Leishmaniasis is a zoonotic infection that is caused by obligate intracellular protozoan of the genus *Leishmania*, family Trypanosomatidae and order Kinetoplastida (Dawit *et al.*, 2013). *Leishmania donovani* is named after its discoverers W.B. Leishman and C. Donovan, who discovered one of the earliest strains of *Leishmania* in 1901 (WHO, 2010). This disease is included in the list of the world’s most neglected diseases, prevalent in developing countries based on the limited resources invested in diagnosis, treatment and control, and its strong association with poverty (Bern *et al.*, 2008). Published disease burden estimate places leishmaniasis second in mortality and fourth in morbidity among all tropical diseases (Schroeder and Aebischer, 2011). On a global scale, approximately 350 million people live in areas of active transmission of *Leishmania*, with 14 million people throughout Africa, Asia, Europe, and the Americas directly affected by the disease (Kimutai *et al.*, 2009). The global burden of leishmaniasis has remained stable for some years, causing a morbidity and mortality loss of 2.4 million disability adjusted life-years (DALYs) and approximately 70,000 deaths, a significantly high rank among communicable diseases (Igineweka *et al.*, 2012). Based on overall significance, leishmaniasis is regarded second to malaria (Jha *et al.*, 2012) among the protozoal diseases of humans.

The leishmaniasis include a complex of vector-borne diseases, caused by more than 20 species of the protozoan genus *Leishmania*, and transmitted by approximately 30 species of phlebotmine sandflies belonging to the *Lutzomyia* subgenus in the New World and *Phlebotomus* subgenus in the Old World. These are classified into two main groups: Old World: *L. major*, *L. tropica*, *L. aethiopica* and the *donovani* complex (*L. donovani*, *L. infantum*, *L. archibaldi* and *L. chagasi*) and New World: *L. mexicana*, *L. amazonensis* and *Viannia* complex (e.g. *L. braziliensis*, *L. guyanensis*, *L. panamensis* and *L. peruviana*) (Roychoudhury and Ali, 2008; WHO, 2010). Disability-adjusted life years (DALYs) lost due to leishmaniasis are close to 2.4 million (Desjeux, 2004a). Despite the scarcity of reliable data, there is little doubt that the case-load worldwide is considerably higher than official reported figures (Desjeux, 2004b). Infection with *Leishmania* spp. results in a wide range of clinical
symptoms that can be classified into four main forms: CL, MCL, VL and PKDL. 
Organism prevalence and disease patterns differ according to the geographical area 
and distribution (Piscopo and Mallia, 2006).

2.1. Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL), first descriptions of which can be traced back 
to the 9th century (Balkh sore), remains a major world health problem in the 21st 
century (Rastogi and Nirwan, 2007). The disease is known by different local names in 
various endemic areas: Baghdad boil, Aleppo boil, Delhi boil, okht (‘little sister’) in 
Arabia, and hashara (‘insect’) in Sudan. The clinical features of cutaneous 
leishmaniasis tend to vary between and within regions, reflecting different species of 
parasite or the type of zoonotic cycle concerned, immunological status and also 
perhaps genetically determined responses of patients (WHO, 2010). Cutaneous 
leishmaniasis is caused by *Leishmania tropica*, *Leishmania major* and *L. infantum* in 
the Old World and by *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania 
panamensis*, *L. peruviana*, *L. mexicana*, *L.amazonensis* and *L.venezuelensis* in the 
New World (Maltezou, 2010). 90% of the new cases of cutaneous leishmaniasis occur 
in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria and they are caused 
principally by *L. major* and *L. mexicana* (Herwaldt, 1999; WHO, 2002; Eslami 
et al., 2012). Increasing cases of CL have been reported during recent years from a border 
area between Iran and Pakistan, a previously non-endemic area (Fazaeli 
et al., 2009). 
CL usually is more common in rural than urban areas. However, it is found in some 
periurban and urban areas (e.g., in Baghdad, Iraq, Kabul and Afghanistan). The 
ecologic settings range from rainforests to arid regions (Weisser et al., 2007). It 
affects 1.5 million individuals per year (Abbas et al., 2009). Over the past ten years, 
the number of reported cases has increased sharply and the geographical distribution 
of endemic regions has expanded considerably due to urbanization and the migration 
of non-immune individuals to endemic areas (Sakthianandeswaren et al., 2009).

The disease may be limited to a single lesion on the skin (localized cutaneous 
leishmaniasis, LCL) or may produce a large number of lesions (diffuse cutaneous 
leishmaniasis, DCL). In LCL, the disease is generally benign and most of the cases 
heal spontaneously (Kemp et al., 1994). DCL is an anergic variant of LCL, in which 
lesions are disseminated to involve most areas of the skin and never heals 
spontaneously (Grevelink and Lerner, 1996). 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 (Goto and Lindoso, 2010). A ‘classical’ lesion starts as a papule or nodule at the site of inoculation; it grows slowly, taking at least 1 week to reach its final size. A crust develops centrally, which may fall away, exposing an ulcer up to 5 cm in diameter with a raised edge and variable surrounding induration, which heals gradually over months or years, leaving a depressed scar with altered pigmentation. Satellite nodules at the edge of the lesion are common (WHO, 2010). Regardless of species, CL is not life threatening, however, lesions can lead to significant disfigurement and social stigmatization. In a small percentage of cases, inadequate treatment of a primary CL lesion may leave an individual at risk for later development of mucocutaneous leishmaniasis (David and Craft, 2009).

2.2. Muco-cutaneous Leishmaniasis

When skin and mucosa are affected the disease is known as mucocutaneous leishmaniasis. This is very rare in East Africa but frequent in South America, where it is known as "espundia" or “white leprosy”. After an initial skin lesion, that slowly but spontaneously heals, chronic ulcers appear after months or years on the skin, mouth and nose, with destruction of underlying tissue (nasal cartilage, for example). Tissue destruction with disfigurement can be very severe. Parasites are usually rare in the lesions. In mucocutaneous leishmaniasis, the lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues. The distribution of mucocutaneous leishmaniasis is very tightly linked to geography and villages even 15 miles apart can have very different rates of cutaneous leishmaniasis. There are about 20 species of *Leishmania* that may cause these diseases. Some *Leishmania* species are closely linked to humans and are therefore found in cities (*L. tropica*), some others are more traditionally associated with animal species and therefore considered zoonoses (*L. major*). Some species that are traditionally considered zoonotic (e.g. *L. panamensis*) becoming primarily human diseases of *Leishmania* are transmitted to human skin by the bite of a sand fly. *Leishmania* invades human macrophages and replicates intracellularly. A raised, red lesion develops at the site of the bite (often weeks or sometimes years afterwards). The lesion then ulcerates and may become secondarily infected with bacteria. In many species (for example, *L. major*) the lesion often heals spontaneously with atrophic scarring. In some species (for example, *L. viannia braziliensis*) the lesion may heal
spontaneously with scarring, but re-appear elsewhere as destructive mucosal lesions (Palumbo, 2010).

**2.3. Visceral Leishmaniasis**

Visceral leishmaniasis (VL) or kala-azar was first described in Greece and subsequently in India in 1882 (Alencar et al., 1997). At that time, emphasis was placed on a typical aspect of the disease found in India, which did not manifest itself in Brazil, i.e. the darkening of the skin. This characteristic basis of the first denomination of this disease, which was called ‘black fever’ or ‘kala-azar’ by Indians (Figueiro-Filho et al., 2004). Visceral leishmaniasis is usually an insidious, chronic disease among the inhabitants of endemic areas, however, the onset may be acute in travelers from Leishmania-free areas. Estimates for the incidence and prevalence of kala-azar cases per year are 0.3 million, respectively (Khan et al., 2013). More than 90 per cent of the cases are in India, Bangladesh, Nepal, Sudan and Brazil (Vyas and Shah, 2012). The incidence of kala-azar in India is among the highest in the World (Bhattacharya, 2006). In Indian sub-continent, about 200 million population is at risk of 25,000–40,000 cases (Hasker et al., 2012) and 200–300 deaths are reported per year, but these are grossly underestimates. Well-designed multicentric studies identified VL burden of 21 cases/10,000 among sampled population in 1 continent (Bangladesh, India and Nepal). This estimate of 4,20,000 cases is conservative, as it is clear that the disease is highly underdiagnosed (Joshi et al., 2008).

![Status of VL endemicity, worldwide. (WHO, 2012)](image-url)
2.3.1. Life cycle of the parasite

Visceral leishmaniasis (VL) is a vector-borne disease caused by replication of parasites in macrophages, mononuclear phagocytic system. The *Leishmania* parasite has a digenetic complex life cycle with an extracellular developmental stage in the insect vector, a female phlebotomine sandfly, and a developmental stage in mammals, which is mostly intracellular. The parasite undergoes major morphological and physiological transformations during the different stages of its life cycle. Phlebotomine female sandflies are dipteran insects of family Psychodidae. The transmission of leishmaniasis is attributed to about 70 of around 1000 known sandfly species. Those belonging to the genus *Lutzomyia* are prevalent in the New World and those belonging to the genus *Phlebotomus* are prevalent in the Old World. Phlebotomine sandflies are not active during the day and seek out cool and relatively humid dark niches which allow them to survive in hot and dry climates. Indeed, they become active at dusk when the temperature drops and humidity rises (Murray *et al.*, 2005).

*Leishmania* parasites need female phlebotomine sand flies not only to complete their life cycle but also to propagate i.e. it acts as a vector. There are many adaptations like secretion of phosphoglycans, which protect the parasite from digestive enzymes; production of chitinases that degrade the stomodeal valve of the sand fly; secretion of a neuropeptide that arrests midgut and hindgut peristalsis; and attaching to the midgut to avoid expulsion (Kamhawi, 2006). 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 multiplies and matures in the insect midgut into an infective metacyclic form which migrates to its proboscis. When sandflies bite mammalian host for blood meal the metacyclic promastigotes reach the punctured wound (Tripathi *et al.*, 2007). A typical inoculum contains around 100–1000 metacyclic promastigotes. Once inside the host they quickly get phagocytised by leucocytes, particularly macrophages, neutrophils and dendritic cells. The parasites which are internalized by these cells through phagocytosis are trafficked to endosomes and lysosomes. The resulting phagolysosome, also called parasitophorous vacuole (PV), is an acidified and hydrolytically active compartment where the *Leishmania* parasite is not only capable to survive but also to multiply (Antoine *et al.*, 1998; Shio and Olivier, 2010). Then
they immediately undergo a further morphological change by shedding it and taking an ovoid shape hence termed ‘amastigote’ and possibly a metab with a switch to anaerobic metabolism under acidic conditions found ch phagolysosomal compartment. The parasite’s persistence in such environment is attributed to its differentiation in amastigotes, whic macrophage hydrolases (Desjardins and Descoteaux, 1997). Amastigotes such a manner that phagocytic cells eventually rupture to infect other sustaining their survival and eventually damaging the whole reticul system (Roberts, 2006). On the one hand, Leishmania parasites antioxidants, such as superoxide dismutase and other ROS scavenge undermine the production of ROS (Olivier et al., 2005). Alternatively, parasites also inhibit several host innate inflammatory functions, such as lysosome maturation and expression of costimulatory molecules required presentation (Shio and Olivier, 2010). Leishmania causes deviation from immune response by inducing the activity of suppressive cytokines, such as PGE2, and TGF-β. More importantly, Leishmania suppresses microbicidal functions by down-regulating NADPH oxidase and iNOS thereby inhibiting ROS and NO production, respectively (Olivier et al., 20

![Schematic diagram of Leishmania digenetic life cycle](image-url)

**Fig. ii.** Schematic diagram of *Leishmania* digenetic life cycle (Kaye and Sc...
2.3.2. Clinical Manifestations of VL

While the factors, which determine clinical outcome, are in part, a feature of the parasite, it is the nature of the host and its genetic makeup that play crucial role. Host genetic factors play an important role in the disease (Blackwell, 1996). The symptomology of *Leishmania donovani* is characteristic. The incubation period is from 6 weeks to 6 months but can be as short as 2 weeks or as long as 9 years. Human VL is typified by glomerulosclerosis, mesangial cell proliferation and interstitial nephritis (Andrade and Labuki, 1972; Brito *et al.*, 1975; Weisinger *et al.*, 1978; Dutra *et al.*, 1985; Davies and Mazboumi, 1999). It instigates fever, weight loss, hepatosplenomegaly, splenomegaly, anaemia and depression of the immune system. Lymphadenopathy is usually observed in Sudan but is rare in other endemic regions (Van Griensven and Diro, 2012). Adverse effects include prolongation of the QT interval, sometimes leading to serious arrhythmias (Hepburn, 1993), pancreatitis (Gasser *et al.*, 1994) and hepatic dysfunction (Hepburn *et al.*, 1994). Edema, cachexia and hyperpigmentation (kala-azar means black fever) are late manifestations. In asymptomatic subjects, who have no manifestations of leishmaniasis, however seroconversion and positive skin tests are evidence of previous infection (Badaro *et al.*, 1986; Carvalho *et al.*, 1992; Mary *et al.*, 1992). Complications further include progressive wasting or intercurrent infections (e.g. pneumonia, tuberculosis and diarrhoea) and these may lead to death (Bora, 1999; Guerin *et al.*, 2002; Singh and Sivakumar, 2003) which is caused by the consequences of pancytopenia and opportunistic infections leading to pneumonia and diarrhoea. In 95% of cases, death can be avoided by timely treatment, even in basic field circumstances (Ritmeijer and Davidson, 2003).

