Analysis of the interaction and proliferative activity of adenocarcinoma, peripheral blood mononuclear and mesenchymal stromal cells after co-cultivation *in vitro*

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Abstract

The tumor microenvironment is a heterogeneous population of cells actively involved in the process of growth and development of a tumor. Research has demonstrated the interactions between the different populations of cells are critical for the formation of the tumour micro-environment and if recapitulated experimentally can be used to produce more effective models for preclinical screening of anticancer drugs. In this study, we demonstrate co-culturing HeLa adenocarcinoma cells, peripheral blood mononuclear cells and mesenchymal stromal cells, results in changes in the proliferative activity of the peripheral blood mononuclear cells and mesenchymal stromal cell populations. This data supports the further development of *in vitro* co-culture systems utilizing these cell types for pre-clinical screening of anticancer drugs.

Keywords: co-culture, intercellular interaction, adenocarcinoma, mononuclear blood cell, mesenchymal stromal cell, test system, drug screening

Introduction

The microenvironment plays a key role in tumor progression, metastasis and the formation of therapeutic resistance [1]. The tumor stroma consists of a mixed-cellular component that includes immune cells, endothelial cells, tumor-associated fibroblasts and myofibroblasts, adipocytes and mesenchymal stromal cells (MSCs), as well as an extracellular matrix that provides structural support for the cell component in the extracellular space and organs [2]. The recruitment of MSCs into the tumor stroma is generally as a result of inflammatory factors produced within the tumor microenvironment [3]. After recruitment to the tumor environment, MSCs can act as precursors of cancer-associated fibroblasts (CAFs), which contribute to the progression of tumors due to the secretions of a number of growth factors, chemokines, interleukins, and matrix metalloproteinases [4]. The migratory response of MSC's to soluble factors such as MIP-1δ and MIP-3α, is evidence of this ability to respond to tumor secreted factors [5]. Using bioluminescent imaging, it has been shown that MSCs after administration to mice within a xenograft model of human glioma, localize to areas of tumor formation and are not detected in healthy areas of the brain [6,7]. At the same time, fibroblasts did not show similar specificity of the ability to migrate, which indicates the selective migration of MSCs to the area of tumor formation [6].

MSCs are involved in the normal tissue regeneration processes, migrating to sites of damage in response to inflammatory mediators. This same process occurs within tumors that express a similar repertoire of factors as the inflamed tissue [1]. Stromal cells attracted by tumor secrete growth factors of various types, such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), epidermal growth factor (EGF) [8] and chemokines, including CXCL12, not only promote the growth and survival of tumor cells, but also act as a chemoattractant for other cells to migrate into the microenvironment of the tumor. In addition, MSCs actively remodel the extracellular matrix, contribute to neoangiogenesis and the formation of resistance to chemotherapeutic drugs [3]. In general it is assumed the changes induced by MSCs collectively result in a pro-tumor growth environment, as illustrated by the incorporation of MSCs as part of both 3D in vitro and in vivo models of human glioblastoma, resulting in an increase in both the proliferative and invasive potential of tumor cells [9]. However, MSCs have been reported to inhibit tumor growth, by attracting cells of the inflammatory response, negatively affecting angiogenesis and the Wnt and AKT signaling pathways, inducing cell cycle arrest and apoptosis [10,11]. The mechanisms underpinning these differential effects on the tumor microenvironment are yet to be fully understood, but may depend on the activation status of these cells and other elements of the stromal microenvironment of the tumor. For example, MSCs isolated from adipose tissue of mole rats (Blind Mole Rat Spalax) showed antitumor activity and reduced migration activity to tumor sites, in contrast to MSCs isolated from adipose tissue of ordinary rats [12]. Despite the multitude of tumor-supporting properties, the ability of MSCs to migrate and reside within the tumor environment makes this cell type a promising vector for the delivery of therapeutic gene products, chemotherapeutic drugs, and immunomodulating agents [1,13].

