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Exploring the Impact of Leishmania Major on Mesenchymal Stem Cells: Evaluating Differentiation, and Immunomodulatory Function

Elham Mashayekh¹, Arezou Khosrojerdi², Ahmad Zavaran Hosseini¹, and Sara Souidi¹

¹ Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

² Infectious Diseases Research Center, Birjand University of Medical Sciences, Birjand, Iran

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ABSTRACT

Pathogen recognition receptors (PRRs), which play a crucial role in responding to pathogens, affect the function of mesenchymal stem cells (MSCs). One important group of PRRs is the toll-like receptors (TLRs). When PRRs are activated, they can alter the expression of specific surface markers, the ability of MSCs to differentiate, and the types of substances they secrete. These modifications in MSC function may have unexpected consequences for patients. In this study, we examined how *Leishmania major* (*L. major*) promastigotes affect the properties of MSCs.

MSCs were isolated from adipose tissue and categorized into two groups: one group left untreated and the other group exposed to *L. major*. Giemsa staining was employed to accurately quantify the number of parasites that entered the cells. After 72 hours, real-time polymerase chain reaction was utilized to assess the expression of TLRs. Additionally, the flow cytometry technique was used to evaluate the expression of surface markers on the MSCs.

Our results showed that MSCs can engulf parasites and increase the expression of TLR4 and TLR6. The pro-inflammatory cytokine increased, and the transforming growth factor- β decreased significantly. The parasite exposure increased reactive oxygen species production. Additionally, the percentage of cluster differentiation (CD) 73 decreased, and the mean fluorescent index of CD29 and CD73 was down-regulated by *L. major*.

Exposure to parasites diminishes the immunomodulatory capacity of MSCs. This discovery holds significance for the application of MSCs in addressing parasite infections and underscores the need for additional research to enhance their therapeutic effectiveness.

Keywords: Cytokines; *Leishmania major*; Mesenchymal stem cells; Oxidative stress

INTRODUCTION

Mesenchymal stem cells (MSCs) are a type of stromal cell that has become a promising tool for treating

many diseases due to their ability to migrate to the site of inflammation, suppress inflammatory responses, and activate pathways for tissue repair.¹ The promising potential of MSCs has prompted the initiation of numerous clinical trials.

Corresponding Authors: Sara Souidi, PhD;
Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, P.o.Box: 14115-111, Tehran, Iran.
Tele/Fax: (+98 21) 8882 0000, E-mail: souidi@modares.ac.ir

Arezou Khosrojerdi, PhD;
Infectious Diseases Research Center, Birjand University of Medical Sciences, P.o.Box: 97178-53076, Birjand, Iran. Tele/Fax: (+98 56) 3238 1258, E-mail: Arezou.khosrojerdi@modares.ac.ir

The participation of over 2000 patients in these clinical trials is a significant milestone in assessing the effectiveness and safety of MSC therapy. By gathering valuable insights from these trials, researchers can better understand the therapeutic potential of MSCs and how they can be integrated into mainstream medical practice.²⁻⁴

MSC therapy has shown promise in treating degenerative diseases such as osteoarthritis, diabetes, cardiovascular diseases,⁵ and neurological disorders. Additionally, MSCs can expedite burn wound healing⁶ and reduce inflammation in sepsis.^{7,8} Infectious diseases pose a significant global health challenge, and the effectiveness of current treatments, such as antibiotics and antiviral drugs, is diminishing due to the rise of drug-resistant pathogens.⁹ However, recent studies have revealed the potential of MSCs as a treatment option for infectious diseases. MSCs can reduce inflammation, enhance tissue repair, and regulate immune responses. They secrete substances that facilitate the polarization of immune cells,¹⁰ decrease detrimental immune reactions, and improve survival rates in animal models of infection.¹¹ MSCs also have antimicrobial properties that inhibit pathogen growth and promote phagocytic activity.¹²

Sandflies carry the parasite *Leishmania*, which is the cause of the tropical disease leishmaniasis.¹³ It is a prominent public health issue, especially in developing countries with poor healthcare systems. The disease can range from skin ulcers to life-threatening organ involvement. Despite efforts to control and treat it, leishmaniasis remains a problem due to limited treatment options and drug resistance.¹⁴ Recently, there has been interest in MSC therapy as a potential treatment for leishmaniasis. MSCs can migrate to affected tissues, interact with immune cells, and modulate the immune response to reduce inflammation and tissue damage.¹⁵ They can promote tissue repair and regeneration by releasing growth factors and cytokines.¹⁶ Nevertheless, the outcomes of preclinical studies and clinical trials have shown variability among patients. This variation may be attributed to the diverse functions of MSCs once they are introduced to the site of inflammation.

