RESEARCH ARTICLE

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A Comparative Study of the Bone Marrow- and Umbilical Cord-Derived Mesenchymal Stem Cells (MSCs) Efficiency on Generating MSC-Educated Macrophages (MEMs)

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Abstract

Background: Mesenchymal stem cells (MSCs) have gained much more attention in cell therapy and regenerative medicine due to their immunosuppressive effects. MSCs have interaction with other immune cells, such as macrophages (MQs). Bone marrow (BM)-derived MSCs can educate MQs toward MSC-educated MQs (MEMs) which possess an anti-inflammatory immunophenotype. Given this and based on the important limitations of BM collection, we hypothesized whether co-culture of MQs with umbilical cord (UC)-derived MSCs can result in the MEM phenotype. **Methods:** First, isolated monocytes cultured for five days to obtain M0 MQs. Then, they were co-cultured with either BM- or UC-MSCs under direct and indirect conditions. After three days of co-culture, MEM-specific surface markers, as well as the gene expression of inflammatory and anti-inflammatory cytokines, were evaluated. **Results:** Surface expression of CD163/CD206, as specific markers for M2 MQs, increased in MEMs after co-culture with BM- and UC-derived MSCs, while CD80/CD86 expression (specific markers for M1 MQs) didn't change significantly. The mRNA expressions of PDL-1 as well as anti-inflammatory cytokines, including IL-6, IL-10, and TGFβ also increased in MEMs after co-culture of UC-MSCs compared to control MQs ($p < .05$), while the expression of IL-12 was significantly decreased ($p < .001$). **Conclusions:** To the best of our knowledge, this study shows for the first time that the co-culture of MQs with UC-derived MSCs efficiently contributes to the generation of MEMs even greater than BM-MSCs; shedding light on the promising potential of UC as an alternative source to educate MQs in vitro.

Keywords: Mesenchymal stem cells (MSCs)- co-culture- Mesenchymal stem cell-educated macrophages

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Introduction

Mesenchymal stromal cells (MSCs) are multipotent stem cells (Kassem and Abdallah, 2008) isolated from various tissues, including adipose tissue, placenta, umbilical cord (UC), amniotic fluid, liver, dental pulp, mobilized peripheral blood, but the most frequently used sources of MSCs remain bone marrow (BM). Many studies indicated that MSCs possess immunomodulatory properties besides the feature of tissue repair (Nauta and Fibbe, 2007). Numerous studies proved that MSCs play an essential role in maintaining the regulation of transplant tolerance. They can reduce the incidence of graft versus host disease (GVHD), improve graft survival, and accelerate the reconstruction of the hematopoietic and immune systems due to their immunological features (Wolff et al., 2011). Also, in the last decades, MSCs infusion has been reported to be effective in adult and pediatric patients with steroid-resistant aGVHD (Ball et al., 2013), however, the field of MSC therapy is comforted with a paradox regarding the utility of MSCs for cell therapy. For example, while clinical-grade MSCs are not yet approved in the United States to treat aGVHD, MSCs are conditionally approved in Canada and some other countries (Kassem and Abdallah, 2008).

Subtle differences in donor source, culture methods, expansion levels, poor homing capacity, and inadequate cell survival are major challenges (Galipeau, 2013) that

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have limited the therapeutic benefits of MSC therapy. Given this, most of the studies have shifted into other aspects, one of which is focusing on the interactions of MSCs with immune cells. Numerous data have shown that MSCs regulate immunity partly through the regulation of monocytes and MQs (Chiossone et al., 2016). In general, MQs are categorized into two main subsets of classical (M1) and alternative (M2) MQs based on the phenotypic pattern and cytokine secretion profile. In 2009, Kima et al. highlighted the ability of MSCs to modulate and skew the phenotype of tissue MQs, so-termed MSC-educated Macrophages (MEMs), toward an anti-inflammatory phenotype (Kim and Hematti, 2009). MEMs have greater potency for anti-inflammatory and tissue-secreting activities than conventional MQs. They increase the survival of patients with GVHD and increase the survival of mice exposed to radioactive damage, respectively, by inhibiting the proliferation of human T cells and enhancing the proliferation of mouse fibroblasts (Bouchlaka et al., 2017). Bouchlaka et al. have also demonstrated that human MQs educated by BM-derived MSCs represent a novel and distinct cell subset of alternatively activated MQs with significant anti-inflammatory benefits. Using xenogeneic GVHD and radiation injury models, the authors reported that ex vivo generated MEMs have therapeutic potential in vivo. In agreement, Abumaree et al. indicated that by changing the differentiation of MQs from M1 to M2, placenta-derived MSCs can induce anti-inflammatory phenotype (Abumaree et al., 2013).

In many studies, MEMs are usually educated by MSCs derived from BM, however, since the collection process of MSCs from BM has some limitations such as invasive acquisition procedure, higher risk of infectious diseases transmission, donor's age, and limited proliferative potential of MSCs (Pittenger et al., 2019), exploiting alternative sources could be beneficial. UC is a prenatal organ which composed of various anatomical parts like the amniotic membrane (UCM), perivascular region (PRV), and the central part of UC constituted by Wharton's Jelly (WJ) (Semenova et al., 2021). MSCs can be rapidly isolated from UC without any risk or discomfort for the donor. Indeed, UC is an excellent source to obtain a considerable number of MSCs as the collection process is non-invasive. Of great importance, MSCs from UC are primitive with a higher potential of expansion in vitro. To the best of our knowledge, the effects of UC-derived MSCs, as an alternative source, on generating MEMs have not been yet described, and this study shows for the first time that the co-culture of monocytes with UC-derived MSCs efficiently contributes to the generation of MEMs; shedding light on the promising potential of UC in the context of MCS therapy.

