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Evaluation of Transgenic Iranian Lizard *Leishmania* Encoding Human IL-12 as a Candidate Vaccine Against Experimental Cutaneous Leishmaniasis in Susceptible BALB/c Mice

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ABSTRACT

Introduction: Leishmaniasis is a neglected tropical disease that causes significant morbidity worldwide, particularly in developing countries. Despite ongoing efforts, there is no widely approved human vaccine against this disease. Live, non-pathogenic organisms such as *Leishmania tarentolae* have emerged as promising candidates for vaccine development due to their safety profile and ability to present antigens in a native context. This study aimed to evaluate transgenic Iranian Lizard *Leishmania* (I.L.L.) encoding human IL-12 (hIL-12) as a candidate vaccine against experimental cutaneous leishmaniasis in susceptible BALB/c mice. **Methods:** BALB/c mice were divided into six groups and immunized subcutaneously with either transgenic *L. tarentolae*-hIL-12 (G1 and G2), wild-type *L. tarentolae* (G3 and G4), or RPMI-1640 medium (G5 and G6). Three weeks post-immunization, animals received a booster injection. Two weeks after the booster, challenged groups (G1, G3, and G5) were infected with *Leishmania major* promastigotes. Lesion progression was measured weekly for 11 weeks. IgG2a responses were assessed using ELISA, while cytokine responses (IFN-γ, IL-17, IL-10, TNF-α) were quantified after stimulation with soluble *L. major* antigen (SLA). Parasite burden in spleens was evaluated by real-time PCR. **Results:** Mice vaccinated with transgenic I.L.L.-hIL-12 developed significantly stronger cellular and humoral immune responses than the control groups. Elevated IFN-γ, IL-17 and IgG2a levels indicated a predominantly Th1-biased immune response, while increased IL-10 production suggested a concomitant regulatory response. Vaccinated mice also exhibited smaller lesion sizes and lower splenic parasite burdens than the control groups, indicating partial protection against *L. major* infection. **Conclusion:** The transgenic *L. tarentolae*-hIL-12 strain effectively induced protective immunity in susceptible BALB/c mice, supporting its potential as a novel and safe live vaccine platform for cutaneous leishmaniasis due to *L. major*.

INTRODUCTION

Leishmaniasis caused by intracellular protozoan parasites of the genus *Leishmania* ranges from self-limited cutaneous leishmaniasis to fatal visceral leishmaniasis (VL) [1]. Leishmaniasis affects over 1 million people annually, particularly in low- and middle-income countries. Furthermore, HIV-*Leishmania* coinfection is a concern as an emerging health problem in endemic regions [2]. While there is no widely approved human vaccine, several veterinary vaccines and experimental candidates have shown promise [3-5]. Surveillance and control of leishmaniasis generally depend on drugs and reducing contact with sand flies using personal protective equipment.

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Current therapeutic options are often expensive, toxic, and can lead to drug resistance [3]. Vaccines are powerful tools, but other factors such as sanitation, vector control, and effective treatments play significant roles in disease elimination, as well [6]. Vaccines can be prepared from inactivated or genetically modified organisms [7]. The use of live attenuated vaccines carries biosafety concerns, including virulence reversion, manufacturing yield issues, and cold-chain requirements [8]. A new approach has been pioneered by Haghdoust *et al.* using a non-pathogenic strain, Iranian Lizard *Leishmania* (I.L.L., also known as *Leishmania tarentolae*), as an experimental vaccine against *L. major* [9]. *L. tarentolae* is capable of entering mammalian macrophages and transiently differentiating into amastigote-like forms, thereby mimicking aspects of the intracellular life cycle of pathogenic *Leishmania* species; however, it does not replicate efficiently and is rapidly cleared, preventing the establishment of disease [9,10]. Since *Leishmania* is an intracellular parasite, an active cellular immune response is necessary to control the disease [11]. Th1 cells support cell-mediated immunity and help in the clearance of intracellular pathogens. The balance between IL-12 and Th1 activation determines the early immune response intensity [12–14]. IL-12 is a cytokine produced by dendritic cells (DCs) [15,16], macrophages, neutrophils, and human B lymphocytes in response to antigenic stimulation. Moreover, *L. tarentolae* activates DC maturation, induces T-cell proliferation, and promotes production of IFN-γ [9]. In a previous study, we produced a transgenic I.L.L.-hIL-12 that secreted hIL-12 [17]. We hypothesized that I.L.L. expressing hIL-12 would enhance Th1 responses and confer protection against *L. major* infection in BALB/c mice. In this study, BALB/c mice were vaccinated with I.L.L.-hIL-12 using a prime-boost regimen. The immunogenicity of the vaccine and protection against *L. major* infection were then evaluated.

