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Evaluation of protective effect of IL-22 and IL-12 on cutaneous leishmaniasis in BALB/c mice

Hajar Ziaei Hezarjaribi¹, Fatemeh Ghaffarifar^{2*}, Abdolhossein Dalimi², Zohreh Sharifi³

¹Department of Parasitology and Mycology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

²Parasitology and Entomology Department, Faculty of Medical Sciences, Tarbiat Modares University, P.O. Box: 14115-331, Tehran I.R. Iran

³Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran

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ABSTRACT

Objective: To investigate the protective effect of IL-22 and IL-12 on cutaneous leishmaniasis in BALB/c mice. **Methods:** The protective effect of IL-22 and IL-12 on cutaneous leishmaniasis in BALB/c mice was evaluated by measurement of IL-4, INF- γ , total IgG, IgG1 and IgG2a after challenge with *Leishmania major*. Clinical evaluations were performed by measurement of lesion diameter, and survival rate of the mice. **Results:** In week 27 post infection, the mortality rates for control groups were 100%. While the survival rates for the IL-12, IL-12 + IL-22, and IL-22(5 ng/g) groups were 100%. The size of lesions decreased in the presence IL-22 (5 ng/g) of mice weight, which was statistically significant in comparison with other groups ($P < 0.05$). Mean of total IgG, IgG1 and IgG2a for IL-22 (5 ng/g) group was more than other groups. In IL-22 group (5 ng/g), INF- γ production was significantly higher than other groups and IL-4 was significantly lower than other groups. **Conclusions:** The results obtained indicate the effectiveness of IL-22 and its effect on IL-12 in protection of cutaneous leishmaniasis.

1. Introduction

Leishmaniasis is a widespread parasitic disease, reported from 98 countries of the world. So far, there is not any effective vaccine or drug to control this disease, or appropriate chemical method for definite fight against the vector available. The annual new cases of leishmaniasis are estimated to be one to two million cases. Leishmaniasis globally threatens 350 millions of people throughout the world. Visceral leishmaniasis is responsible for 20 000 to 40 000 deaths annually^[1-3].

Using the currently available treatment methods, it is not possible to treat cutaneous leishmaniasis completely. Moreover, the treatments are associated with the infection recurrence, secondary bacterial infections, high costs, and being opportunistic in immunocompromised people. Considering these facts and also the high prevalence rate of the disease and the disease distribution, evaluation of novel

effective methods for prevention and treatment of cutaneous leishmaniasis is indicated^[3,4].

Some plasmids encoding exogenous cytokines (such as IL-6, IL-7, or IL-12) can potentially induce the cellular and humoral immune responses. Particularly when application of these plasmids is accompanied by DNA vaccines, they provide a robust method for enhancement or shift of the immune response against the antigen encoded by the DNA^[5-7].

IL-12 is involved in stimulation of adaptive and natural immune responses, and plays an important role in induction of the Th1 response and suppression of the Th2 response. This cytokine increases the cytotoxic activity and production of INF- γ by dendritic cells (DCs). Moreover, IL-12 enhances INF- γ production and Th1 evolution through the signaling pathways increasing the STAT-4 activity. Therefore, the cytokines have been introduced as crucial inducers in evolution of T-helper subpopulations^[8,9].

Studies have shown that IL-12 production is suppressed in contaminated macrophages. Promastigotes and amastigotes of different *Leishmania* strains suppress IL-12 production in contaminated cells. IL-12 is produced by non-contaminated macrophages and dendritic cells that are not suppressed. The mechanism of suppression

*Corresponding author: Fatemeh Ghaffarifar, Parasitology and Entomology Department, Faculty of Medical Sciences, Tarbiat Modares University, P.O. Box: 14115-331, Tehran I.R. Iran.

Tel: +982182884553

Fax: +982182884555

E-mail: ghafarifar@modares.ac.ir

is not thoroughly understood. In this respect, it is known that lipophosphoglycans involve in suppression of IL-12 production. However, other molecules are also effective in the process. Binding of CR3 receptors would decrease IL-12 production. Since *Leishmania* employs this receptor to enter the cell, inhibition of IL-12 production may be explained by this mechanism^[10, 11].

