Chapter 4

Stress-Induced Molecules in Regulation of NK Cell Activity

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1. Introduction

Natural killer (NK) cells are a heterogeneous multifunctional population of immune cells traditionally classified as cytotoxic lymphocytes of the innate immune system [1]. NK cells possess several key characteristics of innate immune cells. They are capable of rapid effector response without prior sensitization and express a range of germline-encoded receptors that do not need any additional rearrangement. On the other hand NK cells share some phenotypic and functional characteristics with cytotoxic T cells: they express common surface markers (CD2, CD7, CD8), recognize infected and tumor-transformed cells of host organism and also mediate the similar mechanism of granule-dependent cytolysis. NK cells are able to interact with major histocompatibility complex class I (MHC-I) self-molecules as distinct from other innate immune cells. Moreover, recent findings show that NK cells possess a form of immunological memory [2]. Thus, NK cells represent evolutionary and functional link connecting innate and adaptive immunity.

In the organism NK cells fulfill two main functions: they provide constitutive cytotoxic activity against infected, malignant and other damaged cells and regulate innate and adaptive immune reactions by production of chemokines and cytokines. At early stage of immune response NK cells are the main sources of IFN-γ, triggering the adaptive immune response [3]. NK cells interact with other cells of immune system, participating in contact-dependent costimulation. Primed dendritic cells (DCs) and macrophages produce a range of cytokines including IL-12, IL-15 and IL-18 which activate NK cells. Contact interaction of NK cells with these cells also induces macrophage and DC maturation [4, 5]. NK cells express receptors for many cytokines and chemokines and therefore might be influenced by other cells producing them [6].

NK cells are classically identified in humans as lineage-negative $CD3^-CD14^-CD19^-CD56^+$ leukocytes circulating in peripheral blood and residing in various organs and tissues of the

body. Two main subpopulations of NK cells based on the expression levels of CD16 and CD56 have been described [7]. Subpopulation $CD56^{dim}CD16^{+}$ is prevalent in peripheral blood. These cells express receptors of killer-cell immunoglobulin-like receptor (KIR) family and possess high levels of cytotoxic activity. Because they express CD16 ($Fc\gamma RIII$), $CD56^{dim}$ NK cells are able to recognize and lyze cells opsonized with IgG antibodies [8, 9]. $CD56^{bright}CD16^{-}$ NK cells consist of approximately 5-10% of peripheral blood NK cells; they reside mostly in secondary lymphoid organs, liver and uterus. $CD56^{bright}$ cells express CCR7 and CD62L (L-selectin) which allow them to migrate to the lymph nodes. They are considered to be a regulatory NK cell subpopulation because they are able to contact with DCs, have low cytotoxicity and high levels of cytokine production compared with $CD56^{dim}$ cells. Due to constitutitive expression of CD25 these cells can easily proliferate in response to IL-2 stimulation.

In general, functional activity of NK cells is regulated by fine dynamic balance between the activating and inhibitory signals providing by the interaction of receptors and co-stimulatory molecules with surface ligands of potential target cells, and depends also on the soluble factors produced by cellular environment [10, 11]. The density of interacting molecules on both effector and target cell surface is crucial for NK cell activity. Low or absence of inhibitory signals leads predominantly to NK cell activation through activating receptors followed by target cell lysis and cytokine release. On the other hand, high expression of activating ligands on target cells may trigger NK cell cytolytic activity, despite normal expression of MHC-I molecules [12]. The repertoire of activating and inhibitory receptors expressed on single NK cells is developed randomly during its maturation and differentiation [13]. The result of this expression is a diverse set of NK cell clones in the body. NK cells also express various adhesion and co-stimulatory molecules that participate in cell interaction and signaling. Diversity of NK cells allows them to identify damaged cells with distinct phenotypic characteristics and display functional response adapted to microenvironment.

NK cells express multiple inhibitory and activating receptors regulating their cytolytic activity. Receptors of the KIR family recognize MHC-I molecules expressed by most normal cells in the body. Interaction of inhibitory ITIM-bearing KIRs with MHC-I results in abrogation of activation. MHC-I expression on the surface of virally infected and tumor cells have often been shown to decrease significantly, making these cells “invisible” to cytotoxic T lymphocyte recognition [14, 15]. In contrast, NK cells are able to recognize and lyse such cells due to diminished inhibitory signals. It is important to note that KIRs are able to distinguish between groups of MHC alleles. In the case of transplantation, graft cells with MHC-I molecules differing from the host proteins can be rejected by host NK cells. Various activating receptors for the most part serve to recognize cell surface molecules whose expression indicates viral infection, tumorigenesis or cell damage caused by cellular stress. Activating receptors of natural cytotoxicity receptor (NCR) family including NKp46, NKp44 and NKp30 drive natural cytotoxicity against many tumor and virally infected cells upon signaling through linked ITAM-bearing CD3ζ, FcRγ, or DAP12 molecules [16, 17]. It is believed that these receptors have the ability to recognize molecules not expressed on the

surface of normal cells. In recent years, most of the ligands recognized by these receptors have been identified. NKp46 and NKp44 bind virus-derived hemagglutinins, and NKp30 is able to recognize human cytomegalovirus-encoded pp65 protein [18, 19]. The normally intracellular proteins vimentin and leukocyte antigen-B-associated transcript 3 (BAT3) are recognized by NKp46 and NKp30, respectively [20]. NKp30 binds a B7 family homolog (B7-H6) expressed by some tumor but not normal cells [21]. A group of heparan sulfate/heparin molecules has been also proposed as ligands for NCR receptors [22]. Another important activating receptor NKG2D recognizes self-molecules which are usually absent or expressed on low level on the surface of normal cells but become expressed in tumor transformation, infection or in conditions of cell stress. A number of highly polymorphic "self" stress-induced proteins such as MICA, MICB and a group of UL16 binding proteins (ULBPs) are ligands of NKG2D [23-25]. Appearance of these molecules on the cell surface serves for detection by NK cells of cell damaging alterations induced by oxidative stress, heat shock, hypoxia, genotoxic stress, virus infection or tumor transformation.

Heat shock proteins (HSPs) represent another group of endogenous stress-induced proteins recognized by NK cells on the surface of target cells. HSPs, normally presented in cytoplasm, can be found on cell surface, in particular on the surface of tumor cells, virus-infected cells and cells subjected to stress. Tumor and stressed cells can also release HSPs in the extracellular milieu. It has been shown that expression of HSPs, in particular HSP70, on the surface of tumor cells increased cytotoxic activity of NK cells towards the targets. However, the activating receptors of NK cells for these molecules have not been identified. Membrane associated HSPs and their extracellular pool may constitute danger-associated molecular patterns (DAMPs) in the context of the "Danger model" proposed by P. Matzinger [26, 27]. Detection of stress-induced molecules as "danger signals" in conjunction with self MHC-I recognition by multiple sensor system possessed by NK cells underlies the complex process of NK cell activity regulation, although a full range of activating ligands recognized by NK cells still remains unknown. Reactive oxygen species (ROS) produced by activated monocytes and neutrophils also affect on NK cell functionality. ROS action leads to lowering of NKG2D and NKp46 expression by NK cells, that should decrease their cytolytic ability. Histamine was shown to prevent this effect of ROS on NK cells. Interesting, that $CD56^{bright}$ NK cells are more resistant to ROS action: NKG2D expression is unchanged on these cells [28, 29].

Simultaneous interactions of various surface molecules of target cells with NK cells lead to the integration of different intracellular signals, which together dictate the quality and intensity of effector NK cell response. Small changes in the target cell surface molecular profile may significantly influence the susceptibility of the cells to NK cell action. Cell stress induces multiple changes in protein synthesis and intracellular localization, and influences oligosaccharide expression. Intracellular perturbations caused by some types of cell stress might influence cell death and survival processes and are in close connection with the process of tumor cell transformation.

Activity of NK cells may be influenced by both contact interactions with target cells and various soluble factors produced by surrounding cells under stress conditions, in particular

by extracellular pool of HSPs and MICA/B molecules. Besides stimulation of stress-induced protein expression, cell stress significantly modifies surface carbohydrate phenotype affecting NK cell effector functions. Membrane-associated oligosaccharides participate in NK cell – target cell interactions. Some of them have been identified to decrease NK cell cytolytic activity, others displayed stimulating effects on NK cells [30]. In this work we discuss our results obtained in the study of surface oligosaccharide influence on NK cell activation utilizing polymeric lipophilic neoglycoconjugates. We also present a study of involvement of surface and extracellular pools of HSP70 and MICA/B in regulation of human NK cells activity. The obtained results demonstrate that surface and soluble HSP70, as well as some fragments of this protein, activate NK cells in contrast to extracellular forms of MICA/B which inhibit cytotoxicity of NK cells. In addition, some characteristics of cell surface MICA/B expression in a model of ethanol-induced cell stress are discussed.

2. Oligosaccharides in regulation of NK cell activity

Oligosaccharides exposed on the plasma membrane of mammalian cells are an essential component of intercellular contacts inhibiting or cooperating with certain molecular interactions. Carbohydrate-carrying molecules also play a role in cell adhesion and signaling and contribute to immune cell activation or inhibition. Considerable changes in cell surface carbohydrate expression can be evoked by malignant transformation, infection or other unfavorable conditions and can be detected by NK cell recognizing system. Membrane-associated glycans by their structural diversity might provide fine tuning of NK cell – target cell interactions and regulate NK cell activity. Positive and negative effects of various carbohydrate compounds including sialo-oligosaccharides, glycosphingolipids, complex and high mannose-type N-glycans, and others on NK cell-mediated effector functions have also been reported [31-33]. However only in rare cases ligand-receptor signaling pathways underlying the saccharide effects were identified. Recently, inhibitory receptor Siglec-7 expressed by NK cells has been revealed to recognize specifically disaccharide Neu5Acα2-8Neu5Ac [34]. It has therefore been suggested that the NK cell recognition of the sialic residues contributes to their tolerance to self-tissues.

In our study we tested synthetic glycoconjugates designed to mimic the biological activities of native oligosaccharides for investigation of the effects of an array of oligosaccharides on NK cell activity. The lipophilic glycoconjugates were incorporated in target cells modifying carbohydrate phenotype of the plasma membrane, and NK cell cytotoxicity and cytokine production were assessed (Fig. 1).

