In Vitro Comparison of Lymphocytes Transduced with a Human CD16 or with a Chimeric Fc Receptor Reveals Potential Anti-TUMOR ACTivity

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Abstract

Natural killer (NK) cells are known to play a role in mediating innate immunity, in enhancing adaptive immune responses, and have been implicated in mediating anti-tumor responses via antibody-dependent cell-mediated cytotoxicity (ADCC) by reactivity of CD16 with the Fc region of human IgG1 antibodies. The NK-92 cell line, derived from a lymphoma patient, has previously been well characterized and adoptive transfer of irradiated NK-92 cells has demonstrated safety and shown preliminary evidence of clinical beneft in cancer patients. The NK-92 cell line, devoid of CD16, has now been engineered to express the affnity CD16 FcγRIIIa receptor，as well as engineered to express 41BB and CD3 fusion gene in intracellular; 41BB and CD3 are commonly used as co-stimulators in CAR structure; expression in NK cells can promote the activation of NK cells, leading to enhanced perforinand granzyme-mediated lysis of tumor cells. The studies reported here show gene modified NK-92 cells lines combined with antibodies mediated ADCC using rituximab and trastuzumab monoclonal antibodies to lysis of tumor cells significantly higher than NK-92 cell lines. Studies also chimera 41BB, CD3 molecular modified CD16 receptor anti-tumor activity was stronger than CD16. These studies thus provide the rationale for the use of gene modified NK-92 cells in combination with IgG1 anti-tumor monoclonal antibodies. Moreover,these studies also provide the rationale for the chimera modified CD16 receptor can improve the anti-tumor effect of NK92 cells through ADCC.

Keywords

Lymphocytes, Chimeric Fc Receptor, Anti-TUMOR ACTivity.

1. Introduction

Natural killer (NK) cells are a subgroup of lymphocytes that play an essential role in the cellular immune defense against virus infected and malignant cells[1-3]. Unlike T cells, which express their own antigen specific receptor, the TCR, NK cells are devoid from the expression of such a receptor[4]. In addition to these mechanisms, NK cells can play a role in anti-tumor immunity alone, or in combination with select antibodies via antibody-dependent cell-mediated cytotoxicity (ADCC) . The potential use of targeted NK cells for adoptive immunotherapy provides an effector cell type with the capacity of recognizing antigen positive as well as MHC-I negative or low tumor cells[5]. Several NK cell lines have been derived from patients with a range of different leukemias and lymphomas. One such NK line, designated NK-92, derived from a patient with non-Hodgkin's lymphoma, has been previously well characterized[6-8]. NK-92 cells are similar to A-NK cells with respect to the expression of typical NK cell surface receptors and functional characteristics, but they do not harbor FcγRIII (CD16). NK-92 cells grow well in culture and are dependent on exogenous IL-2 for propagation[9]. They also lack or have extremely low levels of the inhibitor killer immunoglobulin (Ig)-like receptors (KIR), which allows for lysis of tumor cells expressing major histocompatibility

complex (MHC) molecules. Preclinical studies have shown that NK-92 cells do not form tumors when transplanted into severe combined immunodefciency (SCID) or athymic mice. Several clinical studies employing repeated infusions of irradiated NK-92 cells have been completed. Up to 10 billion cells/m2 have been infused with no severe side effects observed[10,11]. Clinical responses have been observed in patients with melanoma, lung cancer, Merkel cell carcinoma, lymphoma, and kidney cancer[12]. Despite the allogeneic nature of NK-92 cells, the formation of anti-human leukocyte antigen (HLA) antibodies was seen in less than half of the patients. Moreover, the pharmacodynamics of NK-92 clearance did not differ upon repeated doses. NK-92 cells, however, do not express the CD16 Fc receptor, which is necessary for NK-mediated ADCC lysis of tumor cells employing monoclonal antibodies (MAbs) of the immunoglobulin G1 (IgG1) isotype.

The clinical benefts associated with adoptive immunotherapy of some mAbs have established the clinical pertinence of several antigens as immune therapeutic targets. For some therapeutic antibodies such as the anti-CD20 rituximab or the anti-HER2 trastuzumab, cell-mediated immunity has been recognized as one of the mechanisms responsible for their clinical efciency[13,14]. Prior clinical studies[15] employing the IgG1 isotype MAbs trastuzumab (Herceptin), or rituximab (Rituxan) have shown that breast and lymphoma NSG mouse, respectively, whose NK-92 cells express CD16 have improved overall survival compared to patients with NK-92 cell lines. Similarly, after transduction with FcIIIγRa/FεcRI (referred to as CD16/γ) receptor fusion gene, CD4+ and CD8+ cytotoxic T lymphocytes displayed a stable expression of the CD16/γ receptor at their surface and mediated ADCC. Tus, associating a therapeutic mAb and an adoptive transfer of CD16/γ transduced T cells could combine the advantages associated with the functional potential of cytotoxic lymphocytes and recognition of the target cells unrestricted by the major histocompatibility complex[16]. This strategy can significantly improve NK92 cell killing cell activity, however, the single activation signal factor can only cause a weak reaction.

NK-92 cells have now been engineered to express the CD16 high affnity FcγRIIIa (158V) receptor[17]. NK92 cells also need to be co-cultured with IL-2. NK cells have previously been shown[18,19] to be “serial killers,” in that a single NK cell can lyse multiple tumor cells[20] .These studies have also shown that IL-2 can replenish the granular stock of NK cells leading to enhanced perforin- and granzyme-mediated lysis of “exhausted” NK cells[21].

The aim of the present study was to directly compare the single signal structure Fc receptor (CD16, CD16/CD3) and the common stimulus signal receptor (CD16/41BB/CD3) combined antibody ADCC-mediated specific anti-tumor effect. To this end, as a first step in designing a model, we equipped the same cytotoxic lymphocyte line (the human NK cell line NK-92) with either a FcγIIIa (referred to as NK92-CD16) and FcγIIIa/CD3 (referred to as NK92-CD16/CD3) or FcγIIIa/41BB/CD3 (referred to as NK92-CD16/41BB/CD3) and compared their efciency in killing HER2 and CD20 positive tumor target cells in vitro. The study showed that the gene modified NK92 cells did not affect the activity of killing cancer cells. At the same time, CD16 combined with co-stimulative signal 41BB/CD3 cells can significantly improve antitumor activity. Similarly, the presence of 41BB signal factor increases NK92 secretion of perforin and granulase.

