



Expression of Cytokine Genes in *Leishmania major*-Infected BALB/c Mice Treated with Mesenchymal Stem Cells

Sahar Hamoon Navard¹, Hossein Rezvan^{1*}, Mohammad Hossein Feiz Haddad^{2,3}, Mohamadreza Baghaban Eslaminejad⁴, Sakineh Azami¹

¹Department of Pathobiology, Faculty of Veterinary Science, Bu-Ali Sina University, Hamedan, Iran; ²Leishmaniasis Disease Registry Committee, Dezful University of Medical Sciences, Dezful, Iran; ³Infectious and Tropical Diseases Research Centre, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; ⁴Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

ARTICLE INFO

Original Article

Keywords: Cutaneous Leishmaniasis, *Leishmania major*, Inbred BALB/C, Mesenchymal Stem Cells, Cytokines

Received: Dec. 27, 2019

Received in revised form: Jun. 19, 2020

Accepted: Jun. 20, 2020

DOI: 10.29252/JoMMID.8.1.7

*Correspondence

Email: h.rezvan@basu.ac.ir

Tel: +98 8134227350

Fax: +98 8133227475

ABSTRACT

Introduction: Cutaneous leishmaniasis (CL) is an infectious disease with a high rate of prevalence worldwide. No effective vaccine is now available for CL, and the current chemotherapy ensues serious side effects. The potency of Mesenchymal stem cells (MSCs) in immunomodulatory and wound healing have shown promise for the treatment of CL. **Methods:** In the present study, BALB/c mice were infected with *leishmania major* promastigotes and then injected with 1×10^6 MSCs at the lesion sites. After 10, 20, and 30 days, the lesion size in MSC-treated mice was measured and compared with that of controls received only PBS. Also, expression of IFN- γ , TNF- α , IL-12, IL-4, and IL-10 cytokines were assayed in lesions, neutrophils, spleen, and liver of both test and control groups using RT-PCR. **Result:** The lesion size significantly reduced in MSC-treated mice by day 30. Soon after the treatment, the expression of IFN- γ , TNF- α , and IL-10 genes, but not IL-4 was observed in the spleen and liver of the MSC-treated mice. In neutrophils of this group, only the TNF- α gene was expressed. **Conclusion:** our study exhibited the useful role of MSCs in the treatment of CL, which can open a new window to *Leishmania* research.

INTRODUCTION

Cutaneous leishmaniasis (CL), caused by *Leishmania tropica*, *Leishmania aethiopsica*, and *Leishmania major* in the old world, represents 50-75% of all new cases of leishmaniasis worldwide. According to the World Health Organization, 90% of CL cases occur in Afghanistan, Syria, Algeria, Brazil, Iran, Peru, and Saudi Arabia. Due to different causative species, CL clinical symptoms vary from healing to nonhealing cutaneous progressive lesions, which start as a papule or nodule [1]. The factors influencing the difference in clinical symptoms of CL are not entirely apparent; however, the genetic diversity of the parasite is a known factor influencing the clinical manifestations [2]. In Iran, *L. major* strains causing CL showed genetic diversity, microscopical manifestation in human lesions correlated with the genetic heterogeneity of the parasite [3]. Genetic characteristics of the parasite such as the relationship between polymorphism in interleukin 4 gene promoter and CL clinical manifestation [4] or the molecular typing and phylogenetic analysis of *Leishmania* isolates from healing, and nonhealing patients [5] can be helpful in the assessment of the patient's response to treatments.

