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Original Research

Evaluation of infection course in mice induced by *L. major* in presence of positively charged liposomes containing CpG ODN

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Abstract

Objective(s): An inoculation of virulent *Leishmania major* is known as leishmanization (LZ) which is proven to be the most effective control measure against Cutaneous Leishmaniasis (CL). However, using LZ is restricted due to various side effects such as uncontrolled lesion development.

Materials and Methods: In the present research, the efficacy of cationic nanoliposomes containing CpG oligodeoxynucleotides (CpG ODN) as an improved adjuvant delivery system was studied to diminish the lesion development and infection course of *L. major* after inoculation into the mice. BALB/c mice were inoculated subcutaneously (SC) with *L. major* plus empty DSPC, DSPC (CpG ODN), DSPC (Non CpG ODN), empty DMPC, DMPC (CpG ODN), DMPC (Non CpG ODN) or HEPES buffer.

Results: The results showed that group of mice received DMPC (CpG ODN) nanoliposomes developed a significantly smaller lesion and showed minimum number of *L. major* in the spleen and draining lymph nodes. In addition, using DMPC (CpG ODN) liposomes resulted in a Th1 type of immune response with a preponderance of IgG2a isotype which is concurrent with the production of DMPC (CpG) induced IFN-γ in the spleen of the mice.

Conclusion: Taken together, the results suggested that immune modulation using DMPC (CpG ODN) nanoliposomes might be a practical approach to improve the safety of LZ.

Keywords: CpG ODN, DMPC (CpG ODN), Nanoliposomes, Immune response, Leishmanization, *L. major*

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Introduction

Leishmanization (LZ) is the practice of inducing infection by injecting live virulent parasites in an aesthetically acceptable site of the body in healthy individuals. Despite of limited efficacy results seen in clinical trials using first generation *Leishmania* vaccine consists of killed *Leishmania* with or without adjuvant, inoculation of live *L. major* showed to be highly efficient tool to control CL. LZ has been used for centuries in various parts of Asian countries to protect against further infection especially multiple injections in the face. Mass LZ was used in Iran during the 1980s and the results showed that LZ is highly effective. Due to various reasons such as development of uncontrolled lesion, LZ practice was stopped in different countries except in Uzbekistan (1).

The ability of CpG ODN to induce both innate and adaptive cellular immune responses makes it a prospective prophylactic and therapeutic vaccine adjuvant for diseases requiring cellular immunity (2, 3). Unmethylated CpG motifs present at high frequency in bacterial but not vertebrate DNA are recognized by Toll-like receptor 9 expressed in B cells and plasmacytoid dendritic cells (pDCs) (4, 5). A potential method to increase ODN uptake by the cells of the immune system involves liposome encapsulation of ODN (6, 7) as we also showed previously against leishmaniasis (8, 9). Moreover, the efficacy of coadminstration of live *L. major* with liposome–protamine–DNA nanoparticle (LPD) containing immunostimulatory CpG oligodeoxynucleotides (CpG ODN) which is an improved adjuvant delivery system was examined to check *Leishmania* pathology and immune response generated (10). The results suggested that immune modulation using LPD nanoparticles might be a practical approach to improve the safety of inoculation of live *L. major*.Cationic liposomes are used as an adjuvant delivery system for CpG ODN in the current study. Cationic liposomes are particularly attractive due to their favorable characteristics such as biodegradability, minimal toxicity, and relative ease of large-scale production and simplicity of use (11). These particles target antigens for endocytosis by APCs more efficiently as the cationic entities interact with the negatively charged molecules on the surface of APCs (12).

In addition, there have been reports of the use of cationic liposomes for the generation of $CD8^+$ T cell response, which requires antigen presentation in the context of the MHC class I pathway (13).

They have also been successfully used as delivery systems for nucleic acids in gene therapy (14).

In the present study, cationic nanoliposomes containing CpG ODN was used instead of LPD nanoparticle, according to their simplicity during formulation and stability issues, to explore the possibility to induce a milder lesion and infection after live *Leishmania* parasites inoculation in susceptible BALB/c mice.