Material and Methods

Ethics of experimentation

Peripheral blood (PB) and bone marrow aspiration samples were collected from healthy volunteers and healthy BM donors, respectively. All samples were obtained with informed patient consent. The protocol was reviewed and approved by the institutional review board of Tehran University of Medical Sciences.

Human monocyte isolation and MQ differentiation

According to the manufacturer's protocol, monocytes were isolated from human PB based on using magnetic bead separation methods. First, peripheral blood mononuclear cells (PBMCs) were collected from a healthy volunteer by density gradient separation medium (1.077 g/ml, Ficoll-Paque, GE Healthcare, USA). The whole blood of healthy volunteers was diluted by phosphate buffer saline (PBS) and then layered on the top of the Ficoll solution gently. The tube was centrifuged at 400×g for 30 min at 20°C, and mononuclear cells were washed with PBS three times to reduce red blood cells (RBCs) contamination and centrifuged at 300×g for 10 min. To isolate monocytes, the cells' pellet was incubated with anti-human CD14 microbeads (Monocyte isolation kit II, Human, Miltenyi Biotech, Germany) for 15 min at 4°C. After washing unbounded antibodies with the isolation buffer, cell separation was done using auto-MACS Pro Separator (Miltenyi Biotech) using LS columns. Flow cytometry and samples' purity of CD14+ isolated monocytes were ascertained and used with purity greater than 90% for the experiment.

To obtain M0 MQs, monocytes were seeded into a six-well culture plate at a density of 1×106 cell/well using RPMI supplemented by 10% human autologous serum blood type AB. Cells were cultured for 5 to 7 days at 37°C with 5% CO_2 without adding cytokine or growth factors to generate MQs. With one change of media 3 to 4 days after initiation of culture. To determine the best time of M0 generation for co-culture, cells were assessed using flow cytometry for M0 markers at days 5 and 7 of differentiation.

BM and UC-derived MSCs culture

BM aspiration from a healthy donor, referred to BM Transplantation Center, Shariati hospital, Tehran, Iran, was collected into bags containing ACD anticoagulant. Freshly isolated BM was diluted with an equal volume of PBS and layered 2:1 on the top of the Ficoll solution gently (GE Healthcare, Chicago, USA). After centrifugation at 300×g for 20 min, the mononuclear cell layer was isolated from the interface. Then, cells were washed twice with PBS and centrifuged at 300×g for 10 min. Cell's pellet was resuspended in DMEM medium containing 10% fetal bovine serum (FBS) (Gibco, Massachusetts, USA), 1% penicillin (Gibco, Massachusetts, USA), 1% streptomycin (Gibco, Massachusetts, USA), and two mM glutamine (Invitrogen, Merelbeke, Belgium). The cells were seeded at a density of 80,000/cm^2 in 25 cm^2 T-flasks and maintained at 37°C with 5% CO_2. After 72 hours, the non-adherent cells were discarded, and attached cells were allowed to reach near confluency. Cells were then passaged using Trypsin (Invitrogen, Carlsbad, CA, USA) until passage 3 when flow cytometry and differentiation assays were performed to verify MSCs according to established criteria (Dominici et al., 2006). MSCs between passages 3 to 5 were used for experiments.

Besides, UC-derived MSCs were also obtained from Royan Institute for Stem Cell Biology and

Technology, Tehran, Iran, and cultured in DMEM medium supplemented with 10% heat-inactivated FBS (Gibco, Massachusetts, USA), 100μg/ml streptomycin and100U/l penicillin. When cells reached 80–90% confluency, the adherent cells were trypsinized with 0.25% TrypLE (Invitrogen, Carlsbad, CA, USA) and sub-cultured into new flasks for more expansion. MSCs at passage 3–5 was characterized and used for experiments as mentioned above.

Co-culture of M0 MQs with BM/UC-derived MSCs

Two different co-culture methods were used in this study. For MSC/MQs co-cultures in direct cell contact, at day +5, 2×10^5 MSCs were added to each well of MQs (1:10 ratio) in a six-well culture plate incubated for an additional 3-4 days. One well of MQs without MSC addition was used as a control group. Cells were cultured for 3 days in RPMI 10% AB serum, and the cell population was then separated using a cell sorter device.

The MSC-conditioned medium was used for indirect co-culture. To prepare conditioned media, 1×10^5 MSCs were cultured in 75 cm^2 flasks containing DMEM medium with 10% FBS, 100μg/ml of-glutamate, 100μg/ml streptomycin and 100U/l penicillin. Every two days, the medium was removed and replaced with fresh medium. When cells reached 75 % confluency, they were cultured in fresh medium for two days, and conditioned medium was then harvested, centrifuged at 500×g for 10 min, and stored at -80 °C until further use. On day +5, conditioned medium was added to MQs and incubated for an additional 3-4 days. The control group was a 5-day monocyte well with no MSC addition.

Flow cytometry analyses

Cells were harvested using trypsin and resuspended in PBS. On days 5 and 7 MQ-specific markers expression were subsequently analyzed. Three days after direct and indirect co-culture with BM- and UC-derived MSCs, MQ polarization and MEM formation were investigated. For analysis, 1×10^5 cells were stained with antibodies (Table 1) for 30 min at 4°C in the dark, and then cells were washed with cold PBS by centrifugation at 150 × g for 5 min at 4°C. Cell surface staining was analyzed using a BD FACSCalibur (BD biosciences, San Joes, CA, USA) and Partec (GmbH, Germany). FlowJo software version 7.6 was used to analyze data acquired.

