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Mesenchymal stem cells alter macrophage immune responses to *Leishmania major* infection in both susceptible and resistance mice

Safura Dameshghi [a], Ahmad Zavaran-Hosseini [a,*], Sara Soudi [a,*], Fatemeh Jalali Shirazi [a], Shahrzad Nojehdehi [b], Seyed Mahmoud Hashemi [c]

[a] Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[b] Immunology Department, Stem Cell Technology Research Center, Tehran, Iran
[c] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

Mesenchymal stem cells (MSCs) are attracted to inflammation site and switch immune system to modulate inflammatory responses. This ability makes MSCs the best candidate cells for stem cell therapy of infection diseases. Therefore, we aimed to evaluate the modulatory effect of adipose-derived MSCs (AD-MSCs) on macrophages in *Leishmania* (*L.*) *major* infection. Macrophages and MSCs were isolated from both susceptible (BALB/c) and resistance (C57BL/6) strains. After co-culture of AD-MSCs with macrophages using a transwell system, we assessed MSCs-educated macrophage responses to *L. major* infection. Our results indicated suppression in levels of tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10) of MSCs co-cultured macrophages in response to *L. major* infection. To clarify the effects of this suppression on inflammatory conditions, TNF-α/IL-10 ratio was calculated, indicating an increase in TNF-α/IL-10 ratio in MSCs co-cultured groups. The higher TNF-α/IL-10 ratio was observed in BALB/c macrophages co-cultured with BALB/c MSCs. Nitric oxide (NO) assay presented a significant reduction in the supernatant of all MSCs co-cultured groups compared to control. We observed a significant reduction in phagocytosis of MSCs co-cultured groups in response to *L. major* infection without any significant differences in the phagocytic index. In conclusion, our results represented a new spectrum of immunomodulation induced by MSCs co-cultured with macrophages in response to *L. major* infection. The magnitude of immunoregulation was different between BALB/c and C57BL/6 strains. Our findings also showed that MSCs exerted potential effect of M1 polarization due to unequal decrease in levels of TNF-α and IL-10 when we considered TNF-α and IL-10 as representatives of M1 and M2 phenotypes, respectively. Induction of inflammatory cytokine milieu and reduction in level of IL-10 provides a new hope for stem cell therapy of leishmaniasis in susceptible models.

1. Introduction

Leishmaniasis as one the vector borne disease is caused by about 21 *Leishmania* (*L.*) species in different geographical conditions. Of the three different clinical manifestations of leishmaniasis (visceral, cutaneous and mucocutaneous), self-healing ulcers of cutaneous leishmaniasis is not considered as a life-threatening disease. However, due to vast disease distribution in endemic countries and difficulties in vector and reservoir control, this type of leishmaniasis is known as a public health problem [1]. After entering the body, *L.* promastigotes are trapped by phagocytic cells. Macrophages as the main host of *L. major* either engulf the parasite through phagocytosis of infected neutrophils [2,3], or opsonize parasites through complement receptor 3 (CR3) and C-reactive protein receptor (CRPRs) [4,5]. Parasite–host interaction happens as soon as the parasite is entrapped within phagosomes. After phagosome–lysosome fusion, *L. major* converts the harsh environment of phagolysosome to a safe niche through evasion mechanism [6]. *L. major* infection exerts a great diversity in macrophage responses through patterns of gene expression. Down-regulation of about 40% of macrophage genes and induction of several other genes allow the *L. major* to survive [7,8] within macrophages. Proteomic analysis shows the modulation of about 162 proteins in macrophages after *L. major* infection [8]. *L.* species alters not only gene expres-

* Corresponding authors at: Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Ale-Ahmad Avenue, PO Box 14115-331, Tehran, Iran.
E-mail addresses: zavarana@modares.ac.ir (A. Zavaran-Hosseini), Soudi@modares.ac.ir (S. Soudi).

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sion but also different macrophage cell signaling pathways like Ca+2– protein kinase C (PKC), mitogen-activated protein (MAP) kinase, JAK2/STAT1 and interferon-gamma (IFN-γ) mediated pathways. Alterations in these signaling pathways result in an increase in intracellular Ca+2 concentrations, a reduction in PKC activity, suppression of nitric oxide (NO) and major histocompatibility complex (MHC) class II as well as repression of nuclear factor (NF)-kappa B (NF-κB) that lead to enable the parasite to survives [9,10]. All of these modulations affect macrophage inflammatory and destructive effects by repression of inflammatory cytokines production [interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), IL-6 and IL-12] and by induction of immunosuppressive molecules [prostaglandin E2 (PGE2), transforming growth factor-β (TGF-β) and IL-10] [11]. Although *L. major* infection has shown to have suppressive effects on macrophage function, other involved cellular and molecular factors in the infection site, direct macrophages to a beneficial or detrimental condition. Mesenchymal stem cells (MSCs) are one of these factors that are present in all tissues, migrate to inflammation site and exert different modulatory effects on immune cells by both cell to cell contacts and secretion of soluble factors [11]. MSCs cytokine secretions [like macrophage inflammatory protein-1 alpha (MIP-1α)] promote macrophage migration to injured site [12] that leads to engagement of a bidirectional interaction with M1 or M2 phenotype dependent to the inflammatory and anti-inflammatory conditions [13–15]. During these reciprocal regulation, novel types of alternatively activated macrophage appear as both inflammatory and anti-inflammatory phenotypes [15]. This kind of macrophage modulation creates a new opportunity for treating infections by MSCs therapy. In spite of the different macrophage responses to *L. major* in BALB/c and C57BL/6 mice as a representative model of TH2 and TH1 immune responses [16], we designed the current study to evaluate the modulatory effect of adipose-derived MSCs (AD-MSCs) on macrophages in both susceptible and resistant mice.

