



Research Paper

Molecular Investigation of Pro-inflammatory and Anti-inflammatory Cytokines Gene Expression in Macrophages Exposed to *Leishmania major*

Fatemeh Babaei Dehaghi¹ , Sahar Hamoonnavard^{1*} , Hossein Rezvan¹ , Mahya Fekri¹, Faezeh Baniasadi¹ , Atefeh Sharifirad¹ ,
Mohammad Parsa Miadfar¹

1. Department of Pathobiology, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamadan, Iran.



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ABSTRACT

Introduction: Cutaneous leishmaniasis (CL) is an infectious skin disease that affects people all over the world. The innate and adaptive immune response generated against the parasite in the host is effective during the treatment period and wound healing process. The production of pro-inflammatory and anti-inflammatory cytokines plays a central role in susceptibility and resistance to the pathogen.

Materials & Methods: Peritoneal macrophage cells were harvested from the peritoneal cavity of BALB/C mice and exposed to *Leishmania major* parasite (MHOM/IR/75/ER) at three time points (24, 48, and 72 hours). Gene expression of *TNF-α*, *IL-12*, *CXCL-9*, *CXCL-10*, *IL-10*, and *TGF-β* cytokines was analyzed by real-time PCR.

Results: The expression of *IL-10* as an anti-inflammatory cytokine was higher than that of inflammatory cytokines during the three treatment periods (24, 48, and 72). The expression of *IL-p35* was also high, but not *IL-p40*. The expression of *CXCL-9* (crucial for the recruitment of immune T cells) was also upregulated ($P \leq 0.05$). The gene expression of *TNF-α* was low at three different time points, especially after 72 hours of exposure, and the level of *TGF-β* expression increased significantly after 72 hours, and anti-inflammatory cytokine expression was higher than that of pro-inflammatory cytokines ($P \leq 0.05$).

Conclusion: Pro-inflammatory and anti-inflammatory cytokines have a critical role in the treatment of *L. major* infections. Pro- and anti-inflammatory cytokine production is related to the mechanism of suppression of cellular immune responses mediated by Th2 lymphocytes during disease progression. Evaluation of macrophage cytokine gene expression may be indicative of cytokine expression by macrophage cells as a major factor in the host defense involved in CL and is important for studies on the pathogenesis of the disease.

* Corresponding Author:

Sahar Hamoonnavard, Professor Assistant.

Address: Department of Pathobiology, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamadan, Iran.

Tel: +98 (81) 31406017

E-mail: s.hamoonnavard@basu.ac.ir



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1. Introduction

Cutaneous leishmaniasis (CL) is a neglected tropical disease and the most common cause of skin lesions worldwide. The most prevalent clinical form of leishmaniasis is, Visceral leishmaniasis (VL), disseminated leishmaniasis (DL), mucocutaneous leishmaniasis (ML), disseminated cutaneous leishmaniasis (DCL), and CL [1]. Macrophages and neutrophils phagocytose the parasite and play a central role as the first host cells against *Leishmania* infection [2]. Differences in immune responses that produce pro-inflammatory, anti-inflammatory cytokines, and chemokines play a central role in determining the effective immune response to eliminate the *Leishmania* infection [3]. Immune responses triggered by Th1 lymphocytes with cytokines such as TNF- α , IFN- γ , and IL-12 lead to repair of the *Leishmania*-induced lesion, while the action of Th2 lymphocytes and anti-inflammatory cytokines such as TGF- β and IL-10 leads to disease progression and resistance to treatment. The production of chemokines by macrophages may be important for the recruitment of Th1 lymphocytes and other leukocytes to parasite elimination [3]. The critical role of macrophages as the first cells against *Leishmania* parasites has been recognized in targeted therapy and vaccine-related studies aimed at inducing protective immune responses [4]. The interaction between macrophages and primary promastigotes leads to the internalization of promastigotes by phagocytosis and the formation of a parasitic vacuole where the parasites transform into amastigotes. These amastigotes eventually lyse macrophages, and are themselves susceptible to phagocytosis [5]. Parasite species affect the ability of macrophages to interact with CD8⁺ T cells, and the immunopathology of CL results in skin wound healing due to their cytotoxicity effects [6]. The IL-12 cytokine, reactive oxygen species (ROS), and other types of nitrogen radicals are released from infected macrophages to promote the development of IFN- γ production by CD4⁺ Th1 cells. Infected macrophages are activated by IFN- γ from Th1 cells to eliminate intracellular *Leishmania* parasite. On the other hand, infected macrophages and Treg cells produce TGF- β and IL-10 as immunoregulatory cytokines, which inactivate many infected cells and contribute to the destruction of the parasite [7]. Cytokines are effective in generating immune responses with synergistic or antagonistic effects in the control of CL, where the cytokine expression profile is important in susceptibility or resistance to the disease [8]. The study of pro- and anti-inflammatory cytokines from macrophages may be helpful in the development of therapeutic and preventive strategies. In the present

