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Humoral immune responses in DNA vaccine formulated with poly (methyl methacrylate) against *Leishmania major*

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Abstract
DNA vaccines can generate more powerful and broader immune responses than conventional vaccines. In order to increase immunity, the DNA vaccine has been supplemented with adjuvant. In this study a new nano-vaccine containing TSA recombinant plasmid and poly (methyl methacrylate) nanoparticles (acting as adjuvant) was designed and its immunogenicity tested on BALB/c mouse. After three intramuscular injections of nano-vaccine (100 µg), the recombinant TSA protein (20 µg) was injected subcutaneously. Finally as a challenge animals were infected by *Leishmania major*. After the last injection of nano-vaccine, after protein booster injection, and also after challenge antibody responses were evaluated by ELISA method. The findings of this study showed the new nano-vaccine was capable of inducing specific antibody responses.

Keywords: TSA, *Leishmania major*, Poly(methyl methacrylate), Immune response

1. Introduction
Leishmaniasis is widespread in many parts of the world with about 12 millions infected cases [1]. Infection with HIV/AIDS can increase the risk of developing leishmaniasis by 100 to 1000 folds [2]. Chemotherapeutics are available but show high toxicity, costs and are prone to resistance development due to prolonged treatment period [3]. Development of either new anti-*Leishmania* drugs or a vaccine is an attractive alternative. Immunity against reinfection is acquired following cutaneous infection with *Leishmania* spp., suggesting that prophylactic immunization is feasible. A number of *Leishmania* vaccine candidates, including killed parasites, crude parasite fractions, recombinant *Leishmania* antigens, and antigen-encoding DNA, have been investigated in murine models [1-3]. But in spite of several tested vaccine protocols no protective vaccine against any clinical leishmaniasis has been produced commercially. The major advantage of DNA vaccine is inducing the expression of antigens, which are unaltered in their protein structure and antigenicity [3-5]. Most of the works have focused on different antigens among the vaccine candidates, TSA (Thiol-Specific Antioxidant protein) has been introduced as one of the predominant vaccine candidates. TSA is *L major* recombinant protein homologue to eukaryotic Thiol-Specific-Antioxidant protein with molecular weight of 22.1KDa is composed of 200 amino acids and placed in the chromosome of 15. TSA is expressed in *L. major* promastigote and amastigote [6, 7]. TSA DNA vaccine stimulated high titers of specific IgG2a antibody which is the type of immune response required. Many efforts to develop effective *Leishmania* vaccine have been limited due to lack of an appropriate adjuvant [8, 9]. Nanoparticles are solid very small fragments ranging in size from 1 to 1000 nm (1 µm). They consist of macromolecular materials and can be used therapeutically or prophylactically, for example, as an adjuvant in vaccines or drug carriers, in which the active principle is dissolved, drew or encapsulated, or to which the active principle is adsorbed or chemically attached .Nanoparticles are able to enter antigen-presenting cells by different pathways, thereby regulating the immune response to the antigen. Their properties also make them appropriate for the delivery of antigens at mucosal surfaces and for intradermal administration. It is generally agreed that the adjuvanticity of nanoparticles and microparticles is affected by particle sizes, which in turn affects the type of immune responses

caused by antigens carried by particles. Particulate carriers can serve as an effective antigen delivery system and, thus, improve and/or facilitate the uptake of antigens by antigen - presenting cells. Particle-based antigen carriers may attend as a depot for controlled release of antigen, thereby increasing the availability of antigens to the immune cells. Poly (methyl methacrylate) (PMMA) is a synthetic polymer approved by the Food and Drug Administration for specific human clinical applications such as the bone cement [10-11]. *In vivo*, PMMA particles are phagocytosable and have the potential to initiate strong immune responses by stimulating the production of inflammatory cytokines [8-11]. The purpose of this work was to study the DNA-vaccine efficacy in the presence PMMA adjuvant.