IL-22, which is also called interleukin-10-related T cell-derived inducible factor (IL-TIF), is secreted by Th-17, natural killer cells, Th22 and T cells (Th1) immediately after activation of these cells by IL-4. Moreover, the cytokine is secreted by thymic and brain mast cells following their activation by Con-A. This is one of the few cytokines, which has been confirmed to be present in non-mammalian animals such as fish^[12–16].

It has been confirmed that cutaneous keratinocytes have receptors for IL-22. In the skin, IL-22 induces antimicrobial peptides, enhances the proliferation and inhibits the differentiation of keratinocytes, and plays a role in wound healing and innate immunity mechanisms. The role of IL-22 in skin disorders such as atopic eczema and allergic contact dermatitis is unknown^[17]. IL-22 produced by human NK cells inhibits growth of *Mycobacterium tuberculosis* by enhancing phagolysosomal fusion and activation of macrophages^[18].

In a previous study it was shown that resistance to human Kala-azar is related to levels of IL-22^[19]. The present study was fulfilled to evaluate the effect of IL-22 administration solely and accompanied by the IL-12-encoding plasmid on cutaneous leishmaniasis caused by *Leishmania major* (*L. major*) in BALB/c mice.

2. Materials and methods

2.1. Experimental mice

Since BALB/c mice are susceptible to promastigotes of *L. major*, we bought 60 white female inbred BALB/c mice at the age of eight weeks from the Razi Vaccine and Serum Research Institute (Hesarak, Karaj). The mice were kept under standard condition and received standard food and water. The mice were categorized according to the treatments they received. The sixty mice were divided into six groups, each including 10 mice.

2.2. Recombinant plasmid pCAGGS-IL-12

Recombinant IL-12 plasmid containing a part of the gene expressing IL-12, which was constructed in the form of pCAGGS-IL-12, was kindly donated by Prof. Masanori Matsui (University of Tokyo, Japan).

Primary and mass culture of the plasmid containing IL-12 was performed in TG1 bacterium by LB broth culture medium (Merck). The plasmid was extracted by the endo-free plasmid purification Mega Kit (Qiagen). Then, the OD values of the plasmid extraction products were read and

elution was performed according to the injection of 100 μ g to each mouse^[7].

2.3. Preparation and injection of IL-22

Recombinant IL-22 (rIL-22) protein of murine (Cat. Number 582-ML; R&D) was prepared according to the manufacturer's protocol. Single doses of rIL-22 5 or 10 ng per g body weight (ng/g) in the volume of 100 μ L were injected intramuscular before the challenge with promastigotes of *L. major*^[20].

2.4. Cytokine injection

In this study, we divided 60 female BALB/c mice of eight-week age into six groups of 10 mice. One group of 10 mice received PBS (100 μ L) and another group of 10 mice received eukaryotic empty plasmid pCAGGS (50 μ g) as control groups. Twenty mice received the plasmid encoding IL-12 gene (pCAGGS-IL-12) (50 μ g in 100 μ L, eluted in PBS), in three doses with three-week intervals. Among the mice that received pCAGGS-IL-12, a group of 10 mice also received rIL-22 (5 ng/g, IM). The remaining two groups received single dose of rIL-22 (5 and 10 ng/g in the volume of 100 μ L) in the quadriceps muscle using 30-gauge insulin syringes^[7, 20].

Classification of the mice with regard to the treatment they received and the follow up. Based on the treatment received, mice were divided in 6 groups ($n=10$ each group) as follow: Control groups treated with PBS (100 μ L) and empty pCAGGS (100 μ g), rIL-22 treated group with 5 ng/g of body weight, rIL-22 treated group with 10 ng/g of body weight, IL-12 (100 μ g) treated group and IL-12+ IL-22 (100 μ g and 5 ng/g body weight respectively). All the mice were treated intra-muscular (IM) three times with three-week intervals. Further each group was divided in two sub-groups of 5 mice as follows:

(1) Seven weeks after applying the above-mentioned treatments, the animals were challenged by *L. major* promastigote of the MRHO/IR/75/ER strain and then seven weeks later the animals' humoral and cellular responses were measured.