2. Results

2.1 Expression of The CD16 and The Fusion Gene CD16/CD3, CD16/41BB/CD3 on the Surface of NK-92

The chimeric cDNAs were synthesized by GeneCust. The CD16 cDNA comprises the leader (S) and the two extracellular domains (EC1 and EC2) of human and the intact transmembrane (TM) and intracellular (IC) domains (Figure 1A). The CD16/CD3 chimeric cDNA comprises the leader (S) and the two extracellular domains (EC1 and EC2) of human CD16 and the intact transmembrane (TM) and intracellular (IC) domains with CD3 (Figure 1A). Similarly, the CD16/41BB/CD3 chimeric cDNA used leader (S) and intact transmembrane (TM) of CD16 gene, as well as the intracellular (IC1) domains with 41BB and intracellular (IC2) domains with CD3 gene (Figure 1A). One molecule, the ligand for 4-1BB, triggers activation signals after binding to 41BB (CD137), a signaling molecule

expressed on the surface of NK cells. The gene was linked to the lentiviral vector CD713B and the lentivirus particles were expressed in 293T (see Section 4). The purified particles were measured by titer(see Section 4). Afer transduction, 85% of the NK-92 expressed CD16 and 84.7% expressed the CD16/CD3,as well as 84.2% expressed the CD16/41BB/CD3 (Figure 1B). After the infection, the positive rate of the cells was above 90% after 7d of screening with puromycin(Figure 1C). After 14d, the positive rate was stable above 95% (Figure 1D).

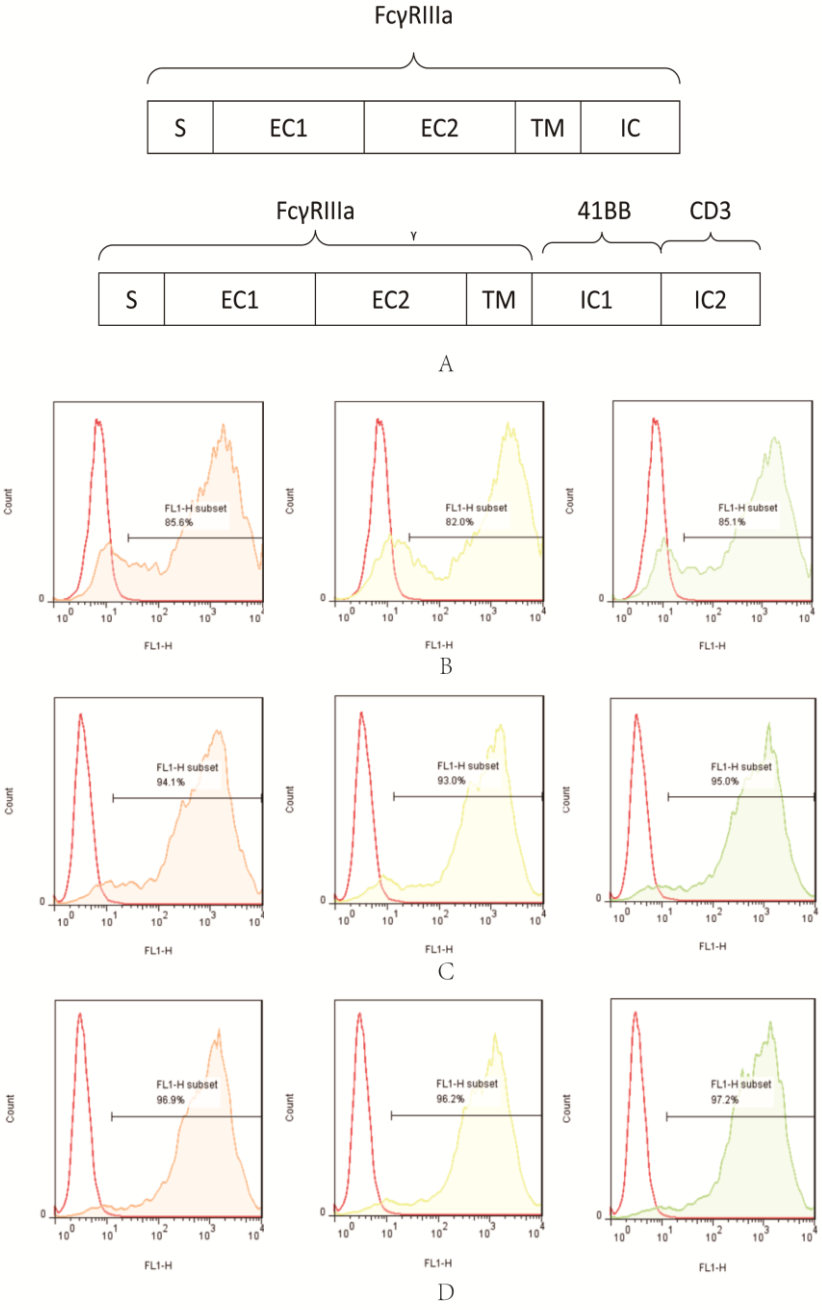


Figure 1:(A)Recombinant plasmid design and infection on NK92 cell lines. The CD16 and schematic representation of the chimeric:the CD16/CD3 and CD16/41BB/CD3 chimeric cDNA comprised the leader (S) , the two extracellular domains (EC1 and EC2) and the intact transmembrane (TM) of human CD16,however, intracellular segments are replaced by CD3 or 41BB/CD3 molecules. (B)Recombinant lentiviral plasmid transiently infects 293T to express lentivirus particles, infects NK92 cells, calculates green fluorescent protein(eGFP) expression, and detects infection efficiency. (C)Screening with puromycin after infection, positive rate by flow cytometry after 7 days. (D)Screening with puromycin after infection, positive rate by flow cytometry after 14 days.

2.2 Gene Modification of NK92 Cell Protein Expression and Activity Analyze

The NK92 cells of lentivirus infection, the cells express chimeric proteins. The CD16 extracellular region was expressed on the surface of the cell membrane, which was combined with the CD16 antibody labeled with PE to fluoresce. The positive rate can be detected by flow cytometry. As seen in Figure 2A, afer immunomagnetic purifcation using anti-CD16-PE, the cell positive rate is up to 95%, essentially pure populations of NK92-CD16, NK92-CD16/CD3 and NK92-CD16/41BB/CD3 were obtained. The CD16 most important activity is the combination with the antibody Fc, and the CD16 activity can be verified by the detection of gene modification of NK92 cells affinity with antibody. As shown in Figure 2B and 2C, the anti-CD20 antibody rituximab was predetermined by the fluorescein Cy5 and combination reaction with the CD20 high expression cell strain Raji and Daudi, the results showed that the antibody was active,the combined positive rate with Raji was 98.7%, and the associative law of Daudi was 60%, as well as, genetic engineering modification of the constructed K562-CD20 cells is also associated with it,and the positive was 45%, as show the Figure 2D. The antibody and cellular combining efficiency can be calculated by enzyme-linked immunoassay (ELISA), as shown in Figure 2E, compared with NK92 cell, genetic modification of NK92 cell CD16, CD16/CD3 and CD16/41BB/CD3 have antibodies binding, and there was no significant difference. It is shown that the gene modification cells can stabilize the expression of the target gene, and the expression protein has biological activity and can be combined with Fc.

