DISCUSSION

Visceral leishmaniasis is a neglected but typically fatal vector-borne protozoan disease reported from all continents except Antarctica and Australia (Van Griensven and Diro, 2012). Ninety percent of all cases occur in 5 countries: India, Bangladesh, Nepal, Sudan, and Brazil. With an estimated 300,000 cases per year, India carries the largest VL burden (Sundar and Chakravarty, 2013). In active VL, macrophages host the replicating amastigotes in phagolysosomal compartments leading to splenomegaly, hepatomegaly, hyperglobulinemia, anemia, weight-loss, incessant fever and ultimately death if not treated (Banjara, 2013).

Following the observation that recovery from infection confers immunity to reinfection in leishmaniasis (Launois et al., 2008), the development of effective vaccines represents one of the most promising approaches for providing cost effective interventions against this disease. While substantial efforts have been made to develop vaccine-induced specific antiparasitic immune responses, no acceptable antileishmanial vaccine exists against this infection (Shakya et al., 2011). The first vaccine to reach the human phase I clinical trials is Leish-F3 for visceral leishmaniasis (Rattue, 2012), and Leishmune is the only licensed vaccine against the canine disease. Therefore, in the absence of an effective vaccine, chemotherapy remains the only option for the control of disease. Although treatment for leishmaniasis was introduced in the early 20th century, parenteral administration of pentavalent antimony compounds (meglumine antimoniate and sodium stibogluconate) remains the first-choice treatment for all forms of leishmaniasis (Godinho et al., 2012). In the case of antimonial resistance, the second-choice treatment includes amphotericin B (deoxycholate or liposomal formulation) (Godinho et al., 2012). However, each of these therapies have important limitations, such as long term parenteral administration, toxic side effects, high cost in endemic countries and an increase in number of resistant cases (Croft et al., 2006). A major breakthrough in chemotherapy of VL was the discovery of miltefosine, an analogue of phosphatidylecholine initially developed as an anticancer agent (Sachdeva et al., 2013). It is not recommended during pregnancy as teratogenicity has been observed in
one species during preclinical development (Obonaga et al., 2014). Moreover, its cost is another limiting factor (Sundar and Chakravarty, 2013). Till date, no ideal drugs are available that fulfil the major requirements for efficient antileishmanial therapy, including high efficacy, low toxicity, easy administration, cost and preventing the occurrence of drug-resistant parasites (Van Griensven and Diro, 2012).

Chemotherapy of leishmaniasis is often compromised due to suppression of immune function during the course of infection. The immunology of infection reveals that during active disease the cell mediated immune responses are suppressed. The fatal visceral disease in humans is the result of intramacrophage infection and the outcome of infection is largely dependent on the ability of the host to mount a Th1 or Th2 type of immune response (Kaur et al., 2011). The factors such as macrophages and effector molecules, dendritic cells, T-helper cells (CD4+ cells), cytotoxic T cells (CD8+ T cells) and cytokines, all play an important role in generating immune response to Leishmania infection (Liese et al., 2008). The primary mechanism for the elimination of parasites is the activation of macrophages by IFN-γ secreted by NK and Th1 cells. Activated macrophages produce different cytokines such as TNF-α, IL-6, IL-18, IL-12, and IFN-γ. IL-12, secreted mainly by dendritic cells (DC), has the essential role of inducing a Th1 type of immune response. The Th1 effector cytokine IFN-γ leads to activation of infected macrophages and parasite killing (Schwarz et al., 2013). Resistance to infection is linked to a Th1 response, with production of IL-12 and IFN-γ, and inhibition of Th2 cytokine production, whereas susceptibility to disease is related to a predominant Th2 response, determined by the presence of IL-4 and abrogation of IL-12 expression (Dey et al., 2007; Kedzierski et al., 2009). Therefore, efficiency of any chemotherapy of leishmaniasis is dependent on the generation of effective cell-mediated immune response suitable for disease resolution (Shakya et al., 2011).

As Leishmania evades the immune response by selectively attenuating proinflammatory signaling pathways, the immunomodulatory potential of an antileishmanial compound is documented by its influence on enhancing macrophage derived proinflammatory cytokines (IFN-γ, IL-12 and TNF-α) or decreasing levels of IL-10. Indeed, this is corroborated by the majority of the conventional antileishmanial drugs and compounds tested in experimental models of leishmaniasis.
(Dalton and Kaye, 2010; Saha et al., 2011; Kulshrestha et al., 2011). So an auxiliary therapeutic measure that might enhance the efficacy of these antileishmanials or reduce the resulting toxicity would be valuable. Synergy between chemotherapy and host immune function was first suggested by observations that immunocompromised patients with VL failed to respond to antimonial drugs (Dalton and Kaye, 2010). The recognition that many anti-leishmanial drugs operate in synergy with host immune mechanisms has fuelled interest in developing combined immunochemotherapy. Combination of one or more of immunotherapeutic agents like BCG, Alum, IFNγ, antigen-pulsed dendritic cells (DC), etc. with chemotherapeutic drugs has been tested raising hopes for a suitable immunochemotherapy against VL and Post Kala-azar Dermal Leishmaniasis (PKDL). Antagonists of IL-10, TGF-β and IL-13 have been effectively used with pentavalent antimonials in treatment of experimental VL. Taken together, screening for compounds having the propensity to modulate the host defense signaling pathways alone or in combination with existing anti leishmanial drugs (El-On, 2009) may well prove to be an effective immunochemotherapeutic strategy in leishmaniasis worthy of pharmacological consideration (Saha et al., 2011).

