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IL-8 and IL-23 Levels in Peripheral Blood Mononuclear Cells of Patients with Cutaneous Leishmaniasis Caused by *Leishmania major*: A Case-Control Study

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Abstract

Background: Several cytokines contribute to cutaneous leishmaniasis outcomes. IL-23 is a key cytokine in inflammation and a central cytokine in controlling Th17. IL-8, also known as a neutrophil chemotactic factor in the lesion site of cutaneous leishmaniasis, indicates disease promotion.
Objectives: This study aimed to evaluate the relationship of *Leishmania major* with IL-8 and IL-23 expression.
Methods: This case-control study was performed on 25 healthy individuals and 25 cutaneous leishmaniasis patients who lived in Southwestern Iran, in 2017. Peripheral blood mononuclear cells (PBMCs) were first isolated. Total RNA was extracted and reverse-transcribed into cDNA. The expression levels of IL-8 and IL-23 were measured by the real-time PCR. The data were statistically analyzed using SPSS version 16.
Results: Our findings indicated that the expression of IL-8 was significantly higher in the leishmaniasis group than in the control group with the median (IQR) of 0.39 (0.92) and 0.03 (0.11), respectively (P = 0.02). The expression of IL-23 was higher in the leishmaniasis group than in the control group with the median (IQR) of 0.13 (0.43) and 0.11 (0.61), respectively (P = 0.48). The increased IL-8 expression accompanied the location sites in face and hand and the increased number of skin lesions.
Conclusions: The expression of IL-8 in patients with cutaneous leishmaniasis is an index of increased activity of local neutrophils that can contribute to leishmaniasis survival or inflammation increase. However, IL-23 appears to be less important in the inflammatory reaction than IL-8.

Keywords: Complementary, Cutaneous, Cytokines, DNA, Human, Interleukin-8, Interleukin-23, Leishmaniasis, *Leishmania major*, Real-Time Polymerase Chain Reaction

1. Background

Cytokines play determining roles in susceptibility or resistance to leishmaniasis.

Interleukin (IL)-8 is a neutrophil-chemoattractant cytokine. Neutrophils play key roles against *Leishmania* protozoa in the lesion site. They are the first cells that reach the bite site of female *Phlebotomus* spp. to protect against the proliferation of Leishman bodies (1). Indeed, neutrophils have protective roles against all *Leishmania* spp. and help survive the disease. Although the exact mechanism of the increased survival of *Leishmania major* (*L. major*) within neutrophils is unclear, it seems that the reduction of dendritic cells and suppression of Th1 cells and CD8+ T cells function are important (2).

IL-23 as a pro-inflammatory cytokine is composed of two subunits, p19 (IL-23 alpha) and p40 (IL-12β) that is a subunit of IL-12 whereas IL-12 induces the development of Th1 cells. Naive CD4+ T cells differentiate into Th17 in the presence of IL-23. The activity of Th17 followed by the production of IL-17 stimulates the expression of IL-1, IL-6, TNF-α, and NOS-2 (3). IL-23 is increased in inflammatory diseases such as psoriasis, rheumatoid arthritis, spondyloarthritis, and Crohn's disease (3, 4). Anti-IL-23 monoclonal antibodies have been developed as a new therapeutic approach for psoriasis (5). Based on our knowledge, a few studies have focused on the interaction between IL23 and *L. major* in human samples.

The World Health Organization (WHO) estimates 0.7 to

1.2 million new cases of cutaneous leishmaniasis (CL) annually (6). Over a three-year period, 56546 people were identified with CL in Iran. Razavi Khorasan ranks third in the incidence rate of CL among 31 provinces of Iran (7).

There is no efficient vaccine for CL because of inadequate knowledge of immunity pathways against CL. Thus, studying immunological routs or responses during leishmaniasis helps find suitable immunotherapy or effective vaccine. It has been proven that IL23 and IL8 can influence leishmaniasis progression via the regulation of neutrophil recruitment in BALB/c mice (8).

2. Objectives

The roles of IL23 and IL8 are not completely clear in human leishmaniasis. Thus, in this study, we evaluated the role of IL-8 and IL-23 in the process of healing or developing infections caused by *L. major*.

