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Research Article

Recurrent stimulation of NK cell clones with K562 expressing membrane-bound IL-21 affects their phenotype, IFN-γ production and lifespan

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Abbreviations: IFN-γ, interferon-γ; IL, interleukin; K562-mbIL21, gene-modified K562 cells expressing membrane-bound IL-21; NK cells, natural killer cells; PBMC, peripheral blood mononuclear cells.

Abstract

A pattern of NK cell heterogeneity in each individual determines proliferative and functional responses of NK cells to activating stimuli. Obtaining the progeny of a single cell by cloning original population is one of the ways to study the NK cell heterogeneity. In this work, we used single cell sorting into a plate and stimulation by IL-2 and gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21) that led to generation of phenotypically confirmed and functionally active NK cell clones. We applied two models of clone cultivation, which differently affected their phenotype, lifespan and functional activity. The first model, which included weekly restimulation of clones with K562-mbIL21 and IL-2, resulted in the generation of relatively short-lived (5-7 weeks) clones of highly activated NK cells. HLA-DR expression in the expanded NK cells correlated strongly with IFN-γ production. The second model, in which NK cells were restimulated mainly with IL-2 alone, produced long-lived clones (8-14 weeks) that expanded up to 10^7 cells with lower ability to produce IFN-γ. Our method is applicable for studying variability in phenotype, proliferative and functional activity of the certain NK cell progeny in response to the stimulation, which may help in selecting NK cells best suited for clinical use.

Keywords: NK cell clones, IL-2, K562-mbIL21, membrane-bound IL-21.

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1 Introduction

The phenotype of natural killer cells (NK cells) substantially changes during their differentiation and activation, forming heterogeneous subpopulations with various expression of surface receptors, effector molecules and signal proteins, and different functional activity [1,2]. The mechanisms of NK cell differentiation are not completely clear. During this process, due to certain epigenetic changes, NK cells lose expression of the NKG2A/CD94 receptor and begin to express inhibitory KIR receptors, and the marker of maturation CD57. Occasionally, often associated with cytomegalovirus infection, highly differentiated NK cells form subsets of adaptive-like cells intensively expressing activating KIR and NKG2C receptors [3,4]. All of this can lead to changes in proliferative and functional activity of NK cells, including cytotoxicity and production of cytokines. Generation and analysis of the individual NK cell progeny helps to better characterize the differentiation and activation processes on the single-cell level, and to study the functional features of NK cells at different developmental stages that may have advantages in the developing of approaches for the expansion of NK cells for clinical applications. Previously, clonally expanded NK cells were used to study distribution of CD94/NKG2A and CD94/NKG2C heterodimers [5] and the dependence of changes in CD94/NKG2 receptor expression on the functional activity of NK cells [6]. It was shown that heterogeneity in cytotoxic activity of NK cells was related to various expression levels of different activating and inhibitory receptors in NK cells. The role of KIR receptors in HLA-uncoordinated hematopoietic stem cell transplantation was studied in a number of works using NK cell clones [7,8]. Level of anti-tumor NK cell cytotoxicity was shown to be dependent on KIR repertoire acquired during NK cell differentiation. Functional tests utilizing NK cell clones may help in defining NK cells most cytotoxic for different tumor variants [8]. Several techniques were designed to produce NK cell clones. One of the first methods for obtaining NK cell clones was based on the use of limiting dilution and cultivation of cells in a medium supplemented with irradiated (EBV)-transformed B cells or irradiated allogeneic lymphocytes as feeder cells [9,10]. In certain studies, the method of co-cultivation of NK cells with dendritic cells was used to obtain NK cell clonal expansion [11,12]. In 2000 a protocol was published on the stable production of NK cell clones by the limiting dilution method using RPMI-8866 feeder cells [13]. Since the method of limiting dilutions does not guarantee obtaining progeny of a single cell, sorting technique in the "single cell" mode was used in some other works to obtain cell clones [14–17]. This stimulation platform using gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21) in combination with IL-2 was previously shown to induce stable proliferation and significant expansion of NK cells [18,19]. IL-2 stimulates proliferation and differentiation of NK cells and increases their functional activity [20,21]. According to various data, IL-21 co-stimulates proliferation, promotes maturation of NK cells, enhances their functional activity, in particular, induces the production of IFN-γ [22] and increases cytotoxicity [20,23]. It is shown that IL-21-stimulated NK cells not only have a direct antitumor effect, but also affect naive and activated T cells, causing their migration and differentiation [24]. Along with IL-2, IL-21 has been described as a promising cytokine that enhances the antitumor properties of NK cells *ex vivo* [25]. Both these cytokines may be useful for obtaining NK cells for immunotherapeutic applications providing innate antitumor response of NK cells. First results of clinical trials (phase I) using mbIL21-expanded haploidentical NK cells for the treatment of leukemia patients after transplantation of allogeneic hemapoietic stem cells demonstrated low toxicity and promising therapeutic effects [26]. Because of the significant heterogeneity, a final NK cell population obtained after in vitro stimulation and expansion may not always satisfy the necessary requirements for cell numbers and anti-tumor activity. NK cell cloning allows to select cells with desirable properties for further adoptive cancer immunotherapy. One of the problems of using high doses of ex vivo expanded NK cells is their decreased cytotoxic activity during storage. The possibility to use thawed NK cells without loss of functional activity after freezing would allow accumulate them in advance and use for treatment as soon as they are needed. Search for strategies for optimal cryopreservation of NK cells was performed by several groups [27–29].

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The method of NK cell cloning we proposed here is based on stimulation of sorted in the "single cell" mode NK cells with gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21) in combination with IL-2. We developed two models for cultivation of NK cell clones obtained using K562-mbIL21 feeder cells, which resulted in different phenotype and lifetime of clones. Model 1 includes the weekly addition of the feeder cells together with partial substitution of the IL-2-supported medium. This model of cultivation leaded to the shorter lifespan (5-7 weeks) of the clones, but resulted in more activated phenotype and higher IFN-γ production compared to model 2 according to which the addition of K562-mbIL21 feeder cells occurs once during incubation, at week 6. This way of cultivation resulted in an increase of the clone lifespan up to 14 weeks. NK cells in this model retain the natural cytotoxicity but in most of clones had lower CD16 and HLA-DR expression levels compared to cells that were cultivated in model 1. We demonstrate that cryopreservation of well proliferated NK cell clones cultivated using model 2 did not change much their phenotype and functional activity after defrosting.

