CHAPTER-2
REVIEW OF LITERATURE

Leishmaniasis is still one of the major public health problem in the tropical and sub-tropical countries of the world including India and the effect of its morbidity on the social and economic development are staggering (Croft and Coombs, 2003). Leishmaniasis constitutes a globally widespread group of neglected diseases caused by an obligatory, intracellular, protozoan parasite of genus *Leishmania*, of which 21 out of 30 species infects human beings (Herwaldt, 1999). *Leishmania* parasite is transmitted through the bite of an insect vector *Phlebotomine* sand fly. Sand flies get infected when they bite an infected vertebrate host. Sand flies are most active from dusk to dawn. Leishmaniasis can also be spread by blood transfusions or infected needles. Leishmaniasis causes wide clinical manifestations including visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis (Murray et al., 2005, Schwartz et al., 2006, Reithinger et al., 2007). The species of genus *Leishmania* causes visceral leishmaniasis and cutaneous leishmaniasis (old world leishmaniasis) are *L. donovani, L. infantum, L. tropica, L. major, and L.aethiopica* respectively are transmitted to humans by the biting of female sand flies of the genus *Phlebotomus* (Gonzalez et al.,2008), and mucocutaneous leishmaniasis caused by(new world leishmaniasis) i.e. *L. brazilliensis, L. mexicana, L.chagasi and L. peruviana* are carried by sandflies of the genera *Lutzomyia* and *Psychodopygus* (Tuon et al., 2008).

Leishmaniasis affects around 12 million people (WHO, 2011) and is endemic in 88 countries (72 of which are developing countries), putting an estimated 350 million people at risk. Annual incidence is estimated at 500,000 new cases of visceral leishmaniasis, and as many as 1 – 1.5 million for cutaneous leishmaniasis (Desjeux, 2004, Dujardin et al., 2008). The leishmaniasis causes an estimated 70,000 deaths each year, and an estimated 2.4 million disabilities every year (Reithinger et al., 2007). Approximately 90% of CL cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia and Syria, while 90% of VL cases occur in Bangladesh, North Eastern India, Nepal, Sudan and north eastern Brazil (Desjeux, 2004).The number of cases reported globally has increased over the past 10 years, impart due to improved diagnosis, but also due to an increase in anti-leishmanial drug resistance and a lack of adequate vector or reservoir control tools (Reithinger et al., 2007).The distribution of leishmaniasis has been modified since the emergence of the HIV/AIDS pandemic, and co-infection of HIV/Leishmania, which can lead to uncommon clinical forms of the disease and resistance against antileishmanial current drugs, has been reported in 35 countries (Cruz et al., 2006). Until the introduction of highly active antiretroviral therapy (HAART) in the late 1990s, up to 70% of all cases of visceral leishmaniasis in the Mediterranean basin were
associated with HIV. Co-infection with HIV/Leishmania is becoming an increasing problem in countries including Ethiopia, Sudan, Brazil and India (Cruz et al., 2006).

In India visceral leishmaniasis is a serious problem in Bihar, West Bengal and eastern part of Uttar Pradesh. Sporadic case have been reported from Tamil Nadu, Pondicherry, Assam, Orissa extending and there has been a sharp increase in the number of recorded cases of the disease (Seaman et al., 1996). The association of VL and human immunodeficiency virus (HIV) infection clearly confirm the fact that VL is an opportunistic infection due to immune suppression (Alvar et al., 2008). HIV/Leishmania co-infection is considered to be a real threat, especially in Southeran Europe, where 700 cases of co-infection have been reported to WHO (WHO, 1998). In Ethiopia visceral leishmanisis is commonly observed as an opportunistic infection in HIV infected adults with documented mortality rates up to 18.5% (Lyon et al., 2003). In western upper Nile, Sudan, the majority of cases reported during a major out brakes from 1984 to 1994 were adult with death rate of 38.57% (Seaman et al., 1996). HIV/AIDS can also slow down diagnosis of VL as antibody based tests may not be indicative of disease in AIDS patients. Interestingly, while HIV may have a profound impact on VL the evolution of purely cutaneous disease would only seem to be only moderately affected and L. major have not been reported to associated with HIV infection (Alvar et al., 2008).