Thus, in an attempt to find a new alternative therapy, we evaluated the protective efficacy of immunochemotherapy with two different drugs (SSG or cisplatin) combined with immunotherapy (KLD or 78kDa antigen or KLD+MPL-A or 78kDa+MPL-A), and compared them with chemotherapy or immunotherapy alone in a mouse model against experimental visceral leishmaniasis. The protective efficacy was assessed by the percentage reduction in the parasite load in liver. Similarly, immunogenicity was assessed through the detection of delayed type hypersensitivity response, production of antileishmanial antibodies (IgG1 and IgG2a) and cytokines such as IFN-γ, IL-2, IL-4 and IL-10. To assess the toxic effects of infection and the drug, various hematological, biochemical and histopathological studies were also carried out.

To test novel therapeutic and immunoprophylactic agents, murine models of leishmaniasis have been extensively used (Gupta and Nishi, 2011). In the present study, inbred BALB/c mice were infected intracardially with 1x10^7 promastigotes of *L. donovani* (Kaur et al., 2008). The mice were kept for 30 days for the progressive infection and then the animals were divided into two different categories where one
category of animals were given all the different therapies (chemotherapy, immunochemotherapy and immunotherapy) for once (single dose) and then the other category of animals were treated with two doses of the above therapies. The parasite load was assessed in all the groups of mice on different post infection and post treatment days in liver as leishman donovan units (Bradley and Kirkley, 1977). Maximum protective efficacy was observed in animals treated with the two doses of immunochemotherapy among all the other treatments. About 99-49% protection was observed in SSG+78kDa+MPL-A treated animals followed by SSG+KLD+MPL-A treated animals which suggested more effectiveness of the drug with a specific antigen along with an adjuvant. These results are consistent with our earlier studies where experimental infection of mice immunized with second generation antigen (78 kDa) along with an adjuvant (MPL-A) induced 92% protection against \textit{L. donovani} infection (Nagill and Kaur, 2010). Similarly, in an earlier study, the therapeutic efficacy of Leishmune, the only licensed vaccine against canine leishmaniasis, was assessed for immunochemotherapy in combination with allopurinol or amphotericin B, in dogs. It was observed that by the end of 8 months, no parasite antigens were observed in lymph nodes of 80% of immunochemotherapy treated dogs. This suggests that the combination therapy not only abolished the symptoms but also the latent infection, curing the dogs (Borja-Cabrera \textit{et al.}, 2010). Similarly, treatment of CL patients in a Phase III clinical trial with imiquimod and antimony showed a higher cure rate (75%) compared to that seen in patients treated with placebo and antimony (58%) (Miranda-Verastegui \textit{et al.}, 2009). Moreover, Murray \textit{et al.} (2003a) tested an immunochemotherapy protocol in \textit{L. donovani} infected mice by the association of amphotericin B with IL-12, anti CD40 and anti IL-10R observed that, despite this drug's direct action against parasites and its independence from host immunity, the combination was more efficient than the monotherapy. In the present study, when the two different drugs i.e. SSG and cisplatin were compared, it was observed that, although the SSG treated combinations (SSG+78kDa+MPL-A or SSG+KLD+MPL-A) at a dose of 40 mg/kg body wt. imparted maximum protection comparable protection was also achieved with cisplatin (cis+78kDa+MPL-A or cis+KLD+MPL-A) at a low dose of 0.5 mg/kg body wt. given for five days. This shows that cisplatin at a low dose can be used as an alternative drug. Earlier studies have shown 90% inhibition of amastigotes in golden hamsters infected with \textit{L. donovani} when treated
with a combination of low doses of both stibanate (5mg/kg body wt continuously for five days) with Poly ICLC plus arginine (continuously for 10 days) (Bhakuni et al., 1996). This is in consistence to an earlier study which showed that both low-dose cisplatin (0.6 mg/ kg) and xenogeneic endoglin (10µg/mouse) resulted in significant tumour growth inhibition. During this treatment, promotion of tumour cell apoptosis and inhibition of tumour cell proliferation without any increase in host toxicity was also observed (Tan et al., 2004). In an earlier study also it was observed that the association between antimony and vaccine (immunochemotherapy) showed the same cure rate when compared with the standard treatment (100%) and but was able to reduce the salt volume by 17.9% and treatment length from 87 to 62 days, thus decreasing the side effects caused by the use of drug alone. Moreover, combination therapy using cisplatin and human leucocyte antigen-A24-restricted human vascular endothelial growth factor receptor 1 (VEGFR1)-1084 and VEGFR2-169 in patients with advanced or recurrent adenocarcinoma of the stomach showed that the disease control rate (partial and stable disease) was 100% after two cycles of the combination therapy (Masuzawa et al., 2012). Moreover, longer progression-free survival with no treatment related deaths was observed in phase I/II clinical trials of advanced gastric cancer patients when treated with S-1 plus cisplatin (Koizumi et al., 2008). Overall, animal treated with SSG+78kDa+MPL-A imparted maximum protection followed by SSG+KLD+MPL-A treated animals (98.50% protection was achieved) and then cis+78kDa+MPL-A treated animals (92.30% protection was achieved). In our study, combination of a drug with 78kDa+MPL-A resulted in maximum elimination of parasites. This might be due to the presence of an adjuvant MPL-A. Several studies have demonstrated that an adjuvant either directly or indirectly stimulates the production of T helper cell type 1 (Th1) cytokines IL-2 and IFN-γ (Gustafson and Rhodes, 1994). In addition, MPL-A activates monocytes and macrophages (Ribi et al., 1984). It is likely that these monokines lead to the recruitment and maturation of dendritic cells in the lymph nodes (Jonuleit et al., 1996) where the dendritic cells can efficiently present antigen to T lymphocytes. Therefore, the combination has been found to be most effective in eliminating the parasites. However, it have been reported earlier that complete clearance of parasites did not occur even from liver when BALB/c mice infected with high dose of L. donovani parasites were treated with anti-IL-10 Ab in combination with pentavalent antimonials or AmB (Murray et
al., 2003; Banerjee et al., 2008). It has been reported that in genetically susceptible (Nrampls) mice (including BALB/c and C57BL/6), parasite numbers rapidly expand in hepatic mononuclear phagocytes, followed over the subsequent 2–4 weeks post-infection by a T cell-dependent decline in tissue parasite numbers (Bradley and Kirkley, 1977) whereas parasite numbers increase more slowly in the spleen, and total splenic parasite burdens usually only reach 5–10% of maximum levels in the liver, often with greater variation between individual mice than in the livers of the same animals (Stanley et al., 2008). Moreover, as BALB/c mice develop self limiting infection, no relapse occurs in them. Hence, it was assumed that neither chemotherapy nor satisfactorily expressed T cell-dependent host immune responses could eradicate all tissue parasites in any form of leishmaniasis (Banerjee et al., 2008).