2.3 Comparison of Tumor Cell Lysis by NK92 Cells with Gene Modified or Natural

The ability of NK92 cells to lysis 8 human tumor cell lines was analyzed, including lung (n=1), breast (n=3), ovarian and leukemia cell cell line. As shown in figure 3A, after modification NK92 cell line for blood cancer cell K562 maintain high sensitivity, the effect of target when the ratio of 1:1 is as high as 35%, percent lysis above 80% when the ratio was 8:1, and there was no significant difference with NK92. However, for solid tumor lung cancer or breast cancer cell lines, etc, there is no strong sensitivity, as shown in Figure 3B-H, the anti-tumor effect of cells at the high efficiency target ratio of 16:1, the highest shows only 40-60% of the lysis rate. The cell activity indicated that the genetically modified NK92 cells maintained the killing effect on hematological tumors such as K562. At the same time, there was no significant difference between the NK92 cells and the lysis effects of the solid tumor cell lines.

2.4 NK92 ADCC Mediated by Trastuzumab and Rituximab Was Evaluated with CCK-8 Assays

As in the example shown in Figure 4A, ADCC activity of the NK92-CD16 against the BT474 cell line was tested in the presence of increasing concentration of trastuzumab and demonstrated demonstrated a plateau (close to 60% specifc lysis) at 12μg/mL (E/T ratio: 8/1). No ADCC was observed in the presence of rituximab and NK92-CD16 or NK92. Note that a background lysis was observed for NK-92+trastuzumab (compared to NK-92+rituximab), likely due to background expression of CD16 by the NK-92 cell line. For all other in vitro experiments, trastuzumab was used at a concentration of 10μg/mL. Next, we compared the efciency of target cell lyses induced either afer direct recognition of the HER2 Ag by the NK-92 or afer indirect recognition by the NK92-CD16, NK92-CD16/CD3 and NK92-CD16/41BB/CD3 in the presence of trastuzumab. To this end, target cells (the HER2 negative MDA-MB-468 and the HER2 positive BT474) were coated or not with trastuzumab, and the NK-92 was used as a negative control. Cytotoxic activities of NK92-CD16, NK92-CD16/CD3 and NK92-CD16/41BB/CD3 against trastuzumab coated or not coated MDA-MB-468 and BT474 are summarized in Figure 4A. Neither CD16 nor recombine CD16 (CD16/CD3 or CD16/41BB/CD3) presented a signifcant level of cytotoxicity against the HER2 negative MDA-MB-468 cell line. When tested against the BT474, NK92-CD16/41BB/CD3 showed a high level of cytotoxic activity (25.81±4.03% at 1:1, $p<0.05$; 52.02±3.66% at 4:1, $p<0.01$; 72.18±3.20% at 8:1, $p<0.01$;92.00±4.27% at 16:1, $p<0.01$), in the presence of trastuzumab. To confrm the differences in cytotoxic performance between NK92-CD16, NK92-CD16/CD3 and NK92-CD16/41BB/CD3, further comparison was performed against 3 different HER2 positive cell lines:SKBR3, SKOV3, MCF-7 (Figures 4B). Tese

data showed that, in vitro, in these experimental conditions, based upon 8hr cytotoxicity assays, the co-existing signaling pathways of killing (by NK92-CD16/41BB/CD3) was always more efcient than the single pathway (by NK92-CD16 and NK92-CD16/CD3). The specific data provided in Table 1 also proves this significant difference.Rituximab experiments also show similar results(Figures 4C).