Materials and Methods

Animals, parasites and CpG ODNs

Female BALB/c mice 4-6 weeks old were purchased from Pasteur Institute (Tehran, Iran). All mice were maintained in animal house of Pharmaceutical Research Center and fed with tap water and standard laboratory diet (Khorasan Javane Co., Mashhad, Iran). Animals were housed in a colony room 12/12h light/dark cycle at 21˚ C with free access to food and water. Animal experiments were carried out according to Mashhad University of Medical Sciences, Ethical Committee Acts.

The *Leishmania major* strain (MRHO/I-R/75/ER) used in this experiment is the one which has been used for experimental Leishmania vaccine and Leishmanin test in Iran (15-17).

The CpG ODNs used in this study was a Nuclease-resistant phosphorothioate-modified sequence 1826 containing two CpG motifs (underlined: 5'-TCC ATG A<u>CG</u> TTC CTG A<u>CG</u> TT -3') with known immunostimulatory effects on murine immune response (2, 18) which was provided by Microsynth (Micro-

synth, Balgach, Switzerland).

Preparation of cationic nanoliposomes

Liposomes containing DMPC or DSPC phospholipids, DOTAP and cholesterol were prepared using lipid film method. Briefly, lipid film consisting of dimyristoyl phosphatidyl cholin (DMPC) or distearyl phosphatidyl cholin (DSPC) (24µmol/ml; Avanti Polar lipids, USA), 1, 2-dioleoyl-3-trimethylammonium propane (DOTAP) (Avanti Polar lipids, USA) and cholesterol (Avanti Polar lipids, USA) (3:1:1 molar ratio) were prepared in a glass vial by evaporating the chloroform: methanol (2:1, v/v) solution under rotary evaporation (Heidolph, Germany). The lipid film was then hydrated and dispersed by adding required amount of HEPES buffer with sucrose 10%. The resulting multilamellar vesicles (MLVs) were sonicated in a bath-type sonicator (Decon, England) at 50˚C for 15 min followed by extrusion (Avestin, Canada) through 400, 100 nm membrane filter to form 100 nm SUVs (small unilamellar vesicles). CpG ODNs (200µg/ml) or Non CpG ODNs (200µg/ml) was then added by drop wise addition to cationic nanoliposomes as it was swirling gently.

Size distribution and zeta potential analysis of nanoliposomes

Liposome and LPD nanoparticles size distribution and zeta potential were measured using a Zetasizer (Nano-zs, Malvern, UK). Particle sizes were reported as the means ± standard deviation and poly dispersity index (PDI) (n=3). Zeta potentials were reported as the means ± zeta deviation (n=3).

Inoculation of BALB/c mice

Different groups of mice, 10 mice per group, were subcutaneously (SC) inoculated in the left hind footpad (LF) with either of the following formulations plus parasites (P) suspension (1×10^6 promastigotes of *L. major* harvested at stationary phase in 25µl buffer/mouse): HEPES buffer (25µl/mouse), empty DSPC (474.1 µg DSPC, 139.6 µg DOTAP, 77.2 µg cholesterol/25 µl/mouse), empty DMPC (406.7 µg DMPC, 139.6 µg DOTAP, 77.2 µg cholesterol/25 µl/mouse), DSPC (CpG ODN) (10 µg CpG ODN, 474.1 µg DSPC, 139.6 µg DOTAP, 77.2 µg cholesterol/25 µl/mouse), DMPC (CpG ODN) (10 µg CpG ODN, 406.7 µg DMPC, 139.6 µg DOTAP, 77.2 µg cholesterol/25 µl/mouse), DSPC (Non CpG ODN) (10 µg Non CpG ODN, 474.1 µg DSPC, 139.6 µg DOTAP, 77.2 µg cholesterol/25 µl/mouse) or DMPC (Non CpG ODN) (10µg Non CpG ODN, 406.7 µg DMPC, 139.6 µg DOTAP, 77.2 µg cholesterol/25 µl/mouse). The progress of infection was followed by weekly measurement the thickness of the footpad using a metric caliper (Mitutoyo Measuring Instruments, Japan).

