Arch. Immunol. Ther. Exp. (2018) 66:55–64
https://doi.org/10.1007/s00005-017-0484-4

ORIGINAL ARTICLE

Comparative Assessment of Induced Immune Responses Following Intramuscular Immunization with Fusion and Cocktail of LeIF, LACK and TSA Genes Against Cutaneous Leishmaniasis in BALB/c Mice

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Received: 6 January 2017 / Accepted: 22 May 2017 / Published online: 4 August 2017

Abstract In the present study, we evaluated induced immune responses following DNA vaccine containing cocktail or fusion of LeIF, LACK and TSA genes or each gene alone. Mice were injected with 100 μg of each plasmid containing the gene of insert, plasmid DNA alone as the first control group or phosphate buffer saline as the second control group. Then, cellular and humoral responses, lesion size were measured for all groups. All vaccinated mice induced Th1 immune responses against *Leishmania* characterized by higher IFN-γ and IgG2a levels compared with control groups ($p < 0.05$). In addition, IFN-γ levels increased in groups immunized with fusion and cocktail vaccines in comparison with LACK ($p < 0.001$) and LeIF ($p < 0.01$) groups after challenge. In addition, fusion and cocktail groups produced higher IgG2a values than groups vaccinated with a gene alone ($p < 0.05$). Lesion progression delayed for all immunized groups compared with control groups from 5th week post-infection ($p < 0.05$). Mean lesion size decreased in immunized mice with fusion DNA than three groups vaccinated with one gene alone ($p < 0.05$). While, lesion size decreased significantly in cocktail recipient group than LeIF recipient group ($p < 0.05$). There was no difference in lesion size between fusion and cocktail groups. Overall, immunized mice with cocktail and fusion vaccines showed stronger Th1 response by production of higher IFN-γ and IgG2a and showed smaller mean lesion size. Therefore, use of multiple antigens can improve induced immune responses by DNA vaccination.

Keywords *Leishmania* · Vaccine · Immunization · Fusion · Cocktail

Introduction

Leishmaniasis is caused by more than 20 different species of *Leishmania* and has three clinical manifestations according to relative parasite tropism to viscera or dermis including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, visceral leishmaniasis, diffuse CL and in rare cases post-kala-azar dermal leishmaniasis (Carneiro et al. 2009; Katara et al. 2011; McLachlan et al. 2009; Reithinger et al. 2007; Salman et al. 1999). Clinical features of leishmaniasis depend on different factors such as specific species of *Leishmania* and the genetic and immunological status of the host (Hepburn 2000). The disease affects 12 million people worldwide with 1.5–2 million new cases annually. CL is the most common form of leishmaniasis with annual incidence of approximately 1–1.5 million, of which more than 90% occur in developing countries including Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. Some leishmaniasis forms are cured difficult and may last for long time and cause severe facial disfigurements (Alvar et al. 2012; Reithinger et al. 2007). Human immunodeficiency virus (HIV)-CL co-infection is lately observed in many endemic regions (Gonzalez et al. 2009). The HIV can increase susceptibility

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to CL and modifies the clinical presentation of leishmaniasis in the co-infected patients leading to the formation of atypical, diffuse, and disfiguring lesions of CL. The HIV-CL co-infection can even affect the bone marrow and internal organs such as the spleen or liver (Laguna 2003; Rabello et al. 2003). In addition, HIV co-infected patients may become resistant to standard treatments and exhibit frequent relapses (Alvar et al. 1997; Talat et al. 2014). Recently, anti-leishmanial therapy depends exclusively on chemical compounds including pentavalent antimonials, pentamidine and amphotericin B which are losing values in endemic areas due to expensive cost, toxicity, difficulty of administration and drug resistance. The details of treatment of all forms of leishmaniasis are presented in WHO (2010) technical report. Vaccines remain important strategies for prevention, control and elimination of leishmaniasis in endemic regions (Das et al. 2014). During the past several decades, different formulations have been investigated to develop effective vaccines against *Leishmania*, including killed or live attenuated parasites, recombinant *Leishmania* proteins and DNA encoding *Leishmania* proteins (Dunning 2009; Kedzierski et al. 2006). None of which were effective enough to be recommended as a vaccine to control leishmaniasis (Okwor and Uzonna 2009). Meanwhile, vaccination with live parasites (leishmanization) was the only successful approach to control CL in Old World (Khamesipour et al. 2005; Nadim et al. 1983). However, this approach has been abandoned mainly because of safety problems (Okwor and Uzonna 2009). Therefore, development of a safe, effective and low-cost vaccine against *Leishmania* remains a crucial health purpose of global priority (Kedzierski et al. 2009).

