Медицинская иммунология
2019, Т. 21, № 4,
стр. 669-688

Оригинальные статьи
Original articles

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2019, Vol. 21, № 4, pp. 669-688

ФЕНОТИПИЧЕСКИЕ И ФУНКЦИОНАЛЬНЫЕ ХАРАКТЕРИСТИКИ МИКРОВЕЗИКУЛ, ПРОДУЦИРУЕМЫХ NK-КЛЕТКАМИ

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Резюме. Среди множества клеток, являющихся источниками микровезикул (МВ), особый интерес представляют естественные киллеры (NK-клетки) – субпопуляция лимфоцитов, осуществляющая контактный цитолиз вирус-инфицированных и опухолевых клеток. Каждая из популяций NK-клеток обладает уникальным репертуаром рецепторов на своей поверхности, а, следовательно, и функциями. Функционирование NK-клеток регулируется широким диапазоном активирующих и ингибирующих рецепторов, которые экспрессируются на их поверхности. Экзоцитоз содержимого литических гранул при контакте с клеткой-мишенью – самый распространенный механизм цитолиза мишени NK-клетками. В настоящее время имеются косвенные данные о способности NK-клеток продуцировать МВ с фенотипом CD56. В плазме периферической крови обнаружены МВ лейкоцитарного происхождения с различным фенотипом. Следует отметить, что до сих пор остаются неразрешенными вопросы о составе и функциях таких МВ. Целью настоящего исследования явилось изучение фенотипа, состава и функциональной активности микровезикул, образуемых естественными киллерами. Сравнительный анализ спонтанной экспрессии рецепторов при помощи проточной цитофлуориметрии показал, что МВ, как и клетки-источники (NK-клетки линии NK-92), обладают сходным профилем экспрессии молекул CD56 и CD16, но отличаются более выраженной экспрессией CD119 и CD11b и менее выраженной экспрессией CD18. Культивирование клеток линии NK-92 в присутствии индукторов (TNFα, IL-1β, IFNγ, PMA) приводило к изменению фенотипа как самих клеток, так и МВ, образуемых ими. По данным проведенного Western Blot analisis в присутствии указанных индукторов

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Образец цитирования:

Д.И. Соколов, К.Л. Маркова, В.А. Михайлова, Л.П. Вязьмина, Ю.П. Милютина, А.Р. Козырева, А.А. Жданова, Д.А. Малыгина, К.В. Онохин, А.Н. Иванова, А.В. Кореневский, С.А. Сельков «Фенотипические и функциональные характеристики микровезикул, продуцируемых NK-клетками» // Медицинская иммунология, 2019. Т. 21, № 4. С. 669-688. doi: 10.15789/1563-0625-2019-4-669-688

For citation:

D.I. Sokolov, K.L. Markova, V.A. Mikhailova, L.P. Vyazmina, Yu.P. Milyutina, A.R. Kozyreva, A.A. Zhdanova, D.A. Malygina, K.V. Onokhin, A.N. Ivanova, A.V. Korenevsky, S.A. Selkov "Phenotypic and functional characteristics of microvesicles produced by natural killer cells", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2019, Vol. 21, no. 4, pp. 669-688. doi: 10.15789/1563-0625-2019-4-669-688

DOI: 10.15789/1563-0625-2019-4-669-688

также изменялось содержание перфорина и гранзима B (GrB) в составе МВ. Анализ цитотоксической активности клеток линии NK-92 в отношении клеток линии K562 показал, что МВ, полученные от активированных TNFα клеток линии NK-92, повышают цитотоксичность активированных TNFα клеток линии NK-92 по сравнению с уровнем их цитотоксичности в отношении клеток линии K562 без МВ, что совпадает с обнаруженным нами повышенным содержанием GrB в МВ, полученных от активированных TNFα клеток линии NK-92. Суммируя полученные данные, следует отметить, что в зависимости от типа индуктора NK-клетки линии NK-92 продуцируют микрочастицы, разные по фенотипу и составу. Изменение состава МВ может приводить к изменению их функциональной активности, в частности к усилению цитотоксической активности NK-клеток.

Ключевые слова: NK-клетки, микровезикулы, гранзим B, перфорин, цитотоксичность, цитокины

PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF MICROVESICLES PRODUCED BY NATURAL KILLER CELLS

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Abstract. Natural killer (NK) cells are of special interest among a multitude of microvesicle (MV) source cells. NK cells are a lymphocyte subpopulation performing contact cytolysis of virus-infected cells and tumor cells. Each of the NK cell populations has a unique receptor repertoire on its surface and, thus, unique functions. During their contact with a target cell, the most common mechanism of cytolysis is an exocytosis of lytic granules. However, some indirect evidence suggests that MV with CD56 phenotype and leukocyte-derived MV with various phenotypes are present in the peripheral blood plasma. This research is aimed to study the phenotype, composition and cytotoxic activity of microvesicles produced by NK cells. The analysis of receptor expression showed that MV, as well as source cells of the NK-92 cell line, had a similar CD56 molecule expression profile. The expression profile in MV differs from the same in source cells by higher CD119 and CD11b expression and by lower CD18 expression. Culturing of NK-92 cells in the presence of PMA, IL-1β, TNFα, IFNγ resulted in alterations of cell phenotypes and MV. Immunoblots revealed a change of perforin and granzyme B (GrB) in MV. The analysis of the cytotoxic activity of NK-92 cells in a natural killer in vitro assay employing K562 target cells demonstrated that MV obtained from TNFα-activated cells of the NK-92 cell line increased the cytotoxicity of the same TNFα-activated NK-92 cells regarding cytotoxicity levels. This coincides with the previously revealed increased content of GrB in MV obtained from TNFα-activated cells of the NK-92 cell line. To sum up depending on the cytokine NK-92 cells produce MV that differ in their phenotype, composition and activity. Any changes in MV composition can result in changes in their functional activity: in particular, changes can increase the cytotoxic activity of NK cells of the NK-92 cell line. Thus, besides a well-known and proved way for GrB delivery to a target cell, we can suggest an additional way – the transportation of GrB within MV.

Keywords: NK cells, microvesicles, granzyme B, perforin, cytotoxic activity, cytokine

The research was supported by the Research and resource center for Molecular and Cell Technologies under St. Petersburg State University. All the authors have read the the journal's authorship agreement. The manuscript has been reviewed and approved by all the authors. All the authors have read the journal's policy on disclosure of potential conflicts of interest. The authors declare that they have no conflicts of interest.

The stages of the research supported by the Russian Science Foundation grant No. 17-15-01230: the evaluation of phenotypic characteristics of unstimulated NK cells of the NK-92 cell line and microvesicles produced by them, the granulometric analysis of microvesicles, the analysis of protein content in microvesicles, Western Blot analysis of unstimulated microvesicles produced by NK

cells, microvesicle sorting, transmission electron microscopy. The stages of the research supported by the Russian Foundation for Basic Research grant No. 17-04-00679: the cell culture, the evaluation of phenotypic characteristics of activated NK cells of the NK-92 cell line and microvesicles produced by them, atomic force microscopy, Western Blot analysis of microvesicles produced by activated NK cells, the assessment of cytotoxicity in cells of the NK-92 cell line and in microvesicles produced by them towards the K562 cell line. The work was partially carried out within the State Program No. AAAA-A19-119021290116-1 (financing of cell culture). The funders did not take any participation in the research design, data collection and analysis, publication or preparation of the manuscript.

Introduction

K cells are a lymphocyte subpopulation performing contact cytolysis of virus-infected cells and tumor cells. This subpopulation is the source of cytokines, stimulates other cells and participates in the immune response implementation [13, 52, 81]. As specific markers of human NK cell differentiation, CD56, KIR [76], and CD16 [38] have been identified. . Currently, literature data describe over 48 NK cell populations. However, only two populations are traditionally distinguished that differ in the ratio of CD56 and CD16 membrane molecules on their surfaces: $CD56^{bright}/CD16^{dim/-}$ and $CD56^{dim/low}/CD16^{bright}$ [12, 38, 66]. Each of the NK cell populations has a unique receptor repertoire on its surface and, thus, unique functions. During their contact with a target cell, the most common mechanism of cytolysis is an exocytosis of lytic granules [23, 31]. These granules include amines, proteoglycans, catecholamines, enzymes, and hormones, but the main components are perforin, granzyme and granulysin [31, 55, 61].

Perforin and GrB are proteins included in lytic granules also called “secretory lysosomes”. These granules are localized in the NK cell cytoplasm, are covered with a bilayered membrane, contain enzymes typical for lysosomes, as well as perforin, granzymes, Fas ligand (FasL; CD178) [5], TNF-related apoptosis-inducing ligand (TRAIL; CD253) [42], granulysin, and small anti-microbial peptides [31]. These granules are divided into three types: type I granules (50-700 nm) are mostly lled with a dense core surrounded by a thin layer of vesicles, while type II granules (200-1000 nm) are characterized by multiple vesicles and membrane whorls. The intermediate granules have a dense core, although smaller than that in type I granules, and multiple vesicles that are not as abundant as those in type II granules [31, 46]. The exocytosis of lytic granules upon their contact with a target cell is the most common mechanism of target cell cytolysis by NK cells. Perforin is localized in secretory granules in the inactivated state at pH < 5. Perforin subunits assemble into a membrane-attacking complex at pH = 7 in the presence of Ca^{2+} ions in the immunologic synapse zone between a cytotoxic lymphocyte and a target cell [72]. As a result, perforin creates pores in the target cell membrane [47, 55]. Granzymes and granulysins are injected into the target cell cytoplasm through the perforin pores. Granzyme initiates the apoptosis of the target cell. After the contents of natural killer granules gets into the target cell, apoptosis and phosphatidylserine on the cell surface as a signal for phagocytes are induced [7, 31, 47]. Granzyme B (GrB) is a serine proteinase initiating the apoptosis in the target cell due to the activation of the mitochondrial pathway, the activation of effector caspases (the most common pathway for a mouse cell), or the splitting of intracellular substrates (ROCKI, α-tubulin, filamin, etc.) [22, 24, 61]. (Patho) physiological properties of GrB were also described: extracellular matrix breakdown (activity toward vitronectin, fibronectin, and laminin), participation in the proinflammatory reaction induction through IL-1α breakdown [24], splitting of C3 and C5 components of a complement [49], and modulation of coagulation processes due to the effect of the von Willebrand factor on the expression [24]. GrB can be stimulated by IL-1β, IL-18, TNFα, IFNα, IFNγ, PMA, and LPS. The irreversible interaction between GrB and PI-9, a proteinase inhibitor, forms a stable inactive serpin-proteinase complex [55]. Thus, just like perforin, GrB in NK cells is located only within secretory granules comparable to MV by their sizes (50-1000 nm) and cannot be located in the cytoplasm as an active enzyme.

