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Up-regulation of CD200/CD200R1 Immunomodulatory Axis of Allogenic Peripheral Blood Mononuclear Cells in a Co-culture with Adipose-derived Mesenchymal Stem Cells

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ABSTRACT

Co-inhibitory molecules modulate immune responses. Immunomodulatory properties of mesenchymal stem cells (MSCs) turn them into ideal candidates for cell therapy. This study was designed to investigate the immunomodulatory effect of adipose-derived stem cells (ASCs) on inflammatory environment of a co-culture of allogenic peripheral blood mononuclear cells (PBMCs) in a two-way mixed leukocyte reaction (twMLR) setting.

ASCs were co-cultured with allogenic PBMCs in twMLR setting for four days. The proliferation of peripheral blood mononuclear cells (PBMCs), levels of interleukin (IL)-10, and expression of interferon-gamma (IFN-γ), B7-1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-ligand 1 (PD-L1), +, and CD200R1 genes, as well as cell surface expression of CD200 and CD200R1, were measured in twMLR as control, and co-culture groups on days 0, 2 and 4 of the experiment.

The proliferation of PBMCs was suppressed on days 2 and 4 of co-culture. The expression of CD200 (p=0.014), CD200R1, CTLA-4, and PD1 genes increased on days 2 and 4 of the co-culture compared to twMLR. CD200 expressing PBMCs decreased by 1.75% on day 2 of the co-culture but increased by 6.23% on day 4 of the co-culture (p=0.013) compared to the same days of twMLR. IL-10 levels increased in the co-culture supernatants on days 2 and 4 compared to twMLR (p<0.05).

Our results showed that ASCs upregulate the CD200/CD200R1 axis more than PD-1/PD-L1 and CTLA-4/B7-1 pathways in the twMLR. Also, elevated expression of CD200R1 in the final day of

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co-culture was similar to PD-1 expression pattern. This finding suggests a role for the CD200/CD200R1 axis in later modulation of the immune response.

Keywords: CD200 protein; CD200R1 protein; Immunomodulation; Mesenchymal stem cells

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent, self-renewing, fibroblast-like cells with the ability to differentiate into chondrogenic, osteogenic, and adipogenic lineages.[1] MSCs can be isolated from a variety of tissues such as adipose tissue, bone marrow, umbilical cord, placenta, and amniotic fluid and can modulate immune and inflammatory responses.[1] Cell to cell contact and paracrine secretion of soluble factors are the main mechanisms through which MSCs exert their immunomodulatory properties,[2] making them a proper candidate for cell-based immunotherapy.[3] Remarkably, the immunosuppressive properties of MSCs are not constitutive, indicating that MSCs acquire immunosuppressive traits due to the presence of excess inflammatory cytokines in their milieu, namely the interferon-gamma (IFN-γ).[4] MSCs can also induce a regulatory phenotype in innate and adaptive immune cells.[5] MSCs have been shown to reduce the expression of co-stimulatory molecules such as B7 complex on the surface of dendritic cells (DCs), preventing the induction of T-cell proliferation in adaptive immune response.[6] Enhancing the polarization of T-cells toward regulatory T-cell (T_{reg}) phenotype[7] and induction of co-inhibitory molecules expression such as the programmed death-ligand 1 (PD-L1)[8] are among other mechanisms by which MSCs exert their immunoregulatory function.

Mixed leukocyte reaction (MLR) is an assay commonly used to investigate alloreactivity and in a co-culture of a set of stimulator T cells and a set of responder T cells that are mixed and cultured for 4–7 days to allow an alloreactive response to develop.[9] Due to the alloreactive nature of the immune response in MLR, it is functionally similar to the recognition of non-self-antigens on major histocompatibility complex (MHC) by T-cells and has been used as a popular assay to study the activation and proliferation of T-cells.[10] During the adaptive immune response and following the activation of T-cells through recognition of antigen-MHC complex, both co-stimulatory and co-co-inhibitory molecules expand to regulate the activation and suppression of immune response.[11] In the absence of other stimuli, T-cells mainly differentiate into T helper 1 cell (Th1),[12] secreting pro-inflammatory cytokines, IFN-γ, interleukin 2 (IL-2) and tumor necrosis factor-alpha (TNF-α), which are responsible for cell-mediated immunity.[13] Meanwhile, Th1 cells are capable of producing IL-10, which inhibits their over activation of Th1 cells and suppresses the production of pro-inflammatory cytokines.[14]

Members of the CD28 family, including cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1) are well-known co-inhibitory receptors of activated T-cells[15] that regulate T-cell survival, activation, and differentiation into various subtypes.[16] CTLA-4/B7-1 signaling pathway is primarily activated in T-cells with strong TCR signaling,[17] whilst PD-1 is expressed on activated T- and B-cells, inducing the apoptosis of T-cells and antigen-presenting cells through PD-L1 and PD-L2 ligands.[18] CD200 receptor 1 (CD200R1) is another co-inhibitory receptor of activated T- cells.[19] CD200R1 expression is mainly limited to myeloid cells and some lymphoid cells, including Th1 cells, while CD200 is expressed on a wide range of cells, including MSCs, thymocytes, B-cells, activated T-cells, neurons and vascular endothelial cells.[20]

Considering the immunoregulatory effects of MSCs on immune responses and the expression of co-inhibitory ligands on these cells, we aimed to investigate the effect of adipose-derived stem cells (ASCs) on the expression of CD200R1, CTLA-4 and PD-1 as main T-cell co-inhibitory receptors as well as their respective ligands in a co-culture of ASCs with allogenic peripheral blood mononuclear cells (PBMCs) in a two-way MLR (twMLR) setting.