DNA vaccination induces Th1 responses and is an attractive strategy to design a vaccine against leishmaniasis (Gurunathan et al. 1997, 2000). Immune response induced by DNA vaccines can improve through different techniques such as combination of several specific antigens (multivalent vaccines), prime-boost regiments, codon optimization and use of natural adjuvants (Ivory and Chadee 2004). A DNA vaccine presents a protein that is usually correctly folded and structurally similar to the native protein. Therefore, if such a vaccine is applied under appropriate conditions, it could become highly immunogenic and might provide effective immunoprotection (Okwor and Uzonna 2009). We have previously showed that DNA-based vaccination with plasmids encoding LeIF, LACK and TSA antigens induces partial protection against *L. major* infection in BALB/c mice (Ghaffarifar et al. 2013; Hezarjaribi et al. 2014; Maspi et al. 2016b; Tabatabaie et al. 2014). In the present study, we tried a multivalent vaccine to improve immune responses and protection against CL. To this end, BALB/c mice were vaccinated using fusion and cocktail of LeIF, LACK and TSA genes and compared their immune responses upon challenging them with *L. major* promastigotes in stationary phase.

Materials and Methods

Parasites

Leishmania major promastigotes (MRHO/IR/75/ER, Iranian strain) were grown at 24 °C in RPMI-1640 (Gibco, BRL, MD, USA (plus L-glutamine (20 mM), supplemented with 10% fetal bovine serum (FBS; Gibco, BRL, MD, USA), 100 µg/mL streptomycin and 100 U/mL penicillin (Sigma, St. Louis, MO, USA). The parasites were kept in a virulent state by regular passage in susceptible BALB/c mice. Stationary-phase promastigotes were harvested and centrifuged at 800×*g* for 10 min. The pellet was washed three times in phosphate buffer saline (PBS) and used for DNA extraction, challenge and antigen preparation.

Construction of Recombinant Plasmids

In this study, as shown (Fig. 1) three whole genes of LeIF, LACK and TSA were fused following: the LACK gene was amplified from pCDNA3-LACK plasmid (Jorjani et al. 2012) using Pfu DNA polymerase and the following primers (forward, 5′-TATGAATTC**ACCATG**AACTACGAG GGTCACCTGAAGGG-3′) containing *Eco*RI restriction site and a kozak sequence (underlined and bold, respectively) and (reverse, 5′-ATCGGTACCCTCGGCGTCGGA GAT-3′) containing *Kpn*I restriction site (underlined). Then, the LACK gene was cloned into the *Eco*RI and *Kpn*I sites of vector pEGFP-LeIF (Maspi et al. 2015, 2016b) downstream of LeIF fragment. Cloning integrity of LeIF–LACK (LL) fusion was confirmed by Colony PCR with LeIF forward and LACK reverse primers and restriction analysis. TSA gene was amplified from pcDNA3-TSA plasmid (Tabatabaie et al. 2007, 2014) using Pfu DNA polymerase and the primers: (forward, 5′-CAATTAGG-TACC**GCCATG**TCCTGCGGTAACGCCAAG-3′) containing *Kpn*I restriction site and a kozak sequence (underlined and bold, respectively) and (reverse, 5′-CATGGGATCCCTGCTTGCTGAAGTATCC-3′)

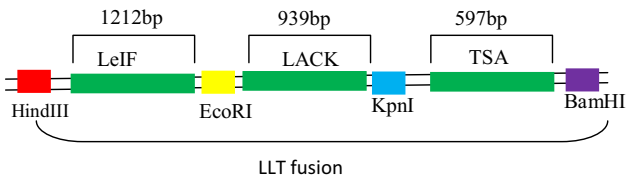

Fig. 1 Schematic diagram of LeIF–LACK–TSA (LLT) construct with restriction sites and fragment length of each gene

including *Bam*HI restriction site (underlined), then cloned into pEGFP-N1 vector. To construct tri-gene fusion, TSA gene was digested by *Kpn*I and *Bam*HI and subcloned in pEGFP–LeIF–LACK downstream of LL fusion. The construction of LeIF–LACK–TSA (LLT) fusion was confirmed by colony PCR with LeIF forward and TSA reverse primers and restriction analysis.

Transfection of Recombinant Plasmids in Hek-293 Cells

Human embryonic kidney 293 (Hek-293) cells were maintained in Dulbecco's modified eagle medium (DMEM; Gibco, USA) supplemented with 10% FBS (Gibco, BRL, MD, USA). Log-phase cells were used to transfect plasmids encoding fusion fragment of LLT antigens. Briefly, 24 h before transfection, approximately 3×10^4 cells/well were seeded into six-well plates. The cells reached 40–60% confluence at the day of transfection and culture medium was replaced with fresh DMEM supplemented with 5% FBS. For transfection, 50 μg plasmid was added to 23 μL 2M$CaCl_2$ and the total volume made to reach 186 μL with double-distilled water. This DNA-$CaCl_2$ mixture was incubated at room temperature for 5 min. Then, the $CaCl_2$-DNA solution was added dropwise to 186 μl of 2× HBS (50 mM HEPES and 1.5 mM Na_2-HPO4, 280 mM NaCl and 20 mM KCl, adjusted to pH 7.12) and formed to a homogeneous calcium phosphate-DNA precipitate. After 30 min incubation at room temperature, the solution was added to Hek-293 cells by dropping slowly. The plates were incubated at 37 °C and 5% CO_2. At 16 h post transfection, the culture medium was changed with fresh DMEM supplemented with 10% FBS and transfected cells evaluated for protein expression after 48 h.

