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The Effect of Direct Co-culture of Adipose-derived MSCs and Murine Macrophages on Response to LPS In Vitro

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Abstract

Introduction: We investigated whether the interaction of mesenchymal stem cells (MSCs) with macrophages could play a significant role in the macrophage response to lipopolysaccharide (LPS) and their anti-inflammatory/immune-modulatory effects.
Methods: BALB/C-adipose-derived MSCs were characterized by assessment of cell surface marker expression (cluster of differentiation (CD) 29, CD11b, CD73, CD105, spinocerebellar ataxia type 1 (Sca1), and CD45), and their capacity of differentiation into osteocytes and adipocytes. Co-culture of isolated MSCs with macrophages was done and RNA expression profiles, cytokine, and Nitric Oxide levels were assessed.
Results: Macrophages co-cultured with MSCs treated with LPS significantly decreased the protein and mRNA expression of inducible nitric oxide synthase (iNOS), compare to macrophages treated with LPS in the absence of MSCs. Furthermore, these LPS treated MSC-educated macrophages expressed high levels of Tumor necrosis factor α (TNF-α) and low levels of Interleukin 10 (IL-10) and transforming growth factor β1(TGF-β1), as determined by inflammatory and anti-inflammatory cytokine, respectively. However, MSC-educated macrophages results showed that the IL-10 and TGF-β1 significantly increased, and TNF-α decreased in the co-cultured system compared with macrophages cultured alone.
Conclusions: We describe a type of macrophage generated in vitro, as MSC-educated macrophages, which may be a unique type of alternatively activated macrophage with a potentially significant role in tissue repair.

INTRODUCTION

Imagine a simple intravenous cell therapy that can restore function to damaged or diseased tissue, avoid hosts rejection, and reduce inflammation throughout the body without the use of immunosuppressive drugs. Such a breakthrough would revolutionize medicine. Fortunately, this approach might not be far off, pending regulatory approval. The last ten years have witnessed a large expansion in stem cell research principally because of the development of new techniques for their isolation. Among the various types of stem cells, those of mesenchymal origin appears to be the most promising. Cell therapy utilizing adult mesenchymal stem cells, multipotent cells with the capacity to promote angiogenesis, differentiate to produce multiple types of connective tissue, and downregulate an inflammatory response, are the focus of a multitude of clinical studies currently underway [1-5].
Mesenchymal stem cells (MSCs), first described in bone marrow in the middle of the last century. Since then, MSCs have been the objects of a myriad of publications, progressively increasing our knowledge on their potentialities and bringing high expectancies for their regenerative properties [6-9]. During the same period, numerous tissues, such as adipose tissue, placenta, or umbilical cord, have been used as al-