To learn more about how *Leishmania major* (*L.major*) and MSCs interact, we investigated the effects of *L.major* on various aspects of MSCs, such as their differentiation ability, impact on the immune system, nitric oxide (NO) production, response to oxidative stress, and Toll-like receptor (TLR) expression patterns.

MATERIALS AND METHODS

Animals

Female BALB/c mice were purchased from the Pasteur Institute of Iran in Tehran, Iran. Animals aged between 6 and 8 weeks were kept under standard conditions and fed a regular diet.

Isolation of MSCs

MSCs were obtained from the abdominal fat tissues of mice, which were anesthetized with intravenous ketamine (25 mg/kg) and xylazine (2 mg/kg). The tissues were collected and cut into small pieces. Subsequently, the dissected pieces were digested with 0.075% type I collagenase (1 mg/mL in DMEM/10 mM HEPES) (Sigma, Germany). After removing the debris, the pellets were dissolved in DMEM/F12 medium (Bioidea, Iran) with 15% FBS (Gibco, USA), seeded in T25 flasks, and incubated at 37°C with 5% CO₂.⁸

Characterization of MSCs

Surface markers of MSCs were analyzed using flow cytometry. The cells were detached from the bottom of the plate using trypsin (Bioidea, Iran), followed by centrifugation for 5 minutes at 1700 rpm to remove the supernatant. The resulting cell pellet was washed with cold PBS and centrifuged at 400 g for 5 minutes. After washing and counting the cells, 100,000 cells were added to separate microtubes for each indicator. In this study, fluorescein isothiocyanate (FITC)-conjugated mouse antibodies, including anti-CD34, anti-CD45, and phycoerythrin (PE)-conjugated mouse antibodies including anti-CD29, anti-CD73, and anti-CD105 (Biolegend, USA) were used. The suggested amount of each antibody was added to the cell plate and incubated for 45 minutes at 4°C in the dark. The microtubes were centrifuged at 400g for 5 minutes, and the supernatant solution was removed. The resulting cell pellets were re-dissolved in cold PBS and analyzed using the BD FACSCanto™ II Clinical Flow Cytometry System device (BD, USA) and Flowjo software (Ver. 10).

To assess adipocytic differentiation, MSCs were cultured in DMEM/F12 differentiation medium supplemented with dexamethasone (250 nM), isobutyl methylxanthine (0.5 mM IBMX), and indomethacin (0.5 mM). After two weeks, the cells were stained using Oil Red O (Merk, USA). Additionally; the MSCs were cultured in a DMEM/F12 differentiation medium that contained 100 nM dexamethasone, 10 mM glycerol

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phosphate, and ascorbic acid (150 g/mL) to promote osteogenic differentiation. Finally, the cells were stained using Alizarin Red (Merk, USA).

Preparation of Parasites

The Center for Research and Training in Skin Diseases and Leprosy, Tehran, Iran, provided promastigotes of *L. major* (HOM/IL/81/Friedlin). Promastigotes were grown in 5% FBS-RPMI-1640 medium (Bioidea, Iran) at 27°C. After four days of incubation, cultures were passaged. An inverted microscope (Olympus CKX 41) was used for tracking the development of promastigotes.

Infecting AD-MSCs with *L. major* Promastigotes

Stationary-phase promastigotes were mixed with AD-MSCs at a ratio of 1:10 and then incubated for four hours at 37°C with 5% CO₂. After four hours, the cells were washed to eliminate free parasites and seeded into a DMEM medium (Bioidea, Iran) containing 10% FBS. Following this, the AD-MSCs were stained with Giemsa at 24-, 48-, and 72-hour intervals after exposure to the parasite. Finally, a light microscope was used to count the number of parasites in each cell and the number of infected cells.

Evaluation of Pro- and Anti-inflammatory Cytokines Concentration

To analyze the effects of adipose (AD)-MSC contact with the parasite, we collected supernatant samples after 72 hours. Using the enzyme-linked immunosorbent assay (ELISA) method, we determined the concentrations of pro- and anti-inflammatory cytokines in both infected and non-infected AD-MSCs. Our analysis focused on four key cytokines: tumor necrosis factor. alpha (TNF.α), IL-10, and transforming growth factor. beta (TGF.β), which were measured according to the protocol provided by the R&D kit from the USA.

