Chitosan Nanoparticles Loaded with Whole and Soluble Leishmania Antigens, and Evaluation of Their Immunogenecity in a Mouse Model of Leishmaniasis

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ABSTRACT

Background: Although there have been numerous attempts to develop vaccines for Leishmaniasis, no vaccine can be found against Leishmania in routine use for an effective global vaccination. It seems that one of the reasons for the low efficacy of such vaccines is the lack of a suitable adjuvant. **Objective:** To evaluate the effects of chitosan nanoparticles containing whole Leishmania lysate antigen (WLL) and soluble leishmania antigens (SLA), a first generation Leishmania vaccine, on the type of immune response generated in BALB/c in a murine model of leishmaniasis. **Methods:** The optimum coating ratio between the polymer and antigens was determined according to their physico-chemical properties such as particle size and zeta potential. Chitosan nanoparticles were loaded with antigens via ionic gelation method. BALB/c mice were immunized subcutaneously three times with various nanoparticulate and free antigens with 2-week intervals. **Results:** There was no significant ($P > 0.05$) difference concerning the footpad thickness of mice immunized with nanoparticulate formulations containing either SLA or WLL during the experiment period; these formulations induced a strong mixed Th1/Th2 type immune response characterized by the production of IFN-γ and IL-4, and high levels of IgG2a IgG1 anti-Leishmania antibody. **Conclusion:** Nanoparticulate formulations (CHT: SLA and CHT: WLL) are not suitable candidates for preferential induction of a pure Th1-type immune response and immunization against Leishmania infection. However, it might be a good strategy in other infectious diseases where a mixed Th1/Th2 immune response is required.

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Keywords: Chitosan nanoparticles, soluble Leishmania antigen, Leishmania, vaccination. , Whole Leishmania lysate

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INTRODUCTION

Among the 350 million people living in countries with a potential of active transmission of Leishmania, about 14 million are prone to active contamination(1). Treatment of leishmaniasis is not readily possible, because a very limited number of drugs is available, most of which are expensive and difficult to administer . Moreover, low efficacy and drug resistance is frequently reported.

Several antigens have been tested for immunization against leishmaniasis, most of which failed to induce efficient immune responses or even led to inappropriate immune responses (Th2 immune responses) and exacerbation of the disease (2). However, strong immune responses and long-lasting protection is still expected, similar to that observed following the recovery of cutaneous leishmaniasis (3-7). Th2 or mixed Th1/Th2 immune responses exacerbate the disease, hence demand the necessity of a proper antigen and delivery system/adjuvant for pure Th1 responses (2, 8-11). Several immunoadjuvants such as BCG, G-CSF (12, 13), CpG-ODN (2, 14-20), and various delivery systems like PLGA nanospheres (2, 11, 21) and liposomes (7, 18, 22, 23) have been experimented.

Efforts to develop vaccines against leishmaniasis resulted in recognizing numerous candidate antigens; however, only whole killed Leishmania or first generation vaccines with or without adjuvant have been successful to reach phase 3 clinical trials (7, 24). However, the results were not conclusive in certain trials, where a restricted prophylactic efficacy was shown (25). It appears that one of the reasons for the restricted efficacy of first generation vaccines is the lack of a suitable adjuvant (26, 27). On the contrary, new candidate vaccines against leishmaniasis, particularly those based on DNA or recombinant proteins (28), seem to be less immunogenic compared to the first generation vaccines. However, some Leishmania antigens in first generation vaccines were able to induce protection in susceptible animals when employed with suitable adjuvant, particularly IL-12 (29). Accordingly, using an appropriate adjuvant is important for almost any modern vaccine, especially those against leishmaniasis (30). On the other hand, there are limited choices when looking for appropriate adjuvants in humans. For example, Mycobacterium bovis, Bacillus Calmette– Guérin (BCG), used in numerous clinical trials, resulted in restricted efficacy (18 , 7). The results of phase 3 trials in humans showed that BCG mixed with ALM induced a Th1 immune response which was not potent enough to protect against Leishmania infection (7, 31).

