

Evaluation of the immunogenicity and protective efficacy of Killed *Leishmania donovani* antigen along with different adjuvants against experimental visceral leishmaniasis

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Abstract Visceral leishmaniasis (VL) caused by *Leishmania donovani* is a life-threatening disease involving uncontrolled parasitization of vital organs. Drugs to treat leishmaniasis have one or more limitations or insufficiencies in the long run. A safe and efficacious vaccine to control this disease is needed. Killed antigens that could be safer as vaccines have shown limited efficacy in clinical trials. Immunogenic enhancement with appropriate adjuvants may thus be required to elicit protective immunity based on antibodies and effector T-cell functions. Therefore, it is essential to search for adjuvant to enhance the immunogenicity of killed vaccines and to induce protection against leishmaniasis. So, the aim of the present study was to compare the effectiveness of four adjuvants, i.e. alum, saponin, monophosphoryl lipid A, cationic liposome in combination with Killed *Leishmania donovani* (KLD) antigen against murine VL. Animals were immunized subcutaneously thrice at an interval of 2 weeks with a final volume of 100 μ l per dose. Challenge infection was given 2 weeks after last booster. Mice were sacrificed 15 days after last immunization and on 30, 60 and 90 post-infection/challenge days. The protective efficacy of vaccines was revealed by significant reduction in parasite burden and enhanced DTH responses in comparison with the infected controls. Immunized animals also generated significant levels of Th1 cytokines and increased production of IgG2a, thus indicating the generation of a protective Th1 response. All the adjuvants imparted significant protection, but liposomal formulation was most effective followed by KLD + MPL-A, KLD + saponin, KLD + alum and KLD antigen.

Keywords Killed *Leishmania donovani* · Adjuvants · Vaccines · Immunization · Visceral leishmaniasis

Introduction

Visceral leishmaniasis (VL) is a vector-borne parasitic disease, which results from systemic infection with *Leishmania donovani* and *L. infantum* in the Old World and *L. chagasi* in the New World. *L. chagasi* clearly belongs to the *L. donovani* complex but it has been synonymized with *L. infantum* on the basis of RAPD and DNA sequence analysis, which suggests the introduction of *L. chagasi* in New World [1]. Parasites of the genus *Leishmania* target preferentially macrophages throughout the viscera and are usually found in spleen, liver and bone marrow [2]. Current treatment relies on handful of drugs. It is unlikely that chemotherapy alone will enable disease eradication, and hence, there is an urgency to develop an effective vaccine. The acquisition of immunity following *Leishmania* infection gives a hope that prevention of leishmaniasis through prophylactic vaccination is feasible.

Killed antigens composed of parasite fractions or whole killed parasites reached phase 3 clinical trials, but showed a limited prophylactic efficacy [3]. Most of the prophylactic immunizations in past have been carried out with autoclaved *L. major* promastigotes. Vaccination with alum-absorbed preparation (alum-ALM) along with BCG has been shown to be more immunogenic, protective and produced a strong and long-lasting Th1 response in mice, monkeys and dogs against VL [4–6] but it did not produce conclusive results in humans [7–9]. In spite of the availability of numerous candidate antigens against leishmaniasis, limited studies are available about the use of formalin-killed promastigotes. Recently, formalin-killed promastigotes of

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L. major, along with Montanide ISA 720 (MISA) adjuvant, has been reported to be an effective antigen candidate against murine cutaneous leishmaniasis [10].

It seems that major limiting factor for the development of killed vaccines is lack of a suitable adjuvant [11, 12]. The accessibility to hundreds of adjuvants has encouraged a need for identifying rational standards for selection of adjuvant formulation based on safety and sound immunological principles for human vaccines. Aluminium salt-based adjuvants, referred generically as ‘alum’, are among few of the licensed adjuvants approved for human use [13]. In relation to leishmaniasis, alum has been used in combination with ALM + BCG for immunization of patients with VL [14] and with ALM + SSG + BCG in PKDL patients [15]. Monophosphoryl Lipid A (MPL-A) is a nontoxic derivative of lipopolysaccharide of *Salmonella minnesota*, a gram-negative bacteria. It activates innate immunity through toll-like receptor (TLR4), enhances phagocytosis, upregulates major histocompatibility complex (MHC) expression and induces a cascade of cytokines [9, 16]. MPL is approved for human use and currently used in several human vaccines including malaria, Epstein-Barr virus, herpes simplex, hepatitis and leishmaniasis [9, 17, 18]. Saponins are chemically a heterogeneous group of sterol glycosides and triterpene glycosides. Saponin-based adjuvants have the ability to modulate the cell-mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity [19]. QS21 fraction of saponin in combination with FML (Leishmune[®]) has been registered in Brazil for immunization in dogs and induced significant protection in various trials [20–22]. Liposomes are versatile and robust delivery systems known for induction of humoral and cell-mediated immune responses. The potential of liposomes to assimilate broad range of antigens (diphtheria, hepatitis A, influenza, tetanus toxoid and leishmanial proteins) exhibits the versatility of these vesicles as an antigen delivery system [23].

The encouraging findings of above studies prompted us to investigate the immunoprophylactic potential of formalin-fixed Killed *Leishmania donovani* (KLD) antigen along with different adjuvants against murine VL.

Materials and methods

Parasite culture and preparation of different vaccine formulations

Promastigotes of *L. donovani* strain MHOM/IN/80/Dd8 were used for the present study and maintained in modified Novy, McNeal and Nicolle’s (NNN) medium by serial subcultures in Minimum Essential Medium (MEM) after

every 48–72 h. For the preparation of vaccination antigen, stationary-phase promastigotes were harvested, counted in Neubauer’s chamber and then resuspended in 1 ml sterile PBS (pH 7.2) at a concentration of 10^8 parasites. Promastigotes were killed by suspending in 0.1 % formalin overnight, and then, they were washed thrice with PBS. About 0.1 ml of this suspension containing 10^7 parasites was used for immunization of mice. Each animal was immunized with KLD antigen alone or mixed with respective dose of adjuvant in 100 μ l of PBS. For each animal, adjuvant dosage was 100 μ g saponin and 40 μ g of MPL-A. For preparation of KLD + alum vaccine, KLD antigen was dissolved in 4 ml of PBS. It was then added to 1 ml of 10 % potash alum (aluminium potassium sulphate). After that, 10 % Na_2CO_3 solution (0.3–0.5 ml) was added gradually to bring the pH of solution between 5.8 and 6.8. The precipitates so formed were shaken well and then injected into mice. For preparation of positively charged liposomes, commercially available kit from Sigma-Aldrich (USA) was used. About 63 μ mol of phosphatidylcholine, 9 μ mol of cholesterol and 18 μ mol of stearylamine in the ratio of (7:1:2) were used for the preparation of positively charged liposomes. Encapsulation of antigen in liposomes was carried out by the method described by Afrin and Ali [24]. The protein content entrapped in the liposome was estimated by the method described by Lowry et al. [25], with bovine serum albumin as the standard, in the presence of 0.8 % sodium dodecyl sulphate and appropriate blanks. The protein content was found to be 46.6 μ g.