In addition to changes caused by MSCs, the tumor microenvironment can also directly and indirectly disrupt the normal function of infiltrating immune cells [14]. Tumor cells and tumor-associated stromal cells express inhibitors of the immune response control points that suppress the function of T-cells, through PD-1 and its ligand (PD-L1), and inhibitors of CTLA-4, which contributes to the avoidance of immune surveillance in both solid and hematological tumors [15]. In addition to direct immunosuppression using regulatory ligands, tumor cells also co-opt microorganism that produce high levels of lactate or kinurenin, which directly inhibit T-cells [16]. In addition,

some populations of immune cells, in particular, T-regulatory cells, are able to exert an immunosuppressive effect on T-effector cells [17].

The tumor-recruited macrophages of the M2-phenotype form a significant contribution to the development of the tumor and suppression of the antitumor immune response. It is known that a decrease in an excessive immune response by M2-macrophages promotes more active tumor growth and its escape from immune surveillance. M2-macrophages respond to cytokines IL-4 and IL-13, which leads to activation of the transcription factor STAT6 and activation of the signal anti-inflammatory genes arginase (Arg1) and resistin-like molecule α [18]. These regulatory pathways contribute to the recruitment of immunoregulatory macrophages, the release of immunoregulatory cytokines, the induction of angiogenesis and the remodeling of basement membranes [19].

Studies of the tumor microenvironment in recent years have shown the need to develop new test systems that can take into account the factors of the microenvironment and the heterogeneity of the natural tumor [20,21]. However, the creation of a model that is able to take into account all aspects of the microenvironment is not currently possible. Nevertheless, the strategy of using several basic elements involved in the tumor formation processes can significantly improve the prognostic value of such test-systems compared to conventional single cell tumor models [22]. Thus, to reproduce the processes occurring in the stromal microenvironment of a tumor, the development of *in vitro* test systems containing several cellular components of the tumor microenvironment is required.

The study reports the beneficial interactions of co-culturing adenocarcinoma cells with peripheral blood mononuclear cells and mesenchymal stromal bone marrow cells *in vitro*. This data highlights the value of including these and similar cell types into complex pre-clinical models for cancer drug testing.

Materials and methods

Isolation and cultivation of cells

Bone marrow samples were provided by the Republican Clinical Hospital for research purposes in accordance with ethical standards and current legislation (the protocol was approved by the Committee on Biomedical Ethics of Kazan Federal University (No. 3, 03/23/2017)). Written informed consent was obtained from donors. MSCs from human bone marrow were isolated in a ficoll density gradient (1.077 g/cm^3, PanEco, Russia), followed by adhesion of cells to the surface of culture plastic. Human adenocarcinoma cells (HeLa) were obtained from the American Type Culture Collection (ATCC number: CCL2, Manassas, VA, USA). Mononuclear cells were isolated from peripheral blood (PBMCs) of a healthy donor in a ficoll density gradient (1.077 g/cm^3, PanEco, Russia) as previously described [23,24]. Written informed consent was obtained from donors. MSCs, HeLa and PBMCs were cultured in DMEM/F12 (PanEco, Russia) supplemented with 10% FBS (Invitrogen, USA), 2 mM L-glutamine (PanEco, Russia), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Biolot, Russia). Cells were maintained at 37 °C in a humid atmosphere with a content of 5% CO_2. Sub-confluent cells (approximately 80% confluent) were passaged using 0.25% trypsin-EDTA solution (Invitrogen, USA).

Immunophenotyping of peripheral blood mononuclear cells (PBMC)

PBMC immunophenotyping was performed using the following CD3 antibodies (Cat. No. 300405, BioLegend, USA), CD4 (Cat. No. 317409, BioLegend, USA), CD8 (Cat. No. MA5-16988, Thermo Fisher Scientific, USA), CD14 (Cat. No. 301813, BioLegend, USA), CD34 (Cat. No. 345802, BD Biosciences, USA), CD45 (Cat. No. 304009, BioLegend, USA). The cell population was analyzed using FACS Aria III (BD Biosciences, USA) and BD FACSDiva™ software version 7.0.

Immunophenotyping of mesenchymal stem cells (MSC) isolated from bone marrow

MSC immunophenotyping was performed using the following antibodies to CD29 (Cat. No. 87106, SONY, USA), CD44 (Cat. No. 51-9007656, BD Biosciences, USA), CD73 (Cat. No. 51-9007649, BD Biosciences, USA), CD90 (Cat. No. 51-9007657, BD Biosciences, USA), CD105 (Cat. No. 323218, BioLegend, USA), CD166 (Cat. No. 10846, SONY, USA), a cocktail of conjugated PE antibodies hMSC Negative Cocktail potential contaminants from the Human MSC Analysis Kit (Cat. No. 562245, BD Biosciences, USA) was used as a negative control. The staining results were analyzed using FACS Aria III (BD Biosciences, USA) and BD FACSDiva™ software version 7.0.

