

SECRETED FACTORS FROM UMBILICAL
CORD-MESENCHYMAL STEM CELLS AFFECT CD86
EXPRESSION ON B LYMPHOCYTES AND INCREASE CCL5
CHEMOKINE SECRETION BY HEALTHY DONORS' PBMC

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Abstract

A growing number of autoimmune diseases, allergies, and atopic conditions in recent years are calling for the development of innovative therapies, such as those using stem cells with immunoregulatory activity. Because of the few and often conflicting results in the scientific literature, it is still uncertain whether mesenchymal stem cells (MSC) could be a useful therapeutic strategy for targeting B lymphocytes and be helpful in treating autoimmune and allergy patients or those with a variety of viral infections. In connection with the aforementioned, our team investigated the influence of umbilical cord MSC-secreted factors on healthy donors' B-cell CD86 expression and CCL5 secretion by peripheral blood mononuclear cells (PBMC) in vitro. When healthy donors' PBMC were cultivated with conditioned medium derived from umbilical cord MSC, CD86 density on the B-cell membrane statistically decreased, but the percentage of B cells expressing this co-stimulatory molecule was slightly but significantly increased. We also found significantly increased secretion of the chemokine CCL5 by PBMC under the influence of the MSC secretome.

Key words: mesenchymal stem cells, B lymphocytes, CD86, CCL5

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1. Introduction. In the rapidly evolving field of modern biology and medicine, where information is constantly increasing, stem cells interactions and effects on various cell types are an integral part of human body knowledge as a unified biological system. A wide variety of stem cells exist, depending on their origin, the period of individual development in which they are isolated, and their potency and efficacy [1]. However, the subject of this short scientific report is mesenchymal stem cells (MSC), especially their paracrine effects. As is well known, MSC can be isolated from a variety of sources, including bone marrow (BM-MSC), adipose tissue (AT-MSC), umbilical cord (UC-MSC), and others. And yet they share common features such as fibroblast-like morphology, positive expression of markers such as CD90, CD105, CD73, lack of expression of hematopoietic markers; participation in processes such as angiogenesis, tissue regeneration, immunomodulation, as well as immunosuppression [2]. MSC have the ability to communicate directly with other cell types or exert their effects through the soluble factors they release, many of which have immunosuppressive function and important biological significance. In this regard, it should be emphasized that MSC are a source of many cytokines, chemokines, growth factors, and enzymes, that have already been identified in the cell culture environment and have been the subject of research by our team too [3]. Attention has also been paid to the effects of MSC on B cells over the last few decades, but the information available so far is quite inconsistent. Some researchers have stated that B-cell activation, proliferation, and differentiation are stimulated under the action of MSC [4], while others have reported the opposite results [5]. This raises many research questions awaiting answers, and the idea of using MSC as a therapeutic tool for B lymphocytes, the cells at the heart of autoimmunity, is one of them. In relation to the foregoing, our team aimed to establish the immunoregulatory effects of soluble factors released from umbilical cord MSC (UC-MSC) on the expression of key molecule CD86 on the membrane of B-lymphocytes from healthy individuals' peripheral blood mononuclear cells (PBMC). The CCL 5 chemokine detection in supernatants of healthy donors' PBMC, cultivated with and without UC-MSC-conditioned medium (UC-MSCcm), is also a part of the objective of our short research study, since this chemokine was reported to be released by MSC [3]. Furthermore, CCL5 is known as a chemoattractant as well as having important immunoregulatory functions, such as non-antigen specific activation of lymphocytes [6].

Materials and methods. Cell isolation and culture. UC-MSC. Human umbilical cord tissue samples from five healthy donors were provided by Tissue Bank BulGen after obtaining signed written informed consent, approved by the Ethics Committee of Ob/Gyn Hospital Dr Shterev, Sofia, Bulgaria. Tissue Bank BulGen confirms and characterizes the isolated MSC according to their clonogenic activity, trilinear differentiation and by the positive expression of the already described MSC markers and lack of hematopoietic markers expression. A small piece of the umbilical cord (2.5 cm) was placed in a sterile Petri dish, and the

