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Induction of Immune Responses by DNA Vaccines Formulated with Dendrimer and Poly (Methyl Methacrylate) (PMMA) Nano-Adjuvants in BALB/c Mice Infected with *Leishmania major*

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Abstract

BACKGROUND: Leishmaniasis is a parasitic disease induced by a protozoan from the genus *Leishmania*. No effective vaccine has yet been developed against the disease.

AIM: In this work, two nano-vaccines, TSA recombinant plasmid and dendrimer and poly (methyl methacrylate) (PMMA) nanoparticles (as adjuvants), were designed and tested for their immunogenicity in BALB/c mice.

METHODS: After the plasmid construction and preparation of adjuvants, three intramuscular injections of the nano-vaccines (100 µg) and the recombinant TSA protein (20 µg) were subcutaneously performed. Eventually, the challenged animals were infected with the parasites ($1*10^6$ promastigotes). After the last injections of the nano-vaccines, the responses of their antibody subclasses and cytokines were assessed via ELISA method before and after the challenge.

RESULTS: This study revealed that the new nano-vaccines were strong and effective in inducing specific antibody and cellular responses and reducing the parasite burden in the spleen compared to the control groups of *Leishmania major*-infected BALB/c mice.

CONCLUSION: Based on the results, we can suggest that the formulated vaccines are suitable candidates for further studies in the field of leishmaniasis control.

Introduction

Leishmaniasis is a widespread parasitic disease in many tropical and subtropical regions of the world, which is transmitted via the bites of infected sand flies. Re-infection is prevented by the immunity achieved via cutaneous infection with *Leishmania* spp. This suggests that prophylactic immunisation is achievable. No vaccines have been approved to be effective against leishmaniasis. DNA vaccination is a recent immunisation plan with many potential benefits over any other vaccine strategies. DNA vaccines can elicit broader immune responses than formal vaccines. Thus, to elevate immunity, DNA vaccine is complemented with adjuvants. Thiol-Specific Antioxidant (TSA) protein is one of the dominant antigens of *L. major* promastigote and amastigote and is considered as a primary DNA vaccine candidate against leishmaniasis. Many attempts to improve an efficient anti-*Leishmania* vaccine have failed due to lacking a suitable adjuvant [1][2].

Nanoparticles represent a group of macromolecular materials that exhibit promising

therapeutic or prophylactic properties to be used as adjuvants for delivering antigens via mucosal surfaces and intradermal routes. However, the size of a particle affects both antigen delivery and the type of immune responses it produces. As antigen carriers, these particles may act as a depot for the regulated release of antigens to enhance immune cell responses [3]. Dendrimers represent another group of repetitively branched molecules with the ability of gene and drug delivery. They can also be used in the synthesis of monodisperse metallic nanoparticles [4][5].

Given the recent developments of nanotechnology in the field of drug delivery and the unique features of carriers, such as dendrimers, which alleviate the problems of low solubility and bioavailability of drugs, we applied biocompatible and biodegradable dendrimers with polyethylene glycol (PEG) core and citric acid branches in this study. Today, thanks to nanotechnology, researchers in the pharmaceutical industry have developed drug carriers, which resolve such problems as low solubility and poor absorption of drugs by cells. They can not only increase drug bioavailability and help targeted delivery to a specific tissue, but also control the amount of drug release. The polyvalent natures of peptide dendrimers enhance their peptide-specific affinities to interact with peptides, proteins, and carbohydrates [6].

Despite its approval by the US Food and Drug Administration (FDA) for certain clinical human uses, poly (methyl methacrylate) (PMMA) as a phagocytised particle may trigger strong immune responses by inducing the production of inflammatory cytokines [7].

Therefore, we appraised the effectiveness of dendrimer and PMMA as nano-adjuvants with the DNA-encoding TSA antigen of *L. major* in BALB/c mice in a bid to obtain a vaccine of improved efficacy against leishmaniasis.

Materials and Methods

L. major promastigotes

L. major MHRO/IR/75/ER, which is an Iranian strain separated by Nadim et al. in 1964, was obtained from Iranian Pasteur Institute. Promastigotes were cultured in RPMI 1640 medium (Sigma®) and supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) (Gibco®, BRL) and 100 lg/ml of gentamicin (Sigma®) at 26°C. The stationary phase was catched by centrifugation and used at $1*10^6$ promastigotes/ml. The procedures of this study were also approved by the Ethical Committee of the Faculty of Medicine (Iran University of Medical Sciences) with code number: IR.IUMS.REC1390.15896.

