ЭКСПАНСИЯ NK-КЛЕТОК *IN VITRO* СОПРОВОЖДАЕТСЯ ПОТЕРЕЙ ЭКСПРЕССИИ ИНГИБИРУЮЩИХ РЕЦЕПТОРОВ KIR

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Резюме. NK-клетки – лимфоциты врожденного иммунитета, которые способны эффективно элиминировать измененные клетки организма, что делает перспективным их применение в иммунотерапии вирусных заболеваний и опухолей. Популяция NK-клеток отличается высоким фенотипическим и функциональным разнообразием. В частности, в пуле высокодифференцированных NK-клеток в присутствии цитомегаловируса (HCMV) может формироваться популяция адаптивных клеток, которые отличаются высокой продолжительностью жизни и высокой цитотоксичностью. Однако для осуществления цитотоксической реакции NK-клетке необходимо пройти процесс обучения, в ходе которого она приобретает экспрессию рецепторов NKG2A и KIR. Ингибирующий сигнал от этих рецепторов предотвращает цитотоксическую реакцию против здоровых клеток организма. В настоящий момент существует множество эффективных методов накопления NK-клеток для последующего применения в терапии, один из них – стимуляция IL-2 и фидерными клетками K562-mbIL21. Высокодифференцированные NK-клетки с адаптивно-подобным фенотипом способны отвечать экспансией на такую стимуляцию. Однако показано, что в ходе активной пролиферации может динамически изменяться фенотип NK-клеток. Потеря экспрессии ингибирующих рецепторов KIR в ходе интенсивной пролиферации NK-клеток в ответ на стимул может негативно сказаться на их цитотоксическом потенциале и способности элиминировать мишени. В этой работе показано, что высокодифференцированные NK-клетки $CD56^{dim}NKG2C^{+}$ HCMV-серопозитивных индивидов отличаются высокой долей клеток $KIR2DL2/3^{+}$. Это может свидетельствовать о высокой стабильности экспрессии рецепторов KIR в этой популяции. Нами было показано, что клональные культуры $CD56^{dim}NKG2C^{+}$, полученные при стимуляции IL-2 и K562-mbIL21, отличаются высокой стабильностью экспрессии KIR2DL2/3 по сравнению с NKG2C-негативными и менее дифференцированными $CD56^{bright}NKG2C^{+}$. Также в гетерогенных культурах предшественников адаптивных NK-клеток $CD57^{-}CD56^{dim}NKG2C^{+}$ наблюдался более высокий уровень экспрессии KIR2DL2/3 в сравнении с NKG2C-негативными

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культурами CD57$^-$CD56^{dim}NKG2C$^-$. Таким образом, накопление NK-клеток при стимуляции IL-2 и фидерными клетками K562-mbIL2 может приводить к потере экспрессии рецепторов KIR и снижению их функциональной активности. Однако культурам высокодифференцированных NK-клеток HCMV-серопозитивных индивидов CD56^{dim}NKG2C$^+$, а также культурам предшественников адаптивных NK-клеток CD57$^-$CD56^{dim} NKG2C$^+$ свойственна большая стабильность экспрессии KIR2DL2/3 в сравнении с культурами NKG2C-негативных и менее дифференцированных NK-клеток. Как следствие, стимуляцию IL-2 и фидерными клетками K562-mbIL21 можно применять для накопления адаптивно-подобных клеток и их предшественников, и при этом цитотоксический потенциал полученных культур, как и экспрессия ингибирующих рецепторов KIR, будет стабилен.