We found that glycoconjugates containing Le^x, HSO_3Le^x and Le^y structural motifs enhanced NK cell-mediated cytolysis of K562 cells as measured by analysis of NK cell-mediated caspase 6 activation in these cells (Fig. 2). The same oligosaccharides exposed on the cell surface displayed a potency to stimulate IFN-γ production with the strongest effect revealed for Le^y-containing glycoconjugate. Only polymeric and not monomeric glycoconjugates displayed the abilities to increase NK cell activity. Le^x antigen expressed as a part of glycoproteins and glycosphingolipids on many hematopoietic cells is mainly involved in

intercellular adhesion. Both Le^x and Le^y are also considered to be tumor-associated antigens [35]. Not all donors exhibited NK cell sensitivity to these glycoconjugates. This suggests a role for individual genetic polymorphism in glycan receptor expression, and also other mechanisms where the expression of molecules interacted with glycans might be regulated in the human organism throughout life. Polymeric glycoconjugates also had more biological activity than monomeric constructs in this system, suggesting that appropriate presentation is critical for carbohydrate recognition and subsequent biological effects. The unique mode of clustered oligosaccharide presentation provided by polymeric glycoconjugates may effectively mimic carbohydrate recognition found in nature. Most carbohydrate-lectin and carbohydrate-carbohydrate bonds require multivalent carbohydrate interactions for proper adhesion and signaling. The possible scenarios of carbohydrate-mediated interaction NK cells with target cells include the intercellular adhesion strengthening or/and the specific NK cell recognition of the saccharide determinants. Interestingly, only polymeric glycoconjugates presented on the cell surface in appropriate microenvironment affected NK cell activity. Glycoconjugates presented alone had no effect.

Figure 1. Modification of K562 cells targeting by NK cells with polymeric glycoconjugates.

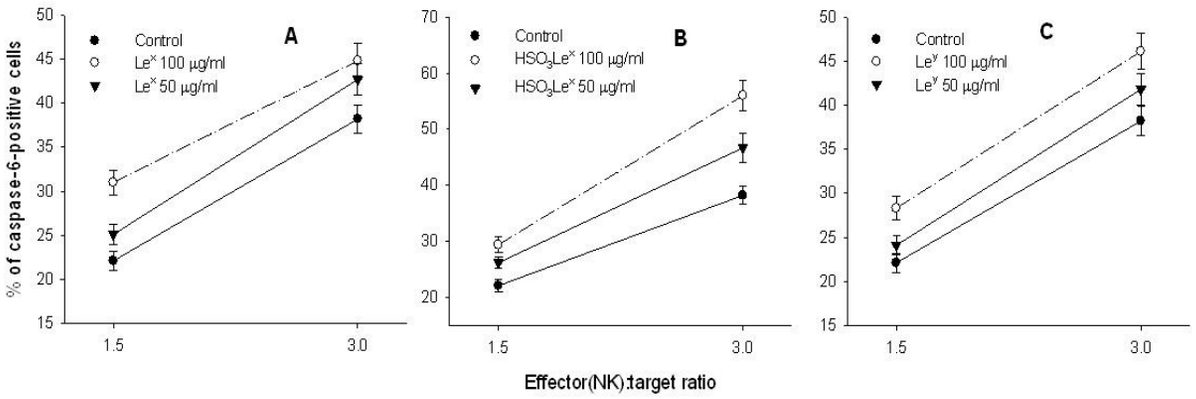

Figure 2. Effects of glycoconjugates containing (A) Le^x, (B) sulfated Le^x or (C) Le^y incorporated in target cell membrane on NK cell-mediated cytotoxicity.

Thus, a proper presentation mode of the oligosaccharides was an essential requirement for biological effects of glycans connected with carbohydrate adhesion and cell recognition. Recently suggested model of a "glycosynapse" involves membrane assembly of different types of glycosylated molecules interacting with carbohydrate partners [36]. According to this model, oligosaccharide effects depend on their clustering and proper orientation of

"glycotopes", and may result in a synergistic effect with protein-dependent interactions. Further investigations may discover additional physiological functions of glycans consisting of certain glycoproteins and glycolipids expressed in specific cell types.

3. Surface and exogenous HSP70 in NK cell – Target cell interaction

3.1. Involvement of membrane-associated HSP70 in NK cell – Target cell interaction

As was mentioned above, stress-induced proteins exposed on surface of cells interacting with NK cells can be recognized by NK activating receptors followed by cytotoxic attack toward target cells. This fact highlights an important role of cell stress in the process of NK cell – target cell interaction. It is known that cellular stress response to any form of damaging factor results in a wide range of protective intracellular processes directed toward cell survival. Of critical importance among these counteracting cell reactions is the induction of high levels of HSP expression. HSPs are highly conserved and ubiquitously expressed proteins that are predominantly located in the cytoplasm and function as molecular chaperones [37]. However, evidence has now accumulated that translocation of HSPs to the cell surface may occur in stressed, infected and transformed cells [38-40]. Although mechanisms of translocation remain unclear, it seems plausible that surface HSPs may play a role as molecular markers of "altered" cells, destined to be eliminated by cytotoxic lymphocytes [39, 40]. Some tumors produce large amounts of HSPs that are expressed on the cell surface and can potentially attract NK cells [41, 42]. Surface expression of inducible form of HSP70 on human lung carcinoma cells was associated with their increased sensitivity to lysis by NK cells [43]. Also, IL-2 activated human NK cells were shown to recognize HSP70 on the surface of K562 erythroleukemia and human sarcoma cells subjected to heat shock [44-46]. Mechanisms by which surface HSPs may mediate recognition and elimination of tumor by cytotoxic lymphocytes remain poorly understood. One possibility is that immunogenic peptides expressed in association with HSP70 and HSP90 on tumor cells can be recognized by T lymphocytes, as well as NK cells [44, 48]. Another possibility is that HSPs themselves, e.g. HSP60 and HSP70 on the surface of tumor cells can be recognized by γδ-T cells in a non-MHC restricted manner [49]. Thus, the available evidence strongly suggests that surface HSPs, in particular HSP70, may be important in mediating recognition and/or elimination of tumor cells by host lymphocytes. Previously, we have shown that culture-adapted EL-4 mouse lymphoma lines express surface HSPs [37]. This fact was accounted for selection of EL-4 cell line in our *in vitro* mouse model aimed at investigation of a role of target cell surface HSP70 in tumor recognition and activation of cytotoxic effector cells. In this study we used EL-4 cells to analyze involvement of surface HSP70 of the target lymphoma cells in interaction with cytotoxic immune effectors.

For these experiments C57BL/6 (H-2^b), CBA (H-2^k) and Balb/c (H-2^d) mice were used. Cytotoxic effector cells were obtained from mouse spleen using standard methods [50]. The cytotoxic effectors were obtained either from intact mice or mice inoculated with

EL-4 lymphoma. In the latter case, C57BL/6 mice were inoculated intraperitoneally with $1x10^6$ EL-4 cells, and splenic effector cells were isolated 10 days after inoculation. Mouse EL-4 (H-2^b) lymphoma cells were maintained *in vitro* by passages in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, 20 mM HEPES, 10^{-5} M 2-ME and 50 μg/ml gentamicin. For flow cytometric analysis FITC- or PE-conjugated anti-CD8, anti-CD4, anti-CD3, anti-γδTCR and anti-NK-1.1 mAb were used for cell staining. Fluorescent and laser scanning confocal microscopy was used to confirm surface localization of HSP70 in EL-4 cells stained with anti-HSP70 antibody (clone BRM22). MTT- and LDH viability assays [51, 52] were applied to evaluate cytotoxic effect of the effectors in the *in vitro* models. To determine whether expression of MHC class I molecules and HSP70 on the surface of EL-4 cells plays a role in cytotoxic response against these targets, we performed antibody blocking assay using following mAb: anti-MHC class I (clones HB11 and HB51), anti-HSP70 (clone BRM22), or anti-Thy-1.2, as a control (clone 53-2). To remove $CD8^+$ cytotoxic T lymphocytes from the effector cell population a magnetic bead separation method was used (Dynal, Germany).

At the first stage of the study the surface localization of HSP70 on EL-4 cells registered previously by flow cytometry was confirmed with confocal microscopy (Fig. 3).

Splenic effector cells from normal C57BL/6 mice demonstrated strong cytotoxicity against EL-4 cells. By MTT assay, after 6 h incubation of EL-4 with effector cells, percent cytotoxicity ranged from 6 ± 1% to 28 ± 3%, at E:T ratios 5 and 20, respectively. Similar levels were detected by LDH release assay (Fig. 4A), thus confirming the validity of the MTT method. After 24 h incubation, cytotoxicity increased up to 18 ± 2% and 61 ± 15%, at E:T ratios 5 and 20, respectively (Fig. 4A). Effector cells from allogeneic mice (CBA and Balb/c) demonstrated similar levels of cytotoxicity against EL-4 cells (data not shown). Splenic effector cells from C57BL/6 mice inoculated with EL-4 cells 10 days prior to the experiment, showed negligible cytotoxicity after 6 h incubation. However, they exhibited a pronounced cytotoxic effect against EL-4 cells after 24 h of incubation with the targets (Fig. 4B).

To determine whether surface expression of HSP70 on EL-4 cells is important for their recognition and elimination by cytotoxic lymphocytes, we first incubated EL-4 cells with anti-HSP70 antibody and then used these cells as targets in a 6 hour cytotoxicity assay. Splenic effector cells from normal C57BL/6 mice showed markedly decreased cytotoxicity against target EL-4 cells pretreated with anti-HSP70 mAb (Fig. 5A). Inhibitory effect of the antibody was also observed in allogeneic co-culture, i.e. with effector cells from CBA (Fig. 5C) and Balb/C mice (data not shown). Similar result was obtained using splenic effector cells from C57BL/6 mice inoculated with EL-4 tumor: percent cytotoxicity 21 ± 5% (target EL-4 cells pretreated with anti-HSP70 antibody) versus 53 ± 11% (untreated EL-4 cells), at E:T ratio 20 and 24 h incubation. In contrast, pretreatment of target EL-4 cells with anti-H-2^b antibody resulted in their enhanced killing by syngeneic effector cells (Fig. 5B). A control anti-Thy1.2 antibody was without effect (Fig. 5D). It should be mentioned that treatment with antibodies alone (anti-H-2^b, anti-HSP70) for up to 24 h, did not affect viability of either target EL-4 cells or splenic effector cells (data not shown).