3. Methods

3.1. Patients

This case-control study was performed on 30 patients with cutaneous lesions before any treatment who were selected from the Valfajr government health center (as a central referral health center) in Shiraz, Iran, as an endemic region of *L. major*. The number and location of lesions were recorded individually. Shiraz is the third most populous city of Iran located in the southwest of the country (https://en.wikipedia.org/wiki/Shiraz).

For each patient, the edge of each skin lesion was smeared using a sterile scalpel on a microscope slide, followed by air-drying, fixation with absolute ethanol, and staining with Giemsa. Some scraping wound was retained in labeled containers in 70% ethanol separately for molecular analysis. After the direct observation of slides, 10 mL of blood was taken from confirmed patients in EDTA tubes. The criteria for inclusion were having $\leq$ 4 cutaneous lesions and receiving no specific leishmaniasis treatment. Patients with treatment, pregnancy, or lactation were excluded from the study.

We also selected 25 age and sex-matched healthy people as the control group. The sample size was determined to include 25 individuals in each group based on the formula for comparison of two independent means with an alpha of 0.05 and a beta of 0.2 according to the mean values reported in similar studies of interleukin assessment (9, 10).

A convenience sampling method was used to select the cases from among all patients that referred to the Valfajr center after primary assessment and obtaining their consent. The control group was selected from among individuals who referred for other chief complaints matched for the age and sex of the cases. All the patients and healthy individuals were negative for intestinal parasitic diseases, diabetes, HIV, HBV, and T-lymphotropic virus type 1 (HTLV-I), or any autoimmune diseases. The study adhered to the tenets of the Declaration of Helsinki as approved by the Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.REC.1394.178). Written informed consent was obtained from all patients and healthy individuals.

3.2. PCR

After preparing direct Giemsa-stained smears from each patient, positive microscopic samples were scrapped for DNA extraction using the GeNet Bio Kit. The PCR was performed with initial denaturation for 5 min at 95°C, followed by 38 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s, ended by a final extension at 72°C for 7 min. In each round of PCR, we used *L. major* standard strain (MRHO/IR/75/ER) and *L. tropica* standard strain (MHOM/01/IR/YAZA) as positive controls and distilled water as a negative control. Statistical analysis of data was performed using the Chi-square test in IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, N.Y., USA). The primers of kDNA of *Leishmania* parasite included F: 5'TCGCAGAACGCCCCTACC 3' and R: 5'AGGGGTTGGTGTAAAATAGG 3', made by the Tuba Negin Company. DNA fragments amplified with these primers were seen at 615 bp for *L. major* and 744 bp for *L. tropica*.

All microscopes, PCR, and real-time PCR devices were calibrated every six months by the Kawsar Biotech Company, and they received certificates.

3.3. Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient Ficoll (Sigma-Aldrich, UK). A hemocytometer was used for cell counting, and 250 μL of lymphocytes in RPMI 1640 (PAA, Austria) was transferred into 2 mL of the culture medium. The cells were then added to 24-well plates and kept for 24 h at 37 °C in 5% CO_2. Next, the supernatant was collected and stored frozen until analysis for cytokines. Beta-2 microglobulin gene (β2M) as the control along with IL-23 and IL-8 was measured by the real-time PCR.

3.4. RNA Extraction and Complementary DNA Synthesis

Total RNA was extracted using Tripura according to the Roche Company protocol (CAT number 11667157001). Complementary DNA (cDNA) synthesis was performed using

the cDNA Synthesis Kit (Bioneer Company, Korea) according to its protocol. Real-time reverse transcriptase-PCR (real-time RT-PCR) analysis with the TaqMan Real-time RT-PCR was used to investigate the expression of β2M as the reference gene (Figure 1), as well as IL-8 and IL-23 (Figures 2 and 3). The qPCRs were performed in a 10-μL volume consisting of 2 μL of cDNA target, 4 μL of each primer (10 pmol) (Table 1), 2.2 μL of distilled water, and 5 μL of TaqMan supermix (Bio-Rad). Reactions were run on the Light Cycler 480 system (Roche). The thermal cycling conditions were as follows: 94°C for 3 min, 35 cycles at 94°C for 15 s, 60°C for 30 s, and 60°C for 30 s.

