Tumor-derived membrane vesicles from the IL-2 overexpression melanoma cells affect on the expression of surface markers of human peripheral blood mononuclear cells in vitro

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Research Article

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Abstract

Nowadays, immunotherapy, in particular immunotherapeutic vaccines, is a promising approach in the treatment of cancer which has already demonstrated its effectiveness. Extracellular vesicles (EVs), which are capable of delivering biologically active agents to the cells, can be a promising candidate for such vaccines. M-14 human melanoma cells were transduced with lentivirus encoding interleukin (IL)-2. Membrane vesicles from M-14 cells expressing IL-2 were isolated using cytochalasin B. The interaction of membrane vesicles with human peripheral blood mononuclear cells has been analysed. Activation of T-cells was shown, as well as a decrease in the number of NK cells, after cultivation with tumor-derived vesicles. However, no cytotoxic activity of T-cells after cultivation with tumor-derived vesicles was observed, which can be explained by their immunosuppressive properties. On the other hand, such vesicles can be a promising source of tumor-specific antigens for dendritic vaccines. Therefore, further studies are required in view of the possible use of tumor-derived vesicles as a target antigen for dendritic cells.

1. Introduction

Intercellular communication plays a key role in the development of the tumor. It is known that tumor cells actively interact with the cells of surrounding tissues, involving them in the process of tumor growth, escape from immune surveillance, and spread to distant parts of the body. In particular, this occurs with the help of extracellular vesicles (EVs). EVs are lipid bilayer membrane-enclosed vesicles secreted by various cell types both *in vivo* and *in vitro* [1]. In addition of origin and size, several types of EVs are distinguished, which include: exosomes (40–100 nm), microvesicles (MVs) (100–1000 nm) and apoptotic bodies (500–2000 nm) [2]. Regardless of type, EVs carry parental cell-specific antigens, proteins, and nucleic acids [3]. It has been shown that tumor-derived EVs are able to modulate the functions of healthy cells and influence the outcome of antitumor therapy. Thus, it was shown that MVs isolated from tumors contributed to the activation of phosphoinositide 3-kinase/Akt pathway in normal endothelial cells, which led to an increase in their proangiogenic properties [4]. Similar results were obtained with esophageal squamous cell carcinoma-derived miR-181b-5p-enriched EVs that dramatically induced angiogenesis by targeting phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and PH domain and leucine rich repeat protein phosphatase 2 (PHLPP2) [5]. In addition, it was shown that human and mouse melanoma cells secreted a large number of EVs during treatment with temozolomide and cisplatin, which contributed to the polarization of macrophages in the M2 phenotype, as well as an increase in the expression of genes responsible for DNA repair in tumor cells [6]. The interaction of tumor-derived EVs and the immune system is of great interest. Although tumor-derived EVs are predominantly immunosuppressive, it has been shown that tumor-derived EVs can nevertheless stimulate an antitumor response of $CD8^+$ cytotoxic T-lymphocytes (CTLs) against autologous tumor cells [7]. Due to the fact that EVs can fuse with the target cell [8], they have the potential to be used as therapeutic agents to modulate the immune system. Natural EVs are produced by body cells in low amounts. In order to increase the yield of tumor-derived EVs, cells can be treated with cytochalasin-B, a

substance that prevents the polymerization of actin filaments, thereby causing disorganization of the cytoskeleton. Thus, it will allow obtaining cytochalasin B-induced membrane vesicles (CIMVs) in increased amounts. Also, CIMVs can be modified to express immunostimulatory cytokines in order to increase immune response. Despite a number of difficulties, the combination of biological properties allows us to consider CIMVs as promising antitumor agents. Therefore, this study explores the potential of *in vitro* activation of human immune cells using CIMVs derived from M-14 tumor cells overexpressing the immunomodulatory cytokine interleukin (IL)-2. The choice of IL-2 as an immunostimulatory cytokine is due to its ability to stimulate the cytotoxic activity of $CD8^+$ T-cells and NK cells [9], [10].

