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Research Article

Effect of the secretome of multipotent mesenchymal stromal cells induced by dexamethasone *in vitro* on the expression of phospho-NF-κB p65 and Ki-67 in mononuclear cells

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Abstract

Introduction: To investigate the influence of secretomes from native and dexamethasone-treated adipose-derived multipotent mesenchymal stromal cells (MMSC) on the proliferation of mononuclear cells (MNCs) and on their expression of phospho-NF-κB p65 *in vitro*.

Materials and Methods: MMSCs were isolated from the fat of 5 healthy donors. The cells were grown in culture up to passage four, then treated with dexamethasone for 3 hours, washed off the preparations, and incubated in a serum-free medium for 48 hours. Some of the cells were not treated with dexamethasone. Supernatants from cell cultures were concentrated by ultrafiltration, standardized by the content of galectin-1, sterilized, and added to MNCs from peripheral blood of 8 healthy donors. MNCs were isolated in a Ficoll density gradient according to a standard protocol. The expression of phospho-NF-κB p65 and Ki-67 in MNCs under the influence of MMSC secretomes in isotypic and negative controls was determined on a flow cytometer.

Results and Discussion: The expression of phospho-NF-kκB p65 and Ki-67 is decreased by the MMSC secretome. At the same time, a statistically significant decrease in phospho-NF-κB p65 by 36.2% ($p < 0.05$) is observed when using a secretome from native cells. Ki-67 expression is reduced by 42.3% ($p < 0.05$) under the influence of a secretome from dexamethasone-treated MMSCs.

Conclusion: The MMSC secretome, as well as MMSCs themselves, has an anti-inflammatory effect due to the effect on the expression of the active form of NF-κB and the proliferative activity of mononuclear cells. At the same time, pretreatment of cells with dexamethasone reduces the effect on phospho-NF-κB expression and increases the inhibitory effect on MNC proliferation.

Keywords

multipotent mesenchymal stromal cells, dexamethasone, mononuclear cells, inflammation, NF-κB, RELA, p65, Ki-67.

Introduction

NF-κB is a ubiquitous transcription factor that is capable to activate the transcription of a lot of genes encoding immunologically relevant proteins. Interfering with NF-κB activation may be useful in suppressing the toxic shock, graft versus host disease, acute inflammatory reactions, acute phase response, and radiation injury (Baeuerle and Henkel 1994, Oeckinghaus and Ghosh 2009). A NF-κB p65 subunit of the NF-κB transcriptional complex plays a crucial role in biological processes, such as inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis. It has been shown that nuclear translocation of p65 is increased in T-cells and monocytes of patients with progressive multiple sclerosis (Yan et al. 2018) and in MMSCs in the tumor environment (Shen et al. 2019). Also, NF-κB is an important molecule that controls the normal development and pathological destruction of cartilage in rheumatoid arthritis and osteoarthritis (Wang et al. 2017, Jimi et al. 2019). AN increase in activated NF-κB p65 and subsequent transactivation of effector molecules is an integral part of the pathogenesis of many chronic diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and neurodegenerative pathologies (Giridharan and Srinivasan 2018, Sayegh et al. 2019). It was shown that adipose-derived MMSCs have an immunosuppressive effect on T-cells by inhibiting phosphorylation of NF-κB (Zhou et al. 2018). Inhibition of NF-κB activation may provide a pharmacological basis for treatment of inflammatory processes. Intracellular marker Ki-67 was chosen to study the effect of the MMSC secretome on the proliferative activity of MNCs, because Ki-67 is expressed only in proliferating cells and plays a role in early stages of rRNA synthesis. (Bullwinkel et al. 2006).

Aim of the Study

The study was aimed to research the effect of a secretome from native and dexamethasone-treated adipose-derived multipotent mesenchymal stromal cells (MMSCs) on the proliferation of mononuclear cells (MNCs) and on their expression of phospho-NF-κB p65 in an *in vitro* experiment.