Cell-derived microvesicles (MV) are a relatively new object of research that represents vesicular fragments of a plasma membrane 100 (150)-1000 nm in diameter, while exosomes are less than 100 (150) nm in size [20, 21, 69, 70]. It was demonstrated that MV are produced by cells both in an unstimulated state and as a result of activation; both at physiological processes and at pathologies [43, 44, 48, 54, 57, 64, 69]. It has been established that MV contain both membrane molecules expressed by a source cell and cytoplasmic molecules: lipids, class I and II MHC molecules, chemokines, cytokines, growth factors, transcription factors, microRNA [8, 14, 21, 63], and messenger RNA [1, 68, 75]. It was demonstrated that MV participate in the regulation of target cell function. Moreover, they are involved in clotting [64, 69], inflammatory [2, 67], immune response [63], neogenesis [8, 32, 45], and carcinogenesis [3, 11, 50] processes. Among numerous cells being the source of MV, leukocytes are of particular interest due to the diversity of their receptor and effector functions. With our study, we provide the evidence of the capacity of NK cells to produce MV. However, some indirect

evidence suggests that MV with CD56 phenotype [40] and leukocyte-derived MV with various phenotypes are present in the peripheral blood plasma [9, 33, 40, 41]. This research is aimed to study the phenotype, composition and functional activity of MV produced by NK cells.

Materials and methods

Cells

NK cells of the NK-92 cell line (ATCC, USA) obtained from large granular lymphocytes of the peripheral blood of a 50-year-old man with a galloping non-Hodgkin's lymphoma were the object of this research [19]. Cells of the NK-92 cell line display the main phenotype and functional characteristics of activated NK cells [19, 28]. To culture them, we used the complete cell culture medium based on the α-modification of the Eagle's minimal essential medium (α-MEME) containing 12.5% inactivated fetal calf serum (FCS), 12.5% inactivated donor horse serum, depleted of MV, 0.2 mM myoinositol, 0.02 mM folic acid, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10 mM HEPES buffer solution, 0.1 mM 2- mercaptoethanol (Sigma-Aldrich Chem. Co., USA), and 500 U/ml recombinant IL-2 (Roncoleukinum, Biotech LLC, Russia). Cells of the NK-92 cell line are a suspension cell culture requiring the subcultivation once every 2 days. The cells were cultured in humid environment at 37°C, and 5% CO_2. Cells of the K-562 cell line were cultured in RPMI 1640, the complete cell culture medium containing 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10 mM HEPES buffer solution (Sigma-Aldrich Chem. Co., USA). Cells of the K562 cell line are a suspension cell culture requiring the subcultivation once every 3 days. The cells were cultured in humid environment at 37°C, and 5% CO_2. Using the trypan blue solution, the cell vitality was evaluated. It was not less than 96%.

Cytokines and inducers

TNFα (10 U/ml, 50 U/ml, 400 U/ml), IFNγ (40 U/ml, 400 U/ml, 1000 U/ml), IL-1β (10 ng/ml, 100 ng/ml, 1000 ng/ml), and phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Chem. Co., USA) (10 ng/ml) were used as inducers. Culturing in the complete cell culture medium without inducers served as controls.

Phenotypic characteristics of NK cells of the NK-92 cell line including microvesicles

NK cells phenotype was evaluated after culturing cells in presence of inducers in humid environment

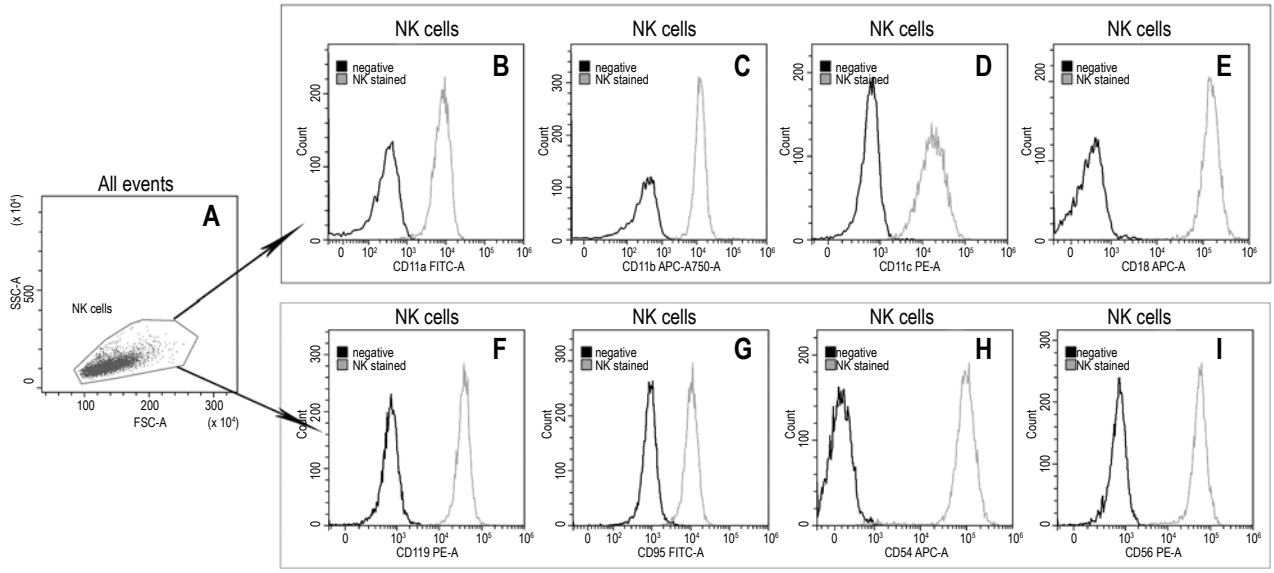

Figure 1. Distribution graphs for NK cells of the NK-92 cell line, unstained and stained with antibodies to CD11a, CD11b, CD11c, CD18, CD119, CD95, CD54, CD56, receptors

Note. For all graphs: isotype controls (BD, USA) were used as negative controls; stained NK cells are NK cells treated with antibodies.
A) Distribution diagram for NK cells of the NK-92 cell line in FSC – SSC coordinates; B) Distribution histogram for NK cells of the NK-92 cell line by the CD11a receptor expression; C) Distribution histogram for NK cells of the NK-92 cell line by the CD11b receptor expression; D) Distribution histogram for NK cells of the NK-92 cell line by the CD11c receptor expression; E) Distribution histogram for NK cells of the NK-92 cell line by the CD18 receptor expression; F) Distribution histogram for NK cells of the NK-92 cell line by the CD119 receptor expression; G) Distribution histogram for NK cells of the NK-92 cell line by the CD95 receptor expression; H) Distribution histogram for NK cells of the NK-92 cell line by the CD54 receptor expression; I) Distribution histogram for NK cells of the NK-92 cell line by the CD56 receptor expression. The compensation matrices were adjusted using BD CompBeads and procedure recommended by the manufacturer of the Cytoflex flow cytometer (Beckman Coulter).
The experiment was repeated twice, using triplicates.

at 37 °C and 5% CO_2 for 24 hours as we described before [39]. There is no single standard for the isolation and characterization of MV, so various methodological approaches are currently used. They allow obtaining microparticle fractions that differ in their purity degree and enrichment level [74]. To isolate MV, we used the differential centrifugation method [58, 60] using Hanks' solution without Ca^{2+} and Mg^{2+} (Sigma-Aldrich Chem Co., USA). The obtained supernatants were consecutively centrifuged as we described before [39]. After the second centrifugation, the pellet was washed twice with PBS and centrifuged again at 20 000 g 10° C for 20 minutes. The supernatant was then discarded and the pellet was resuspended in Hank's solution without Ca^{2+} and Mg^{2+} similarly to the method we described before [29]. This procedure allows separating MV from coarse particles of cellular debris and large apoptotic bodies, as well as from exosomes [15, 30]. After that, the obtained cells of the NK-92 cell line and their MV were treated with monoclonal antibodies to CD11a, CD11b, CD11c, CD18, CD119, CD54, CD95, CD56 (BD, USA) in accordance with the instructions of the manufacturer. We analyzed the intensity of expression of phenotypic marker CD56 by NK cells, as well as the intensity of expression of leukocyte adhesion molecules CD11a, CD11b, CD11c, CD18 typical for all leukocytes. These adhesion molecules can participate in the formation of the immunologic synapse. To evaluate the expression, we chose CD119 and CD95 receptors, and adhesion molecule CD54 as markers of intercellular communication and activation. Isotype controls (BD, USA) were used as negative controls. The receptor expression was assessed using a Cytoflex flow cytometer (Beckman Coulter, USA) as shown before [39]. Working with MV, we filtered all solutions through filters with 0.2 μm pore diameter (Sigma-Aldrich Chem. Co., USA) [70]. The information on the gating strategy for the cells and MV produced by them are specified in Figure 1 and Figure 2. The experiments were repeated twice, using triplicates for each cytokine and control.