2.4. Post Kala Azar Dermal Leishmaniasis

It is a dermatropic form of leishmaniasis developed by the ex-VL patients (WHO, 2013), but there are cases without any previous known history of VL (El-Hassan *et al.*, 1992). The disease is characterized by the development of macules, papules and nodules, which first appear around the mouth; those which do not heal spontaneously become denser and spread over the entire body (Berman, 1997). The interval between the end of treatment of VL and the onset of PKDL is variable: PKDL may appear during or directly after treatment (Zijlstra *et al.*, 2003) to up to 2 years post treatment. PKDL patients may be important sources of infection in VL.
transmission (Addy and Nandy, 1992; Desjeux et al., 2013). Most cases of PKDL occur after infection with *Leishmania donovani* and less commonly after *Leishmania infantum*. However, the PKDL is extremely rare in patients infected with *Leishmania chagasi* (Singh et al., 2011). The disease occurs in all areas endemic for *L. donovani* but is commonest in East Africa and on the Indian subcontinent, where up to 50% and 10% of patients (WHO, 2010) with kala-azar, respectively, develop the condition. PKDL is considered to be triggered immunologically and follows apparently successful treatment of visceral leishmaniasis in a proportion of patients. Histologically, the macular and hypopigmented varieties consist of isolated areas, with a granulomatous reaction and few parasites. The more common erythematous and nodular forms show considerable histiocytic infiltration, oedema, proliferation of capillaries and numerous parasites (Desjeux et al., 2013). The inflammatory cells are mainly CD3+, IL-10 is prominent in the lesions, interferon-γ is found uniformly, and IL-4 is present in varying amounts (Ismail et al., 1999). Diminished expression of interferon-γ receptor 1 and tumour necrosing factor (TNF)-R1 and -R2 receptors during PKDL may interfere with an effective host response (Ansari et al., 2008). IL-10-expressing CD3+CD8+ lymphocytes are prominent, and their level decreases with treatment. Patients with PKDL present raised levels of immunoglobulins G3 and G1 and increased serum levels of IL-10. High serum concentrations of IL-10 during visceral leishmaniasis correlate with subsequent development of PKDL. Antiretroviral treatment during HIV coinfection can lead to PKDL (WHO, 2010).

### 2.5. *Leishmania*/HIV co-Infection

One of the major threats to control of visceral leishmaniasis (VL) is its interaction with HIV infection. VL has emerged as an important opportunistic infection associated with HIV. In visceral leishmaniasis endemic areas, people who are immunocompromised due to HIV infection are more prone to developing clinical VL compared to those without HIV co-infection. In fact, *L. infantum* co-infection is now the third most frequent infection in HIV-infected individuals in VL endemic areas (Desjeux and Alvar, 2003). HIV infection rates are 5 % in Brazil, 2–5 % in India (Sinha et al., 2011) and range between 25 and 40 % in Ethiopia (Diro et al., 2011). Furthermore, even non-pathogenic strains of *Leishmania* and other species of trypanosomatids may cause disease in HIV-infected individuals (Chicharro and Alvar, 2003). The existence of both *Leishmania* and HIV in the same cell has been shown to
influence the multiplication and expression of either one or both organisms (Bernier et al., 1995). In the co-infected patients, *Leishmania* parasites and HIV share a symbiotic relationship. Amastigotes stimulate a chronic immune activation, leading to an increased HIV viral load with faster progression to AIDS. The immunosuppression caused by HIV is particularly favorable for the uncontrolled multiplication of the *Leishmania* parasite (Alvar et al., 2008).

2.6. Animal Models

Since human beings cannot be used to understand the immunological and therapeutic aspects of any vaccine or drug due to ethical considerations, animals remain the best model for the development of new chemotherapy and identification and characterization of potential vaccine targets. An ideal animal model should be similar in physiology to that of human beings. It should mimic the pathological and immunological features of the patients. Also, the animal model should be easily available and its handling should not be difficult. *Leishmania* infections vary markedly between individuals whereas an animal model may always present a uniform disease profile (Awasthi et al., 2004). A suitable laboratory host for the target parasite is very important from the point of view of conducting research on various aspects including host-parasite interactions, pathogenesis, biochemical changes, prophylaxis and maintenance of parasite and above all evaluation of antileishmanial action of newer compounds for development of newer drugs (Garg and Dube, 2006). No animal model is known to mimic the exact pathological features like human beings (Nieto et al., 2011). For *in vivo* testing of drugs or vaccines, the best studied animal models include mice and hamsters as primary screens, dogs as secondary screens and squirrel, vervet, and Indian langur monkeys as tertiary screens (Selvapandian et al., 2012).

2.6.1. Rodent models

Several attempts were made in the past to use small rodents with *L. donovani* infection. These include hamsters (European, Chinese and Syrian); mice (BALB/c, NMRI, C57BL/6), rats, squirrels, gerbils 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 drug and vaccine testing against VL (Garg and
Dubey, 2006). Murine models of leishmaniasis have been extensively used to study the pathogenesis of disease and to test novel therapeutic and immunoprophylactic agents (Mehta et al., 2008). This model also led to the characterization of the immune mechanisms important for the development of organ specific immune responses, which cause the clearance of parasites from liver but not the spleen. Another important contribution of the mouse model has been the discovery that chemotherapy is ineffective in the absence of intact T-cell mediated responses (Handman, 2001). Different *Leishmania* species cause clinically distinct diseases and the severity of disease caused by any given parasite can vary markedly between individual hosts (Bradley, 1974). 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); whereas BALB/c and all T-cell immunodeficient strains manifest a systemic visceral leishmaniasis 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 further growth of the parasite causing self-healing phenotype whereas susceptible BALB/c strain mounts early Th2 response and results in non healing lesion and exaggeration of disease (Latifinia et al., 2013). 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. (L.) donovani* or *L. (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 is then controlled by the host immune response (Garg and Dube, 2006). As mentioned above, human visceral leishmaniasis presents a spectrum of clinical manifestations from a self-controlled infection to a progressive disease. The mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of protective immune response. On the other hand, better model to study progressive disease is hamsters infected with *L. (L.) donovani* or *L. (L.) chagasi* that develop 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, initial immune events at the bite site, parasite tropism, and pathogenesis (Aslan et al., 2013)
2.6.2. Hamsters

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 (Garg and Dube, 2006). Various studies have revealed that the mice model of *L. donovani* does not reproduce the features of active human VL like chronic fever, hepatosplenomegaly, pancytopenia and profound cachexia and have an ineffective antileishmanial cellular response (Melby *et al.*, 2001a). On the contrary, Syrian golden hamster model of active VL closely relates the human counterpart as shown by relentless increase in visceral parasite burden, progressive cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinaemia and ultimately death. While the mice are either intrinsically resistant or susceptible to *Leishmania* infection and offer a well characterized genetic makeup, chiefly by the use of inbred, recombinant and naturally or experimentally mutated strains, hamsters provide an excellent model for an overtly susceptible host. Therefore, hamsters are used for histopathological and vaccine studies despite the lack of fine immunochemicals that limit the mechanistic exploration of immune responses to *Leishmania* infection (Melby *et al.*, 2001a).

2.6.3. 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 (Singh *et al.*, 2010; Gupta and Nishi, 2011); 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 but others will develop a progressive disease ultimately leading to death (Teixeira and Gomes, 2013). 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 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 and vaccination studies could be performed (Mendonca et al., 1995). 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 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).

2.6.4. 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 monkeys (Chapman et al., 1983) and squirrel monkeys (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).

2.7. Diagnosis

The clinical signs and symptoms are not pathognomic of VL or CL. The kala-azar may be confused with other similar conditions such as malaria, tropical splenomegaly, schistosomiasis or cirrhosis with portal hypertension, african trypanosomiasis, milliary tuberculosis, brucellosis, typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma, and leukemia (Singh and Sivakumar, 2003). Similarly, numerous primary and secondary skin conditions are frequently overdiagnosed as early lesions of cutaneous leishmaniasis in endemic countries and in non endemic countries CL is misdiagnosed as other diseases. Some of the common conditions that should be differentiated from cutaneous leishmaniasis are tropical ulcers, impetigo, infected insect bites, leprosy, lupus vulgaris, tertiary syphilis, yaws, blastomycosis, skin cancer, etc. (Herwaldt, 1999; Singh, 2006). Therefore, reliable laboratory methods become mandatory for accurate diagnosis. Early case detection followed by adequate treatment is central to the control of VL (Kumar et al., 2011). Patient management, screening of asymptomatic infections, surveillance including verification of elimination, and epidemiological studies are some of the areas where diagnostic tests play a major role. Ideally, a test should make the distinction between acute disease and asymptomatic infection, as most of the antileishmanial drugs are toxic. In order to provide more sensitive and accurate diagnostic tests, new tools based on parasitological, immunological and molecular approaches have been developed and validated (Gomes et al., 2008).

2.7.1. Parasitological Diagnosis

Parasitological diagnosis remains the gold standard in the diagnosis of leishmaniasis because of its high specificity (Herwaldt 1999; Galai et al., 2011). The amastigote forms (called LD bodies) can be seen in tissue smears from lymph nodes, bone marrow or spleen. Splenic smears have the sensitivity of 93.1–98.7% (Zijlstra et al., 1992; Boelaert et al., 2007). Bone marrow and the lymph node smears have lower sensitivity ranging from 52–85% (Ho et al., 1948) and 52–58% (Zijlstra et al., 1992; Siddig et al., 1988; Srvidya et al., 2012) respectively. Bone marrow aspiration (BMA) or splenic aspirations are painful and risky techniques. Serious or fatal
bleeding after splenic aspiration is not uncommon, however, in skilled and experienced hands, serious bleeding is rare. The use of microscopy in the diagnosis of VL offers the benefits of high specificity and the possibility of grading the parasite on a logarithmic scale (0-6+) in splenic smears (Chulay and Bryceson, 1983). But like all microscopic procedures it suffers from variability of detection, sensitivity and the inevitable need for an expert microscopist. Culture, too, suffers from same deficiencies and the tedious, time consuming nature of technique and high cost are prohibitive and thus, except in dedicated research laboratories, it is seldom used for clinical diagnosis. In a modification in the form of microtitre culture, sensitive and reproducible detection of parasites is possible using buffy coat (WBC rich layer) and peripheral blood mononuclear cells (PBMC) isolated from patient blood (Maurya et al., 2010).

2.7.2. Serological Diagnosis

Serological diagnosis using various techniques is based on detection of *Leishmania*-specific antibodies (Herwaldt 1999; Lakhal-Naouar et al., 2009). A wide range of serological methods varying in sensitivity and specificity are available for the diagnosis of VL. These serological methods can be grouped into non-specific and specific tests. Formal gel test was being used in the past but should be abandoned because of its poor specificity and sensitivity (Srivastava et al., 2011).

2.7.3. Indirect Fluorescent Antibody Test (IFAT)

The test is based on detecting antibodies, which are demonstrated in the very early stages of infection and are undetectable six to nine months after cure. If the antibodies persist in low titres, it is an indication of a probable relapse. It is sensitive (96%) and specific (98%) but the requirement of sophisticated laboratory conditions prohibit its application in the field (Sassi et al., 1999; Kumar and Kumar, 2012).

2.7.4. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA has been used in the serodiagnosis of VL, but its sensitivity and specificity depends upon the type of antigen used. Most promising results are shown by antigen rK39 with sensitivity and specificity of 100% and 96% respectively (Palatnik-de-Sousa et al., 1995; Molinet et al., 2013). The antibody titres to this antigen directly correlate with active disease and have the potential in monitoring chemotherapy and in predicting the clinical relapse (Kumar et al., 2001). In addition,
rK39 ELISA has a high diagnostic and prognostic utility in HIV-infected patients (Houghton et al., 1998). However, up to 32% healthy individuals living in endemic areas test positive with this test (Sundar et al., 1998a; Kumar et al., 2011). Moreover, due to the requirement of skilled personnel, sophisticated equipment and electricity, ELISA is not used in the endemic regions for the diagnosis of VL (Bhargava and Singh, 2012).

### 2.7.5. Immunoblotting

It provides detailed antibody responses to various leishmanial antigens. This test is more sensitive than IFAT and ELISA, but expensive and time consuming. (Srivastava et al., 2011). The band pattern can correlate with disease stages (Singh, 2006). Titers above 1:20 are significant and above 1:128 are diagnostic (Davidson, 1998). It also requires considerable skill and is therefore only sparingly used in the diagnosis of VL.

### 2.7.6. Direct Agglutination Test (DAT)

In this test, Coomassie brilliant blue stained whole promastigotes are incubated with sera of the patients and agglutination observed after an overnight incubation. It is a highly specific, sensitive, inexpensive and simple test. Initially, aqueous antigen was used but it had the drawback of cold chain requirement, and short life. Now, freeze dried antigen has been developed which can be transported at ambient temperature. In a meta-analysis of studies using DAT, it had sensitivity and specificity estimates of 94.8% and 85.9%, respectively (Chappius et al., 2006). However, the major disadvantage of DAT is the relative long incubation time of 18 h and the need for serial dilutions of serum (Singh and Shivakumar, 2003)). Also, DAT has no prognostic value. As with any antibody based test, DAT remains positive for a long time after the disease is cured, and thus cannot be used as a test of cure or for diagnosis of relapses (Kumar and Kumar, 2012). Furthermore, about 20-30% of healthy individuals living in the endemic areas test positive with DAT, and an illness mimicking VL might be mistaken for VL (Sundar et al., 2006).
2.7.8. Immunochromatographic (ICT) strip test

Immunochromatographic strips use K39 antigen containing 39 amino acids encoded by a 117 base pairs gene encoded in the highly conserved kinesin region of *L. chagasi* (Chappius *et al.*, 2007). In micro-ELISA format this antigen showed remarkably high sensitivity. Using its recombinant product, an immunochromatographic based strip test has been developed in which rK39 is fixed on a nitrocellulose paper, and colloidal gold-protein A is used for detection. A drop of serum or blood obtained by finger prick is smeared over the tip of strips and dipped in a small amount of buffer, with the results read within 15 minutes. In the initial clinical evaluation 100% sensitivity and 98% specificity has been observed (Bhargava and Singh, 2012). The rK39 strip test has been found to be highly sensitive and a reliable indicator of kala-azar (Houghton *et al.*, 1998; Burns Jr *et al.*, 1993; Singh *et al.*, 1995; Singh *et al.*, 2002). In a meta-analysis of rK39 strip test studies, the results have been found to be quite uniform with very high sensitivity of 98.40-100% and specificity of 81.20-96.40% (Boelaert *et al.*, 2008). ICT suffers from the same disadvantage as DAT being positive in a significant proportion of healthy individuals in endemic regions and for long periods after cure of VL.

2.7.9. Antigen Detection

Antigen levels are expected to broadly correlate with the parasite load. It is more specific than antibody-based immunodiagnostic tests (De Colmenares *et al.*, 1995; Vinayak *et al.*, 1994; Singh, 2006). This method of diagnosis should be a better alternative to the antibody detection, particularly in HIV-VL coinfection, where antibody response is very poor (Prajapati and Mehrotra, 2013). Two polypeptide fractions of 72-75 kDa and 123 kDa have been detected in the urine of kala-azar patients. The sensitivity of 72-75 kDa fractions was 96% and the specificity was 100%. These antigens were not detectable within three weeks of successful antileishmanial treatment, suggesting that the test has a very good prognostic value (De Colmenares *et al.*, 1995; Sundar and Rai, 2002). Another urinary leishmanial antigen, a low-molecular-weight, heat-stable carbohydrate has been detected in the urine of VL patients (Sarkari *et al.*, 2002). An agglutination test to detect this antigen has been evaluated extensively in clinical trials, using urine collected from well-defined cases and controls from endemic and non-endemic regions. This test shows 79.1-94.1% specificity and sensitivity of 60.4-71.6% in India (Boelaert *et al.*, 2008).
However, the sensitivity of this test was low in clinically suspected patients (Boelaert et al., 2008).

