Archives of Razi Institute, Vol. 77, No. 6 (2022) 2223-2233

DOI: 10.22092/ARI.2022.358499.2238

Original Article

Evaluation of a Novel Multi-Epitope Peptide Vaccine Candidate from LACK, LeIF, GP63, SMT Antigens of *Leishmania major* in BALB/c Mice

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Received 1 May 2022; Accepted 29 May 2022
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Abstract

Leishmaniasis is one of the major health problems in most countries of the world. Millions of people around the world are at risk for the disease. Given the prevalence of this parasite in Iran and developing countries and the emergence of resistance in some cases to existing drugs, developing an effective vaccine against leishmaniasis is necessary. This research aims to design a multi-epitope vaccine derived from LACK, LeIF, GP63, and SMT antigens of *Leishmania major* based on the combination of bioinformatics methods. The synthesized construct with 16.86 KDa was cloned and sub-cloned in pEGFP- N1 and pLEXSY-neo2, respectively. They were then transfected in promastigotes of *L. tarentolae*. After confirmation of expression, immunization was carried out in 8 groups of BALB/c mice (9 mice per group) three times at two-week intervals. Cellular immune responses were assessed before and after the challenge by *L. major*. Furthermore, at 3rd week post-infection, the survival rate, mean lesion size, and parasite burden were assessed. All vaccinated mice demonstrated partial immunity to higher IFN-γ levels than the control groups (P<0.05). Immunized mice with cytosolic complex (G1) indicated the highest levels of IFN-ɤ and ratio of IFN ɤ/ IL-4, the lowest levels of IL-4 and IL-10 compared to control and the other groups (P≤0.05), and produced a partial Th1 immune response. Mean lesion size and parasite burden of G1 and G5 reduced significantly compared to the other and control groups post-challenge (P<0.05). The outputs of our result could be a hopeful sign in the achievement of practical approaches as multi-epitope vaccines against *Leishmania major*.

Keywords: *Leishmania major*, multi-epitope, vaccine

1. Introduction

Leishmaniasis is one of the major health concerns in most countries. Due to its importance, the World Health Organization has ranked leishmaniasis in the top 10 critical diseases in tropical and subtropical regions (1). Existing drugs for leishmaniasis require repeated injections and have limited effectiveness; drug resistance is observed in some cases (2, 3). It is also well established that recovery from leishmaniasis or a lesion caused by the disease is associated with a robust immune response and long-term protection (4). Hence, it seems feasible to produce an effective vaccine against leishmaniasis. An effective vaccine should prevent severe complications of parasitic infection and reduce economic losses. Vaccines were evaluated to control leishmaniasis, including killed or inactivated, live, attenuated subunit, DNA, and recombinant polypeptide (5). Various *leishmania* proteins are identified based on cellular localization and its frequency, T-cell clones, antigen reserves, screening of antigen reserves, and serologic screening of infected animals and humans, such as LACK, CPB, CPA, TSA,

LMST1, LeIF, KMP11, LDI, Gp63, H1 and SMT (6). Induction of long-term protection against leishmaniasis requires the production of memory T-cells, possibly both CD4 and CD8 (7-9). The constant presence of antigens maintains these responses through the live parasite activity or DNA vaccine (10, 11). Vaccination with a strain of a single antigen induces protective immune responses to the same species of *Leishmania* (12). Therefore, a multi-antigen vaccine, rather than a single antigen, can induce protective immune responses against leishmaniasis in many individuals. Hence, using two or more specific antigens is logical to design an effective vaccine against parasitic infections (12, 13).

LeIF is expressed in both promastigote and amastigote forms of all *Leishmania* species. LeIF is a natural adjuvant for Th1 and, as an antigen, induces the production of Th1 protective cytokines, such as IL-12 and IFN-γ, in the peripheral blood mononuclear cells of patients with leishmaniasis. It is also the only *Leishmania* protein capable of inducing the Th1 response in infected cells of patients with leishmaniasis through an IL-12-dependent mechanism (14). LACK antigen is expressed in both promastigote and amastigote forms of the parasite. It triggers the differentiation of promastigotes from amastigotes in infected macrophages, leading to better growth and survival of parasites inside macrophages and keeping amastigotes away from the host immune system (15). GP63, or leishmanolysin, is a zinc metalloprotease, inducing the production of IFN-γ mostly in cutaneous leishmaniasis (6). SMT (sterol 24-c-methyltransferase) is used in the biosynthesis of ergosterol in trypanosomatidae, fungi, and organic plants. In studies, the combination of SMT with MPL-SE (monophosphoryl lipid A) oriented Th1 immune responses to the production of IFN-ɤ and IL-2. It significantly reduced the parasitic load on the liver and spleen of vaccinated mice (16, 17).

