immune system reducing the amount of antigen needed per dose and the number of required doses. Other mechanisms of action involve complement, eosinophil and macrophage activation (Walls, 1977). Aluminium salts can enhance the immune response to some, but not all, protein antigens and they have little effect on peptide and polysaccharide antigens. Aluminium adjuvants have been used in a large number of veterinary vaccine formulations against viral (McDougall, 1969; Wilson et al., 1977; Sellers and Hemiman, 1974; Hyslop and Morrow, 1969; Pini et al., 1965) and bacterial (Thorley and Egerton, 1981; McCandlish et al., 1978; Nagy and Penn, 1974; Ris and Hamel, 1979) infections, as well as in anti-parasitic vaccines (Leland et al., 1988; Monroy et al., 1989; Carlow et al., 1987; Gamble et al., 1986). In human vaccination, aluminium adjuvants have been primarily used in tetanus, diphtheria, pertussis and poliomyelitis vaccines and later also hepatitis A and hepatitis B virus vaccines. In addition, other aluminium-adsorbed vaccines are available for special risk groups. For example, an aluminium adsorbed anthrax vaccine is administered to military servicemen in the USA (Lindblad, 2004). A study in the mouse model indicated the immunity elicited by killed *Leishmania* antigen plus mouse rIL-12 without alum may be relatively short-lived, lasting less than three months (Gurunathan et al., 1998). In another case, it was shown that a vaccine with autoclaved *L. amazonensis* promastigotes using combination of recombinant human IL-12 (rhIL-12) and alum was safe, effective and fully immunogenic in a vaccine against cutaneous leishmaniasis in Rhesus monkey model (Kenney et al., 1999).

Alum-precipitated, ALM vaccine combined with BCG was shown to induce successful vaccination against *L. donovani* infected Indian langurs (Misra et al., 2001). Moreover, a single dose of a vaccine containing alum-precipitated *L. major* (ALM) and BCG has been shown to be safe with 69.3% efficacy rate (Mohebali et al., 2004). Unfortunately, alum salts are relatively weak adjuvants and rarely induce cellular immune responses (Schirmbeck et al., 1994; Traquina et al., 1996; Brewer et al, 1996). Adverse reactions that have been reported with aluminium-containing vaccines are generally local
reactions, including sterile abscesses, erythema, subcutaneous nodules, granulomatous inflammation, increased IgE production, allergenicity and neurotoxicity. Generally, low doses of aluminium are excreted by the kidneys under normal conditions. Under certain circumstances, such as reduced renal function, aluminium is accumulated in the body and becomes highly toxic. Predominately brain and bone tissue are damaged causing fatal neurological syndrome and dialysis-associated dementia by high aluminium levels in the body. Amyotrophic lateral sclerosis and Alzheimer’s disease has also been related to aluminium intoxication (Gupta et al., 1995; Butler et al., 1969; Straw et al., 1985; Audibert and Lise, 1993; Goto et al., 1993).

