Cationic Immune Stimulating Complexes Containing Soluble *Leishmania* Antigens: Preparation, Characterization and in Vivo Immune Response Evaluation

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ABSTRACT

Background: Cationic immune stimulating complexes (PLUSCOMs) are particulate antigen delivery systems. PLUSCOMs consist of cationic immunostimulatory complexes (ISCOMs) derivatives and are able to elicit *in vivo* T cell responses against an antigen. **Objective:** To evaluate the effects of PLUSCOMs containing *Leishmaniamajor* antigens (SLA) on the type of immune response generated in the murine model of leishmaniasis. **Methods:** PLUSCOMs consisting of 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) were used as antigen delivery system/immunoadjuvants for soluble SLA. BALB/c mice were immunized subcutaneously, three times in 2-week intervals. Footpads swellings at the site of challenge and parasite loads were assessed as a measure of protection. The immune responses were also evaluated by determination of IgG subclasses and the level of IFN-γ and IL-4 in cultured splenocytes. **Results:** There was no significant difference ($p<0.05$) between the sizes of lesions in mice immunized with different formulations. Also, there was no significant difference in the number of parasites in the footpad or spleen of all groups compared with the control group. The highest level of IFN-γ secretion was observed in the splenocytes of mice immunized with PLUSCOM/SLA ($p<0.001$) and lower amounts of IL-4 was observed in PLUSCOM group ($p<0.001$) as compared to negative control. **Conclusion:** Our results indicated that SLA in different formulations generated an immune response with mixed Th1/Th2 response that was not protective enough despite the activation of $CD4^+$ T cells with secreting IFN-γ in groups which received PLUSCOM with antigen.

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INTRODUCTION

Leishmaniasis, a disease caused by several species of intracellular protozoan parasites of genus *Leishmania,* is an important public health problem in some endemic regions of at least 98 countries (1). *Leishmania* infections have diverse clinical manifestations, including cutaneous (CL), mucocutaneous (MCL), visceral (VL or kala-azar), post kala-azar dermal leishmaniasis (PKDL) (2).Cutaneous leishmaniasis (CL) is widely distributed in the Middle East, Central Asia, Americas and North Africa. Ninety percent of CL occurs in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (3).

Control of leishmaniasis is difficult, costly and not always effective, and treatment of CL is solely depend on chemotherapy using pentavalent antimonial agents (4)The duration of treatment is long with the need of multiple painful injections which is not fully effective (4). Protection against *L. major* infection depends upon induction of Th1 response, which in turn, results in production of IFN-γ (5). While an effective vaccine against leishmaniasis should elicit a Th1 response, various *Leishmania* antigens such as whole killed parasites, soluble *Leishmaniamajor* antigens (SLA), fractionated parasite and recombinant antigens showed to induce variable levels of protection in animal models when were used with an appropriate adjuvant and/or delivery system (6,7).Some cationic lipids containing an ammonium ion head group are immunostimulatory and could be used as delivery systems due to the ability to form liposome structures which offer efficient protein adsorption and/or encapsulation (8).

Immunostimulatory complexes (ISCOMs) are colloidal, spherical particulate antigen delivery systems composed of antigen, cholesterol, phospholipid and saponin such as QuilA (9). ISCOMs are usually about 40 nm in size and are composed of regularly oriented subunits. The subunits look like rings in electron micrographs of negatively stained preparations. ISCOMs are used as vehicles for amphipathic macromolecules, mostly membrane proteins. Before 1973, their potential as subunit vaccine delivery systems was not recognized Further on, 1SCOMs were 'reinvented' and their application as potent antigen vehicle was established (10). 1SCOMs' negative charge conferred by the glucouronic acid group of the Quil A glycosides means the attachment of anionic antigens such as protein and DNA is only low at best (11). To better facilitate the incorporation of anionic antigens, generation of positive charge 1SCOMs is essential. Hence, lipid component was substituted with a cationic lipid called 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP). The resultant particles demonstrated a cage-like structure similar to classic ISCOMs, but exhibited a net positive charge; therefore these particles were termed “PLUSCOMs”(12).The Quil A saponin, which is used in the ISCOMs and PLUSCOMs adjuvant for the development of human vaccines, is a multicomponent fraction from bark extract of *Quillaia saponaria* tree and able to form complex with lipids and amphiphilic antigens. The complex formation probably increases the adjuvanticity/toxicity ratio (13). ISCOMs vaccines have been approved for veterinary use and are currently undergoing clinical trials inhuman for anti-bacterial, anti-cancer, anti-viral, and anti-parasite purposes(14,15).In the this study, we prepared and characterized PLUSCOMs containing SLA and assessed their type of immune response generated against leishmaniasis in the murine model of leishmaniasis.

MATERIALS AND METHODS

Animals and Ethics Statement. Female BALB/c mice 6-8 weeks old used in this study were purchased from Pasteur Institute (Tehran, Iran). The animals were maintained in animal house of Pharmaceutical Research Center under specific pathogen free conditions and fed with tap water and laboratory pellet chow (Khorasan Javane Co., Mashhad, Iran). Experiments were carried out according to 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 issued in 2005.

Parasites, Soluble *Leishmania* Antigen (SLA) and Quil A. *L. major* strain (MRHO/IR/75/ER) used in this experiment was previously used in leishmanization and for preparation of experimental *Leishmania* vaccine and *leishmanin* preparation (16,17). The parasites were received from Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran. The method of SLA preparation was carried out using the protocol developed by Scott *et al.* (18) with minor modifications. Briefly, stationary phase promastigotes were harvested and washed four times in HEPES buffer (HS buffer; 10 mM, pH 7.5) containing 10% sucrose. The number of promastigotes was adjusted to 1.2×10^9/ml in a buffer solution containing enzyme inhibitor cocktail (50µl/ml; Sigma, St. Louis, MO, USA). The parasites were then lysed using freeze-thaw method followed by probe sonication in an ice bath. The supernatant of centrifuged lysate parasites was collected, dialyzed against buffer solution and sterilized by passage through a 0.22 µm membrane. The total protein concentration of SLA was determined using BCA (Bicinchoninic acid) protein assay kit (Thermo Scientific, USA) (19).The antigen was aliquoted and stored at -70°C until use. QuilA was obtained from Brenntag Biosector, Frederikssund, Denmark.