blood vessels were carefully removed. The tissue was then chopped with sterile surgical scissors into small pieces, placed in a tube with 0.25% Collagenase I (Genaxxon, Germany), 4 mg/ml hyaluronidase (Genaxxon, Germany) and 1% Penicillin/Streptomycin/Amphotericin B Mix – Pen/Strep/Amph (PAN Biotech, Germany) and incubated at 37 °C on a shaker for 3 h in a humidified incubator, containing 5% CO₂. After the incubation period, the enzymatically degraded tissue is mixed with saline, filtered through a 70 µm cell strainer (Greiner bio-one, Austria) and then centrifuged at 876 × g for 10 min. The resulting cell pellet was resuspended in DMEM/F12 (PAN Biotech, Germany), supplemented with 10% fetal bovine serum – FBS (PAN Biotech, Germany) and 1% Pen/Strep/Amph, and seeded in 10 × 25 cm² cell culture flasks at a density of 1 × 10⁴ cells/flask. The cells were kept in the incubator, and the cell culture medium was changed every 48 h, until the cells reached 80–90% confluence in each 25 cm² tissue culture flask. Old medium was then aspirated and replaced, with fresh one, cells were cultured for another 48 h, and the collected medium entitled “UC-MSc-conditioned medium” was stored in aliquots at –80 °C.

PBMC. The study included ten healthy donors, five women and five men, with an average age of 33 years. Following the ethical recommendations of the Declaration of Helsinki, voluntarily signed informed consent forms were obtained from all participants before the study. Peripheral blood, collected from participants (9 ml), was diluted 1:1 with phosphate buffered saline 10X PBS (BioSystems; Barcelona, Spain) and PBMC were isolated by density gradient centrifugation (400 × g for 10 min) with Ficoll Paque™ PLUS (GE Healthcare BioSciences AB; Uppsala, Sweden). PBMC isolated from each healthy donor were then seeded in 6-well plates at a concentration of 1 × 10⁶ cells per well, containing UC-MSc. Another well with 1 × 10⁶ PBMC was kept in control cell culturing medium – DMEM/F12, containing 10% FBS and 1% Pen/Strep/Amph. Then PBMC were further cultured for 72 h under standard conditions (37 °C, 5% CO₂, and 95% humidity). Afterwards, cells were resuspended, centrifuged at 350 × g for 10 min, separated from the medium, and subsequently analyzed by flow cytometry. The supernatants were collected and stored as aliquotes at –80 °C for further analysis.

Flow cytometry. PBMC at a concentration of 1 × 10⁶ cells were washed twice in BD cell wash (Beckton Dickinson, Pharmingen, USA) and B lymphocytes were analyzed by using FITC-conjugated anti-human CD19 antibody (BioLegend, San Diego, CA) and APC-conjugated anti-human CD86 antibody (BD Biosciences, USA). The cells were further processed according to the manufacturer’s instructions, fixed with Cell Fix (BD, USA) and analyzed on FACS Calibur flow cytometer using CellQuest software (BD, USA).

Enzyme-linked immunosorbent assay (ELISA). Previously stored at –80 °C supernatants of UC-MSc, PBMC + UC-MSc and PBMC + Control medium were tested by immunoenzyme assay for the presence of chemokine of interest, namely *CCL 5* (Abnova Ltd; Cambridge, United Kingdom), which is also

known as *Regulated on Activation, Normal T Expressed and Secreted (RANTES)*. ELISA was performed according to the manufacturer's recommendations.

Statistical analysis. Graph design and statistical analysis were performed using data analysis software GraphPad Prism 8 and the Statistical Package for the Social Sciences (SPSS) version 21. Due to the relatively small number of samples, the hypotheses were tested using the non-parametric Wilcoxon signed-rank test. Data are reported as mean $\pm$ SD and the level of significance was set up for $p \leq 0.05$.

Results. PBMC cultivated in UC-MSCcm showed visible phenotypic alterations compared to controls after 72 h of incubation, forming large clusters of live cells communicating with one another (Fig. 1).

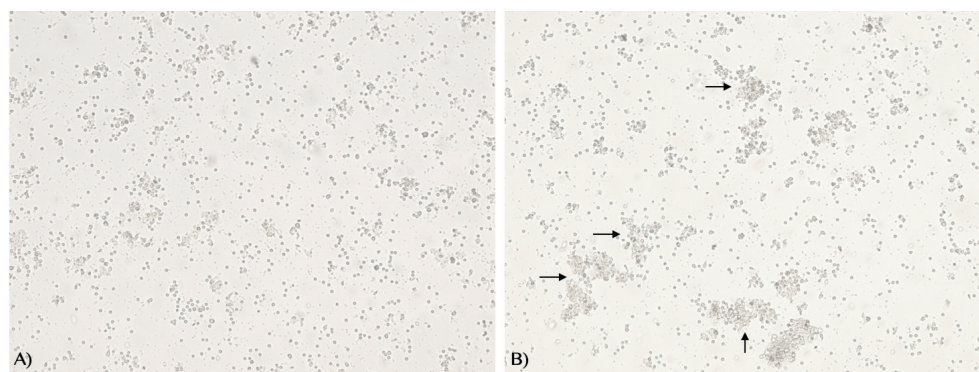


Fig. 1. Representative microphotographs (100 $\times$) of healthy donors' PBMC after 72 h of cultivation. A) PBMC cultured in Control Medium, and B) PBMC cultured in UC-MSCcm with more visualised cell cluster formation (black arrows)

