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Immunomodulatory Effect of Human Umbilical Cord Blood-derived Mesenchymal Stem Cells on Activated T-lymphocyte

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ABSTRACT

Many studies have been performed about regenerative and immunomodulatory properties of mesenchymal stem cells (MSCs) and their application in different treatment approaches. The present study aimed to investigate the immunomodulatory effect of umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) on the gene expression profile of cytokines in stimulated T-lymphocytes.

For this purpose, MSCs were isolated from umbilical cord blood samples and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. The nature of MSCs was identified by flow cytometry analysis and differentiation to the adipocyte and osteocyte lineage. Moreover, to investigate the immunomodulatory effects of MSCs on T cells, a co-culture system was designed and expression levels of *interleukin (IL)-2*, *IL-4*, IL-6, IL-10, *IL-13*, *interferon-gamma (IFN-γ)*, *tumor necrosis factor-alpha (TNF-a)*, and *transforming growth factor-beta (TGF-β)* genes were measured; using the real-time polymerase chain reaction (RT-PCR) technique.

Our results demonstrated the ability of MSCs to differentiate into adipocyte and osteocyte lineages. Further investigation also displayed that although UCB-MSCs could significantly reduce the expression of pro-inflammatory cytokines like *IL-2*, *IL-6*, *IFN-γ*, and *TNF-a* in activated T-lymphocytes, they noticeably potentiated the expression levels of *IL-4*, *IL-10*, *IL-13*, and *TGF-β* in the co-culture setting.

In conclusion, UCB-MSCs have immunomodulatory effects on activated T-lymphocytes in favor of anti-inflammatory responses.

Keywords: Cytokines; Immunomodulation; Mesenchymal stem cells; T-lymphocytes

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INTRODUCTION

Human umbilical cord blood (hUCB) is considered a rich source of pluripotent and hematopoietic stem

cells, which is used to treat various hematopoietic disorders.[1,2] Also, hUCB is known as a non-hematopoietic cells resource, such as mesenchymal stem cells (MSCs).[3] According to the international society of cell therapy (ISCT) criteria; MSCs are defined as fibroblast-like cells because of their ability to adhere to plastic culture dishes, differentiation to adipogenic, osteogenic, and chondrogenic lineages, and expression of CD44 and CD105 surface markers and not expressing CD45 and CD34 hematopoietic markers.[4] Since UCB stem cells express lower levels of MHC class I than bone marrow-derived stem cells, so they have no human leukocyte antigen (HLA) incompatibility problems and represent a low rate of graft versus host disease (GVHD), compared to bone marrow-derived stem cells.[5-7] UCB derived MSCs do not express CD40, CD80 (B7-1), CD86 (B7-2), and MHC class II molecules, which are generally expressed on antigen-presenting cells.[8,9] Therefore, these types of cells are low immunogenic which are considered as a source of cell therapy.[8,10] Many studies have examined the immunomodulatory effect of MSCs on immune cells *in vitro*.[11] MSCs directly affect the chemotactic properties and differentiation capacity of B-lymphocytes.[12] Other studies have displayed that MSCs change the cytokine secretion profiles of dendritic cells, natural killer cells, and T- lymphocytes with several anti-inflammatory mechanisms.[13,14,15] Besides, MSCs modify T-cells' function and suppress their proliferation via soluble mediators and cell-cell contact.[16,17] In the present study, we aimed to investigate UCB-MSCs' effect on pro-inflammatory and anti-inflammatory cytokines genes expression profile in T-lymphocytes.

MATERIALS AND METHODS

Isolation and Culture of hUCB-MSCs

Cord blood samples were collected from the umbilical cord vein attached to the placenta after the umbilical cord has been detached from the newborns of full-term cesarean deliveries. Informed consent was obtained from all participating persons. The heparinized UCB was diluted at 1:3 with 2 mM EDTA-PBS. The Mononuclear cells (MNCs) were isolated from UCB by Ficoll-Paque density gradient centrifugation (density, 1.077 g/cm^3). The isolated MNCs layer was washed by PBS and expanded in low glucose Dulbecco's modified Eagle's medium (DMEM-LG), (Gibco BRL, Gaithersburg MD, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan UT, USA), 10 ng/mL bFGF, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma, St. Louis MO, USA), 2 mM L-glutamine (Gibco BRL, Gaithersburg MD, USA) and incubated at 37°C with 5% CO2 and 95% humidity. After 24 hours of incubation, non-adherent cells were removed, and a fresh medium was added to the flasks. The medium was replaced every 4-5 days until the adherent cells population reached 80% confluence.

