Expression of IL15 and IL18 in NK92 Cells Results in Enrichment Proliferation

Shuying Lin [a], Guangmeng Li [b], Yangyang Dong [c], Gang Liu [d], Hui Zhao [e], Yu Zhang [f], Sheng Xiong [*, g]

Institute of Biomedicine & National Engineering Research Center of Genetic Medicine, College of Life Science and Technology, Jinan University, Guangzhou 510632, China.

[a]287640048@qq.com, [b] 1206027887@qq.com, [c]2289062826@qq.com, [d] 914492912@qq.com, [e]947333358@qq.com, [f]1036387645@qq.com, [*, g] xiongsheng@jnu.edu.cn.

Abstract

The rapid development of NK cell therapy is one of the tumor immunotherapy methods with promising prospects. However, how to improve the killing activity of NK cells and maintain the proliferation ability and reduce the toxic side effects of treatment are currently important research directions. Cytokines are a hot research topic in cell therapy, but the use of exogenously added cytokines to promote the function of cells is a problem that has certain drawbacks. Gene modification technology has been widely used as a technical means that can change the function of cells. Therefore, in this study, NK92 cells were genetically modified to endogenously secrete cytokines, and NK92 cells were used as carriers of cytokine secretion to increase the killing activity of NK92 cells, and systemic toxic side effects caused by systemic infusion of cytokines were reduced,L18 and IL15 were used as cytokines that played a key role in the proliferation and cytotoxicity of NK cells, and NK92 cells were genetically modified. The single cell factor modified, co-modified IL15 and IL18 with NK92 cells were established. The proliferation function of the cells was detected by eFlour and CCK8 cell cycle methods, and the cytotoxicity of the cells was detected by flow cytometry. The results confirmed that NK92-IL15-18 has obvious cell-promoting effect as proliferation , and maintain cytotoxicity, in particular enhance the killing effect of NK cells on SKOV3. The results of this study will provide new design ideas for the clinical application of NK92 cells and its CAR immune cell therapy technology, and at the same time provide important reference for the development of new cell therapy products.

Keywords

NK92, Gene modification, IL18 , IL15, Cell proliferation.

1. Introduction

Natural killer cells, as an indispensable component of the body's natural immune system, are the body’s first natural defense against tumor and virus-infected cells and a bridge between natural and acquired immunity[1]. The appearance of its killing function does not require pre-immunization or sensitization, and no histocompatibility complex (MHC) restriction. In addition, cells also play an important role in transplant rejection, hypersensitivity and autoimmune diseases[2-4]. The killing effect of cells mainly depends on the binding of activating receptors and inhibitory receptors expressed on the cell surface with the corresponding ligands on the cells, identifying non-classical molecules or molecular-related ligands, thereby exerting its own activity and killing. At the same time, cells can secrete a large number of cytokines, such as TNF,IFN-gama, etc., and modulate innate immunity while mediating acquired immune responses by modulating cell activity.

Studies have found that many cytokines are also involved in promoting the differentiation and activity of NK cells and support the homeostasis of NK cells[5]. As mentioned earlier, cytokines also influence the activation state of NK cells, providing clues to the microenvironment to increase or decrease through Surface receptors trigger thresholds required for NK cells, as provided by

exogenous inflammation or immunotherapy. The above functions are an important way to promote the immunotherapy of NK cells.

Cytokine receptors have important effects on various NK cell events, including development, proliferation, homeostasis and activation states, which are the key aspects of biological immunotherapy. It has been demonstrated that human NK cells constitutively express many cytokine receptors, and that they transduce signals when they are linked to intracellular signaling pathways. In addition, selected cytokine receptors or subunits can be induced, or they can be enhanced after activation, providing a mechanism for synergy between different cytokines. In addition, it is known that the discovery of multiple cytokine receptors or cytokines plus a combined signal that activates NK cell receptors produces the most powerful NK cell effector response[6-8].

NK92 cells were obtained in 1992 from the peripheral blood lymphocytes of patients with non-Hodgkin's lymphoma and were successfully established as a type of NK cell line[9]. The immunophenotype of this cell line is CD56+, CD16- Without ADCC effect, it can be amplified in vitro and cultured for a long time[10, 11]. Similar to the peripheral blood CD56+ NK cells, it has become a good tool for many laboratories to study the differentiation, development and killing mechanism of NK cells.

