Original Research

Turk J Immunol 2024;12(1):19-27
DOI: 10.4274/tji.galenos.2024.35219

Effects of Adipose-tissue Derived Mesenchymal Stem Cells on PBMCs in Co-culture with HeLa Cell Line

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Cite as: Dorfaki M, Ghatrehsamani M, Arab FL, Roozbehani M, Khoshmirsafa M, Falak R, Keshavarz F, Faraji F. Effects of Adipose-tissue Derived Mesenchymal Stem Cells on PBMCs in Co-culture with HeLa Cell Line. Turk J Immunol 2024;12(1):19-27

Received: 13.10.2023 **Accepted:** 17.04.2024

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Abstract

Objective: Cervical cancer, the most common reproductive system cancer being women, is the fourth leading cause of cancer-related deaths. The use of mesenchymal stem cells (MSCs) for treating various diseases is being studied, but their use in cervical cancer has not been well explored. In this study we study investigated the effect of adipose tissue-derived MSCs on the apoptosis and proliferation of peripheral blood mononuclear cells (PBMCs) in co-culture with the HeLa cell line. MSCs were isolated from adipose tissue and then co-cultured with PBMCs, and HeLa cells at different time points (24, 48, and 72 hours).

Materials and Methods: The effect of MSC cells on proliferation, apoptosis, and gene expression of the cytokines tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interleukin (IL)-4, IL-2, and interferon-γ in PBMCs cultured together with HeLa cells was investigated using flow cytometry and real-time polymerase chain reaction (PCR), respectively.

Results: Flow cytometry showed that co-culture of PBMCs/MSCs/HeLa significantly increased the proliferation of PBMCs at different time points, with a p-value of 0.0022. In addition, MSCs significantly decreased apoptosis of PBMCs in co-culture with HeLa at 48 h. the p-value was 0.0022. Real-time PCR showed that the expression of TGF-β in PBMCs/MSCs/HeLa co-culture increased after 24 h, with a p-value of 0.006.

Conclusion: These data showed that adipose-derived MSCs can stimulate the proliferation and survival of PBMCs and enhance the apoptosis of HeLa cells, indicating their potential as immunomodulatory therapy for cervical cancer cells. However, further research is required to fully understand the underlying mechanisms and optimize therapeutic approaches involving PBMCs and MSCs.

Keywords: Mesenchymal stem cell, PBMC, HeLa, cancer, apoptosis

Introduction

Cervical cancer is the most common malignancy in women, with approximately 604.127 new cases and 341.831 deaths per year (1). This number is higher in developing countries due to difficulty in accessing screening and appropriate treatment (1). In most cases, the development of cervical cancer is related to infection with high-risk strains of human papillomavirus (HPV) (1). Since the discovery that the HPV virus causes most cases of cervical cancer, the HPV vaccine has been used for prevention since 1990 (2). Treatment is based on the time of diagnosis, extent of involvement, and available resources and usually includes hysterectomy and chemotherapy or a combination of both (3). Radiation therapy is also a method of cervical cancer treatment and one of the research fields along with immunotherapy. The use of immunotherapy is a

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new option for the treatment of patients with cervical cancer (1). Mesenchymal stem cells (MSCs) are being investigated as therapeutic agents in clinical research because of their high self-renewal capacity, paracrine effects, and ability to undergo mesodermal differentiation (3). MSCs can be isolated from various sources such as bone marrow, adipose tissue, peripheral blood, placenta, and umbilical cord. Among these, adipose tissue is the most abundant source and is more easily accessible (4). The role of MSCs in tumor modulating s is still under investigation. Some researchers have shown that these cells stimulate cancer by suppressing the immune system, stimulating angiogenesis and mesenchymal-epithelial transition, and influencing the tumor microenvironment (5). On the other hand, research on the effect of bone marrow-derived MSCss on Kaposi's sarcoma model showed antitumor and pro-apoptotic effects in cell-to-cell contact (6). Human endometrial MSCs can also reduce tumor growth by inducing cell cycle arrest, *in vitro* and *in vivo* inducing apoptosis and reducing angiogenic ability in epithelial ovarian cancer (7). In addition, indirect co-culture with adipose tissue-derived MSCs and hepatocellular carcinoma cells decreased the growth of Huh7 (hepatocellular carcinoma cells line) with increased protein levels of p53/p21 and phosphorylated STAT1 (pSTAT1), without apoptosis (8). Co-culture of HeLa adenocarcinoma cells, peripheral blood mononuclear cells (PBMCs), and bone-marrow-derived mesenchymal stromal cells results in changes in the proliferative activity of the PBMCs and mesenchymal stromal cell population (9). Therefore, determining the effect of these cells on the immune system and tumor cells in the tumor microenvironment helps to better use these cells for treating cancer. Hence, this study aimed to investigate the effect of MSCs isolated from adipose tissue on the proliferation, apoptosis, and cytokine expression of PBMCs of healthy individuals in co-culture with HeLa cervical cancer cells.