2 Results

2.1 NK cell cloning conditions

NK cell cloning was performed by FACS sorting of NK cells into 96-well plates. The advantage of this method in comparison with the method of limiting dilutions was that all wells most likely contained one NK cell per well. Several combinations of cytokines and feeder cells were tested for NK cell cloning in order to maximize the efficiency of clone generation. Stimulation of NK cells with IL-2 alone, IL-2 + IL-21 (10 ng/ml) or IL-2 + unmodified K562 (10^4 cells/ml) resulted in cell proliferation in only 10-15% of the wells containing single NK cells, with minor differences between stimuli combinations (Figure 1A).

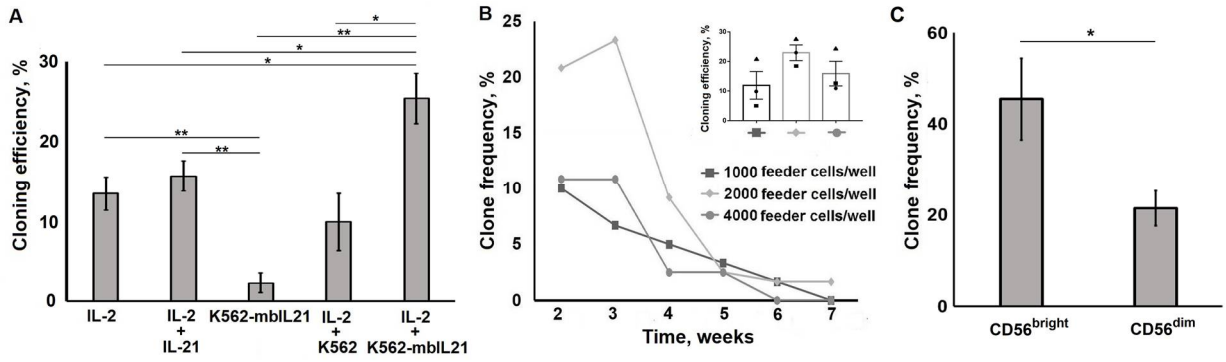

Figure 1. Selection and optimization of NK cell cloning method. (**A**) The efficiency of clone formation (cloning efficiency) using different stimuli. Mean ± SE of *n* independent experiments is presented (n=3 for IL-2; n=4 for IL-2+IL-21; n=3 for K562-mbIL21; n=3 for IL-2+K562; n=5 for IL-2+K562-mbIL21). (**B**) Selection of the number of K562-mbIL21 feeder cells for obtaining human NK cell clones (n=3). Cloning efficiency was calculated as clone frequency at the indicated week, when the greatest number of clones was detected in a collection. (**C**) $CD56^{bright}$ NK cells generate more clones than $CD56^{dim}$. The sub-figure shows the data for 4 clone collections.

Thus, IL-21 or unmodified K562 had no additional impact on clone frequency whereas IL-2 was required for NK cell clone generation. NK cells stimulated with modified K562-mbIL21 feeder cells alone also demonstrated very low clone generation efficiency (Figure 1A). The clones obtained with IL-2 alone, IL-2 + IL-21 or IL-2 + unmodified K562 lived no more than 4-5 weeks. However, when NK cells were cultivated in the presence of IL-2 in combination with K562-mbIL21, the efficiency of clone generation increased significantly, reaching 30% or more in certain experiments. Moreover, we were able to obtain long-lived clones of NK cells (up to 14 weeks) only by this way. Notably, the frequency of clones was higher in the fraction of $CD56^{bright}$ cells among all sorted NK cells (Figure 1C). $CD56^{dim}$ cells also responded to IL-2, but form less clones. It seems that IL-2 stimulation affects mainly $CD56^{bright}$ NK cells.

In order to select the optimal conditions for clone generation, we compared the efficiency of clone formation using several feeder cell concentrations which were added once before cell sorting (Figure 1B). The efficiency was the greatest at 2×10^3 feeder cells per well, and the survival of the obtained NK cell

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clones was also more prolonged compared to other stimulation conditions (Figure 1B).Therefore, the optimal conditions for NK cell clones generation appeared to be the stimulation of single cells with 100 U/ml of IL-2 and 2 × 10^3 K562-mbIL21 cells per well (Figure 1A).

2.2 Restimulation frequency affected NK cell clones lifespan, phenotype and functional state

We studied the influence of restimulation frequency on clone formation and survival, as the effect of feeder cells may depend on the time and duration of their addition [30]. In model 1, K562-mbIL21 feeder cells combined with IL-2 were added to NK cells every week after clonal expansion was registered (usually at week 3). In model 2, feeder cells were added to NK cell clones once during cultivation, at week 6, and IL-2 was added weekly. In both models, initial cloning conditions were the same (100 U/ml IL-2 and 2 × 10^3 K562-mbIL21 cells per well) (Figure 2).

