CHAPTER 5
DISCUSSION

Leishmaniasis is a parasitic infection caused by an obligate intra cellular protozoan parasite belongs to the family Trypanosomatidae and genus *Leishmania*. It is still a major public health problem in tropical and subtropical countries of the world including India (Croft and Coombs, 2003). It 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. It causes wide clinical manifestations including visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis (Murray et al., 2005, Schwartz et al., 2006, Reithinger et al., 2007). Leishmaniasis affects around 12 million peoples world wide and is endemic in 88 countries (72 of which are developing countries), it is estimated that 350 million peoples are on risk. The annual incidence of visceral leishmaniasis is estimated 0.5 million cases and 1 – 1.5 million cases for cutaneous leishmaniasis (Desjeux, 2004, Dujardin et al., 2008) along with 70,000 deaths each year, and an estimated 2.4 million disability every year (Reithinger et al., 2007). In addition, leishmaniasis is spreading to several non endemic areas of the world due to co-infection with human immunodeficiency virus (Alvar et al., 1997, Redhu et al., 2006). The number of cases have been increased globally during the past decades, may be one of the reason is anti-leishmanial drug resistance and lack of adequate vector or reservoir control tools (Reithinger et al., 2007).

In India visceral leishmaniasis is a serious problem in Bihar, West Bangal and eastern parts of Uttar Pradesh. Sporadic cases have been reported from Tamil Nadu, Pondicherry, Assam, Orissa and Gujarat (Pearson et al., 1995). 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 Southern Europe, where 700 cases of co-infection have been reported by World Health Organization, (1998).

The recent emergence of VL in Rampur Bushahr of Himachal Pradesh appears to be due to construction activities for several hydroelectric power projects on River Sutlej, establishment of new residential colonies leading to destruction of forests and intrusion into the sylvatic cycle of the vector and migration of the laborers from Bihar, Jharkhand, Uttar Pradesh and Nepal (WHO, 1990). 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).

In India localized cutaneous leishmaniasis (LCL) is mostly due to *L. tropica* and prevalent in the deserts of Rajasthan. Recently, Kinnaur district of Himachal Pradesh has been identified as new endemic foci for the disease. It is rarely reported as a cause of LCL exists with sporadic VL. *L. donovani* is a predominant pathogen along with *L. tropica* and the number of new cases of LCL has been increasing reaching almost epidemic proportions in Kinnaur district of Himachal Pradesh from last few years (Sharma *et al.*, 2005). The risk of acquiring LCL increases considerably with human activity, especially with developmental projects that have an impact on the environment. 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).

In order to develop a successful parasitic relationship with its host, the *Leishmania* must evade both the innate and adaptive immune responses. The promastigote forms *Leishmania* inoculated by the sand fly into human body at the time of blood feeding. These promastigotes engulfed by macrophages and immediately converted into amastigote forms which are resistant to proteolytic enzymes and degradation inside phagosomes. After inoculation, the promastigote interacts with opsonic serum factors and activates the complement system (Brittingham and Mosser, 1996). The complement protein C3b is one of the most potent immune opsonins. C3b binds to *Leishmania* parasite which results in uptake by the macrophages. *Leishmania* has a special surface glycoprotein called gp63, which converts C3b into iC3b (Hermoso *et al.*, 1991). This conversion favor phagocytic clearance rather than lytic clearance of *Leishmania*, as *Leishmania* is very resistant to degradation once phagocytosed. Therefore, this conversion is crucial for *Leishmania*’s survival.