Quantitative parasite burden

The number of viable *L. major* parasites was enumerated in the spleen and subiliac lymph nodes of the mice using limiting dilution assay method (19). Briefly, the mice were sacrificed at week 14 post inoculation; the spleens and lymph nodes were aseptically removed and homogenized in RPMI 1640 supplemented with 10% v/v heat inactivated FCS (Eurobio, Scandinavie), 2 mM glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin sulfate (RPMI-FCS). The homogenates were diluted with the same media in 8-serial 10-fold dilutions and then placed in each well of flat-bottom 96-well microtiter plates (Nunc) containing solid layer of rabbit blood agar in triplicate and kept at 25 ˚C for 7-10 days. The number of viable parasites per spleen or lymph node was calculated by ELIDA software, a statistical method for limiting dilution assay (20).

Antibody isotype assay

Blood samples were collected from mice at week 6 post inoculation and serum samples were analyzed for specific anti-SLA IgG, IgG1 and IgG2a antibodies using ELISA method (Nunc, Denmark) according to the manufacturer's instructions. Briefly, 96-well microtiter plates were coated with 50 µl of SLA (0.5 µg/ml) overnight at 4 °C. Plates were washed

and blocked for 1 h at 37 °C using 200 µL per well of 1% bovine serum albumin (BSA) in PBS-Tween. Serum samples were diluted to 1:200 with PBS-Tween and applied to the plates. The plates were then treated with HRP-rabbit anti-mouse IgG isotype according to the manufacturer's instructions (Zymed Laboratories Inc., USA). Optical density was determined at 450 nm using 630 nm as the reference wavelength.

In vitro spleen cell response

Three mice in each group were sacrificed at week 14 post inoculation. The spleens were aseptically removed and a single-cell suspension was obtained by homogenization of the tissue. Mononuclear cells were isolated by Ficoll-Hypaque (Biogene, Iran) density centrifugation method from spleen cell suspension. The cells were washed and resuspended in complete medium (RPMI 1640-FCS) and seeded at 2×10^6 cells/ml in 96-well flat-bottom plates (Nunc, Denmark). The spleen cells were then stimulated *in vitro* in the presence of SLA (5 µg/ml), SLA (10 µg/ml), Concanavalin A (ConA) (2.5 µg/ml), or medium alone and incubated at 37˚ C with 5 % CO2. Supernatants were collected at 72h of culture and the concentration of IFN-γ and IL-4 were assayed using ELISA method according to the manufacturer's instructions (MabTech, Sweden).

Statistical analysis

One-way ANOVA statistical test was used to analyze the data. In the case of significant F value, Tukey–Kramer multiple comparisons test was carried out as a post-test to compare the means in different groups of mice. Differences were considered significant when $P < 0.05$.

Results

Physical properties of nanoliposomes

Liposomes used in this study were morphologically unilamellar vesicles with mean diameters of 77.6 ± 16.1, 143.2 ± 15.4, 161.5 ± 17.4, 212.9 ± 20.7, 205.6 ± 24.4 and 209.1 ± 19.2 nm (n=3) and the zeta potential of 71.2 ± 21.4, 54.4 ± 13.2, 69.5 ± 11, 48 ± 5.32, 75.7 ± 8.06 and 47.8 ± 3.74 mV, as calculated by particle size analyzer for empty DSPC, empty DMPC, DSPC (CpG ODN), DMPC (CpG ODN), DSPC (Non CpG ODN) and DMPC (Non CpG ODN), respectively.