Evaluation of Oxidative Stress Using Free Oxygen Radicals (ROS)

Reactive oxygen species (ROS) are reactive chemical molecules containing oxygen. In healthy aerobic cells, ROS are naturally produced as a byproduct of oxidative phosphorylation in a controlled amount. ROS production can increase under environmental pressures such as UV, heat, or certain drugs and cause damage to cell structures such as DNA, proteins, and lipids, ultimately leading to cell death. In this

experiment, 2' and 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Invitrogen™, USA) was used as an indicator to check the ROS in cells. This substance penetrates living cells. Its acetyl group is broken by intracellular esterase. This non-fluorescent compound is trapped inside the cell, and after being regenerated by free oxygen species, it shows its fluorescence property, which is read by a flow cytometry device. In this way, 72 hours after AD-MSC exposure to the parasite, the cells were stained with DCF, and after washing, they were read by flow cytometry. In this study, AD-MSCs were the negative control, and H₂O₂-treated AD-MSCs were the positive control.

Evaluation of Nitric Oxide (NO) Concentration

Nitrite levels were measured in the supernatant of AD-MSCs 72 hours after parasite exposure. Nitrite is the only stable end product of nitric oxide self-oxidation in an aqueous solution. There is a strong correlation between the amount of nitrite or nitrate in the supernatant and the endogenous production of nitric oxide, making it a reliable and quantitative estimate of nitric oxide output. To measure nitrite levels, samples were reacted with 50 μl of Griess reagent (Cib Biotec Co. Iran) in a 96-well microplate. Total nitrite was estimated from a standard absorbance curve using a 592 nm reader.

Evaluation of TLR-2, -4, and -6 Gene Expression

After 72 hours of AD-MSC contact with the parasite, the cells were lysed, and their mRNA was extracted. During the reverse transcription polymerase chain reaction (RT-PCR) process, cDNA was synthesized, and real-time PCR was utilized to determine the gene expression levels of TLR-2, -4, and -6 in both AD-MSCs and parasite-infected AD-MSCs. The list of primers is presented in the Supplementary Table.

Statistical Analysis

The data and the graphs generated were analyzed using GraphPad PRISM 8. The T-test was used in most of the tests to compare the AD-MSC and AD-MSC+L. major groups. However, the ROS test was evaluated by using the one-way ANOVA test to analyze the difference between the groups. To ensure accuracy and consistency, the experiments were conducted three times. The data are presented as mean±standard deviation, and $p \leq 0.05$ is considered significant.

RESULTS

Identification of MSCs

The spindle-shaped morphology of MSCs after being attached to the flask was confirmed by light microscopy (Figure 1A). These cells showed calcium deposits in the osteogenic differentiation medium (Figure 1B) and vesicles containing fatty acids in the adipocytic differentiation medium (Figure 1C) after staining with Alizarin Red S and Oil Red-O, respectively, which confirms the differentiation ability of MSCs. In addition, the absence of CD34 and CD45 markers (blood cell line markers) and the expression of CD29, CD70, and CD105 markers (MSC line markers) on the surface of MSCs were confirmed by flow cytometry (Figure 1D).

The Effect of *L.major* on MSCs' Differentiation Ability CD Markers

After confirming the MSCs, they were infected with

parasites in a ratio of 1 to 10. In the next step, the percentage of infected MSCs and infection index were measured at 24-, 48-, and 72 hours post-infection (Figure 2A). The results showed that after 24 hours, 48 hours, and 72 hours of being exposed to the parasite, 66%, 85%, and 94% of the MSCs were infected (Figure 2B), and the infection index in these cells was 4, 4, and 7 ($p \leq 0.05$) (Figure 2C). Our results showed that parasite-infected MSCs still retained their differentiation ability (Figure 2D). In addition, the percentage of MSC markers and mean fluorescent intensity (MFI) were evaluated 72 hours after infection (Figure 2E). This study demonstrated that the percentage of CD73 decreased in infected MSCs, while other markers did not change significantly (Figure 2F). However, the MFI of CD73 and CD29 decreased significantly after MSCs were infected (Figure 2G).