Flow Cytometry Analysis

The cells of the third passage were harvested, washed, and suspended in phosphate-buffered saline (PBS); approximately 1×10^6 cells/mL were incubated on ice for 20 minutes with conjugated monoclonal antibodies against CD19/ FITC, CD14/PE, CD34/PE, CD45/ FITC, CD73/ FITC, CD90/PE, CD105/PE, CD3/ FITC, CD106/PE, CD349/frizzled FITC and HLA-G/PE (Becton Dickinson, San Jose, CA) and analyzed; using FACS Calibur (*Becton* Dickinson, San Jose CA) flow cytometer with Cell Quest software (BD Biosciences) as previously described by Silva Wilson.[18] Flow cytometry analysis was performed in five groups. Mouse IgG1 antibody was used as isotype control.

Differentiation Capacity of UCB-MSCs

For MSCs identification, fibroblast-like cells from the third passage were cultured at osteogenic medium for about three weeks and adipogenic medium for about one month. The medium was replaced every four to five days over culture days. Differentiation into adipocyte lineage was verified by staining with oil red O to examine the accumulation of lipids in fat vacuoles. Determination of osteogenesis and development of trabecula were done with the detection of calcium compounds and *alkaline phosphatase* staining, respectively. The cells cultured in the DMEM-LG medium were considered as control groups.

Isolation and Stimulation of Peripheral B T-lymphocytes

To determine the effect of UCB-MSCs on T-lymphocytes, peripheral blood was isolated from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation (density 1.077 g/cm^3). T-lymphocyte isolation from peripheral blood

mononuclear population was done by negative immunodepletion of $CD14^+$, $CD15^+$, $CD16^+$, $CD19^+$, $CD34^+$, $CD36^+$, $CD38^+$, $CD56^+$ $CD123^+$, and $CD235a^+$ (glycophorin A) cells; using MACskit (Miltenyi Biotec, Bergisch Gladbach, Germany).

T-lymphocytes Stimulation with Phytohemagglutinin (PHA)

Before co-culture with UCB-MSCs, isolated T-lymphocytes were stimulated with 5 µg/mL PHA (Sigma, St. Louis MO) and incubated for 96 hours at 37°C with 5% CO2 and 95% humidity. Cultures without any PHA stimulation served as controls. For evaluation of the effect of PHA on T-lymphocyte activation, flow cytometry assay for CD3 and CD25 surface markers and also RT-PCR evaluation for *IL-2* gene expression were done to confirm T-lymphocyte activation.

Flow cytometry Analysis of PHA Activated T-lymphocytes

To confirm T-lymphocytes activation by PHA, following 96 hours incubation with PHA, T-lymphocytes were washed with PBS and resuspended at approximately 1×10^6 cells/mL in PBS. Expression of CD3 and CD25 markers were evaluated by FACS Calibur (*Becton* Dickinson, San Jose CA) and the results were analyzed with Cell Quest software (BD Biosciences).

RT-PCR Analysis for Evaluation IL-2 mRNA Level

Following 96 hours of incubation with PHA, T-lymphocytes were harvested and total RNA was extracted using Qiazole (Qiagen, USA) and reverse transcribed into cDNA; using Revert Aid first-strand cDNA synthesis kit (Fermentas, Burlington, Canada). The level of IL-2 mRNA was analyzed by RT-PCR technique on a thermal cycler system (Sunsequest, Germany). For detection of IL-2, the Forward primer 5-AGAACTCAAACCTCTGGAGGAAG-3 and Reverse primer 5-GCTGTCTCATCAGCATATTCACAC-3 were used. For detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the Forward primer 5-TCTCTGCTCCTCCTGTTC-3 and Reverse primer 5-GTTGACTCCGACCTTCAC-3 were employed. The RT-PCR products were separated by electrophoresis in a 2% agarose gel and stained in ethidium bromide for 30 minutes before visualization under UV light.

