ORIGINAL RESEARCH PAPER

DDA/TDB liposomes containing soluble *Leishmania* major antigens induced a mixed Th1/Th2 immune response in BALB/c mice

Mansure Hojatizade [1,8], Ali Badiee [2], Ali Khamesipour [3], Abbas Mirshafiey [1], Javad Akhtari [4], Ahmad Mehravaran [5], Seyedeh Hoda Alavizadeh [2], Azam Abbasi [2], Zahra Saberi [2], Amin Reza Nikpoor [6], Mahmoud Reza Jaafari [7]*

[1]*Pathobiology Department, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

[2]*Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran*

[3]*Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran*

[4]*Immunogenetics Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

[5]*Infectious Diseases and Tropical Medicine Research Center, Zahedan University of Medical Sciences, Zahedan, Iran*

[6]*Immunology Department, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

[7]*Biotechnology Research Center, Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran*

[8]*Department of Basic Medical Sciences, Neyshabur University of Medical Sciences, Neyshabur, Iran*

ABSTRACT

Objective(s): Leishmaniasis is a complex parasitic disease that represents a major public health problem. Despite numerous attempts over the past decades, yet there is no effective vaccine against human leishmaniasis probably due to the lack of suitable adjuvants. In this study, a first generation liposomal-based *Leishmania* vaccine was developed using soluble *Leishmania major* antigens (SLA) and á, Ü-trehalose6, 6'-dibehenat (TDB) as an immunostimulatory adjuvant. In this liposome structure, the cationic lipid Dimethyldioctadecylammonium (DDA) provides intrinsic adjuvant activity and cholesterol was added as a membrane stabilizer. Liposomes containing SLA were prepared.

Materials and Methods: BALB/c mice were subcutaneously (sc) immunized with Lip (DDA/TDB/CHOL)-SLA+, Lip (DDA/TDB)-SLA+, Lip (DDA)-SLA+, Lip (DDA/CHOL)-SLA+, SLA or Tris-HCl buffer. Immunization was done every two weeks for three weeks. The immunized mice were then challenged sc in the left footpad with 1×10^6 stationary phase *L. major* promastigotes (50 ìl), at 2 weeks after last booster injection.

Results: mice immunized with any of the liposomal formulations containing SLA (Lip-SLA+), substantially increased footpad swelling and parasite loads of foot and spleen with no significant difference compared to Tris-HCl buffer or SLA alone. Lip-SLA+ formulations induced a mixed Th1/Th2 immune response characterized by IFN-ã and IL-4 production as well as high levels of IgG1 anti-*Leishmania* antibody.

Conclusion: immunization with liposomes containing DDA and/or TDB in combination with SLA induces a mixed Th1/Th2 immune response and is not an appropriate strategy for preferential induction of a Th1 response and protection against leishmaniasis.

Keywords: *DDA, Liposome, Leishmaniasis, TDB, SLA, Vaccine*

INTRODUCTION

Leishmaniasis is a spectrum of disease ranging from self-healing cutaneous leishmaniasis (CL) to fatal visceral forms [1]. Cutaneous leishmaniasis, the most common type of leishmaniasis worldwide, is representing 50–75% of all new instances. World Health Organization (WHO) announces that the number of CL cases is about 1 to 1.5 million annually, 90% of which is prevalent in several countries including Iran, Saudi Arabia, Afghanistan, Algeria, Brazil, Peru and Syria [2] ; with recently reported cases in East Timor [3] and Thailand [4]. Current treatment modalities of CL need multiple injections of drugs [5]. In addition, cost of drugs, the occurrence of toxicity, unavailability of drugs in different regions of the world [3] as well as drug resistance have limited the efficacy of treatment [5]. Since recovery from CL induces a long-lasting immunity to re-infection [6], the most feasible approach seems the development of an effective vaccine to control various types of leishmaniasis [7]. Despite extensive efforts, there is currently no effective vaccine for human leishmaniasis [7, 8]. Though attempts to develop vaccines against *Leishmania* infections resulted in identifying many candidate antigens, only whole killed *Leishmania* or first generation vaccines with or without adjuvant, succeed to reach phase III trials [9, 10]. The results however, were not definitive in some trials, and in general they showed a finite prophylactic efficacy [11]. It appears that one reason for the limited efficacy of first generation vaccines is the lack of an appropriate adjuvant [9, 12]. On the other hand, new candidate vaccines against *Leishmania* infection, particularly those based on DNA and recombinant proteins [13], appear to be less immunogenic than first generation vaccines. Some *Leishmania* antigens in first generation vaccines showed to promote protection in animal model when used with an appropriate adjuvant, particularly IL-12 [14]. Therefore, it appears that the use of an appropriate adjuvant is essential for almost any modern vaccine, particularly those against *Leishmania* infection [15].

Liposome could act as an adjuvant, and the adjuvancity increases by incorporating immunostimulatory compound in to its structure [16]. Immunostimulatory cationic lipids, bearing an ammonium ion head group, offer efficient protein encapsulation and /or adsorption and could serve as an efficient delivery system [17].

Dimethyldioctadecylammonium (DDA) is an effective adjuvant which belongs to the group of lipophilic quaternary amines [18]. It was previously reported that combination of DDA and synthetic cord factor trehalose dibehenate (TDB), induce strong protective immune response against *Mycobacterium tuberculosis* infection [19] and improves immu-nogenicity of other subunit vaccines [20].

TDB stimulates antigen presenting cells (APC) through the syk-fcR-Card-Bcl-10-Malt1 signaling pathway and is known to induce potent Th1/Th17 responses [21]. TDB in combination with *M. tuberculosis* subunit vaccines significantly enhances antigen specific IFN-ã production [19]. Previous studies showed that the capability of forming depot is crucial for adjuvant activity [17]. By exerting a strong depot effect at the site of injection, DDA/TDB mixtures induce powerful cellular and humoral immune responses [22]. The presence of cholesterol as another essential component in liposome membrane improves lipid packing either by reducing or eliminating the main phase transition temperature [23]. Moreover, incorporation of cholesterol increases the transfection rates [24] and enhances immunogenicity [25].

In the current study, SLA was encapsulated in DDA cationic liposomes incorporating TDB to generate a first generation vaccine for the immunization of BALB/c mice. The extent of protection and the type of immune response were then investigated compared to control groups receiving either Tris-HCl buffer or SLA alone.

MATERIALS AND METHODS
Chemicals

Dimethyldioctadecylammonium (DDA) bromide and á, Ü-trehalose 6, 6'-dibehenate (TDB), were obtained from Avanti Polar Lipids (Alabaster, AL). Methanol (extra pure), Chloroform (extra pure) and HCl (1M), used to adjust pH in the Tris-HCl buffer, were purchased from Merck (Darmstadt, Germany). Tris base and all other chemicals used were of analytical grade.