2. Materials and methods

2.1. Animals

Female BALB/c and C57BL/6 mice (6–7 weeks old) were purchased from the Pasteur Institute, Tehran, Iran. The animals were housed and maintained under standard laboratory conditions. Mice were allowed free access to feed and water; all experiments were carried out according to the guidelines of Tarbiat Modares University, Tehran, Iran, for animal care and handling.

2.2. AD-MSCs isolation and culture

Abdominal fat tissues of BALB/c and C57BL/6 mice were minced and washed in the phosphate-buffered saline (PBS). Extracellular matrix was digested with 0.075% type I collagenase at 37 °C for 30 min that was followed by being centrifuged at 500 × *g* for 5 min. The pellet was then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin, and streptomycin (all reagents were purchased from Invitrogen, USA). After 72 h, non-adherent cells were removed. After adherent cells reached confluency, they were trypsinized, harvested, and expanded. All the experiments were performed using AD-MSCs at passages three.

2.3. Differentiation of AD-MSCs into osteocytes and adipocytes in vitro

Differentiation potential of AD-MSCs was assessed. To induce osteogenic differentiation, the cells were treated with 10 mM beta-glycerophosphate (Merck, UK), 50 mg/ml ascorbic acid biphosphate (Sigma–Aldrich, USA), and 100 nM dexamethasone (Sigma–Aldrich) for 3 weeks. Osteogenic differentiation was assessed with alizarin red (AR) staining. Adipocyte differentiation was achieved in the presence of 250 nM dexamethasone (Sigma–Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma–Aldrich), 5 mM insulin (Sigma–Aldrich), and 100 mM indomethacin (Sigma–Aldrich), for 3 weeks. Oil Red O (ORO) staining was used to determine the accumulation of oil droplets in the cytoplasm.

Table 1
AD-MSCs isolated from BALB/c and C57BL/6 co-cultured with both BALB/c and C57BL/6 peritoneal macrophages, after 72 h, MSCs were removed and macrophage responses were assessed in the presence or absence of *Leishmania major*.

Experimental groups		
BALB/c MSCs	+BALB/c MQ	w *L. major*
		w/o *L. major*
	+C57BL/6 MQ	w *L. major*
		w/o *L. major*
C57BL/6 MSCs	+BALB/c MQ	w *L. major*
		w/o *L. major*
	+C57BL/6 MQ	w *L. major*
		w/o *L. major*

2.4. AD-MSCs phenotyping

All isolated AD-MSCs at passage three were harvested with 0.25% trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA), counted and diluted to 5×10^6 cells/ml. About 100 μl of this cell suspension were incubated in separate tubes with one of the specific monoclonal antibodies against CD45, CD44, CD29, CD105, CD73, CD11b, Sca-1 and the related isotype control antibodies (all were purchased from eBioscience, USA) for 1 h at 4 °C. The cells were then fixed with 1% paraformaldehyde (Sigma–Aldrich) and analyzed using a FACSCalibur flowcytometer (BD Biosciences, USA) and Cyflogic software (CyFlo Ltd., Finland).

2.5. Peritoneal macrophage isolation

Four days after peritoneal injection of 1 ml 4% (w/v) Brewer's thioglycollate medium to BALB/c and C57BL/6 mice, thioglycollate-elicited macrophages were harvested by intraperitoneal injection of 10 ml cold DMEM medium and aspiration of injected fluid. Peritoneal exudate cells were cultured for 1 h at 10 cm adherent tissue culture dish. Non-adherent cells were removed, while adherent cells were detached and centrifuged at 400 × *g* for 10 min. Cells were diluted to 1×10^6 cells/ml in DMEM supplemented with 10% FBS. A sample was analyzed for CD14 marker using flow cytometry method.

2.6. Co-culture of MSCs with macrophages

At 80% confluence, AD-MSCs from third passage of both BALB/c and C57BL/6 mice were inactivated by treatment of 5 μg/ml mitomycin-C (Santa Cruz Biotechnology, USA) for 1 h. MSCs were detached, washed and suspended to 2.5×10^6 cells/ml in DMEM supplemented with 10% FBS. In a co-culture system containing transwell inserts with 0.4-μm pore size (Corning, USA), 500 μl of BALB/c and C57BL/6 macrophages were seeded separately into the 24-well plates. Subsequently 100 μl of MSCs isolated from BALB/c and C57BL/6 were added to the top chambers in order to co-culture with both BALB/c and C57BL/6 macrophages. The co-cultures were incubated for 72 h at 37 °C in a humidified 5% CO2 incubator. The schematic representation of experimental groups was indicated in Table 1.