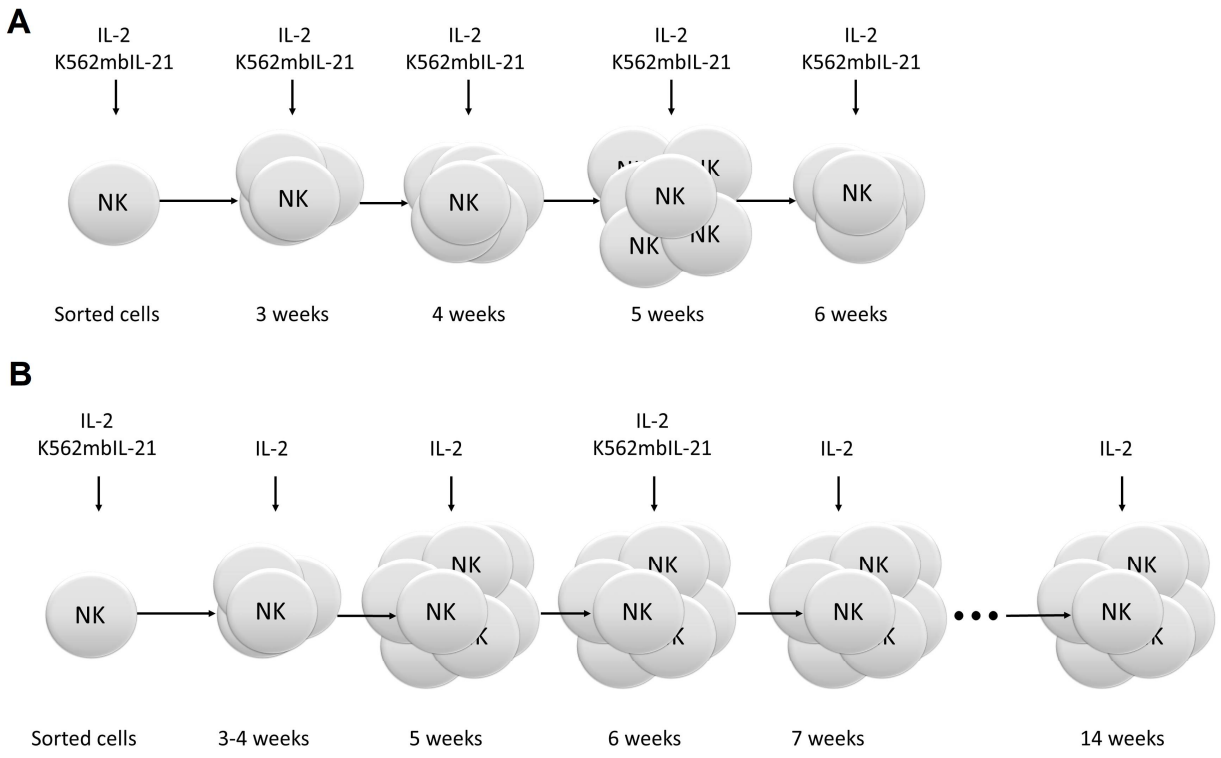

Figure 2. Schemes of NK cell clone cultivation procedures. (**A**) Model 1 – weekly addition of feeder cells, starting from the third week. (**B**) Model 2 – single addition of feeder cells at week 6.

Clones cultivated using model 1 generally had a shorter life-span than clones cultivated by model 2. In three collections of clones obtained from different donors with model 1, the life-span of all clones did not exceed 5-7 weeks. In contrast, when model 2 was used, the life-span of some individual NK cell clones was 8-14 weeks or more. Totally, 550 clones were obtained using model 2. Among them, 86 (15.6%) were long-lived clones which lifespan lasted 8 or more weeks. 10 of these clones (11.6%) showed the greatest lifespan (12-14 weeks).

We then analyzed the phenotype of obtained clones by the expression of different NK cell markers. All of the clones were CD56-positive and CD3-negative. The level of CD56 expression increased during cultivation, regardless of the model of cultivation (Figure 3A).

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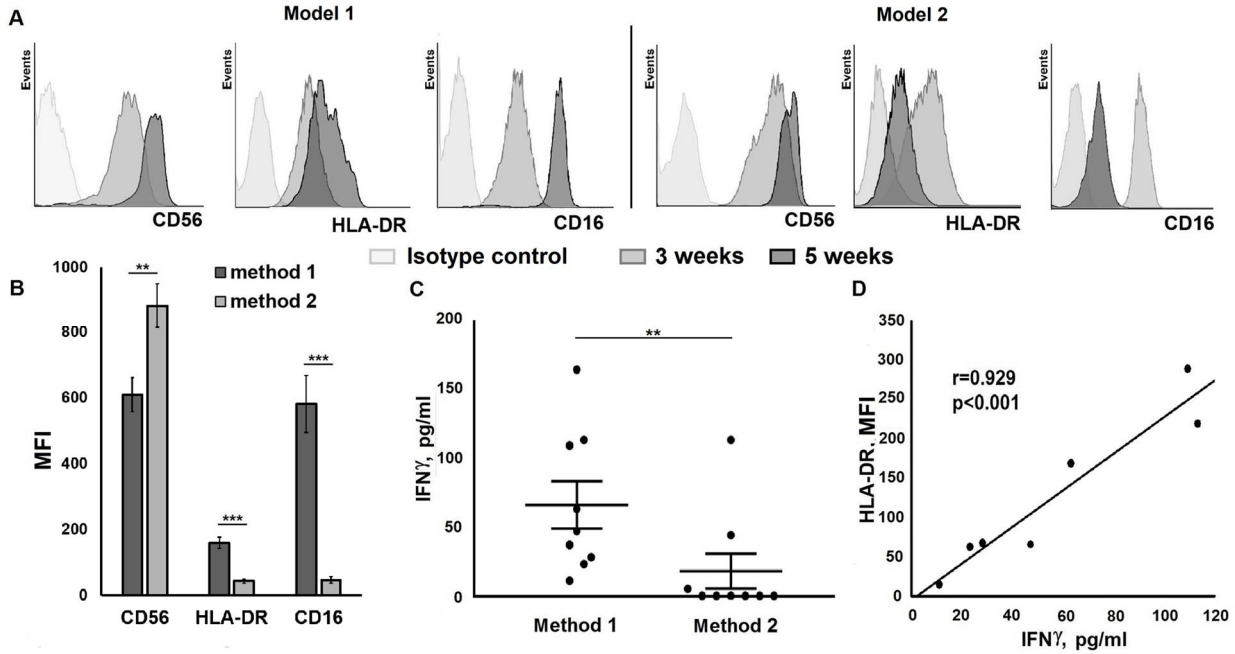

Figure 3. Expression of surface markers and IFN-γ production of NK cell clones depend of cultivation models. (**A**) Dynamics of surface markers expression in NK cell clones obtained in model 1 and model 2. Measurements were carried out at week 3 and 5 of cultivation. (**B**) Differences in the expression of CD56, HLA-DR and CD16 during clones cultivation by models 1 and 2 (measurements were carried out at week 5). (**C**) Comparison of the level of IFN-γ in supernatants of clones obtained in models 1 and 2. (**D**) Correlation between HLA-DR expression level and IFN-γ production in clones cultivated using model 1. (**C**, **D**) - Measurements were carried out at week 4 of clone cultivation.

The expression level of HLA-DR, measured after 5 weeks of cultivation, was higher in clones restimulated weekly with IL-2/K562-mbIL21 (model 1) compared to clones with one feeder cells restimulation at week 6 and weekly restimulation with IL-2 alone (model 2) (Figure 3B). Normally, a part of human peripheral blood NK cells express HLA-DR, and its function is still unknown, but it is considered to be an activation and/or proliferation marker [31]. We observed that repeated additions of the IL-21-expressing feeder cells during cultivation gradually increased the expression of HLA-DR in NK cell clones. In clones cultivated in model 2, high expression of HLA-DR was observed in 3 weeks after culture initiation, but decreased later even in well-proliferating clones (Figure 3A).