2.7.10. Molecular Diagnosis

PCR-based assays form the mainstay of molecular diagnosis especially for HIV-VL coinfections (De Doncker et al., 2005) with primers targeting several multicity genes, e.g., rRNA genes, kinetoplast DNA (kDNA), minicircles (Attar et al., 2001; Santos-Gomes et al., 2000; El Tai et al., 2001; Pizzuto et al., 2001) etc. Studies have shown the sensitivities of the *Leishmania* species-specific PCR to be 95.7% for bone marrow aspirate and 98.5% for peripheral blood samples versus sensitivities of 76.2%, 85.5%, and 90.2% for bone marrow aspirate isolation, serologic testing, and microscopic examination of bone marrow biopsy specimens, respectively (Antinori et al., 2007). In PCR screening of blood samples of suspected cases of VL, sensitivity ranging from around 70% (Osman et al., 1997; Adhya et al., 1995) to around 100% (Salotra et al., 2001; Bossolasco et al., 2003; Srivastava et al., 2011) has been reported. Real-time PCR has made quantification of parasite burden possible, with a high degree of complex-specific diagnostic accuracy for clinical samples (Wortmann et al., 2005). Quantitative nucleic acid sequence-based amplification (QT-NASBA) detects RNA in a background of DNA and may thus serve to measure viable parasites which might significantly increase assay sensitivity and decrease required sample volume (Reithinger et al., 2007). In a report from Kenya the *Leishmania* OligoC-Test showed a sensitivity of 96.4% and a specificity of 88.8%, while the sensitivity and specificity of the NASBA-OC were 79.8% and 100%, respectively. These findings indicate high sensitivity of the *Leishmania* OligoC-Test on blood while the NASBA-OC is a better marker for active disease (Basiye et al., 2010; Mugasa et al., 2010). The sensitivity of PCR using conjunctival swab (CS) as a sampling method for VL diagnosis by PCR of asymptomatic dogs was found to be 90% by kDNA primer and 83.3% by internal transcribed spacer 1 (ITS1) primer. On the other hand, for blood samples, the positivity of ITS1 PCR was significantly higher than the one obtained by the kDNA PCR-hybridization, indicating that sensitivity of PCR methods can vary according to the biological sample examined (Leite et al., 2010).

Reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) assay, based on nucleic acids, has also been developed as a point-of-care diagnostic
tool (Adams et al., 2010). Amplification is visualized by pre-amplification addition of fluorescent detection reagent (FDR) and a simple UV lamp. By using a reverse-transcriptase step, the system detects infections between 10 and 100 parasites per mL (Srivastava et al., 2011). The assay has been tested on a range of nucleic acid extracts from *Leishmania* species of VL patients from Sudan, and cutaneous leishmaniasis (CL) patients from Suriname. The sensitivity of RT-LAMP from the blood of VL patients was 83% (Adams et al., 2010).

### 2.7.11. Diagnosis of HIV-VL coinfection

VL is an important opportunistic infection in AIDS patients and atypical clinical presentations of VL in HIV-infected patients pose a considerable diagnostic challenge. In fact, the clinical symptoms of fever, splenomegaly and hepatomegaly is found in less than half of such patients, though more so in patients with low CD4 counts (less than 50 CD4+cells/mm³) (Albrecht, 1998; Alvar et al., 1997). In these patients, leishmaniasis can present with gastrointestinal involvement (stomach, duodenum or colon); ascites; pleural or pericardial effusion; involvement of lungs, tonsils, and skin; and even as widely disseminated disease (Alvar et al., 1997; Rosenthal et al., 2000). The diagnostic principles remain essentially the same as those for non-HIV-infected patients. The presence of amastigotes may be demonstrated in buffy coat preparation. Sometimes the presence of amastigotes in unusual sites may be demonstrated (e.g., amastigotes may be present in specimens from bronchoalveolar lavage, pleural fluid, or biopsy specimens from the gastrointestinal tract) (Sundar and Rai, 2002). For HIV patients, the sensitivity of antibody-based immunologic tests like the IFA test and ELISA is low (Dhingra and Satapath, 2014). Since the parasite load is quite heavy in these patients, the presence of *leishmania* amastigotes in the bone marrow can often be verified. However, PCR analysis of the whole blood or its buffy coat preparation may prove a useful screening test for these patients, obviating the need for traumatic procedures (Srivastava et al., 2011).

### 2.8. Immunology

Immunology is fundamentally a measure of the living interaction between parasite and host. In order to develop a successful parasitic relationship with its host, the *Leishmania* must evade both the innate and adaptive immune responses.
2.8.1. Innate Immune Response

The infection with *Leishmania* is accompanied by a complex immune response that begins with the innate response, where innate receptors such as TLR-2 present on macrophages, DC and NK cells recognize molecules present on the parasite surface such as lipophosphoglycan, and induce the production of proinflammatory cytokines such as TNF-α, IFN-γ and IL-12, as well as costimulatory molecules (Becker *et al.*, 2003; Ruiz and Becker, 2007).

To establish a protective immune response against *Leishmania*, the presentation of appropriate antigens by antigen presenting cells (APCs) is a crucial step responsible for T-cell activation. The immune response is initiated at the site of pathogen entry by sentinel cells, including DCs, macrophages and T lymphocytes. Such cells are well equipped with toll-like receptors (TLRs) (Teixeira *et al.*, 2006; Muzio *et al.*, 2000) and phagocytic receptors (Ross, 2000), enabling the detection of pathogen-associated molecular patterns (Gordon, 2002) and uptake of pathogens and opsonized particles. Sentinel cells also express various receptors for cytokines and, together with tissue cells, produce numerous chemokines initiating a cascade of innate responses (Spellberg, 2000). Infection with *Leishmania* begins when an infected female sand fly takes a blood meal from a human host. The sand fly injects the mammalian host with *Leishmania* within its saliva. Sand fly saliva contains well-characterized molecules that have several activities, including vasodilation, inhibition of coagulation and immunomodulatory effects (Sacks and Kamhawi, 2001). It also contains uncharacterized molecules that attract PMNs as well as macrophages (Zer *et al.*, 2001; Anjili *et al.*, 1995). The parasite itself also produces a chemoattractant protein called *Leishmania* chemotactic factor, which can attract PMNs (Teixeira *et al.*, 2006). It has been shown that, two hours after saliva injection, an intense and diffuse inflammatory infiltrate comprising PMNs, eosinophils and macrophages is induced only in mice pre-exposed to saliva (Silva *et al.*, 2005). PMNs are the first cells to arrive at the site of *Leishmania* infection (Muller *et al.*, 2001). In humans, PMNs containing *Leishmania* start secreting chemokines such as IL-8 (also known as CXCL8) (Laufs *et al.*, 2002) that are essential in attracting more PMNs to the site of infection. Upon experimental infection with *L. major*, MIP-2 and keratinocyte-derived cytokine (KC; also known as CXCL1), the functional murine homologs of human IL-8 (Modi and Yoshimura, 1999; Kumar *et al.*, 2010), are rapidly produced in the skin (Muller *et al.*, 2001). *In vitro* studies have also shown that *L. major* promastigotes
induce rapid and transient expression of KC by murine macrophages (Racoosin and Beverley, 1997) and of IL-8 by human macrophages (Badolato et al., 1996). All of these chemokines are chemoattractants for PMNs (Baggiolini, 2001). PMNs can function as phagocytic cells, taking up and killing Leishmania, and they have been implicated in early parasite control (Rebbestad et al., 2012). After being ingested by PMNs, Leishmania induce the release of MIP-1β, recruiting macrophages to the site of infection (Van Zandbergen et al., 2004). Infected PMNs taken up by macrophages do not activate macrophage microbicidal function (Van Zandbergen et al., 2004; Meagher et al., 1992). After ingesting apoptotic PMNs, macrophages undergo inhibition of their proinflammatory cytokine production, through mechanisms involving transforming growth factor-β, prostaglandin E2 and platelet-activating factor (Fadok et al., 1998; Ribeiro-Gomes et al., 2004). These events contribute to a ‘silent’ entry of Leishmania into macrophages, its main host cell type (Laskay et al., 2003).

2.8.2. Macrophages

The interaction between the parasite and the macrophage is likely to be a critical event both in determining the type of immune response which develops and in influencing the ability of macrophages to be activated to eliminate the parasites. A successful treatment of all the forms of leishmaniasis depends on efficient elimination of parasites by activated macrophages. Paradoxically, Leishmania utilizes their phagocytic function as a strategy for internalization and replication within the phagolysosomes (Kedzierski, 2010). Thus, macrophages act as both the host cells and effector cells that kill the parasites. Macrophages produce a large repertoire of cytokines following Leishmania infection that may both positively and negatively regulate these events (Nacy and Meltzer, 1991). In all Leishmania spp. infections, the macrophages are colonized by the amastigote parasites, which multiply intracellularly until the cells are disrupted. In this disease, macrophages play a triple role since they are host cells, antigen-presenting cells (APCs) that activate specific T cells and effector cells whose leishmanicidal activity depends on the presence of activating cytokines such as IFN-γ and TNF-α (Bogdan and Rollinghoff, 1999). During Leishmania promastigote infection, macrophages respond by producing reactive oxygen intermediates (ROI), together with reactive nitrogen intermediates (RNI), lysosomal hydrolases and neutral peptidases (Xu et al., 2011). Nitric oxide is a regulatory molecule that is important in host protective responses, acting not only as
an anti-microbial effector molecule (Brunet, 2001), but also as a potential host-destructive mediator in several pathologies, mainly in infectious diseases (Holzmuller et al., 2006).

Macrophages can kill ingested micro-organisms and thus help eliminate the threat from infection, but some pathogens have adapted to survive within macrophages and use the intracellular niche they provide as a means of immune evasion (Phillips et al., 2010). 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 include 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 of 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 entry 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). LPG is known to be a multifunctional virulence factor that not only help the promastigotes for attachment and entry into macrophages, but also promote the induction of neutrophil extracellular traps (NETs) and inhibit protein kinase C (PKC) and maturation of phagosome (Ibraim et al., 2013). The ability to use a variety of different receptors makes it easier for the promastigotes to enter the macrophages

2.8. 3. NK Cells

Together with phagocytes, NK cells represent the first line of defense against pathogens by two principal mechanisms, cytolitic destruction of infected cells, and secretion of proinflammatory cytokines (e.g., IFNγ, TNFα) (Nylen et al., 2003). Activated NK cells promote DC maturation along with clearance of autologous
immature DCs (Das and Ali, 2012). Natural killer (NK) cells are known for their ability to directly lyse host cells that express an altered cell surface phenotype following a viral infection, malignant transformation, or stress reaction (Bogdan 2012). In addition to their cytotoxic potential NK cells also function as producers of cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF), which contribute to the development of type 1 T helper (Th1) cells and activate macrophages for the expression of antimicrobial killing mechanisms (e.g., inducible or type 2 nitric oxide synthase (iNOS,NOS2)) (Bogdan, 2001; Stetson et al., 2003; Laouar et al., 2005; Prajeeth et al., 2011). Studies have demonstrated that the inhibition of nitric oxide production from inducible nitric oxide synthase (iNOS) (by *Leishmania*) renders macrophages powerless against *Leishmania* infection (Dunning, 2009). In cutaneous *L. major* infections of self-healing mouse strains, NK cell cytotoxic activity and IFN-γ production becomes readily detectable in the draining lymph node at day one of infection (Scharton and Scott, 1993; Bajenoff et al., 2006; Liese et al., 2007). 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).

2.8.4. Neutrophils

The best-characterized function of neutrophils is their prominent role in the phagocytosis and killing of invading microorganisms acting as a first line of defense against invading pathogens. They act via the generation of oxygen intermediates and the release of lytic enzymes stored in their granules. Neutrophils, by promoting tissue injury, also contribute to the initiation of inflammation, an essential step in the launching of immunity. Neutrophils may also be implicated in immunoregulation as a source of cytokines, such as interleukin-2 (IL-12), interleukin-10 (IL-10), gamma interferon (IFN-γ) and TNF-α (Romani et al., 1997; Bliss et al., 1999), thus establishing a link between innate and adaptative immunity during parasitic infection (Appelberg, 2006; Nathan, 2006). The role of neutrophils in infection with *Leishmania* has primarily been studied using experimental models of cutaneous
leishmaniasis caused by *Leishmania major*. In resistant mice, transient depletion of neutrophils has been shown to lead to an increase in the parasite load (Chen *et al.*, 2005; Lima *et al.*, 1998). However, neutrophil depletion in susceptible mice (BALB/c) has been shown to lead to the opposite effect, as demonstrated by an increase in parasite elimination (Ribeiro-Gomes *et al.*, 2004; Tacchini-Cottier *et al.*, 2000).

Neutrophils have also shown to behave like Trojan horses, sponsoring the invasion of macrophages by *L. (L.) major* *in vitro* (Carmo *et al.*, 2010) and *in vivo* (Peters *et al.*, 2008). *In vivo* visualization of the interaction between neutrophils and macrophages revealed that neutrophil-containing *Leishmania* were not directly phagocytosed by macrophages, but rather, neutrophils released the parasites that subsequently entered macrophages (Charmoy *et al.*, 2010). A role for neutrophil in protective immune responses to *L. (Viannia) braziliensis* (Novais *et al.*, 2009) and to visceralizing *Leishmania* species has also been reported (McFarlane *et al.*, 2008; Rousseau *et al.*, 2001).

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).