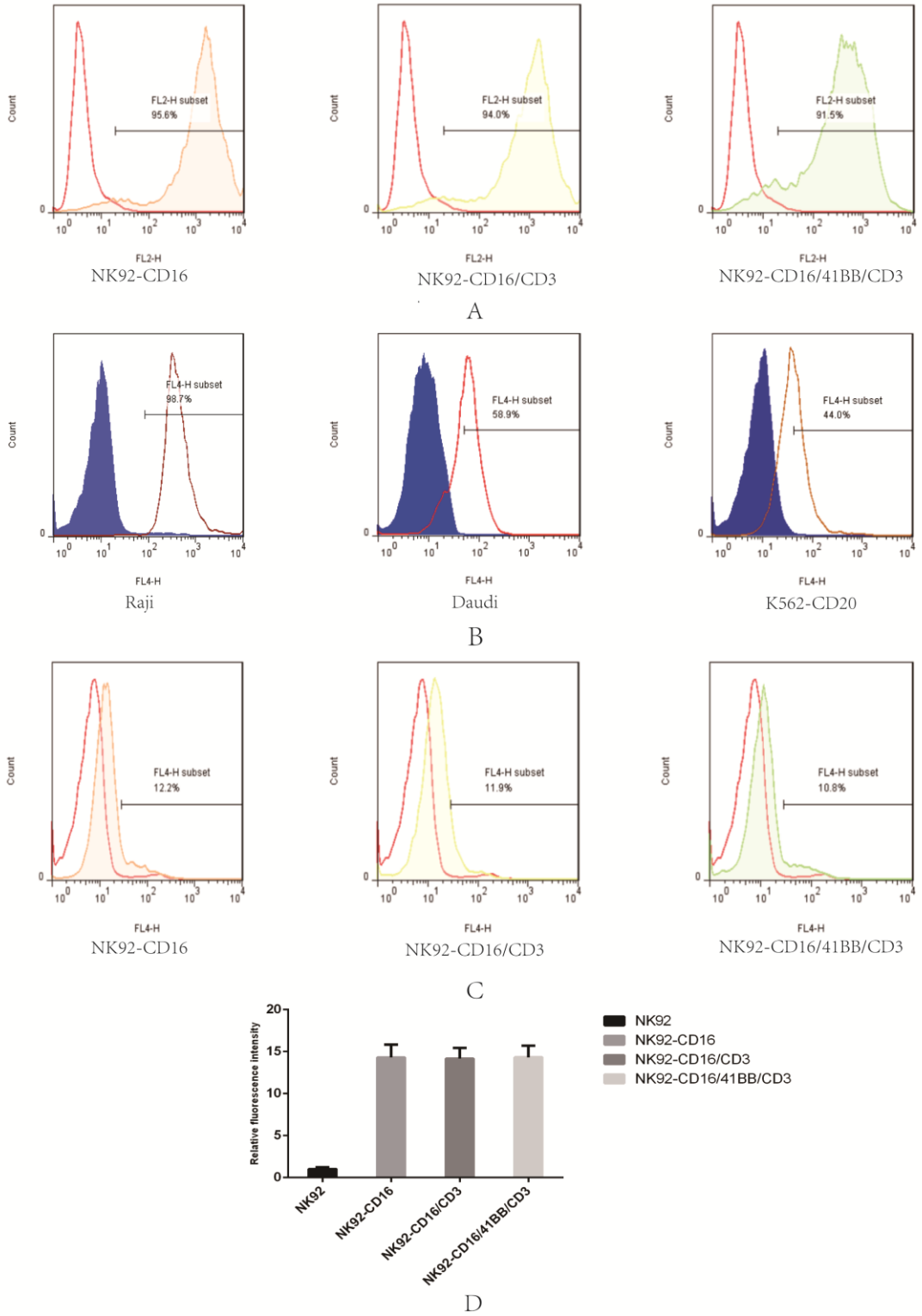


Figure 2:CD16 expression and activity detection. (A)The positive rate can be detected by flow cytometry using anti-CD16-PE. (B)The antibody-fluorescein(Cy5) coupled protein was detected using the Raji strain highly expressing CD20 protein and the Daudi cell strain, and the exogenously constructed K562-CD20 cell line. (C) CD16 affinity antibody Fc was detected with rituximab-Cy5.

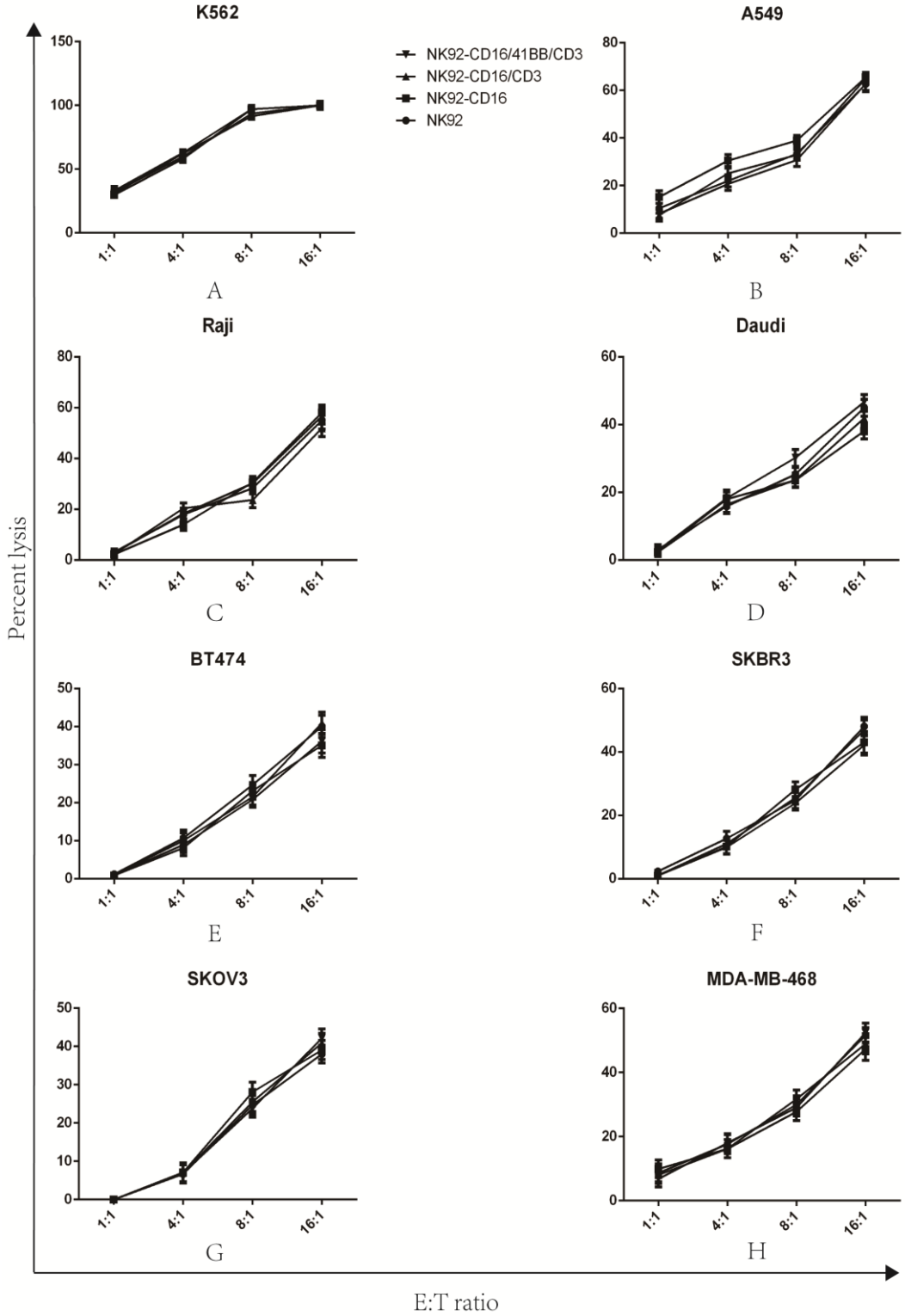
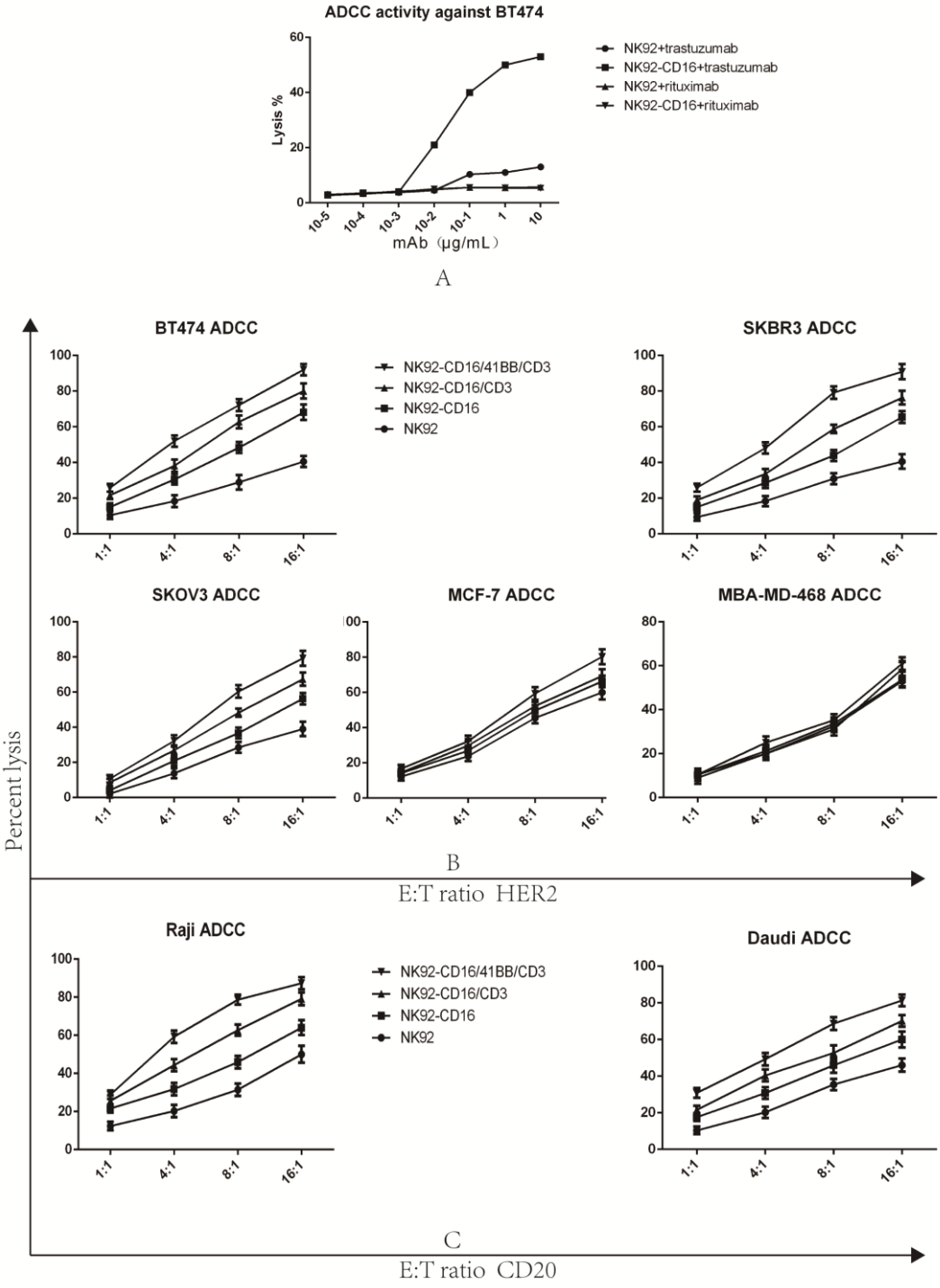


