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## Original Article

# The Comparison of the IFN-ɣ, TNF-α and IL-10 Cytokines in Healing and Non-healing Cutaneous Leishmaniasis

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***Abstract***

***Background:*** Leishmaniasis is one of the main vectors borne and neglected tropical parasitic diseases. T cell cytokine responses are highly important in the presentations of disease such as control or progression, and understanding of the host immunological response is valuable in diagnosis, follow-up, and vaccine designs. In the current study, the profile of IFN-ɣ, TNF-α, and IL-10 cytokines was investigated through the ELISA technique in PBMCs isolated from antimony resistance and susceptible patients.

***Methods:*** In this experimental study, 54 patients with healing (n=27) or non-healing (n=27) CL were recruited. Lesion samples were collected to determine the genotype of *Leishmania* spp. and peripheral blood mononuclear cells (PBMCs) were obtained to evaluate the cytokines profiles using soluble *Leishmania* antigen (SLA) and phytohaemagglutinin (PHA) mitogen. Cytokines were assessed by the ELISA technique

***Results:*** The IFN-ɣ and TNF-α cytokines were significantly increased in the healing group treated with both SLA antigen and PHA mitogen ($P$<0.001). The level of IL-10 was significantly increased in non-healing and significantly declined in healing groups ($P$<0.001).

***Conclusion:*** The profile of IFN-ɣ, TNF-α, and IL-10 cytokines are crucially associated with the response of treatment.

## Introduction

Leishmaniasis is a neglected tropical disease caused by intracellular parasites belonging to the *Leishmania* species. Visceral (VL), cutaneous (CL), mucosal (MCL), and diffuse cutaneous leishmaniasis (DCL) are the four clinical forms of the disease (1, 2). Leishmaniasis causes a high rate of morbidity and mortality with 0.7 to 1.0 million annually new cases (3). CL is the most prevalent clinical form of leishmaniasis reported from 98 countries (4). Brazil, Colombia, Afghanistan, Algeria, and the Middle Eastern countries, especially Iran are the most important endemic areas. *L. major* and *L. tropica* (old world leishmaniasis) and *L. mexicana* complex (new world leishmaniasis) are the most etiological cause of CL worldwide (3-5).

Female sand flies are the main vector for leishmaniasis and cause localized lesions (6). Pentavalent anti- monials (Sbv), glucantime ®, and miltefosine ® are the first-line choices of treatment against all forms of leishmaniasis worldwide that are treated within a few weeks or months (7). In the non-healing form of cutaneous Leishmaniasis (NHCL), the lesions persist for more than 2 years and do not respond to conventional chemotherapy (8). Variations in the immune responses to infection and immunological mediators such as cytokines are the most effective parameter in different clinical outcomes. Serological conversion in endemic areas for leishmaniasis is resistant to the disease and can control the disease (8-11).

The progression and pathogenesis of the disease depend on the immune response and cytokines delivered from Th1 and Th2 cells. The exact role of Th1 and Th2 profile in human leishmaniasis is not fully described but, in some animal model studies on Blab/c mice, the immune response has been clearly explained as a Th2 profile during progression and Th1 in the control of the disease. Interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) delivered from Th1 cells activate the macrophages and induce inducible nitric oxide synthase (12-14), whereas Interleukin 10 (IL-10) commonly release from Th2 cells and plays an important role in decreasing the ability of macrophages and is consequently effective in pathogenesis and facilitates parasite growth and replication (15-17).

Due to the increasing incidence of drug resistance and the high-pathogen strain of *Leishmania* (18) in some patients and the unidentified cytokine profile in patients with non-healing and healing CL, this study was conducted to evaluate the levels of IFN-ɤ, TNF-α, and IL-10 cytokines in non-healing and healing CL.