(2) Seven weeks after applying the treatments, the animals were challenged by *L. major* promastigote of the MRHO/IR/75/ER strain and then lesion size and weight and survival rate of the mice were evaluated.

2.5. Challenge with promastigotes of *L. major*

Seven weeks after the last immunization, the BALB/c mice were infected with *L. major*. The mice were challenged with at least 2×10^6 *L. major* promastigotes of the Iranian standard strain MRHO/IR/75/ER. The promastigotes were in the stationary phase, and were inoculated in the tail base by subcutaneous injection (100 μ L). Three to four weeks after the injection, a nodule was developed at the injection site, and a lesion resulted from the growth of the parasite was formed in the tail base^[20, 21].

2.6. Evaluation of humoral immunity

The humoral immunity was evaluated by measurement of total IgG in the sera samples of the immunized and control groups using ELISA kits. Furthermore, the antibody subtypes (IgG1 and IgG2a) were measured by Mouse Monoclonal Antibody Isotyping Reagent Kit (Sigma).

To determine the antibody levels, optimized volumes of the antigen, serum concentration, and conjugated antibody concentration were determined using checker board titration as previously defined^[21]. Then, all sera samples were examined by the ELISA method. Then, 100 μ L of the crude soluble Leishmania antigen (SLA) at the concentration of 20 μ g/mL in the carbonate–bicarbonate buffer was prepared. Then, 100 μ L of the sera eluted in PBS buffer (1:10) was used.

2.7. Extraction and culture of lymphocytes for cytokine evaluation

In the mice of sub–group 1, spleen was collected and meshed–up to extract the lymphocyte. 2.5×10^6 /mL lymphocytes were plated in 24 well tissue culture plate with complete RPMI media (Gibco, BRL, Maryland, USA) containing 20% FCS (Gibco, BRL, Maryland, USA), stimulated with 40ug/ml SLA and cultured at 37 degree, 5% CO₂ for 72 hours to access the cytokine release. Seventy two hours later, the cellular supernatant was collected and kept at -70°C until cytokine assay^[22].

2.8. Cytokine assay

INF- γ and IL-4 levels in the cellular supernatant prepared from the spleen lymphocytes of immunized and control mice, which were kept at -70°C , were measured by the ELISA method^[21], using U–CyTech biosciences (Netherlands). According to the standard measures included in the kit, the standard diagrams were drawn and the samples were evaluated.

2.9. Evaluation of number of parasites in spleen of the mice after the challenge

Seven weeks after challenge by the parasites, the treated and control group mice were sacrificed; the spleens were removed under sterile conditions and weighed and large volumes of the spleen cellular supernatant was used for lymphocyte extraction. Almost 60 μ L of the spleen cellular suspension was cultured under sterile condition in 1.5 mL microtube containing 1 mL RPMI 1640 (containing 20% FCS, 100 unit/mL penicillin, and 100 μ g/mL streptomycin). After eight days of incubation at $24\text{--}25^\circ\text{C}$, the presence or absence of mobile promastigotes and the number of promastigotes in each ml was microscopically evaluated^[21].

2.10. Measurement of lesion size and body weight of mice

In subgroup 2, after the challenge and creation of the lesion, the lesions size were measured weekly by a digital caliper and considered as the lesionsize. To confirm the lesion resulted from Leishmania, we obtained samples from the lesions. The body weight of mice measured by a digital scale weekly. After the parasite challenge, the life span and survival rate of the immunized and control mice were weekly measured^[21].

2.11. Statistical analysis

The results were analyzed using SPSS software. In this regards, INF- γ , IL-4, MTT, IgG and its subtypes, parasite loads in spleen, lesion size and survival rate of mice were analyzed. Non–parametric data were compared by Mann–Whitney test.

3. Results

3.1. Survival rate

In all groups, the survival rate was 100% up to week six. Mortality of the control groups started from week seven on (week seven: mortality 20%; survival rate 80%).

