SUMMARY AND CONCLUSIONS

Visceral leishmaniasis (VL), is a neglected tropical disease caused by Leishmania donovani (L. donovani) that is transmitted by the bite of an infected sandfly (Chappius et al., 2007). The main clinical features of VL are prolonged fever, anemia, splenomegaly, prominent wasting and when untreated death from organ failure and opportunistic infections (Tiuman et al., 2011). There are an estimated 3,60,000 new cases of VL each year worldwide and over 60% of these occur in the Northern Indian state of Bihar and bordering regions of Nepal and Bangladesh (Matlashewski et al., 2013). Chemotherapy potentially leads to toxicity and other undesirable side effects and due to the problem of emerging resistance, vaccination remains the only hope for control of disease but no vaccine is available till date. Since host immune responses have a direct impact on the efficacy of chemotherapy against leishmaniasis, the use of immunomodulators in combination with conventional chemotherapy will enhance host immune responses thereby improving the current therapeutic regimens.

The present study was carried out to test the combinative immunotherapeutic potential of 78kDa and KLD of L. donovani with SSG or cisplatin against murine visceral leishmaniasis.

6.1. Identification and electro-elution of 78kDa

The parasite proteins were run on SDS-PAGE, after staining and destaining the gel, the band of interest was taken in electrophoresis buffer in the electro-eluter. The protein was eluted by applying constant voltage through the gel. After elution, the protein was dialyzed, suspended in PBS and quantified.

6.2. Treatment of animals

Three different types of therapies that is chemotherapy, immunochemotherapy and immunotherapy were tested for their therapeutic efficacy in L. donovani infected inbred BALB/c mice. Animals were also tested with two different treatments that is one group of animals was treated with a single dose of all the different therapies and another group was treated with two doses of all the therapies with a gap of 14 days between two doses. The efficacy was assessed by analyzing the parasite load in liver and by the generation of cell mediated and humoral immune responses. To check the
drug induced side effects different hematological, biochemical and histopathological studies were carried out.

6.2.1. Parasite load

To check the antileishmanial efficacy of immunochemotherapy, the parasite burden was assessed in terms of Leishman Donovan Units (LDU) on geimsa stained impression smears of liver and spleen.

The parasite load in groups of animals treated with two doses was significantly reduced (p<0.001) when compared with animals treated with a single dose. However, maximum protection was conferred by immunochemotherapy treated animals since least parasite burden was observed in these animals. SSG+78kDa+MPL-A treated animals imparted maximum protection followed by SSG+KLD+MPL-A and then cisplatin+78kDa+MPL-A treated ones.

6.2.2. Humoral immune responses

Antibody responses in treated and control mice were analysed by IgG1 and IgG2a antibodies in their respective serum samples by ELISA. The IgG1 antibodies were determined for the assessment of Th2 type, whereas IgG2a was determined for the assessment of Th1 type of immune responses.

The Th2 specific antibody, IgG1 was found to be maximum in the infected controls and immunotherapy treated animals. However, minimum levels were observed in immunochemotherapy treated ones.

All the three different therapies (chemotherapy, immunotherapy and immunochemotherapy) induced good IgG2a antibody response which is an indicator of protective Th1 type of immune response. However, maximum IgG2a antibody response was induced by animals treated with the two doses of immunochemotherapy. Treatment with SSG+78kDa+MPL-A induced maximum IgG2a and minimum IgG1 antibody levels followed by SSG+KLD+MPL-A treated ones. In addition cis+78kDa+MPL-A treatment also produced a considerable antibody response followed by treatment with cis+KLD+MPL-A.
6.2.3. Cell mediated immune responses

I. DTH Responses

The delayed type hypersensitivity responses (DTH) were assessed by measuring the percentage increase in footpad thickness of leishmanin injected footpad in comparison to the control (PBS) footpad. Among all the treated animals, a profound DTH response was induced by animals treated with immunochemotherapy. Maximum DTH responses were elicited by animals treated with two doses of SSG+78kDa+MPL-A then SSG+KLD+MPL-A followed by cisplatin+78KDa+MPL-A and then cisplatin+KLD+MPL-A treated animals.

II. Cytokine Responses

The cellular immune responses (protective Th1 or non-protective Th2) generated by various therapies were assessed by quantifying the cytokines (IFN-γ, IL-2, IL-4 and IL-10) produced by splenocytes of treated animals. Cytokines IFN-γ and IL-2 indicate the generation of Th1 type of immune responses whereas IL-4 and IL-10 points towards the generation of Th2 type of immune responses.

IFN-γ responses were significantly higher in treated groups as compared to the infected controls. However, animals treated with immunochemotherapy produced maximum levels of this cytokine as compared to chemotherapy and immunotherapy treated ones. The levels were further increased when the animals were treated with two doses of all the treatments. Taken together, maximum concentration of IFN-γ was observed in animals treated with two doses of SSG+78kDa+MPL-A, then SSG+KLD+MPL-A followed by cis+78kDa+MPL-A and then cis+KLD+MPL-A treated animals.