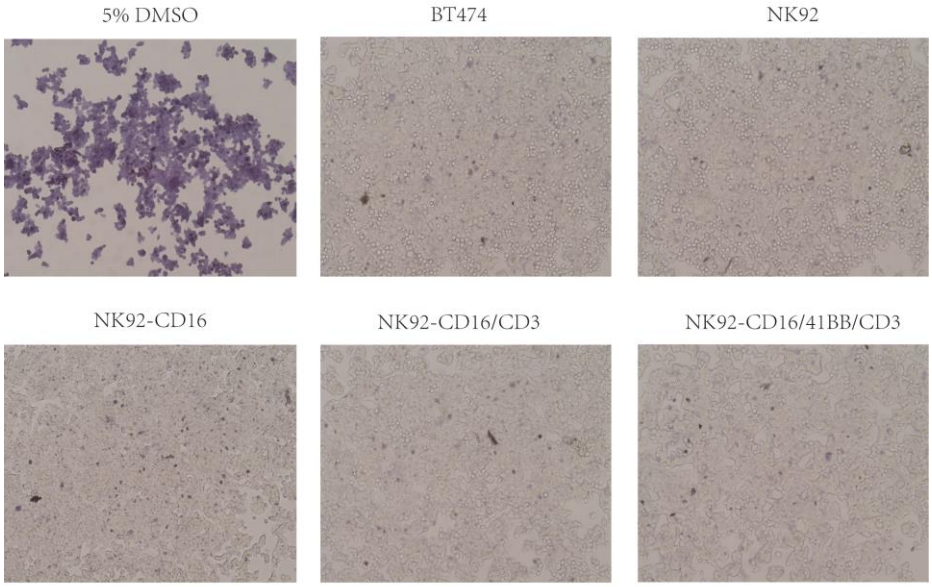
Figure 3: NK92 lysis of human tumor cell lines.haNK cells were kept in culture for 24 h prior to an 8-h lysis assay at different E:T ratios.Analysis of blood(A), lung(B), lymphoma(C,D), breast(E,F) and uterine tumors(G,H), respectively by CCK-8 assay.Results shown are the means (SD) of triplicate measurements from one of at least three comparable repeat experiments.