Quantitative RT-PCR

Total RNA was extracted from MQ after direct/indirect treatment and also MQ at day +5 as a control group using TRIZOL (Sigma Aldrich, Missouri, USA). The quality of isolated RNA was verified by Nanodrop 1000 (Fisher scientific, Pittsburgh, PA, USA), and one to 5μg of the resultant RNA reversely transcribed to cDNA using a reverse transcription kit according to the manufacturer's instruction (BIO FACT, Korea)(Khakpour et al., 2017; Eskandari et al., 2019; Motaei et al., 2019; Abedi et al., 2020). The quantitative polymerase chain reaction was then performed for cDNA using SYBR green master mix (BIO FACT, Korea) on Corbett Rotor gene 6000 instruments (QIAGEN, Germany) using standard protocols. Human-specific primers for IL10, IL12a, Programmed Death-Ligand 1(PD-L1), Arginase-1(Arg-1), IL-1β, IL6, and TGF-β were purchased from Pishgam (Tehran, Iran) which the sequences are listed in Table 2. Each reaction consisted of a 2x master mix, 0.5 μM forward primer, and 0.5 μM reverse primer in a total volume of 10 μL. Each gene's relative mRNA level was normalized with the ABL1 (Abelson murine leukemia viral oncogene homolog 1) housekeeping gene. Ct values for the housekeeping genes and the genes of interest were determined, and the difference between the Ct values was calculated (ΔCt). Reverse transcriptase-PCR (RT-PCR) results are presented as fold change expression = $2^{-\Delta\Delta Ct}$ of each gene compared to the control MQ group. A dissociation melt curve at the end of RT-PCR was also run to verify the PCR products' homogeneity and the absence of primer-dimers.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significance of results, and Sidak was the post-test. The results were analyzed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA) and were reported as the mean ± standard deviation of three replicates. A $P<.05$ was considered significant.

Results

BM- and UC-derived MSCs were successfully isolated and characterized

All MSCs derived from BM and UC were more than 95% positive for MSC markers such as CD44, CD90, CD73, CD29, and CD105 and negative for hematopoietic

Table 1. Antibodies Used to Analyze Surface Markers of MQs

Antibody	Conjugate	Manufacturer	Expressed on
CD80	PE	Miltenyi Biotech, Germany	M1 MQ
CD86	FITC	Miltenyi Biotech, Germany	M1 MQ
CD163	FITC	Miltenyi Biotech, Germany	M2 MQ
CD206	PE	Miltenyi Biotech, Germany	M2 MQ
CD1c (BDCA)	PE	Miltenyi Biotech, Germany	DCs
CD68	FITC	Miltenyi Biotech, Germany	M0 MQ
CD39	APC	Miltenyi Biotech, Germany	MEMs
CD14	PE	BD Pharmingen, USA	Mono
CD11b	PE	BD Pharmingen, USA	M0 MQ

DCs, Dendritic cells; Mono, Monocytes; MEMs, MSC-educated MQs; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

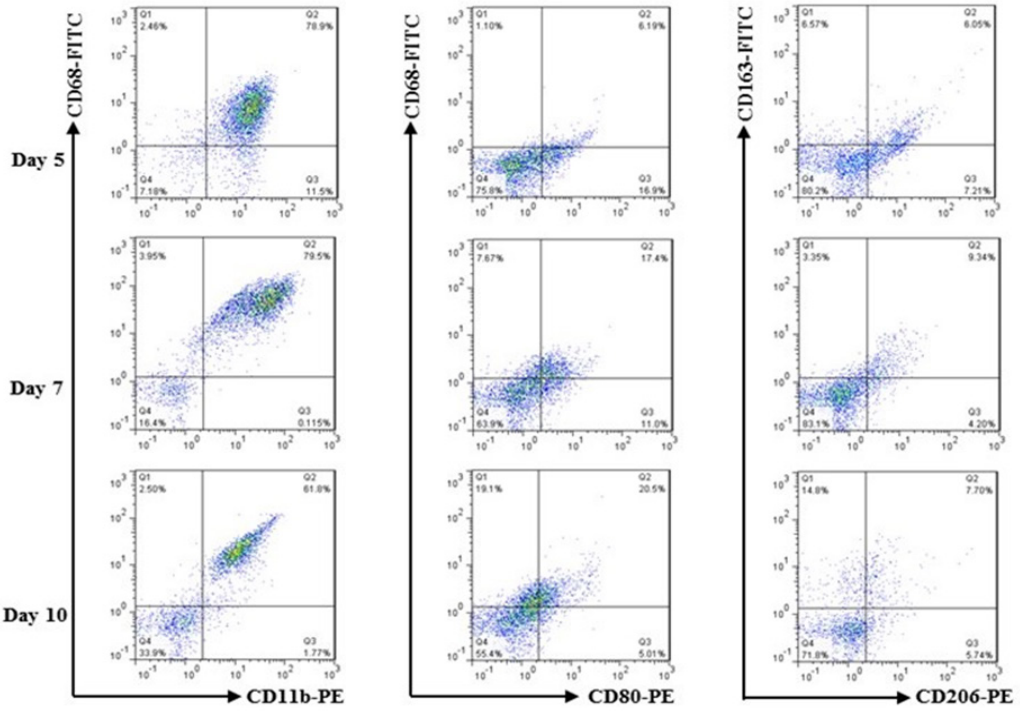

Figure 1. Comparison of MQ CD Markers on Day 5, 7 and 10. Monocytes on day +5 express high level of CD11b/CD68 while expressing low level of CD80, CD86, CD206 and CD163. High expression of CD11b/CD68 indicated the monocyte differentiation to MQs during in vitro culture. Low expression of polarization markers also indicated a lack of differentiation into inflammatory/anti-inflammatory phenotype and, in fact, remaining the state of resting (M0) MQs. (A) Unstained cells; (B) Cells expressing CD11b/CD68; (C) Cells expressing CD80/CD86; (D) Cells expressing CD206/CD163. PE, phycoerythrin; FITC, fluorescein isothiocyanate.

endothelial markers including CD31, CD45, CD34, CD11-b, and CD14 at passage 2. They were also negative for HLA-DR molecules. The cells were able to differentiate into osteocytes and adipocytes (Schmelzer et al., 2019). According to the above criteria, we used MSCs at passage 2 in the subsequent experiments.