A complication of visceral disease is post-kala-azar dermal leishmaniasis (PKLD). PKLD is characterized by a nodular rash and has been reported in patients from India and Sudan after treatment for VL (Zijlstra et al., 2003). It is develops after the apparent cure of VL and is characterized by the occurrence of macules, papules, or nodules on the skin of the face, limbs, or trunk (Hassan et al., 1992). The recent emergence of VL in Rampur Bushahr of Himachal Pradesh appears to be due to construction activities for several hydroelectric power projects with the help of River Sutlej (figure 2.1), horticulture development, and establishment of new residential colonies leading to destruction of forests and intrusion into the sylvatic cycle. The laborers employed are mainly from the known endemic areas of Bihar, Jharkhand, Uttar Pradesh and Nepal. It is still not clear whether the parasite has come from sylvatic cycle or introduced by migrant population. The cattle kept close to the houses by people in rural areas are also known to attract both anthropophilic, as well as zoophilic vectors (WHO, 1990).
Figure 2.1 Sutlej River in Kinnaur valley, Himachal Pradesh, India
Localized cutaneous leishmaniasis (LCL) in India is mostly due to *L. tropica* and prevalent in the deserts of Rajasthan. Recently, Kinnaur district in Himachal Pradesh has been identified as new endemic foci for the disease. *L. donovani* is a predominant pathogen in Sutlej river valley along with *L. tropica* (Sharma *et al*., 2005), the number of new cases of LCL has been increasing reaching almost epidemic proportions in Kinnaur district of Himachal Pradesh from last few years. The risk of acquiring LCL increases considerably with human activity, especially with developmental projects that have an impact on the environment (Sharma *et al*., 2005). Epidemics of LCL have been associated with deforestation, road construction, wars, or any activity through which humans intrude the habitat of the vector (McHugh *et al*., 1994). The LCL endemic in western Thar deserts of Rajasthan, India decreased during the implementation of malaria control programs, but it has re-emerged in recent years after the discontinuation of these programs. In addition, a few cases of LCL among travelers have been documented in other part of India which is not disease-endemic areas including, Kerala, Assam, and Haryana, (Sharma *et al*., 2003).

Despite the adopting of impressed control measures, Leishmaniasis still a major public health problem due to the development of drug resistance in parasite and insecticide resistance in vectors. It is an alarming feature that the spread of drug resistance strain of parasite and insecticide resistant strain of vectors.

Initially trivalent antimonials (SbIII) were used, but subsequently pentavalent antimonials (SbV) were found to be effective against visceral leishmaniasis (kala azar) while also being less toxic. Pentavalent antimonials were introduced in 1922 as therapeutic drug for leishmaniasis (WHO, 2010). They remained the first-line treatment for about 70 years and had a treatment success rate of up to 95% (Van *et al*., 2010). The efficacy of one of the current first-line drugs, sodium stibogluconate (Pentostam), was proven more than 50 years ago. Meglumine antimoniate (Glucantime) is the other pentavalent antimonial formulation currently being used in the clinic. A generic form of Pentostam produced in India and cheaper than the branded version of the drug was recently found to be safe and effective for the treatment of visceral leishmaniasis (Moore *et al*., 2001, Ritmeijer *et al*., 2001). To be active against *Leishmania*, SbV has to enter the host cell, cross the phagolysosomal membrane and act against intracellular amastigotes. A reduction or loss of drug activation could be one mechanism by which the parasite might become resistant. This drug interferes with *Leishmania* DNA synthesis, modifying the morphology of the kinetoplast, and promotes fragmentation of the mitochondrial membrane, killing the parasite. This drug was shown to
present the same efficacy as antimonials. Hypoglycemia and hyperglycemia are the main adverse effects of pentamidine (Goto et al., 2010).

Since the early 1980s, poor treatment responses have increasingly been reported from Bihar (Peters, 1981, Sunder, 2001), resulting in WHO's recommendations to increase treatment dosage and duration. Initially improved the results, the effects were only temporary (Thakur et al., 1984, 1991). The worst treatment outcome for pentavalent antimonials was reported from Bihar in 1997 with a treatment success rate of only 35% (Sundar et al., 2000). The rapidly increasing treatment failure rate in Bihar is an alarming feature because increased treatment failure has also been reported from Nepalese districts neighboring Bihar (Rijal et al., 2005, 2010).

In 2005, the governments of India, Nepal, and Bangladesh agreed to participate in a regional ‘VL elimination program’ to reduce the annual incidence of VL from about 22 cases per 10,000 inhabitants to only one case per 10,000 inhabitants by 2015. This programme was based among others on the replacement of antimonials as first-line treatment by the oral drug miltefosine. So far, the treatment success rate of this drug does not seem to be affected by antimony-resistance. The action mechanism of antimonials is still poorly understood (Haldar et al., 2011), but they seem to have a dual mode of action. On the one hand, they perturbate the redox-balance of the parasites (Wyllie et al., 2004) and on the other hand, they impose extra oxidative and nitrosative stress upon the parasite through interaction with the host cell (Mookerjee et al., 2006, Rais et al., 2001).