After being engulfed, the *Leishmania* endure harsh conditions inside the phagosome like the oxidative burst used by the macrophage to destroy foreign material inside the phagosome. Macrophage plays a primary role in the host defense and regulation of immune response upon activation (Unane and Allen, 1987). This process consists of an attack by superoxide and hydroxyl radicals on the parasite. *Leishmania* parasite produces acid phosphateses on its surface which inhibits the oxidative burst (Alexander and Russell, 1992). In addition to the oxidative burst, macrophages often degrade parasites with acidic enzymes. This occur when lysosome fuse with the phagosome. The *Leishmania* parasite resists this attack through a proton pump present on the surface and allows its intracellular pH to remain close to neutral. Also, the lipophosphoglycan (LPG) plays an active role by inhibiting
lysosomal enzymes. The parasites perform a complex host parasite interaction inside the severe environment of the phagolysosomes and eventually evade this immune defense mechanism (Alexander and Russell, 1992). Infection of macrophages with *Leishmania* results in impaired microbicidal machinery as evidenced by decreased responsiveness to the lipopolysaccharide required for induction of interleukin-1(IL-1) production (Reiner *et al*., 1990). By continuing to live inside the macrophages *Leishmania* effectively avoids the humoral branch of immune system.

During early infection with *Leishmania*, both resistant and susceptible host have been shown to exhibit mixed Th1/Th2 responses of CD4\(^+\) cell population with IL-2, IL-4 and IL-13 production, while IL-6 is induced often together with the pro-inflammatory cytokines TNF-\(\alpha\) and IL-1 in many alarm conditions, and circulating IL-6 plays an important role in the induction of acute phase reactionstranscripts were variable in different strain of mice (Heinzel *et al*., 1993). IL-6 has been proposed to favor the development of Th2 responses. When IL-6 deficient mice on a susceptible BALB/C were infected with *Leishmania major*, the course of infection was not different from control animals (Roger *et al*., 2002). The absence of IL-6 led to down regulation of both Th1(IL-12) and Th2 (IL-4, IL-10 and IL-13) associated cytokines. Thus, in mice infected with *L. major*, IL-6 may promote the development of both Th1 and Th2 responses.

The levels of IFN-\(\gamma\) and IL-4 are elevated during active VL and decline significantly after cure. In active VL disease, PBMCs exhibits a poor proliferative response to parasite antigen and fail to generate IFN-\(\gamma\) in vitro (Haldar *et al*., 1983). This lack of IFN-\(\gamma\) production by PBMCs seems to predict progression of the infection into fulminant VL (Carvalho *et al*., 1985, Carvalho *et al*., 1989). IL-4 is considered to be the signature cytokine of Th2 response (Alexander *et al*., 2000, Wilson *et al*., 2005). VL have a strong expression of mRNA for IL-4 and sera from VL patients have IL-4 levels, IL-4 is involved in the down- regulation of the Th1 type of response in human leishmaniasis (Alexander *et al*., 2000, Wilson *et al*., 2005).IL-4 also did not suppress IFN – \(\gamma\) production in cure of leishmaniasis (Ribeiro *et al*.,1998). 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). It is an ‘anti-inflammatory cytokine and able to block the production of IL-1, IL-6, TNF-\(\alpha\) and other cytokines (De Waal Malefyt *et al*., 1989).A role for IL-10 in human VL pathology is supported by studies indicating that IL-10 blockade can enhance VL PBMC IFN-\(\gamma\) responses and inhibit VL serum promoted
parasite replication in macrophages (Nylen et al., 2007, Carvello et al., 1994, Ghalib et al., 1993).

Infection of *Leishmania* is characterized by the appearance of anti-leishmanial antibodies in the sera of the patients. In CL, usually they are present at low levels during active phase of the disease (Behin and Jacques, 1989). In contrast, strong anti-leishmanial antibody titers are well documented in VL (Bray, 1976, Neogy et al., 1987). Critical analysis of *Leishmania* antigen specific immunoglobulin isotypes revealed elevated levels of IgG, IgM, IgE and IgG subclasses during disease (Ghosh et al., 1995, da Matta et al., 2000, Ryan et al., 2002, Atta et al., 2004). IgG not only fails to provide protection against this intracellular pathogen, but it actually contributes to disease progression (Miles et al., 2005).