Co-culture of UCB-MSCs and Peripheral Blood T-lymphocytes

UCB-MSCs from the third passage were plated at 2×10^4 cell/mL into 12-well plates for 6 hours, to prevent cell proliferation, UCB-MSCs were gamma-irradiated (30 Gy). Subsequently, 2×10^5 cell/mL PHA activated T-lymphocyte was added to each well (UCB-MSCs to activated T-lymphocyte ratio, 1:10). The wells containing T-lymphocytes without any hUCB-MSCs were considered as control.

Evaluation of Pro-inflammatory and Anti-inflammatory Cytokine Gene Expression Profiles in Activated T-lymphocytes

Following 24 hours of co-culture, T-lymphocytes were harvested, and total RNA was extracted using TRIzol reagent (Invitrogen, Karlsruhe, Germany). First-strand cDNA synthesis was done using the First-Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA)according to the standard protocol.[19] The *GAPDH* gene (endogenous housekeeping gene) was used as an internal calibrator, and the changes in gene expression fold change were calculated based on the internal calibrator. The Interleukin (IL)-2, IL-4, IL-6, IL-10, IL-13, interferon (IFN)- γ, tumor necrosis factor (TNF)-α and TGF-β primers (Table 1) were used to target the DNA sequence.

Statistical Analysis

Statistical analysis was done by GraphPad Prism version 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) test was done to determine the statistical significance between co-cultured groups. p values<0.05 or less were considered statistically significant. (**P<0.05, **** p<0.0001)

RESULTS

Immunophenotyping of UCB-MSCs

To investigate mesenchymal characterization, flowcytometry results showed UCB-MSCs were positive for CD90 (98.46%), CD105 (91.24%), CD106 (97.03%), CD73 (93.93%), HLA-G (44.96%) and negative for CD34 (6.81%), CD45 (3.81%), CD19 (3.11%), CD14 (1.71%) and CD3 (10.82%), CD349L/frizzled (6.01%) markers (Figure 1).