Granulometric analysis of microvesicles

The granulometric analysis of microvesicles produced by NK cells of the NK-92 cell line was carried out by the dynamic light scattering method

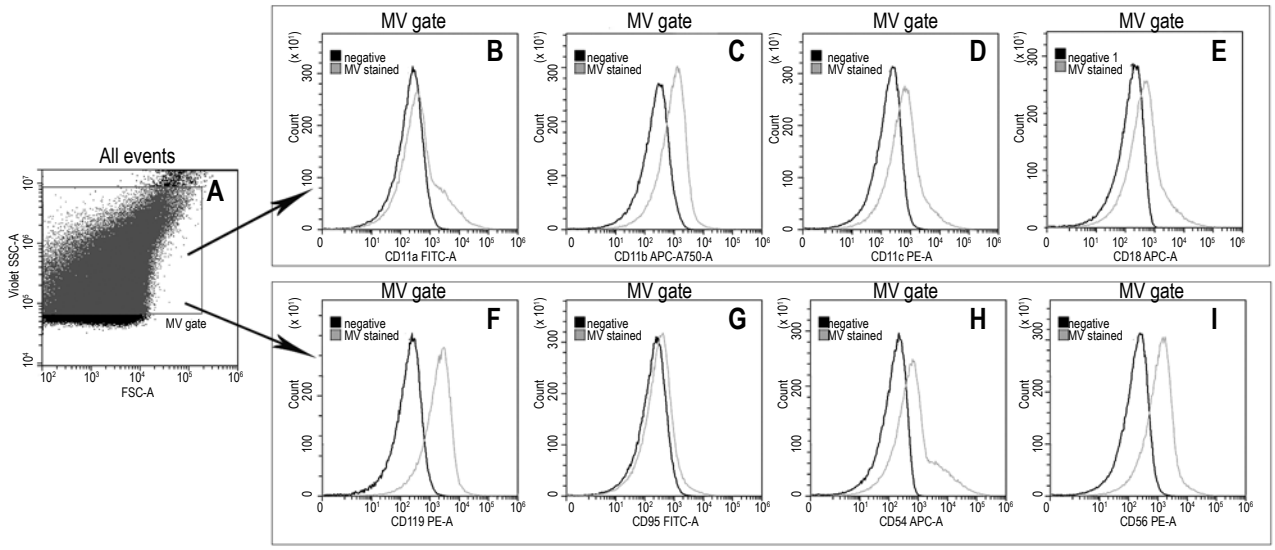

Figure 2. Distribution graphs for microvesicles of NK Cells of the NK-92 cell line, unstained and stained with antibodies to CD11a, CD11b, CD11c, CD18, CD119, CD95, CD54, CD56, receptors

Note. For all graphs: isotype controls (BD, USA) were used as negative controls; negative control (microvesicles untreated with antibodies); stained MV are microvesicles treated with antibodies. A) Distribution diagram for microvesicles produced by NK cells of the NK-92 cell line in FSC – Violet SSC coordinates. The parameter of lateral light scattering (SSC) was detected using 405 nm laser radiation in order to facilitate the determination of small particle sizes; B) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD11a receptor expression; C) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD11b receptor expression; D) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD11c receptor expression; E) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD18 receptor expression; F) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD119 receptor expression; G) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD95 receptor expression; H) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD54 receptor expression; I) Distribution histogram for microvesicles produced by NK cells of the NK-92 cell line by the CD56 receptor expression. The compensation matrices were adjusted using BD CompBeads and standard protocol recommended by the manufacturer of the Cytoflex flow cytometer (Beckman Coulter). The experiment was repeated twice, using triplicates.

using Zetasizer NanoZS, the laser correlation spectrometer (Malvern Instruments, UK). MV were isolated as described above.

Atomic force microscopy of microvesicles

Morphometric analysis of MV produced by the NK-92 cell line was carried out by atomic force microscopy method. The suspension of isolated MV in Hanks' solution without Ca^{2+} and Mg^{2+} was spread on grease-free cover glasses covered with polylysine (Sigma-Aldrich Chem. Co., USA) with addition of an equal volume of 10% formalin and dried at 37 °C. Next, the samples were rinsed in distilled water and dried at room temperature. The Integra Aura scanning probe microscope (NT-MDT, Russia) was used to scan the surface of samples in tapping mode in air. The scanning was performed with NSG01-A proximity silicon high-resolution probes (NT-MDT, Russia) with hardness of 5.1 N/m and an average resonating frequency of 150 kHz. The images were processed using Nova software (NT-MDT, Russia).

Transmission electron microscopy (TEM)

10 μl portions of suspension of MV of the NK-92 cell line in Hanks' solution without Ca^{2+} and Mg^{2+} obtained by means of centrifugation at 20 000 g were mixed with 5% glutaraldehyde of an equal volume. 300 mesh copper grids (Electron Microscopy Sciences, USA) with a formvar carbon-shadowed coating were superimposed on the resulting drop and incubated for 5 minutes. The grids were then washed by transferring them to the surface of deionized water drops. After that, the samples were contrasted for 1 minute in the 2% uranium acetate solution. The excess of the contrastor was removed with blotting paper. The images were registered with the Jeol JEM-1400 transmission electron microscope (Japan) at an accelerating voltage of 90 kV and a beam current of 50 mA using the Olympus Veleta siding camera (Japan) with a 100 000-250 000× zoom.

Analysis of protein content in microvesicles

The analysis of protein content in microvesicles was conducted through Bradford protein assay using the Qubit fluorometer (Life technologies, USA) [6].

Western Blot analysis

The cells of the NK-92 cell line were cultured in flasks in the complete cell culture medium at a concentration of 400 000 c/ml at 37 °C in the humid environment with 5% CO_2 for 24 hours in the presence of inducers (50 U/ml TNFα, 400 U/ml IFNγ, 100 ng/ml IL-1β, 10 ng/ml PMA). Culturing in the complete cell culture medium without inducers served as controls. After 24 hours, the cell suspension was centrifuged at 200 g, 22 °C for 10 minutes and washed twice with PBS (pH = 7.4). MV were obtained from supernatant by method specified above and washed twice with cold PBS solution at 20,000g. The obtained cells and MV were lysed in the RIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 1 mM EDTA (Sigma-Aldrich Chem. Co., USA)) with addition of Complete protease inhibitor mixture (Roche Diagnostic GmbH, Germany) for 30 minutes with intermittent shaking. The cell lysates were centrifuged at 16 000 g for 10 minutes. Supernatants were stored at -80 °C before the assay. The cell lysate samples with equal protein content were divided using electrophoresis in 10% polyacrylamide gel under denaturing conditions by Laemmli method and were transferred onto PVDF membranes (BioRad, USA). The membranes were blocked with 2% albumin solution (Sigma-Aldrich Chem. Co., USA) in TBST solution (50 mM Tris-HCl; 150 mM NaCl; 0,1% Tween20), and then incubated with primary monoclonal antibodies to granzyme B (GrB) (Purified anti-Granzyme B, mouse Ab, 1:1000, Biolegend, USA) or to perforin (Anti-Human Perforin Purified, mouse Ab, 1:1000, eBioscience Inc., CA) at 4 °C for one night in a shaker. Actin (β-actin rabbit Ab, 1:1000, Cell Signaling Technology, USA) was used as a load control. After the reaction with corresponding HRP conjugated secondary antibodies (1:1000, Cell Signaling Technology, USA), the signals were visualized by the enhanced chemiluminescence method (ECL, GE Healthcare, Uppsala, Sweden). The intensity of bands obtained by Western blotting method was determined using the ImageLab software. The data obtained on cell lysates were normalized using β-actin. When studying MV, the samples containing equal volume of total protein were also approved for analysis. All experiments were independently carried out five times. The experimental data were represented as M±m ratios between the induced samples and corresponding controls.

Sorting

Microvesicles were sorted using a flow cytometer sorter BD FACS Aria III (BD, USA). The sensitivity of the sorter was increased due to a 405 nm laser and 450/10 filter for the side scatter analysis [70] in compliance with the manufacturer's recommendations (BD, USA). The unit was adjusted as described above using standard gauge particles of 200 nm and 1000 nm in size (Invitrogen, USA) in accordance with the instructions of the unit manufacturer (BD, USA). To start working with MV, all the solutions were filtered through filters with 0.2 μm pore diameter (Sigma-Aldrich Chem. Co., USA). The information on the unit adjustment with gauge particles is specified in Figure 3. Microvesicles obtained from the cells of the NK-92 cell line by the differential centrifugation method described above were treated with antibodies to CD119 and CD11b marked with PE (BD, USA). Positively stained microvesicles were sorted in the Purity protocol (70μm nozzle) with the aim of subsequent analysis of the granzyme B (GrB) concentration in the pool of microvesicles expressing

the stated receptors by Western blotting method. Isotype controls (BD, USA) were used as negative controls.

Assessment of cytotoxicity in cells of the NK-92 cell line and microvesicles produced by them in towards cells of the K562 cell line

Cells of the NK-92 cell line were cultured in 75 cm^2 flasks (BD Falcon, USA) in a volume of 40 ml. The cell concentration was 4×10^5 per 1 ml. We added IL-1β at a concentration of 1000 U/ml or TNFα at a concentration of 400 U/ml to some flasks with cells of the NK-92 cell line and incubated them for 22 hours at 37 °C and 5% CO_2. Then, both activated and non-activated cells of the NK-92 cell line were centrifuged at 200 g 22 °C for 10 minutes at 22 °C in order to separate the cells from the medium containing MV. After that, the cells were washed with Hanks' solution. As the result, we obtained a sediment containing the cells and supernatant containing MV. MV were obtained from this supernatant by the abovementioned method. We resuspended the sediment containing MV in 700 mcl of cold α-MEME and measured the protein concentration.