Flow cytometry was used to assess the expression of CD86 on B-lymphocytes' membranes (Fig. 2A). We observed that under the influence of the factors secreted by UC-MSC, the mean fluorescence intensity (MFI) of CD86 was significantly reduced (145.40 ± 30.32) compared to the level of expression in B lymphocytes from PBMC cultured in a control medium (163.00 ± 28.59), ($p \leq 0.05$). However, the percentage of B lymphocytes from the PBMC cultured in UC-MSCcm was moderately increased (3.85 ± 3.40) than the control group of B lymphocytes (2.65 ± 2.20), with statistical reliability between these two groups ($p \leq 0.05$) (Fig. 2B).

After performing the enzyme-linked immunosorbent assay, our results showed that when PBMC are cultivated in UC-MSCcm, there are significantly higher ($6.24 \text{ ng/ml} \pm 9.02$) levels of CCL5/RANTES compared to the control group of PBMC ($1.34 \text{ ng/ml} \pm 0.66$), ($p \leq 0.05$), and the UC-MSCcm group alone ($0.73 \text{ ng/ml} \pm 0.11$). The results for CCL 5 levels are summarized and graphically presented in Fig. 3.

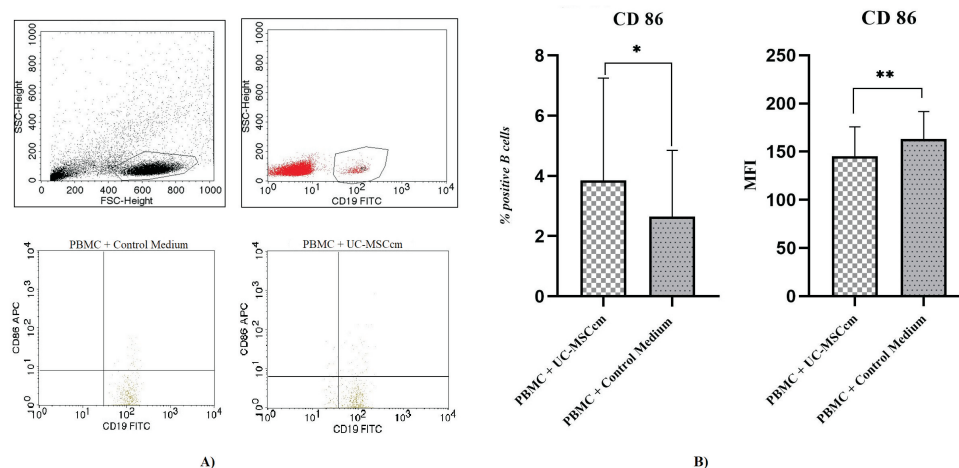


Fig. 2. A) Representative flow cytometry dot plot analysis of the percentage of CD19⁺ B cells expressing CD86 molecule in the pool of healthy donors' PBMC cultured in Control Medium and in UC-MSCCm. B) Graphical representation of the percentage of CD19⁺ B cells positive for CD86 expression (* $p \leq 0.05$) and the mean fluorescence intensity (MFI) of the CD86 molecule expressed on B-lymphocytes' membrane (** $p \leq 0.005$)

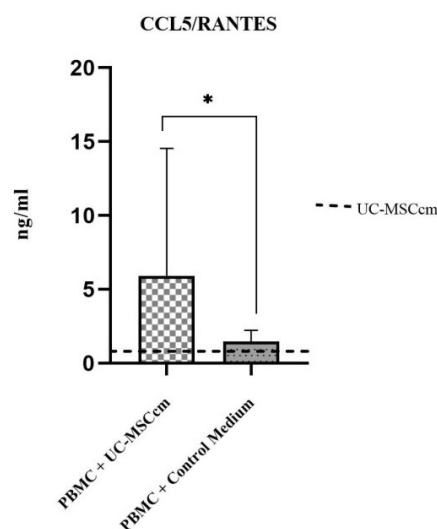


Fig. 3. Changes in the chemokine levels of CCL5 in PBMC samples obtained from healthy donors, cultured in UC-MSCCm (PBMC+UC-MSCCm) and PBMC from the same healthy donors, cultured in Control Medium (* $p \leq 0.05$). The levels of CCL5 in UC-MSCCm are represented by a black dash line on the graph