2. Material and Methods

The period of study was 6 months (during the first week of April to October)

2.1 *Leishmania major* Promastigotes

An Iranian strain, MHRO/IR/75/ER of *L. major* was provided by Pasteur Institute of Iran. Promastigotes were grown at 26 °C in RPMI 1640 medium (Sigma®) supplemented with 10% heat inactivated fetal calf serum (Gibco®, BRL), and 100 µg/ml gentamicin (Sigma®). Stationary phase of the promastigotes was harvested at a density of $1x10^6$ /ml.

2.2 Plasmid constructions

The TSA recombinant plasmid DNA was prepared in a previous study transformed into *E. coli* DH5-α and purified by plasmid extraction Kit (Bioneer, Germany), dissolved in sterile deionizer distilled water and stored at -20 °C until use. Then the EndoFree plasmid purification Giga Kit (Qiagen, CA, USA) was used according to the manufacturer's instructions. DNA concentrations were measured by taking absorbance at 260 nm. The OD260/280 ratios for the purified DNA were 1.80-1.95, indicating that the preparations were free from protein contamination.

2.3 Preparation of vaccine

The PMMA polymeric nanoparticles used as adjuvant were produced by gamma irradiation polymerization method in the absence of antigen. In order to prepare the nano-vaccine candidate, pcDNA3/*TSA* recombinant plasmid was loaded to PMMA nanoparticles. In brief, 10 Mm EDAC 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was added to 1Mm of PMMA nanoparticles solution and incubated for 10 minutes at room temperature with gentle stirring. After that, 1ml Plasmid DNA solution of 100 µg/ml was added (equal volumes of the two solution were mixed) and then left in the cold room overnight. For purification, the solution was further subjected to extensive dialysis. The resulting PMMA-plasmid DNA nanoparticles were preserved in suspension form in double-distilled water. Size of nanoparticles was determined using a Zeta Sizer (Malvern, UK)(data not shown).The TSA recombinant peptide booster (22 KD) was gifted by Miss Narges Khabaz zadeh Tehrani from Faculty of Basic Sciences, Science and Research Branch of Islamic Azad University of Tehran.

2.4 Immunization and experimental infection of the mice

Inbred Female BALB/c (6-8 weeks old) mice were purchased from Lab. Animal Center (Pasteur Institute, Karaj, Iran) and handled in accordance with the National Animal Care and Use protocol at Iran University of Medical Sciences. Mice were divided into two test (T) and three control (C) groups (20 mice/group) and respectively received DNA Vaccine (pcDNA3/*TSA*), nano-vaccine (pcDNA3/*TSA*+PMMA) and pcDNA3 (as control group) in dose of 100 µg. Other control groups were injected with PMMA and PBS. The mice in each group were anesthetized with 25 μLg^{-1} of mixture of ketamin 10% and xylazin 2% via intraperitoneal (i.p.). All injections were done intramuscularly (i.m.) and the injection sites were immediately pulsed using tweezer-type electrodes (CUY650, Sonidel Limited®, Ireland) to administer eight 60 V pulses each of 20 ms duration with a 200 ms interval using a BTX ECM830 generator (Harvard Apparatus, USA).The mice were immunized (i.m.) injection into both quadriceps's with 100 µl of PBS, 50 µl in each anterior tibialis muscle [12-14]. Three inoculations were employed with the same DNA and PMMA doses and the same immunization schedule was applied at three weeks interval. Two weeks after the last injection of nano-vaccines, 20 µg booster peptide plus incomplete Freund's adjuvant was injected subcutaneously. Three weeks after final immunization mice were challenged with 1×10^6 promastigotes of *L. major* (*strain* MHRO/IR/75/ER) at the base of tail by the intradermal route. Two weeks after the last injection of nano-vaccine, two weeks after the peptide booster injection and five weeks after parasite challenge the animals were sacrificed and serum samples were harvested for immunological analysis.

