DNA vaccination with a plasmid encoding LACK-TSA fusion against *Leishmania major* infection in BALB/c mice

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Abstract

Vaccination would be the most important strategy for the prevention and elimination of leishmaniasis. The aim of the present study was to compare the immune responses induced following DNA vaccination with LACK (*Leishmania* analogue of the receptor kinase C), TSA (Thiol-specific-antioxidant) genes alone or LACK-TSA fusion against cutaneous leishmaniasis (CL). Cellular and humoral immune responses were evaluated before and after challenge with *Leishmania major* (*L. major*). In addition, the mean lesion size was also measured from 3rd week post-infection. All immunized mice showed a partial immunity characterized by higher interferon (IFN)-γ and Immunoglobulin G (IgG2a) levels compared to control groups (p<0.05). IFN-γ/Interleukin (IL)-4 and IgG2a/IgG1 ratios demonstrated the highest IFN-γ and IgG2a levels in the group receiving LACK–TSA fusion. Mean lesion sizes reduced significantly in all immunized mice compared with control groups at 7th week post-infection (p<0.05). In addition, there was a significant reduction in mean lesion size of LACK-TSA and TSA groups than LACK group after challenge (p<0.05). In the present study, DNA immunization promoted Th1 immune response and confirmed the previous observations on immunogenicity of LACK and TSA antigens against CL. Furthermore, this study demonstrated that a bivalent vaccine can induce stronger immune responses and protection against infectious challenge with *L. major*.

Keywords: *Leishmania*, vaccine, LACK, TSA, fusion

INTRODUCTION

Leishmaniasis caused by different species of genus *Leishmania* is a major health problem in large parts of the world. More than 98 countries are endemic for leishmaniasis putting about 350 million people at risk. The annual global prevalence of leishmaniasis is about 1.5-2 million new cases.1,2 Different chemical drugs have been applied for treatment of cutaneous leishmaniasis (CL) including pentavalent antimonials, amphotericin B, pentamidine3,4, topical formulations of paramomycin5, oral miltefosine6 and some anti-fungal drugs (ketoconazole).7 However, the use of these drugs is limited because of problems (e.g. high toxicity, expensive cost and ineffectiveness in some endemic regions).8,9 Vaccination remains the most appropriate opportunity for the prevention and safe treatment of all forms of the disease.10-12 Currently there is no effective vaccine for prevention of leishmaniasis.13 Therefore, development of an effective drug or vaccine against leishmaniasis is one of the main global public-health priorities.14,15 TSA antigen is a 22.1 kDa protein that is conserved in all species of *Leishmania* and expressed in both amastigote and promastigote forms of the protozoa.16-19 In previous studies, TSA antigen singly or in combination with other antigens have induced protection against leishmaniasis.17,19-21 LACK is a 36kD protein that is highly conserved among different species of genus *Leishmania* and is also expressed in both promastigote and amastigote forms of the protozoa.22 The exact function of LACK protein in *Leishmania* is not completely understood.23 However, it is clearly demonstrated...
that LACK differentiates promastigotes to amastigote forms into host macrophages and is essential for the viability of the parasite. The L. major LACK protein plays a key role in the induction of immune responses in susceptible BALB/c mice and acts as a vaccine candidate against human leishmaniasis. Immunization with either protein or DNA of LACK antigen conferred protection in susceptible BALB/c mice infected with L. major and reduced disease progression and parasite burden through increased IFN-γ production.

Vaccination with a single antigen induces protective immune responses against infectious challenge with the same Leishmania species and is not able to induce protection against different species of genus Leishmania in people with different genetic backgrounds and different parasite stages. Therefore, it is more likely that a vaccine composed of multiple antigens rather than a single gene product would show a protective immunity against leishmaniasis in a broad spectrum of individuals. Multivalent vaccines may confer stronger immune responses and protection. Therefore, the use of two or multiple specific antigens is a rational strategy to develop an effective vaccine against parasitic infections. In the present study, we tried a bivalent vaccine to improve immune response against CL. Hence, immune responses in susceptible BALB/c mice vaccinated using LACK, TSA genes alone or with LACK-TSA fusion were compared after infectious challenge with L. major promastigotes in the stationary phase.