RNA Extraction, RT-PCR

Transfected cells were harvested after 48 h, and subjected to three step washing with PBS followed by centrifugation at 1500×*g* for 10 min. Extraction of RNA of transfected cells was performed using RNX-Plus™ kit (Sina Clon, Tehran, Iran). The plasmid DNA was removed by addition of DNase to extracted mRNA. In addition, precluded contamination with DNA was checked via the absence of amplicon following PCR upon extraction of mRNA. Then, the corresponding cDNA was synthesized in accordance with the procedure of Easy™ cDNA Synthesis Kit (Parstous, Tehran, Iran) with oligo dT primers. The cDNA of LLT fusion was amplified by specific primers of LeIF forward and TSA reverse. PCR products were visualized using agarose gel electrophoresis after staining with DNA safe stain.

SDS-PAGE and Western Blotting Analysis

After 48 h, the transfected cells were trypsinized and washed in PBS by centrifuging at 1500×*g* for 10 min. The cell pellets were lysed using 200 μL ice cold lysis buffer with protease inhibitor and incubated on ice for 30 min. Then, the lysate was centrifuged at 800×*g* for 10 min at 4 °C. The protein mix in supernatant was transferred to a fresh tube and mixed with sample buffer (4.5 mM Tris–HCl, pH 6.8, 5% v/v 2-mercaptoethanol, 10% v/v glycerol, 0.05% w/v bromophenol blue, 2% w/v SDS). The samples were placed into a boiling water bath for 5 min at 100 °C. Then, the samples were run on a 10% SDS-PAGE until the dye front reaches the bottom of the gel. Then, the protein bands were transferred to a nitrocellulose membrane and the blocking of non-specific sites was carried out using 5% bovine serum albumin (BSA) in TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris base and 0.1% Tween 20) before incubating at 4 °C overnight. 5 mL of serum samples of mice vaccinated with LLT fusion in 5% BSA was then added to the membrane and incubated for 3 h on a shaker. The membrane was washed three times with TBST for 10 min. The blot was treated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000; Sigma, USA) for 2 h and the protein bands were visualized with diaminobenzidine tetrahydrochloride substrate (DAB; Sigma, USA) after about 2–3 min.

Immunization Schedule and Challenge

Animals

Female BALB/c mice were from Pasteur Institute of Iran and maintained under suitable conditions for experiments. The mice were put in experiments at 6–8 weeks of age.

Immunization Schedule and Infection

Mice were allocated to seven groups for immunization experiments (15 animals/group): group I for plasmids encoding LeIF, group II for LACK, group III for TSA, group IV for LLT fusion, group V for a cocktail of three genes, group VI for pEGFP-N1 (empty vector) as first control group and group VII for PBS alone as second control group. Then, each mouse vaccinated intramuscularly three times at intervals of three weeks with 100 μg of recombinant plasmids containing the gene of insert or with plasmid DNA alone (empty vector) in a final volume of 100 μL PBS and group VII injected with100 μL PBS. Three weeks after the latest immunization, five mice per group were killed to assay pre-challenge cytokine levels. The remaining ten mice of each group were infected by subcutaneous injection of 2×10^6 stationary-phase

promastigotes of *L. major* (MRHO/IR/75/ER) into their tail bases. The challenged mice of each group were then divided into two equal subgroups; five mice for measurement of lesion size and five mice for cytokine assay post-challenge.

Evaluation of Lesion Size

The measurement of lesion size in mice (5 animals/group) was undertaken within a period of 3–12 weeks post-infection. The lesion diameter including lesion length and width were measured on a weekly basis using a digital metric caliper within accuracy of one mm. In addition, percentage reduction of lesion size was calculated for immunized groups than PBS group in the 12th week post-infection.

Antigen Preparation

Soluble *Leishmania* antigen (SLA) was prepared from stationary-phase promastigotes of *L. major* as described by Coelho et al. (2003). Briefly, the number of 2×10^8 promastigotes/mL was washed three times in PBS. After ten times of freezing and thawing in liquid Nitrogen and 37 °C water bath, the suspension was centrifuged at $8000 \times g$ for 30 min at 4 °C. The supernatant containing SLA was then collected and stored at −70 °C. The protein concentration was assayed by Bradford method (Bradford 1976).