study, pro- and anti-inflammatory cytokines secreted by macrophage cells were measured in response to *Leishmania* infection.

2. Materials and Methods

2.1. Parasite

Leishmania major strain (MHOM/IR/75/ER) from the Pasteur Institute of Iran was cultured in T25 flasks containing RPMI-1640 medium, 5,000 IU/mL penicillin, 5,000 μ g/mL streptomycin, and 10% fetal bovine serum (FBS) at 24 °C.

2.2. Isolation and cultivation of peritoneal macrophage cells

Peritoneal fluid was collected from the peritoneal cavity of BALB/c mice using cold normal saline containing penicillin/streptomycin (5%). After centrifugation and counting of macrophage cells, 1×10^5 cells were cultured in each well of a 12-well plate with RPMI-1640 medium supplemented with 20% FBS. After 24 hours, the supernatant was replaced, and the macrophage cultures were kept in an incubator at 37 °C for three days.

2.3. Exposure of macrophage cells to *Leishmania* parasites

After counting *L. major* parasites, 2×10^5 parasites were added to each of well containing macrophage cells. Exposure was carried out for 24, 48, and 72 hours at 37 °C in 5% CO₂. Macrophage cells were trypsinized and, after centrifugation, resuspended in 500 μ L of phosphate-buffered saline (PBS) and stored at -20 °C.

2.4. RNA extraction of macrophage cells and complementary DNA (cDNA) synthesis

To extract total RNA from macrophage cells, the RNA extraction kit of Rana Biotechnology Company (RNA Biotech Co., Isfahan, Iran) was used. 1,000 μ L of extraction buffer was added to 2×10^5 infected cells. After sonication of the cells, 200 μ L chloroform was added. Contents were precipitated in ethanol 80 and 100% ethanol and centrifuged at $10,000 \times g$; total RNA was then resuspended in 20 μ L distilled water. cDNA was synthesized from total RNA (10 μ g) using a reverse transcription kit (RB MMLV reverse transcriptase, RNA Biotech Co., Isfahan, Iran). Briefly, 500 ng of total RNA was added to 200 U M-MLV RT, 1 μ g oligo (dT), 10 Mm dNTPs, and 5 $\times$ RT buffer. Nucleic acids and other components were incubated at 50 °C for 50 min, and the reaction inactivated at 72 °C for 15 min.