To analyze the cytotoxic activity, the sediment containing cells of the NK-92 cell line, which was obtained after the first centrifugation at 200 g, was washed twice with Hank's solution and resuspended in α-MEME containing 10% FCS, 0.2 mM myoinositol, 0.02 mM folic acid, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10 mM HEPES buffer solution, and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich Chem. Co., USA). Cells of the K562 cell line were stained with CFSE green fluorescent dye (Sigma-Aldrich Chem. Co., USA) as described earlier [78]. The dyed cells of the K562 cell line were added at a concentration of 3×10^4 in 25 mcl per one well of a round-bottom 96-well plate (BD, USA). Some wells containing the cells of the K562 cell line were supplemented with cells of the NK-92 cell line at a concentration of 3×10^5 cells in 25 mcl per one well of a round-bottom 96-well plate. Some wells with cells of both NK-92 and K562 cell lines were supplemented with prepared microvesicles so that the total protein

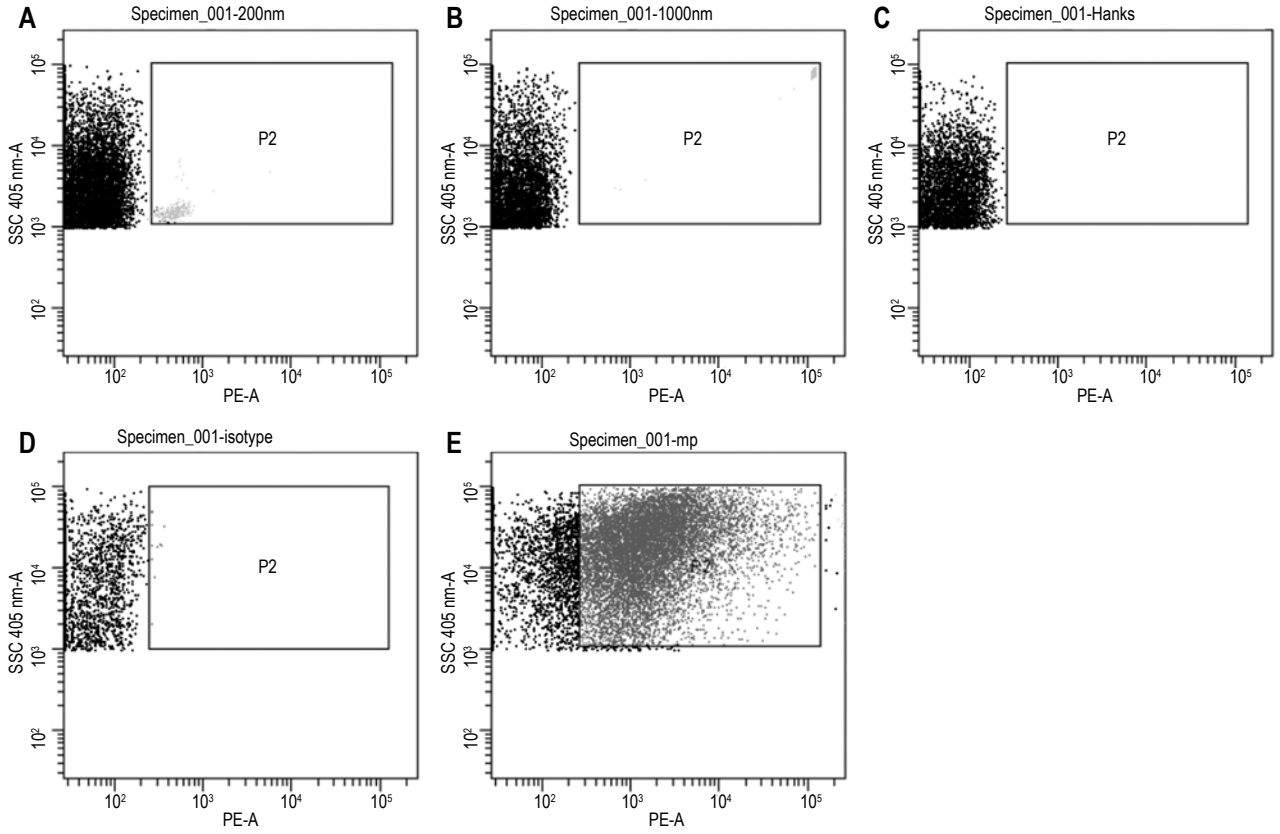

Figure 3. Distribution graphs for gauge particles and microvesicles produced by cells of the NK-92 cell line with the use of the BD FACS Aria III Flow cytometer sorter (BD, USA)

Note. The parameter of lateral light scattering (SSC) was detected using 405 nm laser radiation in order to facilitate the determination of small particle sizes. A) Distribution graph for gauge particles 200 nm in size (PE – SSC); C) Distribution graph for gauge particles 1000 nm in size (PE – SSC); C) Hanks' solution; D) Distribution graph for microvesicles produced by cells of the NK-92 cell line treated with isotype control antibodies (PE – SSC); E) Distribution graph for microvesicles produced by cells of the NK-92 cell line treated with antibodies to CD119 and CD11b, PE-marked, in PE – SSC coordinates.

concentration in them amounted to 400 μg/ml, 200 μg/ml, and 100 μg/ml. Cells of the K562 cell line served as controls (constitutive death). Then, the cells were cultured for 22 hours at 37 °C and 5% CO_2. After that, the cell suspension was treated with Propidium iodide (Sigma-Aldrich Chem. Co., USA) and antibodies to CD45 (FITC) (BD, USA). The level of the K562 cell line death was assessed using the FACS Canto II flow cytometer (BD, USA) by Propidium iodide inclusion, as described above [10, 73, 78]. The experiments were repeated three times with six wells per each combination of cells and MV (n = 3).

Statistical analysis

Statistical analysis was performed using the Statistica 10 software (Russia). We tested the data distribution for normality using the Shapiro-Wilk's test and then the parametric Student's t-test. The differences under $p < 0.05$ were considered to be significant. For the Western Blot analysis, we conducted a paired comparison of band intensity values in constitutive culture and in the presence of an inducer that were obtained from each sedimentation using the Wilcoxon method.

Results

Granulometric analysis

The granulometric analysis showed that sizes of MV produced by NK cells of the NK-92 cell line lay in the range of 192-458 nm. The peak of MV quantity distribution fell at 297 nm that was in accordance with our previously published data [29]. The analysis of particles in the supernatant obtained after MV sedimentation at 20 000g demonstrated that their sizes lay in the range of 20-184 nm. The peak of the distribution of particle quantity fell at 37 nm that also corresponds to our previously published data [29].

Atomic force microscopy of microvesicles produced by the NK-92 cell line

The population of spherical objects with the height of 39-588 nm and with the mode in the area of 314 nm (Figure 4) was determined on the surface relief and its height distribution histogram within a 10 × 10 μm visual field. The height of the major population of microvesicles lay in the range of 196-392 nm. The sample containing only the Hanks' solution without Ca^{2+} was used as a control. The average size of particles in the control sample was about 22 nm with the mode in the area of 8 nm. Atomic force microscopy was done using MV only from intact cells.

Transmission electron microscopy

Transmission electron microscopy (TEM) of the MV sediment fraction obtained after centrifugation at 20 000 g allowed to reveal the spherical objects 150-500 nm in diameter (Figure 5), which corresponds to the data obtained by other methods described in the article. TEM was done using MV only from intact cells.

Analysis of protein content in microvesicles and in cells in separate experiments with different inducers

In the experiment with PMA as an inducer, the total protein content in unstimulated cells of the NK-92 cell line (constitutive level) amounted to 60.2±6.1 ng/10^3 cells. In the presence of PMA, this content amounted to 91.3±40.0 ng/10^3 cells. At that, the total protein content in MV obtained from unstimulated cells of the NK-92 cell line (constitutive level) amounted to 2.5±0.3 ng per 10^3 source cells, while in the presence of PMA, this content amounted to 5.0±0.7 ng per 10^3 source cells.

In the experiment with TNFα as an inducer, the total protein content in unstimulated cells of the NK-92 cell line (constitutive level) amounted to 94.0± 0.9 ng per 10^3 cells. In the presence of TNFα, this content amounted to 191.0±18.1 ng/10^3 cells. At that, the total protein content in MV obtained from unstimulated cells of the NK-92 cell line (constitutive level) amounted to 5.0±0.8 ng per 10^3 cells, while in the presence of TNFα, this content amounted to 19.6±3.7 ng per 10^3 cells.

In the experiment with IL-1β as an inducer, the total protein content in unstimulated cells of the NK-92 cell line (constitutive level) amounted to 61.2±6.3 per 10^3 cells. In the presence of IL-1β, this content amounted to 66.3± 6.0 ng/10^3 cells. At that, the total protein content in MV obtained from unstimulated cells of the NK-92 cell line (constitutive level) amounted to 4.6±0.6 ng per 10^3 cells, and with IL-1β it amounted to 2.6± 0.4 ng per 10^3 cells.

Phenotypic characteristics of NK cells of the NK-92 cell line including microvesicles

Almost all NK cells in the NK-92 cell line (96-99%) expressed CD11a, CD11b, CD11c, CD18, CD119, CD54, CD95, CD56 on their surface. The activation of the NK-92 cell line by specific cytokines at different concentrations resulted in a change in the expression of only certain molecules (Figure 6). Thus, the introduction of TNFα resulted in dose-dependent increase in the CD54 and CD95 expression level. The incubation of the NK-92 cell line in the presence of different IL-1β concentrations increased the CD54 and CD95 expression level. However, IL-1β at a concentration of 1000 U/ml decreased the CD18 expression level. The incubation of the NK-92 cell line in the presence of IFNγ decreased the CD11b expression level. The incubation of the NK-92 cell line in the presence of PMA increased the CD11c, CD54, CD95, and CD119 expression level, but decreased the CD56 expression level.