In recent years, *Leishmania tarentolae* was very popular for cloning and expression of genes since features such as high growth rate, non-pathogenicity for mammals, cheap growth conditions, proper glycosylation of glycoproteins, and successful expression of several proteins of the parasite, have made it a potential host for the expression of heterologous glycoproteins. It is also anticipated that this species will be raised as an alternative to expression systems (18-23). The present study aimed to evaluate the immunogenicity of epitopes derived from genes of GP63, LACK, LeIF, and SMT presented by a live vector (*L. tarentolae*) in the BALB/C mouse model.

2. Materials and Methods

2.1. Prediction of Epitopes

Amino acid sequences of LACK (GenBank ID: AAB88300.1), Gp63 (GenBank ID: ACL01096.2), LEIF (GenBank ID: XP_001681281.1), SMT (GenBank ID: XP_001686815.1) of were selected and using IEDB (Immune Epitope Database), epitopes were defined with a great affinity for MHC II in BALB/c mice. The mentioned epitopes were linked, and the final construct with 16.82 kDa molecular weight was obtained for the subsequent analysis. The secondary structure of the multi-epitope was predicted by the usage of Garnier-Osguthorpe-Robson (GOR). Furthermore, the three-dimensional tertiary structure of the construct was stimulated by SWISS-MODEL. ANTIGENpro and VaxiJen v.2.0 were employed to predict antigenicity, and solubility was calculated with the AlgPred server. Also, Physico-chemical parameters such as theoretical isoelectric point (pI), the total number of positive and negative charged residues, molecular weight (MW), aliphatic index, grand average of hydropathicity (GRAVY), and estimated in vitro and in vivo half-life were assessed by the usage of the Expasy ProtParam online server. NetPhos 3.1 and CSS-Palm were computed for the phosphorylation and acylation of the multi-epitope, respectively.

2.2. Expression of Multi-Epitope Construct in *Leishmania tarentolae*

After final approval of the designed fragment via bioinformatics software, the multi-epitope was synthesized, then cloned and sub-cloned in two sites (cytosolic and secretory), in pEGFP-N1 and pLEXY–

neo2 as an expressing eukaryotic system, respectively. Primers (F: 5´TTCTATGTGCGTCGCAACCG 3´, R: 5´GCCGCTTCTTCCAGCACCT 3´) are designed according to gene sequence and cloning strategy. Then this construct transferred to the pLEXSY-neo as a vector, according to our previous study (24). Afterward, the multi-epitope construct was transfected into *L. tarentola*. cDNA synthesis and RT-PCR were performed according to the manual of manufacture, and western blot analysis was done to confirm gene expression.

2.3. Immunization Schedules of Animals and Challenge

In this research, female BALB/c mice with 6-8 week-old were purchased from the Pasteur Institute of Iran. Then, they were kept for two weeks under the standard nutrition, temperature, and moisture conditions. Immunization experiments of mice in 8 groups (n= 9 at each group) were performed subcutaneously, 3 times at 3-week intervals. For this purpose, 50 µg/ml of recombinant antigen and 107 parasites were used for secretory and cytosolic samples, respectively. The vaccinated groups were categorized according to vaccinated (G1-5) and control groups (G6-8), including G1 (*L. tarentolae*-cytosolic complex), G2 (*L. tarentolae*-secretory complex), G3 (*L. tarentolae*- secretory-Montanide-70 complex), G4 (*L. tarentolae*- secretory-cytosolic- Montanide-70 complex), G5 (*L. tarentolae*-secretory- cytosolic complex), G6 (RPMI-1640), G7 (wild *L. tarentolae*) and G8 (*L. tarentolae*- Montanide-70 complex) (Table 1.). Three weeks after the last immunization phase, 2 mice from each group were euthanized for cytokine assay (pre-challenge). Afterward, the remaining mice were challenged by intradermal injection (tail base) with (MRHO/IR/75/ER) promastigotes in the stationary phase (2×10^6). The challenged BALB/c mice were classified into 2 sub-groups: post-challenge cytokine assay (2 mice) and evaluation of lesion diameter size (5 mice).