Preparation and Characterization of PLUSCOMs. PLUSCOMs were prepared by lipid film hydration in solid sugar matrices. The ratio of DOTAP, QuilA, cholesterol was 2:2:1, respectively. The total lipid concentration was 6.7 mg/ml. DOTAP (8mg) and cholesterol (4mg) were first dissolved in chloroform in a sterile tube and then the solvent was removed by rotary evaporator (Hettich, Germany). In the next step, SLA (1mg/ml) and sucrose (200mg) were added to lipid film and dissolved in a 4 mL mixture of tert-butanol and water (V/V 1:1). Snap freezing of the resulting monophase solution was carried out in nitrogen tank followed by overnight freeze drying (VD-800F, Taitech, Japan) to remove the solvents completely. Four milliliters of PBS (0.01 M, pH 7.4) and 8mg of Quil A were then added to hydrate the solid matrices followed by 15 min sonication to facilitate dispersion. The PLUSCOMs dispersion were subsequently extruded through 400, 200, 100 nm polycarbonate membranes (Avestin, Canada) (20).PLUSCOM/SLA will stand for PLUSCOMs incorporated SLA, while PLUSCOM + SLA will stand for a mixture of empty PLUSCOMs with SLA just before injection. Zeta potential and mean diameter of prepared formulations were determined using a Zetasizer (Nano-ZS, Malvern Instruments, UK) (21).SLA concentration encapsulated in PLUSCOMs was determined using BCA protein assay kit after removing lipids (Thermo Scientific, USA). The PLUSCOMs structures were checked by transmission electron microscopy (TEM) (22).Briefly, samples were coated onto glow-discharged; carbon coated copper grids and negatively stained with 2% phosphotungstic acid (pH=5.2). Then, the samples were scanned using a Phillips

CM100 electron microscope with an acceleration voltage of 100 kV and a magnification of 93,000X.

SDS-PAGE Analysis. The polyacrylamide gel electrophoretic analysis (SDS-PAGE) was carried out to estimate qualitatively the concentration of antigen encapsulated in formulations containing SLA. The gel consisted of running gel (10.22%, w/v, acrylamide) and stacking gel (4.78%, w/v, acrylamide) at the thickness of 1 mm. The electrophoresis buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH=8.3. Electrophoresis was carried out at 140 V constant voltages for 45 min. Then, the gels were stained with silver for protein detection (23).

Immunization of Mice. Mice (ten per group) were subcutaneously (SC) immunized three times in 3-week intervals with one of the following formulations: PLUSCOM/SLA(50 µg SLA/50 µl PLUSCOM/mouse), PLUSCOM + SLA (50 µg SLA+50 µl PLUSCOM/mouse), PLUSCOM(50 µl PLUSCOM/mouse), SLA (50 µg SLA/50 µl /mouse), Buffer (HEPES 10 mM, sucrose 10% w/v, pH 7.4) alone.

Challenge with *L. major* Promastigotes. Two weeks after the last booster injection, 1×10^6 late stationary phase *L. major* promastigotes in 50 µl PBS were inoculated SC into the right footpad of immunized and control group of mice. Lesion development and progression were monitored by measuring footpad thickness using a digital caliper (Mitutoyo Measuring Instruments, Japan) at weekly intervals for 8 weeks. Grading of lesion size was done by subtracting the thickness of the uninfected contra lateral footpad from that of the infected ones (24).

Quantitative Parasite Burden after Challenge. Spleens and footpad of infected mice were removed. The number of viable *L. major* parasites was estimated using limiting dilution assay method as described previously (25,26). Briefly, the mice were sacrificed at week 8 post-challenge, the infected foot and spleen were removed and homogenized in RPMI 1640 supplemented with 10% v/v heat inactivated FCS (Eurobio, Scandinavie), 2 mM glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin sulfate (RPMI-FCS). The homogenate was diluted with the media in 8 serial 10-fold dilutions and then was placed in each well of flat-bottom 96-well microtiter plates (Nunc, Denmark) containing solid layer of rabbit blood agar in tetra plicate and incubated at 25 ± 1°C for 7-10 days. The positive and negative wells were detected by presence and absence of motile parasite, respectively using an inverted microscope.

Antibody Assay. Serum-specific anti-*Leishmania* IgG subclasses were determined by a standard ELISA method. Blood samples were collected from mice before and at week 8 after challenge and the sera were isolated and kept at -20°C until use. Assessment of anti-SLA IgG total, IgG1 and IgG2a antibodies were assessed (26).Briefly, microtiter plates (Nunc, Denmark) were coated with 50 µl of SLA (10 µg/ml) in PBS buffer (0.01 M, pH 7.3) at 4°C for overnight and then a serial dilutions of serum was added to the plates after blocking with bovine serum albumin (BSA). The plates were treated with HRP-rabbit anti-mouse IgG isotype according to the manufacturer's instructions (Invitrogen Inc., USA). Optical density (OD) was determined at 450 nm using 630 nm as the reference wavelength.

Cytokine Assay with ELISA. The level of IL-4 and IFN-γ were determined in culture supernatants using ELISA method. Briefly, three mice in each group were sacrificed at week 3 after the last booster. The spleens were aseptically removed and mononuclear cells were isolated using Ficoll-Hypaque (Biogene, Iran) density centrifugation method (25).The cells were washed and resuspended in complete medium (RPMI 1640-FCS)

and seeded at 2 × 10^6/mL in 96-well flat-bottom plates (Nunc, Denmark). Then, the spleen cells were cultured and stimulated with SLA (10 μg/mL) or medium alone as a control and incubated at 37°C with 5% CO2 for 72 h. The culture supernatants were collected and the level of IFN-γ and IL-4 were determined using ELISA method according to the manufacturer's instructions (MabTech, Sweden).