Leishmania promastigotes are transmitted to the vertebrate hosts via the infective bite of sandflies. Once inside the phagocytic host cells, they differentiate into the amastigotes, which then proliferate and establish the infection [6]. The cytokines IFN- γ , TNF- α , IL-12, IL-4, and IL-10, play critical roles in the host's immune responses against *Leishmania* infection [7, 8]. In leishmaniasis, IL-12 is crucial for the differentiation of naive T cells into Th1 effectors [9]. IFN- γ and TNF- α are two key cytokines in *Leishmania* infection; IFN- γ activates the infected macrophages by enhancing the respiratory burst in the cells and improving their parasite killing potency [10]. TNF- α induces Th1/IFN- γ responses against *L. major* infection and stimulates nitric oxide (NO) synthesis and increases macrophage activity, which finally results in *Leishmania* clearance [11, 12]. IL-4 instead inhibits the generation of Th1 cytokines and chemokines that recruit Th1-type cells to the site of infection by downregulation of IL-12 production. This process results in the reduction of leishmanicidal activity and parasite stability [13]. IL-10 is another cytokine, which plays a vital role in susceptibility to *Leishmania*

infection. This cytokine renders an immunoregulatory effect, which helps the parasite viability in the site of infection [14]. Prevention of leishmaniasis using a potent vaccine has always been the ultimate goal in *Leishmania* studies [15]. However, no licensed vaccine against leishmaniasis is available yet, and developing a vaccine with long-lasting immunity is still in the early stages [16]. At present, chemotherapy is the primary approach for the treatment of CL. Antimonial drugs are generally effective but are costly and associated with side effects in long-term treatment [17].

MSCs are assumed to be of potency in tissue repair, immune modulation, and cell-based therapies [18]. Availability, ease of expansion, and amenability to genetic engineering have made MSCs as a novel candidate for cell therapy and regenerative medicine [19]. The therapeutic feature of MSCs is related to their ability to secrete a range of bioactive molecules with anti-inflammatory, anti-apoptotic, anti-fibrotic, immune regulatory, pro-angiogenic, and chemotactic effects [20]. These cells are also able to induce repair at the site of inflammation and injured tissues by releasing enzymes, chemokines, and growth factors resulting in the activation of resident progenitor cells in damaged tissue [18]. Mesenchymal stem cells (MSCs) have shown to have a positive effect on the reduction of scarring [21] by increasing collagen production [22]. These cells also increase the M2 (anti-inflammatory)/ M1 (pro-inflammatory) macrophages ratio in the lesion [23].

Although some studies have reported the MSCs' beneficial effect on the host's immune responses to infectious diseases, the therapeutic effects of these cells on *Leishmania* infection is not apparent yet. In an in-vivo assay on BALB/c mice using MSCs, no effect on the treatment of cutaneous infection caused by *Leishmania amazonensis* was observed [24]. In another in-vitro study, MSCs reduced the potency of phagocytosis in macrophages of *L. major*-infected mice. Besides, the MSCs induced an anti-inflammatory effect in the infection [25, 26].

The present study investigates the efficiency of bone marrow-derived MSCs in inducing changes in the expression pattern of crucial cytokine genes in *Leishmania* infection.

MATERIAL AND METHODS

Animals, parasite infection and sampling. Eight-week female BALB/c mice were purchased from Razi Institute (Tehran, Iran) and housed in Bu-Ali Sina University. All animals were treated according to the standard codes of animal studies, and the ethical committees of BU-Ali Sina University and Dezful University of Medical University approved the animal work (code Number: IR.DUMS.REC.1397.043)

Leishmania major strain (MHOM/IR/75/ER) was obtained from Pasteur Institute of Iran. All animals were injected intradermally with $2 \times 10^6/100 \mu\text{l}$ of promastigotes. When the lesions appeared, the animals were randomly divided into test and control groups with 15 mice in each group. The test group was intralesionally treated with $1 \times 10^6/100 \mu\text{l}$ of mouse MSCs, and the control group received the same amount of PBS. On days 10, 20, and 30

post-treatment 5 mice in each group were sacrificed, and spleen, liver tissues, besides lesion tissues, were obtained. Blood samples were used to isolate neutrophils, and peritoneal macrophages were collected after incision from the peritoneal cavity.