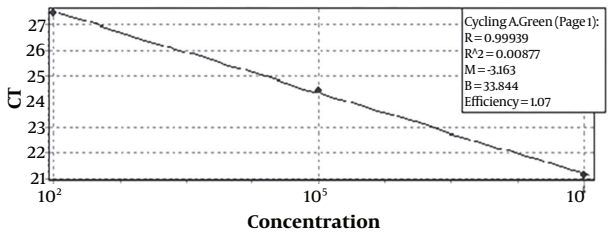

Figure 1. Representative standard curves for the β2M gene using the Light Cycler device

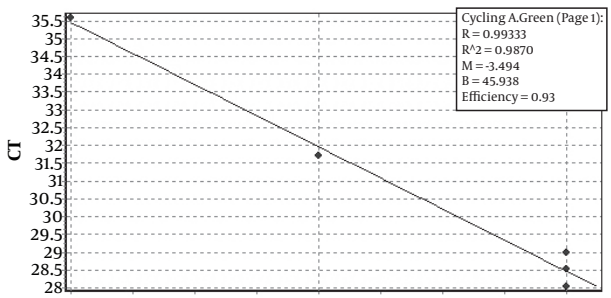

Figure 2. Representative standard curves for IL-8 using the Light Cycler device. All samples are shown in duplicate.

3.5. Statistical Analysis

The normality of quantitative data was assessed by the Shapiro-Wilk test. The mean/standard deviation (SD) was used for normally distributed quantitative variables and median/inter-quartile range (IQR) for non-normally distributed variables. For comparing quantitative data between the two groups, *t*-test or Mann-Whitney test was used based on the Shapiro-Wilk test P values using SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, ILL, USA). P values of < 0.05 were considered significant.

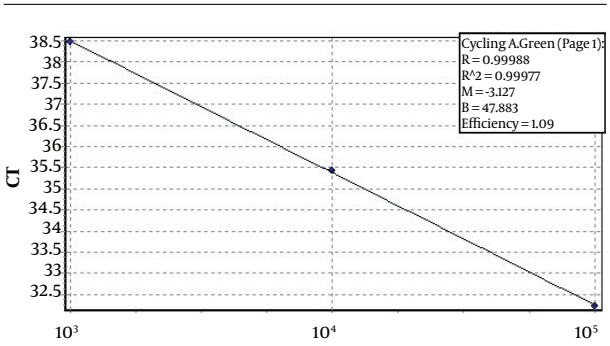

Figure 3. Representative standard curves for IL-23 using the Light Cycler device. All samples are shown in duplicate.

Table 1. Primer Sequences (5'→ 3') of IL- 8, IL-23, and β2M Used in the Real-time PCR

Primer Sequences	Length
β2M	
Forward: TTGTCTTTCAGCAAGGACTGG	
Reverse: CCACTTAACTATCTTGGGCTGTG	
IL- 8	77 bp
Forward: CGGAAGGAACCATCTCACTGTG	
Reverse: AGAAATCAGGAAGGCTGCCAAG	
Probe: TGACTTCCAAGCTGGCCGTGGCTC	
IL-23	122 bp
Forward: GCCTTCTCTGCTCCCTGATAG	
Reverse: TGGGACTGAGGCTTGGAATC	
Probe: TCTCCCAGTGGTGACCCTCAGGCT	

4. Results

Among suspected individuals who referred to the study setting, 25 (83%) patients were infected with *L. major* based on the appearance of the 615-bp band (Figure 4). These 25 patients (eight women (32%) and 17 men (68%) with a mean age of 32 $\pm$ 13 years) and 25 controls (matched for age, sex, and geographical location) were involved in further analysis.

The expression of IL-8 was significantly higher in the patient group than in the control group (P = 0.038). All samples were shown in duplicate with an SD of 0.3 for the patient group and SD of 0.041 for the control group (Figure 5). The median (IQR) and P values of IL-8 and IL-23 expression are shown in Table 2.

The locations of skin lesions are shown in Table 3. Lesions were most prevalent in hands (in 14 cases), faces, and feet. The median number (IQR) of lesions was 2 (2) in a range of 1 to 6 lesions. The location and the number of skin lesions had direct relationships with IL-8 expression.