MATERIAL AND METHODS

Parasites. The wild-type Iranian Lizard *Leishmania* strain [18], transgenic I.L.L.-hIL-12 [17], and *L. major* (MRHO/IR/75/ER) promastigotes were grown in RPMI-1640 medium (Sigma, Germany) supplemented with 7% heat-inactivated fetal bovine serum (FBS) (Sigma, Germany) (pH 7.2), 100 U/mL penicillin, and 100 µg/mL streptomycin (Jaber Ebne Hayan, Iran), and cultured at 26°C in a shaking incubator (110 rpm; DAIKI Scientific, Japan). *L. major* promastigotes were used to infect mice.

Preparation of soluble *L. major* antigen (SLA). Thirty millilitres of stationary-phase promastigotes (4–6 days old) were collected in a 50-mL tube and centrifuged at 2,000 × *g* for 15 min at 4°C. The resulting pellet was washed three times with cold 0.02 M phosphate-buffered saline (PBS; pH 7.2) and subjected to ten cycles of freezing (at −80°C) and thawing (at 37°C), then centrifuged at 2,000 × *g* for 20 min [19]. The supernatant was collected, and protein concentration was assayed using the Bradford method [20]. Protein samples were stored at −80°C for further use.

Animals. Four-week-old female BALB/c mice were obtained from Royan Research Institute (Tehran, Iran). The animals were divided into six groups, each comprising 6 mice (G1–G6). Animals were kept in pathogen-free conditions at 20°C, with a 12 h light/12 h dark cycle, and in a humidity-controlled environment. All invasive procedures were performed in accordance with ethical and biosafety guidelines approved by the Shahid Beheshti University of Medical Sciences Ethics Committee.

Vaccine administration and experimental infection. All groups were immunized subcutaneously (SC) at the base of the tail [21]. Groups G1 and G2 were immunized with 3 × 10^6 transgenic I.L.L.-hIL-12 promastigotes in 150 µL of RPMI-1640 (test groups). Groups G3 and G4 were immunized with 3 × 10^6 wild-type I.L.L. promastigotes in 150 µL of RPMI-1640 (positive controls). Groups G5 and G6 were injected with 150 µL of RPMI-1640 alone (negative controls). Three weeks after the primary injection, all mice received a booster. Two weeks after the booster, groups G1, G3, and G5 were infected (/ challenged) with *L. major* by SC inoculation with 2 × 10^5 stationary-phase *L. major* promastigotes [22, 23] in a volume of 30 µL of RPMI-1640. Groups G2, G4, and G6 were left unchallenged. For pre-challenge evaluation, blood samples (from retro-orbital plexuses [24]) were collected from groups G2, G4, and G6 two weeks following the booster injection. For post-challenge assessment, blood samples were obtained from groups G1, G3, and G5 eleven weeks after challenge (day 112). Following blood collection, all mice were sacrificed for the assessment of cellular immune responses and splenic parasite burden. Splenocytes were isolated and stimulated with SLA for further analysis. To assess clinical changes in challenged groups, lesion size was measured using a Vernier caliper weekly for 11 weeks.