## Materials and Methods

### *Screening of healing and non-healing CL and Sampling*

Peripheral blood (10ml) was collected by venipuncture into EDTA-containing tubes (BD Vacutainer) from healing (n=27) and non-healing (n=27) patients with CL referred to the different health care centers of Golestan and Isfahan provinces, Iran, from March 2018 to January 2020. In addition to these patients, 56 samples were collected from healthy individuals, including 27 healthy individuals who received phytohemagglutinin and 27 individuals who received no treatment as positive and negative controls. CL patients with the active lesions, which last for more than 2 years without any decrease in lesion size were considered as non-healing and patients with CL and completely treated lesion in less than 1 year and without any clinical sign of CL during a 9-month follow-up were considered as healing.

### Human and animal rights

All patients confirmed ethical consent and entered the study with full knowledge. All parts of this work are ethically approved by the Ethical Committee of Islamic Azad University, Hamedan branch, and sampling was not done only for the current study.

### DNA extraction and Genotyping

DNA was extracted from the lesion samples and purified using QIAamp Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. ITS1-PCR was applied to detect genus *Leishmania* species (18) by preparation of a 50 µl reaction mix with adding 1 mM MgCl2 (GIBCO™), 10% DMSO (Merck, Germany), 10 mM Tris-HCl pH 8. 4 (GIBCO™, Invitrogen™ Life Technologies, CH), 25 mM KCl, 1µM of each primer (LITSR (5-CTGGATCATTTTCCGATG-3) and L5.8S (5- TGATACCACTTATCGCACTT-3)), 20 mM tetramethylammonium chloride (Carl Roth GmbH, FRG), 0.1 mM dNTPs (Takara Bio-Company, Japan), and 1 U *Taq* polymerase (GIBCO™). PCR reaction was carried out in a thermal cycler (Eppendorf, Germany) based on the following condition: initial denaturation at 95 °C for 5 min and continued in 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s; and final extension at 72 °C for 5 min. The RFLP procedure was performed to the determination of the species/strains using the *HaeIII* restriction enzyme (Jena Bioscience, Germany) (19). In this step, 2.5 µL universal buffer, 1.5 µL enzyme, 5 µL PCR product, and 6 µL sterile deionized water were added to a PCR tube and incubated at 37ºC for 2 h (18).

### Preparation of Soluble Leishmania Antigen (SLA)

The promastigotes of *L. major* were grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% FBS (Gibco Laboratories, Grand Island, NY) and 50 µg/mL of Penicillin/streptomycin (Gibco, Paisley, Scotland) at 23 ºC for 72 h. The 5 × $10^6$ cells were washed two times in 1 ml of sterile PBS by centrifugation at 1000g for 5 min. The parasites were frozen at -70 ºC and thawed at 37 ºC and then sonicated with pulses of 50– 62 kHz for 40 s at time intervals of 20 s (seven times). The lysed cells were centrifuged at 30,000 g for 25 min, and the supernatant was stored at -70 °C until the use. The concentration of protein was determined by the Micro BCA Protein Assay Kit (Thermo Fisher Scientific) (8, 20).

### PBMCs purification

A volume of 10 mL of peripheral blood containing EDTA was collected from 27 healing and non-healing patients with CL mixed to an equal volume of PBS (pH 7.2), and the PBMCs were isolated over Histopaque 1077 (Sigma-Aldrich, Germany). The harvested cells were washed three times with PBS and resuspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing L-glutamine (2g/L), inactivated fetal calf serum (iFCS, Gibco Laboratories, Grand Island, NY), sodium bicarbonate (10%), and Penicillin/streptomycin (50 mg/mL). The viability of the cells was evaluated by eosin 0.01% (20).

### Culture of PBMCs

The PBMCs (1 × $10^6$ cells) were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) at 37 ◦C with 5% CO2 into 48-well culture plates in a final volume of 500 µL and were then stimulated with 1 µg/mL of SLA. PBMC cultures were incubated at 37°C with 5% CO2. After 72h of stimulation, the supernatant was collected and kept at -70 °C for cytokine evaluation. Moreover, PBMCs were extracted from healthy control receiving phytohemagglutinin 2 µg/mL, and the no-treatment group was used as positive and negative controls in all experiments (21).