Materials and methods

Isolation and culturing of cells

MMSCs were isolated from human fat from 5 donors using collagenase type 2. To confirm the MMSCs phenotype, cells were stained with antibodies to CD105 (cat. No. 562408, BD, USA), CD90 (cat.No. 562556, BD, USA), CD73 (cat.No. 561260, BD, USA), CD31 (cat. No. 563651, BD, USA), CD45 (cat.No. A07783, Beckman Coulter, USA), CD34 (cat.No. IM1870, Beckman Coulter, USA); mouse IgG1 conjugated to BV 421 (cat. No. 562438, BD, USA) were used for isotype control. MMSCs were grown up to 4 passages in α-MEM medium (cat.No. P0440, Biowest, USA) with 10% fetal bovine serum (cat.No. S140B-500, Biowest, USA) under the standard conditions (humid atmosphere 5% CO_2, 37 °C). MNCs from peripheral blood of 8 healthy volunteers were isolated by Ficoll density gradient according to the standard protocol.

Obtaining the MMSC secretome

MMSCs were seeded in plates and treated with 10 μmol/ml dexamethasone (CSPC Ouyi Pharmaceutical, China) for 3 hours. Then the cells were washed from dexamethasone, incubated for 48 hours under standard conditions in serum-free DMEM/F12 medium (cat.No. 1.3.7.1., Biolot, Russia). Some of the MMSCs were not treated with dexamethasone; they were incubated for 48 hours under standard conditions in serum-free DMEM/F12 medium. Before adding to the plate, supernatants from MMSCs cultures were concentrated using Vivaspin20 tubes (Sartorius, Germany), MWCO = 3 kDa, standardized by the galectin-1 protein content (6 pg/ml) (Gieseke et al. 2010). The concentration of galectin-1 was determined using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Clone Clouds. Corp., USA). The optical density of the content of EIA plates is read with an ELISA plate reader Multiskan FC (Thermo Scientific, Finland), λ = 450 nm. The concentrated and standardized secretome was sterilized by filtration and added to the plates with MNCs.

Design of the experiment

MNCs were seeded into a 96-well plate, and 20 μl of the previously concentrated MMSC secretomes were added. Phytohemagglutinin (PanEco, Russia) 10 μg/ml, lipopolysaccharide (LPS) (Merk, USA) 100 ng/ml were added to the plate to stimulate MNCs. After 48 hours, the plate was centrifuged, and the cell suspension was sampled for studying the amount of phospho-NF-kB p65.

Flow cytometry

The cells were stained with the supravital dye 7-aminoactinomycin D (7-AAD) (cat.No. A07704, Beckman Coulter, USA). Then the cells were fixed using Fix&Perm Medium A (Life Technologies, Netherlands). After that, the cells were stained with primary rabbit antibodies to Phospho-NFκB p65 (Ser536) Monoclonal Antibody (T.849.2) (cat.No. MA5-15160, Thermo Scientific, Germany), then with secondary antibodies Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, APC-marked (cat.No. A-10931, Thermo Scientific, Germany). The cells were analyzed on a flow cytometer in red laser (λ = 633 nm). A comparative analysis of the amount of active intracellular phospho-NF-κB p65 was carried out according to the intensity of APC expression by cells that do not express 7-AAD. LPS-stimulated

MNCs, stained only with Goat anti-Rabbit IgG (H+L) Cross-adsorbed secondary antibody APC- marked in a permeabilizing solution, were used as a control for non-specific binding of secondary antibodies. To study the proliferation marker Ki-67, another 96-well plate with MNCs and secretomes was incubated for 4 days under standard conditions. The cells were then permeabilized with buffer (cat.No. 5115, Merck-Millipore, USA), stained with Ki-67 monoclonal antibodies conjugated to BrilliantViolet 421 (cat.No. 562899, BD, USA) and analyzed in violet laser (λ = 405 nm). A FACSCanto II flow cytometer with BD FACSDiva Version 8.0 software (BD, USA) was used for this experiment.