Many of the clones produced IFN-γ in response to IL-2. The intensity of production varied significantly between clones. Statistical analysis revealed significant difference in the production of IFN-γ between clones grown in models 1 and 2 (Figure 3C). All analyzed clones cultivated in model 1 produced IFN-γ. In contrast, clones cultivated in model 2 demonstrated IFN-γ production in less than 50% of the analyzed clones. Importantly, we found a strong correlation between IFN-γ production and HLA-DR expression in NK cell clones grown in model 1 (Figure 3D). Thus, weekly restimulation with IL-2 + K562-mbIL21 feeder cells, but not with IL-2 alone induced coordinated increase in HLA-DR expression and IFN-γ production. The increase of HLA-DR expression was observed in some but not all proliferating NK cell clones, suggesting that HLA-DR expression was somehow connected with IFN-γ production but was not a reliable proliferation marker for NK cell clones.

In addition to HLA-DR, CD16 expression also increased during clone cultivation by model 1. In clones grown by model 2, CD16 expression level measured at week 5 was lower than at week 3 (Figure 3A). On average, at week 5 the expression level of CD16 in clones grown by model 1 was significantly higher compared to clones cultivated by model 2. Interestingly, despite the similar averaged dynamics of HLA-DR and CD16 expression in the clones cultivated by the same method, we did not find significant correlation between CD16 and HLA-DR expression intensity in individual clones.

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After four weeks of cultivation, total number of cells in clonal cell cultures obtained by both model 1 and model 2 reached values ranging from 5×10^4 to 3×10^6 cells in one clone. In 8 individual clones cultured by model 2 total cell number reached $1\text{-}2\times10^7$ cells.

2.3 The changes in some clone characteristics are associated with the addition of feeder cells

To determine the effect of feeder cells on clone phenotype, several clones cultivated using model 2 were divided into two equal parts at week 6. Then these clones were cultivated for two weeks in the presence of IL-2, 100 U/ml, and to one half of the cells of each clone, feeder cells were added weekly. Then the phenotype of the clones was assessed (Figure 4A).

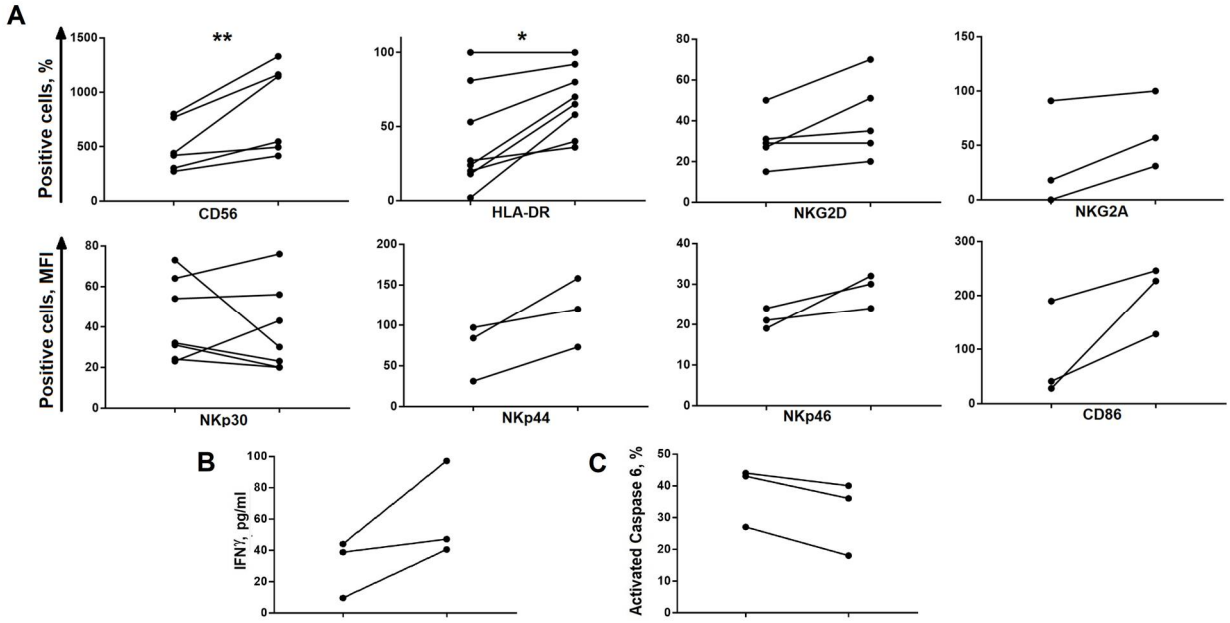

Figure 4. Phenotypic and functional analysis of clones stimulated by IL-2 with or without K562-mbIL21 feeder cells. (**A**) Phenotypic analysis of clones stimulated by IL-2 with or without K562-mbIL21 feeder cells for two weeks. The clones were divided into two equal parts after 6 weeks of cultivation by model 2, and then one part was restimulated by feeder cells. The changes in surface markers cultured clones without (left) and with the addition of feeder cells (right). Wilcoxon signed rang test was used for the determination of statistical differences. (**B**) Natural cytotoxicity of clones stimulated by IL-2 without (left) or with (right) K562-mbIL21 cells. (**C**) IFN-γ levels in supernatants of clones stimulated by IL-2 without (left) or with (right) K562-mbIL21 feeder cells.

Clone cells cultured with the addition of feeder cells were more activated compared to the cells stimulated with IL-2 alone, as seen by an increase in the activation markers HLA-DR and CD86, and NKp44. The surface levels of CD56, of the activating receptor NKp46, and the inhibitory NKG2A receptor also increased. Thus, it can be concluded that contact interactions between NK cells and feeder cells lead to additional activation.

In the further experiments, functional activity of clones obtained with cultivation models 1 and 2 was compared. For this, the selected clones were divided into two parts at week 2 of cultivation. One part was cultivated according to model 1 with weekly restimulation by feeder cells, and the second part – by model 2 without the addition of feeder cells. The levels of IFN-γ production and natural cytotoxicity were evaluated. To determine the potency to produce IFN-γ, the cells were transferred to the medium without interleukins for 24 h, then restimulated with IL-12+IL-15 for 18 h. We found that contact interactions with feeder cells during cultivation increased the level of IFN-γ secretion (Figure 4B), which confirms the data in Figure 2C. At the same time, there was no statistically significant difference in the level of natural cytotoxicity between clones cultivated with IL-2 alone or with the addition of feeder cells (Figure 4C). However, there was a slight tendency that clones restimulated with feeder cells had lower natural cytotoxicity response.