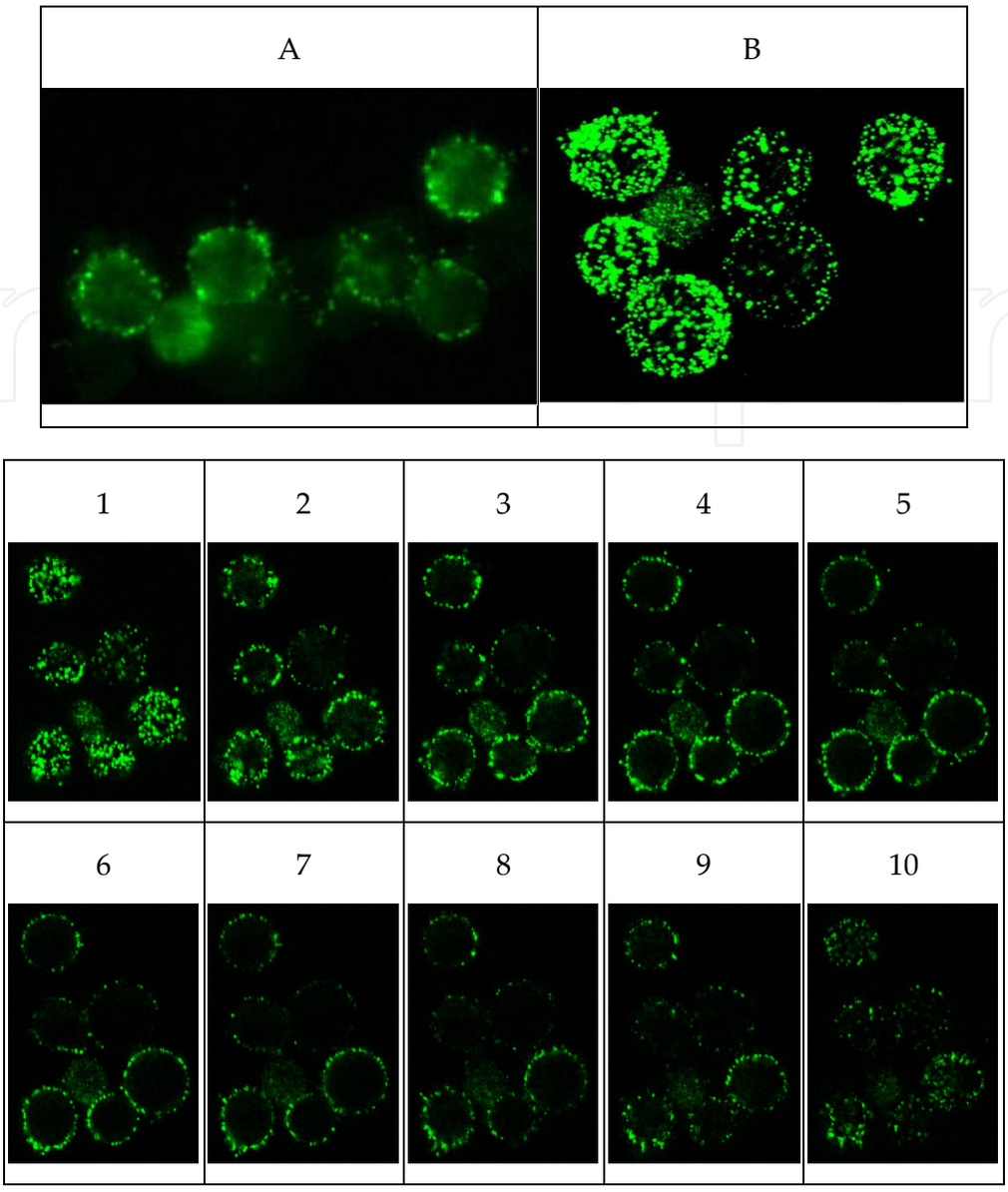

Figure 3. Visualization of cell surface expression of HSP70 in *in vitro* cultures of EL-4 cells performed by using fluorescent (A) and laser scanning confocal (B) microscopy (images were recorded using a Nikon TE-2000 confocal microscope; excitation was at 488 nm argon-ion laser) ; fluorescence emission was detected in the 500-550 nm for Alexa Fluor 488. Cells were imaged in a Z-stack and both sections and the reconstructed images examined for HSP localization. These images clearly demonstrate the cell surface localization of HSP70.

Next, we addressed the question of whether killing of EL-4 cells in our model was mediated by NK cells or cytotoxic T lymphocytes. To this end, we depleted $CD8^+$ cells from the splenic effector cell population obtained from intact C57BL/6 mice. Typically, effector cell population contained 5% sIg^+, 36% $CD8^+$, 48% $CD4^+$, 88% $CD3^+$, 2% $\gamma\delta TCR^+$, and 10% $NK1.1^+$ cells. After depletion of $CD8^+$ cells, the population contained 7% sIg^+, 60% $CD4^+$, 57% $CD3^+$, 3% $\gamma\delta TCR^+$, and 25% $NK1.1^+$ cells. Thus, it was relatively enriched for NK cells. As is shown in Fig. 6, cytotoxicity against EL-4 cells increased, rather than decreased, after depletion of $CD8^+$ cells from the effector cell population. Moreover, cytotoxicity was significantly

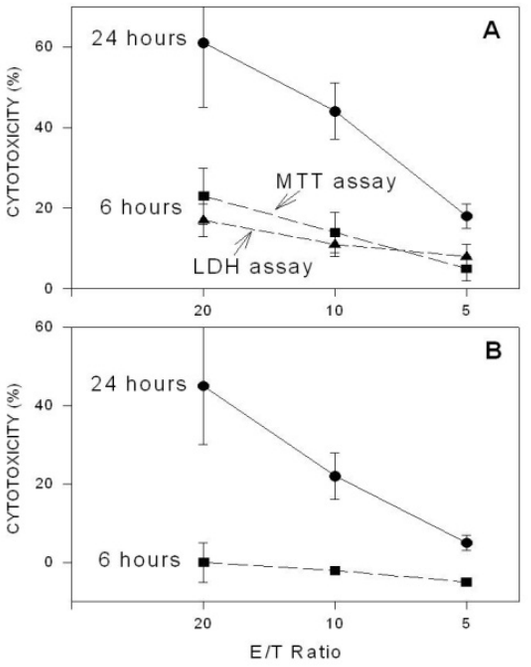

Figure 4. Cytotoxicity of C57BL/6 splenic effector cells after 6 and 24 h of incubation with the target EL-4 cells measured by MTT and LDH assays. (**A**) Cytotoxic effector cells were isolated from spleens of intact mice. (**B**) Cytotoxic effector cells were isolated from spleens of mice inoculated with EL-4 cells 10 days prior to experiment. Results are the mean of triplicate cultures ±S.E.M.

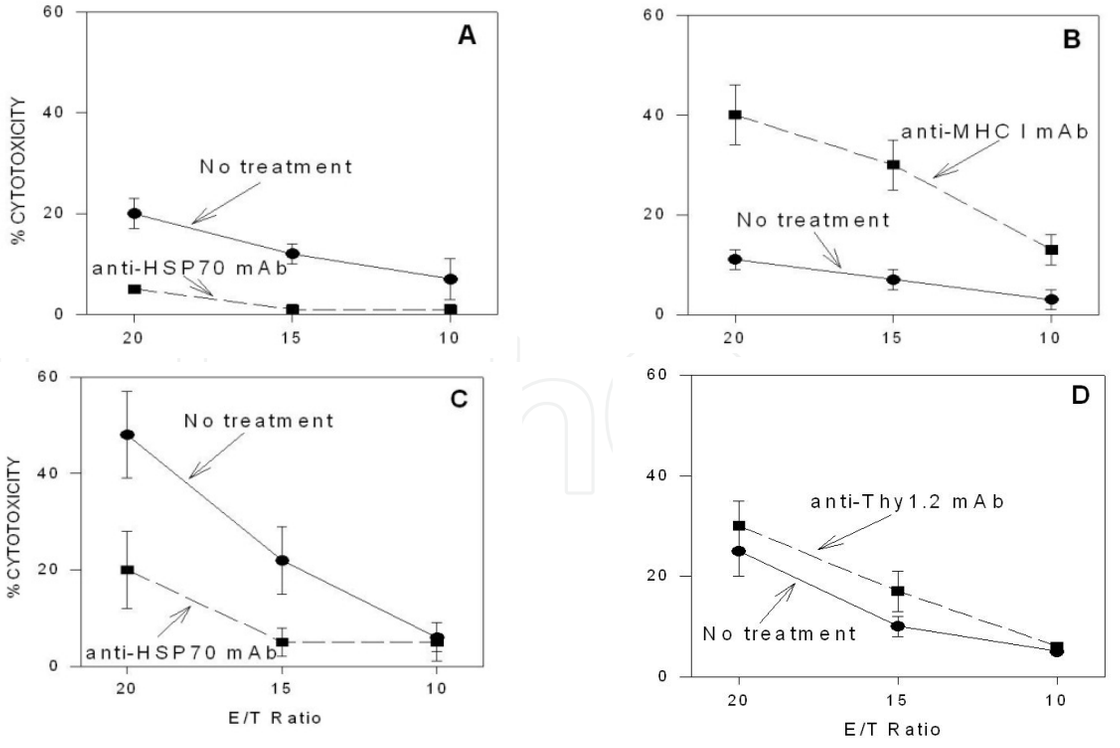

Figure 5. The effect of treatment of EL-4 target cells with antibodies to HSP70, MHC class I molecules and Thy1.2 on their sensitivity to killing by splenic effector cells. Cytotoxic effector cells were isolated from syngeneic C57BL/6 mice (**A**, **B**, and **D**) or allogeneic CBA mice (**C**). Results are the mean of triplicate cultures ±S.E.M.

inhibited by pretreatment of target EL-4 cells with anti-HSP70 mAb. Hence, lysis of EL-4 cells in this system seems to be predominantly mediated by $CD8^-$ cells, possibly NK cells, and may involve recognition of surface HSP70 present on EL-4 target cells.