Cytokine Measurement

Interferon (IFN)-γ and interleukin (IL)-4 cytokines were measured following stimulation of splenocytes with SLA for uninfected and infected mice. Briefly, mice were killed before challenge and at 4 weeks after challenge and their spleens were removed aseptically and homogenized (by the base of a sterile syringe) in PBS. The re-suspended cells were washed twice by centrifuging in PBS at $300 \times g$ for 10 min and discarded the supernatant. Then, cell pellet was resuspended in 5 mL cold ammonium–chloride–potassium lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) and incubated for 5 min at room temperature to lysis erythrocytes. Hemoglobin and insoluble cell debris were removed by washing in PBS with 2% FBS. Spleen cells were cultured with RPMI medium (Gibco, BRL, MD, USA) supplemented with 10% FBS plus 100 U/mL penicillin and 100 μg/mL streptomycin in the presence of SLA at 37 °C and 5% CO_2. Briefly, cell viability was determined by 0.4% Trypan blue. After counting the cells by a hemocytometer slide, the number of 3×10^6 cells/well was seeded into 24-well plates for each mouse in triplicates. Splenocytes were stimulated with 25 μg/mL SLA. After 72 h, the supernatant of splenocytes was harvested for cytokine analysis. Measurement of IL-4 and IFN-γ was performed using sandwich based enzyme-linked immunosorbent assay (ELISA) kits (U-CyTech biosciences, Netherlands) according to manufacturer's recommended procedure.

Measurement of IgG Levels

Blood samples were obtained from the retro-orbital plexus of the immunized mice just before challenge and at 4 weeks after challenge. Then, the pooled sera from each group were stored at −20 °C until use. Total IgG and its isotypes (IgG1 and IgG2a) were measured by ELISA. Briefly, 96-well microtiter plates (Nunc, USA) were coated with SLA at a concentration of 20 μg/well prepared in coating buffer (0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4 °C. After three times washes, plates were blocked with 200 μL of blocking solution (2% BSA in PBS) per well and incubated at 37 °C for 2 h to reduce background interferences in ELISA. Serum samples were diluted 1:100 in PBST buffer (PBS-0.05 Tween-0.5% BSA) and added 100 μL/well. The plates were then incubated at 37 °C for 2 h followed by three washes in PBST. A volume of 100 μL HRP-conjugated goat anti mouse IgG (1:1000, Sigma, USA), IgG1 or IgG2a (1:10,000; eBioscience, Germany) was added to the plates and incubated at 37 °C for 1 h. After six washes, TMB substrate at 100 μL/well was added and the plates were incubated in a dark place for 20 min for detection. Finally, the reaction was stopped with 2 N H_2SO_4 and the optical density was read at 450 nm. All experiments were performed in triplicate.

Statistical Analysis

Statistical analysis was performed using GraghPad Prism version 5.0 for Windows (Graphpad Software Inc 2007, San Diego, CA, USA) and SPSS version 20. The mean of cytokine levels, antibody production, and lesion size were compared between all groups with one-way-ANOVA and Tukey post test. The statistical difference was concluded when p values were less than 0.05 ($p < 0.05$). The percentage reduction of lesion size in immunized groups than PBS group was calculated at 12th week post-infection. Furthermore, IFN-γ/IL-4 and IgG2a/IgG1 ratios were compared for immunized groups than PBS group. The graphs were plotted using Graphpad prism and Microsoft Office Excel 2013.

Results

Expression of LLT Fusion in Eukaryotic Cells

Hybrid protein expression of LLT into transfected cells was tested by RT-PCR and western blot techniques. The

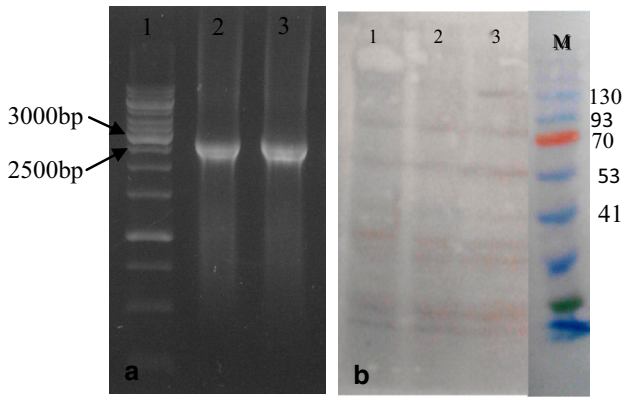

Fig. 2 Protein expression of LeIF–LACK–TSA (LLT) fusion. Hek-293 cells were transfected with recombinant plasmids encoding LLT fusion using calcium phosphate. Untransfected cells were used as negative control. 48 h post-transfection, transfected cells were harvested for RT-PCR and western blotting analysis. **a** LLT fusion was amplified from synthesized cDNA by specific primers of LeIF forward and TSA reverse. *Lane 1* 1 Kb DNA ladder, *lane 2* and *3* PCR product of LLT cDNA was amplified by specific primers. **b** Western blot analysis using anti-LLT antibody showed a recombinant hybrid protein about 130 kDa. *Lane 1* untransfected cells, *lane 2* transfected cells with empty plasmid, *lane 3* transfected cells with plasmids encoding LLT, *lane MW* 8–165 kDa

