**MALDI-TOF MASS SPECTROMETRIC PROTEIN PROFILING OF MICROVESICLES PRODUCED BY THE NK-92 NATURAL KILLER CELL LINE**

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**Introduction**

Microvesicles (MVs) are subcellular structures that are shed from the plasma membrane and may participate in intercellular communication. MVs transfer various proteins, nucleic acids, lipids, and sugars from cell to cell and are involved in the regulation of various biological processes, including angiogenesis, placentation, regeneration, and malignancy [18].

Of particular interest among various MV sources are natural killer (NK) cells, which are a lymphocyte subpopulation that carries out contact cytolysis of virus-infected and tumor cells, and exerts a regulatory function. There is indirect evidence that NK cells are able to produce MVs, since the cells with the CD56 phenotype, as well as those of leukocyte origin with different phenotypes, were found in peripheral blood plasma [29].

The cytotoxic activity of exosomes derived from activated peripheral blood NK cells against various tumor cell lines has been shown in a number of studies devoted to the isolation and description of various extracellular MVs released from NK cells. According to the authors, this cytotoxicity is exerted due to Fas ligand molecules being expressed on their surface [16], as well as effector molecules, namely perforin [8, 16], granzymes, and granulisin, being transferred [8]. The authors of these studies consider promising the use of extracellular MV preparations obtained from NK cells in the treatment of various tumors.

In the above studies, activated peripheral blood NK cells were mainly used, however, the use of their MVs for immunotherapy is fraught with considerable difficulties, among which are the search for suitable donors and the isolation of a sufficient amount of biomaterial [16]. The use of MVs obtained from the NK-92 cell line (NK-92 cells) for this purpose seems more promising due to the ease of their preparation in large quantities and the severity of their cytotoxic effect [16].

Facing the potential prospect of using NK-92 cell-derived MVs for delivery of antitumor drugs and regulatory molecules to target cells [36], the study of functional properties, phenotype, and biochemical cargo of these MVs seems to be an important area of research, which will possibly allow evaluating both positive and negative effects of such therapy.

Cytotoxic proteins of NK cells (Fas ligand, perforin, granzymes, granulisin, and LL-37 polypeptide) play a key role in the implementation of their cytolytic functions [7, 8, 11, 15-17, 26]. The protein profiling of MVs capable of contributing to the cytotoxic effect of NK cells against target cells is one of the important steps in understanding the distant communication of cells and the mechanisms of its regulation. Data to be obtained on the proteomic composition of the NK cell-derived MVs would allow assessing previously unknown mechanisms of interaction between NK and target cells under physiological and pathophysiological conditions.

Earlier, using two-dimensional gel electrophoresis coupled with Q-TOF mass spectrometry, we identified a number of proteins in MVs released from NK-92 cells, among which were found granzyme A, heat shock proteins, components of the ubiquitin-proteasome system, protein biosynthesis and energy metabolism enzymes, nuclear and serum proteins, and cytoskeleton proteins [12]. Given the limitations of our own data obtained, as well as the incompleteness of information on the proteome of the source cells [15, 17, 26], this study was aimed at expanding the existing knowledge of the proteomic profile of the MVs. To undertake this, MALDI-TOF mass spectrometry was used to identify tryptic peptides in protein fractions obtained from NK-92 cell-derived MV lysates by isoelectric focusing in the liquid phase.