Plasmid construction

After preparation, TSA recombinant plasmid DNA was transmuted into *E. coli* DH5-α, purified by plasmid extraction Kit (Bioneer, Germany), dispersed in sterile deionised distilled water, and kept at -20°C until used. Then, a purification step was followed by using Endo-Free plasmid purification Giga Kit (Qiagen, CA, USA) according to the manufacturer's instructions. DNA concentration was concluded by taking the dimensions at the Optical Density (OD) of 260 nm. To ensure that the purified DNA was protein-free, the OD260/OD280 ratio was obtained to be 1.80-1.95 [8].

Preparation of adjuvants

Here, we introduced a new method for the synthesis of G2 dendrimer with PEG core and citric acid branches. The method was characterised by simplicity and the use of non-toxic materials. Also, in this approach, consecutive steps of purification were taken, and impurity removal was done in one run using Sephadex column without a previous G1 purification. The method was thus highly fast, cheap, and efficient. In this approach, 2 ml of PEG 600 equivalent to 3.7 mmol and a dry dimethyl sulfoxide (DMSO) solvent were utilised in a test tube. An amount of 3.7 x 2 mmol of dicyclohexylcarbodiimide (DCC) was then added to the test tube to activate the reaction, and the lid was immediately closed. The reaction tube was stirred for 15 min before the addition of an amount of 3.7x2 mM of citric acid followed by one h of stirring. Upon skipping a reaction stop for G1 purification, we added 3.7x6 mM of DCC and the reacting components were further stirred for 15 min. The stirring was continued again for one h after the addition of 3.7 x 6 mM of citric acid and 10 ml of DMSO. The reaction was ended by the addition of 30 ml of double-distilled water. For G2 dendrimer purification, we utilised Sephadex column G-75 (Merck, Germany). To this end, an amount of 6.0 g of Sephadex powder was dissolved in 20 ml of double-distilled water and maintained at ambient temperature for 24 h. The Sephadex was then transferred to a column and eluted once with double-distilled water. Afterwards, G2 dendrimer solution was separated from the sedimented DCC using a filter paper and transferred to the Sephadex column. The eluted and purified solution was thus collected. This step was repeated to remove all the impurities and obtain the purified water-soluble G2 dendrimer, which was lyophilised.

The required amount of the contributing substance was determined with the aid of its corresponding stoichiometric relationship, density, and molecular weight. Since PEG has two functional carboxyl groups capable of binding to citric acid, two moles of citric acid was applied per one PEG besides DCC as its activator. To ensure the synthesis of the required dendrimer, Thin-Layer Chromatography

(TLC) was performed using a solvent system of gradient methanol-chloroform. The size and surface charge of G2 dendrimer was determined by Dynamic Light Scattering (DLS) using double-distilled water as a solvent. Gamma irradiation polymerisation method was applied to produce PMMA nanoparticles in the absence of antigens [4]. A nano-vaccine candidate was then prepared by loading pcDNA3/TSA recombinant plasmid into PMMA nanoparticles. In short, 1 Mm of PMMA nanoparticle solution was used to cross-link to 10 Mm of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) reagent under incubation with soft mixing at room temperature for 10 min. Then, 1 ml of plasmid DNA (100 lg/ml of the solution) was added to an equal volume of the former and placed in a cold room overnight. The solution was finally purified by comprehensive dialysis, and the resulting PMMA-plasmid DNA nanoparticles were suspended in double-purified water. The nanoparticle size was determined by using a Zeta Sizer (Malvern, UK) (data not shown) [1]. TSA recombinant peptide booster (22 KD) was a gift from Miss Nargestehrani, a faculty member of the Islamic Azad University of Tehran [4][5][6].

