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Enhancement of immune response induced by DNA vaccine cocktail expressing complete LACK and TSA genes against *Leishmania major*

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Leishmaniasis is an important disease in humans. Leishmania homologue of receptor for Activated C Kinase (LACK) and thiol specific antioxidant (TSA) as immuno-dominant antigens of *Leishmania major* are considered the most promising molecules for a DNA vaccine. We constructed a DNA cocktail, containing plasmids encoding LACK and TSA genes of *Leishmania major* and evaluated the immune response and survival rate in BALB/c mice. IgG and Interferon gamma values were noticeably increased in the immunized group with DNA cocktail vaccine, which were significantly higher than those in the single-gene vaccinated and control groups ($p < 0.05$) following the immunization and after challenging with *Leishmania major*. Interleukin 4 values were decreased in all immunized groups, but only in DNA vaccine cocktail and single-gene vaccination with pc-LACK there were statistical differences with control groups ($p > 0.05$). The immunized mice with the cocktail DNA vaccine presented a considerable reduction in diameter of lesion compared to other groups and a significant difference was observed ($p < 0.05$) in this regard. The survival time of the immunized mice with the cocktail DNA vaccine was significantly higher than that in the other groups ($p < 0.05$) after their being challenged with *Leishmania major*. The findings of this study indicated that the cocktail DNA vaccine increased the cellular response and survival rate and induced protection against infection with *Leishmania* in the mice.

Key words: DNA vaccine; *Leishmania major*; cocktail pcTSA + pcLACK; immune responses.

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Leishmaniasis is caused by the intracellular protozoan parasite *Leishmania major* (1) and several species of the genus *Leishmania* are responsible for this parasitic disease. The disease is endemic in many parts of the world with about 12 million infected cases. Also, 1.5–2 millions of new cases of cutaneous leishmaniasis are reported annually (2).

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The drugs of choice against leishmaniasis are toxic and have side effects and resistance (3). Development of either new anti-*leishmania* drugs or a vaccine is thus much important.

Lasting immunity against re-infection is acquired following a cutaneous infection with *Leishmania* spp., suggesting that prophylactic immunization is feasible (4). There is a marked development of cell-mediated reaction, but a weak specific antibody response can be detected (5).

In recent years, significant progress has been made in the identification of protective vaccines. To develop a protective and effective anti-*leishmania* vaccine, most of the works have focused on different antigens (6–8). Among the vaccine candidates, Leishmania homolog of receptors for activated c-kinase (LACK) and thiol-specific-antioxidant (TSA) have been introduced as the predominant vaccine candidates (9, 10). TSA is *Leishmania major* recombinant protein homologue to eukaryotic thiol-specific-antioxidant protein. TSA gene is 600 bp and is located on chromosome 15. TSA is expressed in *Leishmania major* promastigote and amastigote, and it is much conserved among *Leishmania* species (11).

The protein of LACK antigen is highly conserved among related *Leishmania* species, and is expressed in both promastigote and amastigote forms of the parasite (12). LACK gene is located on chromosome 28 and is composed of 939 bp (13).

In some studies, TSA DNA–vaccine stimulated high titers of specific IgG2 antibody, high levels of interferon gamma (IFN-γ) and low levels of interleukin 4 (IL-4), phenotypic markers of Th1 responses, which are the type of immune responses required for the control of this parasite (1, 4, 14).

The purpose of this research was to arrange a DNA cocktail vaccine, containing plasmids encoding the full length of LACK and TSA genes of *Leishmania major* and to examine its immune response and protective efficacy in comparison with single-gene vaccines (LACK and TSA) and control groups (PBS, pcDNA3).

Some reports have shown that epitopes of the two or more antigens could stimulate the immune system more than one antigen and maybe produce effective protection against *Leishmania major* (15).

According to this background, we constructed DNA cocktail, containing plasmids encoding the full-length LACK and TSA genes and evaluated its immune response and protective efficacy in BALB/c mice before and after challenging with Leishmania *major* compared to single-gene vaccines and control groups (PBS, pcDNA3).

MATERIALS AND METHODS

Leishmania major

The MRHO/IR/75/ER of *Leishmania major* was provided by the Pasteur Institute of Tehran, Iran. Promastigotes were grown at 24 °C in RPMI 1640 medium (Gibco, BRL, Maryland, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, BRL), and 100 μg/mL Gentamicin (Sigma-Aldrich, Deisenhofen, Germany). Stationary phase of the promastigotes was harvested at a density of 2×10^6/mL.