Ключевые слова: NK-клетки, натуральные киллеры, ингибирующие рецепторы KIR, экспансия NK-клеток, иммунотерапия, цитомегаловирус, HCMV

NK CELL EXPANSION *IN VITRO* IS FOLLOWED BY LOSS OF INHIBITORY KIR EXPRESSION

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Abstract. NK cells are innate lymphocytes that are able to eliminate altered cells, which makes them promising for the immunotherapy of viral diseases and tumors. The NK cell population is characterized by high phenotypic and functional diversity. In particular, in the pool of highly differentiated NK cells in the presence of cytomegalovirus (HCMV), a population of adaptive cells can be formed, characterized by a high lifespan and high cytotoxicity. However, in order to carry out a cytotoxic reaction, a NK cell must undergo a licensing process, during which it acquires the expression of NKG2A and KIRs. Currently, there are many effective methods of NK cell accumulation for subsequent use in therapy, one of them is the stimulation with IL-2 and K562-mbIL21 feeder cells. Highly differentiated adaptive-like NK cells are able to expand in respond to such stimulation. However, the phenotype of actively expanding NK cells dynamically changes. Loss of inhibitory KIR expression during intense proliferation of NK cells may adversely affect their cytotoxic potential. This work shows that highly differentiated CD56^{dim}NKG2C$^+$ NK cells from HCMV-seropositive individuals have a high proportion of KIR2DL2/3$^+$ cells. This may indicate a high stability of KIR receptor expression in this population. We have shown that CD56^{dim}NKG2C$^+$ clonal cultures obtained by stimulation with IL-2 and K562-mbIL21 are characterized by high stability of KIR2DL2/3 expression compared to NKG2C-negative and less differentiated CD56^{bright}NKG2C$^+$. Also, in heterogeneous cultures of adaptive NK cells precursors CD57$^-$CD56^{dim}NKG2C$^+$, a higher expression level of KIR2DL2/3 was observed in comparison with NKG2C-negative cultures of CD57$^-$CD56^{dim}NKG2C$^-$. Thus, the accumulation of NK cells upon stimulation with IL-2 and K562-mbIL2 feeder cells can lead to loss of expression of KIR receptors and a decrease in their functional activity. However, cultures of highly differentiated NK cells of HCMV-seropositive individuals CD56^{dim}NKG2C$^+$, as well as cultures of precursors of adaptive NK cells CD57$^-$CD56^{dim}NKG2C$^+$, are characterized by a greater stability of KIR2DL2/3 expression. As a result, stimulation with IL-2 and K562-mbIL21 feeder cells can be used to accumulate adaptive-like cells and their progenitors with stable inhibitory KIR expression and high cytotoxic potential.

Keywords: NK cells, natural killers, inhibitory KIR, NK cells expansion, immunotherapy, HCMV

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Introduction

NK cells are innate lymphocytes that are capable of exhibiting cytotoxic activity against altered cells without prior sensitization. They are characterized by high phenotypic and functional diversity. In NK cell population, there are both regulatory cytokine-producing cells and effector cells with a high density of cytolytic granules. The population diversity of human NK cells is resulted from both their interaction with the internal environment of the organism and by

external factors, including a wide range of pathogens. Pathogens can influence the differentiation and maturation of NK cells, as well as promote the formation of a pool of so-called adaptive NK cells in the subset of highly differentiated $CD56^{dim}$NK cells. These adaptive NK cells are distinguished by a high lifespan and specialized functional activity.

Among adaptive NK cells the best studied are cytomegalovirus (HCMV)-specific ones, which have high expression levels of the NKG2C activating receptor [8], capable of recognizing the HCMV-derived peptide presented on the surface of infected cells in context of HLA-E [2, 6] engagement of inhibitory killer immunoglobulin-like receptors (KIR. However, these cells show cytotoxic activity only after the licensing, when inhibitory receptors KIR or NKG2A begin to be expressed on the surface of NK cells. These receptors are able to recognize HLA-I on surrounding cells, which allows NK cells to inhibit the cytotoxic response towards healthy cells. NKG2A expression is typical of poorly differentiated cells and decreases during maturation, while the proportion of KIR-positive cells increases during differentiation. In addition, the NKG2A receptor, in contrast to KIR, is more sensitive to the expression level of HLA-I molecules than to the presented peptide repertoire [3]. Thus, KIR receptors can enhance the specificity of the cytotoxic pathogen response of NK cells.