IL15 belongs to the IL-2 family and was isolated and purified from the culture supernatant of the monkey kidney epithelial cell line CV-1/EBNA[12]. It is secreted mainly by fibroblast cell strains and adherent peripheral blood mononuclear cells, and can regulate the activation and proliferation of T cells and NK cells. Because IL15 is very similar to IL2 and shares signal receptor subunits IL-2/15Rβ and γc, which have biological effects similar to IL2, as a The functional cytokines, IL15 and IL2, represent the best cytokines for NK cell research and have many positive functions for enhancing antitumor responses. Downstream signaling of IL15, including Jak1 / 3 and STAT3 / 5, PI3K pathway, MAPK pathway, and ultimately activation of NF-κB. These signals regulate NK cell development and homeostasis, induce proliferation, co-stimulate cytokine production, and enhance cytotoxicity [13, 14].

IL18 factor is a pro-inflammatory mononuclear factor[15]and is a family of pro-inflammatory IL-1. It was originally discovered because of IFN-gamma inducing factors. It is produced by various cells, including dendritic cells, adipocytes, and giant phagocytes [16, 17]. IL18 is a pleiotropic cytokine that regulates both humoral and cellular immunity and plays an important role in the inflammation cascade[18]. IL18 has a certain role in the function, the current study found that IL18 combined with other cytokines can enhance the relevant functions of the cell, in NK cells, IL18 was seen as a co-stimulatory cytokine synergistic effect of IL12 and IL15[6].IL18 is constitutively expressed on unstimulated NK cells and can induce the proliferation of NK cells alone, whereas the addition of IL15 greatly enhances cell proliferation. Among them, studies have reported that the combination of IL18 and IL15 can enhance cytotoxicity and interferon secretion [19].

At present, cytokines are stimulated by exogenously added cytokines in NK cells, but there are certain side effects. Therefore, it is hoped that NK92 cells will secrete cytokines endogenously through genetic modification and increase the killing activity of NK92 cells. Using NK92 cells as a carrier of cytokine secretion to reduce systemic side effects caused by systemic infusion of cytokines, IL18 and IL15 were used as cytokines that play a key role in the proliferation and cytotoxicity of NK cells. The effects of different genetic modifications on the proliferation and antitumor effects of NK92 cells were studied and the mechanism of action was explored. The results of this study will provide new design ideas for the clinical application of NK92 cells and its CAR immune cell therapy technology, and at the same time provide important reference for the development of new cell therapy products.

2. Materials and Methods

2.1 Cells and Culture Conditions

SKOV3 cells were maintained in RPMI 1640 medium. 293T cells were cultured in DMEM medium. All the medium were supplemented with 10% FBS,100U/ml penicillin, 100ug/ml streptomycin.In

addition human NK92 cells were maintained in RPMI-1640 medium supplemented with 12.5% FBS, 12.5% horse serum,100U/ml penicillin, 100ug/ml streptomycin and 100 IU/ml IL-2.

2.2 Production of Lentiviral Vectors and Transduction of NK Cells

VSV-G pseudotyped lentiviral vector particles were produced by co-transfecting 293T cells with the respective lentiviral transfer plasmid together with packaging and envelope plasmids psPAX and pMD2.G by standard calcium phosphate transfection. For transduction, vector-containing supernatants were incubated overnight at 4°C and centrifuged for 30 min at 4°C and 4,000 g to concentrate. Add the concentrated supernatant to NK-92 in the presence of 8 g/ml polybrene. Then, the samples were incubated 24h or 48h before replacing the medium with regular growth medium.IL18 and IL21-expressing cells were identified by addition of puromycin and GFP-expressing cells by diret flow cytometry using

2.3 RNA Isolation and Reverse Transcription-Quantitative PCR

Total RNA was extracted using Trizol Reagent from cells following to the manufacturer's instructions.PCR was performed with specific primers in 20 ul PCR mixtures for 40 cycles.The levels of mRNA were measured by SYBR Green quantitative PCR performed on the Real-Time PCR Detection System(Bio-Rad,Hercules,CA,USA).Quantification data were normalized to GAPDH.