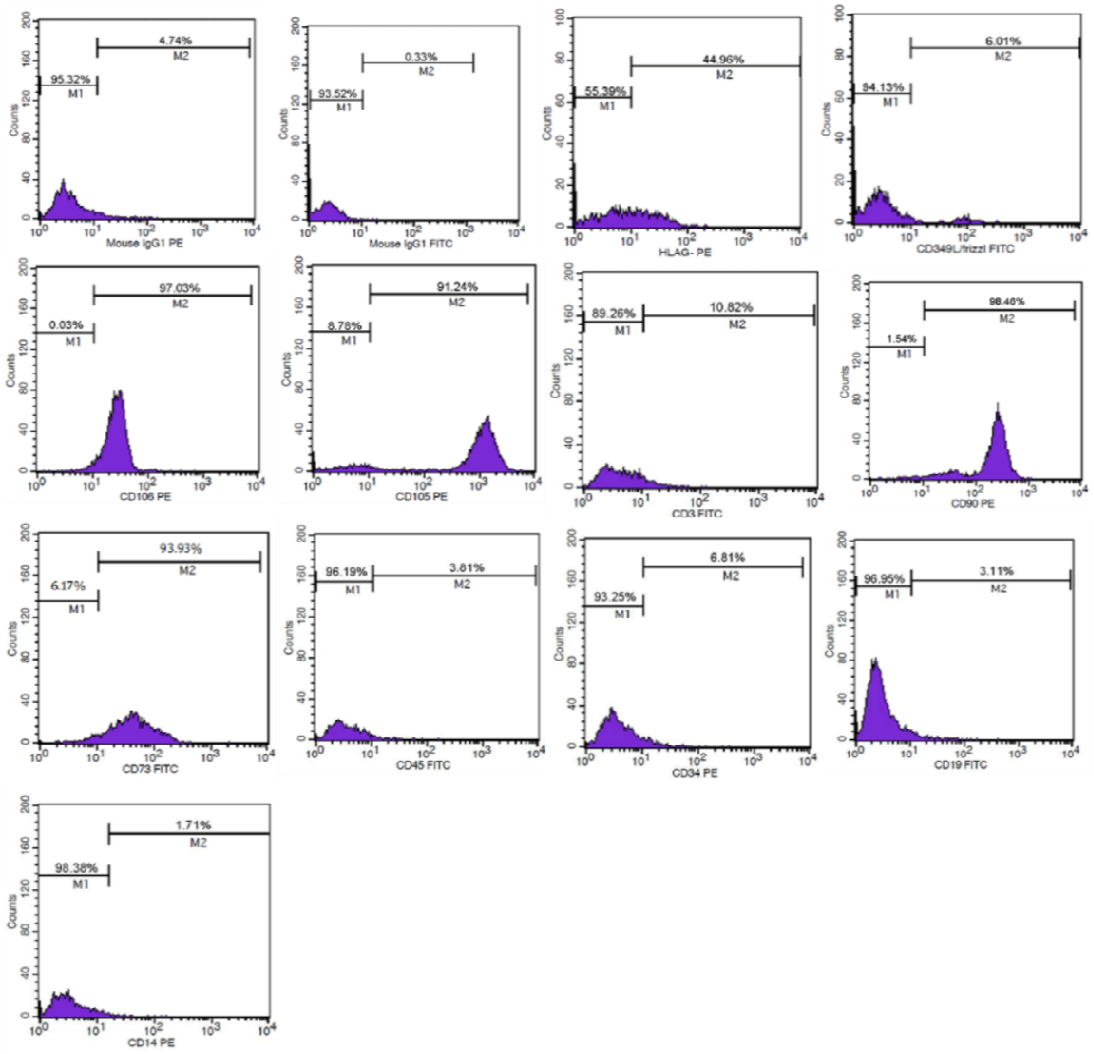

Figure 1. Immunophenotypic characterization of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) using flow cytometry analysis. Plot showing markers CD90, CD105, CD106, CD73, CD349L/frizzled, HLA-G, CD34, CD45, CD19, CD14 and CD3.

Osteogenic Differentiation

To identify differentiation capacity to osteocyte lineage; cells were cultured at osteogenic medium. Osteogenic differentiation was identified by calcification of the matrix as recognized by alkaline phosphatase staining (Figures 2A and B).

Adipogenic Differentiation

For confirmation adipogenic differentiation, the initial lipid vacuoles formation was observed on the fifth day of culture in adipogenic induction medium. At the end of 29 days of culture, oil red-O staining determined the presence of lipid accumulations but in the control condition, fat vacuoles were not formed following one month culture at DMEM-LG medium, Figures 2C, and D.

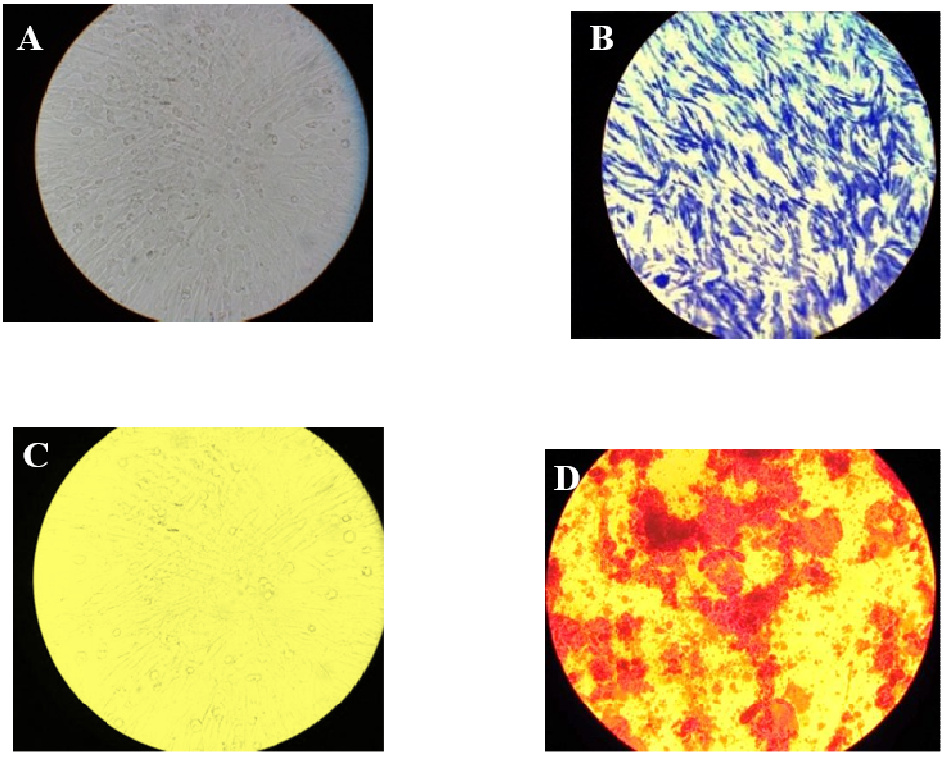

Figure 2. Osteogenic and adipogenic differentiation of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). (A), (C) Cells cultured in the regular low glucose Dulbecco's modified Eagle's medium (DMEM-LG) medium served as control. (B) Osteogenesis identified by Alizarin red after three weeks of induction. (D) Adipogenesis was evidenced by the formation of lipid vacuoles via oil-red O staining. Color images were captured using an inverted microscope (40X) (Nikon, Japan)