2.4. Cytokine Assays

The cytokines were evaluated pre and post-challenge (4 weeks) after stimulation with Leishmania soluble antigen (SLA). SLA was provided with promastigotes in the stationary phase. The protein concentration of SLA was calculated via the Bradford method. Two mice of each group were sacrificed, and afterward, spleens were removed under aseptic conditions and homogenized in PBS. The counting of the cells was assessed by the usage of 0.4% of trypan blue. Then cells were seeded with 3 × 106 cells/ml and 25 µg/ml SLA. Afterward, incubation of plates was done at 37ºC with 5% CO2. Eventually, after 72 hours of incubation, the supernatants were harvested for cytokine assay. In this stage, IL-4, IL-10, and IFN-γ levels related to lymphocyte cell supernatants were calculated via ELISA kits (Invitrogen Thermo Fisher Scientific, United States) according to the manufacturer's procedure. Then, the ratio of IFN-γ/IL-4 was calculated.

Table 1. Groups and strategies for immunization

Groups name	No. mice in immunological studies	No. mice in survival and lesion size	Dose
G1 (rP)	4	5	10^7 rP/ 500 µl
G2 (S.Co)	4	5	50 µg/500 µl S.Co
G3 (S.Co+M-70)	4	5	50 µg/250 µl S.Co+250 µl M-70
G4 (rP+ S.Co+M-70)	4	5	5×10^6 rP+ 25 µg/250µl S.Co+ 250µl M-70
G5 (rP+ S.Co)	4	5	5×10^6 rP+25 µg/500 µl S.Co
G6 (R)	4	5	500 µl R
G7 (wP)	4	5	10^7 wP/500 µl
G8 (wP+M-70)	4	5	10^7 wP/250+ 250µl M-70

rP: recombinant parasite, S.Co: Secretory Complex, S.Co+M-70: Secretory Complex+ Montanide-70, rP+S.Co+M-70: recombinant Parasite+ Secretory Complex+ Montanide-70, rP+S.Co: recombinant Parasite+ Secretory Complex, R: RPMI, wP: wild Parasite , wP+M-70: wild Parasite+ Montanide-70

2.5. Statistical Analysis

Lesion size and average cytokine levels of all groups were compared by a two-way-ANOVA test (Multiple comparisons Tukey's post hoc test). Then, the graphs were sketched via "GraphPad Prism" (version 6.07) and Microsoft Office Excel. The *P* values<0.05 were considered to be significant.

2.6. Estimation of Parasite Burden in the Spleen

For assessment of parasite burden, 4 weeks after the challenge, two mice from each group were sacrificed, then their spleens were removed and weighed. Subsequently, the serial dilution was prepared for a specific spleen parasite load. The final titer of a well with at least a motile parasite is the final titer. The number of parasites per gram was calculated and computed using the following formula: parasite burden = $-\log^{10}$ (parasite dilution/tissue weight).

3. Results

3.1. Properties of Multi-Epitope Sequence

Based on the IEDB online service, the functional epitopes for MHCII alleles were chosen, then connected by SAPGTP linker, and the final construct with 162 amino acids was designed (Table 2).

3.2. Physico-Chemical Properties

The number of negatively (Asp + Glu) and a total number of positively charged residues (Arg+Lys) were specified as 13 and 19, respectively. The prediction of Physico-chemical features of this multi-epitope indicated MW of 16.82 kDa, theoretical pI=9.55. Bio half-life was calculated at 3.5 hours (mammalian reticulocytes, in vitro), >10 hours (*Escherichia coli*, in vivo), and 10 min (yeast, in vivo). The GRAVY, aliphatic, and instability index (II) were assessed at 0.03, 85.12, and 38, respectively. The extinction coefficients in units of M-1 cm-1 at 280 nm were measured in water.

3.3. Secondary and Tertiary Structure

162 amino acids in sequence length were computed by GOR IV online server and then obtained the secondary structure of multi-epitope such as extended strand (6.8%), alpha-helix (39%), and random coil (54.39%) (Figure 1). Distribution of solvent availability regions including polarity and hydrophobic aspects of residual patterns predicted by SCRATCH server. The results showed that solvent availability aspects of multi-epitope were acceptable. Also, one of the best-created sequence identities from three 3D models (92.86%) was chosen (Figure 2). The prediction of disulfide bond outcomes via DiNNNA software revealed 3 cysteines in sequence at 46-65 positions to create the disulfide bond (Table 3).