The relative number of MV produced by NK cells of the NK-92 cell line and expressing the analyzed receptors was significantly lower compared to the number of source cells: only 2% of MV expressed CD95 molecule; only 10-20% of MV expressed CD11a, CD11c, and CD18 molecules; only 20-39%

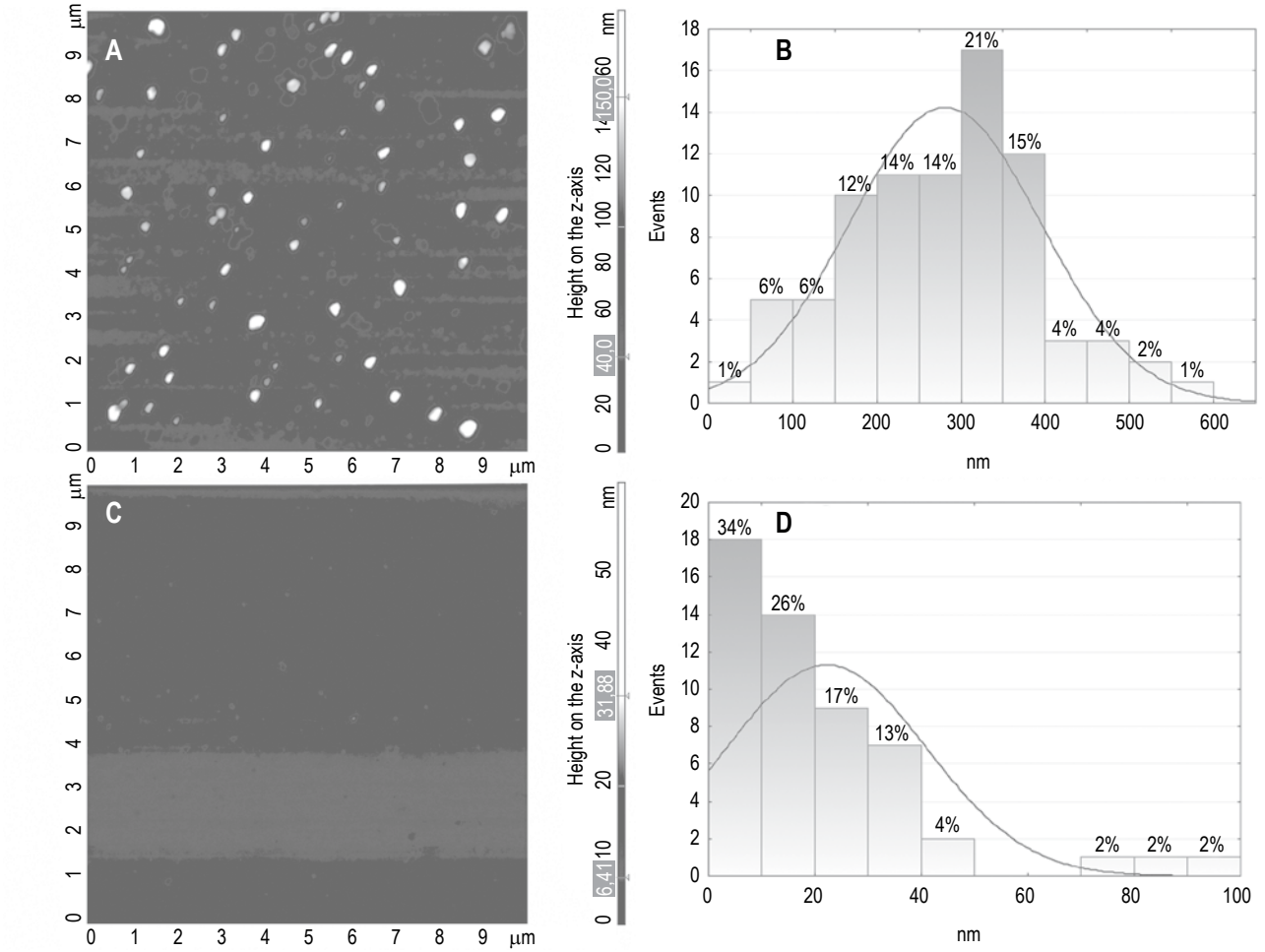

Figure 4. Atomic force microscopy of samples of MV produced by NK-92 cells (n = 5)

Note. Visualization of MV (A) and height distribution histogram (B) within a 10 × 10 μm field of a sample of MV produced by NK-92 cells. IImage of a control sample in Hanks' solution (C) and respective height distribution histogram e (D).

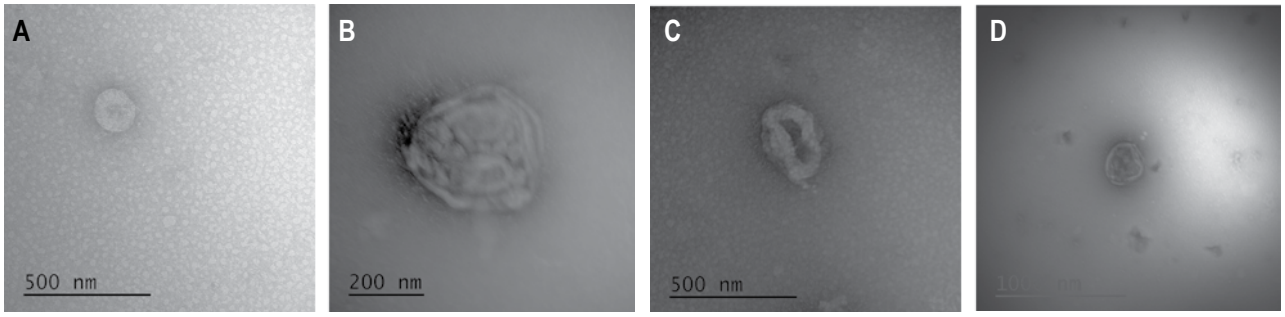

Figure 5. Transmission electron microscopy (TEM) of MV produced by NK-92 cells

of MV expressed CD11b, CD119, CD54, and CD56 molecules. The intensity of the analyzed receptor expression by MV was also lower than the intensity of the expression on the surface of source cells (Supplementary data Figure 6). The activation of the NK-92 cell line by specific cytokines resulted in a change in the expression of only certain molecules by MV produced by the cells of the NK-92 cell line (Supplementary data Figure 7). With that, the activation of cells of the NK-92 cell line by cytokines had no effect on the relative quantity of MV expressing the analyzed receptors. The comparative profile of the constitutive intensity of the surface molecule expression on both cells of the NK-92 cell line and the MV produced by them is given in the Figure 3. The Figure shows that MV, as well as source cells, have a similar profile of CD56 molecule expression. However, the expression profile in MV differs from

the same in source cells by higher CD119 and CD11b expression and by lower CD18 expression.

Western Blot analysis

Figure 9 shows the change in levels of GrB and perforin in lysates of cells of the NK-92 cell line in the presence of different inducers. For MV content analysis by Western blot method, we used the inducers, which showed the prominent influence on MV phenotype (PMA, TNFα и IL-1β). It has been established that when cultured in the presence of PMA, the expression of GrB and perforin in NK cells of the NK-92 cell line increases (1.97-fold

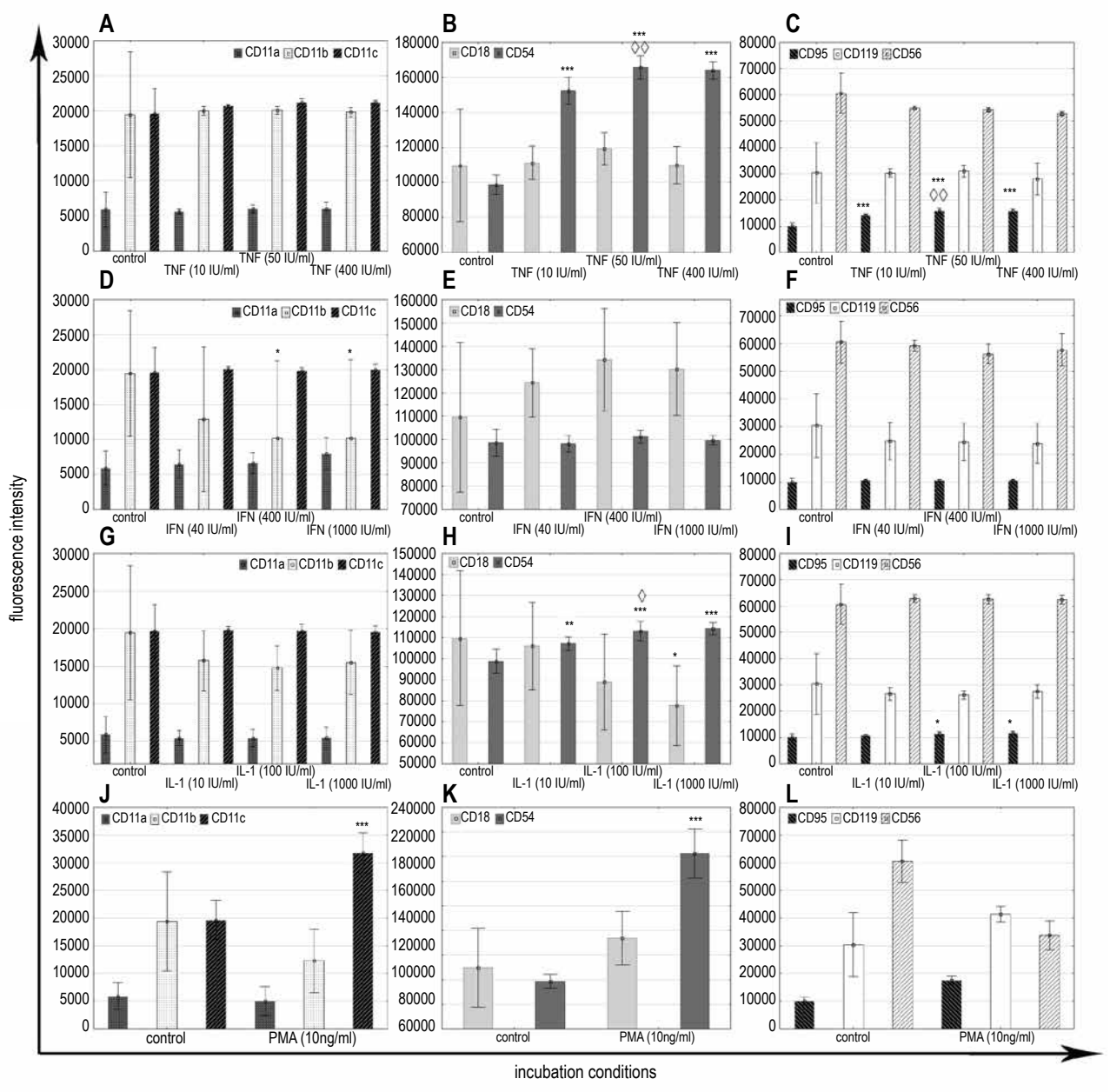

Figure 6. Intensity of surface receptor expression by NK cells of the NK-92 cell line, unstained and stained with antibodies to CD11a, CD11b, CD11c, CD18, CD119, CD95, CD54, CD56 receptors