Evaluation of humoral immune response. IgG2a levels were measured to assess the humoral immune response. The mouse IgG2a Ready-SET-Go ELISA kit (eBioscience, USA) was used to measure IgG2a according to the manufacturer's instructions.

Evaluation of cellular immune response. Splenocytes were aseptically isolated from spleens and separated using Ficoll (Histopaque 1077; Sigma, USA) and centrifugation at 2,000 × *g* for 30 min at 4°C. The mononuclear cells were washed twice in DMEM (Sigma, France) [25, 26] and then counted using a Neubauer chamber. The separated mononuclear cells were suspended in DMEM supplemented with 20% (*v/v*) heat-inactivated FBS. Cell suspension (3.5 × 10^6/mL) was plated in duplicate in 48-well culture plates and incubated for 96 h in the presence of 10 µg/mL of SLA (prepared as described above). LPS (50 ng/mL)-treated and untreated media were used as positive and negative controls, respectively [27]. Culture supernatants were collected to assess levels of IL-10 and TNF-α after 24 h, IL-17 after 72 h, and IFN-γ after 96 h. All supernatants were stored at −80°C. Assays were performed by sandwich ELISA (DuoSet ELISA Mouse IFN-γ, TNF-α, IL-10, and IL-17; R&D Systems, USA) according to the manufacturer's instructions.

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Determination of parasite burden. To detect and quantify *L. major* burden in mouse spleen tissue, real-time PCR was performed to quantify *L. major* kinetoplast DNA (kDNA) [28].

Cloning of *L. major* kDNA into pTG-19T vector. A fragment of the conserved region of *L. major* minicircle kDNA (120 bp) was amplified [28, 29] using JW11 as the forward primer (5′-CCTATTTTACACCAACCCCCAGT-3′) and JW12 as the reverse primer (5′-GGGTAGGGGCGTTCTGCGAAA-3′). *L. major* promastigote genomic DNA (20 ng) was mixed with 5 µL of Master Mix (Ampliqon, Denmark) and 40 pmol/µL primers in a 30 µL final volume. After initial denaturation (4 min at 94°C), 30 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 61°C, and elongation for 30 s at 72°C were carried out, and the cycling was terminated by a final extension at 72°C for 10 min. Negative controls showed no amplification, confirming the absence of contamination. Following electrophoresis, PCR products were visualized on a 2% agarose gel, purified with a PCR purification kit (Qiagen, Germany), and inserted into the pTG-19T plasmid. The resulting construct was transformed into *E. coli* Top10 cells according to a standard protocol [30]. The desired recombinant clone was selected by colony PCR using M13 forward and reverse primers. The PCR product was sequenced using M13 forward and reverse primers. The recombinant plasmid was named pTG-19T-kDNA.

Preparation of recombinant pTG-19T-kDNA serial dilution. In order to draw a standard logarithmic concentration curve of *L. major* kDNA, recombinant pTG-19T-kDNA was purified using a plasmid purification miniprep kit (Bioneer, Korea), and its concentration was determined using a spectrophotometer. The copy number of the recombinant plasmid in a volume of 1 µL was determined using the following formula [31]:

$$\text{DNA copies}/\mu\text{L} = \frac{C_{DNA} \times N_A}{l \times ng \times w_{bp}}$$

C_{DNA} = DNA concentration (ng/µL)
N_A = Avogadro's constant (6.022×10^{23})
l = Length of the template (bp)
ng = Conversion factor (nanogram)

w_{bp} = Average molecular weight of a base pair (660 Da)

Ten-fold serial dilutions of the recombinant plasmid (from 10^8 copies/µL) were prepared using nuclease-free water (Sigma, USA) according to the $C_1V_1 = C_2V_2$ equation [32].