Mortality for the IL-12, IL-12+IL22, and IL-22-5 ng/g groups started from week 26 and 27.

The mortality rates for PBS and pCAGGS control groups were 100% in weeks 26 and 27 after the challenge with *L. major*. This is while at this point, the survival rates for IL-12, IL-12+IL-22, and IL-22-5 ng/g groups were 100%. The survival rate of 80% for IL-12 and IL-12+IL-22 groups was observed at week 28, while this was observed at week 26 for the IL-22-5 ng/g group (Figure 1).



Figure 1. Survival rate of the mice that received IL-12, IL-22, and IL-12+IL-22 in comparison with the control groups during the 30 weeks of evaluation after the challenge with 2×10^6 promastigotes of *L. major*.

3.2. Body weight of mice

The groups that received 5 and 10 ng of rIL-22 were not significantly different with regard to the weight of mice. However, rIL-22-5 ng/g was significantly different from the control groups (pCAGGS and PBS) in this respect ($P < 0.05$).

(Figure 2).

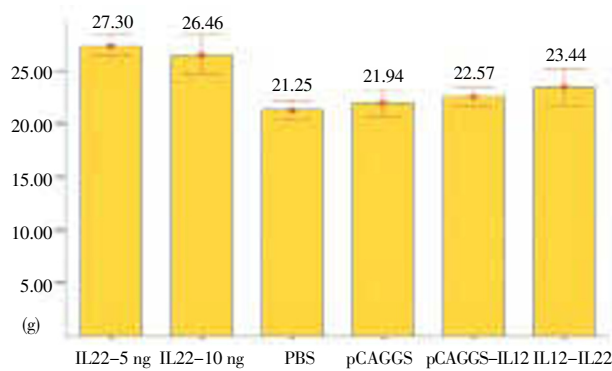


Figure 2. Mean and standard deviation of weight (g) in treated and control groups of BALB/c mice at week 7 after the challenge with 2×10^6 *L. major* promastigotes.

Statistically significant differences were observed among the IL-12, IL-12+IL-22, and IL-22-5 ng/g groups ($P < 0.05$). The results did not show any significant difference between IL-12 and IL-12+IL-22 groups in the weight of mice ($P > 0.05$).

3.3. Lesion status

The mean lesion size in the IL-22-5 ng/g was smaller than that in the IL-12 group, and as shown, the differences in different weeks of the study were statistically significant ($P < 0.05$).

Furthermore, the lesion size in IL-12+IL-22 group was smaller than that in the IL-12 group. In different weeks of the study, both groups were significantly different in this respect ($P < 0.05$) (Figure 3).

3.4. IgG total, IgG1 and IgG2

In the mice of sub-group 1 the humoral immunity was determined. Comparison between mean and SD of IgG total, IgG1 and IgG2 in different groups of mice is given in Table 1.

3.5. INF- γ

The mean INF- γ level in the IL-22 (5 ng/g) was significantly higher than that in the IL-22 (10 ng/g). Also in IL-22 (5 ng/g) group, INF- γ production was significantly

higher than other groups.

The mean level of INF- γ in the IL-22-10 ng/g was significantly different from the levels in the pCAGGS and PBS groups ($P < 0.05$). The mean level of INF- γ in the IL-22-5 ng/g group was significantly higher than the values obtained for other groups ($P < 0.05$) (Table 2).

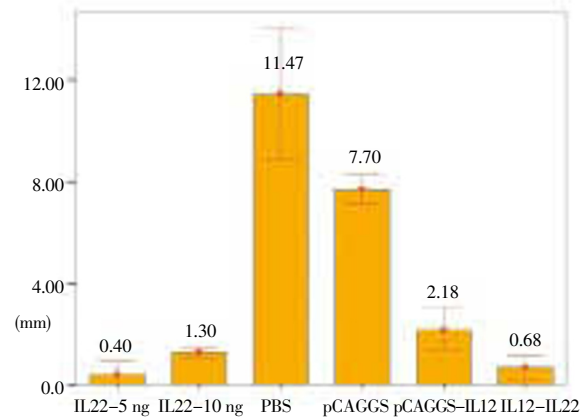


Figure 3. The mean and SD of lesion size (mm) in treated and control groups of BALB/c mice in three doses with three-week intervals, 7 weeks after the challenge with 2×10^6 *L. major* promastigotes.