2.11.6 Monophosphoryl Lipid A (MPLA)

MPLA (3-deacylated monophosphoryl lipid A) is isolated from bacterial cell walls and is a detoxified form of lipid A derived from the lipopolysaccharide (LPS) of Salmonella minnesota R595. The compound has an average molecular weight of 1718. The studies that inspired the development of MPL as an adjuvant began 60 years ago when it was noticed that the immune response to diphtheria and tetanus toxoid vaccines was improved when gram-negative bacteria were included (Greenberg and Fleming, 1947). LPS a major component of gram-negative bacteria cell walls, was eventually isolated and confirmed to be the material responsible for the enhanced immune response (Landy, 1955; Webster et al., 1955). Since then many studies have demonstrated the potent adjuvant activity of LPS, yet its inherent toxicity precludes its use as a vaccine adjuvant (Johnson et al., 1956). The lipid A portion of LPS was shown to be responsible for both the adjuvant and the toxic properties of LPS (Galanos et al., 1985; Takada and Kotani, 1989). Ribi and co-workers (Ribi et al., 1984; Qureshi et al., 1982) established that removal of the phosphate group from the reducing end sugar of the lipid A disaccharide decreased the toxicity of the molecule 100 to 1000 fold without appreciably affecting the immunostimulating activity. The resulting derivative, which had only one phosphate group, was called monophosphoryl lipid A (Myers et al., 1990). Subsequently it was determined that removal of an ester-linked fatty acid group from the 3-position further reduced the pyrogenic properties without substantially affecting the adjuvant properties. The resulting 3-O -deacylated monophosphoryl lipid A (MPL), which is isolated and structurally derivatized from LPS of Salmonella minnesota R595, has proven to be a safe and effective vaccine adjuvant (Fries et al., 1992).
Until recently, no adjuvants were available for use in human T-cell vaccines. However, monophosphoryl lipid A has been shown to be safe and effective (Reed et al., 2003). MPL was shown to have no adverse effects on cardiovascular/respiratory function, reproduction, and genotoxicity (Baldrick et al., 2002). The adjuvant activity of MPL is attributed primarily to its ability to activate antigen presenting cells and induce cytokine cascades. Several studies have demonstrated the ability of MPL to activate monocytes and macrophages (Ribi et al., 1984; Masihi et al., 1986). Presumably through the activation of these cells, vaccine antigens are more readily phagocytized, processed, and presented. These cells also release tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and granulocyte–macrophage colony stimulating factor (GM-CSF) in response to MPL (Arend and Massoni, 1986; Astiz et al., 1994). It is likely that these monokines lead to the recruitment and maturation of dendritic cells in the lymph nodes (Cumberbatch and Kimber, 1992; Jonuleit et al., 1996) where the dendritic cells can efficiently present antigen to T lymphocytes. In addition, MPL either directly or indirectly stimulates the production of the T helper cell type 1 (Th1) cytokines IL-2 and IFN-γ (Gustafson and Rhodes, 1994). It is probable that MPL is able to further influence the development of cellular immunity to vaccine antigens through the action of these cytokines. This capacity of MPL to stimulate the cytokine cascades necessary for the induction of cellular immunity makes it an effective adjuvant by itself. But MPL can also be used to enhance and complement the activity of vehicles that act as depots for antigen such as alum adsorbates, liposomes, microparticles, and oil-containing emulsions (Schneerson et al., 1991). A study by Skeiky et al., in 2002 reported that, MPL-SE formulated with rLeish-111 elicited protective immunity against L. major infection. A chimeric vaccine made of three recombinant leishmanial antigens (LeIF, LmSTI-1 and TSA) in the form of a fusion protein combined with monophosphoryl lipid A in stable emulsion MPL-SE has been developed and is the first subunit vaccine to be evaluated in humans. The vaccine is protective against both cutaneous and visceral leishmaniasis in mice. Analysis of the cellular immune responses of immunized, uninfected mice demonstrated that the vaccine induced a significant increase in CD4+ T cells producing interferon-gamma (IFN-γ), IL-2, and tumor necrosis factor cytokines, indicating a Th 1-type immune response (Coler et al., 2002; Coler et al., 2007) and has been demonstrated to be safe and well-tolerated in humans (Velez et al., 2009). MPL-SE serves as an efficacious adjuvant to produce...
protective Th1 responses and is more affordable than rIL-12 (Reed et al., 2003). In a recent study it was investigated that immunotherapy with subunit vaccine Leish-11f + MPL-SE is better than chemotherapy. This vaccine has undergone meticulous preclinical testing and been demonstrated to be safe in human clinical trials (Trigo et al., 2010).