Mouse tissue DNA extraction. Spleen tissue (10 mg) was sampled from infected or uninfected BALB/c mice and mechanically homogenized. Tissue DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

Real-time PCR. The *Leishmania* burden in BALB/c mice was evaluated by quantitative real-time PCR. Serial dilutions of the pTG-19T-kDNA plasmid, ranging from 10^3 to 10^7 copies/µL, were used to generate a standard curve. Each reaction for the standard curve contained 1 µL of the appropriate dilution of the plasmid standard, 10 µL of SYBR Green Master Mix 2x (TAKARA, Japan), 2 µL of primers (JW11 and JW12, 40 pmol/µL each) targeting a 120 bp fragment of the kDNA of *L. major*, and nuclease-free water to a final volume of 20 µL. Each reaction was performed in duplicate.

To assess the parasite burden in the spleens of the experimental mice, real-time PCR was performed as follows: 0.1 µL of extracted splenic DNA (approximately 75 ng) was combined with 10 µL of SYBR Green Master Mix 2x, 2 µL of specific primers targeting the same 120 bp kDNA fragment (JW11 and JW12), and nuclease-free water to a final volume of 20 µL. All reactions were performed using the LightCycler system (Roche, Germany). The reaction conditions were optimized to ensure efficient amplification while minimizing background signal. The standard curve for parasite quantification exhibited linearity across a DNA concentration range of 10^3 to 10^7 copies, with a correlation coefficient of 0.97.

Statistical analysis. Statistical analyses were performed using IBM SPSS Statistics version 18.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD). Depending on the distribution of the data, appropriate parametric or non-parametric statistical tests were applied. Differences among groups were evaluated using one-way analysis of variance (ANOVA) or the Kruskal–Wallis test, as appropriate. When one-way ANOVA showed a significant difference, pairwise comparisons were performed using the least significant difference (LSD) post hoc test. Analysis of covariance (ANCOVA) was used to compare post-challenge cytokines (IFN-γ, TNF-α, IL-10, and IL-17) and IgG2a levels among groups while adjusting for the corresponding pre-challenge values as covariates. Effect sizes were expressed as partial eta squared (ηp^2). A two-sided *P* value < 0.05 was considered statistically significant.

RESULTS

Vaccination and clinical follow-up. To assess the ability of transgenic I.L.L.-hIL-12 to protect BALB/c mice against *L. major*, 36 inbred mice were divided into six groups (G1–G6). The G1 and G2 groups were vaccinated subcutaneously (SC) with two doses of transgenic I.L.L.-hIL-12 (test vaccine), the G3 and G4 groups received two doses of wild-type I.L.L. (positive controls), and G5 and G6 received two doses of RPMI-1640 (negative controls). The G1, G3, and G5 groups were designated as post-challenge (received *L. major* promastigotes) and G2, G4, and G6 as pre-challenge (received no *L. major* promastigotes). Groups G1, G3, and G5 were followed for 11 weeks to evaluate clinical symptoms of leishmaniasis as well as humoral immune responses. Transgenic I.L.L.-hIL-12 was well tolerated, and there were no reactions at the site of injection.

For the analysis of the specific humoral response elicited in vaccinated and control groups, serum from all mice was obtained at different time points throughout the experiments (T0: pre-challenge at day 35; T1: 11 weeks after challenge at

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day 112). IgG2a production was determined by ELISA. The mean IgG2a levels in G5, G3, and G1 (post-challenge) were 201.24 ng/mL, 426.97 ng/mL, and 336.73 ng/mL, respectively. ANCOVA was used to assess the effect of I.L.L.-hIL-12 vaccination on post-challenge IgG2a levels, using pre-challenge levels as covariates. The results are presented in Table 1. Levene's test and normality checks were carried out, and the assumptions were met by the one-sample Kolmogorov–Smirnov test. There was a significant difference in mean IgG2a [F (2, 14) = 13.503, P = 0.001] between the groups. LSD post hoc tests showed that there was a significant difference between G1 and G5 (P = 0.008) but not between G1 and G3 (P = 0.069).