### Cytokine assay

Cytokine analysis of IFN-γ, TNF-α, and IL-10 was performed using the sandwich ELISA method through the employment of the e-Bioscience commercial kit (Ready-SET-Go, USA) according to the manufacturer's instructions. The levels of cytokines were evaluated on triplicate wells for each sample. Optical density was measured with an ELISA reader (Anthos 2020) at 450 nm of wavelength. The standard curve for each cytokine was estimated using a computer-generated four-parameter logistic curve-fit with the program MyAssays (http://www.myassays.com/) (8).

### Statistical analysis

Data were analyzed using SPSS ver. 22.0 software (IBM Co., Armonk, NY, USA). Mann-Whitney and Friedman's tests were used to compare the levels of cytokine and control groups. For comparison of the nominal variables, the Pearson chi-square test and Fisher's exact test were applied. $P<0.05$ was considered statistically significant.

## Results

### Clinical findings

Our two groups (healing and non-healing) were matched for age and sex and did not differ significantly. The BMI index, lesion locations, and season of onset were not significantly different between healing and non-healing groups. Smoking habit was significantly more prevalent in the non-healing group ($P=0.026$). The lesion size (mm) in healing and non-healing groups was 24.57±7.13 and 38.26±14.09 mm (mean±SD), respectively ($P=0.017$) (Table 1). The results of genotyping assay indicated that the *Leishmania* species from all patients were *L. major*.

**Table 1:** The clinical characteristics of the healing and non-healing patients with CL

| ***Characteristics*** | | ***Healing N= 27*** | ***Non-healing N= 27*** | ***P- value*** |
|---|---|---|---|---|
| Age N (%) | <25 | 6 (22.22) | 6 (22.22) | 0.999 |
| | 26-50 | 16 (59.25) | 16 (59.25) | |
| | >50 | 5 (18.51) | 5 (18.51) | |
| Sex N (%) | Male | 25 (92.59) | 25 (92.59) | 0.999 |
| | Female | 2 (7.40) | 2 (7.40) | |
| BMI N (%) | < 18.5 | 1 (3.70) | 2 (7.40) | 0.637 |
| | 18.6-24.9 | 14 (51.85) | 16 (59.25) | |
| | 25-29.9 | 7 (25.92) | 5 (18.51) | |
| | >30 | 5 (18.51) | 4 (14.81) | |
| Smoking habit N (%) | Yes | 8 (29.62) | 12 (44.44) | 0.026 |
| | No | 19 (70.37) | 15 (55.55) | |
| Area of living N (%) | Urban | 6 (22.22) | 4 (14.81) | 0.072 |
| | Rural | 21 (77.77) | 23 (85.18) | |
| Lesion location N (%) | Face | 5 (18.51) | 5 (18.51) | 0.962 |
| | Hand and foot | 18 (66.66) | 15 (55.55) | |
| | Multiple locations | 4 (14.81) | 7 (25.92) | |
| Lesion size (mm) | (mean±SD) | 24.57±7.13 | 38.26±14.09 | P= 0.017 |
| Number of lesion N (%) | 1 | 16 (59.25) | 12 (44.44) | P= 0.035 |
| | >1 | 11 (40.74) | 15 (55.55) | |
| Season of Onset N (%) | Spring | 1 (3.70) | 1 (3.70) | P= 0.096 |
| | Summer | 5 (18.51) | 4 (14.81) | |
| | Autumn | 16 (59.25) | 18 (66.66) | |
| | Winter | 5 (18.51) | 4 (14.81) | |

### Cytokine assay

Supernatant levels of IFN-ɣ, TNF-α, and IL- 10 cytokines produced by PBMCs incubated with SLA, PHA, and non-treated groups were compared. The production of IFN-ɣ and TNF-α in the presence of SLA and PHA in the healing group was significantly higher than the non-healing group ($P< 0.001$), whereas the IL-10 production in the healing group was significantly lower than non-healing group ($P< 0.001$) (Fig. 1).