Chitosan is a biocompatible, low cost, highly tolerable polymer capable of inducing Th1 responses (32, 33). Chitosan mucoadhesivity prolongs antigen presence time at mucosa, and its ability to open the epithelial tight junctions augments the transport of antigens and induces more immune responses (33). Various kinds of chitosan-based delivery systems, such as micro/nanoparticles, hydrogels, and microneedles have been employed in immunization studies (19, 34, 35).

Nanoparticles, as drug delivery/adjuvant, are able to increase the immune responses against loaded antigen through several mechanisms. Encapsulation of soluble antigens in nanoparticles gives them a particulate nature, increasing their interaction with antigen presenting cells (APCs) and macrophages. Nanoparticles co-encapsulate both antigen and adjuvant and co-deliver them to the same APC (2, 36, 37).

In the present study, chitosan nanoparticle was used as an adjuvant delivery system, in which whole Leishmania lysate (WLL) and soluble Leishmania antigens (SLA) were encapsulated to generate a first generation vaccine. Chitosan nanoparticles (NPs) loaded

with SLA or WLL antigens were prepared with a simple method, and their immunoadjuvant potential was studied in BALB/c mice.

MATERIALS AND METHODS

Chemicals. Chitosan (degree of deacetylation: 95%; viscosity of 1% solution: 11 cP) was purchased from Primex, Avaldsnes, Norway. Cell culture reagents were bought from Invitrogen (USA). Goat anti-mouse IgG, IgG1 and IgG2a conjugated with horseradish peroxidase were purchased from Zymed laboratories (San Francisco, USA). ELISA assay kit for mouse Interferon-γ and IL-4 were bought from Mabtech AB (Sweden). BCA protein assay kit was purchased from Pierce (Thermo Scientific, Rockford, IL).

Parasites, soluble Leishmania antigen (SLA), whole *Leishmania* lysed (WLL). Leishmania major strain (MRHO/IR/75/ER) (used in this investigation was formerly employed for leishmanization in Iran. The method of SLA preparation was based on the earlier published protocol with slight modifications (38). In brief, log phase promastigotes were harvested and washed 4 times with PB buffer (pH 6.7, 8 mM). The number of L. major parasites was adjusted to 1.2 × 109/ml in buffer solution containing enzyme inhibitor cocktail (50µl/ml) (Sigma, St. Louis, MO, USA). The preparation was then incubated in ice-water bath for 10 minutes and lysed via freeze–thaw method followed by probe sonication in an ice bath. The supernatant of the centrifuged lysate promastigotes was collected, dialyzed against PB buffer, sterilized by passage through a 0.22 µm membrane and stored at −70 °C until further use. The protein concentration of the SLA was determined using BCA protein assay kit (Thermo Scientific, USA).
To prepare whole Leishmania lysate (WLL), the promastigotes of L. major were isolated at log phase, and washed 4 times with PB buffer; the parasites pellet was then resuspended in buffer solution containing octyl-β-d-glucoside (OG) with gentle shaking, which formed complexes containing all membrane spanning proteins, a spectrum of hydrophobic components and water-soluble proteins.

Animals and ethics statement. 6-8-week old female BALB/c mice were bought from Pasteur Institute (Tehran, Iran). The mice were kept in the animal house of Pharmaceutical Research Center and fed with tap water based on a standard laboratory diet (Khorassan Javane Co, Mashhad, Iran). Animal experiments were performed according to Mashhad University of Medical Sciences, Ethical Committee Acts.

Preparation and characterization of CHT NPs encapsulated with WLL and SLA. In order to prepare CHT:SLA NPs and CHT:WLL NPs, equal volumes of CHT solution were added dropwise to SLA or WLL dispersion and gently mixed for about 5 s (35). To find the best mixing ratio, 50 µl of different concentrations of CHT solution in PB buffer (pH 6.7, 8 mM) were mixed with 50 µl of SLA and WLL (1 mg/ml) dispersions. Ten polymer/antigen ratios (ranging from 1:1 to 10:1, w/w) were mixed and characterized for their size, PDI and zeta potential with dynamic light scattering (Zetasizer Nano, Malvern Instruments, Malvern, UK).
So as to study the NPs stability in terms of size, PDI and zeta potential, three batches of each NP were kept at 4°C for a month. Every five days, NPs were sampled and

characterized for their size, PDI and zeta potential by Dynamic Light Scattering (DLS) Instrument (Nano-ZS; Malvern, UK).