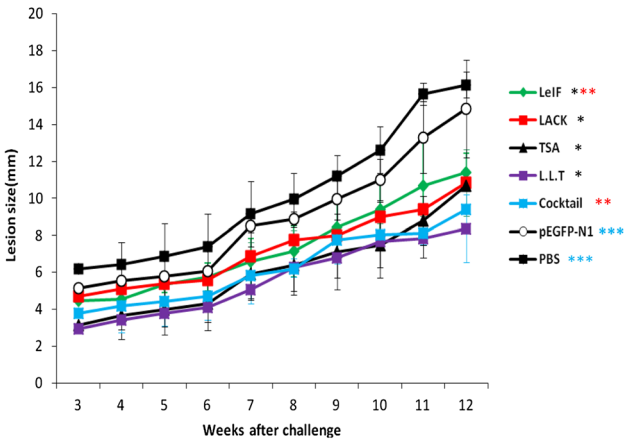

Fig. 3 Mean ± SD of lesion size at 3–12 weeks after infectious challenge with *L. major* promastigotes in stationary phase. Lesion progression delayed for immunized groups compared with control groups from 5th week post-infection ($p < 0.05$) (*triple asterisk*). Mean lesion size decreased only significantly in cocktail recipient group than LeIF recipient group ($p < 0.05$) (*double asterisk*). Mean lesion size decreased in immunized mice with LLT fusion than those immunized with LeIF, LACK and TSA antigens alone ($p < 0.05$) (*asterisk*). There was no significant difference in mean lesion size of fusion and cocktail groups

synthesized cDNA of Hek-293 cells was amplified by specific primers and indicated a product size of about 2745 bp as expected (Fig. 2a). Western blot with serum of mice vaccinated with plasmids encoding LLT fragment showed that transfected cells produced a recombinant hybrid protein as represented by a band of about 130 kDa (Fig. 2b). For this experiment, untransfected Hek-293 cells and transfected cells with empty vector were used as negative control.

Effect of DNA Vaccination on Lesion Size

Lesion size was evaluated between 3 and 12 weeks post-infection with *L. major* (Fig. 3). Lesion progression delayed significantly in immunized mice compared with both control groups and the mean lesions size was significantly different between all immunized groups and both control groups from 5th week post-infection ($p < 0.05$). The mean lesion size indicated considerable reduction between groups immunized by plasmids encoding LLT fusion than groups immunized by plasmids encoding LeIF, LACK and TSA genes individually ($p < 0.05$). While, mean lesion size of immunized animals with cocktail DNA showed only significant difference with LeIF group ($p < 0.05$). There is no significant difference in mean lesion size of fusion and cocktail groups. Our results showed that mean lesion size at 12th week post-infection was 48.14% and 41.82% less for fusion and cocktail groups, respectively, compared with PBS group (Table 1).

Effect of DNA Vaccine on Cytokine Production

To characterize the cellular immunity during *L. major* infection, splenocytes obtained from spleens of immunized and unimmunized mice stimulated in vitro with SLA for 72 h. Then, the supernatants were harvested to assay IFN-γ and IL-4 levels. The immunized mice showed higher levels of IFN-γ in comparison with unimmunized mice ($p < 0.05$). In addition, the fusion and cocktail DNA recipient groups produced higher levels of IFN-γ and lower levels of IL-4 compared with TSA and LeIF recipient groups ($p < 0.05$) but not from LACK recipient group after challenge There is no significant difference in IFN-γ and IL-4 levels between fusion and cocktail groups after challenge (Fig. 4a, b).The IFN-γ/IL-4 ratio increased about 34.08 and 30.17 times in fusion and cocktail groups, respectively, compared with the baseline ratio in PBS group (Fig. 4c).

Effect of DNA Vaccination on Ag-Specific Antibody Response

To monitor antigen-specific humoral immune response, serum samples of immunized and control groups were assayed just before and at 4 weeks after challenge (Fig. 5). There was no significant difference in total IgG before challenge. Total IgG levels increased in immunized groups than control groups post-infection ($p < 0.05$). In addition, total IgG increased significantly in LLT fusion group than

Table 1 Mean ± SD of lesion size at 12th week post-infection (mm) and percentage reduction of lesion size for different immunized groups compared to PBS group at 12th week post-infection

Groups	Lesion size at 12th week post-infection (mm)	Percentage reduction of lesion size than PBS
LeIF	11.41 ± 1.026	29.30
LACK	10.84 ± 1.126	32.83
TSA	10.63 ± 1.819	34.13
LLT	8.37 ± 1.392	48.14
Cocktail	9.39 ± 0.888	41.82
pEGFP-N1	14.85 ± 1.92	7.99
PBS	16.14 ± 2.69	0

LACK, TSA and cocktail groups after challenge ($p < 0.001$) (Fig. 5a). All immunized groups produced higher IgG2a and lower IgG1 levels in comparison with control groups after challenge. In addition, vaccinated mice with fusion showed higher IgG2a levels than vaccinated groups with cocktail and a gene alone after challenge ($p < 0.001$). In addition, IgG2a levels increased in cocktail group in comparison with TSA ($p < 0.01$) and LeIF groups ($p < 0.05$) while there is no significant difference in IgG2a levels between cocktail and LACK groups post-challenge (Fig. 5b, c). Ratio of IgG2a/IgG1 increased about 1.97 and 2.7 times in immunized groups with plasmids encoding cocktail and LLT DNAs, respectively, in comparison with PBS group after challenge. Collectively, mice vaccinated with LLT fusion elicited the highest ratio of IgG2a/IgG1 compared with other immunized groups after challenge (Fig. 5d).