Immunization and experimental infection of the mice

Inbred female BALB/c mice matured 6-8 weeks were obtained from the Animal Center of Pasteur Institute of Iran (Karaj) and treated by the National Animal Care and Use protocol adopted by the Iranian University of Medical Sciences. The mice were divided into 3 test (T) and four control (C) groups (20 mice/group). The test group received DNA vaccine (pcDNA3/TSA), nano-vaccine (pcDNA3/TSA+dendrimer), and nano-vaccine (pcDNA3/TSA+PMMA), while the control group received pcDNA3, dendrimer, PMMA, and PBS at the doses of 100 µg. For experimentation, the mice were anesthetized by an intraperitoneal injection of 25 µl g^{-1} of a combination of 10% ketamine and 2% xylazine. All the treatments were intramuscularly administrated, and the injection sites were immediately subjected to 8 electric 60-V pulses for 20 ms at a 200-ms interval by using a BTX ECM 830 generator (Harvard Apparatus, USA) equipped with tweezer-type electrodes (CUY 650, Sonidel Limited, Ireland). Immunization of the mice was done by injecting 50 µl of PBS into each anterior tibialis muscle. The immunisation schedule was performed with three inoculations of equal doses of DNA, dendrimer, and PMMA at 3-week intervals. The booster peptide (20 µg) and incomplete Freund's adjuvant was subcutaneously injected two weeks after the injection of the last nano-vaccines. The immunised mice were intradermally challenged with $1*10^6$ promastigotes of *L. major* at the base of their tails three weeks later. The animals were then sacrificed after five post-challenge weeks, and their serum samples and spleens were immunologically analyzed [7][8][9].

Lymphocyte proliferation assay

The spleen of each sole mouse was dismembered and suspended in sterile, cold Phosphate-Buffered Saline (PBS) containing 2% Fetal Bovine Serum (FBS). The Red Blood Cells (RBCs) were lysed, and a single-cell suspension was prepared in RPMI 1640 (Gibco, Germany) at $3*10^6$ cells/ml, which was complemented with 10% of FBS, 4 mM of L-glutamine, 1 mM of sodium pyruvate, 50 µm of 2-ME, 100 µg/ml of streptomycin, and 100 IU/ml of penicillin. Flat-bottom 96-well culture plates were used to dispense 100 µl of the cell suspension motivated with ten µg/ml of the recombinant TSA protein expressed in *E. coli* cells for antigen recall. Phytohemagglutinin-A (5 µg/ml, Gibco) and unmotivated wells were utilized as the positive and negative controls, respectively. The whole culture medium was similarly applied as blank. All the tests were done in triplicate. The plates were incubated for 72 h before supplementing 100 µl of 5-Bromo-2-deoxy-uridine (BrdU) labelling solution into each well and incubating them for 18 further hours. The plates were then subjected to centrifugation to remove the culture medium before drying and fixing the wells with 100 µl of fixation/ permeabilisation buffer. Subsequently, each well received 100 µl of anti-BrdU antibody before washing the plates four times and supplementing them with tetramethylbenzidine (TMB) substrate. The test was halted by concluding 100 µl of 2 NH_2SO_4. The OD of each well was assessed at 450 nm. The stimulation indices were estimated in accord with the following formula: OD of the stimulated well/OD of the unstimulated well.

Cytokine evaluation before and after the challenge with L. major

The single-cell suspension ($3*10^6$ cells/ml) derived from each mouse spleen was dispensed into the 24-well plates, aroused *in vitro* with ten µg/ml of recombinant TSA protein, and incubated in 5% CO_2 at 37°C. After 72 hrs of antigen recall, the supernatants were obtained by centrifugation at 300*g for 10 min and supplied at -70°C for cytokine analysis. Then, using commercial ELISA Kits (Mabtech, Sweden) according to the manufacturer's instructions, IFN-γ and IL-4 cytokines were quantified. Each cytokine was quantified as pg/ml based on the plotted standard bend.