M0 MQs generated from monocytes at day +5 of primary culture

To confirm the induction of M0 MQs phenotype, we evaluated monocyte- and MQ-specific markers by flow cytometry on days 5, 7, and 10 following monocyte isolation. The surface markers of M0 MQs, CD11b/CD68, were expressed among cells on days 5 and 7, indicating successful differentiation of monocyte to MQ. However, cells on day 7 showed elevated surface marker expression of both CD80/CD86 and CD206/CD163, which are used to define M1 and M2 polarization, respectively, as compared to cells on day 5. After 10 days' culture of monocyte-derived MQs, not only was the number of viable cells reduced but the expression of M1 and M2 differentiation markers were also increased (Figure 1). Taken together, cells at day 5 appear to expressed M0 surface markers while the expression of markers

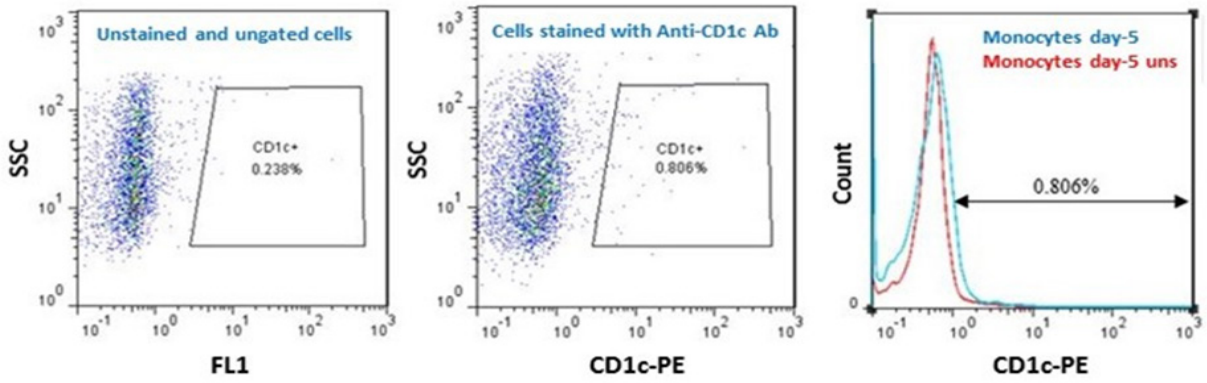

Figure 2. Monocytes on day 5 Did not Express DCs' Surface Marker. By using CD1c staining for monocytes on day 5 of primary isolation, the presence of any DCs did not detect at the end of day 5 by flow cytometry, highlighting that monocytes did not differentiate to DC without adding any cytokine or growth factor. PE: phycoerythrin, uns: unstained.

Table 2. The Primer Sequences for Target Genes

Genes	Primer sequence (5′–3′)	Amplicon size
IL-10	F: GCTGGAGGACTTTAAGGGTTACCT	109 bp
	R: CTTGATGTCTGGGTCTTGGTTCT	
IL-12a	F: AATGTTCCCATGCCTTCACC	110 bp
	R: CAATCTCTTCAGAAGTGCAAGGG	
PD-L1	F: GTGGCATCCAAGATACAAACTCAA	147 bp
	R: TCCTTCCTCTTGTCACGCTCA	
Arg-1	F: GGAATCTGCATGGGCAACCTGTGT	140 bp
	R: AGGGTCTACGTCTCGCAAGCCA	
IL-1β	F: CCTGTCCTGCGTGTTGAAAGA	150 bp
	R: GGGAACTGGGCAGACTCAAA	
IL-6	F: GGTACATCCTCGACGGCATCT	81 bp
	R: GTGCCTCTTTGCTGCTTTCAC	
TGF-β	F: CTGGATTGTGGTTCCATGCA	122 bp
	R: TCCCCGAATGCCTCACAT	
ABL	F: GGAATCCAGTATCTCAGACGAAGTG	227 bp
	R: GAGGGAGCAATGGAGACACG	