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2.4 *Long-lived NK cell clones obtained in model 2 were capable of natural cytotoxicity*

We have analyzed natural cytotoxicity of well proliferating clones, generated using model 2. Even after 10-14 weeks of cultivation these NK cell clones retained the ability to degranulate (Figure 5A), and showed a significant cytotoxic potential against standard K562 target cells (Figure 5B).

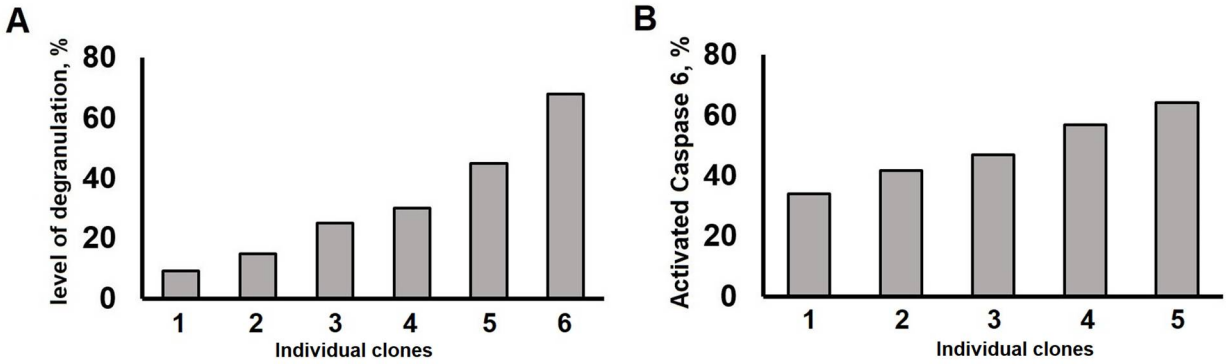

Figure 5. Analysis of the functional activity of long-lived NK cell clones at week 14 of cultivation. (**A**) Degranulation of the clones, measured by the level CD107a expression on the cell surface after incubation in the presence of K562 target cells. (**B**) The level of natural cytotoxicity of the clones, measured by caspase-6 activation in K562 cells.

These clones were $CD56^{+}NKG2D^{+}$, most of them express NKp30 and NKp46 at significant levels. The clones were also positive for CD16 and HLA-DR, however the expression levels of these markers varied widely.

2.5 *Freezing of NK cell clones retain their functional potential*

To study the effect of freezing on the phenotype and functional status, some of the long-lived clones obtained by model 2 were frozen and, then, defrosted after a year. It was found that freezing of the clones at the concentration below 1.5 million/ml led to unstable outcome of NK cells from the frozen state, which negatively affected the lifetime of the clone after defrosting. Clones with different proliferative activity were used for freezing. The level of proliferation was assessed by the increment in the number of cells per week of incubation, which was calculated according to the formula N_2/N_1, where N_1 is the initial number of cells per ml of medium and N_2 is the number of cells per ml of medium after 1 week of incubation. The best survival after defrosting was shown by clones that had the increment index of 2 or higher. The lifetime of clones after defrosting was at least 3 weeks. As a result, optimal conditions for the freezing of clones were selected, which provided stable proliferation of clones after defrosting.

Defrosted clones were cultured for a week in complete medium with IL-2 (100 U/ml), and then were phenotypically characterized (Figure 6A).

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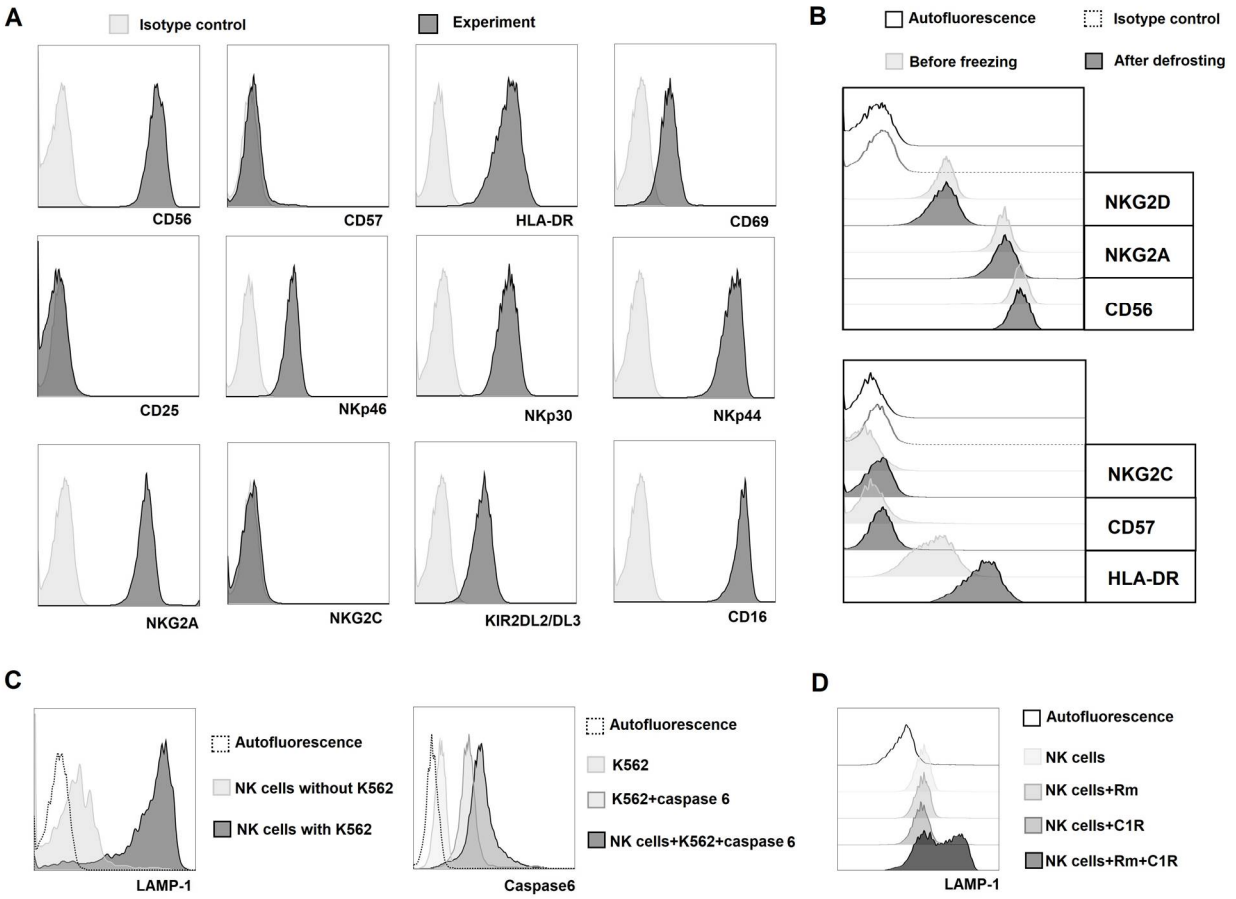