Materials and Methods

Cell Culture

The HeLa cell line was purchased from the Pasteur Institute of Iran and antibodies (PE/Cyanine7-antiCD73, APC-antiCD90, PE-antiCD34, and FITC-antiCD45) from ebioscience (Palo Alto, CA). The HeLa cell line was cultured in DMEM low glucose medium (Anacell, Iran) containing 10% FBS (Gibco, USA) and 1% penicillin/streptomycin (PAN biotech, Germany) in a T75 flask and incubated at 37°C with 5% CO_2 until 80-90% confluency was achieved. All experiments were performed in duplicate.

Isolation, Culture, and Characterization of MScs

MSCs were isolated from adipose tissue. After the completion of written consent forms, adipose tissue was collected from 3 healthy women via liposuction surgery. MSCs were isolated from adipose tissue as previously described and pooled together (8,10). Briefly, fresh adipose tissue was washed with phosphate-buffered saline (PBS) and cut into small pieces. After adding the collagenase I enzyme solution (Worthington, USA), it was incubated at 37°C in a water bath for 30-45 min. During this time, the container was shaken several times. To neutralize the enzyme, DMEM containing 10% FBS was added and centrifuged. Fat and cell debris were removed, and the sediment from the bottom of the tube was transferred to the cell culture flask with DMEM medium containing 10% FBS and 1% streptomycin/penicillin.

Cell Surface Biomarker Analysis by Flow Cytometry

MSCs were detached from the flask using trypsin-EDTA solution and collected into tubes. After centrifugation, the supernatant was removed and replaced with PBS and 5% FBS solution. Cells were examined for cell surface biomarker expression using the conjugated antibodies PE/Cyanine7-antiCD73, APC-antiCD90, PE-antiCD34, and FITC-antiCD45 and incubated in the dark for 45 min. Isotype-matched antibodies were used to validate antibody staining. Finally, the fluorescence of the cells was analyzed using a FACSCalibur flow cytometer (Becton Dickinson, UK), and the collected data were analyzed using the FlowJo software. Adipogenic and Osteogenic Differentiation of MSCs.

The ability of MSCs to differentiate into adipocytes and osteocytes was demonstrated as previously described (10). Briefly, cells were detached from the flask with trypsin-EDTA solution, seeded in 6-well cell culture plates (1×10^5 cells/well) in the presence of DMEM containing 10% FBS and incubated at 37°C with 5% CO_2 for 48 h. Subsequently, the medium of cells was replaced with adipogenic induction medium (AdipoDiff, StemMACS, USA) or osteocyte differentiation medium (OsteoDiff, StemMACS, USA) and incubated at 37°C with 5% CO_2 for 21 days. Finally, lipid droplets were identified using oil red O staining (11) and calcium deposits were identified using alizarin red and alkaline phosphatase according to previously described protocols (10-12).

Isolation of PBMCs

For the isolation of PBMCs, peripheral blood samples were collected from 3 healthy donors in tubes containing EDTA anticoagulant after providing written informed consent. PBMCs were isolated by density gradient centrifugation with Ficoll Isopaque (Pars Azmaye Teb, IRAN). Fresh PBMCs were used in the experiments.

Cell Proliferation Assay

The carboxyfluorescein diacetate succinimidyl ester (CFSE) kit (BioLegend, USA) was used to examine the

effects of MSCs on the proliferation of PBMCs in the presence of HeLa cells, according to the kit instructions. Briefly, PBMCs isolated using ficoll solution were washed twice with PBS solution. After removing the supernatant and adding 1 mL of PBS, 1 µL of 5 mM CFSE stock solution was added, and the tube was incubated at room temperature in the dark for 20 min. Then, DMEM culture medium containing 10% FBS (5 times the original staining solution) was added, and the tube was centrifuged. After discarding the supernatant, a fresh cell culture medium containing 10% FBS and 1% antibiotics was added, and the cell suspension was used for co-culture. Stained PBMCs (1×10^6 cells/well) were seeded with HeLa cells (2×10^5 cells/well) and MSCs (2×10^5 cells/well) in the presence of 2 mL of fresh cell culture medium in 6-well cell culture plates. After 24 and 48 h of incubation at 37°C with 5% CO_2, the supernatant containing stained PBMCs was collected and cell proliferation was analyzed with a FACSCalibur flow cytometer.