**Materials and methods**

**Cells and cell culture**

The cells of the NK-92 cell line (American Tissue Culture Collection, USA), obtained from large granular peripheral blood lymphocytes of a 50-year-old man with rapidly progressive non-Hodgkin's lymphoma, reproduce the main phenotypic and functional characteristics of activated NK cells. The NK-92 cell line is a suspension culture, requiring subculturing every 48 hr. The cells were cultured spontaneously using the complete cell culture medium based on the α-modification of the Minimum Eagle medium (α-MEME; BioloT Ltd., Russia) supplemented with 12.5% inactivated fetal calf serum, 12.5% inactivated donor horse serum (both depleted of their own MVs using membrane filters with a pore diameter of 0.1 μm), 0.2 mM myoinositol, 0.02 mM folic acid, 2 mM L-glutamine, 100 μg/mL streptomycin, 100 IU/ml penicillin, 10 mM HEPES buffer solution, 0.1 mM 2-mercaptoethanol (Sigma-Aldrich Chem. Co., USA), and 500 IU/ml recombinant IL-2 (Roncoleukinum; Biotech Ltd., Russia). The cells were cultured using standard cell culture procedures under the damp atmosphere at 370C and 5% CO2. Using the trypan blue solution, the cell vitality was evaluated, which was not less than 96%.

**Isolation of biomaterial**

Due to no single standard protocol available for the isolation and characterization of MVs, various methodological approaches are currently used to obtain MV fractions with a proper degree of purity and enrichment. The MVs separated from NK-92 cells were isolated by the modified step-wise centrifugation method [29] in Hanks's solution without Ca2+ and Mg2+ (Sigma-Aldrich Chem. Co., USA), for which the supernatants were sequentially centrifuged at 200g (room temperature, 10 min) and 9,900g (40С, 10 min). The pellet after the second centrifugation was washed twice with cold phosphate buffer solution (PBS; Sigma-Aldrich Chem. Co., USA) and recentrifuged at 19,800g (40С, 20 min). The supernatant was discarded, the pellet being washed several times with cold PBS, each time precipitating the MVs by centrifugation at 19,800g (40С, 20 min). The purified pellet was resuspended in MilliQ deionized water, with the protease inhibitor mixture (cOmplete, EDTA-free; Roche Diagnostics GmbH, Germany) added at the concentration specified by the manufacturer, and was then stored at –800С until being analyzed. This protocol allows isolating MVs with a diameter of 100-200 nm with sufficient purity and minimal losses of the biomaterial, while the MVs are sequentially separated from coarse particles of cellular debris and large apoptotic bodies, as well as from exosomes.

**Laser correlation analysis**

The granulometric analysis of the MVs released from NK-92 cells was performed by the dynamic light scattering method described elsewhere [12]. The MV diameter was calculated using Zetasizer Software 7.11 (Malvern Instruments, UK). The dimensions of the MVs were shown to lie in the 190-460 nm range corresponding to the diameter of ectosomes (100-1000 nm), the peak size being equal to 295 nm (40.8%, n=3). The data obtained by us are consistent with the results of other researchers who evaluated the size of MVs produced by different cells [32]. Besides, the analysis of the supernatant obtained after the MV isolation showed that sizes of remnant particles lay in the range of 15-190 nm, the peak size being equal to 28 nm (less than 30%, n=3).

**Biomaterial preparation**

The frozen MVs were thawed and subjected to repeated “freeze-thaw” cycles five times, and were then intensively homogenized in a glass homogenizer for 5 min. The debris was removed by centrifugation at 16,000g (40С, 10 min), and the supernatant collected for further investigation.

**Spectrophotometric analysis**

The analysis of the MV protein content was performed through the Bradford protein assay using the NanoDrop One spectrophotometer and NanoDrop One Viewer software (Thermo Scientific, USA).

**Isoelectric focusing**

To separate the MV proteins, the isoelectric focusing method in the liquid phase was used. This method was performed on an OFFGEL High Resolution IPG strip (24 cm) with the immobilized pH 3-10 gradient in the 3100 OFFGEL Fractionator chamber (Agilent Technologies, USA) under active rehydration and subsequent separation at 200-3,400 V (20⁰C, 24 hr). The strip was loaded with the MV lysate so that the protein content (150 μg) in the strip was sufficient to obtain valid data. The analysis was performed under denaturing conditions in accordance with the manufacturer's protocol (Agilent Technologies, USA). For isolation from the environment, the strip in the chamber was covered with mineral oil.