2.5 ELISA of total antibodies and IgG1, IgG2a subclasses

To evaluate the humoral immune responses, before and after challenge with *L. major*, sera of experimental groups were collected and specific antibodies were determined by an optimized indirect ELISA method. Briefly, 100 µl of 10 µg/ml of antigen in PBS buffer were added into 96-well ELISA Maxisorp plates (Nunc, Naperville, IL) and incubated for 24 hrs at 37 ˚C. The wells were washed with PBS containing 0.05% Tween 20 (washing buffer) and blocked for 1 hr at 37 ˚C with 5% skimmed milk in PBS (blocking buffer). Plates were washed with washing buffer and 100 µl of 1/100 diluted sera were added to each wells and incubated at 37 ˚C for 2 hrs. The wells were washed five times with washing buffer and incubated for 2 hrs with 100 µl of 1/7000 dilution of anti-mouse conjugated to HRP (Sigma, USA). The wells were washed five times and incubated 30 min with 100 µl of TMB substrate in the dark and reaction was stopped with 2N H2SO4 and color density was measured at A_{450} nm with ELISA plate reader. Specific IgG1and IgG2a subclasses were detected using goat anti-mouse IgG1and IgG2a secondary antibodies (Sigma, USA) according to the manufacture's instructions [15].

2.6 Statistical analysis

One-way ANOVA statistical test was used to assess the significance of the differences among various groups and Post Hoc LSD test was used to compare the means of different treatment groups. Results with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1 Antibody and subclasses responses

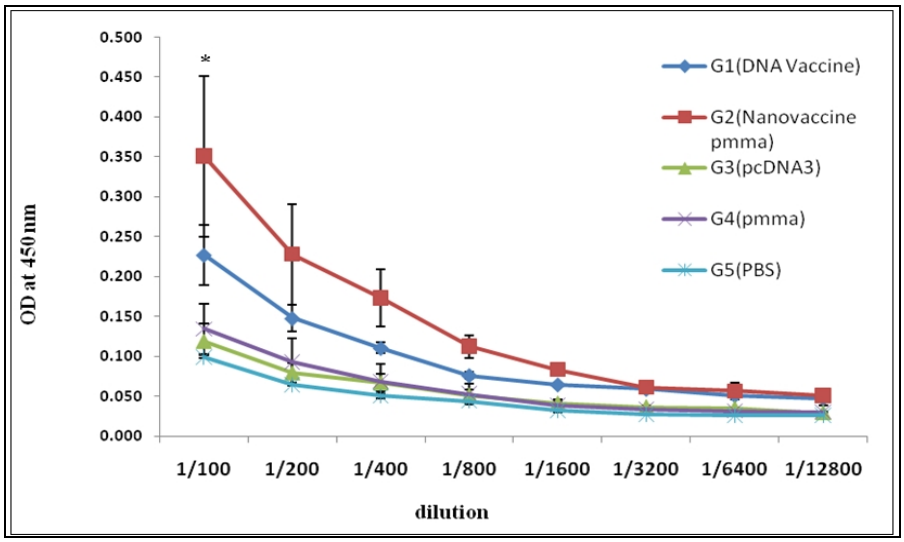
Specific changes total IgG, before and following booster injection and after challenge, were shown (fig. A, B, C**)**, respectively. Specific total IgG, IgG1 and IgG2a were measured by ELISA method. Sera from each group were diluted 1:200 and evaluated for the presence of IgG1 and IgG2a. Specific changes of IgG1 and IgG2a levels during the study were shown in figures D & E respectively. Detection was done with the substrate TMB and optical density at 450 nm was determined. All data represent mean ± SD (95% C.I.).

The results indicated the significant difference of total antibody between G2 and control groups after 3 injection of vaccine (i.m) (* p < 0.003) (A). After peptide booster injection and after challenge the significant difference of total antibody was observed between G2 and control groups (* p < 0.048) (B, C.).