Chemicals and reagents

IgG1 and IgG2a (Serotec), MEM (Hi-Media), alum, saponin, liposomes, monophosphoryl lipid A, Primers (Sigma-Aldrich, USA), Maxima SYBR Green qPCR Master Mix (Thermo Scientific), Cytokine ELISA kits (Diacclone, France), 96-well ELISA plates (Tarson).

Immunization and challenge infection of experimental animal groups

Female BALB/c mice, weighing 20–25 g, were acquired from the Institute of Microbial Technology, Chandigarh, India, and then maintained in the central animal house of Panjab University, Chandigarh. They were housed in clean cages and fed with water and mouse feed ad libitum. Experimental groups consisted of 4–6-week-old inbred BALB/c mice. Three immunizations were carried out at an interval of 2 weeks. Animals immunized with PBS only and challenged with 10^7 promastigotes of *L. donovani* served as infected controls. Normal control mice received only PBS. Two weeks after last booster dose, mice of control and

Table 1 Forward and reverse primers sequence

	Primer sequence (5'–3')	Annealing temperature (°C)	Product size (bp)	T_m
FP	GGTAGTGGGTAGTAACGTGGATGTAG	62	70 bp	57.9
RP	GGCCGGGACCCATT			58.5

immunized groups were challenged with 1×10^7 promastigotes. Various parasitological and immunological studies were carried out 15 days after last immunization and on 30, 60 and 90 post-infection/challenge days (p.c.d.)

Ethical clearance

Experiments were 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). The ethical clearance for conducting the experiments was obtained from the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh, India (Approval No. IAEC 284-295/3.09.2012). Mice were handled according to their guidelines.

Assessment of parasite burden

Six animals from each group were killed on different post-challenge days. Liver and spleen of all animals were removed and weighed. Impression smears were made on clean glass slides. The smears were air dried, fixed in methanol and stained with Giemsa. The parasite load was assessed in terms of Leishman Donovan Units (LDU) by the method prescribed by Bradley and Kirkley [26] and calculated as follows:

Number of amastigotes/Number of macrophages
 \times weight of organ in milligrams

For real-time PCR analysis, DNA was isolated from liver and spleen (100 mg) using commercially available Genomic DNA mini spin kit (Cat# RKN 32, Chromous Biotech) according to manufacturer's instructions. Eluted DNA was quantified, analysed and stored at -20°C till further use. A real-time PCR was performed using Maxima SYBR Green Master Mix to amplify kinetoplast DNA of *L. donovani* using specific primers (Table 1). Primers were designed using Primer 3 input software. The 10- μl reaction contained 50 ng of genomic DNA (2 μl), 5 μl of Maxima SYBR Green qPCR Mastermix, 0.12 μl of each primer and 2.76 μl of nuclease-free water. Negative control tubes that received 2 μl of water instead of DNA extract were included in each PCR run to detect any amplicon contamination. Times and temperatures of cycling programme are shown in Table 2. The efficacy of vaccine formulation was determined in terms of fold change decrease in parasitic load as compared to infected controls.

Table 2 Time and temperature used for running real-time PCR

Parameter	Temp (°C)	Time (m/s)	Acquisition mode
Denaturation	95	10 m	None
Amplification (40 cycles)	95	45	None
	62	30	None
	72	45	Single
Melting	95	1	None
	69	5 m	None
	95	–	Continuous
Cooling	40	30	None

Delayed-type hypersensitivity (DTH) responses to leishmanin

DTH response is a classic hallmark of cell-mediated immunity. The induction of such measurable immunological parameter may have important predictive value with regard to resistance to infection. For evaluation of DTH responses, 48 h before the day of killing, mice were challenged in the right foot pad with a subcutaneous injection of leishmanin (prepared in PBS) and left foot pad with PBS (leishmanin was prepared by harvesting stationary-phase promastigotes from modified NNN medium and washed thrice with PBS. The final pellet was then suspended in 5 ml PBS containing 0.5 % phenol and kept at room temperature for 10 min. The phenol was then removed by washings with PBS, and the final concentration was adjusted to 2×10^8 promastigotes/ml). After 48 h, the thickness of right and left foot pad was measured using a pair of vernier callipers. Results were expressed as mean \pm SD of percentage increase in the thickness of the right footpad as compared to the left footpad of mice [27].

ELISA for parasite-specific IgG1 and IgG2a isotypes

Conventional ELISA was performed to determine the levels of serum immunoglobulin G (IgG) isotype antibodies (IgG1 and IgG2a) by the method described by Ravindran et al. [28]. Briefly, 96-well plates were coated with crude antigen (5 $\mu\text{g}/\text{well}$) and incubated overnight at 4°C . Serum samples were added at twofold serial dilutions. It was followed by addition of isotype-specific HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 or IgG2a) after which the H_2O_2 substrate and DAB chromogen were added. Absorbance was read on an ELISA plate reader (Lisa Plus, India) at 450 nm.

Cytokine assays

Cytokines play an important role in shaping the host immune response to *Leishmania* infection and leading the progression towards protective or non-protective immunity during infection. For the evaluation of cytokine levels, cell suspensions from spleen of normal controls, infected controls and immunized mice were prepared and cultured in 96-well plates in RPMI-1640 containing 20 mM NaHCO₃, 10 mM HEPES, 10 U/ml of penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and 10 % FCS. Cells were stimulated with 50 µg/ml of crude antigen of *L. donovani* [29]. Cells were incubated at 37 °C in a humidified chamber containing 5 % CO₂ for 72 h and supernatants were collected and stored at –20 °C [30]. This supernatant was assayed for the concentration of IL-12, IL-4, IL-10 and IFN-γ by commercially available cytokine ELISA kits from Diaclone, France according to the manufacturer's instructions.