Flowcytometry Analysis. Splenocytes were isolated 2 weeks after the last booster and stained for intracellular cytokine IFN-γ (anti-IFN-γ–FITC) and IL-4 (anti-IL-4-FITC) according to BD protocols Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit. Briefly, Splenocytes (10^6 cells/ml) in medium containing GolgiPlug™ (1 μl/ml) were stimulated with PMA/ionomycin cocktail (2 μl/ml) for 4 h at 37°C. After stimulation, 10^5 splenocytes were transferred into flow cytometry tubes and washed two times with stain buffer (2% FCS in PBS). Splenocytes were stained with 1 μl anti-CD8a-PE-cy5 antibody and 1 μl anti-CD4-PE-cy5 antibody in separate tubes for 30min at 4°C. The cells were washed with stain buffer and fixed using Cytofix/Cytoperm™ solution. Fixed cells were washed two times with Perm/Wash™ buffer and then stained with 1 μl anti-IFN-γ- FITC antibody for 30 min at 4°C. CD4 cells were also stained with 1 μl anti-IL-4-PE antibody. The cells were washed with Perm/Wash™ buffer and suspended in 300 μl stain buffer for flowcytometric analysis Calibur (BD Biosciences, USA).

Statistical Analysis. One-way ANOVA statistical test was used to assess the significance of the differences among the various groups. The mean and standard deviation of all experiments were determined. In case of significant F value, Turkey-Kramer multiple comparisons test was carried out as a post-test to compare the means in different groups of mice. p value of ≤ 0.05 was considered as significant.

RESULTS

Characterization of PLUSCOM. The mean diameter and charge of prepared formulations calculated by zeta sizer (Malvern, UK) for each preparation and is shown in Table 1. The polydispersity index (PDI) was ~0.2 that indicates all formulations are fairly homogenous. Zeta potential of all preparations was positive due to the presence of positively charged DOTAP lipid (Table 1).

Table1. Particle size distribution, polydispersity index (PDI) and zeta potential of prepared formulations (Mean ± SD, n = 3).

Formulation	Size (nm)	PDI	Zeta potential (mv)
PLUSCOM	128.4 ± 21.5	0.2± 0.1	49.6 ± 12.5
PLUSCOM/SLA	163.8 ± 36.4	0.3 ± 0.1	35.3 ± 6.8
PLUSCOM + SLA	93.7 ± 10.8	0.3 ± 0.1	37.2 ± 5.4

The entrapment of SLA in PLUSCOM/SLA formulation was 55 ± 15%. The concentration of SLA in final formulation was 1 μg/μl. The presence of SLA in different formulations was confirmed using SDS-PAGE gel electrophoresis (Figure 1).

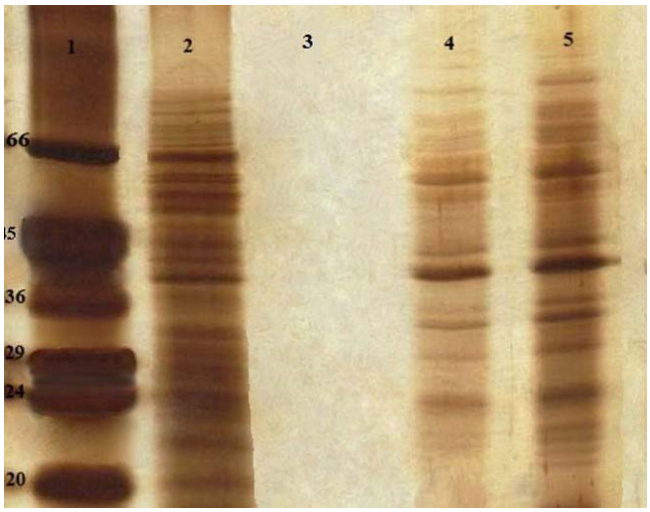


Figure1. SDS-PAGE analysis of SLA alone, PLUSCOM, PLUSCOM/SLA and PLUSCOM + SLA. Lane 1, Low-range protein standard (Sigma, USA); Lane 2, SLA (10 µg); Lane 3, PLUSCOMs without SLA; Lane 4, SLA incorporated in PLUSCOM; Lane 5, physical mixture of PLUSCOMs and SLA.

SDS-PAGE analysis of SLA revealed several protein bands with molecular weight ranges from 10 to 70 kDa. After purification, SDS-PAGE analysis of PLUSCOM/SLA revealed bands similar to free SLA. TEM picture (Figure 2) was used to confirm the size as well as the morphology of the prepared particles. The size of PLUSCOM was in the range of 60-70 nm as measured for almost 100 particles.

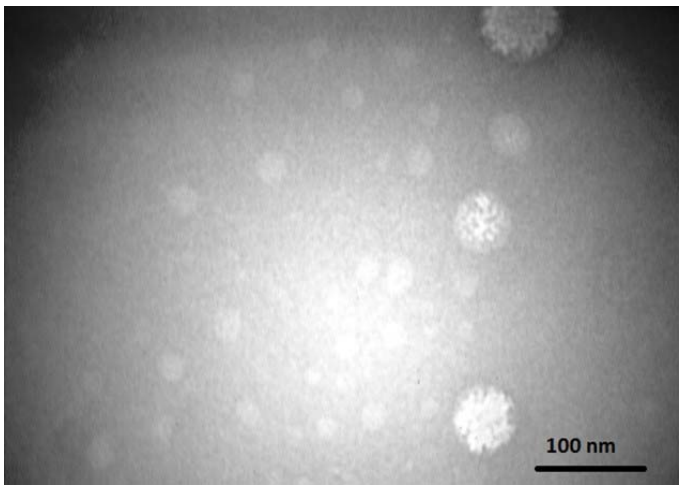


Figure2. TEM image of PLUSCOM consisting DOTAP:Chol:Quil A.

Challenge Results. Lesion development and progress were recorded in each mouse weekly by measuring of footpad thickness (Figure 3). Footpad thickness progressed similarly in all groups of mice up to two weeks after the challenge. Footpad swelling in all groups was progressed continuously and no protection was observed in any groups. Interestingly, the group of mice received PLUSCOM showed the smallest lesion size compared to the groups of mice received SLA at week 6, but there was no significant difference compared with PLUSCOM/SLA group. In all the groups, the footpad

swelling reached a plateau after 6 weeks but the disease progressed by metastasis to other organs and some of the mice lost their foot.