Table 1. Summary of the ANCOVA results of the effects of transgenic I.L.L.-hIL-12 on IgG2a (ng/mL) levels in sera.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	155077.910[a]	3	51692.637	9.479	.001	.670
Covariate (IgG2a)	163.734	1	163.734	.030	.865	.002
Group	147273.440	2	73636.720	13.503	.001	.659
Error	76349.725	14	5453.552			
Total	2093626.743	18				

[a] R^2 = .670.

Pre-challenge IgG2a levels showed no significant difference between groups (P = 0.865). The partial eta squared (ηp^2 = 0.659) indicated a very large effect size according to Cohen's benchmarks, suggesting that the treatment explained a substantial proportion of the variance in post-challenge IgG2a levels.

Assessment of IFN-γ, TNF-α, IL-10, and IL-17 levels in splenocytes. IFN-γ, TNF-α, IL-10, and IL-17 levels were measured in culture supernatants stimulated with SLA at different time points in pre- and post-challenge groups. The results are presented in Table 2. Levene's test and normality checks were carried out, and the assumptions were met by the one-sample Kolmogorov–Smirnov test. There was a significant difference in mean IFN-γ between groups [F (2, 14) = 38.184, P < 0.0001]. LSD post hoc tests showed a significant difference in IFN-γ levels between G1 and G5, and between G1 and G3 (P < 0.0001). There was a significant difference in TNF-α levels between groups [F (2, 14) = 210.067, P < 0.0001]. LSD tests showed a significant difference in TNF-α between G1 and G5, and between G1 and G3 (P < 0.0001). There was a significant difference in IL-10 levels between groups [F (2, 14) = 998.680, P < 0.0001]. LSD tests showed a significant difference in IL-10 between G1 and G5, and between G1 and G3 (P < 0.0001). There was a significant difference in IL-17 production between groups [F (2, 14) = 6.191, P = 0.012]. LSD tests showed a significant difference in IL-17 between G1 and G5, and between G1 and G3 (P = 0.004).

The pre-challenge comparison showed no significant difference in mean IFN-γ, TNF-α, IL-10, and IL-17 levels (pg/mL) between groups (P = 0.906, 0.700, 0.373, and 0.322, respectively). The partial eta squared (ηp^2) values indicated large effect sizes for the treatment effects. According to Cohen's benchmarks for partial eta squared (0.01 = small, 0.06 = medium, and 0.14 = large), all observed effect sizes were in the large range, indicating that vaccination with transgenic I.L.L.-hIL-12 accounted for a substantial proportion of the variance in cytokine responses.

Development of cutaneous leishmaniasis in BALB/c mice. Two weeks after challenge (inoculation with *L. major* promastigotes), nodules developed at the base of the tail in all challenged groups (G1, G3, and G5). Two weeks later (after four weeks of the study), the nodules transformed into ulcers. Lesion diameter was measured weekly for 11 subsequent weeks. In all groups, the diameter of the lesions increased over time, as shown in Figure 1. During the 11 weeks of lesion evaluation, lesions were monitored and differentiated among groups. Lesions in group G1 did not expand as much as those in the other groups (G3 and G5). At week 11, mean lesion sizes were 0.69 cm² (G1), 1.06 cm² (G3), and 1.89 cm² (G5). A one-way ANOVA was conducted to compare mean lesion sizes among the three challenged groups. The results are shown in Table 3. There was a significant difference in mean lesion size between the groups [F (2, 15) = 46.387, P < 0.0001]. LSD post hoc tests showed a significant difference in lesion size between G1 and G5, and between G1 and G3 (P < 0.0001).