Apoptosis Assay

The FITC Annexin V Apoptosis Detection Kit (BioLegend, USA) was used to investigate the induction of apoptosis in PBMCs in the presence of MSCs and HeLa cells, according to the manufacturer's instructions. PBMCs (1×10^6 cells/well) were co-cultured with HeLa cells (2×10^5 cells/well) and MSCs (2×10^5 cells/well) in the presence of 2 mL fresh cell culture medium in 6-well cell culture plates for 24, 48, and 72 h at 37°C with 5% CO_2. The supernatant was then collected and centrifuged. After discarding the supernatant, 500 µL of buffer solution was added and gently pipeted. Then, 2 µL of Annexine dye was added to each microtube and incubated in the dark for 15 min. Finally, 2 µL of PI reagent was added and cell apoptosis was analyzed using a FACSCalibur flow cytometer. Annexin V-FITC positive plus PI negative cells (early apoptotic cells) and double positive cells for annexin V-FITC and PI (late apoptotic cells) were considered apoptotic cells. Also, annexin V-FITC negative plus PI-positive cells were considered as necrotic cells.

Assessment of Cytokine Expression

To study the gene expression of cytokines, including interleukin (IL)-2, IL-4, tumor necrosis factor alpha (TNF-α), interferon (IFN)-γ, and transforming growth factor beta (TGF-β), the real-time polymerase chain reaction (PCR) was performed using SYBER Green technology and specific primers for each cytokine. PBMCs (1×10^6 cells/well) were co-cultured with HeLa cells (2×10^5 cells/well) and MSCs (2×10^5 cells/well) in the presence of 2 mL fresh cell culture medium in 6-well cell culture plates for 24 h at 37°C with 5% CO_2. The supernatant was then collected and centrifuged. After discarding the supernatant and adding 500 µL of TRIzol reagent (Sinaclon, Iran), total RNA was extracted according to the manufacturer's instructions (Parstous, Iran). RNA quality was determined by measuring absorbance at 260 and 280 nm. RNA concentration was calculated using absorbance measurements at 260 nm using a Nanodrop ND-1000 spectrophotometer (Scrum Inc., Japan). Total RNA was converted to cDNA according to the manufacturer's instructions (Parstous, Iran). Cytokine gene expression was quantified using a *Rotor*-gene Q thermal cycler (Qiagen, Q, Germany). The total volume of the reaction was 20 µL including 8 µL of SYBR Green PCR Master Mix (Amplicon, Denmark), 1 µL of both forward and reverse primers, 1 µL of cDNA, and 10 µL of DEPC water (CinnaGen, Tehran, Iran).

The *GAPDH* gene was used as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of genes. Data are presented as fold change compared to the control (untreated cells).

Statistical Analysis

To interpret the results and determine the differences between the control and treatment groups, the collected data were analyzed using GraphPad Prism version 8 software. The independent t-test and one-way analysis of variance (ANOVA) test were used. A p-value of less than 0.05 was considered significant. All experiments were performed in triplicate.

Results

Cell Surface Biomarker Analysis

To confirm the mesenchymal nature of the stem cells, the cell surface biomarkers of these cells were analyzed with flow cytometry. The results showed that these cells were negative for the biomarkers CD45 and CD34, whereas they strongly expressed the biomarkers CD90 and CD73 (Figure 1).

Table 1. Oligonucleotide primers sequences of selected cytokine genes and internal control

Gene	Primer sequence
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC Reverse: TGGTGAAGACGCCAGTGGA
TNF-α	Forward: GCCCATGTTGTAGCAAACCC Reverse: TATCTCTCAGCTCCACGCCA
IL-2	Forward: AACTCCTGTCTTGCATTGCA Reverse: TGCTCCAGTTGTAGCTGTGTT
IFN-γ	Forward: TGTGGAGACCATCAAGGAAG Reverse: TGCTTTGCGTTGGACATTCAG
IL-4	Forward: ACCATGAGAAGGACACTCGC Reverse: GTTCCTGTCGAGCCGTTTCA
TGF-β	Forward: CCCTGGACACCAACTATTGC Reverse: TGCGGAAGTCAATGTACAGC

IL: Interleukin, TNF: Tumor necrosis factor, IFN: Interferon, TGF: Transforming growth factor

Differentiation of MSCs into Adipocytes and Osteocytes

To further analyze and confirm the mesenchymal nature of MSCs, these cells were cultured in specific differentiation media for adipocytes and osteocytes (Figure 2A). After three weeks, the cells were stained with oil-red O to identify lipid droplets, and the cells were viewed under a light microscope, showing lipid vacuoles (Figure 2B). To investigate the differentiation of MSCs to osteocytes, MSCs were stained with alizarin red after three weeks of culture, and calcium crystals were observed under a microscope in dark red or black (Figure 2C).