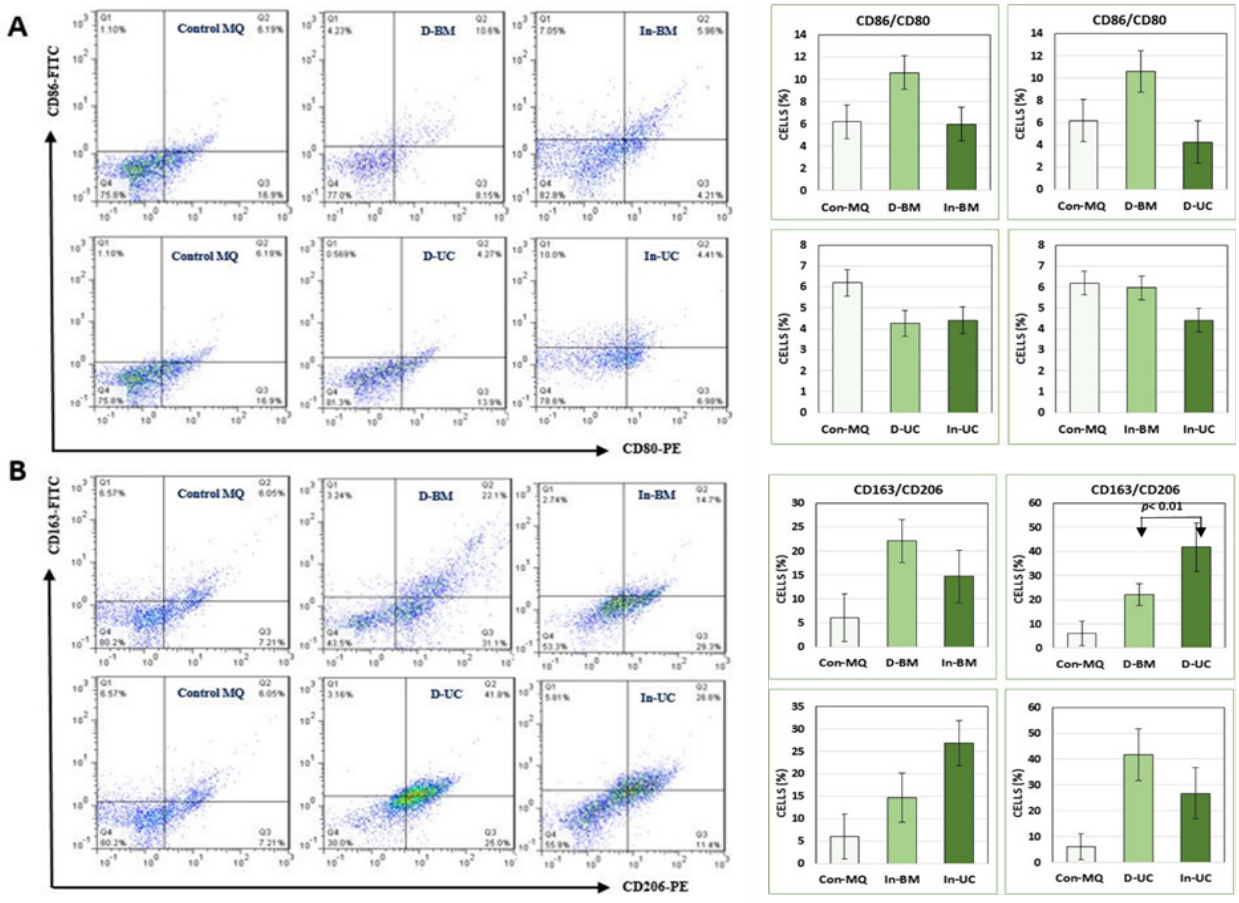

Figure 3. The Comparison of Alternatively and Classically Activated MQs Surface Markers on MEMs Educate from UC- and BM-derived MSCs. A) The expression of classically activated MQs markers, CD80/86 did not change or increase significantly after direct and indirect co-culture with BM and UC-derived MSCs. B) The expression level of CD206/CD163 markers on MQ after direct co-culture with both MSCs is increased which is also statistically higher in MQs educated by UC-MSc rather than BM-MSCs. It also indicates that indirect co-culture of both MSCs elevates the expression of alternatively activated markers with higher expression in In-UC groups. Moreover, the comparison of alternatively and classically activated MQs markers between direct and indirect co-culture of each MSC source separately displays that the effect of direct co-culture to increase CD206/CD163 expression is more significant than indirect one. D-BM: MQ after direct co-culture with BM-MSC; In-BM: MQ after indirect co-culture with BM-MSC; D-UC: MQ after direct co-culture with UC-MSC; In-UC: MQ after indirect co-culture with UC-MSC.

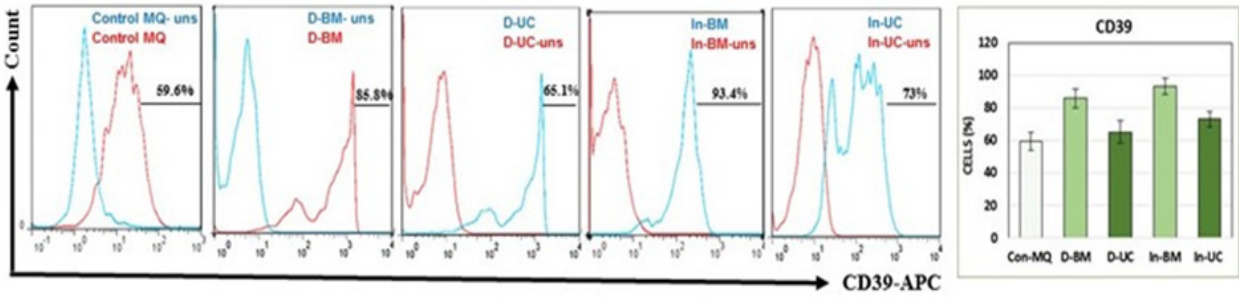

Figure 4. CD39 Expression is Elevated after Direct and Indirect Co-Culture in MQs Educated by Both BM- and UC-Derived MSCs. Both direct and indirect co-culture of both sources increase the expression surface marker of CD39 on educated MQs compared to control group. D-BM: MQ after direct co-culture with BM-MSC; In-BM: MQ after indirect co-culture with BM-MSC; D-UC: MQ after direct co-culture with UC-MSC; In-UC: MQ after indirect co-culture with UC-MSC; uns: unstained.

associated with MQ polarization was insignificant. Notably, using CD1c staining, we did not find the presence of any DCs at day 5 (Figure 2).