Statistical processing

SPSS Statistics 17.0 (IBM, USA) was used for analyses. The data obtained were checked for normal distribution using the Shapiro–Wilk test. Descriptive statistics are represented by median, lower and upper quartiles – Me [q_{25}-q_{75}]. Statistically significant differences were calculated using the Mann–Whitney U test and the Kruskal–Wallis test; the significance level was taken as $p < 0.05$.

Results and discussion

The secretome from untreated MMSC significantly ($p < 0.05$) reduces the expression of phospho-NF-κB p65 in MNCs (Fig. 1). Expression of an activated form of the protein when incubating MNCs with the MMSC secretome pretreated with dexamethasone and when adding dexamethasone to stimulated MNCs decreases statistically insignificantly ($p > 0.05$) (Fig. 2). A pairwise analysis did not reveal any significant differences between treatments with dexamethasone or with one of the MMSC secretomes ($p > 0.05$).

The amount of Ki-67 was investigated to analyze the proliferative activity of lymphocytes (Figs 3, 4). Figure 3 shows that Ki-67 was significantly lower ($p < 0.05$) when exposed with a secretome of dexamethasone-treated MMSCs than of those without treatment, which indicates the suppression of the proliferation of immune cells by the corresponding secretome.

It is well known that dexamethasone inhibits NF-κB activation in various cell types (Chang et al. 2018) and reduces expression of CD200 on the surface of MMSCs (Pontikoglou et al. 2016). Exposure of breast cancer cells MCF-7 to the synthetic glucocorticosteroid dexamethasone significantly increased miR-708 expression through transactivation of the glucocorticoid receptor alpha (GRα), which led to inhibition of cell proliferation, suppression of the activity of NF-κB and its subsequent target genes, including cyclooxygenase-2, cMYC, cyclin D1, matrix metalloproteinase (MMP)-2, MMP-9, CD24, CD44 (Senthil Kumar et al. 2019). A similar mechanism is shown in pancreatic cancer cells (Yao et al. 2020). Dendritic cells created *in vitro* made