Figure 6. Analysis of the phenotypic and functional characteristics of defrosted NK cell clones generated using model 2. After defrosting, clones were incubated in NK cell medium with 100 U/ml IL-2 for one week. The figure shows the data of one representative clone. (**A**) Analysis of several surface marker expression in clones, measured by flow cytometry after defrosting the clone. (**B**) Comparison of surface expression of surface receptors and markers before freezing (light gray) and after defrosting (dark gray). (**C**) Analysis of natural cytotoxicity in NK cell clones by evaluating CD107a expression (left) and the level of activated caspase 6 in K562 target cells (right). (**D**) Analysis of antibody-dependent cell cytotoxicity. The targets were CIR cells (B-cell lymphoma line). Humanized antibody Rituximab against CD20 (Rm) was used.

All clones were $CD56^{+}CD57^{-}NKG2A^{+}$ that indicates these clones have the phenotype of less differentiated NK cells. After defrosting, the surface expression of main markers in the clones was similar to the expression measured before freezing (Figure 6B). Besides, defrosted clones were functionally active in both natural (Figure 6C) and antibody-dependent cellular cytotoxicity tests (Figure 6D).

3 Discussion

NK cell clones are commonly used for studies of NK cell differentiation [16,32], phenotypic and functional characteristics of individual NK cells [5,6], and for selection of NK cells with increased anti-tumor activity [7,8]. Development of an effective method of generation and expansion of NK cell clones for NK cell-based immunotherapy can help to avoid the problem of heterogeneity of functional and phenotypic characteristics of donor NK cells.

A variety of stimulation options can be applied to activate and expand NK cells [18,33,34]. Some of them can be used to obtain NK cell clones [13,16]. Most of the stimulation approaches are based on combinations of cytokines acting cooperatively on NK cells. Previously a combination of IL-2 and K562mbIL-21 had been shown to induce the wide proliferation of NK cells [18,19] and thus this approach can be successfully used for immunotherapy [26]. This method of stimulation had been chosen for generation of NK cell clones in current work.

Because of the significant heterogeneity of initial NK cells fraction a final NK cell population obtained after *in vitro* stimulation and expansion may not always satisfy the necessary requirements for cell numbers and anti-tumor activity. NK cell cloning allows to select cells with desirable properties for further adoptive cancer immunotherapy. At this time, there are a few protocols described for the stable production of NK cell clones. Our clone production method allows to obtain a homogeneous populations of cells with the better accuracy than the limiting dilution method described in the Cella and Colonna protocol [13]. The acquisition of long-lived clones has been described in work of Carr with coworkers [17]; they reported that the clones were cultivated for 8-16 weeks. However, their technique requires the weekly addition of two types of feeder cells (allogeneic PBMC and Epstein–Barr virus (EBV)-transformed B lymphoblastoid cell line) into wells with growing clones. The production of allogeneic PBMC is associated with additional difficulties. In contrast to this method, we propose a model for the stable production of long-lived clones, which requires only two-fold introduction of one type of feeder cells. Feeder cells based on the cell line are more accessible and easier to use. Since feeder cells are eliminated within a week after their addition it is possible to obtain a pure culture of NK cells, which does not require special purification from feeder cells on the ficoll gradient.

IL-2 and IL-21 receptors contain common IL-2 receptor (IL-2R) γ-chains, which explains the similarity in the effect of these cytokines on NK cells, although there are also a number of differences in signaling pathways [39]. The balance of signaling components underlies the complex regulation of NK cell activation and functioning mediated by these cytokines. In our stimulation system IL-21 was presented to NK cells on the surface of K562 cells. These feeder cells expressed as well the 1-4BBL ligand, whose receptor may be expressed by NK cells [40]. Clones obtained with such combined stimulation lived longer than clones obtained with IL-2 alone. Empirically, we defined the optimal dose of feeder cells (2×10^3 cells per well of 96-well round-bottom plate), sufficient to stimulate NK cell expansion (Figure 2B). The results agree with data from earlier works describing co-stimulatory effects of IL-21 on IL-2-induced proliferation of NK cells [30,41].

The highest cloning frequency has been found in $CD56^{bright}$ subset of NK cells. These cells are thought to respond better to IL-2 by expressing the high affinity receptor for this cytokine. The longest-living clones (up to 14 weeks or more) were obtained using model 2, at which feeder cells expressing IL-21 were added to NK cells at the beginning and then once at week 6. More frequent addition of feeder cells leaded to the lower life-span of the clones that comprised about 5-6 weeks. Several studies have already demonstrated that the effects of IL-21 on the expansion rate and phenotypic characteristics depend on the dose and, most interestingly, on the timing and duration of exposure. For instance, addition of IL-21 only during the first week of cultivation caused an increase in the number of NK cells at the initiation of culture [30], whereas IL-21-mediated restriction of proliferation was observed during repeating stimulation with IL-21 in combination with IL-2 [30,42]. Continuous exposure to IL-21 can make the proapoptotic effect of this cytokine more pronounced [22]. Multiple additions of feeder cells can also lead to excessive degranulation of NK cells, which depletes their intracellular resources and adversely affects their survival. The most significant expansion of NK cells (10^{11}-fold after six weeks) described in literature was observed with a single initial stimulation with IL-21 [43]. In our work, more than 2×10^7 cells per clone were obtained in certain clones when membrane-bound IL-21 was added twice: at the beginning of cultivation and at week 6. Single stimulation of NK cells by feeder cells also resulted in a significant increase in the number of cells up to 6 weeks, but without restimulation, the rate of proliferation of NK cells after 6 weeks of cultivation slowed, and death of clones was registered (data not shown).