Fluorescent staining of cells

Fluorescent staining of MSCs, HeLa and PBMCs was performed in a serum-free medium using the Vybrant Cell Labeling Solutions kit (Thermo Fisher Scientific, USA) [25] using the dyes DiD (red spectrum), DiI (yellow spectrum) and DiO (green spectrum), respectively, according to the method recommended by the manufacturer. A qualitative assessment of staining was performed on an AxyObserver.Z1 fluorescence microscope (Carl Zeiss, Germany) and using the software AxyoVision Rel. 4.8.

Co-cultivation of cells

For co-culture, fluorescently stained MSCs, HeLa and PBMCs were mixed in a 1:1:1 ratio in a 12-well plate (6×10^4 cells/well). MSCs, HeLa and PBMCs were co-cultured in DMEM/F12 (PanEco, Russia) supplemented with 10% FBS (Invitrogen, USA), 2 mM L-glutamine (PanEco, Russia), penicillin (100 U/ml) and streptomycin

(100 µg/ml) (Biolot, Russia). Cells were maintained at 37 °C in a humid atmosphere with a content of 5% CO_2. The self-organization of co-culture cells was analyzed using a fluorescence microscopy on an AxyObserver.Z1 microscope (Carl Zeiss, Germany) and using the software AxyoVision Rel. 4.8.

FACS analysis

After 72 hours of co-culture, the cells were trypsinized and washed using Dulbecco's phosphate-buffered saline solution (PanEco, Russia). The cells were then sorted according to the fluorescence spectrum and size on a FACS Aria III flow cytometer (BD Biosciences, USA) using BD FACSDiva™ software version 7.0.

Analysis of cell proliferation activity

After separation, individual cell populations of MSCs, HeLa and PBMCs were sown in a 96-well plate and cultured for 24 hours in DMEM/F12 (PanEco, Russia) supplemented with 10% FBS (Invitrogen, USA), 2 mM L-glutamine (PanEco, Russia), penicillin (100 U/ml) and streptomycin (100 µg/ml) (PanEco, Russia) at 37 °C in a humid atmosphere with a content of 5% CO_2. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA) was used to determine cell proliferation according to the method recommended by the manufacturer.

Statistical analysis

The analysis of the obtained data was carried out using the GraphPad Prism 7 software (GraphPad Software) using the Shapiro-Wilk criterion and the Student's t-test.

Results and discussion

Mononuclear cells were isolated from the peripheral blood of a healthy donor. Immunophenotypic analysis of human PBMCs showed the presence of a large population (82.6%) of cells carrying a CD45 leukocyte marker on their surface (Figure 1B), among which 81.8% were T-lymphocytes (CD3 +, CD45 +) (Figure 1C), of which33.4% were T-cytotoxic lymphocytes (CD3+, CD8+, CD45+) and 44.1% T-helper cells (CD3+, CD4+, CD45+) (Figure 1D), together with 0.9% hematopoietic progenitor cells (CD34+ and CD45+) (Figure 1E) and 2.2% CD14+ monocytes (Figure 1F), consistent with published data [26]. It is known that T-cell populations are actively involved in the development of a cellular immune response to cancer, as shown in the study of the microenvironment of colorectal cancer, melanoma and breast cancer, in which the high content of infiltrate activated CD8+/CD4+ T cells in the early stages is a positive prognostic sign for patients [27-30]. In addition, cells carrying a CD14 marker on their surface can differentiate into macrophages with M1 and M2 phenotypes; involved in both the development and suppression of the immune response.