Note. The data are presented as Mean±SD. For all graphs: unstimulated cells – the basic level of surface receptor expression by NK cells; TNF (10 IU/ml; 50 IU/ml; 400 IU/ml) – the level of surface receptor expression by NK cells in the presence of TNFα as an inducer at an appropriate concentration; IFN (40 IU/ml; 400 IU/ml; 1000 IU/ml) – the level of surface receptor expression by NK cells in the presence of IFNγ as an inducer at an appropriate concentration; IL-1 (10 IU/ml; 100 IU/ml; 1000 IU/ml) – the level of surface receptor expression by NK cells in the presence of IL-1β as an inducer at an appropriate concentration; PMA (10 ng/ml) – the level of surface receptor expression by NK cells in the presence of PMA as an inducer at an appropriate concentration. The significance of differences: the expression of surface receptors by cells in all groups differs from the constitutive expression ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. The expression of surface receptors in the presence of cytokines differs from that one in the presence of the same cytokines at a lower concentration ◊◊, $p < 0.01$; ◊, $p < 0.05$.

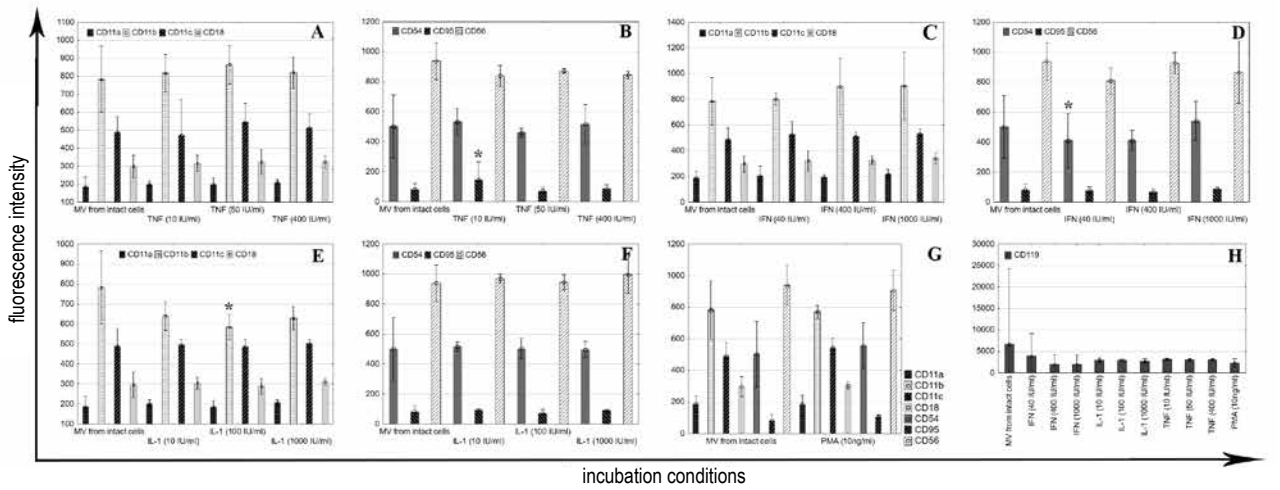

Figure 7. Intensity of surface receptor expression on microvesicles produced by NK cells, unstained and stained with antibodies to CD11a, CD11b, CD11c, CD18, CD119, CD95, CD54, CD56 receptors

Note. The data are presented as Mean±SD. For all graphs: MV obtained from unstimulated cells – the basic level of surface receptor expression on MV produced by NK cells; TNF (10 IU/ml; 50 IU/ml; 400 IU/ml) – the level of surface receptor expression on MV produced by NK cells in the presence of TNFα as an inducer at an appropriate concentration; IFN (40 IU/ml; 400 IU/ml; 1000 IU/ml) – the level of surface receptor expression on MV produced by NK cells in the presence of IFNγ as an inducer at an appropriate concentration; IL-1 (10 IU/ml; 100 IU/ml; 1000 IU/ml) – the level of surface receptor expression on MV produced by NK cells in the presence of IL-1β as an inducer at an appropriate concentration; PMA (10 ng/ml) – the level of surface receptor expression on MV produced by NK cells in the presence of PMA as an inducer at an appropriate concentration. The significance of differences: the expression of surface receptors by cells in all groups differs from the constitutive expression *, $p < 0.05$.

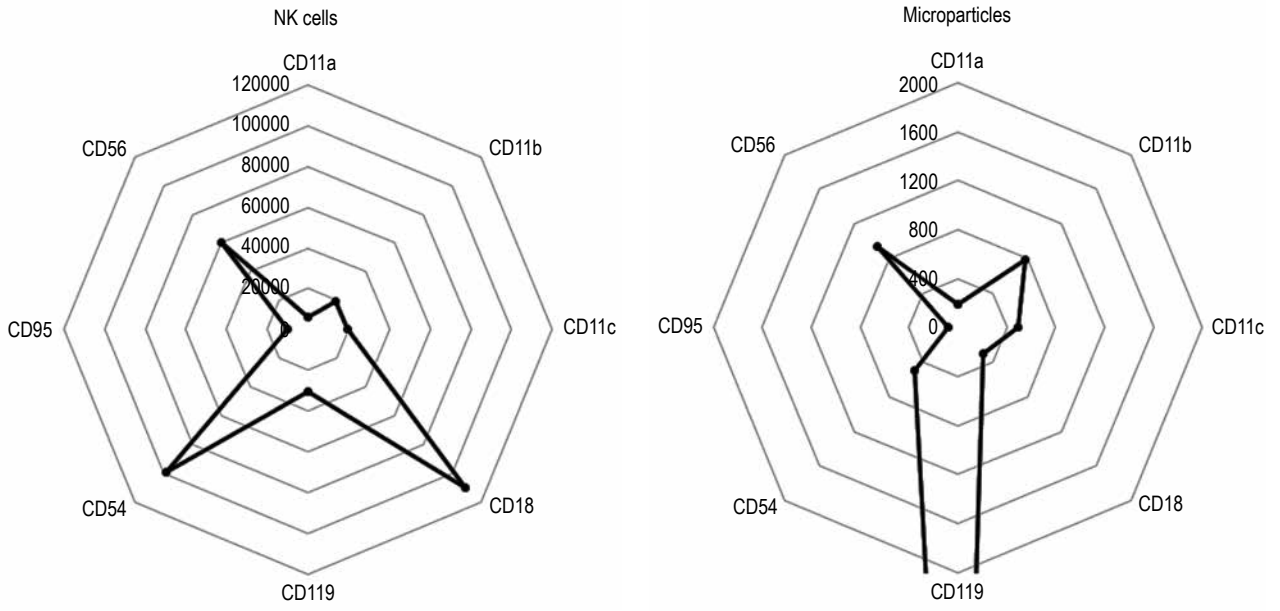

Figure 8. Comparative profile of the constitutive intensity of the surface molecule expression in cells of the NK-92 cell line and in microvesicles produced by them

and 9.7-fold, correspondingly) compared to their constitutive expression level. In addition, the pairwise comparison revealed that the expression of GrB increased (1.53-fold), and the expression of perforin decreased (1.23-fold), when NK cells were cultured in the presence of IL-1β. When TNFα was added to the culture of NK cells of the NK-92 cell line, no significant changes in the expression of GrB and perforin were revealed.

Figure 9 shows the change in the expression of GrB and perforin in MV obtained from NK cells of the NK-92 cell line cultured in the presence of various inducers. It has been established that when cultured in the presence of TNFα, the expression