### 2.8.5. Dendritic cells

Dendritic cells (DCs) are a family of professional antigen presenting cells, which sit in an immature state, capable of antigen uptake and processing, in all peripheral non-lymphoid tissues and therefore function as sentinel of the immune system. Upon recognition of foreign antigen for DCs via the TLR-PAMP interaction (Tuettenberg *et al.*, 2011; Kumar *et al.*, 2011), immature DCs resident in tissues mature into professional antigen-presenting cells (APCs) to induce effector and memory T-cell responses in lymphoid organs (Jin *et al.*, 2012). They can initiate and regulate adaptive immunity by the induction of both CD4+ and CD8+ T cells thereby leading to a long lasting memory response (Okwor and Uzonna, 2009). DCs take up
Review of Literature

antigens via different groups of receptor families, such as Fc receptors for antigen–
antibody complexes, C-type lectin receptors (CLRs) for glycoproteins (Geijtenbeek et al., 2004) and pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs), which enable DCs to recognise a wide range of microbial stimuli (Takeda and Akira, 2004). The interactions between Leishmania parasites and dendritic cells (DCs) are complex and involve paradoxical functions that can stimulate or halt T cell responses, leading to the control of infection or progression of disease (Soong, 2008). 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 (Dunning, 2009). 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 plays an essential role in both initiation and regulation of antimicrobial immune responses to Leishmania (Schleicher et al., 2007). Depending on the nature of the microbial stimulus, they can direct the generation of antigen specific Th1 T cell responses and offers a promising target for vaccination against Leishmania (Matos et al., 2013).

2.8.6. Role of CD4+ cells

Cell mediated immunity is known to play a central role in the host response to control leishmaniasis. The primary immune response is initiated by the presentation of antigen to naive CD4+ T cells. 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 to perform their key roles in controlling humoral, CTL-mediated and inflammatory immune responses (Sandberg and Glas, 2001). 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 an 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; Broere et al., 2011). These cytokines further interact with specific receptor molecules on their target cells.
like macrophages. For example, on presentation of *Leishmania* antigens to CD4+ T 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 (Ruiz and Becker, 2007). Conversely, the secretion of IL-4 during antigen presentation to CD4+ T cells drives Th2 cell development that inhibits Th1 responses and promotes B lymphocyte growth and development (Dunning, 2009). 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).

### 2.8.7. 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+ 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+ and CD4+ cells can on their own prevent reactivation of disease (Belkaid et al., 2002; Murray et al., 2005).

CD8+ 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+ 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 (Tsagozis et al., 2003). These cells have been found to be associated with both cure and pathology in human leishmaniasis. An expanded CD8+ cell population was observed in the draining lymph node prior to ulcer development, implicating CD8+ cell mediated immunity in the early containment of *Leishmania* infection (Bomfim et al., 2007). It was found that CTL’s were involved in the elimination of *L. major* as well as establishment and maintenance of immunity,
since the inhibition of CTL with monoclonal antibodies rendered resistant mice susceptible to \textit{L. major} (Dunning, 2009). Elevated numbers of CD8 have been reported in blood and lesions of patients infected with \textit{L. major} and \textit{L. mexicana} and their protective role has been associated with IFN\gamma production (Hernandez-Ruiz \textit{et al.}, 2010). Exacerbated CD8$^+$ cell activity, in addition to a poor regulatory response, could however, underlie an unfavorable fate with regard to MCL. Basu \textit{et al.} (2007) demonstrated that CD8$^+$ T cells are vital in a protective response against \textit{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$^+$ T cells expressing granzyme A is associated with lesion progression in CL caused by \textit{L. braziliensis} (Faria \textit{et al.}, 2009). Accumulation of CD8$^+$ cells has also been linked to PKDL (Ghosh \textit{et al.}, 1995; Nylen and Gautam, 2010).

2.8.8. Role of Cytokines

2.8.8.1. IL-10

IL-10 is an anti-inflammatory cytokine. It was initially characterized as a Th2 cytokine but later on it was proved to be a pleiotropic cytokine, secreted from different cell types including the macrophages (Sharma and Singh, 2009). Experimental models have clearly demonstrated the central role played by IL-10 in pathology and parasite persistence (Anderson \textit{et al.}, 2007; Kane and Mosser, 2001). Originally identified as a Th2 cell-derived factor, it is now known to be secreted also by regulatory T cells (Treg), Th1 cells, CD8$^+$T cells, B cells (Ronet \textit{et al.}, 2010; Schwarz \textit{et al.}, 2013), macrophages, DC, mast cells, eosinophils, NK cells and some cell types not belonging to the immune system (Moore \textit{et al.}, 2001; Bouabe \textit{et al.}, 2011; Hedrich and Bream, 2010). During infection it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for optimal pathogen clearance but also contribute to tissue damage (Couper \textit{et al.}, 2008). 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 \textit{et al.}, 1993; Ghalib \textit{et al.}, 1993; Kenney \textit{et al.}, 1998). The suppressive function of IL-10 also involves the inhibition of the expression of MHC class II and costimulatory molecules (Moore \textit{et al.}, 2001; Gazzinelli \textit{et al.}, 1996; O’Garra and Vieira, 2004). The regulation of IL-10 production by macrophages infected with \textit{Leishmania donovani} was linked to phosphorylation and inactivation of
glycogen synthase kinase-3β (GSK-3β) which negatively regulates CREB (transcription factor), and in turn positively regulates IL-10 expression thus leading to disease exacerbation (Nandan et al., 2012). IL-10 has been shown to block Th1 cell activation and consequently a cytotoxic response by down-regulating IL-12 and IFN-γ production (Tripathi et al., 2007). IL-10 has been suggested to be mainly produced by regulatory T (CD4⁺ (or CD8⁺) CD25⁺) cells (T reg cells) rather than by Th2 cells during Leishmania infection in mice. Thus, exacerbation of infection may not be due to the action of Th2 cells, but to another type of T lymphocyte (Sacks and Anderson, 2004). Although IL-10 responses are typically generated to balance excessive Th1 and CD8⁺ T-cell responses and prevent immunopathology, overproduction of IL-10 has been shown to inhibit proinflammatory responses leading to susceptibility to infectious pathogens such as Leishmania (Anderson et al., 2005; Belkaid et al., 2001).

2.8.8.2. IL-4

IL-4 is considered to be the signature cytokine of Th-2 response. 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 down regulating 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 (Saha et al., 2006).

2.8.8.3. IFN-γ

The control of all forms of leishmaniasis requires the IL-12 driven differentiation and recruitment of IFN-γ producing T- helper cells (Mougneau et al., 2011), the presence of IFN-γ activated macrophages capable to kill intracellular amastigotes (Lykens et al., 2010), and mechanisms that prevent hyperinflammation and mediate resolution of the tisse damage (Peters and Sacks, 2006). Seminal studies on experimental infections of Leishmania major in C57BL/6 and BALB/c inbred
mouse strains correlated parasite control with the elaboration of interferon gamma (IFN-γ) by CD4+ T cells and uncontrolled infections with the absence of IFN-γ in the C57BL/6 and BALB/c mice, respectively (Kima and Soong, 2013; Reiner and Locksley, 1995). Delivery of IFN-γ at the time of infection can modulate T-cell subset development but does not affect the eventual outcome of disease (Scott, 1991). Sustained IFN-γ delivery to macrophages \textit{in vivo} by \textit{L. major} parasites themselves is also unable to change the course of infection (Tobin \textit{et al.}, 1993).

Cells infected with either the promastigote or amastigote forms of the parasite are less responsive to stimuli such as LPS that elicits IFN-γ production or IFN-γ itself that activates the antiparasitic effector response of macrophages (Abu-Dayyeh \textit{et al.}, 2010; Matte and Descoteaux, 2010). In visceral leishmaniasis also IFN-γ induces microbicidal activity against both the promastigotes and amastigotes of \textit{L. donovani} in monocyte derived human macrophages, in H2O2-dependent (Murray \textit{et al.}, 1983; Das and Ali, 2012) as well as in NO mediated pathway (Vouldoukis \textit{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 \textit{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 \textit{in vitro} (Sharma and Singh, 2009). 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 \textit{et al.}, 1989; Goto and Prianti, 2009). It is also produced in higher amounts by PBMC from ML than from CL patients (Carvalho \textit{et al.}, 2007). It has been found to be effective in the chemotherapy of VL or CL patients (Badaro \textit{et al.}, 1990; Harms \textit{et al.}, 1989).

\textbf{2.8.8.4. 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 (Malek, 2008). It mediates several lymphocyte functions. In experimental visceral leishmaniasis, acquired resistance is T cell-dependent, involves IFN-γ activated macrophages, and is expressed in the tissues by granuloma formation (Murray \textit{et al.}, 1983; Das and Ali, 2012).
Resistance is also correlated with antigen-stimulated IL-2 secretion. Continuous administration of IL-2 to L. donovani-infected BALB/c mice was found to reduce 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 has been 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). Resistant strains of mouse which exhibit a self-limiting infection develop an immune response dominated by CD4+ Th1 cells secreting IFN-γ, IL-2 and TNF (Lonardoni et al., 2000). It is also found that IL-2 limits the expansion of Th1 CD4+ T cells even in the presence of IL-12 during Leishmania amazonensis infections and promote the survival of an unpolarized T cell population (Ramer-Tait et al., 2011).

2.9. Vaccines

Leishmaniasis exists in three major forms cutaneous, mucocutaneous and visceral leishmaniasis; but the field of vaccine designing has focused on all the forms because of the conserved nature of molecules in all the species of Leishmania that have been selected as the targets (Singh and Sundar, 2012). Although considerable progress has been made over the last decade in understanding immune mechanisms underlying potential candidate antigens, including killed, live attenuated parasites, crude parasites, pure or recombinant Leishmania proteins or DNA encoding leishmanial proteins, as well as immunomodulators from sand fly saliva, very few candidate vaccines have progressed beyond the experimental stage (Kedzierski et al., 2006; Dunning, 2009). As such there is no vaccine against any form of human leishmaniasis (Duthie et al., 2012).

2.9.1. First generation vaccines

The first vaccine against leishmaniasis was developed by Professor Adler at the Hebrew University of Jerusalem, Israel, who had observed that mothers of Lebanon exposed their children's arms to the bite of sand flies because they intuitively knew that the development of a self-healing single first lesion would protect them from the severe disease in future. Therefore, the ancient practice was to
inoculate uninfected individuals with infectious material from lesions, in the body where the scar would be hidden. After a method for axenic cu parasites was established, “leishmanization” became usual in Israel and further evolved to the use of first generation vaccines composed of w parasites or crude extracts. Leishmanization process was discontinu uncontrolled long-lasting skin lesions, the spread of HIV and tl immunosuppressive drugs, ethical reasons, parasite persistence, and diffict quality inoculum control. Its use at present is limited to one vaccine r Uzbekistan and to live challenge in vaccine efficacy trials in humans in Ira de-Sousa, 2008).

The early trials with killed *Leishmania* as a vaccine were co Brazil in 1940s (Khamesipour *et al.*, 2006). Vaccination with killed dates back to early 1940s and was pioneered by Brazilian scienti Mayrink and his colleagues developed a killed vaccine composed of fi of *Leishmania* (Mayrink *et al.*, 1979; 1985; Ganero *et al.*, 1996; Nagill 2011). The vaccine was shown to be safe and immunogenic as m leishmanin skin test (LST) conversion, but conferred only a small protection (50%). A simplified vaccine composed only of *L. amazonen* as Leishvacin, was tested for prophylactic potential in Columbia (V 2000; 2005) and Ecuador (Armijos *et al.*, 2004) and showed that the v safe but not efficacious.

Convit and his colleagues (Sharples *et al.*, 1994) in Venezuela the use of immunotherapy with a combination of killed *L. amazomexicana* and Bacillus Calmette Guerin (BCG) for the treatment o cutaneous leishmaniasis and observed healing rate of more than 95% was associated with a Th1 like immune response in the patients (Co 1987; Convit *et al.*, 2003; Alvar *et al.*, 2013). The combination o *amazonensis* promastigotes with a half-dose regimen of meglumine was also shown to be highly effective for the treatment of leishmaniasis in Brazil (Machado-Pinto *et al.*, 2002). Additionally, preparation consisting of *L. braziliensis*, *L. guyanensis* and *L. amazo* evaluated in Ecuador and showed significant protection against leishmaniasis (Armijos *et al.*, 1998; 2003; Noazin *et al.*, 2008). I modified form of the vaccine using pasteurized *L. braziliensis* promas live BCG was effective in the treatment of refractory muc
leishmaniasis and early cases of diffuse cutaneous leishmaniasis (Convit et al., 2004). In dogs, the use of merthiolated sound-disrupted promastigotes of *L. braziliensis* along with BCG was usually accompanied by strong cellular reactivity, as observed by lymphocyte proliferation (Mayrink et al., 1996). A vaccine comprising killed *L. braziliensis*, sand fly gland extract (SGE) and saponin elicited a strong antigenicity against *L. chagasi* infection which related to the increase of anti-*Leishmania* IgG isotypes and increased nitric oxide production after in vitro stimulation with *L. chagasi* (Giunchetti et al., 2007; 2008). In ACL patients, administration of two doses of killed *Leishmania (Leishmania) amazonensis* vaccine was found to efficiently confer protection in the Caratinga microregion, south-east Brazil (Mayrink et al., 2013).

An epidemiological study in eastern Sudan suggested that previous exposure or infection with *L. major* protected against visceral leishmaniasis caused by *L. donovani* (Zijlstra et al., 1994). Keeping this in mind, various studies have been carried out with autoclaved *L. major* and *L. donovani* along with BCG or CpG oligonucleotides against cutaneous and visceral leishmaniasis in primates and murine models and encouraging results have been observed by many workers (Misra et al., 2001; Rhee et al., 2002; Srivastava et al., 2003; Michel et al., 2006) that provides the basis for further human trials.

Vaccination with autoclaved *L. major* (ALM) along with BCG has been found to be safe but did not induce significant protective immune response in healthy volunteers against cutaneous leishmaniasis in Iran (Momeni et al., 1998; Sharifi et al., 1998) and against visceral leishmaniasis in Sudan (Khalil et al., 2000; Satti et al., 2001). However, its alum-absorbed preparation (alum-ALM) along with BCG was shown to be more immunogenic, protective and produced a strong and long-lasting Th1 response in mice, monkeys and dogs against visceral leishmaniasis (Kenney et al., 1999; Misra et al., 2001; Mohebali et al., 2004). but it did not produce conclusive results in humans (Kamil et al., 2003; Khalil et al., 2006; Ghalib and Moaddabber, 2007). In a study, co-administration of intrarectal BCG prior to subcutaneous ALM+alum induced protective type 1 immune responses against *L. major* infection in susceptible BALB/c mice (Soudi, et al., 2011).

First generation vaccines composed of whole killed parasites have been proposed as both prophylactic and therapeutic vaccines. The therapeutic application
may be particularly important in cases of drug resistant refractory disease. In theory, these vaccines should be easy to produce at a low cost in endemic countries; however, standardization of cultured parasite-derived vaccines is a drawback in the way to their registration. In general, the whole-cell, killed vaccines have been rather poorly defined and variable in potency, hence they have rendered inconclusive results (Noazin et al., 2008). Nevertheless, the trials completed so far demonstrated their good safety profile, and despite poor prophylactic outcomes, showed encouraging results as therapeutic vaccines in South America and Sudan (Kedzierski, 2010).