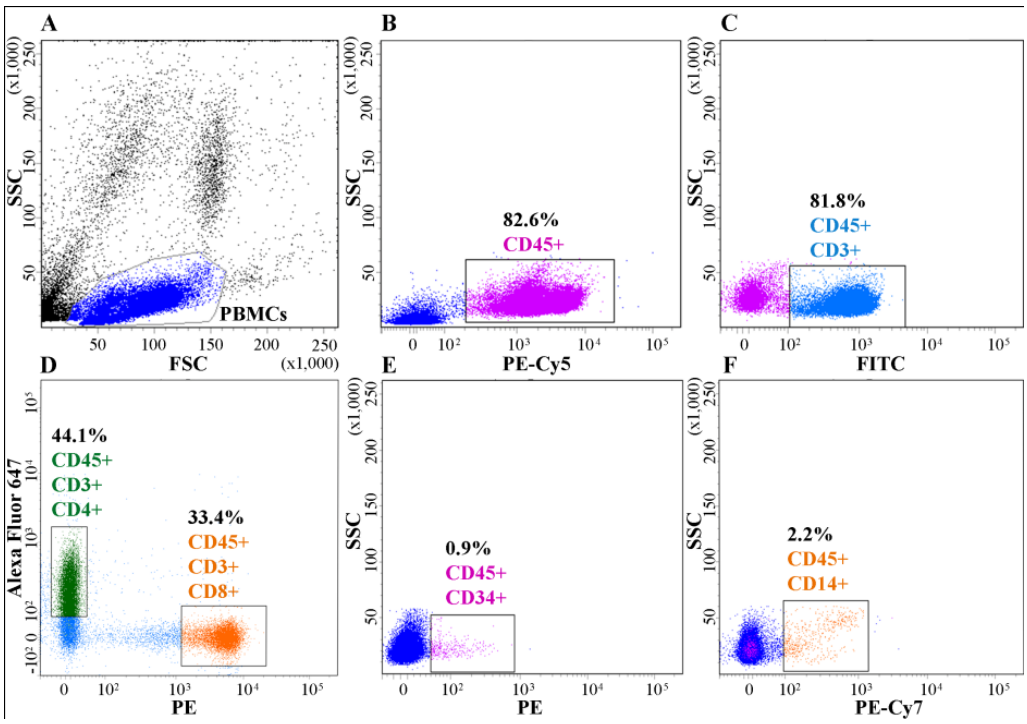

Figure 1. Immunocytometric characterisation of surface antigens (CD-markers) on isolated PBMCs. (A) The total PBMC population. (B-F) Population of cells carrying the leukocyte marker (B) CD45 (82.6%). (C) CD3+/CD45+ T-lymphocytes (81.8%). (D) CD3+/CD4+/CD45+ T-helper (44.1%), CD3+/CD8+/CD45+ T-cytotoxic lymphocytes (33.4%). (E) CD34+/CD45+ hematopoietic progenitor cells (0.9%). (F) CD14+/CD45+ monocytes (2.2%).

The MSCs isolated from bone marrow had a mesenchymal stromal cell phenotype, expressing surface markers CD90, CD166, CD29, CD73, CD44, CD105, lacking surface markers characteristic of hematopoietic cells (CD11b, CD19, CD34, CD45, HLA-DR) (Figure 2).