Recent molecular studies showed that (i) antimony-resistant parasites emerged from L. donovani populations with different genetic background (Downing et al., 2011) and (ii) that molecular mechanisms of drug resistance may vary with that genetic background (Decupere et al., 2012). Antimony-resistant L. donovani parasites can inhibit the patient's immune response even more than antimony-sensitive L. donovani (Murray and Nathan, 1999) and are hereby able to prevent the drug from inducing an effective antileishmanial response through the host cell (Haldar, 2010). These adaptations seem to protect them not only against antimony-induced stress, but also against natural stress such as immune response of hosts that resistant parasites may have a higher general fitness under drug-free conditions than sensitive ones (Vanaerschot et al., 2010, Vanaerschot et al., 2011).

Since there is no antileishmanial vaccine available to use, control of VL relies almost exclusively on chemotherapy. For almost seven decades pentavalent antimonials constituted the standard drug of choice worldwide, however the last two decades their clinical value was jeopardized due to the widespread emergence of resistance to these drugs in Bihar,
India, where half of VL cases occurs globally of individual. From the last decade novel formulations of conventional antileishmanials as well as new drugs, including the oral drug miltefosine, became available for the treatment of parasite. It however, their wide use in poor countries is hampered mainly due to high costs and also due to concerns of toxicity and emergence of resistance (Sundar et al., 2007). Most of the drugs used to treat leishmaniasis must be given parenterally. Visceral leishmaniasis in AIDS patients is often resistant to antileishmanial drugs, and relapse occurs. Inadequate treatment in terms of dosing, duration, and poor compliance promote the widespread antimonial resistance in India. In the country, the high incidence rate of unresponsiveness to antimonials is further sustained by the anthroponotic transmission of leishmanial infection, which increases the chances of the rapid spread of resistant parasites among humans once they emerge (Sundar et al., 1994, Singh et al., 2006).

The situation has been complicated even more, due to the spread of the strains of this parasite which are resistant to Stibogluconate (V) ((Thakur et al., 1998, Sundar, 2000, 2001)). The alternative drugs available are comparatively more toxic, less effective and above all are very expensive (Singh et al., 2001).The newer insecticides like malathione and pyrethroids are several times more expensive. Thus the control of *Leishmania* has become a costly affair due to high cost of alternative antileishmanial drugs and insecticides. So *Leishmania* control with only existing means, is very difficult and cost intensive for poor developing nations. So development of *Leishmania* vaccine is very important and urgently needed. Vaccine, if could be developed, will be a cost effective adjunct to the existing *Leishmania* control measures. Preventive vaccines are recognized as the best and most cost effective protection measure against pathogens and save millions of lives across the globe each year. *Leishmania* vaccine development has proven to be a difficult and challenging task and is hampered by an inadequate knowledge of disease pathogenesis, the complexity of immune responses needed for protection, and the cost of vaccine development (Evans et al., 2012).

In order to survive in the vertebrate host, *Leishmania* parasites use several immune evasion strategies: the most prominent is intracellular replication, which hides the parasites by the surrounding host cell from direct contact to the immune system. Many tactics of the parasites most probably are not yet known, however a few selected strategies are known which used by parasite. While these selected strategies are by far not all, these strategies show the ability of the parasite to modulate the immune response at many different levels, from complement resistance to triggering the type of immune response towards higher susceptibility of the host. The latter tactic has great impact: a common rule is that a Th1 type
cytokine milieu yields in clearing of the parasite load, whereas a Th2 type cytokine milieu leads to susceptibility of the host (Alexander et al., 1999, Launois et al., 1998).

All natural Leishmania infections start when Leishmania promastigotes are injected into the skin dermis of humans and other warm-blooded animals. To survive, the parasite must resist exposure to host serum component and destruction by innate immune cells present in or rapidly recruited to the skin. The skin is a complex immunological organ in which multiple innate immune cells function to protect the host from infectious pathogens. Normal skin of adult humans also contains a substantial number of T cells, nearly twice that present in the circulation, (Clark et al., 2006) which may play an important role in the local immune response. The interaction with the complement system depends on the developmental stage and the species of the parasite. In short, exponentially multiplying log-phase promastigotes are sensitive to complement mediated lysis and perform poorly in experimental infections while metacyclic promastigotes and amastigotes are more resistant and more infective. No parasite is completely resistant to physiological plasma complement levels (Sack and Perkins, 1984, Ueno et al., 2009). Prompt infection of susceptible host cell may be essential for survival (Moreno et al., 2007). While surface deposition of complement can cause destruction, Leishmania can use deposited C3b, which is rapidly converted into iC3b, to facilitate parasite entry into macrophages and neutrophils via complement receptor (CR) 3(Wozencraff and Blackwell 1987).