It has been well established that the success of any chemotherapy is often dependent on the type of immune response generated by the infected host, and in leishmaniasis, a drug is considered successful if it results in generation of antigen-specific T cells and delayed type hypersensitivity responses. Delayed-type hypersensitivity (DTH) is the cell mediated immune response for clearance of pathogen from the infected host that potentiates the infiltration of lymphocytes and macrophages into the infected tissue (Khabiri et al., 2007) and has been frequently used as a correlate for protection against or sensitization to Leishmania antigen (Mahmoudzadeh-Niknam et al., 2007). The DTH responses to leishmanin were apparent during L. donovani infection in BALB/c mice as evident by an increase in the foot pad swelling after injection of leishmanin. Our results revealed that DTH responses to leishmanin after treatment were much higher in mice treated with a combination of drug and an immunomodulator than the groups of animals treated with chemotherapy or immunotherapy alone. Moreover, the increase was more pronounced in animals treated with the two doses than the animals treated with a single dose. This suggests that the mice treated with drug (SSG or cisplatin) and immunotherapy (KLD or 78kDa) with or without adjuvant (MPL-A) developed a strong cell mediated immune response indicating that drug treatment followed by vaccine therapy was helpful in reversal of immunosuppression caused by the parasite. Treatment of animals with SSG and immunotherapy elicited strong DTH responses and SSG+78kDA+MPL-A treated animals induced maximum DTH response followed by
animals treated with SSG+KLD+MPL-A. Lower but significant levels of DTH responses were also seen in animals treated with SSG alone or along with 78kDa or KLD suggesting a correlation between cell mediated immune responses and immunity to infection. When the two different drugs, SSG and cisplatin were compared, the increase in footpad thickness in cisplatin treated animals was found to be comparable to SSG treated mice. The efficacy of treatment with cis+78kDa+MPL-A or cis+KLD+MPL-A was superior to that of SSG alone or cis+78kDa or cis+KLD treated animals in generating DTH responses. The DTH responses also show an inverse correlation with parasite burden. The higher the DTH response, the lesser is the parasite load and greater is the efficacy of the drug (Sharma et al., 2012). Our results also demonstrate a positive correlation between enhanced DTH responses and reduced parasite load for all the therapeutic treatments (Sachdeva et al., 2013). Our results were also in agreement with the study where patients with mild or healing forms of cutaneous leishmaniasis exhibited positive DTH responses and successful cure of individuals with progressive forms of leishmaniasis (Nabors and Farrell, 1996) is usually accompanied by expression of positive DTH responses, as well as other positive correlates of a protective cell mediated immunological response. Moreover, in another study, untreated patients with VL were unable to control their visceral infection with L. donovani and it was attributed to a defective cell mediated immune response to leishmanial antigens. These patients were unable to respond to DTH skin tests (de Andrade et al., 1982). Progressive infection is associated with poor delayed-type hypersensitivity (DTH) responses and high antibody production, whereas containment is associated with a strong cell-mediated DTH response (Hailu et al., 2001; Banerjee et al., 2011). In the present study we used first generation (KLD alone or along with MPL-A) and second generation vaccines (78kDa alone or along with MPL-A) to check their immunotherapeutic efficacy alone or with the drugs. However, it has already been established that successful vaccination of humans and animals is often related to antigen induced DTH responses in vivo and T- cell stimulation with antigen in vivo (Kushawaha et al., 2012). A study by Nagill et al. (2009), revealed that immunization of BALB/c mice induced significantly higher levels of delayed type hypersensitivity (DTH) responses in mice immunized with heat-killed antigen followed by autoclaved antigen. In another study immunization of alum precipitated autoclaved L. major along with BCG (Kamil et al., 2003) and the photodynamic
vaccination of hamsters with inducible suicidal mutants of *L. amazonensis* (Kumari *et al.*, 2009) induced significant DTH responses. Similarly immunization of mice with 78kDa antigen along with autoclaved *L. donovani* when used as an adjuvant in BALB/c infected with VL had shown a significant increase in DTH responses in immunized mice as compared to the controls (Nagill and Kaur, 2010). These studies are in consistence to our present work where immunotherapy also revealed significant levels of DTH responses and these levels were more pronounced in animals treated with 78kDa+MPL-A. Therefore, the findings of the present study suggest that purified antigens are highly immunogenic and induce significant protective cellular immune responses.