Size distribution and zeta potential analysis of particles. DLS was utilized to estimate the mean diameter and zeta potential of the NPs. Particle size and polydispersity index (PDI) and zeta potentials were reported as the means ± SD (n = 3).

Immunization of BALB/c mice. Immunizations were performed in 9 groups of mice, 9 mice per each group. The following formulations were injected subcutaneously (SC) 3 times at 2-week intervals: 1- PB buffer pH 6.7; 2- CHT solution in PB pH 6.7; 3- SLA dispersion; 4- WLL dispersion; 5- CHT:WLL NPs (25 µg antigen); 6- CHT:WLL NPs (50 µg antigen); 7- CHT:SLA NPs (25 µg antigen); 8- CHT:SLA NPs (50 µg antigen).

Antibody isotype assay. To assess the type of immune response induced in immunized mice in week 2 after the last booster and before the challenge, blood samples were collected from mice and sera anti-Leishmania IgG total, IgG1 and IgG2a antibody titers were determined by an ELISA method (Zymed Laboratories Inc., San Francisco, USA). Briefly, 96-well plates (Nunc) were coated with 50 µl of 10 µg/ml of SLA dispersion overnight at 4 °C. Plates were washed and blocked with BSA (1% w/v) + Tween 20 (PBST). Further added were serial dilutions of serum samples (in PBST). Absorbances were read at 450 nm using 630 nm as the reference wave length following the addition of TMB substrate (11, 39, 40).

Cytokine ELISA. In week 2 after the last booster and prior to the challenge, 3 mice were sacrificed in each group and their spleens were aseptically isolated. After homogenization, erythrocytes were disrupted using ammonium chloride. The splenocytes were washed and resuspended in complete medium (RPMI 1640-FCS) and seeded at 106/ml in 96-well flat-bottom plates (Nunc). SLA (10 µg/ml) or Con A (2.5 µg/ml), or the medium alone were used for the stimulation of the spleen cells and incubated at 37 °C in 5% CO2. After 72 h, culture supernatants were collected and the concentration of the IL-4 and IFN-γ was determined by an ELISA kit (Bender Med Systems GmbH, Vienna, Austria) (39).

Challenge with L. major promastigotes. Six mice in each group were challenged SC with 1×106 L. major promastigotes harvested at the stationary phase. Fifty µl of promastigotes was injected into the left footpad, two weeks after the last booster. As control, right footpads were injected with the same volume of PBS. Footpad thickness was measured by a metric caliper (Mitutoyo Measuring Instruments, Japan) and lesion development was recorded in each mouse. Lesion size was graded by subtracting the thickness of the uninfected contralateral footpad from that of the infected one (41).

Quantitative parasite burden after challenge. To determine protection against parasites, titration of viable L. major in the infected footpad and spleen was carried out in week 7 post-challenge using limiting dilution assay, as earlier described (18). Mice were sacrificed and infected footpad tissues and spleen were removed in each group. Spleens were isolated, homogenized and deposited in RPMI 1640, containing 2mM glutamine, 10% v/v heat inactivated FCS (Eurobio, Scandinavie), 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate (RPMI–FCS). The infected footpad tissues were homogenized completely in 1 ml RPMI-FCS using bead beating for 20 s (Bead Beater, Biospec, Bartlesville, OK) (42). Serial 10-fold dilutions of cell homogenates were placed in triplicate onto 96-well flat-bottom plates (Nunc) over a solid layer of rabbit blood agar. Next, the plates were incubated for 7–10 days at 25°C. Presence and absence of motile parasites per well were estimated as positive and negative wells using an invert microscope (CETI, UK). The number of viable promastigotes per infected

footpad and spleen were calculated based on the highest dilution at which the parasites could grow out after the incubation time. Ultimately, the statistical results were obtained through Graph Pad Prism software.