ternative sources of MSC in comparison with bone marrow. In particular, considering the accessibility and ease to harvest fat tissue, adipose-derived MSCs have gained interest above bone marrow-derived MSC [10-15].
More recently, MSCs have been described to possess anti-inflammatory and immunomodulatory properties, which may represent an additional contributing factor to tissue repair through the reduction of immune-mediated damage and without requiring long-term engraftment [16-18]. The discovery that MSCs do not express Human Leukocyte Antigen (HLA) class II encoded antigens led to the assumption that MSCs confer only low immunogenicity if transplanted in an allogeneic setting [19]. Upon studying the MSCs' interaction with non-related immune cells, it turned out that, in addition to their low immunogenicity, MSCs can suppress the function of various immune effector cell types and promote regulatory immune functions. According to these features, MSCs became a very attractive cell source in regenerative medicine and immune therapy. In vitro usual demonstration of MSC immunosuppressive function is based on the capacity of MSC to reduce the proliferation of immune cells in co-culture, affecting both innate and adaptive immunity [20, 21]. In these experiments, immune cells [total splenocytes, peripheral blood mononuclear cells (PBMC), or purified populations of cells] undergo polyclonal or antigen-specific activation, using phytohemagglutinin (PHA), lipopolysaccharides (LPS), cluster of differentiation 3 (CD3), or a specific antigen CD28 [21, 22].
The immunomodulatory activity of MSCs on B and T lymphocytes, macrophages, dendritic cells (DCs), and Natural killer (NK) cells has been extensively studied in vitro. This leads to effector cell energy, as testified by the secretory profile of these cells, with a decrease of the pro-inflammatory cytokines such as interferon γ (IFN-γ), tumor necrosis factor (TNF)-α, Interleukin (IL) 17, and an increase of IL-10 and IL-4 (switch to Th2 and/or regulatory phenotype). The two main and well-described soluble factors responsible for these effects are (1) indoleamine 2,3 dioxygenase (IDO), an enzyme whose activation depletes the surrounding environment in the essential amino acid tryptophan, which is catabolized into kynurenine, leading to the accumulation of breakdown toxic products and (2) inducible NO synthase (iNOS) activation, with NO release, resulting in cytotoxicity on neighboring immune cells (i.e., T lymphocytes, NK and Macrophages) [23-29].
As we know, macrophages are phagocytic cells resident in the tissue, which plays important role in the steady-state of tissue homeostasis, removing cellular debris and apoptotic cells, and in the defense against intracellular pathogens. Macrophages respond rapidly to environmental signals using a multitude of receptors, which results in a specific and optimized activation state and also possessing high plasticity regulates cell proliferation, mediate immunomodulation, and promotes tissue regeneration, in response to specific environments. In general, macrophages are divided into two subtypes, immune-suppressive or anti-inflammatory M2 or activated macrophages, and immune-reactive or proinflammatory M1 or classically activated macrophages. The alternatively activated M2 macrophages play a critical role in the immune system regulation and tissue remodeling, like wound healing [30-35].
MSCs are known as macrophages stimulator to produce immunosuppressive and anti-inflammatory cytokines such as IL- 10, and in this way induce polarization toward an M2 subtype expressing CD206. Several studies have evaluated the effects of MSCs on the immune cells, including macrophages, dendritic cells, natural killer cells, and T lymphocytes previously; nevertheless, few of them focused on macrophages.
Hence, it will be important to have a better understanding of the effects of adipose-derived MSCs (Ad-MSCs) on macrophages for improving more effective treatment strategies in the future. Therefore, we evaluated whether the interaction of MSCs with macrophages could play a significant role in the macrophage response to LPS and their anti-inflammatory/immune-modulatory effects, and also we investigated this hypothesis that the interaction between macrophages and Ad-MSCs in response to LPS could reduce iNOS synthase expression or not.

METHODS

Isolation of Adipose tissue MSCs

In this experimental study, adipose tissue was obtained from BALB/C male mice 6-8 weeks old (Pasteur Institute of Iran). Before the collection of the adipose tissue, mice were killed, and the adipose tissue was excised from the ventral region and kept on ice and PBS mixture until downstream enzymatic digestion. Then the tissue was minced thoroughly by scalpel and digested with 1 mg/ml collagenase type I solution (Sigma-Aldrich, St. Louis, MO, USA). After 30 minutes at 37 ° C, Dexmedetomidine (DMED) (low glucose, Gibco-BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, NY, USA) was added to neutralize collagenase type 1 and then centrifugation at 1500 rpm for 5 minutes was carried out. The cell pellet was cultured in a cell culture flask containing DMEM plus 10% FBS. After 24 hours of incubation at 37 ° C and 5% CO_2, Non-adherent cells were removed, and adherent cells were cultured to become confluent. Then, the cells were sub-cultured 2-3 times to have a pure population of MSCs.

Quantitative flow cytometry (FACS) analysis of Adipose Tissue MSCs Surface Markers

To analyze surface markers, the cultured MSCs were harvested with trypsin, then collected in PBS solution. Then, 10^5 cells were washed twice with 0.05% tween-20. The fluorescence conjugated monoclonal antibodies used in the study were anti-CD29, anti-CD11b, anti-CD73, anti-CD105, and anti-CD45 and anti-sca1 (all from Biosciences), which were added to the cells and incubated for 45 minutes at 4 ◦C. Then, the mixture was centrifuged for 5 minutes at 500 rpm to remove the unbound antibodies. Data were obtained using the Flow Cytometer.

