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Vaccination with Liposomal Leishmanial Antigens Adjuvanted with Monophosphoryl Lipid–Trehalose Dicorynomycolate (MPL-TDM) Confers Long-Term Protection against Visceral Leishmaniasis through a Human Administrable Route

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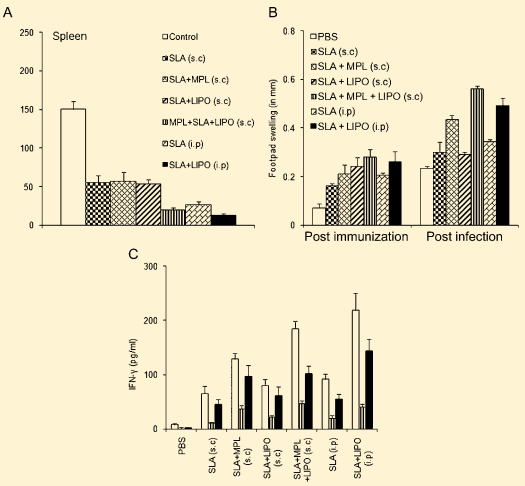
ABSTRACT: The development of a long-term protective subunit vaccine against visceral leishmaniasis depends on antigens and adjuvants that can induce an appropriate immune response. The immunization of leishmanial antigens alone shows limited efficacy in the absence of an appropriate adjuvant. Earlier we demonstrated sustained protection against *Leishmania donovani* with leishmanial antigens entrapped in cationic liposomes through an intraperitoneal route. However, this route is not applicable for human administration. Herein, we therefore evaluated the immune response and protection induced by liposomal soluble leishmanial antigen (SLA) formulated with monophosphoryl lipid–trehalose dicorynomycolate (MPL-TDM) through a subcutaneous route. Subcutaneous immunization of BALB/c mice with SLA entrapped in liposomes or with MPL-TDM elicited partial protection against experimental visceral leishmaniasis. In contrast, liposomal SLA adjuvanted with MPL-TDM induced significantly higher levels of protection in liver and spleen in BALB/c mice challenged 10 days post-vaccination. Protection conferred by this formulation was sustained up to 12 weeks of immunization, and infection was controlled for at least 4 months of the challenge, similar to liposomal SLA immunization administered intraperitoneally. An analysis of cellular immune responses of liposomal SLA + MPL-TDM immunized mice demonstrated the induction of IFN-γ and IgG2a antibody production not only 10 days or 12 weeks post-vaccination but also 4 months after the challenge infection and a down regulation of IL-4 production after infection. Moreover, long-term immunity elicited by this formulation was associated with IFN-γ production also by $CD8^+$ T cells. Taken together, our results suggest that liposomal SLA + MPL-TDM represent a good vaccine formulation for the induction of durable protection against *L. donovani* through a human administrable route.



KEYWORDS: *cationic liposomes, leishmanial antigen, MPL-TDM, long-term protection, visceral leishmaniasis, adjuvants*

INTRODUCTION

Protozoan parasites of the genus *Leishmania* cause a broad spectrum disease complex known as leishmaniasis which occurs predominantly in tropical and subtropical regions. Clinical manifestations range from self-limiting cutaneous leishmaniasis to life-threatening visceral leishmaniasis. Visceral leishmaniasis is a chronic and progressive systemic disease characterized by fever, weight loss, and hepatosplenomegaly.[15] Clinical and experimental evidence suggests that a cell-mediated immune response is responsible for the control and resolution of leishmaniasis.[35,36] Recovery from natural or experimental infection confers immunity to reinfection[45,53] and strongly suggests that control of leishmaniasis by vaccination is possible. Thus, the development of a safe and effective *Leishmania* vaccine could prevent new cases of leishmaniasis worldwide.

Investigations of the immune protection mechanism against *Leishmania* spp. revealed that a Th1 type cell response and IFN-γ production are important to resist infection whereas the Th2 cell response favors the disease. To date, there is no effective long-lasting vaccine system for the control of leishmaniasis except for the use of live virulent parasite vaccines (leishmanization).[37] It is widely believed that the persistence of parasites at the site of infection is critical for the maintenance of established antileishmanial immunity.[5,54] However, due to reports of adverse reaction and safety issues this protocol of immunization has fallen out of favor. As an alternative various leishmanial antigens from crude to defined proteins have been used for vaccination against various form of leishmaniasis.[19,22,28] Still there is no successful vaccine available against the disease due to the poor immunogenecity of the protein antigens.[16] Using immunopotentiating adjuvants such

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as rIL-12, CpG, and MPL, immunogenecity could be enhanced, resulting in a robust immune response to provide significant protection against subsequent challenge infection.[8,25,32,44,48,58] However, these vaccines were unable to generate a sustained immune response for long-term protection.[25,26,44] It has recently been observed that repeated immunization of leishmanial antigens can generate and maintain antileishmanial effector (and/or effector memory-like) T-cells.[21,52] Moreover, the persistent presence of these antigens could confer protection to levels achieved so far only with live parasites. While adjuvants like rIL-12, CpG, and MPL can promote strong Th1 type immune responses, delivery vehicle like liposomes allow the slow release of encapsulated antigens. So, we speculate that a combination of a liposomal antigen with such an adjuvant could promote the longevity of the *Leishmania*-specific effector and memory T cell responses.

Liposomes are lipid−bilayer membranes capable of encapsulating antigens and act as an efficient slow-releasing antigen delivery vehicle with depository effects.[4] They have emerged as a promising adjuvant system with low toxicity, can protect antigens from damage, and are capable of enhancing the uptake and presentation of encapsulated antigens by antigen-presenting cells through MHC II pathways.[29] The adjuvant effect of liposomes depends mainly upon the surface charges of the vesicles.[50,51] Recent studies showed that cationic or positively charged liposomes are more suitable in comparison to anionic or neutrally charged liposomes due to their ability to elicit strong humoral as well as cell-mediated immune responses through the activation of MHC-I and MHC-II pathways by the activation of both $CD8^+$ and $CD4^+$ T-cells, respectively.[17,27,49−51] Our earlier studies reported long-term protective immune responses using cationic liposome encapsulated leishmanial antigen[11] and gp63[12] against the challenge with *Leishmania donovani* infection. But the intraperitoneal route of immunization employed for vaccination in these studies is not the preferred route for human administration. The administration of liposomal leishmanial antigen through subcutaneous route however failed to elicit protection.[10] Therefore, there is a need for a formulation which can trigger immune responses equivalently through the clinically relevant route.

A recent approach for generating sustained cellular immunity *in vivo* is the use of toll-like receptor agonists as vaccine adjuvants in association with liposomes. Monophosphoryl lipid (MPL) signals via TLR-4 and is generally reported to promote IFN-γ production by Ag specific $CD4^+$ T-cells to enhance the immune response toward a Th1 profile.[14] MPL administered through a subcutaneous route has also been found to be successful for vaccination against leishmaniasis.[18] To optimize the route of immunization we used soluble leishmanial antigens (SLA) which are more immunogenic than *L. donovani* promastigote antigens (LAg).[9,13] Thus, to fulfill the need for a vaccine that can be immunized through a route safe for humans, we investigated the effectiveness of monophosphoryl lipid−trehalose dicorynomycolate (MPL-TDM) on SLA in its free as well as liposome-encapsulated form to impart short- and long-term protection using a well-established live *L. donovani* infection model.