Stimulation with IL-2 and/or IL-21 results in the increase of HLA-DR expression on the surface of NK cells [44–46]. Thus, HLA-DR has been proposed as a marker of NK cell activation. Besides, IL-21 in both soluble and membrane-bound form augments the production of IFN-γ in NK cells [30,47]. In this and earlier studies by our group, HLA-DR expression has been detected in NK cell clones obtained by combined stimulation with IL-2/K562-mbIL21 [23]. Higher level of HLA-DR expression has been observed in clones grown with weekly addition of IL-2/K562-mbIL-21, not IL-2 alone (Figure 3C), which indicates the significant role of IL-21-expressing feeder cells in increasing the expression of HLA-DR. CD16 surface level was also higher in clones cultivated by model 1 compared to model 2.

Interestingly, HLA-DR level, but not CD16 level, correlated positively with intensity of IFN-γ production in these clones. In clones cultivated with the single addition of feeder cells at week 6 and weekly addition of IL-2 (model 2) IFN-γ production significantly decreased throughout cultivation alongside with the decrease in HLA-DR and CD16 expression (Figure 3D). A slight decrease in CD16 expression was observed in another study when IL-21 was applied at the initial stage of stimulation [30,45]. Significant change in CD16 expression intensity observed in both cultivation models indicates that CD16 should not be considered as a differentiation marker *in vitro*.

The low level of proliferative activity and short lifetime of clones obtained by model 1 makes this method less attractive for immunotherapy. However the increase of lifespan of clones generated using model 2 leaded to decrease of surface expression of CD16 and production of IFN-γ that also hinder the use of this method in immunotherapy. We have tried to find a way to increase functional activity of clones generated by model 2 during their growth. It is known that restimulation of NK cells by cytokines leads to increase of their functional activity. In particular, cytokine-induced NK memory cells can be obtained by such method [48]. We assumed that feeder cells can affect the NK cell functions by the same way. We have investigated whether NK cell clones can be activated at later period of clone growth by restimulation with feeder cells. Twice-repeated addition of feeder cells significantly increased expression levels of activation-associated markers in clones (Figure 4). The possibility to increase the expression level of surface molecules at various time periods makes possible to change the functionality of clones generated using method 2, taking advantage at the same time of more intense proliferation and longer lifespan of such clones, which can be useful for personalized therapy. Also, long-lived clones cultivated using model 2 retained natural cytotoxicity and demonstrated the ability to kill targets even after 14 weeks of cultivation. All this makes model 2 a quite perspective approach for generation of NK cells with desired properties. With regard to model 1, the weekly restimulation of clones by feeder cells may lead to the decrease in natural cytotoxicity apparently because of exhausting of cytolytic granules in NK cells (Figure 4C). It can also be assumed the functional activity is somehow connected to the proliferative potential of the clone.

The use of cloning technique to obtain a homogeneous population of NK cells for personalized immunotherapy is possible only if the cells retain their characteristics after freezing/defrosting. This is a potential limitation for the use of NK cells in immunotherapy, because unlike T cells, they are very sensitive to the process of freezing and thawing, which leads to loss of their functional activity. Our results showed that even prolonged freezing (within one year) does not affect the phenotype and does not reduce the functional activity of NK cell clones. Recovered after cryopreservation clones demonstrated high expression levels of a number of surface markers and good proliferative capacity (Figure 6). They also demonstrated a high level of cytotoxic activity. It can be concluded that the freezing/defrosting of clones, generated with IL2+K562mbIL-21 does not affect their phenotypic and functional characteristics.

Thus, in this work we have developed two models of NK cell clone generation that lead to different phenotype and functional abilities of resulting cells. Weekly restimulation with IL-2 and K562-mbIL-21 feeder cells (model 1), caused higher expression of HLA-DR, CD16, and high production of IFN-γ in NK cell clones, i.e. led to development of clones with activated phenotype. Such clones demonstrated shorter lifetime, lower levels of proliferative activity and natural cytotoxicity. On the contrary, model 2 (weekly restimulation of clones with IL-2 in the absence of feeder cells) led to generation of long-lived highly proliferating clones with lower level of IFN-γ secretion, but high natural cytotoxicity. Depending on the objectives of the study, a choice can be made in favor of a particular model of NK cell cloning and culturing. Method of human NK cell clone generation with the use of K562mbIL-21 cells can be considered as a way to obtain homogenous NK cell populations to increase the effectiveness of immunotherapeutic treatment. The possibility of freezing of the cells without the loss of functional activity allows creating banks of NK cells for personalized immunotherapy.

4 Material and methods

4.1 Cell lines

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K562 cells and K562-mbIL21 cells expressed CD64, CD86, CD137L (4-1BBL), and truncated CD19 [18] were cultivated using complete RPMI-1640 medium, i.e. RPMI-1640 supplemented with 10% fetal calf serum (FCS) (HyClone, USA), 2 mM of L-glutamine (PanEco, RF) and antibiotic-antimycotic solution (Millipore-Sigma, USA), at the concentration of 2-6×10^5 cells/ml. Before using for NK cell stimulationK562-mbIL21 cells were irradiated with γ-radiation (100 Gy) and immediately frozen in FCS containing 10% DMSO (Sigma, USA) at -135°C or -150°C. C1R cell line was cultivated using complete RPMI-1640 medium at the concentration of 2-6×10^5 cells/ml.

4.2 NK cell isolation

Blood samples were taken from healthy volunteers of different age and sexes. All participants gave their informed consent prior to the study in accordance with the recommendations of local ethics committee (Pirogov Russian National Research Medical University). NK cells were isolated by ficoll density gradient centrifugation of peripheral blood samples followed by negative magnetic separation using human NK cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's protocol.

4.3 Preparation of plates with feeder cells for cell sorting

Complete NK cell medium was mixed from 80% complete DMEM medium (PanEco, RF), 20% Ex-vivo medium (Lonza, USA) supplemented with 100 unit/ml of recombinant IL-2 (Sigma, USA), or AIM-V medium (Gibco, USA) supplemented with 100 unit/ml of recombinant IL-2 (Sigma, USA). This medium and 10^4 of irradiated K562-mbIL21 feeder cells per ml were added. Sixty central wells of a 96-well plate were filled with 200 µl of this suspension. The marginal wells were filled with RPMI-1640 medium to minimize evaporation from the central wells during cultivation of sorted NK cells.