MSCs Differentiation Assay

To assess whether MSCs have the capacity of differentiating into osteocytes and adipocytes. To induce the MSCs differentiation into adipocytes, 10μM dexamethasone, 0.5 mM IBMX, 66 nM insulin, and 10 μM indomethacin were added to culture media. After 21 days of incubation at 37◦C and 5% CO_2 (the medium was replaced every three days), the cells

were fixed with 4% paraformaldehyde solution for 20 minutes. Furthermore, to induce the MSCs differentiation into osteocytes, the cells were treated with 10μM dexamethasone, 10 mM beta-glycerol-phosphate, and 50μg/ml ascorbic acid, and finally, after 21 days of incubation at 37◦C and 5% CO_2 (the medium was replaced every three days), the cells were fixed with 4% paraformaldehyde solution for 20 minutes. After the incubation period, osteogenesis was evaluated by alizarin red S staining, and adipogenesis was determined by staining the cells with Oil Red O. Images of the stained cells were taken using an Olympus IX71 microscope (Olympus, Tokyo, Japan).

Isolation of Peritoneal Macrophages

To isolate peritoneal macrophages, the first 2ml of 4% thioglycolate solution was injected into the peritoneal region of mice. After 3 days, the mice were sacrificed, then 10ml of DMEM was injected into mice peritonea, and then the solution containing mice macrophages was cultured (37° C, 5% CO_2) in DMEM plus 10% FBS.

Co-culture of MSCs with Macrophage

For coculture, 5 × 104 MSCs and 25 × 104 macrophages were seeded in a -12well plate as describes below.

A. Macrophage (control group)

B. Macrophages treated with LPS

C. Co-culture of MSCs and macrophages (1:5 ratio)

D. Co-culture of MSCs and macrophages (1:5 ratio) treated with LPS

After 72 hours, the supernatant of cultured cells was collected to analyze the cytokine and Nitric Oxide levels. Also, the total RNA was extracted from macrophages for downstream procedures.

Nitric Oxide Evaluation

The produced level of NO was evaluated in control and test groups via Griess colorimetric assay. In this method, 100μl of supernatant were added to 96-well plates. Then, 100μl of Griess reagent was added to each well, and after 5 minutes, the absorbance was read at 540 nm using Biotek-Elx800 ELISA reader.

Quantitative real-Time PCR (qPCR)

Total RNA was extracted using TRIZOL reagent (GeneAll, Seoul, Korea). Then the RNA was treated with DNase I to remove any DNA contamination. The RNA was reverse transcribed into cDNA using PrimeScript cDNA synthesis kit (Takarabio, Japan), according to the manufacturer's instruction. Quantitative real-Time PCR was performed in 96- well plates using the RealQ Plus Master Mix Green (Ampliqon, Netherland) on a StepOnePlus real-Time PCR System (ABI) under the following conditions: 95° C for 10 Min and 95° C for 25 s, 50 cycles of 60° C for 25 s, and 72° C for 15 s. The sequences of the primers used are listed in Table 1. Gene expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed using Livak method. The data are expressed as the mean ± SD of three independent experiments.

Cytokinetics Analysis

The collected supernatant of control and test groups was centrifuged at 300 rpm for 5 minutes. The obtained supernatant was collected and stored at - 70 ◦C for downstream cytokine evaluation. To evaluate the TNF-α, IL-10, and TGF-β1 cytokine, R&D kits were used according to the manufacturer's instructions.

Statistical Analysis

All data are expressed as the mean ± SD. Statistical comparisons were performed by one-way ANOVA with post hoc Tukey correction and by Student's t-test. A P value < 0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software 13 (SPSS Inc., Chicago, IL, USA) and Graphpad prism 7.0.