3.6. IL-4

The mean level of IL-4 showed a decreasing trend in the groups that received IL-22. These groups were significantly different from the other groups.

In IL-22 group (5 ng/g), IL-4 was significantly lower than other groups, whereas the IL-4 for IL-22 (10 ng/g) group was significantly lower only with control groups (Table 2).

3.7. Spleen weight and number of parasites in spleen after challenge

Enlargement of the spleen (as a lymphatic organ) and increase in its weight in the control mice are parameters indicating the increase in number and accumulation of immune cells in the organ. The accumulation could demonstrate the presence of the parasite in the spleen or exposure of the immune cells with infectious factors.

The data related to the spleen weight and parasite count in week 7 after the challenge by *L. major* demonstrated

Table 1

Mean and SD of IgG total, IgG1 and IgG2 in different groups of mice seven weeks after challenge with *L. major* promastigotes of the MRHO/IR/75/ER strain.

Group	Cytokine regimen	IgG1		IgG2		IgG-total	
		Mean \pm SD	Sig($P < 0.05$)	Mean \pm SD	Sig($P < 0.05$)	Mean \pm SD	Sig($P < 0.05$)
1	PBS	0.354 0 \pm 0.001 9	3, 4, 5, 6	0.422 0 \pm 0.000 8	3, 4, 5, 6	0.417 0 \pm 0.000 4	3, 4, 5, 6
2	pCAGGS	0.374 0 \pm 0.002 6	3, 4, 5, 6	0.441 0 \pm 0.000 5	3, 4, 5, 6	0.424 0 \pm 0.000 5	3, 4, 5, 6
3	IL-22 (5 ng/g)	0.732 0 \pm 0.001 7	1, 2, 4, 5, 6	0.998 0 \pm 0.000 8	1, 2, 5, 6	0.785 0 \pm 0.000 7	1, 2, 5, 6
4	IL-22 (10 ng/g)	0.595 0 \pm 0.000 6	1, 2, 3	0.916 0 \pm 0.000 4	1, 2, 5	0.714 0 \pm 0.000 5	1, 2, 5, 6
5	IL-12	0.540 0 \pm 0.001 3	1, 2, 3	0.815 0 \pm 0.000 5	1, 2, 4	0.582 0 \pm 0.000 4	1, 2, 3, 4
6	IL-22 (5 ng/g)+IL-12	0.563 0 \pm 0.000 6	1, 2, 3	0.845 0 \pm 0.000 1	1, 2, 3	0.594 0 \pm 0.000 7	1, 2, 3, 4

Table 2Mean comparison of INF- γ and IL-4 levels (pg/mL) of the groups after the challenge.

Group	Cytokine regimen	Mean \pm SD (INFN - γ)	Sig($P<0.05$) [*] (IFN - γ)	Mean \pm SD (IL-4)	Sig($P<0.05$) [*] (IL-4)
1	PBS	176.25 \pm 34.49	3, 4, 5, 6	203.75 \pm 14.34	3, 4, 5, 6
2	pCAGGS	123.75 \pm 20.56	3, 4, 5, 6	229.75 \pm 33.67	3, 4, 5, 6
3	IL-22 (5 ng/g)	1744.50 \pm 157.95	1, 2, 4, 5, 6	3.75 \pm 4.50	1, 2, 4, 5, 6
4	IL-22 (10 ng/g)	271.25 \pm 41.82	1, 2, 3, 6	14.25 \pm 5.12	1, 2, 3
5	IL-12	441.75 \pm 89.41	1, 2, 3	15.50 \pm 3.70	1, 2, 3
6	IL-22 (5 ng/g)+IL-12	632.25 \pm 168.35	1, 2, 3, 4	12.25 \pm 3.69	1, 2, 3

* indicates significance ($P<0.05$) according to the Mann Whitney test.**Table 3**

Mean and standard deviation of spleen weight (g) and parasite count in week 7 after the challenge.