Footpad thickness post inoculation

In order to establish the efficacy of cationic nanoliposomes at the site of *L. major* inoculation, the evolution of lesions was monitored by weekly measurement of footpad thickness (Figure 1). The group of mice received DMPC (CpG ODN) along with *L. major* showed significantly ($P<0.001$) the smallest lesion size compared to untreated control group (HEPES) throughout the study period. While the group of mice received empty DMPC or DMPC (Non CpG ODN) showed no significant difference compare with those inoculated with HEPES ($P>0.05$). However, there was a significant difference in lesion size in mice immunized with DSPC (CpG ODN) and those received HEPES buffer during 8 weeks post injection ($P<0.001$). Moreover, the results of footpad measuring during 14 weeks showed that there was no significant difference between mice treated with DMPC (CpG ODN) and those received DSPC (CpG ODN) ($P>0.05$). The results of footpad measuring showed that there was a significant difference between mice treated with empty DSPC and those received HEPES buffer ($P<0.001$). However, no significant difference between the group of mice immunized with empty DSPC and those received DSPC (CpG ODN) was observed during 14 weeks post injection ($P>0.05$). Although the group of mice received empty DSPC showed no significant difference compare with those inoculated with DMPC (CpG ODN) during 11 weeks post injection ($P>0.05$), later the difference became sign-

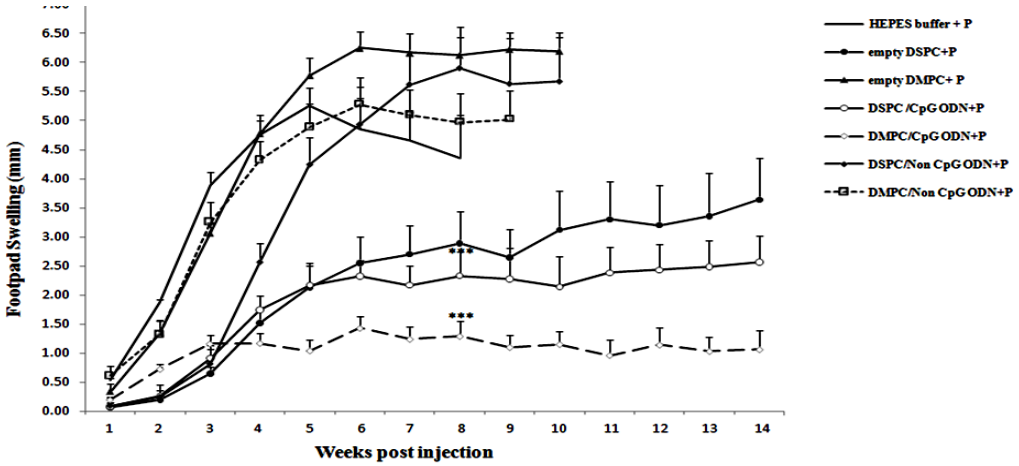

Figure 1. Footpad swelling in BALB/c mice inoculated subcutaneously (SC) in the left footpad with empty DSPC, empty DMPC, DSPC (CpG ODN), DMPC (CpG ODN), DSPC (Non CpG ODN), DMPC (Non CpG ODN) or HEPES in combination with 10^6 *L. major* promastigotes. The values represent the mean lesion diameters ± standard error mean (SEM) (n=10). (***), $P<0.001$ indicates that the values of mice received DMPC (CpG ODN) or DSPC (CpG ODN) nanoliposomes are significantly different from those received HEPES.

ificant until the end of measuring period ($P<0.01$). The group of mice immunized with DSPC (Non CpG ODN) showed significant lower footpad thickness compare with those inoculated with HEPES buffer during 3 weeks post injection ($P<0.001$) but later the difference became insignificant until the week 8 of measuring period ($P<0.01$). The measurement of footpad swelling was stopped in some control groups (Figure 1) at week 8 or 9 because the mice had lost their footpads so that the measurement was not accurate.

Parasite burden

The number of viable *L. major* parasites was determined in the subiliac lymph nodes and spleens of different groups of mice at week 14 post inoculation (Figure 2A and B). As noted, the group of mice inoculated with DMPC (CpG ODN) or DSPC (CpG ODN) showed almost no parasites in the lymph nodes and spleens compared with the control group received HEPES (P<0.001). Mice immunized with empty DSPC, empty DMPC, DSPC (Non CpG ODN) or DMPC (Non CpG ODN) showed significantly lower parasite burden than those received HEPES buffer (P<0.001). Although, the parasite number in these groups were higher than the groups of mice immunized with DMPC (CpG ODN) or DSPC (CpG ODN), but the differences were not significant (except for empty DMPC group in which the significant higher number of parasites were observed in spleen ($P<0.001$) and lymph nodes ($P<0.05$) comared with DMPC (CpG ODN) or DSPC (CpG ODN) groups).