2.4 Analysis of IL18 and IL15 Expression and Activity

Expression of IL18 and IL15 mRNA were analyzed by quantitative RT-PCR using total RNA from NK-92/IL18 or IL15-GFP or parental NK-92 cells as templates ,and the IL18 or IL15 oligonucleo-tides indicated above as primers.The amount of IL18 and IL15 protein in culture supernatants collected after each day in 1-5d of culture of 1X106 IL18 expressing NK92 cells in 10ml of IL2 withward growth medium was quantified in triplicate samples using a human IL18 and IL15 ELISA kit following the manufacturer's recommendations. Supernatants of non-transduce parental cells served as controls.

2.5 Cell Proliferation and Cycle Analysis

Cell proliferation was measured using a cell counting kit-8(CCK-8,Dojindo, kumamoto,Japan) according to the manufacturer's instructions.The cells were cultured for 24,72 and 144 hrs,and the absorbance was measured at a 450 nm wavelength.

Another way to measure the cell proliferation was used the Cell Proliferation Dye eFlour 670 ,labled the cells with the eFlour 670.Pepar the cells to be labeled,wash twice with PBS,resupend cells at 2X the desired final concentration in PBS.Prepare a 10 μM solution of Cell Proliferation Dye eFlour 670 in PBS,while vortexing cells,add an equal volume of the 10 μM solution,incubate for 10 minutes at 37℃ in the dark,and stop labeling by adding 4-5volumes of cold complete media.Wash cells 3 times and culture as desired. During the 5 days of culture, an equal amount of cells were drawn daily for flow detection.

2.6 Cytotoxicity Assays and Apoptosis

Cytotoxic activity of NK cells toward target cells was analyzed in CCK-8 metabolization assays as described. Target cells were incubated for 12h at 37°C in 100uL medium in FluoroNunc 96-well plates.NK cells washed,and co-cultured with effectors cells at various effectors to target(E/T) ratios for 12 h at 37°C and the well without NK cells as control.After co-culture, added CCK-8 dye exclusion and tested the absorbance value .The cytotoxicity of NK cells was measured based on the absorbance value.

Before doing experiment the viability was derermined by vital dye exclusion using a standard hemocytometer count star. For the apoptosis of tumor cells which incubated with NK-92 cells was using the Annexin V-PE/7-AAD assays kit.The nucleic acid dye 7-aminoactinomycin D-(7-AAD) was used in flow cytometric assays for the estimation of nonviable cells. Annexin V-PE in conjunction with 7-AAD was used in flow cytometric assays for the identification of early apoptosis cells. Cells that stain positive for Annexing V-PE and negative for 7-AAD are undergoing apoptosis.

Cells that stain positive for both Annexin V-PE and 7-AAD are either in the end stage of apoptosis, are undergoing necrosis or are already dead.

2.7 Statistical Analysis

Differences between values were evaluated using the two-tailed unpaired Student's t test. Differences between groups were evaluated by two-way ANOVA followed by Bonferroni tests. p values < 0.01 were considered significant. Statistical calculations were done using Prism 5 software. (GraphPad Software, La Jolla, CA

3. Results

3.1 Generation of IL15 and IL18-expressing NK cells

Human IL-15 , IL18 and overlapping sequence of IL15 and IL18 cDNA(Fig.1-a) was inserted into the lentiviral transfer vector pCDH that also encodes GFP as a maker, the puromycin gene for the screeing(Fig.1-b),and lentiviral particles were generated. After transduction of IL-2-dependent human NK-92 cells, the positive cells were screened by adding puromycin.The GFP and puro expressing NK-92 cells were enriched by two weeks puromycin sorting. In both the NK-92/IL15 ,NK-92/IL15-18 and NK-92/IL18-GFP cell population,the IL15 as well as IL18 mRNA expressing was verified by realtime fluorescence quantitative PCR analysis(Fig.1-c-f). NK92 cells modified with IL15 or IL18 expressed high levels of IL15 and IL18 . nevertheless,NK92/IL15 and NK92/IL15-18 cells secreted low levels of IL15 into the culture supernatant(<70 pg/ml).compare with NK92/IL15-18 cells , NK92/IL15 cells secreted more IL15, reaching 70 pg/ml,but another secreted only 50 pg/ml.In the meanwhile , NK92/IL18 and NK92/IL15-18 cells secreted high levels of IL18 into the culture supernatant, but NK92/IL15-18 cells secreted more IL18, reaching 3,5ng/ml.