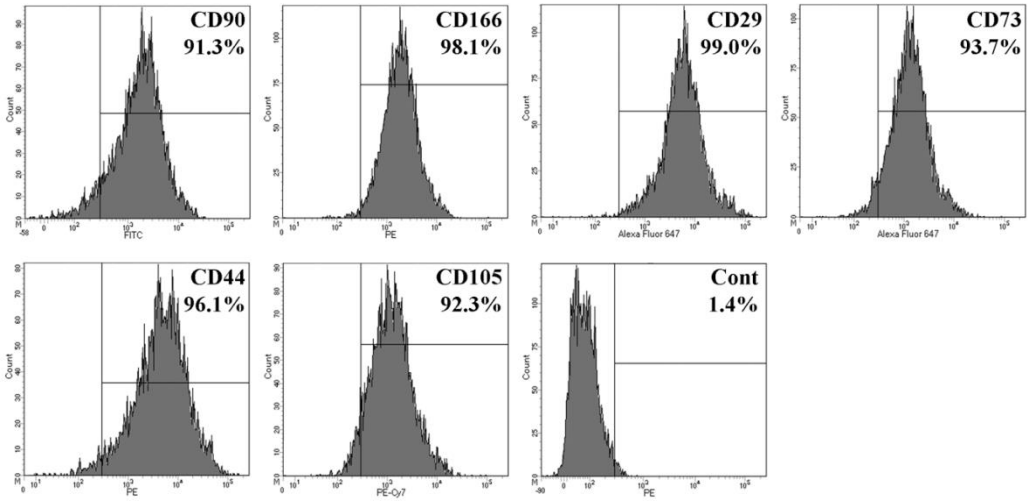

Figure 2. Immunocytometric characterization of CD surface antigens CD90, CD166, CD29, CD73, CD44, CD105. Cont – negative marker cocktail CD11b, CD19, CD34, CD45 and HLA-DR.

To analyze the behavior of adenocarcinoma cells, PBMCs and bone marrow MSCs were co-cultures with HeLa adenocarcinoma cells. Vital fluorescent staining of cells was carried out and tracing to determine the self-organization of individual cell populations in the tumor microenvironment model was conducted. The approach is based on the previously developed technology for creating pseudo-two-dimensional models of the interaction of tumor and stem cells using various extracellular matrices [25]. The cells were subsequently separated by cell-type by FACS.

The co-culture of MSCs, HeLa adenocarcinoma cells and PBMCs, revealed a high-degree of interaction between the cells types. Figure 3D, demonstrates cells with mixed fluorescence spectra (indicated by arrows), this potentially demonstrates an active exchange of membrane components, or the fusion and formation of hybrid cells. Similar phenomena are described in the literature [31,32]. The potential phenomena of tumour-stroma cell fusions and uptake of tumor cell fragments by immune cells and vice-versa, would contribute significantly to the heterogeneity of cells within the tumor and environment as we all potentially contributing to suppression of the immune response and the formation of resistance to chemotherapeutic drugs [33,34].

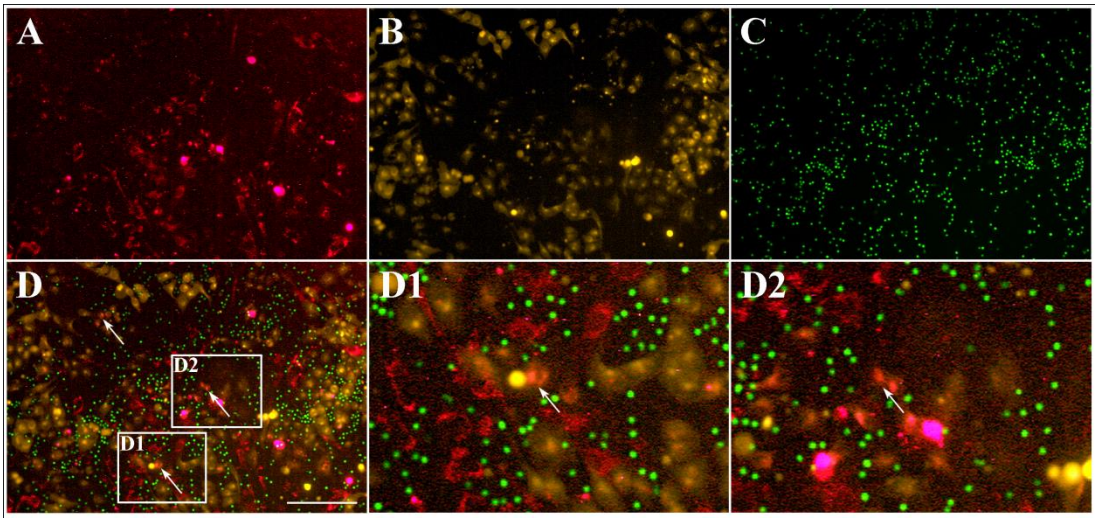

Figure 3. Analysis of self-organization of MSCs, Hela and PBMCs after 72 hours of co-culture. Arrows indicate fused cells. A – Alexa Fluor 647 (MSCs), B – PE (HeLa), C – FITC (PBMCs), D, D1, D2 – merge of Alexa Fluor 647, PE and FITC spectra. Scale: 200 microns.