Antibody response

To assess the type of immune response generated in different groups of mice, serum levels of anti-SLA specific IgG, IgG1 or IgG2a antibodies were evaluated at week 6 post infections. As shown in Figure 3A, B and C, the sera of mice immunized with DSPC (CpG ODN), DMPC (CpG ODN) and DSPC (Non CpG ODN) showed a significantly ($P<0.001$, $P<0.05$ and $P<0.01$ respectively) lower level of IgG total antibody titer compared with the group received HEPES. Moreover, the level of IgG1 antibody in the above groups of mice was significantly ($P<0.001$) lower than the control group received HEPES.

The highest level of IgG1 was observed in sera of mice received HEPES buffer compared to other groups. In case of IgG2a, the sera of mice immunized with DMPC (CpG ODN) showed no significant difference with the group received HEPES. Interestingly, the highest ratio of IgG2a/IgG1 antibodies was seen in the sera of mice immunized with DMPC (CpG ODN) com-pared with all other groups.

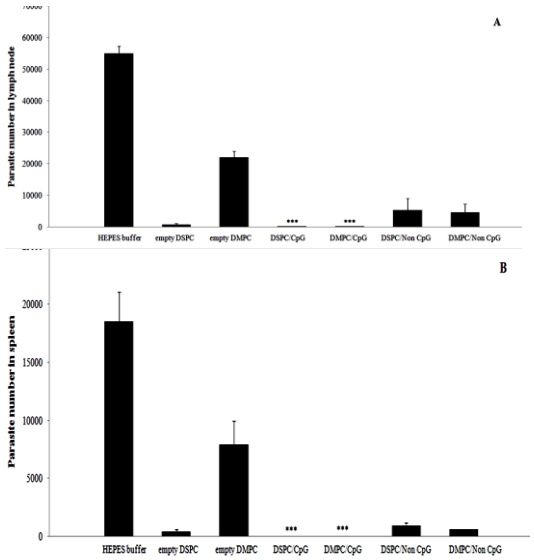

Figure 2. Parasite burden in (A) local draining lymph nodes and in (B) spleens of BALB/c mice inoculated subcutaneously (SC) in the left footpad with empty DSPC, empty DMPC, DSPC (CpG ODN), DMPC (CpG ODN), DSPC (Non CpG ODN), DMPC (Non CpG ODN) or HEPES in combination with 10^6 *L. major*. A limiting dilution analysis was performed at week 14 post infection on the cells isolated from three mice (n=3) and cultured in the RPMI-FCS for 10 days at 25ºC in serial 8-fold dilutions. The wells were assessed microscopically for *L. major* growth, and the number of viable parasite was determined by ELIDA software. The values represent mean ± SD. (***), P<0.001 indicate that the values of mice received DMPC (CpG ODN) or DSPC (CpG ODN) nanoliposomes are significantly different from those received HEPES.

The level of IgG2a antibody in the sera of mice received DSPC (CpG ODN) or DSPC (Non CpG ODN) was significantly lower than those received HEPES (P<0.001). In terms of the group of mice received empty DSPC or empty DMPC, the results showed no significant differences in the level of IgG or IgG1 antibodies compared with HEPES.

In the case of IgG2a, the sera of mice immunized with empty DSPC showed significantly lower level compared with the group received HEPES (P<0.05), however no significant difference between the group of mice immunized with empty DMPC with those received HEPES was observed (P>0.05).