2.9.2. Subunit vaccines

Despite the knowledge about various life stages of the parasite and the ongoing work, designing an effective vaccine against leishmaniasis is still a matter of research, there are hundreds of potent vaccine candidates but issues regarding the cost, antigenic complexity along with the variability of organisms and the mixed type of responses produced in the host are limiting the progress in the relevant direction. Thus the technical challenges and the complexity in the immunity against the parasites clearly contribute to the absence of vaccines. The major hurdle in developing a potent vaccine is lack of more than one experimental model for studies which do not provide us all the facets of immune responses in humans and the safety issues further limits their development (Mayrink et al., 2013).

2.9.2.1. Cysteine protease

These are of major two types in eukaryotes–cysteine proteases (papain like) and serine proteases (trypsin like) while in case of Leishmania cysteine protease is the major protease involved in survival of parasite within the host cell and adaptation to the changing environment (Olivier et al., 2005). These proteases cause nucleophilic attack cleaving the peptide bond. 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., 2003a). Three classes of CPs have been identified; Type I (CPB), Type II (CPA) and Type III (CPC) (Robertson and Coombs, 1993). Type I CP (CPB) is encoded by multicy copy genes arranged in tandem arrays and is characterized by the presence of a long C-terminal extension (CTE) rich in proline, serine and/or threonine residues, both in L. major and L. mexicana (Mottram et al., 2004). Type II (CPA) has been described in L.
major, L. mexicana, and L. infantum (Denise et al., 2006; Williams et al., 2006) and Type III (CPC) is reported in L. mexicana, L. major, L. donovani, and L. chagasi and present in all life cycle stages of Leishmania (Rafati et al., 2006b) is encoded by a single copy gene (Khamesipour et al., 2006). Immunization of mice with cysteine proteinases elicit a protective response induced by T cell proliferation with increased IFN-γ production against L. major infection (Aebischer et al., 2000; Rafati et al., 2000). Further studies on immunization with recombinant CPA and CPB along with poloxomer 407 as an adjuvant revealed that only recombinant CPB, and not CPA was able to confer partial protection in mice against L. major challenge and protection relied on IFN-γ producing CD8+ T lymphocytes (Rafati et al., 2002). Furthermore, vaccination with pDNA encoding cysteine proteinase Type II (CPA) and Type I (CPB) with its unusual C-terminal extension (CTE) along with cationic solid lipid nanoparticles (cSLN) as an adjuvant showed highest parasite inhibition at 3-month post vaccination against L. major in BALB/c mice (Doroud et al., 2011). It has also been shown that a hybrid fusion protein of CPA/B induced partial protection against experimental cutaneous infection in mice with increased level of IgG2a and IFN-γ, pointing towards Th1 type of immune response (Zadeh-Vakili et al., 2004). However, in patients of cutaneous leishmaniasis due to L. guyanensis, purified CPA and CPB induced Th1 response (Pascalis et al., 2003). Leishmania mexicana cysteine protease is a T-cell immunogen, causes the development of protective Th1 cell lines by inducing the expression of IL-12 and in turn IFN-gamma by the macrophages that leads to the killing of parasite (Singh and Sundar, 2012). Finally, cysteine proteinases are attractive drug and/or vaccine targets as they are involved in parasite survival, replication and onset of disease (McKerrow et al., 1999; Nagill and Kaur, 2011).

2.9.2.2. LACK

The LACK antigen (Leishmania homologue of receptor for activated C kinase) (36 kDa) is highly conserved among Leishmania species and is expressed by both promastigote and amastigote forms of the parasite (Nagill and Kaur, 2011). LACK and its homologue from mammals called as RACK1 belongs to the WD repeat protein family. These macromolecules are highly conserved proteins with tryptophan aspartate motif and have variety of functions in the eukaryotes, including signal transduction, RNA processing, and cell cycle control (Singh and Sundar, 2012). In L. infantum, LACK is localized near the kinetoplast, and interacts with sequences from
proteins and performs a variety of tasks from DNA replication, RNA synthesis and processing to signal transduction and regulation of cell cycle (Gonzalez-Aseguinolaza et al., 1999). The threshold level of protein is required for establishing parasitism or disruption of regulatory regions responsive for the phagolysosomal environment (Kelly et al., 2003). Vaccination using LACK has been found to be protective against *L. major* infection by a number of workers (Mougneau et al., 1995; Gurunathan et al., 1997; Soussi et al., 2002; Evans and Kedzierski, 2012). However, vaccine strategies involving LACK against other species like *L. mexicana* and *L. donovani* have been unsuccessful (Melby et al., 2001; Aguilar-Tormentera and Carlier, 2001; Duthie et al., 2012). Although immunization of mice with LACK, delivered in DNA form, could induce a robust parasite-specific Th1 immune response, it was unable to protect mice against *L. donovani* challenge infection (Melby et al., 2001).

### 2.9.2.3. PSA-2/GP46/M-2

GP46/M-2, a promastigote surface membrane glycoprotein, ranging from 96, 80 to 50 kDa, elicits a protective immune response; this molecule, when administered with an adjuvant, can elicit a protective response against infection in susceptible mice against challenge infection with *L. amazonensis* (Champsi and McMahon-Pratt, 1988; Lohman et al., 1990), *L. major* and *L. mexicana* (McMahon-Pratt et al., 1993). Three distinct polypeptides on the surface of promastigotes, collectively known as promastigote surface antigen 2 (PSA-2) complex, and only one on amastigotes, have been identified that encoded the genes of the same family as gp46/M-2 (Symons et al., 1994). The *L. infantum* PSA-2 consists predominantly of 15 leucine-rich repeats (LRR) that are shared with an unrelated surface proteophosphoglycan (PPG) (Kedzierski et al., 2004). 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). One of the PSA-2 genes was cloned and expressed in both *E. coli* and *L. mexicana* promastigotes. In a recent study it was shown that parasite surface antigen-2 is involved in macrophage invasion through the interaction of its leucine rich repeats (LRR) with complement receptor 3 (CR3) (Mutiso et al., 2013). 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 \textit{L. major} PSA-2 expressed episomally in \textit{L. mexicana}
promastigotes conferred protection against cutaneous leishmaniasis in mice
(Handman \textit{et al.}, 1995). It has also been shown that vaccination with native PSA-2
plus \textit{Corynebacterium parvum} as adjuvant protects mice from \textit{Leishmania} through a
Th1 mediated response, but the recombinant PSA-2 purified from \textit{Escherichia coli}
and administered in immunestimulating complexes (ISCOMs) or mixed with \textit{C. parvum}
as an adjuvant, does not induce protective immunity despite the induction of
Th1 responses. However, protection of susceptible BALB/c mice by vaccination with
secreted/excreted \textit{L. major} antigens has been attributed to a combination of PSA-2
and LPG (Tonui \textit{et al.}, 2004).

2.9.2.4. Kinetoplastid membrane protein (KMP-11)

KMP-11, a highly conservative surface membrane protein associated to
lipophosphoglycan (LPG), (King \textit{et al.}, 1987) is expressed differentially in
amastigote and promastigote stages of \textit{Leishmania} (Jardim \textit{et al.}, 1995;
Berberich \textit{et al.}, 1997; Carrillo \textit{et al.}, 2008). The increased expression in
metacyclic promastigotes and especially in amastigotes indicate a role for this
molecule in parasite survival in the mammalian host (Matos \textit{et al.}, 2010). Patients
cured from visceral leishmaniasis caused by \textit{L. donovani} showed cellular
proliferation against KMP-11 and also production of IFN-\(\gamma\) and IL-4 (Kemp \textit{et al.},
1993; Kurtzhals \textit{et al.}, 1994). It has been described that the DNA vaccine of
KMP-11 protected hamsters experimentally infected with \textit{L. donovani} (Basu \textit{et al.},
2005). Peripheral blood mononuclear cells from american tegumentary
leishmaniasis patients stimulated with KMP-11 produce high levels of IL-10 (de
Carvalho \textit{et al.}, 2003, Carvalho \textit{et al.}, 2005), a cytokine responsible for pathogenesis
and parasite persistence in leishmaniasis (Belkaid \textit{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 \textit{et al.}, 1991, Russo \textit{et al.}, 1992). Recently, it was demonstrated
that immunization with polyester poly(lactide-co-glycolide acid) (PLGA)
nanoparticles loaded with the kinetoplastid membrane protein 11 (KMP-11)
significantly reduced parasite load \textit{in vivo} by the production of pro-inflammatory
cytokines and chemokines and reactive oxygen intermediates and by triggering of the
2.9.2.5. Histones

Histones are evolutionarily conserved proteins which associate with DNA to form the chromatin structural unit in eukaryotes, the nucleosome. *Leishmania* histone H2B protein is present in both the amastigote and the promastigote forms of the parasite and is highly conserved among *Leishmania* spp (Singh and Sundar, 2012). *Leishmania* histones are intracellular components that behave as pathoantigens related to the virulent *Leishmania* phenotype and pathological stage of disease (Chang and McGwire, 2002). 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 (Carrion et al., 2008). The protection was only associated with a significant reduction in the cytokine levels of IL-4. In contrast, mice immunized 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 (Iborra et al., 2004). *Leishmania* histone H2B has been reported to be a promising candidate for both vaccination and serodiagnosis. 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 (Meddeb-Garnaoui et al., 2010). Considerable leishmanicidal properties (dose-dependent killing promastigotes of *L. amazonensis*, *L. major*, *L. braziliensis*, and *L. mexicana*) have also been demonstrated for histones H2A and H2B (but not histone H1β) (Wang et al., 2011) which show a new possibility of using these histone proteins for therapeutic vaccinations against leishmaniasis (Das and Ali, 2012).

2.9.2.6. Glucose regulated protein 78 (GRP 78)

Glucose-regulated protein 78 (GRP78), also known as BiP (Binding protein) is a 78 kDa Ca^{2+} binding chaperone molecule and belongs to heat shock protein 70 family (HSP70). The HSP70s are well known for their immunogenicity in many parasitic infections such as leishmaniasis (de Andrade et al., 1992; Wallace et al., 1992; Skeiky et al., 1995), malaria (Behr et al., 1992), schistosomiasis (Moser et al., 1990) and onchocerciasis (Rothstein and
Rajan, 1991). They are highly abundant proteins, which, although similar to their human homologues, possess many foreign determinants that induce strong humoral and cellular immune responses during infection. Proteins of the HSP70 family are central components of many fundamental cellular processes, including the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organellar and secretory proteins, proteolytic degradation of unstable proteins, and control of the activity of many regulatory proteins (Bukau and Horwich, 1998; Mayer and Bukau, 2005). HSP70s constitutes the most conserved protein group, being present in all organisms (Boorstein et al., 1994). HSP70 was initially discovered as one of the most prominent heat shock protein exhibiting markedly increased expression when the cell is stressed by an increase in temperature or other stresses (Lindquist and Craig, 1988). It was later demonstrated that certain HSP70s are constitutively expressed and others are localized to a variety of cellular compartments: the cytoplasm, the ER, mitochondria and chloroplasts. The members of HSP70 family that are located in mitochondria and the ER are also required for the translocation process of proteins from cytoplasm to cellular compartments (Hartl, 1996).

In kinetoplastids, the first homologue of BiP, the ER resident member of the HSP70 family, was characterized in T. brucei (Bangs et al., 1993). It was demonstrated that the protein localizes specifically in the ER of this trypanosome. Shortly thereafter, the T. cruzi homologue, named GRP78, was also characterized (Tibbetts et al., 1994). The L. donovani homologue of BiP, also named GRP78, has been characterized by Jensen et al. (2001). Genes encoding GRP78 have also been characterized in a number of species including yeasts, trypanosomes, rats (Pehlam, 1986; Normington et al., 1989; Rose et al., 1989; Tibbetts et al., 1994) and other species of Leishmania viz. L. major (Ivens et al., 2005), L. infantum and L. braziliensis (Peacock et al., 2007). The GRP78 gene of L. donovani is localized to chromosome 15 and that of L. major strain Friedlin, L. braziliensis and L. infantum on chromosome 28. GRP78 of L. donovani share 98%, 97%, 89% and 82% homology with GRP78 of L. major, L. infantum, L. braziliensis and T. cruzi respectively. It has been shown that GRP78 is also expressed on the surface of cells from a lineage ranging from cancer cells and endothelial cells to activated macrophages (Davidson et al., 2005; Misra et al.,
2005; Misra and Pizzo, 2005). Cell surface GRP78 plays a role in cell signaling, viral entry and antigen presentation (Triantafilou et al., 2001; Triantafilou et al., 2002; Misra et al., 2002; Jindadamrongwech et al., 2004). Nagill and Kaur (2010) showed that 78 kDa antigen in combination with various adjuvants imparts different degrees of protection in BALB/c mice against visceral leishmaniasis, maximum protection being observed in mice immunized with 78 kDa antigen in combination with rIL-12.

2.9.2.7. Amastigote protein A2

Amastigote protein A2 when used as a vaccine in either protein or DNA form against murine VL could significantly reduce parasite burden in spleen with increased production of IFN-γ (Ghosh et al., 2001). Since A2 is expressed solely in the intracellular amastigote stage of Leishmania, it represents a prospective vaccine candidate against VL. A commercially available vaccine, canine leishmaniasis known as Leish-Tec, consists of adenovirus expressing the L. donovani A2 antigen. The recombinant adenovirus encoding the A2 gene has been found to be capable of inducing strong Th1-type of immune responses in vaccinated mice and reduced parasite burden following challenge with VL parasites (Resende et al., 2008). In another study, recombinant Leishmania tarentolae expressing the A2 virulence gene against L. infantum challenge (Azizi et al., 2009) was associated with the high levels of IFN-γ production (Mizbani et al., 2009).