MATERIALS AND METHODS

Isolation and Expansion of ASCs

Adipose tissue samples were obtained from the omentum of patients (20-40 years old) undergoing any kind of abdominal surgery after obtaining informed written consent and explaining the goal and protocol of

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the study [Ethics board approval number: IR.MUK.REC.1397.106]. The samples were extracted under aseptic conditions in the operating room and immediately transferred to cold Hank's Balanced Salt Solution (HBSS) containing penicillin (300 U/mL) and streptomycin (300 µg/mL). To isolate the ASCs, samples were cut into small pieces (~ 5 mg in weight and <1 mm in diameter) under a sterile laminar hood in the laboratory, washed twice with phosphate buffer saline (PBS), and explanted in the corners of a 6-well cell culture plate. The surface of each sample was covered with 50 µL of fetal bovine serum (FBS) (Life Technologies, UK).

Plastic adherence of ASCs was used to purify and isolate single ASCs through a previously described method.[21] For this purpose, the culture plates were incubated for 24 hours at 37°C and 5% CO_2 with the subsequent addition of low-glucose Dulbecco modified Eagle's medium (DMEM-LG) (Life Technologies, UK) containing penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% FBS to the wells. Adipose tissue specimens were monitored daily by microscope for mobilization of ASCs until the fibroblast-like cells appeared at margins of the sample pieces. The culture medium was replaced every three days until the cells reached a confluency of 70-80%. The cells were trypsinized and subsequently passaged 4-5 times.

Immunophenotyping of Adipose-derived Cells

To evaluate the immunophenotype of adipose-derived cells, 2×10^5 cells were harvested after the third passage and suspended in PBS containing 1% FBS. The cells were incubated at 4°C in dark for 45 minutes with conjugated antibodies, including anti-human CD34-PE, anti-human CD73-PE, anti-human CD45-FITC, anti-human CD90-percp, and anti-human CD105-PE (eBioscience, USA). The incubated cells were washed twice with PBS and cells stained with anti-isotype antibodies (eBioscience, USA) was used as the control group. Expression of cell surface markers was evaluated by FACS Calibur flow cytometry (Becton Dickinson, USA) and later analyzed by the FLOWJO V.7.6 software (FLOWJO, LLC, USA).

Osteogenic Differentiation of ASCs

The ASCs were further characterized through assessment of tri-lineage differentiation of adipose tissue mesenchymal stem cells (i.e. ASCs), which was verified by culturing the passage 3 of cells in a specific differentiation media. 5×10^3 cells/cm^2 were cultured in a 24-well plate containing DMEM-LG. After 24 hours, the culture medium was discarded and replaced with the osteogenic differentiation medium (Life Technologies, USA), which was replaced every three days afterward. On Day 21, the cells were washed with PBS, fixed with 4% formaldehyde and stained with 2% Alizarin red solution. The presence of calcium deposits in wells was investigated by an optical microscope (Olympus, Japan).

Chondrogenic Differentiation of ASCs

Five µL of the cell suspension containing 1.6×10^7 cells/mL was seeded in the center of a 96-well microplate. After 2 hours of incubation at 37°C, the chondrogenic differentiation medium (Life Technologies, USA) was added to the wells and the mixture was further incubated for 14 days. The differentiation medium was changed every three days. On day 14, the cells were washed with PBS, treated with 4% formaldehyde and incubated with 1% Alcian blue solution, and the wells were evaluated for proteoglycan formation under an optical microscope.

Adipogenic Differentiation of ASCs

An amount of 3×10^4 cells/cm^2 were cultured in a 24-well plate. After reaching 80% confluency, the culture medium was replaced with the adipogenic differentiation medium (Stem Cell Technology Research Center, Iran). The differentiation medium was changed every three days. After 21 days, the cells were washed with PBS, treated with 4% formaldehyde, and stained with Oil red O solution. The wells were visualized for fat vesicles under an optical microscope.

Co-culture and Leukocyte Proliferation Assay

PBMCs were isolated from two healthy donors using Ficoll-paque (1.077 g/mL) density gradient centrifugation and were used to perform twMLR assay as described below. To investigate the immunomodulatory effect of ASCs two sets of experiments were designed: twMLR setting containing allogenic PBMCs (1×10^5) in a ratio of 1:1 as a control group and co-culture of ASCs and allogenic PBMCs (in a twMLR setting) as a test group, referred as co-culture group throughout the text. All tests were performed in 96-well microplates and for the co-culture group, the ratio of ASCs to PBMCs is considered 1 to 20. For co-cultures, a day before the isolation of PBMCs, 1×10^4

ASCs were cultured in co-culture wells. After 3 hours, the attached cells were treated with DMEM-LG containing 20 µg/mL mitomycin C for 2 hours to inhibit the proliferation of ASCs as explained before.[22,23] The mitomycin dose was enough to inhibit the proliferation of the ASCs but not to induce apoptosis or interfere with the gene expression profile of ASCs.[24] The ASCs were subsequently washed 5 times with DMEM solution and incubated at 37°C and 5% CO_2 for 24 hours. The proliferation of PBMCs was evaluated on days 0, 2, and 4 of co-cultures (test group) and twMLRs (control group); using XTT (PromoCell GmbH, Germany) according to the manufacturer's instructions. We considered twMLR proliferation as 100%, and the proliferation ratio of co-cultures was calculated as the percentage of the twMLR. The proliferation ratio was calculated as the mean of nine independent experiments, all of which were performed in triplicate. Proliferation ratio= [Co-culture-OD/twMLR-OD] ×100