MATERIALS AND METHODS

Animals and Parasites. BALB/c mice 4−6 week old reared in the institute facilities under pathogen-free conditions were used for the experiment with prior approval from the Animal Ethics Committee of Indian Institute of Chemical Biology. *L. donovani* strain AG83 (MHOM/IN/1983/AG83) was cultured as promastigotes at 22 °C in medium 199 supplemented with penicillin G sodium (100 U/mL), streptomycin sulfate (100 μg/mL), and 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO).[2]

Preparation of Antigens. Soluble leishmanial antigen (SLA) extracted from *L. donovani* promastigotes membranes were prepared as described earlier.[2] Briefly, stationary-phase promastigotes, harvested after the third or fourth passage, were washed four times in cold 20 mM phosphate buffered saline (PBS), pH 7.2 and resuspended at a concentration of 1.0 g of cell pellet in 50 mL of cold 5 mM Tris−HCl buffer (pH 7.6), lysis buffer, containing 5 μg of leupeptin/mL, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide (Sigma-Aldrich). The suspension was vortexed and centrifuged at 2310 *g* for 10 min. The membrane pellet was resuspended in 10 mL of lysis buffer and sonicated three times for 1 min each in an ultrasonicator (Misonix, Farmingdale, NY). The suspension thus obtained was solubilized with 1% (w/v) octyl-β-D-glucopyranoside (Sigma-Aldrich) in lysis buffer with overnight incubation at 4 °C and was finally centrifuged for 1 h at 100 000 *g*. The supernatant containing SLA was then dialyzed against 1 mM Tris-HCl buffer (pH 7.6) and stored at −70 °C until use. The amount of SLA obtained from 1.0 g cell pellet was approximately 2 mg, assayed by the method of Lowry et al.[38]

Entrapment of Soluble Leishmanial Antigens (SLA) in Liposomes. Liposomes were prepared with egg lecithin, cholesterol (Sigma-Aldrich), and stearylamine (Fluka, Buchs, Switzerland) at a molar ratio of 7:2:2 as described previously.[2] Empty and SLA-containing liposomes were prepared by the dispersion of lipid film in 1 mL PBS alone or containing 1 mg/mL of SLA. The mixture was then vortexed and the suspension sonicated for 30 s in an ultrasonicator (Misonix, Farmingdale, NY). Liposomes with entrapped antigen were separated from excess free antigen by three successive washings in PBS with centrifugation at 105 000 *g* for 60 min at 4 °C. The protein amount entrapped in liposomes was estimated by Lowry's method using BSA as a standard in the presence of 0.8% SDS and appropriate blanks. The liposomal phospholipid content was 15.5 mg/mL as determined using the Stewart assay.[62] The amount of SLA associated with per milligrams of egg lecithin was 35 μg.

Measurement of Size and Zeta Potential of Liposomes. The mean diameter and zeta potential of liposomes were measured at room temperature by photon correlation spectroscopy (PCS) on Nano Zs ZetaSizer (Malvern Instruments, Worcestershire, UK) by diluting the dispersion to the appropriate volume in doubly filtered (0.22 μm pore size) distilled water. The polydispersity index was used as a measure of the size distribution of the liposomes. A polydispersity index value of 0.0 represents a homogeneous particle population, while a value of 1.0 indicates the heterogeneity of the liposome preparations.

Immunization of Mice and Challenge Infection. BALB/c mice (eight animals/group) were immunized subcutaneously (into the lower left or right quadrant of abdomen) or intraperitoneally twice with 15 μg of free or SLA entrapped in liposomes. In parallel, groups of mice were immunized subcutaneously with MPL-TDM (purchased from Sigma-Aldrich Corp., St. Louis, MO) mixed with 15 μg of SLA or

SLA in liposomes. Animals receiving PBS served as controls. Briefly, each vial of MPL-TDM (2 mL) was reconstituted with 1 mL of saline and mixed with 1 mL of free SLA or SLA in liposomes. The booster immunization was given on day 22. In the short-term case , 10 days, and in the long-term case, 12 weeks after the last booster, and 4 months after infection (for both short-and long-term), serum samples were collected, and spleens were removed aseptically for the analysis of humoral and cellular responses. Immunized animals were challenged intravenously with 2×10^7 freshly transformed promastigotes 10 days and 12 weeks after last booster, for short- and long-term studies, respectively. After 4 months of challenge infection, mice were sacrificed to determine the parasite load in liver and spleen.[12] Infection was monitored by the microscopic examination of Giemsa-stained impression smears of the liver and spleen. The parasite load was expressed as Leishman−Donovan units and was calculated by the following formula: number of amastigotes per 1000 cell nuclei × organ weight (mg).

Assessment of Delayed Type Hypersensitivity Response (DTH). The delayed type hypersensitivity (DTH) was determined as an index of cell-mediated immunity as described earlier. The responses were evaluated at 24 h by measuring the difference in hind footpad swelling following intradermal inoculation of the test footpad with 50 μL of SLA (800 μg/mL) and the control footpad with 50 μL of PBS using a constant pressure calliper (Starrett Company, Athol, MA).

Antibody Responses. Sera from immunized and infected animals were analyzed by an enzyme-linked immunosorbent assay (ELISA) for the presence of antigen specific antibodies. In brief, 96-well microtiter plates (Maxisorp, Nunc) were coated with SLA (15 μg/mL) in 20 mM phosphate buffer (pH 7.5) overnight at 4 °C. The plates were blocked with 1% BSA in PBS at room temperature for 3 h to prevent nonspecific binding. After washing with PBS containing 0.05% Tween-20 (Sigma-Aldrich) (PBST), the plates were incubated overnight with 1:1000 dilution of mice sera at 4 °C. Next day, the plates were washed again with PBST and incubated further for 3 h at room temperature with peroxidase-conjugated goat antibody directed against mouse IgG, IgG1, and IgG2a (Sigma) at 1:5000 dilution in blocking buffer. The plates were washed, and 100 μL of substrate solution (*o*-phenylene diamine dihydrochloride, 0.8 mg/mL in 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.04% H_2O_2) was added for 30 min and the absorbance read in an ELISA plate reader (Thermo, Waltham, MA) at 450 nm.[1]

***In Vitro* Production of IFN-γ and IL-4 from Splenocytes of Immunized and Infected Mice.** Spleens were removed aseptically from individual mice of each group at 10 days or 12 weeks after vaccination and at 4 months post-infection. Each spleen was filtered through a 20 μm pore-size sieve for preparation of a single-cell suspension in complete medium of RPMI 1640 containing 10 mM $NaHCO_3$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin (100 U/mL), streptomycin (100 μg/mL), 50 μM β-mercaptoethanol, and 10% FBS. Erythrocytes were removed by lysis with 0.14 M Tris-buffered NH_4Cl. The splenocytes were washed twice and resuspended in the culture medium, and viable mononuclear cell numbers were determined by trypan blue exclusion. Aliquots of viable cells were incubated with anti-$CD4^+$ (GK 1.5, ATCC TIB 207) and anti-$CD8^+$ (53-6.72 ATCC TIB 105) monoclonal antibodies or the respective control IgGs at a concentration of 15 μg/mL for 1 h at 4 °C and washed twice in complete medium. Total and CD4- or CD8-depleted splenocytes were stimulated *in vitro* with medium alone or SLA (10 μg/mL) for 72 h. The supernatants collected were stored at −70 °C for cytokine analysis. The measurement of IFN-γ and IL-4 levels was carried out as detailed in the instructions supplied with the cytokine ELISA kit (BD Biosciences).