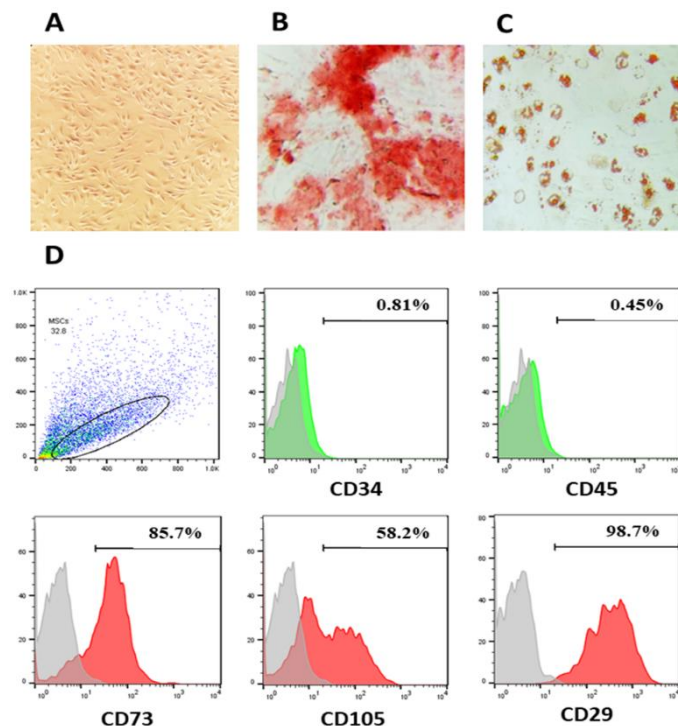


Figure 1. Characterization of mesenchymal stem cells (MSCs). MSCs were isolated from the adipose tissue of mice, and the morphology of MSCs was confirmed by a light microscope (A). The MSCs' differentiation capability into osteocytes (B) and adipocytes (C) was assessed using alizarin-red and oil-red stains. The immunophenotype of the adipose tissue-derived MSCs was analyzed by conjugated antibodies against the surface markers of MSCs (D). Fluorescein isothiocyanate (FITC)-conjugated mouse antibodies, including anti-cluster differentiation (CD)-34, anti-CD45, and phycoerythrin (PE)-conjugated mouse antibodies including anti-CD29, anti-CD-73, and anti-CD105 were used.

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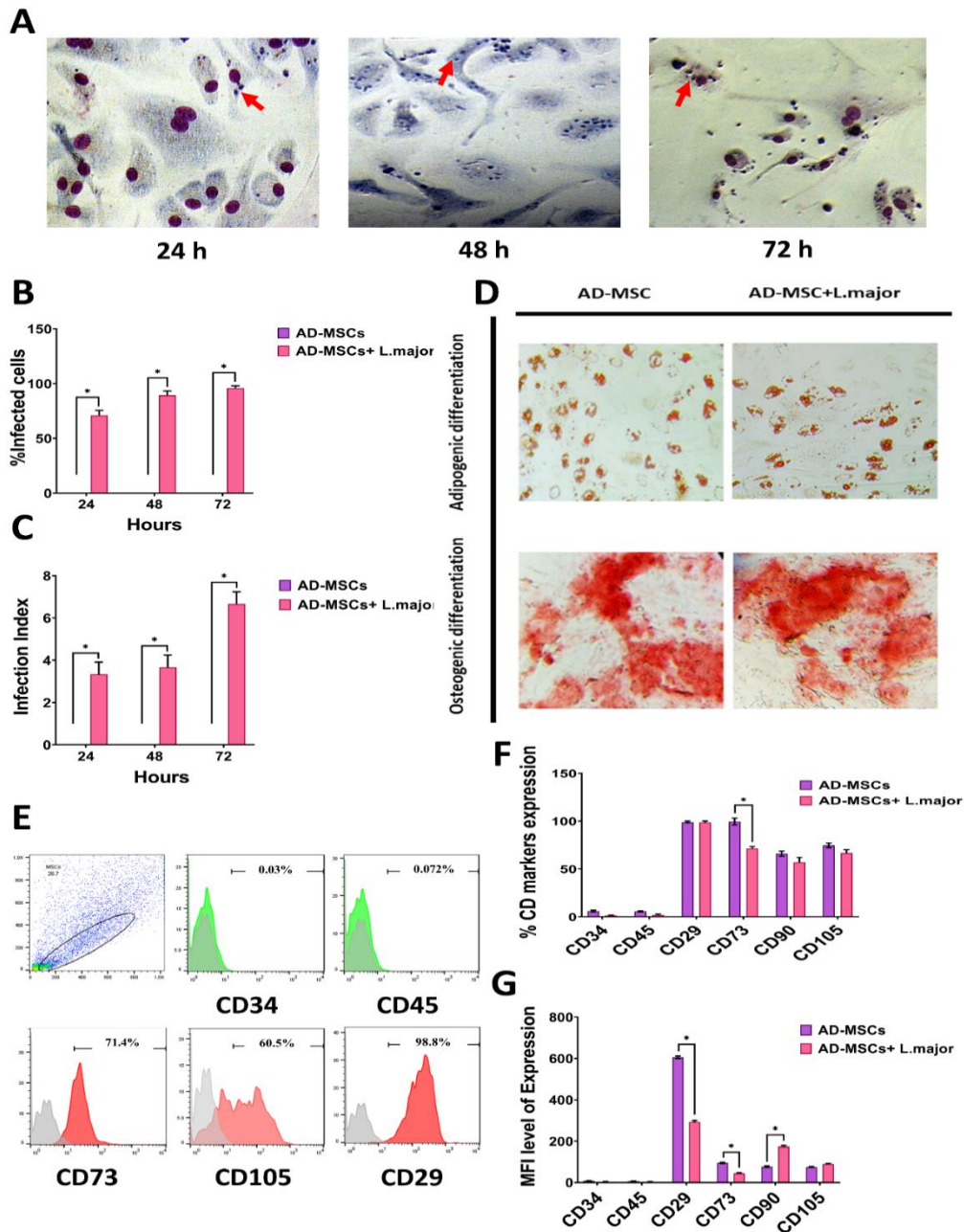


