Molecular investigation of pro-inflammatory and anti-inflammatory cytokines gene expression in macrophages exposed to *Leishmania major*

Running title: Cytokines gene expression in macrophages exposed to *Leishmania major*

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Abstract

Cutaneous leishmaniasis (CL) is an infectious skin lesion that affects people all over the world. The innate and specific 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 play a central role in susceptibility and resistance to the pathogen. Peritoneal macrophage cells were harvested from the ventricular 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 analysed by real-time PCR. The expression of the IL-10 as anti-inflammatory cytokines 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-value*≤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 gene increased significantly after 72 hours and anti-inflammatory cytokines was higher than that of inflammatory cytokines (*P-value*≤0.05). Inflammatory and anti-inflammatory cytokines have an critical role in the treatment of *Leishmania 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 macrophage gene expression of cytokines 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.

Keywords: Inflammatory and anti-inflammatory cytokines, macrophage, *Leishmania major*

1. Introduction

Cutaneous leishmaniasis (CL) is a neglected tropical disease and most common cause skin lesions of people worldwide. Visceral leishmaniasis (VL), disseminated leishmaniasis (DL), mucocutaneous leishmaniasis (ML), disseminated cutaneous leishmaniasis (DCL) and CL (1). Macrophages and neutrophils phagocytos the parasite and play an central role as the first host cell against Leishmania infection (2). Differences in immune responses to 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, IL-12 and 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 other leukocytes (Th1) to parasite elimination (3). The critical role of macrophages as the first cells against Leishmania parasites has been recognised in targeted therapy and vaccine-related studies to induce 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 change to amastigotes, which eventually lyse macrophages, and these amastigotes are susceptible to phagocytosis (5). Parasite species affected on the ability of macrophages to absorb $CD8^+$ T cells and the immunopathology of CL lead to skin wound healing due to their cytotoxicity effect (6). The IL-12 cytokine, reactive oxygen species and other types of nitrogen radicals release from infected macrophages to development of IFN-γ from $CD4^+$Th1 cells. Infected macrophages activated by IFN-γ from Th1 cells to eliminate the intracellular Leishmania parasite. On the other hand, infected macrophages and Treg cells produce TGF-β and IL-10 as immunoregulatory cytokines which inactivate most infected cells and lead to 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 was measured against Leishmania infection.

2. Materials and methods

2.1. Parasite

Leishmania major strain (MHOM/IR/75/ER) from the Pasteur Institute of Iran were cultured in flasks $_{T25}$ containing RPMI-1640 medium, 5000 IU/ml penicillin, 5000 µg/ml streptomycin antibiotics and 10% of fetal bovine serum(FBS) at 24 ºC.

2.2. Isolation and cultivation of peritoneal macrophage cells

Peritoneal fluid were collected from peritoneal cavity of BALB/c mice using cold normal saline containing 5% penicillin/streptomycin. 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 with 20% FBS. After 24 hours, the supernatant of the macrophage cells was changed and kept in an incubator at 37ºC for 3 days.

2.3. Exposure of macrophage cells to Leishmania parasite

After counting *Leishmania major* parasites, 2×10^5 parasites were added to each of the wells containing macrophage cells. Exposure was done for 24, 48 and 72 hours at 37ºC and 5% CO2. Macrophage cells were trypsinized and after centrifugation were dissolved in 500 ul of phosphate buffer saline (PBS) and stored at -20ºC.

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

To extract total RNA from macrophage cells, the RNA extraction kit of Rena Biotechnology Company (RNA Biotech, Co, Isfahan, Iran) were used. 1000ul of extraction buffer was added to 2×10^5 infected cells, after sonication of the cells, 200ul chloroform was added. Contents resolved in Ethanol 80 and 100% and centrifuged at 10000g then total RNA resuspended in 20ul distilled water. cDNA was synthesized from total RNA (10 μg) using a reverse transcription (RB MMLV reverse transcriptase kit, RNA Biotech, Co, Isfahan, Iran). Briefly, 500ng of total RNA was added to 200U/ul M-MLV RT, 1ug/ul oligo T, 10Mm dNTP, 5X RT buffer. Nucleic acids and other compounds incubated at 50ºC for 50 min, then reaction inactivate at 72ºC for 15min.

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 performed by the SYBR Green reverse transcription (RT)-PCR with 5 ml of 2x Master Mix kit (Applied Bio-systems), 10ug cDNA, 0.3ul (500nM) each primer (table 1) in a total up to 10ul with D.W. The PCR amplification were done on the following program: 95ºC for 5min and 35 cycles consisting of 94˚C for 30s, 54˚C/58 for 30s. Finally, ΔΔCt calculation has been used for this approach.

