SUMMARY AND CONCLUSIONS

VL is a potentially fatal human disease and is endemic in the Indian subcontinent and East Africa (WHO, 2015). The available treatment for VL is based on chemotherapy. Most traditional and low-cost treatment options for VL are toxic and have many side effects, and the use of more effective drugs is limited mainly by high cost. Resistance to chemotherapy is also a growing problem in many regions (Roatt et al., 2014). So, alternate strategies such as vaccination need to be developed for the control of this disease. It is thus imperative to optimize vaccine targets so that the infection in a susceptible host is controlled.

The present work was carried out to evaluate the protective efficacy of three different killed antigens (ALD, KLD, FTP) in combination with four different adjuvants (alum, saponin, MPL-A and cationic liposomes) against Leishmania donovani infection in inbred BALB/c mice. To estimate the protective immunity of different vaccine formulations cohorts of naive BALB/c mice went through a prime-boost immunization regimen. To facilitate broad clinical applicability, we selected the minimally invasive subcutaneous route. The protective efficacy and immunogenicity of all vaccine formulations was analyzed by the assessment of parasite burden and generation of cell mediated and humoral immune responses.

Protective efficacy

The efficacy of all formulations was evaluated by analyzing parasite burden in liver and spleen.

Parasite load

Six animals from each group were sacrificed on 30, 60 and 90 post infection/challenge days. Liver and spleen of all animals were removed and weighed. Impression smears were made and the parasite load was assessed in terms of Leishman Donovan Units.

A significant reduction in parasite load was observed in all immunized groups on all post challenge days. Maximum reduction in hepatic parasite burden was observed in group of animals immunized with liposome encapsulated KLD antigen where parasite load declined by 93.7% as compared to infected controls. It was followed by group immunized with KLD+MPL-A in which parasite burden declined by
92.8% on 90 post challenge day. Lowest protection was observed in animals immunized with FTP antigen alone without any adjuvant.

Maximum splenic parasite load was observed in infected control animals. Parasite burden decreased significantly in immunized animals and maximum decline was observed on 90 post challenge day. Among immunized groups least reduction in splenic parasite load was observed in group of animals immunized with FTP antigen alone followed by animals immunized with ALD antigen and KLD antigen respectively. Maximum reduction in parasite burden was observed in animals immunized with liposome encapsulated KLD antigen (80.5%). It was followed by group immunized with liposome encapsulated ALD antigen (76.19%), KLD+MPL-A (73.6%) and group immunized with liposome encapsulated FTP antigen (73.3%).

**Immunogenicity of vaccines**

The immune responses, whether Th1 or Th2 type, induced by different vaccine formulations were also evaluated in the present study. All the preparations were found to be immunogenic as seen by enhanced delayed type hypersensitivity (DTH) response, anti-leishmanial antibodies and increased cytokine production.

**Delayed type hypersensitivity responses**

DTH responses were assessed by measuring the percentage increase in footpad thickness of leishmanin injected footpad in comparison to the control (PBS) footpad. All the immunized animals showed enhanced DTH responses in comparison to the infected controls. Among immunized groups maximum DTH response was elicited by animals immunized with liposome encapsulated KLD antigen. Animals immunized with KLD+MPL-A also induced strong DTH responses and it was followed by group immunized with liposome encapsulated ALD antigen, liposome encapsulated FTP antigen and then ALD+MPL-A, FTP+MPL-A immunized groups. Significant DTH responses were also induced by saponin adjuvanted formulations.

**Cytokine responses**

The cellular immune responses were assessed by quantifying the cytokines (IFN-γ, IL-12, IL-4 and IL-10) in serum samples of immunized groups. Cytokines like IFN-γ and IL-12 indicate the generation of Th1 type of immune responses whereas IL-4 and IL-10 points toward the generation of Th2 type of immune responses.