2. Materials And Methods

2.1. Cells and culture conditions

Mononuclear cells were isolated from human peripheral blood (PBMCs) of a healthy donor in a Ficoll density gradient (1.077 g/cm^3, PanEco, Russia) in accordance with approved ethical standards and current legislation (the protocol was approved by the Committee on Biomedical Ethics of Kazan Federal University (No. 3, 03/23/2017). An informed consent was obtained from the healthy donor. M-14 melanoma cell line was received from John Wayne Cancer Institute Specimen Repository (JWCI). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator in RPMI 1640 medium (PanEco, Russia), supplemented with 10% fetal bovine serum (Invitrogen, USA), 2mM L-glutamine (PanEco, Russia), and 5000 µg/ml mixed penicillin-streptomycin (Rosmedbio, Russia). All cell lines were tested for mycoplasma routinely.

2.2. Lentivirus production

Lentiviral vector plasmid encoding human *IL-2* gene (pLX304-IL-2) was obtained from the Harvard Plasmid Database (#HsCD00421565-4). The production of lentiviruses encoding *IL-2* genes was carried out by calcium phosphate co-transfection of a packaging HEK293T cell line with three plasmids: an expression plasmid (pLX304-IL2); packaging plasmid (psPAX2, AddGene #12260); and enveloping plasmid (pCMV-VSV-G, AddGene #8454). The concentration of lentiviral particles was carried out by ultracentrifugation for 2 h at 26,000 rpm at 4°C.

2.3. Genetic Modification and Selection

M-14 cell line was transduced by lentivirus encoded *IL-2* gene (vector plasmid pLX304-IL-2 #HSCD 00421565-4, Harvard University, USA) in serum-free medium using 10 µg/ml protamine sulfate (#P4020, Sigma, USA). Antibiotic selection was performed with 7.5 µg/ml Blasticidin S HCl (#R21001, Gibco, USA) for 7 days. Cell culture was tested and resulted negative for *Mycoplasma.*

2.4. Isolation of cytochalasin B-induced membrane vesicles

Cells were washed from medium with Dulbecco's Phosphate Buffer Saline (PanEco, Russia) and supplemented with serum-free medium. After counting $2 \cdot 10^6$ cells were suspended with 10 ml serum-

free medium containing Cytochalasin B from *Drechslera dematioidea* (#C6762-5mg, Sigma-Aldrich, USA) in concentration 10 µg/ml. Cells were incubated for 30 min in a humidified incubator at 37°C and 5% CO_2 with gentle shaking every 10 minutes. After incubation cells were mixed by vortex during 1 min and then centrifuged for 10 min at 100 g (Biosan Laboratory centrifuge LMC-3000), supernatant was collected in the new tube and centrifuged for 10 min at 350 g, supernatant was collected into the clean tube and filtered through polyvinylidene difluoride (PVDF) membrane filter (GVS filter technology, UK) with pore size 1µm. After filtration supernatant was centrifuged for 30 min at 8064 g. The precipitate containing CIMVs was collected. The determination of protein concentration was carried out using Pierce BCA Protein Assay Kit (#23225, Thermo Scientific™, USA) according to manufacturer's manual.

2.5. Western blot analysis

Cell samples were lysed by RIPA buffer (Thermo Fisher Scientific, USA) with addition of protease and phosphatase inhibitor cocktail (#78444, Thermo Fisher Scientific, USA), after which the protein concentration was quantified. Equal amounts of protein were loaded and separated on 12% SDS-PAGE gels and then transferred onto PVDF membranes. The membranes were incubated with primary antibodies at 4°C overnight and then washed in PBS-T and incubated with secondary antibody at room temperature for 2 h. Then membranes were washed in PBS-T and targeted band of proteins were visualized using HRP (BioRad, USA) and analyzed using ChemiDoc XRS + system (BioRad Laboratories, USA). The primary rabbit polyclonal antibodies to IL-2 (1:500) were purchased from Abcam, UK (ab180780). The secondary goat anti-rabbit IgG H&L antibodies (Alkaline Phosphatase) (1:2000) were purchased from Abcam, UK (ab97048).