Extraction of RNA and Quantitative Reverse Transcription PCR (RT-qPCR)

To extract the total RNA and perform RT-qPCR, twMLRs and ASCs/twMLR co-cultures were performed in 24-well plates, whereas in twMLRs, 6×10^5 PBMCs from each donor were added to the wells and the ratio of ASCs to PBMCs was considered 1 to 20 in the co-cultures. 6×10^4 ASCs were seeded in each well of the co-culture and inactivated with mitomycin C. 6×10^5 PBMCs from each donor were added to co-culture wells 24 h later and harvested on days 0, 2 and 4. The total RNA content of PBMCs was extracted using the RNA extraction kit following the manufacturer's protocol (Bio Basic Inc., Canada) and cDNA was constructed using a cDNA synthesis kit (Takara, Japan). mRNA levels of *CD200*, *CD200R1, PD-1, PDL-1, CTLA-4*, and *B7-1* genes were measured using specifically designed primers (Table 1) through the SYBR Premix EX Taq II (Takara, Japan) in Rotor-Gene 6000 sequence detection system (Corbett Life Sciences, Australia). The level of β-actin mRNA was used as an internal control. The expression ratio of genes was calculated using $2^{-\Delta CT}$ according to the following formula: ΔCT=CT target gene–CT β-actin

Cell Staining and Flow Cytometry

PBMCs were removed from twMLR and co-culture wells on days 0, 2, and 4 of the experiment and incubated with anti-human CD200-FITC and anti-human CD200R1-PE (eBioscience, USA) at 4°C for 45 minutes according to the manufacturer's protocol. The cells stained with isotype antibodies (eBioscience, USA) were used as the control group. Cell surface

Table 1. Sequences of designed primers, length of amplicons, and annealing temperature of *β-actin*, *CD200*, *CD200R1*, *PD-1*, *PDL-1*, *CTLA-4*, *B7-1* genes for evaluation of their mRNA levels with real-time PCR.

Genes	Primer sequence (5'-3')	Product size (bp)	Annealing Temperature
Actin-β	Forward: AGATCATTGCTCCTCCTGAG Reverse: CTAAGTCATAGTCCGCCTAG	161	58°C
CD200	Forward: ACTCTGTCTCACCCAAATG Reverse: CTTAGCAATAGCGGAACTG	173	55°C
CD200R1	Forward: ACAGATTACACAGAACTACTCG Reverse: TCTTAATGCGATAGGAGGGC	106	58°C
PD-1	Forward: GGCCAGGATGGTTCTTAGAC Reverse: TGCGGTACCAGTTTAGCAC	150	61°C
PDL-1	Forward: AAGTCAATGCCCCATACAAC Reverse: CAGGACTTGATGGTCACTG	140	60°C
CTLA-4	Forward: CCTGAAGACCTGAACACCG Reverse: CACGTGCATTGCTTTGCAG	152	59°C
B7-1	Forward: TGATATGCTGCCTGACCTACTG Reverse: GCTTCTGCGGACACTGTTATAC	108	61°C

cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-ligand 1 (PD-L1), programmed cell death-1 (PD-1)

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expression of CD200 and CD200R1 were evaluated by flow cytometry and the data were analyzed using the FLOWJO V.7.6 software.

Enzyme Linked-immunosorbent Assay (ELISA)

The supernatant of cell cultures was collected from 24-well plates of twMLR and co-culture on days 0, 2, and 4 separately and stored at -70°C. Levels of IL-10 and IFN-γ were measured by ELISA kits according to the manufacturer's instructions (Mabtech, Inc., Sweden). Expression levels of the two mentioned cytokines were shown to be altered in co-cultures of MSCs with PMBCs in previous publications.[25-27]

Statistical Analysis

Data were entered into the SPSS V.16 software (IBM Analytics, USA). The mean and standard deviation of the data were calculated in all groups. The proliferation ratio of PBMCs in twMLR and co-culture, gene expression of *CD200*, *CD200R1, PD-1, PDL-1, CTLA-4*, and *B7-1*, along with IL-10 and IFN-γ levels were compared between groups using Student's t-test or Mann-Whitney U test where applicable and the significance level was considered as $p<0.05$.

RESULTS

Characterization of ASCs

Adherent and spindle-shaped cells were observed in the cultures (Figure 1A). Flow cytometry analysis of ASCs confirmed that these cells were CD90, CD73, and CD105 positive and CD34 and CD45 negative (Figure 1B). Furthermore, three-lineage differentiation of ASCs into osteogenic, adipogenic, and chondrogenic lineages was assessed through specific differentiation media and confirmed by specific staining of each lineage (Figure 1C).