Parasites and SLA

Leishmania major strain (MRHO/IR/75/ER (was previously used for leishmanization in Iran and for the preparation of experimental old world *Leishmania* vaccine [10, 26, 27]. SLA was prepared according to

the previously published protocol with some modifications [28], Briefly, promastigotes were isolated at the stationary phase, washed four times with Tris-HCl buffer (10 mM Tris-HCl, 10% sucrose, ... [9] per ml in the same buffer containing enzyme inhibitor cocktail (50 µl/ml) (Sigma, St. Louis, MO, USA). The preparation was then incubated in ice-water bath for 10 minutes and lysed by the freeze-thaw method followed by prob sonication in an ice bath. The supernatant of the centrifuged lysate promastigotes was isolated, dialyzed against Tris-HCl buffer, sterilized by passage through a 0.22 µm membrane and stored in small aliquot at -70 °C until use. The protein concentration of SLA was determined with BCA protein assay kit (Thermo Scientific, USA).

Liposomes Preparation

Different formulations of DDA/TDB liposomes (Table 1) with or without cholesterol were prepared by thin film method followed by sonication. Briefly, DDA, TDB and cholesterol were dissolved in chloroform/methanol (9:1, by volume). The organic solvent was removed with a rotary evaporator (Hettich, Germany), forming a thin lipid film at the bottom of the test tube.

The lipid film was freeze-dried (TAITEC, Japan) overnight under low pressure for complete removal of the organic solvent. The lipid film was then hydrated for 20 min with Tris-HCl buffer containing 1 mg/ml SLA at 10 °C, which was above the main phase transition of DDA (Tm: 47 °C) [29]. The final concentrations of DDA, TDB and cholesterol were 4, 0.5 and 0 or 3µmol/ml, respectively. The resulting multilamellar vesicles (MLVs) were sonicated in a bath-type sonicator and then dialyzed against Tris-HCl buffer. The concentration of SLA in the purified liposomes was determined with BCA protein assay kit (Thermo Scientific, USA). The encapsulation efficiency of SLA in liposomes was calculated with the following equation:

$$\text{encapsulation efficiency} = \frac{\text{protein content of liposome after purification}}{\text{protein content of liposome befor purification}} \times 100$$

Size Distribution and Zeta Potential Analysis of Particles

Dynamic Light Scattering Instrument (Nano-ZS; Malvern, UK) was used to measure the mean size and zeta potential of the liposomes. Particle size and poly dispersity index (PI) were reported as the means± standard deviation (n = 3). Zeta potentials were reported as the means ± zeta deviation (n = 3).

SDS-PAGE Analysis of SLA and Liposomal SLA

The polyacrylamide gel electrophoretic analysis (SDS-PAGE) was carried out to characterize and qualitatively estimate the concentration of SLA encapsulated in various liposomal formulations. The gel consisted of running gel (10.22%, w/v, acrylamide) and stacking gel (4.78%, w/v, acrylamide) with 1 mm thickness.

The electrophoresis Tris- glycine buffer was 25 mM Tris, 192 mM glycine, pH 8.3 plus 0.1% SDS. Electrophoresis was carried out at 140 V constant voltages for 45 min.

After electrophoresis, the gels were stained with silver for protein detection [30].

Immunization of BALB/c Mice

Female BALB/c mice, 6 to 8 weeks old, were obtained from Pasteur Institute (Karaj, Iran). Mice were housed in a colony room 12/12 h light/dark cycle at 21 °C and fed with tap water and laboratory pellet chow (KhorasanJavane Co, Mashhad, Iran). Animal experiments were performed in compliance with Mashhad University of Medical Sciences Ethical Committee Acts (Education Office dated March 31, 2010; proposal code 88527), based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry Of Health and Medicinal Education (MOHME) of Iran [31-33].

Different groups of 10 mice were sc immunized three times in a 2-week interval with 50 µl liposomal formulations per mouse according to Table 1.

Table 1. Formulations of liposomal SLA and controls

Liposomal Formulations	Amount of lipids and SLA associated in liposomes
Lip (DDA/TDB/CHOL)-SLA+	Lip DDA 4µmol/ml, TDB0.5µmol/ml, CHOL 3µmol/ml, SLA 1mgl/ml
Lip (DDA/TDB)-SLA+	Lip DDA 4µmol/ml, TDB0.5µmol/ml, SLA1mg/ml
Lip (DDA)-SLA+	Lip DDA 4µmol/ml, SLA 1mg/ml
Lip (DDA/CHOL)-SLA+	Lip DDA 4µmol/ml, CHOL 3µmol/ml, SLA 1mgl/ml
SLA	SLA 1mg/ml
Tris-HCl buffer	Tris-HCl-sucrose buffer (10 mM, 10% w/v, pH 7.5)

Enzyme-Linked Immunospot (Elispot) Assay

ELISPOT assays were performed with mouse ELISPOT kit from U-cytech (Utrecht, The Netherlands) as directed by the manufacturers' instruction. At week 2 after the last booster injection, 3 mice of each group were sacrificed; the spleens were harvested, homogenized and splenocytes were used according to the manufacturer's instructions (U-cytech, Utrecht, Netherlands). Briefly, Splenocytes (10^6 cells/well) were cultured in triplicate in anti-IFN-ã or anti-IL-4 antibody-coated ELISPOT plates and stimulated *in vitro* with SLA (10 ìg/ml). Culture media and Concanavalin A (2.5 μg/ml) were added to the negative and positive control wells, respectively. After 24 h incubation at 37 °C, 5% CO_2, spot counting was done with a Kodak 1Dsoftware package (Version 3.5, Eastman Kodak, and Rochester, New York). The mean number of spots± SD in triplicate wells were enumerated and expressed as spot-forming units (SFU) per 10^6 splenocytes.

Challenge with L. Major Promastigotes

The immunized mice (7 per group) were challenged sc in the left footpad with $1×10^6$ stationary phase *L. major* promastigotes (50 ìl), at day 14 after last booster injection. The increase in footpad thickness was weekly measured with a metric caliper (Mitutoyo Measuring Instruments, Japan) and the size of lesion was estimated by subtracting the thickness of contralateral uninfected footpads from the infected ones.

Cytokine ELISA

The levels of IFN-ã and IL-4 were measured at week 6 after challenge by ELISA method. Briefly, three mice from each group were sacrificed and spleens were aseptically isolated. Mononuclear cells were harvested using Ficoll-Hypaque (Biogene, Iran) density centrifugation method [34]. The splenocytes were suspended in complete medium (RPMI 1640-FCS) and seeded at $2×10^6$ cells/ml in 96-well flat-bottom plates (Nunc, Denmark). The splenocytes were then cultured and stimulated with SLA (10 μg/mL), Con A (2.5μg/mL) or medium alone as control and incubated at 37 ºC with 5% CO_2 for 72 h. The culture supernatants were collected and the levels of IL-4 and IFN-ã were assayed by ELISA method according to the manufacturer's instructions (MabTech, Sweden).

Quantitative Parasite Burden after Challenge

To evaluate protection against *L. major*, titration of live parasites in the infected footpad and spleen were performed at week 6 post-challenge by limiting dilution assay [35]. Mice were sacrificed and spleen and infected footpad tissues were isolated in each group. Spleens were collected, homogenized and deposited in RPMI 1640 containing 10% v/v heat inactivated FCS (Eurobio, Scandinavie), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (RPMI–FCS). The infected footpad tissues were homogenized completely in 1 ml RPMI-FCS by bead beating for 20 s (Bead Beater, Biospec, Bartlesville, OK) [36]. Serial 10-fold dilutions of cells homogenates were placed in triplicate into 96-well flat-bottom plates (Nunc) over a solid layer of rabbit blood agar. The plates were then incubated for 7–10 days at 25 °C. Presence and absence of motile promastigotes per well were detected as positive and negative wells by an invert microscope (CETI, UK). The number of live parasites per spleen and infected footpad were estimated based on the highest dilution at which promastigotes could be grown out after the incubation time. Finally the obtained results were statistically analyzed using Graph Pad Prism software.