Co-cultured MQs with UC-derived MSCs increased expression of cell surface markers associated with alternatively activated MQs

Based on the results, monocytes at day 5 were used in the co-culture experiment as M0 MQ. MQs cultured in direct and indirect contact with UC-derived MSCs and BM-derived MSCs, as well. For direct co-culture, 2×10^5 MSCs were added to MQ at a ratio of 1:10, and for indirect contact, MQ was cultured with MSCs-conditioned media for 3 days of co-culture. On day 10 of primary culture, MSCs were eliminated, and CD14+/CD105- cells were characterized as educated MQs and used for further analysis. Overall, MQs after direct and indirect co-culture with either BM- or UC-derived MSCs elevated the expression of CD206/CD163 as specific markers for M2 MQs (Martinez et al., 2009; Tarique et al., 2015) while reducing the expression of CD80/CD86 (specific markers for M1 MQs) compared to the control group. However, we found that the direct interaction was more effective in increasing the expression of M2 MQs markers. Concerning the source of MSCs, we also noted that

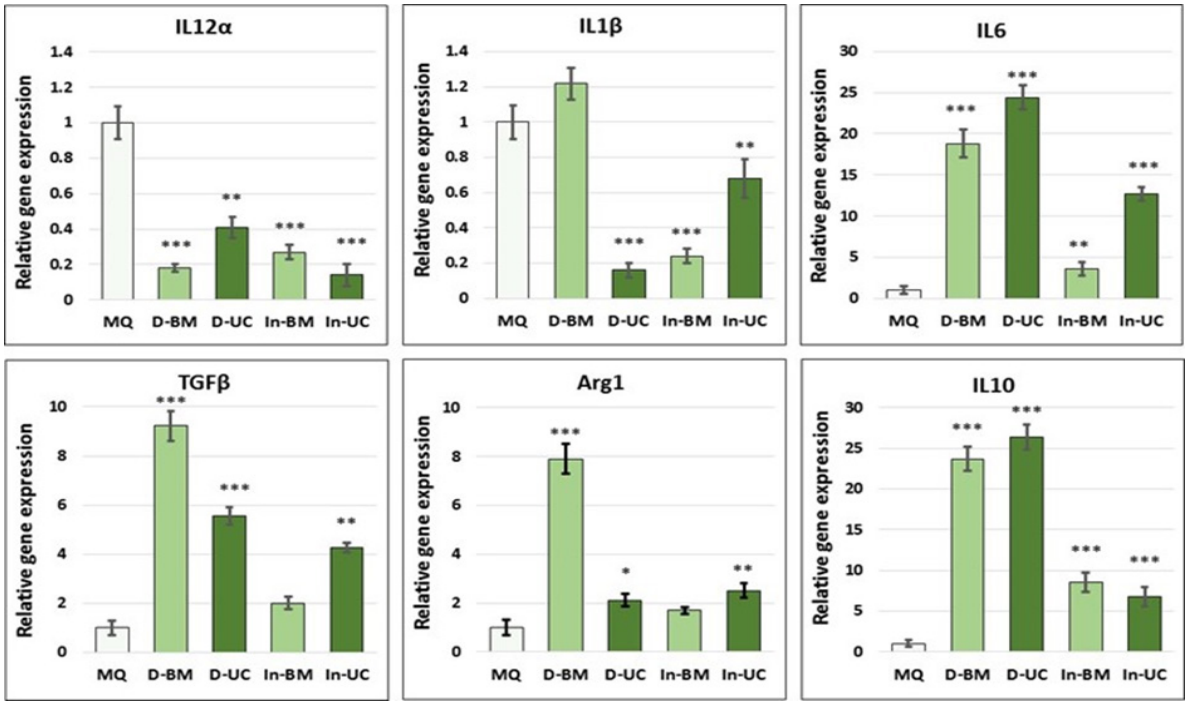

Figure 5. Comparison of the Gene Expression of Inflammatory and Anti-Inflammatory Cytokines in Different Study Groups. On day +10 of ex vivo expansion, CD14+ sorted MEMs after direct co-culture and MQs after indirect co-culture were analyzed by RT-PCR for gene expression. Anti-inflammatory genes increased significantly after co-culture with both MSC sources. Direct co-culture of BM-MSCs accounts for the highest expression of Arg-1 and TFG-β in MEMs, which is the highest expression among other sample populations. The gene expression of inflammatory cytokine IL12α decreased in all sample groups compared to control MQs. Moreover, the gene expression of IL1-β significantly decreased in all groups but after direct co-culture with BM-MSCs. Mean ± SEM analyzed by 2-way ANOVA/ Sidak post-test with multiple comparisons. P values of D-UC, In-UC, D-BM, In-BM were respectively <.0001, .0001, < 0.0001, and 0.1189. MQ: Control MQ; D-UC: MQs after direct co-culture with UC-derived MSCs; In-UC: MQs after indirect co-culture with UC-derived MSCs; D-BM: MQs after direct co-culture with BM-derived MSCs; In-BM: MQs after indirect co-culture with BM-derived MSCs.

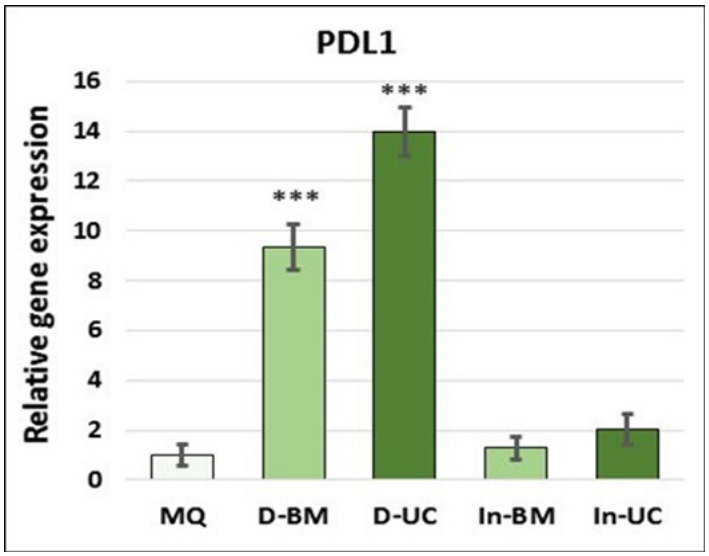

Figure 6. The Gene Expression of PD-L1. The gene expression of PD-L1 after direct co-culture with BM and UC-derived MSCs was statistically increased. MQ: Control MQ; D-UC: MQs after direct co-culture with UC-derived MSCs; In-UC: MQs after indirect co-culture with UC-derived MSCs; D-BM: MQs after direct co-culture with BM-derived MSCs; In-BM: MQs after indirect co-culture with BM-derived MSCs.