Figure 2. Mesenchymal stem cells (MSCs) after exposure to *Leishmania major*. MSCs were infected with parasites in a ratio of 1 to 10 (A). The percentage of infected MSCs (B) and infection index (C) were measured at 24-, 48-, and 72 hours post-infection. The MSCs' differentiation capability into osteocytes and adipocytes was assessed using alizarin-red and oil-red stains (D). The cluster differentiation (CD) markers of MSCs were analyzed by flowcytometry (E). The percentage of CD markers (F) and their mean fluorescent index (MFI) level (G) were measured. Our data showed that the infection index increases with time as the number of parasite-infected cells rises. The analysis revealed notable differences in the expression of CD markers between MSCs that were treated and those that were not treated. The data is presented as mean \pm standard deviation. * $p \leq 0.05$ is considered significant.

The Effect of *L.major* on Pro-and Anti-inflammatory Cytokine Production Pattern of MSCs

We took the supernatant 72 hours after parasitically treating MSCs and used the ELISA method to measure the levels of pro-inflammatory cytokines (IL-6 and

TNF- α) and anti-inflammatory cytokines (IL-10 and TGF- β). Our findings indicate a significant increase in the production of IL-6, TNF- α , and IL-10 (Figure 3A-C) and a decrease in TGF- β production (Figure 3D) in MSCs due to the presence of parasites.).

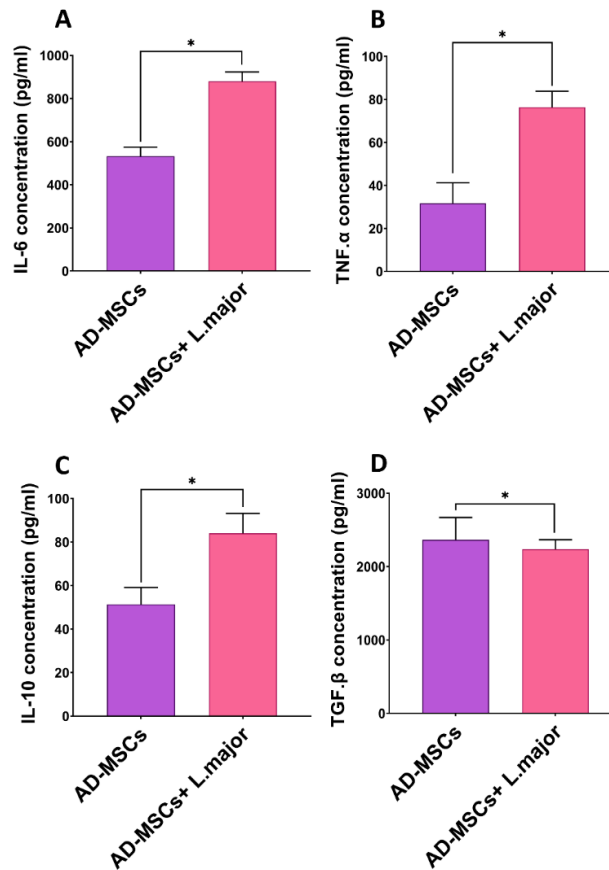


Figure 3. The concentration of cytokines produced by mesenchymal stem cells (MSCs) before and after exposure to *Leishmania major*. 72 hours after the treatment of MSCs with parasites, the supernatant was collected, and the interleukin (IL)-6 (A), tumor necrosis factor (TNF)- α (B), IL-10 (C), and transforming growth factor (TGF- β) (D) concentration were measured by the enzyme-linked immunosorbent assay (ELISA) method. Our findings indicate that parasites cause increased production of IL-6, TNF- α , and IL-10 significantly and decreased TGF- β production in MSCs. The data is presented as mean \pm standard deviation. * $p \leq 0.05$ is considered significant.