Isolation of neutrophils. Briefly, 2 ml of heparinized whole blood was taken from the heart of euthanized mice and diluted with an equal amount of normal saline. The diluted blood was slowly added to the 25% meglumine compound (Daroupakhsh, Iran) and centrifuged for 350 g for 15 min. For lysis of red blood cells, the cell pellet was washed twice with cold distilled water. Then, serum 2.55% was used to neutralize the cell lysis, and neutrophil cells were obtained through centrifugation at 350 g for 5 min of contents [27].

Isolation, culture, and characterization of MSCs. MSCs were isolated from the bone marrow of the femur and tibia of BALB/c mice. After washing with PBS, the bones were chopped into small pieces, and the bone marrow was flushed out using DMEM medium (Invitrogen, USA) by a syringe. The extracted cells were centrifuged at 400 g for 10 min and cultured in fresh DMEM containing 5,000 IU/ml penicillin, 5,000 $\mu\text{g/ml}$ streptomycin, and 15% FBS followed by incubation at 37°C and 5% CO₂. The culture media was changed every 3 days, and the cells were passaged three times using 0.25% trypsin-EDTA solution before performing characterization tests.

MSCs were evaluated by their ability to differentiate into adipocytes and osteoblasts. For adipocyte differentiation, the cells were cultured in DMEM media containing 10% FBS, 50 $\mu\text{g/ml}$ Ascorbyl-2-Phosphate (Sigma, USA), 100 nM dexamethasone (Sigma, USA) and 50 $\mu\text{g/ml}$ indomethacin (Sigma, USA). After incubation for 21 days at 37°C and 5% CO₂, Oil Red O staining was applied to confirm the differentiation of the MSCs into adipocytes. To induce osteoblast differentiation, the MSCs were cultured in osteogenic media, including DMEM medium containing 10% FBS, 50 $\mu\text{g/ml}$ Ascorbyl-2-Phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA), 10 mM β -Glycerol phosphate (Sigma, USA). After three weeks of incubation at 37°C and 5% CO₂, the cells were analyzed by alizarin red S staining for calcium deposition [28].

Isolation of peritoneal macrophages and Nitroblue Tetrazolium Test (NBT). The mice were euthanized, and peritoneal macrophages were collected by injection and aspiration of 5ml cold physiological serum containing 5% penicillin/streptomycin to the peritoneal cavity of *L. major*-infected mice.

The isolated cells were cultured in 96-well plates with RPMI-1640 containing 10% FBS and 20 μl zymosan (5 mg/ml) as a stimulator and incubated at 37°C with 5% CO₂ for 24 h. Fifteen μl of NBT solution (1 mg/ml) was then added to each well and incubated again for 1 h. Then 70 μl of dimethylformamide was added to the wells, and the optical density of the wells was measured by spectrophotometer at 450 nm (Biotek, American, ELX808) [29].

RNA extraction and cDNA synthesis. Total RNA was extracted from neutrophils from the blood of the heart, skin,

spleen and liver tissues by using an Irozol kit (RNA Biotech, Co, Isfahan, Iran) according to the manufacturer's instructions. Briefly, 1000µl of the extraction buffer was added to 100-500 mg of crushed tissues and sonicated with a frequency of 40KHz for 2 min. Then, 200 µl of chloroform was added to the mixtures, followed by the addition of 1000 µl ethanol 100%. The suspensions were centrifuged at 10000 g, the supernatant was decanted, and the resulting RNA was resuspended in 50 µl RNase-free water containing 0.2 µl DNase enzyme.

Complementary DNA (cDNA) was prepared from the total RNA using a reverse transcription system (RB MMLV reverse transcriptase kit). Briefly, 500 ng of mRNA was dissolved in 10 µl RNase-free water. Then, 200U/µl M-MLV RT, 1 µg/µl Oligo dT, 10 Mm dNTPs and 5X RT buffer were added followed by incubation at 50°C for 50 min and an additional incubation at 72°C for 15 min.