Table 1. Primer sequences used for quantitative RTPCR

No	Genes		Sequence
1	<i>IL-12 p40</i>	Forward	CTGCTGCTCCACAAGAAGGA
		Reverse	ACGCCATTCCACATGTCACT
2	<i>IL-12 p35</i>	Forward	ATGATGACCCTGTGCCTTGG
		Reverse	CACCCTGTTGATGGTCACGA
3	<i>IFN-γ</i>	Forward	GCTCTGAGACAATGAACGCT
		Reverse	AAAGAGATAATCTGGCTCTGC
4	<i>TNF-α</i>	Forward	TATAAAGCGGCCGTCTGCAC
		Reverse	TCTTCTGCCAGTCCACGTC
5	<i>IL -10</i>	Forward	AGCCGGGAAGACAATAACTG
		Reverse	CATTTCGATAAGGCTTGG
6	<i>TGF-β</i>	Forward	CTTGGTGTGAGAGCCTCACC
		Reverse	GGGGTCTCCCAAGGAAAGGT
7	<i>CXCL-9</i>	Forward	CTTTCTCTTGGGCATCAT
		Reverse	GCATCGTGCATTCTTATCA
8	<i>CXCL-10</i>	Forward	GCTGCCGTCATTTTCTGC
		Reverse	TCTCACTGGCCCGTCATC
9	<i>GAPDH</i>	Forward	GCCAAAAGGGTCATCATCTC
		Reverse	CACACCCATCACAAACATGG

2.5. Real-time polymerase chain reaction (RT-PCR)

The primers of, *TNF- α* , *IFN- γ* , *IL-12*, *IL-10*, *TGF- β* , *CXCL-9*, *CXCL-10*, and *GAPDH* genes were synthesized and used for real-time PCR. Quantitative RT-PCR was performed using the SYBR Green reverse transcription PCR with 5 μ L of 2x Master Mix kit (Applied Biosystems), 10 μ g cDNA, 0.3 μ L (500 nM) of each primer (Table 1), in a total reaction volume of 10 μ L with distilled water. The PCR amplification was carried out under the following program: 95 °C for 5 min, followed by 35 cycles consisting of 94 °C for 30 s and 54 °C/58 for 30 s. Finally, the $\Delta\Delta C_t$ method was used for data analysis.

2.6. Statistical analysis

Data were analyzed using GraphPad Prism software, version 8 by two-way ANOVA followed by Tukey's post-hoc test ($P \leq 0.05$).

3. Result

3.1. Culture of peritoneal macrophage cells

After aspiration of the peritoneal cavity fluid with cold sterile saline, the macrophage cells attached to the flasks after 24 hours of incubation. After three days of growth in the culture medium, they were used for parasite exposure (Figure 1).

3.2. Gene expression of pro-inflammatory and anti-inflammatory cytokines in macrophages exposed to *L. major* parasite

Pro-inflammatory and anti-inflammatory cytokine gene expressions were affected at different exposures, *TNF- α* , *TGF- β* and, *CXCL10* had a very low expression level at different times from 24 to 72 hours, so the ΔC_T data showed that the expression level of these genes was negative (Figure 2). The expression of *IL-10* and *CXCL9* genes was significantly increased compared to other cy-

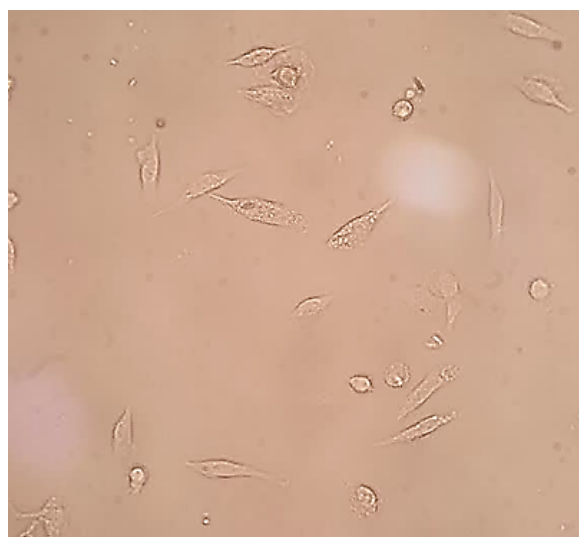


Figure 1. Macrophages cultured from the peritoneal cavity of BALB/c mice after exposure to *L. major* parasite