**MALDI-TOF mass spectrometric analysis**

After isoelectric focusing, the protein fractions were mixed with ice-cold acetone (“extra pure grade,” Khimmed Ltd., Russia) (3:1, *v*/*v*) and were incubated at –20⁰С for 30 min. The precipitated proteins were then centrifuged at 15,000g (40C, 10 min), with the obtained supernatant discarded and the precipitates washed with a cooled acetone-water mixture (4:1, *v*/*v*), followed by being recentrifuged and dried up in the air at room temperature until the smell of acetone was gone. Thereafter, 10 μl of modified bovine trypsin solution (Promega, USA) were added to the dried samples at a concentration of 20 ng/ml and incubated first for 1 hr on ice and then for 16-18 hr at 37⁰С. Mixtures of tryptic peptides in the solutions were dried up in the air at 40C and were then dissolved in 50 μl of 50% acetonitrile-water solution (Sigma-Aldrich Chem. Co., USA) containing 0.1% trifluoroacetic acid (Sigma-Aldrich Chem. Co., USA). The contents of the tubes were thoroughly mixed on a Vortex shaker until completely dissolved. The resulting solutions were applied to standard steel target plates for MALDI analysis based on the following protocol: 2 × 0.5 μl of the matrix solution and 5 × 0.5 μl of a protein sample solution (in order to concentrate it on the substrate as much as possible). The 2,5-dihydroxybenzoic acid solution at a concentration of 10 mg/ml in 10 mM sodium chloride (Sigma-Aldrich Chem. Co., USA) was used as the matrix. The mixtures were dried up in the air.

MALDI-TOF mass spectra of tryptic peptides were acquired on the Axima Resonance MALDI mass spectrometer (Shimadzu/Kratos Analytical Ltd., UK) in the range of 200-3000 m/z with mass accuracy of all measurements within 0.01 m/z unit, selecting the laser power which is optimal for achieving good results. The measurements were carried out in the positive ion mode.

Proteins were searched against the UniProt (SwissProt) database (https://www.uniprot.org) and the NCBI database (https://www.ncbi.nlm.nih.gov) with a taxonomic restriction for the species *Homo sapiens* using the Mascot search engine (www.matrixscience.com) by peptide mass fingerprinting. Parallel search was performed using the database of inverted and random amino acid sequences (decoy). After the peptides were identified, the determined proteins were checked for their compliance with their actual positions on the strip.

**Functional analysis**

The established proteins were divided into groups depending on molecular function, cellular component, and biological process. It should be noted that such a division is largely arbitrary, since many of the proteins we have discovered show multiple functions in the cell, and in most cases, it corresponds to the division principle accepted in the SwissProt and NCBI databases.

The functions of proteins and their positions in the cell were also determined using the GeneGO database with the algorithms of the DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov>). The resulting list of all functional groups, along with the data on the number of proteins in each group, was further processed by REVIGO ([http://revigo.irb.hr](http://revigo.irb.hr/)), which simplifies long GO term lists by clustering similar groups and avoids the distribution of the same protein in different groups. Meanwhile, the identified proteins were compared with the data of MV proteomes from different sources using the EVpedia database algorithms (http://evpedia.info).

**Results**

The total protein content in the MVs produced by NK-92 cells was found to be 2.5±0.30 μg/106 source cells. The data obtained subsequently allowed calculating the protein load of the strip for isoelectric focusing to obtain valid results.

During the isoelectrophoretic separation of the MV proteins, 24 fractions were obtained in the pH range from 3 to 10 with a step between fractions of approximately 0.3.

The MALDI-TOF mass spectrometric analysis determined a total of 986 proteins having a variety of functions (see Supplement).

Thus, the largest group of the proteins identified is represented by hypothetical proteins, proteins with unknown functions, and domains (117 entries). The most representative groups (comprising more than 40 proteins each) are also formed by transcription regulators; intracellular signaling proteins; RNA translation, transcription, processing, and utilization regulators; receptors; protein processing and proteolysis regulators; amino acid metabolism enzymes, as well as transport proteins and transport regulatory proteins.