Group	Cytokine regimen	Spleen weight(g)	Parasite in spleen $\times 10^5$
1	PBS	0.890 \pm 0.017	13.85 \pm 0.25
2	pCAGGS	0.840 \pm 0.015	14.11 \pm 0.22
3	IL-22 (5 ng/g)	0.300 \pm 0.002	–
4	IL-22 (10 ng/g)	0.320 \pm 0.002	–
5	IL-12	0.360 \pm 0.062	2.26 \pm 0.09
6	IL-22 (5 ng/g)+IL-12	0.760 \pm 0.022	1.90 \pm 0.11

Data were expressed as Mean \pm SD.

that the control groups were significantly different from the experiment groups in this respect (Table 3).

4. Discussion

In the current study, for the first time, we evaluated the effect of IL-22 together with IL-12 on cutaneous leishmaniasis caused by *L. major* in BALB/c mice. The results indicated higher effectiveness of IL-22 in treatment of cutaneous leishmaniasis. This is supported by the lowest lesion size among the groups in the IL-22-5 ng/g group in week seven. Furthermore, mice in the IL-22-5 ng/g group had the highest weight and survival rate.

The results of INF- γ and IL-4, evaluations in BALB/c mice demonstrated that IL-22 evidently increased the INF- γ production and reduced the IL-4 production after the challenge. Administration of IL-22 caused a statistically significant difference between this group and other groups in this respect. This shows that IL-22 resulted in development of Th1 cytokine responses and indicates that the cytokine is effective in treatment of cutaneous leishmaniasis. Co-administration of cytokines with other treatments is currently performed in treatment of many infectious and parasitic diseases to enhance the protective responses of T cell subpopulations[7, 18–20, 23–24]. In this study, it was observed that IL-22 has a dose-dependent effect; such as 5 ng/g produced better responses in comparison with 10 ng/g. At some doses, IL-22 has synergistic effect with other cytokines; for instance, IL-22 at 200 ng/mL together with IL-17A (20 ng/mL) is effective in treatment of psoriasis. This is caused by activation of S100A8 and S100A9 pathways through the synergistic effect of IL-17A and IL-22[25].

In some studies, long-term and high doses of IL-22

have been reported to be associated with adverse effects and there are some reports on the carcinogenicity of the cytokine[25]. However, such reports are not available for the permitted doses (2–20 ng/mL).

There is some evidence indicating that IL-17 and IL-22 play the complement role in natural immunity against *Leishmania donovani* (*L. donovani*). This hypothesis is consistent with the effect of Th17 on monocytes and neutrophils and the ability of Th17 cells in infiltration of Th1 cells and formation of granulomas[19]. Furthermore, IL-17 and 22 hasten tissue regeneration and strengthen the epithelial cells; thus, contributing to the immunity and protection[25].

Mean lesion size in the groups that received IL-22 was significantly different from that in other groups. The lesion size in the group that received IL-12+IL-22 was close to the values obtained for IL-22 groups. Therefore, it could be probably stated that IL-22 performed better than IL-12 in reducing the lesion growth and wound healing in cutaneous leishmaniasis. It has been found that skin keratinocytes have receptors for IL-22. It was shown in a study that, IL-22 induces antimicrobial peptides in skin, enhances proliferation of keratinocytes and inhibits their differentiation, and plays a role in skin regeneration in innate immunity mechanisms[17].

The protective role of IL-22 against human Kala-azar has been reported previously[19]. In our own previous study, we found that IL-22 as an adjuvant could show effective role in DNA vaccine with plasmid encoded LACK gene against cutaneous leishmaniasis[26]. The mean levels of INF- γ in the groups that received IL-22 were significantly higher than those in other groups. This indicates that IL-22 resulted in development of Th1 cytokine response in mice suggesting its protective role in cutaneous leishmaniasis specially when used at 5 ng/g concentration.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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