IFN-γ levels were particularly elevated in immunized groups as compared to the infected controls. Taken together, maximum levels of this cytokine were observed in
animals immunized with liposome encapsulated KLD antigen followed by group immunized with KLD+MPL-A, liposome encapsulated ALD antigen, liposome encapsulated FTP antigen and then ALD+MPL-A immunized groups.

Like IFN-γ, IL-12 levels were also higher in the immunized animals as compared to the infected controls. The levels were observed to be maximum in mice immunized with liposome encapsulated KLD antigen followed by those immunized with liposome encapsulated ALD antigen, KLD+MPL-A and ALD+MPL-A immunized groups. An enhanced IL-12 response was also observed in KLD+saponin, ALD+saponin and FTP+saponin immunized groups. Since maximum levels of Th1 cytokines were observed in animals immunized with KLD antigen along with liposomes and MPL-A as an adjuvant it suggests that this combination is more efficient in generating Th1 type of immune responses.

In contrast, IL-10 cytokine, an indicator of Th2 type of immune response was high in the infected control animals. It was significantly low in immunized animals, being minimal in groups immunized with liposome encapsulated KLD antigen and KLD+MPL-A. Significantly low IL-10 levels were also observed in group immunized with liposome encapsulated ALD antigen, liposome encapsulated FTP antigen and then ALD+MPL-A, FTP+MPL-A immunized groups. Among adjuvanted vaccine formulations maximum IL-10 levels were observed in group immunized with FTP+alum.

Similar to IL-10, maximum levels of IL-4 were observed in infected control animals. IL-4 levels were found to decline significantly in all immunized groups. Animals immunized with liposome encapsulated KLD antigen and liposome encapsulated FTP antigen showed minimum levels of this cytokine.

**Humoral responses**

Serum samples of all immunized and control animals were examined for the detection of IgG1 and IgG2a antibodies. The isotype IgG1 antibody was detected as a marker for Th2 type of immune response whereas IgG2a antibody was detected for the assessment of Th1 type of immune response.

IgG1 antibody response was found to be higher in the infected controls as compared to the immunized animals. Minimum levels of this antibody were observed in group immunized with liposome encapsulated KLD antigen. On the contrary, minimum levels of IgG2a antibody were observed in infected control animals.
Maximum levels of this antibody were observed in KLD+MPL-A immunized group which was followed by groups immunized with liposome encapsulated KLD antigen, ALD+MPL-A and liposome encapsulated ALD antigen. A significant level of IgG2a antibody response was also exhibited by groups immunized with KLD+saponin and ALD+saponin. Among all immunized groups minimum levels of IgG2a antibody were observed in group immunized with FTP antigen alone.

From these results, following conclusions can be drawn:

1. A considerable protective efficacy was shown by all vaccine formulations against experimental murine visceral leishmaniasis. It was evident from significant reduction in parasite load.
2. Significant DTH responses were generated in all immunized groups which suggests the generation of cell-mediated immune responses.
3. The higher levels of IgG2a, IFN-γ and IL-12 in all immunized animals suggests the generation of protective Th1 type of immune responses.
4. The lower levels of IgG1, IL-4 and IL-10 suggest the abolishment of the non-protective Th2 type of immune responses in immunized animals.
5. Among three antigens used in the study a comparable protective efficacy was shown by animals immunized with KLD and ALD antigen. However, the efficacy was superior as compared to the FTP antigen.
6. Animals immunized with combination of antigen and adjuvant showed better protection as compared to animals immunized with killed antigen alone.
7. Out of four adjuvants used in the present study, liposomal encapsulation contributed maximum to enhance the efficacy and immunogenicity of antigens followed by MPL-A, saponin and alum.
8. Studies on different post challenge days revealed that maximum efficacy was observed on 90 post challenge day.
9. The protective Th1 type of immune responses were most pronounced in the group of animals immunized with liposome encapsulated KLD antigen followed by group immunized with KLD+MPL-A.

For future investigations combination of these potential adjuvants can thus be tried to further improve the anti-leishmanial potential of killed antigen based vaccines.