Minor functional groups (including less than 10 entries each) are represented by vitamin metabolism and mineral metabolism enzymes, membrane microdomain proteins, hormones, hemostatic regulators, sensory system proteins, specific mitochondrial and Golgi apparatus proteins, and intercellular signaling proteins.

The intermediate position (from 10 to 40 entries in each group) is occupied by various functional groups, including cytoskeleton and motor proteins; centriole proteins; ion channels and their regulators; proteins of the ubiquitin-proteasome system; lipid, steroid and fatty acid metabolism enzymes; nucleic acid base metabolism enzymes; carbohydrate metabolism enzymes, as well as energy metabolism enzymes and other proteins involved in intermediate metabolism; proteins of the immune response and inflammation; antigens and histocompatibility complex proteins; cytokines and growth factors; regulators of apoptosis, autophagy, endocytosis, and exocytosis; cell cycle and division regulators; cell proliferation and differentiation regulators; developmental proteins; cell adhesion and matrix proteins; nuclear transport proteins; transposition proteins; DNA replication and repair proteins, as well as inactive proteins.

The identified proteins were also distributed into functional groups by the DAVID GO analysis algorithm, the proteins showing a number of functions in the cell appearing simultaneously in several groups. The resulting list of 654 functional groups was further reduced using REVIGO. Thus, the most common groups were obtained, being distributed by molecular function (Fig. 1a), cellular component (Fig. 1b), and biological process (Fig. 1c).

**Figure 1a. Functional groups of proteins obtained from lysates of microvesicles produced by the NK-92 natural killer cell line: molecular function (\**P* < 0.05, \*\**P* < 0.005, \*\*\**P* < 0.001; DAVID 6.8 and REVIGO).**

**Figure 1b. Functional groups of proteins obtained from lysates of microvesicles produced by the NK-92 natural killer cell line: cellular component (\**P* < 0.05, \*\**P* < 0.005, \*\*\**P* < 0.001; DAVID 6.8 and REVIGO).**

**Figure 1c. Functional groups of proteins obtained from lysates of microvesicles produced by the NK-92 natural killer cell line: biological process (\**P* < 0.05, \**P* < 0.005, \*\*\**P* < 0.001; DAVID 6.8 and REVIGO).**

The analysis of the identified proteins distributed by molecular function showed that the bulk of them is involved in protein (66%) and carbohydrate derivative (16%) binding, while the minor components altogether account for only 18% and include anion, carboxylic acid, magnesium ion, SNARE, syntaxin, GDP, and modified amino acid binding, as well as transferase activity, transferring acyl groups other than amino-acyl groups, and anion channel activity (Fig. 1a).

The distribution of proteins by cellular component showed that the most representative (7-20%) groups include proteins of the intracellular part, organelles and membrane-bounded organelles, cytoplasm, nucleus, and macromolecular complex, while proteins of the mitochondrial part, entire membrane, and membrane protein complex, as well as anchoring and adherens junctions, are minor components, which account for only 9% of the total number of the identified proteins (Fig. 1b).

The functional groups distributed by biological process were found to be uniform, though. There are approximately equal proportions (10-14%) in the groups of proteins responsible for the cellular developmental and single-organism metabolic processes, and regulation of molecular function and macromolecule organization. The groups of proteins with slightly smaller fractions (6-9%) are represented by molecules involved in the regulation of catalytic activity, organonitrogen compound and phosphate metabolic process, and biological adhesion, as well as the regulation of transport and vesicle-mediated transport (Fig. 1c).

Additionally, DAVID Functional Annotation Clustering analysis of the identified proteins was performed, combining similar functional groups into one cluster (in this case, all the proteins were counted, but not totalized). As in the case of the distribution by functional groups, the most common groups of the proteins were selected for the subsequent analysis, being distributed by molecular function (Fig. 2a), cellular component (Fig. 2b), and biological process (Fig. 2c).