5.1 Determination of Anti-leishmanial whole IgG antibody and its subclasses (IgG1, IgG2, IgG3 and IgG4)

In the present study we have detected the antileishmanial IgG and its subclasses viz., (IgG1, IgG2, IgG3 and IgG4) antibodies in suspected leishmaniasis individual by ELISA. The ELISA is a sensitive technique like IFAT for the diagnosis of leishmaniasis (Choudhary et al., 1990, Hommel et al., 1978). The disease is also characterized by high levels of *Leishmania* specific antibodies (Bray et al., 1976). Some reports have shown a high concentration of serum antileishmanial antibodies in VL patients, individuals with the SCL form however shows the low titers of these immunoglobulins (Badaró & Duarte, 1997). While still limited in ability to distinguish between active and past or subclinical infection, antibodies have proven useful in diagnosis of VL disease (Sundar et al., 2002, Sundar and Rai, 2002, Clements et al., 2010, Gidwani et al., 2011).

In the present study, we have analysed 100 serum samples for Whole IgG antibody, whole IgG antibody was found predominant in individuals infected with *Leishmania* species, and it was detected in 42% serum samples against leishmanial promastigote antigens. Several studies have also been reported that the predominance of the antileishmanial IgG antibodies in leishmaniasis (VL) patients was found from different part of the world (Atta et al., 1998, Anam et al., 1999). IgG class is found to be the major class of antibodies present in the sera of Indian KA and PKADL patients (Ghose et al., 1980, Haldar et al., 1981), which is in accordance to the present study, though PKDL patients have been excluded from the study, hence the observation of the present study could not be correlated with PKDL. The earlier studies have 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).
Subclasses of IgG Abs were also measured in all 42 positive samples, of which 40.5% were of males and 59.5% were of females. The highest frequency of IgG1 38.1% subclass was detected among the individuals followed by IgG3 26.2% and IgG4 9.52% respectively. However IgG2 subclass was not detected in any of the sample. Hence it can be assumed that IgG2 subclass is not prevalent among the people of this region. In accordance to the present study, earlier studies have been reported the absence of IgG2 antibodies among the population of endemic region for leishmaniasis (Iskander et al., 1981).

In support of our findings the prevalence of IgG subclasses had been reported in early studies from Bihar and West Bengal situated in Eastern part of India (Anam et al, 1999, Da Matta, 2000, Ryan et al., 2002, Ravindran et al., 2004). In the present study, the IgG4 subclass was less frequent among the study population. Similar observation was made by El Amin et al., (1986) from Sudan, and Zwingenberger et al., (1990) from de Sao Paulo, Brazil, that observed minimal involvement of IgG4 in VL patients among the individual infected with *L. donovani*. The high level of IgG1 was reported by Shiddo et al., (1996) from Somalia which is accordance to the present study as we have detected IgG1 antibody in high level among the study population infected with VL. The subclasses of IgG antibody revealed differential patterns in the sera of leishmaniasis patients with respect to their patterns of reactivity with antigens may be indicative of disease activity and pathogenicity. In the present study predominance of IgG1 followed by IgG3 was observed while IgG4 was detected in low levels. High level of IgG1 and IgG3 production has been associated with IL-10 activity in human VL (Garraud et al., 2003, Calds et al., 2005). Similar to the present study, IgG1, and IgG3 subclass have been shown to increase in patients with active VL from Somalia (Shiddo et al., 1996). The role of IgG4 in parasitic infections is not clear but it has been suggested to play a blocking role in parasitic killing and clearance (Jassim et al., 1987, Dafa’aalla et al., 1992). In the present study, the presence of low level of IgG4 in individuals residing in Sutlej river valley suggested that the parasite prevalence in this area is highly virulent and may be able to cause more pathogenicity. Contrarily, significantly high level of IgG4 subclass was detected in Sudanese patients along with low level of IgG1 while high level of IgG3 subclass was observed in the same patients (Ellassad et al., 1994) which is similar to those of our finding. Conversely, Venezuelan patients have a dominant IgG1 response followed by IgG4 (Ulrich et al., 1995). Predominance of IgG1 subclass antibody response, followed by IgG3, IgG4, and IgG2 was detected in kala-azar patients in India (Jefferis et al., 1990).