Recovery and protection against leishmaniasis result from a strong and specific cellular immune response, followed by the development of long-lasting protection (El-On, 2009). Control of *Leishmania* infection relies on cell-mediated immune response, as IFN-γ induced activation of macrophages is known to be the main mechanism of parasite destruction within these cells. It has been observed that the effector memory (CD45RA-CCR7+) CD4+ T cells are the main population of cells producing IFN-gamma in cured CL and ML individuals who responded *in vitro* to SLA (soluble *L. braziliensis* antigen) (Carvalho *et al.*, 2013). The major biological function of IFN-γ is to activate macrophages and enhance the microbicidal activity of these cells to kill intracellular pathogens. Treatment of animals with different therapies induced preferential production of IFN-γ and a massive increase was seen in the splenocytes of immunochemotherapy treated animals. In addition, animals treated with the two doses produced higher levels of Th1 cytokines than the animals treated with a single dose. In our study highest level of this cytokine was observed in SSG+78kDa+MPL-A treated animals followed by SSG+KLD+MPL-A. It is well known that cell mediated immunity is suppressed in neoplastic diseases and same happens with leishmaniasis. Therefore drugs which can modulate the immune response are applicable. *cis*-diaminedichloroplatinum (cisplatin) has been reported to function as an immunomodulator, especially when used in low dose in combination with 5-Fluourouracil (5-FU). When cisplatin and UFT, which is a prodrug of 5-FU, were administered with an immunomodulator polysaccharide K (PSK) to ten patients with colorectal cancer, an increased concentration of IFN-γ was observed with
reduced production of IL-10 after 2 months of treatment demonstrating the immunomodulatory potential of this combination (Shibata et al., 2002). Cisplatin is known to boost the cytotoxic T-lymphocyte mediated antitumor immunity which plays a key role in protection against Leishmania species. Therefore, cisplatin may enhance the CD8+ T-cell mediated killing of the parasite. Hae-Ran et al. (2009) also showed that cisplatin treatment increased the level of IFN-γ and IL-2. Our results are in contrast to a study where chemotherapy treated group produced more IFN-γ at the end of treatment, while a significant decrease in this cytokine production was associated with healing in the immunochemotherapy treated patients (Toledo et al., 2001). Lower but significant levels were also produced by splenocytes of cis+78kDa+MPL-A and cis+KLD+MPL-A treated animals. Interestingly, considerable levels of IFN-γ were also secreted by immunotherapy treated animals on different days post treatment. Substantial levels of IL-2, a principal T cell growth factor for Th1 type of immune response, was also produced by all the treated animals. However, the profile of IL-2 was same as that of IFN-γ in the treated animals. A preliminary study suggested that low-dose CDDP and IL-2 in association with the pineal hormone MLT, given as a second line therapy, is an effective and well-tolerated treatment for patients with metastatic melanoma, with a clinical efficacy at least comparable to that obtained with a first-line therapy of dacarbazine plus interferon-alpha (Lissoni et al., 2002). In contrast, IL-4 and IL-10 response was high in the immunotherapy treated animals but declined significantly in chemotherapy and then immunochemotherapy treated animals. The minimal levels of IL-4 and IL-10 were seen in the cultures of splenocytes obtained from mice treated with the two doses of immunochemotherapy (SSG+78kDa+MPL-A and SSG+KLD+MPL-A). This is in consistence to a study carried out by Toledo et al. (2001) where the continuous administration of Leishmania antigen in immunochemotherapy (Glucantime plus Leishvacin) treated ACL patients may have contributed to a greater decrease in IL-10 production which lead to a faster elimination of the parasites by IFN-γ activated macrophages. The results demonstrate that the cytokine profile in all the mice was driven towards Th1 type of response which contributes to the resistance against Leishmania infection.
In the earlier studies, it has been well established that profound impairment of the immune system of the infected host in VL is a major cause for partial success of the antileishmanial chemotherapy, and success of cure depends on the combined effect of drug and immune status of the host (Banerjee et al., 2008). Recovery from leishmaniasis is strongly co-related with the development of distinct T helper type 1 (Th1) cell mediated immune responses manifested by the production of cytokines such as IFN-γ and IL-2 by T cells that activate macrophages and kill intracellular parasites (Dey et al., 2013). In contrast, Th2 cell-mediated immune response limits the action of Th1 functions via IL-10 which deactivate macrophages helping intracellular parasite growth and disease progression (Awasthi et al., 2004). IL-10 has been suggested to play a role in counterbalancing the exacerbated polarized response that may develop following cure (Tripathi et al., 2008). It has been well established that the leishmanicidal effect of pentavalent antimony (Sb), requires T cells and endogenous IFN-gamma (Murray et al., 2000). Our results are in correspondence with the previous studies which showed that biological immunomodulators, such as interferon gamma (IFN-γ), enhance the activity of antimonials in the treatment of VL by inducing macrophages to accumulate pentavalent antimony, the conventional therapeutic agent for leishmaniasis, and augments the efficacy of antimony both in vitro and in vivo (Murray et al., 1988; 1989; Sundar et al., 1997). In addition, if given during the early stages of infection, combined treatment, with recombinant IL-12 and antimonials, inhibits the appearance and progression of cutaneous lesions in BALB/c mice by promoting the development of a protective Th1 immune response (Nabors et al., 1995). Also, it was found that sodium stibogluconate (SSG) and IFN-alpha synergized to overcome IFN-alpha resistance in various human cancer cell lines in culture and eradicated IFN-alpha-refractory WM9 human melanoma tumors in nude mice with no obvious toxicity (Yi et al., 2002). Furthermore, in a study it was observed that a chimeric fusion protein (OX40L-Fc) which stimulates T cells through OX40, as well as an agonist monoclonal antibody (mAb) which blocks CTLA-4, an inhibitory receptor on T cells (CD40), promoted host protective immunity when given in combination with antimonials and supported low-dose Sbv therapy in mice against VL (Zubairi et al., 2004). Similarly, treatment with mAb against the IL-10 receptor allowed a 35-fold reduction in the effective dose of Sbv compared with drug alone, as well as considerably shortening the time for effective therapy (Murray et al., 2002;
This suggests that the combination therapy (immunochemotherapy) enhances the establishment of CD4+ Th1 type of immune response. Moreover, comparative analysis of mononuclear cells from peripheral blood, prior to and 30 days after the treatment with SbV, Leishvacin or SbV and Leishvacin, revealed that the different therapies did not alter significantly the cellular profiles of the patients suffering from ACL suggesting that that the development of a combination treatment regimen should take into account the shortest period over which each individual component needs to be applied in order to minimise the amount of drug used and, consequently, to reduce the possibility of side effects and adverse events (Botelho et al., 2009). In another study, after several unsuccessful chemotherapy treatment regimens and many relapses of patients suffering from diffused cutaneous leishmaniasis (DCL), 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. In the study it was observed that the frequencies of CD16+CD56+ NK cells and CD14+CD16+ proinflammatory monocytes increased in peripheral blood, and CD56+ lymphocytes were found infiltrating the lesions, suggesting that immunochemotherapy reduced the parasite load and activated NK cells and monocytes which can lyse infected macrophages and parasites, and in addition, release IFNγ and TNF to activate macrophages for parasite elimination (Pereira et al., 2009).