ELISA of the total antibodies and the subclasses of IgG1, IgG2a

Assay of the sera of the empirical groups was done using an optimised indirect ELISA approach to assess humoral immune responses based on the specific antibodies before and after *L. major* challenge. In short, 100 µl of antigen (10 µg/ml) in PBS buffer was supplemented into 96-well ELISA MaxiSorp plates (Nunc, Naperville, IL) and incubated

at 37°C for 24 h. After being washed with PBS containing 0.05% Tween 20 (washing buffer), the plates were blocked with 5% skimmed milk in PBS (blocking buffer) at 37°C for one h. The plates were again rinsed with a rinsing buffer before the addition of 100 µl of the diluted sera (1/100) to each well and then incubated at 37°C for two h. The wells were rinsed five times and incubated with 100 µl of the diluted (1/7,000) anti-mouse sera conjugated to HRP (Sigma, USA) for two h. The wells were again rinsed five times before further incubation with 100 µl of TMB substrate in the dark for 30 min. The reaction was then stopped by the addition of 2 N H2SO4. The ODs were estimated with an ELISA plate reader at λ 450 nm. Using the secondary antibodies of goat anti-mouse IgG1 and IgG2a (Sigma, USA) based on the manufacture's instruction, the specific subclasses of IgG1 and IgG2a were detected [10][11].

Parasite load distinction

The parasite burden was determined by sacrificing three mice per each group, which had been challenged seven weeks earlier. Then, their spleens were drained using the limiting dilution method. Briefly, a slice of a spleen was removed and weighed to be homogenised with a tissue grinder in 2 ml of RPMI 1640 medium (Gibco, Germany) complemented with 20% heat-inactivated FCS and Gentamicin (0.1%). Serial dilutions were prepared in 96-well micro titration plates under sterile conditions. After seven days of incubation at 26°C, the plates were examined using an inverted microscope at a magnification of 40*. The presence or absence of mobile promastigotes was recorded in each well. The last titer was the last dilution, in which the number of parasites per gram was estimated in the following way: _log10 (parasite dilution/tissue weight) [2][10].

Statistical analysis

The significance of the differences among various groups was tested using the one-way ANOVA test. Besides, Post-hoc LSD test was utilised to compare the means of the different groups under treatment. The statistics were regarded significant at $P < 0.05$.

Results

Lymphocyte proliferation assay

Lymphocyte proliferation analysis before the challenge through BrdU method revealed no significant differences between the vaccinated groups after three injections of DNA and the nano-vaccines (pcTSA+dendrimer and pcTSA+PMMA and pcTSA) at $P = 0.337$ although 3 test groups demonstrated significantly different proliferative activities from those of the control groups at $P < 0.001$. After challenging with *L. major*, the immunised groups displayed no significant differences among themselves ($P > 0.549$), but all produced higher degrees of proliferation responses than those of the control groups ($P < 0.001$) (Fig. 1).

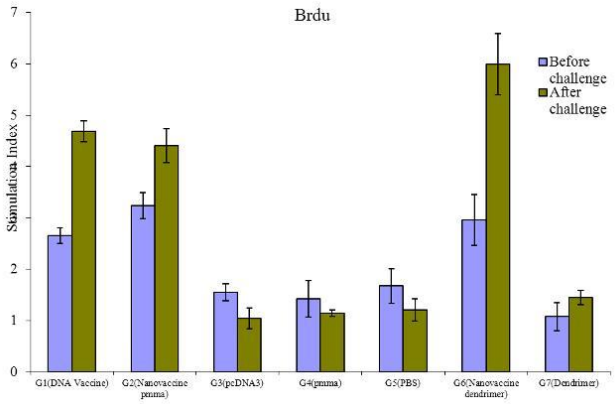

*Figure 1: Lymphocyte proliferation responses before and after the challenge: The mice were immunised with DNA vaccine with or without a dendrimer and PMMA (n = 5 mice per group) in the DNA prime/peptide raising plan. The four groups of mice were injected with pcDNA3 vector, dendrimer, PMMA, and PBS as the negative controls (n=4 mice per group). Proliferative reactions were followed for the unique mice in triplicate and evaluated using BrdU method as represented in the section of "Materials and Method". The data depict mean ± SD (95% C.I.). *P < 0.001 considered for the vaccinated groups before and after the challenge showed a significantly higher rank for rapid growth compared to those of the control groups*