Polymerase chain reaction (PCR). IFN- γ , TNF- α , IL-4, IL-12 p35, IL-12 p40, and IL-10 primers were designed using NCBI primer design software and synthesized by a commercial company (RNA Biotech, Isfahan, Iran) (Table 1). The 25 µl reactions contained 1 µL of DNAs from tissues, 100 pmol of forward and reverse primers, 2 µl 5x *Taq* buffer, 0.5 µl *Taq* DNA polymerase (5U/µl), 0.2 mM of dNTPs, 1.5 MgCl₂ and RNA/DNA free water to the final volume. The amplifications were programmed for an initial denaturation at 95°C for 1 min followed by 35 cycles of 45 s at 95°C, 45 s at 54°C, and 45 s at 72°C. For IFN- γ , the annealing temperature was increased to 56°C. The amplifications were finalized with a final extension at 72°C for 10 min. Five µL of the PCR products were run on 1.5% (w/v) wide range agarose gel and stained with 0.2 µg/mL ethidium bromide (EtBr) (Sigma Aldrich, USA, Country). The gel was visualized under ultraviolet light and photographed by computerized gel documentation (Canon, Japan).

Table 1. The primers used in this study

Cytokine	Oligonucleotide primers	Product size	References of the primers
IFN- γ	F: 5' GCTCTGAGACAATGAACGCT 3' R: 5' AAAGAGATAATCTGGCTCTGC3'	227	Present study
TNF- α	F: 5' TATAAAGCGGCCGTCTGCAC 3' R: 5' TCTTCTGCCAGTTCACGTC 3'	239	Present study
IL-12 p40	F: 5' CTGCTGCTCCACAAGAAGGA 3' R: 5' ACGCCATTCCACATGTCCT 3'	208	Present study
IL-12 p35	F: 5' ATGATGACCCGTGTCCTTGG 3' R: 5' CACCCTGTTGATGGTCACGA 3'	282	Present study
IL-4	F: 5' GATCCCGGGCAGAGC 3' R: 5' TGTCGCATCCGTGGATATGG 3'	165	Present study
IL-10	F: 5' AGCCGGGAAGACAATAACTG 3' R: 5' CATTCCGATAAGGCTTGG 3'	189	Present study

Lesion size measurement. The lesions were photographed every ten days using a Canon digital camera (Canon, Japan), and their size was measured by the Image J software (imagej.nih.gov/ij/download).

Statistical analysis. The data were statistically analyzed using SPSS (version 20). Due to the normal distribution of the data, one-way ANOVA followed by post hoc Tukey's test were performed to analyze the results in all groups. *P*-values ≤ 0.05 were considered significant in the assays.

RESULTS

Leishmania lesion sizes in MSC-treated mice. By day 30, significant regression in *Leishmania* lesions of MSC-treated mice compared to the control group that received only PBS was observed (*P*-value ≤ 0.05) (Fig. 1).

Evaluation of phagocytosis (NBT test). The phagocytic potency of peritoneal macrophages was evaluated in both groups of *L. major*-infected BALB/c mice treated with either MSCs or PBS. The results clearly showed no significant increase in the potency of phagocytosis in MSC-treated mice compared to the controls (*P*-value ≤ 0.05) (Fig. 2).