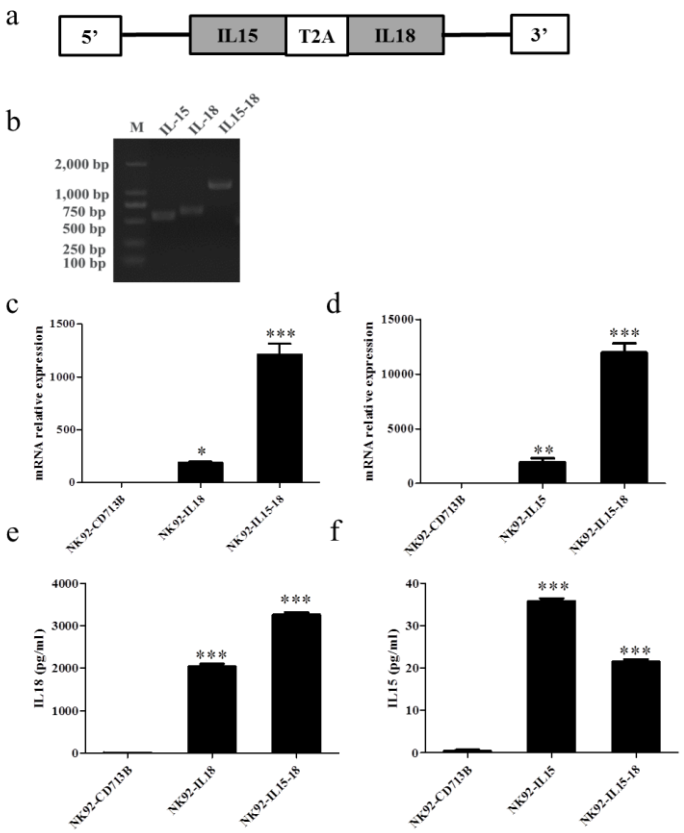

Fig.1. Expression of IL-15 and IL18 in NK-92 cells.
(a)Schematic of overlapping PCR of IL15 and IL18;(b) Amplification of IL15 and IL18 Sequences; (c): Gene expression of IL18; (d): Gene expression of IL15; (e): Protein expression of IL18; (f): Protein expression of IL15(**p<0.01 vs NK92-CD713B)

3.2 Cell Poliferation Activity of NK-92 Modified Cells

The study found that IL15 can promote cell proliferation. In this study, to test the combined effect of IL15 and IL18 about the cell poliferation. Using the eFlour 670 dye staining method to detect the cell division rate and determine the cell proliferation ability.

The results on day 0 of staining showed that the cells successfully labeled , stained evenly and an equal amount of cells were pipetted daily for flow cytometry. It can be seen that when the cells were cultured on the 5th day(Fig 2-b), the fluorescence intensity of the cells in each group decreased significantly, showing that the cell proliferation was active. Obviously, the figure of the NK-92 IL15-18 cellis shifted to the left, the fluorescence intensity was weakened more. There is no significant difference between the fluorescence intensity of IL15 and IL18 alone and the control. Thus, the division rate of NK92-IL15-18 cells was slightly faster than that of the control group, and the co-expression of IL15 and IL18 promoted the proliferation of NK92.