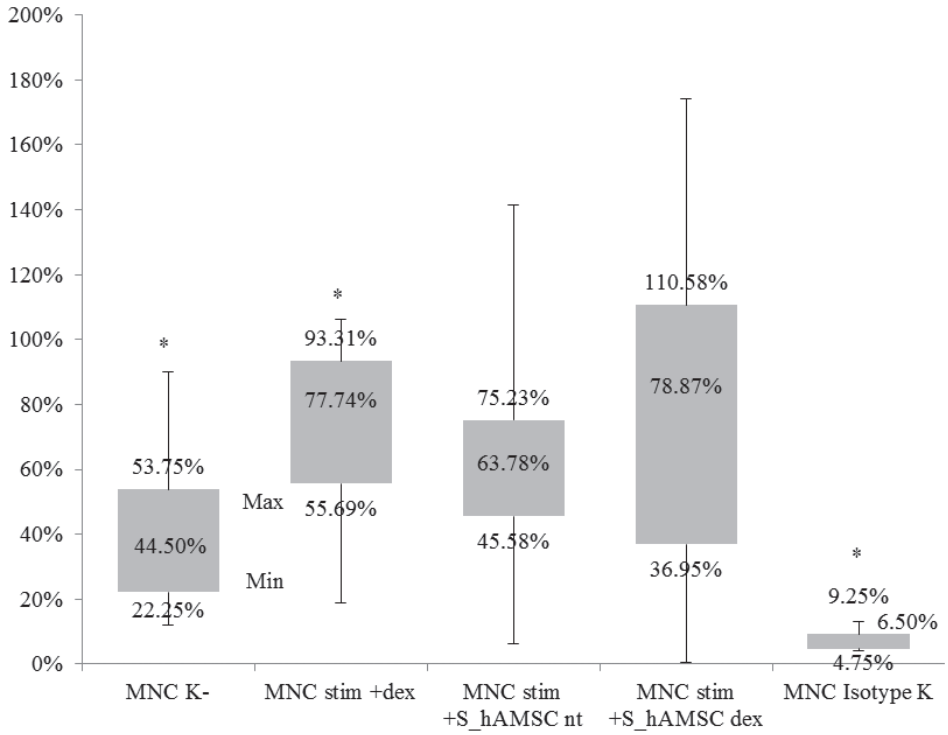

Figure 1. Effect of MMSC Secretome on Phospho-NF-κB Expression in MNCs. **Note:** Error limits: maximum and minimum values (excluding outliers). MNC K- – unstimulated MNCs; MNCstim+dex – stimulated MNCs + dexamethasone; MNCstim+S_hAMSC nt – stimulated MNCs+secretome from native MMSCs; MNCstim+S_hAMSCdex – stimulated MNCs+secretome from dexamethasone-treated MMSCs; MNC Isotype K – isotype control. * – statistical significance of the difference in comparison with stimulated MNCs, taken as 100% ($p < 0.05$).

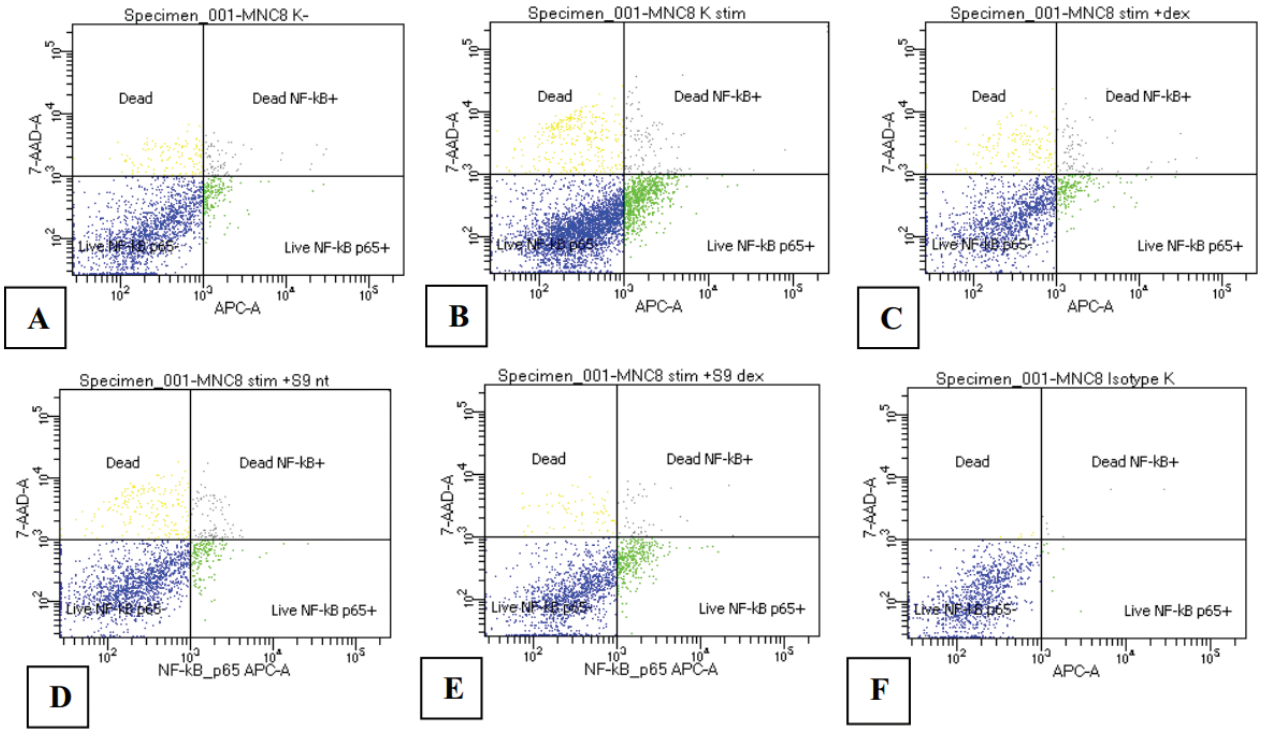

Figure 2. Effect of MMSC secretome on phospho-NF-κB expression in MNCs. Flow cytometry results. **A** – unstimulated MNCs; **B** – stimulated MNCs; **C** – stimulated MNCs + dexamethasone; **D** – stimulated MNCs + secretome from native MMSCs; **E** – stimulated MNCs + MMSC secretome treated with dexamethasone; **F** – isotype control.