Leishmania infection has been assumed to be initiated by direct parasitization of skin resident macrophages (Locksley et al., 1988) whereas uptake by skin dendritic cells has been linked to priming and shaping the T cell response (Leon et al., 2007). Recent studies have, however, slightly changed this view; in vivo imaging of sandfly transmitted L. major infection revealed that the neutrophil is the first cells to be infected by the promastigote (Peter et al., 2008). Infiltrating neutrophils did not destroy the parasites, instead they facilitated infection as depletion of neutrophils prior to infection reduced the parasites load and delayed onset of disease. Infection of neutrophils is transient and within a week post infection macrophages or monocytes take over the parasite primary host cell (Peter et al., 2008). However, the in vivo images by Peters et al. were not able to capture neutrophils uptake by monocytes or macrophages (Peter et al., 2008). Instead the parasites were observed to egress dying neutrophils, to invade macrophages. Nevertheless, when appropriately activated, neutrophils can kill intracellular pathogens (Pearsons and Steigbrigel, 1981) and there are several reports suggesting that neutrophils play an important role in early protection against Leishmaniasis (Lima et al., 1998, Rousseau et al., 2001, McFarlane et al., 2008).
The capacity of neutrophils as an immune evasion targets probably depends on the genetic background of the host of the developmental stage of the parasite used (Ritter et al., 2009). In both human and murine leishmaniasis neutrophils are prominent infiltrates in lesions (Palma et al., 1997, Donnelly et al., 1998), their presence at the site of infection can cause immune mediated tissue pathology (Smelt et al., 2000, Lopez et al., 2009). For a productive infection, the Leishmania parasites need to establish in macrophages. Macrophages possess potent antimicrobial functions and activated macrophages can kill Leishmania. To survive the parasite need to avoid macrophage activation and recognition by T cells. The ability to survive in macrophages is partly stage specific with metacyclic promastigotes having better capacity to survive compare to pro-cyclic promastigotes. However, most targets for Leishmania would appear to be infiltrating monocytes/macrophages, which enter the site of infection one to two days post infection (Peter et al., 2008, De Trez et al., 2009). Interestingly, recent studies show that macrophages may not be the main host cells for the parasites in chronic stages of healing disease. In chronic self-healing L. major infection (in C57Bl/6 mice) TNF–α and iNOS producing CD11b+CD11c+Ly6C+MHC-II+DC (TIP-DC), which most likely are derived from monocytes, host the majority parasites in the skin (De Trez et al., 2009, Filip-Santos et al., 2009). If this is related to cure and generation of a protective Th1 response and/or preparing the parasite for transmission to blood feeding shadflies, is not known.

In human cells L. donovani has been suggested to block maturation of human dendritic cells (DC) (Tejle et al., 2008). IL-12 produced by DC, is essential for the initiation of a protective immune response in mice and probably also in humans. It is differently affected by L. donovani and L. major; while uptake of L. major by human monocyte derived DC efficiently prime DC for IL-12 production but uptake of L. donovaniis not well known (Ma Dowell et al., 2002). Together with phagocytes, NK cells represent the first line of defense against pathogens by two principal mechanisms, cytolytic destruction of infected cells and secretion of pro-inflammatory cytokines (e.g. IFN-γ and TNF-α) (Laurenti et al., 1999).

NK cell number and activity has mainly been associated with protection against or healing of disease. Patients with active leishmaniasis (cutaneous and visceral) have been reported to have a reduction in the frequency of peripheral NK cells (Nylen et al., 2007) and recently an increased frequency NK cells, following immunotherapy, in a L. amazonensis diffuse cutaneous leishmaniasis (DCL) patients was associated with cure (Pereira et al., 2009).
Th1 dependent mechanisms are involved in control of human disease. Self-healing forms of leishmaniasis and cure of VL is typically accompanied by parasite specific proliferation and IFN-\(\gamma\) production. Human macrophages are activated to kill intracellular parasites by IFN-\(\gamma\) and exogenous IFN-\(\gamma\) can promote cure of human CL. Th2 responses might act in favor of the parasite; polarized Th2 response has not been able to elucidate non-curative or dissemination of human disease (Anderson et al., 2005). The IFN-\(\gamma\) production by CD4\(^+\) cells, alone, in response to *Leishmania* antigens is not predictive of protection or disease development human CL (Nylen et al., 2006). This indicates that other mechanisms acting in synergy with IFN-\(\gamma\) or counteracting the effects of IFN-\(\gamma\) are important. Th17 and T-reg cells are widely accepted subsets with important functions in induction and control of the inflammatory response. Both Th17 and T-reg cells have a greater degree of plasticity in their differentiation decision, as compared to conventional Th1 and Th2 cells. These two cells enable to response to signals provided by the environment in which they are residing (Lee et al., 2009). Th17 cells are pro-inflammatory T helper cells, and have ability to secrete IL-17 cytokine. IL-17 is involved in recruitment, migration and activation of neutrophils and Th17 cells have an important function in protecting surfaces against certain extracellular bacterial and fungal pathogens, but may be also mediated severe immune pathologies (Korn et al., 2009). It has been demonstrated experimentally by using Th-17 deficient Balb/c mice that Th-17 cell have been associated with tissue destruction in leishmaniasis. It has been established that the IL-17, IL-22 and IFN-\(\gamma\) enhanced in protection from kala-azar. The IL-27 produced by macrophages and DC play an important role in regulation of Th-17 cells as wells as less anti-inflammatory (Anderson et al., 2009) and induction of naïve human CD4 cells to IL-10 production (Murugaiyan et al., 2009). Studies showed that CD4 T cells were linked to pathology and IL-27 was found to regulate both IL-10 and IL-17, and tissue pathology was associated with IL-17 producing T cells. Thus, the elevated levels of IL-27 in human VL may serve an important function suppression of IL-17 producing CD4 T cells and subsequent tissue damage by neutrophils (Anderson et al., 2009).