**Figure 2. Most common clusters of proteins obtained from lysates of microvesicles produced by the NK-92 natural killer cell line: a. Molecular function; b. Cellular component; c. Biological process (percentage of the total number of identified proteins; \**P* < 0.05, \*\**P* < 0.005, \*\*\**P* < 0.001; DAVID 6.8).**

In contrast to the clustering of GO terms by molecular function (Fig. 2a), which did not reveal significant discrepancies with the results of functional analysis (Fig. 1a), cluster annotation analysis of the identified proteins based on information about their biological function (Fig. 2c) revealed 1.5-2.0-fold higher fractions of the functional groups and even higher ones exceeding the data of functional analysis by 3.5-4.0 times, when the proteins were analyzed based on cellular component (Fig. 2b).

An analysis performed using the EVpedia database algorithms compared the currently known MV proteomes (produced by mesenchymal bone marrow stem cells, cerebral endothelial cells, glioblastoma, hepatocarcinoma, and blood platelets) with the data obtained in our study (Fig. 3). Thus, we were able to obtain another set of functional groups of the identified proteins that were significantly (*P* < 0.001) better represented in the MVs released from NK-92 cells.

**Figure 3. Specific functional groups of proteins obtained from lysates of microvesicles produced by the NK-92 natural killer cell line (*P* < 0.001; EVpedia).**

It was shown that the main difference between the MVs produced by NK-92 cells and other sets of MVs was in metabolic peculiarities and cellular component organization or biogenesis (400-788 entries). The next most significant difference was the increased number of proteins involved in developmental process, cellular response to stimulus, multicellular organismal, and cell communication (227-355 entries). The smallest difference was revealed in the regulation of biological process, molecular function, and catalytic activity (98-139 entries).

**Discussion**

NK-92 cells used by us allow *in vitro* analyzing the main biochemical processes that occur in NK cells *in vivo*, including those leading to spontaneous secretion of MVs into the extracellular space. The data obtained using MALDI-TOF mass spectrometry indicate that the MVs produced by NK-92 cells are characterized by a wide range of proteins with various functions and properties, providing the possibility of multilateral regulation of cell metabolism.

In the last 10-15 years, international research teams studied in detail the proteome of NK cells with the CD56dim or CD56bright phenotype [15, 17, 26]. However, the most detailed of them only mentions approximately 3,000 proteins [26], which is significantly less than the number of proteins contained in any eukaryotic cell. That strongly indicates the incompleteness of the NK cell proteome described to date. Nevertheless, the main functional groups of proteins presented in the above work matched those found in our study. However, from the list of proteins presented in that work, only 120 entries (4.1%) matched those found by us in the MVs derived from the source cells. Meanwhile, the remaining 869 entries of the proteins identified by us in the MVs give a piece of new information on the NK-92 cell proteome.

In concordance with the biogenesis pathways of the studied MVs, their proteome constitutes a set of protein molecules of cellular origin. Proteomes of MVs produced by platelets, mature lymphocytes, endotheliocytes, mast and some other types of cells are currently studied, and data from these studies are presented by a number of authors [9]. Thanks to these works, it is known that in MVs derived from blood and vascular cells, there are both non-specific proteins characteristic of any type of cells and specific proteins involved in the functioning of a particular cell. Proteins specific for cells of a certain type are, as has been shown, a T-cell receptor expressed primarily on T-lymphocytes, platelet P-selectin, and other proteins capable of being involved in the immune response. Regardless of their cellular origin, MV proteins, are most often involved in the formation of vesicles. Undoubtedly, such proteins are tetraspanins (CD9, CD63, CD81, and CD82), heat shock proteins (HSP70, HSP90), cytoskeletal elements, enzymes of various metabolic pathways, adhesion molecules, as well as proteins of the main histocompatibility complex (MHC) [30].