Statistical Analysis. The significance of the differences between groups was analyzed by one-way ANOVA statistical test. As for the significant F-value, multiple comparison Tukey test was used to compare the means of different treatment groups, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Characterization of CHT: WLL and CHT: SLA NPs. Particle size and surface charge of WLL and SLA dispersions were primarily characterized (Table 1). CHT:WLL and CHT:SLA NPs were prepared by a simple method. Briefly, an equal volume of CHT solution was added to WLL or SLA dispersion. To find the optimal w/w ratios of the polymer/antigen, different ratios (1:1–10:1, w/w ratios) were mixed and the resulted NPs were further characterized (Tables 2 and 3). The smallest size and PDI and the highest zeta potential was observed for 4:1 ratio of CHT: WLL and CHT: SLA NPs. As shown in Tables 2 and 3, when the negatively charged particles are titrated by the increase in the concentration of positively charged polymer, more and more polymers are adsorbed on the surface of the antigen particles until the saturation of antigen surface. Prior to saturation, more chitosan creates more condensed NPs with smaller sizes and higher zeta potentials. After saturation, a constant size and zeta potential are expected.

Table 1. Characteristics of antigen dispersions used for preparation of CHT: WLL and CHT:SLA NPs.

Antigen	Mean size (nm)	PDI	Zeta potential (mV)
SLA	170.6 ± 2.45	0.344 ± 0.01	-10.9 ±2.25
WLL	289.9 ± 15	0.448 ± 0.07	-9.2 ± 5.18

Table 2. Characteristics of NPs prepared with different weight ratio of CHT and SLA

Chitosan:SLA	Mean size (nm)	PDI	Zeta potential (mV)
1:1	654.8 ± 176.5	0.510 ± 0.01	-9.2 ± 2.6
2:1	366.8 ±167.4	0.391 ± 0.06	-6.2 ± 1.6
3:1	421.8 ± 57.5	0.412 ± 0.02	-2.6 ± 1.2
4:1	268.4 ± 27.8	0.450 ± 0.05	6.7 ± 2.3
5:1	589.5 ± 106.3	0.455 ± 0.03	6.8 ± 3.05
6:1	424.8 ± 210.2	0.382 ± 0.05	5.04 ± 4.1
7:1	496.6 ± 161.6	0.465 ± 0.02	7.9 ± 3.2
8:1	439.1 ± 79.1	0.387 ± 0.05	8.6 ± 3.3
9:1	547.1 ± 57.4	0.472 ± 0.01	7.8 ± 5.3
10:1	468.1 ± 195.2	0.439 ± 0.03	8.1 ± 3.4

Table3. Characteristics of NPs prepared with different weight ratio of CHT and WLL

Chitosan:WLL	Mean size (nm)	PDI	Zeta potential (mV)
1:1	533.2 ± 11.9	0.474 ± 0.1	7.3 ± 1.8
2:1	681.9 ± 2.7	0.578 ± 0.1	7.9 ± 0.9
3:1	484.8 ± 6.7	0.458 ± 0.2	8.7 ± 1.4
4:1	394.9 ± 68.3	0.426 ± 0.4	8.1 ± 0.9
5:1	359.5 ± 0.6	0.501 ± 0.1	6.3 ± 2.6
6:1	446 ± 68.4	0.486 ± 0.4	7 ± 0.8
7:1	366.5 ± 113.3	0.423 ± 0.9	7.7 ± 2.3
8:1	417.5 ± 76.2	0.434 ± 0.7	9.8 ± 3.3
9:1	338.5 ± 55.5	0.470 ± 0.5	6.7 ± 1.6
10:1	482.9 ± 195.2	0.537 ± 0.8	8.8 ± 2.8

For 30 days, both CHT: WLL and CHT: SLA NPs were characterized for their size, zeta potential and PDI every five days right after preparation (Figures. 1A, 1B and 1C). In terms of size and PDI, the CHT: SLA NPs were more stable than CHT: WLL NPs, and the zeta potentials of both NPs showed similar fluctuations.