2.9.2.8. Paraflagellar rod (PFR) protein

A Paraflagellar rod (PFR) protein is a potent immunogen, being able to induce a protective Th1 response against the experimental infection with Trypanosoma cruzi (Miller et al., 1996). Though the PFR proteins are highly conserved among kinetoplastid parasites, there are few studies about the immunogenicity of this antigen against Leishmania (Carrillo et al., 2008). In cutaneous leishmaniasis, PFR-2 from Leishmania mexicana induced protection against the experimental infection with L. panamensis, showing that PFR-2 is a conserved immunogen between Leishmania species (Saravia et al., 2005).
2.9.2.9. Fucose Mannose Ligand (FML)

FML, a *L. donovani* promastigote glycoprotein complex has been identified as potent immunogen and gp36 has been identified as an immunogenic component of FML (Santos *et al.*, 1999; Paraguai de Sousa *et al.*, 2001). It consists of a glucoprotein fraction containing a ligand for fucose and mannose (FML) and inhibits penetration of promastigote and amastigote forms in mice macrophages *in vitro* (de Lima *et al.*, 2010). It is present on the surface of the parasite throughout the life cycle (Palatnik-De-Sousa *et al.*, 1993) and is a potent immunogen for mice, rabbits and a specific antigen for human and canine serodiagnosis (Palatnik-De-Sousa *et al.*, 1995; Cabrera *et al.*, 1999). The FML antigen as a vaccine is proved to be safe, protective and highly immunogenic for dogs (da Silva *et al.*, 2001). In a Brazilian area endemic for both human and dog visceral leishmaniasis, a phase III trial showed that 7 months after vaccination with FML associated with saponin, used as adjuvant, dogs presented 97% seropositivity for FML and 100% presented positive DHR (delayed hypersensitivity reaction) against *L. donovani* lysate (da Silva *et al.*, 2001). The FML-saponin vaccines promote significant, specific and strong protective effects against murine visceral leishmaniasis. BCG-FML induced minor and non-specific protection while IL-12-FML, although enhancing the specific antibody and DTH response, failed to reduce the parasitic load of infected animals (Santos *et al.*, 2002).

2.9.2.10. 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 (Jain and Madhubala, 2008). 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.*, 2004; Tewary *et al.*, 2005). ORFF expressed as a chimeric ubiquitin conjugate was tested against antimony-sensitive and antimony-resistant strains of *L. donovani* as a
vaccine candidate. This study demonstrated better protective efficacy of ubiquitin-
conjugated ORFF than unconjugated one against experimental VL (Sharma and Madhubala, 2009).

2.9.2.11. Heterogeneous family of acidic surface proteins (HASPs)

A heterogeneous family of acidic surface proteins (named HASPs) expressed only in metacyclic and amastigote stages of the *Leishmania* life cycle have been investigated as vaccine candidates against murine VL (Ravindran and Ali, 2004). HASPB has been recognized as a marker for metacyclogenesis in *L. major* and both HASPB and SHERP (small hydrophilic endoplasmic reticulum associated protein) play vital role either in differentiation process and/or in the metacyclic parasite (Singh and Sundar, 2012). The HASP lipid anchors have been shown to be essential in intracellular trafficking and export to the parasite surface (Denny *et al.*, 2000). Although the functions of HASPs are yet unknown, earlier studies have demonstrated the ubiquity of proteins of this type in all *Leishmania* spp. tested, suggesting their suitability as candidate antigens in the development of vaccines against leishmaniasis in general (Stager *et al.*, 2000). Immunization with recombinant HASPB1 led to significant protection against *L. donovani* infection in both liver and spleen. Protection correlated with the production of IFN-γ by CD4+ and CD8+ T cells and an enhancement of *Leishmania* specific IgG1 levels. Protection induced by rHASPBl did not require an adjuvant. However, the level of protection did not exceed 50% at the peak liver burden (Bhowmick *et al.*, 2008).

2.9.2.12. Leishmune

Leishmune, the first prophylactic vaccine licensed against canine visceral leishmaniasis (CVL), has been used in Brazil since 2004, where seropositive dogs are sacrificed in order to control human visceral leishmaniasis (VL) (Palatnik-de-Sousa *et al.*, 2009). Among the many formulations tested for anti-*Leishmania* vaccination, Leishmune is one of the only three vaccines licensed for commercialization all over the World, the only one of second generation kind and the only one against the canine disease (Singh and Sundar, 2012). It consists of an industrial formulation of FML-saponin vaccine which was immunoprophylactic and immunotherapeutic in mice, hamsters and dogs (da
Siva et al., 2001; Borja-Cabrera et al., 2002, 2004; Palatnik-de-Sousa et al., 1994; Santos et al., 2002, 2003, 2007) and showed safety (Parra et al., 2007), 92–95% of protective effect in vaccinated dogs and 76–80% of vaccine efficacy in previous field assays in Brazil (da Siva et al., 2001). Leishmune was designed to induce a strong humoral response against the FML antigen, to facilitate the monitoring during field assays, there was a concern that vaccinated dogs could not be differentiated serologically from infected dogs (Marcondes et al., 2013). Dogs vaccinated with Leishmune showed reduced exposure of parasites to sand flies (Nogueira et al., 2005) and it behaves as a transmission blocking vaccine, raising antibodies in protected dogs which impede the binding of Leishmania to the sand fly’s midgut curtailing the transmission of the infection in nature (Borja-Cabrera et al., 2010; Saraiva et al., 2006). Preliminary results of xenodiagnosis also support these findings (Palatnik-de-Sousa et al., 2008). An assay with the Leishmune commercial preparation on 550 seronegative dogs of endemic areas disclosed 98.8% asymptomatic dogs (at the end of first year) and 99% healthy survivors (at the end of the second year) among vaccinated dogs, compared to the 79.4% asymptomatic and 61% survivor dogs (p<0.001) monitored in the untreated exposed cohort (Borja-Cabrera et al., 2008). Leishmune induced an immunological pattern characterized by enhanced levels of IFN-γ, NO, and anti-L. chagasi IgG2 (Araújo et al., 2009; Palatnik-de-Sousa, 2012), the early and persistent activation of neutrophils and monocytes, and increased the CD8+ T-cells expressing IFN-γ (Araújo et al., 2008). In spite of the low vaccine coverage in Brazil, it was possible to detect 66.1% and 80.2% reduction of CVL incidence among vaccinated dogs of Belo Horizonte and Aracatuba, respectively, when compared to the global incidence of each town (Borja-Cabrera et al., 2008). While immunotherapy with the saponin-enriched Leishmune reduces the symptomatology, the rate of obits and the parasite load in lymph nodes, immunochemotherapy with Leishmune, allopurinol, and amphotericin B promotes the sterile cure, turning to negative the PCR reactions for Leishmania DNA (Borja-Cabrera et al., 2010).
2.9.3. Polyprotein vaccines

Apart from defined single molecules, multicomponent vaccines have been shown to protect against VL in experimental infection systems.

2.9.3.1. Q Protein

Recombinant Q protein, a chimeric antigen composed of the genetic fusion of four fragments of the acidic ribosomal proteins from *L. infantum* (Lip2a, Lip2b, P0, and histone H2A) coadministered with live BCG protected 90% of immunized dogs by enhancing parasite clearance (Molano *et al.*, 2003; Evans and Kedzierski, 2012). Protein Q has also been investigated as a potential activator of immune response in combination with different adjuvants (HSP70, BCG, CpG-ODN, pUC18, pcDNA3, alum and Freund’s adjuvant). It has been observed that the Protein Q-CpG-ODN induced higher level of protection than other formulations in mice against *L. infantum* infection. A 99% protection was achieved in Q-CpG-ODN vaccinated group that was marked by enhanced production of IgG2a/IgG1, IFN-γ and increased lymphoproliferation (Parody *et al.*, 2004).

2.9.3.2. Leish IIIf

The vaccine, called Leish-F1+MPL-SE, is composed of the recombinant *Leishmania* polyprotein Leish-F1 (formerly known as Leish-11f) antigen and the MPL-SE adjuvant, which is a potent TLR-4 agonist (Casella and Mitchell, 2008). The antigen component of the vaccine includes three proteins derived from *L. major* but conserved across various *Leishmania* species, including *Leishmania donovani*, which causes Old World VL; *Leishmania chagasi*, which causes New World VL; and *L. braziliensis*, which causes both CL and MCL in the New World (Webb *et al.*, 1996, 1998). The three proteins are: *Leishmania* elongation initiation factor (LeIF), thiol-specific antioxidant (TSA), and *L. major* stress-inducible protein 1 (LmSTI1) (Skeiky *et al.*, 2002; Kobets *et al.*, 2012). The adjuvant component is monophosphoryl lipid A derived from the lipopolysaccharide of *Salmonella minnesota* and formulated in a stable emulsion (MPL-SE) (Persing *et al.*, 2002; Reed *et al.*, 2003; Chakravarti *et al.*, 2011). The individual proteins expressed in both life cycle stages of *Leishmania* have been investigated as potential vaccine candidates. Immunization of mice with
recombinant LeIF stimulates the induction of Th1 type of immune response with increased production of IFN-γ (Skeiky et al., 1998). Mice immunized with individual antigen alone (TSA or LmSTI1) did not induce protection and developed only IgG1 but not IgG2a antibody response to the immunizing agent. In contrast, the use of IL-12 as an adjuvant conferred strong and partial protection by LmSTI1 and TSA, respectively, with increased concentrations of IFN-γ and IgG2a (Webb et al., 1998; Campos-Neto et al., 2001). Moreover, immunization with both antigens plus IL-12 resulted in significant protection in both the mouse and monkey model of cutaneous leishmaniasis (Campos-Neto et al., 2001). In animal studies, the vaccine has been shown to elicit a T-helper cell type 1 (Th1) immune response, believed necessary for control of leishmaniasis (Coler et al., 2002, 2007). The mixture of recombinant components of Leish-111f has been found to be highly immunogenic in dogs (Fujiwara et al., 2005) but failed to protect them against *L. infantum* infection and did not prevent disease development in a phase III trial in dogs (Gradoni et al., 2005). A Phase 1 trial of the Leish-F1+MPL-SE vaccine in 45 volunteers in the United States showed that the vaccine was safe and immunogenic in subjects from Colombia, a region not endemic for *Leishmania* (Velez et al., 2009). These trials successfully demonstrated the safety and immunogenicity of the Leish-F1+MPL-SE vaccine and support the plan to move its clinical development forward for prophylaxis of human CL (Velez et al., 2009; Chakravarti et al., 2011).

### 2.9.4. First Vaccine trial against Visceral leishmaniasis

The Infectious Disease Research Institute (IDRI) has launched the first human trial of a new vaccine for visceral leishmaniasis. The trial was conducted in Washington State, and a Phase 1 trial in India, where VL is prevalent. The IDRI vaccine, known as LEISH-F3 + GLA-SE, is a highly purified, recombinant vaccine. It involves 2 fused *Leishmania* parasite proteins and a strong adjuvant to activate an immune response against the parasite (Rattue, 2012).

### 2.9.5. 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 has been 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 has been compared with that of LACK protein and IL-12. It has been 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). 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. When followed by a booster with a vaccinia virus recombinant expressing p36/LACK, it further enhanced this immunity by expanding the CD8+ T cell 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.
review of literature

Amazonensis challenge (Pinto et al., 2004). To date, the protective efficacy of LACK has been mainly reported in cutaneous leishmaniasis and it 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 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 were thought 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).

2.1. Treatment

Certain parasitic infections are considered to be amongst the most prevalent diseases in the world, attacking millions of people, often endangering lives. Due to social, economic, dietary and environmental variations in the living habits of the different groups of the world's population, the control of parasitic diseases has become complicated. At present, prevention of these diseases depends mainly on ecological measures, such as sanitation and vector control, and also on antiparasitic
drugs. Drug treatment necessitates a thorough knowledge of the life cycle of the parasite as well as its biology in general. Two aspects must be considered in drug selection: adequate knowledge of the physiology and biochemistry of the parasite could lead to the rationale design of new and more effective drugs; and, conversely, studies on mechanisms of action of effective antiparasitic drugs provide insight into the physiology and biochemistry of the parasite (Frayha et al., 1997).

*Leishmania* are protozoan parasites shuttling between sandfly vector and mammalian host (Handman, 1999; Yao et al., 2013), and cause a disease which ranges in severity from skin lesions to serious disfigurement and fatal systemic infection (Kedzierski et al., 2009). Drug treatment is effective but has significant toxicity and morbidity. Finally, as the prognosis of the disease varies with the species, the choice of treatment also depends on the causative *Leishmania* species (Blum et al., 2004; Minodier and Parola, 2007). The drug treatment modifies the response from a Th2 or mixed Th1/Th2 antiparasitic response towards the Th1 pole (Hailu et al., 2001). Thus, the primary goal for clinical management is straightforward-to prevent death from visceral leishmaniasis and morbidity from cutaneous and mucosal leishmaniasis. However, even tropical medicine clinicians are often baffled by the complexities of leishmaniasis: by the apparently innumerable possible combinations of different leishmanial syndromes, species and geographical areas of acquisition of infection, each combination varying by clinical presentation, ease of diagnosis, natural history and response to therapy. Translation of the increased interest in leishmaniasis and the advances in the understanding of the immunoregulation of this disease into field-applicable methods for diagnosing, treating and preventing infection is challenging. Rapid methods for diagnosis and species identification are needed, as are therapies, prophylactics, and control measures that are effective, safe, affordable and easily administered (Berman, 1997).

2.10.1. Pentavalent Antimonials

The pentavalent antimonials such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) have been recommended for the treatment of leishmaniasis for over 70 years although they elicit a variable therapeutic response (Romero et al., 2001). These compounds are non-covalent chelates of Sb(V), so considered as a prodrug which is further converted to trivalent antimony (SbIII) an active form of drug albeit to the parasite (Ephros et al., 1999; Haldar et al., 2011). Data suggests that pentavalent antimony (SbV), enters the host cell crosses the
phagolysosomal membrane and is converted to trivalent antimony (S reduction can be non enzymatic under acidic conditions such as those phagolysosome by glutathione, glycylcysteine and trypanothione or enzy thiol dependent reductase (TDR) (Denton et al., 2004) and antimoniate ion (ACR) (Ashutosh et al., 2007). ACR also increases the sensitivity of Leish Sb" (Zhou et al., 2004). Pentavalent antimony may also kill parasite by mechanisms, such as increasing cytokine levels (Pathak and Yi, 2001). The at the DNA level, inducing damage in vivo (Lima et al., 2010) and inhibit topoisomerases (Chakraborty and Majumder, 1988). Both forms of antimo and Sb" kills Leishmania species by DNA fragmentation, suggesting th apoptosis, β oxidation of fatty acids and adenosine diphosphate phosph Treatment of Leishmania infantum amastigotes with Sb(III) at low concentr been found to induce DNA fragmentation, suggesting the appearance of late programmed cell death (apoptosis) (Sereno et al., 2001). Moreover the an inhibit glycolysis and metabolic ATP binding cassette (ABC) transporter drug resistance protein A (MRP A) (El-Fadili et al., 2005).