Table 2. Selected epitopes of *L. major* antigens

	Epitope	Allele	Peptide Rank	Start Position	Length
MHCII	MCVATERSLSVYDLE[1]	H2-IAd	2.56	250	15
	LESKAVIAELTPDGA[1]	H2-IAd	5.32	263	15
	RRRCVAARLVRLAAA[2]	H2-IAd	1.80	10	15
	RCVHDAMQARVRQSV[2]	H2-IAd	5.85	47	15
	EHLKAVEIMKLRHLV[3]	H2-IAd	6.8	176	15
	LNGAAIMDAALLLIA[3]	H2-IAd	21.6	150	15
	IFTPSFYIRARKPSK[4]	H2-IAd	24	337	15
	PKGTYKATEVLEEAA[4]	H2-IAd	18.6	312	15

[1] LACK, [2] GP63, [3] LEIF, and [4] SMT antigens

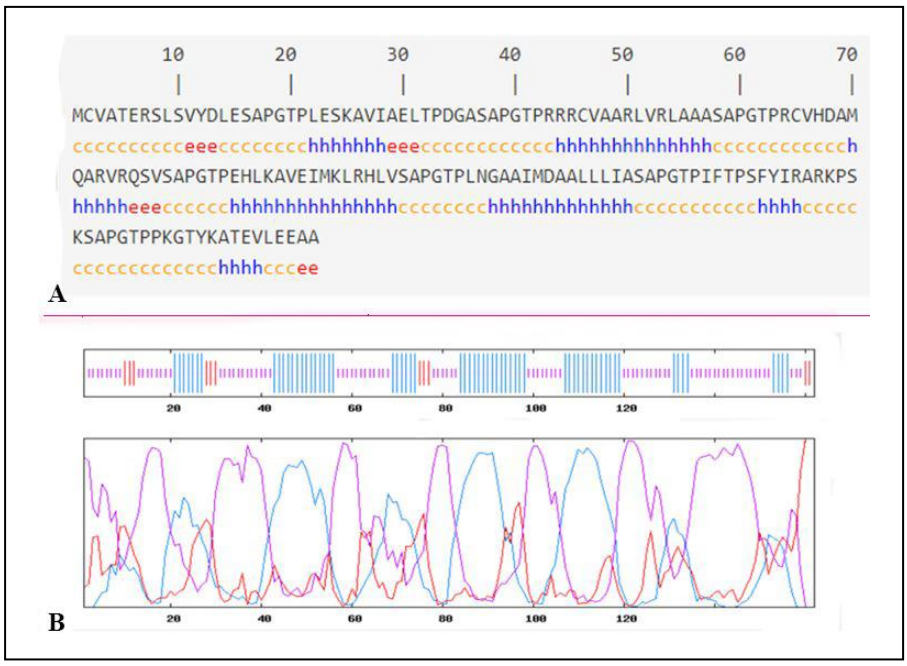

Figure 1. **A**: prediction of secondary structure with GOR IV. e=extended strand, h=helix, c=coil; **B**: Prediction of graphical outputs for the secondary structure of multi-epitope via GOR

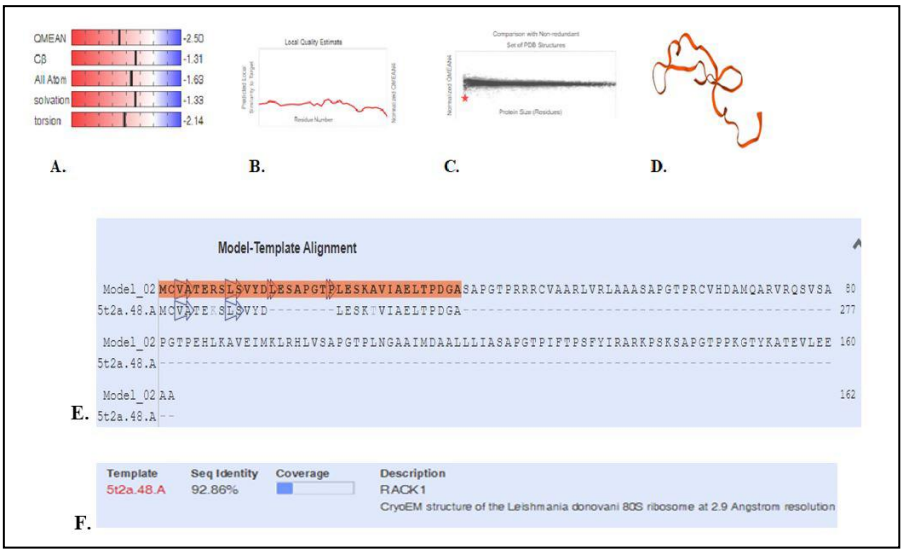

Figure 2. **A:** Global quality estimate; **B:** Local quality estimate; **C:** Comparison with a non-redundant set of PDB structures; **D:** The 3D model constructed; **E:** Model-template alignment; **F:** Sequence identity and coverage

