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RESEARCH

To investigate the protective efficacy and immunomodulatory potential of single dose combination of SSG along with KLD/78kDa antigen +MPL-in inbred BALB/c mice

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ABSTRACT

Visceral Leishmaniasis (VL) is a disease of poor and neglected populations; it affects 79 countries of the world and accounts 58,000 new cases to each year. However, no effective treatment for cure of the disease is yet available. Therefore, the present study was designed to assess the curative efficacy of single dose of sodium stibogluconate in combination with Killed *Leishmania donovani* (KLD) antigen/78kDa antigen along with MPL-A in murine visceral leishmaniasis. After 30 days infection, infected mice were treated with a single dose of chemotherapy or immunochemotherapy. These animals were then sacrificed after 30 days post treatment and determined for hepatic parasite load and their immunological profile. It was observed that animals treated with a combination of SSG and immunotherapy not only reduced the parasite load but the immune profile was shifted to protective Th1 type (elevated levels of IFN-gamma and IL-2 were observed) of immune response. Hence our study lay emphasis on the use of short course (single dose) combination therapy of SSG and 78kDa/KLD along with MPL-A.

Key words: visceral leishmaniasis, KLD, 78kDa, immunotherapy, sodium stibogluconate.

1. INTRODUCTION

Leishmaniasis, a vector-borne disease characterized by both diversity and complexity, is endemic in large areas of the tropics, subtropics and the Mediterranean basin. (Chappius et al., 2007). Of the three different forms of the disease, visceral leishmaniasis produces high fever,

substantial weight loss, swelling of the spleen and liver, and anemia, and results in death if not treated. More than 90% cases of VL occur in 5 countries: India, Sudan, Bangladesh, Nepal and Brazil (Topno et al., 2010). The control of this disease relies mainly on chemotherapy since there are no licensed vaccines available in the market. The primary treatment or the first line drug treatment includes pentavalent antimonials. 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 phosphatidylcholine 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

Since, progression of VL infection is generally associated with down regulation of the host immune system and the treatment of leishmaniasis probably seldom eradicates all parasites in tissue macrophages. Therefore, compounds/agents that boost host cell activation by Th1 biased immune response might be useful as potential therapeutic agents for treatment of experimental VL (Shivahare et al., 2014). Recently, several studies have reported benefits of co-administration of antileishmanial drugs with immunostimulants as they shorten the course of treatment, delay or prevent the emergence of resistance and increase the efficacy of current therapeutic regimen. Previously, we studied the two doses of immunochemotherapy and found that the combination of drug and immunotherapy not only resulted in parasite elimination but also caused the reversal of the immune response from Th2 to protective Th1 type (Joshi and Kaur, 2014). Therefore, the current study was planned to further reduce the dose and assess the protective efficacy of single dose of sodium stibogluconate along with immunotherapy against murine visceral leishmaniasis.

2. MATERIALS AND METHODS

Animals and parasites

Inbred BALB/c mice (4-6 weeks old) used in the experiments were obtained from Institute of Microbial Technology, Chandigarh, India and then maintained in the Central Animal House, Panjab University, Chandigarh. This study was carried out according to the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA, Registration No. 45/1999/ CPCSEA). *L. donovani* strain Dd8 (MHOM/IN/80/Dd8), originally obtained from London School of Hygiene and Tropical Medicine, London, was maintained *in vitro* at 22^{o} C $\pm$ 1^{o} C in modified NNN medium by serial culturing after every 48-72 h (Rao et al., 1984).

In vivo infection and treatment

All the mice were infected intracardially with 10^{7} promastigotes/0.1ml (Kaur et al., 2010). After 30 days infection, these animals were divided into four different groups. Group1 animals served as infected controls. Group 2 animals were treated intraperitoneally with sodium stibogluconate (SSG) at a dose of 40mg/kg body wt. continuously for five days. Animals ingroup 3 and group 4 were given a combination of chemotherapy (SSG) at the same dose and immunotherapy (Killed *Leishmania donovan* antigen/78kDa antigen along with MPL-A) for one day only (Joshi and Kaur, 2014; Joshi et al., 2014).

Parameters studied

Assessment of Infection

To assess the parasite load in liver, all the treated mice were sacrificed on 30 day post treatment. Livers of all the animals were aseptically removed and their impression smears were microscopically examined after fixing and staining the slides with Giemsa. In order to quantitate levels of infection, Leishman Donovan units (LDU) were calculated as: Number of amastigotes/Number of cell nuclei X weight of organ in milligrams (Bradley and Kirkley 1977).