Like IFN-γ, IL-2 levels were also greater in the immunochemotherapy treated animals as compared to the infected controls. The IL-2 levels were observed to be maximum in SSG+78kDa+MPL-A treated animals followed by those treated with SSG+KLD+MPL-A and then cis+78kDa+MPL-A followed by cis+KLD+MPL-A treated ones. Maximum cytokine concentration was observed in the animals treated with SSG followed by those treated with cisplatin suggesting that the combination of drug and vaccine is more efficient in generating Th1 type of immune responses.

In contrast IL-4, an indicator of Th2 type of immune response was significantly lesser in the treated animals as compared to the infected controls. SSG+78kDa+MPL-A
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treated animals revealed minimum levels of this cytokine followed by SSG+KLD+MPL-A, cis+78kDa+MPL-A and then cis+KLD+MPL-A treated ones.

Similar to IL-4, least concentration of IL-10 was observed in mice treated with immunochemotherapy which declined further in animals treated with the two doses. The infected controls showed significantly higher concentration of IL-10 when compared with the treated animals. Among all the treated animals SSG+78KLD+MPL-A produced least cytokine levels followed by SSG+KLD+MPL-A and then cis+78kDa+MPL-A followed by SSG and then cis+KLD+MPL-A treated animals.

6.3. Hematological investigations

The induction of hematological responses was studied by estimating Hemoglobin (Hb) and Total Leucocyte Count (TLC) in different groups of animals on different days post treatment.

6.3.1. Hemoglobin Estimation

A decrease in hemoglobin levels was observed in infected and immunotherapy treated animals as compared to the normal controls. However, in animals treated with chemotherapy and immunochemotherapy, the hemoglobin levels were found to be in the normal range of 8-10g/dl.

6.3.2. Total Leucocyte Count

Leucopenia was observed in the infected animals and animals treated with immunotherapy alone. TLC was found to be in the normal range of 7000-12000/mm$^3$ in chemotherapy and immunochemotherapy treated animals.

6.4. Biochemical investigations

The induction of biochemical responses by the drug was studied by evaluating liver and kidney function tests in the sera samples of different groups of animals on different days post treatment.

6.4.1. Liver function tests

Quantitative estimation of SGOT and SGPT activity revealed that enzyme levels were raised in animals treated with two doses when compared with animals treated with a single dose of their respective therapy (chemotherapy or immunochemotherapy). However, when the doses were compared enzymatic activity was more pronounced in cisplatin treated animals than SSG treated animals.
The alkaline phosphatase and acid phosphatase activity was found to be in the normal range of 4-11KA units and 0 to 0.6U/L respectively in all groups of animals on different days post treatment.

6.4.2. Kidney Function Tests

The urea, BUN and creatinine levels were raised in animals treated with two doses (p<0.001) when compared with animals treated with a single dose of their respective therapy (chemotherapy or immunochemotherapy). The increase was more pronounced in animals treated with cisplatin alone or along with immunotherapy.

Also, the electrolytes were within the normal range in all the different groups of treated animals.

6.5. Histopathological analysis

The histopathological studies of different organs (kidney, liver and spleen) of all the groups of animals was done under light microscope and photography was done using phase contrast microscope fitted with digital camera.

6.5.1. Kidney

Treatment of animals with two doses of either of the drugs (i.e. SSG or cisplatin) showed no major significant changes. In animals treated with two doses of cisplatin the kidney showed contracted glomerulus, decreased lumen and damaged brush border at some places while no major significant changes were observed when the animals were treated with a single dose. Moreover, in SSG treated animals normal appearance of kidney architecture was observed except tubular necrosis at some places.

6.5.2. Liver

Hematoxylin/eosin stained transverse sections of liver of infected and drug treated animals showed focal reaction changes in liver and mild kupffer cell hyperplasia was also observed. However, when the animals were treated with the single dose, liver morphology was normal in appearance.

6.5.3. Spleen

In animals treated with two doses of cisplatin, spleen showed mild expansion and enlargement of marginal zone at numerous places called hyper-reactive spleen. However, no change was observed in animals treated with the two doses of SSG or single dose of cisplatin.
From these results, the following conclusions can be withdrawn:

1. The mice treated with immunochemotherapy showed considerable therapeutic efficacy against *L. donovani* as revealed by the significant reduction in parasite load. Moreover, among the two different doses, animals treated with two doses were found to be better in imparting protection than the animals treated with a single dose. In addition, animals treated with cisplatin in combination with immunotherapy showed comparable parasite reduction when compared with SSG treated animals, therefore, can be an alternate option for the treatment of VL without any side effects.

2. The animals treated with two doses of immunochemotherapy elicited the generation of heightened DTH responses suggesting the generation of cell mediated immune responses.

3. When the two antigens are compared i.e. KLD or 78kDa, maximum protective efficacy was generated in animals treated with 78kDa+MPL-A as compared to KLD+MPL-A.

4. Immunochemotherapy treated animals showed remarkable increase in the levels of IFN-γ and IL-2 pointing towards the generation of protective Th1 type of immune responses.

5. Decreased levels of IL-4 and IL-10 in immunochemotherapy treated animals as compared to the chemotherapy and immunotherapy treated animals suggested the suppression of non-protective Th2 type of immune responses.

6. Mild increase in liver and kidney function tests of cisplatin treated animals with no change in the histological appearance showed the antileishmanial potential of drug with no toxicity.