Table 3. Disulfide bonds prediction

Cysteine sequence position	Distance	Bond	Score
2 – 46	44	XXXXMCVATER-TPRRRCVAARL	0.01039
2 – 65	63	XXXXMCVATER-PGTPRCVHDAM	0.01037
46 – 65	19	TPRRRCVAARL-PGTPRCVHDAM	0.011
Weighted matching			
46 – 65		TPRRRCVAARL-PGTPRCVHDAM	

3.4. Analysis of Phosphorylation and Acylation

Antigenicity of the multi-epitope construct was estimated via ANTIGENpro, and VaxiJen v. 2.0 were 0.8 and 0.6, respectively (Threshold for this model: 0.5). The solubility of this protein was calculated at 0.7 by the SOLpro server.

3.5. Expression of Construct -pLEXY-neo2 in *L. tarentolae*

Transfection of construct-pLEXY-neo2 in promastigotes of *L. tarentolae* was performed successfully, and expression of the protein was measured and approved by RT-PCR and western blot procedures. The complementary data is shown in figure 3. The expression of cytosolic and secretory construct-pLEXY-neo2 in promastigotes of *L. tarentolae* was confirmed.

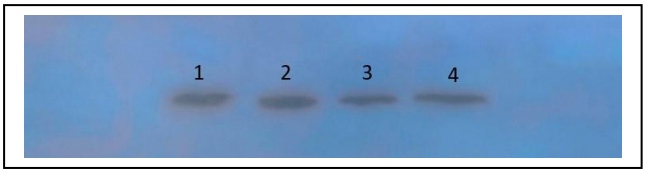

Figure 3. Western blot analysis. The expression of the multi-epitope construct in *L. tarentolae* was performed. Lane1: Logarithmic phase of the cytosolic specimen, Lane2: Logarithmic phase of the secretory specimen, Lane3: stationary phase of the cytosolic specimen, and Lane4: Stationary phase of the secretory specimen

3.6. Measurement of Cytokines

After stimulation of cellular immune responses, the vaccinated groups indicated a relative immunity with the production of higher IFN-γ levels and lower IL-4 and IL-10 levels than the control groups pre and post-challenge (P<0.05). IFN-γ levels in the cytosolic group (G1) were considerably higher than the both vaccinated and control groups pre and post-challenge (P<0.001). In pre and post-challenge, the highest increase in IFN-γ levels was in the cytosolic group (G1), and the lowest one was related to the secretory group (G2) as compared with control groups (P<0.001) (Figure 4, Panel A).

Further, the production of IL-4 levels illustrated that the lowest levels were in the cytosolic group (G1) compared with all of the groups (P<0.001) (Figure 4, Panel B). The ratio of IFN-γ/IL-4 of immunized groups as an index of potential immunization was estimated (Figure 4, Panel D). The highest ratio was related to G1 with 3.9. In this study, IL-10 levels were also estimated for all groups. Accordingly, the highest and lowest level of IL-10 was related to G6 and G1 groups (P<0.001) (Figure 4, Panel C).