Statistical analysis

The data comparisons were tested for significance using Sigma plot (12.5) software. All the data were analysed using two-way analysis of variance (ANOVA). Post hoc test was used for multiple comparisons using Holm–Sidak method. *p* values of <0.05 and <0.001 were considered significant. Results were expressed as mean ± SD of one of three independent experiments.

Results

Assessment of parasite burden

Quantification of parasite load was done for all groups on 30, 60 and 90 post-challenge days. Parasite load was calculated by studying giemsa-stained impression smears of liver and spleen. As compared to infected controls, we observed a trend of decreased parasite burden in immunized groups (*p* < 0.001). The combination of liposome-encapsulated KLD antigen imparted maximum protection with 86–93.7 % reduction in LDU from 30 to 90 post-challenge day. Groups immunized with KLD + MPL-A and KLD + saponin also conferred good protection with 83.4–92.8 % and 79.9–88.8 % parasite reduction, respectively. Immunization with KLD + alum resulted in 73.78–82.9 % reduction in parasite load. Between the immunized groups, the difference in LDU was found to be significant (*p* < 0.05, *p* < 0.001) on all post-challenge days (Fig. 1a). Furthermore, immunization with KLD antigen also significantly (*p* < 0.001) reduced the parasite burden (66.3–71.7 %, respectively) as compared to infected controls. In

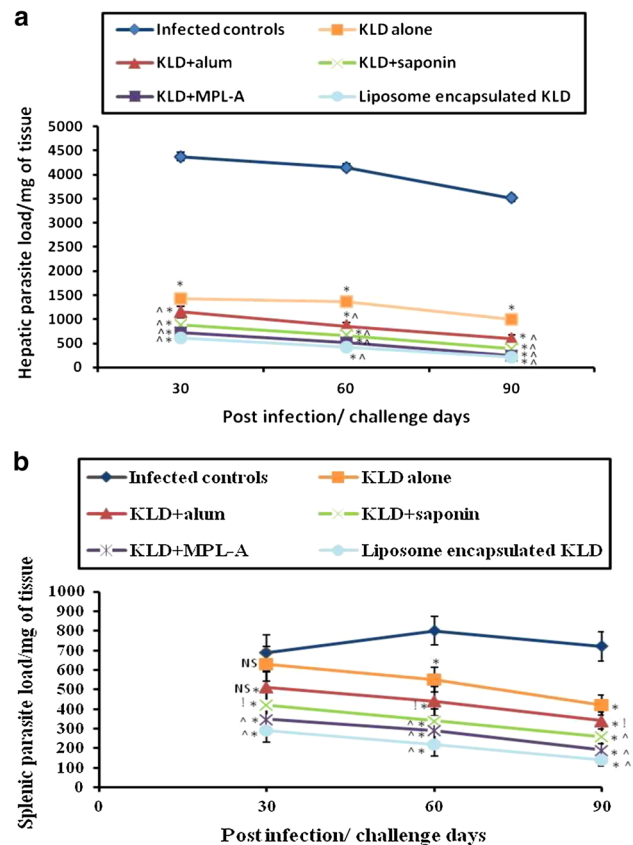


Fig. 1 Estimation of hepatic and splenic (a, b) parasite load in all groups of BALB/c mice. The data are presented as mean ± SD of six mice per group. ^{α*}*p* value: infected controls versus KLD; KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. ^Δ*p* value: KLD versus KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. ^{*}*p* value <0.001, ^α*p* value <0.05, NS nonsignificant

VL, the spleen acts as a reservoir for parasitic persistence, which is further associated with failure to clear the disease. We therefore wanted to observe the effect of immunization with different vaccine formulations on the splenic persistence of *L. donovani* following challenge infection. The splenic parasite burden was also found to be significantly reduced in all the immunized groups as compared to infected controls (Fig. 1b).

To confirm the accuracy of parasite load calculated by giemsa-stained slides, we also did qPCR analysis at 90th post-challenge day. Real-time PCR analysis also displayed similar trend of decrease in parasite burden for all groups of immunized animals (Fig. 2a). Maximum protection was achieved with the use of liposome-encapsulated KLD antigen with 13.69-fold decrease in hepatic parasite burden as compared to infected controls. This group was followed by group immunized with KLD + MPL-A (12.82), KLD + saponin (7.12), KLD + alum (5.5) and KLD alone (3.89). Similar trend of decrease in parasite burden was also seen in spleen.

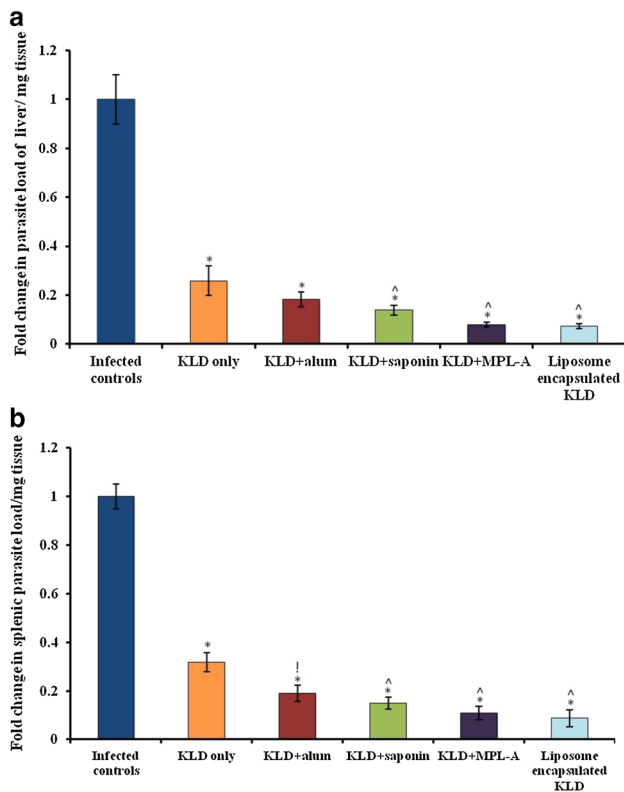


Fig. 2 Estimation of fold change decrease in hepatic and splenic (a, b) parasite burden in all groups of BALB/c mice. The data are presented as mean \pm SD of six mice per group. α^* *p* value: infected controls versus KLD; KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. $^{\wedge}$ *p* value: KLD versus KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. ** *p* value <0.001, $^{\alpha^1}$ *p* value <0.05, NS nonsignificant

Since spleen is a site of persistent infection, so parasite number decreased slowly as compared to liver. However, the parasite load declined significantly when compared with infected controls at 90th post-challenge day (Fig. 2b).