Antibody Isotype Assay

To determine the type of immune response, blood samples were collected from BALB/c mice before and at week 6 after challenge. The sera were separated to assess anti-SLA IgG1, IgG2a and IgG total antibodies by ELISA method [35]. Briefly, 96-well plates (Nunc) were coated with 50 ìl SLA (10 ìg/ml) in PBS buffer (pH 7.4) and incubated overnight at 4 °C before being blocked with 1% bovine serum albumin in PBS–Tween (200 μl) for 1 h at 37 °C. Then, serum samples were diluted in PBS-Tween and applied to each well. The plates were treated with HRP-rabbit anti-mouse IgG isotype according to the manufacturer's instructions (Invitrogen Inc., USA). Optical density (OD) was read at 450 nm with background subtraction at 630 nm.

Statistical Analysis

Statistical analysis of data was recorded in Graph Pad Prism software and One-way ANOVA statistical test was used to indicate the significance of the differences among different groups. $P<0.05$ was recorded as statistically significant.

Table 2. Particle size distribution, polydispersity index (PDI), zeta potential, and encapsulation
efficiencies (%EE) of SLA in different liposomal formulations (mean ± SD, n = 3)

Liposomal Formulations	Particle size± SD (nm)	PDI	Zeta potential ±SD (mV)	EE (%)±SD
Lip DDA/TDB/CHOL/SLA	762±114	0.493	54.5±0.7	67%±2
Lip DDA/TDB/SLA	1395±100	0.143	39±2.11	93%±2.3
Lip DDA/SLA	1200±115	0.479	34.6±2.65	95%±3
Lip DDA/CHOL/SLA	736±50	0.355	57.8±0.92	78%±2.1

RESULTS

Liposome Characterization

he particle sizes and zeta potentials of liposomes are shown in Table 2. All formulations showed a positive zeta potential due to the presence of positively charged lipid (DDA). As shown in Table 2, the entrapment efficiency of SLA in DDA/TDB/CHOL, DDA/CHOL and DDA/TDB formulations were 67%±2, 78%±2.1 and 93%±2.3, respectively. SLA concentration was determined by BCA protein assay Kit after dialysis against Tris-HCl buffer from three technical triplicates of each of the liposomal preparation (Mean ± SD, n=3).

Characterization of Particles by SDS-PAGE

Characterization of free and liposomal SLA was carried out by SDS-PAGE as shown in Fig. 1. SDS-PAGE analysis of soluble SLA showed several protein bands with a broad range of molecular weight between 10 to 70 kDa (lane 2, 3). The SDS-PAGE analysis of liposomes containing SLA after purification (lane 4-7) revealed the same bands as in the soluble SLA, corresponding to their liposomal encapsulation efficiency.
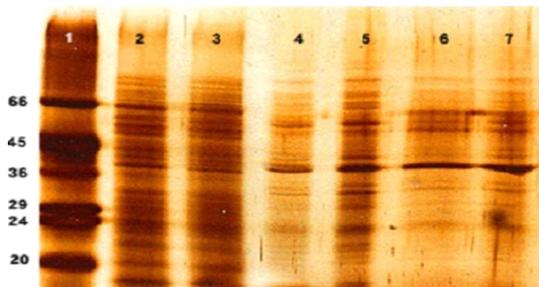
Fig.1. SDS–PAGE analysis of SLA alone and liposomal SLA Lane 1 low-range protein standard (Sigma, USA), Lane 2 purified SLA (2.5 ìg), Lane 3 purified SLA (5 ìg), Lane 4 Lip (DDA/CHOL)-SLA+ (2.5 ìg), Lane 5 Lip (DDA/TDB/CHOL)-SLA+ (5 ìg), Lane 6 Lip (DDA/TDB)-SLA+ (2.5 ìg), Lane 7 Lip (DDA)-SLA+ (2.5ìg)
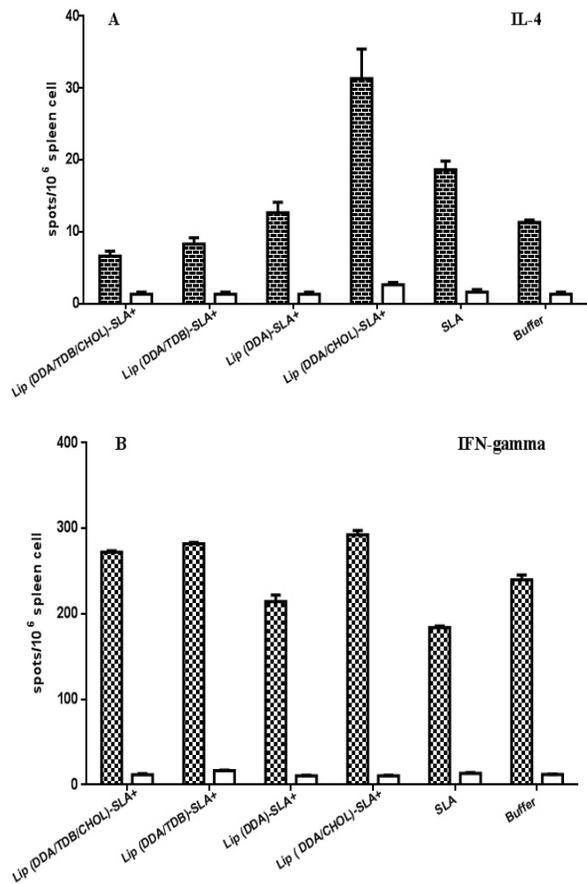
Fig. 2. Cytokine levels in immunized mice at week 2 after the last booster injection and before challenge
Groups of 10 mice were vaccinated three times with different liposomal formulations and control groups. 14 days later, the spleens of three mice from each group were harvested and mononuclear splenocytes were cultured in the presence of SLA (10 ìg/ml). The level of IL-4 (A) or IFN-γ (B) were then evaluated using ELISPOT method. Results are shown as Mean ± SEM (n = 3). White columns are negative control without any stimulation

In Vitro IFN-γ and IL-4 Assay

To evaluate the expressions of IFN-y and IL-4, splenocytes of immunized mice were harvested at week 2 after the last booster injection. The results of ELISPOT assays showed that splenocytes of mice immunized with Lip (DDA/CHOL)-SLA+, released the highest levels of IL-4 compared to other formulations (Fig.2 A).

There was no significant difference among mice received either SLA, Lip-SLA+ or Tris-HCl buffer (p>0.05). Results also demonstrated that splenocytes of Lip-SLA+ immunized mice released higher levels of IFN-ã compared to SLA alone (Fig. 2B). However, there was no significant difference between mice immunized with Lip-SLA+, SLA or Tris-HCl buffer in terms of IFN-ã secretion.