In the MVs studied by us, proteins belonging to the above classes were also found, in particular tetraspanin-32, chaperones, structural and motor proteins, numerous proteins involved in metabolism (those forming the cytoskeleton, along with enzymes involved in various metabolic pathways, constituted a more significant part of the identified proteome), alpha-catulin, cadherins, liprin, as well as proteins associated with the MHC. Besides, we identified some specific proteins, in particular those responsible for the implementation of the defense mechanisms, chemokines and cytokines, as well as their receptors, semaphorins, defensins, collectin, ficolin, interferon β, TGFβ, TNF ligands, growth factors, regulators of apoptosis (including granzyme A, a serine protease expressed in cytotoxic T-lymphocytes and NK cells), as well as specific nuclear proteins, which could not be quite expected in the studied MVs [34].

Among the proteins identified by us in the MVs produced by NK-92 cells, cytokines, receptors, and regulatory proteins (Table 1) should be especially distinguished for two reasons. First, most of them have been found in NK cells isolated from both tissues or peripheral blood and transplantable NK cell lines. Secondly, the expression of such proteins in the MVs can have a certain signaling or regulatory function in relation to target cells. For instance, the transfer of granzymes to target cells as part of the MVs can enhance the cytotoxicity of NK cells [28]. Cytotoxic protein granzyme A being found in the studied MVs can be accounted for by the fact that in the cell, granzymes are densely packed in membrane lytic granules, which then enter the MVs either in whole or in part and thus can participate in the attack on target cells.

**Table 1. Some functions of proteins of microvesicles produced by the NK-92 natural killer cell line.**

We also identified galectin-3 in the MVs released from NK-92 cells. It was shown earlier that this protein expressed by tumor cells increases their viability and proliferation, and helps them suppress the cytotoxicity of NK cells by binding to the nkp30 receptor [39]. It could be possible that NK-92 cells can thus increase their viability and regulate their cytotoxic activity using negative feedback. It is also worth noting that transmission of cell surface receptors (CCR2, CCR3, CCR7, CD215, CD49f, CD16a, CD150, THRA, CD100, and CD252) from the MVs to target cells, as a result of the membrane fusion, can underlie the emergence of new characteristics in the target cells and play a role in tumor metastasis.

The detection of the MHC class II antigens (DQ), as well as the α and β subunits of the T-cell receptor, in the MVs produced by NK-92 cells (Supplement) was unexpected, despite the fact that it had been previously shown that individual subpopulations of NK cells could express the MHC class II molecules and T-cell receptors [37]. We also obtained data on the detection in the MVs of proteins not previously described in NK cells, for instance, CCR3, CD150, CXCL10 (found in mouse NK cells [27]), CXCL11, interferon β, interleukin-7, interleukin-12 (Table 1), and some others (Supplement). These data must be validated later using the immunoblotting and (or) real-time polymerase chain reaction methods in both NK-92 cells and NK cells isolated from various tissues, as well as at different stages of differentiation of NK cells.

The data obtained by us on histones and histone-lysine N-methyltransferases, as well as a number of other specific nuclear proteins, represented in the studied MVs, indicate the presence of a small admixture of apoptotic bodies therein (or even MVs released from those apoptotic bodies), sizes of which (1000-5000 nm) can partially overlap with the MV dimensions (100-1000 nm) within the 1000 nm border region, rather than the presence of nuclear proteins in the fraction [32]. It should be noted that we succeeded in achieving reproducible results on granzyme A, histones, and a number of other proteins being identified in the studied MVs by two different methods of mass spectrometry [12].

There is literature data on granzymes being found in exosomes (40-150 nm) [16], while larger ectosomes mainly contain cytoplasmic isoforms of proteins. At the same time, there is evidence that large MVs may include other compartments and cell organelles [31]. Thus, the detection in the studied MVs of granzymes differentiated by size does not allow to unambiguously indicate the origin of these particles. In studying this issue, it might be useful to use, along with the approach presented by us, additional techniques, including the determination of specific exosomal proteins, for example, Alix, or, conversely, cytoplasmic membrane proteins. In this way, the question of the possible inclusion of other organelles or their parts in the MVs is still open.