Plasmid constructions

Genomic DNA was extracted from promastigotes in stationary phase, then LACK and TSA genes were amplified by PCR (16–18).

DNA sequence of the gene encoding TSA of *Leishmania major* was obtained from GenBank (accession number EU194915.1) with 600 base pairs and we designed a pair of primers.

For confirmation of gene expression, six-histidine tag (6-His tag) sequence was designed in the forward primer.

Forward primer**,** 56 nt: introduced *Hin*dIII recognition site, underlined:

5′ - CAATTAAAGCTTATATGCATCACCATC ACCATCACATGTCCTGCGGTAACGCCAAG- 3′.

Reverse primer, 31 nt: introduced EcoRI recognition site, underlined:

5′ - CATGGAATTCTTACTGCTTGCTGAAG TATCC-3′ (579–600 nt).

Also, we designed a pair of primers based on LACK gene sequence of *Leishmania major* obtained from GenBank (accession number AF363975) with 939 base pairs.

Forward primer, 35 nt: introduced Hind III recognition site, underlined:

5′ ATTAAGCTTATGAACTACGAGGGTCACC TGAAGGG 3′

Reverse primer, 27nt: introduced EcoRI recognition site, underlined:

5′ TTAGAATTCTTACTCGGCGTCGGAGAT 3′

We used pcDNA3 as eukaryotic expression vector. The expression in eukaryotic cells (CHO) was

confirmed using SDS-PAGE and Western blotting (19–24).

Antigen preparation of *Leishmania major*

Soluble *Leishmania* antigen was prepared from stationary–phase promastigote of *Leishmania major* after a few passages in RPMI medium. About 2×10^6 *Leishmania major* promastigotes were washed five times in cold sterile PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma, Steinheim, Germany) as anti-protease. After five cycles of freezing and thawing, the suspension was centrifuged at $8000 \times g$ for 30 min at 4 °C and the supernatant was collected. The protein of supernatant was quantified using Bradford method, filtrated in a 0.2 μm pore filter (Nalge-Nunc International, Rochester, NY, USA), and stored at −80 °C (25).

Immunization and challenge

Female BALB/c mice aged 6–8 weeks were purchased from the Razi Serum and Vaccine Production Research Institute (Karaj, Iran) and maintained under standard conventional conditions.

The mice were grouped based on the administration content as follows:

A: Control groups (PBS and pcDNA3), 15 mice per group;

B: Vaccinated groups (pc-TSA, pc-LACK, pc-TSA+ pc-LACK), 21 mice per group;

All groups were divided into the following three subgroups of assay:

1 Immunological assay without parasite challenge.
2 Immunological assay with parasite challenge 4 weeks after the final inoculation.
3 Survival assay.

The injection amount for each mouse was 100 μg of each purified plasmid.

The mice in each group were anesthetized with 25 μL/g of the mixture of ketamin 10% and xylazin 2% via intraperitoneal injection and immunized via intramuscular injection into both quadricepses with 100 μL of administration content according to their grouping. The mixture was not injected more than 50 μL per muscle. Mice were immunized three times at the 3-week interval.

Four weeks after the final inoculation, the spleens from 1/3 of mice in each group (seven of immunized and five of control mice) were collected under aseptic conditions for immunological assay (MTT and cytokine assay).

Three weeks subsequent to the last immunization, 2/3 of mice in each group (14 of immunized and 10 of control mice) were challenged at the base of tail by the intradermal route with 2×10^6 promastigotes of high virulent strain of *Leishmania major* (MRHO/IR/75/ER). The measuring of the lesion diameter at the site of inoculation was monitored by a Vernier caliper thereafter weekly. The challenged group was divided into two subgroups: in one subgroup the animals were sacrificed 7 weeks after being challenged and the spleens and serum samples were harvested for immunological analysis. In the other subgroup, the mice were checked for survival assay.