MQ educated by UC-MSCs increased the expression of CD206/CD163 more significantly than BM-derived MSCs (Figure 3). Next, to confirm the generation of MEMs, we evaluated the expression of CD39 whose upregulation was previously showed on MQs educated by BM-MSCs (Bouchlaka et al., 2017). According to our study, the expression of CD39 increased in MEMs after direct and indirect co-culture with both sources of MSCs compared to the control MQs (Figure 4).

Gene expression analysis of MEMs generated after co-culture with BM- and UC-derived MSCs

To confirm the result of flow cytometry analysis, we performed molecular techniques to analyze the gene expression of certain cytokines at mRNA level. The gene expression of specific cytokines associated with inflammatory and anti-inflammatory effects in control MQs (MQs at day +5) and MQs after direct and indirect co-culture with either BM-MSCs or UC-MSCs were analyzed by using RT-PCR. MQs after both direct and indirect co-culture demonstrated a statistically significant increase in the expression of IL-6 with the highest expression after direct co-culture with UC-MSCs ($p< .0001$). The level of IL-10 as an anti-inflammatory cytokine secreted by MQs was also significantly increased following direct and indirect co-culture with both BM- and UC-derived MSCs ($p< 0.05$). In agreement, direct and indirect co-culture with BM- and UC-MSCs significantly decreased the expression of IL-12α and IL-1β, as inflammatory cytokines, compared to control MQs. Notably, we figured out that the expression of the Arginase-1 gene and TGF-β increased significantly in MQs following direct and indirect interaction with UC-derived MSCs, while it did not happen upon indirect co-culture with BM-MSCs (Figure 5); further highlighting the fact that UC-derived MSCs may probably generate MEMs greater than BM-MSCs. We also showed an upregulated PD-L1 (CD274) expression in MEMs generated from direct and indirect co-culture of both mentioned MSCs, but it was not statistically significant after indirect co-culture. The highest and statically significant PD-L1 expression was observed in MQs after direct co-culture with UC-derived MSCs ($p< 0.0001$) as compared to the control MQs and also MQs after direct co-culture with BM-MSCs ($p= 0.0062$) (Figure 6).

Discussion

MQs are key innate immune cells that are widely located in many tissues of the body (Gordon, 2003). These cells are generally classified into two main subgroups; M1 MQs, which are known as inflammatory MQs that act to secrete inflammatory interleukins as well as Th1 responses and M2 MQs that have intense anti-inflammatory and phagocytic activity (Martinez and Gordon, 2014). They are classified into several subgroups M2a, M2b and M2c according to their cytokine expression pattern. This classification is based on low secretion of inflammatory cytokines including interleukin 12 and high secretion of anti-inflammatory cytokines such as IL-10, but, the M2b subgroup is still associated with high expression of IL-6 and TNF-α production (Mosser, 2003).

In 2009, a new subset of MQs was introduced as trained MQs, created from co-culture with MSCs in vitro (Kim and Hematti, 2009). Due to their immunomodulatory and repairing properties of damaged tissues, these MQs have been highly regarded for the prevention and treatment of inflammatory diseases. Interaction of MSCs with M0 MQs induces MQs with anti-inflammatory and regulatory phenotype or M2-like phenotype. Although M2 MQs usually have low levels of IL-6 production, educated MQs produce large amounts of IL-6. This cytokine is commonly known as a pro-inflammatory cytokine. However, the results of some studies show that IL-6 has anti-inflammatory properties and plays an important role in increasing the repair of damaged tissues. It also strengthens and supports BM hematopoiesis and

reduces radiation-induced inflammation. This cytokine is also known to induce alternative MQs and improves healing of colitis-damaged mucosa and spontaneous repair of cartilage by MSCs (Scheller et al., 2011). In fact, the cytokine expression profile of educated MQs is more similar to that of M2c. In general, MEMs with high IL-10, low IL-12, high IL-6 and low TNF-α are known as a new subset of alternative M Qs and distinct from other anti-inflammatory subsets.

In this study the expression of IL-6 gene in educated MQs after co-culture of BM and UC-MSCs were increased, which confirms the results of the similar studies. In addition to examining the effects of BM-MSCs, UC-MSCs as a new source of MSCs was also used to induce in vitro educated MQs. UC is a significant source of MSCs as the collection process is painless and non-invasive, with no harm or discomfort to the mother or fetus, and is usually a disposable source (Bieback et al., 2004). In addition, the growth and proliferation kinetics of UC-MSCs are significantly higher than those of BM-MSCs, and these cells will continue to proliferate longer in culture medium. The increased proliferative capacity allows these cells to proliferate in a shorter time in vitro to produce a sufficient number of MSCs for clinical aplications (Jin et al., 2013). To perform co-culture, day 5 M0 MQ was selected due to the higher expression of CD11b and CD68 and also low expression of CD80, CD86, CD206 and CD163 markers. Day 5 was also selected as a pre-ship control to compare results after three days of direct and indirect co-culture. In addition, in order to confirm the lack of differentiation of monocytes into DCs, the monocytes were examined for the expression of the marker CD1 on day 5c, which is one of the markers related to DCs, and the expression percentage of this marker was close to zero.