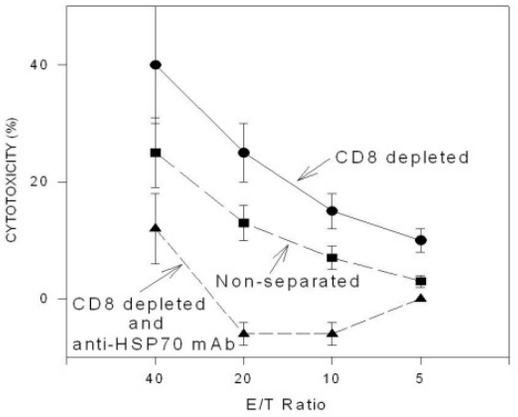

Figure 6. The effect of depletion of $CD8^+$ cells from splenic effector cell population obtained from intact C57BL/6 mice on cytotoxicity against EL-4 target cells, treated or untreated with anti-HSP70 antibody. Results are the mean of triplicate cultures ±S.E.M.

3.2. Exogenous HSP70 as mediators of NK cell activation

As stated above HSPs are intracellular proteins functioning as molecular chaperones; at the same time evidence has now accumulated that HSPs can localize on cell surface and can exit to the extracellular space, including blood. These two forms of HSPs – membrane-associated and extracellular soluble proteins – have immunomodulatory capabilities realizing by different mechanisms. Translocation of HSPs to plasma membrane has been observed in stressed, infected and transformed cells. It seems plausible that surface HSPs may represent molecular targets for recognition and elimination of 'altered' cells by cytotoxic lymphocytes, in particular NK cells. In this report we describe some models enabling to study mechanisms of interaction of NK cells with the target tumor cells bearing HSP70 on their surface.

Extracellular HSPs act as soluble immunostimulators affecting different immune cells such as DCs, macrophages, T and NK cells. HSPs can bind extracellular peptides from dead cells. Such HSP-peptide complexes become internalized by professional antigen-presenting cells – DCs or macrophages – where peptides release from complexes with HSP and become reassociated with MHC molecules. This process is called "cross-presentation"; HSPs act here as extracellular chaperones able to bind peptides of different structure [53-55]. On the other hand HSPs can also directly stimulate immune cells. For example, HSP70 added to DCs and macrophages induces their maturation and cytokine secretion. Exogenous HSP70 also stimulates proliferation and cytotoxicity of human NK cells, moreover, NK cells were shown to move by the gradient of HSP70 concentration [56, 57]. Thus, HSPs can act on immune cells similar to cytokines or chemokines. Due to such dualism extracellular HSPs were named "chaperokines", i.e. chaperones plus cytokines [58].

To clearly elucidate any immunostimulatory action of HSP70 on NK cells, we tested whether exogenous HSP70 influences NK cell cytokine secretion. In our experiments we analyzed effects of HSP70 on IFN-γ production by human NK cells. NK cells were isolated from peripheral blood samples by centrifugation on Ficoll density gradient with subsequent magnetic separation of mononuclear cell population using MACS NK cell isolation kit (Miltenyi Biotec, Germany) or cell sorting using fluorescent labeled CD3, CD16 and CD56 antibodies. Recombinant human HSP70 expressed in *E.coli* (#NSP-555, Stressgen, Canada) was used in experiments. HSP70 was added to isolated NK cells without or with 500 U/ml IL-2 (Hoffmann-La-Roche, Switzerland). After incubation for 18 h in 37°C, 5% CO_2, supernatants were collected and analyzed for IFN-γ content using Human IFN-γ ELISA kit (Pierce Endogen, USA). Effect of HSP70 on IFN-γ production by NK cells was also analyzed by flow cytometry. NK cells incubated with HSP70 and IL-2 were collected and fixed by paraformaldehyde. Cells were then labeled intracellularly with fluorescent conjugated antibody to human IFN-γ (BD Pharmingen, USA). Samples were then analyzed by flow cytometry (BD FACScan, BD Biosciences, San Jose, CA USA) and the percentage of NK cells producing IFN-γ was measured.

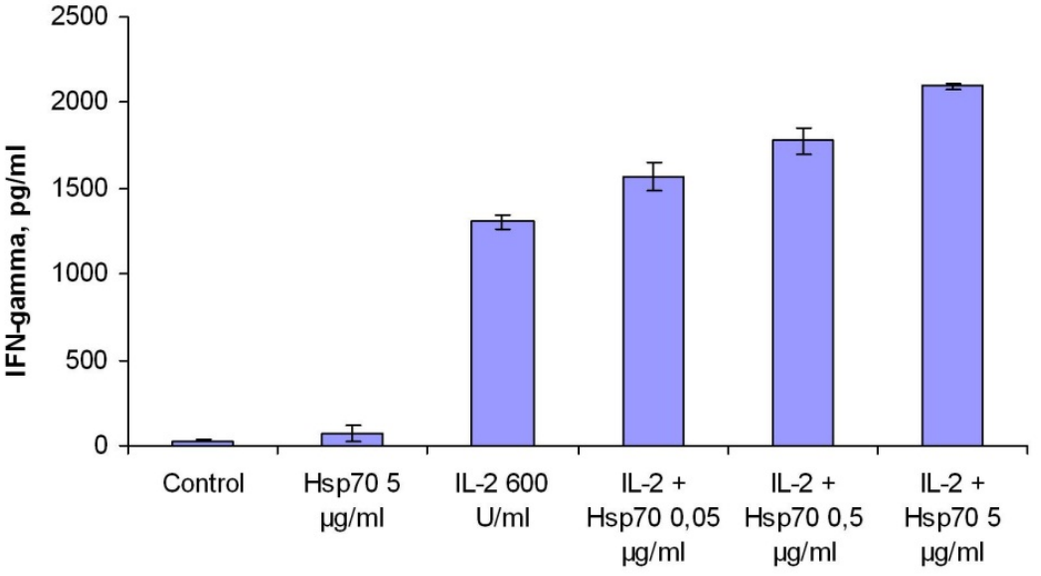

Figure 7. Recombinant human HSP70 stimulated IFN-γ production in NK cells activated by IL-2. IFN-γ production was measured by ELISA. Similar results were obtained with intracellular IFN-γ measurement by flow cytometry (data not shown).

HSP70 in dose 5 μg/ml did not induce IFN-γ production in native NK cells (Fig. 7). However HSP70, when added together with IL-2, did markedly stimulate it. Similar data were obtained with intracellular staining of NK cells. Thus HSP70 was shown to stimulate cytokine production in IL-2 activated NK cells. This fact is very consistent with the assumed role of HSPs as danger-associated molecular patterns (DAMPs) able to activate a range of cellular mediators of innate immunity. It seems HSP70 exerts a cytokine-like action on NK cells; however, it does not work by itself. It needs in initial activation by cytokines like IL-2

(similar results were obtained using IL-12 and IL-15, data not shown). It is important to note that no receptors for HSP70 on NK cells were found. Based on presented data we can speculate about a putative HSP70 receptor which expression is triggered under cytokine-mediated NK cell activation. There is also a possibility that some NK cell subpopulations are more sensitive to HSP70 action. To test this we investigated whether HSP70 induced IFN-γ production in sorted $CD56^{bright}$ and $CD56^{dim}$ human NK cells.

Cell sorting of $CD3^{-}CD16^{-}CD56^{+}$ and $CD3^{-}CD16^{+}CD56^{+}$ NK cells was performed on a fluorescence-activated cell sorter (FACSVantage DiVa, BD Biosciences, USA), with final purities at 99%. HSP70 was added to sorted NK cells simultaneously with IL-2 (600 U/ml). After 18 h incubation supernatants were collected and analyzed by ELISA as described above.

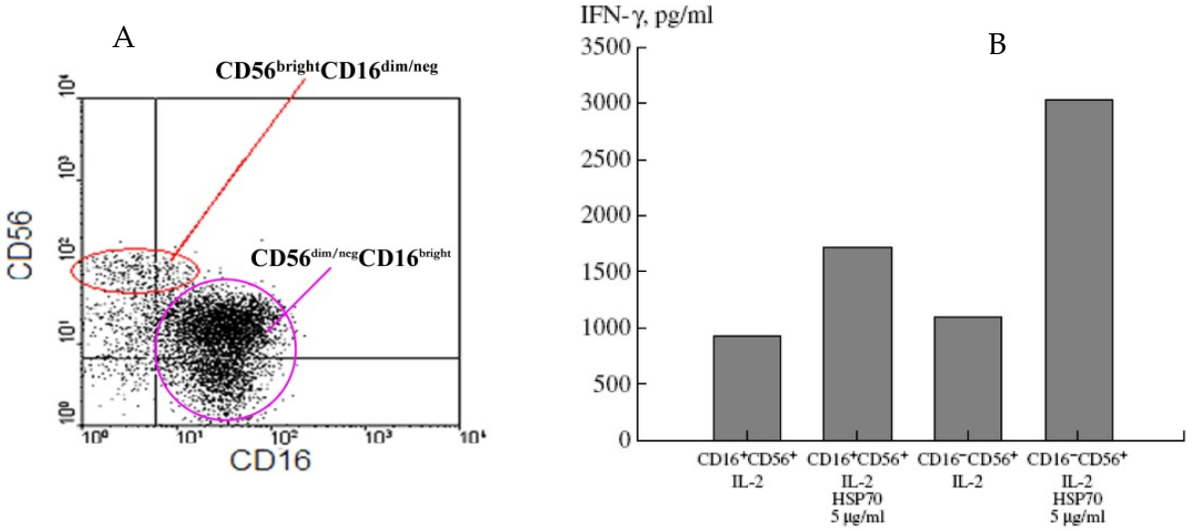

Figure 8. Effect of HSP70 on IFN-γ production of two major NK cell subpopulations. (A) Flow cytometric analysis of sorted NK cell subpopulations. (B) Recombinant human HSP70 stimulates IFN-γ production in both NK cell subpopulations $CD16^{-}CD56^{+}$ and $CD16^{+}CD56^{+}$ in presence of IL-2 (600 U/ml).

HSP70 enhanced IFN-γ production in both NK cell subpopulations (Fig. 8), and $CD16^{-}CD56^{+}$ cells produced larger amount of that cytokine. It is known $CD56^{bright}$ NK cells produce markedly higher amount of cytokines than $CD56^{dim}$. So, possibly the higher effect of HSP70 on $CD56^{bright}$ cells was due to properties of this NK cell subpopulation.

The next step of our work was the elucidation of HSP70 fragments which are responsible for apparent effects of that protein. Previously Botzler and coworkers demonstrated that substrate-binding domain of HSP70 is responsible for stimulating HSP70 effect on NK cells [44]. Then they had shown NK cells could be activated by a 14 aa fragment of HSP70 with sequence TKDNNLLGRFELSG. This fragment named TKD-peptide corresponds to 450-463 aa residues belonging to substrate-binding domain of HSP70 [59].