2.11.7 CpG Oligodeoxynucleotide (CpG ODN)

Unmethylated CpG dinucleotide motifs present in bacterial genomes or synthetic oligodeoxynucleotides (ODNs) have been described to function as natural adjuvants because they promote professional antigen presenting cell (APC) function and co-stimulate lymphocytes. ODNs which contain immunostimulatory CG motifs (CpG ODN) can promote Th1 responses, an adjuvant activity that is desirable for vaccination against leishmaniasis. They are proving very successful at stimulating innate and adaptive immune responses in a variety of species (Kumar et al., 2004) to coadministered antigens. It has been shown that CpG DNA, acting as a TLR9 ligand, turns the outcome from fatal disease to protective long-lasting immunity in murine leishmaniasis (Verthelyi et al., 2002; Zimmermann et al., 1998; Walker et al., 1999). These effects are strongly dependent on IL-12 and TNF-α which in turn enables the adaptive immune system to mount a protective Th1-biased immune response. Even established Th2 responses could be reverted by a triple injection of CpG DNA within an early phase of infection. In another study it was shown that BALB/c mice vaccinated with crude soluble leishmanial antigen (SLA) plus CpG ODN modulates Leishmania-specific immunity towards a protective immune response (Stacey and Blackwell, 1999). Similar to IL-12, the addition of ODNs (CpG) to the leishmanial antigens, TSA, LeIF and LmSTI-1, resulted in complete protection of susceptible mice against progressive disease, whereas no protection was observed in the absence of adjuvant (Coler and Reed, 2005). CpG ODN along with heat-killed Leishmania vaccine provided significantly increased protection against CL infection in macaques (Verthelyi et al., 2002). In a recent study it was suggested that coencapsulation of CpG ODN with rLmSTI1 in liposomes enhances the Th1 type of immune response and the induction of protection against leishmaniasis in a murine model (Badiee et al., 2008). Immunostimulatory effect of CpG ODN is often transient because of its rapid degradation and absorption and hence, repeated administration or a much higher dose of CpG ODN is required to achieve the desired effects (Mutwiri et al., 2004). Moreover, the use of CpG ODN might be limited by the
difficulty of delivery to the intracellular compartments because CpG ODN are recognized by Toll-like receptor 9 as a synthetic ligand (Akira et al., 2006) and must be delivered to the endosomes before signaling is possible (Ahmad-Nejad et al., 2002). This severely limits the potential clinical application of the CpG ODN technology (Mutwiri et al., 2004).

2.11.8 Liposomes

Liposomes are synthetic closed vesicles composed of concentric lipid bilayers that can encapsulate antigens and are utilized as delivery systems for vaccines, drugs and hormones. They are used as immunoadjuvants to induce immune responses to various antigens (O’Hagan and Singh, 2003; Alving, 1995). Liposomes are safe, biodegradable, FDA-approved compounds. The adjuvanticity of liposomes depends on the number of lipid layers (Heath et al., 1976), electric charge (Tyrrell et al., 1976), composition (van Rooijen and van Nieuwmegen, 1983) and method of preparation (Eldridge et al., 1991; Kramp et al., 1982). Liposomes help extend the half-life of antigens in blood ensuring a higher antigen exposure to antigen presenting cells after vaccination (Kramp et al., 1982).

The composition of a liposome might be designed to preferentially induce a humoral or cellular immune response against a specific antigen (Alving, 1995). Liposome-based vaccines based on virosomes are approved in Europe for hepatitis A and influenza (Ambrosch et al., 1997). In earlier studies membrane antigens of \textit{L. donovani} promastigotes (LAg) in liposomes of different charges induced significant but varied levels of protection against infection in BALB/c mice (Afrin and Ali, 1997; Afrin et al., 2000). These protected animals elicited profound DTH and increased levels of \textit{Leishmania}-specific IgG antibodies. The variation was shown as a result of an adjuvant-induced difference in the immune responses to LAg or the differential entrapment of the various components of LAg. Further the vaccine efficacy of soluble leishmanial antigens (SLA) from \textit{L. donovani} promastigote membrane, entrapped in negative, neutral and positively charged liposomes was evaluated in experimental VL. The best vaccine formulation was SLA in positively charged liposomes (Bhowmick et al., 2007).

In another study, liposomes containing Autoclaved \textit{L. major} (ALM) mixed with BCG were used as a vaccine against leishmaniasis in resistant C57BL/6 mice. The results indicated that vaccine could selectively induce Th1 response in resistant mice. The
result of cytokine assays showed that IFN-γ which is an indicator of a Th 1 response was significantly higher in the group that received vaccine than in the control groups. This study concluded that liposomes containing *Leishmania* antigens mixed with BCG could be used to induce a Th1 response in resistant C57BL/6 mice (Sohrabi *et al.*, 2005).

Liposomes have suffered from manufacturing difficulties, stability, quality assurance and high cost, which have limited their use. Furthermore, they are more antigen vehicles than true adjuvants and hence require addition of immunostimulatory components such as MPL for potent adjuvant action. Injection site pain can be a major limitation in some liposome vaccines (Davidsen *et al.*, 2005).