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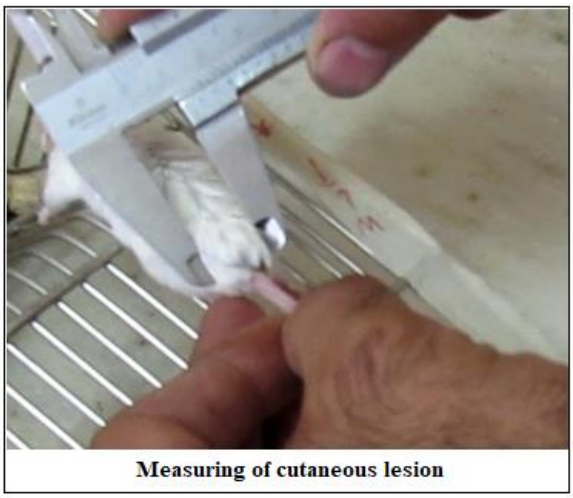
Table 2. Summary of the ANCOVA results of the effects of transgenic I.L.L.-hIL-12 on IFN-γ, TNF-α, IL-10, and IL-17 levels (pg/mL) in supernatants of splenocytes at 10 µg/mL SLA exposure

Source	Type III sum of squares	Df	Mean square	F	Sig.	Partial eta squared
IFN-γ (pg/mL)						
Corrected Model	1.954E6[a]	3	651419.700	43.926	.000	.904
Covariate (IFN-γ before)	214.408	1	214.408	.014	.906	.001
Group	1132539.042	2	566269.521	38.184	.000	.845
Error	207620.118	14	14830.008			
Total	3651365.430	18				
TNF-α (pg/mL)						
Corrected Model	1.422E6[a]	3	473949.635	279.191	.000	.984
Covariate (TNF-α before)	262.862	1	262.862	.155	.700	.011
Group	713212.172	2	356606.086	210.067	.000	.968
Error	23766.157	14	1697.583			
Total	2392381.038	18				
IL-10 (pg/mL)						
Corrected Model	2.050E6[a]	3	683431.624	669.263	.000	.993
Covariate (IL-10 before)	863.161	1	863.161	.845	.373	.057
Group	2039643.923	2	1019821.961	998.680	.000	.993
Error	14296.382	14	1021.170			
Total	3803939.997	18				
IL-17 (pg/mL)						
Corrected Model	3.455E6[a]	3	1151539.682	100.963	.000	.956
Covariate (IL-17 before)	12028.106	1	12028.106	1.055	.322	.070
Group	141214.735	2	70607.367	6.191	.012	.469
Error	159678.522	14	11405.609			
Total	5479173.726	18				

[a] R^2 values: IFN-γ = .904, TNF-α = .984, IL-10 = .993, IL-17 = .956.



Measuring of cutaneous lesion

Fig. 1. Weekly changes in lesion diameter over the 11-week observation period. Lesion diameter increased progressively in all groups throughout the study period.

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Table 3. Summary of the ANOVA results of the effects of transgenic I.L.L.-hIL-12 injection on lesion size (cm²).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.810	2	.405	46.387	.000
Within Groups	.131	15	.009		
Total	.941	17			

The post-challenge comparison showed a significant difference between groups (P = 0.0001). A descriptive table (Table 4) is presented to show the mean and standard deviation for each group.

Table 4. Mean and standard deviation of lesion size (cm²) for each group at week 11.

Group	N	Mean	Std. deviation	Std. error
G5 (RPMI-1640)	6	1.898	0.10169	0.04125
G3 (Wild-type I.L.L.)	6	1.0682	0.10233	0.04178
G1 (Transgenic I.L.L.-hIL-12)	6	0.6915	0.07336	0.02995
Total	18	0.9832	0.23527	0.05545

Determination of splenic parasite load in post-challenge groups (G1, G3, and G5). The number of parasites was determined by real-time PCR in spleen samples 11 weeks post-challenge. Results were compared with a standard curve constructed from ten-fold dilutions of pTG-19T-kDNA. Data are represented as the number of parasites per µg of extracted spleen DNA, with the corresponding results visualized in Figure 2. The standard curve, calculated from the experiment, was linear over at least a five-log range of DNA concentrations, with a correlation coefficient of 0.97 (Figure 2).