Assessment of DTH responses

To evaluate cell-mediated immune responses, we monitored the delayed-type hypersensitivity (DTH) responses in mice after 15 days post-immunization and at 30, 60, 90 post-infection/challenge days. On day 15 post-immunization, all the animals displayed elevated DTH responses ($p < 0.001$) in comparison with the control mice. DTH responses were further found to increase from 30 to 90 post-infection/challenge days. A profound DTH response was induced by immunization of mice with KLD alone. Adjuvants used along with KLD antigen for immunization further heightened the DTH responses. Peak DTH response was observed in group of animals immunized with liposome-encapsulated KLD and KLD + MPL-A suggesting the generation of protective cell-mediated immunity. A significant increase ($p < 0.001$) in footpad thickness

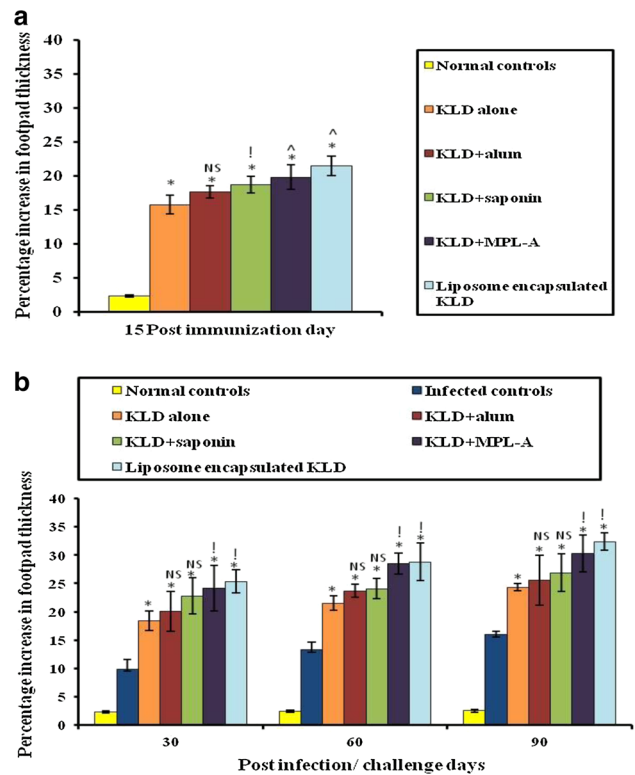


Fig. 3 DTH responses (a, b) in BALB/c mice following immunization and *L. donovani* challenge infection. The data are presented as mean \pm SD of six mice per group. α^* *p* value: Control mice versus KLD; KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. $^{\wedge}$ *p* value: KLD versus KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. ** *p* value <0.001, $^{\alpha^1}$ *p* value <0.05, NS nonsignificant

was observed in immunized mice as compared to infected control mice on all post-challenge days (Fig. 3).

Humoral immune responses

Serum samples of immunized and control mice were analysed for detection of IgG1 and IgG2a antibodies by ELISA. The relative production of these isotypes can thus be used as a marker for the induction of Th1-like and Th2-like immune responses. At 15th day post-vaccination, all the immunized groups displayed significantly higher levels of parasite-specific IgG isotypes, i.e. IgG1 and IgG2a in comparison with control mice ($p < 0.001$) that persisted even after challenge infection. Least IgG1 levels were observed in the animals immunized with liposome-encapsulated KLD antigen. IgG1 levels in immunized mice were lesser as compared to the infected control animals. The difference was significant ($p < 0.001$) on all post-challenge days. IgG2a antibody levels indicate the generation of Th1 type of immune response, which is protective. Immunization of mice with KLD antigen alone significantly raised the level of IgG2a as compared to infected controls