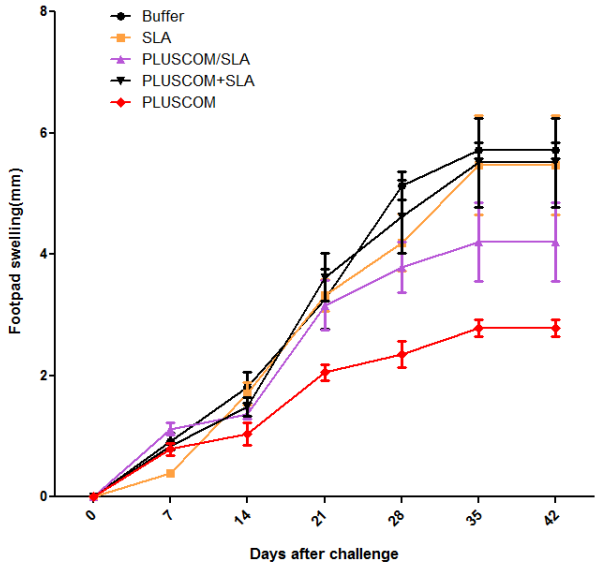


Figure 3. Footpad swelling in BALB/c mice immunized SC, three times in 3-week intervals, with SLA, PLUSCOM, PLUSCOM/SLA, PLUSCOM + SLA or buffer alone. The footpad thickness of each mouse was measured on both footpads for 42 days. Each point represents the average increase in footpad thickness ± SEM (n=7).

Splenic Parasite Burden after Challenge. At day 42 after challenge, the number of viable *L. major* was quantified in spleen of different groups of mice (Figure 4A). The group of mice immunized with PLUSCOM showed the least parasite burden, but was not significant ($p > 0.05$) compared to the control group which received PBS. Also, there was no significant difference among the groups in terms of parasite number.

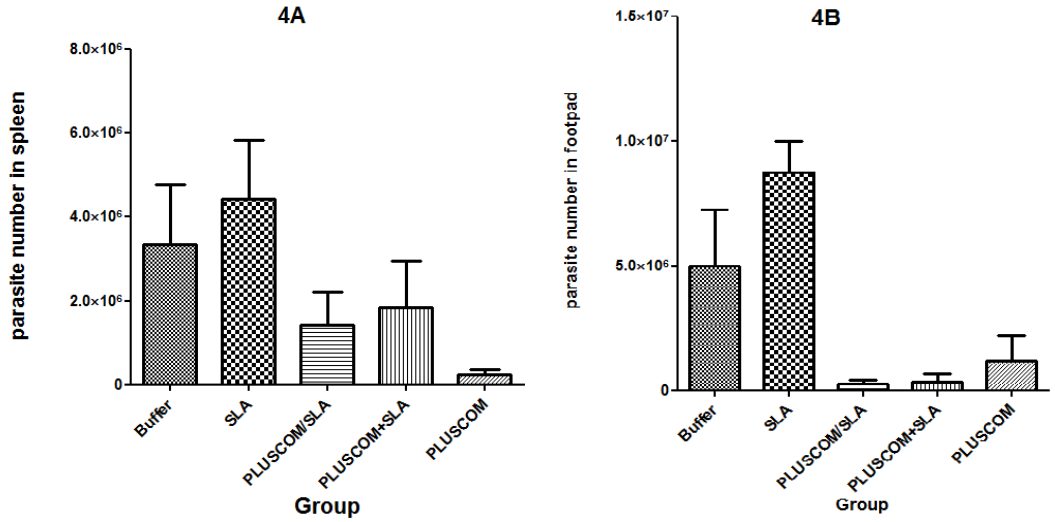


Figure 4. Parasite burden in spleen (A) and footpad (B) of infected BALB/c mice. Mice immunized SC, three times in 3-week intervals with SLA, PLUSCOM, PLUSCOM/SLA, PLUSCOM + SLA or buffer alone after challenge with *L. major*. A limiting dilution analysis was performed at week six post infection on the spleen and foot of individual mouse. The wells were assessed microscopically for *L. major* growth, and the number of viable parasite per spleen was

determined by GraphPad Prism5 software. The bar represents the average number ± SEM (n=3).

Parasite Burden in Foot after Challenge. The number of viable *L. major* was quantified in the infected foot-pad of different groups of mice at day 42 after challenge (Figure 4B). As shown, however, the group of mice immunized with PLUSCOM/SLAshowed the least parasite burden compared with the other groups, but there was no significant difference in the number of footpad parasites among all groups compared with the control group received PBS.

Antibody Response. To determine the type of immune response generated, the serum samples were collected prior and post-challenge and anti *Leishmania* IgG isotypes were tested. As shown in Figure 5A-C, there was a significant ($p<0.0001$) higher level of IgG2a, IgG1,IgG antibodies in the sera of mice immunized with PLUSCOM/SLA or PLUSCOM + SLA compared with the control group before challenge, respectively.