RESULTS

Identification of Isolated MSCs and Macrophages

To confirm the mesenchymal state of the isolated cells, these cells were differentiated into adipocytes and osteocytes. After 21 days of MSCs culture in the adipogenic or osteogenic selective media, the oil-red and Alizarin red S staining showed the adipose and osteocytic status of the cells, respectively (Fig.1)

Table 1: Primer Sequences used for Quantitative Real-time RT-PCR.

Primers	Sequences (5′-3′)
B- actin	F: ATGATGATATCGCCGCGCTC
	R: TACTTCAGGGTGAGGATGCC
iNOS	F: GCGAAGTGTCAGTGGCTT
	R: TCCTTTGAGCCCTTTGTG

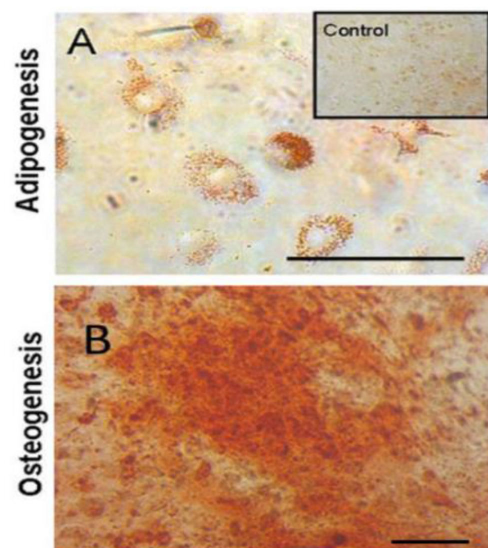

Figure 1: Confirmation of the differentiating properties of MSCs cells. A: Differentiation of MSC into adipocytes using oil-red staining, B: Differentiation of MSC into osteocytes using Alizarin red s staining . (× 200)

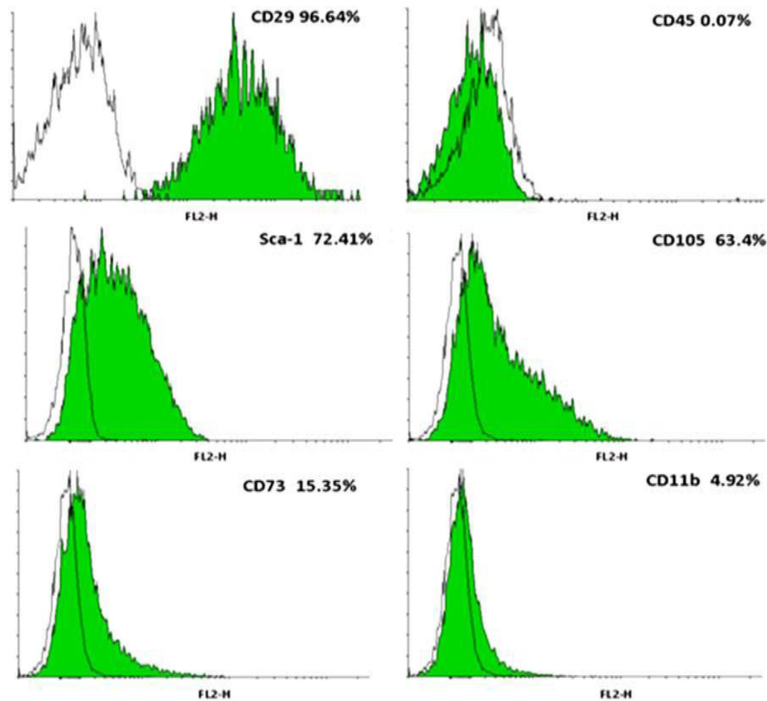

Figure 2: Flow cytometry analysis of adipose tissue mesenchymal stem cells for markers CD45, CD105, CD73, Scs-1, CD11b, CD29. , the average percentage of CD29, CD105, Sca-1, CD73, CD45, CD11b expression were 96.63, 63.4, 72.41, 15.35, 0.07, and 4.92, respectively.