In India, pentavalent antimonials (Sb"), meglumine antimoniate an stibogluconate (SSG) have been the main first line drugs for the treatment of of leishmaniasis including VL since 1940. Sodium stibogluconate (sodium gluconate) was developed in the 1940s, under the name Pentostam (Goodw: It is available commercially as solustibosan and stibanate. Generic sodium gluconate has been used extensively in India. In an earlier resurgence of I which assumed epidemic proportions by 1977, an estimated 2,50,000 pati affected in Bihar of which 30% cases were unresponsive to Sb" (Peter Sodium stibogluconate (SSG) and meglumine antimoniate daily injection of body weight for 28-30 days has been the standard treatment for VL in mos the world (Sundar and Chakravarty, 2013). They require injectable admin can be intravenous (IV), intramuscular (IM) and intralymphatic (IL) (Matou 2007). It has been observed that children’s tolerance for higher and prolong of SSG is better than adults (Jha, 2006). Pharmacokinetic studies of Sb(V) d patients have suggested that almost about 80 - 95% of the drug appears in within 6 h (Frezard and Demicheli, 2010). It has lost its efficacy, beca development of a high resistance (50-60%) in Bihar (India) (Thakur et al., et al., 2001; Croft and Seifert, 2006) and some extent in adjoining Nepa al., 2003) which has led to adoption of alternative treatment strategies
regions. However, in rest of the world, it continues to be effective (Sundar and Chakravarty, 2013). Resistance appears in Bihar due to consulting unqualified medical practitioners (Croft and Seifert, 2006). Chances of response to further courses of antimonials diminish once there is a relapse after the initial Sb(V) treatment (Haldar et al., 2011). Diminishing biological reduction of Sb (V) to Sb (III) has been demonstrated in *Leishmania donovani* amastigote resistant to SSG (Shaked-Mishan et al., 2001). Also, SSG retreatment regimens are lengthy (30-60 days), are difficult to tolerate and are not always successful (Ritmeijer et al., 2006).

Meglumine antimoniate is marketed as glucantime and prostib by Sanofi Aventis. It eliminates systemic amastigotes, which are responsible for persistence of infection (Bodgan et al., 2000; Nicolas et al., 2000). But its treatment requires multiple injections; also persistant variations in efficacy and drug treatment are reported (Croft and Coombs, 2003).

Antimonials are notorious for their toxic effects, which include life-threatening cardiac arrhythmias arising as a result of prolongation of the electrocardiographic QT interval (Chulay et al., 1985; Sundar et al., 1998b; Thakur et al., 1998); Sundar and Chakravarty, 2010), pancreatitis (Gasser et al., 1994; Van Griensven and Diro, 2010) and hepatic dysfunction (Hepburn, 1993). While some data suggest serious side effects are frequent, requiring treatment interruption, discontinuation or dose modification (Aronson et al., 1998; Chulay et al., 1985), other studies have reported the drugs to be well tolerated (Ballou et al., 1987; Seaton et al., 1999).

### 2.10.2. Pentamidine

Pentamidine has been extensively used as a second line drug for SSG unresponsive cases since seventies till 2003. It is not only known for its antileishmanial activity (Chance, 1980), but also for their antiviral, tumoricidal, antibacterial and fungistatic properties (Bell et al., 1990). The exact mechanism by which pentamidine and its analogues exert various microbicidal activities is not known, although their active role in the inhibition of DNA/RNA synthesis cannot be ruled out (Jones et al., 1990). The cytotoxic properties against the promastigotes are due to the induction of a higher amount of programmed cell death (PCD) which is also associated with the inhibition of DNA synthesis and cell-cycle arrest in the G2/M phase. The drug faces resistance among the patients. Pentamidine drug-resistance has been shown to be linked to a decreased accumulation of this compound, accompanied
by a decreased transport of structurally unrelated molecules such as pyrimidine nucleotide as well as by alterations of the polyamine levels (Basselin and Robert-Gero, 1998; Basselin et al., 1998). It gets well distributed in tissues and is excreted very slowly (Salame and Cunha, 1988).

2.10.3. Amphotericin B

Amphotericin B, an antifungal macrolide polyene is characterized by the hydrophilic polyhydroxyl and hydrophobic polyene faces on its long axis. Presently, it is the only drug with highest cure rate. Selectivity is due to the higher affinity of amphotericin B for ergosterol over cholesterol, the predominant sterol in the mammalian host cells (Croft and Seifert, 2006). It acts on membrane sterols resulting in parasite cell lysis. Its lipid formulations have been developed to minimize side effects. Other anti-fungals like ketoconazole, fluconazole and terbinafine are less effective (Pardasani, 2000).

It has been known that amphotericin-B potentiates the antimicrobial and tumoricidal activities of macrophages either directly or via induction of cytokines such as tumour necrosis factor–α and interleukin 1-β, as well as generation of respiratory burst (Washington et al., 2007). At higher concentrations (≥0.1M) it induces the formation of aqueous pores in cell membrane of promastigotes that results in osmotic changes leading to cell lysis (Singh et al., 2012). In India, amphotericin B deoxycholate regimens include infusions of 1 mg/kg given either daily for 20 days (Thakur et al., 1999) or, because of concerns about tolerability, on alternate days over a 30-day period (Murray and Delph-Etienna, 2000; Sundar et al., 2002; Murray, 2001). Efficacy is high e.g. long-term cure rates of >95% (Sundar, 2001; Murray, 2001; Thakur, 1999). The use of AmB as an antileishmanial and antifungal drug is limited by its toxicity, which has been attributed to increased TNF, NO, and free radical production (Brajtburg et al., 1990; Tokuda et al., 1993). At present amphotericin B is extensively used in Bihar for all SSG unresponsive cases and even as a first line drug. However, AmB therapy is limited because of its negligible solubility in aqueous solution and its toxicity for renal cells due to internalisation of amphotericin B-lipoprotein complexes mediated by low-density lipoprotein receptors (Mishra et al., 2013). Primary unresponsiveness and relapses are uncommon (Jha et al., 1995; Thakur et al., 1996; Sundar et al., 2002). To ameliorate this, reformulation of amphotericin B has been developed to alter the pharmacokinetics. Liposomal drug delivery system is an efficient mode of targeting the antileishmanial agent to site of
Review of Literature

infection (Alving, 1983). Lipid formulations of amphotericin B (LFAB) have been developed in an attempt to improve both efficacy and tolerability, especially renal toxicity (Martino, 2004) and are more selective in damaging fungal or parasitic cells than mammalian cells. They are concentrated within phagocytic cells and increase the local drug concentrations in the infected tissues (Alving, 1983).

Lipid formulations of amphotericin B: liposomal amphotericin B (AmBisome; Gilead Sciences), amphotericin B lipid complex (Abelcet; Enzon pharmaceuticals) and amphotericin B cholesterol dispersion (Amphotec Intermune) have also been successfully applied in VL in India and elsewhere (Murray, 2000). AmBisome consists of ribbons of lipids interspersed with amphotericin B. Amphotec is a sterile, pyrogen-free, lyophilized powder for reconstitution and intravenous (IV) administration. It consists of a 1:1 (molar ratio) complex of amphotericin B and cholesteryl sulphate and Abelcet is a sterile, pyrogen-free suspension for intravenous infusion, consists of amphotericin B complexed with two phospholipids in a 1:1 drug-to-lipid molar ratio. Lipid formulations are rapidly concentrated into organs such as liver, spleen and remain there for long periods (Lestner et al., 2010).

Studies in India demonstrated that AmBisome and Abelcet when given together at a dose of 2 mg/kg/day each for 5 days produced far fewer infusion-related reactions versus amphotericin B (1mg/kg infusions for 15 days on alternative pattern) and reduced toxicity of the latter drug, for example renal insufficient hypokalemia and anemia (Sundar et al., 2001). A very high therapeutic index, short treatment courses and the absence of side effects makes AmBisome the most attractive existing drug for VL treatment (Bern et al., 2006).

Nevertheless, the lipid formulations have particular clinical appeal in the treatment of VL, because they have largely remedied the drawbacks of conventional amphotericin B deoxycholate. These agents are well tolerated and remarkably efficient therapeutically, resulting in 90-100% cure rates when administered for 5-7 days or even less (Murray, 2000, 2001; Sundar et al., 2002). Such short day regimens produce an abbreviated inpatient stay, which (Sundar et al., 2000) is important to both patients and accompanying families for whom any hospitalization with lost wages is a hardship, (Guerin et al., 2002) increases hospital bed availability in a setting with chronic shortages, (Sundar et al., 2001) reduces laboratory monitoring, and lowers overall hospital-related expenditures. High cost, however, has put all three preparations beyond the reach of patients in regions of developing countries where
VL is endemic (Murray, 2001). Therefore to successfully take advantage of these regimens in India, the drug has been offered at a preferential price for the treatment of VL by its producer, Gilead. This has raised considerable interest in its use either as a single dose treatment or as part of a combination treatment (Edwards et al., 2011). Recently, single-dose liposomal amphotericin B at 10 mg/kg body wt. was observed with a cure rate of 97%, and fewer side-effects that were easily manageable at the primary-health care level in Bangladesh against VL (Mondal et al., 2014).

2.10.4. Paromomycin

Paromomycin or Aminosidine belongs to an aminoglycoside family of antibiotics and it has been used as a broad spectrum parenteral antibiotic against bacteria and an oral agent against intestinal protozoa. It was first used successfully in human VL in Kenya in the 1980s (Chunge et al., 1990). It was considered to be a great achievement because of its relatively low cost and easy application. Paromomycin ointments have contained different major components, including methylbenzethonium chloride, wool fat, white soft paraffin, and 10% urea. A regimen of 20 mg/kg is recommended in treating patients (Bern et al., 2006) and a regimen of 21 days of 15 mg/kg given as intramuscular injection has been found to be highly effective with safe profile (Sundar et al., 2007). In India, a single dose of 5 mg/kg was effective in 91% patients (Sundar et al., 2001). It accumulates in tissues and is only slowly released and excreted (Bekersky et al., 2002). It causes side effects like nephrotoxicity and ototoxicity. Some cases of tetany have been detected in patients of kala-azar treated with paromomycin. In a phase III study of VL in India, this drug was associated with 94.6% cure rates, similar to amphotericin B (Sundar et al., 2007). In a phase IV trial paromomycin is used intramuscularly singly or in combination with SSG in Indian kala-azar patients (Jha, 2006). In phase II randomized controlled trial of aminosidine (paromomycin) vs sodium stibogluconate, paromomycin was used in the dose of 16 mg/kg/day or, 20 mg/kg/day for 21 days, giving cure rates of 93.3% and 96.7% respectively at six month follow up (Sundar et al., 2007). Paromomycin inhibits protein synthesis and modifies membrane fluidity and permeability. An in vitro study showed that following a 72-hour exposure of L. donovani promastigotes and amastigotes to paromomycin, the mitochondrial potential was decreased, which indicates that mitochondria are the targets of the drug (Jhingran et al., 2009). However, as paromomycin is an aminoglycoside, resistance can emerge rapidly if used as monotherapy (den Boer et al., 2009).
2.10.5. Sitamaquine

Sitamaquine is a primaquin analogue; the derivative of 8-amino quinoline (Yeates, 2002) used against VL. Preliminary clinical studies in Kenya and Brazil showed satisfactory efficacy against different species of *Leishmania* (Sherwood *et al.*, 1994; Dietze *et al.*, 2001). According to some studies sitamaquine targets succinate dehydrogenase causing oxidative stress in *L. donovani* promastigotes (Carvalho *et al.*, 2011). Sitamaquine at high concentrations affects parasite motility, morphology and growth (Duenas-Romero *et al.*, 2007). Mechanism of its action involves electrostatic interaction between phospholipid anionic polar head groups and positively charged sitamaquine and then with phospholipid acyl chains leading to drug insertion within biological membranes (Coimbra *et al.*, 2010). After binding to the membrane, sitamaquine accumulates in *Leishmania* cytosolic acidic compartments, acidocalcisomes (Lopez-Martin *et al.*, 2008). A few side effects like vomiting, dyspepsia, cyanosis, nephritic syndrome and glomerulonephritis have also been observed (Wasunna *et al.*, 2005). Although resistance against this drug has not yet been reported in clinical practice but *in vitro* resistance against *L. donovani* promastigotes has been reported (Bories *et al.*, 2008).

2.10.6. Imiquimod

Imiquimod (Aldaras) an antiviral compound (1-(2-methylpropyl)-1H-imidazo (4.5-c) quinolin-4-amine) is used for the tropical treatment of genital warts, caused by the human papilloma virus or premalignant conditions. This imidazoquinoline amine is an immune response modifier that targets preferentially monocytes and macrophages, and induces a T-helper 1 response with an increasing release of interferon-α (IFN-α), tumor necrosis factor-α (TNF-α) and interleukins (IL)-1β, IL-6 or IL-8. In an *in vitro* infection assay and in infected mice, imiquimod demonstrated a leishmanicidal activity by inducing the expression of the inducible nitric oxide synthase (iNOS) gene and the release of nitric oxide (Buates and Matlashewski, 1999). It induces the production of cytokines and NO in macrophages (Buates and Matlashewski, 2001).

2.10.7. Miltefosine

Anticancer alkylphosphocholines have been most effective oral compounds. These act as membrane synthetic ether-lipid analogues, and consist of alkyl chains in the lipid portions. Most promising of these is miltefosine (Mishra *et al.*, 2007). It (hexadecylphosphocholine; marketed by Zentaris GmbH as Impavido) is a new oral
drug to treat leishmaniasis, with relatively high efficacy rates reported for treatment of cutaneous (Soto et al., 2001, 2004), mucocutaneous (Soto and Toledo, 2007) and visceral leishmaniasis (Bhattacharya et al., 2004, 2007; Sundar et al., 2002). It is now registered in many countries and is the first orally administered breakthrough therapy (More, 2003). In October 2006, it received orphan drug status from the US Food and Drug Administration. Recently, three countries in South Asia (India, Nepal, and Bangladesh) have decided to use miltefosine regularly as the first line drug in their VL elimination program by the year 2015 (Sundar et al., 2012).