The models of IL-4 and IFN-γ cytokines

In an attempt to appraise the pattern of cytokine secretion caused by vaccination, the special mouse splenocyte culture was re-stimulated *in vitro* with recombinant TSA protein and cleansed in *E. coli* cells (data not shown). The collected supernatants were tested for IFN-γ and IL-4 quantities causing the types of the induced immune responses (T helper one vs T helper 2). The results represented that before the challenge, IFN-γ secretion level was significantly higher in the vaccinated than in the control groups ($P<0.001$) meaning that immunisation increases IFN-γ production by lymphocytes. Behind the challenge, IFN-γ level remarkably augmented in the mice immunized with the nano-vaccines (pcTSA+dendrimer, pcTSA+PMMA) compared to the control groups and those immunized with DNA vaccine (PcTSA) ($P<0.001$) (Fig. 2a). Before the challenge, the mice immunized with the DNA vaccine and formulated with the nanoparticles revealed a significant enhancement of IL-4 level produced by lymphocytes compared to the control and DNA-vaccinated mice groups ($P < 0.001$). However, after the challenge with *L. major*, no statistically significant differences between the test and control groups were

noticed (P > 0.733) regarding IL-4 production (Fig. 2b).

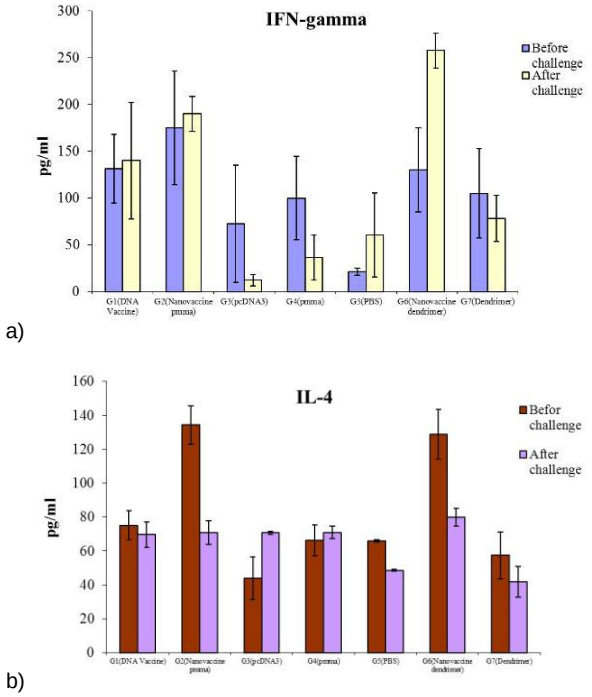

*Figure 2: Cytokine yields (IFN-γ (a) and IL-4 (b)) by the spleen cells of BALB/c mice after the immunization periods and the challenge: The mice were immunized with DNA vaccine with or without the dendrimer and PMMA (n = 5 mice per group) in the DNA prime/peptide life plan. The four groups of mice were injected with pcDNA3 vector, dendrimer, PMMA, or PBS as the controls (n=4 mice per group). Cytokine analyses were observed during the study by using ELISA approach as mentioned in the section of "Materials and Methods". The tests were carried out in duplicate for the special mice. The merits represent mean ± SD (95% C.I.). *P < 0.005 was considered for the nano-vaccine groups compared to all the other groups. *P < 0.001 was regarded for the immunised groups compared to the control groups. *P < 0.001 for the groups of pcTSA+dendrimer and pcTSA+PMMA was considered to be similar to all the other groups after the challenge with L. major (Fig. 2a). *P < 0.025 was taken for the nano-vaccine groups compared to all the other groups before the challenge. *P < 0.011 for the vaccinated groups was regarded to be similar to the PBS group after the challenge (Fig. 2b)*

Antibody responses

In general, the immunised groups showed a significant rise in their total antibody productions before the challenge compared to the control groups (P < 0.003). After the challenge, the mice produced elevated levels of antibodies and had been thus immunised with the nano-vaccines, but they did not indicate statistically significant differences among themselves (P > 0.059). The nano-vaccines exhibited a significant rise in the total antibody production after the challenge in comparison to the control and vaccinated groups (P < 0.003) (Fig. 3a,b). The effects of IgG isotyping demonstrated that the test and control groups had similar IgG1 levels before the challenge with no significant differences between them (P > 0.059). However, after the challenge, all the immunised groups produced significantly increased IgG1 isotypes as compared to the control groups (P<0.030). Before and after the challenge, IgG2a titer was more significant in the immunised than in the control groups (Fig. 3c,d).