2.6. Confocal microscopy

To avoiding losing CIMVs in staining process, M-14 cells were previously stained by CellTracker™ Green CMFDA Dye (#C7025, Invitrogen, USA) according to manufacturer's instructions. After staining CIMVs were isolated using previously described protocol. PBMCs were stained by Vybrant™ Multicolor Cell-Labeling Kit (#V-22889, Thermo Fisher Scientific, USA) by DiD spectrum according to manufacturer's instruction and seeded on 12-well culture plate. PBMCs were incubated with CIMVs for 3 h incubation in a humidified incubator at 37°C and 5% CO_2.

PBMCs were pelleted by centrifugation at 250 g for 5 min. After that, cells were washed from the growth culture medium using PBS and pelleted onto the bottom of the culture plate wells on the coverslip. At this moment it is important to ensure that coverslip remains on the well. This procedure followed by incubation for 30 min at room temperature for sedimentation and adherence of PBMCs onto the coverslips. After PBMCs have been adherent the PBS was gently removed and cells were fixed with 500 µl 10% formalin for 10 min at room temperature. Fixed cells were, firstly, washed once with 500 µl PBS for 5 minutes and then permeabilized with 500 µl 0.5% Triton X-100 for 10 min. Permeabilized cells were washed once again with 500 µl PBS for 5 min. Nuclei were stained with Dapi (dilution 1:10,000) for 10 min and were then washed twice with PBS for 5 min to remove excessive dye. In order to prepare samples coverslips were gently carefully mounted onto glass microscope slides using aqueous mounting medium

(ab128982, Abcam, UK). Samples were analyzed by confocal microscopy using LSM 780 confocal microscope and Zen black 2012 software (Carl Zeiss, Germany) at the KFU Interdisciplinary Center for Analytical Microscopy.

2.7. PBMC activation

Isolated PBMCs were seeded on 6-well culture plate ($2.5 \cdot 10^6$ cells per well). CIMVs from native or IL-2 modified M-14 cells were added to the immune cells in concentration 145 μg/ml. Native PBMCs were used as a control. The flow cytometry assay was performed after 72 h of incubation. Also, part of these PBMCs was used for apoptosis analysis after 72 h of cultivation.

2.8. Flow cytometry assay of activated PBMCs

For identification of activated immune cell populations conjugated antibodies staining was performed. Cells were separated by panels and stained with the next conjugated antibodies: FITC anti-human CD8a Antibody (#300906, Biolegend, USA), APC anti-human CD4 Antibody (#357408, Biolegend, USA), PE/Cy7 anti-human CD38 Antibody (#356608, Biolegend, USA), PE anti-human HLA-DR Antibody (#307605, Biolegend, USA), Brilliant Violet 421™ anti-human CD107a (LAMP-1) Antibody (#328626, Biolegend, USA), FITC anti-human CD3 Antibody (#300306, Biolegend, USA), Pacific Blue™ anti-human CD4 Antibody (#317429, Biolegend, USA), PE anti-human CD127 (IL-7Rα) Antibody (#351304, Biolegend, USA), PE/Cyanine7 anti-human CD25 Antibody (#356108, Biolegend, USA), PE anti-human CD196/CCR6 Antibody (#353410, Biolegend, USA), APC anti-human CD183/CXCR3 Antibody (#353708, Biolegend, USA), PerCP/Cyanine5.5 anti-human CD56/NCAM Antibody (#362506, Biolegend, USA) according with manufacture's instruction. Data was analyzed using FACS Aria III (BD Biosciences, USA) and BD FACSDiva™ software version 7.0.

2.9. Apoptosis analysis

Native M-14 melanoma cells were seeded on cultural 12-well plate ($2 \cdot 10^5$ cells per well) previously before 48 h for performing apoptosis analysis. Then, CIMV-activated PBMCs were added to M-14 melanoma cells ($2 \cdot 10^5$ cells per well). After 24 h of cultivation M-14 cells were collected and washed by DPBS. For performing apoptotic analysis FITC Annexin V Apoptosis Detection Kit with PI (#640914, Biolegend, USA) was used according to manufacturer's instruction. Data was analyzed using FACS Aria III (BD Biosciences, USA) and BD FACSDiva™ software version 7.0.

2.10. Statistical analysis

Statistica 10.0 version software was used for statistical analysis. One-way ANOVA analysis was used to compare independent groups by quantitative trait. Differences between groups were considered statistically significant at $p < 0.05$.