tokines ($P \leq 0.05$). *IL-p35*, a subunit of the *IL-12* protein, was also expressed more than other inflammatory cytokines. A high expression level of *IL-p35* was observed at 24 h after exposure, but *IL-p40* was not expressed. The expression level of *IL-p35* was similar to that of *IL-10* and *CXCL10* (Table 2). The change in the expression of the cytokines *IL-p40*, *TNF- α* , *CXCL9*, *CXCL10*, and *IL-10* was not significant at the three time points, but the change in the expression level of *TGF- β* and *IL-p35* across the three different time points showed significant differences (Table 2, Figure 3). *IL-p40* decreased from a positive level at 24 h of exposure, with a significant change compared to 48 and 72 h ($P \leq 0.05$, Figure 3). *CXCL9* and *IL-10* had an unchanged expression level at three different treatment time points. *TNF- α* , as one of the pro-inflammatory cytokines, showed a decrease

in expression at three different time points, but the p35 subunit of *IL-12* increased its expression at 24 h after exposure and then decreased significantly at 48 h and 72 h ($P \leq 0.05$). In addition, an increase in the expression of suppressive and anti-inflammatory cytokines, including *IL-10*, was observed with increasing exposure time, but pro-inflammatory cytokines were decreased. The expression of *CXCL9*, but not *CXCL10*, was increased at the three exposure times (Table 3).

4. Discussion

Macrophages and neutrophil cells are the first line of phagocytic cells against *Leishmania* promastigotes and initiate an innate immune response with pro-inflammatory mediators [9]. Macrophages are capable of secret-

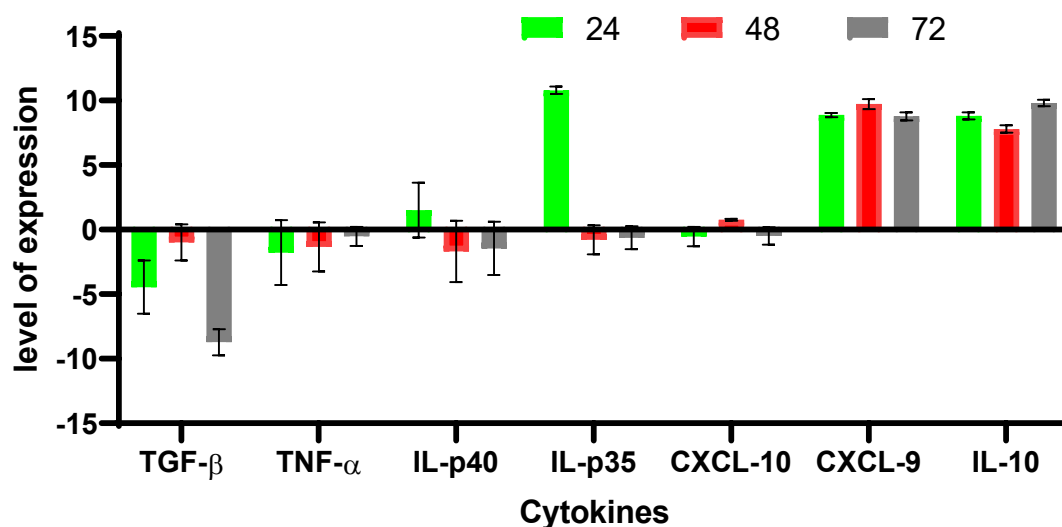


Figure 2. The expression level of cytokines at different times of exposure to *L. major*