Moreover, the productions of cytokines in healing and non-healing groups in the treatment of different stimulators were assessed separately. In treatment with SLA and PHA, the production of IFN-ɣ and TNF-α was significantly increased. The production of IL-10 was significantly decreased in treatment with SLA ($P<0.001$), while in treatment with PHA, the difference was not significant. In the non-treated group, there was no significant difference in the production of IFN-ɣ, TNF-α, and IL- 10 cytokines (Fig. 1).

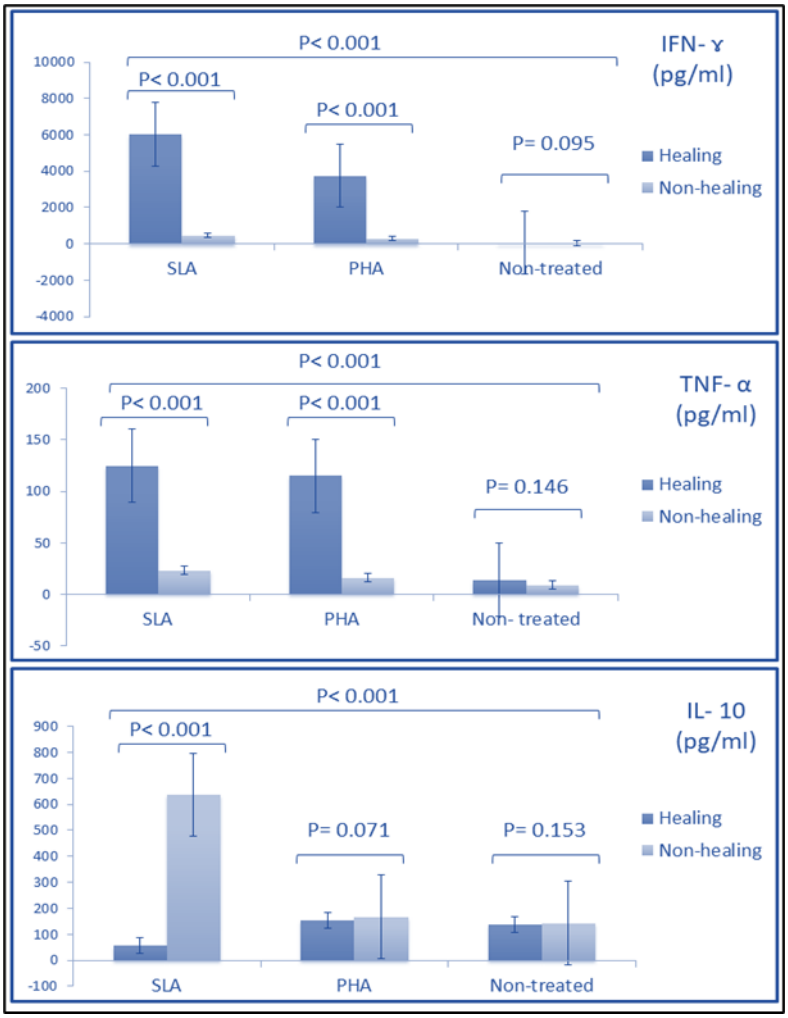


**Fig. 1:** The production of IFN-ɣ, TNF-α and IL-10 (pg/ml) by PBMCs isolated from healing and non-healing patients with CL treated with SLA, PHA and non-treated groups

## Discussion

The pathogenesis and response to the treatments of the *Leishmania* parasites are highly associated with the response of the host immune. Helper T cells play important roles during leishmaniasis by producing several cytokines and chemokines (10, 22). Cytokines have a very complex manner and have multifaceted associations to other cytokines. So, the investigation of cytokine profiles is advantageous to recognize the exact response of the immune system in different situations (23).