Statistical Analysis. Data are represented as the mean ± standard error of mean. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test were used for the analysis of data using Prism-Graphpad version 5.0 (Graphpad Software, v.5.0, SanDiego, CA). *P* values of <0.05 were considered to be statistically significant.

RESULTS

Characterization of Liposomes. The mean size of SLA entrapped liposomes was 331.7 ± 20.61 nm as determined by Zetasizer Nano-ZS (Malvern Instruments). The presence of antigen could not influence the size of the vesicles (empty vesicle size 286.7 ± 27.09 nm). The polydispersity index of empty (0.714 ± 0.12) and SLA incorporated (0.705 ± 0.1) liposomes indicate the presence of a heterogeneous population. The observed zeta potential of empty (38.60 ± 3.86 mV) and SLA encapsulated liposomes (43.97 ± 2.59 mV) revealed that both the formulations carry a positive charge. The protein (SLA) content encapsulated into liposomes was 61.67% as estimated by the method described by Lowry et al.[38] (Table 1).

Table 1. Particle Size, Zeta Potential, and SLA Incorporation in Liposomes[a]

formulations	vesicle size (nm)	zeta potential (mV)	SLA incorporation (% of total)
only liposomes	286.7 ± 27.09	38.60 ± 3.86	
liposomal SLA	331.7 ± 20.61	43.97 ± 2.59	61.67 ± 6.7

[a]Vesicles were formulated with egg lecithin, cholesterol, and stearylamine at a molar ratio of 7:2:2 either alone or in combination with SLA. Results represent mean ± S.E. of three independent liposome preparations.

Immunization against Experimental Visceral Leishmaniasis with Liposomal SLA Reconstituted with MPL-TDM through Subcutaneous Route Confers Durable Protection. Earlier we had reported that liposomal SLA was able to impart strong protection against hepatic (93%) and splenic (98%) parasitic burden after 4 months of infection with *L. donovani* promastigotes.[13] But the results were obtained by intraperitoneal immunization. To overcome the need for intraperitoneal immunization we, in the present study, investigated the effectiveness of the adjuvant activity of MPL-TDM with SLA in its free as well as liposome encapsulated form to impart short- and long-term protection against challenge infection through subcutaneous route. BALB/c mice were therefore immunized subcutaneously with either free SLA, SLA entrapped in cationic liposomes, SLA + MPL-TDM alone, or liposomal SLA with MPL-TDM to compare their efficacies with intraperitoneal immunization with free and SLA in liposome to protect against challenge infection with *L. donovani* promastigotes 10 days and 12 weeks after the last immunization. Control mice were injected with PBS alone. We and others have found that infection with the Indian strain *L. donovani* AG83 in BALB/c mice results in a progressive

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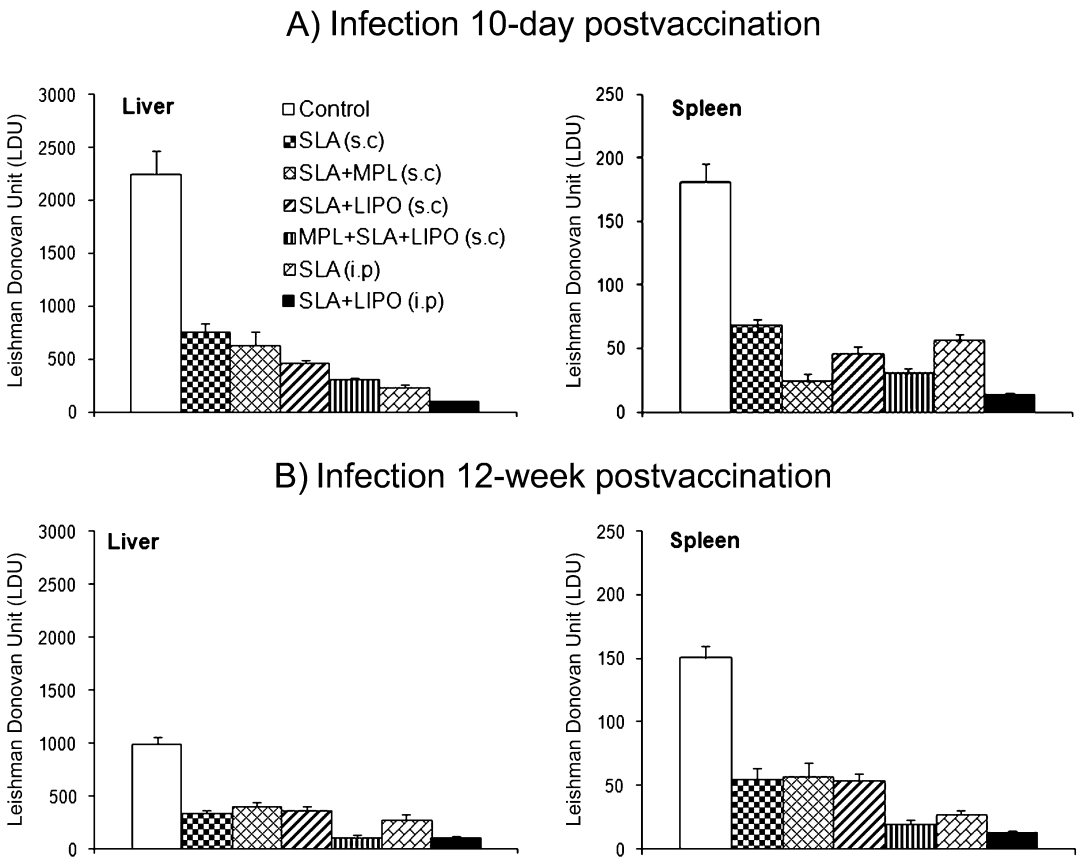

Figure 1. Evaluation of sustained protective immunity in BALB/c mice elicited by liposomal SLA + MPL-TDM through a subcutaneous route against visceral leishmaniasis. Mice were infected intravenously with 2×10^7 freshly transformed *L. donovani* promastigotes either 10 days (A) or 12 weeks (B) after vaccination with free SLA or SLA entrapped in cationic liposomes through intraperitoneal or subcutaneous route, MPL-TDM mixed with SLA alone or SLA entrapped in cationic liposomes through a subcutaneous route. Control mice received only PBS. The parasite burden in liver and spleen were determined 4 months post-infection. Data represent mean LDU ± SE for four individual animals.