In accordance to this study, we have observed the predominance of IgG1 and IgG3 subclasses while contrarily, IgG4 was observed in low level but the prevalence of IgG2 was
not found among the individuals residing in study area. This may be due to the non-endemic area for VL leishmaniasis or the parasite may be transmitted through migrant population coming for labour work for developmental projects from endemic area and the native population of this area is not acted as native host for the parasite. These subclasses of human IgG are endowed with unique biological and functional properties, including their response to different types of antigens (Jefferis et al., 1990).

Our findings also revealed the prevalence of high frequency IgG antibodies among adult age group i.e. between 21-40 years old, suggested that this age group is more susceptible to Leishmania parasite infections and are on high risk. Furthermore we found that females are more susceptible for Leishmania species infection as the frequency of detection of antileishmanial IgG antibody was high amongst the females in comparison to those of males. The reason may the development of strong cell-mediated (Th1) immune response in males against Leishmania species infection or might be females have more exposure for Leishmania infection in comparison to those of males. On the other hand, females develop stronger humoral (Th2) immune response again suggesting that males are more resistant to infection then those of females (Beer et al., 1991, Jahn et al., 1986).

In conclusion, our findings revealed that the Leishmania species strains causing VL leishmaniasis prevalent in study area is not native parasite it may be migrated from other endemic areas along with migrant population involved in developmental projects because the mixed type of antibody responses in native population demonstrated that the immune response against the parasite could not developed properly and in developmental stage. Furthermore, the serological data demonstrated that the IgG1 antibody may be used as a diagnostic marker for Leishmania species infection. In contrast the IgG antibody response against CL was found strong in native population and week in migrant population suggested that the migrant population doesn’t have earlier exposure to the infection of L. tropica. In the present study, two different populations (migrant and native) have mixed type of antibody response against L. donovani as well as L. tropica which is vice versa.

5.2 Measurement of expression of Pro and anti - inflammatory Cytokines by Flowcytometry

T-cells and other immune cells exert their effector function partly through the production and release of cytokines. Th1 and Th2 cells are characterised by their distinct cytokine patterns (Mosmann and Coffman, 1989). In the present study, we have measure the expression level of anti and pro-inflammatory cytokines which helps in protection and progression of the disease by using different cytokines viz. IL-2, IL-4, IL-6,
1L-10, 1L-17A, TNF-α and IFN-γ, to know whether these cytokine play role in protection or pathology. The findings indicates that the parasite prevalent in this region is highly virulent and cause more pathogenic effect to the patients, as only IL-10 anti-inflammatory cytokines was expressed during the infection while IL-4 could not detected in any individual.

5.2.1 Expression level of pro-inflammatory cytokines: The expression level of pro-inflammatory cytokines IL-2, IL-6, 1L-17A, TNF-α and IFN-γ was analyzed. Among all pro-inflammatory cytokines, IL-2 cytokine showed highest level of expression followed by TNF-α, IFN-γ, IL-6 and IL-17A has shown minimum level of expression.

(i) Interleukin-2 (IL-2)

IL-2 produced by Th-1 cells, is a potent inflammatory cytokines mediating multiple immune responses on activated B cells, monocytes, and natural killer (NK) cells (Beadling et al., 1993, Sarfro et al., 2011).