The role of CD8\(^+\) T cells is still not understood completely defined in *Leishmania* infection. Though, a number of early reports suggested a role for CD8\(^+\) cells in immunity against *L. major* infection (Farrell et al., 1989, Holiday et al., 1991, Belkaid et al., 2002, Murray, 2005). CD8\(^+\) T cells protect against pathogens by using two major mechanisms: production of cytokines (IFN-\(\gamma\) and TNF-\(\alpha\)) and by direct killing of infected cells. As *Leishmania* is an intracellular parasite, the contribution of CD8\(^+\) T cells to control of *Leishmania* infection through IFN-\(\gamma\) production. Cytotoxic T-lymphocyte (CTL)-mediated
mechanisms in the regulation and control of Leishmania infection remain largely unexplored (Ruiz and Becker, 2007). Both murine and human Leishmania infection can lead to the killing of antigen-pulsed macrophages (Kima et al., 1997).

2.1.1 Interleukin-2 (IL-2)

IL-2 produced by Th-1 cells, is a potent inflammatory cytokine mediating multiple immune responses on activated B cells, monocytes, and natural killer (NK) cells (Beadling et al., 1993, Sarfro et al., 2011). It is a necessary cytokine for the development of T cell immunologic memory, one of the unique characteristics of the immune system, which depends upon the expansion of the number and function of antigen selected T cell clones (Beadling et al., 1993). Antigen binds to the T cell receptor (TCR) and stimulates the secretion of IL-2 as well as the expression of IL-2 receptors (IL-2R). The interaction of IL-2/IL-2R stimulates the growth, differentiation and survival of Cytotoxic T (Tc) Cell, (Beadling et al., 1993) and regulatory T cells in thymus (Thornton et al., 2004, Sarfro et al., 2011). These T reg cell are suppressor T cells and play an important role to prevent other T cells from recognizing and reacting against “self antigen”, which could result in “auto inflammatory”.

2.1.2 Interleukin-4 (IL-4)

IL-4 is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells and it promotes the growth of differentiated Th2 cells resulting in the production of an antibody response. The human Interleukin 4 (IL-4) gene located on the chromosome 5 (5q31-33) encodes for an anti-inflammatory cytokine produced by CD4+ Th2 cells, basophils and mast cells. IL-4 regulates variety of cell types playing an essential role in differentiation of Th2 effector cells and suppression of Th1 signaling (Gyan et al., 2004). IL-4 has a pleotropic effect. IL-4 has been shown to act as a growth factor for activated T cells, thymocytes, natural killer cells, and B cells (Defrance et al., 1987). Several mechanisms are involved in reducing blood stage parasite immunity and one of them is that IL-4 which interferes with Th1 cell development and reduces the production of IFN-γ (Zhu et al., 2006). Another study has revealed that IFN-γ levels were significantly elevated during early stages of infection (malaria, leishmaniasis), where as the IL-4 levels were elevated during intermediate and late stages (Tangteerawatana et al., 2007). IL-4 also plays a role in promoting the differentiation of naive T cells to Th2 cells. A key anti-inflammatory action of IL-4 results from its ability to inhibit release of pro-inflammatory cytokines by innate immune cells and to up-regulate the synthesis of IL-1 receptor antagonist (Brown and Hural, 1997). Several cells express IL-4 receptors, including hematopoietic, endothelial, and
epithelial cells (Nelms et al., 1999), but robust evidence indicating their presence on neurons is lacking, although expression on cultured glial cells has been reported (Szezeparnik et al., 2001).