In Vitro Cytokine Production by Splenocytes

The supernatant of cultured splenocytes were analyzed at week 6 after challenge to determine the amounts of IFN-ã and IL-4 cytokines, indicative of Th1 and Th2 responses, respectively. Results demonstrated that the highest amounts of IL-4 was detected in mice immunized with Lip (DDA/TDB/CHOL)-SLA+ (Fig. 3A), which was significantly higher than Tris-HCl buffer or SLA alone (p<0.05). Interestingly, Lip (DDA/TDB/CHOL)-SLA+ immu- nized mice also produced significantly higher amounts of IFN-ã compared to Tris-HCl buffer (p<0.05) (Fig. 3B). Generally, mice immunized with Lip-SLA+ showed higher amounts of IL-4 and IFN-ã compared to Tris-HCl buffer.

Antibody Response

To assess the type of immune response in mice, the anti-SLA IgG, IgG1 and IgG2a subclasses were assayed before (Figs. 4A–C) and after challenge with *L. major* promastigotes (Figs. 5A–C). Challenge with *L. major* increased IgG1, IgG2a and IgG antibody levels in mice received SLA or Tris-HCl buffer (Figs. 5A–C).

As shown in Fig. 4A, the sera of all Lip-SLA+ immunized mice, showed significantly higher levels of IgG1 antibody than SLA alone in serum dilution of 1:200 (p<0.001) and also Tris-HCl buffer. In terms of IgG2a, the antibody level in mice immunized with Lip (DDA/TDB/CHOL)-SLA+ was significantly (P<0.001) higher than Tris-HCl buffer in serum dilution of 1:2,000 (Fig. 4B). On the whole, mice immunized with Lip-SLA+, showed higher levels of IgG total (Fig. 4C), than Tris-HCl buffer (p<0.001).

After challenge, the sera of mice immunized with Lip-SLA+ produced elevated levels of specific IgG1 antibody than buffer control groups (p<0.001). The level of IgG1 in the sera of mice immunized with Lip (DDA/TDB/CHOL)-SLA+ was significantly (P<0.05) higher than all other groups in serum dilution of 1/20000 (Fig. 5A). On the other hand, IgG2a serum levels of mice immunized with Lip-SLA+ were increased after challenge except for Lip (DDA/TDB/CHOL)-SLA+ which showed a lower level of IgG2a compared to Lip (DDA)-SLA+ or Lip (DDA/CHOL)-SLA+ in serum dilution of 1/2000, 1/20,000 and 1/200,000 (P<0.05) (Fig. 5B) .Generally, mice immunized with Lip-SLA+ showed higher levels of IgG total compared to Tris-HCl buffer (Fig. 5C).
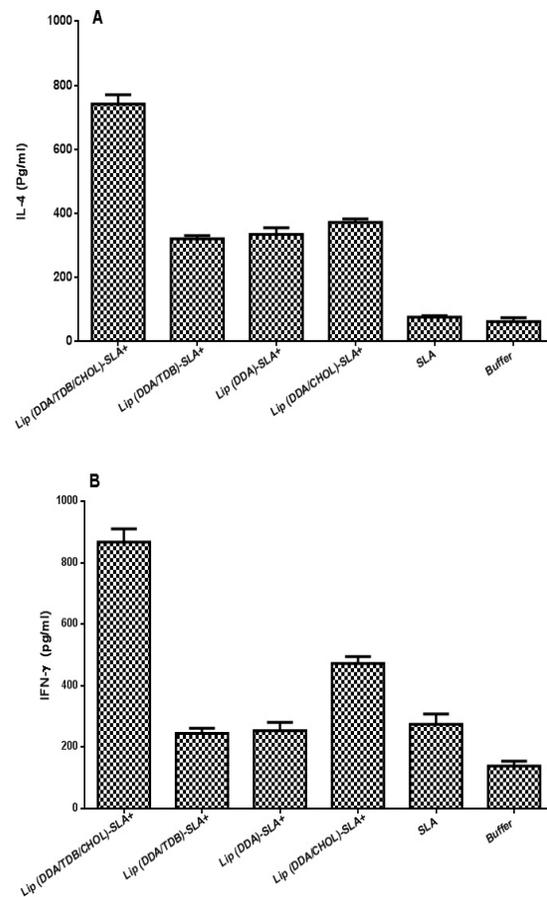
Fig.3. Cytokine levels in immunized mice at week 6 after challenge

Groups of 10 mice were vaccinated 3 times with different liposomal formulations at 6 weeks after challenge, the spleens of mice in each group were harvested; mononuclear splenocytes were cultured in the presence of SLA (10 ìg/mL) and the levels of IL-4 (A) or IFN-ã (B) in the culture supernatants were detected with ELISA method. Results are shown as the mean ± SEM (n = 3)
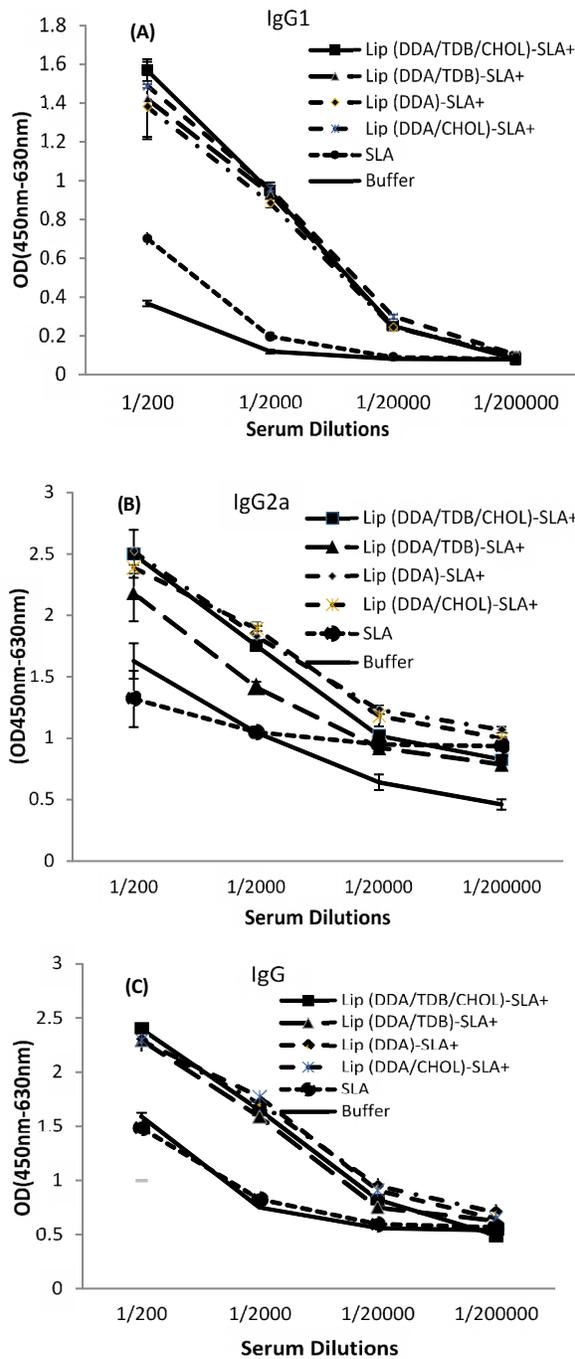
Fig. 4. Levels of anti-SLA IgG1 (A), IgG2a (B), and IgG (C) in pooled sera of BALB/c mice before challenge