NK cells are currently used in cell therapy of several diseases. The lifespan along with the high level of antibody-dependent cytotoxicity of adaptive NK cells make them promising for the immunotherapy of various diseases, both viral and oncological. Therefore, the search for effective approaches to the accumulation of adaptive NK cells with a stable phenotype is especially important. For *ex vivo* expansion NK cells are stimulated with cytokines in the presence of feeder cells, made from modified and irradiated cell lines. K562 with membrane-bound interleukin 21 (K562-mbIL21) cells have shown their effectiveness and can induce an active proliferation of NK cells and increase their functional activity [5]. However, during cultivation, the cell phenotype changes dynamically. For example, when cultured with K562-mbIL21 feeder cells, NK cells are able to acquire the expression of the terminal differentiation marker CD57 or the NKG2A inhibitory receptor *de novo* [11] we analyzed the phenotype and growth of human NK cell clones obtained by the stimulation of individual NK cells with IL-2 and gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21).

In this work, we analyzed the stability of KIR2DL2/3 expression in cultures of NK cell subsets which differ in the differentiation stage and the level of NKG2C expression. The stability of KIR2DL2/3 expression in the subset of potential adaptive NK cell progenitors [7] was evaluated as well. NK cell *in vitro* cultures were obtained by stimulation with IL-2 and K562-mbIl21 feeder cells.

Materials and methods

NK cells for following experiments were isolated from peripheral blood mononuclear cells (PBMC) by negative magnetic separation (NK cell isolation kit, MiltenyiBiotec, Bergisch Gladbach, Germany). PBMC were previously obtained by gradient centrifugation from blood samples of healthy donors, who gave their informed consent. *Ex vivo* NK cells were stained with anti-human monoclonal antibodies: CD56-BrilliantViolet (clone HCD56), NKG2C-AlexaFluor (AF) 488 (clone 108724), KIR2DL2/3-PE-Vio615 (clone REA1006) (Miltenyi Biotec, Germany) and CD57-PE-Vio770 (clone REA769) and then sorted into wells of 96-well plate. Heterogeneous cultures were obtained via planting by 100 cells into each well, clonal cultures by planting a single cell into each well. Sorted NK cell subsets differed in differentiation stage and NKG2C expression level: $CD56^{bright}NKG2C^{-}$, $CD56^{bright}NKG2C^{+}$, $CD56^{dim}NKG2C^{-}$, $CD56^{dim}NKG2C^{+}$. Subsets of adaptive NK cells precursors $CD57^{-}CD56^{dim}NKG2C^{+/-}$ were sorted as well.

These sorted cells were cultivated in medium for clones (80% DMEM medium (PanEco, Moscow, Russia), 20% x-vivo medium (Lonza, Swiss), 2 mM L-glutamine, 2 mM sodium pyruvate (PanEco, Moscow, Russia), 2 mM antibiotic-antimycotic (Sigma-Aldrich, St. Louis, MO, USA)) with 100 U/ml IL-2 and feeder cells K562-mbIL21 (37 °C, 5% CO2), for 2 weeks (heterogeneous cultures) or 3 weeks (clonal cultures). *Ex vivo* NK cells and NK cell cultures obtained after the stimulation were stained with following anti-human monoclonal antibodies: NKG2C-AF 488, KIR2DL2/3-PE-Vio615 and CD57-PE-Vio770, CD56-APC, KIR2DL2/3-PE (clone DX27). Then flow cytometry data was obtained on MACSQuant 10 cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with 405 nm, 488 nm, and 635 nm lasers. Flow cytometer data was processed in FlowJo software version X (TreeStar Williamson Way, Ashland, OR, USA). Statistical analysis was performed using the GraphPad Prism 7 software (StatSoft Inc., Tulsa, OK, USA). Student's t-test was applied for data that passed the Shapiro–Wilk normality test and the Mann–Whitney test for data not normally distributed. Means ± SEM are presented throughout the paper (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Results and discussion