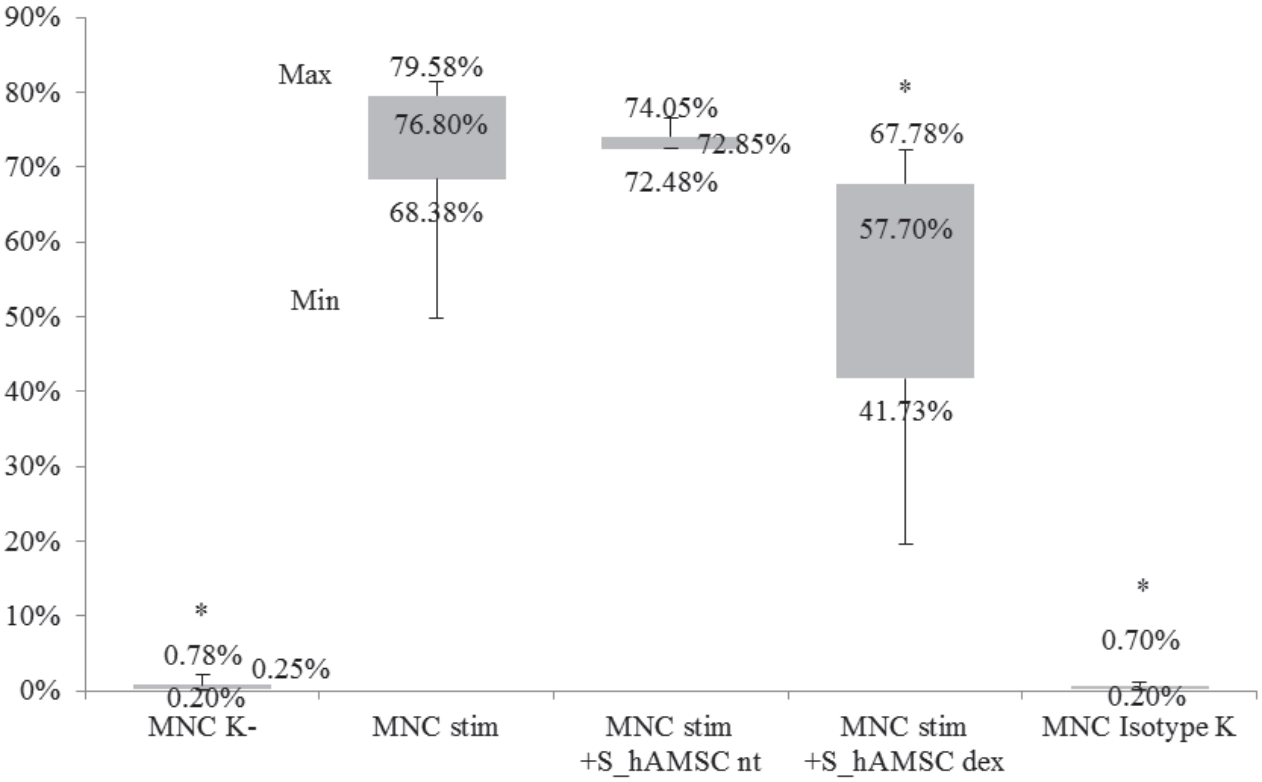

Figure 3. Influence of MMSC secretome on Ki-67 expression in MNCs. **Note:** Error limits: maximum and minimum values (excluding outliers). MNC K- – unstimulated MNCs; MNCstim – stimulated MNCs; MNCstim + S_hAMSC nt – stimulated MNCs + secretome from native MMSCs; MNCstim + S_hAMSCdex – stimulated MNCs + secretome from dexamethasone-treated MMSCs; MNC Isotype K – isotype control. * – statistical significance of the difference in stimulation of MNCs and secretomes from native MMSCs ($p < 0.05$).