Lymphocyte proliferation assay using MTT Method

In each well of microtitre plates, 3×10^5 lymphocytes per well were cultured in RPMI 1640 (Gibco, BRL) and 20% FCS (Gibco, BRL) and allowed to multiply for 72 h in the medium alone (control group), in the presence of 40 μg/mL of SLA or 10 μg/mL of PHA (Sigma-Aldrich) as mitogen and incubated at 37 °C and 5% CO_2. Afterwards, 20 μl of tetrazolium (Roche, Mannheim, Germany) (5 mg/mL) was added to each well and incubated at 37 °C for 4 h and then centrifuged in 1000 *g* for 10 min. The supernatant was discarded and 100 μL of DMSO (Merck, Germany) was added to each well and resuspended. The optical density (OD) was detected by ELISA reader in 540 nm.

Cytokines assay following the immunization and after challenge infection with *L. major*

The splenocyte cultures and cytokine assays were performed. Briefly, the cell preparations from the spleen tissue were cultured in RPMI 1640 (Gibco, BRL) and 20% FCS (Gibco, BRL) plated in duplicate in 24-well plates (Nalge-Nunc International) at 2×10^6 cells/mL, then allowed to multiply for 72 h in the medium alone (control group), in the presence of SLA with concentration of 40 or 10 μg/mL of PHA(Sigma-Aldrich) as mitogen and incubated at 37 °C and 5% CO_2.

Levels of IFN-γ and IL-4 in the supernatants were assessed by ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

Detection of antibody responses

The sera were collected from blood samples by retro-orbital puncture from the immunized mice 4 weeks after the final booster injection and 7 weeks after the challenge infection; then the samples were centrifuged in 3000 rpm for 15 min for serum preparation. The sera were frozen at −20 °C until use. They were tested for the presence of total anti-*Leishmania major* IgG antibodies by ELISA method. The microtiter plates were coated overnight at 4 °C with soluble *Leishmania major* antigens in concentration of 10 μg/

mL prepared in 100 mM carbonate-bicarbonate buffer pH 9.6 (100 μL per well), and sealed. The plates were washed in PBS (pH 7.4) and blocking was carried out with 5% dried skimmed milk in PBS (pH 7.2) for 1 h at 37 °C. After being washed with PBS containing 0.05% Tween 20 (Merck KGaA, Darmstadt, Germany) (PBST20), the sera were diluted 1/100 in 5% dried skimmed milk-PBST20 (100 μL per well) and incubated for 1 h and 30 min at 37 °C. After being washed, the bound antibodies were detected by incubation at 37 °C for 1 h and 30 min with horseradish peroxidase-labeled goat anti-mouse IgG (Dako, Denmark) at 1/2000 dilution in 5% dried skimmed milk – PBST20 (100 μL per well). Peroxidase activity was revealed by adding 100 μL per well of tetra methyl banzidine (Sigma-Aldrich) substrate. The reaction was stopped after 15 min by adding 100 μL of 2 M H_2SO_4 and the OD was read at 450 nm using an ELISA microplate reader (Bio-Rad, USA) (26).

Determination of parasite burden

A piece of spleen was excised, weighed, and then homogenized using a tissue grinder in 2 mL of Schneider's Drosophila medium supplemented with 20% heat-inactivated fetal calf serum and Gentamicin(Sigma-Aldrich) (0.1%). After 7 and 15 days of incubation at 26 °C, the plates were examined using an inverted microscope at magnification of ×40. The number of parasites per gram was calculated in the following way:

Parasite burden = $\log_{10}$ (parasite number/tissue weight) (27).

Statistical analysis

Statistical comparisons between the experimental groups were made using analysis of variance (ANOVA) and post-hoc Tukey test and Mann–Whitney test. Statistical analysis of survival time per each group was carried out using Kaplan–Meier (Log-Rank) test.

Differences were considered statistically significant when p-values was less than 0.05 ($p < 0.05$).

RESULTS

Evaluation of the extracted plasmid

The extracted plasmids from the bacterial colonies (recombinant plasmid) were sequenced and submitted to GenBank under the accession number: EU 194915.

Sequence analysis showed that the sequence from MRHO/IR/75/ER (an Iranian strain) *Leishmania major*, TSA gene with accession number LmjF15.1080 (TSA gene), strain 'LV39' (accession number AF069386 isolated by Levick et al. in 1998) and strain 'Friedlin' (accession number AF044679 isolated by Webb et al. in 1998) had high homology 97%, 96% and 95%, respectively.