infection in the liver and spleen, corresponding with hepato and splenomegaly.[9,61] Mice were therefore sacrificed after 4 months of infection when parasite loads were well-expressed in both liver and spleen. Control mice had high levels of liver and spleen parasite burden. Immunization with the free form of SLA injected either through subcutaneous or intraperitoneal route could confer significant ($P < 0.001$) protection to 66% and 90% in the liver and 62% and 69% in the spleen, respectively, in comparison to controls (Figure 1A). Subcutaneous immunization of liposomal SLA and SLA + MPL-TDM could marginally enhance the protection imparted by subcutaneous immunization of free SLA in both liver and spleen. Liposomal SLA + MPL-TDM, however, could induce higher levels of protection (86% in liver and 83% in spleen), and the results were significant not only in comparison to controls ($P < 0.001$) but also to the level of protection induced in spleen by subcutaneously immunized free SLA in the short term study (Figure 1B). Though mice immunized with liposomal SLA through intraperitoneal route showed the highest level of protection in both liver and spleen, interestingly, the difference with liposomal SLA + MPL-TDM was not statistically significant in both organs. Mice immunized with SLA, SLA + MPL-TDM, and liposomal SLA through a subcutaneous route and challenged 12 weeks later induced similar levels of protection in both liver (65, 59, and 64%) and spleen (62, 61, and 64%), respectively, which were lower than that induced in short-term immunized mice (Figure 1A and B). Mice immunized subcutaneously with liposomal SLA + MPL-TDM and challenged for long-term protection, however, demonstrated higher levels of protection (89% vs 86%) in the liver and (87% vs 83%) in the spleen in comparison to the short-term protection (Figure 1A and B). More importantly, the level of protection induced by this vaccine was not only comparable to that induced by intraperitoneal immunization of liposomal SLA (89% for both in liver, 87 and 91%, respectively, in spleen), but also the difference was insignificant. Thus our data demonstrated that vaccination with liposomal SLA + MPL-TDM formulation through the subcutaneous route could induce sustained and long-term protection in both liver and spleen similar to that induced by intraperitoneal immunization of liposomal SLA.

Vaccination with Liposomal SLA Reconstituted with MPL-TDM through a Subcutaneous Route Confers Durable DTH Responses in Immunized Animals. The cure for visceral leishmaniasis is generally associated with the acquisition of a DTH response and, consequently, a classical cell-mediated immunity.[3] Hence, to investigate the possible DTH responses induced, BALB/c mice were immunized through intraperitoneal or subcutaneous routes with free SLA or in combination with liposome and/or MPL-TDM as an adjuvant. The DTH responses elicited by all of the immunized groups apart from SLA injected subcutaneously exhibited significantly higher levels ($P < 0.05$) of protection in comparison to the PBS immunized control animals. The

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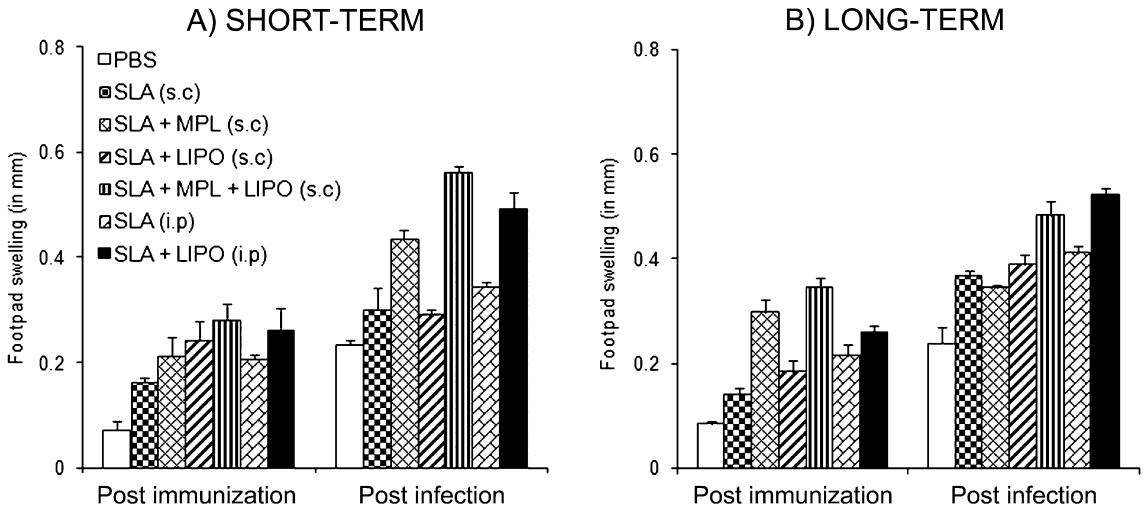

Figure 2. DTH responses in BALB/c mice post-immunization and post-infection in short-term (A) and long-term (B) studies. Groups of four mice were immunized intraperitoneally or subcutaneously with free SLA or SLA entrapped in cationic liposomes, MPL-TDM mixed with SLA alone, or SLA entrapped in cationic liposomes through a subcutaneous route; control mice received only PBS. Ten days, short-term protection (A), or 12 weeks, long-term protection (B), after the last immunization, and 4 months post-infection SLA-specific DTH responses were measured. The response is expressed as the difference (in mm) between the thickness of the test (SLA-injected) and control (PBS-injected) footpads at 24 h. The bars represent the footpad thickness ± standard error of means.

highest and significant levels of DTH were, however, exhibited by mice immunized with liposomal SLA injected either through subcutaneously or intraperitoneally or in combination with MPL-TDM injected subcutaneously (Figure 2A). Following infection the DTH responses increased in all groups. The responses in liposomal SLA + MPL-TDM injected mice were highest and statistically significant compared to infected controls ($P < 0.001$), as well as to subcutaneous and intraperitoneal immunization of free SLA, subcutaneous immunization of SLA + MPL-TDM ($P < 0.01$), and liposomal SLA ($P < 0.001$). The strong DTH response elicited by the liposomal SLA + MPL-TDM correlating with enhanced protection especially in the spleen of these animals (Figure 2A). In the long term study recall responses were evaluated 12 weeks after vaccination and at 4 months after challenge infection. Immunization with the different formulations of SLA could elicit DTH responses before and following infection. However, mice immunized subcutaneously with liposomal SLA + MPL-TDM showed the strongest elicitation of recall responses which persisted even after 4 months of infection (Figure 2B). Following infection, the responses in all groups increased. Still the DTH response elicited by the animals immunized subcutaneously with liposomal SLA + MPL-TDM were significantly higher ($P < 0.01$) than all of the other immunization groups and were almost equal to intraperitoneal immunization of liposomal SLA. These results indicate the persistence of cellular responses following short- and long-term immunization which correlated with the highest levels of protection in these groups (Figure 1A,B).