In the present study, among all pro-inflammatory cytokines, IL-2 showed highest expression level and was positive in 10% samples. The frequency of detection of IL-2 was high amongst the males. Expression level of IL-2 was significantly very high in male comparison to those of females. Ganguly et al., (2008) has been demonstrated that the elevated level of IL-2 in VL and PKDL patients from Kolkata (India) and also observed that the helper T cells in treated individuals with anti-leishmanial drugs failed to synthesized IL-2 in PKDL patients.

However, IL-2 level was high among asymptomatic and cured patients than those of VL patients from Sao Luis MA USA (Costa et al., 2012). Similarly, low levels of IL-2 were detected in VL patients from Bihar, India (Ansari et al., 2006) and Brazil (Peruhype-Magalhaes et al., 2005), suggesting the absence of effective lymphocyte activation during the disease. Contrarily, the high level of IL-2 was detected from the individual residing in our study area and suffering with VL. The detection of high level of IL-2 cytokines in our subject might be due the low exposure of disease.

This cytokine is necessary 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).

(ii) Interleukin-6 (IL-6): IL-6 is a pro-inflammatory cytokine that plays an important role in intraprostatic inflammation and carcinogenesis (Mandic et al., 2013). It is involved in the acute phase response, B cell maturation, and macrophage differentiation. A novel functions of IL-6, the control of T helper (Th1)/ (Th2) differentiation (Diehl and Rincon, 2002).
We observed IL-6 in 9% serum/plasma samples and expression level was 23.85 pg/ml observed. In regards to gender, we observed significantly high expression of IL-6 in males whereas expression level in females was less. In concurrence of our finding the levels of IL-6, was high in patients with self-healing lesions, but was not detectable or very low in DCL lesions (Caceres-Dittmar et al., 1993, Convit et al., 1993, Ritter et al., 1996).

Elevated level of IL-6 during disease in Indian kala azar was reported by Ansari et al., (2006), he has observed low level of IL-6 in PKDL then those of VL patients. Kurkjian et al., (2006) and Nylen et al., (2007) has also been reported the elevated levels of IL-6 in VL which is in accordance to the present study in which we have observed high level of IL-6 in VL and CL patients. Here the limitation of our study that we have not included the PKDL patients in this study so the correlation of our study is not possible.

(iii) Interleukin-17A (IL-17A): 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). In humans, IL-17 secretion by CD4+ T cells cultured under Th17 polarizing conditions (Murugaiyan et al., 2009).

The results showed no significant change in males in comparisons to females for IL-17A cytokines. Interestingly this cytokine expression was found to be lowest among all the cytokines detected. Recent finding has also shown a very low expression of IL-17A in the splenic biopsies of both pre- and post-treatment VL patients (Ansari et al., 2011). However, few studies so far done on IL-17A cytokine expression in leishmaniasis.

IL-17 act as a classic effector of innate immunity and induces expression of many innate inflammatory mediators, including IL-6, acute phase proteins, granulocyte colony stimulating factor (G-CSF), and prostaglandin E2, as such Th17 cells have been implicated in a number of immune-mediated disorders (Tesmer et al., 2008).

(iv) Tumor Necrosis Factor–α (TNF-α): Cytokines play a major role in imparting pathogenicity to leishmaniasis. One of the most prominent cytokines is 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). It 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 A et al., 2002).
The expression level of TNF-α cytokine was observed second highest amongst pro-inflammatory cytokine after IL-2 and it was detected in 9% samples. The frequency of detection of TNF-α was high among the males. Significant difference of expression was not observed in between male and females. In the present study the level of expression of IL-2 in samples collected from CL was observed high. In support of our finding some studies showed the level of TNF-α is high in patients with CL, but are not detectable or very low in DCL lesions (Caceres-Dittmar et al., 1993, Ritter et al., 1996). VL patients, however, have been found to have elevated plasma protein levels of TNF-α from Bihar, India (Ansari et al., 2006; Kurkjian et al., 2006; Nylen et al., 2007). We limited our studied to VL and CL patients therefore we cannot correlate our study with DCL data.