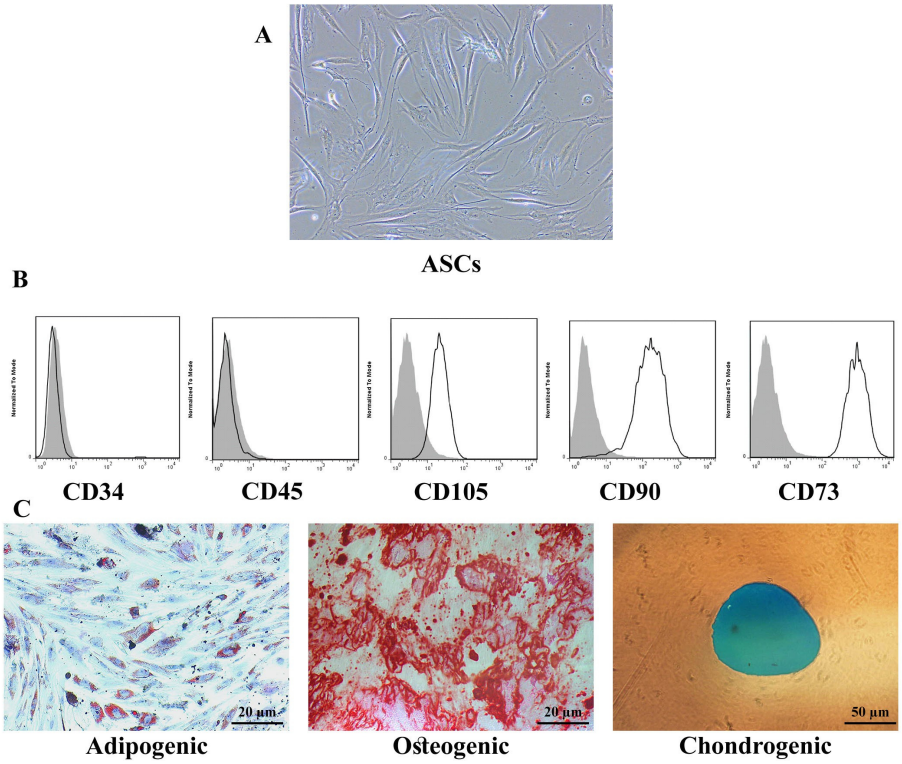

Figure 1. Characterization of adipose-derived stem cells (ASCs). A) Representative illustration of adherent and spindle-shaped isolated cells from adipose tissue in passage 4. B) These cells were negative for CD34 and CD45 and positive for CD105, CD90, and CD73 in flow cytometry analysis. C) Representative microscopic images indicating the differentiation of cells into tri-lineages of adipogenic, osteogenic, and chondrogenic. Magnification 100X and 40X.

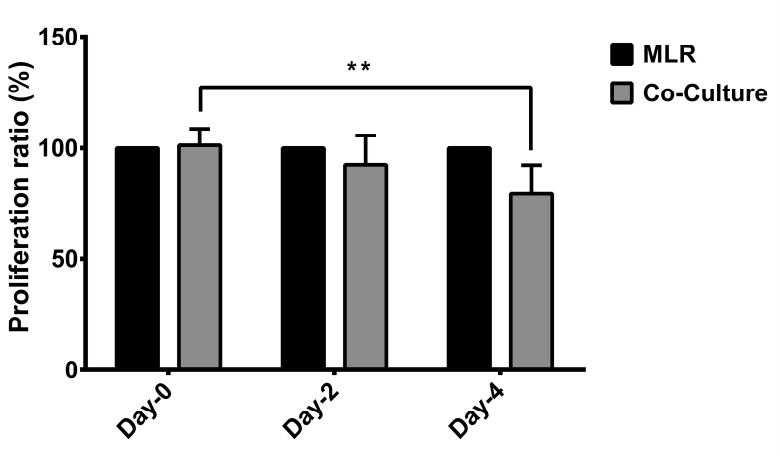

Figure 2. Effect of adipose-derived stem cells (ASCs) on the proliferation of peripheral blood mononuclear cells (PBMCs) in two-way mixed leukocyte reaction (twMLR). ASCs reduced the proliferation of PBMCs. Decreased proliferation of PBMCs in co-culture was significant on day 0 compared to day 4 (p=0.002). The proliferation ratio was calculated according to the following formula: Proliferation ratio = [Co-culture-OD/twMLR-OD] × 100

ASCs Suppressed the twMLR Proliferation

To investigate the effect of ASCs on PBMCs proliferation, the proliferative ability of ASCs was temporarily inhibited by mitomycin C and the ASCs were co-cultured with twMLR for 4 days. Beyond this period, ASCs would undergo apoptosis and lose their immunomodulatory potentials. The mean proliferation ratios of PBMCs in the co-culture over twMLR on days 2 and 4 of the co-culture were 92.61%±13.09 and 79.04%±12.91, respectively. Moreover, the proliferation of PBMCs on day 4 of the co-culture reduced significantly compared to day 0 of co-culture (p=0.002) (Figure 2). Overall, ASCs could suppress the proliferation of PBMCs, which peaked on day 4 of the co-culture.

ASCs Altered the Expression of CD200, CD200R1, PD-L1, PD-1, B7-1 and CTLA-4 Genes in Co-cultures

The mRNA levels of co-inhibitory genes were measured in twMLR and co-culture samples using real-time PCR on days 0, 2, and 4 of twMLR and co-culture using $2^{-\Delta CT}$ (Table 2). The expression of *CD200* increased on days 2 and 4 of the co-culture compared to day 0 (p=0.001), as well as compared to the expression of *CD200* on the same days of twMLR (p=0.014). Moreover, the expression of *CD200R1* increased significantly on days 4 (p=0.008) and 2 (p=0.034) of co-culture compared to day 0 and also on day 4 of the co-culture compared to the same day in twMLR (p=0.033). The expression of *PD-1* increased on day 4 of the co-culture compared to day 0 (p<0.001) and day 2 of co-culture (p=0.001), as well as on day 4 of the co-culture compared to the same day of twMLR (p=0.033). The expression of *PD-L1* increased on days 2 and 4 of the co-culture compared to day 0 (p=0.001). Finally, the expression of *CTLA-4* and *B7-1* increased on days 4 and 2 of the co-culture compared to day 0 (p<0.05) (Figure 3).