Humoral Responses in Vaccinated Mice. To understand whether the protection afforded by subcutaneous immunization of liposomal SLA + MPL-TDM was also associated with the elicitation of a humoral response, we assayed the leishmanial antigen specific total IgG in sera from mice 10 days and 12 weeks after immunization, and 4 months after infection for both short- and long-term studies, respectively. Following immunization, the levels of antigen-specific IgG, IgG1, and IgG2a were high in animals immunized subcutaneously with liposomal SLA + MPL-TDM in both short- and long-term studies and were comparable to those immunized intraperitoneally with liposomal SLA (Figure 3A,B). The responses of IgG, IgG1, and IgG2a in liposomal SLA + MPL-TDM (s.c.) immunized animals were significantly higher, not only in comparison to the PBS controls, but also to all other subcutaneous immunizations ($P < 0.05$) in the short term. Although in the long term the significant difference between liposomal SLA + MPL-TDM (s.c.) and other subcutaneous immunization groups was restricted to IgG2a, these results indicate that the liposomal SLA + MPL-TDM are capable of inducing strong, sustained, and long-term humoral responses following subcutaneous immunization.

Following infection there was no evident difference in the total IgG response between the different groups (Figure 3A,B) in short- and long-term studies. However, parasite infection induced differential elevation of both IgG1 and IgG2a isotypes in all of the groups. Interestingly, mice immunized subcutaneously with liposomal SLA + MPL-TDM in a short-term investigation showed a decrease in IgG1 and an increase in IgG2a levels which become significant in long-term immunized animals in comparison to all of the other subcutaneously immunized groups ($P < 0.05$). The IgG2a level resulted with subcutaneous immunization of liposomal SLA + MPL-TDM were similar to the IgG2a levels obtained with intraperitoneally immunized liposomal SLA. These results were reflected in IgG2a/IgG1 ratios (Figure 3A,B), which demonstrated that mice immunized subcutaneously with liposomal SLA + MPL-TDM showed significantly higher ($P < 0.05$) ratios than subcutaneous immunization with SLA and SLA + MPL-TDM in the short term as well as against subcutaneous immunization with liposomal SLA in long-term, at 4 months post-infection. Overall, these results indicate that the immunization with liposomal SLA + MPL-TDM through a subcutaneous route stimulates a strong and durable Th-1 type response and is comparable with intraperitoneal immunization of liposomal SLA.

$CD4^+$ and $CD8^+$ T Cell Responses in Immunized Animals. It is well-established that MHC class II restricted $CD4^+$ T cells are dominant during the development of

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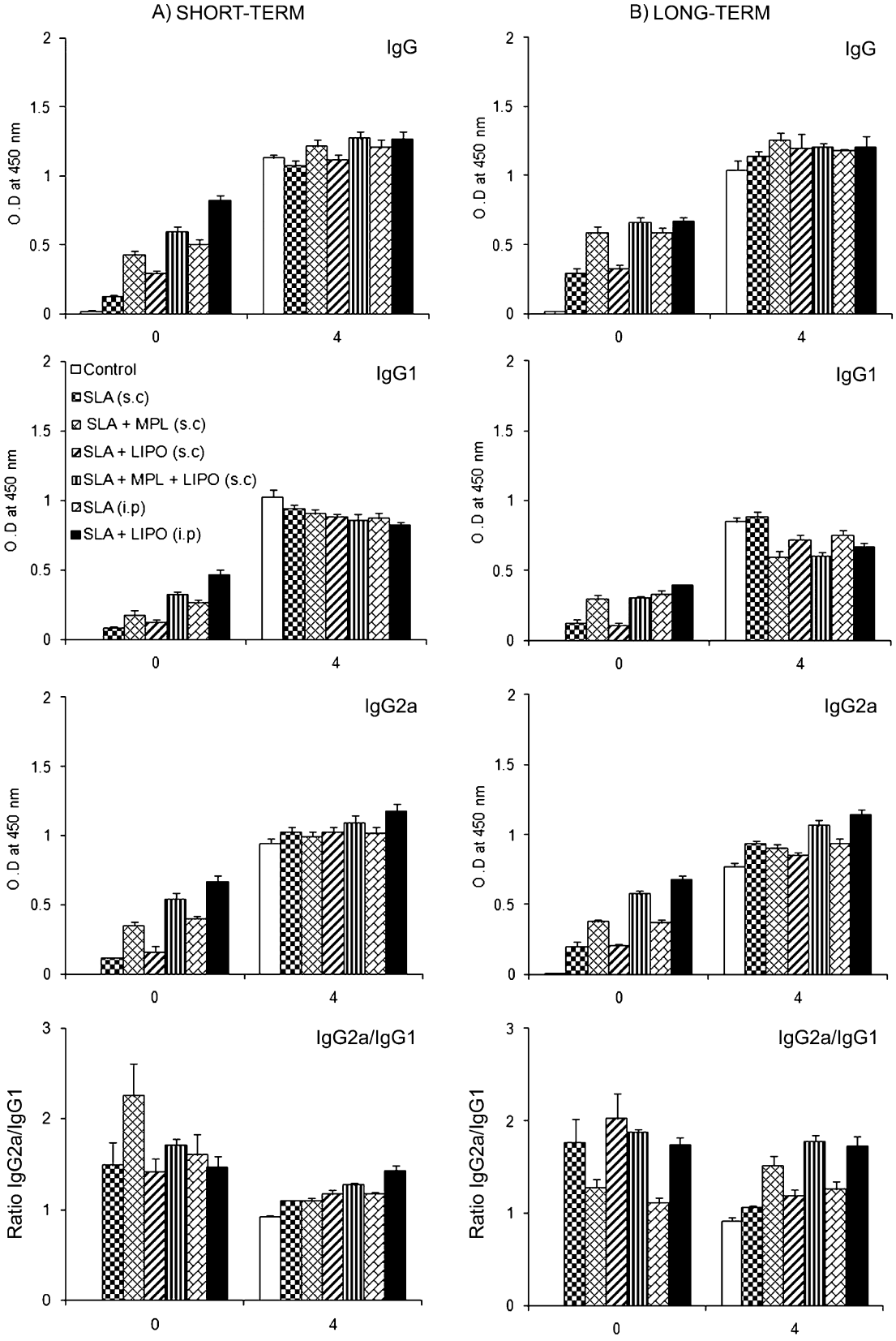

Figure 3. SLA-specific IgG, IgG1, and IgG2a production in sera of BALB/c mice against visceral leishmaniasis in short-term and long-term studies. Microtiter plates coated with SLA were incubated overnight with 1:5000 (IgG, IgG1, and IgG2a) dilution of sera from groups of four mice immunized with free SLA or SLA entrapped in cationic liposomes intraperitoneally or subcutaneously, MPL-TDM mixed with SLA alone, or SLA entrapped in cationic liposomes through a subcutaneous route. Control mice received only PBS. Sera were collected 10 days after immunization and 4 months after infection for short-term protection (A) and 12 weeks after immunization and 4 months after infection for long-term protection (B). The ELISA was carried out as described in Materials and Methods. Data are presented as the mean absorbance at 450 nm ± SE.