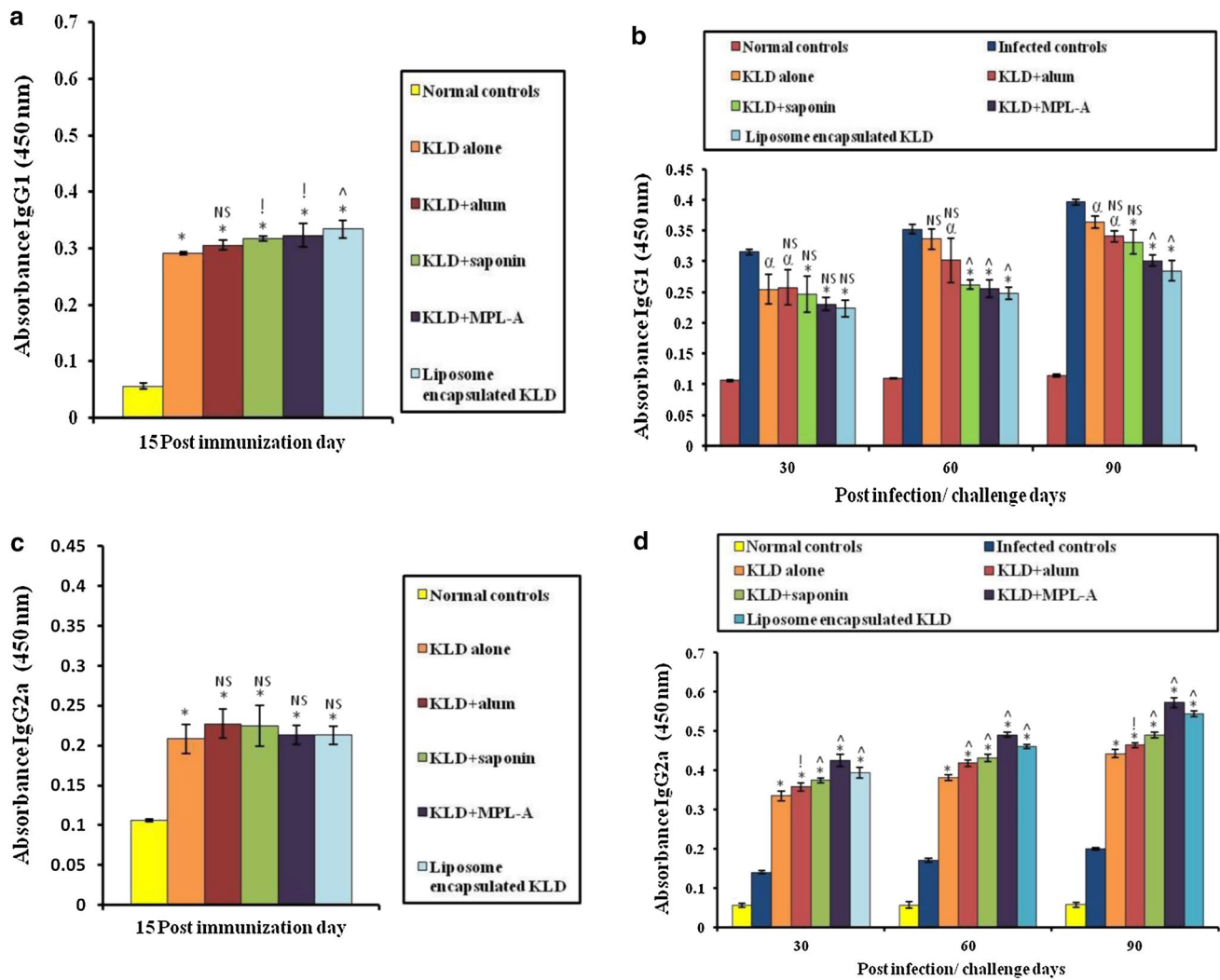


Fig. 4 Antibody level (a, b—IgG1, c, d—IgG2a) in serum samples of BALB/c mice following immunization and *L. donovani* challenge infection. The data are presented as mean \pm SD of six mice per group. α^* *p* value: Control mice versus KLD; KLD + alum;

($p < 0.001$). Inclusion of adjuvants to KLD antigen further enhanced the IgG2a production ($p < 0.001$). Peak absorbance values were observed in animals immunized with a combination of KLD + MPL-A followed by groups immunized with combination of liposome-encapsulated KLD, KLD + saponin and KLD + alum with IgG2a/IgG1 ratio 1.91, 1.9, 1.48 and 1.36, respectively (Fig. 4). All immunized groups maintained a trend of high IgG2a/IgG1 ratio till day 90 post-challenge, thus reflecting stimulation of a Th1-biased immune response.

Determination of vaccine-induced cytokine production

To get a further insight into the protective efficacy of different vaccine formulations, we cultured splenocytes on 15 day post-immunization as well as on 30, 60, 90 post-infection/challenge days. Immune responses (Th1 or Th2) induced by different

KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. $^{\Omega}$ *p* value: KLD versus KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. * *p* value < 0.001 , $^{\wedge}$ *p* value < 0.05 , NS nonsignificant

vaccine formulations were assessed by quantifying the cytokine levels (IFN- γ , IL-12, IL-4 and IL-10) in splenocyte cultures of all groups of animals. On 15th day immunization, we found that splenocytes from immunized groups secreted significantly higher cytokines as compared to normal control mice. It was interesting to note that level of Th1 cytokines in immunized groups was high as compared to Th2 cytokines.

We also wanted to assess the cytokine production after infection, and we found that immunized animals maintained high levels of Th1 cytokines over infected controls. Mice immunized with liposome-encapsulated KLD antigen elicited highest concentration of Th1 specific cytokines, i.e. IFN- γ and IL-12 (Fig. 5) followed by those immunized with a combination of KLD + MPL-A, KLD + saponin and KLD + alum. Among immunized groups, lowest levels of Th1 cytokines were found in mice immunized with KLD