Our results also indicated that *CTLA-4* had the highest level of expression and that *PD-L1* and *PD-1* had the lowest expression levels on days 2 and 4 of twMLR and co-culture. The expression of *CD200R1* was rather constant during twMLR, while the expressions of *PD-1* and *CTLA-4* co-inhibitory receptors indicated a slight increment from day 0 to day 4 of twMLR. The mRNA level of *CTLA-4* showed its highest increase from day 0 to 2 of co-culture, while the highest increase in *PD-1* and CD200R1 expression was observed later and from day 2 to 4 of co-culture (Figure 4).

ASCs Altered CD200 and CD200R1 Expression on the Surface of PBMCs

Flow cytometry analysis showed that the percentage of CD200 positive PBMCs was 8.8±0.87, 8.7±0.4 and 4.2±0.81 on days 0, 2 and 4 of twMLR, respectively, while the percentage of CD200 positive PBMCs was 8.27±1.08, 6.95±3.6 and 10.43±4.49 on days 0, 2 and 4 of co-culture, respectively.

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Table 2. Mean and SD of the relative expressions of *CD200*, *CD200R*, *PD-L1*, *PD-1*, *B7-1,* and *CTLA-4* genes in the study groups. Relative expressions of the genes were calculated using the following equation: ΔCT=CT target gene - CT β-actin. Abbreviations: cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-ligand 1 (PD-L1), programmed cell death-1 (PD-1)

Groups		CD200	CD200R	PD-L1	PD-1	B7-1	CTLA-4
twMLR-0	Mean	0.000036	0.000421	0.000042	0.000046	0.000101	0.002493
	Std.deviation	0.000025	0.000225	0.000003	0.000014	0.000033	0.000812
twMLR-2	Mean	0.000326	0.000395	0.002025	0.000068	0.006884	0.004494
	Std.deviation	0.000121	0.000253	0.001321	0.000051	0.007869	0.002383
twMLR-4	Mean	0.000206	0.000410	0.001053	0.000116	0.004225	0.007198
	Std.deviation	0.000052	0.000242	0.000324	0.000016	0.001987	0.001434
Coculture-0	Mean	0.000018	0.000489	0.000039	0.000042	0.000090	0.002972
	Std.deviation	0.000018	0.000210	0.000010	0.000016	0.000029	0.000810
Coculture-2	Mean	0.002314	0.000543	0.002062	0.000057	0.002663	0.010783
	Std.deviation	0.001845	0.000371	0.001732	0.000028	0.001944	0.008609
Coculture-4	Mean	0.002270	0.001704	0.000779	0.000320	0.006142	0.013471
	Std.deviation	0.001695	0.001675	0.000639	0.000172	0.005845	0.009859

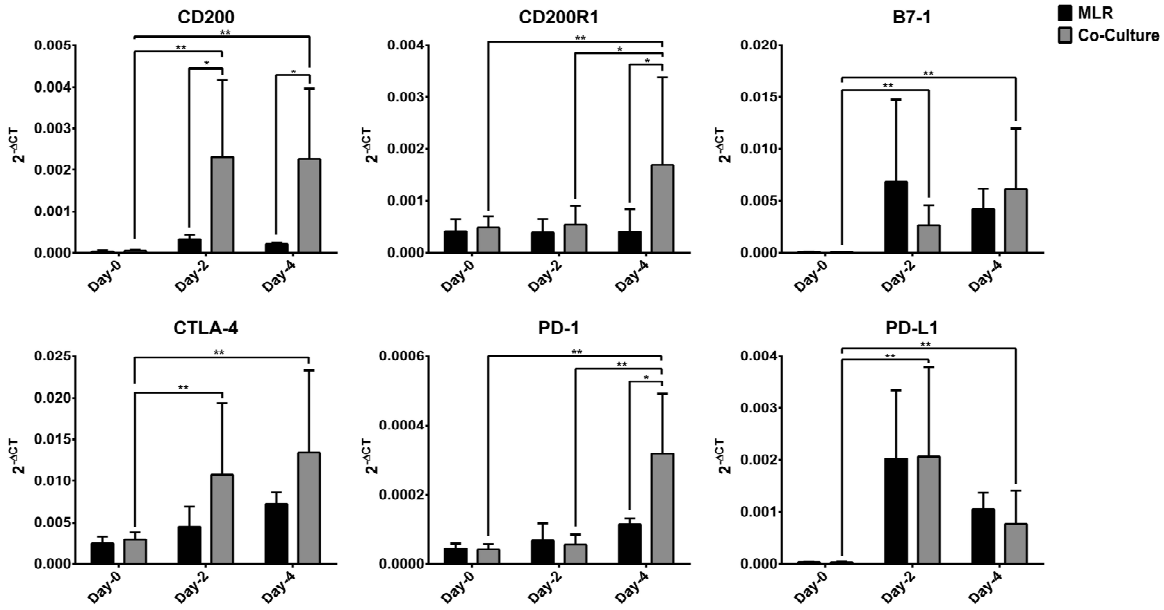

Figure 3. The relative expressions of co-inhibitory genes in two-way mixed lymphocyte reaction (twMLR) setting and adipose-derived stem cells (ASCs)/twMLR (co-culture) with real-time PCR. The gene expression was calculated using the $2^{-\Delta CT}$ formula according to the following equation: ΔCT=CT target gene - CT β-actin. Increased expression of CD200 between days 2 and 4 of the co-culture was significant compared to twMLR on the same days. Also, this increment was significant in days 2 and 4 compared to day 0 of co-culture. *CD200R1* mRNA level in the co-culture on days 2 and 4 was significantly increased compared to day 0, an increase that was also observed on day 4 of the co-culture compared to the twMLR at the same day. Expression of programmed cell death-1 (*PD-1)* gene showed a significant increment on day 4 compared to days 0 and 2 of the co-culture, and also on day 4 of the co-culture compared to day 4 of the twMLR. The expression of programmed death-ligand 1 *(PD-L1)*, cytotoxic T-lymphocyte-associated protein 4 *(CTLA-4),* and *B7-1* genes on days 2 and 4 of the co-culture demonstrated a significant increase compared to their expression on day 0 of the co-culture. *Indicates a significance level of <0.05 and ** indicates a significance level of <0.01.