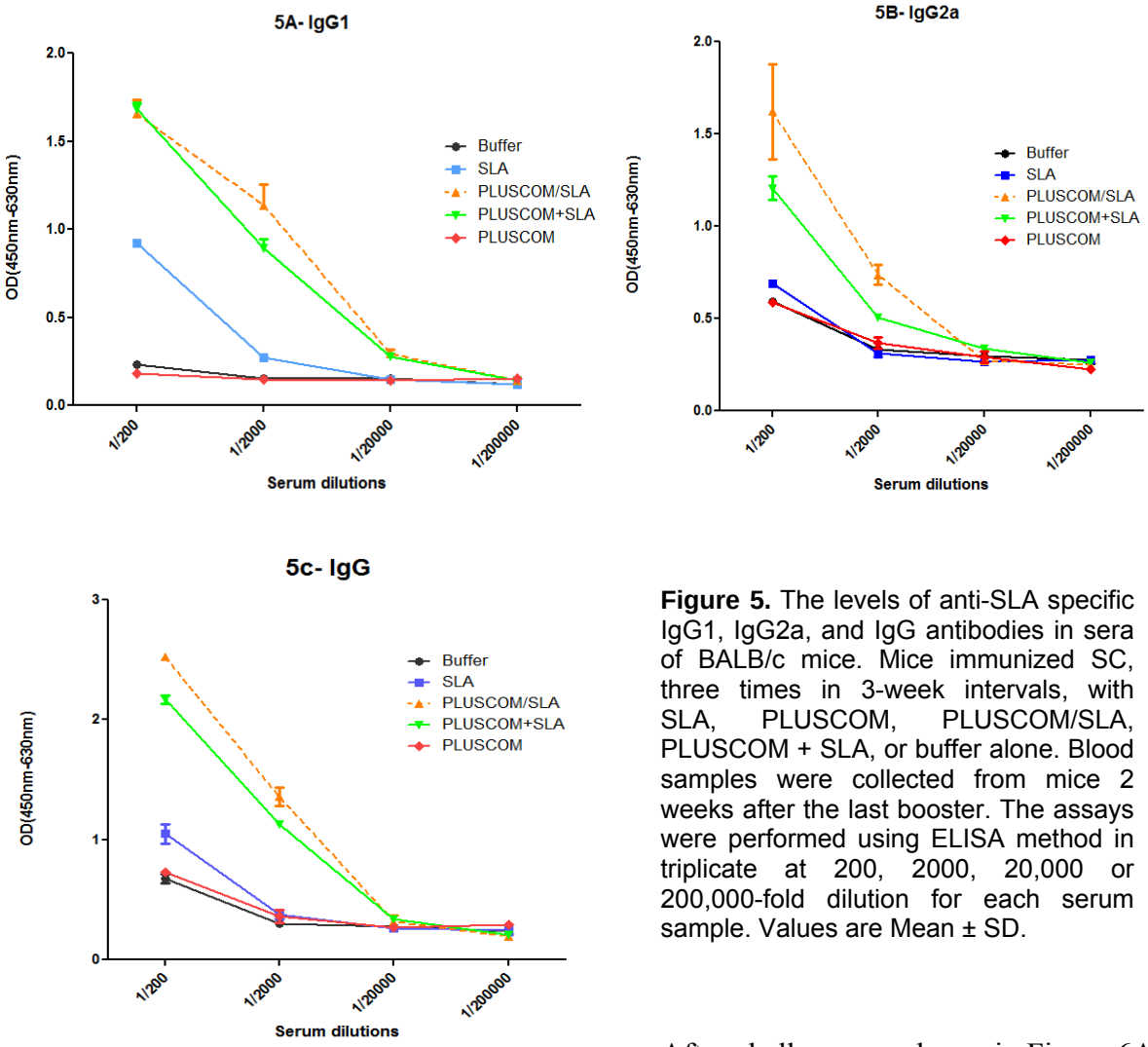


Figure 5. The levels of anti-SLA specific IgG1, IgG2a, and IgG antibodies in sera of BALB/c mice. Mice immunized SC, three times in 3-week intervals, with SLA, PLUSCOM, PLUSCOM/SLA, PLUSCOM + SLA, or buffer alone. Blood samples were collected from mice 2 weeks after the last booster. The assays were performed using ELISA method in triplicate at 200, 2000, 20,000 or 200,000-fold dilution for each serum sample. Values are Mean ± SD.

After challenge, as shown in Figure 6A-C, although the group PLUSCOM + SLA showing the highest antibody titer, there was no significant difference in level of IgG total and IgG1 antibodies compared to the other groups. Interestingly, the sera of mice immunized with PLUSCOM/SLA or PLUSCOM

showed significantly ($p<0.0001$) the lowest IgG2a antibody titer compared with the other immunized groups.

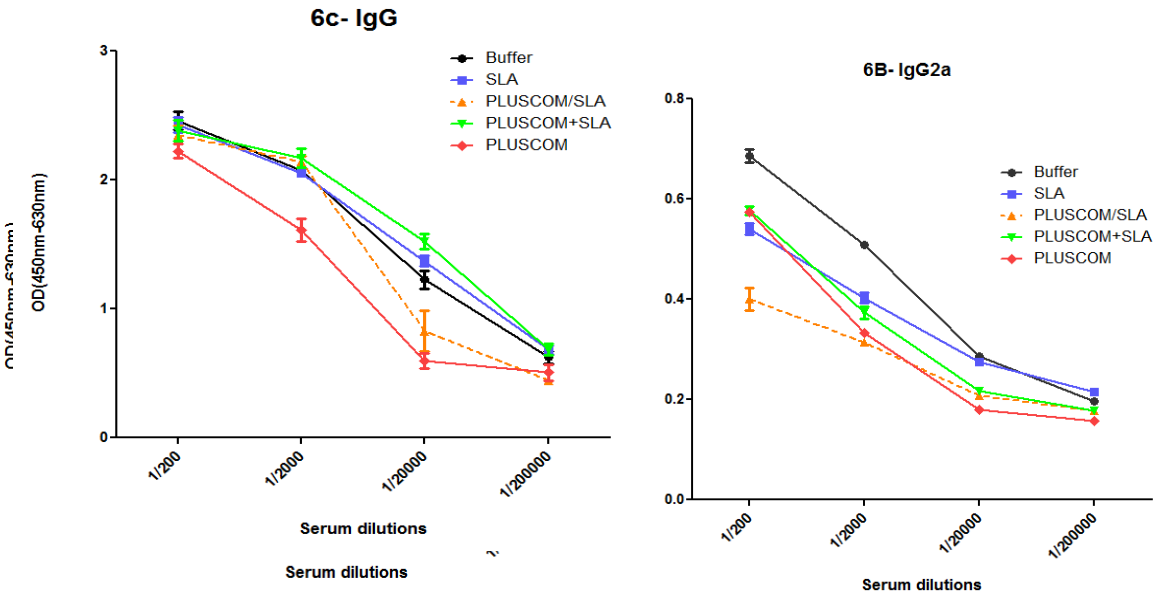
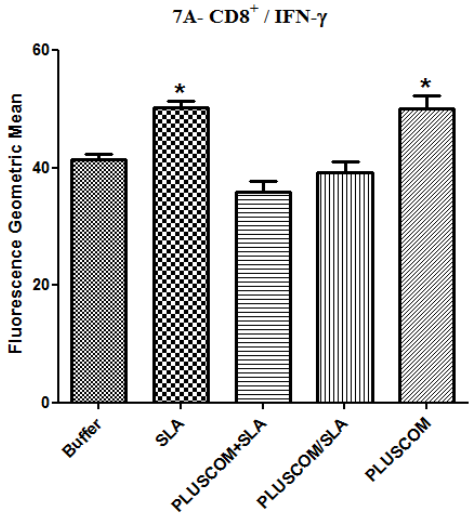
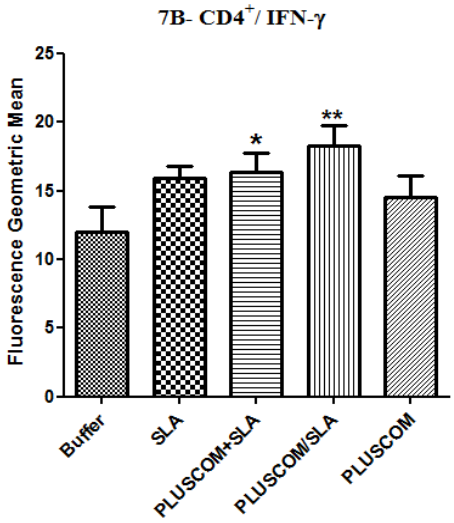


Figure 6. The levels of anti-SLA specific IgG1, IgG2a, and IgG antibodies in sera of BALB/c mice. Mice immunized SC, three times in 3-week intervals, with SLA, PLUSCOM, PLUSCOM/SLA, PLUSCOM + SLA, or buffer alone. Blood samples were collected from mice at week 6 after challenge. The assays were performed using ELISA method in triplicate at 200, 2000, 20,000 or 200,000-fold dilution for each serum sample. Values are Mean ± SD.