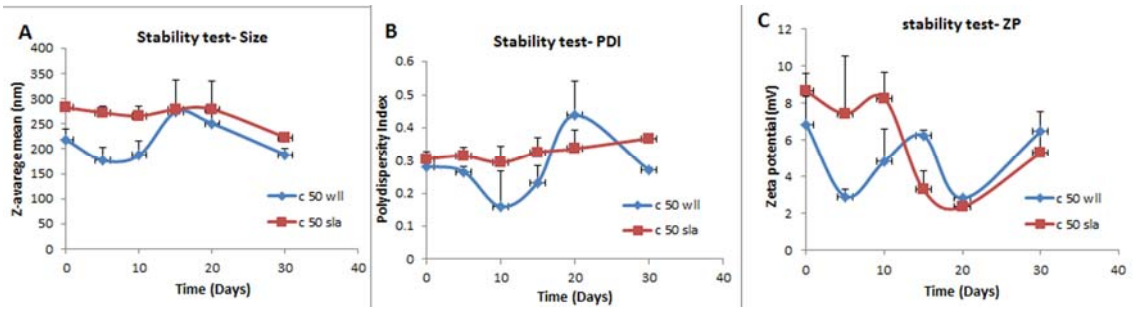

Figure 1. After preparation and every 5 days for 30 days storage in 4 °C, both CHT: WLL and CHT: SLA NPs were characterized for their size (A), PDI (B) and zeta potential (C). Error bars represent the SD (n=3).

Antibody response. In week 2 after the last booster, the anti-SLA IgG, IgG1 and IgG2a subclasses were tested by ELISA method (Figures. 2A, 2B and 2C).

As shown in Fig. 2A, the sera of all immunized mice with nanoparticulate formulations containing SLA or WLL, showed higher levels of IgG1 antibody compared to SLA or buffer in serum dilution of 1:200 ($p<0.001$). Also, in terms of IgG2a, the antibody levels in mice immunized with these formulations were higher than SLA or WLL in all serum dilution (Figure. 2B). On the whole, mice immunized with nanoparticulate formulations containing SLA or WLL showed higher levels of IgG1 and IgG2a and IgG total (Figure. 2C), comparisons with SLA or WLL dispersions. However, buffer, SLA or WLL dispersion failed to induce antibody titers.

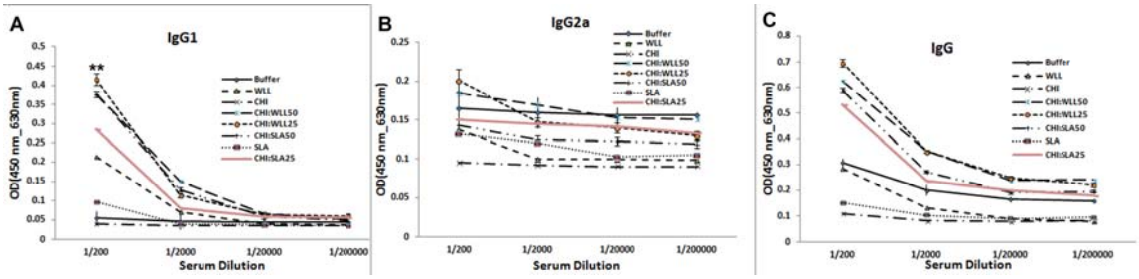

Figure 2. Levels of anti-SLA IgG1 (A), IgG2a (B), and IgG (C) in pooled sera of BALB/c mice before challenge. BALB/c mice immunized subcutaneously with CHT:SLA 50 µg NPs, CHT:SLA 25 µg NPs, CHT:WLL 50 µg NPs, CHT:WLL 25 µg NPs, SLA, WLL, CHT solution and PBS. After 3 times of injection at 22-week intervals, Titers of anti-SLA-specific IgG, IgG2a, and IgG1 in sera were measured using ELISA method. Blood samples were collected in week 2 after the last booster. Each assay was performed in triplicate at 200, 2000, 20,000 or 200,000-fold dilution for each serum sample. Values are the mean ± SD (n=9). ** (P<0.01) denote significant difference from buffer and all other liposomal formulations.

In vitro cytokine production by splenocytes. Splenocytes extracted from immunized mice were cultured, and the concentrations of IFN-γ and IL-4 in their supernatant were specified through the use of ELISA method in week 2 after the last booster injection. Based on the results, groups immunized with nanoparticulate formulations containing SLA or WLL showed higher amounts of IFN-γ compared to the control groups. The highest amounts of IFN-γ were detected in mice immunized with CHT: WLL NPs (25 µg antigen) (Figure. 3A), which was significantly higher than PB buffer ($p<0.05$). However, there was no significant difference between other vaccinated groups. In the case of IL-4, the highest amounts of IL-4 were detected in mice immunized with buffer, which was significantly higher than WLL ($p<0.05$); lower amounts of IL-4 were detected in mice receiving WLL (Figure. 3B), yet there was no significant difference among other vaccinated groups.