Using a special *in silico* approach we analyzed five peptide fragments belonging to substrate-binding domain in comparison with TKD-peptide for their ability to activate NK

cells. The method of analysis of informational structure (ANIS) proposed by A.N. Nekrasov [60, 61] was used for search of potentially active fragments of HSP70 sequence. Five peptides were chosen and synthesized (Fig. 9). All of them were tested for ability to induce IFN-γ production in human NK cells isolated from peripheral blood by magnetic separation. Peptides were added to NK cells together with IL-2 in concentration 2 μg/ml. Cells were incubated for 18 h and then intracellular staining with antibody to IFN-γ was performed as described above.

Most peptides including TKD-peptide developed by G. Multhoff did not significantly enhance the amount of IFN-γ producing cells. Only peptide comprising residues 526-543 of HSP70 (P2) markedly induced IFN-γ production. Its effect was even greater than HSP70 full-length protein. It can be explained by difference in molar concentrations: 2 μg/ml solution of such peptide corresponds to the greater molar concentration than 5 μg/ml solution of HSP70 protein. Similar data were obtained from experiments with cytotoxicity of NK cells against K562 target cells measured using assay for caspase 6 activity (Oncoimmunin, USA). Only P2 peptide significantly stimulated lysis of K562 by NK cells.

399-408	P1	LSLGLETAGG	
411-424	P2	TALIKRNSTIPTKQ	
450-463	P3	TKDNNLLGRFELSG	TKD-peptide
461-470	P4	LSGIPPAPRG	
509-515	P5	RLSKEEI	
526-543	P6	KAEDEVQRERVSAKNALE	active peptide fragment

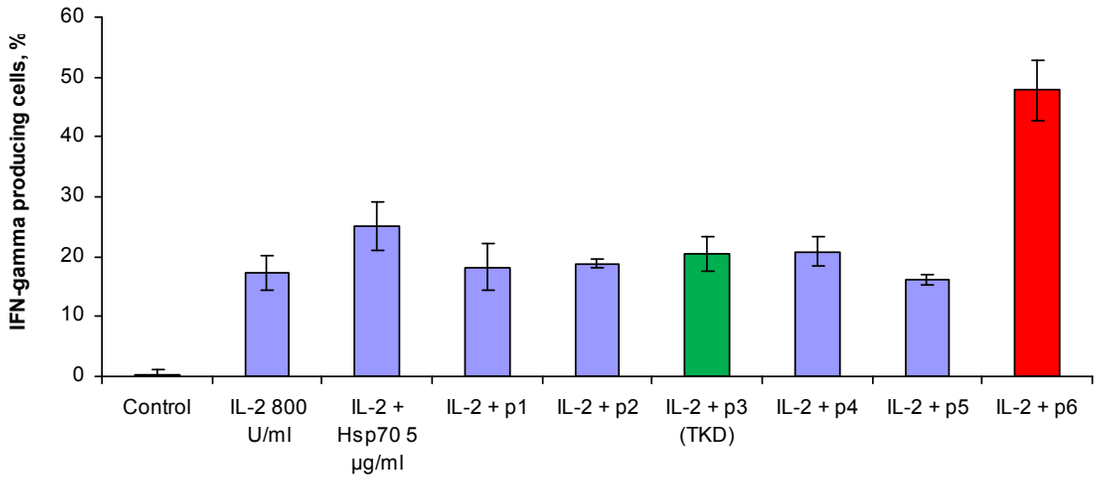

Figure 9. Testing of HSP70 peptide fragments for an ability to stimulate IFN-γ production in NK cells. Peptides were added in concentration 2 μg/ml. Amount of IFN-γ producing NK cells was determined by flow cytometry using intracellular staining with anti-IFN-γ antibody. Testing of the peptides in cytotoxicity assay provided similar results (data not shown).

In human peripheral blood there are many cells which have shown to be activated by heat shock proteins, including monocytes and blood DCs. Even small numbers of such cells can

exert an influence on the apparent effect of HSP70 on NK cells. We therefore analyzed whether the presence of additional cell populations in NK cell preparations can affect HSP70 induced IFN-γ production. For that purpose NK cells and DCs were isolated by fluorescence activated cell sorting. PBMCs were stained with antibodies CD3-TC, CD11c-APC (Caltag, USA), CD16-FITC and CD56-PE (Beckman Coulter, USA). PBMCs were sorted into $CD3^-CD11c^-CD16^+CD56^+$ (NK cell) and $CD3^-CD11c^+CD16^-CD56^-$ (myeloid DC) fractions using FACSVantage DiVa. Purity was 97-98%. NK cells were then mixed with DCs in different proportions in presence of suboptimal dose of IL-2 (300 U/ml) and HSP70 was added. After 18 h incubation supernatants were collected and analyzed for IFN-γ content by ELISA (Pierce Endogen, USA).

Low doses of IL-2 did not markedly induce IFN-γ production (Fig. 10A). Mixtures of NK cells with DCs produced larger amounts of IFN-γ proportional to the numbers of DCs. This is fully consistent with current view on NK-DC interactions. It seems that DCs in the mixture with NK cells provided a stimulating signal which is additional to suboptimal dose of IL-2. Effect of HSP70 was more noticeable if it was added to the cell mixtures and increased proportionally to the elevation of basal level of IL-2 induced IFN-γ production. Thus, the amount of DCs in NK cell preparations was very important factor affecting NK cell functions, though in our experimental conditions DCs did not influence HSP70-mediated induction of NK cell activation.

Other important problem linked to HSP70 physiological action is its potential association with bacterial lipopolysaccharides (LPS). LPS can activate a range of cells of immune system via Toll-like receptor 4 (TLR4) [62]. Many reports describe LPS contamination causing apparent HSP effects on different immune cells [63-65]. HSP70 protein used in our experiments was recombinant, expressed in *E.coli*, so we might assume it was contaminated by LPS. To investigate it we have performed amoebocyte Limulus lysate (LAL) assays for LPS activity in HSP70 samples (E-Toxate kit, Sigma-Aldrich, USA). HSP70 samples were shown to contain more than 10000 U/mg of LPS. Thus, additional experiments were necessary to investigate the role of LPS in NK cell activation. NK cells isolated by magnetic separation were incubated with LPS, HSP70 known to be LPS-negative ((#ESP-555, Stressgen, Canada) and IL-2 for 18 h. Cells were collected, fixed and stained with fluorescent labeled antibody to human IFN-γ (BD Pharmingen, USA).

As with HSP70, LPS did not markedly stimulate IFN-γ production in native NK cells (Fig. 10B). However it did lead to a significant increase of IFN-γ producing cells then added together with IL-2. Thus, LPS stimulated NK cells only in cooperation with IL-2. Interestingly, HSP70 purified from LPS (HSP70 low endotoxin, HSP70-LE) added together with IL-2 did not stimulate IFN-γ production in contrast to non-purified protein. Moreover, HSP70-LE added together with LPS inhibited LPS-induced IFN-γ production showing effect opposite to LPS. One explanation for the inhibitory effect of HSP70-LE is that LPS and HSP70 compete for the same receptors on the cell surface. However it does not explain the activating effects of p6 and TKD peptides on NK cells. Additional experiments are required to characterize the regulatory role of exogenous HSP70 in NK cell functioning.

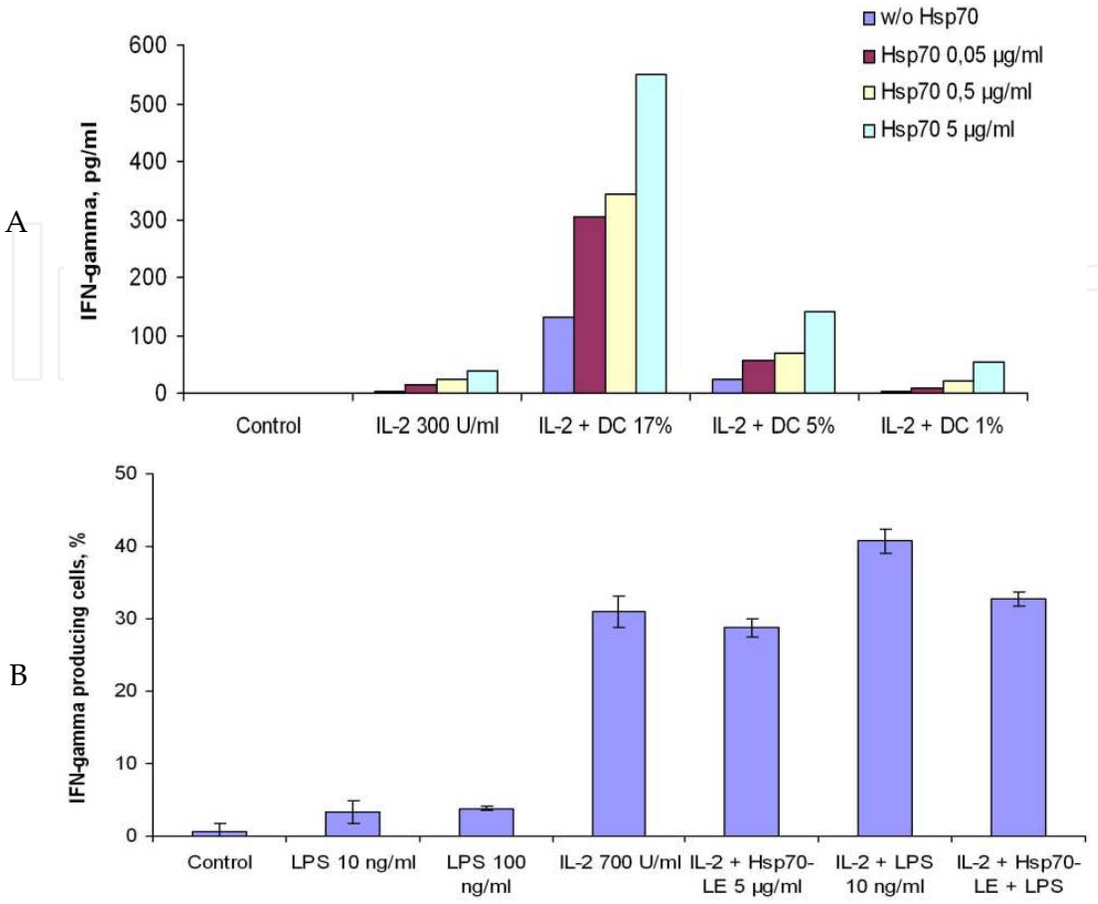

Figure 10. (A) Admixture of blood DCs affects IFN-γ production by NK cells. DCs and NK cells were isolated by cell sorting and mixed in different proportions with IL-2 in suboptimal dose, HSP70 was added. IFN-γ concentration in supernatants was analyzed by ELISA. (B) LPS stimulates IFN-γ production by NK cells in presence of IL-2. LPS, IL-2 and HSP70-LE were added to isolated NK cells, amount of IFN-γ producing NK cells was determined by flow cytometry using intracellular staining with anti-IFN-γ antibody.