Table 2. Median (IQR) and P Values of IL-8 and IL-23 Expression in the Patient and Control Groups

	Patients		Controls		PV
	Median	IQR	Median	IQR	
IL-8	0.39	0.92	0.03	0.11	0.02
IL23	0.13	0.43	0.11	0.61	0.48

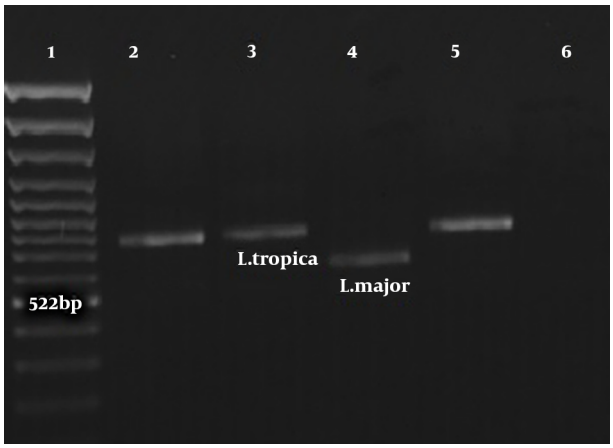

Figure 4. Lanes 1, ladder 100 pb; lane 2, *L. tropica* (positive control); Lane 3 and 5, *L. tropica*, lane 6: negative control; lane 4, *L. major*.

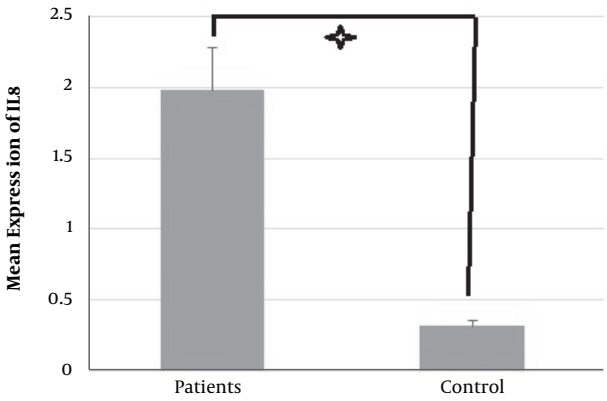

Figure 5. Comparison of mRNA expression levels of IL-8 in patients and healthy controls in human peripheral blood mononuclear cells analyzed by the real-time PCR; significant differences at $P < 0.05$.

Patients with lesions in face and hand had significantly higher levels of IL-8 ($P = 0.042$). Finally, the expression of IL-23 was insignificantly higher in the patient group than in the control group ($P = 0.48$). All samples are shown in duplicate with an SD of 0.2 for the patient group and SD of 0.03 for the control group (Figure 6).

Table 3. Location of Skin Lesions in Patients with Cutaneous Leishmaniasis Caused by *Leishmania major*

Location	Frequency[a]
Face	4 (16.0)
Body	2 (8.0)
Hand	8 (32.0)
Foot	5 (20.0)
Face and hand	2 (8.0)
Hand and foot	4 (16.0)
Total	25 (100.0)

[a]Values are expressed as No. (%).

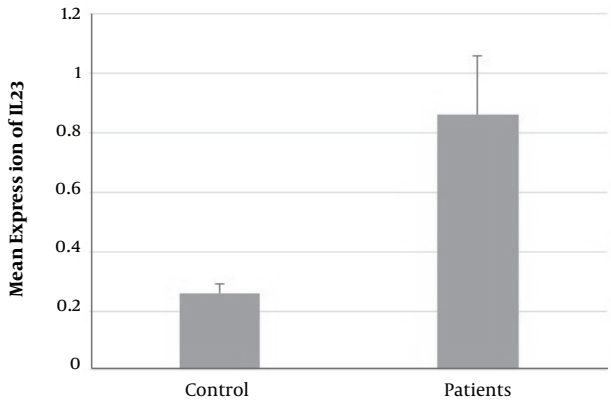

Figure 6. Comparison of mRNA expression levels of IL-23 in patients and healthy controls in peripheral blood mononuclear cells analyzed by the real-time PCR.

5. Discussion

In this study, we evaluated the expression of IL-8 and IL-23 in patients infected with *L. major*. In a previous study, the level of IL-23 was significantly higher in the healing form of CL than in the non-healing form of lesions ($P < 0.001$) (11). It was shown that *L. major* strongly stimulated IL-17 and IL-23 could also induce the production of IL-17 (5). IL-17 contributes more than IFN-γ in the pathology and inflammation of experimental leishmaniasis in mice (12). In C57BL/6 mice, the secretion of IL-17A and IL-17F does not influence the disease progression. It appears that, depending on the genetic background, cytokines of the IL-17 family might be

responsible for disease progression primarily in susceptible mice (13). IL-23 stimulated IL-17 production following disease progression to protect patients against *Leishmania* infection along with inflammatory responses (8, 11). IL-23 production by *L. major* in infected BALB/c mice maintains IL-17+ cells that influence disease progression via the regulation of neutrophil recruitment (8).