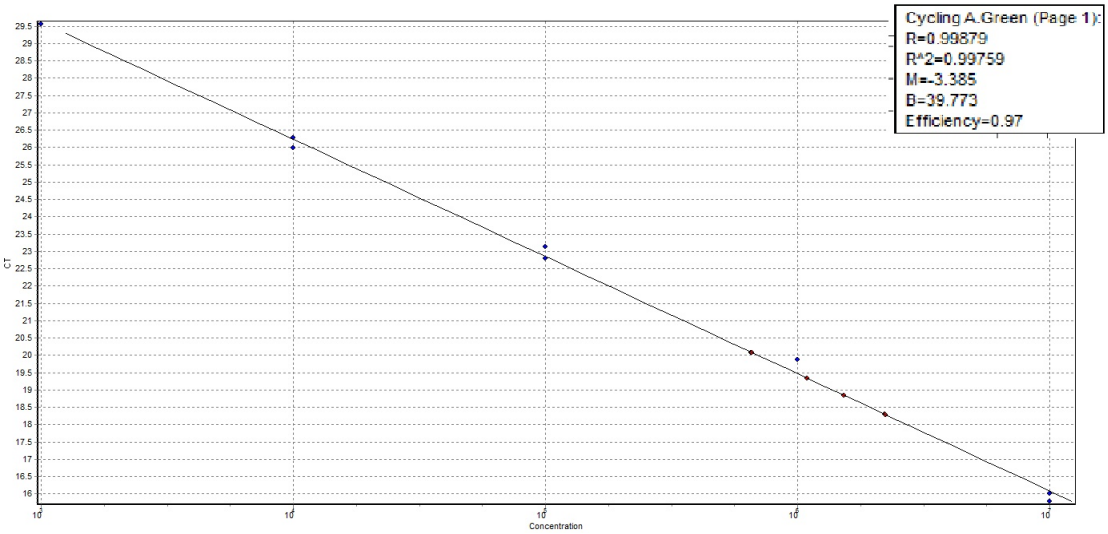

Fig. 2. Sensitivity of the real-time PCR assay with ten-fold dilutions of pTG-19T-kDNA. A plot of mean Ct values versus the logarithmic concentration of parasite kDNA (ranging from 10^3 to 10^7 copies per reaction) is shown. Data are presented as mean ± 1 SD.

DISCUSSION

In this study, we vaccinated BALB/c mice with a live transgenic vaccine against cutaneous leishmaniasis using the non-pathogenic protozoan parasite, transgenic I.L.L.-hIL-12, expressing hIL-12, and tested its immunogenicity and protective potential against *L. major* infection.

Haghdoust *et al.*, in a recent study, showed the effects of recombinant I.L.L. as a live non-pathogenic vaccine with chitin microparticles against pathogenic *Leishmania* strains [9]. The IL-12 produced by DCs plays a vital role in orchestrating the initial immune response against *Leishmania* [33]. The production of IL-12 is critical during the early phase of cutaneous leishmaniasis. It should be noted that DCs from the highly susceptible BALB/c mice, which are generally Th2-biased, produce lower amounts of IL-12 under certain stimuli, and their T cells respond poorly to this cytokine, partially due to reduced expression of the IL-12Rβ chain [34–39]. Thus, IL-12 is an efficient adjuvant for cell-mediated immunity against leishmaniasis [40]. Dinc *et al.* also showed that the *L. tarentolae*–DC interaction activates DC maturation and induces T-cell proliferation and IFN-γ production, thus skewing $CD4^+$ T cells toward a Th1 cell phenotype and acting as an immunostimulatory adjuvant [7].