Flow Cytometry Analysis of Activation T-lymphocytes Surface Markers

For assessing T-cells activation, following T-cell isolation and PHA stimulation, the flow cytometry technique was used. The results demonstrated high expression levels of surface CD25 marker by activated CD3+T-lymphocytes (Figure 3).

RT-PCR Assay to Confirm T-lymphocytes Activation

The results of the RT-PCR assay showed an increased IL-2 mRNA expression level as a T-lymphocytes activation indicator, following PHA stimulation in comparison with untreated control samples (Figure 4).

Cytokines Genes Expression in T-lymphocytes

To evaluate the effect of UCB-MSCs on the cytokine profile of T-cells in the co-culture system, we analyzed cytokine genes expression in T-cells co-cultured with UCB-MSCs. The results (Figure 5) displayed that UCB-MSCs could significantly increase anti-inflammatory cytokine genes expression and decrease pro-inflammatory cytokine genes profiles in T-lymphocytes at co-culture with UCB-MSCs ($p<0.05$). The fold induction of IL-13, TGF-β, and IL-10 increased from 1.00 to 1.51 (±0.1), 5.68 (±0.07) and 8.43 (±0.05), respectively. In contrast, UCB-MSCs decreased IFN-γ, IL-2, IL-6, and TNF-α from the baseline of 1.00 to 0.15 (±0.05), 0.01 (±0.0005) and 0.17 (±0.04) and 0.21 (±0.02), respectively.

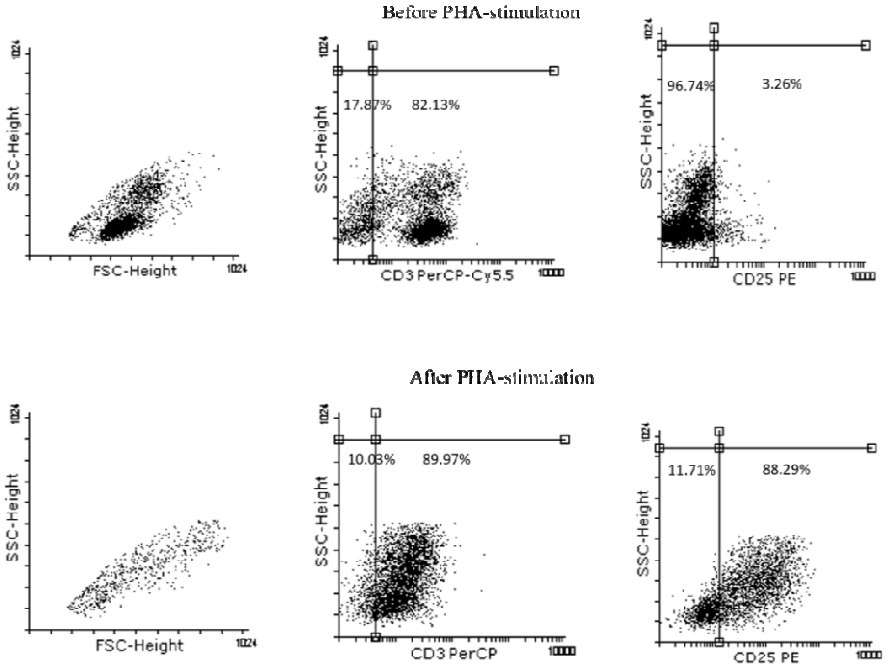

Figure 3. Flow cytometry analysis of CD3 and CD25 markers in the presence and absence of phytohemagglutinin (PHA). Mononuclear cells were gated on a forward and side scatter plot. PHA induced expression of CD25 on the surface of CD3+ T-lymphocytes.