Flowcytometery Results. Splenocytes were isolated to determine antigen-specific T cell responses in different groups of mice 2 weeks after the last booster. Extracellular staining was used for CD4 and CD8 surface markers and intracellular cytokine staining was used for IFN-γ and IL-4 cytokines followed by flowcytometery analyses. $CD4^+$ and $CD8^+$ cells were studied concomitantly. CD8 and CD4 markers represent the frequency of IFN-γ and IL4 producing cells in Th1 and Th2 population, respectively. As shown in Figure 7A-C, the group of mice which was immunized with SLA and PLUSCOM generated a significantly higher number of $CD8^+$/IFN-γ T cells compared to all the other groups. The frequency of $CD4^+$/IFN-γ cells in the group of mice immunized with PLUSCOM/SLA and PLUSCOM + SLA were significantly greater than that of other groups, while flow cytometric results also showed IL-4 production in $CD4^+$ cells that implies T cell-dependent humoral immunity was not induced significantly in all groups.





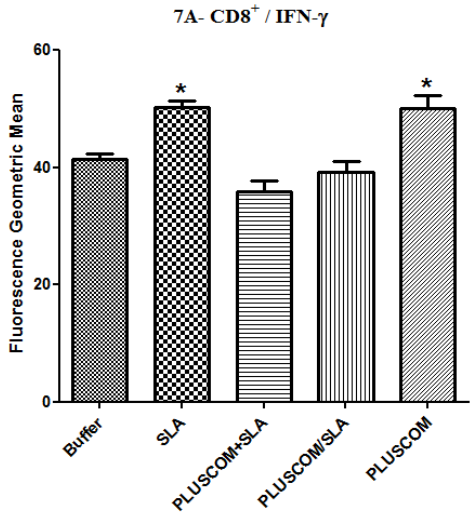
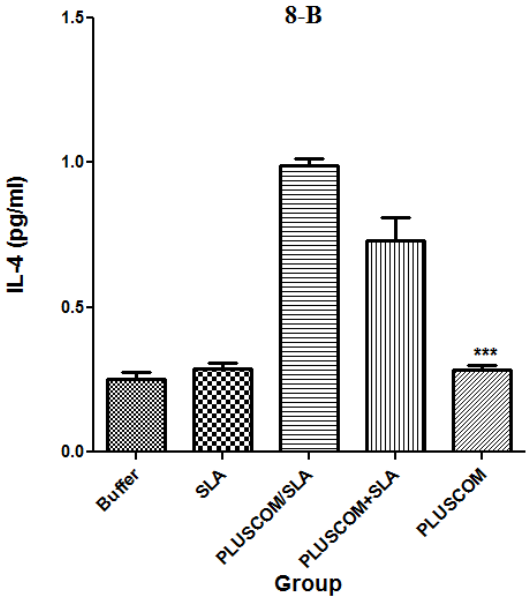


Figure 7. After last booster, splenocytes were isolated, restimulated, and then stained for surface CD8, CD4 and intracellular IFN-γ and IL-4 cytokines. Splenocytes were gated by side vs. forward scatter light followed by staining with CD8 Pe-cy5 and CD4 Pe-cy5. Plots show log fluorescence intensity for IFN-γ- FITC and IL-4-PE. The data indicate the mean ± S.E.M, (n = 3). ** ($p<0.01$) and * ($p<0.05$) denote significant difference from buffer and all other formulations, respectively.

***In vitro* Cytokine Production by Splenocytes.** One day before challenge, splenocytes of immunized mice were isolated and cultured to detect the concentration of two related cytokines. The results showed that the level of IFN-γ in the supernatant of splenocytes in the group of mice immunized with PLUSCOM/SLA was significantly ($p<0.001$) higher than the other groups. There was no significant difference in the level of IFN-γ among the other groups (Figure 8A). The least amount of IL-4 was detected in the group of mice immunized with PLUSCOM, this group produced a lower significance ($p<0.001$) level of IL-4 compared to the other SLA formulated groups (Figure 8B).



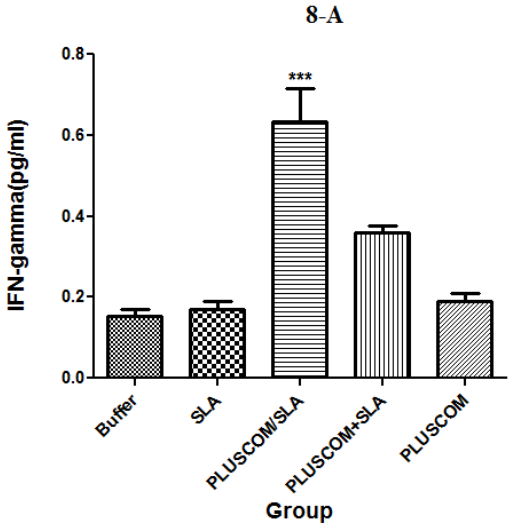


Figure 8. Splenic cell responses of BALB/c mice immunized SC, three times in 3 weeks intervals, with SLA, PLUSCOM, PLUSCOM/SLA, PLUSCOM+SLA, or buffer alone. Two weeks after the last booster, their spleens were removed and stimulated in vitro with SLA (10 µg/ml). Production of IFN-γ (A) and IL-4 (B) were assessed by sandwich ELISA with supernatants removed after 72 h of in vitro incubation. Cells from 3 mice per group were pooled. Each bar represents the mean ± SD of triplicate wells. ***p< 0.001 when the mice immunized with PLUSCOM and PLUSCOM/SLA are compared with the mice received buffer.