It has been developed as an anti-neoplastic agent that interferes with cell-signal transduction pathways and inhibits phospholipids and sterol biosynthesis and is now used topically in dermal metastases of breast cancer. Phase III clinical trials in India showed that miltefosine is very effective for treating VL in both adults and children, including those who experienced failure with antimonials (Monge-Maillo and Lopez-Velez, 2013). Phase IV trials have also been carried out to evaluate the suitability of miltefosine in an outpatient setting for the kala-azar elimination program of the Indian government (Sundar and Olliaro, 2007). It has been shown to block the proliferation of Leishmania and to alter phospholipid and sterol composition (Urbina, 1997; Meena et al., 2010). The currently recommended dose for miltefosine as monotherapy for either CL or VL is 2.5 mg/kg/day for a total of 28 days (Dorlo et al., 2012). The mechanism of action is probably based on interference with synthesis and degradation of parasite membrane lipids (Dorlo et al., 2006). Protein binding is approximately 95% in human plasma. Miltefosine is metabolized mainly by phospholipase D, releasing choline, choline-containing metabolites and hexadecanol, which are likely to enter the intermediary metabolism. The terminal elimination half-life is reported to be 150-200 hours in adults (Berman, 2005; Dorlo et al., 2008) and expected similar value in children (Sundar et al., 2003). Only less than 0.2% of the applied dose is excreted unchanged in the urine (Sindermann and Engel, 2006). Because VL is currently often incurable in HIV-patients and many co-infected persons will ultimately experience relapse (Ritmeijer et al., 2006), the safety profile of miltefosine makes it a suitable drug for the treatment of HIV co-infected patients. However, higher infectivity of L. donovani being associated with both relapse after miltefosine treatment and SSG treatment failure, challenges the current view on visceral leishmaniasis treatment failure in the Indian subcontinent and other endemic areas (Rai et al., 2013).
2.10.8. Cisplatin

M. Peyrone first described the compound cis-PtCl$_2$(NH$_3$)$_2$ in 1845, and was known for a long time as Peyrond’s salt (Peyrone, 1844). The structure was deduced by Alfred Werner in 1893 (Trzaska, 2005). It was approved for clinical use by the United States Food and Drug Administration (FDA) in 1978 (Trzaska, 2005) and it revolutionized the treatment of certain cancers including sarcomas, some carcinomas (e.g. small cell lung cancer and ovarian cancer), lymphomas and germ cell tumors. It is a neutral inorganic, square planar complex that reacts with DNA to induce its characteristic biological effects, which culminate in either repair of the DNA damage and cell survival or activation of the irreversible apoptotic program (Siddik, 2003). However, for interaction to occur with DNA, the neutral cisplatin has to be activated through a series of spontaneous aquation reactions, which involve the sequential replacement of the cis-chloro ligands of cisplatin with water molecules (el Khateeb et al., 1999; Kelland, 2000). The monoaquated form is recognized as a highly reactive species, but its formation is rate limiting in the interaction with many endogenous nucleophiles, such as glutathione (GSH), methionine, metallothionein, and protein. Thus, when cisplatin enters cells, it is potentially vulnerable to cytoplasmic inactivation by these and other intracellular components. The platinum complexes can react with DNA forming both intrastrand and interstrand cross-links. The N7 of guanine is a particular reactive site, leading to platinum cross-links between adjacent guanines on the same DNA strand; guanine-adenine cross-links also readily form and may be critical to cytotoxicity (Ratanaphan et al., 2009; Parker et al., 1991). The formation of interstrand cross-links is less favoured.

The optimal use of cisplatin as a chemotherapeutic drug has been limited by its toxicity to non-cancerous tissue (Pinzani et al., 1994), especially to kidneys, which are very sensitive (Price et al., 2004). Cisplatin was in danger of being discarded after phase-I trials in the early 1970's as it showed marked gastro-intestinal and renal toxicities. It is generally recognised as a DNA-damaging drug. It causes nausea, vomiting, kidney or liver damage, peripheral sensory polyneuropathy (numbness or tingling of the extremities), hearing loss, ringing in the ear, loss of appetite, abnormal taste sensations, hair loss and rarely focal encephalopathy (Alberts et al., 1995).

After a single dose of cisplatin, there is preferential sequestration of the drug in kidneys, liver, intestine and testes, with concentration in the kidneys being as high as 37 times those of plasma (Litterst et al., 1976). Hepatotoxicity has been reported in
the cisplatin treated group with increased serum levels of alanine (ALT) and aspartate (AST) aminotransferases. The mechanism of cisplatin-induced hepatotoxicity has been found to involve membrane rigidification; decreased GSH/GSSG ratio, ATP, GSH and NADPH levels; lipid peroxidation; oxidative damage of cardiolipin and protein sulfhydryl groups. Moreover, cell death by apoptosis has also been demonstrated and the participation of the mitochondrial signaling pathway in the process has been suggested (Martins et al., 2008).

Interestingly, and in spite of the well established toxicity of cisplatin, quality of life, when studied, appeared improved since specific system relief overshadowed the proper side effects of cisplatin (Bunn, 1998). Substantial improvements in the management of cisplatin-induced toxicities has been achieved (Marty et al., 1990; Marty and Rosenthal, 2002). The anti-leishmanial activity of some cis-DDP derivatives i.e. cisplatin on promastigotes and amastigotes has also been reported along with its anticancerous effects. The amastigotes are more sensitive to cis-DDP when compared to promastigotes (Tavares et al., 2007). Later on, in vivo studies also demonstrated the leishmanicidal potential of two low doses of cisplatin (1 mg/kg body weight and 0.5 mg/kg body weight). The drug was more effective at a concentration of 1 mg/kg body weight with minimum side effects (Kaur et al., 2010). Since nephrotoxicity is a dose limiting factor of cisplatin, the protective efficacy of antioxidants (Silibinin, vitamin C and vitamin E) was studied which effectively reversed the toxic side effects caused by high dose of the drug (Sharma et al., 2012). In a study, patients treated with a combination of gemcitabine and cisplatin showed moderate efficacy and safety as second-line treatment for advanced biliary tract cancer that is refractory to gemcitabine-based first-line chemotherapy (Sasaki et al., 2013). Also, combination treatment of the siRNAs and cisplatin encapsulated in CD44-targeting hyaluronic acid (HA)-based self-assembling nanosystems, delayed the tumor growth significantly (growth inhibition increased from 30 to 60%) in cisplatin-resistant tumors causing, no abnormalities in body weights, liver enzyme levels or histopathology of liver/spleen (Ganesh et al., 2013).

2.11. Immunochemotherapy and therapeutic vaccines

Protective immunity usually follows recovery from all forms of leishmaniasis in immunocompetent individuals, but the behaviour of leishmaniasis in these individuals suggests that the immunity is not sterile. Hence, the host immune system and its components play a crucial role in this
infection healing process by the activation of macrophages and the increased nitric oxide production among other mechanisms, to eliminate the infection (Monzote, 2009). The success of any chemotherapeutic regimen is often dependent on the potential or latent immunologic response by the infected host. Successful chemotherapy of leishmaniasis in humans results in generation of antigen-specific T cells and delayed type hypersensitivity responses. Because of this close association between chemotherapy and cell-mediated immunity, treatment of *L. donovani* infection has been thought to be more amenable to combined therapy, that is, immunochemotherapy, as the infection involves primarily those organs where there is an activated macrophage system (Allison, 1978). Therefore, one of the strategies that has been exploited lately in the treatment of leishmaniasis is enhancement of the efficacy of standard antileishmanial drugs by administrating them in combination with microbial or synthetic products that provide specific or nonspecific immunostimulation (Haidaris and Bonventre, 1983; Badaro et al., 1994). The basis for this strategy stems from the fact that intracellular *L. donovani* would be attacked simultaneously by two different mechanisms: one using direct toxicity to the parasite within the parasitized macrophage (antimonials) and the other involving effects on the macrophage itself, such as with IFN-γ or Poly ICLC (Murray et al., 1988; Bhakuni et al., 1996). Moreover, success of recombinant IFN-γ in combination with pentavalent antimony (Badaro et al., 1990; Sunder et al., 1994) in both experimental and clinical studies has demonstrated the potential of immunochemotherapy in the treatment of leishmaniasis. Immunotherapy with or without chemotherapy has been used for treatment of cutaneous leishmaniasis in Venezuela for more than a decade. Three injections of autoclaved *L. mexicana* mixed with BCG are given; if the patient does not respond, chemotherapy is initiated. The cost of the vaccine is much lower than that of chemotherapy. It appears to be safe and is not associated with the adverse effects of the antimonials, but it produces a lesion which may last for a few weeks (Convit et al., 2003). An association seems to exist between the increased frequency of innate immune system cells and the healing of lesions, suggesting that this protocol of immunotherapy reduced the parasite load and activated NK cells and monocytes. In DCL patients with several unsuccessful chemotherapy treatment regimens and many relapses, a monthly immunotherapy scheme of *L. amazonensis* PH8 plus *L. (Viannia) braziliensis* M2903 monovalent vaccines associated with BCG was established, one round of which also included an M2903 vaccine associated with intermittent antimonial
treatment. Temporary healing of all lesions was achieved, although *Leishmania* skin tests were negative and interferon-γ was not detected in mononuclear cell cultures stimulated with *Leishmania* antigens (Pereira et al., 2009). In Brazil, repeated daily doses of killed *L. amazonensis* vaccine ('Mayrink vaccine') are given with a low dose of antimonials (8 mg/kg body weight per day) for four cycles of 10 daily injections, followed by a 10-day rest. The vaccine has been registered in Brazil as an adjunct for low-dose chemotherapy (Mayrink et al., 1992; WHO, 2010). In the Sudan, a trial involving patients with persistent PKDL showed that the cure rate with immunochemotherapy was significantly higher than that with chemotherapy alone (final cure rates: 87% and 53%). The vaccine was a mixture of killed *L. major* adsorbed on alum plus BCG, given four times at weekly intervals (Musa et al., 2008). Murray et al. (2003a) tested immunochemotherapy protocol in *L. donovani* infected mice by the association of amphotericin B with IL-12 anti-CD40, or anti-IL10 and observed that, despite this drug's direct action against parasites and its independence from host immunity, the combination was more efficient than the monotherapy and led to reduction of the amphotericin B dose. Moreover, no alterations in phenotypic profiles of CD3+, CD4+, CD19+, CD16+ or CD56+ cells or the CD4/CD8 and CD3/CD19 cells were observed in patients with ACL following glucantime and leishvacin treatment. This suggested that the therapeutic methods exerted a similar effect on the immune response of the patient. So, combination treatment regimen should take into account the shortest period over which each individual component needs to be applied in order to minimize the amount of drug used and, consequently, to reduce the possibility of side effects and adverse events (Botelho et al., 2009).

2.12. Rationale of the present study

Leishmaniasis designates a human disorder produced by protozoan species and subspecies of the genus *Leishmania*. Visceral leishmaniasis caused by *Leishmania donovani* represents a major public health problem in tropical and subtropical regions of the world. The Indian subcontinent (India, Nepal and Bangladesh) is one of the main areas affected by VL worldwide. It accounts for about 67% of total cases reported with almost 200 million people at risk of contracting the disease (Nagill and Kaur, 2011). In India, highest incidence has been reported from the states of Bihar, Assam, West Bengal and eastern UP where resistance and relapses are on the increase (Van Griensven and Diro,
2012). No effective vaccine is yet available against this parasite and its control relies primarily on chemotherapy. Current challenges in anti-leishmanial chemotherapy include the increasing resistance of parasites to the front-line drug, sodium antimony gluconate (SAG), high costs and drug toxicity, and also a limited repertoire of new drugs (Mukhopadhyay et al., 2012). Since the discovery of the pentavalent antimonials, until today, the search for newer drugs with antileishmanial activity, without toxic effects, and the drugs able to overcome the emergence of drug resistant strains, still remains as the current goal. So far no antileishmanial therapy is available which does not have any side effects.

A pivotal pathogenic event in leishmaniasis is the harboring of the causative Leishmania parasite within phagolysosomes of macrophages for which the parasite deviously initiates mechanisms to modulate the macrophage microbicidal machinery. As macrophages are also critical sentinels of the immune system, establishment of infection critically hinges on the parasite’s ability to modulate the hosts signaling systems, the end point being immunosuppression. Therefore, the quest for expanding the limited therapeutic armamentarium against Leishmania should focus on identifying compounds having direct parasiticidal and/or indirect immunomodulatory activity. In view of the emerging resistance of parasites to SAG, combination therapy with immunomodulators (Immunochemotherapy) is being tested as an alternative therapeutic option against VL. In suboptimal doses, SAG along with diperoxovanadate reduced the parasitic load in BALB/c mice, achieved by expanding the anti-leishmanial T-cell repertoire, along with increased generation of superoxide and NO and an altered ratio of IFN-γ:IL-10 (Saha et al., 2011). Several attempts have been made to combine pentavalent antimony with immunomodulators, such as IFN-γ or IL-2, and although it proved beneficial in T-cell-deficient hosts, it only showed moderate improvement in clinical studies (Sundar et al., 1995). In experimental VL, another approach attempted was the removal of the endogenous immunosuppression by the use of anti-IL-10 combined with SAG/amphotericin B, and the results were promising (Murray et al., 2002; 2003b). Dendritic cell-based immunotherapy combined with antimony has been found to be effective against murine VL (Ghosh et al., 2003), as was the addition of GM-CSF with antimonials in American CL and muco-cutaneous leishmaniasis (Almeida et al., 2005). Imiquimod combined with SAG or paromomycin also showed marked improvement in patients with CL (El-On,
Similarly, when miltefosine was combined with liposomal CpG ODN, a strong enhancement of Th1 cytokines was observed, along with raised levels of NO, reactive oxygen species and H$_2$O$_2$ (Sane et al., 2010).

To the best of my knowledge no studies are available as regards the immunochemotherapeutic potential of SSG and cisplatin with 78kDa antigen and KLD with adjuvant MPL-A against VL. It is hoped that with the clinically relevant data generated out of this study, one more step towards the elimination of this disease from the Indian subcontinent will be taken.

2.13. Aims and Objectives of the study

**Aim:** To study the protective efficacy of sodium stibogluconate/cisplatin in combination with 78kDa+MPL-A antigen/heat killed *Leishmania donovani* promastigote antigen+MPL-A.

The aim was met by the following objectives:

a) To study the parasite load in experimentally infected animals before and after treatment.

b) To monitor the haematological and various biochemical parameters (kidney and liver function tests) before and after treatment in different groups of animals.

c) To check the specific antibody levels (IgG1 and IgG2a) by ELISA before and after treatment in experimentally infected animals.

d) To check the cell mediated immune response by
   i) studying the DTH response in various groups of animals.
   ii) cytokine responses (IFN-γ, IL-2, IL-4 and IL-10) in animals before and after treatment.

e) To study the histopathological changes in kidneys, liver and spleen in different groups of animals.