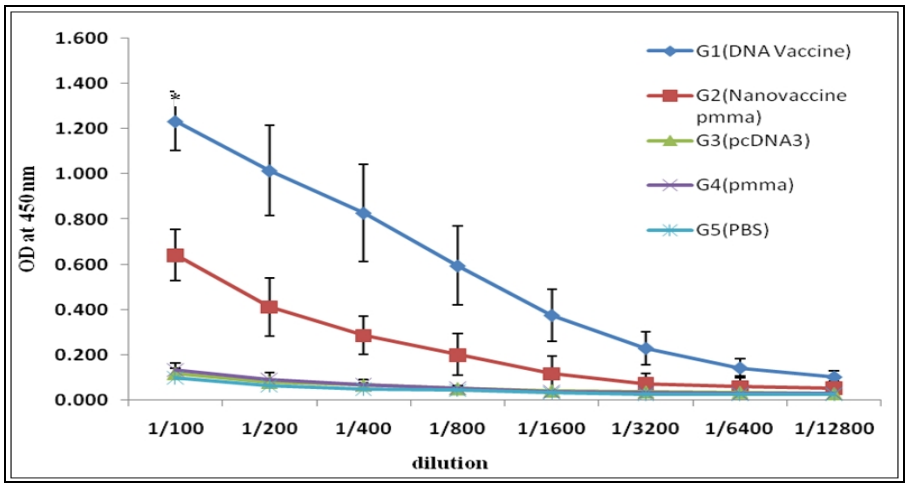
Results of total antibodies in the experimental groups showed that before recombinant peptide booster injection in immunized group with nano-vaccine, significantly were increased total antibodies as compared to the control groups (P<0.003). Following the booster injection and after challenge, mice were immunized with DNA vaccine and boosted with recombinant peptide significantly were increased total antibodies as compared to control groups (groups 3, 4 and 5) (P<0.031). Immunization of mice with vaccine candidate formulated with PMMA (group 1) after booster injection and also after challenge showed high induction of total antibodies as compared to the negative control groups but no statistical significant difference was observed (p>0.059) (fig. 1A, 1B and 1C).

Results of IgG isotyping showed that following the booster injection IgG1 level in the group of mice which received DNA vaccine was significantly increased compared to nano-vaccine and control groups (p=0.012 and p<0.002 respectively). Moreover all immunized groups (groups 1, 2) significantly increased IgG2a isotype as compared to the control groups (groups 3, 4, 5) (P<0.030) and immunization of mice with DNA vaccine candidate (group 1) significantly increased IgG2a titer as compared to nano-vaccine group (group 2) (p=0.041). After challenge IgG1 levels in vaccinated groups showed increased significantly compared to control groups (P<0.018) and high level of IgG2a in mice were immunized with DNA vaccine /peptide booster in comparison to control groups was observed (p <0.001). (Figure. 1D, 1E).

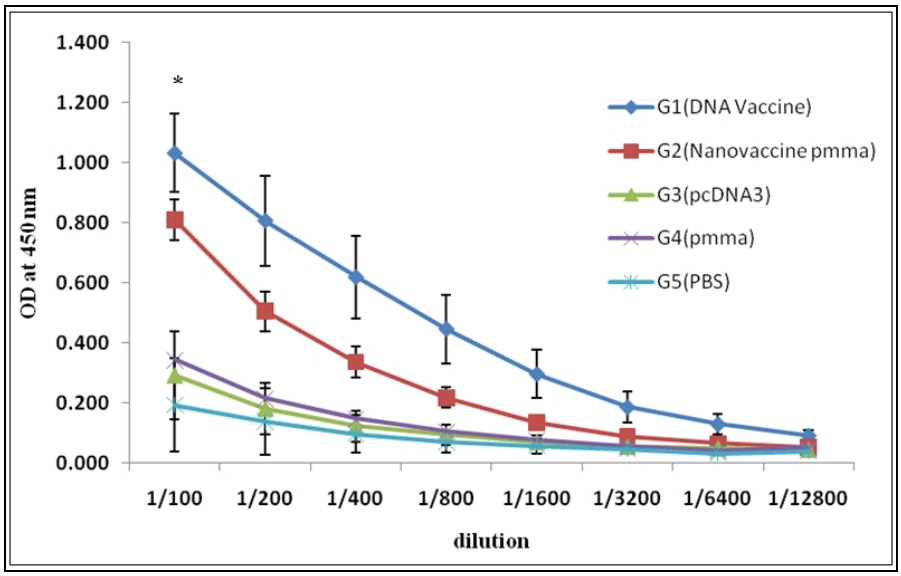
*p < 0.041 for DNA vaccine group compared to all other groups after booster injection (D, E), *p < 0.030 for vaccinated group compared to control groups following the booster injection (E), *p < 0.018 for G1and G2 compared to control groups after challenge with *L. major* (D), *p < 0.001 for DNA vaccine/peptide booster compared to other groups after challenge (E) were detected.