Discussion

DNA vaccines have some advantages in comparison to alternative vaccine platforms (1) DNA vaccines have intrinsic safety because the vectors are non-replicating, express only the target antigen and do not have reversing risk to disease—causing forms unlike live vaccines; (2) unlike viral vectors, DNA vaccines do not induce immune responses against vector. Therefore, prime—boost regiments and multiple products can be used for one patient; (3) DNA vaccines can induce protection in infants even in the presence of neutralizing antibodies (Premenko-Lanier et al. 2004); (4) design and construct of DNA vaccines is comfortable and fast; (5) DNA vaccines show a great safety in human clinical trials (Kutzler and Weiner 2008; Liu 2011; Lu et al. 2008; Williams 2013).

A large number of studies have demonstrated that IFN-γ-dominant Th1 response phenotype induces protection against *L. major* infection, while the Th2 response phenotype (with IL-4) is related to host susceptibility and aggravation of the disease (Heinzel et al. 1989; Kemp 2000; Locksley and Scott 1991). IFN-γ/IL-4 ratio determines the phenotype (Th1 vs Th2) of the immune response against leishmaniasis. Increase of IFN-γ relative to IL-4 shows the promotion of immune responses toward cellular immunity and may lead to delay in the development of lesion while reduction in the ratio promotes Th2 responses that may leads to the disease progress and development of progressive lesions (Abdoli et al. 2017; Maspi et al. 2016a, 2017; Sacks and Noben-Trauth 2002; Scott and Novais 2016). Here, we vaccinated BALB/c mice with a cocktail or fusion DNA of LeIF, LACK and TSA genes and tested their immunogenicity and protective potential against infectious challenge. The results of the present study indicated that upon infectious challenge, a partial immunity was induced by all immunized mice against *L. major* infection characterized by higher IFN-γ levels and lower IL-4 levels. However, vaccinated mice with LLT fusion and cocktail DNA vaccines exhibited more potent type I immunity than immunized groups with a single gene and IFN-γ/IL-4 ratio increased almost two-times in LLT and cocktail groups than LeIF, LACK and TSA groups alone. Therefore, the results of cytokine profiles of them showed that immune responses promoted toward type 1 immunity following DNA vaccination which may be the reason of reduction in lesion size and induction of protection in the two groups. Antibody measurements were in conformity with the results of cytokine assessment. Multivalent vaccines consisting of multiple antigens present more epitopes to immune system and induce more potent immune responses and better protection against *Leishmania* infection in experimental models in comparison with single antigen vaccines (Coler et al. 2007; Ghaffarifar et al. 2013; Goto et al. 2011; Maspi et al. 2017). In the present study, all immunized groups showed delay in lesion progression than control groups which may be due to induction of cellular immunity following DNA vaccination. However, fusion and cocktail recipient mice exhibited smaller lesions than mice immunized with single antigen that may be due to increase of IFN-γ/IL-4 ratio in them. The results of the present study revealed that fusion construct developed more protection and smaller lesions than TSA and LeIF separately. In general, fusion recipient mice had the smallest lesion diameter amongst all experiment animals. These results emphasize previous studies in stimulation of immune responses following DNA immunization against leishmaniasis and support the idea that a single polygenic plasmid DNA construct can improve specific immune responses against several distinct parasite antigens which lead to more protection and the development of smaller lesions. Ahmed et al. (2009) showed that DNA vaccine with plasmids encoding cocktail of LACKp24, TSA, LmSTI1 and CPa genes induced stronger