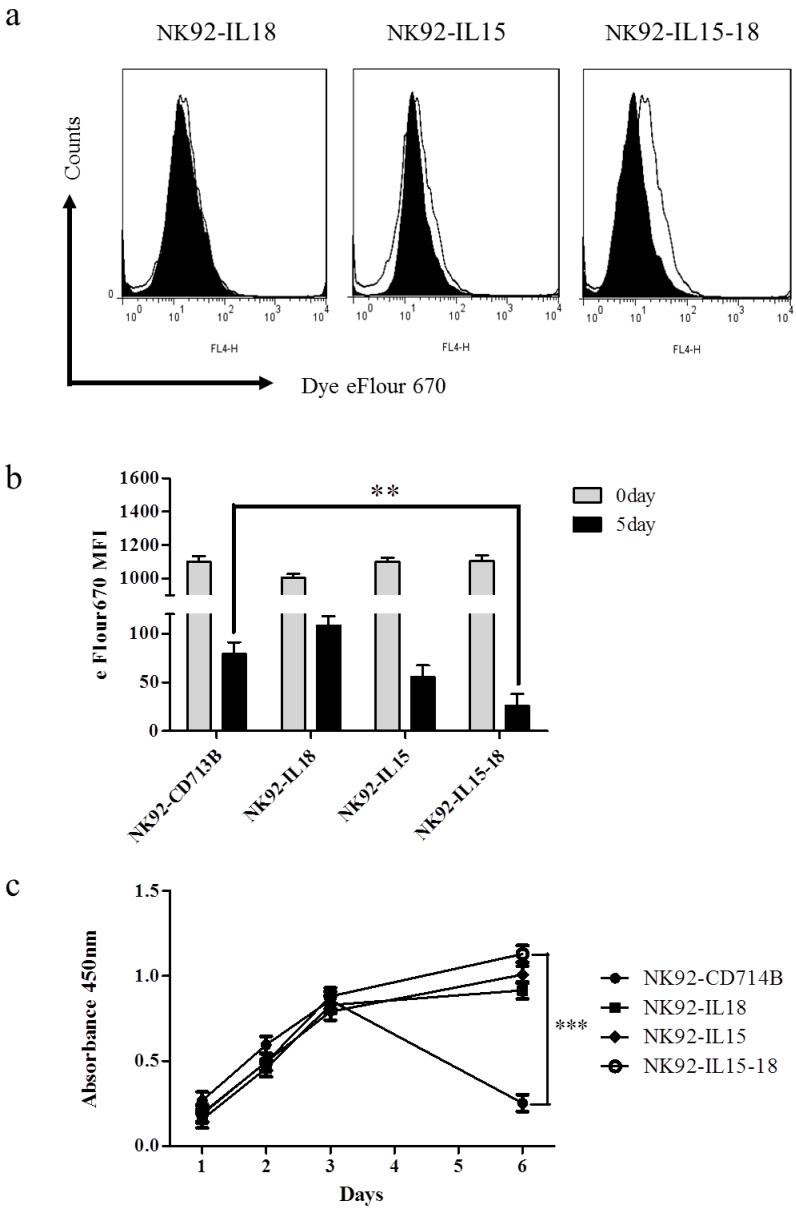

Fig.2. IL-15 and IL18 expressed in NK-92 cells promote cell poliferation.
(a) The eFlour 670 flow fluorescence assay on the fifth day, the fluorescence intensity of NK92-IL15-18 decreased more significantly;(b) Detection of cell proliferation by eFlour670 staining.;(c)CCK8 detection of cell proliferation.(**p<0.01 vs NK92-CD713B)

To test whether the IL15 and IL18 levels produced can fully support normal growth of NK92/IL15-18, NK-92/IL18 and NK92/IL15 cells ,all these cells and parental NK-92 were placed in growth medium containing IL-2 ,and proliferation was followed by CCK-8 metabolization assays(Fig.2-c).Thereby,irrespective of the presence of exogenous IL-2, NK92/IL15-18, NK-92/IL18 and NK92/IL15 cells continued to proliferate with growth characteristics indistinguishable from parental NK-92 cells in IL-2-containing medium.In the present of both IL15 and IL18 ,the growth of NK-92 was maintained until the six day ,and contained the higest absorbance ,while parental NK-92 cells showed a decreasing trend from the third day.

In addtio, to test whether the stimulation of the secreted protein affects the cell cycle and enhance cell proliferation , the cell cycles in all these cells and parental NK-92 were determined and compared differences between groups(Fig.3).

The S and G2 phases of NK92-IL15-18 cells were slightly higher than the control, G1 phase was the lowest of the four , and the control difference of nearly 7%($p<0.05$).NK92-IL18 compared with the control cycle the change was not obvious, and the G1 phase of NK92-IL15 was even higher than control. It can be seen that the interaction of IL15 and IL18 has a certain influence on the cell cycle of NK92, thereby affecting the cell proliferation ability.