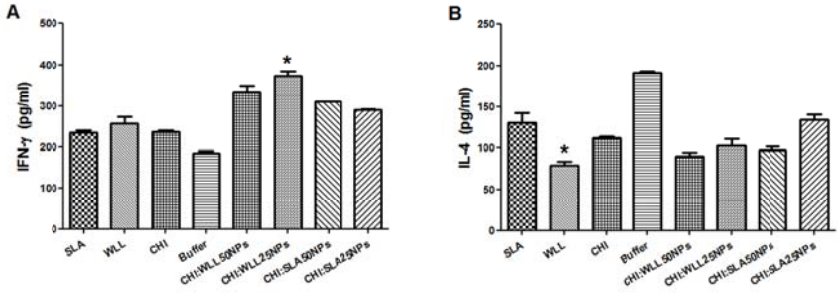

Figure 3. Cytokine levels in immunized mice in week 2 after the last booster injection and before challenge. Concentrations of cytokines in the supernatant of cultured splenocytes extracted from BALB/c mice immunized SC, 3 times at 2-week intervals with CHT: SLA 50 µg NPs, CHT:SLA 25 µg NPs, CHT:WLL 50 µg NPs, CHT:WLL 25 µg NPs, SLA, WLL, CHT solution and PBS. Two weeks following the last booster, the spleens were removed and the splenocytes were stimulated in vitro with SLA (10 µg/ml), concanavalin A (2.5 µg/ml), or with no stimulation. Concentrations of IFN-γ (A) and IL-4 (B) were determined using a sandwich ELISA method. Cells from 3 mice per group were pooled. The bars represent the mean and SD of triplicate wells. * (P<0.05) denote significant difference in formulations and buffer.

Lesion development after challenge. To evaluate the protection rate, immunized mice were challenged with L. major and lesion development was monitored by a weekly measurement of footpad swelling for 7 weeks (Figure. 4). As shown in Figure. 4, based on the swelling trends over the 7-week course of the study, the least swelling was observed in week seven in mice immunized with SLA and WLL antigens alone. There was no significant difference regarding the footpad thickness of mice immunized with nanoparticulate formulations containing either SLA or WLL. Footpad swelling progressed continuously in these groups and no protection was observed.