Thus, multiple data support that activity of NK cells can be modulated by HSP70 both bound to the target cell surface and extracellular soluble HSP70. However, exact mechanisms of HSP70 reception by NK cells and HSP70-induced signaling are still not known. Moreover, a role of LPS in NK cell activation remains to be investigated.

4. Role of proteins MICA/B in regulation of NK cell activity

Another type of stress-induced proteins that play an important role in NK cell regulation is represented by molecules reacting with the activating receptor NKG2D. NKG2D is expressed by most NK cells; it recognizes two groups of ligands – ULBPs and MIC proteins distinct by structure and membrane anchoring [23, 25]. MICA and MICB, the most extensively studied NKG2D ligands, are highly glycosylated transmembrane proteins with one site of S-acylation necessary for anchoring in plasma membrane. They share high homology with MHC-I but are not associated with β_2-microglobulin and do not bind antigenic peptide. Like MHC-I, they consist of three extracellular domains (α1, α2 and α3),

with two of them, α1 and α2, forming a site for NKG2D binding [66]. Oppositely, ULBPs have less homology with MHC-I molecules. They have neither an extracellular α3-domain nor a transmembrane domain, anchoring to the plasma membrane through GPI. This feature determines constitutive localization of ULBPs in lipid rafts of the plasma membrane [25, 67]. MIC proteins in their turn are also found in lipid rafts, however, their physical link to these membrane domains is less tight: they are able to transfer between rafts and the detergent-sensitive, "usual" bilipid zone [67].

Previous data demonstrates that in humans the MICA-NKG2D pair participates in regulatory interactions between NK cells and other cells involving in immune response. Interactions of membrane bound MICA/B with NKG2D lead to activation of NK cells and elimination of aberrant cells. MICA/B proteins exposed on the cell surface serve as a "danger signal" for cytotoxic lymphocytes and mark the cells that should be eliminated (Fig. 11).

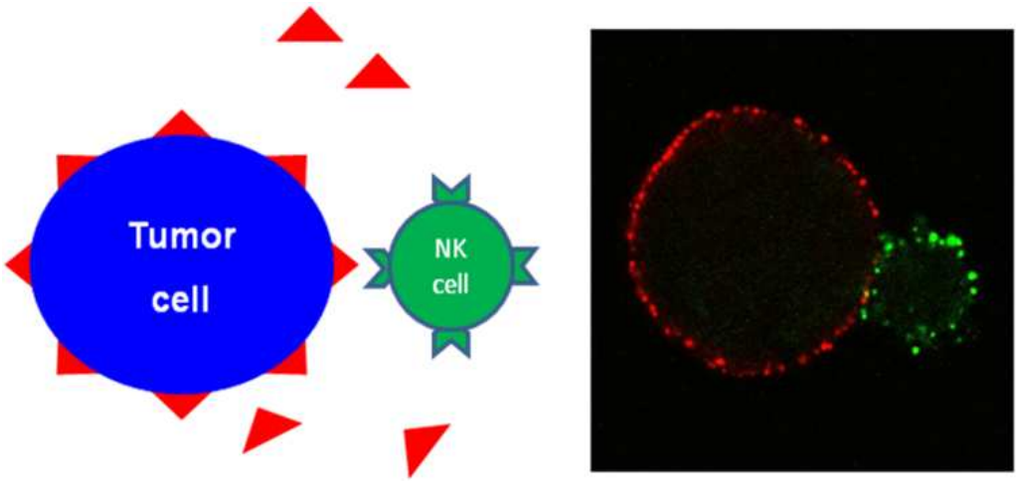

Figure 11. Receptor NKG2D recognizes proteins MICA/B. NK cells and K562 target cells were labeled with anti-NKG2D-Alexa Fluor 488 antibody and primary anti-MICA/B and secondary antibody conjugated with Alexa Fluor 555, respectively.

In our experiments we found a role for MICA/B in NK cell-mediated cytotoxicity using both intact human NK cells and cells of NK-cell-like line NKL. NK cells were isolated from human peripheral mononuclears by magnetic separation. Cytotoxicity was measured by flow cytometry method based on estimation of caspase 6 activity in target cells. Antibodies against both NKG2D and MICA/B significantly inhibited cytotoxic activity of effector cells against erythroleukemia cell line K562 and B-lymphocytic cell line 721 (Fig. 12). The values of cytotoxicity and levels of the inhibition varied for different target cell lines not depending directly on MICA/B expression. The results suggested that NKG2D-MICA/B interactions were not the only way of NK cell recognition of these target cells. Expression of NKG2D and cytotoxic activity of NK cells increased remarkably following their incubation with IL-2. Finding ways of additional induction of surface expression of MICA/B on the cell surface might be useful for developing new approaches for increase of tumor cell sensitivity to cytotoxic lymphocyte action.

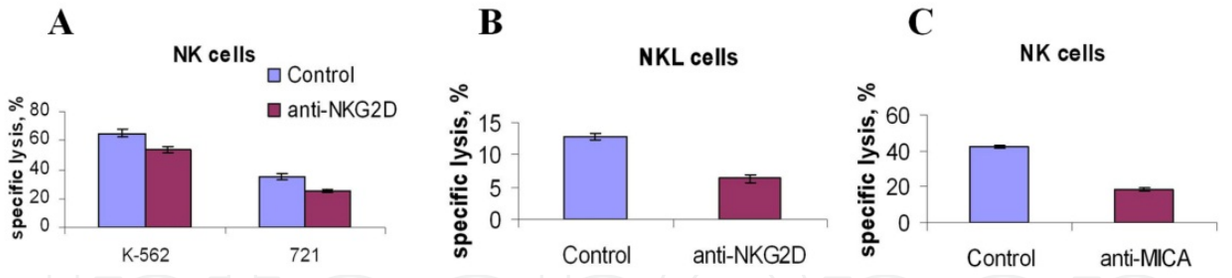

Figure 12. Cytolytic action of NK cells is mediated by NKG2D-MICA interaction. (A) Measurement of cytotoxicity of NK cells against K562 and 721 target cells in presence of antibody to NKG2D. (B) Measurement of cytotoxicity of NKL cells against 721 target cells in presence of antibody to NKG2D. (C) Cytotoxicity of NK cells against K562 cells treated with antibody to MICA.

MIC proteins can be released from the cell surface into the extracellular space. At least two pathways of release were documented: proteolytic shedding of extracellular part of MIC molecule and formation of exosomes containing MICA/B [68, 69]. Both mechanisms result in accumulation of soluble form of MIC (sMIC) in the extracellular space. Proteolytically cleaved sMICA/B circulate in human blood and can be found at remarkable levels in sera of tumor patients. This type of sMIC is also generated during viral infections. In contrast to membrane-associated MIC proteins, circulating forms of MICA/B are able to inhibit activation of NK cells and lead to anergy. It is hypothesized that tumor cells can use this mechanism to evade immune surveillance [25]. Proteolysis of MIC proteins occurs by a number of metalloproteinases, at least three of them were recently identified: two proteins of ADAM (A Disintegrin And Metalloproteinase) family, ADAM10 and ADAM17, and matrix metalloproteinase MMP14 [70, 71]. Metalloproteinases cleave MICA in a linker sequence between the transmembrane domain and the α3 domain. There is no definite site of hydrolysis, but sufficient length of the linker region is essential [72]. MICA is expressed on the cell surface in complex with proteins originally found in endoplasmic reticulum (ER): ERp5, GRP78, and two proteins of 47 and 48 kDa of thioredoxin family. ERp5 reduces a unique disulfide bond in α3 domain of MICA and induces conformation changes of the domain rendering the MICA molecule accessible for metalloproteinases. The roles of GRP78 and two other proteins associated with MICA on cell surface in sMICA shedding remain to be elucidated [73].

We estimated the amounts of sMIC in peripheral blood sera of patients with lymphoproliferative diseases. Levels of sMIC were measured in seven groups of patients with different types of lymphoproliferative diseases consisted of from 5 to 20 patients in a group (Fig. 13). sMICA/B concentrations were measured by ELISA (R&D Systems). The analysis of the data in every group was performed using a nonparametric Mann-Whitney test. The mean value of sMIC in the control group equaled 40 pg/ml and was not greater than 250 pg/ml. In all studied groups with the exception of Burkitt-like lymphoma group the mean sMIC serum levels exceeded significantly the levels in the group of normal donors. The highest levels of sMIC content were found in patients with T-cell anaplastic lymphomas.

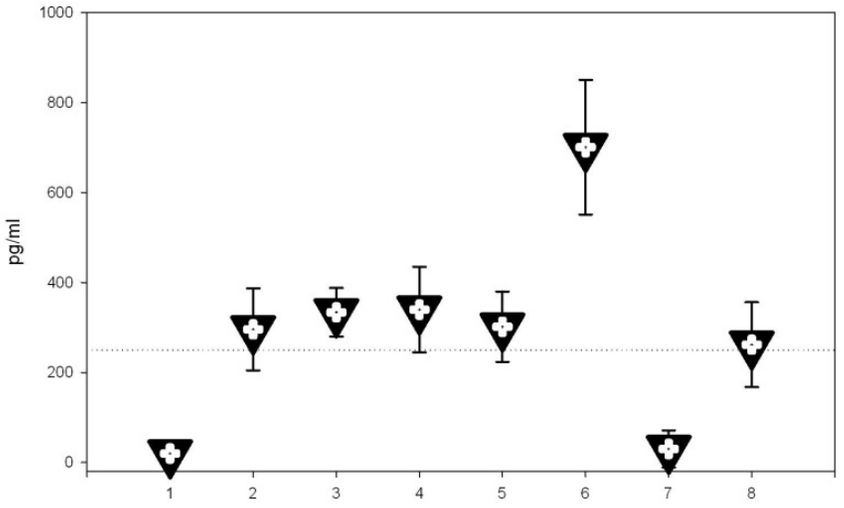

Figure 13. sMICA/B content determined in sera of patient with lymphoproliferative diseases. 1 – healthy donors, 2 – chronic lymphocytic leukemia, 3 – diffuse large B-cell lymphoma, 4 – lymphatic plasmacytoma, 5 – follicular lymphoma, 6 – T-cell anaplastic lymphoma, 7 – Burkitt-like lymphoma, 8 – lymphocytoma.