The immune system is a truly amazing constellation of responses to attack from outside the body. Cellular immunity is thought to be essential for controlling *Leishmania*; however *Leishmania*-specific humoral immune responses are also present during the infection (Miles et al., 2005; Mukbel et al., 2006). The CD4+ Th1 and Th2 subsets participate in B-cell differentiation and immunoglobulin isotype switching. In mice, the level of IgG1 antibody correlates well with an overall Th2 type of immune response whereas IgG2a antibody is indicative of an overall Th1 profile (Ebrahimpoor et al., 2013). In active visceral leishmaniasis, high IgG levels are predictive of disease. Patients with ongoing disease had high IgG antibody titers and no delayed-type hypersensitivity (DTH) responses to *Leishmania* antigens. This pattern was reversed upon disease resolution after treatment, resulting in a decrease in
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total IgG, which was accompanied by a progressive increase in DTH responsiveness (Miles et al., 2005). The possible role of antibody-mediated protection against *Leishmania* signifies the importance of identification of antigens that may elicit protective antibodies (Ozbilge et al., 2006). Analysis of anti-*Leishmania* IgG isotypes in the present study disclosed a dichotomous response to visceral infection. As Indian kala-azar elicits a mixed Th1-Th2 response, an increase in IFN-γ (Th1) would account for the increased IgG2 levels. The enhanced IL-4 (Th2) induces an increase in IgG4 levels, whereas the raised levels of IL-10 (Th2) are reflected in increased IgG1 and IgG3 levels. Similarly with parasite disappearance, the levels of IFN-γ, IL-4 and IL-10 decrease, accounting for the decreased IgG1, IgG2, IgG3 and IgG4 levels (Chatterjee et al., 1998). Although humoral response is also present during the *Leishmania* infection, antibodies play no role in protection and are associated with the non-healing disease (Kedzierski and Evans, 2013). Our results revealed higher IgG2a and lower IgG1 levels in the treated animals when compared with the infected controls. However, maximum levels of IgG2a and minimum levels of IgG1 were observed in animals treated with two doses of immunochemotherapy. Highest levels of IgG2a were observed in serum samples of mice treated with SSG+78kDa+MPL-A followed by SSG+KLD+MPL-A treated animals. In addition, a marked elevation was also observed in cisplatin treated animals (cisplatin+78kDa+MPL-A and cis+KLD+MPL-A treated animals). Our results are in consistence with our earlier study where immunization of mice with 78 kDa+MPL-A resulted in significant increase in IgG2a response (Nagill and Kaur, 2010). Moreover, a significant reduction in specific antibody titres was observed after treatment with immunochemotherapy (Glucantime+Leish-110f/MPL-SE) in dogs suffering from canine leishmaniasis (Miret et al., 2008). Our results are in consistence with a study in dogs that determined higher IgG2a levels in PCR positive dogs than those that were negative (Quinnell et al., 2003). In the present study, as number of post treatment days increased, the antibody levels also started decreasing which further showed success of all therapies in all the groups of animals. Similarly, Todoli et al. (2010) also found that the levels of specific antibodies decrease after cure, this phenomenon has been used to evaluate the success of therapy.
To assess the drug induced side effects, various haematological biochemical and histological studies were carried out. In our study, anemia was observed in infected animals and animals treated with immunotherapy alone (KLD or 78kDa alone or along with adjuvant MPL-A). The hematological and serum biochemical parameters are very useful in evaluating the clinical status of the animal and the extent of lesions and might give indications of the disease prognosis (Freitas et al., 2012). Anemia was the most frequently observed hematological abnormality in VL infected animals. Hematological findings indicate that the development of anemia was because of sequestration and destruction of red blood cells (RBC) in enlarged spleen, immune mechanism and alterations in RBC membrane permeability. Leucopenia is another early and striking manifestation of VL. The main cause of the development of leucopenia has been attributed to hypersplenism (Bajaj et al., 2013). In our study also, anemia and leucopenia was observed in infected mice and the animals treated with immunotherapy alone (KLD or 78kDa alone or along with adjuvant MPL-A). This is in consistence with a study by Chakrabarti et al. (2013) who reported leucopenia in 61.1% cases of visceral leishmaniasis (VL) patients over a 2-year study period among immunocompetent patients in West Bengal, India. In a retrospective study of 50 children with VL, fever, nonproductive cough and splenomegaly were found in all patients whereas anemia, leukopenia, neutropenia, thrombocytopenia and pancytopenia were found in 100, 80, 60, 60, and 60% of cases, respectively (Mehmet et al., 2003). However, when the animals were treated with SSG alone or in combination with immunotherapy, an increase in TLC count in the treated animals, when compared to the infected controls was observed which is in accordance with the study of Das et al. (2005) where at the end of SSG treatment, variables like Hb, DLC, TLC were found to be significantly improved in patients who responded to treatment which justified our study. Also, al Khawajah et al. (1992) studied subacute toxicity of pentavalent antimony (Sb) compounds, sodium stibogluconate (SSG) and meglumine antimoniate (MA) in rats and observed that both drugs caused a dose-related reduction in hemoglobin concentration but significantly raised the WBC (white blood cell) count. Similarly, the hematological parameters were found to be in the normal range when the animals were treated with 40mg/kg body wt. of SSG continuously for five days (Sharma et al., 2012). Also in a study conducted by Sinha et al. (2006) treatment with SAG alone as well as with SAG, folic acid and Vit B12, significant improvement
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in the Hb and TLC was reported in all 50 cases of visceral leishmaniasis, which is in consistency with our study where anemia was observed before treatment whereas after treatment, Hb was found to be in normal range (9-11.2g/l) on all post treatment days. Moreover the results are in consistence with a study where patients suffering from VL produce comparable levels of WBC count, TLC, platelet count and Hb levels when treated with Pentostam or Pentostam plus IFN-γ (Squires et al., 1993).