DISCUSSION

The main goal of this study was to evaluate the protection rate and type of immune response induced in BALB/c mice immunized using SLA in PLUSCOMs as a vaccine and adjuvant delivery system. The immunized groups of mice along with control groups were challenged at week 3 after the last booster injection. Several parameters such as lesion size, parasite burden in foot and spleen, cytokine production and antibody responses were evaluated. Although the size of footpad swelling after challenge and splenic parasite burden in mice showed that PLUSCOM group had the lowest size footpad swelling, the treatment did not protect BALB/c mice against leishmaniasis. In this study, PLUSCOMs consisting with DOTAP were used. In pharmaceutical point of view, we prepared for the first time PLUSCOMs containing SLA with a reasonable entrapment efficiency (55%). A similar high incorporation efficiency of pFITC-OVA (Ovalbumin) into cationic structures of PLUSCOM is showed elsewhere (~ 85%) (27).
SLA is a crude soluble *Leishmania* antigen which induces partial protection when used alone; however, in association with delivery systems such as cationic liposomes possessing adjuvant activity, SLA enhances antibody and antigen-specific T-cell proliferative responses (28).In the current study, SLA alone induced more IgG1 than IgG2a antibody, and low titer of IFN-γ that means it could not induce Th1 type of immune response. However, the mice which received PLUSCOM/SLA or PLUSCOM + SLA showed a high titer of IgG2a and IFN-γ that are indicator of Th1 type of immune response to some extent. Although we detected a high amount of IFN-γ in those groups, they induced a significant high amount of IL-4 that was correlated to a Th2 type of immune response. It seems that these formulations induced a mixed Th1/Th2 response that has been confirmed in challenge results as well. ISCOMs are effective delivery systems which transform soluble antigens into a particulate form thereby prolong the *in vivo* half-life of the antigens. Net anionic charge of ISCOMs is due to the glucouronic acid group present on QuilA glycosides. This may limit the effectiveness of the ISCOMs as a delivery system for anionic antigens such as protein or DNA (13,27). Among different types of ISCOMs, cationic ones (termed PLUSCOMs) are premier in inducing antigen-specific CTL and antibody response due to their tightly bound

complex formation with negatively charged antigens Therefore, the positive charge on the surface of the resulting particle strongly improves the uptake of both PLUSCOMs and the entrapped antigen by APCs and their subsequent presentation to T cells (12).
For assessment of protection rate, size of lesion and footpad parasite burden were checked in different groups of mice. The results indicated that the group of mice received PLUSCOMs showed the smallest lesion size compared to other groups, but there was no significant difference between this group and those received PLUSCOM/SLA. The results of cytokine assay demonstrated that highest level of IFN-γ secretion was observed in the splenocytes of mice immunized with PLUSCOM/SLA as compared to buffer group($p<0.001$). Vaccination of BALB/c mice with PLUSCOMs produced strong antigen-specific proliferative responses in the spleen, confirming previous reports of the ability of ISCOMs to prime for recall proliferative responses *in vitro* after immunization (29).It is known that IFN-γ, produced by Th1 type cells, both suppresses the induction of IgG1 and boosts the production of IgG2a antibody subtype, and similar types of protection have been reported for many parasite infections (30-32). Moreover, flowcytometery results indicated that PLUSCOM/SLA and PLUSCOM + SLA generated considerably a higher number of $CD4^+$/ IFN-γcells in groups which received PLUSCOM/SLA or PLUSCOM + SLA. However, these two groups induced a high amount of IL-4 that means our formulations may induce a mixed Th1/Th2 response, the fact that was reported by others (Ref needed here). It was shown that parasite surface Ag-2 (PSA) in immune-stimulating complexes generated an immune response with mixed Th1/Th2 response that was not protective despite the activation of large numbers of CD4+ T cells secreting IFN-γ (33).In another study, although ISCOMs containing gp63 formulations modulated the immune response of vaccinated BALB/c mice preferentially towards a Th1 rather than a Th2 type response, induced a partial protection against *Leishmania* challenge (34).A reason for induced mixed response in this study might be the presence of Quil A. QuilA is a semi-purified preparation of *Quillaia* saponin that is composed of a heterogeneous mixture of closely related saponins. Quil A is suitable for veterinary applications but has been unacceptable for human applications (35).However, further characterization of QuilA has identified several saponin fractions, which have adjuvant activity and retain the capacity to form ISCOMs (36). Exclusive saponins from Quil A such as QS-7, QS-17, QS-18, and QS-21 have been investigated for their adjuvant properties (35).Therefore, because of this impurity, our formulations showed an immune response with mixed Th1/Th2 properties (30,37). Although there are some studies which have shown a Th1 response after using ISCOMs, there are some other reports which show they induce a concomitant Th2 response, resulting in a mixed Th1/Th2 response (38,39).It seems that the type of immune response reported after ISCOM immunization is vary with the type of antigens used (40). In the current study, despite the activation of high numbers of T cells secreting IFN-γ, immunization of mice with PLUSCOM containing SLA did not protect them against *L. major* infection. Some findings propose that the activation of Th2-like T cells by ISCOMs vaccination is sufficient to abrogate the protective Th1 effects (Reference needed here). It can be derived that ISCOMs have induced potent T cell responses of a Th1 type immune response but are also able to induce a concomitant Th2 response giving rise to T cell response with mixed Th1/Th2 properties (30).In summary, our results indicated that PLUSCOMs formulations containing SLA induced a mixed Th1/Th2 response in mice that could not protect mice against *Leishmania* infection but it might be helpful for other infections which need a mixed Th1/Th2 responces.