2.1.3 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that plays an important role in intraprostatic inflammation and thus carcinogenesis (Mandic et al., 2013). IL-6 is a cytokine produced by several cell types including antigen presenting cells (APC) such as macrophages, dendritic cells, and B cells. IL-6 is involved in the acute phase response, B cell maturation, and macrophage differentiation. A novel function of IL-6: the control of T helper (Th1)/ (Th2) differentiation. IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization through two independent molecular mechanisms. IL-6 activates transcription mediated by nuclear factor of activated T cells (NFAT) leading to production of IL-4 by naive CD4+ T cells and their differentiation into effector Th2 cells. While the induction of Th2 differentiation by IL-6 is dependent upon endogenous IL-4, inhibition of Th1 differentiation by IL-6 is IL-4-and NFAT-independent. IL-6 inhibits Th1 differentiation by up-regulating suppressor of cytokine signaling (SOCS)-1 expression to interfere with IFN γ signaling and the development of Th1 cells. Since IL-6 is abundantly produced by APC, it is a likely source of early Th1/Th2 control during CD4+ T cell activation. Thus, by using two independent molecular mechanisms, IL-6 plays a dual role in Th1/Th2 differentiation (Diehl and Rincon, 2002).

IL-6 act as both pro-inflammatory and anti-inflammatory cytokine. IL-6 is secreted by T cells and macrophages to stimulate immune response. Nitric oxide (NO) and interleukin-6 (IL-6) are highly reactive mediators that have been shown to play different roles in a variety of different biological process (Carmeli et al., 2009). Interleukin (IL)-6 contributes to a myriad of physiologic and pathophysiologic processes. IL-6 plays an active role in immunology, inflammatory responses, bone metabolism, arthritis and neoplasia. Overproduction of IL-6 has been implicated in the disease pathology of several inflammatory and autoimmune disorders, including rheumatoid arthritis, Castleman's disease, Crohn's disease and systemic-onset juvenile idiopathic arthritis. Interception of the IL-6 signaling pathway could thus represent a new treatment option for these diseases, given their refractory status to conventional therapy (Paul-Pletze, 2006).

2.1.4 Interleukin-10 (IL-10)

IL-10 is an important immune-modulatory cytokine that can be produced by monocytes, macrophages mast cells, natural killer cells, B cells, CD4+, CD8+ T cells, T reg,
Th1, Th2 and Th-17 cell subset (Moore et al., 1993, Kubo et al., 2012). IL-10 is an ‘anti-inflammatory cytokine and able to block the production of IL-1, IL-6 and TNF-α and other cytokine (De Waal Malefyt et al., 1989). The IL-10 also prevents the maturation of dendritic cells, rendering these cells ineffective in activating T and other immune cells (De Waal Malefyt et al., 1989). Furthermore, IL-10 regulates the proliferation and differentiation of Th1 cells by acting on antigen presenting cells to suppress the defense and many crucial effector immune responses such as anti-tumor immunity and auto-immunity (Fiorentino et al., 1991). In addition, IL-10 was also shown to induce the proliferation of B lymphocytes and most notably their differentiation into plasma cells secreting immunoglobulins at high rate (Rousset et al., 1992). The studies carried out in mice model and in vitro have suggested a regulatory role of IL-10 in the mediation of susceptibility to acute parasitic infection (Bogdan et al., 1991, Ghalib et al., 1993). In particular, IL-10 inhibits the microbicidal activity of interferon–gamma (IFN-γ) treated macrophages against intracellular parasite (Bogdan et al., 1991). IL-10 and IL-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation. The host response to Leishmania infection is regulated by a specific pattern of local cytokine production. Bamford et al., (1997) investigated the effect of interleukin (IL)-10 and IL-4 on the leishmanicidal activity of human macrophages. IL-10 was more potent than IL-4 in inhibiting the leishmanicidal activity of human macrophages. Inhibition of Leishmania killing by IL-4 and IL-10 correlated with decreased NO generation from macrophages and was reversed when exogenous NO was added to cell cultures. Therefore, IL-10 and IL-4 down-regulate leishmanicidal activity of human macrophages in part by inhibiting NO generation by these cells.

IL-10 is a cytokine intimately linked with disease progression of both murine and human Leishmania infection (Nylen and Sacks, 2007). Experimental models have clearly demonstrated the central role played by IL-10 in pathology and parasite persistence (Murphy et al., 2001, Anderson et al., 2007). In human VL, elevated levels of IL-10/IL-10 mRNA are found systemically as well as in spleen, bone marrow and lymph nodes. A role for IL-10 in human VL pathology is supported by studies indicating that IL-10 blockade can enhance VL PBMC IFN-γ responses and inhibit VL serum promoted parasite replication in macrophages (Ghalib et al., 1993, Carvello et al., 1994, Nylen et al., 2007). However, if the IL-10, as assumed, is a major suppressor of effector T cell in VL patients, remains to be proved. In human CL, elevated IL-10 has been demonstrated in lesions (Salhi et al., 2008). A recent genetic analysis of IL-10-819C/T polymorphism, in the IL10 promoter, showed that the C
allele, which is linked to higher levels of IL-10 production, is associated with increased risk of developing cutaneous lesions in populations exposed to *L. braziliensis* (Salhi *et al.*, 2008).