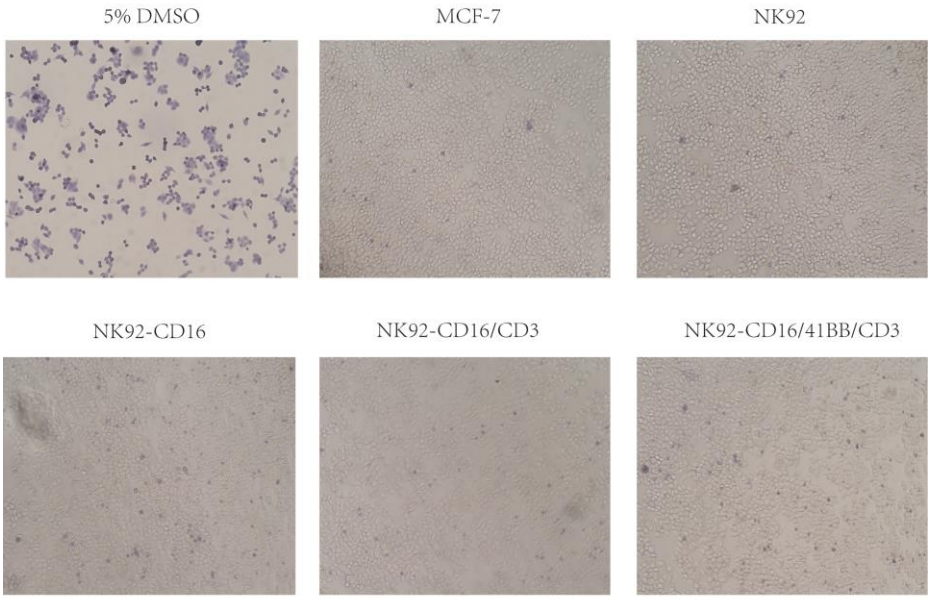
Figures 4:NK92 cell ADCC mediated by trastuzumab and cetuximab was evaluated with 8 h CCK-8 assays. (A)Te effector cells NK92-CD16 were tested against the HER2 positive BT474 cell line preincubated in the presence of increasing concentration of trastuzumab (mean of two experiments). (B)To compare the killing effects of different modified NK92 cells in combination with trastuzumab on HER2-expressing cells.Highly expressed cells BT474 and SKBR3, medium expressing cells SKOV3, low expressing cells MCF-7, and negative expressing cells MDA-MB-468 was used the antibody of 10μg/mL. (C) Compare with rituximab(10μg/mL) on CD20-expressing cells of Raji and Daudi cell line.Results shown are the means (SD) of triplicate measurements from one of at least three comparable repeat experiments.

2.5 Trypan Blue Staining to Observe The Killing Activity of NK92 Cells Targeting Tumor Cells

In order to more intuitively respond to NK92 combined antibody-mediated ADCC targeting tumor cell killing effect. The NK92 cells were at a ratio of 8:1 at the target effect ratio and 10μg/mL at the antibody concentration. After 8 hours of action, effector cells were washed out and the target cells were stained with trypan blue, and the target cell viability was observed under a microscope. As shown in Figures 5A, 5B shows that NK92-CD16/41BB/CD3 has a stronger effect in antagonizing BT474 and MCF-7 with trastuzumab.



A



B

Figure 5:Trypan blue staining to observe the killing activity.(A)BT474 was killed by effector cells in combination with trastuzumab(10μg/mL) for 10 hours. (B)MCF-7 was killed by effector cells in combination with trastuzumab(10μg/mL) for 10 hours.Positive controls were treated with 5% DMSO.

2.6 Detection of Granzyme-Associated Protein CD107a

ADCC is the main way for NK cells to kill tumors. The killing response is mainly caused by NK cells releasing granzymes, perforin disrupting the morphology of target cells, and inducing apoptosis of target cells. CD107a (lysosome-associated membrane protein-1), with the occurrence of cell degranulation, CD107a molecules will be transported to the surface of the cell membrane, and the up-regulation of CD107a molecules is consistent with perforin secretion. As shown in Figure 6A, BT474 was targeted with a combination of HER2 antibodies, NK92-CD16/41BB/CD3 cells detected higher amounts of CD107a than other cells, and there was no significant difference for negatively expressing cells (Figure 6B). As shown in Figure 6C, Calculate percentage of apoptosis statistical analysis.

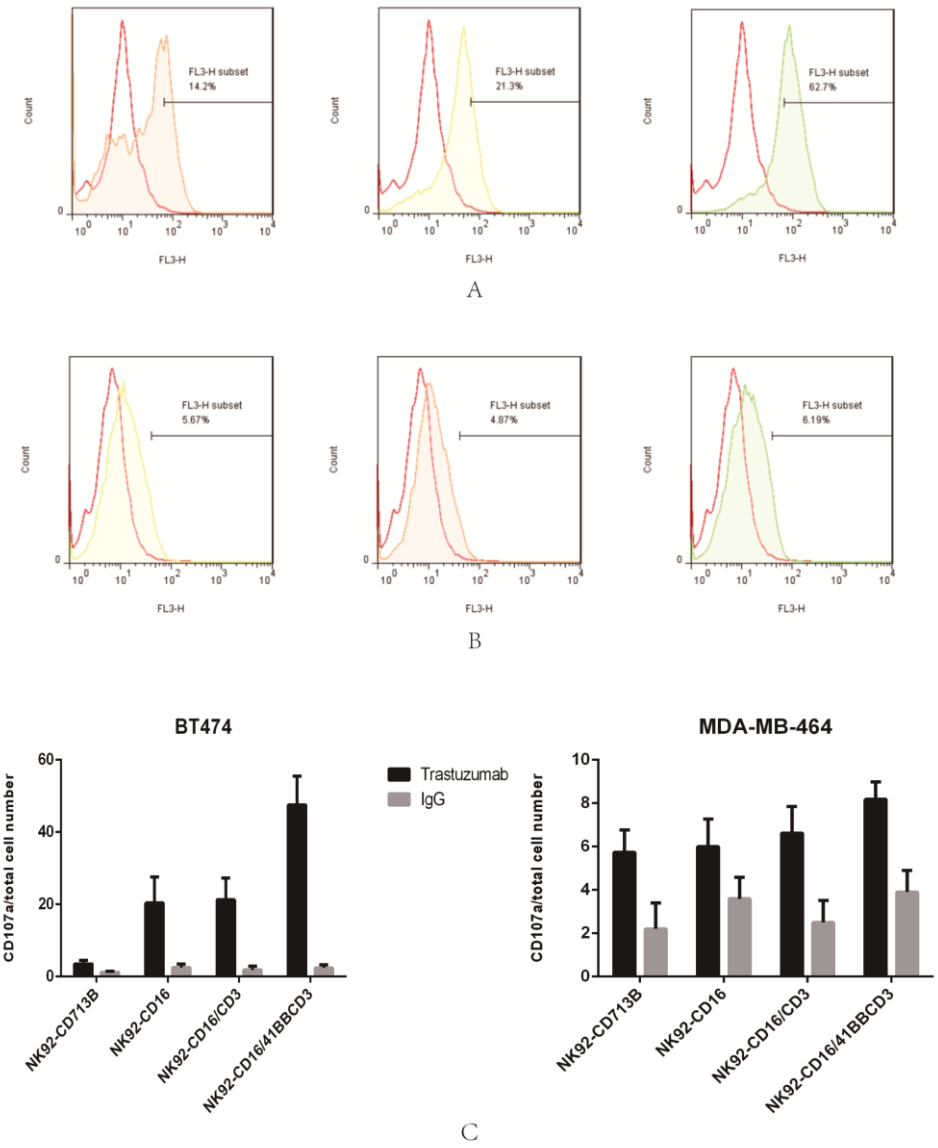


Figure. 6 :Detection of granzyme-associated protein CD107a. BT474(A) and MDA-MB-468(B) was killed by effector cells which NK92-CD16(left), NK92-CD16/CD3(middle) and NK92- CD16/ 41BB/CD3(right) in combination with trastuzumab(10μg/mL) for 10 hours.Flow cytometry detected CD107a expression using anti-CD107a-APC. (C)Calculate percentage of apoptosis using SPSS for statistical analysis.Results shown are the means (SD) of triplicate measurements from one of at least three comparable repeat experiments.