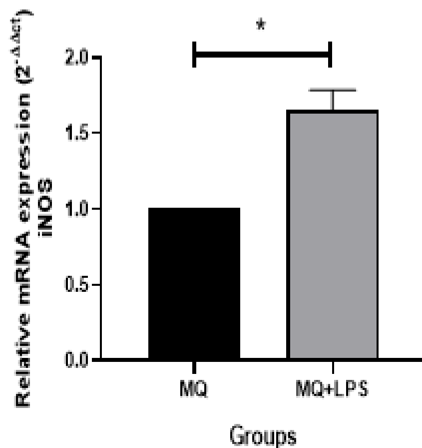

Figure 3: The CT Mean changes of iNOS gene in ipopolysaccharide-treated macrophages compared to non-treated cells.

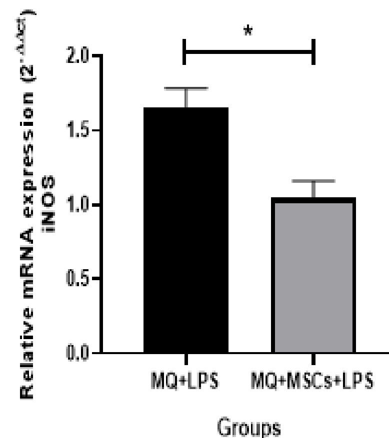

Figure 4: The CT Mean changes of iNOS gene in ipopolysaccharide-treated co-cultured macrophages with MSCs cells compared to non-treated cells.

Also, by using the flow cytometer, the isolated MSCs were identified. Based on results, the average percentage of CD29, CD105, Sca-1, CD73, CD45, CD11b expression were 96.63, 63.4, 72.41, 15.35, 0.07, and 4.92, respectively (Fig.2).
MSCs Modulate the Macrophage iNOS Activity
The results of qPCR and Griess results confirmed that LPS can significantly increase the iNOS mRNA expression in macrophages (Fig.3). Also, the results of qPCR showed that mRNA expression of iNOS in the co-culture of MSCs and macrophage treated with LPS significantly decreased compared to the groups without MSCs (Fig.4). Also, the Griess iNOS assay verified the qPCR results in which the group treated with LPS revealed a significant increase in iNOS level (Fig.5). Moreover, the co-cultured macrophages with MSCs treated with LPS demonstrated lower iNOS activity and expression in comparison with the macrophages treated with LPS (Fig.5).

MSC Regulates LPS-Induced Inflammatory Response of Macrophages

The co-culture of MSCs and macrophages diminishes the anti-inflammatory responses of macrophages. The enzyme-linked immunosorbent assay (ELISA) results showed

that the TNF-α level is decreased in macrophages co-cultured with MSCs compared with macrophages cultured alone. Similarly, the treatment of macrophages with LPS produced a significantly higher level of TNF-α compared with the co-culture of MSCs and macrophages plus LPS (Fig.6). Also, the ELISA results showed that the IL-10 and TGF-β1 level is significantly increased in macrophages cocultured with MSCs compared with macrophages cultured alone (Fig. 7, 8). More, treatment of macrophages with LPS produced a significantly lower level of IL-10 and TGF-β1 compared with co-culture of MSCs and macrophages plus LPS (Fig. 7, 8)

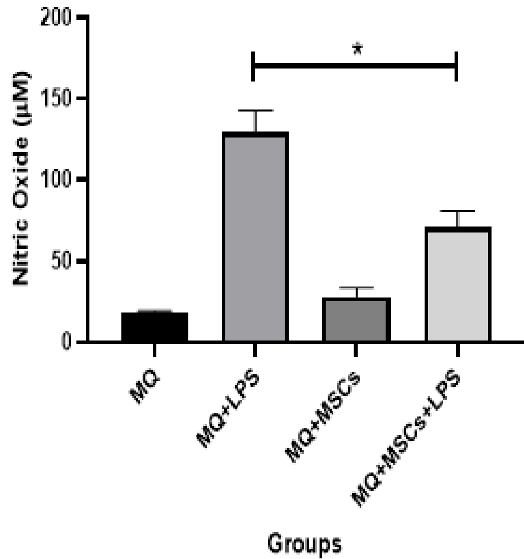

Figure 5: The results of Griess iNOS assay. The mean ± SD of nitric oxide in different groups.