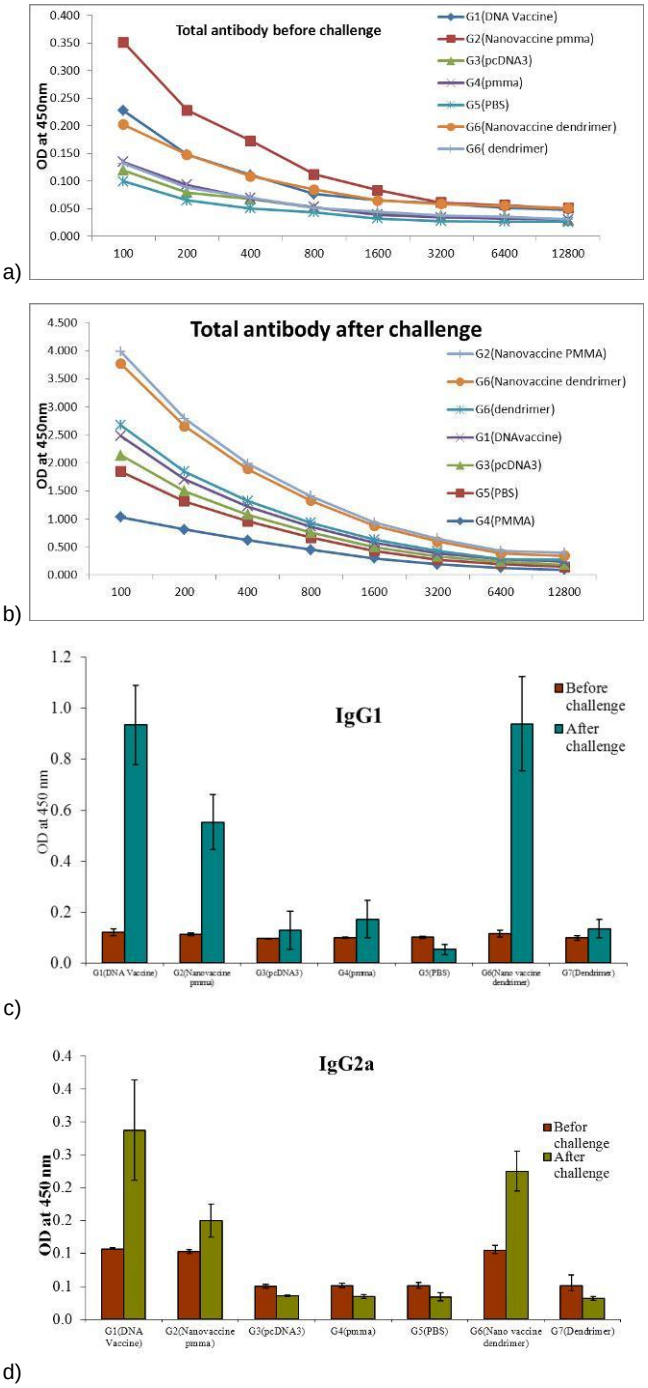

Figure 3: Specific antibody production against TSA recombinant protein in BALB/c mice immunized with DNA vaccine and nano-vaccines before the challenge (a) and after the challenge (b): The specific total IgG, IgG1, and IgG2a were measured through ELISA approach as mentioned in the section of "Materials and Methods". The sera obtained from each group were diluted 1:200 and assessed for the presence of IgG1 and IgG2a. Specific changes in IgG1 (c) and IgG2a (d) levels were detected throughout the study. TMB substrate was employed for the detection and OD was determined at 450 nm. Mean ± SD (95% C.I.) is represented throughout the data

Splenic parasite burden

The splenic parasite burden assays of all the empirical groups revealed that the numbers of viable splenic parasites were different among the vaccinated and unvaccinated groups following the immunisation and seven weeks after the challenge with *L. major*. The immunised mice displayed a significantly lower number of alive parasites compared to the control groups ($P < 0.001$) (Fig. 4).