The Effect of *L.major* on Oxidative Stress in MSCs

In this study, we used the fluorescent probe emission of 2,7-dichlorofluorescein diacetate (DCFH-DA) to measure the production of intracellular reactive oxygen species (ROS) to see how *L. major* affected oxidative stress in MSCs. According to the results, the percentage of ROS and its MFI in *L. major*-infected MSCs was higher than in untreated MSCs (Figure 4A-B).

The Effect of *L.major* on Nitric Oxide (NO) Production from MSCs

After 72 hours of exposure to the parasite, the cell supernatant was collected to study how *L. major* affected MSCs' ability to produce NO. There was, however, no apparent difference between the groups (Figure 4C).

The Effect of L.major on TLR Expression Level on MSCs

To examine the influence of L. major on the expression pattern of TLRs in MSCs, the expression

levels of TLR2, TLR4, and TLR6 were measured using real-time PCR. Our research showed that the levels of TLR4 and TLR6 were much higher in infected MSCs than in MSCs that had not been treated (Figure 4D–E).

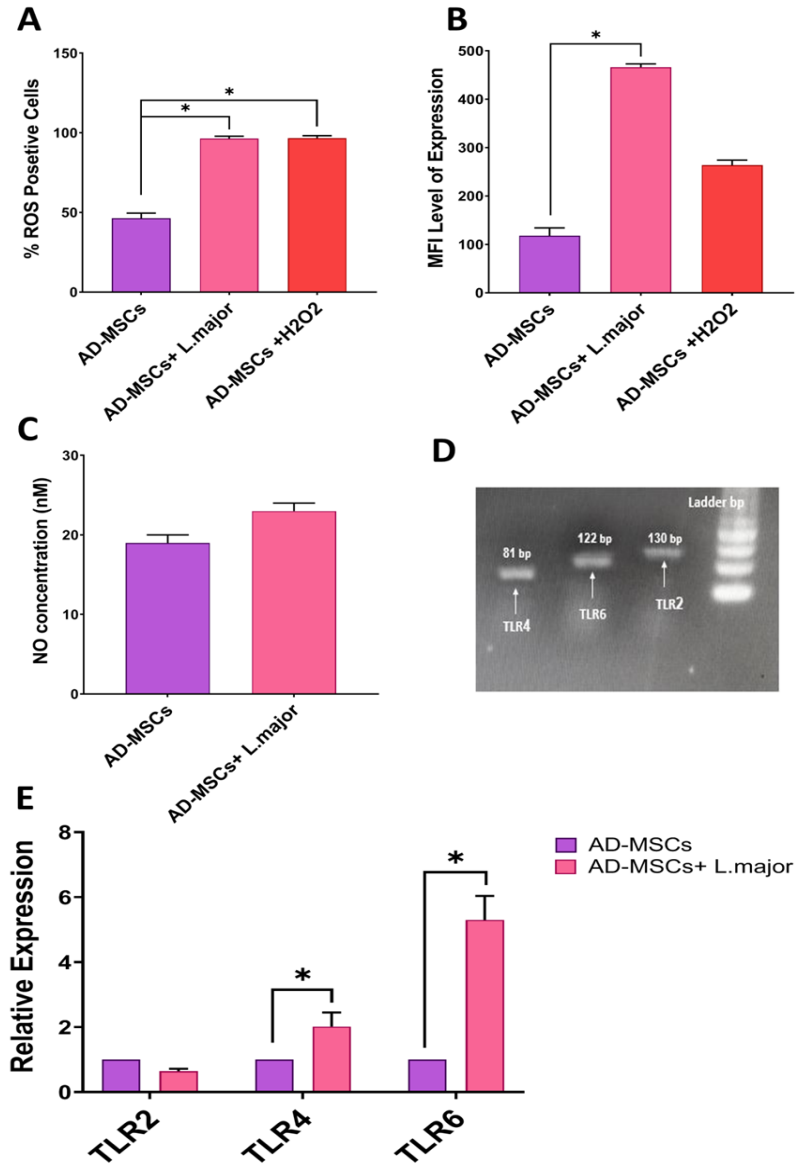


Figure 4. Evaluation of toll-like receptor (TLR) gene expression, oxidative stress, and nitric oxide (NO) production of mesenchymal stem cells (MSCs). After 72 hours of exposure to the parasite, the percentage of reactive oxygen species (ROS)-positive cells and their mean fluorescent index (MFI) level were measured. NO concentration was measured in the supernatant. TLR expression levels were analyzed by Real-time PCR. According to the obtained results, the percentage of ROS and its MFI in Leishmania major infected-MSCs was higher than that in untreated-MSCs. There was, however, no apparent difference between the groups and the expression level of TLR4 and TLR6 was significantly higher in infected MSCs when compared to untreated MSCs. The data is presented as mean \pm standard deviation. * $p \leq 0.05$ is considered significant.