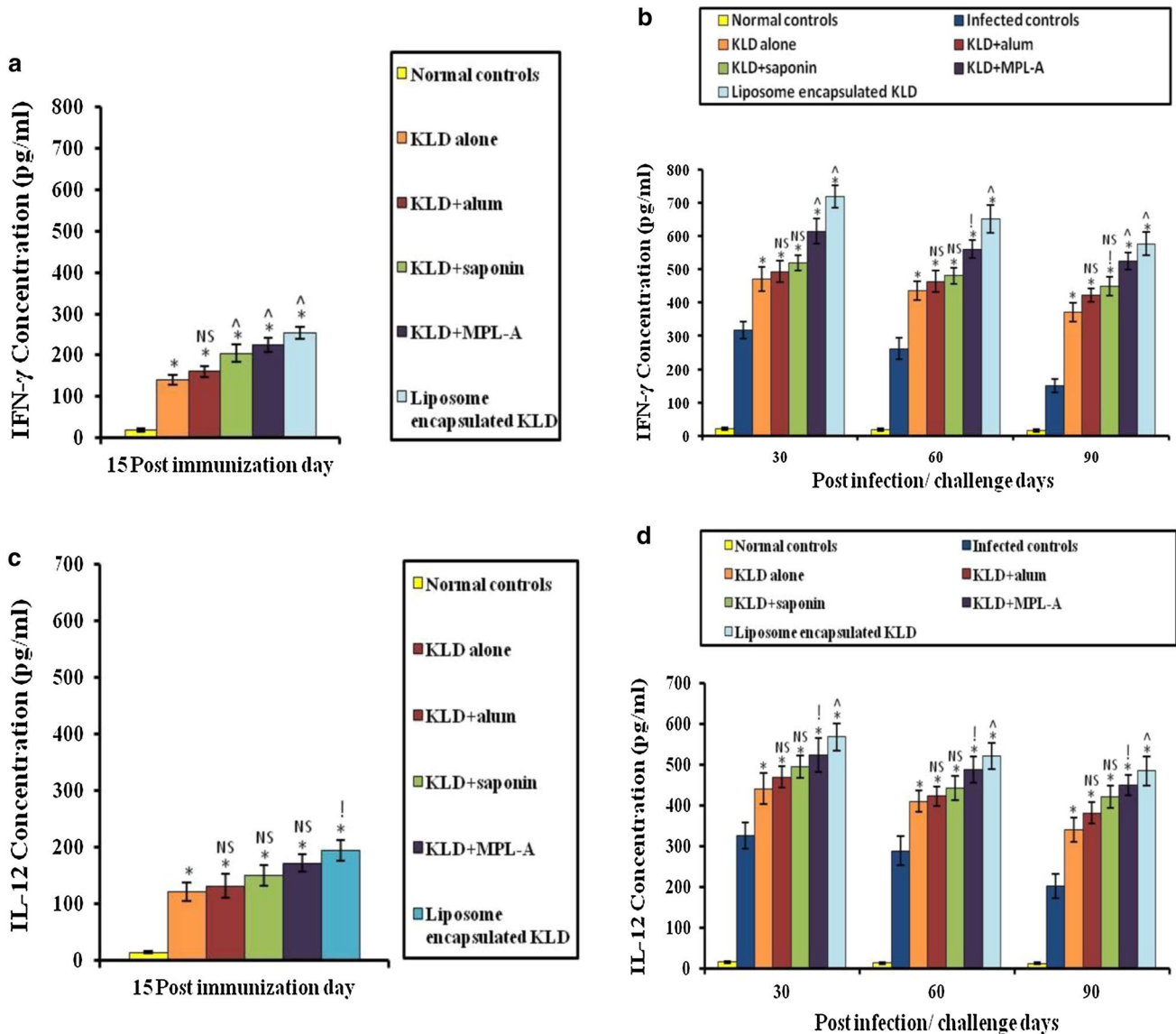


Fig. 5 IFN- γ and IL-12 concentration (a, b—IFN- γ , c, d—IL12) in BALB/c mice following immunization and *L. donovani* challenge infection. The data are presented as mean \pm SD of six mice per group. ^{*}*p* value: Control mice versus KLD; KLD + alum;

KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. ^{!^}*p* value: KLD versus KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. [^]*p* value <0.001, ^{!^}*p* value <0.05, NS nonsignificant

alone. In sum, we could say that high IFN- γ and IL-12 levels correlated with protective immunity.

On the contrary, maximum levels of Th2-regulated cytokines, i.e. IL-10 and IL-4 (Fig. 6), were observed in the infected controls. Levels of IL-10 (*p* < 0.05) and IL-4 (*p* < 0.001) were significantly lesser in immunized animals as compared to the infected controls except for mice immunized with KLD antigen where the difference was insignificant on 90th p.c.d (IL-10). Among immunized groups, peak levels of IL-4 and IL-10 were seen in groups of mice immunized with KLD antigen alone. Addition of adjuvants significantly downregulated IL-4 and IL-10 levels except

for KLD + alum where the difference was insignificant on all post-infection/challenge days (p.c.d). Minimum level of Th2 cytokines was observed in mice immunized with liposome-encapsulated KLD antigen.

We also checked IFN- γ /IL-10 ratio and found that highest IFN- γ /IL-10 ratio was found in liposome-encapsulated KLD antigen (4.61) followed by group immunized with KLD + MPL-A, KLD + saponin, KLD + alum and KLD alone with IFN- γ /IL-10 ratio 3.74, 2.57, 1.9, 1.86 respectively. The ratio implicated that a strong Th1 bias may be an important correlate of protection observed within these groups. Lowest IFN- γ /IL-10 (0.81) ratio was observed in

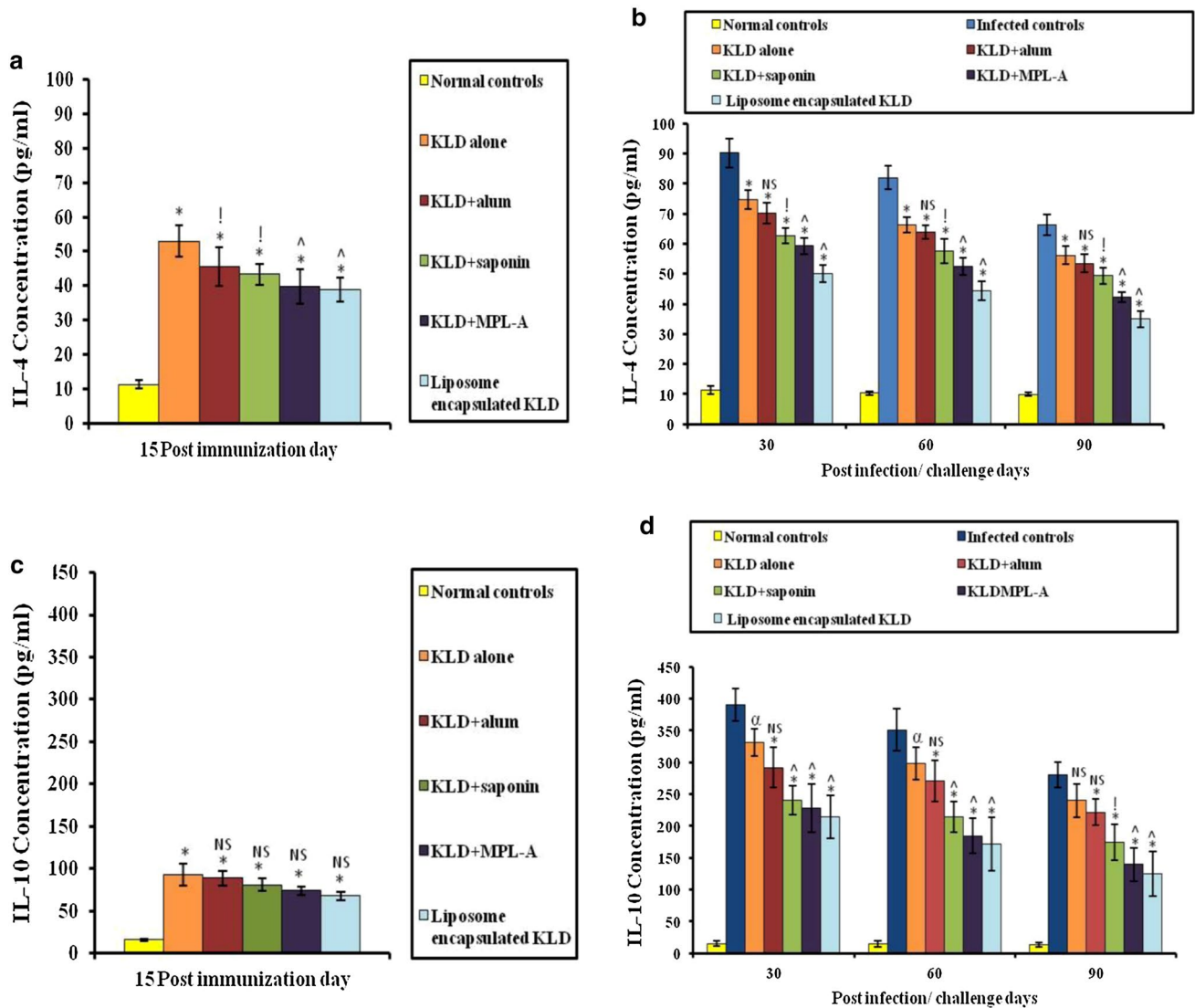


Fig. 6 IL-10 and IL-4 concentration (**a**, **b**—IL10, **c**, **d**—IL4) in BALB/c mice following immunization and *L. donovani* challenge infection. The data are presented as mean \pm SD of 6 mice per group. ^α*p* value: Control mice versus KLD; KLD + alum; KLD + saponin;