Table1: Sequence of the primers

No	Genes		Sequence
1	**IL-12 p40**	Forward	CTGCTGCTCCACAAGAAGGA
		Reverse	ACGCCATTCCACATGTCACT
2	**IL-12 p35**	Forward	ATGATGACCCTGTGCCTTGG
		Reverse	CACCCTGTTGATGGTCACGA
3	**IFN-γ**	Forward	GCTCTGAGACAATGAACGCT
		Reverse	AAAGAGATAATCTGGCTCTGC
4	**TNF-a**	Forward	TATAAAGCGGCCGTCTGCAC

		Reverse	TCTTCTGCCAGTTCCACGTC
5	**IL -10**	Forward	AGCCGGGAAGACAATAACTG
		Reverse	CATTTCCGATAAGGCTTGG
6	**TGF-β**	Forward	CTTGGTGTCAGAGCCTCACC
		Reverse	GGGGTCTCCCAAGGAAAGGT
7	**CXCL-9**	Forward	CTTTTCCTCTTGGGCATCAT
		Reverse	GCATCGTGCATTCCTTATCA
8	**CXCL-10**	Forward	GCTGCCGTCATTTTCTGC
		Reverse	TCTCACTGGCCCGTCATC
9	**GAPDH**	Forward	GCCAAAAGGGTCATCATCTC
		Reverse	CACACCCATCACAAACATGG

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106 **Statistical analysis**

107 Data were analyzed using GraphPad prism 8 by Two-way ANOVA following Tukey's Post-Hoc
108 test (*P-value*≤0.05).

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110 **3. Result**

111 **3.1.Culture of peritoneal macrophage cells**

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

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120 **Figure 1: Macrophages cultured from the peritoneal cavity of BALB/c mice after treatment with**
121 ***Leishmania major* parasite**

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

Pro-inflammatory and anti-inflammatory cytokines gene expression were affected at different exposures, TNF-α, TGF-β and the CXCL-10 had a very low expression level at different times from 24 to 72 hours, so the ΔCT data showed that the expression level of these genes is negative (Fig. 2). The expression of IL-10 and CXCL-9 genes was significantly increased compared to other cytokines (*P-value*≤0.05). IL-p35 of IL-12 protein was also expressed more than other inflammatory cytokines, a high expression level of IL-p35 was observed in 24h after exposure, but IL-p40 was not expressed, the expression level of IL-p35 was similar to that of IL-10 and CXCL-10 (Table 2). The change in the expression of the cytokines IL-p40, TNF-α, CXCL-9, CXCL-10 and IL-10 was not significant in the three time points, but the change in the expression level of TGF-β and IL-p35 in the three different time points showed significant differences (Table 2)(Figure. 3). IL-p40 decreased from a positive level in the 24 exposure with a significant change compared to the 48 and 72 hour. (*P-value*≤0.05)(Fig.3). CXCL-9 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 fragment of IL-12 increased its expression at 24 hours after exposure and then decreased significantly at (48h and 72h) (*P-value*≤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 CXCL-9 but not CXCL-10, was increased at the three exposure times (Table 3).

Table 2- Expression of cytokine genes in different treatments with *Leishmania major* parasite

Tukey's multiple comparisons test	24H Significant	24H P Value	48H Significant	48H P Value	72H Significant	72H P Value
TGF-β vs. TNF-α	NS	0.4130	Ns	>0.9999	****	<0.0001
TGF-β vs. IL-p40	**	0.0027	Ns	0.9979	***	0.0003
TGF-β vs. IL-p35	****	<0.0001	Ns	>0.9999	****	<0.0001
TGF-β vs. CXCL-10	NS	0.0814	Ns	0.8269	****	<0.0001
TGF-β vs. CXCL-9	****	<0.0001	****	<0.0001	****	<0.0001
TGF-β vs. IL-10	****	<0.0001	****	<0.0001	****	<0.0001

TNF-α vs. IL-p40	NS	0.2038	NS	>0.9999	NS	0.9904
TNF-α vs. IL-p35	****	<0.0001	NS	0.9994	NS	>0.9999
TNF-α vs. CXCL-10	NS	0.9579	NS	0.6770	NS	>0.9999
TNF-α vs. CXCL-9	****	<0.0001	****	<0.0001	****	<0.0001
TNF-α vs. IL-10	****	<0.0001	****	<0.0001	****	<0.0001
IL-p40 vs. IL-p35	****	<0.0001	NS	0.9919	NS	0.9950
IL-p40 vs. CXCL-10	NS	0.7083	NS	0.5171	NS	0.9878
IL-p40 vs. CXCL-9	***	0.0002	****	<0.0001	****	<0.0001
IL-p40 vs. IL-10	***	0.0003	****	<0.0001	****	<0.0001
IL-p35 vs. CXCL-10	****	<0.0001	NS	0.8914	NS	>0.9999
IL-p35 vs. CXCL-9	NS	0.7610	****	<0.0001	****	<0.0001
IL-p35 vs. IL-10	NS	0.7292	****	<0.0001	****	<0.0001
CXCL-10 vs. CXCL-9	****	<0.0001	****	<0.0001	****	<0.0001
CXCL-10 vs. IL-10	****	<0.0001	***	0.0004	****	<0.0001
CXCL-9 vs. IL-10	NS	>0.9999	ns	0.7530	NA	0.9823