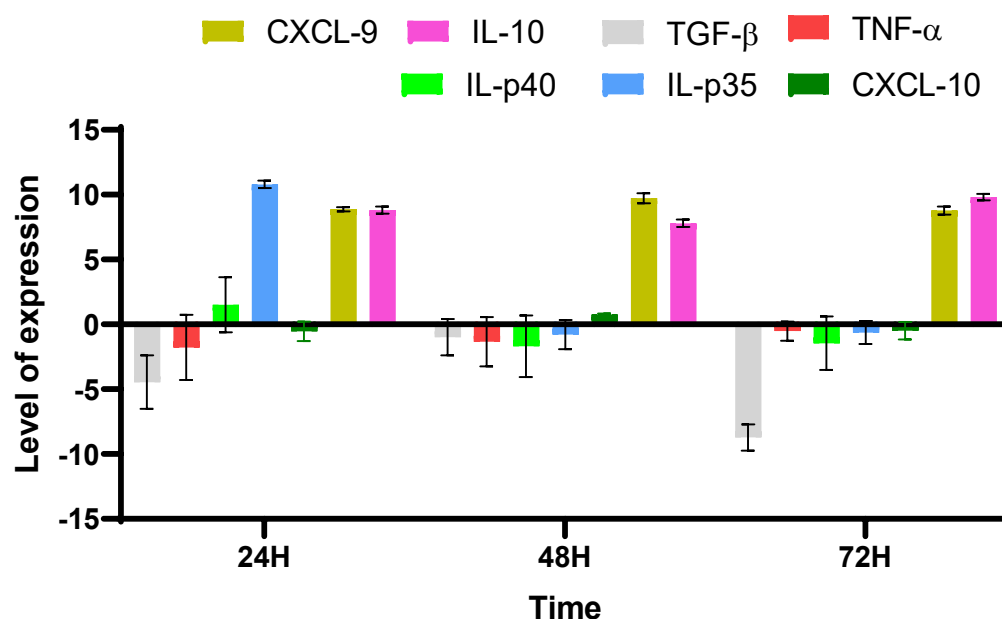


Figure 3. The expression level of each cytokine at different times of exposure to *L. major*

ing a variety of cytokines such as $TNF-\alpha$, IL-1, IL-6, IL-12, IL-8, leukotrienes, and prostaglandins, which are directed against microbial pathogens and, in some cases, can lead to septic shock if produced inappropriately [10]. In our previous studies, in vivo pro-inflammatory and anti-inflammatory gene expressions were investigated after CL treatment with mesenchymal stem cells. We observed changes in the expression and production of cytokines in the control of *Leishmania* infection [11, 12].

IL-12 consists of two subunits (p35 and p40) produced by monocytes and macrophages, which are paired together after synthesis, and the absence of one of the subunits leads to the progression of infection. IL-12 plays an essential role in the control of *Leishmania* infection by promoting the Th1 lymphocyte-mediated response in leishmaniasis through IFN- γ production to enhance macrophage function. IL-12 deficiency leads to a shift in the Th2 lymphocyte-mediated response, and immune responses fail to develop Th1 cells [13]. IL-12 is secreted under the influence of Cdc42, which also influences the secretion of $TNF-\alpha$ [14]. In this study, the expression of the $TNF-\alpha$ gene was very low, and the IL-12 subunits were not expressed in a regular pattern. The secretion of IL-12 was detected after 24 h of exposure, and the expression of IL-12 was suppressed with increasing exposure time. Studies have suggested that CR3 (complement receptor 3, involved in phagocytosis) reduces the release of IL-12 during silent macrophage invasion by *L. major*, and that internalization receptors on macrophages are responsible for differences in IL-12 release

[15]. $TNF-\alpha$ is an inflammatory cytokine involved in the elimination of parasites within macrophages through increased nitric oxide and polarization of macrophages to type 1 (M1) in CL infection. M1 macrophages eliminate *Leishmania* in the phagolysosome and accelerate the wound healing process by increasing oxidative function [16], our results also confirmed these findings at three different exposure times, so that $TNF-\alpha$ expression was reduced after 24, 48, and 72 hours of exposure to macrophages with *Leishmania* parasites. M2, or alternatively activated macrophages, are divided into four subgroups (M2a, M2b, M2c, and M2d). M2d secretes angiogenic and anti-inflammatory factors such as vascular endothelial growth factor (VEGF), IL-10, as well as CCL5, CXCL10, and CXCL16 chemokines, and produces low levels of $TNF-\alpha$, IL-12, and TGF- β cytokines [17]. The expression of $TNF-\alpha$ and TGF- β in macrophages exposed to *Leishmania* was also low in our study. Thus, the activation and function of macrophages depend on their polarization and proliferation and may influence the immune system response through the production of different types of cytokines [18]. Persistence of the parasite in M1 macrophages has also been observed with increased oxidative activity, and this may be one reason for *Leishmania*'s resistance to nitric oxide [4]. TGF- β promotes the progression and persistence of *Leishmania* infection by suppressing and regulating inflammatory responses. TGF- β inhibits the differentiation of Th1 cells and macrophage function by reducing and preventing the production of IFN- γ by Th1 lymphocytes. In one