Sequence of LACK gene showed 89% homology with *Leishmania major* strain Abdou p36 LACK gene (accession number AF363975.1), *Leishmania major* LACK gene (accession number AF034804.1) and *Leishmania major* activated protein kinase c receptor (LmjF28.2750, accession number XM_001684509.1).

The other results of plasmid constructions of these genes have been reported previously (28, 29).

Expression of LACK and TSA in eukaryotic cells

For expression of LACK and TSA genes, we could detect the protein bands in 22 kDa for TSA (Fig. 1) and 36 kDa for LACK (Fig. 2) by using Western blotting method.

Evaluation of the effect of pc-LACK, pc-TSA and pc-LACK+ pc-TSA immunization on the size of lesion

Developed lesions were followed by weekly measurement using a Vernier caliper. The

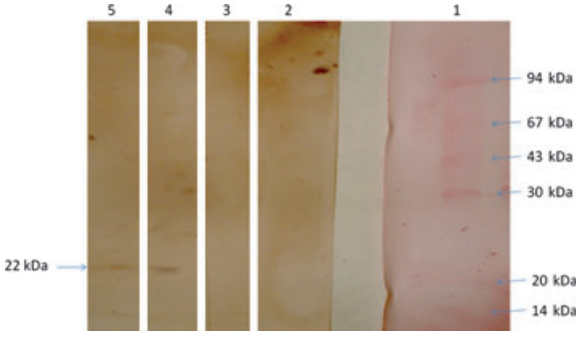

Fig. 1. Western blot analysis of expressed TSA gene. Western blotting showed anti-His tag monoclonal antibody recognizing TSA protein from transfected CHO cells. It was not detected in non-transfected control cells. Lane 1, protein molecular weight marker (top to down 94, 67, 43, 30, 20, and 14 kDa); Lane 2, CHO cells (negative control); Lanes 3, transfected cells containing plasmid pc-DNA 3; Lane 4 and 5, transfected cells containing pc-TSA, using *Leishmania major* antibody-positive mice sera and anti-His tag monoclonal antibody, respectively.

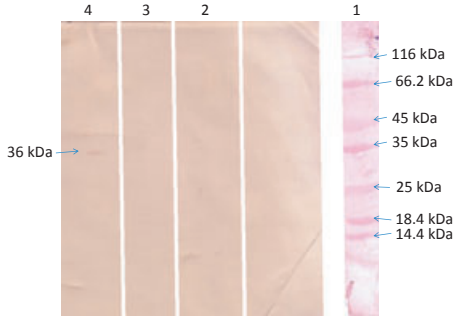

Fig. 2. Western blot analysis of expressed LACK gene. Western blotting showed mouse *L. major* positive sera recognizing LACK protein from transfected CHO cells. It was not detected in non-transfected control cells. Lane 1, protein molecular weight marker (top to down 116, 66.2, 45, 35, 25, 18.4, and 14.4 kDa); Lane 2, CHO cells (negative control); Lanes 3, transfected cells containing pc-DNA 3 (negative control); Lane 4, transfected cells containing pc-LACK plasmid.

evaluation of lesion size in the BALB/c mice following the infection with 2×10^6 *Leishmania major* promastigotes showed statistically significant differences between all immunized mice with the DNA vaccine and control group as well as statistically significant differences between pc-LACK+ pc-TSA cocktail and each pc-LACK and pc-TSA ($p < 0.05$). The mean of lesion size during 10 weeks in all groups is shown in Fig. 3.

Evaluation of the survival rate in the immunized animals

To assess the effect of vaccine in the studied groups (immunized and control) and those challenged with 2×10^6 *Leishmania major* promastigotes, the results indicated that the survival time in vaccinated group was significantly longer than that in the control groups ($p < 0.05$). The cocktail vaccine containing pc-LACK+pc-TSA could increase the survival rate more than single genes and the difference was significant ($p < 0.05$). The survival rate difference between pc-LACK and pc-TSA ($p < 0.05$) was significant too, and this result shows that pc-LACK is more effective than pc-TSA ($p < 0.05$).

The survival rate for all groups is shown in Fig. 4. All the mice in the control groups died within 11 weeks after the challenge.