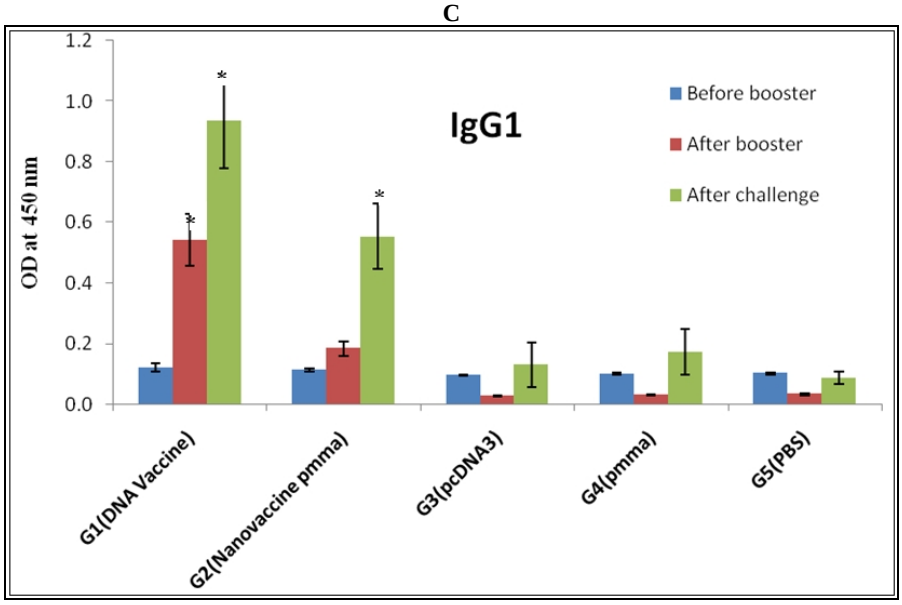
A



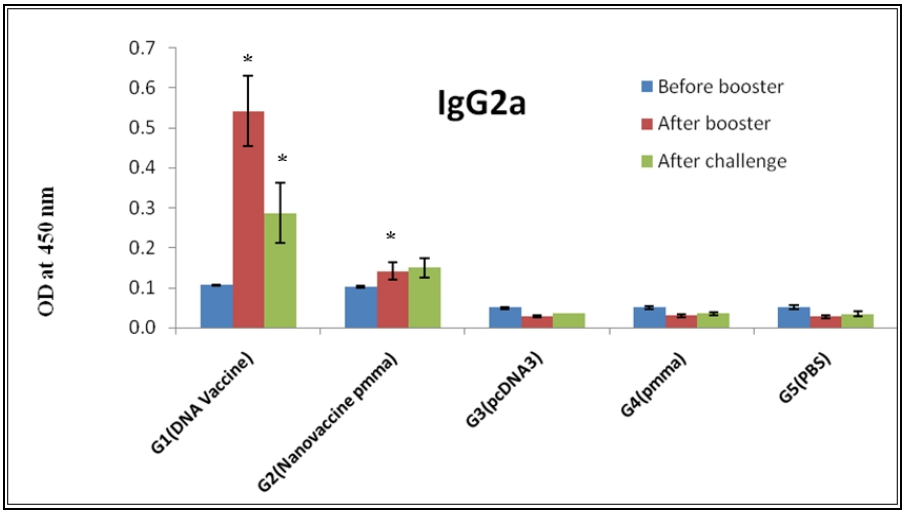
B



C



D



E

Fig 1(A-E): Specific antibody production against TSA recombinant protein in BALB/c mice immunized with DNA vaccine and nano-vaccine