3. Results

3.1 Obtaining of genetically modified melanoma cells overexpressing IL-2

Genetically modified M-14 human melanoma cells were obtained by lentiviral transduction. Membrane vesicles were isolated from both native and genetically modified M-14 cells using cytochalasin B treatment. The presence of IL-2 protein in cells and CIMVs was confirmed by Western blot analysis (Fig. 1). The data obtained indicate that the modified M-14 tumor cells and CIMVs isolated from them contain IL-2 protein with a molecular weight of 16 kDa. This protein was absent in native M-14 tumor cells and CIMVs isolated from them.

3.2 Analysis of the interaction of CIMVs and immune cells

In order to analyze the interaction of PBMCs with tumor-derived CIMVs, cells and vesicles prestained with vital dyes were added to the coverslip at the bottom of the well of the culture plate for 3 h. After that, the PBMCs were washed of vesicles and prepared for confocal microscopy. As a result, the fusion of immune cells with CIMVs was shown (Fig. 2, B), which indicates the interaction of PBMCs with CIMVs.

3.3 Activation of immune cells after co-cultivation with CIMVs

Flow cytometer analysis revealed populations of activated HLA-DR^+/$CD38^+$ CTLs (Fig. 3). After short-term cultivation of PBMCs with native M14-derived CIMVs, a slight increase in the population of CTLs compared to control (9%) was shown. However, after incubation of PBMCs with M14-derived CIMVs-IL-2, an increase by 33% in the CTL population was shown. In a population of T-helper 2 (Th2) cells an increase in the amount of Th2 was observed both after M14-derived CIMVs and M14-derived CIMVs-IL-2 by 40% and 32%, respectively. Also, in the regulatory T-cell (Treg) population, there was an increase in Treg number after native M14-derived CIMVs by 7% and after M14-derived CIMVs-IL-2 by 36%. In the NK cell population, there was a decrease in the number of cells compared to control cells of 44.5% and 31%, respectively.

3.4 Apoptosis analysis

To determine the cytotoxic activity, non-activated PBMCs and activated by native CIMVs and CIMVs-IL-2 PBMCs were added to M-14 cells and cells were cultured for 24 h. Untreated M14 were used as a control. A slight increase in the number of apoptotic M-14 cells was shown after cultivation with both PBMCs activated by native CIMVs (6%) and PBMCs activated by CIMVs-IL-2 (7.7%). However, cultivation with unactivated PBMCs also resulted in an increase in the number of apoptotic M-14 cells (8.45%) (Fig. 4).

4. Discussion

Melanoma is a malignant tumor that develops from melanocytes, melanoma accounts for 75% of skin cancer deaths [11]. It is known that the recombinant IL-2 protein is used to treat patients with metastatic melanoma and has shown its effectiveness [12], [13]. The relatively high efficacy of IL-2 against melanoma may be associated with both the expression of IL-2 receptors (IL-2R) [14], stabilization of IL-24 mRNA [15], activation of NK cells [16], which, ultimately, enhances the patient's immune activity against melanoma. However, high-dose IL-2 therapy is known to cause serious side effects, which has been shown in clinical trials [17]. We obtained M-14 melanoma cells with IL-2 overexpression and isolated CIMVs containing IL-2. It has been shown that stimulation of PBMCs with IL-2 promotes polyclonal activation of PBMCs and an increase in the expression of IL-24, which in turn is a tumor suppressor [18]. Besides, high doses of IL-2 resulted in endogenous induction of IL-24 in the A375 human melanoma cell line, resulting in growth suppression of these cells [15]. Thus, CIMVs containing IL-2 as an immunostimulatory agent were used in this work. We have obtained data on the interaction between CIMVs-IL-2 and peripheral blood mononuclear cells. These data are consistent with literature data, where the authors were able to transfer siRNA from exosomes to monocytes and lymphocytes by co-cultivation [19].