2.11.9 **Glucan**

Glucan, a reticuloendothelial stimulant, is a β 1, 3 polyglucose derivative of baker's yeast. Macrophages have glucan receptors, activation of which stimulates phagocytosis and cytokine secretion (Susuki *et al.*, 1994). Protective efficacy of glucan has been studied, by immunizing BALB/c mice with a series of intravenous injections of formalin-killed *L. donovani* promastigotes alone and in combination with glucan. Mice which received dead parasites plus glucan exhibited protection against challenge with *L. donovani* parasites. A lesser degree of protection was found in animals receiving only glucan but no measurable protection was seen in animals receiving only killed parasite against infection (Holbrook *et al.*, 1981). In a different study CF1 mice were immunized with dead promastigotes of *L. donovani* along with glucan and results showed an elicited protective resistance, positive skin test responsiveness before and after challenge and increased antipromastigote antibody levels. In this study, administration of glucan alone induced a lesser degree of resistance against infection without significant skin test or humoral responsiveness (Holbrook and Cook, 1983).

2.11.10 **Corynebacterium parvum (C. parvum)**

*C. parvum* has been shown to be an effective adjuvant in various vaccine studies. *C. parvum* has been reported to enhance the T helper cell-antibody response to erythrocytes (IgG2>IgM>IgG1) (Warr and James, 1975). *C. parvum* has also been reported to enhance the level of natural killer (NK) and macrophage cell activity. NK cell
activity has been indicated to affect the course of leishmanial infection (Kirkpatrick and Farrell, 1982). Since the NK activity stimulated by *C. parvum* may last for several weeks (Thatcher *et al.*, 1979), NK cell activity may be involved in or facilitate the protective response against leishmaniasis. The macrophage activation by *C. parvum* appears, to be short-lived (Sljivic and Watson, 1977), and *C. parvum*-elicited macrophages have been reported to be tumoricidal but not leishmanicidal (Haidaris and Bonventre, 1981).

Champsi and McMahon-Pratt in 1988 compared the efficacy of 46-kilodalton membrane glycoprotein (M-2) of *Leishmania amazonensis* along with different adjuvants such as Freund incomplete and complete adjuvants, saponin, and *C. parvum* in both susceptible and resistant strains of mice. The level of protection varied with the mouse strain although all animals received identical preparations of antigen and adjuvant. In this study *C. parvum* appeared to be the most effective adjuvant in all the mouse strains tested. In a comparable study it was demonstrated that, immunization of mice three times intraperitoneally at 2 weeks interval with recombinant parasite surface antigen 2 of *L. major* purified from *E. coli* in combination with *C. parvum* as an adjuvant did not induce protective immunity, despite the induction of strong Th1 responses (Sjolander *et al.*, 1998b). In another study a successful vaccination was done against *L. chagasi* infection in BALB/c mice utilizing *C. parvum* as an adjuvant. It caused a significant reduction in parasite load in liver and spleen and induced a high production of IFN-γ and IL-4 by spleen cells (Vilela *et al.*, 2007).

### 2.11.11 Saponins

Saponins (Quil-A, ISCOM and QS-21) are triterpene glycosides isolated from plants (Sharples *et al.*, 1994). Quil-A and its derivatives are the most widely used saponin in adjuvant research. These are extracted from the bark of the Quillaja saponaria tree (Kensil *et al.*, 1995). Quil-A is composed of a heterogeneous mixture of triterpene glycosides that vary in their adjuvant activity and toxicity. Saponins have been widely used as an adjuvant in veterinary vaccines. QS-21 is a purified component of Quil-A associated with low toxicity and maximum adjuvant activity. Several authors have shown that *Quillaja* saponins (including QS-21) stimulated the production of CTLs and induce Th1 cytokines (IL-2 and IFN-γ) and antibodies of the IgG2a isotype to protein antigens.
(Takahashi et al., 1990; Kensil, 1996; Kensil et al., 1995). In addition, QS-21 can be applied in subunit vaccines to produce variant-specific antibody responses, inducing CTL also to an additional unidentified epitope outside antigen-specific region (Newman et al., 1997). QS-21 has also been claimed to perform as an adjuvant for DNA vaccines, following both systemic and mucosal administration (Sasaki et al., 1998). Saponin was used as an adjuvant in combination with L. braziliensis promastigote protein in a vaccine using dog as an animal model. This vaccine candidate elicited strong antigenicity related to the increases of anti- *Leishmania* IgG isotypes, together with higher levels of lymphocytes, particularly of circulating CD8 T-lymphocytes, and *L. chagasi* antigen. The FML antigen of *Leishmania donovani*, in combination with either QuilA, QS21 saponins, IL12 or BCG, was used in vaccination of an outbred murine model against visceral leishmaniasis (VL). Significant and specific increases in anti-FML IgG and IgM responses were detected for all adjuvants. The novel particles or complexes known as ISCOMs were first described by Morein and co-workers in 1984. The immunostimulating complex or ISCOM is one adjuvant with multiple adjuvant properties. ISCOMs are open cage-like complexes typically with a diameter of about 40 nm that are built up are composed of saponin, cholesterol, phospholipid, and immunogen, usually protein. They were originally designed to form a vaccine delivery system that combined certain aspects of virus particles such as their size and orientation of surface proteins, with the powerful immunostimulatory activity of saponins (Morein et al., 1984). ISCOMs have only been used in veterinary vaccines, partly due to their haemolytic activity and some local reactions (Scheibner, 2000). The hemolytic activities of saponins are related to their chemical composition (Santos et al., 1997). The degree of hemolytic activity was shown to be related to the presence of side chains bearing aglycone (sugar chains) (Santos et al., 1997) acyl residues or the epoxy framework system (Oda et al., 2000).