4.4 Fluorescence-activated single cell sorting and generation of NK cell clones

Purified NK cells were labeled with mouse anti-human monoclonal antibodies (mAbs) CD3-PE-Cy7 (Beckman Coulter, USA, clone UCHT1) and CD56-Brilliant Violet 421 (Sony, USA, clone HCD56). $CD3^-CD56^+$ cells were sorted into 96-well round-bottom plates containing feeder cells suspended in complete NK cell medium, one cell per well in the "Single cell" mode. Cell sorting has been performed with FACSVantage DiVa machine (BD Biosciences, San Jose, CA, USA) equipped with 405, 488, 643 nm lasers and an appropriate set of detectors and filters. The plates were then put into a CO_2-incubator (5% CO_2, 37°C) for 2 weeks. After 8 weeks of incubation, clones were transferred to a new 96-well round-bottom plate.

4.5 Cultivation of NK cell clones

Model 1

Every week beginning from week 3, 100 µl of culture media was substituted with the same volume of suspension consisting of complete NK cell medium with 100 unit/ml of recombinant IL-2 (Sigma-Aldrich) and 10^4/ml of irradiated K562-mbIL21 feeder cells (Figure 2A).

Model 2

Substitution of 100 µl of fresh complete NK cell medium with 100 unit/ml of recombinant IL-2 was performed weekly starting from the third week of incubation.10^4/ml of irradiated K562-mbIL21 feeder cells were added once after 6 weeks of clone cultivation (Figure 2B).

4.6 Estimation of NK cell clone frequency and lifespan

Clone generation frequency were calculated according to the formula Fc=Fa/Fb*100% (Fc - clone generation frequency, Fa - number of wells with clones, Fb - total number of occupied wells). Depending on the donor, the maximal number of clones could be obtained at different time points, from 2 to 4 weeks of incubation. The percentage of surviving clones was calculated as the ratio of the number of clones detected at a certain week to the maximum number of clones detected throughout the whole experiment (usually at week 2, 3, or 4, depending on the collection).

4.7 Surface fluorescent immunostaining and flow cytometry

To study the marker surface expression in NK cells, the following mAbs were used: CD3-PE-Cy7 (clone UCHT1), CD56-APC (clone N901), CD56-PE (clone N901 (HLDA6)), HLA-DR-FITC (clone B8.12.2), from Beckman Coulter, USA; CD56-Brilliant Violet 421 (clone HCD56), CD16-PE (clone 3g8), from eBioscience, USA; CD57-PE (clone TB01), NKp46-FITC (clone 9E2), NKp30-PE (clone P30-15), NKp44-PE (clone 44.189), CD107a-PE-Cy5 (clone H4A3), NKG2D-PE (clone 1D11), CD69-PE (clone FN50), from Sony, USA; CD57-FITC (clone TB03), CD57-APC (clone TB03), anti-KIR2DL2/DL3-PE (clone DX27), from Miltenyi Biotech, Germany; anti-NKG2A-PE (clone 131411), anti-NKG2C-PE (clone 134591), anti-NKG2A-PE (clone 131411), R&D Systems, USA; CD25-FITC (clone M-A251, BD Biosciences, USA), and CD86-PE (clone IT2, BioLegend, USA).

Surface fluorescent immunostaining was performed on ice using staining buffer PBA (PBS containing 0.5% BSA (bovine serum albumin) (Serva, Germany) and 0.01% sodium azide (Amresco, USA)). After double washing with PBA samples were analyzed using FACSCalibur flow cytometer (BD Biosciences, USA) equipped with 488 and 640 nm lasers. At least 30000 events in lymphocyte gate for total NK cells and 5000 events for NK cell clones were recorded. Acquired data was analyzed using FlowJo program version 7.6 (FlowJo LLC, Ashland, OR, USA) and Flowing Software version 2.5.1 (PerttuTerho, Turku Centre for Biotechnology, Finland).

4.8 Natural cytotoxicity evaluation

The K562 cell line was used as target cells for the analysis. The experiments were carried out at a ratio 1:1 of NK cells:targets. Killing effectiveness was measured by two methods.

a. Registration of caspase-6 activity in target cells

Analysis of cytotoxicity based on count of percentage of target cells with active caspase-6 was performed using kit CyToxiLux (CTL602, Oncoimmunin, USA). Briefly, K562 cells were pre-stained with vital TFL4 dye in RPMI-1640 medium, and then washed. NK cells and K562 cells were mixed and centrifuged at 240 g for 5 min. After decantation of the supernatant, 50 µl of caspase-6 substrate (CS) was added, cells were pelleted at 240 g for 30 s and then incubated for 30 min at 37°C, 5% CO_2. After washing with PBA, samples were analyzed with FACSCalibur flow cytometer, percentage of TFL^+CS^+ K562 cells was evaluated.

b. Registration of NK cell degranulation by CD107a expression

The experiments were carried out according to the procedure described earlier [23]. Briefly, NK cells were mixed with K562 cells in the RPMI-1640 medium with anti-human CD107a-PE-Cy5 (eBioscience, USA, clone H4A3) and brefeldin A (Sigma, USA) at a concentration of 10 µg/ml. Cells were precipitated at 240g for 30 s and then incubated for 2.5 h at 37°C, 5% CO_2. Analysis was performed on the FACSCalibur flow cytometer, the percentage of $CD107a^+$ NK cells was evaluated.

4.9 Antibody-dependent cytotoxicity evaluation

C1R target cells were added to NK cells at a ratio 1:3 (target:effector) in RPMI-1640 medium containing brefeldin A, 10 µg/ml, CD107a-PE-Cy5 mAb, 0.5 µg/100 µl, and Rituximab (CD20 mAb, Roche, Switzerland), 2.5 µg/ml. Cells were precipitated at 240 g for 30 s and incubated for 2.5 h at 37°C, 5% CO_2. Analysis of NK cell degranulation was performed on FACSCalibur flow cytometer.

4.10 Analysis of IFN-γ production by ELISA

NK cell clone supernatants were collected at week 5 of clone cultivation. Stimulation of NK cells was performed using two methods. In first method, 10^5 cells were incubated with fresh complete medium with 100 U/ml IL-2 overnight. The second method were performed with incubation with fresh complete medium without 100 U/ml IL-2 overnight and restimulation with 10 ng/ml IL-12 и 10 ng/ml IL-15 within 16 hours. IFN-γ secretion level was analyzed as described earlier [50] using ELISA kit (Vector-Best, RF).

4.11 Statistical analysis

For determining the statistical significance of results, Student's t-test was used for data with normal distribution and Mann-Whitney U-test and Wilcoxon signed rang test were applied to data having non-normally distribution. P-values of < 0.05 were considered significant.

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