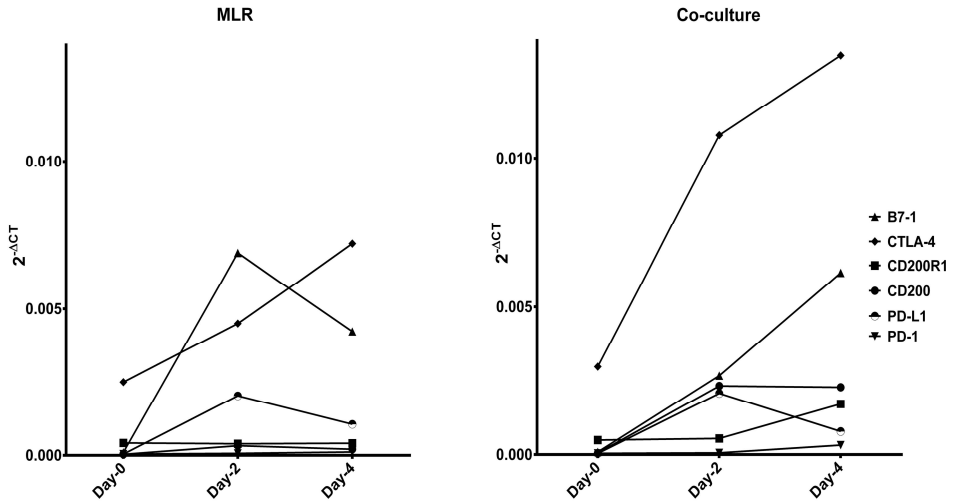

Figure 4. Simultaneous illustration of the co-inhibitory genes' expressions in two way mixed lymphocyte reaction (twMLR) setting and adipose-derived stem cells (ASCs)/twMLR (co-culture) groups. The expressions of *CD200*, *CD200R1*, programmed cell death-1 *(PD-1)*, programmed death-ligand 1 *(PD-L1)*, cytotoxic T-lymphocyte-associated protein 4 *(CTLA-4),* and *B7-1* genes on days 0, 2 and 4 of the twMLR and co-culture groups showed that although the *CTLA-4* and *B7-1* had the highest expressions in both groups, *CD200* and *CD200R1* had the highest expression changes in the presence of ASCs

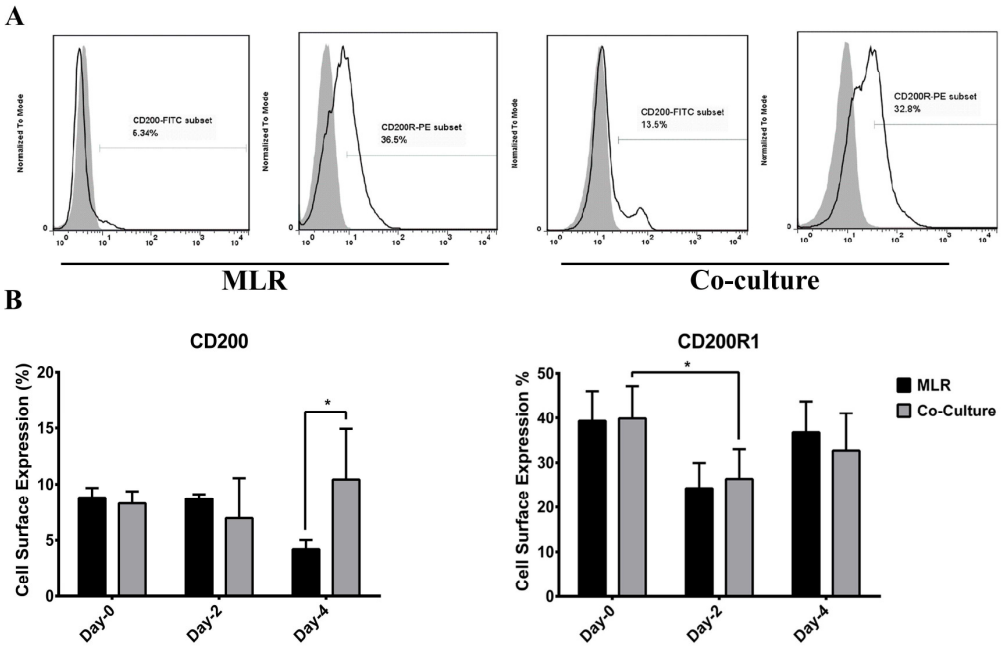

Figure 5. Flow cytometry results of CD200 and CD200R1 expressions on the surface of Peripheral blood mononuclear cells (PBMCs) in two way mixed lymphocyte reaction (twMLR) setting and adipose-derived stem cells (ASCs)/twMLR (co-culture) groups. A) Representative images of CD200 and CD200R1 expressions on PBMCs in twMLR and co-culture. B) The percentage of CD200 positive and CD200R1 positive cells were compared at days 0, 2, and 4 of twMLR and co-culture, indicating the increase in CD200 positive cells at day 4 of the co-culture compared to twMLR of the same day. The number of CD200R1 positive cells decreased on day 2 of the co-culture compared to day 0 co-culture. *Indicates a significance level <0.05