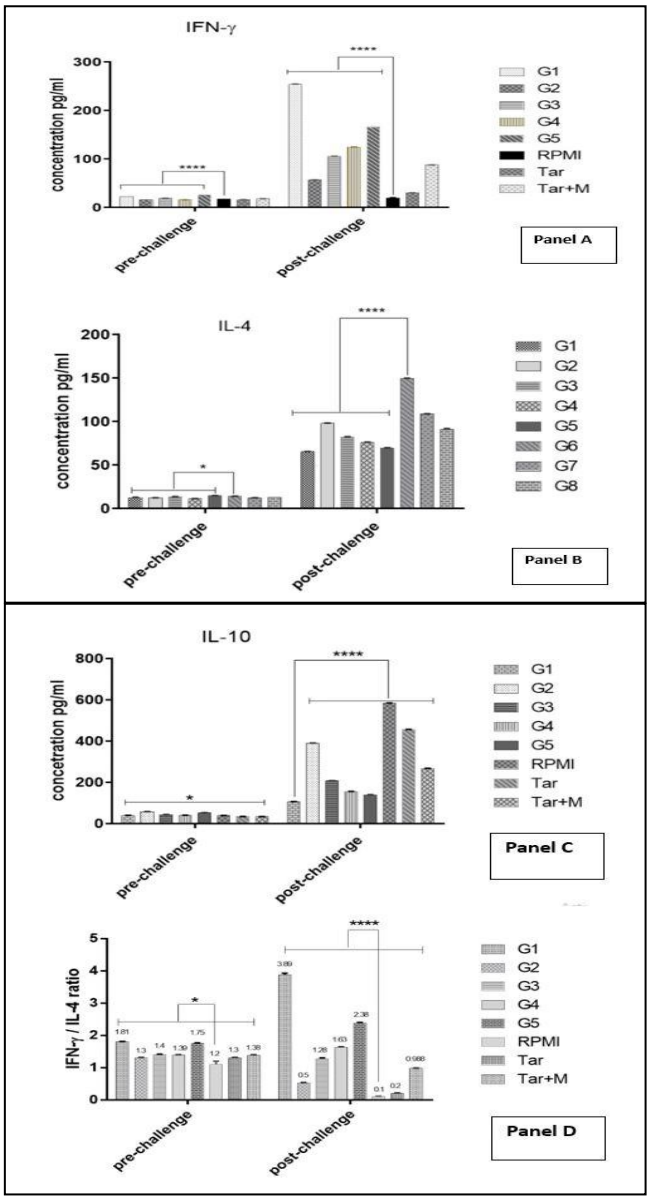

Figure 4. Cytokine evaluation in test and control groups. Lymphocytes of splenocytes from two sacrificed mice were harvested 4 weeks after infection and provoked in vitro via 20 µg/ml of SLA for 72h. **Panel A**, IFN-γ pre-challenge post-challenge; **Panel B**, IL-4 pre-challenge post-challenge. **Panel C**, IL-10 pre-challenge post-challenge, **Panel D**. IFN-γ/IL-4 ratio pre-challenge post-challenge. The asterisk elicits a significant difference between values compared by two-way-ANOVA (P<0.05 indicated as *, P<0.0001 indicated as ****)

3.7. Lesion Size Evaluation

Lesion exacerbation was postponed significantly in all vaccinated animals compared to the control groups at the 7th week after infection (P<0.05). Further, the average lesion size demonstrated a significant decrease in immunized mice by cytosolic group (G1) and cytosolic-secretory complex (G5), respectively, in comparison with the control groups at the 7th week post-infection (Figure 5). The mean lesion size reduced by about 36% in G1, 35% in G5, 33.5% in G4, 31% in G3, 30% in G8, and 6.1% for G2 compared to the control groups at various weeks after infection.

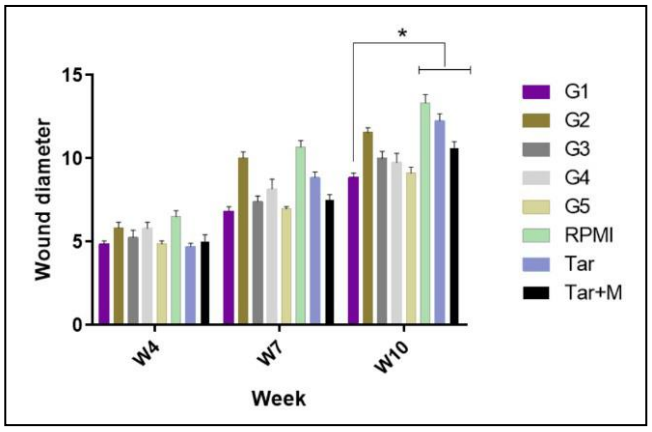

Figure 5. Mean±S.D. of lesion size (3 mice per group) at various weeks of post-infection. The asterisk sign (*) indicates that mean lesion size decreased significantly in all immunized groups than in control groups at various weeks of post-infection (P<0.05). The asterisk sign (*) shows a significant reduction in mean lesion size in G1 compared to control groups

3.8. Assessment of Parasite Burden

The degree of conservation against infection was specified by evaluation of the parasite burden in the spleen at 4 weeks after challenge with *L. major*. The final titer was the last dilution containing at least one live parasite. The highest and the lowest parasite burden in all groups were calculated in RPMI and G1, respectively (Figure 6).