KLD + MPL-A; liposome-encapsulated KLD. [!]*p* value: KLD versus KLD + alum; KLD + saponin; KLD + MPL-A; liposome-encapsulated KLD. ^{*}*p* value <0.001, ^α*p* value <0.05, NS nonsignificant

infected controls suggesting bias towards nonprotective Th2 immune response (Fig. 7).

Discussion

Vaccination seems to be the most cost-effective way of controlling infectious diseases. Therefore, the study and designing of safe and effective vaccine regimens for clinical use remains critical. Killed vaccines offer the advantage of being safe and have been tested in clinical trials but they lack immunogenicity. Development of an effective vaccine depends not only upon selection of appropriate vaccine

candidates but also on choosing the right adjuvant or delivery vehicle. Thus, a successful anti-leishmanial vaccine will need to be assessed with human-compatible adjuvants. So the main goal of the present study was to evaluate the immunoprophylactic efficacy of different adjuvants in combination with KLD antigen. Our findings clearly indicate that all the adjuvants used in the study exerted adjuvant effects against *L. donovani* challenge infection. However, the extent of protection varied according to the type of adjuvant used.

Quantification of parasite load was done in all the groups of mice on different post-infection/challenge days. *L. donovani* infection is described by distinct organ-specific immune interactions, where liver is the site of infectious resolution.

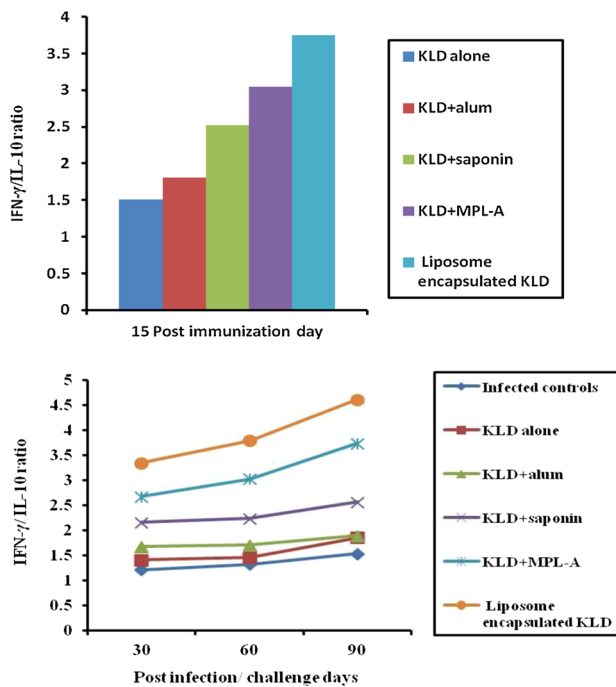


Fig. 7 IFN- γ and IL-10 ratio in BALB/c mice following immunization and *L. donovani* challenge infection

Since infection is self-limiting in BALB/c mice, maximum hepatic parasite burden was seen on 30 days post-infection/challenge in all groups of mice. After that, the parasite load declined significantly in all groups of animals. Among immunized groups, we found that mice immunized with liposome-encapsulated KLD antigen showed maximum reduction in parasite load (93.7 %). Our results are in agreement with the study of Shargh et al. [31] where cationic liposomes containing soluble *Leishmania antigen* + CpG ODNs showed significantly lower parasite burden. The antigenicity of KLD antigen in combination with MPL-A was also studied, and it was found that mice immunized with KLD + MPL-A showed a significant reduction in the parasite load. Results are in accordance with study of Nagill and Kaur [32] where 78 kDa antigen along with an adjuvant (MPL-A) induced 92 % protection against *L. donovani* infection. Several studies have demonstrated that this adjuvant either directly or indirectly stimulates the production of the T helper cell type 1 (Th1) cytokines IL-2 and IFN- γ [33], which plays a major role in elimination of parasites. Mice immunized with KLD + saponin also resulted in reduction in parasite burden. Saponin as an adjuvant can affect the generation of immune response at several levels. These may include mobilization of antigen-presenting cells to the site of injection, enhancing efficient processing of antigen, prompting the cytokine response including IFN- γ and other co-stimulatory signals needed for an optimal immune response [34, 35]. These studies underline the immunostimulatory

potential of saponin that may have led to the enhanced level of protection found in our study. So, there is much hope for a saponin-adjuvanted killed leishmanial vaccine in veterinary and clinical research. Significant level of protection was also conferred by mice immunized with KLD + alum. This is in agreement with another study which showed that vaccination with alum-precipitated autoclaved *L. major* + BCG conferred significant protection in mice [36].

Successful vaccination of animals and humans is often related to antigen-induced DTH responses in vivo and T-cell stimulation with antigen in vitro [37, 38]. In the present study, results obtained upon vaccination with killed leishmanial antigen in association with different adjuvants demonstrated induction of an appreciable DTH response suggesting the activation of cell-mediated immunity. This measure of cellular immunity did correlate well with enhanced resistance to infection in immunized groups. In our study, immunization of mice with KLD antigen alone significantly raised the DTH responses in comparison with the infected controls. The addition of adjuvants further increased the DTH responses significantly. Since the infection is associated with depressed cell-mediated immune responses, lowest DTH responses were observed in infected controls. The significantly higher DTH response induced by liposome-encapsulated KLD antigen over other adjuvant before and after challenge infection demonstrates elicitation of strong and persistent cell-mediated immunity by this vaccine. Our results are in agreement with the studies, which showed that mice immunized with liposome-encapsulated gp63 [39] induced good DTH response. Further results are in line with a previous study in which mice immunized with recombinant *Leishmania* antigen plus MPL showed significant elevation of DTH response against *L. donovani* infection [40].