After 72 hours of co-cultivation, cytofluorimetric analysis showed a high degree of cell interaction through the exchange of membrane components, which was expressed as the mixing of fluorescence spectra. The highest percentage of mixed fluorescent spectra were observed between MSCs and PMBCs (Alexa Fluor 647 and FITC) – 47.9% (Figure 4B), a smaller percentage of exchanged membrane components was observed between MSCs and

HeLa (Alexa Fluor 647 and PE) (Figure 4A) – 18.5%, and between HeLa and PBMCs (PE and FITC) (Figure 4C) – 5.7%, while the percentage of PBMCs that did not interact with both MSCs and HeLa cells accounted for about 5.6% (Figure 4C). To note, a relatively high percentage of cells lost the fluorescent labels during the co-cultivation process – overall decrease by 24.1% (HeLa and MSCs), 69.9% (PBMCs and MSCs) and 18.8% (HeLa and PBMCs) in total number of fluorescent cells in both cell populations in co-culture (Figure 4A, B, C). The active interaction of cells with each other and the exchange of membrane components can be explained by extracellular vesicle transport and direct cell-to-cell contacts. A recent study was showed the transfer of vesicle material from prostate cancer cells to bone marrow derived stromal cells [35]. It is known that extracellular vesicles transfer components such as proteins, nucleic acids (DNA, RNA), non-coding RNA, as well as proteins [36]. Due to this content, the phenomenon of extracellular communication between cells of the tumor microenvironment contributes to the development of resistance to chemo-and radiotherapy as well as contributing to tumor progression [37]. However, in addition to negative effects, promising options for the use of microvesicles for therapeutic purposes have been shown in a number of publications [38]. In addition, it has been shown in the literature that when co-cultivated, it is possible to transfer functional mitochondria from mesenchymal stromal cells to tumor cells. This process led to increased oxidative phosphorylation in tumor cells and subsequent increases in their proliferative and invasive ability [39].

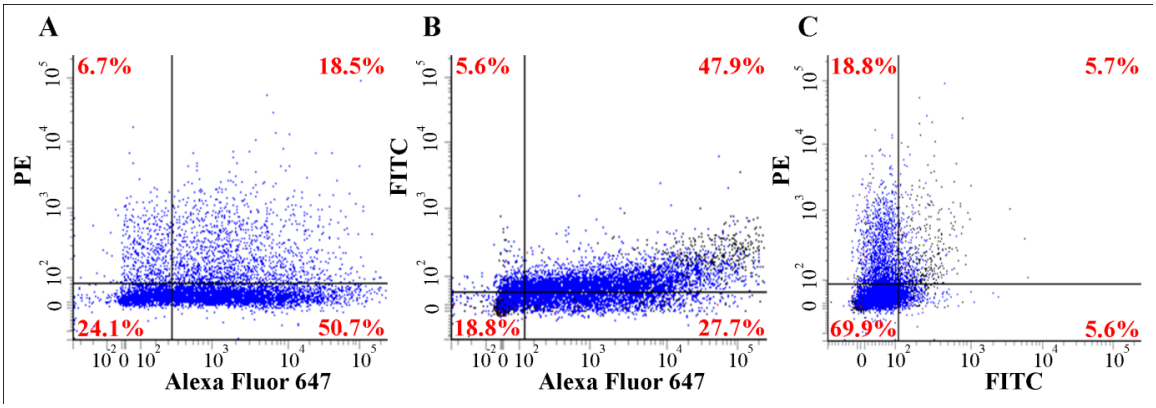

Figure 4. Cytofluorimetric analysis of individual cell populations of HeLa (PE), MSCs (Alexa Fluor 647) and PBMCs (FITC) after 72 hours co-culture. Analysis of cell populations from the fluorescence spectra of (A) PE and Alexa Fluor 647 (HeLa and MSCs); (B) FITC and Alexa Fluor 647 (PBMCs and MSCs); (C) PE and FITC (HeLa and PBMCs).

To analyze the effect of co-culture on the proliferation of the individual cell populations, following 72 hours co-culture cells were sorted based on their fluorescence spectra and the proliferation of each sorted cell population determined. The effect of co-culture on the proliferation of MSCs was significantly increased (more than 2-fold, $p < 0.0001$). This result can be explained by the response of MSCs to inflammatory cytokines secreted by co-culture cells, which leads to the induction of their proliferation [3].