NS: No Significant

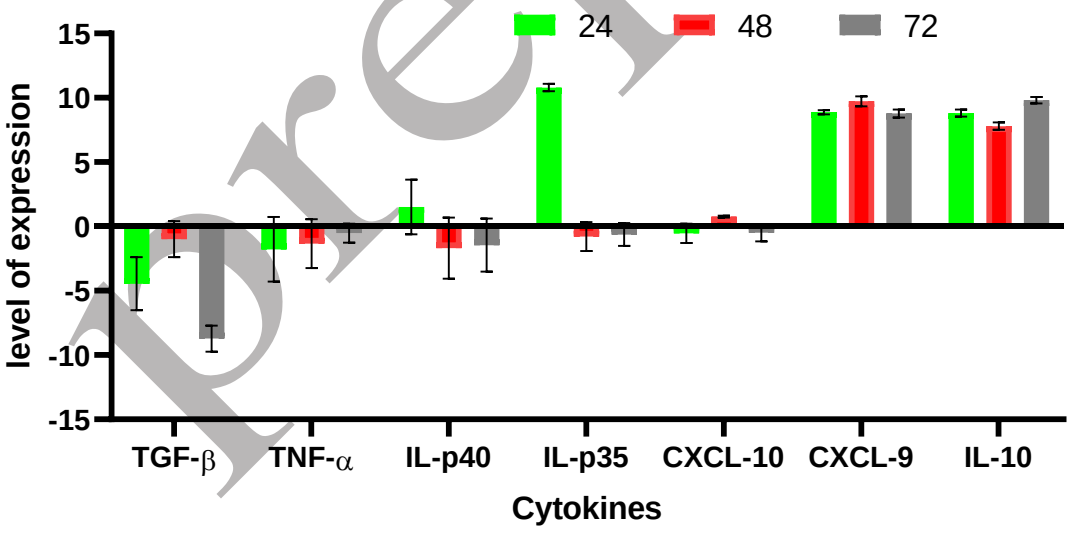

Figure 2: The expression level of cytokines at different times of exposure to *Leishmania major*

Table 3-Tukey's multiple comparisons test

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

NS: No Significant

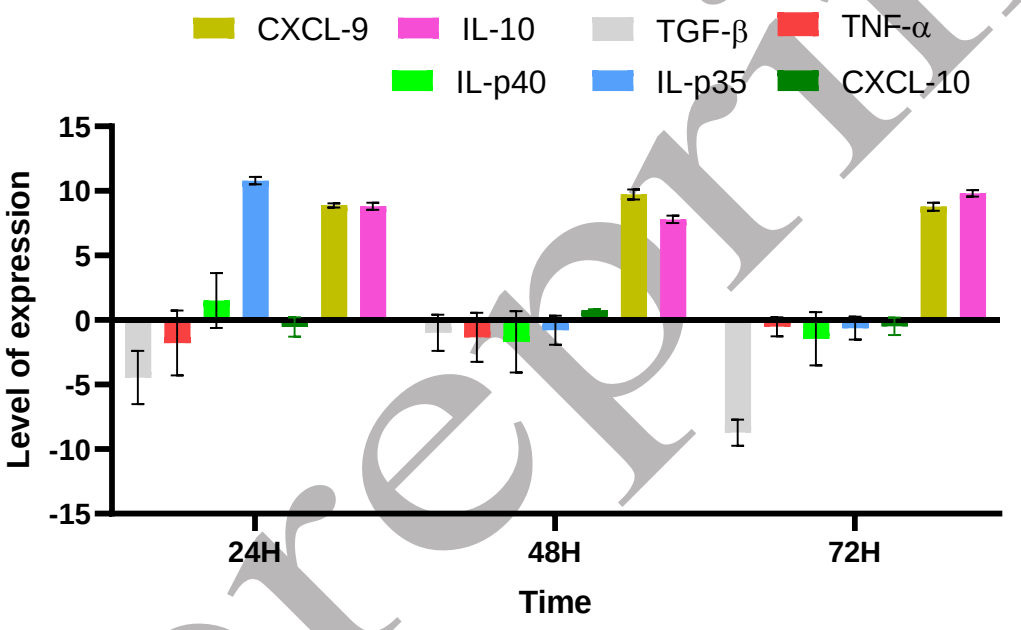

Figure 3- The expression level of each cytokine at three different treatment times

4. Discussion

Macrophage and neutrophils cells are the first line of phagocytic cells against Leishmania promastigotes and lead to an innate immune response with pro-inflammatory mediators (9). Macrophages are capable of secreting a variety of cytokines such as TNF-α, 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 expression were investigated after CL treatment with mesenchymal stem cell, we observed changes in the expression and production of cytokines to control of Leishmania infection (11, 12).