Since IgG1 and IgG2 responses are strictly T cell dependent, we used them as marker to assess the overall immunogenicity of different vaccine formulations [41]. IgG1 and IgG2 subtypes have been traditionally used as surrogate markers of the Th2 and Th1 type of immune responses, respectively. Serological data showed that mice immunized with different adjuvants in combination with KLD antigen induced strong humoral responses after immunization that persisted after challenge infection. All immunized groups showed almost similar level of IgG1 antibodies. Higher levels of IgG2a antibody were detected in serum samples of immunized animals again pointing towards the control of the infection in these animals. Maximum levels of IgG2a were seen in serum samples of mice immunized with MPLA vaccine followed by liposome-encapsulated KLD antigen, KLD + saponin and KLD + alum. High antibody titres generated by KLD + alum confirm that this adjuvant is a potent inducer of antibodies in vaccines [5]. Further our results are in agreement with study of Tafaghodi et al. [42] where vaccination with ALM + QS (autoclaved *L. major* + Quillaja saponin) showed highest IgG2a/IgG1 ratio against experimental *L. major* infection.

Recovery and protection against leishmaniasis result from a strong and specific cellular immune response, followed by the development of long-lasting protection [43]. Studies have shown that control of VL infection in mice requires the development of IL-12 dependent IFN- γ production by splenic cells that drives the immune response towards a protective Th1 type [44–47] with low levels of IL-4 [48]. These inflammatory cytokines are required for the generation of reactive oxygen and nitrogen species by infected macrophages that enables killing of intracellular parasites. Similar trend of cytokine production was seen in our study as splenic T cells of immunized animals produced higher amount of IFN- γ and IL-12 with downregulation of IL-4 and IL-10. Highest level of IFN- γ and IL-12 were produced by liposome-encapsulated KLD antigen followed by group immunized with KLD + MPL-A. These findings may suggest that cationic liposomes and MPL-A are potent inducers of Th1 cytokines and thus are suitable for vaccines intended to use against intracellular pathogens. Moderate levels of IFN- γ and IL-12 were seen in mice immunized with KLD + saponin and KLD + alum. It is in agreement with a study which showed that alum-precipitated proteins can also induce CD8⁺ T cells to produce Th1-associated IFN- γ [49].

Recent advances have also been made in understanding immunoregulatory mechanisms that suppress parasite-specific CD4⁺ T-cell responses in human VL patients, and it was found that interleukin-10 produced by CD4⁺ T cells is a potential, autocrine inhibitor of IFN- γ production and promotes parasite persistence in visceral organs [50]. Our results are in accordance with this study as infected control mice displayed highest levels of interleukin-10. The negative regulatory role of IL-10 in vaccine failure is indeed well established [51]. Interestingly, it was found that after immunization IL-4 was produced in the spleen cells of all vaccinated groups. However, level of IL-4 declined significantly on 90th post-challenge day as compared to the infected controls. The evidences that early IL-4 is needed to drive Th1 differentiation [52] and to enhance IFN- γ secretion [53, 54] imply that early IL-4 production does not hinder the Th1 response. It is in line with observations revealing that IL-4 is crucial for the priming of long-term CD8⁺ T-cell memory responses [55–57].

Moreover, previous studies have shown that SSG chemotherapy of *L. donovani* in IL-4^{-/-} [58] or IL-13^{-/-} [59] BALB/c mice compared with wild-type mice resulted in downregulated IFN- γ production and increased IL-10 production. This suggests a requirement for IL-4 and IL-13 in instructing a type-1 response. Another study has also shown that successful immunotherapeutic intervention in the BALB/c mouse using a hybrid cell vaccine was associated not only with downregulation of IL-10 but also with significantly enhanced IL-4 and IL-13 expression as well as IFN- γ production [60] providing further circumstantial

evidence for these Th2 cytokines in facilitating the generation of type-1 immunity.

Thus, in the present study, it was found that all adjuvanted killed antigen formulations were immunogenic and imparted significant protection. Protective efficacy of cationic liposomes as an adjuvant was superior when compared to alum and saponin and it was comparable to MPL-A. Liposome vaccine technology has advanced in recent years, and now several vaccines containing liposome-based adjuvants have been approved for human use or have reached late stages of clinical evaluation [61]. In contrast to a report [62] preferring intra-peritoneal route for liposome-encapsulated antigen, our liposomal formulation given through subcutaneous route was effective. Subcutaneous route is always considered as a slow release route in case of antigen presentation, allowing the body to generate enough resources to counter the invading microorganisms. Moreover, it is a compatible route for human vaccination. Our findings correlate very well with these assumptions. High levels of Th1 cytokines and IgG2a antibody were seen in group immunized with KLD + MPL-A. It is well known that MPL acts upon TLR-4, promoting the maturation of APCs. Ultimately, activation of TLR-4 not only triggers innate immunity, but also enhances both humoral and cellular immune responses [63, 64]. Recently, Mutiso et al. [65] indicated good safety levels of a vaccine comprising *L. donovani* sonicate antigen delivered with either monophosphoryl lipid A or montanide ISA 720 in the vervet monkey model. Saponins are natural glycosides of steroid or triterpene, which are known to activate immune system. From our results, we found that vaccine formulation composed of KLD + saponin also showed promising results. We observed that vaccine formulation comprising KLD + alum also induced protection, although it was low when compared with other adjuvants. Therefore, in order to improve efficacy of this vaccine, it is better to use alum with another Th1-promoting adjuvant.

Conclusion

Most studies have focussed on the use of autoclaved vaccine formulations with or without adjuvants against VL, and few studies are available on the use of formalin-killed antigens. The present study highlights the greater efficacy of formalin-fixed KLD antigen as potential vaccine candidate along with promising role of cationic liposome and MPL-A as adjuvants to enhance stronger immune response against murine VL. Considering the easy preparation of killed antigen vaccines, their lower production cost and promising results, this approach might prove to be useful in controlling leishmaniasis in endemic areas, particularly in developing countries. For future perspective, more studies are required in higher animal models in order to ensure the efficacy of above vaccine formulations.

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Conflict of interest The authors hereby declare that they have no conflicts of interest.

Ethical standard The authors hereby declare that the experiments comply with the current laws in India.

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