Evaluation of the number of parasites in the spleen cells

For evaluation of the number of parasites in the spleen cells, all groups of mice were challenged with 2×10^6 *Leishmania major* promastigotes. The immunized mice had significantly lower parasite loads compared to the control mice ($p < 0.05$). Among the immunized groups, the group vaccinated with cocktail of pc-LACK + pc-TSA showed the most reduction in parasite burden in comparison with the other groups and the difference was significant ($p < 0.05$) (Fig. 5).

Evaluation of the level of anti- Leishmania major IgG

The sera were collected from the immunized and control mice 4 weeks after the final booster injection and 7 weeks after the challenge infection for measurement of total IgG

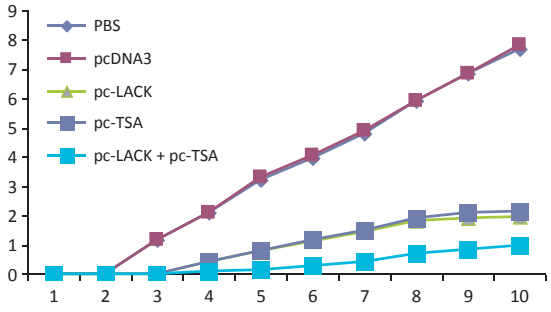

Fig. 3. The mean of lesion size of immunized animal groups during 10 weeks after challenge with 2×10^6 *Leishmania major* promastigotes.

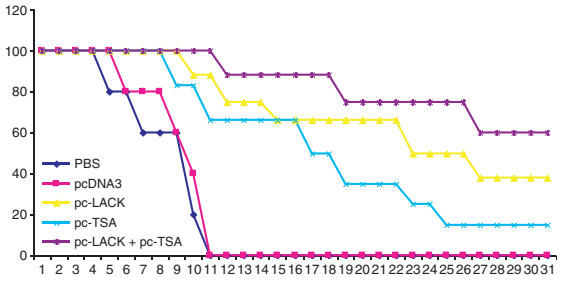

Fig. 4. Survival curves of immunized BALB/c mice after challenge with 2×10^6 promastigotes of *L. major* 4 weeks after the last immunization until 32 weeks after challenge.

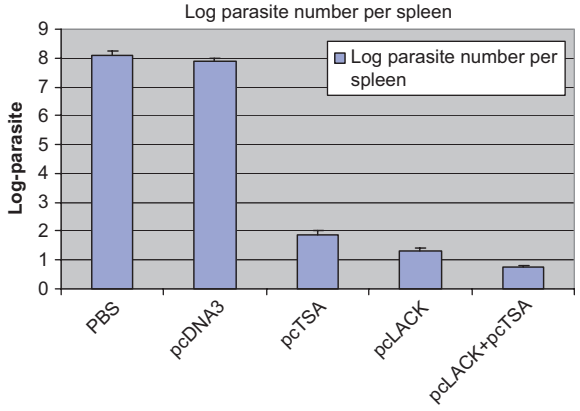

Fig. 5. The logarithmic number of the *Leishmania major* promastigotes in the spleen cells of the BALB/c mice in vaccinated and control groups and challenged with 2×10^6 *Leishmania major* promastigotes.

antibodies by using indirect ELISA method. Figure 6 shows that the humoral responses were elicited by the immunization pre- and post- challenge infection. Anti–*Leishmania major* IgG values increased significantly in vaccinated groups in comparison with control groups pre- and post-challenge ($p < 0.05$) and the difference between cocktail vaccine and single vaccine was significant pre- and post-challenge as well ($p < 0.05$) (Fig. 6).

The positive cutoff value of IgG relative to PBS control was measured as 0.18 according to Mean + 3 × SD of OD. All vaccinated samples were positive and the OD was more than the measured cutoff.

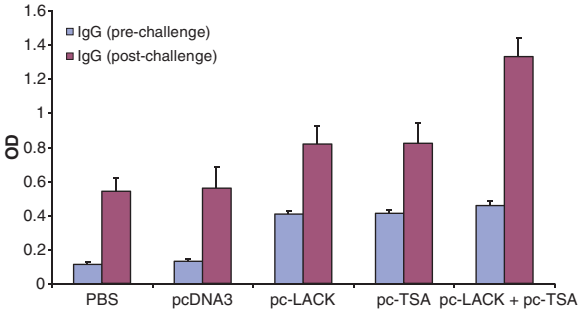

Fig. 6. Humoral responses (IgG) against *L. major* infection induced by DNA vaccination 3 weeks after the last vaccination and 7 weeks after the challenge infection. All the sera were diluted 1:200 and the results are expressed as the optical density (OD) at 450 nm.