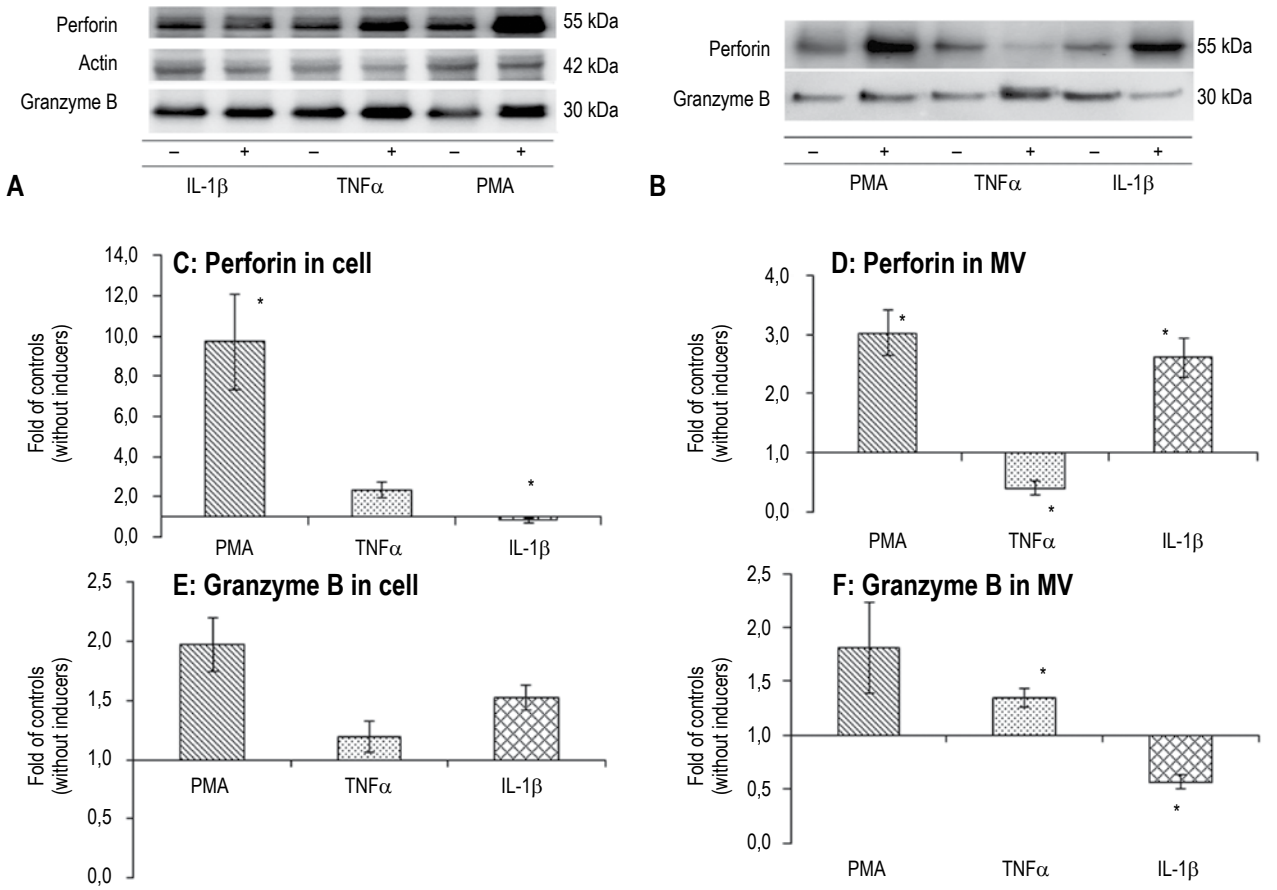

Figure 9. Analysis of perforin and GrB content: Western blotting in lysates of cells of the NK-92 cell line (A) and their microvesicles (B) obtained upon the constitutive cell culturing (–) or in the presence of various inducers (+)

Note. The ratio of Western blotting results by GrB and perforin expression in cells of the NK-92 cell line (C, E) or their microvesicles (D, F) in the presence of inducers to the results of corresponding controls (without inducers). *, $p < 0.05$ – difference from controls (culturing in the presence of the complete cell culture medium only).

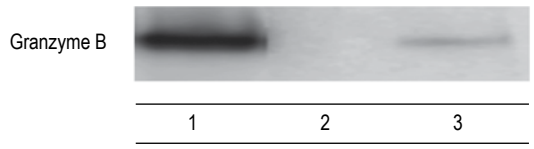

Figure 10. Qualitative analysis of GrB content in lysates from the pool of microvesicles expressing CD119, or CD11b obtained by sorting using the BD FACS Aria III flow cytometer sorter (BD, USA) from the Initial mixture of microvesicles produced by the unstimulated cells of the NK-92 cell line

Note. 1, NK-92 cell line (positive controls); 2, lysing buffer solution (negative controls); 3, pool of microvesicles expressing CD119 or CD11b.

of GrB increased (1.34-fold), and the expression of perforin decreased (2.54-fold) (compared to the constitutive level) in MV obtained from the cultured NK cells of the NK-92 cell line. In addition, the pair-wise comparison revealed that the expression of GrB decreased (1.76-fold), and the expression of perforin increased (2.60-fold), when NK cells were cultured in the presence of IL-1β. When cultured in the presence of PMA, the expression of perforin increased (3.00-fold) (compared to the constitutive level) in MV obtained from the cultured NK cells of the NK-92 cell line.

Western Blot analysis of the pool of microvesicles expressing CD119 or CD11b (obtained by sorting with the BD FACS Aria III flow cytometer sorter (BD, USA) from the initial mixture of MV produced by unstimulated cells of the NK-92 cell line) showed the presence of GrB in them (Figure 10).

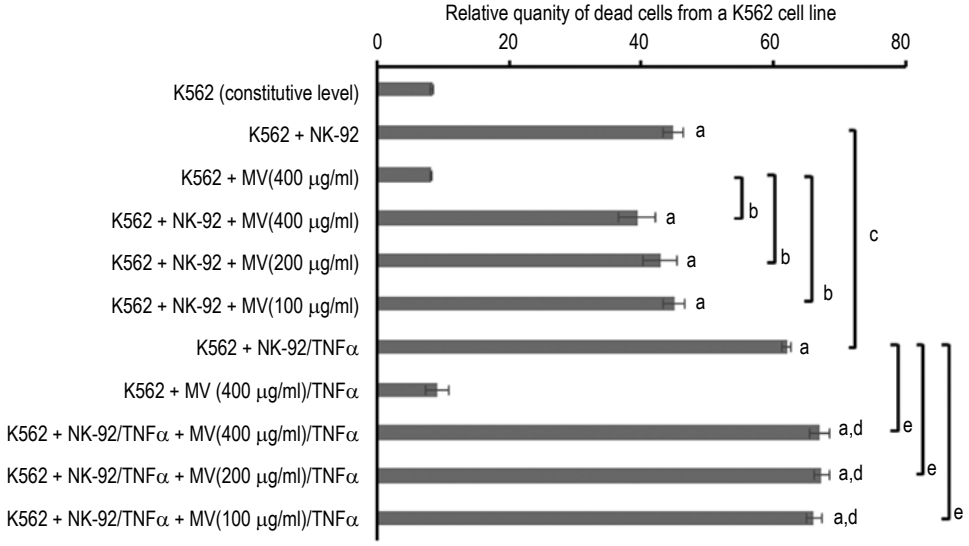

Figure 11. Cytotoxic activity of NK cells of the NK-92 cell line towards cells of the K562 cell line in the presence of microvesicles and TNFα

Note. Legend: K562 (constitutive level) – the quantity of dead K562 cells after the incubation without inductors; K562 + NK-92 – co-culturing of the K562 Cell Line and NK-92 Cell Line; MV – culturing in the presence of MV produced constitutively by cells of the NK-92 cell line; MV/TNFα – culturing in the presence of MV produced by cells of the NK-92 cell line as a result of the TNFα activation; NK-92/TNFα – TNFα-activated cells of the NK-92 cell line. The differences in the figure are shown as follows: a, difference ($p < 0.001$) from the constitutive death of K562; b, difference ($p < 0.001$) from the death while culturing K-562 + MV (400 μg/ml); c, difference ($p < 0.001$) from the death while culturing K-562 + NK-92; d, difference ($p < 0.001$) from the death while culturing K-562 + MV (400 μg/ml)/TNF; e, difference ($p < 0.01$) from the death while culturing K-562 + NK-92/TNF.

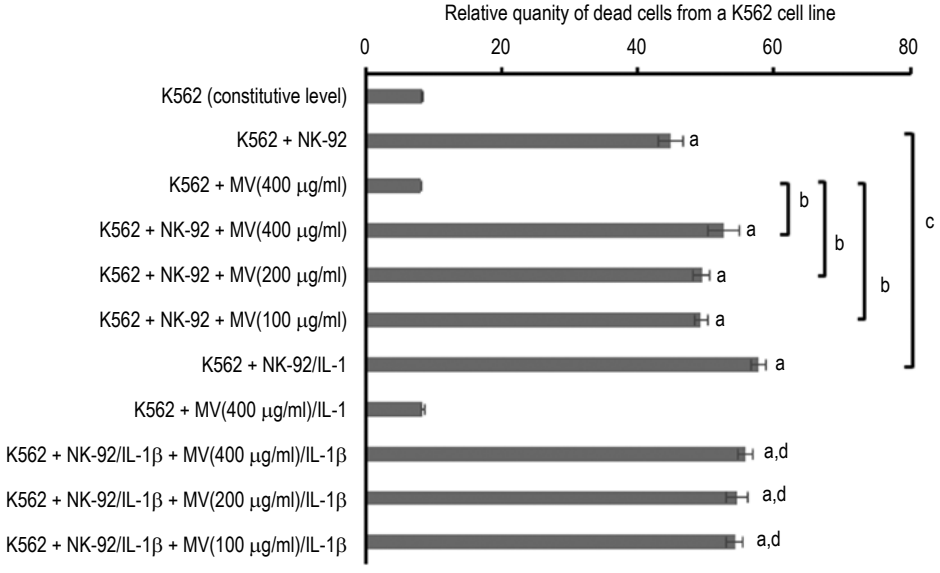

Figure 12. Cytotoxic activity of NK Cells of the NK-92 cell line towards cells of the K562 cell line in the presence of microvesicles and IL-1β

Note. Legend: K562 (constitutive level) – the quantity of dead K562 cells after the incubation without inductors; K562 + NK-92 – co-culturing of the K562 Cell Line and NK-92 Cell Line; MV – culturing in the presence of MV produced constitutively by cells of the NK-92 cell line; MV/IL-1β – culturing in the presence of MV produced by cells of the NK-92 cell line as a result of the IL-1β activation; NK-92/ IL-1β – IL-1β-activated cells of the NK-92 cell line. The differences in the figure are shown as follows: a, difference ($p < 0.001$) from the constitutive death of K562; b, difference ($p < 0.001$) from the death while culturing K-562 + MV (400 μg/ml); c, difference ($p < 0.001$) from the death while culturing K-562 + NK-92; d, difference ($p < 0.001$) from the death while culturing K-562 + MV (400 μg/ml)/IL-1β.