It is known that tumor membrane vesicles play a key role in the development of tumor processes, in particular, due to their effect on immune cells. MicroRNAs are the key mechanisms of the influence of membrane vesicles from tumor cells on other cells of the tumor microenvironment. Exosomes isolated from colorectal cancer have been shown to promote disease progression by polarizing macrophages into the M2 type. This effect is due to exosomal miR-934, which downregulates PTEN expression and activates the PI3K/AKT signaling pathway, as well as positive feedback CXCL13/CXCR5/NFκB/p65/miR-934 between M2 macrophages and colorectal cancer cells [20]. Moreover, components of the tumor microenvironment, in particular the exosomes of tumor-associated fibroblasts, inhibit mitochondrial apoptosis in colorectal cancer cells [21]. It has been shown that exosomal miR-1468-5p contained in cervical cancer exosomes promotes the launch of an immunosuppressive program in lymphatic endothelial cells [22]. Thus, we have demonstrated a direct interaction of PBMCs with CIMVs-IL-2. We obtained data on the activation of T-killers, Tregs and Th after co-cultivation with CIMVs and their cytotoxic activity.

It is known that melanoma cells produce a wide range of tumor antigens, including tumor-associated antigens (TAA) and tumor-specific antigens (TSA), which provide a high level of immunogenicity [11]. However, one study showed that exosomes isolated from the plasma of melanoma patients were enriched with immunosuppressive proteins and also suppressed $CD8^+$ T-cell proliferation and NKG2D expression in NK cells [23]. In addition, it has been shown that melanoma-derived EVs carry PD-L1 on their surface, which also suppresses the function of $CD8^+$ T-cells and promotes tumor growth [24]. Our data are consistent with the literature, according to which tumor-derived CIMVs increased the number of Tregs [25]. However, according to these data, they have also inhibited other lymphocytes. Also, the immunosupressive effects of pancreatic ductal adenocarcinoma-derived EVs on NK cells was expressed in decreasing of cytotoxic activity against pancreatic cancer stem cells [26]. Thus, we have shown that

CIMVs derived from native M-14 melanoma cells are immunogenic, and the use of CIMVs-IL-2 increases the number of activated CTLs. However, the cytotoxic activity of T-cells after cultivation with CIMVs-IL-2 was a tendency to decrease and did not differ significantly from the cytotoxic activity of native T-cells.

Conclusion

Despite the presence of a population of activated T-cells after interaction with CIMVs, their effect, in general, is immunosuppressive. Thus, further investigations are required on the possibility of using tumor-derived CIMVs for immunotherapy. The most promising approach is to use CIMVs as antigenic material for professional antigen-presenting cells, in particular, dendritic cells.

Declarations

Ethics approval and consent to participate

An informed consent was obtained from all subjects involved in the study. All manipulations were carried out in accordance with approved ethical standards and current legislation (the protocol was approved by the Committee on Biomedical Ethics of Kazan Federal University (No. 3, 03/23/2017).

Consent for publication

Not applicable.

Availability of data and material

Authors can confirm that all relevant data used to support the findings of this study are including within the article.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Conceptualization, F.I.Y., K.K.V. and V.V.S.; methodology, F.I.Y., K.K.V., V.V.S.; formal analysis, F.I.Y. and K.K.V.; investigation, D.S.C. (flow cytometry analysis), E.R.A. (confocal microscopy); writing—original draft preparation, F.I.Y. and K.K.V.; writing—review and editing, V.V.S. and D.S.C.; visualization, K.K.V.; supervision, A.A.R. All authors have read and agreed to the published version of the manuscript.