4. Discussion

A number of vaccine strategies have been tested, ranging from killed parasites to recombinant antigens or DNA vaccines. DNA vaccines effectively engage both MHC-I and MHC-II pathways allowing for the induction of CD8+ and CD4+ T cells whereas antigen present in soluble form, such as recombinant protein, generally induces only antibody responses [16-19]. Using the prime-boost immunization strategy was a method which was able to affect the quality and quantity of immune responses. Moreover, some approaches such as application of *In vivo* electroporation that improve the efficacy of DNA vaccine is needed to be considered. In the present study a new nano-vaccine containing TSA DNA plasmid was designed and evaluated for it's immunogenicity in BALB/c mice. TSA is an immune-dominant antigen of *Leishmania major* and is antigenic in both murine and human systems. Immunization of BALB/c mice with a TSA plasmid DNA confers high levels of protective immunity. This DNA stimulates high titers of specific IgG1, IgG2a antibodies. Further, it also induces a predominant IFN-γ production and low levels of IL-4, phenotypic markers of Th1 responses. The recombinant leishmanial antigens LmSTI1 and TSA have been shown to induce excellent protection in both murine and nonhuman primate models of human cutaneous leishmaniasis. It seems that the use of an adjuvant and/or delivery system is necessary for almost any modern vaccine particularly vaccines against leishmaniasis [11, 20]. In this research PMMA nanoparticles were utilized as adjuvant to improve specific humoral immune responses to our candidate vaccine. PMMA nanoparticle adjuvant achieved good antibody responses and good protection against challenge with a number of antigens. In addition, the PMMA adjuvant seems to lead a high stability of vaccines, which are containing polymeric particulate. These nanoparticles can easily be manufactured in a reproducible manner in the described particle sizes and with specific surface properties. Among the numerous advantages of the nanoparticles can be pointed simple and easy to produce, process low degradation, less adverse effects during use. The Use of Polymethyl methacrylate adjuvant for Split Influenza vaccines showed safety record and excellent and stronger protection. In this research the animals were sacrificed and their spleen cells were obtained and cultured in the presence of specific antigen. The findings of this study showed that immunization of mice with nano-vaccine or DNA vaccine enhanced total antibody responses as compared with control groups .Before booster injection high level of total antibody was observed in mice immunized with nano-vaccine and following the booster injection and after infection with *L. major* the level of total specific antibody in the group of mice immunized with DNA vaccine was higher than the other vaccinated group. Previous findings demonstrated that PMMA nanoparticle enhanced humoral responses in Hiv-2 Split Whole virus and there was safety against adverse effects during use. Other studies suggested that PMMA adjuvant may represent an attractive alternative to increase the efficacy of candidate vaccines toward antibody production [21-25]. While exploring IgG isotypes our results revealed that both specific IgG_1 and IgG_{2a} were augmented. Considering that IgG_1 is a Th_2 and IgG_{2a} is a Th1 markers respectively. This funding indicated that after peptide booster injection levels of IgG_1 and IgG_{2a} isotypes were increased in immunized groups. Studies of Campos-Neto *et al* showed that Immunization of BALB/c mice with a TSA plasmid DNA induced high titers of specific IgG_1, IgG_{2a} antibodies against *Leishmania*. Our studies showed that using the prime-boost vaccination to mice, resulted increasing of protection against *Leishmania* infection. The results of previous studies showed that use of nanoparticles and prime boost strategy enhanced protective in animal models of *Leishmania* infection [22, 23, 26].

5. Conclusion

In this study, we demonstrated that PMMA could effect on efficacy of a DNA vaccine encoding TSA against *L. major* infection and elicited humoral immune responses to the antigen delivered. In this work, we showed the vaccine formulation described here may be an excellent candidate for further vaccine development against *Leishmania*.

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