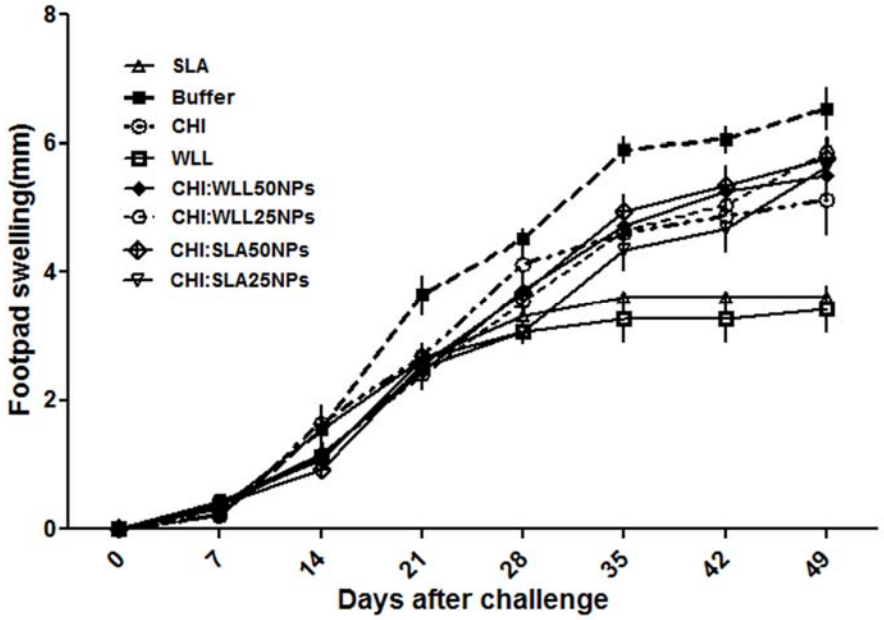

Figure 4. Footpad swelling in BALB/c mice. BALB/c mice immunized subcutaneously with CHT:SLA 50 µg NPs, CHT:SLA 25 µg NPs, CHT:WLL 50 µg NPs, CHT:WLL 25 µg NPs, SLA, WLL, CHT solution and PBS. After 3 rounds of injection at 2-week intervals, footpad swelling was measured. Two weeks following the last booster, both immunized and control groups of mice were challenged in the left footpad with 106 L. major promastigotes. For 7 weeks, the footpad thickness was measured on both footpads every week. Each point represents the average increase in footpad thickness ± SEM (n = 6).

Estimation of parasite loads in the foot and spleen. In week 7 after the challenge, the number of viable L. major in the infected footpads and spleen was quantified. All immunized mice revealed live L. major in their footpads; however, the number of viable parasites in the footpads of mice immunized with WLL or SLA were significantly lower than the control group receiving PB buffer ($p< 0.05$; Figure. 5A).

Besides, mice immunized with CHT: WLL NPs (25 µg antigen) showed the highest number of live parasite in their spleen compared to other mice (Figure. 5B). Nonetheless, mice receiving buffer showed the highest number of live L. major in the foot and spleen, as compared to other vaccinated groups, which was significantly higher than the mice receiving WLL or SLA ($p<0.05$) (Figures. 5A, 5B).

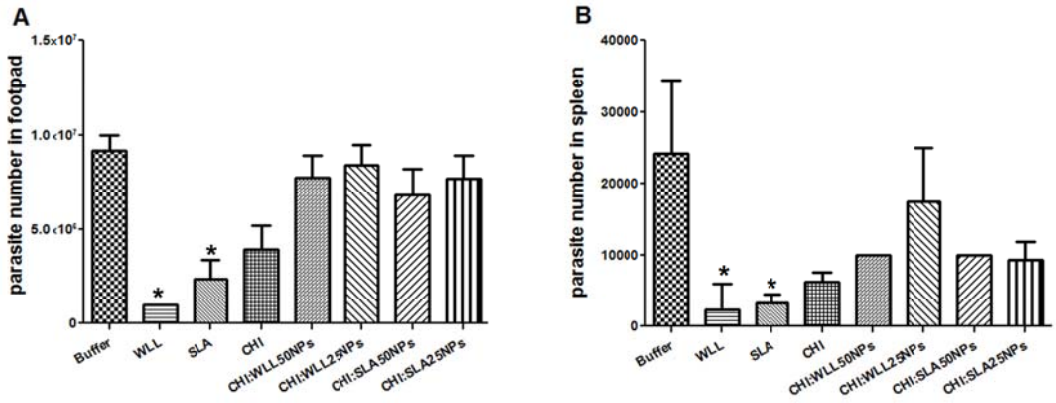

Figure 5. Parasite burden in BALB/c mice. Mice were 3 times immunized SC at 2-week intervals, with CHT: SLA 50 µg NPs, CHT: SLA 25 µg NPs, CHT:WLL 50 µg NPs, CHT:WLL 25 µg NPs, SLA, WLL, CHT solution and PBS . A limiting dilution analysis was performed in week 7 after challenge on the footpads (A) and spleens (B) of mice. The number of viable parasites per spleen and footpad of different groups of mice were further specified. The bar represents the average score ± SEM (n = 3). * ($P<0.05$) denote significant difference in buffer and SLA or WLL.

DISCUSSION

Since solid protection is achievable following recovery from cutaneous leishmaniasis (6), the development of an effectual vaccine is also feasible. Some Leishmania antigens have proved to induce potent immune responses and protection in murine models of Leishmania infection when used with IL-12 (3). However, the first generation Leishmania vaccines in phase 3 trials have shown a very low efficacy, partly due to the absence of an appropriate adjuvant (7).

In the current study, chitosan nanoparticles were used as immunostimulatory adjuvant based on three main purposes: (a) to prolong the in vivo half-life of the antigen (43), (b) to enhance the immune responses against Leishmania antigen and (c) to benefit from the potential for induction in Th1 responses as was shown previously (32, 33).