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REFERENCES

1. Word Health Organization (WHO). Control of the Leishmaniases, Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases. WHO Technical Report Series 949, Geneva 2010; pp. 1-187.
2. Alvar J, Croft S, Kaye P, Khamesipour A, Sundar S, Reed S. Case study for a vaccine against leishmaniasis.Vaccine. 2013; 31: 229-244.
3. Hadighi R, Mohebali M, Boucher P,Hajjaran H, Khamesipour A, Ouellette M.Unresponsiveness to Glucantime Treatment in Iranian Cutaneous Leishmaniasis due to Drug-Resistant Leishmania tropica Parasites. PLoS Medicine. 2006; 3: e162.
4. Heinzel F, Sadick M,Mutha S, Locksley R. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. Proceedings of the NationalAcademy of Sciences of the United States of America. 1991; 88: 7011-7015.
5. CoplandMJ, RadesT, DaviesNM, Baird MA. Lipid based particulate formulations for the delivery of antigen. Immunology and Cell Biology. 2005; 83: 97–105.
6. Badiee A, Heravi SV, Khamesipour A, Jaafari MR. Micro/nanoparticle adjuvants for antileishmanial vaccines: present and future trends. Vaccine. 2013; 31: 735-749.
7. Lovgren K, Morein B. The requirement of lipids for the formation of immunostimulating complexes (ISCOMs). BiotechnolApplBiochem. 1988; 10:161-72.
8. Morein B, Sundquist B, Höglund S, Dalsgaard K, Osterhaus A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature. 1984; 308:457-60.
9. KerstenGF, SpiekstraA, Beuvery EC, Crommelin DJ. On the structure ofImmune-stimulating saponin-lipid complexes (ISCOMs). BiochimBiophys Acta.1991; 1062:165–71.
10. Lendemans DG, MyschikJ, Hook S, Rades T. Cationic cage-like complexes formed by DC-cholesterol, Quil-A, and phospholipid. Journal of pharmaceutical sciences. 2005; 94:1794-1807.
11. Sanders MT, Brown LE, Deliyannis G, Pearse MJ. ISCOM-based vaccines: the second decade. Immunology and cell biology. 2005; 83:119-28.
12. Morein B, Hu KF, Abusugra I. Current status and potential application of ISCOMs in veterinary medicine. Advanced drug delivery reviews. 2004; 56:1367-82.
13. Khamesipour A, Rafati S, Davoudi N, Maboudi F, Modabber F. Leishmaniasis vaccine candidates for development: a global overview. Indian Journal of Medical Research. 2006;123: 423–438.
14. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, et al. First generation leishmaniasis vaccines: a review of field efficacy trials. Vaccine. 2008;26: 6759–6767.
15. 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. Journal of immunology. 1987;139: 221-227.
16. BainorA, ChangL, McQuadeTJ, Webb B, Gestwicki JE. Bicinchoninicacid(BCA) assay in low volume. Analytical Biochemistry. 2010; 410: 310–402.
17. LiangMT, Toth I, DaviesNM. A novel method for preparing immune stimulating complexes (ISCOMs) by hydration of freeze-dried lipid matrix. European Journal of Pharmaceutics and Biopharmaceutics. 2008;68: 840–845.
18. Jaafari MR, GhafarianA, Farrokh-Gisour A, SamieiA, KheiriMT, MahboudiF, et al. Immune response and protection assay of recombinant major surface glycoprotein of leishmania (rgp63) reconstituted with liposomes in BALB/c mice. Vaccine. 2006;24:5708–5717.

19. TitusRG, MarchandM, Boon T, Louis JA. A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. Parasite Immunology. 1985;7:545–555.
20. BadieeA, Jaafari MR, KhamesipourA. Leishmani amajor:immune responsein BALB/c mice immunized with stress-inducible protein1 encapsulated in liposomes. Experimental Parasitology. 2007; 115:127–134.
21. Taswell C. Limiting dilution assays for the determination of immuno competent cell frequencies. 1. Data analysis. Journal of Immunology. 1981;126:1614–1619.
22. Jaafari MR, Badiee A, Khamesipour A, Samiei A, Soroush D, Kheiri MT, et al. The role of CpGODN 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: 6107–6117.
23. HejaziH, TasbihiM, et al. The role of liposomal CpGODN on the course of *L. major* infection in BALB/C mice. Iranian journal of parasitology. 2010;5:47-54.
24. McBurney WT, Lendemans DG, Myschik J, Hennessy T, Rades T, Hook S. In vivo activity of cationic immune stimulating complexes (PLUSCOMs). Vaccine. 2008; 26:4549-56.
25. BhowmickS, RavindranR, AliN.Leishmanial antigens in liposomes promote protective immunity and provide immunotherapy against visceral leishmaniasis via polarized Th1 response. Vaccine. 2007;25: 6544- 6556.
26. MaloyK, Donachie AM, Mowat AM. Induction of Th1 and Th2 CD4+ T cell responses by oral or parenteral immunization with ISCOMS. European journal of immunology. 1995;25: 2835-41.
27. Pond L, Wassom D, Hayes CE. Evidence for differential induction of helper T cell subsets during Trichinella spiralis infection. J Immunol. 1989; 143:4232-7.
28. Passero LF, Laurenti MD, Tomokane TY, Corbett CE, Toyama MH. The effect of phospholipase A2 from Crotalus durissus collilineatus on Leishmania (Leishmania) amazonensis infection. ParasitolRes. 2008 ;102:1025-33.
29. PapadopoulouG, KaragouniE, Dotsika E. ISCOMs vaccine against experimental leishmaniasis. Vaccine. 1998; 16 : 885-892.
30. Cox JC, Sjölander A, Barr IG. ISCOMs and other saponin based adjuvants. Advanced drug delivery reviews. 1998; 32:247-271.
31. Kersten GF, Teerlink T, Derks HJ, Verkleij AJ, Van Wezel TL, Crommelin DJ, et al. Incorporation of the major outer membrane protein of Neisseria gonorrhoeae in saponin-lipid complexes (iscoms): chemical analysis, some structural features, and comparison of their immunogenicity with three other antigen delivery systems. Infection and Immunity. 1988; 56: 432-8.
32. RönnbergB, FekaduM, Morein B. Adjuvant activity of non-toxic Quillaja saponaria Molina components for use in ISCOM matrix. Vaccine. 1995; 13:1375-82.
33. Sjölander A, Drane D, Maraskovsky E, Scheerlinck JP, Suhrbier A, Tennent J, et al. Immune responses to ISCOM formulations in animal and primate models. Vaccine. 2001;19:2661-5.
34. Hu K, Lövgren-Bengtsson K, Morein B. Immunostimulating complexes (ISCOMs) for nasal vaccination. Advanced drug delivery reviews. 2001; 51:149-59.
35. Kersten GF, Crommelin DJ. Liposomes and ISCOMS as vaccine formulations. BiochimBiophysActa. 1995; 1241:117-138.