Mice were immunized sc, three times in 2-week intervals, with Lip-SLA+, SLA or Tris-HCl buffer. Blood samples were collected 2 weeks after the last booster injection. The serum samples were pooled. The anti-SLA IgG1, IgG2a, and IgG titers were assessed with ELISA method. The assay was performed in triplicate at 200, 2,000, 20,000, or 200,000-fold dilution for each sample. Values are the mean ± SD. ***p<0.001 when the mice immunized with Lip-SLA+ are compared to buffer
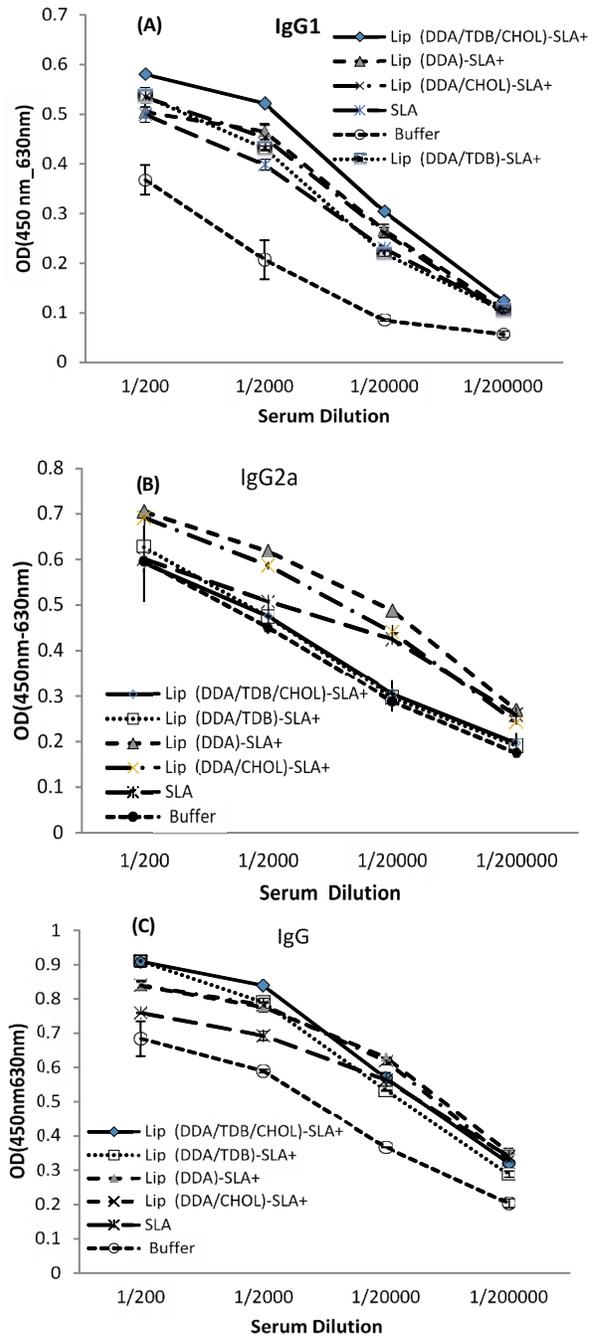
Fig. 5. Levels of anti-SLA IgG1 (A), IgG2a (B), and IgG (C) in pooled sera of BALB/c mice after challenge

Mice were immunized sc, 3 times in 2-week intervals, with Lip-SLA+, SLA or Tris-HCl buffer. Blood samples were collected 6 weeks after challenge. The serum samples were pooled and anti-SLA IgG1, IgG2a, and IgG titers were assessed with ELISA method. The assay was performed in triplicate at 200, 2,000, 20,000, or 200,000-fold dilution for each sample. Values are the mean ± SD. ***p<0.001 when the mice immunized with Lip-SLA+ are compared to Tris-HCl buffer. Besides, mice immunized with Lip (DDA/CHOL)-SLA+ or Lip (DDA)-SLA+ or SLA only showed significant difference in term of IgG2a

Challenge Results

To investigate the extent of protection, immunized mice were challenged with parasites and lesion development was monitored by weekly measurement of footpad thickness. As shown in Fig. 6, the lesion size progressed at a rapid rate in all vaccinated groups. There was no significant difference in footpad swelling of mice either immunized with Lip-SLA+ or SLA alone and Tris-HCl buffer during the observation period. Footpad thickness was continuously progressed in all groups and no protection was observed. Swelling of the footpad reached plateau in all immunized mice after 6 weeks.

Estimation of Parasite Loads in the Foot and Spleen

The number of live *L. major* parasites in the spleen and infected footpads was quantified at day 42 after challenge .All immunized mice showed live parasites in their footpads. The number of live parasites in the

footpads of Lip (DDA/CHOL)-SLA+ or Lip (DDA/TDB/CHOL)-SLA+ immunized mice was more than other groups. However, no significant difference was observed (Fig. 7A). Besides, mice immunized with Lip (DDA/CHOL)-SLA+ or Lip (DDA/TDB/CHOL)-SLA+ showed the highest number of viable parasite in their spleen compared to other vaccinated groups with no observed significant difference (Fig. 7B).