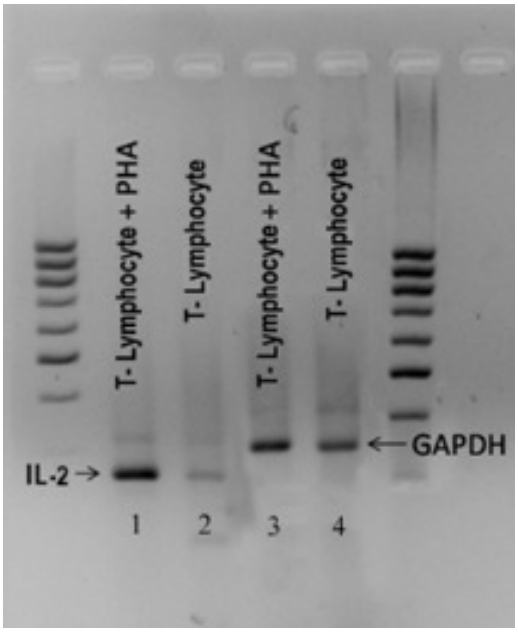

Figure 4. Relative mRNA expression levels of interleukin (IL)-2 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the presence and absence of phytohemagglutinin (PHA). RT-PCR reaction product was separated on an agarose gel containing ethidium bromide. The relative intensities of the bands were revealed under UV radiation.

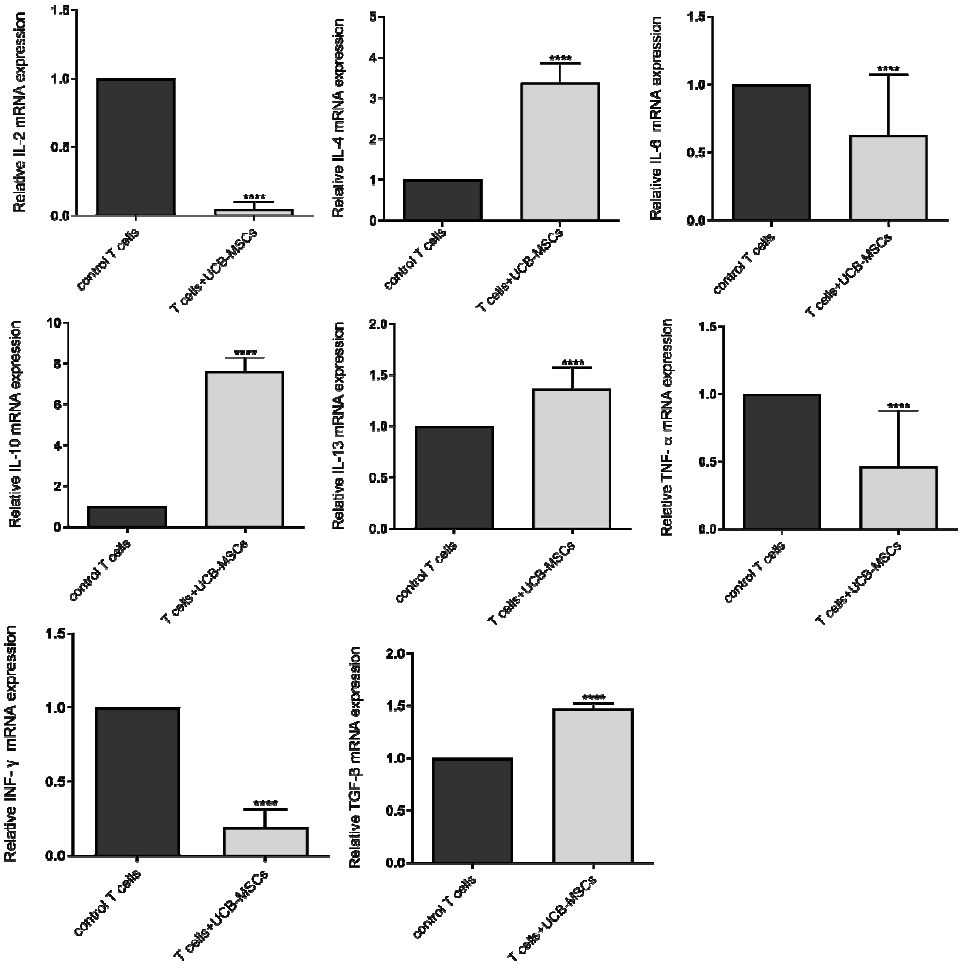

Figure 5. Relative mRNA expression levels of anti-inflammatory and pro-inflammatory cytokines of activated T-lymphocytes co-cultured with human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). Gene expression results displayed a significant increase in mRNA expression levels of interleukin (*IL)-13*, *IL-4*, *transforming growth factor-beta (TGF-β)*, and *IL-10* cytokines in activated T-lymphocytes co-cultured with hUCB-MSCs and decreased mRNA expression levels of *IFN-γ*, *IL-2*, *IL-6*, and tumor necrosis factor-alpha (*TNF-α)* cytokines in T-lymphocytes co-cultured with hUCB-MSCs (**p<0.0001). The assessments were done by 3 independent experiments per group.**