### 2.11.12 Freund's Adjuvants

There are two Freund's adjuvants; incomplete and complete (Scheibner et al., 2000). Freund's complete adjuvant (FCA) is a mixture of non-metabolizable oil (mineral oil), a surfactant (Arlacel A), and mycobacteria (*M. tuberculosis* or *M. butyricum*). When mixed with aqueous solutions or suspensions of antigens, the adjuvant forms a viscous
water-in-oil emulsion, with the antigens in the water phase. In an emulsion, antigen is distributed over a large surface area thereby increasing the potential for interaction with relevant cells (Osebold, 1992). Mechanisms of action include formation of depot and nonspecific immunopotentiation of macrophages by surfactant and the mycobacteria. FCA was used as an adjuvant in combination with killed _L. infantum_ promastigotes for vaccinating dogs subcutaneously (Panaro et al., 2001). Vaccination significantly increased phagocytosis, killing capacity and nitric oxide production by macrophages for a long time. In addition, the amount of IFN-γ in PBMC supernatants was significantly higher after vaccination, suggesting the proactive potential of this antigen-adjuvant combination (Panaro et al., 2001). Although FCA is a very effective adjuvant for production of antibodies, there are problems and hazards associated with its use (Broderson, 1989; Kleinman et al., 1993; Claassen et al., 1992; Steiner et al., 1960; Stills and Bailey, 1989; Stills, 1994). At the site of injection, FCA causes a chronic inflammatory response that may be severe and painful for the animal depending on the site as well as the quantity and quality of adjuvant injected. The inflammatory response may result in formation of chronic granulomas, sterile abscesses, and/or ulcerating tissue necrosis. Adjuvant-induced lesions may appear to be metastatic when excessive amounts of the emulsion are injected in a single site (Scheibner, 2000).

Freund's Incomplete Adjuvant (FIA) has the same oil/surfactant mixture as FCA but does not contain any mycobacteria. It is frequently used to boost animals that received a primary antigen injection in FCA, but it can be used as the adjuvant for the primary injection as well. It has adjuvant properties that favor humoral immunity without cell-mediated immunity, but is generally considered to be less potent than FCA (although exceptions exist). FIA is capable of causing abscesses and granuloma formation, but such reactions are generally less severe than those that accompany the use of FCA (Scheibner, 2000).

### 2.12 Rationale of the Present Study

VL caused by _Leishmania donovani_ represents a major public health problem in tropical and sub-tropical regions of the world. In India, high incidence has been reported from the states of Bihar, Assam, West Bengal and eastern Uttar Pradesh where resistance
and relapse are on the increase. A survey in Bihar has recorded an alarming 100,000 cases with 10,000 unresponsive to antimonials (Sundar, 2001). The available antileishmanial drugs are toxic, have serious side effects and are associated with numerous relapses due to the increasing incidence of drug resistance. In the absence of suitable anti leishmanial drugs an alternative choice for controlling infection is the development an effective and affordable vaccine. Recovery from leishmaniasis results in long-lasting immunity which yields a strong evidence of the possibility of achieving protection against *Leishmania* infection through vaccination (Connell *et al.*, 1993). Hence, it is important to evaluate leishmanial antigens to study their protective immune responses. A number of defined antigens as mentioned in literature have been tried as vaccine candidates. However, no effective vaccine against leishmaniasis is yet available.