Analysis of cytotoxicity in both cells of the NK-92 cell line and microvesicles produced by them towards cells of the K562 cell line

It has been established that TNFα and IL-1β cytokines amplified the cytotoxic activity in NK cells of the NK-92 cell line towards cells of the K562 cell line. This coincides with the literature data [18, 26, 27, 53, 62]. MV obtained from unstimulated cells or activated cells of the NK-92 cell line had no effect on the K562 cell line death. MV obtained from unstimulated cells of the NK-92 cell line had no effect on the cytotoxicity of unstimulated cells of the NK-92 cell line. On the contrary, the cytotoxicity of TNFα-activated cells of the NK-92 cell line in the presence of MV obtained from the same TNFα-activated cells of the NK-92 cell line was higher than their cytotoxicity without MV (Figure 11). The analysis of the NK-92 cell line cytotoxic activity towards the K562 cell line showed that MV obtained from unstimulated cells or IL-1β-activated cells of the NK-92 cell line had no effect on the NK-92 cell line cytotoxic activity (Figure 12).

Discussion

To study the MV produced by NK cells, we used the NK-92 cell line (ATCC, USA). The NK-92 cell line represented the main phenotype and functional characteristics of activated NK cells [19, 28]. The use of the cell line introduced specific restrictions in the extrapolation of the results obtained in the in vivo situation. However, due to the inability to extract enough NK cells from the peripheral blood using a cell sorter, we had to use the NK-92 cell line in order to obtain a large number of MV for the study. The granulometric analysis of MV produced by NK cells of the NK-92 cell line showed that their sizes varied from 190.1 nm to 458.7 nm, while most of MV were 295.3 nm in size. These data complied with the information on sizes of MV produced by various cells [17, 60, 70]. The data obtained by atomic force microscopy method indicated that the major population of microvesicles lay in the range of 196-392 nm in size. The transmission electron microscopy of MV produced by the NK-92 cell line allowed to identify the spherical objects with a diameter of 150-500 nm, which corresponded to the data obtained during the granulometric analysis and atomic force microscopy. It is worth noting that the images obtained using TEM coincided with those presented in the world scientific literature [51, 71]. For example, the Figure 5C shows a typical cup-shaped structure. It was considered a native one for the extracellular vesicles for a long time, but cryoelectron microscopy data showed that this form is a consequence of sample preparation for TEM [71].

The analysis of phenotype of NK cells of the NK-92 cell line in unstimulated state demonstrated that almost all cells expressed CD11a, CD11b, CD11c, CD18, CD119, CD54, CD95, CD56 with different levels of intensity. These data coincide with the literature data [19, 36, 56, 77, 79, 80]. The activation of NK cells of the NK-92 cell line by inducers resulted in their phenotype alteration: TNFα and IL-1β stimulated the CD54 and CD95 expression, IL-1β decreased the CD18 expression, IFNγ decreased the CD11b expression, PMA increased the CD11c, CD54, CD95, and CD119 expression and decreased the CD56 expression. At that, most of the specified data on constitutive and induced expression of receptors by NK cells coincide with the literature data [16, 36, 59].

It should be noted that changes in the phenotype of NK cells of the NK-92 cell line in the presence of inducers had little effect on the phenotype of MV produced by these cells. NK cells differed from MV in the number of CD54, CD18 and CD11b receptors. The analysis of MV phenotype showed that, unlike the source cells, 33% of MV carried the CD119 marker. Other markers were expressed on less than 30% of MV. The abovementioned analysis also showed that, unlike the source cells, the number of double-positive MV was always about two times less than the number of mono-positive MV. Despite the fact that MV formation is an energy-dependent process accompanied by the mobilization of intracellular Ca^{2+} and the activation of proteins of the Rho/ROCK-dependent signal pathway regulating the dynamics of actin microfilaments in a cell, our findings concerning the phenotype of cells of the NK-92 cell line and MV produced by them provide evidence of the accidental inclusion of the analyzed surface proteins into a MV membrane. At the same time, small changes in the phenotype of MV produced by NK cells of the NK-92 cell line in the presence of inducers were accompanied by a rapid change in the amount of proteins contained in both the cells and MV produced by them. Thus, it has been established that the amount of proteins in cells increased in the presence of the inducers used by us. The analysis of protein content in MV showed that the protein content in MV doubled, when cells of the NK-92 cell line were cultured in the presence of PMA; that the protein content in MV increased fourfold, when cells of the NK-92 cell line were cultured in the presence of TNFα; and that the protein content in MV decreased 1.5-fold, when cells of the NK-92 cell line were cultured in the presence of IL-1β (compared to constitutive levels). This can be connected with changes in the protein amount and composition in MV and with changes in the expression level of some other non-analyzed receptors on the surface of MV. Previously, it was demonstrated that the cell culturing in the presence of inducers could not only increase the number of MV produced by a cell [35], but also alter the MV composition depending on the inducer type [4].

We analyzed changes in perforin and GrB content in cells of the NK-92 cell line and MV produced by them in the presence of PMA and proinflammatory cytokines TNFα and IL-1β. Western Blot analysis requires a large amount of proteins for detection, which, in turn, requires the accumulation of source cells when working with MV. Therefore, considering that IFNγ, as well as TNFα and IL-1β, refers to proinflammatory cytokines, we decided not to evaluate the effect of IFNγ on the content of perforin and GrB in cells of the NK-92 line and in MV produced by them by Western blotting method. We established that, depending on the inducer type (PMA, TNFα, IL-1β), the content of perforin and GrB changed in cells and in MV produced by them. Thus, despite the fact that the content of GrB and perforin in cells of the NK-92 cell line did not change in the presence of TNFα, their content in MV changed variously: the expression of GrB increased, but the expression of perforin decreased. In the presence of IL-1β, the expression of GrB increased and the expression of perforin decreased in cells of the NK-92 cell line. However, the expression of GrB decreased and the expression of perforin increased in MV produced by cells of the NK-92 cell line. In the presence of PMA used as an inducer, the expression of GrB and perforin increased in cells of the NK-92 cell line, and only perforin content increased in MV. Our findings concerning the production of MV with various GrB and perforin content by NK-92 cells in the presence of different types of inducers (PMA, TNFα, IL-1β) provide evidence of the existence of specific mechanisms in a cell that regulate the MV contents. This enables further discussions of the issues related to the regulation of such processes.

We used the procedure of MV isolation from the supernatant of cultured cells through differential centrifugation. The death rate of unstimulated cells and activated NK cells of the NK-92 cell line in the culture not did not exceed 6-8%. This cuts the likelihood of the contamination of our MV sample with cytoplasmic secretory granules. To confirm this suggestion, we used the BD FACS Aria III flow cytometer sorter and isolated a fraction of MV carrying CD119 and/or CD11b from the MV sample obtained by differential centrifugation. Thus, we obtained a sample containing a pool of MV expressing any of these markers on their surface. This approach was required to increase an output of MV positively expressing a surface marker to perform the further analysis by Western blotting method that confirmed the GrB presence in the obtained fraction of MV. However, the question concerning the state, in which GrB is included in these MV, remains open: within a secretory granule in its active state, within MV as an inactive serpin-proteinase complex, or as an active free protein? Previously, it was demonstrated that large MV could include both mitochondria and granules [65], which supported the first suggestion. Currently, the question concerning the way of GrB delivery into the target cell cytoplasm remains open. Besides the classical theory of cell cytolysis considered to be proved and stating that perforin creates a pore in the cytoplasmic membrane and GrB enters the cytoplasm through this pore [34, 72], there was another theory stating that perforin and GrB were endocytosized by the cell, then perforin created a pore in the endosome membrane, and GrB entered the cytoplasm through this pore and initiated the apoptosis [22, 37, 55]. We carried out the analysis of cytotoxic activity of cells of the NK-92 cell line towards cells of the K562 cell line. The results revealed that MV obtained from unstimulated or IL-1β-activated cells of the NK-92 cell line had no effect on the cytotoxic activity of cells of the NK-92 cell line. This fact coincides with our findings concerning the decreased GrB content in MV obtained from IL-1β-activated cells of the NK-92 cell line. However, MV obtained from TNFα-activated cells of the NK-92 cell line increase the cytotoxicity of TNFα-activated cells of the NK-92 cell line compared to their cytotoxic activity towards cells of the K562 cell line without MV. This coincides with our findings concerning the increased GrB content in MV obtained from TNFα-activated cells of the NK-92 cell line. This fact proves that NK cells have an additional pathway to deliver a cytotoxic signal to a target cell through MV containing GrB. Our findings concerning the increase of the cytotoxic activity of activated NK cells by microvesicles obtained from activated cells of the NK-92 cell line coincide with the data on the cytotoxicity of extracellular vesicles derived from activated human natural killer cells published by Jong A.Y. et al. [25] in 2017.

Conclusion

To sum up the obtained information, it should be noted that MV produced by NK cells of the NK-92 cell line contain perforin and GrB. Depending on the inducer type, NK cells of the NK-92 cell line produce microvesicles that differ in their composition, but hardly differ in their phenotype. Any changes in MV composition can result in changes in their functional activity: in particular, changes can increase the cytotoxic activity of NK cells of the NK-92 cell line. Thus, besides a well-known and proved way for GrB delivery to a target cell, we can suggest an additional way – the transportation of GrB within MV.

Authors' contributions

Dmitriy I. Sokolov, Kseniya L. Belyakova, Valentina A. Mikhailova, Larisa P. Vyazmina, Yulia P. Milyutina, Anastasiya R. Sheveleva, Anna A. Zhdanova, Darja A. Malygina, Kirill V. Onokhin, Alexandra N. Ivanova, Andrey V. Korenevsky, Sergey A. Selkov contributed equally to this work.

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Поступила 07.05.2019
Принята к печати 27.05.2019

Received 07.05.2018
Accepted 27.05.2019