In vitro cytokine production by splenocytes

The supernatant of cultured splenocytes restimulated *in vitro* at week 14 post inoculations with SLA to analyze the level of IFN-

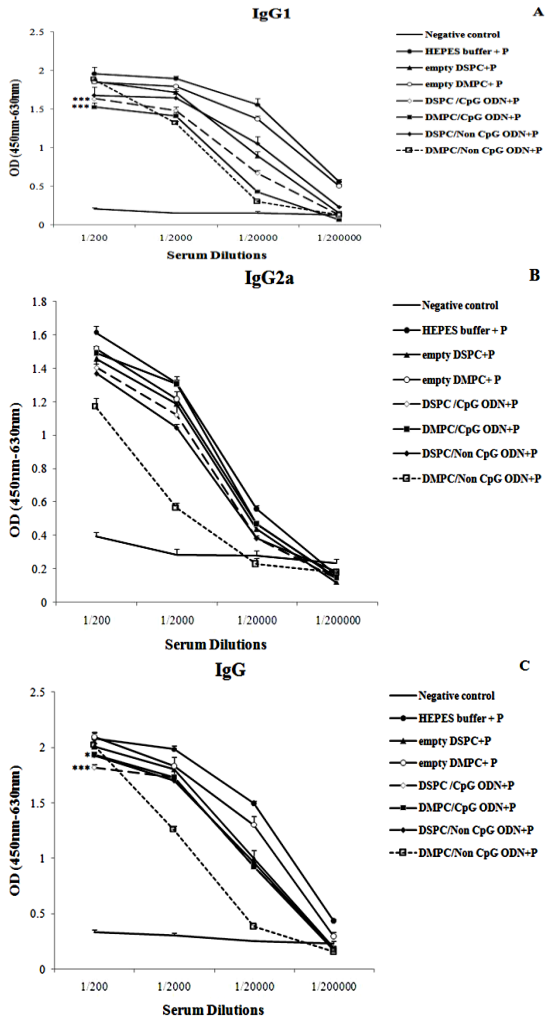

Figure 3. The levels of anti-SLA specific IgG1(A), IgG2a (B) or IgG (C) antibodies based on mean absorbance in the sera of BALB/c mice inoculated subcutaneously (SC) in the left footpad with empty DSPC, empty DMPC, DSPC (CpG ODN), DMPC (CpG ODN), DSPC (Non CpG ODN), DMPC (Non CpG ODN) or HEPES in combination with 10^6 L. major promastigotes. The assays were performed in triplicate with 200, 2000, 20000 or 200000-fold diluted serum samples. Values represent the mean ± standard deviation (SD). (*), P<0.05 and (***), P<0.001 indicate that the values of mice received DMPC (CpG ODN) or DSPC (CpG ODN) nanoliposomes are significantly different from the group received HEPES.

γ and IL-4, cytokine markers of Th1 and Th2 immune responses, respectively. As shown in figure 4A and B, even though DMPC (CpG ODN) or DSPC (CpG ODN) inoculated mice indicated a statistically significant reduction in the level of IL-4 comparing PBS inoculated mice (P<0.001). Regarding the group of mice immunized with DMPC (CpG ODN) the level of IL-4 was comparable to that of DSPC (CpG ODN) (P>0.05), and also a significant high

amount of IFN-γ over the cells compared to DSPC (CpG ODN) (P<0.001). Although the group of mice immunized with empty DSPC showed significant lower level of IL-4 compared with those inoculated with DSPC (CpG ODN) (P<0.05), the level of IFN-γ was significantly lower than that of DSPC (CpG ODN) (P<0.001). In terms of empty DMPC inoculated mice, although they indicated comparable level of IFN-γ to DMPC (CpG ODN) treated ones (P>0.05), they induced a significantly enhanced level of IL-4 (P<0.01), too.

Discussion

Induction of protection against future lesion following recovery from CL was used as a rationale to inoculate children with materials from active lesions to induce a lesion, which is then known as "leishmanization" (LZ) (1). LZ showed to be the most effective control measure against CL; However, LZ was stopped due to several reasons such as development of large troublesome lesions in 2-3% of inoculated persons (21). According to the nature of immune response to the live parasite vaccines, the current study is mainly focused on using cationic nanoliposomes containing CpG ODN adjuvant to explore the possibility of inducing a Th1 type of immune response and reduce pathology of *L. major* infection in BALB/c mice. Inoculation of mice with virulent *L. major* promastigotes mixed with DMPC (CpG ODN) or DSPC (CpG ODN) cationic nano-liposomes showed to induce a significantly smaller lesion size compared with all other groups. Besides the kinetic of lesion development, the results also showed differ-rences in the immune response generated in group of mice received *L. major* mixed with DMPC (CpG ODN) versus the group of mice received *L. major* in the other groups.