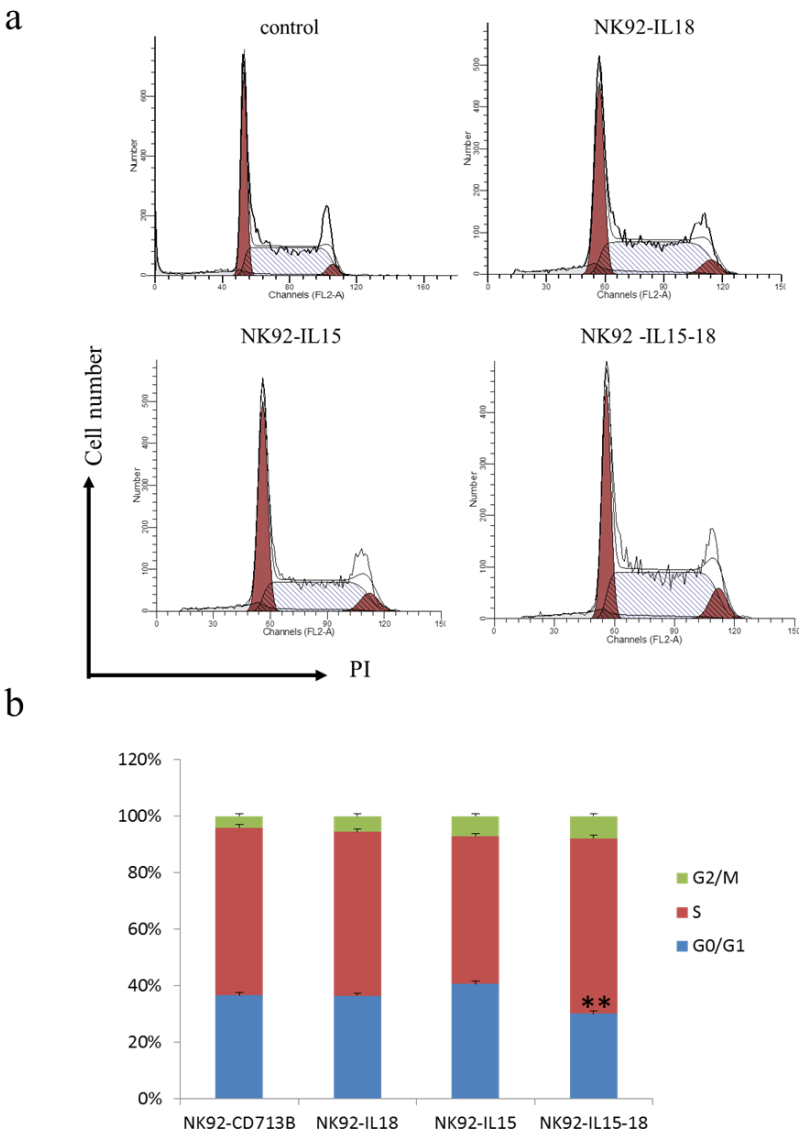

Fig.3.Cell cycle detection of NK92-IL18, NK92-IL15, and NK92-IL15-18 cells.
**$p<0.01$ vs NK92-CD713B

Table 1. Statistics of cell cycle data of NK92-IL18, NK92-IL15, NK92-IL15-18

Group	G0/G1	S	G2/M
NK92-CD713B	36.61%±2.93	59.39%±1.80	4.00%±0.75
NK92 IL18	36.45%±1.25	58.04%±1.28	5.51%±0.23
NK92 IL15	40.71%±0.25	52.26%±0.20	7.03%±0.38
NK92 IL15-18	30.10%±0.47	62.10%±0.47	7.80%±0.37

3.3 Cytotoxicity Activity of NK-92 Modified Cells

Next,we investigated whether ectopic IL15 expressing affcets natural cytotoxicity of NK-92 cells. Cell-killing activity of NK92/IL15-18, NK-92/IL18 and NK92/IL15 cells against NK-sensitive SKOV3 cells were assessed in FACS-based assays upon co-incubation of effector and target cells at different ratios for 6h. The expression of IL15 and IL18 alone had no significant effect on cell killing, whereas NK92-IL15-18 had a more effective killing effect than the 2.5:1 ratio on the premise of maintaining the cell's original killing effect(Fig.4). And the difference with the control group is extremely significant. It can be seen that the combined effects of IL15 and IL18 not only promote the proliferation of cells, but also promote the cytotoxicity of SKOV3 cells.