**MATERIAL AND METHODS**

**Parasite growth**

L. major parasites (MRHO/IR/75/ER, Iranian strain) were grown at 22-26°C in RPMI-1640 (Gibco, BRL, Maryland, USA) plus L-glutamine (20 mM), supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL, Maryland, USA), 100 U/ml penicillin and 100μg/ml streptomycin (Sigma, USA). L. major promastigotes in stationary phase were used for antigen preparation and infectious challenge.

**Animals**

In this study, female BALB/c mice (6 to 8 week-old) were purchased from the Pasteur Institute of Iran and housed in an animal facility which was appropriate to the species, with controlled temperature and humidity, receiving food and water.

**Construction of LACK-TSA fusion**

In the present study, three recombinant plasmid of pEGFP-LACK, pEGFP-TSA, pEGFP-LACK-TSA from our previous studies were used. For construction of LACK-TSA fusion, LACK gene was amplified by Pfu DNA polymerase and specific primers. LACK forward: (5’-TATGAAATCACCATGAACCTCGAGGGTCACCTGAGGAGG-3’) containing a restriction site for EcoRI enzyme and kozak sequence and LACK reverse: (5’-ATCGGATCCCTCGGCGTGGAGAT-3’) containing a restriction site for KpnI following program: initial denaturation at 95°C for 5min; denaturation at 95°C for 1min, annealing at 59.7°C for 1min, extension at 72 °C for 1min with 30 cycles; and final extension at 72°C for 10min. Then, TSA gene was digested from recombinant pEGFP-TSA plasmid obtained from previous study and subcloned in downstream of LACK gene. Construct of LACK-TSA fusion was confirmed by colony PCR with LACK forward and TSA reverse primers in accordance with following program: initial denaturation at 95°C for 5min; denaturation at 95°C for 1min, annealing at 58°C for 1.5 min, extension at 72 °C for 2 min with 30 cycles; and final extension at 72°C for 10min.

**Immunization schedules and challenge**

Immunization of mice was done intramuscularly three times at three week intervals with 100 μg of each plasmid DNA prepared in 100μL phosphate-buffered saline (PBS). The mice were allocated into five groups (15 mice per group) for DNA vaccination. Group I, group II, group III, group IV and group V were vaccinated with pEGFP-LACK, pEGFP-TSA, pEGFP-LACK-TSA fusion, pEGFP-N1 (empty vector) and with PBS only, respectively. Three weeks after the last immunization, five mice from each group were sacrificed for cytokine assay. The remaining animals were subcutaneously challenged through the tail base with L. major (MRHO/IR/75/ER) promastigotes in the stationary phase (2×10⁵/ mouse). The challenged mice were divided into two sub-groups; a sub-group was allocated for cytokine assay and another sub-group was allocated for assessment of lesion size.

**Cytokine profile**

Cytokine assay was performed before challenge and at 4 weeks after challenge following stimulation with Soluble Leishmania Antigen (SLA). SLA was prepared with L. major
promastigotes in the stationary phase. Briefly, the promastigotes were washed three times in PBS and re-suspended at a concentration of 2×10⁶ parasites/ml. Then, freezing and thawing procedure was performed in liquid nitrogen and a 37°C water bath for 10 times. The suspension was then centrifuged at 8,000 × g for 30 min at 4°C and supernatant containing SLA was harvested and kept at −70°C.35 The protein concentration was determined by Bradford method.36

Five mice of each group were sacrificed and spleens were then removed under antiseptic condition and homogenized in PBS. After erythrocyte lysis in Ammonium-Chloride-Potassium (ACK) buffer (0.15 M NH₄Cl, 10 mM KHCO₃ and 0.1 mM Na₂EDTA), splenocytes were re-suspended in RPMI 1640 (Gibco, BRL, Maryland, USA) supplemented with 10% FBS (Gibco, BRL, Maryland, USA) plus 100 U/ml penicillin and 100µg/ml streptomycin. Viability and counting of the cells were performed using 0.4% of trypan blue and a Neubauer Chamber under an optical microscope. Cells were then seeded at a concentration of 3 × 10⁶ cells/ml and stimulated with 20µg/ml SLA. Then, plates were incubated at 37°C with 5% CO₂. After 72 hours, the supernatant was harvested for cytokine assay. IL-4 and IFN-γ levels were measured by sandwich based enzyme-linked immunosorbent assay (ELISA) kits (U-CyTech biosciences, Netherland) according to the manufacturer’s instructions.