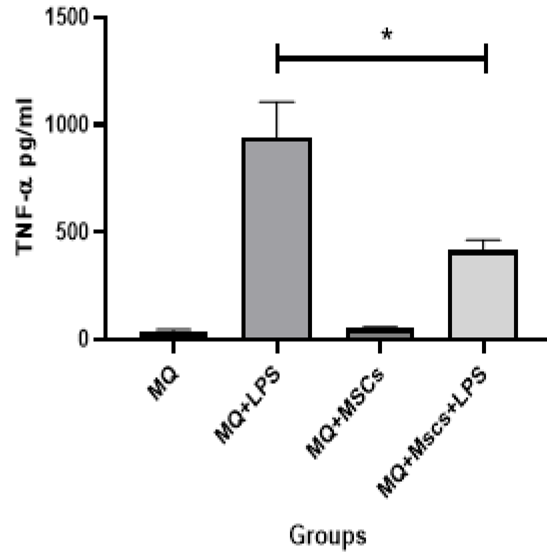

Figure 6: The ELISA results of TNF-α (inflammatory cytokine) level. The mean ± SD of TNF-α level in different groups.

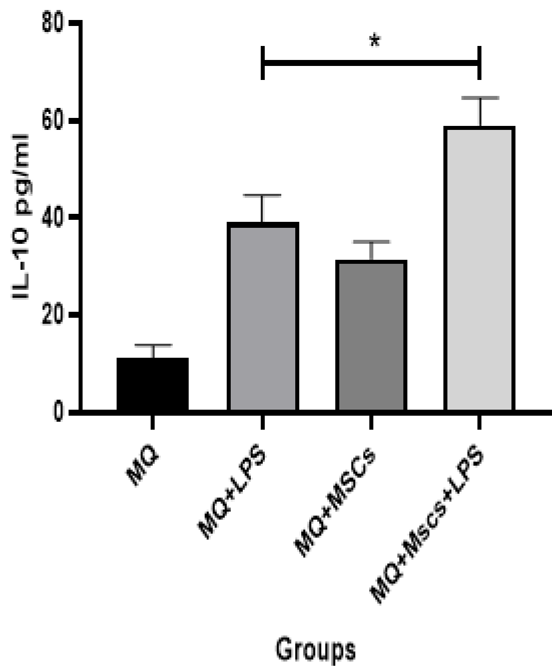

Figure 7: The ELISA results of IL-10 (anti-inflammatory cytokine) level. The mean ± SD of IL-10 level in different groups.

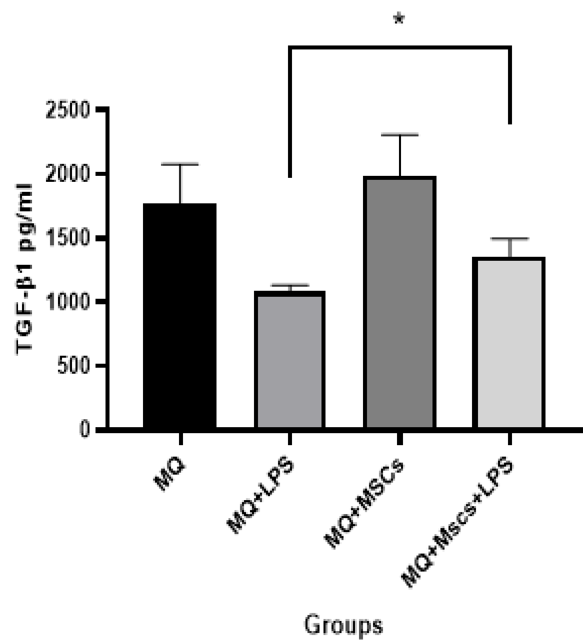

Figure 8: The ELISA results of TGF-β1 (anti-inflammatory cytokine) level. The mean ± SD of TGF-β1 level in different groups.