2.7 Real-Time Fluorescence Quantitative PCR Detection of Post-Reaction Gene Expression Changes

The NK cell kills tumor cells by releasing the cytokine TNF-α and expressing the apoptotic ligand FasL. To test whether the exogenous CD16-expressing NK92 cells can enhance the killing effect of other pathways. The NK cell-activated receptor NKG2D, tumor necrosis factor TNF-α, and apoptotic ligand FasL QPCR primers were designed to detect the mRNA expression level. As shown in the figure 7, the results showed no significant difference between NKG2D, TNF-α, FasL and NK92 cells. Explain that the main way of killing is through the release of granzymes, perforin's ADCC effect.

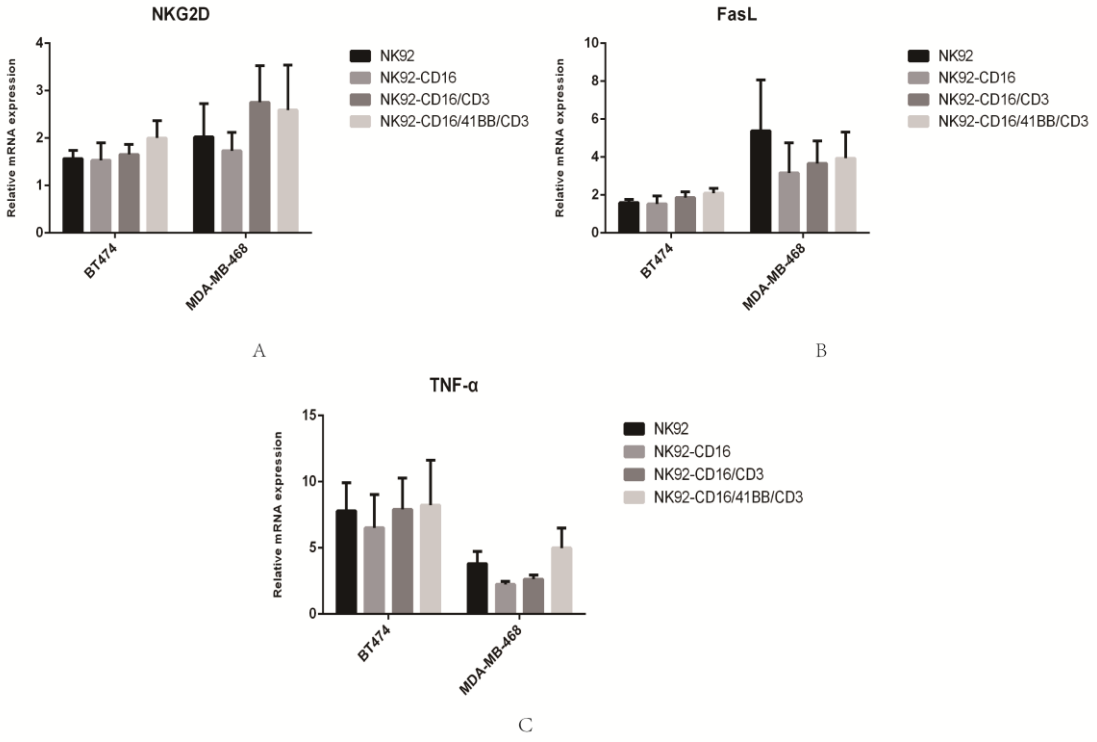


Figure 7:Detection of anti-tumor activity related protein expression.BT474 and MDA-MB-468 was killed by effector cells in combination with trastuzumab(10μg/mL) for 10 hours, Real-time fluorescent quantitative PCR was used to detect the expression of NKG2D(A), pro-apoptotic ligand FasL(B) and tumor necrosis factor TNF-α(C).Results shown are the means (SD) of triplicate measurements from one of at least three comparable repeat experiments.

3. Discussion

We compared in the present study two constructs allowing us to implement two strategies relying on an adoptive transfer of cytotoxic lymphocytes to improve the targeting of HER2 and CD20 positive tumors. In the first, the lymphocytes are equipped with a human CD16 receptor to permit ADCC in the presence of trastuzumab. In the second, We modified the CD16 gene by chimerism and replaced its intracellular signal region with 41BB or CD3 molecules to construct two other chimeric Fc receptor molecules CD16/CD3 and CD16/41BB/CD3 (Figure 1).

After transduction with CD16 and recombinant gene, the human NK cell line NK-92 displayed stable cell surface expression of CD16 and the chimeric receptor of CD16/CD3 and CD16/41 BB/CD3(Figure 2). By analyzing the protein expression and activity of the stable strain cells, the results showed that the gene-modified cell line can stably express the CD16 protein and has the biological activity of binding to the Fc fragment of the antibody (Figure 3). In this study, different human tumor cell lines are shown (Figure 4) to be lysed by NK92 cells in vitro, including lung, colon, breast, blood and lymphoma, cervical, and ovarian carcinoma lines; a range of lysis was observed among lines. These results are similar to those observed with the parental NK-92 line[22,23].

Specifc cytotoxic activity against HER2 positive target cells was demonstrated in both cases, and when we compared their potency against four HER2 positive target cell lines, NK92-CD 16/41BB/CD3 always performed better than NK92-CD16 and NK92-CD16/CD3. Similarly, there are similar results for CD20 positive cell. CCK-8 experiments, target cell apoptosis experiments, and trypan blue experiments have all demonstrated this result(Figure 5-8). The lytic target cell protein granzyme perforin-related protein CD107a in the ADCC pathway was also examined, the results showed that (Figure 9) the chimerically modified NK92-CD16/41BB/CD3 could release higher levels in the combined antibody-cleavable target cell reaction. Granzyme, indicating that the killing effect will be better.