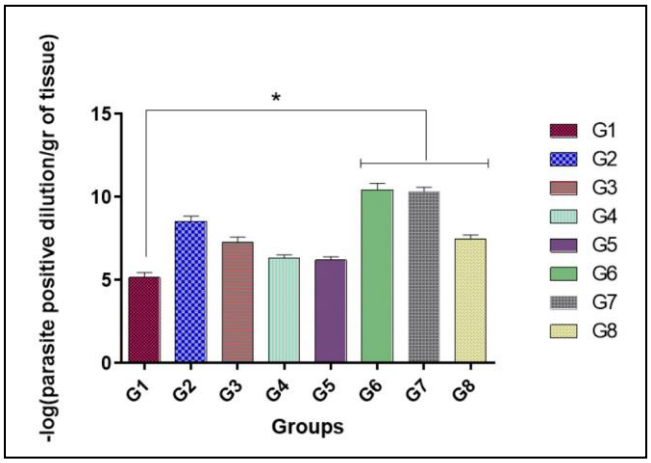

Figure 6. Parasite burden of the spleen. The parasite number in the spleen was calculated 4 weeks after the challenge (*P<0.05 compared to control groups)

4. Discussion

Due to the increasing prevalence of worldwide leishmaniasis, using essential strategies such as effective vaccines to prevent infection and reduce disease has been a priority (12).

In vaccine research, the main aim of vaccination is to create a robust immune response that can protect against infectious agents for a long time (13). Attenuated live vaccines may be activated and cause disease. On the other hand, using these vaccines is associated with a high cost and the need for a suitable temperature chain (14). Therefore, developing a recombinant subunit vaccine is necessary due to its advantages over other vaccines. Recent advancements in designing chimeric vaccines or polypeptide vaccines, and Nano vaccines based on attached or formulated particles with antigenic components and peptides have been increasingly used to elicit an effective immune response (12). Some advantages of peptide vaccines include relatively good stability, low potentially harmful substances, low antigen complexity, and low cost (12). However, recombinant vaccines usually do not have much immunogenicity alone, and therefore they are better to be accompanied by appropriate

adjuvants to stimulate the immune system and thus create an acceptable response. In recent years, bioinformatics methods have been very effective in designing vaccines to analyze and predict proteins. *Leishmania* contains many antigens, some of which were used in this study (LACK, GP63, LEIF, SMT). These antigens have already been tested individually or combined with other antigens or as DNA vaccine candidates (13), but in this study, we used them in the multi-epitope approach. Analysis of epitopes by bioinformatic tools helps us in designing multi-epitope vaccines. The number of amino acids and molecular weight of the construct were estimated at 162 and 16.82 kDa, respectively. The aliphatic index revealed that this fragment has a high-temperature tolerance. So, the GRAVY index showed the hydrophilic property. Antigenicity and allergenicity information indicated that the synthesized peptide is immunogenic and non-allergenic.

In this research, *Leishmania tarentolae* was used as a vector. Various benefits have been reported for the use of vector-based vaccines, such as de novo synthesis, activation of the classical complement cascade, increase in the lethal activity of macrophages, induction of specific humoral responses, prevention of parasite binding to host surface receptors, induction of T-helper and T-cytotoxic responses (15). The usage of non-pathogenic live vectors, even in the absence of adjuvants, evokes an acceptable immune response. The recombinant *L. tarentolae* that is injected as a vaccine into BALB/c mice produces our protein of vaccine candidate in the secretory (secretory) and cytosolic (cytosolic) form actually (16). The outputs of the present study indicate that mice immunized with the *L. tarentolae* vector containing the multi-epitope structure have higher levels of IFN-γ than the control groups in post-infection. It is worth noting that there is a significant difference in the lesion size. This difference is between the Montanide-secretory complex group (G3) (with a decrease of 5.24%) and the secretory group (G2) (with a decrease of 2.13%) compared to the G6 group, which indicates the partial positive effect of montanide-70 adjuvant in the composition of the secretory complex. Between the Montanide-secretory complex (G3) with a 5.24% decrease and the secretory group (G2) with a 2.13% decrease compared to G6, which indicates the partial positive effect of Montanid-70 adjuvant in the composition in the secretory complex.

Various cytokines such as IL-2, IFN-γ, and TGF-β produce from Th1 lymphocytes and induce cellular immune responses against intracellular pathogens (17). The protective immune response in cutaneous leishmaniasis is mediated by other inflammatory cytokines, such as IL-1α, IL-12, and TNF-α (18). On the other hand, Th2 cells produce IL-10, IL-4, IL-5, IL-9, and IL-13 cytokines, which proliferating B cells cause a humoral response in the host immune system and thus change the antibody class (19). Also, eosinophils' activity begins, eventually exacerbating the disease (17). In cutaneous leishmaniasis, regulatory T cells, TGF-β- regulating cytokines, and IL-10 are dual in skin lesions: induction of macrophage activity and limit of immune responses. Therefore, they prevent the disappearance of parasites and cause the persistence of infection in the host (20). IFN-γ can activate macrophages, which increases nitric oxide synthesis and eventually removes intracellular pathogens (21). In this study, the highest ratio of IFN-γ/IL-4 was observed in the cytosolic group (G1), which indicates that this compound can adequately stimulate the cellular immune system before and after challenge and has been effective in increasing cellular immunity.