Cell Proliferation Assay

CFSE method was used to measure cell proliferation. Amine-reactive dye CFSE is non-fluorescent before entering living cells. After entering living cells, it is hydrolyzed by cytoplasmic esterase enzymes and converted to fluorescent amine-reactive dyes. Then, with cell division, CFSE fluorescence in progeny cells is gradually halved. Therefore, there is an indirect relationship between CFSE fluorescence and cell proliferation, as cell proliferation increases, CFSE fluorescence intensity decreases. To investigate the effects of MSCs on the proliferation of PBMCs and HeLa cells, co-culture was performed at different time points (24 and 48 h). It was found that the proliferation of PBMCs was significantly increased in the PBMCs/MSCs co-culture at 24 h (Figure 3A) but noticeably decreased at 48 h compared to the PBMC control culture alone, (p=0.0022) (Figure 3A). PBMCs/MSCs/HeLa co-culture had a significantly higher proliferation of PBMCs than the controls (PBMC/HeLa) at 24 and 48 h (Figure 3A and B).

Apoptosis Assay

Apoptosis of PBMCs in PBMCs/MSCs co-culture decreased significantly at 24 h (Figure 4A) but was increased at 72 h (Figure 4B) compared to the control (PBMC culture alone). However, the difference between the control and PBMCs/MSCs at 48 h was not significant (Figure 4B). In the co-culture of PBMCs/MSCs/HeLa, apoptosis of PBMCs was significantly lower than that of the control (PBMCs/MSCs) at 48. However, apoptosis of PBMCs in the co-culture of PBMCs/MSCs/HeLa

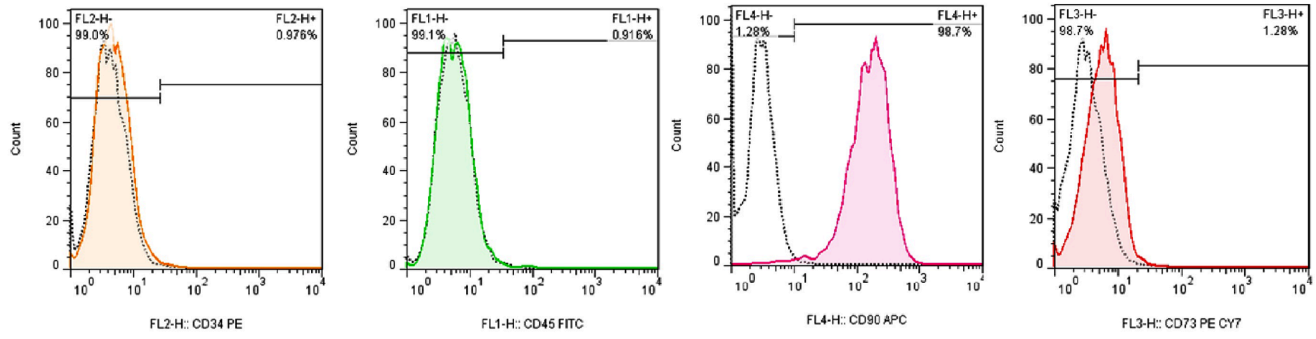

Figure 1. Analysis of cell surface biomarkers of MSCs by flow cytometer.
MSCs: Mesenchymal stem cells

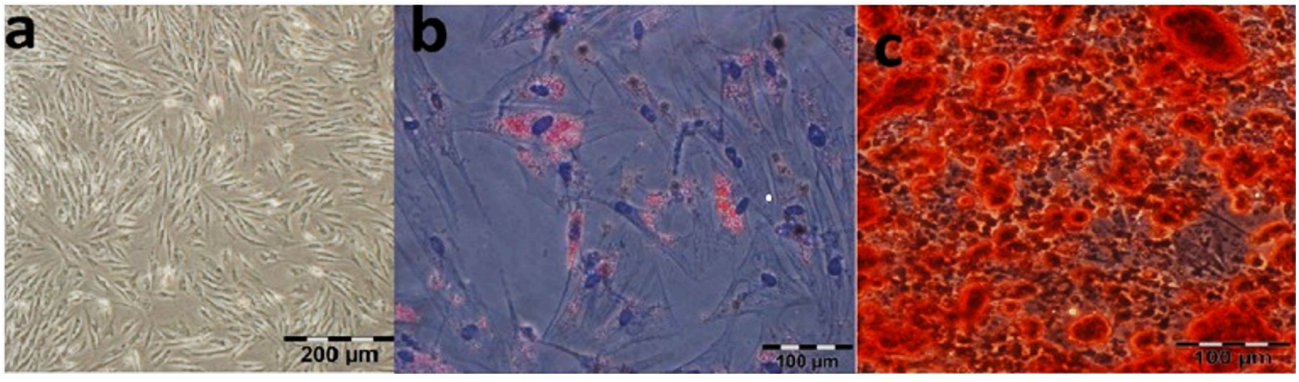

Figure 2. Differentiation of MSCs into adipocytes and osteocytes. a: Mesenchymal stem cells in passage one, which are fibroblast-like and stretched in parallel, b: Differentiation of mesenchymal stem cells into fat cells using Oil Red O staining and the presence of fat vacuoles, c: Differentiation of mesenchymal stem cells into bone cells after Alizarin Red staining and appearance of calcium crystals.
MSCs: Mesenchymal stem cells

at 24 and 72 h did not show any significant changes (Figure 4A and B).