IFN-ɤ delivered from CD4+ and CD8+ T cells can stimulate the generation of the NO and superoxide anion from active macrophage, which both of them are essential to control *Leishmania* parasites (24, 25). The development of Th2 response in CL infection caused by *L. major* triggered severe and progressive disease while responses of Th1 cells in another group increased the production of IFN-ɤ and controlled the multiplication of *Leishmania* parasites (26). In the current study, the production of IFN-ɤ was significantly higher in the healing group compared to the non-healing group, and our results indicated that in the non-healing group or antimony resistance patients, the low production of IFN-ɤ might be the main cause of this defect.

Ajdary et al. revealed an increase in CD4+ cells in PBMCs isolated from patients with leishmaniasis that is a source of TNF-α and IFN-ɤ cytokines (27). There was an association between the size of the CL lesion and the level of T CD4 + cells expressing IFN-γ (28-30). Contrary to the current study, a high level of IFN-ɤ production in non-healing cases was reported (31). Therefore, the outcome of leishmaniasis might be affected by multi pro-inflammatory cytokines such as TNF-α, IFN-ɤ, IL-17a, or/and IL-12.

Similar to IFN-ɤ, TNF-α is another Th1 delivered cytokine that can stimulate the activation of the macrophage and kill the intracellular parasites. TNF reduces the development of regulatory T cell networks. The deficiency of TNF signaling decreases the activities of the IFN-γ and iNOS, and consequently, the immune system will be incapable to inhibit the progression of the infection (32). The lesion size in CL was significantly associated with the level of TNF-α in CL lesion biopsy (33). In the current study, the production of TNF-α was significantly higher in antimony susceptible patients with CL compared to the antimony resistant patients in treatment with both SLA and PHA. All results of these studies have shown that TNF-α has a positive role in macrophage activity and treatment of cutaneous leishmaniasis, whereas in mucocutaneous forms of leishmaniasis, the levels of serum TNF-a have been suggested to be related to the severity of the disease (34, 35). Signaling pathways of the TNF were necessary for creating an inflammatory response to intracellular infection (36, 37). TNF-α as pro-inflammatory cytokines have a strong expression in Patients with chronic lesions of CL (38). The clinical outcome of the disease and determination of the progression or control of the infection are depending on the type of secreted cytokines. By our knowledge, IFN-ɤ and TNF-α are responsible to control the CL, while an increase in IL-10 and decrease in IFN-ɤ and TNF-α production cause progression of the CL.

IL-10 suppresses the immune response by decreasing the reactive nitrogen intermediates, decreasing the creation of IL-12 and TNF, and turning the Th response into a Th2 response (39-41). The production of IL-10 is associated with macrophage deactivation, parasite survival, lesions development, and treatment failure (42). An increase in the level of IL-10 heightens the risk of a skin lesion in CL. Also, by the determination of the SNPs, the results revealed that IL-10-819 was strongly correlated with CL (17). Based on our findings, the level of IFN-γ, TNF-α, and IL-10 cytokines have a decisive role in response to treatment. The patients with antimony re-

sistance profile showed a high production level of IL-10 and low level of IFN-γ and TNF-α, and this balance is completely changed in antimony susceptible patients. Regulatory T cells are the crucial and vital part of the immune system that its exact role has not been characterized during *Leishmania* infection (43).

The results of this study suggest that the profiles of other cytokines and cells involved in the immune response should be examined in future studies on patients with CL and other types of leishmaniasis. Having adequate knowledge of the role of the immune system in controlling or/and in the progression of the disease can be helpful in the treatment of patients and the preparation of appropriate vaccines.

## Conclusion

Our observations related to IFN-γ, TNF-α, and IL-10 cytokines in PBMCs isolated from a patient with CL recommended that these cytokines are essential regulators of immunity against *Leishmania* in humans. Due to the lack of a well-known immunological profile related to a cure, finding the special role of the host's immune response such as CD4+ cells responses and cytokines production in the control and pathology of leishmaniasis are highly important.

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## Conflict of interest

The authors are declared that there is not any conflict of interest.

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