The increased expression of CD206 and CD163 in MQs after co-culture with UC-MSCs was greater than BM-MSCs. In general, direct cell-cell interactions are more likely to increase the expression of anti-inflammatory markers in educated MQs than in indirect co-culture. In addition, direct and indirect co-cultures did not differentiate MQ to the M1 phenotype, because the expression of M1 MQ markers, after co-cultures, were not significantly different from the control group. Educated MQs after co-culture with both MSC sources are associated with increased CD39 expression. CD39 is an actonucleotidase that converts ADP / ATP to AMP and AMP to adenosine (Antonioli et al., 2013). Previous studies have shown that MSCs increase the expression of CD39 and CD73 and ultimately, adenosine increase inhibits T cell proliferation. Thus, MEMs, which are CD39 / CD73 +, inhibit T cell proliferation ex vivo (de Oliveira Bravo et al., 2016). The results of our study indicate that in addition to the direct effect of BM and UC-MSCs, MSC-conditioned media also increases CD39 expression in educated MQs. Indirect co-culture also increases CD39 expression more than direct co-culture.

PDL-1 belongs to the family of B7 membrane-permeable proteins that generally inhibits T cells (Keir et al., 2008). This molecule, as well as its receptor (PD1), is actually protein associated with the programmed cell death signaling pathway or apoptosis (Xing et al., 2018). PDL-1 is mainly expressed by B and T lymphocytes, MQs and DCs (Latchman et al., 2001). Interaction of this molecule with its receptor (PD1) on the surface of immune cells leads to inhibition of proliferation, migration and production of cytokines in antigen-activated lymphocytes and ultimately suppresses the function of executive T cells and immune tolerance. In this study, the expression of PDL-1 gene in educated MQS after direct co-culture with UC-MSCs had the highest expression compared to control group ($P < 0.0001$), whereas increased expression of this gene after indirect co-culture of both MSCs sources compare to control MQs was not statistically significant.

In addition to examining the co-culture of human MSCs and MQs, many studies have examined these effects in mouse models. In a 2014 study by Gao S et al., mouse MSCs were able to induce RAW264.7 MQs (mouse MQ cell line) into the M2 phenotype. IL-10 expression was increased in these MQs and inflammatory cytokines, including TNF-α, were decreased. Unlike our study and other studies that introduce educated MQs as a new and distinct subset of M2, in Gao S et al. study, IL-6 expression decreased, possibly due to differences in mouse and human cells. In our study, the results of IL-10 gene expression also confirmed Gao S et al study. IL-10 is widely produced as an inhibitory cytokine by alternative and anti-inflammatory MQs. IL-10 expression was significantly increased after co-culture with UC-MSCs while the direct co-culture is more effective in increasing IL-10 gene expression in educated MQs. In addition to IL-10, the expression of TGF-β gene, as another inhibitory and anti-inflammatory cytokine, was evaluated by the full PCR technique. This cytokine plays an important role in the development, wound healing and immune responses through regulatory effects on many cell types (Blobe et al., 2000). Educated MQs after co-culture with BM and UC-MSCs showed a significant increase in TGF-β gene expression compared to control MQs, except for indirect co-culture with BM-MSCs. In the present study, the expression of Arg-1 gene as one of the genes involved in the signaling pathway of IL-6 production was also investigated. Arg-1 is a cytosolic enzyme that is permanently expressed in liver cells and its main function in the liver is to remove nitrogen by breaking down arginine and converting it to urea and ornithine (Gordon, 2003) . In addition to the liver, this enzyme is also expressed in MQs, but its expression is not permanent and is regulated by external stimuli of Th2 cytokines such as IL-4 and IL-13 (Pauleau et al., 2004). Increased expression of this enzyme in M2 MQs is used as a specific marker to differentiate inflammatory MQs from anti-inflammatory. The results of studies in this field show that the production of Arg-1 in MQs increases the production of Th2 cytokines and ultimately helps to eliminate inflammation and repair damaged tissues (Martinez et al., 2009). It also leads to early wound healing as well as inhibition of GVHD (Highfill et al., 2010). A similar study showed that educated MQs also have tissue protection roles in vivo by increasing the expression of potential molecules, including Arg-1. In our study, the expression level of Arg-1 gene increased after co-culture with MSCs of both groups compared to the control group.

In addition to the expression of anti-inflammatory

cytokine gene expression, some inflammatory cytokines, including IL-12α, were examined. Evidence from previous similar studies has shown that the expression of this cytokine is decrease in alternatively activated MQs, as well as educated MQs. In this study, the expression of IL-12α gene after co-culture with BM and UC-MSCs showed a significant decrease compared to control MQs. The direct co-culture group with UC-MSCs was associated with less significant but less decreased expression than the other groups (P = 0.0003). In addition, IL-1β gene expression was assessed, which showed a decrease in expression compared to control MQs in all co-culture groups of both MSC sources except BM-MSCs. IL-1β is generally described as a reactive cytokine at the beginning of the acute phase of inflammation, but in some immunological contexts it plays a major role in tissue repair. Elevated IL-1β levels may promote early bone healing as well as the preparation of MSCs to enhance repair of damaged airway epithelial cells (Wu et al., 2016). In addition, in a similar study, after examining the gene expression profile of educated MQs, the expression of IL-1β in these cells was associated with increased expression compared to control MQs, and this increase may be related to the tissue repair characteristic of MEMs (Bouchlaka et al., 2017). However, in our study, only educated MQs after direct co-culture with BM-MSCs showed an increase in IL-1β expression compared to control group, although this increase was not statistically significant (P = 0.2613).

In summary, both direct and indirect co-culture of UC-MSCs with human M0 MQs were effective at inducing educated MQs, increasing the anti-inflammatory and decreasing inflammatory cytokines. MQs educated by UC-derived MSCs increase the expression of CD39 marker after direct and indirect co-culture. Based on our consideration, UC-MSCs can be used as an alternative source to educate MQs with anti-inflammatory properties.

Author Contribution Statement

All authors contributed equally in this study.

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