TNF-α plays an important role in progression of infectious diseases because it’s induced the excessive production of nitric oxide. In addition, TNF-α is responsible for the symptoms of the disease, such as fever, anorexia, weight loss, increased energy expenditure and cutaneous and mucosal pallor, and mediates the polyclonal activation of B cells (Engwerda et al. 2004). It is generally increased during the active disease. In addition, this cytokine has been reported to be a reliable marker of VL that has been cured (Barral-Netto et al. 1991). The high expression level of TNF-α in our subjects determine the high virulent nature of strains prevalent in Sutlej river valley of Himachal Pradesh.

It has been demonstrated experimentally that the elevated expression level of TNF-α has been detected in *L. major* infected mice (Tumang et al. 1994, Arnoldi and Moll, 1998, Luster, 2002).

(v) Interferon-γ (IFN-γ): IFN-γ is a type-II Interferon and structurally unrelated to type I Interferon, 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 (Beach et al., 1997).

We have detected IFN-γ in 16% serum/plasma samples. It is observed significantly high frequency among females in comparison to those of males. While the level of expression in males was detected high whereas expression level in females was less. In accordance to our findings the high expression levels of IFN-γ had been reported from Kolkata in VL patients (Ganguly et al., 2008). The earlier studies, reported form several parts of world also documented the elevated level of IFN-γ but they have also observed that the presence of IFN-γ fails to control the progression of parasite in patients (Zwingenberger et al., 1990, Karp et
al., 1993). Our finding and the earlier studies suggested that the IFN-γ help is progression of disease and unable to control the disease.

In accordance to our finding, the higher concentrations of IFN-γ were detected in the patients with the active disease (Hailu et al. 2004, Caldas et al. 2005, Antonelli et al., 2005, Ansari et al. 2006). It has been found the increased levels of IFN-γ mRNA in the spleen and bone marrow during the acute phase of infection (Ghalib et al., 1993, Karp et al., 1993, Kenney et al., 1998, Nylen and Sacks, 2007). The recent studies established the fact that the blood cells maintain the capacity to produce IFN-γ in response to soluble Leishmania antigen (Ansari et al., 2011, Gidwani et al., 2011). In accordance to our study, Costa et al., (2012) has also observed elevated level of IFN-γ in VL patients from Sao Luis MA USA. The high expression level of IFN-γ was observed in native population of study area suffering with VL suggested that the progression of disease among the population as INF-gamma is known to kill intracellular parasite while in the present study INF failed to control the progression of disease.

5.2.2 Expression level of anti-inflammatory cytokines: We have studied the expression level of anti-inflammatory cytokines i.e. IL-4 and IL-10. It is known that IL-4 and IL-10 can down-regulate inflammatory responses in animal models like murine, mice, hamster etc (Hedrick et al., 1998, Zlotink and Moore, 1991).

(i) 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. Interleukin 4(IL-4) gene located on the chromosome 5 (5q31-33) encodes for 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 signalings (Gyan et al., 2004).

We have detected IL-4 in 3% serum/plasma samples. The frequency of detection of IL-4 was high in the females, and low expression level was observed in males. Hence the expression level of IL-4 is very –very low or negligible in individuals residing in study area. Similar observation was observed Ansari et al., (2006) from Bihar and Ganguly et al., (2008) from Kolkata showed very low expression level of IL-4 in VL pateints. The low level or not detectable expression of IL-4 cytokines suggested that the parasite prevalent in this region is highly virulent and causes more pathogenic effect. Undetectable level of IL-4 cytokine may be associate with progression of disease.