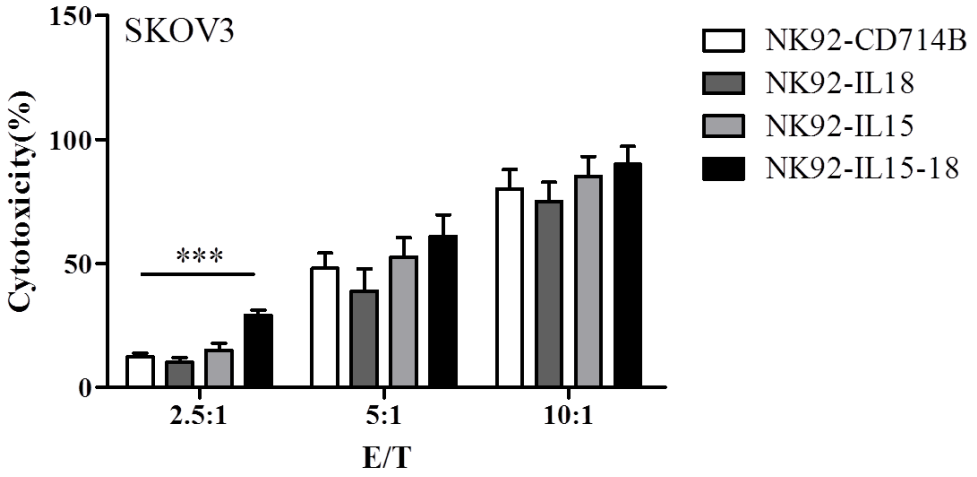

Fig.4. Killing of SKOV3 by NK92-IL18, NK92-IL15, and NK92-IL15-18 cells.
***p<0.01 vs NK92-CD713B

4. Discussion

NK cells are the first line of defense of the body's natural immune system, which are very protective against disease and external invasion. Because of no MHC, allogeneic treatment,so is a prospective research object for immune cell therapy. The function of NK cells are still being constantly enhanced and studied. Genetic modification is a technical means that can change the function of cells, and genetic modification of immune cells is an important research direction, can be used to enhance the function of a variety of cells, has now been widely used in a variety of cells such as T cells, DCs, monocytes, NK cells, etc.[20-23], and achieved good results. Gene modification of NK cells can give cells more new functions, enhance cytotoxic activity, and make them more suitable for tumor immunotherapy requirements. After genetic modification of NK cells, not only can it enhance its anti-tumor function, but also can secrete some related factors, affect peripheral cells, and achieve more

functional effects. At present, there are already many cytokines used to modify NK cells, including IL-2, IL15, IFN, SCF, etc.

Through analysis, it was found that IL18 and IL15 have anti-tumor and proliferative abilities, and there have also been preliminary results obtained by the exogenous addition of this cytokine. IL18 is a kind of cytokine with anti-tumor research foreground, which can promote the secretion of IFN gamma, promote cytotoxicity, and activate cells. Now it has been combined with anti-tumor research to enhance the cell's Activity, to achieve better results. IL15 is a widely used cytokine and has great potential for development. It has similar biological activity as IL-2, promotes cell proliferation and killing activity, and plays an important role in promoting cell proliferation and killing.

NK92 cells are currently the most widely used type of NK cell line and have strong anti-tumor activity. NK92 cells need the addition of IL2 to maintain cell growth and have certain growth limitations. It is hoped that it will be able to break its own restrictions and play a more effective role. Although NK92 has obvious anti-tumor efficacy, its main research is mostly directed against non-solid tumor cells, but there are few reports on the killing effect of solid tumor cells. In order to make NK cells have stronger biological function activity, this study combined IL18 and IL15 to act on NK92 cells to obtain three "enhanced" NK92 cell lines that can continuously express cytokines IL18 and IL15.