IL-12 consists of two subunits (IL-p35 and IL-p40) produced by monocytes and macrophages, which are paired together after synthesis and absence of one of the subunits leads to progression of infection. IL-12 play an essential function in the control of Leishmania infection by promoting the Th1 lymphocyte-mediated response in leishmaniasis by 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-α (14). In this study, the expression of TNF-α gene was very low and the IL-12 subunits were not expressed in a regular pattern. The secretion of IL-12 was expressed after 24 hours exposure and the expression of IL-12 was suppressed with increasing exposure time. Studies suggested that CR3 (Complement Receptor 3-involved in phagocytosis) reduces the release of IL-12 during silent macrophage invasion by *L. major* that internalization receptors on macrophages Leishmania responsible for differences in IL-12 release (15). TNF-α 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 in three different exposure time, so that TNF-α expression was reduced after 24, 48, and 72 hours of exposure of macrophages with Leishmania parasites. M2 or alternatively activated macrophages are divided into 4 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, CXCL-10 and CXCL-16 chemokines and produces low level of TNF-α, IL-12 and, TGF-β cytokines (17). The expression of TNF-α and TGF-β in macrophages exposed to Leishmania was also low in our study. Thus, the activation and function of macrophages depends 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 macrophage has also been observed with increased oxidative activity, and this may be one reason for Leishmania's resistance to nitric oxide (19). TGF-β promotes the progression and persistence of Leishmania infection by suppressing and regulating the inflammatory responses. TGF-β inhibits the

differentiation of Th1 and macrophages function by reducing and preventing the production of IFN-γ by Th1 lymphocytes. In one study, TGF-β expression was measured in skin tissue with Leishmania infection to be highly expressed in skin, spleen and liver tissue (20). In addition, several studies have reported that the expression of TGF-β and IL-10 increases in long-lasting lesions of cutaneous leishmaniasis (21). Hence, the expression of TGF-β was low at three different exposure time in our study due to the evaluation of TGF-β *in vitro* exposed to macrophages. IL-10 cytokine is associated with the progression of leishmaniasis and is one of the reasons for host susceptibility to the Leishmania parasite by anti-inflammatory effects and causes a Th2 lymphocyte mediated response in BALB/c mice (22). The expression of inflammatory cytokines were lower than that of anti-inflammatory factors in this study. IL-10 cytokine was upregulated with increasing duration of exposure and was significantly higher than inflammatory cytokines such as TNF-α and IL-12 (Fig. 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 associated with parasite persistence (23).

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. CXCL-10 is mainly produced by monocytes, endothelial cells, fibroblasts, recruits macrophages and monocytes to the site of inflammation. Th1 lymphocytes recruited by CXCL-9 and CXCL-10 during active leishmaniasis infection (24). Although the positive correlation between CXCL-9, CXCL-10 chemokines in pulmonary tuberculosis was revealed similar to the pathogenesis of leishmaniasis, this correlation was not observed in the present study. IFN-γ affect the function of macrophage phagocytosis and eliminate the pathogen (25), although in some studies, the stability of the parasite in the macrophage phagosome is considered necessary for the maintenance of long-term memory (19), but 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 of Leishmania wound healing process as a cell therapy method.

The interaction between pro- and anti-inflammatory cytokines, as well as chemokines, are effective in recruitment of Th1 lymphocytes in the pathogenesis of cutaneous leishmaniasis is 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 or treatment of cutaneous leishmaniasis and it is possible that their accurate assessment is important for multifaceted investigation in prevention and treatment.

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Authors' contributions

Study concept and design: H. R. and S. H.

Interpretation and analysis of manuscript data: S. H.

Statistical analysis: S. H and A. SH.R.

Experimental studies: F. B.D., M. F., F. B. and M.P. M.

All authors approved the manuscript.

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Ethics approval: These guidelines were approved by the Research council of Bu-Ali Sina University (permission number: IR.BASU.REC.1403.056).

Conflict of Interest

We declare that there is no conflict of interest.

Data Availability

Data for this finding are available on request from the corresponding author.

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