Cisplatin is known to cause nephrotoxicity at higher doses. Therefore, in the present study, we selected a low dose of cisplatin (i.e. 0.5 mg/kg body wt) and combined it with 78kDa or KLD alone or along with an adjuvant MPL-A, and studied its antileishmanial effect in vivo in BALB/c mice. In addition we studied the hematological and biochemical changes brought about by the drug. Normal levels of Hb were observed in all groups of animals treated with cisplatin alone or in combination (immunochemotherapy). This is in accordance with an earlier study by Khynriam and Prasad, (2001) where cisplatin+cysteine treatment of tumor bearing mice caused an increase in hemoglobin levels. Moreover Ampollini et al. (2009) reported that the blood count, renal and hepatic parameters in the blood samples taken at different time points in the rat model of malignant pleural mesothelioma (MPM) were similar between treatment and control group when treated with cisplatin- fibrin + CpG. Also in our earlier studies, normal levels of Hb were obtained after cisplatin treatment in L. donovani infected BALB/c mice (Kaur et al., 2010).

To assess the damage caused to liver, various activities of enzymes like SGOT, SGPT, ALP, acid phosphatase and concentration of bilirubin were measured. Among the most sensitive and widely used liver enzymes are the aminotransferases such as aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT). These enzymes are normally contained within liver cells and spillage of these enzymes by liver cells into the blood raised the SGOT, SGPT levels. In the present study, it was observed that the levels of ALP and acid phosphatase were within the normal range in all the animals treated with either single dose or two doses. This is in accordance with the study carried out by Sharma et al. (2012) where cisplatin along with antioxidants and SSG treatment did not alter the ALP and acid phosphatase activity in infected L. donovani BALB/c mice.
The activity of SGPT was found to be relatively higher in liver than in other tissues as compared to the activity of SGOT. This might perhaps suggest that SGPT might possibly be a more specific index of liver cell damage than the SGOT, because of its relative concentration in the hepatic tissue (Henry, 1959). When the animals were treated with SSG alone or in combination with a vaccine, a transient increase was observed in SGOT and SGPT levels which increased further when the animals were treated with the two doses. However, maximum increase was observed in infected animals and immunotherapy treated ones. In contrast, when the animals were treated with cisplatin alone or in combination with immunomodulators, the increase in SGOT activity was more pronounced in animals treated with chemotherapy (cisplatin) and immunochemotherapy (cisplatin along with immunotherapy) than the infected ones. Our study was in consistence with an earlier study from our laboratory where cisplatin at low dose resulted in higher SGOT levels (Kaur et al., 2010). The enzyme activity in immunochemotherapy treated animals was comparable to those of chemotherapy treated animals. One reason for the increase in SGOT levels in immunotherapy treated animals may be the killing of parasite by the drug that might lead to an increase in the concentration of hepatic enzymes (Sundar et al., 2001). The other reason for the increase may be that the parasite causes structural and functional derangements in the liver. It has already been observed that all the medications used to treat visceral leishmaniasis may be associated with significant increase in levels of liver enzymes during treatment which may be due to the killing of parasite in liver, rather than to direct medication induced hepatotoxic effects (Waikar et al., 2006) and thus hepatocyte damage is considered a non desirable side effect. Moreover, an increase in SGOT, SGPT, alkaline phosphatases and bilirubin in VL patients has already been reported (Mathur et al., 2008).