Ag-specific antibody responses
Mice were bled before challenge and 4 weeks after challenge. The pooled sera from each group were kept at -20°C until use. Total IgG, IgG1 or IgG2a levels were measured by ELISA on the sera obtained from immunized and control mice. Briefly, the 96 well plates (Nunc MaxiSorp, eBioscience) were coated (100µl/well) with 20µg/ml SLA in 50mM carbonate-bicarbonate coating buffer, pH 9.6 and incubated overnight at 4°C. After three times washes, blocking was done using 100 µl of 1% BSA in PBS per well for 2 hours at 37°C. 100µl/well (1:20 dilution) of sera were added and incubated at 37°C for 2 hours. After three washes, blocking was done using 100 µl of 1% BSA in PBS per well for 2 hours at 37°C. 100µl/well (1:20 dilution) of sera were added and incubated at 37°C for 2 hours. After three washes, 100µl of horse radish peroxidase (HRP) -conjugated goat anti-mouse IgG (1:1000, Sigma), IgG1 or IgG2a (1:10,000, eBioscience) were added and incubated at 37°C for 1 h. After six times washing, detection was done with 100µl of TMB substrate. Finally, the reaction was stopped with 2N H₂SO₄ and optical density was measured at 450 nm. All experiments were run in triplicate.

Measurement of lesion size
Lesions were observed in mice tail base three weeks post-infection. Lesion sizes for all groups (5 mice per group) was measured weekly with a digital metric caliper in mm from weeks 3 to 12. Mean lesion sizes were compared using one-way-ANOVA test and LSD Post Hoc at 7th week post-infection.

Statistical analysis
Mean cytokine and antibody levels and also lesion size were compared for all groups with one-way-ANOVA test and LSD Post Hoc. The mean groups were different for p values < 0.05. In addition, IFN-γ/IL-4 and IgG2a/IgG1 ratios were calculated for immunized groups than PBS group. The graphs were plotted by ‘GraphPad Prism’ and ‘Microsoft Office Excel’.

Ethics statement
All animal protocols including maintenance, animal handling and blood sample collection were approved by the Medical Ethics Committee of Tarbiat Modares University of Iran (ID: 52/5101 dated March 16, 2013).

RESULTS
IFN-γ and IL-4 production following DNA vaccination
Cellular immune response showed that immunized mice induced a partial immunity with higher IFN-γ levels and lower IL-4 levels than control groups before and after challenge (p<0.05). IFN-γ levels increased for immunized animals with LACK-TSA fusion as compared with LACK alone before challenge (p<0.001). IFN-γ levels in the LACK group was significantly higher than in the groups immunized with TSA and LACK-TSA after challenge (p<0.001) (Fig. 1, Panel A). In addition, the one-way-ANOVA analysis and comparison of the mean of IL-4 levels demonstrated lower IL-4 levels in LACK-TSA fusion group than LACK (p<0.001) and TSA (p<0.01) groups alone before and after challenge (Fig. 1, Panel B).

The ratio of IFN-γ/IL-4 increased for all immunized groups compared with control groups after challenge (Fig. 1, Panel C). The ratio was 4.72 for LACK, 3.32 for TSA and 13.89 for LACK-TSA fusion while the ratio was only 0.338 for empty vector and 0.225 for PBS group. The IFN-γ/IL-4 ratio increased 16.78, 20.97 and 61.73 times for mice immunized with TSA, LACK and LACK-TSA respectively than PBS group post-infection.
FIG. 1: Cytokine assay in different groups. Splenocytes from five sacrificed mice were harvested before challenge and at 4 weeks post-infection and stimulated \textit{in vitro} with 20 µg/ml of SLA for 72h. IFN-γ and IL-4 cytokines were assessed by ELISA. Each bar represents mean ± S.D in pg/ml. Panel A, IFN-γ before and after challenge; Panel B, IL-4 before and after challenge. Panel C, IFN-γ/IL-4 ratio. The asterisk elicits significant difference between values compared by one-way-ANOVA (p<0.05 indicated as *, p<0.01 indicated as **, p<0.001 indicated as *** and ns. indicated as non significant)
**Antibody production following DNA vaccination**

Total IgG levels increased significantly in immunized groups in comparison with those in control groups after challenge (p<0.05) (Fig. 2, Panel A). There was no significant difference in IgG1 levels between immunized groups post-infection (Fig. 2, Panel B). While, IgG2a levels increased in all immunized groups compared with the control groups in both groups before and after challenge (p<0.05). Furthermore, IgG2a levels increased significantly for LACK and LACK-TSA groups in comparison with those...
in TSA group post-infection (p<0.001) (Fig. 2, Panel C). IgG2a/IgG1 ratio increased about 3.7 times for mice immunized with LACK and TSA DNAs and five times for immunized group with LACK-TSA fusion compared with that in PBS group after challenge (Fig. 2, Panel D).