Cytokine gene expression. Evaluation of IFN- γ , TNF- α , IL-12 p35, IL-12 p40, IL-4, and IL-10 genes in the liver, spleen, and lesion tissues, and neutrophils in both groups on

the days 0, 10, 20, and 30, revealed expression of IFN- γ on the day 10 in the liver and spleen of MSCs-treated mice (Table 2). At the end of the experiment (day 30), IFN- γ showed more expression in the liver and spleen of the control group compared to the test group. In the MSC-treated group, the TNF- α gene was expressed in the liver, spleen, and neutrophils at higher levels, but not in the lesions at the early stages of the experiment. In control mice, the IL-4 gene in neutrophils was expressed over the whole course of the assay. It was also expressed in the liver and spleen at the middle and end stages of the assay, respectively. However, in the MSC-treated group, this cytokine gene was only expressed in the lesion on days 10 and 20 (Table 2). In the spleen, the expression of the IL-10 gene was observed in MSC-treated mice on day 10, but in the control mice, this gene was expressed by day 30. IL-12 P35 and IL-12 P40 had no expression in either group (Fig. 3).

DISCUSSION

The immune response against *Leishmania* is a very complex phenomenon. Early studies in C3H/HeN mice indicated that the generation of protective immune responses was associated with the production of IFN- γ by NK cells. Thus, NK cells and IFN- γ play a crucial role in the control of *Leishmania* infection during the early phases of infection [30, 31].

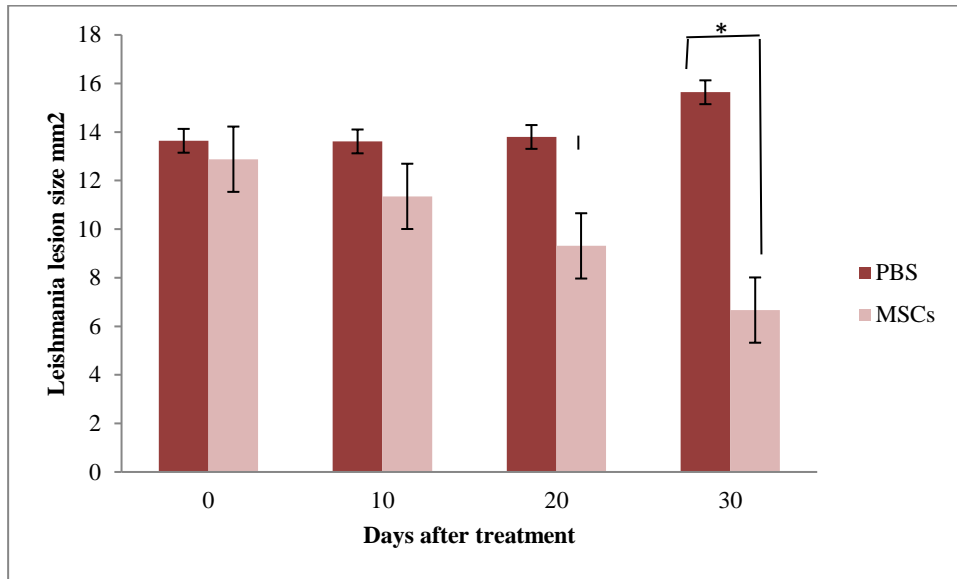


Fig. 1. Lesion size in *L. major*-infected BALB/c mice received intraslesionally either 1×10^6 MSCs or PBS. *, *P*-value ≤ 0.05 .