At the same time, a noticeable decrease in the proliferation of PBMCs was shown, namely by 62% ($p < 0.0001$) (Figure 5). The results obtained are consistent with experimental data from the literature, which showed that the tumor microenvironment, which is rich in inflammatory cytokines, suppresses antitumor immune functions, preventing the recruitment, survival and functioning of immune effector cells [40,41]. In addition, early research showed MSCs exert an immunosuppressive effect both by paracrine regulation and by direct cellular interactions [42]. It has been shown that MSCs have a suppressive effect on populations of CD3+, CD4+ and CD8+ subpopulations of T-lymphocytes [43].

A slight decrease (by 10%, $p < 0.05$) of the proliferation of HeLa cells after co-culture with MSCs and PBMCs (Figure 5) is also shown. It is possible that MSCs and PBMCs under these culture conditions had a slight inhibitory effect on the growth of HeLa adenocarcinoma cells, which can be explained by the high density of the cell monolayer and the presence in the culture medium of a large number of different metabolites. In addition, it is known that MSCs can have antitumor effects under certain conditions and depending on the source of origin [12]. Further research is needed to fully understand the mechanisms that lead to the inhibition of the growth of HeLa adenocarcinoma cells in co-culture with MSCs and PBMCs.

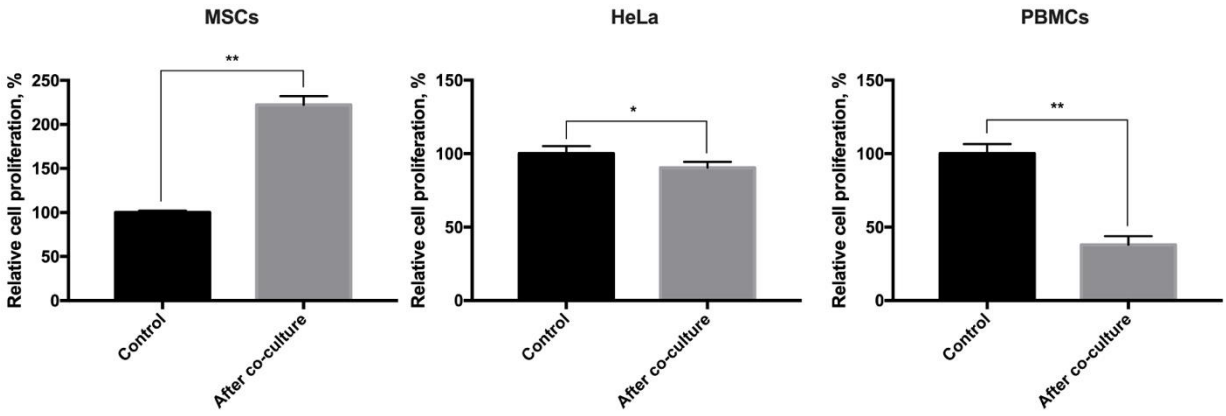

Figure 5. Analysis of changes in proliferation of the individual cell populations following culture as individual cell types (control) or after 3D-co-culture (HeLa, MSCs and PBMCs).
* – $p < 0.05$, ** – $p < 0.0001$.

Conclusions

The results of the study showed that HeLa adenocarcinoma cells, PBMCs and bone marrow derived MSCs in co-culture actively interact with each other not only by paracrine regulation, but also potentially by direct cell-to-cell contact, extra-cellular vesicular transport and fusion into hybrid cells. The co-culture of the three cell types together, resulted in modulation of the viability of MSCs and PBMCs, likely due to the intercellular interactions and exposure to various biologically active molecules and metabolites secreted within the co-culture environment. A possible antitumor effect of MSCs and PBMCs on adenocarcinoma cells is also described. However, it is currently unclear if this is due to the origin of the cell types within the co-culture e.g. MSCs derived from the bone marrow or the physical parameters of the culture system e.g. the high-density of cells. Therefore, further studies are needed to confirm the antitumor effects described. Together the data generated supports the active interaction of adenocarcinoma cells, MSCs and PBMCs when co-cultured, confirming the use of multi-cell type co-cultures is not only a relevant approach, but also essential for the development of potential *in vitro* preclinical screening systems for antitumor drugs.

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