Kidney function tests include estimation of urea, BUN and creatinine. Measurement of the blood levels of other elements regulated in part by the kidneys can also be useful in evaluating kidney function. These include sodium, potassium, calcium, magnesium and phosphorus (Waikar et al., 2006). On studying the renal parameters a transient increase in urea and BUN and creatinine concentration was found in animals treated with either chemotherapy or immunochemotherapy. The increase was more pronounced in animals treated with the two doses of cisplatin as
compared to SSG treated animals. However, the normal levels were restored within 15 to 30 post treatment days. The animals did not show any nephrotoxicity and the kidney parameters were within the normal range in all the mice treated with immunotherapy and in infected controls. The creatinine levels in mice treated with SSG (chemotherapy) alone or along with immunotherapy (immunochemotherapy) were within normal range on all the post treatment days. The results are in consistence to our earlier study where a transient increase in biochemical parameters was observed at a dose of 0.5mg/kg body wt., but when histopathological studies were done, no morphological changes were observed at a lower dose (Kaur et al., 2010). So, the increase in the concentration of urea and creatinine in the cells in cisplatin treated animals might be due to the enhancement of cytotoxicity and may be partly due to increased drug uptake in the cells, increased DNA cross-link formation, alterations in drug metabolism and/or inhibition of DNA repair (Ohno et al., 1992).

Our study is also in consistence with the study of Kashani et al. (2007) where mean serum levels of BUN, creatinine, sodium, bilirubin and alkaline phosphatase increased significantly after treatment, although most of them were within the normal range with no notable differences in serum levels of potassium, amylase, lipase before and after treatment. Also, Lawn et al. (2006), reported both cardiac and biochemical adverse effects of pentavalent antimonial treatment in CL patients, but described the treatment as well tolerated overall. Our study is also in consistence with the studies of Miret et al. (2008) who reported that the indicators of renal function such as creatinine, urea retaining, as well as liver function evaluated by enzyme alanine amino transferase were not different among all the groups at all times of evaluation. The increase in renal parameters after cisplatin treatment points towards the nephrotoxic effect of the drug. However, in contradiction, Durak et al. (2002) reported a fall in kidney enzymatic activities and increase in blood urea nitrogen and creatinine after 7 days post treatment.

The histopathological changes in the kidneys, liver and spleen were evaluated in different groups of animals. Histology of normal mice revealed the presence of glomeruli, proximal convoluted tubules, (PCT) Distil convoluted tubules (DCT) in the cortex and renal pyramids in the medulla. Morphology of the infected animals showed many foci of lymphocytic infiltration in the interstitium depicting focal interstitial
nephritis similar to the studies of Costa et al. (2003) where glomerulonephritis was observed in naturally infected canine leishmaniasis. Immune complex deposition, T cells and adhesion molecules activation have been shown to be important mechanisms of injury in the glomerulonephritis occurring in visceral leishmaniasis (Clementi et al., 2011). Proliferative glomerulonephritis has also been observed in VL patients (Oliveira et al., 2010). In the present study, we took two different drugs SSG and cisplatin and combined it with immunotherapy. SSG treated animals either alone or in combination with vaccine (KLD or 78kDa) showed normal appearance in kidney architecture except at some places where tubular necrosis was observed. Our study is in consistence to a study by Rodrigues et al. (1999) where functional and histopathological alterations of the acute tubular necrosis were found after receiving a total of 53 ampoules of Glucantime of cutaneous leishmaniasis patients. Although animals treated with highest dose (24mg/kg body wt. i.p. for 45 days) had increased kidney to body weight ratio, histological and biochemical (serum urea nitrogen, creatinine, urinary N-acetylglucosaminidase activity) results did not indicate a kidney effect (Poon et al., 1998). Moreover, no significant histopathological alterations were observed in the kidneys of the rats treated with the 30 mg/100mg of wt./day of Sbv (Glucantime) for 30 days whereas when the rats were treated with a high dose of Pentostam (200 mg/100 grams of weight/day) acute tubular necrosis was observed (Veiga et al., 1990). Since nephrotoxicity is a major limitation of cisplatin, we selected a low dose of cisplatin and combined it with immunotherapy for our immunochemotherapeutic purpose. In our study, we found that the kidneys of animals treated with two doses of cisplatin exhibited contracted glomerulus, decreased lumen and damaged brush border while no major significant changes were observed when the animals were treated with a single dose. However, normal appearance of the kidneys was observed on 15 and 30 d.p.t. The kidney glomeruli, PCT, DCT and Bowman’s capsule appeared to be normal. The nucleus attained a normal structure showing no sign of pyknosis. The lumen of tubules was very clearly visible and nuclei were clearly distinct. Proximal and distal convoluted tubules were distinguishable, PCT having small lumen as compared to DCT. The results are in consistence to a previous study from our laboratory where animals treated with 0.5 mg/kg body wt. of cisplatin revealed some tubules lined with flattened cells having diminished cell size and reduced cytoplasmic volume (Kaur et al., 2010). Histological changes in the rat
kidneys after cisplatin treatment at the dose of 0.4mg/kg body wt. i.p. for 8 weeks showed acute tubular necrosis, severe atrophy of glomerulus, marked dilation of proximal convoluted tubules with slogging of almost entire epithelium with cellular debris in the tubular lumen (Ravindra et al., 2010). It has been suggested that cisplatin nephrotoxicity is associated with oxidative stress, DNA damage and apoptosis (Chaney et al., 2004). ROS has been considered to play a central role in injury caused to kidneys (Matsushima et al., 1998).