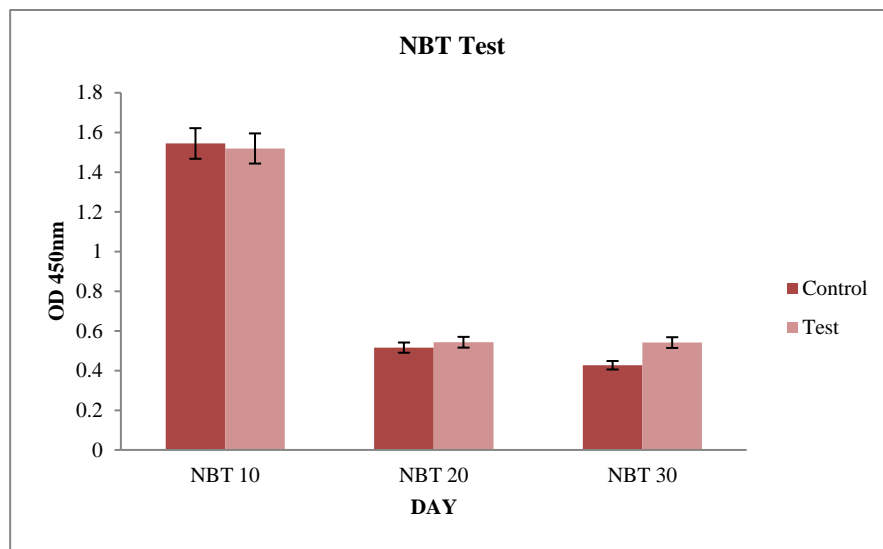


Fig. 2. Phagocytosis potency of macrophages isolated from the peritoneal cavity of *L. major*-infected mice receiving either MSCs or PBS (control).

Table 2. Expression of cytokine genes in *L. major*-infected mice treated with either MSCs or PBS.

	Group	Day 10						Day 20						Day 30					
		IFN- γ	TNF- α	IL-P35	IL-P40	IL-4	IL-10	IFN- γ	TNF- α	IL-P35	IL-P40	IL-4	IL-10	IFN- γ	TNF- α	IL-P35	IL-P40	IL-4	IL-10
Lesion	C	-	-	-	-	-	-	C	-	+	-	-	-	-	-	-	-	-	-
	T	-	-	-	-	+	-	T	-	-	-	-	+	-	-	-	-	-	-
Neutrophil	C	-	-	-	-	+	-	C	-	+	-	-	+	-	-	-	-	+	-
	T	-	++	-	-	-	-	T	-	+++	-	-	-	-	-	-	-	++	-
liver	C	-	+	-	-	-	-	C	-	-	-	-	+	-	-	+	++	-	-
	T	+	++	-	-	-	-	T	-	-	-	-	-	-	-	+++	-	-	-
spleen	C	+	++	-	-	-	-	C	-	+++	-	-	+	-	-	+	+++	-	-
	T	++	+++	-	-	-	++	T	-	+++	-	-	-	-	-	+++	-	-	+

T: Test group (treated with MSCs)
 C: Control group (treated with PBS)

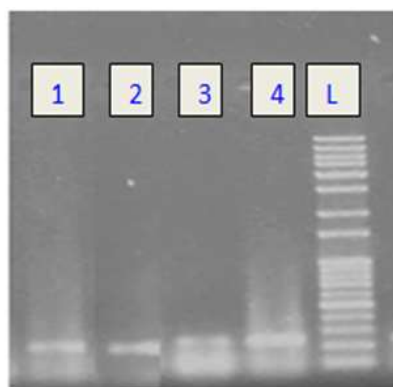


Fig. 3. Expression of genes in *L. major*-infected mice treated with MSCs. Lane 1, IL-4; lane 2, IL-10; lane3, IFN- γ ; lane4, TNF- α ; L, 100 bp-10000 bp DNA ladder

In self-healing lesions, DC produces IL-12, which stimulates the generation of Th1 effector and memory cells. Th1 produces IFN- γ , which in turn induce protective immune responses in the self-healing strain of mice (C57BL/6). IFN- γ , by activation of macrophage and upregulation of iNOS and NO production, kills the parasites and control the disease [32]. On the other hand, the production of Th2 cytokines such as IL-13, IL-5, and IL-4 in CL infections leads to parasite persistence [10]. Other T cells, such as regulatory T (Treg) cells, contribute to disease progression via the production of regulatory cytokines such as IL-10 [33]. The production of IL-10 by Tregs via antigen-induced Foxp3 T cells is assumed to play a role in the progression of leishmaniasis [34]. However, another study showed that placenta-derived MSC resulted in protection against scar formation through increasing the production of IL-10 and inhibition of the proliferation and migration of fibroblasts [35]. Therefore, IFN- γ , IL-4, IL-10, and TGF- β cytokines play an essential role in susceptibility or resistance to leishmaniasis [24].