**Lesion size assessment**

Lesion progression delayed significantly in all immunized mice compared with those in control groups at 7th week post-infection (p<0.05). In addition, the mean lesion size showed significant reduction in immunized mice with LACK-TSA fusion and TSA in comparison with that in LACK group at 7th week post-infection (Fig. 3). The mean lesion size decreased 25%, 35.69% and 39.62% for LACK, TSA and LACK-TSA groups respectively compared to the control group at 7th week post-infection.

**DISCUSSION**

Although development of a vaccine is the best and the most cost-effective technique for protection against leishmaniasis, there is as yet no effective vaccine to prevent leishmaniasis because of insufficient knowledge on the immune-pathogenesis of the disease. DNA vaccines were used for protective and therapeutic targets. These vaccines contain bacterial plasmids encoding antigens whose release into living cells leads to expression of recombinant proteins.

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**FIG. 3:** Three weeks after the last immunization, mice were subcutaneously challenged with $2 \times 10^6$ *L. major* promastigotes in stationary phase into tail base. Panel A. Lesion caused by *L. major* on tail base of BALB/c mice. Panel B. Mean ± S.D. of lesion size in BALB/c mice (5 mice per group) at 7th week post-infection. The asterisk sign (⋆) indicates that mean lesion size decreased significantly in all immunized groups than control groups at 7th week post-infection (p<0.05). The asterisk sign (★★) indicates significant reduction in mean lesion size in LACK-TSA and TSA groups in comparison to LACK group.
The design of DNA vaccine is relatively rapid, cheap, and simple compared to other forms of vaccines. Multivalent vaccines promote stronger specific immunity because they present more epitopes to the major histocompatibility complex (MHC). DNA vaccines can induce both humoral and cellular immunity without requiring complex biochemical techniques. In addition, DNA vaccines contain CpG ODN sequences in plasmid DNA which lead to nonspecific activation of the innate immunity characterized by up-regulation of costimulatory molecules, production of inflammatory cytokines and oxidant radicals by antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs).

Therefore, DNA vaccines can be recommended against intracellular pathogens such as Leishmania. Up to now, immunogenicity and protection induced following vaccination with different antigens have been evaluated against Leishmania infection including LeIF, LmSTI1, SMT, CP49, LACK, TSA, A2, that applied individually or in combination with other antigens. In this study, we found that IFN-γ levels increased in all immunized groups in comparison with control groups post-infection. IFN-γ/IL-4 ratio in immunized mice with LACK, TSA or LACK-TSA was 14.75-61.73-fold higher compared with the PBS group at 4 weeks post-infection. Additionally, lesion progression was delayed in all immunized groups compared to the control groups. The immune responses shifted toward Th1 immunity as characterized by the increased levels of IFN-γ and decreased levels of IL-4 as well as reduction in lesion size of immunized animals. Furthermore, mice immunized with LACK-TSA and LACK showed higher levels of IFN-γ. The LACK antigen appeared to be a stronger immunogenic agent that induces Th1 response than the TSA antigen and has been confirmed by previous studies.

The IFN-γ/IL-4 ratio in vaccinated mice with LACK-TSA fusion increased about 4.18 and 2.94 fold compared to mice immunized with TSA and LACK respectively post-infection. Also, IgG2a/IgG1 ratio for LACK-TSA group was about 1.35 fold higher than immunized groups with one gene alone. In addition, immunized group with LACK-TSA fusion showed the smallest lesion diameter compared with each of these genes alone. Overall, these results showed that fusion group developed stronger Th1 responses and may be due to presentation of more epitopes to the MHC. However none of them could induce full protection in preventing development of lesions in immunized mice. The present study is in agree with other studies that have used two or more antigens to improve protective immune responses and induce long-term immunity compared with one gene alone against the disease.

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REFERENCES