DISCUSSION

Despite numerous studies, MSC therapy has not yet gained approval from the FDA due to inconsistent results.¹⁷ Factors affecting patient response include cell quality, genetics, and the environment. One important attribute of MSCs is their ability to regulate immune responses, making them useful for treating disorders by suppressing immune responses and reducing inflammation. MSCs produce anti-inflammatory cytokines, transform macrophages,¹⁸ promote the development of regulatory T-cells,¹⁹ and inhibit the function of natural killer cells, all contributing to their immunosuppressive properties.²⁰

The immunomodulatory property of MSCs is not constant, though, and can shift in response to environmental factors.²¹ According to research by Xu et al, MSCs lose their immunosuppressive properties in the presence of either a high concentration of anti-inflammatory mediators or a low concentration of inflammatory mediators.²² Other investigations indicate that TLRs can also promote polarity in MSCs.²³ For instance, Waterman et al, demonstrated the importance of TLR-3 and TLR-4 in the polarization of MSC type-2 (anti-inflammatory) and type-1 (pro-inflammatory) MSCs, respectively.²⁴ Zhao et al, additionally showed an enhancement in MSCs' immunosuppressive function following TLR-3-ligand binding.²⁵ Finally, it can be deduced that MSCs may behave differently in response to environmental influences after being administered to a patient. This difference in behavior could potentially explain why therapeutic trials have produced contradictory findings. Since MSC therapy has been considered for various parasitic infections, including leishmaniasis, this study discusses the effect of the *L. major* parasite on the phenotype and function of MSCs.

Our finding determined that MSCs possess the remarkable ability to engulf *L. major* parasites, and the percentage of infected cells increases gradually over time. This discovery suggests that MSCs play a crucial role in phagocytosing these parasites from the surrounding environment. Notably, our findings align with previous research conducted by Costela Ruiz et al, which also highlights the versatile nature of MSCs in phagocytosing particles of different types and sizes.²⁶ A study conducted by Lopes et al, in 2016 demonstrated that *L. infantum* parasites can infect bone marrow (BM)-MSCs. According to a recent study, parasites may use

MSCs (mesenchymal stem cells) to evade anti-parasitic drugs. This is because MSCs have a high expression of drug-release pumps.²⁷ However, it is not yet clear how parasites invade MSCs. Some studies suggest that adipose tissue MSCs have phagocytic properties.²⁸

Our study found that the presence of parasites in MSCs did not affect their ability to differentiate. However, the parasite did impact the expression of positive markers on the surface of MSCs. Specifically, we observed a decrease in CD73 % and MFI. CD73 is an ectoenzyme that converts ATP/ADP to adenosine (Ado). Its mechanism of action involves reducing the amount of free ATP in the environment, which in turn changes a pro-inflammatory environment into an anti-inflammatory one. The immunomodulatory properties of CD73 on MSCs are highly relevant in regenerative medicine. Studies have demonstrated that MSCs that express CD73 can effectively suppress the immune response by inhibiting the proliferation and functions of immune cells such as T cells and natural killer cells. This immunosuppressive effect is attributed to the production of adenosine, which acts on adenosine receptors found in immune cells. According to the findings, the levels of pro-inflammatory cytokines have decreased, while the levels of anti-inflammatory factors have increased. Thus, it can be concluded that the presence of parasites reduced the ability of MSCs to lower free ATP and generate an anti-inflammatory environment.²⁹ It can be concluded that *L. major* decreased the immunosuppression ability of MSCs and polarized them to MSCs type 1, which are inflammatory MSCs.

Our findings also demonstrated a decline in the MFI of CD29 in addition to CD73. CD29 plays a critical role in the function and therapeutic potential of MSCs. Its presence in MSCs enables their adhesion, migration, immunomodulatory effects, and differentiation into various cell types. According to Ode et al, the decrease of CD73 and CD29 impacts the MSCs' migration.³⁰ Therefore, the reduction of CD29 and CD73 by the parasite also diminishes the ability of MSCs to migrate and adhere to the extracellular matrix.