DISCUSSION

Since solid protection could be achieved following recovery from CL, development of an effective vaccine is feasible [6]. Various *Leishmania* antigens showed to induce effective immune response and protection in murine model of leishmaniasis when used with IL-12 [37]. However, first generation *Leishmania* vaccines in phase III trials revealed little efficacy partially due to the lack of a suitable adjuvant [9].
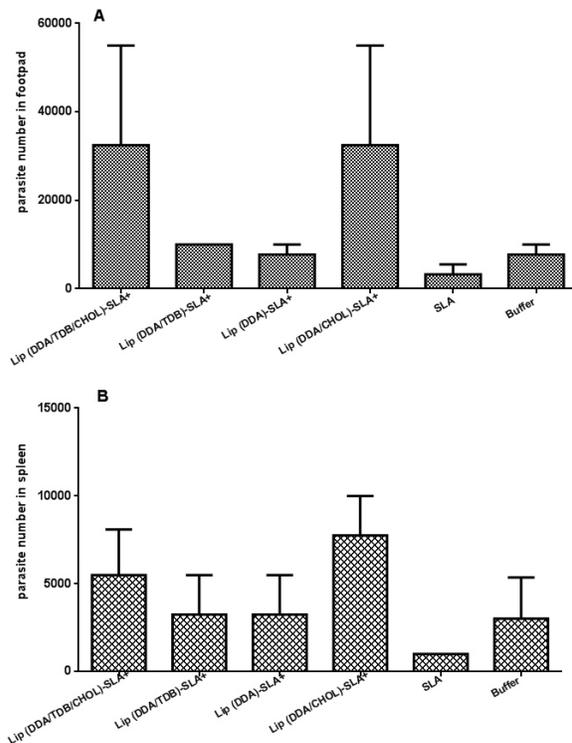
Fig. 6. Footpad swelling in BALB/c mice
Mice immunized 3 time in 2-week interval, with Lip (DDA/TDB/CHOL)-SLA+, Lip (DDA/TDB)-SLA+, Lip (DDA)-SLA+, Lip (DDA/CHOL)-SLA+, SLA or Tris-HCl buffer after challenge with 10^6 virulent *L. major* promastigotes in left hind footpads. The footpad thickness was measured weekly for 6 weeks. Each point represents the average footpad thickness± SD (n=7)
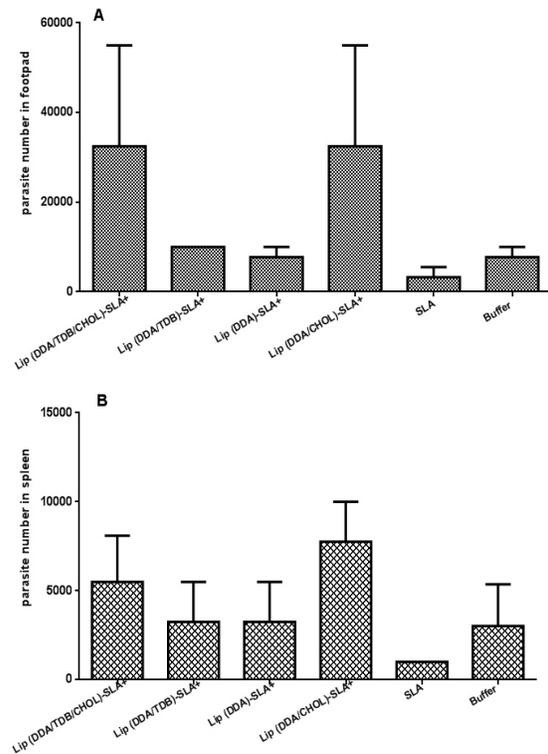
Fig. 7. Parasite burden in BALB/c mice
Mice immunized sc, 3 times in 2-week interval, with Lip (DDA/TDB/CHOL)-SLA+, Lip (DDA/TDB)-SLA+, Lip (DDA)-SLA+, Lip (DDA/CHOL)-SLA+, SLA or Tris-HCl buffer. A limiting dilution analysis was performed at week 6 after challenge on the footpads (A) and spleens (B) of mice. The number of viable parasites per spleen and footpad of different groups of mice were determined. The bar represents the average score ± SEM (n = 3)

In the current study, the cationic lipid DDA was used as an intrinsic adjuvant based on 3 main reasons; (a) to facilitate endocytosis due to its positive charge and through efficient interaction of target antigens with anionic species on the APCs surface [38], (b) to benefit from the intrinsic adjuvancy property of the synthetic quaternary ammonium compound [18] and (c) to exert strong depot effect at the site of injection following sc administration [39].

The combination of cationic DDA liposomes with immunostimulatory agent TDB revealed a significantly elevated immune response against tuberculosis (TB). Indeed, DDA increased IFN-ã levels and induced a persisted memory response due to depot effect compared to DOTAP and 3β-[N-(N2 , N2 - Dimethyl amino ethane) carbomyl]-Cholesterol (DC-Chol) [17]. In our previous report, we investigated the role of DOTAP cationic liposomes as an adjuvant to evaluate immune response against SLA. Our findings showed promising role of DOTAP nanoliposomes containing SLA in inducing a strong Th1 immune response and protection against *L. major* challenge in murine model of leishmaniasis [40]. Recent evidences also suggest that DOTAP liposomes could raise the immunogenic profiles of peptide or protein antigens and induce CTL (Cytotoxic T lymphocyte) and Th1 responses [41].

In the present study, SLA was used as a crude parasite antigen and a first generation vaccine. Previous studies reported that effective vaccine against leishmaniasis requires a multivalent cocktail of various antigens composed of a spectrum of protective epitopes which cover a broad range of MHC types in a population [42]. This is in fact in consistent with the leishmanization results that crude *Leishmania* antigens such as SLA with many antigen epitopes, are appropriate candidates for vaccine development [43]. In addition, *Leishmania* soluble antigens (SLA) induced more protection when used in liposomal form compared to recombinant antigens such as gp63 [44].

In our previous studies we showed that SLA bears a net negative charge at pH 7.5, thus could electrostatically interact with positively charged lipids in liposome bilayers. This interaction reduces the surface zeta potential, resulting in the high entrapment efficiency of SLA in cationic liposomes [45]. In the current study, the entrapment of SLA in DDA/TDB/CHOL or DDA/CHOL formulation was 67% and 78%, respectively and the entrapment of SLA in DDA/TDB was more than 90%. This is actually in agreement with Perrie et al. 2013, which showed that by increasing the cholesterol content of DDA/TDB/CHOL liposomes, antigen loading is decreased [39]. Cholesterol was used in liposome formulation to increase the *in vivo* stability and rigidity of liposomes. Besides, the presence of cholesterol in the vesicles facilitates cytoplasmic release of the antigens and prevents lysosomal degradation [46]. Increased *in vivo* stability of the vesicles may also further trigger stimulation of CD8+ T-cell responses [47].

Vaccine development against *Leishmania* infection requires a Th1-type immune response induced by IFN-γ production, which leads to macrophage activation and parasite killing [48]. The main purpose of this study was to develop an effective vaccine against leishmaniasis to induce a powerful Th1-type immune response.

Vesicle particle size is a major factor influencing the behavior of liposome particulates following administration [49]. A number of studies demonstrated that small particles are usually exchanged via the lymphatic vessels and the larger ones trapped in the interstitial space for a longer period of time [50]. Brewer et al. showed that contrary to small particles, large vesicles induce IL-12 production [51]. Previously, effect of particle size on cytokine production was reported for DDA/TDB liposomes. The most interesting finding was that the larger liposomes promoted higher splenic IFN-ã cytokine production. Henriksen-Lacey et al. have also investigated the immune response to an adsorbed protein antigen derived from *M. tuberculosis* antigen incorporated in DDA/TDB vesicles in different size [17]. The most obvious finding to emerge from this study was that vesicles, 250-700 nm in diameter, elicited the greatest IFN-γ secretion by restimulated splenocytes. These vesicles also induced a Th1 immune response and increased both persistence at the injection site and accumulation in the draining lymph nodes [49]. Previously, we found that contrary to larger size liposomes (400 nm or larger), the smaller ones (<200 nm) could not protect BALB/c mice against leishmaniasis [52]. Particle characteristics results in Table 2 have also shown that Lip (DDA/TDB/CHOL)-SLA+ formulations used in this study had an appropriate size of around 700 nm.

Based on the results of lesion size and the number of live *L. major* parasites in footpad and spleen, immunization with SLA alone induced no effective immune responses and no protection in BALB/c mice. Besides, SLA in combination with DDA/TDB/CHOL liposomes elicited no protective efficacy against murine model of leishmaniasis, and liposomal SLA showed no significant differences compared to SLA alone in terms of protective efficacy. Deterioration of infection with SLA in the absence of an appropriate adjuvant is not surprising in BALB/c mice, since BALB/c mice have an inherent tendency toward developing Th2 responses [53]. Previous studies suggested that DDA induced a mixed Th1/Th2 immune response when used with some antigens [54]. Results of our study also demonstrated that mice immunized with formulations of Lip-SLA+ containing DDA induce a mix Th1/Th2 immune response with no protective responses against murine model of *Leishmania* infection.