### 2.1.5. Interleukin-17(IL-17)

IL-17 family of cytokines consists of five structurally related isoform: IL-17A, IL-17F, IL-17 B, IL-17C AND IL-17D. Recently, a sixth reported IL-17E has been identified and renamed as IL-25, as it does not have significant homology and functional similarity with other IL-17 family members (Dubin *et al.*, 2008). Among all the isoforms of IL-17, IL-17A and IL-17F are characterized because both IL-17A and IL-17F have 55% structural and functional similarities with each other and secondly, both are produced by Th17 cells. However, the other IL-17A has a potential pro-inflammatory role in mediating inflammation to the infection (Luzza *et al.*, 2000, Aggarwal *et al.*, 2003). It was clearly evidenced that IL-17 mediates granulopoiesis, infiltration of neutrophils and recruitment of T cells into peripheral tissues through the induction of chemokines and cytokine expression (Ishida *et al.*, 2010).

The IL-17 mediates its function via various receptor which includes IL-17RA, IL-17RB, IL-17RC and IL-17RD, while, IL-17A and IL-17F exert its effect via IL-17RA and IL-17R respectively (Dubin and Koll, 2008).

### 2.1.6 Tumor Necrosis Factor–α (TNF-α)

Cytokines play a major role in imparting pathogenicity to leishmaniasis. One of the most prominent cytokines is tumor necrosis alpha (TNF-α). TNF-α is a cytokine involved in systemic inflammation and is a member of cytokines that stimulate the acute phase reaction (Depinay *et al.*, 2011). The primary sources of TNF-α are monocytes/macrophages activated by various parasite products. TNF-α is not just a pro-inflammatory cytokine, It has also been proposed to be an immunoregulatory molecule that can alter the balance of T regulatory cells (Wu *et al.*, 2002). Several lines of investigation in humans and animals suggest that low TNF-α activity is associated with pathogenesis of some forms of autoimmunity. Low TNF-α activity might result from gene polymorphisms reducing TNF-α expression or disrupting its production. Low TNF-α activity also might result from excess production of soluble TNF receptors. Soluble TNF receptors bind to and inactivate TNF-α (Loetscher *et al.*, 1991), effectively lowering TNF-α levels available to bind to membrane-bound receptors, which is a necessary step for activating intracellular signaling pathways. Tumor necrosis factor (TNF-α) alpha is a key cytokine involved in Th1-dependent granuloma formation, explaining why patients treated with TNF antagonists have an increased risk of granulomatous infectious diseases.
2.1.7 Interferon-γ (IFN-γ)

IFN-γ is a type II IFN. It is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus. Initially, it was believed that CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, and NK cells exclusively produced IFN-γ (Young, 1996).

However, there is now evidence that other cells, such as B cells, NK T cells, and professional antigen-presenting cells (APCs) secrete IFN-γ (Gessani et al., 1998, Carnaud et al., 1999, Harris et al., 2000). IFN-γ production by professional APCs (monocyte/macrophage, dendritic cells (DCs) acting locally may be important in cell self-activation and activation of nearby cells (Gessani et al., 1998, Fruct et al., 2001). IFN-γ secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFN-γ in the adaptive immune response (Fruct et al., 2001, Sen, 2001).

The protective immunity is associated with the production of IFN-γ by peripheral blood mononuclear cells (PBMC) in addition to the induction of parasite-specific cytotoxic T cells (Moreno and Alvar, 2002). The activation of macrophages by IFN-γ has been shown to result in nitric oxide production which mediates the killing of intracellular parasites (Pinelli et al., 1999, Gradoni and Asceni, 2004). On the other hand, the progression of disease is associated with Th2-type immune response and, in particular with production of IL-4 (Sundar et al., 2001, Nylen and Sacks, 2007). Studies have shown that large CL lesions were correlated with a higher frequency of lymphocytes producing Leishmania soluble antigen specific inflammatory cytokines (IFN-γ or TNF-α) (Antonelli et al., 2005). A major consequence of signaling by many PRRs is the rapid elaboration of inflammatory type I IFN cytokines (Baccala et al., 2007, Mc Cartney and Colonna, 2009) and many viral and bacterial infections lead to IFN-α/β production (Bogdan, 2000 and Mancuso et al., 2009). Type I IFN has been previously characterized to modulate antibody production both in vitro and in vivo including promoting class-switch recombination and polarizing antibody responses toward IgG2a/c production (Finkelman et al., 1991, Le Bon et al., 2001).