The NK cell kills the target cell pathway mainly through the surface expression of the apoptotic ligand FasL, which binds to the apoptosis receptor Fas on the target cell and promotes the apoptosis of the target cell [24]; the secreting cytokines IFN-γ, TNF-α, etc. Target cells [25]; release granzymes and perforin, lyse target cells [26]; kill tumor cells by CD16 (FcγRIII) binding to anti-tumor antibody ADCC. After combined antibody reaction, the activation of NKG2D, pro-apoptotic ligand FasL, and tumor necrosis factor TNF-α expression levels were examined to compare whether the modified NK92 cells could enhance these pathways for anti-tumor responses. The results showed that in the presence of antibodies, the modified cells mainly acted by ADCC (Figure 10).

As far as we know, only three studies directly compared these two methods. Boissel et al. using the two different therapeutic monoclonal anti-CD20 mAbs rituximab and ofatumumab to demonstrate cytotoxic activity of NK-92 cells in the presence of the anti-CD20 monoclonal antibody, which is superior to ADCC over NK-92 CD16. Tassev et al. used a detailed description of the relocalization of NK-92 cells using the HLAA2-restricted EBNA3C-specifc chimeric receptor (a TCR like antibody) and also directly compared the two systems and concluded that: "the CAR mediated approach proved far more effective at killing target cells compared with ADCC "[27]. Beatrice et al. compared their potency against four HER2 positive target cell lines, NK92-CAR always performed better than NK92-CD16[28].

In conclusion, the NK92 cells modified with the gene CD16/41BB/CD3 have higher efficiency, specific tumor cell killing activity and higher antibody affinity than NK92 cells. In the anti-tumor response of the combined antibody, intracellular segment signal molecules 41BB and CD3 can be used to achieve cell proliferation and activation. Our research proposes a new strategy for improving anti-tumor effects by modifying NK92 cells.

4. Materials and Methods

4.1 Cell Lines

NK-92, the human NK cell line (ATCC, Rockville, MD), was grown in RPMI 1640 culture medium (Gbico, Cergy Pontoise, France) supplemented with 10% FBS (PAA Laboratories, Les Mureaux, France), 100 IU/mL IL-2 (Proleukin) (Chiron Corporation, Emeryville, US), 2mM L-glutamine (Gbico), penicillin (100 IU/mL), and streptomycin (0.1μg/mL) (Gbico). The HER2 negative MDA-MB-468 and the HER2 positive BT-474, SKBR3, SKOV3 and MCF7 breast cancer cell lines were obtained from ATCC. Cell lines were cultured in complete medium consisting of DMEM (Sigma Aldrich, St. Quentin Fallavier, France), 10% heat-inactivated foetal calf serum, 2mM glutamine (Sigma Aldrich), 100 U/mL penicillin, and 10 μg/mL streptomycin (Sigma Aldrich).

4.2 Lentiviru Vector Production

Transient retroviral supernatants were produced by PEI precipitation with 15μg of plasmid. Two million HEK 293T cells were seeded into 10 cm diameter dishes 24h prior to transfection. The transfection was performed with 15μg CD713B-CD16, CD713B-CD16/CD3 or CD713B-CD16/41BB/CD3 plasmid DNA using PEI precipitation (Invitrogen). The medium (10mL) was replaced 6h after transfection. The conditioned medium was collected 48h and 72h after transfection, fltered through 0.45μm pore-size filters, and kept at 4∘C until use. The viral titer was determined by the transduction of 293T cells (1×106 cells per well in 6-well plates) with serial dilutions of virus and

analyzed for eGFP expression 4 days afer infection. The retroviral supernatant titers were typically 1-5 × 107 IU (Infectious Units)/mL.

4.3 NK-92 Cell Line Transduction Using Lentiviru Supernatant

The NK-92 cell line was resuspended in RPMI 1640 culture medium supplemented with 10% FBS and 100 IU/mL of recombinant IL-2, seeded at 1×106 cells in 1 mL per well into 6-well plates, and exposed to 2×2 mL of lentivirus supernatant purified (overnight at 4◦C with PEG800 and 4000g, 40min) in the presence of 4μg/mL polybrene (Sigma, St. Quentin Fallavier, France). The culture medium was changed 24h after infection. Mock (nontransduced) controls were performed in parallel, by which the supernatant of untransfected packaging cells was added to the NK-92 cell line. The transduction efciencies were assessed 2 days later by flow cytometry, Screening with puromycin for flow detection at 7 and 14 days, respectively.

4.4 Flow Cytometry

The state of the cells was adjusted in advance and cultured in complete culture medium at 37°C in a humidified incubator with 5% CO2. Take logarithmic growth phase cells, count, and resuspend the cells in fresh complete medium. Take 1× 106 cells into a 1.5mL EP tube. 800g, centrifuge 5min. Wash twice with 1× PBS. Resuspend the cells in 100 μL PBS. In the dark, add 5 μL of flow antibody anti-CD16-PE to the cell suspension. Mix thoroughly and protect from light at 37°C in the dark for 30 minutes or at 4°C overnight. 800g, centrifuge 5min. Wash twice with 1× PBS. Resuspend in 500 μL PBS and inspect with flow cytometry.

4.5 Cytotoxicity and ADCC Assay

Adjust the cell state in advance. The target cells were digested with trypsin and 2mL of medium was used to stop digestion. 10μL of cell suspension was stained with trypan blue and counted. When the cell viability reached 90% or more, the cell density was adjusted to 105 cells/mL. The cell suspension, 90μL/well, was added to a 96-well cell culture plate. Incubate overnight at 37°C in a 5% CO2 saturated humidity incubator. The following day, the antibody was diluted with 1×PBS at a concentration of 100μg/mL. 20μL of antibody solution was added to the experimental group in the air, and 20μL of 1×PBS was added to the control group. Place in a saturated humidity incubator at 37°C, 5% CO2 for 30 min. Draw the effector cells in the logarithmic phase to ensure that the cell viability reaches more than 90%. Adjust cell density and prepare effector cell suspension. Effector cells were added to the target cell wells at a effect/target ratio of 1:1; 4:1; 8:1; 16:1, 90μL/well. Final volume 200μL/well. Set control, blank control group; effector cell control group; target cell control group; antibody control group. Each group has 3 replicates. Tap the 96-well plate and allow cells to mix thoroughly. Placed in 37°C, 5% CO2 saturated humidity incubator for 8h. After the reaction, 20μL/well of CCK-8 solution was added to the 96-well plate, and placed in a saturated humidity incubator at 37°C with 5% CO2 for 1.5h. Measure absorbance at 450 nm and 630 nm with a microplate reader. Cell killing efficiency (%) = [experimental group-effector cell control group]/[control group-blank control group] ×100%.

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