MSCs showed to have useful roles in wound healing due to the secretion of biomaterials associated with the healing process [36]. In the previous study, we showed that MSC biomaterials also increase the rate of neutrophil phagocytosis in comparison with MSCs alone [37]. In this regard, the source of isolation, the administration route, doses, and duration of treatment affect the behavior of MSCs in the healing process [38]. Our study revealed that, in *L. major*-caused CL, local injection of MSCs resulted in a significant regression of the lesions in 30 days. We previously showed that *Leishmania* infection actively down-regulated pro-inflammatory genes in the lesions [39]. Our results clearly showed that the expression of IFN- γ , IL-12, IL-4, and IL-10 cytokines in the lesions was suppressed in both MSCs and PBS-treated mice. IL-4 was first expressed in MSC treated mice and then stopped expressing. In comparison to the control group, in mice that received MSCs in the lesion. IFN- γ and TNF- α were expressed on day 10 in the spleen, liver, and neutrophils in the test group. Surprisingly, in neither group, these genes were expressed in the lesions. This indicates that MSCs injected in the lesions systemically affected the immune system and upregulated the expression cytokine genes in spleen and liver, but not in the lesion. We

previously showed that early expression of IFN- γ in *L. major*-infected mice after treatments results in a protective immune response against the parasite. Other studies have indicated that IFN- γ and TNF- α play crucial roles in the control of intracellular pathogens [40]. These cytokines provide synergic effects against *L. major* infection, increasing the activity of Nitric oxide (NO) and synthesis of macrophages [41]. Production of NO and reactive oxygen species after activation of the cells by IFN- γ are two primary mechanisms to control *Leishmania* infection [42, 43]. So, the upregulation of these cytokines contributes to the regression of *Leishmania* lesions [44]. The MSCs did not affect the expression of IL-12. It seems MSCs cannot induce macrophages to secrete IL-12.

Our results showed that the expression of IL-4 in the lesions was different from the liver, spleen, and neutrophils. In controls, IL-4 was expressed in neutrophils during the whole course of the experiment. In the test group, the expression of this cytokine in the liver and spleen was detected on day 20 and later. These results showed that as expected in BALB/c mice, the immune response shifted toward Th2 in response to the upregulation of IL-4. Instead, in MSC-treated mice, IL-4 did not express in the liver and spleen during the whole course of experiments. In neutrophils, it expressed at the end stages (on day 30), which indicate suppression of IL-4 in these mice, confirming the systemic influences of MSCs on the immune responses.

IL-10 was expressed in the MSCs-treated group on day 10 and downregulated afterward. These findings are not in-line with some reports indicating the down-modulation of IL-10 in clinical cure with a sustained Th1 response in CL patients compared to the patients with active lesions [8]. However, some studies indicated the regulatory effects of this cytokine on inhibition of Th2 cell and production of IL-4 [45]. Perhaps, expression of IL-10 downregulates Th2 immune response in MSC-treated mice resulting in more resistance to the infection.

The results showed that MSCs were useful in healing the lesions caused by *L. major*. Intralesionally injection of MSCs in *L. major*-infected mice resulted in the upregulation of IFN- γ and suppression of IL-4 gene expression in the animals.

ACKNOWLEDGMENT

The authors would like to thank Bu-Ali Sina University for financial support and Mr. Hossein Hoshrouzi for his technical assistance and animal work.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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Cite this article:

Hamoon Navard S, Rezvan H, Feiz Haddad MH, Baghaban Eslaminejad M, Azami S. Expression of Cytokine Genes in *Leishmania major*-Infected BALB/c Mice Treated with Mesenchymal Stem Cells. *J Med Microbiol Infect Dis*, 2020; 8 (1): 7-13. DOI: 10.29252/JoMMID.8.1.7.