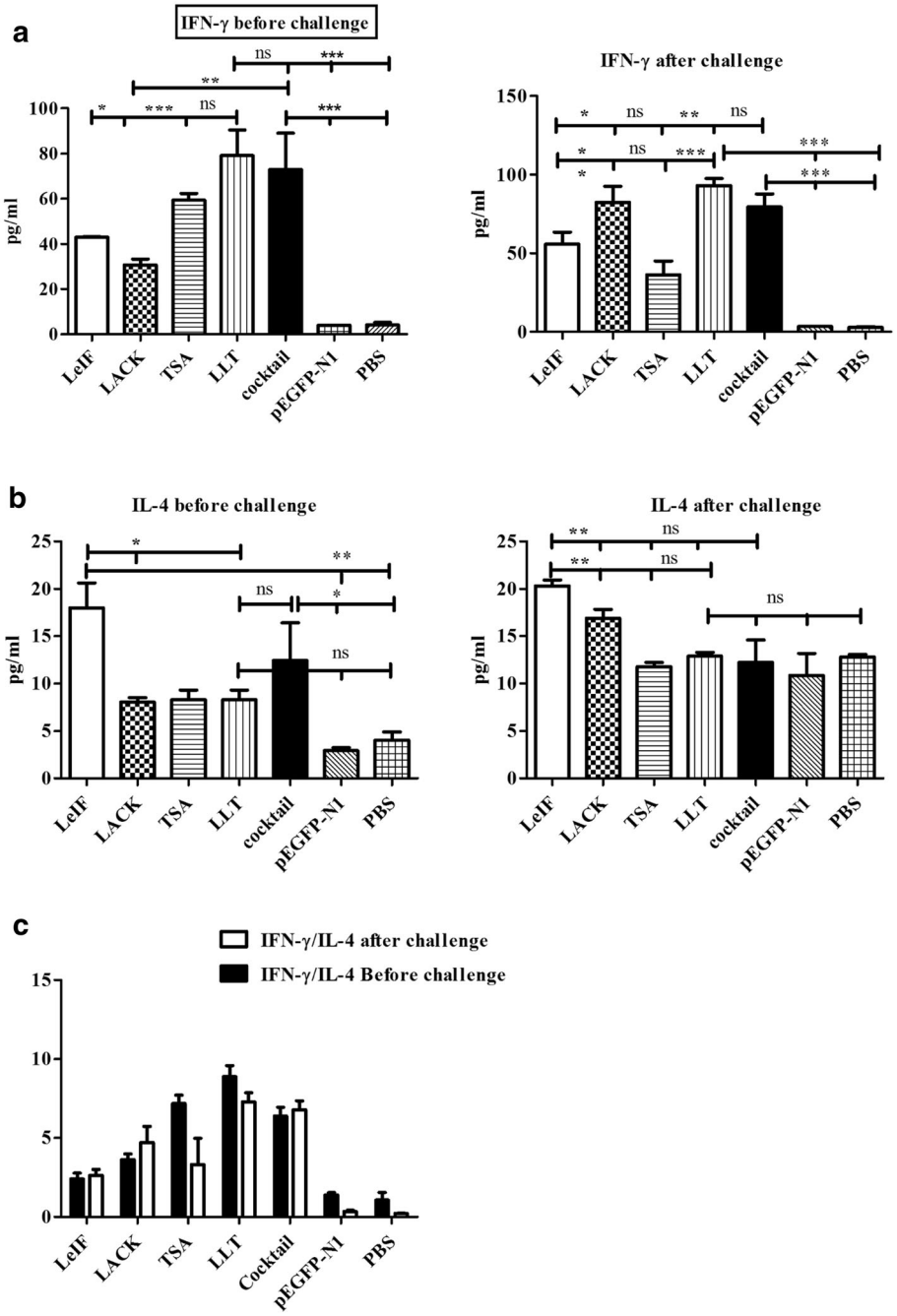

Fig. 4 Mean ± SD of produced cytokines in vaccinated and control mice before (**b**) and after (**a**) challenge. Mice (five mice per group) were immunized three times intramuscularly (at 3-week intervals) with 100 µg of LeIF, LACK, TSA, and LLT fusion or cocktail. The pEGFP-N1 and PBS groups were as control groups. Three weeks after the last immunization, the animals were killed, and their splenocytes were obtained and cultured in the presence of SLA. After 72 h, the supernatants were harvested and assayed for IFN-γ and IL-4 levels. **a** IFN-γ before and after challenge. **b** IL-4 before and after challenge. **c** IFN-γ/IL-4 ratio before and after challenge. The *asterisk* indicates the significant difference between cytokine values as determined by one-way-ANOVA ($p < 0.05$ denoted as *asterisk*, $p < 0.01$ denoted as *double asterisk*, $p < 0.001$ denoted as *triple asterisk* and *ns* denoted as non significant). All tests were done for each mouse in triplicates

immune response and protection against *L. major* infection in comparison with each antigen alone (Ahmed et al. 2009). In addition, in another study, immunized dogs with viruses expressing a cocktail of LACK, TSA, and LmSTI1 genes or with each gene alone showed a partial immunity against CL when compared with unimmunized animals. However, immunized animals with cocktail DNA showed stronger immune responses by more effective suppression of the progression of nodule formation than immunized dogs with a single antigen (Miura et al. 2015). In addition, Th1 responses increased following DNA vaccination with a fusion construct of the TSA and LmSTI1 genes against *L. major* infection characterized by production of higher IFN-γ and lower IL-4 levels compared with each gene alone (Campos-Neto et al. 2002). In another study, immunization with a polyprotein vaccine (leish-111f) containing LeIF, LmsTI1 and TSA antigens induced protection against leishmaniasis by the development of Th1 responses characterizing by increased levels of IFN-γ, IL-2 and TNF-α cytokines (Coler et al. 2002, 2007) and also significant reduction of lesion size and parasite load in mice and non-human primates (Coler et al. 2002).