Cytokine Expression

The expression of TNF-α, TGF-β, IL-4, IL-2, and IFN-γ was evaluated in the cell cultures of the experiment and the control after 24 h, using specific primers (Table 1). In the PBMCs/MSCs co-culture, the TGF-β level was significantly lower than in the PBMCs culture alone, with a p-value of 0.008 (Figure 5E), while it was higher in the PBMCs/MSCs/HeLa co-culture than in the control (PBMCs/MSCs), with a p-value of 0.006. In addition, the IFN-γ level was significantly higher in the PBMCs/MSCs co-culture than in the PBMCs culture alone, (p=0.0001) (Figure 5C). However, TNF-α, IL-4, and IL-2 levels were not significantly different between the experiments and controls (Figure 5A, B, and D).

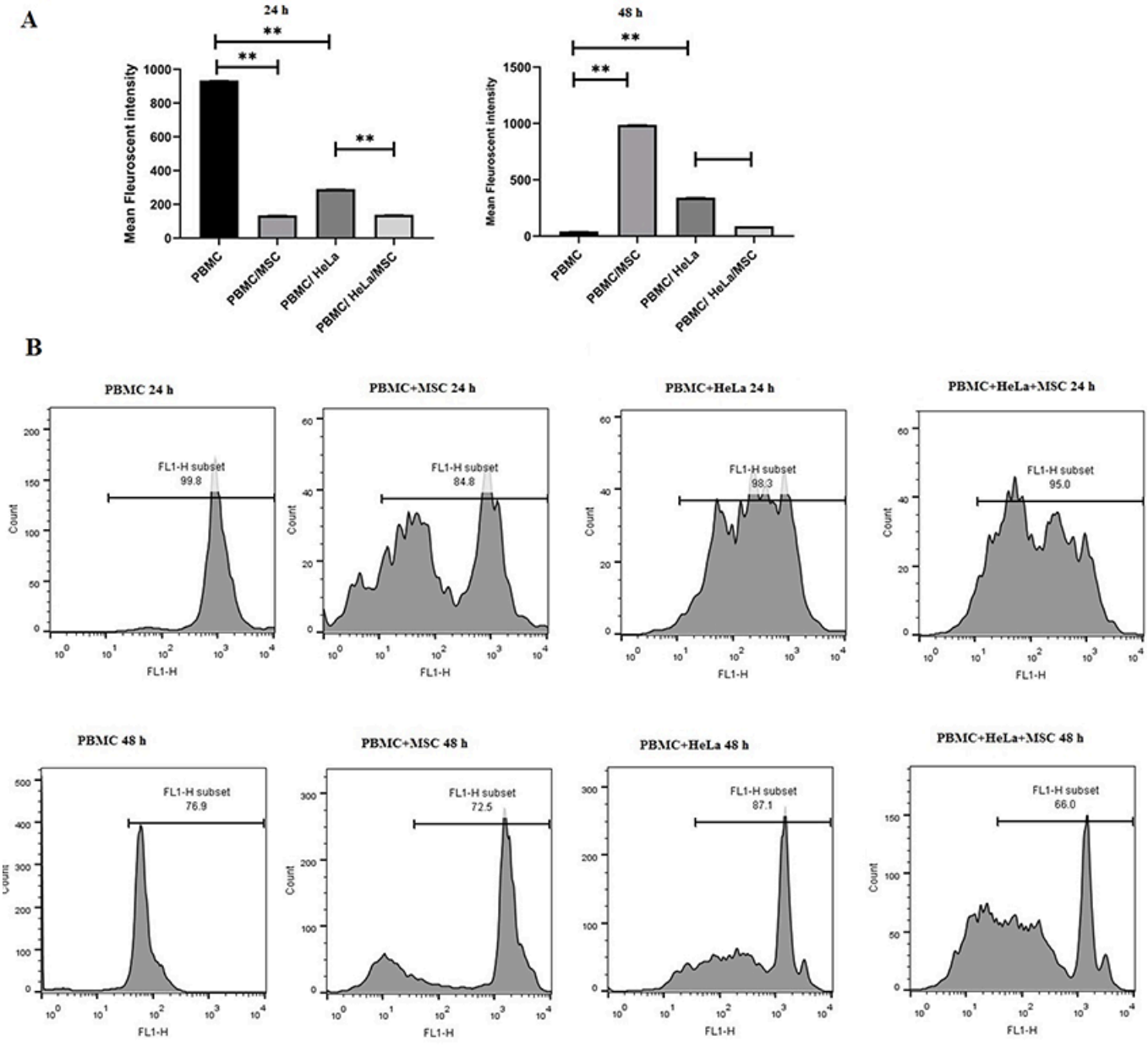