Adaptive NK cell population belongs to the pool of highly differentiated $CD56^{dim}$ cells and is characterized by a high expression level of the terminal differentiation

marker CD57 and the NKG2C activating receptor. To study the relationship between the expression of KIR in highly differentiated NK cells, the individual HCMV serological status and proportion of NKG2C-expressing cells, the KIR2DL2/3 expression in newly isolated $CD56^{dim}NKG2C^{+/-}$ NK cells was analyzed. In the highly differentiated cell subset of HCMV-seronegative individuals, the proportion of $KIR2DL2/3^{+}$ cells was independent of NKG2C expression (Figure 1A), while HCMV-seropositive donors had a higher proportion of $KIR2DL2/3^{+}$ cells in the $CD56^{dim}NKG2C^{+}$ NK cell subset (Figure 1B). This indicates that not only the presence of a pathogen contributes to the accumulation of licensed cells and the emergence of a pool of adaptive ones, but also that these two processes are linked together.

It is known that cytomegalovirus infection induces not only the expansion of the $NKG2C^{+}$ cells, but also the expansion of cells expressing inhibitory KIRs specific to the individual's own HLA-I [4]. This phenomenon is more pronounced in subsets of $NKG2C^{+}$ cells [1]. We have shown that this process is most notable in the subset of highly differentiated $CD56^{dim}NKG2C^{+}$ cells. The role of inhibitory KIR in the formation of adaptive NK cells is currently poorly understood. It is possible that the recognition of viral peptides in HLA-I by inhibitory KIR receptors also leads to the expansion of KIR^{+} cells within the subpopulation of adaptive cells. High proportion of $KIR2DL2/3^{+}$ cells in the subset of adaptive NK cells

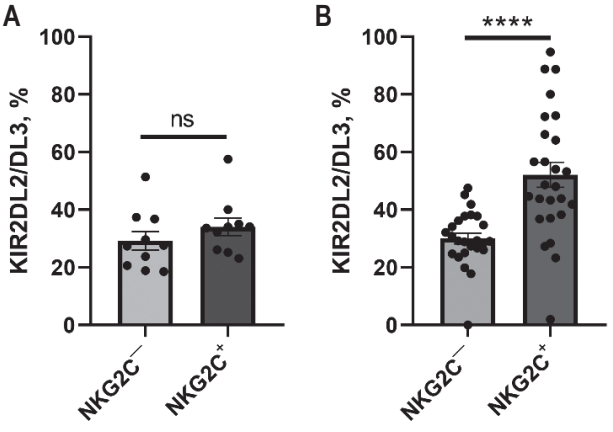

Figure 1. Proportion of $KIR2DL2/3^{+}$ cells in subsets of highly differentiated $CD56^{dim}$ *ex vivo* NK cells differing in the level of NKG2C expression

Note. (A) Proportion of $KIR2DL2/3^{+}$ cells in subsets of highly differentiated $CD56^{dim}$ NK cells differing in the level of NKG2C expression in HCMV seronegative individuals (n = 10). (B) Proportion of $KIR2DL2/3^{+}$ cells in subsets of highly differentiated $CD56^{dim}$ NK cells differing in the level of NKG2C expression in HCMV seropositive individuals (n = 26). Statistical analysis was performed using a paired t-test (**** $p < 0.0001$). Columns represent means ± SEM.

may be also due to the high expression stability of these receptors in the presence of infection.

To assess the stability of KIR expression by NK cells of HCMV-seropositive individuals at different stages of differentiation and with distinct NKG2C expression levels, freshly isolated cells with the phenotype $CD56^{bright}NKG2C^{-}$, $CD56^{bright}NKG2C^{+}$, $CD56^{dim}NKG2C^{-}$ and $CD56^{dim}NKG2C^{+}$ were sorted. Cells were cultured under stimulation with IL-2 and K562-mbIL21 feeder cells, and after two weeks of cultivation, KIR2DL2/3 expression was detected on the surface of cells from the resulting cultures. In all KIR2DL2/3-positive subsets, a decrease in the proportion of $KIR2DL2/3^{+}$ cells was detected. There was a tendency towards a higher proportion of $KIR2DL2/3^{+}$ cells in NKG2C-positive subsets regardless of the level of differentiation (Figure 2A).