4.1. MICA/B expression under ethanol-induced cell stress conditions

The mechanisms of MICA/B regulation are still not completely clear. Expression of *MICA/B* genes on the mRNA level is found in virtually all tissues of the organism. However considerable constitutive expression of membrane-associated proteins was detected only on intestine epithelial cells [74]. It is suggested that MIC proteins participate in maintaining the integrity of the gut epithelium promoting NKG2D-dependent recognition and prompt elimination of damaged epithelial cells by intraepithelial lymphocytes [75]. There are evidence that some cellular miRNA can impair translation of $mRNA^{MICA/B}$, setting up a threshold for MICA/B expression. In stress conditions as well as in cell transformation, viral infection and surgical procedures including transplantation, *MIC* gene transcription markedly increased, specific miRNA was degraded and MIC proteins appeared on the cell surface [76, 77]. Transcription factors NFκB, Sp1, Sp3 were shown to stimulate MIC surface expression. *MIC* expression induced by heat shock is dependent on HSF1 binding to heat shock response element (HSRE) located in promoter region of *MIC* genes [25]. Genotoxic stress causes MIC surface expression via ATM/ATR kinases, sensors of DNA damages, triggering signaling cascade which leads to cell cycle arrest, induction of DNA reparation and expression of NKG2D ligands [78]. In many cases mechanisms of MIC expression triggering remain unclear.

In our work we produced a reliable model of induction of MICA/B surface expression based on cell treatment with ethanol. Using this model we analyzed MICA/B expression in transcriptional, surface and extracellular levels of MICA expression.

It is well known that ethanol can exert damaging effects on many types of cells. It influences membrane fluidity and induces oxidative stress in cells [79]. Ethanol can also affect immune response, particularly by triggering apoptosis in lymphoid and myeloid cells [80]. All these

data characterize ethanol as a good inductor of cell stress conditions. We applied ethanol to induce cell stress in a range of hemopoietic cell lines in order to analyze its influence on MICA/B expression. Surface MIC expression was analyzed by flow cytometry (FACScan, BD Biosciences) and confocal microscopy (Nikon TE-2000 confocal microscope). Monoclonal antibodies to MICA/B (R&D Systems) were used for protein visualization. Expression of *MICA/B* genes was registered by RT-PCR. Percentages of apoptotic and necrotic cells were measured by cell staining with fluorescent-labeled annexin V and propidium iodide using flow cytometry. Soluble MICA/B forms were detected by ELISA.

For the study we chose several human hemopoietic cell lines of different origin: acute T cell leukemia Jurkat, erythroblastoid leukemia K562, monocytic leukemia THP-1 and melanoma cell line MelJuSo. The cell line C1R-MICA transfected with MICA gene was used as a positive control. The cell lines differed in spontaneous MICA/B surface expression (Fig. 14). The maximal MICA/B expression comparable with expression in C1R-MICA was registered in THP-1 cells. Even those cells, which had no spontaneous MICA/B expression on the cell surface had high levels of $mRNA^{MIC}$ (data not shown).

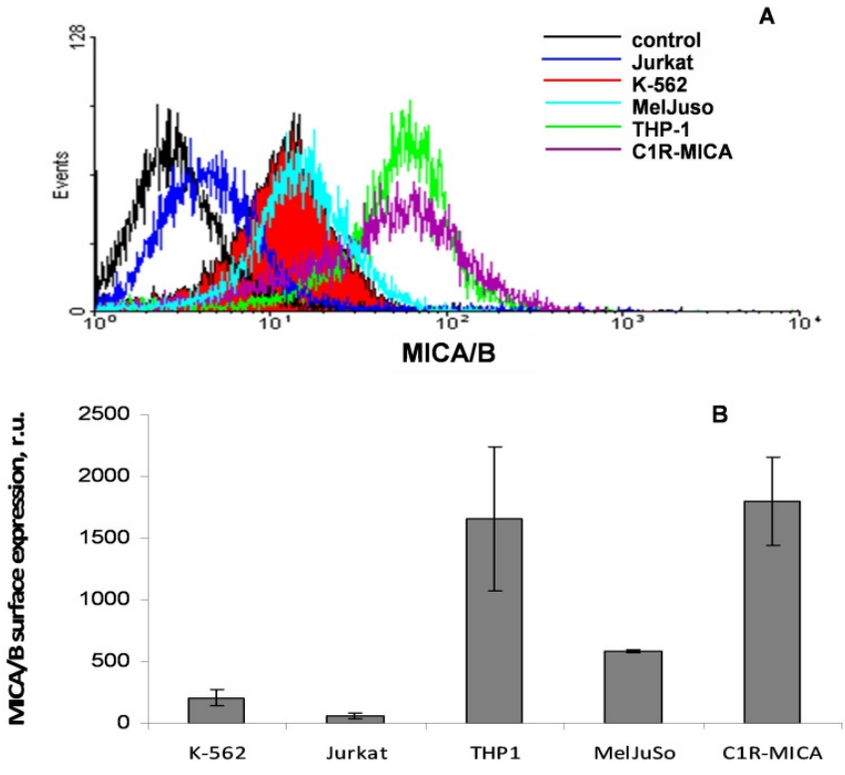

Figure 14. Spontaneous MICA/B surface expression in different hemopoietic tumor cell lines: K562 – human erythroblastoid leukemia, Jurkat – human T cell leukemia, THP-1 – human acute monocytic leukemia, MelJuSo - human melanoma , C1R-MICA – transfected cells (positive control).

We found that treatment of cells with ethanol modulated MICA/B cell expression. Short term cell treatment with ethanol (1 h) leaded to partial elimination of MICA/B from the cell surface. In the same time incubation of cells in the presence of ethanol in concentration range 0.25-3% for 18 h resulted in increased expression of MICA/B on the cell surface (Fig. 15).

Ethanol at high concentrations resulted in a reduction of MICA/B surface expression and an elevation of apoptosis and necrosis in cell culture (Fig. 16). Ethanol-induced increase of MICA/B surface expression preceded cell death caused by higher ethanol concentration. We can assume that the exposure of MICA/B proteins on the cell surface under cell stress conditions allows cytotoxic lymphocytes expressing receptor NKG2D to identify stressed cells in which the program of cell death has not yet been activated.

We also checked expression of MICA/B at the mRNA level in the hemopoietic cell lines and normal human leukocytes. $mRNA^{MICA/B}$ was easily detected in all cells tested without cell stress conditions. Increase of $mRNA^{MICA/B}$ level in cells was registered at 3 h of ethanol treatment (Fig. 17). One of the mechanisms of damaging effects of ethanol on cells is the induction of oxidative stress in cells that can cause DNA damage. We found out that ethanol-induced MICA/B upregulation did not depend on ATM/ATR kinase activity, suggesting that genotoxic effects were not the mechanism of this process (data not shown).

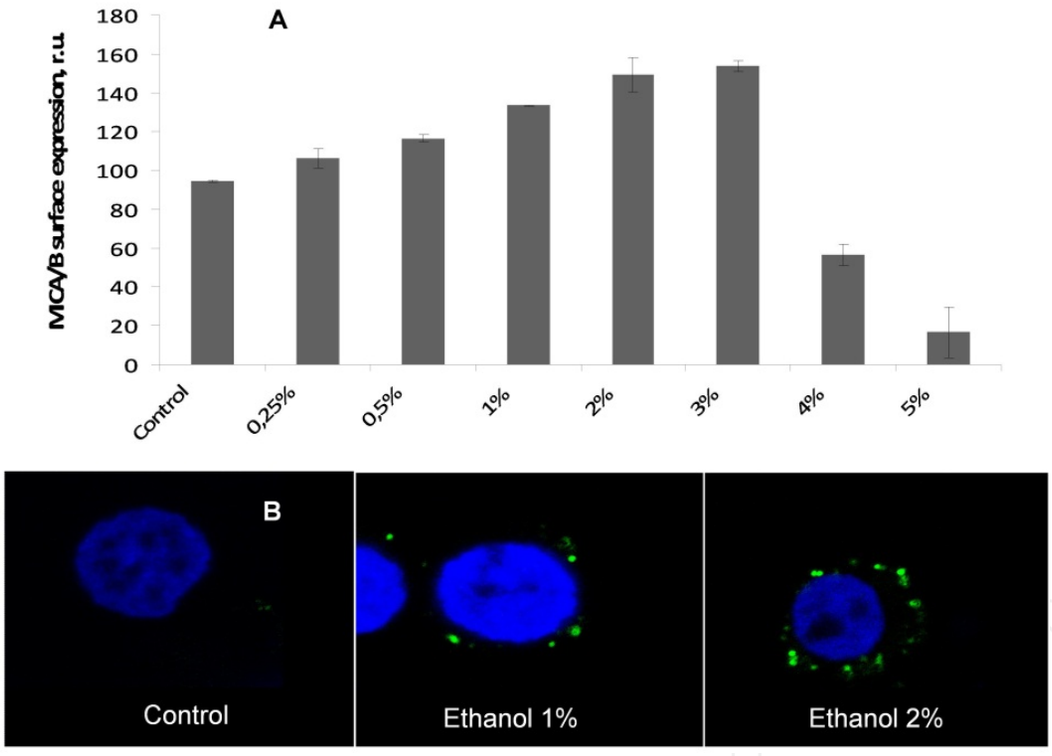

Figure 15. MICA/B surface expression alterations in Jurkat cells followed ethanol treatment for 18 hours. Incubation of cells ($5x10^5$ cells/ml) was performed in RPMI medium containing 10% FCS. Images were collected with a Nikon TE-2000 confocal microscope equipped with 405, 488 and 543 nm lasers and standard set of detectors. A. MICA/B surface expression measured by flow cytometry. Cells were labeled with primary MICA/B antibody and secondary antibody conjugated with AlexaFluor488. Nuclei were identified with Hoechst 33342. MICA/B expression levels were calculated as ((exp/2*ab*)-1)*100%, where exp – mean of fluorescence intensity of experimental sample, 2ab – mean of fluorescence intensity of control sample labeled only with secondary antibody. B – MICA/B surface expression after ethanol treatment analyzed by confocal microscopy.