DISCUSSION

The presence of fibroblast-like cells in hUCB resources has been well documented.[20] In the present study, we successfully isolated fibroblast-like cells from hUCB resources and demonstrated basic MSC characteristics following a prior report[8] and ISCT minimum criteria: the positive expression of endothelial cell surface proteins and multilineage differentiation potential.[4] Several adult tissue resources of MSCs have been explained and many experimental and preclinical investigations have been done to investigate their immunomodulatory properties. However, MSCs derived from neonatal associated tissues such as umbilical cord, placenta, umbilical cord blood, and Wharton's jelly has attracted much attention because they may present exclusive advantages e.g., ease of access and non-invasive accessibility which make them a valuable resource. More considerably, MSCs from these resources have increased *in vitro*

proliferation capability, especially under hypoxic conditions, in comparison with other MSCs derived from different adult tissues.[21-24] MSCs are well distinguished by immunomodulatory effects on mitogen-activated lymphocyte proliferation in co-culture conditions. Although the exact mechanism in which MSCs modulate T-lymphocyte was not fully addressed, but the contribution could be mediated by cell-cell contact and soluble factors.[8,25] The results of the current study showed that pro-inflammatory cytokines mRNA levels such as IL-2, IL-6, IFN-γ, and TNF-α were significantly decreased compared to the control group. In contrast, anti-inflammatory cytokines including *IL-4*, *IL-10*, *IL-13*, and *TGF-β* genes expression were remarkably increased. Furthermore, these data may suggest that UCB-MSCs may induce differentiation of regulatory T-cells or Th2 subtypes in the co-culture system. It has been shown that anti-inflammatory cytokines such as TGF-β, IL-10, and IL-4 are the major cytokines that are secreted by regulatory T-lymphocytes and Th2 subsets.[26,27] Since the main aim of this study was to determine the immunomodulatory effects of hUCB-MSCs on T-cells cytokine pattern, the T-lymphocyte lineage differentiation has not been assessed. Other research in this field have been demonstrated that MSCs modulate immune cells responses by reduction of TNF-α and IFN-γ[15] and also they can alter the Th1 subset response to reduction IFN-γ and caused the Th2 subset to increase production IL-4, leading to the anti-inflammatory milieu.[28-30] Several investigations revealed that human MSCs in co-culture condition can mediate suppression of T-lymphocytes proliferation via soluble factors as prostaglandin E2 (PGE2), Indolamine deoxygenase (IDO), TGF-β.[31,32] In this study, we examine the effect of direct cell-cell contact of hUCB-MSCs on activated T-lymphocytes. We did not evaluate the mechanism of this direct effect. We speculate that engage the Notch family of transmembrane receptors and other cell surface receptors contribute to the immunosuppressive properties of MSCs.[33-35] Toll-like receptors (TLRs) are important in MSCs and immune cells function.[36,37] The activation of the transcription factor nuclear factor-kB (NFkB) is the main signaling factor by TLRs to controlling the production of inflammatory cytokines and suppress T-lymphocyte proliferation.[38] In addition it has been demonstrated that programmed death ligand-1 (PD-L1), which is expressed on MSCs, plays an important role in the differentiation of Th1 subsets into regulatory T-lymphocytes.[39] Due to the impressive immunomodulatory nature of hUCB-MSCs on immune cells and their ability to migrate toward damaged tissues and homing at inflammation sites,[40] they are considered as a promising resource for cell-based therapies. In conclusion, with successful isolation and characterization of hUCB-MSCs, we showed immunomodulatory effects of hUCB-MSCs on PHA-activated T-lymphocytes. Furthermore, additional investigations are required to provide the exact mechanisms in which hUCB-MSCs modulate T-lymphocyte response.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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