The molecular mechanisms of MV formation suggest the inclusion therein of both components of the actin network adjacent to the cell membrane and other cytoskeletal elements. The data obtained using mass spectrometry suggest that the MVs isolated by us may have an ectosomal nature. Thus, we identified proteins of the actin-myosin and tubulin-dynein systems and regulators of the cytoskeleton dynamics (myosin and related factors ARF1, ARF3, and ARF4; tubulin, dynein, coronin, ezrin, gamma-parvin, profilin-3, rho-related GTP-binding protein RhoV, etc.) involved in MV formation and budding from the cell membrane [18]. Various isoforms of cytoskeletal proteins, as well as proteins of the mitochondria, endoplasmic reticulum, or Golgi apparatus that we identified, have not been found in exosomes according to the available literature [2, 4]. This assumption can also be attributed to the presence of a number of ion channels in the studied MVs. The presence of endosomal proteins therein (Ras-related proteins Rab-7a, -21, and -23; WASH complex, nexins, ubiquitin-conjugating enzyme E2 D3, clathrin, etc.) can be accounted for by the fact that the mechanism of MV formation is similar to that of the formation of intraluminal vesicles and the same proteins are involved in the direct transport of molecules in MVs. However, it was previously shown that the cytotoxic protein granzyme A identified by us in the studied MVs is contained in the lytic granules of NK cells and is inactivated after it enters the cytoplasm, forming a complex with serpin, which we also identified [11].

Other proteins commonly found in MVs include cytoplasmic glycolysis and protein folding enzymes. The matrix of the MVs derives from the cytoplasm, which accounts for the presence of these proteins therein.

Thus, the communication function can be performed by both the already listed proteins and other proteins involved in signal transduction (protein kinases and their activators, small GTPase activating proteins, Wnt proteins, etc.).

In addition to the proteins characteristic of the MV proteome, some proteins that cannot belong to the studied structures (chondroadgerin, endothelin, erythropoietin, neurospecific proteins, hemostatic regulators, sensory system proteins, etc.) were found in the samples, indicating their possible contamination. The various isoforms of cytoskeletal keratin identified by us may also indicate a contamination of the samples with cytokeratin, which is often detected by mass spectrometry in fractions that do not comply with its isoelectric point and molecular weight. Similarly, this protein has been found elsewhere by other researchers who analyzed a variety of biological material [22].

Functional and cluster annotation analyzes revealed the predominant molecular functions of the proteins identified in the MVs produced by NK-92 cells, and showed possible participation of these proteins in biological processes. Meanwhile, the comparison of the data obtained with known proteomes of other sets of MVs showed the distinctive features of the studied MVs.

To date, a number of good proteomic research strategies have been described that are based on a combination of different approaches and techniques. Unfortunately, protein identification is a rather complex multi-stage process and does not always lead to reproducible results. The scheme proposed in this study can lead to good results with a qualitative description of the protein profile of the MVs released from NK-92 cells. However, the search for the target protein using this approach causes considerable difficulties. For a more detailed analysis of the MV proteome using mass spectrometric methods, it would be advisable to include in the study additional preparation steps at the stage of the isolation of the MVs, such as ultracentrifugation and immunoprecipitation. It is also worth noting that Q-TOF mass spectrometry gives more accurate results, since it uses the MS/MS technique. At the same time, this method gives a lower protein entry yield than MALDI-TOF mass spectrometry.

**Conclusion**

The conducted study showed that the MVs produced by NK-92 cells, along with proteins universal for body cells, also contain granzyme A and other specific proteins of cytotoxic cells, with which these MVs contribute to the cytotoxic effect of NK cells. The data obtained can expand the understanding of the distant communication of cells and indicate a variety of interactions between NK and target cells, which are not limited to cytotoxic effects only. The presented results will be useful for further proteomic studies of MVs produced by cells involved in immunological tolerance, which is established both under physiological conditions and in the conditions of inflammation.

**Supplement. MALDI-TOF mass spectrometric proteome profiling of microvesicles derived from the NK-92 natural killer cell line (*P* < 0.05).**