Lymphocyte proliferation assay using MTT Method

The pattern of MTT is according to the calculation of SI (SI = mean of OD in each group/mean of OD in PBS group pre-challenge). As indicated by MTT, the lymphocytes proliferation assay results in all immunized groups compared to control groups were significantly higher ($p < 0.05$) in both pre-challenge and post-challenge conditions. Besides, the differences between pc-LACK + pc-TSA group and separate pc-LACK and pc-TSA groups were significant ($p < 0.05$), but there was no significant difference between pc-LACK and pc-TSA groups ($p > 0.05$). The mean and SD of SI are shown in Fig.7.

Measurement of the cytokines IFN-γ and IL-4

The cytokine assay was carried out in immunized and control groups for pre- and post-challenge.

We analyzed the levels of IFN-γ and IL-4 production in the supernatants of the spleen cells stimulated with SLA in all groups 4 weeks after the final booster injection and 7 weeks post-challenge infection with *Leishmania major*. The spleen cells from the immunized mice showed an increased IFN-γ production before and after the challenge infection with *Leishmania major* and the differences with control groups were significant ($p < 0.05$). The difference between cocktail vaccinated group and both single vaccinated groups was significant too ($p < 0.05$) (Fig. 8).

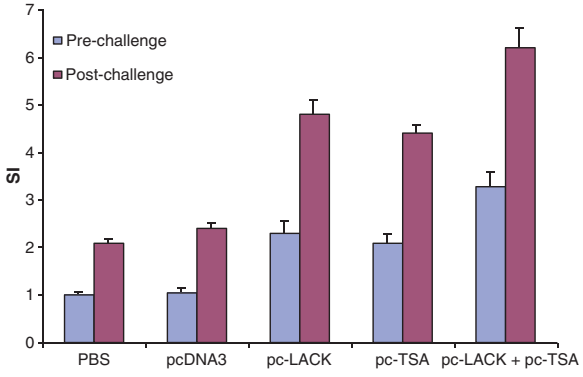

Fig. 7. Mean ± SD of the SI of MTT lymphocyte proliferation assay of spleen cells stimulated with SLA in immunized mice and control groups.

In comparison with that in control groups, IL-4 produced by spleen cells in both pre-challenge and post-challenge of cocktail group and pc-LACK group were significantly decreased ($p < 0.05$), but for pc-TSA group this difference was significant only after challenge ($p < 0.05$) (Fig. 9).

DISCUSSION

DNA vaccine in comparison with traditional vaccines is easy to produce, relatively inexpensive, heat stable, and can induce strong, long lasting and powerful humoral and cellular immunity (30, 31).

DNA vaccines effectively engage both MHC-I and MHC-II pathways, allowing for the induction of $CD8^+$ and $CD4^+$ T cells, whereas the antigens present in soluble form such as recombinant protein generally induce only antibody responses (32).

DNA vaccines may be especially useful for protection against cutaneous leishmaniasis, as the development of naturally acquired immunity to a primary exposure to *L. major*, involving low dose infection in the skin has recently been shown to depend on both $CD4^+$ and $CD8^+$ T cells [12].

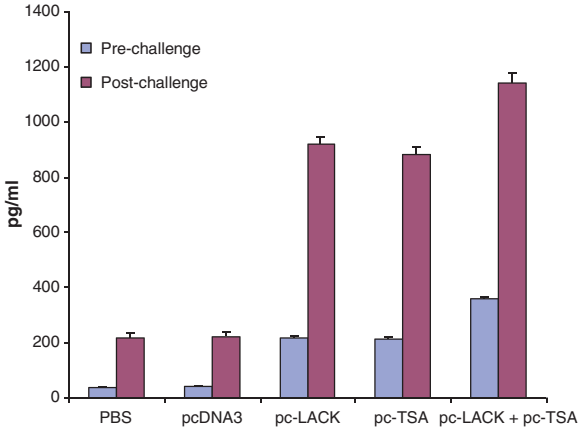

Fig. 8. Cytokine production (IFN-γ) by the stimulated splenocytes with *L. major* antigens from vaccinated and control mice 3 weeks after the last vaccination and 7 weeks after the challenge infection using ELISA. Results are expressed as means of the OD_{450nm}± SD.