Moreover, the IFN-γ/IL-4 ratio in immunized animals with a cytosolic group (G1) and cytosolic- secretory (G5) was 30-18.3 -fold higher than the RPMI group in 4th weeks post-infection. In a study, the vaccinated mice by pleish-dom/pIL-12 indicated the lowest levels of IL-10 and highest IFN-γ levels with the least LST (*Leishmania* skin test) reaction eight weeks after the challenge (22). The results of the mentioned study were somewhat coordinated with the present study.

In a recent study, the measurement of IL-10 levels showed that its concentration increased after challenge

in vaccinated and control groups, which is significantly higher in control groups than in vaccinated groups, so the lowest increase in IL-10 levels was observed in the cytosolic group (G1). Overall results indicate lesion progression was delayed in all immunized groups compared to the control groups.

In immunized mice, the reduction in skin lesion size was associated with the immune system's tendency to Th1, increased production of IFN-γ as the main cytokine, and decreased IL-4 levels. Decreased lesion sizes were observed in vaccinated groups such as the cytosolic group (G1) and the secretory-cytosolic group (G5) compared to the control groups (G6 and G7) during different weeks after the challenge. In recent years, the polytope increased the subcutaneous injection of pCDNA3.1/LAKJB93 DNA containing LACK, GP63, and CPC epitopes of Leishmania spp. IgG2a and IFN-γ levels (23). According to our results, the vaccinated mice with GP63, Kmp-11, and Leishmania Amastin epitopes after a challenge by *L. infantum* were suggested to decrease IL-4 significantly and TNF-α cytokines and parasitic burden lower than the control group.

In a recent study, the measurement of IL-10 levels showed that its concentration increased after challenge in vaccinated and control groups, which is significantly higher in control groups than in vaccinated groups, so the lowest increase in IL-10 levels was observed in the cytosolic group (G1). Overall results indicate lesion progression was delayed in all immunized groups compared to the control groups.

Our trial was shown that increasing the ratio of IFN-γ/IL-4 in the *L. tarantula*- Montanide group (G8) compared to control groups (G6, G7) increases the production of IFN-γ levels somewhat. The immune system tends toward cellular immunity but does not have immunogenicity. It does not prevent the spread of lesion size, which has also been observed in immunized groups with Montanide 70. Montanide 70 can increases the production of IFN-γ and the tendency of the immune system to cellular immunity to some extent, but it does not have immunogenic properties and does not prevent the spread of wound diameter, which was also observed in the vaccinated groups with Montanide 70. In this regard, Montanide ISA 720 had good results in non-human mammalian vaccination studies and was immunogenic. It uses for experimental usage in humans as an alternative to aluminum hydroxide (25-28) and induces both cellular and humoral Th1 immune responses in humans (25). However, in another study on vaccination using exogenous antigens of *L. major* with Montanide ISA 720 in mice, no increase in protection was observed with this adjuvant (29). Montanide 70 as an adjuvant did not inhibit the production of IFN-γ. The results of our study demonstrated that a high level of IFN-γ and ratio of IFN-γ/IL-4 have occurred due to increased cellular immune response in the cytosolic group (G1).

5. Conclusion

Since immunization with an antigen from different species of *Leishmania* is not appropriate. Therefore, a combination of selected immunogenic antigens is recommended to produce an effective and protective immunity. Bioinformatics methods can design these antigens. Hopefully, with the development of multi-epitope vaccines, a promising future was achieved for an effective vaccine against leishmaniasis.

Authors' Contribution

Study concept and design: N. B. A. and A. D.
Acquisition of data: N. B. A.
Analysis and interpretation of data: A. D. and M. P.
Drafting of the manuscript: A. D. and M. P.
Critical revision of the manuscript for important intellectual content: A. D.
Statistical analysis: A. D.
Administrative, technical, and material support: A. D., M. J, and J. S.

Ethics

This study was confirmed by the Ethical Committee of Tarbiat Modares University, Tehran, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

Tarbiat Modares University financially supported this study.

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