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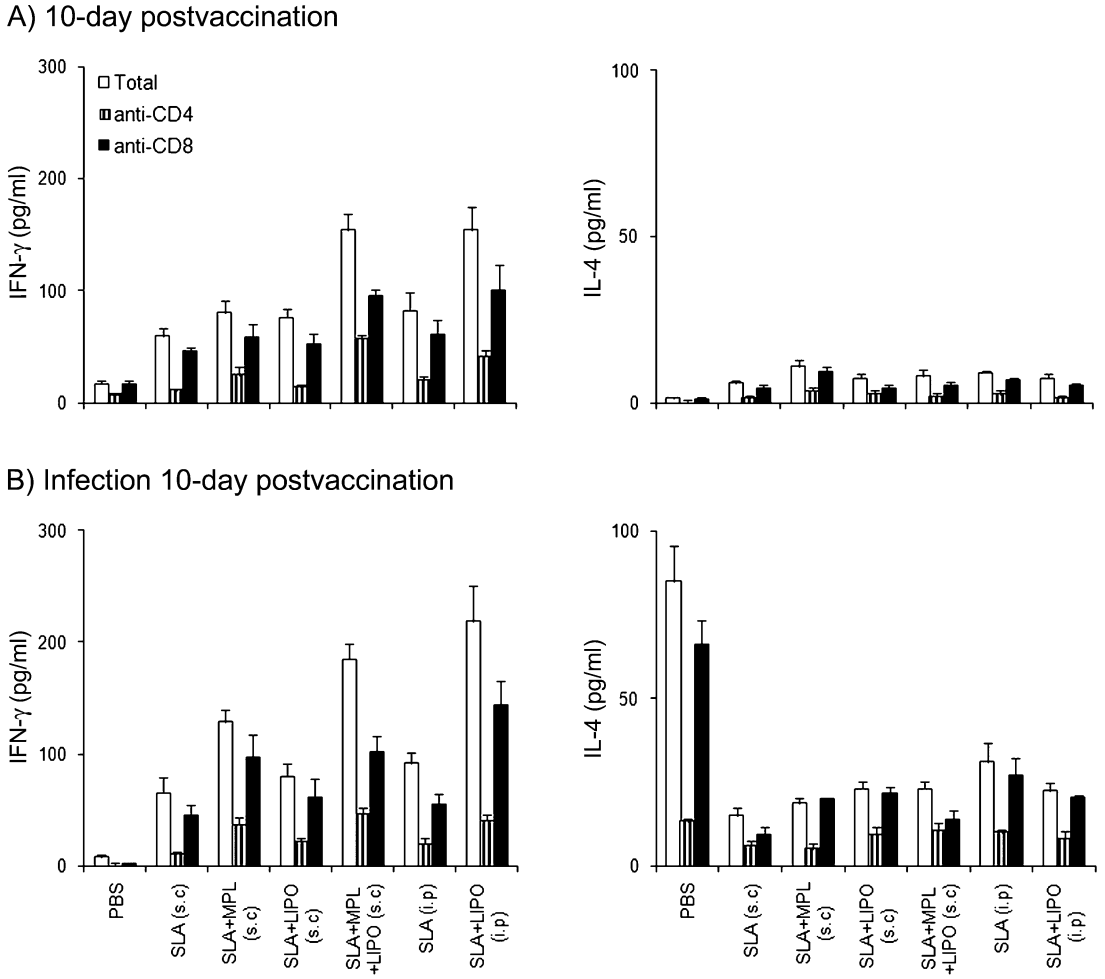

Figure 4. IFN-γ and IL-4 responses against *L. donovani* in vaccinated BALB/c mice with different formulations. Ten days after immunization and 4 months after challenge infection, splenocytes were harvested from vaccinated mice, stimulated with SLA (10 μg/mL), and cultured for 72 h. The supernatants were collected and assayed for IFN-γ and IL-4 through ELISA. Figures represent *in vitro* blocking experiments and production of IFN-γ and IL-4 from $CD4^+$ or $CD8^+$ T cells before (A) and after (B) *L. donovani* infection. Data represent the mean of triplicate wells ± SE of four individual mice per group.

immunity against the *Leishmania* species. Despite these findings a few studies do, however, point to a role for $CD8^+$ cells as essential in immunity to primary infection with *Leishmania major*[6] and also for the induction of long-term vaccine induced resistance against many intracellular pathogens.[59] As an immune correlate of biological protection against experimental visceral leishmaniasis, the incidence of antigen specific $CD4^+$ and $CD8^+$ cells producing cytokines was determined following 10 days and 12 weeks after immunization and 4 months following infection, respectively, for short- and long-term studies. In all experiments splenocytes isolated from mice were blocked *in vitro* with anti-$CD4^+$ or anti-$CD8^+$ monoclonal antibodies, and levels of IFN-γ and IL-4 in culture supernatants were measured following restimulation with SLA.

Ten days and 12 weeks post vaccination, splenocytes from animals immunized subcutaneously with liposomal SLA + MPL-TDM and liposomal SLA immunized intraperitoneally secreted significantly higher levels of IFN-γ not only in comparison to the PBS controls ($P < 0.001$) but also to mice immunized subcutaneously with SLA ($P < 0.001$), SLA + MPL-TDM ($P < 0.01$), and liposomal SLA ($P < 0.01$), indicating the strong induction of protective cellular responses in these groups (Figure 4A and 5A). An analysis of the contribution of $CD4^+$ and $CD8^+$ T cells toward the total response revealed that IFN-γ responses in all of the SLA containing vaccination groups in the short term were primarily through the $CD4^+$ positive cells, as IFN-γ levels were drastically reduced following the addition of anti-$CD4^+$ cells to the cultures. In contrast IFN-γ was produced by both $CD4^+$ and $CD8^+$ cells in animals vaccinated subcutaneously with SLA + MPL-TDM, liposomal SLA + MPL-TDM, and intraperitoneally immunized liposomal SLA (Figure 4A) indicating the involvement of CTL responses in the effector mechanisms governing protection in these groups. With infection, SLA in different adjuvant vaccinated groups produced substantially increased amounts of IFN-γ. The most pronounced increase in IFN-γ level observed in an intraperitoneally immunized liposomal SLA group was interestingly comparable to that induced by subcutaneous immunization of liposomal SLA + MPL-TDM, and the responses were significantly higher to all the other SLA containing vaccination groups except for subcutaneously immunized SLA + MPL-TDM. An exciting observation in long-term study was the fact that the BALB/c mice immunized subcutaneously with liposomal SLA + MPL-TDM maintained the $CD4^+$ and $CD8^+$ IFN-γ levels not only after immunization but also 4 months post-infection (Figure 4B and 5B).