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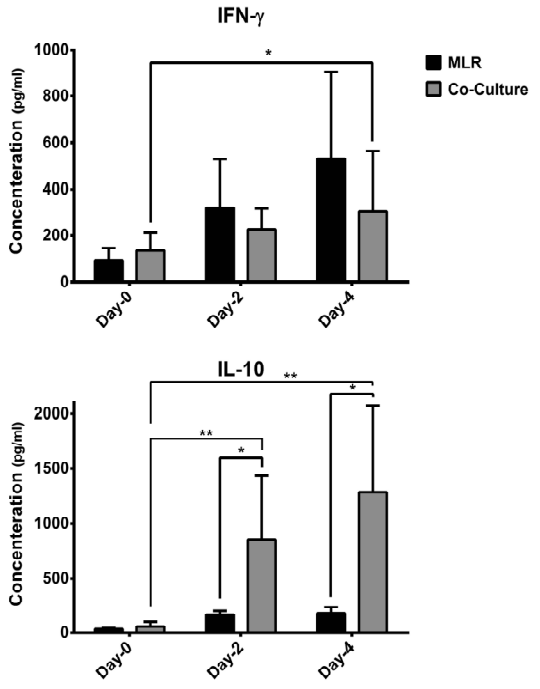

Figure 6. Enzyme Linked-immunosorbent Assay (ELISA) assessment of IFN-γ and IL-10 levels in the supernatants of two way mixed lymphocyte reaction (twMLR) setting and adipose-derived stem cells (ASCs)/twMLR (co-culture) groups. The increase in IFN-γ during the experiment was lower in the co-culture compared to twMLR. The difference in the levels of this cytokine was significant between days 0 and 4 of the co-culture. IL-10 levels in the co-culture were higher compared to twMLR, which was significant on days 2 and 4 of the co-culture compared to the same days of twMLR. Moreover, this increase was significant in days 2 and 4 compared to day 0 of co-culture. * Indicates a significance level <0.05. ** indicates a significance level <0.01

The percentage of CD200 positive PBMCs was significantly higher on day 4 of co-culture compared to the same day of twMLR (p=0.013), but not on days 0 and 2. The percentage of CD200R1 positive PBMCs was 39.30±6.73, 24.26±5.65 and 36.73±7.01 on days 0, 2 and 4 of twMLR, respectively, while the CD200R1 positive PBMCs comprised 39.85±7.31%, 26.35±6.63% and 32.67±8.38% of the co-culture on days 0, 2 and 4, respectively. There was a significant reduction in the number of CD200R1 positive PBMCs in the co-culture between days 0 and 2 (p=0.02) (Figure 5).

ASCs Increased IL-10 and Decreased IFN-γ Levels

Levels of IFN-γ and IL-10 in the supernatants of twMLR and co-culture on days 0, 2, and 4 were measured by ELISA. The concentration of IL-10 was 35±7 pg/mL on day 0 of the twMLR, reaching 166 ± 34 and 180 ± 54 pg/ml on days 2 and 4, respectively. This is while the concentration of IL-10 was 56±41 pg/mL on day 0, reaching to 852±580 pg/mL on day 2 and 1281±791 pg/mL on day 4, respectively. The increase in IL-10 in co-culture was significant on days 2 and 4 compared to its twMLR levels on the same days (p<0.05). Moreover, the increase in IL-10 from day 0 to 2, and 2 to 4 of co-culture was significant (p<0.001). Unlike IL-10, IFN-γ was reduced in the co-culture compared to twMLR. The concentration of this cytokine was 92±52 pg/mL on day 0 of twMLR, reaching 320±209 and 530±374 pg/mL on days 2 and 4, respectively. In the co-culture, IFN-γ concentration was 134±77 pg/mL on day 0, reaching 226±91 pg/Ml on day 2 and 305±257 pg/mL on day 4, indicating a significant increase from day 0 to 4 (p=0.04) (Figure 6).

DISCUSSION

It has been shown that hyperthermia,[28] the age of MSCs donors, and the number of culture passages affect the immunomodulatory properties of MSCs,[29] the main feature of MSCs making them proper candidates for the treatment of autoimmune diseases and prevention of graft rejection.[30] Considering the importance of co-inhibitory pathways in modulating the immune responses, we evaluated the effect of ASCs on the expression of two well-known co-inhibitory pathways of CTLA-4/B7-1 and PD-1/PD-L1, as well as the CD200/CD200R1 axis in twMLR assay. Following the recognition of specific antigens, activation of co-inhibitory pathways result in a balance between T-cells activation, and proliferation with tolerance induction.[31] It has been shown that CTLA-4 activation regulates the more initial steps of immune response while PD-1 affects later steps.[32]

Upregulation and activation of the CD200/CD200R1 axis can inhibit the proliferation of leukocytes through cell-cell contact. Furthermore, it can induce the differentiation of T-cells into induced T_{reg} (iT_{reg}) and type 1 T_{reg} (Tr1) cells, which collectively produce IL-10 and tumor growth factor-β1

(TGF-β1). This axis also reduces the synthesis of inflammatory cytokines, including IFN-γ.[20] In line with this phenomenon, a relationship between CD200/CD200R1 axis and reduced proliferation of leukocytes and the production of IL-10 and IFN-γ was also observed in our results. Increased expression of this axis in the co-culture was accompanied by rising levels of IL-10, decreasing levels of IFN-γ, and reduced proliferation of PBMCs.