Determination of cytokine responses

The lymphocytes from spleens of infected and drug treated mice were cultured in 24 well plates in 1 ml of RPMI-1640 containing 20 mM $NaHCO_3$, 10 mM HEPES, 10 U/ml of penicillin, 100µg/ml streptomycin and 2mM L-glutamine and 10% FCS. Cells were stimulated with 50µg/ml of the parasite antigen and then cells were incubated at 37ºC for 72h and supernatants were collected and stored at -20ºC. This was then assayed for IL-2, IL-10 and IFN-γ by using ELISA kits (BenderMed Systems, Diaclone, France) (Kaur et al., 2008).

Statistical Analysis

The statistical significance of the difference between various groups was determined by ANOVA. Differences were considered statistically significant for $p<0.05$.

3. RESULTS AND DISCUSSION

In the present study maximum reduction in the parasite load ($p<0.001$) was observed in animals treated with immunochemotherapy (95.55% reduction was observed in animals treated with SSG+78kDa+MPL-A) as compared to the chemotherapy alone. Similarly,, in a recent study by Joshi and Kaur, 2014 it was observed that using two doses can led to significant reduction of parasites from the liver. This is in consistence to a study, where 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 et al., 2009). A combination of Z-100, a polysaccharide obtained from *Mycobacterium tuberculosis* combined with pentavalent antimonial, was found effective against *L. amazonensis in-vitro* (Barroso et al., 2007).

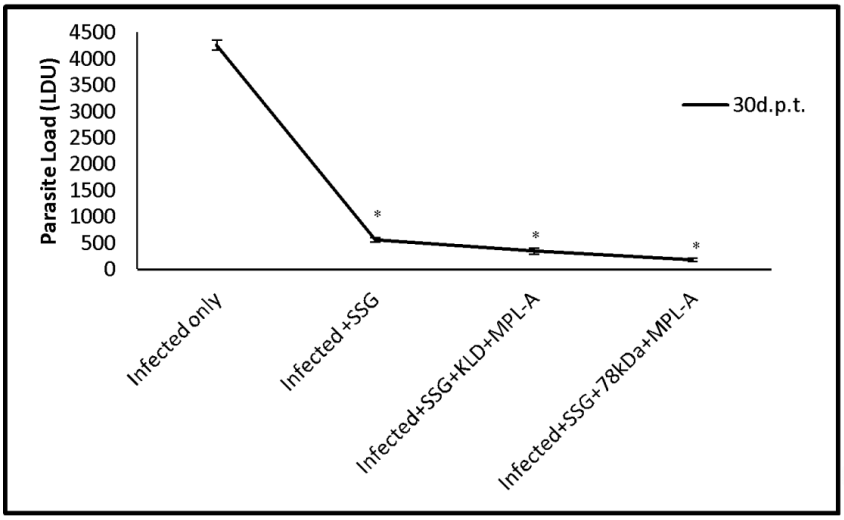

Figure 1

Hepatic parasite load in terms of Leishman Donovan Units (LDU) in different groups of animals

* p value Infected vs Infected+SSG/ Infected +SSG+KLD+MPL-A / Infected +SSG+78kDa+MPL-A

* -(p<0.001)

Also, it was observed that protection against VL in Balb/c mice was very effective using Dendritic cells based immunotherapy combined with antimony-based chemotherapy and resulted in a complete deletion of the parasites (Ghosh et al., 2003).

The control of VL infection depends on a successful cell-mediated immune response, in which IFN-gamma, produced mainly by CD4+ T cells and natural killer(NK) cells stimulated by IL-12, leads to stimulation of microbicide action mediated by nitricoxide (NO) (Roatt et al., 2014). In the present study, Maximum levels of Th1 cytokines (IFN-γ and IL-2) and minimum levels of Th2 cytokines (IL-4 and IL-10) were observed in animals treated with immunochemotherapy. Moreover, maximum elevation of Th1 cytokines was observed in animals treated with SSG+78kDa+MPL-A. This is in consistence to the earlier studies where using two doses of immunochemotherapy led to a significant increase in the Th1 cytokines (Joshi and Kaur, 2014) thus resulting disease resolution.