In contrast to our study, a high level of IL-4 was found in patients with the VL from Sao Luis MA USA Costa et al., (2012).
(vii) Interleukin-10 (IL-10): IL-10 has pleiotropic, primarily anti-inflammatory properties that include suppression of dendritic cell functions and rendering macrophages unresponsive to activation signals (Moore et al., 2001). It 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). It 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 expression level of IL-10 was observed highest amongst all anti-inflammatory cytokine and it was detected in 21% samples. The frequency of detection of IL-10 was significantly high in the females. Expression level in males was observed significantly high in comparison to those of females. In support of our findings the high expression levels of IL-10 has been reported from Kolkata, the authors observed the expression of IL-10 was significantly high among VL patients, before and after treatment (Ganguly et al., 2008). Another study was conducted in Sao Luis MA USA, showing high level of IL-4 in patients with the VL (Costa et al., 2012).

In accordance to our finding, the high expression of IL-10 was detected in the VL patients (Ansari et al., 2006) and they have also detected the low level of IL-10 in PKDL patients. We limited our studied to VL, CL and MCL patients therefore we cannot correlate our study with PKDL data.

Although it’s up-regulation is considered a homeostatic mechanism to limit the tissue damage caused by excessive inflammation, effective clearance of Leishmania can also be compromised (Melby et al., 1994, Gasim et al., 1998). It has broad suppressive activity, functioning to limit collateral tissue damage during inflammation (Anderson et al., 2007). Recently, Gautam et al (2011) from Muzafarpur, Bihar, India has been demonstrated the anti-parasitic effect of IL-10 in spleen, IL-10 is able to block the parasite in spleen of VL patients. We observed a mixed cytokine profile (Th1 and Th2) in the patients with the leishmaniasis. Similar findings were reported from various parts of the world (Goto and Lindoso 2004, Peruhype-Magalhães et al. 2005, Goto & Prianti 2009).

The study revealed that the high level expression of IL-10 in males amongst the study population is able to protect the parasitic infection and more resistant in comparison to those of females.

Flowcytometric analysis of pro and anti inflammatory cytokines reveals that the parasite prevalent in this region is highly virulent and because more pathogenic effect to the
patients, as only IL-10 anti inflammatory cytokines was expressed during the infection while IL-4 could not be detected in any samples.

1.3 Genotyping of Parasite

In the present study, we have characterized the strain prevalent in this region at genetic level. We have identified the prevalence of both the species viz. *L. donovani* and *L. tropica* in Sutlej river valley of Rampur, Himachal Pradesh.

The skin scraping and blood samples were collected in NNN media from patients and cultured. Out of 100 samples only 40 could be adopted in continuous culture which further maintained in RPMI-1640 medium. The *Leishmania* promastigotes were used for species identification by using molecular methods. The ITS-1 region was amplified by using specific primers. The amplicones obtained have shown size variation on agarose gel. Three different type of DNA pattern was observed. Recently, Al- Nahhas and Kaldas (2013) from Syria, have also reported the similar banding pattern of amplicones on agarose gel by using similar region.

Six amplicones were subjected for sequencing. The sequencing analysis of *Leishmania* species revealed that the *Leishmania* strains prevalent in this region belongs to *L. donovani* and *L. tropica* hence both species *Leishmania* are coexist in this region. Further, the consensus sequences were generated from the forward and reverse sequences obtained by using Codon Code Aligner software and submitted to the NCBI Gen Bank database. The sequences were published by NCBI and available in gene bank with accession numbers “KJ397261 to KJ397266.

The study revealed that both the species of *Leishmania, L. donovanai* and *L. tropica* are prevalent in this area and have co-existence in this region of Himachal Pradesh. Both the species have diverse clinical manifestation and pathogenic effect. Sutlej river valley is not the native endemic region for *L. donovani* because it is a colder region and situated on high altitude in western Himalayas while donovanian species of *Leishmania* usually prevalent in tropical and warm region. The prevalence of *L. donovanai* in this region is the point of debate. The parasite may be migrated with migrant population or labours came from endemic region for major developmental projects in Sutlej river valley like hydroelectric projects or developing new foci by adopting new climatic condition, which an alarming feature and need attention to develop control strategies to eradicate VL from new endemic region of VL.