As expected, this moderate dermal pathology was correlated with a substantial reduction in parasite burden in the spleen and subiliac lymph nodes of immunized mice as shown previously (8, 22). Immunostimulatory activity of CpG motifs was also supported by

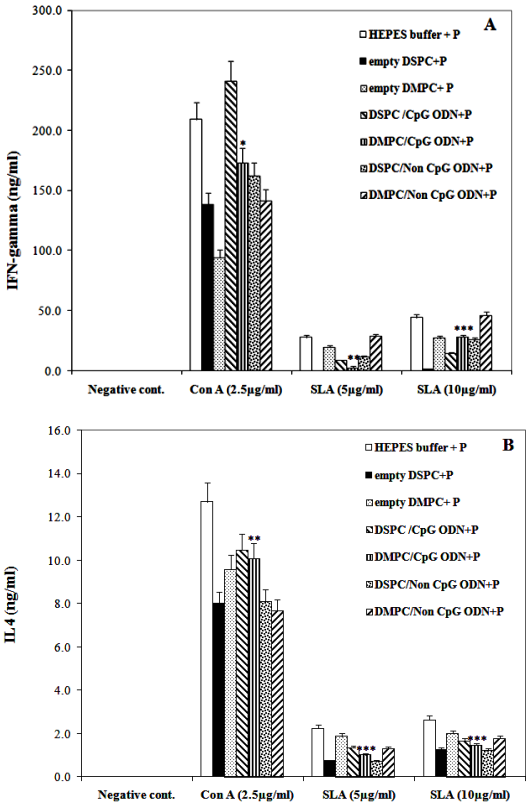

Figure 4. Splenic cell response in BALB/c mice inoculated subcutaneously (SC) in the left footpad with empty DSPC, empty DMPC, DSPC (CpG ODN), DMPC (CpG ODN), DSPC (Non CpG ODN), DMPC (Non CpG ODN) or HEPES in combination with 10^6 L. major promastigotes. At week 14 post inoculation, the spleens were removed and the splenocytes were stimulated *in vitro* with either SLA (5 µg/ml), SLA (10 µg/ml), Concanavalin A (2.5 µg/ml) or medium alone. Amount of IFN-γ (A) and IL-4 (B) were assessed by ELISA method in supernatants collected at 72 h of *in vitro* incubation. Values represent mean concentration of triplicate assays ± SD. (*), P<0.05, (**), P<0.01 and (***), P<0.001 indicate that the values of mice received DMPC (CpG ODN) or DSPC (CpG ODN) nanoliposomes are significantly different from those received HEPES.

the observation that applying CpG sequences at the site of live vaccination with *L. major* in C57BL/6 mice enhances primary immunity and thereby moderates the pathology associated with *L. major* infection (23).

Moreover, coadminstration of CpG ODN with *L. major* in susceptible BALB/c mice also induced a significantly smaller lesion size and a lower death rate compared with the group which received *L. major* parasites alone (24). Inoculation of mice with virulent *L. major* promastigotes mixed with LPD (CpG) nanoparticles also induced a significantly

smaller lesion size compared with those received *L. major* alone.

In the current study, cationic nanoliposomes containing a neutral phospholipid (DMPC or DSPC), DOTAP and cholesterol were used as a delivery system for CpG ODN. DOTAP as a cationic lipid was used in liposome formulation based on two main purposes. The first one was to prepare positively charged liposomes which interact more efficiently with the negatively charged molecules such as CpG ODNs so increasing the entrapment of CpG ODN into the liposomes, and the second one was using the intrinsic adjuvanticity of this synthetic quaternary ammonium compound (25).