DISCUSSION

Mesenchymal stromal cells (MSCs) have emerged as a promising tool for application in regenerative medicine and cell therapy. Stem cells have already been used in individual cases, as well as in clinical studies. Since bacterial infections are a typical complication in wound healing, the behavior of MSC in response to pathogens could be influenced. In this regard, the anti-inflammatory capacity of MSC towards affected tissue might also be influenced by bacterial components, as they might be present in infected tissue [36]. To this end, we analyzed the influence of the anti-inflammatory activity of Ad-MSCs on LPS (the typical bacterial cell wall components) activated Macrophages in vitro by calculating the level of some secreted molecules such as iNOS, NO, TNF-α TGF-β, and IL-10.

MSCs have long been considered immune-privileged since they display no or low expression of class I MHC and of co-stimulatory molecules (CD40, CD80/CD86) and do not induce potent alloreactivity when infused into another organism [37].

Transplantation using MSCs for cell therapy in many clinical studies, autologous or allogeneic, are potentially efficacious for the treatment of a wide variety of clinical conditions, such as acute graft-vs-host disease after allogeneic hematopoietic stem cell transplantation, myocardial, infarction, amyotrophic lateral sclerosis, stroke, Crohn's disease, diabetes mellitus, and refractory wounds; however, no transdifferentiation to tissue-specific cells was observed in vivo [3, 38-42]. This means that the beneficial effects of MSCs occur via paracrine factors, including exosomes and secreted molecules.

There are several cellular targets of MSC therapy that span both the innate and adaptive arms of the immune system. MSCs altered the cytokine secretion profile of dendritic cells, naive and effector T cells [Th1&Th2], and natural killer cells; to induce more anti-inflammatory or tolerant phenotype Specifically, MSCs caused [17, 21, 43, 44]:

1) mature DCs type 1 (DC1) to decrease TNF-α secretion and mature DC2 to increase IL-10 secretion

2) Th1 cells to decrease IFN-γ and caused Th2 cells to increase secretion of IL-4

3) an increase in the proportion of regulatory T suppressor cells

4) decreased secretion of IFN-γ from NK cells

Some studies have examined the in vitro effects of MSCs as well as adipose-derived stem cells (ASCs) on macrophage polarization [35, 45, 46]. MSCs have been shown to inhibit M1 markers such as TNF-α, iNOS, and promote M2 polarization. Recently, a comprehensive study examined the in vitro crosstalk between MSCs and macrophages across different activation stages. The authors reported that MSCs facilitated monocyte to macrophage transition, potentiated microbial responses, skewed naive macrophages to an M1 state, and attenuated already activated M1 macrophages while enhancing M2 activation [47].

Accumulating evidence has indicated that MSCs induce tissue repair by reducing inflammation through their paracrine effects. According to mentioned studies, previous efforts have displayed an increasing number of positive outcomes using the MSCs paracrine activity without cell engraftment. Recently, Ulivi et al. demonstrated that the MSCs are able to change the polarization of pro-inflammatory M1 macrophages into anti-inflammatory M2 phenotype via soluble factors and secreting cytokines [35]. Although the mechanisms controlling MSC-induced macrophage polarization across different stages remain unclear, a few studies have identified some key factors. Nemeth et al. reported that LPS or TNF-α preconditioned MSCs can reprogram macrophages by releasing prostaglandin E2 (PGE2), which acts on the macrophages via the PGE receptors EP2 and EP4 [48]. Moreover, Takizawa et al. revealed that cell-to-cell direct co-culture of MSCs and blood cells can promote M2 macrophage polarization [49]. The polarization of M2 macrophages into educated macrophages is known by a decrease in the secretion of pro-inflammatory cytokines (TNF-α and IL-6) and an increase in IL-10 levels. Conversely, Chen et al. reported that it had no significant effect on the levels of TNF-α [50].