Table 2. Expression of cytokine genes in different times of exposures to *L. major* parasite

Tukey's Multiple Comparisons Test	24 H		48 H		72 H	
	Sig.	P	Sig.	P	Sig.	P
<i>TGF-β</i> vs <i>TNF-α</i>	NS	0.4130	Ns	>0.9999	****	<0.0001
<i>TGF-β</i> vs <i>IL-p40</i>	**	0.0027	Ns	0.9979	***	0.0003
<i>TGF-β</i> vs <i>IL-p35</i>	****	<0.0001	Ns	>0.9999	****	<0.0001
<i>TGF-β</i> vs <i>CXCL-10</i>	NS	0.0814	Ns	0.8269	****	<0.0001
<i>TGF-β</i> vs <i>CXCL-9</i>	****	<0.0001	****	<0.0001	****	<0.0001
<i>TGF-β</i> vs <i>IL-10</i>	****	<0.0001	****	<0.0001	****	<0.0001
<i>TNF-α</i> vs <i>IL-p40</i>	NS	0.2038	NS	>0.9999	NS	0.9904
<i>TNF-α</i> vs <i>IL-p35</i>	****	<0.0001	NS	0.9994	NS	>0.9999
<i>TNF-α</i> vs <i>CXCL-10</i>	NS	0.9579	NS	0.6770	NS	>0.9999
<i>TNF-α</i> vs <i>CXCL-9</i>	****	<0.0001	****	<0.0001	****	<0.0001
<i>TNF-α</i> vs <i>IL-10</i>	****	<0.0001	****	<0.0001	****	<0.0001
<i>IL-p40</i> vs <i>IL-p35</i>	****	<0.0001	NS	0.9919	NS	0.9950
<i>IL-p40</i> vs <i>CXCL-10</i>	NS	0.7083	NS	0.5171	NS	0.9878
<i>IL-p40</i> vs <i>CXCL-9</i>	***	0.0002	****	<0.0001	****	<0.0001
<i>IL-p40</i> vs <i>IL-10</i>	***	0.0003	****	<0.0001	****	<0.0001
<i>IL-p35</i> vs <i>CXCL-10</i>	****	<0.0001	NS	0.8914	NS	>0.9999
<i>IL-p35</i> vs <i>CXCL-9</i>	NS	0.7610	****	<0.0001	****	<0.0001
<i>IL-p35</i> vs <i>IL-10</i>	NS	0.7292	****	<0.0001	****	<0.0001
<i>CXCL-10</i> vs <i>CXCL-9</i>	****	<0.0001	****	<0.0001	****	<0.0001
<i>CXCL-10</i> vs <i>IL-10</i>	****	<0.0001	***	0.0004	****	<0.0001
<i>CXCL-9</i> vs <i>IL-10</i>	NS	>0.9999	ns	0.7530	NA	0.9823

NS: Non significant.