Hsps are one of the widely studied vaccine candidates. They are known to induce very strong humoral and cellular immune responses in numerous infections caused by bacteria, protozoa, fungi, and nematodes, as well as in various experimental infection models (Cohen and Young, 1991). They can stimulate macrophages and dendritic cells to secrete the proinflammatory cytokines IL-6, IL-12, and TNF-α (Asea *et al.*, 2000; Basu *et al.*, 2000). Therefore, they are attractive candidates for formulating vaccines. Many members of the Hsp70 family have been described as dominant antigens in several infectious diseases due to their high conservation among various microbial pathogens. They elicit immunity to cancers and infectious pathogens (Udono and Srivastava, 1993) and are powerful immunogens and attractive candidates for subunit vaccines against infectious diseases in man (Suzue and Young, 1996). Hsp70 family attracts much attention because of its versatile functions in immune system. It is considered as workhouse of the chaperones because of its promiscuity to assist in folding new polypeptide chains (Beckmann *et al.*, 1990; Hartl, 1996). Hsp83 has an adjuvant effect to accompanying protein when it is fused to some other protein and co-administered to BALB/c mice (Rico *et al.*, 1999). It has also been shown that rLbHsp83 contains a potent T-cell epitope which stimulates PBMCs from all *L. braziliensis* infected individuals to proliferate and to produce IL-2, IFN-γ and TNF-α (Skeiky *et al.*, 1995). *Leishmania infantum* Hsp83 has been found to be a powerful immunogen producing humoral response during canine leishmaniasis (Angel *et al.*, 1996). Apart from defined single
molecules, multicomponent vaccines have been demonstrated to afford protection against VL in experimental animals (Requena et al., 2000a; Requena et al., 2000b; Carcelen et al., 2009). Many of the purified protein antigens are small and poor immunogens. Addition of Th1-driving adjuvants results in enhancement of their protective efficacy. Therefore adjuvants are needed to enhance the immunogenicity of poorly immunogenic vaccine proteins (Vogel and Powell, 1995; Nagill and Kaur, 2010). Therefore, this work was planned to observe the efficacy of cocktail of Hsp70 and Hsp83.

To date, there has been no reliable diagnostic test for active infections of VL. In fact, parasitological methods involving bone marrow aspiration and splenic aspirations have many drawbacks and some times record high rates of false negative results (Sunder and Rai, 2002). All these factors necessitate the development and application of reliable serological tests. Various serological tests include IFAT, ELISA and CIEP for diagnosis of VL. The sensitivity of ELISA (99.5%) and IFAT (99.04%) is more than that of CIEP (96.6%) while specificity of these two tests is lesser than CIEP (Mittal et al., 1991). Direct Agglutination Test (DAT) is a simple, sensitive and specific test in this category (Singh and Sivakumar, 2003) but is unable to distinguish between active disease and past infection. Since total Leishmania promastigotes are used as antigen in routine assay, false–positive reactions are frequent due to cross–reaction with the sera from other diseases. Therefore, an antigen that determines less cross reactivity is the best suggested for the serodiagnosis of leishmaniasis (Sunder and Rai, 2002).

To the best of my knowledge no studies are available as regards the immunoprophylactic potential of Hsp70 and Hsp83 in combination with adjuvants against VL and also their role in diagnosis. Therefore the aim of present study was to study the immunoprophylactic and diagnostic potential of Hsp70 and Hsp83 during murine VL.

2.13 AIMS AND OBJECTIVES:

1. To study the protective efficacy of Hsp70 and Hsp83 antigens in combination with two adjuvants (Leishmania autoclaved antigen and MPL-A) in Leishmania donovani infected BALB/c mice.

2. To evaluate the delayed type hypersensitivity (DTH) response to specific antigen in different groups of animals.
3. To determine the vaccine-induced cytokine production in different groups of animals.

4. To monitor the parasite-specific IgG1 and IgG2a isotypes in different groups of animals.

5. To evaluate the diagnostic potential of Hsp70 and Hsp83 by ELISA and western blotting.