Further, to assess the stability of KIR surface expression on the NK cells of HCMV seropositive individuals, clonal cultures of $KIR2DL2/3^{+}$ subsets differing in NKG2C expression and differentiation stage were obtained. The cells were cultured for 3 weeks with stimulation of IL-2 and K562-mbIL21, thereafter KIR2DL2/3 expression on the surface of NK cells in the obtained cultures was evaluated by flow cytometry.

One individual (Figure 2C) showed a high stability of expression of inhibitory KIR2DL2/3 receptors in the $CD56^{dim}NKG2C^{+}$ population compared to NK cells from a less differentiated $CD56^{bright}NKG2C^{+}$ subset and compared to $CD56^{dim}$ cells not expressing the NKG2C receptor. Another individual showed the same tendency (Figure 2B). This observation does not contradict the data obtained in the previous experiment when cultivating heterogeneous, not clonal cultures. Thus, a high proportion of KIR2DL2/3-positive cells in the $CD56^{dim}NKG2C^{+}$ population may be due to the high stability of this receptor when co-expressed with NKG2C. In addition, the differentiation stage can also affect the stability of the expression of the KIR2DL2/3 receptors.

Adaptive NK cells are able to respond by intense proliferation to a repeated encounter with a pathogen; however, adaptive NK cells obtained from the blood of healthy individuals also have a low expression level of the adapter molecule FcεRIγ. Low expression of FcεRIγ is a feature of NK cells with poor proliferative activity [10]. Therefore, the accumulation of adaptive NK cells in the absence of HCMV infection appears to be a challenging task. Nevertheless, the precursors of adaptive NK cells with the $CD57^{-}CD56^{dim}NKG2C^{+}$ phenotype retain many properties of adaptive cells, while being highly proliferative [7]. It has been previously shown that NK cells with the $CD57^{-}CD56^{dim}KIR^{+}NKG2C^{+}$ phenotype are able to respond with intense expansion

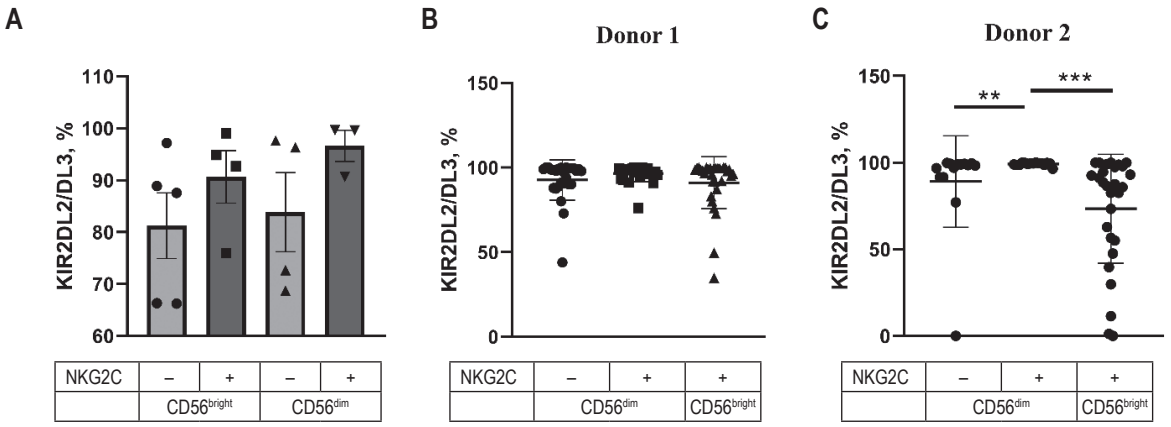

Figure 2. Stability of KIR2DL2/3 expression in heterogeneous and clonal cultures of KIR2DL2/3$^+$ NK cells of HCMV-seropositive individuals differing in differentiation stage and the level of NKG2C expression

Note. (A) Proportion of KIR2DL2/3$^+$ cells in cultures derived from KIR2DL2/3$^+$ NK cell subsets differing in differentiation stage and NKG2C expression level. The number of individuals N = 5. Cultivation time t = 2 weeks. (B) and (C) Proportion of KIR2DL2/3$^+$ cells in NK cell clones of two individuals after cultivation under stimulation with IL-2 and K562-mbIL21. Statistical analysis was performed using an unpaired Mann–Whitney (** $p < 0.01$, *** $p < 0.001$). Columns represent means ± SEM.