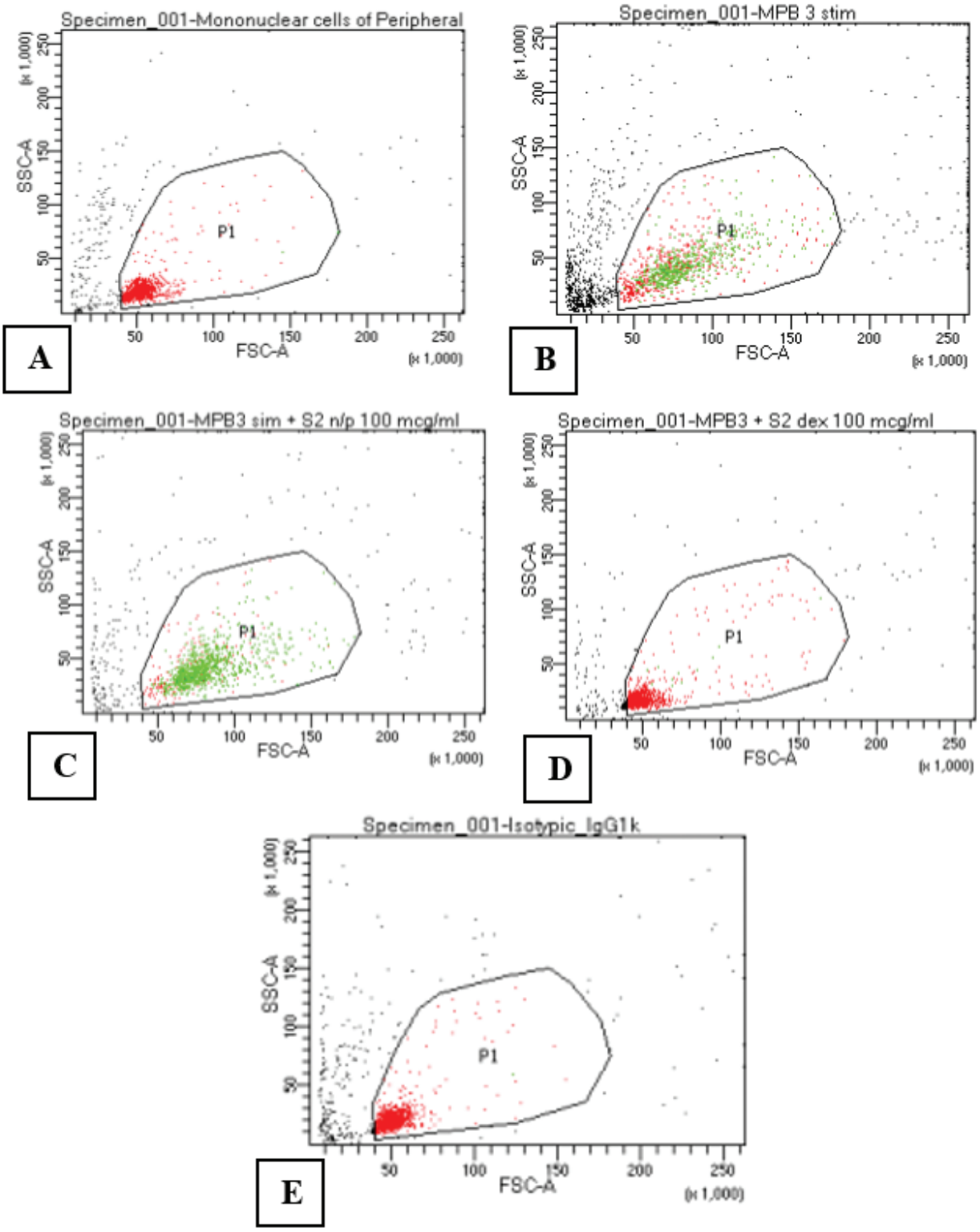

Figure 4. Influence of MMSC secretome on Ki-67 expression in MNCs. Flow cytometry results. **Note:** Ki-67 positive cells are marked in green. The entire population of MNCs (P1) is highlighted in red. **A** – unstimulated MNCs; **B** – stimulated MNCs; **C** – stimulated MNCs + secretome from native MMSCs; **D** – stimulated MNCs + secretome from dexamethasone-treated MMSCs; **E** – isotype control.