The liver is considered as a monitor organ, being able to represent in its ultrastructure the biological responses to environmental alterations (Braunbeck et al., 1987; de Brito-Gitirana, 1988; Miguel and de Brito-Gitirana, 1998; Storch, 1985, 1993). Histological studies were carried out in different groups of animals. Normal controls exhibited large polygonal cells with prominent round nuclei, few spaced hepatic sinusoids arranged inbetween the hepatic cords with fine arrangement of kupffer cells. In infected controls, the liver showed focus of hyperplasia. In one of the first histological study by Meleney, (1925) the relationship between hepatic parasitism and progressive alterations in the parenchyma of the liver was outlined. Andrade and Andrade (1966) described the dilation of the venous sinusoids, swelling of the hepatocytes, frequent parasitization of kupffer cells with diffused infiltration of plasma lymphocytes into the parenchyma, with the formation of sparse granulomas. Similar to our study, the hypertrophy and hyperplasia of kupffer cell was also found in naturally and experimentally L. chagasi infected animals (Giunchetti et al., 2008). Similarly, experimental dogs that had been infected with L. (L.) infantum exhibited moderate hepatitis characterised by periportal infiltration of the lymphoplasmacytic cells, as well as macrophages, often infected by amastigote forms of the parasite (Binhazim et al., 1993). Dogs naturally infected with L. (L.) chagasi, additionally presented intralobular and intravascular granulomas in the liver, consisting predominantly of parasitised and non-parasitised macrophages, epitheloid cells and a small number of lymphocytes together with rare granulocytes (neutrophils), (Tafuri et al., 1996). In the present study, animals treated with cisplatin with two doses, tiny focus of lymphocyte aggregate depicting focal reaction changes in liver, mild kupffer cell hyperplasia and the accumulation of red blood cells around the draining pathways of the central vein was observed. However, when the animals were treated with the
single dose, normal liver morphology was observed. This is in accordance to previous study from our lab where cisplatin treatment at a low dose of 0.5 mg/kg body wt. did not affect the spleen and liver morphology (Kaur et al., 2010). The hypertrophy and hyperplasia of kupffer cell was also found in SSG treated animals with liver necrosis. The reason behind the liver necrosis might be due to the gradual accumulation of SSG III in the cells. Our study is supported by Grimaldi et al. (2010) where he compared the efficacies of two different doses of N-methylglucamine antimoniate (MA) (20mg MA/kg/day and 5 mg MA/kg/day) for treating macaques infected with Leishmania braziliensis and observed that, treating macaques with 20mg/kg body wt, liver tissue necropsied on 95 days after the end of treatment. The reason for the drug-induced hepatic injury could be related to the conversion of SbV to SbIII, which has been demonstrated to be considerably more toxic than SbV in different test systems (Poon et al., 1998).

Histological studies of spleen of normal animals revealed well demarcated red and white pulp area, separated by marginal zone. In infected control animals the spleen showed the reactive enlargement of follicles, brown pigment (haemozoin) within the cells was also observed indicating intravascular hemolysis which could be due to the presence of Leishmania in the spleen tissue. Both red and white pulps were indistinguishable and proliferation of marginal zone was observed. Similarly, Sharma and Kaur, (2013) from our lab also reported changes in the spleen tissue of infected animals where profound modifications were observed in the red pulp due to the considerable increase in cellular proliferation. The intense proliferation of the marginal zone consisting of small cells with dense nuclei and sparse cytoplasm (macrophages) was observed. In animals treated with two doses of cisplatin, spleen showed mild expansion and enlargement of marginal zone at numerous places with excess of megakaryocytes. The above changes suggest that the spleen is reactive suggesting septicemia. No change was observed in animals treated with two doses of SSG or single dose of cisplatin. This study was supported by Sharma et al. (2012) from our lab where SSG treatment at a dose of 40 mg/kg body wt. did not alter splenic architecture.

Visceral leishmaniasis poses the strongest challenge given its behavior as a reemerging disease at a fast deteriorating rate with urban epidemics (Githeko et al., 1998).
In this scenario, the present study highlighted the use of immunochemotherapy for the control of visceral leishmaniasis in BALB/c mice. In our study, use of SSG or cisplatin with a combination of first or second generation vaccine resulted in parasite elimination via the direct parasiticidal activity of the drug and by the switching-on of Th1 based protective cell-mediated immunity. Moreover, as the standard antileishmanials used to treat leishmaniasis are met with various side effects, the low dose of cisplatin in combination with KLD alone or along with MPL-A and *Leishmania donovani* specific 78kDa antigen along with adjuvant MPL-A can prove to be a good alternative for the treatment of visceral leishmaniasis. Our study demonstrated the greater efficacy of two doses of immunochemotherapy over single dose for the treatment of VL. However, further studies should be performed in higher animal models for a better understanding of the immune response before it is to be tested in leishmaniasis patients.