to stimulation by IL-2 and K562-mbIL21 feeder cells [9]. To evaluate the stability of KIR2DL2/3 expression in the adaptive NK cell progenitor subpopulation, heterogeneous CD57$^-$CD56^{dim} cultures of KIR2DL2/3-positive subsets were obtained. NK cells were cultured for 2 weeks, after which KIR2DL2/3 expression was detected. As in cultures of highly differentiated CD56^{dim} cells, there was a tendency towards greater stability of KIR2DL2/3 expression in the NKG2C-positive subset (Figure 3A). Also, in the CD57$^-$CD56^{dim}NKG2C$^+$ subset, a higher level of KIR2DL2/3 expression was observed, compared to the CD57$^-$CD56^{dim}NKG2C$^-$ subset (Figure 3B).

Conclusion

Earlier studies have shown that KIRs have a high expression stability [11] we analyzed the phenotype and growth of human NK cell clones obtained by the stimulation of individual NK cells with IL-2 and gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21. However, we found that NK cells, when expanded in response to stimulation by feeder cells, are able to lose KIR expression. Although stimulation with K562-mbIL21 feeder cells allows efficient accumulation of NK cells in large numbers with high expansion ratios, it is important for proliferating cells to retain their original phenotype during the expansion. Inhibitory KIR expression is necessary for the functional activity of cells, since it is associated with the accumulation of a large amount of cytotoxic granules near the center of microtubule organization. Loss of KIR expression may lead to a decrease in the effector function of NK cells. In this work, we have shown that highly differentiated cells with the phenotype of adaptive NK cells and their progenitors, CD56^{dim}NKG2C$^+$ and CD57$^-$CD56^{dim}NKG2C$^+$, are characterized by high stability of expression of inhibitory KIR receptors, which makes possible to use the IL-2 stimulation method in combination with K562-mbIl21 feeder cells for accumulation of adaptive-like NK cells with a stable phenotype for subsequent immunotherapy.

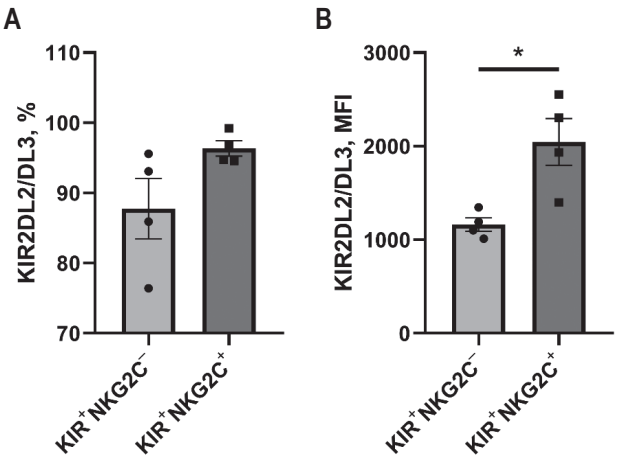

Figure 3. Proportion of KIR2DL2/3$^+$ cells in cultures from the pool of HCMV-specific adaptive NK cells precursors CD57$^-$CD56^{dim} differing in the level of NKG2C expression and derived from HCMV-seropositive individuals

Note. (A) Proportion of KIR2DL2/3$^+$ cells in cultures derived from populations of KIR2DL2/3-positive adaptive NK cell precursors with the CD57$^-$CD56^{dim} phenotype, differing in the level of NKG2C expression. N = 4, t = 2 weeks. (B) Expression level of KIR2DL2/3 in cultures derived from populations of KIR2DL2/3-positive adaptive NK cell precursors with the CD57$^-$CD56^{dim} phenotype, differing in the level of NKG2C expression. N = 4, t = 2 weeks. Statistical analysis was performed using an unpaired Mann–Whitney (* $p < 0.05$). Columns represent means ± SEM.

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