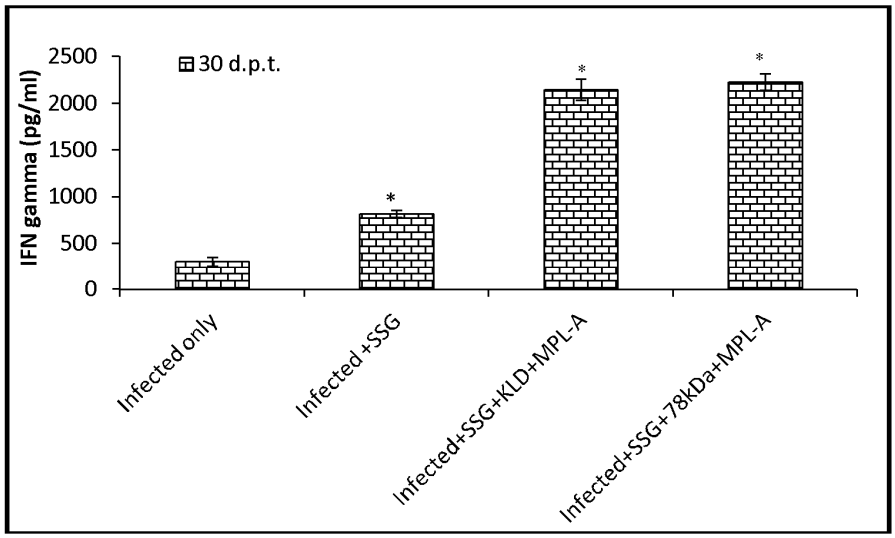

Figure 2a

IFN-γ levels in different groups of animals

* p value Infected vs Infected+SSG/ Infected +SSG+KLD+MPL-A / Infected +SSG+78kDa+MPL-A

* -(p<0.001)

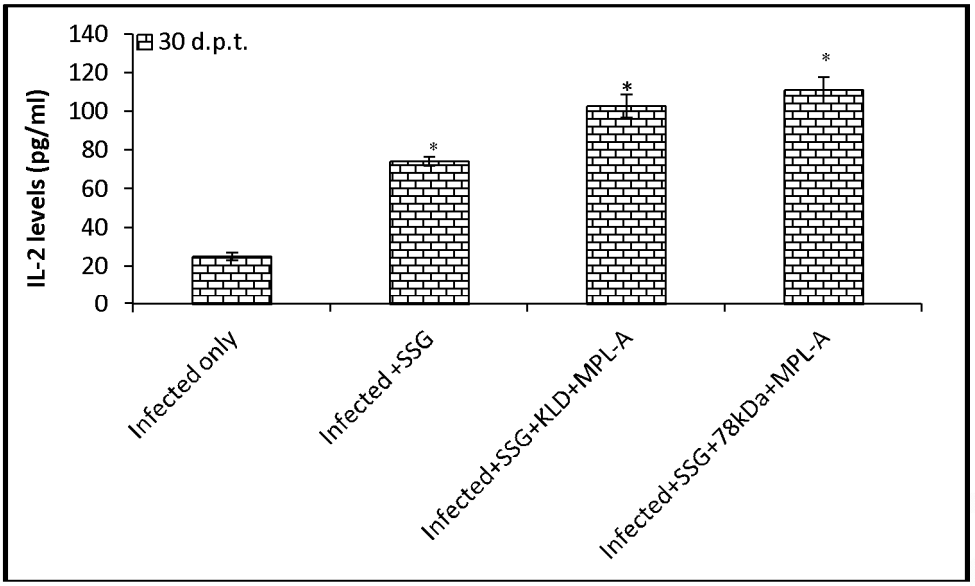

Figure 2b

IL-2 levels in different groups of animals

* p value Infected vs Infected+SSG/ Infected +SSG+KLD+MPL-A / Infected +SSG+78kDa+MPL-A

* -(p<0.001)

It has been well established that IL-10 plays a central role in the pathogenesis and parasite growth in VL. Our results support this and it is observed that minimum IL-10 levels were observed in animals treated with immunochemotherapy. In consistence to our study, treatment of *L. donovani*-infected wild-type mice with a single dose anti-IL-10R mAb and daily low doses of Sb^v resulted in rapid control of the *L. donovani* infection and dramatically enhanced the therapeutic effects of Sb^v namely (Murray ,2005). Similar results were observed in *L. donovani*-infected BALB/c mice treated with a suboptimal single dose (0.1 mg) of an anti-IL-10R mAb and low-dose Amphotericin B (2 mg/kg total dose) (Murray et al., 2003). The combination therapy induced a 76% liver parasite killing, compared with a 16% observed with the anti-IL-10R mAb alone.