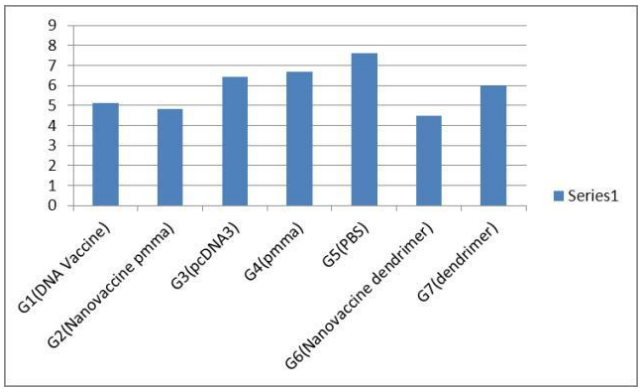

*Figure 4: Parasite burden associated with DNA vaccine and nano-vaccines in the prime/boost strategy and control groups seven weeks after the challenge: Calculations of the total numbers of the viable parasites within the spleens of the infected animals were done based on parasite dilution per tissue weight. Mean ± SD was used to represent the values obtained for the individual mice (n = 3). A significantly less parasite burden was found in the spleens of the vaccinated mice compared to those of the control groups (*P < 0.001)*

Discussion

In the present study, we attempted to design novel nano-vaccines containing TSA plasmid DNA and estimate their immunogenicities in BALB/c mice. Currently, there are no effective vaccines available to produce a protective response against leishmaniasis. This is despite the fact that many vaccination strategies ranging from involving the killed parasites to recombinant antigens or DNA vaccines have been tested. Antigens, such as recombinant proteins induce only antibody responses while DNA vaccines involve both MHC-I and MHC-II pathways. Therefore, DNA vaccines can cause strong, long-lasting, and effective humeral and cellular immunities. The prime-boost immunisation method can influence the quality and quantity of immune responses. However, certain approaches are needed to increase the qualities and efficacy of DNA vaccines, such as *in vivo* electroporation. TSA as the immune-dominant antigen of *L. major* is antigenic in murine and human systems and induces CTL activity and safety against the parasite. Excellent protection against leishmaniasis has been reported for the recombinant leishmanial antigens LmSTI1 and TSA [12][13][14]. Nevertheless, using an adjuvant seemed necessary to boost any modern vaccines against leishmaniasis [15]. The results of this research revealed that the mice immunization with the nano-vaccines and DNA vaccine increased humoral and cellular responses as compared with the control groups.

In this investigation, we used dendrimer nanoparticles as adjuvants to elicit stronger immune responses to the candidate vaccines. This is because dendrimers had nowadays several practical applications in medicine and attracted the attentions of many researchers to obtain novel synthetic designs with reduced toxicities.

An anionic PEG-citrate G2 dendrimer was chosen because of its biocompatibility and biodegradability. Dendrimers are nano-carriers with a high potential to carry hydrophobic drugs and increase their solubilities in water as well as their cellular uptakes. The unique properties of dendrimers, including monodispersity and surface modification capability along with their sizes and structure sets, have made them ideal candidates for drug delivery. Scientific research during the last two decades has shown that dendrimers are appropriate and effective carriers for drug delivery and enhancement of the mobilities of hydrophobic drugs. Another feature of a dendrimer is its possession of suitable spaces, within which various hydrophobic and hydrophilic drug molecules can be accommodated. The presence of multiple well-known functional groups on the surface of these spherical particles make them as suitable carriers to fit various drug molecules or ligands and consequently enable them to help a targeted drug delivery. The results of the studies performed on these dendrimers have shown that these chemicals have great potentials for use in drug delivery systems. Shafiee Alavijeh et al. examined cell death mechanisms (apoptosis and necrosis) caused by a dendrimer in HT1080 cell lines. Based on their results, dendrimers had no significant detrimental effects at a concentration of 0.5 mg/ml. They stated that these hybrid structures would be a very large potentiality for application in the various fields of nano-medicine. In another study carried out by Haririan et al., two conjugates were prepared from cisplatin in aqueous solutions with the two generations (G1 and G2) of biodegradable anionic citric acid dendrimers. Based on the *in vitro* results obtained from their research, a conjugate of G2+platinum had higher toxicity to cancer cells than that of G1+platinum and cisplatin and hence showed a better therapeutic effect. They stated that these conjugates with such a high potentiality and minimum hemolysis are good candidates as new and effective anti-tumour agents. It should be noted that some types of dendrimers, such as viologen with a chemical structure containing bipyridinium salts or other structures like Caminade, PAMAM, polyanionic phenyl dicarboxylic acid (BRI6195), or carbosilane have shown antiviral properties, particularly against HIV-1 with EC [50] = 0.26 ± 0.08 μM (e.g., viologen). The mechanism