As was mentioned before, DDA cationic lipid belongs to the group of lipophilic quaternary amines, just as DOTAP. However, DOTAP liposomes containing SLA prompted a strong Th1 immune response and protection against *L. major* challenge in murine model of leishmaniasis [40]. Based on previous studies, cationic lipids containing a quaternary ammonium group are more potent than those with a tertiary ammonium group [55]. Moreover, DOTAP alone could induce cellular immunity; as recent data indicates that specific cellular signaling pathways and several kinases from the MAPK pathways are activated by cationic lipids. Activation of these kinases explains chemokines production and induction of CD80/CD86 cell surface expression [56].

In conclusion, the current data revealed that liposomes containing DDA and/or TDB in combination with SLA are not appropriate formulations to induce Th1 type of immune responses and protect mice against leishmaniasis. However, it might be a good strategy against infectious diseases which need a mixed Th1/Th2 response.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

REFERENCES

1. Dumonteil E, Jesus R-SM, Javier E-O, del Rosario Ga-MM. DNA vaccines induce partial protection against Leishmania mexicana. Vaccine. 2003; 21(17): 2161-2168.
2. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, Team WLC. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012; 7(5): e35671.
3. Mutiso JM, Macharia JC, Kiio MN, Ichagichu JM, Rikoi H, Gicheru MM. Development of Leishmania vaccines: predicting the future from past and present experience. J Biomed Res. 2013; 27(2): 85-102.
4. Kongkaew W, Siriarayaporn P, Leelayoova S, Supparatpinyo K, Areechokchai D, Duang-ngern P, Chanachai K, Sukmee T, Samung Y, Sridurongkathum P. Autochthonous visceral leishmaniasis: a report of a second case in Thailand. Southeast Asian J. Tropic Med. Public Health. 2007; 38(1): 8-12.
5. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microbiol Rev. 2006; 19(1): 111-126.
6. Khamesipour A, Dowlati Y, Asilian A, Hashemi-Fesharki R, Javadi A, Noazin S, Modabber F. Leishmanization: use of an old method for evaluation of candidate vaccines against leishmaniasis. Vaccine. 2005; 23(28): 3642-3648.
7. Alvar J, Croft SL, Kaye P, Khamesipour A, Sundar S, Reed SG. Case study for a vaccine against leishmaniasis. Vaccine. 2013; 31: B244-B249.
8. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, Sharifi I, Khalil EA, Bernal IDV, Antunes CM. First generation leishmaniasis vaccines: a review of field efficacy trials. Vaccine. 2008; 26(52): 6759-6767.
9. Khamesipour A, Rafati S, Davoudi N, Maboudi F, Modabber F. Leishmaniasis vaccine candidates for development: a global overview. Indian J Med Res. 2006; 123(3): 423-438.
10. Momeni AZ, Jalayer T, Emamjomeh M, Khamesipour A, Zicker F, Ghassemi RL, Dowlati Y, Sharifi I, Aminjavaheri M, Shafiei A. A randomised, double-blind, controlled trial of a killed L. major vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. Vaccine. 1999; 17(5): 466-472.
11. Noazin S, Khamesipour A, Moulton LH, Tanner M, Nasseri K, Modabber F, Sharifi I, Khalil E, Bernal IDV, Antunes CM. Efficacy of killed whole-parasite vaccines in the prevention of leishmaniasis—a meta-analysis. Vaccine. 2009; 27(35): 4747-4753.
12. Valian HK, Kenedy LKA, Rostami MN, Mohammadi AM, Khamesipour A. Role of Mycobacterium vaccae in the protection induced by first generation Leishmania vaccine against murine model of leishmaniasis. Parasitol Res. 2008; 103(1): 21-28.
13. Marques-da-Silva EA, Coelho EA, Gomes DC, Vilela MC,

Masioli CZ, Tavares CA, Fernandes AP, Afonso LCC, Rezende SA. Intramuscular immunization with p36 (LACK) DNA vaccine induces IFN-ā production but does not protect BALB/c mice against Leishmania chagasi intravenous challenge. Parasitol Res. 2005;98(1):67-74.

14. Coler RN, Reed SG. Second-generation vaccines against leishmaniasis. Trends Parasitol. 2005; 21(5): 244-249.

15. Badiee A, Shargh VH, Khamesipour A, Jaafari MR. Micro/ nanoparticle adjuvants for antileishmanial vaccines: present and future trends. Vaccine. 2013; 31(5): 735-749.

16. O'Hagan DT, MacKichan ML, Singh M. Recent developments in adjuvants for vaccines against infectious diseases. Biomol Eng. 2001; 18(3): 69-85.

17. Henriksen-Lacey M, Bramwell VW, Christensen D, Agger E-M, Andersen P, Perrie Y. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. J Control Release. 2010; 142(2): 180-186.

18. Hilgers LT, Snippe H. DDA as an immunological adjuvant. Res. Immunol. 1992; 143(5): 494-503.

19. Holten-Andersen L, Doherty TM, Korsholm KS, Andersen P. Combination of the cationic surfactant dimethyl dioctadecyl ammonium bromide and synthetic mycobacterial cord factor as an efficient adjuvant for tuberculosis subunit vaccines. Infect Immun. 2004; 72(3): 1608-1617.

20. Vangala A, Bramwell VW, McNeil S, Christensen D, Agger EM, Perrie Y. Comparison of vesicle based antigen delivery systems for delivery of hepatitis B surface antigen. J Control Release. 2007; 119(1): 102-110.

21. Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, Agger EM, Stenger S, Andersen P, Ruland J. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J.Immunol. 2010; 184(6): 2756-2760.

22. Kirby DJ, Kaur R, Agger EM, Andersen P, Bramwell VW, Perrie Y. Developing solid particulate vaccine adjuvants: Surface bound antigen favours a humoural response, whereas entrapped antigen shows a tendency for cell mediated immunity. Curr Drug Deliv. 2013; 10(3): 268-278.

23. Moghaddam B, Ali MH, Wilkhu J, Kirby DJ, Mohammed AR, Zheng Q, Perrie Y. The application of monolayer studies in the understanding of liposomal formulations. Int. J. Pharm. 2011; 417(1): 235-244.

24. Xu L, Anchordoquy TJ. Cholesterol domains in cationic lipid/ DNA complexes improve transfection. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2008; 1778(10): 2177-2181.

25. Bakouche O, Gerlier D. Enhancement of immunogenicity of tumour virus antigen by liposomes: the effect of lipid composition. Immunol. 1986; 58(3): 507-513.

26. Sharifi I, Fekri AR, Aflatonian M-R, Khamesipour A, Nadim A, Mousavi M-RA, Momeni AZ, Dowlati Y, Godal T, Zicker F. Randomised vaccine trial of single dose of killed Leishmania major plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. The Lancet. 1998; 351(9115): 1540-1543.

27. Javadian E, Nadim A, Tahvildare-Bidruni G, Assefi V. Epidemiology of cutaneous leishmaniasis in Iran: B.

28. Scott P, Pearce E, Natovitz P, Sher A. Vaccination against cutaneous leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes. J.Immunol. 1987; 139(1): 221-227.