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Figures

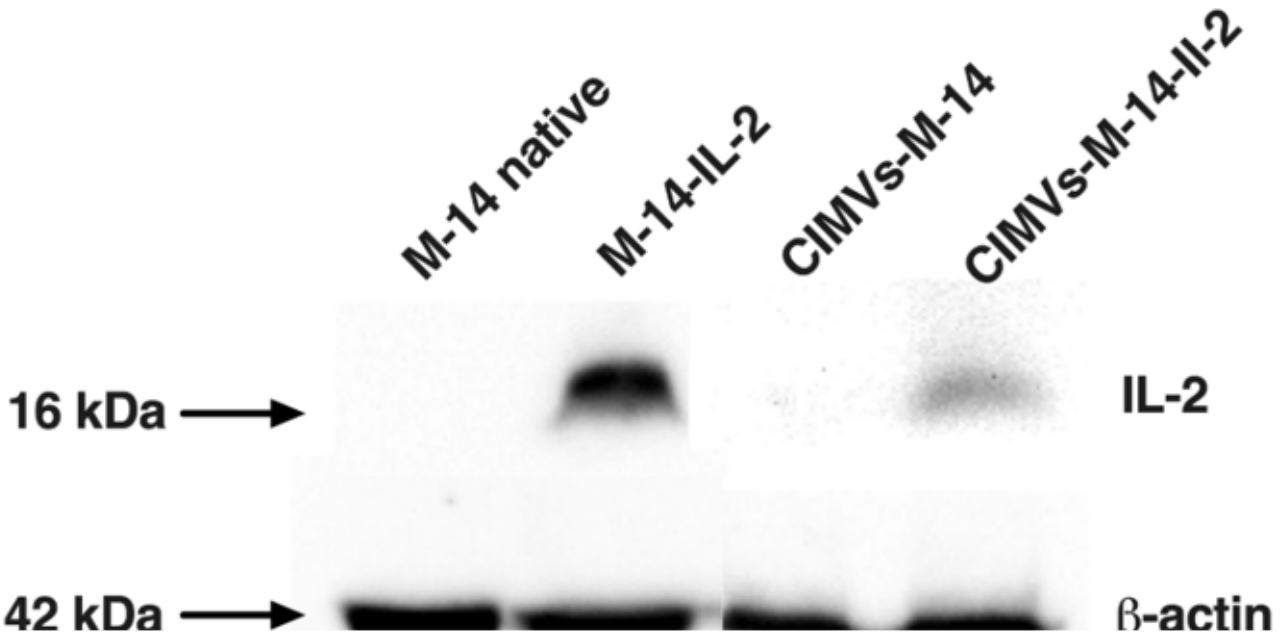

Figure 1

Western blot analysis of IL-2 expression in M-14 melanoma cells and CIMVs with β-actin serving as an internal control. The ratio of target protein (IL-2) to reference protein (β-actin) in cells and CIMVs was 1.02 and 1.32, respectively.

Figure 2

Confocal microscopy. A – control PBMCs (DID red, DAPI blue) (x40); B – PBMCs (DID red, DAPI blue) after cultivation with CIMVs (DIO green) (x63).

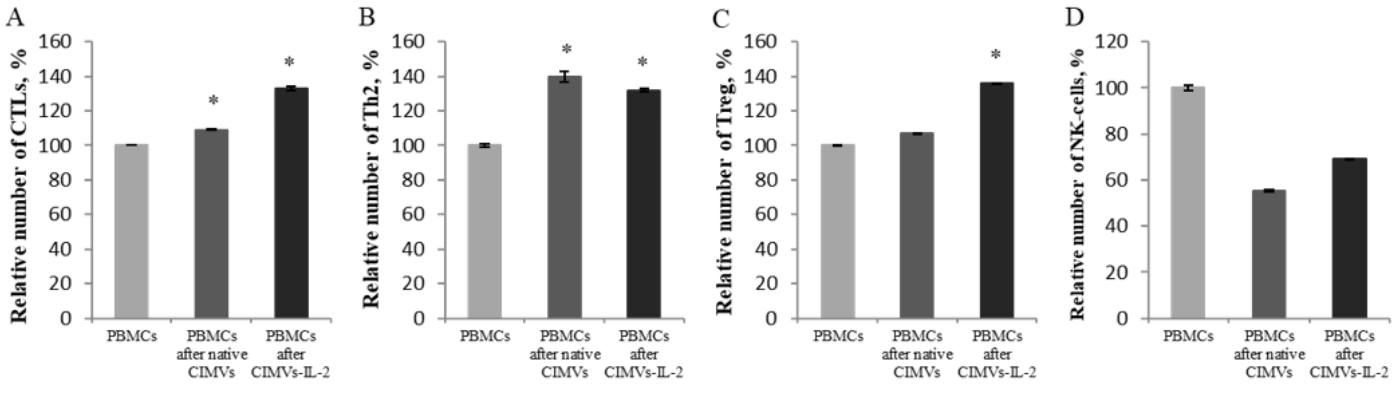

Figure 3

The effect of incubation of CIMVs with PBMCs on expression of surface markers of CTLs (A), Th2 (B); Tregs (C) and NK cells (D). * $p < 0.05$.