Liposomes by themselves act as delivery system, but to further increase the adjuvanticity of liposomes, immune-stimulatory adjuvants should be incorporated into the lipid structures (26). The stronger immune response is seen when CpG is encapsulated in an appropriate delivery system (9). Liposomes protect CpG ODNs from nuclease activity and hamper the distribution of CpG ODNs to tissue, consequently increasing the CpG ODNs half-life (27). At the APCs level, CpG ODNs through TLR9, augment activation and maturation of DC as well as the induction of proinflammatory cytokines (28). Thus, the endogenous production of IL-12, IL-18, and other soluble mediators from activated DC induced by CpG ODNs are likely to result in a more physiologic cognate interaction between the DC and T cell, resulting in both a qualitatively and quantitatively different type of $CD4^+$ and $CD8^+$ T cell response (22). The current results elucidated the effect of the presence of both cationic lipid and CpG for full immune-stimulation activity required for counteracting the drawbacks of *L. major* inoculation. As compared with control groups, DMPC (CpG ODN) demonstrated a significant control on the progression of the growth of the footpad and desired immune response.

To develop a leishmanization protocol with a mild lesion, co-administration of live parasites with neutral liposomes containing CpG ODN was studied before (23, 24).

Interestingly, the presence of CpG ODN decreased lesion size and there was no significant difference between mice receiving CpG ODN in liposomal form and those receiving CpG ODN in soluble form, particularly when DSPC was used as neutral lipid. However, it was not true for the liposomes consisting DMPC (Fig. 1). It might be due to the transition temperature of lipid used in liposome formulation (*i.e.* DSPC). As DSPC has a very high transition temperature (Tm 55 ºC) and produces a very rigid and stable bilayer structure in liposome formulation which resists releasing a suitable amount of CpG ODN for interaction with TLR9 receptor located in the phagosomes (24) while DMPC has low Tm (23 ºC) was not true for. Previously, when we used 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) (Tm ~0 ºC) in LPD nanoparticles, the results showed a significant difference in protection compared with CpG ODN in soluble (10). The difference may be correlated with the availability of the free form of CpG ODN in phagosomes due to the very low Tm of the lipid component. On the other hand, the nanoparticles were destabilized more easily in phagosomes and as a result CpG ODN is available for interaction with the receptors.

The level of both IFN-γ and IL-4 cytokines was high in HEPES received group due to the inoculation of parasite alone without any adjuvants, whereas mice inoculated with DMPC (CpG ODN) or DSPC (CpG ODN) plus parasites significantly suppressed the IL-4 production. Although DMPC (CpG ODN) inoculated mice indicated a comparable level of IL-4 compared to DSPC (CpG ODN) treated ones ($P>0.05$), but they induced a significantly enhanced level of IFN-γ ($P<0.001$). The highest level of IFN-γ was detected in mice treated with HEPES that it might not be due to the induction of Th1 response, because of the high amount of IL-4, IgG1, and IgG titer observed in this group. Moreover, generalization of infection to the spleen was detected by parasite burden experiment. The same trend was reported when LPD nanoparticles was used in combination with *L. major* (10). In group of

mice received DMPC (CpG ODN), though the level of IFN- γ was lower than those received HEPES, the significant lower IL-4 production and the highest IgG2a/IgG1 ratio was also seen. Altogether, the results of cytokine and Ab production analysis show that DMPC (CpG ODN) nanoliposomes prevent to induce a Th2 type of response compared to other groups.

It is proposed that inoculation of live parasites into BALB/c mice induces a specific IgG response in the absence of adjuvant, with a preponderance of the IgG1 isotype (a marker of Th2 response) (29).

In this study, DMPC (CpG ODN) nanoliposomes enhanced the production of IgG2a and particularly IgG2a/ IgG1 ratio, as a hallmark of Th1 type of immune response which is essential for counteracting intracellular pathogens (6).Taken together, the current results suggested that immune modulation using DMPC (CpG ODN) cationic nanoliposomes might be a practical approach to improve the safety of inoculation of live *L. major* and can be utilized instead of LPD nanoparticles.

Conclusion

In conclusion, the current results suggested that immune modulation using DMPC (CpG ODN) cationic nanoliposomes might be a practical approach to improve the safety of inoculation of live *L. major* and can be utilized instead of LPD nanoparticles.

There are limited studies using live *Leishmania* parasites concurrently with adjuvants, thus, further studies are needed to explore the detailed role of co-administration of cationic nanoliposomes with live Leishmania.

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