IL-8 has an important role in neutrophil recruitment. Neutrophils play a dual role at the site of infection with *L. major*. In susceptible mice, they decreased to 2% within 72 h post-infection but in resistant mice, they were detected for more than 10 days after parasite inoculation at the lesion site (14). Therefore, they can fight *L. major* in the early stages of infection. In another study, IL-8 was elevated by 22.5 folds in leishmaniasis patients caused by *L. tropica* compared to healthy individuals. (15). Moreover, neutrophil cells could promote *L. major* survival (16, 17). The higher number of polymorphonuclear cells was detected in the lesion site of *L. major* than in ulcers generated by other *Leishmania* spp. probably because of the higher levels of IL-8 mRNA within Zoonotic Cutaneous Leishmaniasis (ZCL).

It seems that the elevation of IL-8 is more important than the elevation of IFN-γ in the immunopathogenesis of ZCL (18). The level of IL-8 in visceral leishmaniasis elevated after infection and decreased 30 days after infection (19). In 1996, it was shown that the high expression of IL-8 was induced within one-hour post-infection with *L. major* (20). In another study, the level of IL-8 decreased after treatment of *L. tropica*. It seems that IL-8 is an effector cytokine of immune regulation in the progression of CL (21).

In addition, it was shown that infection with *L. major* could lead to the enhanced production of LTB4 (pro-inflammatory lipid mediator for the recruitment of neutrophils) along with IL-8 following acute inflammation (16). In an array study of immune responses against *L. tropica*, both IL-23 and IL-8 showed highly significant elevated expression, in agreement with histochemistry report that showed infiltration of neutrophils and strong IL-8 expression in lesion patients (15, 21). In our study, IL-23 was higher in the patient group than in controls but not significantly. Th17 activity may be more associated with *L. tropica* than with *L. major*.

We used the beta-2 microglobulin (β2-MG) gene as reference for the normalization of our method. Boeuf et al. in 2005 showed that β2-MG was a more suitable gene for data normalization in the real-time PCR than UBC and YWHAZ endogenous genes. They showed that β2-MG transcripts are stable with less than 12% variation, whereas transcript levels of UBC and YWHAZ had up to 47% of the variability. Thus, β2-MG can be chosen as a reliable internal standard gene for reference (22).

The limitations of the study included the unclear duration of lesions and the limited size of the sample. In addition, it would be better to evaluate immune responses in the site of infected tissue not in cells from the peripheral blood. The strong point of the study was the clarification of the roles of IL-8 and IL-23 in CL caused by *L. major* despite a few studies available. Performing further studies on the change of IL-23 expression after treatment of persistent CL lesions and changes of IL-8/IL-23 in patients with multiple widespread lesions is suggested in the future.

5.1. Conclusions

Since IL-23 as a pro-inflammatory cytokine did not change significantly in CL patients, the inflammation in this population might not be associated with Th17 activity. On the other hand, IL-8 expression was around seven times higher in CL patients than in healthy controls. Furthermore, as the higher mean number of lesions was associated with more IL-8 expression, it is more likely that neutrophils and lymphocytes both are responsible for the development and progression of CL. Taken together, these results demonstrate that neutrophils after being stimulated with the parasite content can release IL-8 and other neutrophil activators. In addition, chemoattractants can mediate monocyte and T cell accumulation (J. Clin. Invest. 1996, 97:1931 - 1941) at the site of inflammation, which can worsen the activities. In such a situation, neutrophils release the granule content including lytic enzymes and oxygen active radicals to the extracellular milieu when neutrophils are stimulated with IL-8.

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Footnotes

Authors' Contribution: Aliakbar Shamsian was involved in study concept, design, and critical revision of the manuscript. Seyed Abdolrahim Rezaee helped in the analysis and interpretation of data. Elham Moghaddas contributed to the writing of the manuscript. Narges Khazaee performed all laboratory examinations.

Conflicts of Interests: The authors declare that they have no conflicts of interest.

Ethical Approval: The study adhered to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.REC.1394.178).

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Patient Consent: Written informed consent was obtained from all patients and healthy individuals.

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