Another mechanism of MICA/B surface expression increase under ethanol action was revealed in K562 cells. With confocal microscopy, it was shown that under the influence of ethanol-induced cellular stress the inner pool of MICA/B decreased and the surface pool increased as compared with the control cells indicating that under the alcohol influence MICA/B moved from the cytoplasm to the cell surface (Fig. 18).

Thus, ethanol affects MICA/B expression in cells at several levels resulting in induction of MICA/B *de novo* expression, translocation of intracellular protein pool to the cell surface and release of MICA/B proteins from the cells. MICA/B exposure on the cell surfaces and shedding of these proteins with formation of the extracellular pool are the latest stages of MICA/B cell expression. Both surface and extracellular forms of MICA/B proteins may take part in immune regulation acting on NKG2D-expressing cytotoxic lymphocytes.

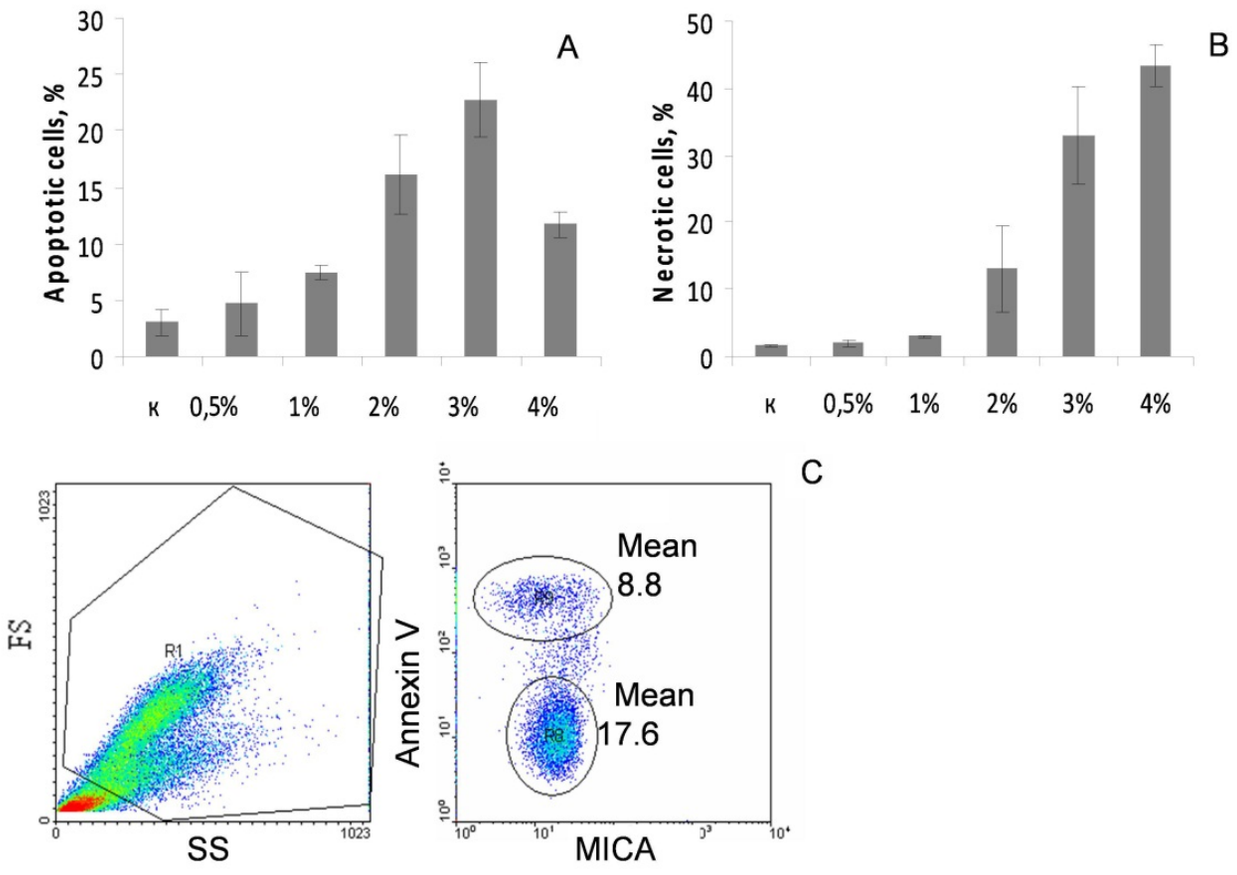

Figure 16. Ethanol influence on cell viability of THP-1 cells. **A** - Percentage of apoptotic cells in cell culture treated with ethanol for 18 h. **B** - Percentage of necrotic cells in cell culture treated with ethanol for 18 h. **C** - Flow cytometry analysis of MICA/B surface expression. THP-1 cells were stained with anti-MICA/B antibodies with FITC-labeled secondary antibodies, Alexa Fluor 647-labeled annexin V and propidium iodide.

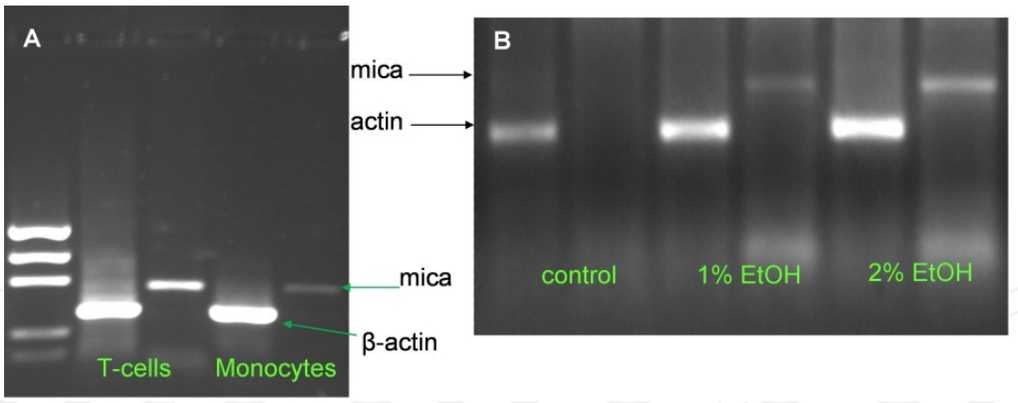

Figure 17. mRNA$^{MICA/B}$ expression estimated by RT-PCR in different kinds of cells. A. mRNA$^{MICA/B}$ expression in human T cells and monocytes. B. mRNA$^{MICA/B}$ expression in Jurkat cells subjected to ethanol treatment.

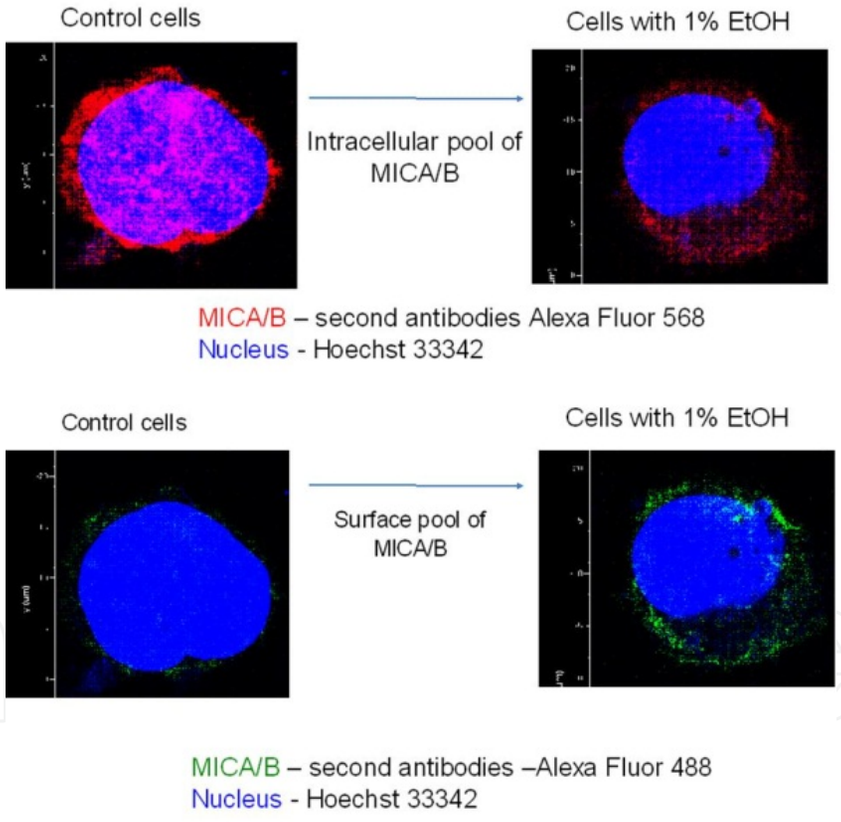

Figure 18. Intracellular and surface pool of MICA/B proteins in control K562 cells and the cells after ethanol treatment. MICA/B expression was detected with secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 568 for surface and intracellular proteins, respectively. Images were recorded with Nikon TE-2000 confocal microscope equipped with 405, 488 and 543 nm lasers and filters appropriate for Alexa Fluor 488, Alexa Fluor 568 and Hoechst 33342.

5. Conclusion

These experiments confirmed an important role of stress-induced modification of target cell surface for the process of NK cell – tumor cell interaction. Our data showed that this interaction is modulated by both alteration of cancer cell surface molecular pattern (including surface oligosaccharide profile) and extracellular pool of stress-induced proteins released by the target cells. Noteworthily, our results show that surface and soluble HSP70 activate cytolytic NK activity in contrast to extracellular form of MICA/B which inhibits cytotoxicity of NK cells. We hypothesize that stress-induced HSP70 and MICA/B protect target cells from NK-mediated effects on two levels: HSP70 serves as protective molecule for key cell components (in the cell space) and MICA/B provide a distant resistance inhibiting NK cell cytotoxic activity before the interaction with a target.

An important point of the obtained results is connected with HSP70 fragments identified by a special *in silico* approach. Testing of synthetic analogs of the fragments demonstrated in our models an essential activity of some peptides, similar to the effect of whole molecule. This data has important implications for future application of stress-induced proteins in cancer diagnostic and therapy.

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