29. Feitosa E, Barreleiro P, Olofsson G. Phase transition in dioctadecyldimethylammonium bromide and chloride vesicles prepared by different methods. Chem Phys Lipids. 2000; 105(2): 201-213.

30. Jaafari MR, Ghafarian A, Farrokh-Gisour A, Samiei A, Kheiri MT, Mahboudi F, Barkhordari F, Khamesipour A, McMaster WR. Immune response and protection assay of recombinant major surface glycoprotein of Leishmania (rgp63) reconstituted with liposomes in BALB/c mice. Vaccine. 2006; 24(29): 5708-5717.

31. Mehravaran A, Jaafari MR, Jalali SA, Khamesipour A, Ranjbar R, Hojatizade M, Badiee A. The role of ISCOMATRIX bilayer composition to induce a cell mediated immunity and protection against leishmaniasis in BALB/c mice. Iran J Basic Med S. 2016; 19(2): 178-186.

32. Mehravaran A, Jaafari MR, Jalali SA, Khamesipour A, Tafaghodi M, Hojatizade M, Abbasi A, Badiee A. Cationic Immune Stimulating Complexes Containing Soluble Leishmania Antigens: Preparation, Characterization and in Vivo Immune Response Evaluation. Iran J .Immunol. 2015; 12(4): 274.

33. Mehravaran A, Jaafari MR, Jalali SA, Khamesipour A, Tafaghodi M, Hojatizade M, Badiee A. The role of surface charge of ISCOMATRIX nanoparticles on the type of immune response generated against Leishmaniasis in BALB/c mice. Nanomed J. 2015; 2(4): 249-260.

34. Jaafari MR, Badiee A, Khamesipour A, Samiei A, Soroush D, Kheiri MT, Barkhordari F, McMaster WR, Mahboudi F. The role of CpG ODN in enhancement of immune response and protection in BALB/c mice immunized with recombinant major surface glycoprotein of Leishmania (rgp63) encapsulated in cationic liposome. Vaccine. 2007;25(32): 6107-6117.

35. Badiee A, Jaafari MR, Khamesipour A. Leishmania major: immune response in BALB/c mice immunized with stress-inducible protein 1 encapsulated in liposomes. Exp Parasitol. 2007; 115(2): 127-134.

36. Huang Z, Jaafari MR, Szoka FC. Disterolphospholipids: nonexchangeable lipids and their application to liposomal drug delivery. Angew. Chem. 2009; 121(23): 4210-4213.

37. Modabber F. Vaccines against leishmaniasis. Ann. Trop. Med. Parasitol. 1995;89:83-88.

38. Yan W, Chen W, Huang L. Mechanism of adjuvant activity of cationic liposome: phosphorylation of a MAP kinase, ERK and induction of chemokines. Mol Immunol. 2007; 44(15): 3672-3681.

39. Kaur R, Henriksen-Lacey M, Wilkhu J, Devitt A, Christensen D, Perrie Y. Effect of Incorporating Cholesterol into DDA: TDB Liposomal Adjuvants on Bilayer Properties, Biodistribution, and Immune Responses. Mol Pharm. 2013; 11(1): 197-207.

40. Firouzmand H, Badiee A, Khamesipour A, Heravi Shargh V,

Khorassan Part V: Report on a focus of zoonotic cutaneous leishmaniasis in Esferayen. Bull. Soc. Pathol. Exot. Filiales. 1975; 69(2): 140-143.

Alavizadeh SH, Abbasi A, Jaafari MR. Induction of protection against leishmaniasis in susceptible BALB/c mice using simple DOTAP cationic nanoliposomes containing soluble Leishmania antigen (SLA). Acta Trop. 2013; 128(3): 528-535.

41. Brgles M, Habjanec L, Halassy B, Tomašiæ J. Liposome fusogenicity and entrapment efficiency of antigen determine the Th1/Th2 bias of antigen-specific immune response. Vaccine. 2009; 27(40): 5435-5442.

42. Skeiky YA, Coler RN, Brannon M, Stromberg E, Greeson K, Thomas Crane R, Campos-Neto A, Reed SG. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. Vaccine. 2002; 20(27): 3292-3303.

43. Bhowmick S, Ali N. Recent developments in leishmaniasis vaccine delivery systems. 2008.

44. Afrin F, Rajesh R, Anam K, Gopinath M, Pal S, Ali N. Characterization of Leishmania donovani antigens encapsulated in liposomes that induce protective immunity in BALB/c mice. Infect Immun. 2002; 70(12): 6697-6706.

45. Shargh VH, Jaafari MR, Khamesipour A, Jaafari I, Jalali SA, Abbasi A, Badiee A. Liposomal SLA co-incorporated with PO CpG ODNs or PS CpG ODNs induce the same protection against the murine model of leishmaniasis. Vaccine. 2012; 30(26): 3957-3964.

46. Hafez I, Maurer N, Cullis P. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther. 2001; 8(15): 1188-1196.

47. Ignatius R, Mahnke K, Rivera M, Hong K, Isdell F, Steinman RM, Pope M, Stamatatos L. Presentation of proteins encapsulated in sterically stabilized liposomes by dendritic cells initiates CD8+ T-cell responses in vivo. Blood. 2000; 96(10): 3505-3513.

48. Nylen S, Gautam S. Immunological perspectives of leishmaniasis. J. Glob Infect Dis. 2010; 2(2): 135-146.

49. Watson DS, Endsley AN, Huang L. Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine. 2012; 30(13): 2256-2272.

50. McLennan DN, Porter CJ, Charman SA. Subcutaneous drug delivery and the role of the lymphatics. Drug Discovery Today: Technol. 2005; 2(1): 89-96.

51. Brewer JM, Tetley L, Richmond J, Liew FY, Alexander J. Lipid vesicle size determines the Th1 or Th2 response to entrapped antigen. J. Immunol. 1998; 161(8): 4000-4007.

52. Badiee A, Khamesipour A, Samiei A, Soroush D, Shargh VH, Kheiri MT, Barkhordari F, Robert Mc Master W, Mahboudi F, Jaafari MR. The role of liposome size on the type of immune response induced in BALB/c mice against leishmaniasis: rgp63 as a model antigen. Exp Parasitol. 2012; 132(4): 403-409.

53. Bhowmick S, Ravindran R, Ali N. Leishmanial antigens in liposomes promote protective immunity and provide immunotherapy against visceral leishmaniasis via polarized Th1 response. Vaccine. 2007; 25(35): 6544-6556.

54. Davidsen J, Rosenkrands I, Christensen D, Vangala A, Kirby D, Perrie Y, Agger EM, Andersen P. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis(trehalose 6, 62 -dibehenate)—A novel adjuvant inducing both strong CMI and antibody responses. Biochimica et Biophysica Acta (BBA)-Biomembr. 2005; 1718(1): 22-31.

55. Vangasseri DP, Cui Z, Chen W, Hokey DA, Falo Jr LD, Huang L. Immunostimulation of dendritic cells by cationic liposomes. Mol Membr Biol. 2006; 23(5): 385-395.

56. Lonez C, Vandenbranden M, Ruysschaert J-M. Cationic liposomal lipids: from gene carriers to cell signaling. Prog Lipid Res. 2008; 47(5): 340-347.