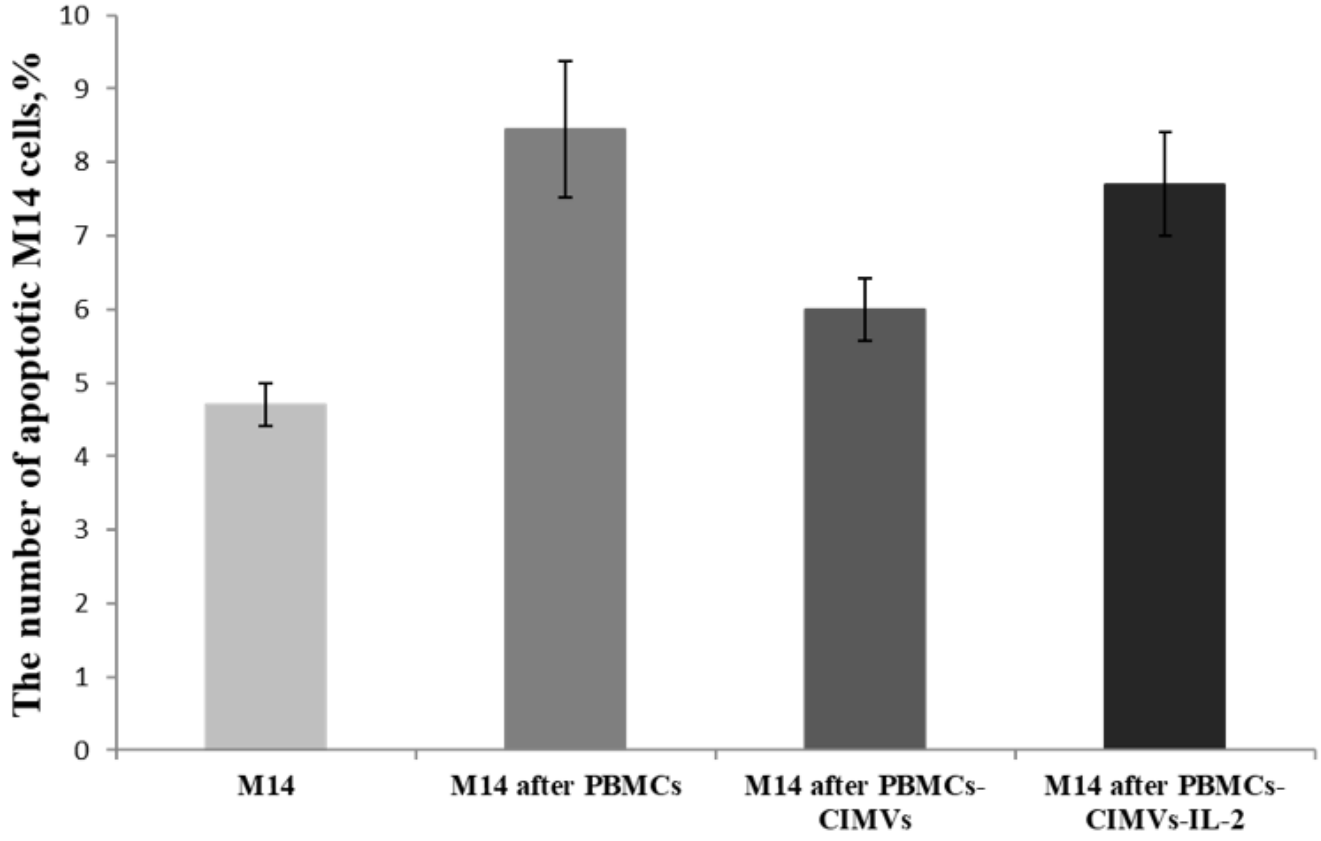

Figure 4

The number of apoptotic M14 cells (Annexin V surface receptors) after 24 h of cultivation with unactivated PBMCs or with PBMCs activated by CIMVs or CIMVs-IL2.