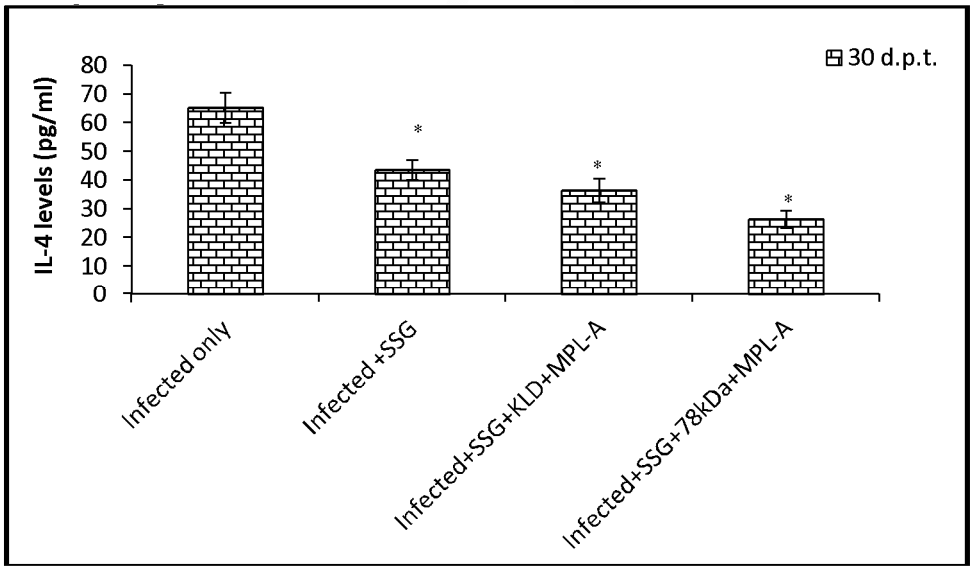

Figure 3a

IL-4 levels in different groups of animals

* p value Infected vs Infected+SSG/ Infected +SSG+KLD+MPL-A / Infected +SSG+78kDa+MPL-A

* -(p<0.001)

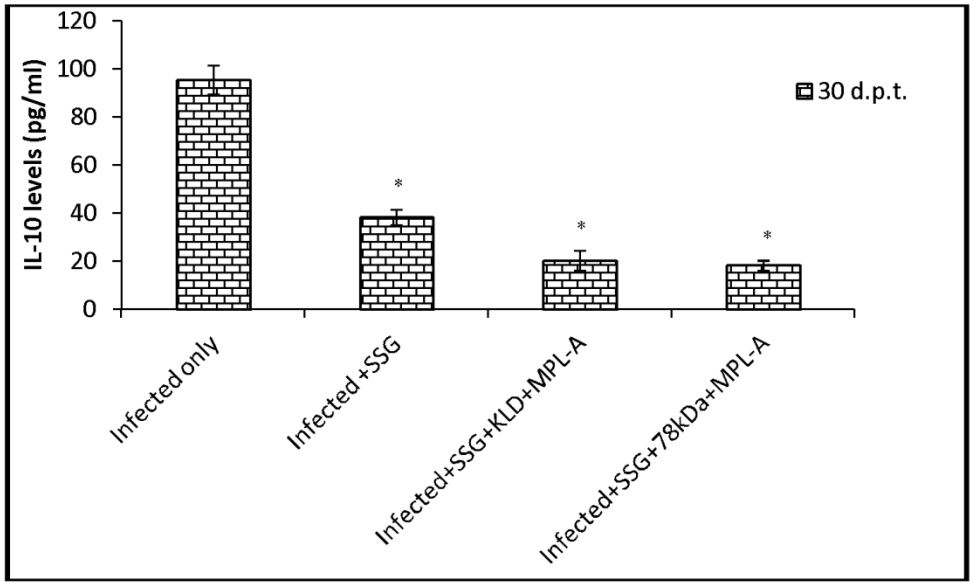

Figure 3b

IL-10 levels in different groups of animals

* p value Infected vs Infected+SSG/ Infected +SSG+KLD+MPL-A / Infected +SSG+78kDa+MPL-A

* -(p<0.001)

To conclude, the current study highlighted the use of single dose of immunochemotherapy over other treatments. As the main purpose of the immunochemotherapy is to reduce the dose of the drug, this prompted us to further reduce the course of the therapy and it was observed that a significant 95.5% elimination of parasite was achieved using SSG along with the immunotherapy. Hence the combination of conventional drugs along with an immune modulator can be beneficial for long term cure of visceral leishmaniasis.

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