Discussion. It is well known that MSC can have ambivalent anti-inflammatory or pro-inflammatory properties due to a variety of conditions, including the presence of an inflammatory environment and communication with other cell types. In addition, MSC can spontaneously secrete biologically active substances, cytokines, growth factors, and chemokines or be stimulated by other cytokines or growth factors [3]. This is where we state that cultured umbilical cord stem cells are undifferentiated and “naive” for the purposes of our experiments. They are cultured under standard cell culture conditions without being pre-stimulated to exert a particular effect. The same applies to PBMC isolated from healthy donors. In the context of our research on the expression profile of the cell surface marker CD86, we should briefly mention that this molecule is also known as B7-2, a part of the B7 complex, along with CD80 (B7-1). The B7 complex is expressed by antigen-presenting cells such as B lymphocytes, macrophages, and dendritic cells, and it delivers the co-stimulatory signals required for T-cell activation and survival by binding to its T-cell membrane partner CD28 [7]. The protein CTLA4 (CD152), which is a negative regulator of T-cell activation [8] and contributes to the maintenance of B cell resistance [9], is another equally essential ligand for the B7 complex. As a result, CD86 can send both activating and inhibitory signals. However, MSC have not been shown to influence CD86 expression in B lymphocytes [5], and we found a slightly increased percentage of B cells that express B7-2 co-stimulatory molecule in the pool of PBMC cultured in UC-MS Cem. It was interesting that at the same time, the mean density of expression of this molecule on the B-cell membrane was significantly lower than that of the control group B cells. As a molecule with very important function for antigen presenting cells (APC), it was reported that CD86 is expressed at lower levels on resting B cells [10] and stimulation via CD86 of B cells influences their proliferation, secretion of immunoglobulins, and production of molecules that affect their programmed cell death [11]. On the other hand, regulatory T cells (Tregs), which constitutively express the checkpoint inhibitor CTLA4 and have the ability to exert immunosuppressive activity, are selectively dependent on CD86 for their overall homeostasis, survival, proliferation, and activation status [12]. With that in mind, we need to share some fairly impressive data on the role of CTLA4 in Tregs suppression. CTLA4 expressed on Tregs promoted the binding of Tregs to APC, resulting in CTLA4-dependent trogocytosis of CD80/CD86 and the concomitant transfer of membrane fragments from APC to the surface of Treg cells [13]. Some authors suggest that this is the reason why certain CD4⁺ T cells have co-stimulatory molecules like CD80/CD86 found on their membrane [14]. Since the expression density of the CD86 molecule is reduced in our experiments, it can be speculated at this time that it may be indirectly induced by the Treg-induced regulatory mechanism “trogocytosis” that leads to the interruption of CD28 co-stimulation. Along with this, indirect evidence of the available communication between PBMC is their significant clustering under the influence of factors se-

creted by MSC in our experiments. And we cannot fail to mention that one of the most important immunomodulatory functions of MSC is the ability to convert conventional T cells into Tregs [15,16]. Another viewpoint is that MSC factors released into the medium can directly induce this reduction in the expression on B cells. The other object of our research, RANTES, is known for its role in tumour progression and, as a chemokine, has the ability to attract immunosuppressive cells to inflammatory sites, such as regulatory T cells, for example [17]. According to one study, CCL5 can increase B-cell proliferation and antibody production [18]. Interestingly, B lymphocytes can also produce CCL5, which in an autocrine way through binding to CCR5, could improve their proliferation, differentiation, and subsequent secretion of Ig M [19]. Unfortunately, at this time, there is no data on the effect of this chemokine on the expression of co-stimulatory molecules in B lymphocytes, and moreover, we do not know the origin of CCL5 secreted in the media, whether it is autocrine, paracrine, or both. CCL5 is a unique chemokine, not only a chemoattractant but, depending on the concentration and its monomeric or dimeric form, RANTES can activate T lymphocytes and have an antiviral effect [20]. Therefore, we can hypothesize that significantly higher levels of this chemokine in supernatants of PBMC cultivated with UC-MSCcm, compared to those secreted by UC-MSC alone or PBMC in the control group, are related to one of MSC's natural functions in the body, namely the restoration of conditions caused by infection or trauma.

Conclusion. Our results indicate that secreted factors from UC-MSC could affect the CD86 expression profile on B cells and stimulate PBMC to release chemokine CCL5. However, the disadvantage of our study is the small number of participants, and also, due to the fact that CD86 and CCL5 do not have an unambiguous effect, further studies in the field are needed.

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