behind these observations was reported to be caused by an interference with viral replication or gp-120 protein function, especially in the case of PAMAM. This would strengthen the hypothesis that the simultaneous use of dendrimeric structures and curcumin can produce synergistic or additive effects. In this research, PEG was chosen due to its environmental compatibility and biodegradability, as well as its easy reaction with citric acid and constitution of the core of a dendrimer. The presence of citric acid in the structure of this dendrimer as a macromolecule, which can be subjected to metabolism by the cellular citric acid cycle upon entering the cell, has made it environmentally compatible and biodegradable. The dendrimer is not cytotoxic in therapeutic doses depending on the study type because of its size (about 80 nm) and negative charge. Cells have negative charges similar to a subjected dendrimer and thus cell surface absorption as the main cause of cell toxicity may not occur. In this study, DCC was applied instead of chlorinated compounds, such as dichloromethane, which is usually used to activate synthetic reactions. Chlorinated compounds are highly toxic and cause a serious damage to human respiratory system, but DCC has a much lower toxicity and can be thus considered advantageous in our method. The obtained product was a two-generation dendrimer (G2), which was completely soluble in water and could, therefore, be a good candidate to increase the solubility of water-insoluble drugs. Studies have shown that this dendrimer has no unfavourable effects on cells at a concentration of 5.0 mg/ml. In this research, we employed a new method to synthesize nanoparticles, which not only shortened the reaction and production times but also enabled the use of less toxic materials [6][16].

The nanoparticle conjugates of the dendrimer produced valid antibody responses and protections against some antigens. Among numerous nanoparticles, those which are biodegradable, safe, and simple and easy to be produced can be selected for the drug delivery under study [17][18][19][20]. The findings of the previous studies indicated that dendrimers have antibacterial effects. In this work, PMMA nanoparticle was utilised as an adjuvant to enhance specific humoral and cellular immune responses to our candidate vaccines due to its good antibody responses in addition to its conferring higher stability to the vaccines. The utilisation of PMMA adjuvant in split influenza vaccines demonstrated a safety record and excellent and powerful protection [21][22]. This nanoparticle may also enhance humoral responses against Hiv-2. Some authors suggested that PMMA adjuvant can increase antibody production and hence the efficacy of candidate vaccines [23][24]. Our findings revealed that both the specific IgG1 and IgG2a were augmented upon immunisation with PMMA nano-vaccine and dendrimer. Considering that IgG1 and IgG2a are Th2 and Th1 markers, respectively, this funding was of prime importance. Campos-Neto et al. reported that immunisation of BALB/c mice with a TSA plasmid DNA influenced elevated titers of particular IgG1 and IgG2a antibodies vs. *Leishmania* [25][26][27]. Other studies have shown that the use of nanoparticles and a major raising plan increases protective immunity against *Leishmania* infection in animal models [26][28]. In this research, we showed that a dendrimer and PMMA can boost the efficacy of DNA vaccines encoding TSA against *L. major* disease and bring out immune responses to the delivered antigen. Our nano-vaccines were productive for lowering parasite load in the spleens of BALB/c mice infected with *Leishmania major* as compared to the control groups.

In conclusion, the vaccine formulation suggested in this investigation may provide a way to be paved for obtaining excellent candidates against *Leishmania* through further research.

Acknowledgments

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Ethics approval

The procedures of this study were also approved by the Ethical Committee of the Faculty of Medicine (Iran University of Medical Sciences) with code number: IR.IUMS.REC1390.15896.

Authors' contributions

Somayeh Zarrati searched the literature and performed experiments. Mehdi Shafiee Ardestani designed the study and analyzed the data. Sayed Hussain Mosawi and Fatemeh Tabatabaie have participated in drafting the manuscript and supervised the research. Fatemeh Tabatabaie and Nasim Samarghandi wrote the final manuscript. All authors read and approved the final manuscript.

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