There is plenty of evidence confirming that the cationic polysaccharide chitosan is capable of driving potent cell-mediated immunity; a previous study showed that the chitosan/IL-12 adjuvant system is capable of inducing a strong Th1-type response in a protein-based vaccine (44). Furthermore, it has been demonstrated that chitosan stimulates dendritic cells (DC), and maturation by inducing IFN-γ production, and enhances antigen-specific Th1-type response (45)

Moreover, viscous chitosan solution not only enhances immune stimulation but also exerts a strong depot effect at the site of injection following subcutaneous administration (46).

A previous study showed that chitosan is able to increase superoxide dismutase B1 (SODB1) immunogenicity in BALB/c mice by the adjuvant properties of chitosan and increasing its size and enhances stimulation of the immune system, both triple-dose and single-dose vaccination with SODB1. Chitosan-loaded nanoparticles were effective in inducing Th1-type response that is responsible for Leishmania eradication and indicated

by high level of IgG2a in mice than control and other groups which received the soluble form of SODB1 antigen. Aside from being safe, biodegradable and cost-effective, chitosan nanoparticles can improve protein stability in administration and transportation, and increase the shelf life(47).

SLA and WLL parasite antigens were used as a first generation vaccine. Previous studies have reported that effectual vaccine against Leishmania infection requires a multivalent cocktail of diverse antigens composed of a broad range of protective epitopes which cover a wide array of MHC molecules in a population (48). This is in line with the leishmanization results, where crude Leishmania antigens such as SLA, containing plenty of antigen epitopes, are proper candidates for vaccine development (49). WLL is comprised of all integral membrane proteins, a spectrum of water-soluble proteins and hydrophobic components.

Soluble antigens of Leishmania donovani in vesicles form are considered as vaccine candidates for future studies. In addition, wide ranges of soluble Leishmania antigens, generated more protection when used in vesicles form, compared to recombinant antigens such as LAg and gp63 (50).

However, developing a vaccine against leishmaniasis requires a pure Th1 immune response mediated by IFN-γ production, entailing the activation of macrophage and parasite killing (51). The main aim of this experiment was to develop an effectual vaccine against Leishmania infection, and induce a potent Th1 immune response with IFN-γ production.

To determine the type of induced immune response and extent of protection rate, we analyzed parameters such as titration of antibodies in the sera, IFN-γ and IL-4 production, footpad swelling, and parasite burden in the spleen or footpad of infected BALB/c mice.

The results of cytokine assay demonstrated that higher amounts of IFN-γ were detected in mice immunized with nanoparticulate formulations containing SLA or WLL compared to mice receiving buffer (Fig. 3A). Also, based on the results of IgG subclasses, the sera of mice immunized with these nanoparticulate formulations showed higher titers IgG2a and IgG1 antibody compared to SLA or buffer or WLL. This elucidatess the potential of nanoparticulate adjuvant system to induce cellular and humoral immune responses.

However, mice immunized with chitosan nanoparticles containing SLA or WLL showed a high level of IgG2a and IFN-γ, that are markers of Th1 type of immune response. Although a high amount of IFN-γ was detected in those groups, they induced a high amount of IgG1 isotype indicative of a Th2 type of immune response.

Taken together, the current results suggested that these formulations induced mixed Th1/Th2 properties, confirmed in challenge results with no protection rate during the experiment period, whereas in Leishmania infection, a pure Th1 response is required.

Moreover, based on the results of lesion size and the number of live L. major in footpad and spleen, the group receiving WLL or SLA showed the smallest lesion size compared to other groups, which is significantly ($P < 0.05$) smaller compared to the control group receiving PB buffer in week 4 after challenge. Indeed, SLA or WLL generated a weak immune response which was not able to protect BALB/c mice against Leishmania infection. Generally, immunization with no formulation was able to reveal a protective immune response in mice. SLA, in the absence of an effective adjuvant, exacerbated the disease in BALB/c mice, a result also confirmed by earlier studies, since BALB/c mice have an innate tendency to induce aTh2 immune responses (23)

In conclusion, nanoparticulate formulations (Chitosan-SLA and Chitosan-WLL) are not suitable candidates for inducing a pure Th1 type of immune response and protecting the mice against Leishmania infection. However, they might be conducive to other infectious diseases which need a mixed Th1/Th2 immune response.

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