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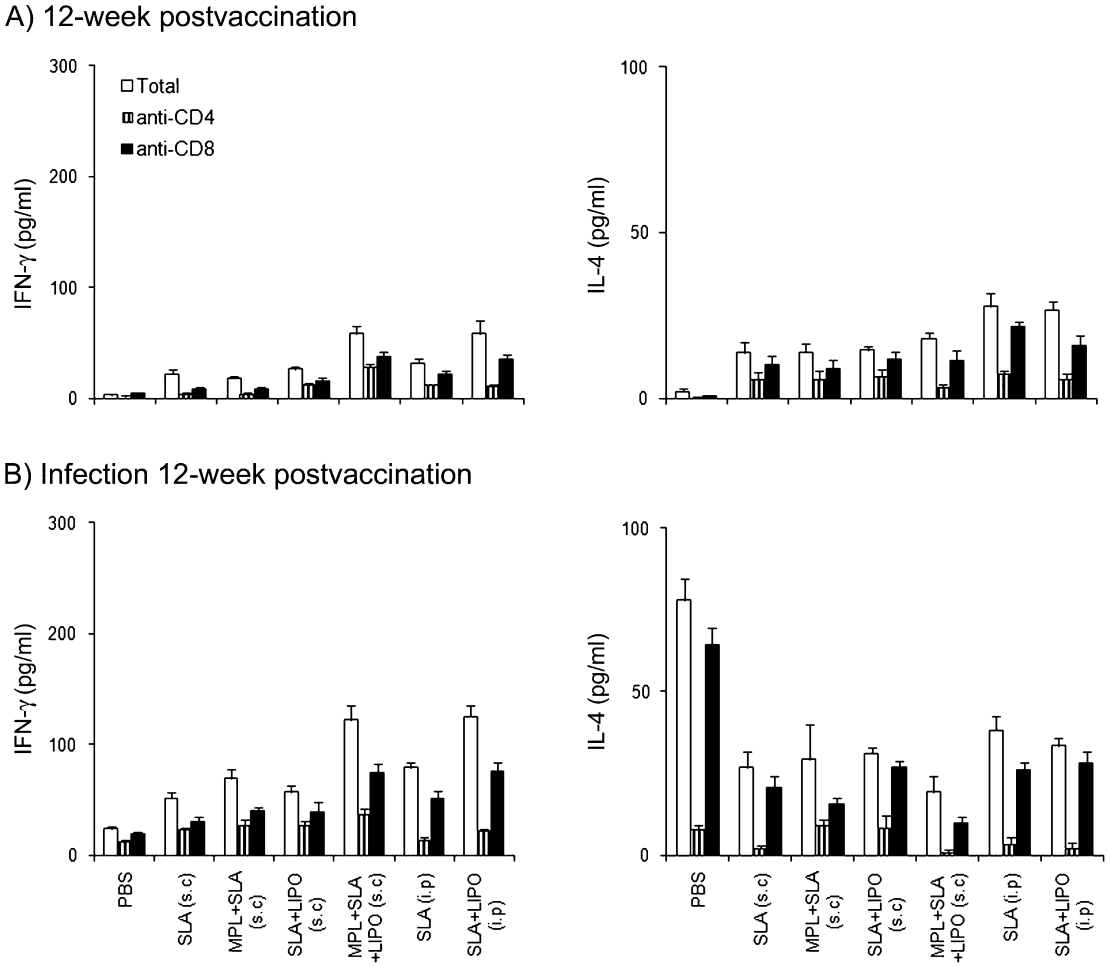

Figure 5. Durable antigen specific protective cytokine responses against visceral leishmaniasis. Spleen cells of mice vaccinated at 12 weeks with free SLA or SLA entrapped in cationic liposomes through intraperitoneal or subcutaneous routes, MPL-TDM mixed with SLA alone or SLA entrapped in cationic liposomes through subcutaneous routes, or PBS control were harvested before and after infection with *L. donovani*, treated with anti-$CD4^+$ or anti-$CD8^+$ monoclonal antibodies, and stimulated with SLA (10 μg/mL) for 72 h. Figures represent total, $CD4^+$ and $CD8^+$ T cell production of IFN-γ and IL-4 before (A) and after (B) infection. Data represent the mean of triplicate wells ± SE of four individual mice per group.

All mice immunized with SLA in different vaccine formulations had low but significant levels of $CD4^+$ cells producing IL-4, 10 days (Figure 4A) and 12 weeks after immunization (Figure 5A), and the levels were similar to intraperitoneal immunization of free and liposomal SLA. After infection all of the groups had higher levels of IL-4 in both short- and long-term studies (Figure 4B and 5B). However, the IL-4 levels were significantly lower ($P < 0.001$) in all of the groups when compared to the infected controls, indicating down regulation of Th2 responses following infection in the SLA vaccinated animals. The significantly higher levels of protection observed in mice immunized subcutaneously with liposomal SLA + MPL-TDM and intraperitoneally with liposomal SLA thus corresponds to the potentiation and maintenance of higher levels of IFN-γ from both $CD4^+$ and $CD8^+$ T cells by these vaccine formulations.

DISCUSSION

In this report we have shown for the first time that subcutaneous immunization with a liposomal leishmanial antigen adjuvanted with MPL-TDM can successfully induce long-term protective immunity against experimental visceral leishmaniasis, mediated by the sustained production of IFN-γ from both $CD4^+$ and $CD8^+$ T cells.

The induction and maintenance of sustained immune responses for the life-long protection of individuals from a variety of diseases is the primary goal of a successful vaccine. Numerous attempts have been made to develop such a vaccine against leishmaniasis. However, despite these efforts there is still no licensed vaccine available against the disease. To date, deliberate inoculation of live parasites (leishmanization) is the only vaccination strategy capable of achieving long-term protection against leishmaniasis. However, observations of a number of adverse side effects together with the development of large ulcerating lesions have led to the discontinuation of the process.[19,28] Therefore, the failures of leishmanization, due to safety concerns, have shifted the focus of vaccine development from the classical live vaccines to the development of subunit vaccine. These subunit vaccines with protein antigens are safer than live vaccines, but they lack strong immunogenicity. Over the past few years an extensive investigation demonstrated the efficacy of adjuvanted protein vaccine to protect against cutaneous leishmaniasis.[20,24,25,44,55,58,65] However, these vaccines, successful against short-term infectious challenges, often failed to produce durable protection in both susceptible BALB/c mice[25] as well as in self-curing C57BL/6 mice.[44] However, vaccination with more immunogenic Leish-111f demonstrated substantial amount of long-term protection against cutaneous