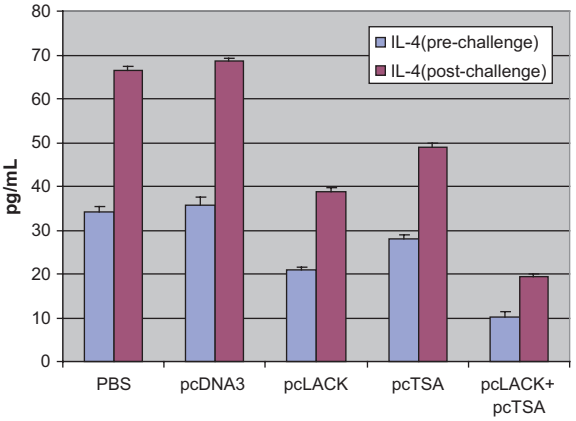

Fig. 9. Cytokine production (IL-4) by the stimulated splenocytes with *L. major* antigens from vaccinated and control mice at 3 weeks after the last vaccination and 7 weeks after the challenge infection using ELISA, Results are expressed as means of the OD_{450nm}± SD.

The results of DNA vaccine are dependent on candidate antigen, parasitic strain, amount, and number of vaccine, and animal model.

Leishmania have different kinds of antigens and could reflect very clearly the complexity of the immune responses against the *leishmania* parasite.

Among candidates of DNA vaccine for *Leishmania major*, the most promising gene is LACK and it has been shown to be more protective when used as a p24 truncated form (5, 6, 33).

LACK antigen, the *leishmania* homologue for receptors for activated C kinase, is a 36 kDa protein highly conserved among related *leishmania* species expressed in both promastigote and amastigote forms of the parasite.

LACK vaccination trials using protein or DNA vectors show protection against cutaneous *L. major* infections by redirecting the early IL-4 responses to a protective Th1 response (12, 34). This protective effect is mediated by IL-12 dependent INF-γ production (10).

LACK gene is constitutively expressed in *L. major* promastigotes and amastigotes (1). In this study the mice that immunized three times with cocktail DNA vaccine contain LACKp36 and TSA genes, showed longer survival rate in comparison with Sami et al. study that used cocktail vaccine with single dose of cocktail

DNA vaccine contain LACKp24 and TSA genes (15).

Immunization of BALB/c mice with recombinant TSA protein showed the development of strong cellular immune responses and conferred protective immune responses against *Leishmania major* (35, 36).

In this study, we found that cocktail DNA vaccine containing pcTSA+pcLACK showed significant protective response. Recombinant TSA protein elicited proliferative responses from peripheral blood mononuclear cells of human leishmaniasis patients.

Thiol specific antioxidant protein can detect specific antibody titers in sera of both cutaneous and visceral leishmaniasis patients. DNA vaccine encoding TSA/LmSTI1 leishmanial fusion proteins conferred protection against *Leishmania major* infection in BALB/c mice (1, 4).

Vaccination with a cocktail of DNA encoding cystein proteinases has been shown to confer protection against experimental cutaneous leishmaniasis. Kochan et al. in 2006 described MVA-LACK as a safe and efficient vector for vaccination against leishmaniasis (6, 8, 32).

In the present study, we constructed a DNA cocktail containing plasmids encoding the full-I LACK and TSA genes of *L. major* and then investigated the ability of pcLACK+pcTSA to elicit protective immunity in a DNA vaccine strategy.

The results show that pcLACK+pcTSA immunization was able to strongly enhance IFN-γ production and IL4 reduction compared to mice immunized with single-gene plasmids, PBS or empty plasmid.

Also the results indicate that improved immune responses driven by pcLACK+pcTSA appear to give protection from *L. major* challenge. When challenged with 2×10^6 *L. major* parasites, mice immunized with pcLACK +pcTSA had a longer average survival time than other groups. However, the vaccination with pcLACK+pcTSA showed no complete protection whereas the survival rate was longer than the other groups and the difference was significant.

Mice immunized with pcLACK+pcTSA elicited stronger Th1-type cellular immune responses than those immunized with single-gene plasmids, empty plasmid or PBS.

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