In the present study, the gene expression of co-inhibitory receptors showed a minor variation in comparison to their ligands in twMLR. Previous studies have demonstrated that MSCs increase the expression levels of *CTLA-4*,[33] *PD-1*[34] and *CD200R* genes[35] in lymphocytes. These findings were consistent with our results in which ASCs upregulated all three co-inhibitory receptors in co-culture compared to twMLR, among which the *CD200R1* showed the highest increment in expression throughout the experiment. We also observed a similarity in expression patterns of *CD200R1* and *PD-1* as their expression was upregulated on day 4 of the co-culture. Regarding the expression of ligands, *CD200* had the highest increase of expression in co-culture compared to twMLR. Since the expression of *CTLA-4*, *PD-1,* and *CD200R1* is limited to hematopoietic-derived cells, we investigated the expressions of B7-1, CD200, and PD-L1 ligands in ASCs. In line with previous studies,[35,36] the results of our real-time PCR showed that *CD200*, *PD-L1,* and *B7-1* are overexpressed in ASCs and that the expression level of *B7-1* is in a low level (data not shown). Importantly, the levels of any given protein cannot be determined based only on the mRNA level of its respective gene. The translation rate, protein's half-life, and protein synthesis delay can all affect the direct relationship between protein and mRNA levels.[37] In our experiments, the expression of CD200/CD200R1 axis mRNA did not match their respective protein levels in the inflammatory condition of twMLR. Indeed, CD200 and CD200R1 mRNA levels increased over the four days of the experiment, while their cell surface protein expression fluctuated within this period.

MSCs have an active interaction with innate and adaptive immune cells during an inflammatory response [38] MSCs induce tolerance in immune cells by cell-cell contact, secretion of soluble factors, and induction of anergy and apoptosis.[39] The pro-inflammatory cytokines secreted by PBMCs, especially IFN-γ can induce an immunosuppressive trait in MSCs [40] to which they respond through the expression of IFN-γ receptor.[36] IFN-γ is known for its effect in stimulating and activating innate immune cells such as monocytes and macrophages resulting in the exacerbation of local inflammatory responses.[41] Meanwhile, IFN-γ has a dual function by which it mitigates the inflammatory response via induction of co-inhibitory molecules like PD-L1, PD-L2, and CTLA-4.[42] The immunomodulatory ability of MSCs depends on the type and concentration of inflammatory mediators available in their microenvironment. During a strong inflammatory response, MSCs exhibit immunosuppressive properties and exert these properties by secreting anti-inflammatory cytokines such as IL-10 and TGF-β^3 as well as induction of these cytokines in T-cells.[43] Todorova et al showed that ASCs induced the secretion of IL-10 in T_h cells[44] and that this cytokine inhibits the expression of co-stimulatory molecules on APCs, leading to Th cells anergy.[45] In our study, the presence of ASCs suppressed the proliferation of PBMCs on days 2 and 4 of the co-culture. Moreover, the IL-10 levels increased significantly in co-culture compared to twMLR, which indicates the lower proliferation of PBMCs in the co-culture. Increased expression of IL-10 in co-culture suggests that IL-10 might underlie the upregulation of co-inhibitory molecules such as PD-1, CTLA-4, and CD200R1.

In the present study, the expression of *B7-1* decreased in co-culture on day 2 compared to the same day of twMLR, which could be due to the inhibitory effect of ASCs on its expression. MSCs decrease the expression of co-stimulatory molecules such as B7 complex on APCs, resulting in T-cell anergy.[38] In fact, they suppress the IFN-γ and TNF-α expression and upregulate IL-10, leading to a downregulation of co-stimulatory molecules such as B7-1 on DCs and loss of DCs ability to activate T-cells.[46]

Similar to PD-1, PD-L1 also interacts with B7-1 on the T-cell surface, thereby linking the PD-1/PD-L1 pathway to B7-1: CD28/CTLA4 pathway.[47] Expression of PD-L1 is related to the level of IFN-γ in the microenvironment.[48] Our results comparing day 4 of co-culture and twMLR showed that *PD-L1* expression reduced in the co-culture, which was accompanied by the reduced level of IFN-γ. As MSCs reduce the production of IFN-γ,[46] the decreased expression of PD-L1 is likely to be associated with a reduced level of IFN-γ. PD-1/PD-L1 pathway regulates the balance

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between positive and negative signals required to maintain tolerance,[49] and the interaction of this receptor/ligand leads to apoptosis and energy of T-cells.[50] Similarly, the proliferation reduction in PBMCs in the co-culture was associated with increased expression of the PD-1/PD-L1 pathway in the microenvironment in our sample.

Experimental and clinical studies have revealed the importance of these co-inhibitory molecules in the amelioration of inflammatory and autoimmune diseases.[51,52] Preclinical and clinical studies have demonstrated promising results with the administration of MSC in alleviating inflammatory responses during graft rejection.[30] MLR is an *in vitro* assay to evaluate the ability of T-cells alloreactivity[53] and has also been used to examine the immunomodulatory effects of MSCs on immune cells.

In conclusion, our results indicated that the presence of ASCs in twMLR upregulated the CD200/CD200R1 axis to a higher extent compared to the PD-1/PD-L1 and CTLA-4/B7-1 pathways. In addition, the elevated expression of *CD200R1* in the final day of co-culture and its similar expression pattern to *PD-1* corroborates the role of this receptor as a late regulator of the immune response. Overall, our results shed light on the CD200/CD200R1 axis as a main modulatory axis in ASC/twMLR co-culture.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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