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leishmaniasis when adjuvanted with MPL-SE.[20] Various levels of protection have also been achieved against the visceral form of leishmaniasis using protein-based vaccines.[7,11,12,18,23,56,61,63] Of these, rHASPB1, rORFF, Leish-111f, and Leish-110f, alone or in combination with various adjuvants, have been reported to show long-term protective efficacy when immunized through routes compatible for human administration.[7,18,61,63] However, vaccination with rORFF antigens conferred protection when coadministered with IL-12 DNA,[63] an approach not applicable for human administration. Protection induced by rHASPB1 did not require any adjuvant, but the level of protection did not exceed 50% at the peak liver parasite burden.[61] On the other hand, Leish-111f and Leish-110f formulated with MPL-SE and immunized through a subcutaneous route showed significant protection in the livers of C57BL/6 mice infected with *L. infantum*. However, no protection data were provided for the spleen.[18] Additionally, Leish-111f-MPL-SE, although it showed some protection in the spleen, failed to protect in the liver of hamsters.[18] Moreover, in all of these studies animals were challenged 3−4 weeks after the last immunization. Our earlier reports with crude leishmanial antigen (LAg) and defined protein antigen (gp63) entrapped in cationic liposomes showed significant protection when infected after 10−12 weeks following immunization.[11,12] However, one of the main drawbacks of these studies was the need for intraperitoneal route of immunization, since immunization through a subcutaneous route failed to protect against *L. donovani* infection.[10] Notably, in contrast to LAg a pool of approximately 33 polypeptides of which many may not be potential vaccine candidates,[2] all of the components of SLA have been found to be immunogenic.[2] Thus, to overcome the route problem we used the more immunogenic SLA[10,13] entrapped in cationic liposomes along with MPL-TDM. Subcutaneous immunization of susceptible BALB/c mice with SLA alone or in the presence of either cationic liposomes or MPL-TDM resulted in a partial level of protection against *L. donovani* infection. Interestingly, immunization with cationic liposomal SLA formulated with MPL-TDM resulted in almost complete elimination of parasites from both spleen (83% to 87%) and liver (86% to 89%) in short- as well as long-term challenges with virulent *L. donovani* parasites, respectively. The levels of protection thus achieved were comparable with those through intraperitoneal immunization of liposomal SLA. In addition, the protective efficacy was maintained for at least four months after infection. These results therefore emphasize the usefulness of MPL-TDM formulated cationic liposomal SLA for vaccination against visceral leishmaniasis. Similar levels of long-term protection achieved through subcutaneous administration of protein vaccines have not been observed so far in laboratory based trials against VL.

The DTH response *in vivo* often related to the success of vaccines against human and animals and is an indication of the activation of cell mediated immunity.[43] We and others earlier reported the correlation of elevation of sustained DTH responses with resistance against VL after immunization through an intraperitoneal route.[12,34] In this article we have highlighted the role of MPL-TDM with cationic liposomal formulation of SLA which significantly induced an appreciable DTH response through human administrable route before and after challenge infection, clearly suggesting the strong and persistent activation of cell-mediated immune response with greater resistance against disease. Successful vaccination corresponded with an increase in IgG2a antibody isotype and decrease in IgG1. The relative production of IgG2a and IgG1 isotype can be used as a marker for the induction of Th1 and Th2 type of immune responses, respectively. We observed that mice immunized subcutaneously with liposomal SLA + MPL-TDM induced strong humoral responses (IgG2a > IgG1) after immunization which were sustained after challenge infection and were comparable with that of intraperitoneally immunized liposomal SLA. Thus, these results demonstrated elicitation of a successful cell mediated as well as humoral immunity through subcutaneous immunization with a liposomal SLA + MPL-TDM vaccine.

Protection against leishmaniasis is believed to be dependent upon production of IFN-γ, which drives the immune response toward a Th1 type phenotype. Earlier it was found that the immunization of liposomal protein through an intraperitoneal route stimulated Th1 responses by inducing sustained IFN-γ production.[12] Herein, mice vaccinated subcutaneously with liposomal SLA + MPL-TDM also exhibited significant enhancement of IFN-γ production at both 10 days and 12 weeks after immunization, as well as four months after infectious challenge, and the level of cytokine production was similar with that of intraperitoneally immunized liposomal SLA. Furthermore, MPL-TDM had a comprehensive adjuvant effect on SLA in inducing IFN-γ production when immunized through subcutaneous route. In different models of leishmaniasis, $CD4^+$ T cell production of IFN-γ has been found to be necessary and sufficient for inducing protection,[30,46] and some of the earlier studies reported that $CD8^+$ T cells were ineffective in providing efficient control against challenge infection with *L. major*.[31] However, the present perception of $CD8^+$ cells has changed with significant observations of failure of $CD8^+$ T cell deficient mice to control parasitic growth suggesting the role of $CD8^+$ T cells in immunity against parasitic infection with *L. major*.[6,60,64] Furthermore, recently it has been observed that induction of long-term protection against leishmaniasis requires the generation of memory T cells, probably of both $CD4^+$ and $CD8^+$ T cell lineages.[26,33,66] Thus, to analyze the relative contribution of $CD4^+$ and $CD8^+$ T cells in inducing IFN-γ production, *in vitro* blocking with anti CD4 or anti CD8 Abs were performed in both short- as well as in long-term studies. After blocking we demonstrated that subcutaneous vaccination of liposomal SLA + MPL-TDM led to higher IFN-γ production from $CD8^+$ T cells as compared to other strategies. In addition, production of IFN-γ from $CD4^+$ T cells was also markedly enhanced in animals immunized with this formulation, indicating the involvement of both $CD4^+$ and $CD8^+$ T cells in the effector mechanism resulting in higher levels of protection. These results were comparable with those obtained through intraperitoneal immunization of liposomal SLA. Recently it has been observed that regimens consisting of two different adjuvants are much more efficient in terms of expansion of protective $CD8^+$ T cells than immunization with a single adjuvant.[47,59] Thus, our results are in agreement with the above observations and liposomal SLA in combination with MPL-TDM was capable enough of priming $CD8^+$ T cells along with $CD4^+$ T cells leading to both short-term and long-term protection. But higher levels of IFN-γ production are not the only criteria that can induce protection against *L. donovani* infection.[42] In our earlier studies we have found that protection against visceral leishmaniasis always corresponded with production of IL-4, along with IFN-γ, following successful immunization through an intraperitoneal route.[11,12,39−41,57] In this study also IL-4 was produced from spleen cells of mice

immunized subcutaneously with liposomal SLA+ MPL-TDM. However, the expression of IL-4 was down-regulated significantly four months after challenge infection. Thus, the early immunological responses evidenced by increased DTH, IgG2a/IgG1, and IFN-gamma level induced in this group post-immunization was sustained for long-term successful protection against challenge with virulent *L. donovani* parasites.

CONCLUSION

In conclusion, liposomal SLA formulated with the immunopotentiating adjuvant, MPL-TDM, appears to be a potential vaccine for subcutaneous immunization against *L. donovani* infection. This formulation, capable of eliciting sustained cell-mediated immunity with strong antibody responses and long-term protection, holds promise for future vaccination strategies against visceral leishmaniasis.

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ABBREVIATIONS

SLA,soluble leishmanial antigen; IFN-γ,interferon gamma; rIL12,recombinant interleukin 12; MPL-TDM,monophosphoryl lipid−trehalose dicorynomycolate; FBS,fetal bovine serum; BSA,bovine serum albumin; CTL,cytotoxic T lymphocyte; Th1/2,type 1/2 T helper; MHC-I/II,major histocompatibility complex-I/II; s.c.,subcutaneous; i.p.,intraperitoneal; DTH,delayed type hypersensitivity

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