In this study, we evaluated both cellular and humoral immunity post-vaccination against *L. major* using I.L.L.-hIL-12 as an adjuvant. We confirmed that vaccination with transgenic I.L.L.-hIL-12 induced an antibody response against *L. major*. These antibodies may reduce parasitic load by facilitating macrophage-mediated

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phagocytosis [41]. Although IgG2a levels increased after infection across all groups, data presented in Table 1 indicated that the G5 group exhibited lower IgG2a levels than the G1 and G3 groups. This discrepancy might reflect biological variation or experimental conditions, and further validation is necessary to clarify these observations in future studies.

There are several studies demonstrating the association between high IgG2a production and asymptomatic infections [42]. Our data indicated that the levels of IFN-γ and TNF-α increased after infection in the vaccinated group (G1) compared with the RPMI-1640 group (G5). This suggests that transgenic I.L.L.-hIL-12 directs a Th1 response and may contribute to the elimination of *L. major* through macrophage activation. These results are in conformity with previous studies that demonstrated nitric oxide-mediated activation of macrophages by IFN-γ and TNF-α and the subsequent intracellular killing of amastigotes [43]. Our results showed that the levels of IL-10 increased significantly in G1 compared with G3 and G5 ($P < 0.05$). Elevated levels of IL-10 may suggest a regulatory mechanism to prevent immunopathology, consistent with IL-12-induced immune modulation. These findings suggest that transgenic I.L.L.-hIL-12 has the potential to serve as a vaccine against *L. major*.

Furthermore, our data indicated that the levels of IL-17 increased significantly in G1 compared with G3 and G5 ($P < 0.05$). The pro-inflammatory cytokine IL-17 plays an important role in the clearance of intracellular pathogens [44]. Additionally, lesion size decreased post-challenge in the G1 group compared with the G3 and G5 groups ($P < 0.05$). Furthermore, the results indicated that the parasite burden in the spleen of G1 mice was significantly lower than in G3 and G5.

Despite these encouraging findings, several limitations of this study should be acknowledged. First, the protective efficacy of the vaccine was evaluated exclusively in the highly susceptible BALB/c mouse model, which may not fully reflect the complexity of human immune responses or those of genetically resistant hosts. Second, the study focused on immune responses shortly after challenge and did not assess the development or persistence of long-term immunological memory. Future studies should investigate memory T-cell and B-cell responses to determine the durability of vaccine-induced protection. Third, the use of hIL-12 in a murine model warrants further investigation to confirm cross-species bioactivity and to evaluate a murine IL-12 construct for translational relevance. Finally, although vaccination significantly reduced lesion progression and parasite burden, sterile immunity was not achieved, as parasites remained detectable in vaccinated animals. Therefore, the protective effect observed in this study should be interpreted as partial rather than complete protection.

In conclusion, this preclinical study demonstrates a potential protective effect of transgenic I.L.L.-hIL-12 against *L. major*. For the future of this vaccine candidate, we propose larger animal studies for greater statistical power and**,** perhaps**,** early-phase clinical investigation to establish the efficacy of transgenic I.L.L.-hIL-12.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests associated with this manuscript.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI DISCLOSURE

The authors confirm that no generative artificial intelligence or AI-assisted technologies were used in the preparation, drafting, or revision of this manuscript.

AUTHORS' CONTRIBUTIONS

TD conducted the laboratory work. RC prepared the manuscript draft. MB served as the laboratory manager. SMY measured parasite burden and analyzed real-time PCR results. HZZ performed animal handling and blood collection. MN performed the statistical analysis. BK proposed and managed the project. All authors have read and approved the final version of the submitted manuscript.

ETHICS STATEMENT

All procedures, including maintenance, animal handling, and blood sample collection, were approved by the Ethics Committee (IR.SBMU.RETECH.REC.1398.092) based on the National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy, Ministry of Health and Medical Education (MOHME), Iran (2005).

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