Figure 3. The effects of MSCs on the proliferation of PBMCs and HeLa cells, co-culture was performed after co-culture for 24 and 48 hours. A) Results of data analysis. B) Flow cytometry histograms. Differences at $p<0.05$ were considered significant p=0.0022. The proliferation of PBMCs along with MSCs and HeLa cells was compared with the PBMCs/HeLa and PBMC control culture alone using one-way analysis of variance (ANOVA). FACS Plot.
MSCs: Mesenchymal stem cells, PBMCs: Peripheral blood mononuclear cells

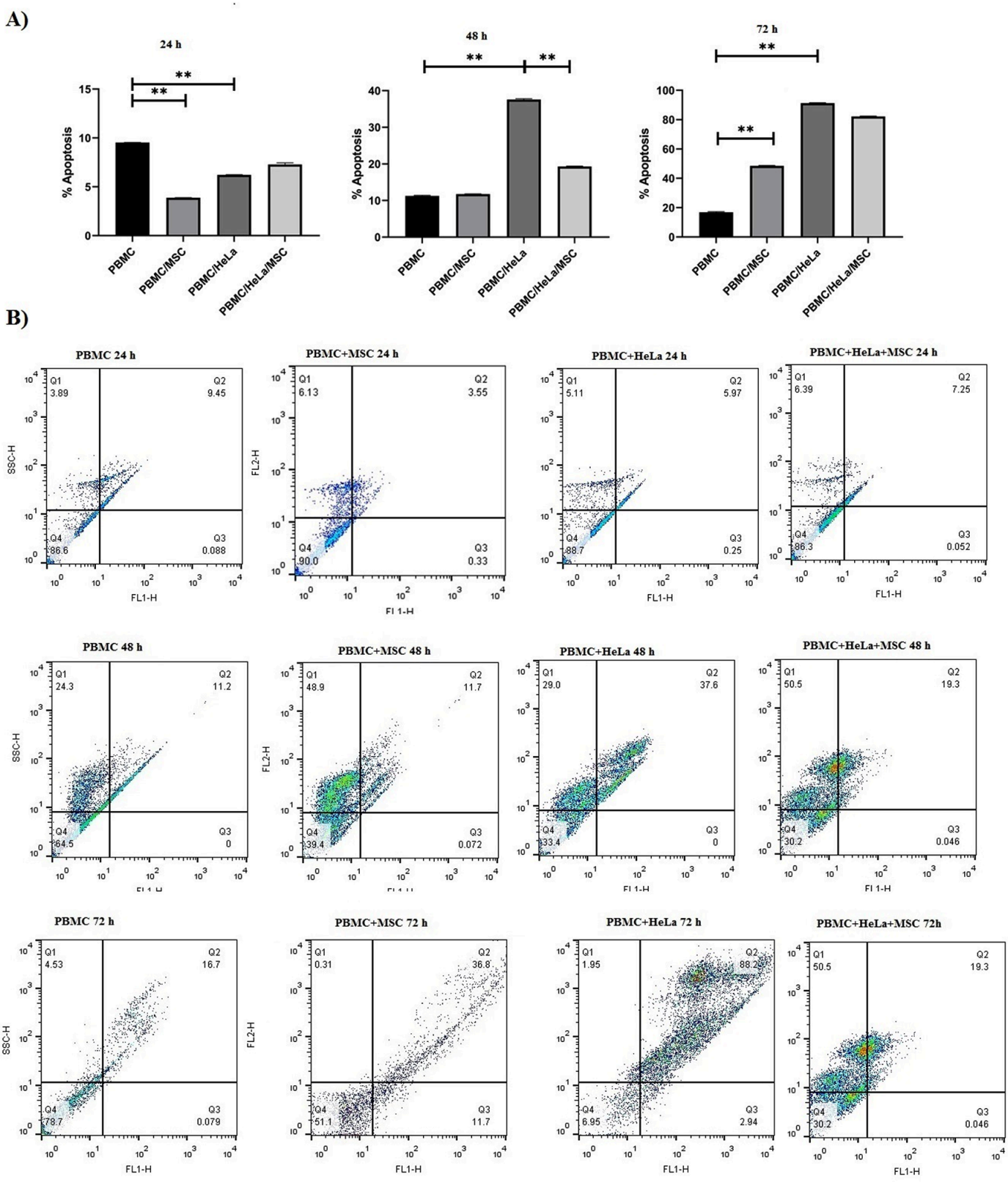

Figure 4. The effect of MSC cells on the apoptosis of PBMCs and co-cultured HeLa cells after 24, 48, and 72 hours was determined by flow cytometry. A. Results of data analysis. B. Flow cytometry plots. Differences at $p<0.05$ were considered significant $p=0.0022$.
MSCs: Mesenchymal stem cells, PBMCs: Peripheral blood mononuclear cells