with addition of dexamethasone have a reduced ability to stimulate T-cell proliferation, which is mediated through the STAT-3 and NF-κB signaling pathways (Kotthoff et al. 2017). Inhibition of NF-κB using dexamethasone regulates the intracellular domain of the Notch signaling pathway, which reduces macrophage differentiation, and it is a potential target for intervention, for example, in atherosclerosis (Binesh et al. 2019). In another study,

the authors showed a decrease in NF-κB expression and an increase in IL-10 expression when dexamethasone was previously injected to mice before stimulating lung inflammation with LPS (Al-Harbi et al. 2016). Dexamethasone was chosen as an inductor of MMSCs, based on the above facts. Our hypothesis regarding the enhancement of the MMSC secretome action by dexamethasone was not confirmed (Fig. 1). However, when only dexamethasone was added to MNCs, a tendency towards a decrease in the expression of the nuclear factor was observed.

A statistically significant decrease in the expression of phospho-NF-κB p65 was observed when MNCs were incubated with the secretome from native MMSCs ($p < 0.05$), rather than from cells treated with dexamethasone. This is consistent with literature data that dexamethasone promotes nuclear localization of NF-κB p65, p50 and p52, and is ineffective in inhibiting an inflammatory response in cultured tenocytes stimulated by TNF-α (Ji et al. 2019).

The revealed decrease in Ki-67 expression suggests that the dexamethasone-treated MMSC secretome can contribute to suppression of proliferation and synthetic activity of MNCs *in vitro*. Since dexamethasone is a drug with an antiproliferative effect on cells, its direct effect on MNCs is natural. The indirect effect of dexamethasone on MNCs through the MMSC secretome is promising. However, protein mediators of this effect are not yet known. Possibly, antinoceptive protein lipocortin-1 (annexin A1), acting through suppression of the production of proinflammatory cytokines and other mechanisms, is involved in such an effect (de Almeida et al. 2019). Recently the role of annexin A1 secreted by MMSCs on regenerative processes in the liver and pancreas has been demonstrated (Zagoura et al. 2019). In addition, it was shown that the annexin-1 mimetic peptide Ac2-26 inhibits the synthesis of inflammatory mediators (TNF-α, IL-1β, MCP-1, MIP-1α) in astrocyte culture, and reduces pain hypersensitivity in a rat pain model (Luo et al. 2020).

Conclusion

A significant decrease in the expression of phospho-NF-κB p65 *in vitro* in MNCs under the action of the native MMSC secretome in contrast to the dexamethasone-treated MMSC secretome, was shown. Based on the analysis of the expression of phospho-NF-κB p65, it can be concluded that the MMSC secretome suppresses one of the main intracellular pathways of the immune response. However, the anti-inflammatory activity of the secretome of pharmacologically stimulated MMSCs is mediated in the cell not via this signaling pathway. Moreover, treatment with dexamethasone reduces the inhibitory effect of the MMSC secretome on the expression of phospho-NF-κB p65. A significant decrease in the expression of Ki-67 was revealed when the secretome of dexamethasone-treated MMSCs was added to MNCs, which indicates a greater suppression of MNC proliferation by this secretome compared to the secretome of native MMSCs.

Thus, the secretome of both native and dexamethasone-induced MMSCs has anti-inflammatory effects, which are realized both through suppression of lymphocyte proliferation, a decrease in the activation of the pro-inflammatory factor NF-κB in MNCs, and through other, not yet specified, mechanisms.

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Conflict of interests

The authors of this paper report no conflicts of interest.

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