The co-expressed approach was used in this study,T2A was used to overlap the IL18 and IL15 cytokines genes to obtain the IL15-18 gene sequence. The obtained IL18 ,IL15 and IL15-18 was ligated with lentivirus vector pCDH-Puro to construct three novel lentiviral expression plasmids.For this design can be compared between single factor and two factors at NK92, its function on NK92 production was explored.

In this study, we used the lentiviral expression system, first transfected the obtained lentiviral recombinant plasmid into HEK-293T to prepare three recombinant viruses: including V-IL18, V-IL15, and V-IL15-18, concentrated with PEG8000, followed by dilution titration for detection of virus titers,and infection of HEK-293T cells.After analysis by flow cytometry, calculation of the prepared virus concentration, all above 107, indicating that the virus higher titer can be used for the next infection experiment.After 72 hours of infection and integration, the virus was efficiently integrated into the cells, and the puromycin resistance gene on the vector was used as a condition for resistance screening. After two weeks of puromycin selection, the expression purity was 90%. The subsequent qPCR assays of NK92 cells in each group also demonstrated that the IL18 and IL15 cytokines were successfully expressed in the three types of cells and that they were not expressed in the control group, demonstrating that this study successfully constructed NK92-IL18, NK92-IL15, NK92-IL15-18 cell line.

In order to test cell proliferation function, we used eFlour 670 dye staining method to analyze the proliferation and division rate of cells by the change of fluorescence intensity. As can be seen from the figure, NK cells can maintain their growth activity, and the fluorescence intensity gradually decreases as the culture time increases. On the fifth day, the fluorescence intensity of eFlour670 on the surface of NK92-IL15-18 was weaker, indicating that the cell proliferation rate was faster. However, the stimulation of single cytokines did not significantly promote the proliferation of NK92. The cell lines constructed in this study were able to maintain the consistent and stable expression of IL18 and IL15, and the cytokines released in the culture supernatant could act on the cells themselves, and play a role in promoting proliferation. At the same time, the proliferative effect of IL15 was also verified, and the effect was more pronounced when combined with other cytokines. Here, we also used the CCK-8 method to further verify proliferation function of cells. Through five days of continuous detection, we can observe that the cells which have added IL15 cytokines maintain their growth state and continue to proliferate, and the proliferation of NK92 -IL15-18 cells was faster, while the control group showed a decreasing trend in cell volume on the third day. The result indicated that NK92-IL15-18 can promote cell proliferation while maintaining cell growth. It can be seen that the co-stimulatory effects of IL18 and IL15 are more obvious. On the basis of cell proliferation, the cell cycle was also examined. Compared with the control, NK92-IL15-18 showed a slight increase in

S phase and G2 phase, and there was a statistical difference. Thus we can see that the cell proliferation of NK92-IL15-18 has a certain effect on the cell cycle, which makes the cells divide more quickly.

It is of great significance whether cells can have a final killing effect after promoting proliferation. Therefore, after studying cell proliferation, we further examined the cytotoxic activity of the cells. This study mainly focuses on the killing of SKOV3. In the killing effect of SKOV3, it can be seen that under the same ratio, the effect of two-factor modification of NK92 was stronger than that of single-factor modification, indicating that the co-expression of IL15 and IL18 can promote mutualism in the anti-tumor effect., and it verified that IL18 together with other factors can produce a more effective anti-tumor effect, but also shows that the IL18 and IL15 constructed in this study have biological activity, and the joint action of the two cytokines exert their biological activity better, making the anti-tumor effect of the cells more obvious.

It can be seen that the new type of NK92 expressed by the IL18 and IL15 cytokines designed in this study can be successfully expressed in NK cells through Lentiviral infection and has continuous secretion. Cytokines undergo autocrine action on peripheral cells and autologous cells, promoting the proliferation and anti-tumor effects of cells. This study explored the function of NK cells expressed by two cytokines at the cellular level, and initially clarified the effects of the expression of IL18 and IL15 on the effects of NK92, in order to improve the therapeutic effect of NK cells and explore novel NK cell therapy and related clinical applications. It provides experimental evidence and also provides a new idea for CAR-NK cells.

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