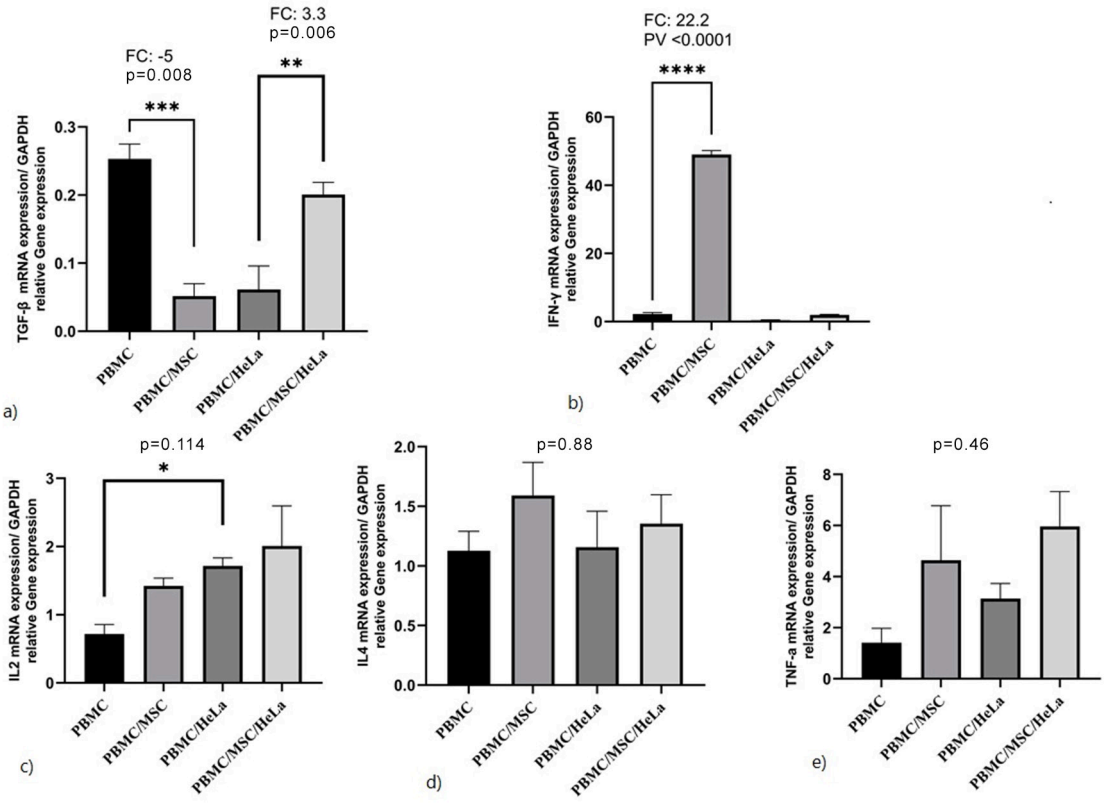

Figure 5. The expression of cytokines A: TNF-α, B: IL-2, C: IFN-γ, D: IL-4, and E: TGF-β was evaluated in PBMC cells in the presence of MSCs and HeLa cells after 24 h by real-time PCR.
IL: Interleukin, TNF: Tumor necrosis factor, IFN: Interferon, TGF: Transforming growth factor, PBMCs: Peripheral blood mononuclear cells, PCR: Polymerase chain reaction, MSCs: Mesenchymal stem cells