B cells and antibodies are generally not considered to be of major importance in protective immunity against Leishmania. Antibodies are not effective at killing the parasite as it hides inside the parasitophorous vacuole and antibody responses in self-healing cutaneous disease are very modest. High levels of Leishmania specific antibodies are observed in patients with VL and other severe forms of leishmanial disease and there are accumulating evidence that B cells and antibodies correlate with pathology. A model where
immunoglobulin (IgG) promotes infection by inducing IL-10 was proposed by Kane and Mosser, who showed that IgG coated amastigotes (*L. major*), could ligate Fc-receptors on murine macrophages and induce IL-10 production (Kane and Mosser, 2001). In support of this model *in vivo* studies found that Fc-deficient mice infected with *L. amazonensis* produce less IL-10 and are less susceptible to infection (Buxbaum and scoot, 2005). Moreover, a regulatory role for B cells has been suggested in a VL model demonstrating that B cell depleted animal exhibit extensive neutrophil mediated pathology (Smelt *et al.*, 2001). There is still much to learn about how antibodies function in leishmaniasis and it should not be ruled out that certain antibodies might contribute to protection. Immunization with, the for dogs licensed vaccine, Leishmune, which confers some protection against leishmaniasis, result in seroconversion and an increase in the proportion of B cells (Borja-Cabrera *et al.*, 2008).

**2.1.8 IgG and its subclasses (IgG1, IgG2, IgG3, and IgG4)**

The role of anti-leishmanial immune response underlying the susceptibility/resistance during canine visceral leishmaniasis (CVL) has been recognized throughout ex vivo and in vitro investigations (Alexander *et al.*, 2006). B-cell immunity is mediated by the immunoglobulins and is commonly referred to as humoral immunity. Humoral immunity is differentiated from T-cell immunity, which is commonly referred to as cellular immunity, and from phagocytic cell immune function. Immunoglobulins, which are protein molecules that contain antibody activity, are produced by the terminal cells of B-cell differentiation known as plasma cells. Immunoglobulins have important roles in humoral immunity. It has been shown that IgG not only fails to provide protection against this intracellular pathogen, but that it actually contributes to disease progression (Miles *et al.*, 2005). Analysis of *Leishmania* antigen-specific immunoglobulin isotypes and IgG subclasses in VL patient sera revealed elevated levels of IgG, IgM, IgE and IgG subclasses during disease (Atta *et al.*, 1998, Anam *et al.*, 1999, da Matta *et al.*, 2000, Ryan *et al.*, 2002, Ravindran *et al.*, 2004). The differential patterns of immunoglobulin isotypes observed during disease progression, drug resistance and cure were specific for antigens of *Leishmania donovani*. IgG subclass analysis revealed expression of all of the subclasses, with a predominance of IgG1 during disease (Anam *et al.*, 1999). The role of antibody titres in resolution of CL and protective immunity is largely unknown. Although some studies have shown the advantage of using specific subclass antibodies for the diagnosis of VL, only a few reports are available for CL. The goal of this study is to evaluate serum IgG levels and IgG subclass distribution and the correlation between them in CL patients, and to find out whether this may be used as a helpful diagnostic
tool for this disease. ELISA is one of the most sensitive tests for serodiagnosis of leishmaniasis, the sensitivity and specificity is influenced by the antigen used. ELISA has sensitivity 83-100% and specificity 96-100% (Felix, 1998, Daniel et al., 1999). Role of Immunoglobin (Ig) isotypes and IgG subclasses in the diagnosis of parasitic diseases is well documented (Short et al., 1990, Daniel et al., 1999, Malla et al., 2006, Yadav et al., 2005).  

2.2 Internal transcribed spacer (ITS)

Internal transcribed spacer (ITS) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. In eukaryotes, the genes encoding ribosomal RNAs are organized in arrays which contain repetitive transcriptional units involving 16 – 18S, 5.5S, and 23 – 28S rRNAs, two transcribed intergenic spacers ITS-1 and ITS-2 and two external spacer sequences (5 and 3’ ETS). High levels of inter and intra species variation have been observed in Old and New World Leishmania species in the DNA internal transcribed spacers (ITS-1 and ITS-2) present in the multi-copy ribosomal operon. Characterisation of Leishmania species in clinical infections is important, as different species may require distinct treatment regimens. Furthermore, such information is also valuable in epidemiologic studies where the distribution of Leishmania species in human and animal hosts, as well as in insect vectors, is a prerequisite of designing appropriate control measures (Janeiro, 2009).

A universal PCR method targeting the internal transcribed spacer-1 (ITS-1) region lying between the genes coding for 18S rRNA and 5.8S rRNA proved to be useful for direct diagnosis and identification of Leishmania parasite, due to its high conservation among species (Davila an Momen, 2000, Schonian et al., 2001, 2003, Al-Jawabreh et al., 2006, Bensoussan et al., 2006, Abda et al., 2011). ITS1-PCR can be used for direct species identification of Leishmania in patient tissues, blood, or other samples preventing the use of microscopic examination and cultivation (Davila An Momen, 2000, Schonian et al., 2001, 2003, Bensoussan et al., 2006). The ITS-1 PCR proved to be slightly more sensitive and more practical than the mini-exon (Jeron et al., 2011).