TLRs are surface receptors that MSCs use to understand their environment and conduct various tasks as a result. Fisher et al, conducted initial studies on the expression of TLRs by MSCs in mice. They demonstrated that mouse MSCs express TLR1-8 but do not express TLR9.³¹ According to a study by Raicevic et al, inflammatory conditions caused MSCs to alter the

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expression pattern of TLRs.³² Our findings demonstrated a significant rise in TLR-4 and TLR-6 expression in infected MSCs. According to research by Faria et al, TLR-4 activation led to the elimination of intracellular *L. major* and inhibited the proliferation of the residual parasites.³³ According to Karmakar et al, the glycosphingophospholipid (GSPL) on *Leishmania*'s surface is a potent TLR-4 ligand, and this binding increases intracellular inflammatory signaling that kills the parasite.³⁴ The activation of TIR-domain-containing adapter-inducing interferon (TRIF) and myeloid differentiation primary response 88 (MYD88) and subsequent phosphorylation of PKR, which boosts the generation of TNF- α and interferon-gamma (IFN- γ) and the ability of the cell to fight parasites, are other effects of TLR-4 recognizing *L. major*.³⁵ To successfully fight bacterial, fungal, and viral infections, TLR-6 forms a heterodimer with TLR-2.³⁶ According to the research by Pandey et al, *L. major* infections in BALB/c mice became worse when TLR-6 expression was inhibited by shRNA.

In addition, our results indicated an increase in the production of IL-6, TNF- α , and IL-10 cytokines and a decrease in the production of TGF- β by infected-MSCs. As previously explained, TNF- α production can increase due to TLR-4 stimulation, which initiates the TRIF/MYD88/PKR signaling pathway.³⁵ Moreover, the GSPL identification by the TLR-2-TLR-6 heterodimer of the parasites could activate the MYD88 signaling pathway and contribute to the increase in IL-6 synthesis.³⁷ Several studies have reported results similar to ours, indicating that the injection of MSCs into various animal models infected with parasites increases inflammatory cytokines,^{15,16,38} and decreases TGF- β . For instance, Soudi et al injected MSCs into mice infected with *L. major*, which increased TNF- α production in the mice's serum.³⁸ In addition, Hamoon Navard et al, demonstrated an increase in IL-10 and TNF- α in the spleen and liver of mice infected with *L. major* following MSC injection.³⁹ The pro-inflammatory activity of MSCs during parasite infection was indicated by a rise in TNF- α and IL-6 production as well as a concurrent decline in TGF- β . An increase in IL-10 may be a compensatory mechanism to limit this inflammation.

Our findings showed that the infected MSCs had higher ROS levels. It might be a valuable pathogen-eradication effort by MSCs. According to an article by Reverte et al, an increase in oxidative stress not only attempts to eliminate parasites but also leads to the

release of inflammatory mediators from the host cell. Additionally, this inflammation may harm nearby tissue.⁴⁰

NO generation plays a crucial role in inhibiting the growth of the *Leishmania* parasite.⁴¹ However, our research findings have shown no significant change in NO production. This contrasts with the studies conducted by Reva S. Thakur et al, which demonstrated that MSCs infected with the malaria parasite produce a lower amount of NO.⁴² However, according to the findings of Li, W et al, there is a belief that MSCs unable to produce NO tend to exhibit more inflammatory properties, whereas MSCs that do produce NO demonstrate immunosuppressive characteristics.⁴³ This aligns with our conclusion, which suggests that after parasite infection, the inflammatory features of MSCs increase while there is no change in their ability to produce NO.

Finally, our data showed that MSCs identify and engulf parasites from the environment through TLRs, and to eliminate existing parasites, they must increase the ROS level. The activation of signaling pathways by TLR-4 and TLR-2-TLR-6 receptors affects the immunomodulatory function of MSCs. MSCs increase pro-inflammatory cytokines and reduce anti-inflammatory ones. In addition, changes in the expression of surface markers on these cells can also affect their migration. All the evidence indicated the polarity of MSCs into type-1 MSCs after infection with *L. major*.

MSCs have fascinating abilities similar to macrophages. They can engulf *L. major* and eliminate it through various strategies. It is important to note that the parasite can alter the functionality of MSCs. This alteration leads to the production of inflammatory cytokines and mediators such as NO and ROS. By continuously examining and studying the impact of *L. major* on MSC functionality, we can advance our understanding of leishmaniasis and develop better strategies to harness the potential of MSCs in combating this disease.

STATEMENT OF ETHICS

The Tarbiat Modares University Ethics Committee, Tehran, Iran, accepted this work under reference code (IR.MODARES.REC.1398.111).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable

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