Discussion

Cervical cancer is the fourth most common cancer among women and the fourth leading cause of death worldwide (13,14). Studies have shown that HPV plays an important role in the development of this disease and causes more than 80% of cases of this cancer (12). Immunotherapy is a new generation of treatments compared to traditional treatments such as surgical therapy, radiotherapy, and chemotherapy (15). Its goal is to stimulate the body's immune system and increase its ability to kill the tumor (15). In general, immunotherapy of cervical cancer can be divided into four groups: immune checkpoint blocking, cell transfer therapy, therapeutic vaccines, and cytokine therapy (15). MSCs are one of the most commonly used cells in regenerative medicine (15). Many studies have demonstrated the beneficial effects of MSC-based treatments in various diseases (16-18). However, the therapeutic potential of MSCs in cancer remains controversial (19,20). While some studies suggest that MSCs may play a role in carcinogenesis, there are emerging data reporting the suppressive effects of MSCs on cancer cells (21,22). When the effect of MSC cells on the proliferation of PBMCs alone and in concurrent culture with HeLa cells was examined, it was found that the presence of MSCs in co-culture with PBMCs led to a significant increase in the proliferation of PBMCs compared to PBMCs control only at 24 h, whereas, co-culture with HeLa cells resulted in a significant increase in the proliferation of PBMCs compared to PBMCs/HeLa control at 24 and 48 h. Kitaeva et al. (9) studied the proliferative activity of bone-marrow-derived MSCss, PBMCs, and HeLa cells after co-culture. They analyzed the effect of co-culture on the proliferation of each cell population after 72 h. The co-culture increased the proliferation of MSCs significantly. At the same time, a noticeable decrease (by 62%) was detected in the proliferation of PBMCs. In addition, a slight decrease (by 10%) was found in the proliferation of HeLa cells after co-culture with MSCs and PBMCs (9).

In addition, our study showed that apoptosis of PBMCs in PBMCs/MSCs co-culture decreased significantly at 24 h but increased at 72 h compared to the control (PBMC culture alone). Moreover, co-culture of PBMCs/HeLa/MSCs showed that the presence of MSC cells significantly decreased the PBMC cell apoptosis at 48 h. Taken together, the current experiment has exhibited that MSC cells have a positive influence on PBMCs, including their proliferation

and apoptosis, depending on the time of co-culture. Our result is consistent with other studies that investigated the effect of mesenchymal cells isolated from amniotic fluid on the survival of HeLa cells in cell culture for 3 and 5 days. They were treated for five days, which was associated with increased expression of *BAX*, *TP53*, and *CDKN1A* genes and decreased expression of BCL-2 (23). Moreover, Bu et al. (7) investigated the effect of endometrial mesenchymal cells isolated from menstrual blood on ovarian epithelial cancer. They concluded that these cells inhibit tumor growth by cell cycle arrest, apoptosis stimulation, and reduction of angiogenesis ability. Therefore, they showed the antitumor potential of these mesenchymal cells *in vitro* and *in vivo* (7). In addition, previous studies reported an increase in the proliferation and invasion of MSC cells on the growth of tumor cells (24). In the current study, we also examined the expression of the cytokines TNF-α, TGF-β, IL-4, IL-2, and IFN-γ in PBMC cells to investigate the effect of the presence of MSC cells in the culture at 24 h. The analysis exhibited that the presence of MSC with PBMC compared to PBMC control caused a significant increase in IFN-γ but a significant decrease in TGF-β in PBMC cells. In the co-culture of PBMC/HeLa/MSC compared to the PBMC/HeLa control, the presence of MSC cells significantly increased *TGF-β* gene expression. In the study by Kwon et al. (25), co-culture of PBMCs with MSCss for 24 h increased IFN-γ and IL-10 mRNA levels in PBMCs, which is similar to our study. In addition, the levels of IL-4 and TGF-β increased significantly in PBMCs after 72 h, which was inconsistent with our results. In another study, direct cell-cell contact between MSCs and PBMCs induced the expression of many cytokines, including IL-6 and IL-8. These findings suggest that direct cell-cell interactions between MSCs and PBMCs can induce the expression of cytokines that may play a role in immune regulation. In the case of co-culture, the results obtained differ from the normal, although it should be emphasized that MSCs are not always immunosuppressive. It is assumed that their effect is determined by the local conditions of the microenvironment. In addition, anti-inflammatory cytokines do not always play such a role (11).

Conclusion

The study concludes that MSCs have different effects on PBMCs depending on the presence and absence of cancer cells and the time of co-culture. This study suggests that MSCs have the potential to show anti-tumor and immune-boosting effects. However, further research is still needed to fully understand the underlying mechanisms and optimize therapeutic approaches involving MSCs.

Ethics

Ethics Committee Approval: The study approved by Ethics Committee of Shahrekord University (approval number: IR.SKUMS.REC.1400.236).

Informed Consent: Informed consent was obtained from healthy volunteers who donated adipose tissue.

Authorship Contributions

Surgical and Medical Practices: M.G., F.F., Concept: M.G., R.F., Design: F.F., Data Collection or Processing: F.F., M.G., M.R., M.K., R.F., M.D., F.L.A., F.K., Analysis or Interpretation: F.F., M.G., M.R., M.K., R.F., M.D., F.L.A., F.K., Literature Search: F.F., M.G., M.R., M.K., R.F., M.D., F.L.A., F.K., Writing: F.F., M.G., M.R., M.K., R.F., M.D., F.L.A., F.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declare that they have no relevant financial.

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