Table 3. Tukey's multiple comparisons test

Time	Genes						
	<i>TGF-β</i>	<i>TNF-α</i>	<i>IL-p40</i>	<i>IL-p35</i>	<i>CXCL-10</i>	<i>CXCL-9</i>	<i>IL-10</i>
24 H vs 48 H P	* 0.0373	NS 0.9406	NS 0.0584	**** <0.0001	NS 0.5919	NS 0.7962	NS 0.7213
24 H vs 72 H P	** 0.0099	NS 0.6086	NS 0.0834	**** <0.0001	NS 0.9992	NS 0.9964	NS 0.7213
48 H vs 72 H P	**** <0.0001	NS 0.8068	NS 0.9824	NS 0.9920	NS 0.6156	NS 0.7498	NS 0.2876

NS: Non significant.

study, *TGF- β* expression was measured in skin tissue with *Leishmania* infection and was found to be highly expressed in skin, spleen, and liver [19]. In addition, several studies have reported that the expression of *TGF- β* and *IL-10* increases in long-lasting lesions of cutaneous leishmaniasis [20]. Hence, the expression of *TGF- β* was low at three different exposure times in our study due to the evaluation of *TGF- β* in vitro in macrophages. *IL-10* is associated with the progression of leishmaniasis and is one of the reasons for host susceptibility to the *Leishmania* parasite through anti-inflammatory effects, and it causes a Th2 lymphocyte-mediated response in BALB/c mice [21]. The expression of inflammatory cytokines was lower than that of anti-inflammatory factors in this study. *IL-10* was upregulated with increasing duration of exposure and was significantly higher than inflammatory cytokines such as *TNF- α* and *IL-12* (Figure 2, Table 2). In the absence of *IL-10*, the severity of *Leishmania* infection in the skin is reduced and healing is accelerated. This cytokine suppresses macrophage function and is associated with parasite persistence [22].

Phagocytosis of parasites leads to the production of various chemokines by macrophages. Chemokines increase the activity of integrins in the migration of leukocytes to peripheral inflammatory tissues as part of the immune response. CXCL10 is mainly produced by monocytes, endothelial cells, fibroblasts, and it recruits macrophages and monocytes to the site of inflammation. Th1 lymphocytes are recruited by CXCL9 and CXCL10 during active leishmaniasis infection [23]. Although the positive correlation between CXCL9, CXCL10 chemokines in pulmonary tuberculosis was revealed, similar to the pathogenesis of leishmaniasis, this correlation was not observed in the present study. IFN- γ affects the function of macrophage phagocytosis and helps to eliminate the pathogen [24], although in some studies the stability of the parasite in the macrophage phagosome is considered necessary for the maintenance of long-term memory [4], the profile of cytokines produced determines the type of response (susceptibility or resistance) to *Leishmania* infection. Future studies are proposed to investigate the mechanism of the immune response induced by macrophages treated with *Leishmania* parasites in vivo, to alter the wound healing process in *Leishmania* as a potential cell therapy method.

5. Conclusion

The interaction between pro- and anti-inflammatory cytokines, as well as chemokines, is effective in recruitment of Th1 lymphocytes in the pathogenesis of cutaneous leishmaniasis and represents a critical factor in the

healing process of CL, parasite clearance, and acceleration of treatment. Our results showed an upregulation of anti-inflammatory cytokine production in macrophages exposed to *Leishmania* parasites at three different time points. Considering the quantitative expression of cytokine genes, this provides a good perspective for the study of macrophages as key cells in the prevention and treatment of cutaneous leishmaniasis, and it is possible that their accurate assessment will be important for a multifaceted investigation in prevention and treatment.

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Compliance with ethical guidelines

This study was approved by the Research Ethics Committees of Bu-Ali Sina University, Hamadan, Iran (Code: IR.BASU.REC.1403.056).

Data availability

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

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Authors' contributions

Conceptualization and study design: Hossein Rezvan and Sahar Hamoonnavard; Experiments: Fatemeh Babaei Dehaghi, Mahya Fekri, Faezeh Baniasadi, and Mohammad Parsa Miadfar; Data interpretation and analysis: Sahar Hamoonnavard; Statistical analysis: Sahar Hamoonnavard and Atefeh Sharifirad; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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