Kim and Hematti created the concept of educated macrophages for the first time [51]. They revealed that after 3 days of co-culture of macrophages with BM-ASCs, they adopted the phenotypes for M2. Gonzalez et al. co-cultured ASCs with colitis-derived macrophages and found a reduction in the pro-inflammatory cytokines TNF-α and IL-12 along with an increase in the anti-inflammatory IL-10 [52]. Moreover, the effects of ASCs on cytokines were inverted by PGE2 blockage. Another group displayed that BM-MSCs significantly affect the function of macrophages. Educated macrophages had a decrease in the inflammatory cytokines TNF-α, IL-6, IL-12p70, and IFN-γ and increase the level of IL-10 and IL-12p40, when provoked with LPS. Zhang et al. demonstrated that when macrophages co-cultured with gingival ASCs, macrophages acquired an anti-inflammatory phenotype (M2 phenotype) characterized by an increased expression of IL-10 and CD206 as well as a suppressed production of tumor TNF-α [53]. In vivo, gingival ASCs immigrated to the wound site, promoted M2 polarization, and significantly enhanced wound repair. It seems the gingival ASCs-induced suppression of TNF-α secretion by macrophages is correlated with impaired activation of NFκB p50 [54-56]. In the present study, we displayed that ASCs were able to increase the expression of M2 macrophages and modify cytokine expression without direct cell-to-cell contact. The data suggest that ASCs secreted exosomes to act on macrophages, inducing high levels of M2-related TGF-β1 and IL-10 and inhibiting macrophage inflammatory responses stimulated by LPS by reducing iNOS, NO, and TNF-α.

Our findings were supported by reports from other studies. In an animal model of Post-spinal cord injury, encapsulated ASCs were able to modulate the function of inflammatory macrophages and promoted the alternative M2 macrophage phenotype [57]. In vitro, this was evidenced by a reduction in macrophage iNOS expression. In another study, macrophages cultured with ASCs-conditioned medium showed an M2 phenotype, which is characterized by high increased production of IL-10 upon re-stimulation, and potent immunosuppressive activity on T cells and macrophages [58-61]. Although, our results demonstrate that administration of ASCs educated macrophages reduces systemic inflammation induced by LPS [62-64]. The data also reveal that ASCs favor the expression of M2 phenotype and anti-inflammatory cytokines independent of direct cell-to-cell contact. Furthermore, educated macrophages may function via IL-10 pathways. Further studies are warranted to delineate the mechanisms of educated macrophages on systemic inflammation.

CONCLUSION

In summary, this study demonstrated that Ad-MSCs could play an important role in the alteration of macrophage response to LPS, through the crosstalk between Ad-MSCs and macrophages. These effects might be through affecting cytokine expression levels, such as increasing IL-10 and TGF-β. Our results demonstrated that Ad-MSCs promote the M2 phenotype possessing anti-inflammatory and immunomodulatory functions in a cell contact-independent manner, and also the Ad-MSCs derived exosomes inducing M2 polarization could serve as an applicable stem cell-based cell-free tool for the therapy of the inflammatory disease through decrease NO, iNOS, and TNF-α. Moreover, exosome therapies could overcome several clinical risks and regulatory hurdles such as tumor formation by transplantation of stem cells. Future research will define whether Ad-MSCs secreted exosomes have anti-inflammatory effects in inflammation-related disease models like wound healing.

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ETHICAL STATEMENTS

All experiments in these studies were approved by the Shahid Beheshti University guidelines for animal care (number of approval ethics committee:IR.SBMU.MSP.REC.1395535).

COMPETING INTERESTS

The authors declare no competing interests.

FUNDING

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AUTHORS' CONTRIBUTIONS

Shokoofe Noori conceived and designed the study. Massoumeh Hotelchi performed the experiments. Sara Soudi analyzed and interpreted the data. Paniz Hazrati participated as a Native English editor. Zahra Shokati Eshkiki, as the corresponding author, managed the study. All authors approved the final version.

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