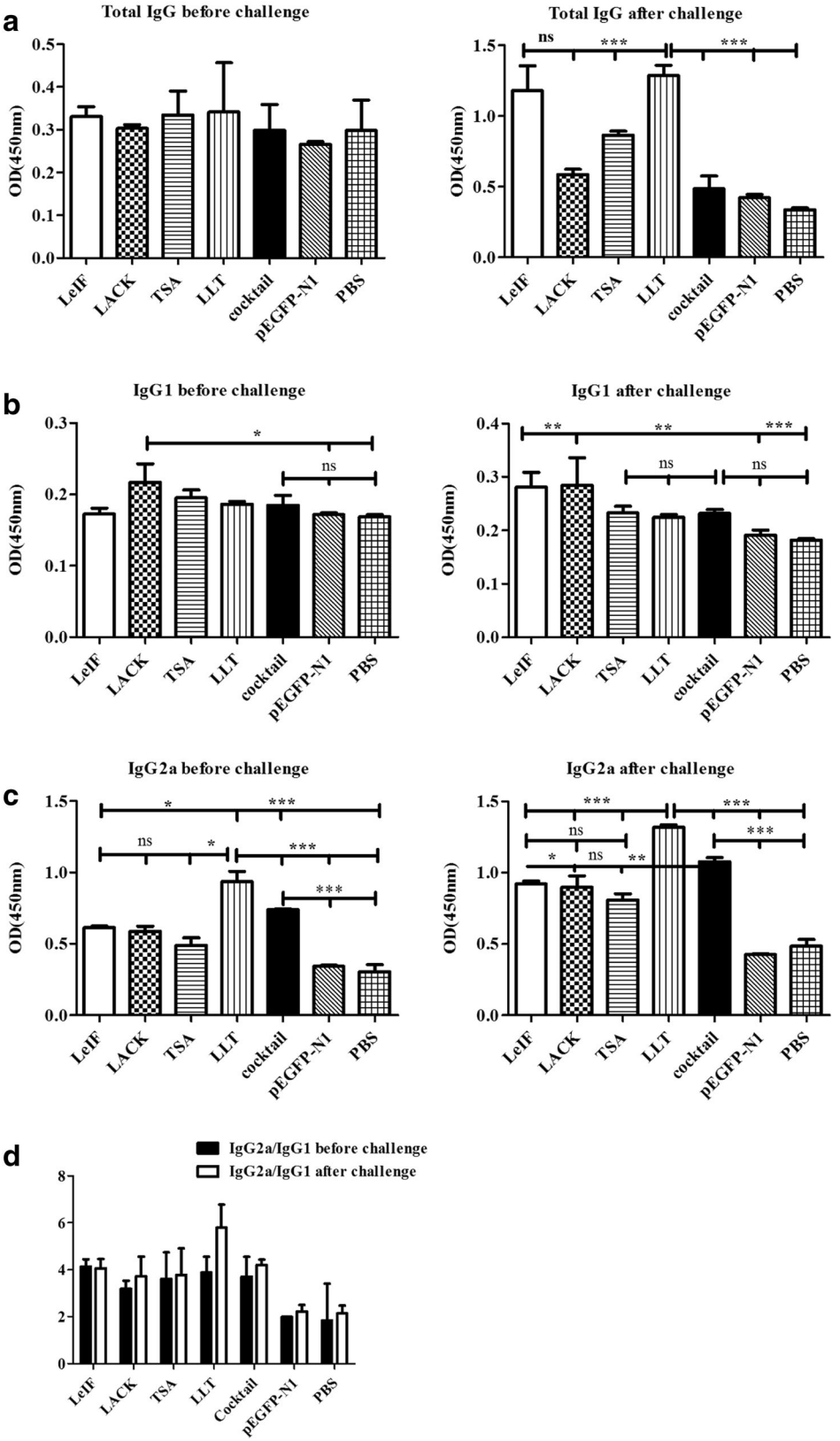

Fig. 5 Mean ± SD of produced antibodies in immunized and unimmunized mice before and after challenge. The optical density (OD) of IgG1 and IgG2a for pooled sera (1:100 dilution) was measured by ELISA at 450 nm. **a** Total IgG levels before and after challenge. **b** IgG1 levels before and after challenge. **c** IgG2a levels before and after challenge. **d** IgG2a/IgG1 ratio before and after challenge. The *asterisk* indicates the significant difference between antibody values as determined by one-way-ANOVA ($p < 0.05$ denoted as *asterisk*, $p < 0.01$ denoted as *double asterisk*, $p < 0.001$ denoted as *triple asterisk* and *ns* denoted as non significant). All tests were done in triplicates

In summary, our results indicated that DNA vaccination with cocktail or fusion of LeIF, LACK and TSA genes can elicit stronger immune responses which decelerated the disease development and delayed progression of lesion size compared with a single antigen. The present study suggests that a combination of DNA-based vaccines expressing different antigens with diverse effects on immune systems may be utilized as a polyvalent vaccine to induce stronger immune responses against CL.

Acknowledgements This research was financially supported by Faculty of Medical Sciences, Tarbiat Modares University, and Tehran, Iran.

Compliance with Ethical Standards

Conflict of interest Authors declare that they have no conflict of interest.

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

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