Specific growth inhibition of ErbB2-expressing human breast cancer cells by genetically modified NK-92 cells

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Abstract. The natural killer cell line NK-92 shows great cytotoxicity against various types of cancer. Several types of solid tumor cells, however, can effectively resist NK-mediated lysis by interaction of major histocompatibility complex (MHC) molecules with NK cell inhibitory receptors. To generate a eukaryotic expression vector encoding chimeric antigen receptor scFv anti-erbB2-CD28-ζ and to investigate the expression and action of this chimeric antigen receptor in cancer cells both *in vitro* and *in vivo*, NK-92 cells were genetically modified with an scFv anti-erbB2-CD28-ζ chimeric receptor by optimized electroporation using the Amaxa Nucleofector system. The expression of the chimeric receptor was evaluated by RT-PCR and immunofluorescence. The ability of the genetically modified NK-92 cells to induce cell death in tumor targets was assessed *in vitro* and *in vivo*. The transduced NK-92-anti-erbB2 scFv-CD28-ζ cells expressing high levels of the fusion protein on the cell surface were analyzed by fluorescence-activated cell-sorting (FACS) analysis. These cells specifically enhanced the cell death of the erbB2-expressing human breast cancer cell lines MDA-MB-453 and SKBr3. Furthermore, adoptive transfer of genetically modified NK-92 cells specifically reduced tumor size and lung metastasis of nude mice bearing established MDA-MB-453 cells, and significantly enhanced the survival period of these mice. The genetically modified NK-92 cells significantly enhanced the killing of erbB2-expressing cancer and may be a novel therapeutic strategy for erbB2-expressing cancer cells.

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Introduction

Natural killer (NK) cells are important effectors of the innate immune system and play an essential role in the body's first line of defense against virus-infected and malignant cells (1,2). NK cells rapidly recognize different pathogens through a variety of activation and inhibition receptors, and mediate spontaneous tumor cytotoxicity in a major histocompatibility complex (MHC)-unrestricted manner (3). The importance of NK cells in antitumor immunity is further demonstrated by the fact that patients with dysfunctional NK cells readily suffer leukemia (4). NK cells may be promising candidates for cancer cell therapy with the potential to target a wide range of malignancies that exhibit defective or altered MHC class I. Currently however, no major benefits have been reported from the use of original NK protocols, partly as tumor cells have developed several mechanisms, including the expression of ligands that interact with NK cell inhibitory receptors, thereby hampering NK cell function (5). Furthermore, the amplification of original NK cells is limited and poorly standardized between different research centers.

A novel approach, different to the adoptive transfer of endogenous NK cells, involves the permanent IL-2-dependent NK-92 cell line, which mediates strong cytotoxic responses against a variety of tumor cells *in vitro* and in humanized mouse models *in vivo* (6,7), most likely as a result of the absence of inhibitory NK cell receptors (8). NK-92 cells possess the characteristics of activated NK cells but do not harbor Fc-γRIII (CD16) and lack the general killer inhibitory receptors (KIRs), except KIR2DL4 (8,9), which interacts with human leukocyte antigen (HLA) molecules on target cells and inhibits NK cell cytolytic activity (10). NK-92 cells are cytotoxic to tumor cells, but have no effect on nonmalignant allogeneic cells. Another major advantage of NK-92 cells in adoptive therapy, in contrast to autologous or allogeneic NK cells, is the ease of the maintenance and expansion in culture and the availability on demand in standardized quality. Nevertheless, similar to primary NK cells, NK-92 cells show little or no activity against many solid tumor cell lines.

In the immunotherapeutic approach, genetically modified NK cells were generated with chimeric single-chain variable fragment (scFv) receptors that recognize tumor-associated

Ags (TAA). This strategy can enhance tumor recognition by primary T cells (11-14) and generate specific killing of tumor target cells by NK cell lines (15-18) or primary human NK cells (19-21).

The aim of the present study was to investigate the potential role of genetically modified NK-92 cells in the therapeutic treatment of cancer cells expressing erbB2 (HER2/neu). It was hypothesized that the method of introducing genetically modified NK-92 cells into cancer cells may inhibit tumor cell proliferation both *in vivo* and *in vitro*. In contrast to parental NK-92 cells, the genetically modified NK-92-anti erbB2 scFv-CD28-ζ cells specifically and effectively lysed erbB2-expressing human breast cancer cells. Furthermore, adoptive transfer of gene-modified NK-92 cells specifically reduced tumor size and lung metastasis of nude mice bearing established breast cancer cells, and significantly enhanced the survival of these mice. Consequently it is proposed that using genetically modified NK-92 cells could be a potent cell-based therapeutic treatment of erbB2-expressing cancers.

Materials and methods

Cell lines and culture conditions. Human breast cancer cell lines MDA-MB-453 (erbB2-expressing) and MDA-MB-231 (erbB2-negative), provided by the Tumor Laboratory of our Hospital, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Human erythroleukemic K562 cells were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. NK-92 cells and transduced NK-92-scFv-CD28-ζ cells were propagated in α-Minimum Essential Medium negative for ribonucleosides and deoxyribonucleosides. The medium contained 2 mM L-glutamine and 1.5 g/l sodium bicarbonate. To produce the final growth medium, the following components were added to the base medium: 0.2 mM inositol; 0.1 mM 2-mercaptoethanol; 0.02 mM folic acid; 1000 U/ml recombinant IL-2; adjusted to a final concentration of 12.5% horse serum and 12.5% FBS.

Construction of a chimeric antigen receptor carrying erbB2-specific scFv. The recombinant chimeric antigen receptor gene was generated by means of oligo chemic synthesis and PCR amplification as previously described (15). The chimeric antigen receptor consisted of heavy- (VH) and light-chain variable domains (VL) of the erbB2-specific antibody and linked by overlap extension with a GS-linker, c-myc tag, CD8a (ENST00000283635), CD28 (ENST00000374478) and CD3ζ (ENST00000392122) chain. The resulting PCR product was re-amplified adding *Hind*III and *Eco*RI restriction sites and cloned into the plasmid PCDNA3.1.

Gene modification of NK-92 cells. NK-92 cell genes were modified by electroporation using the Amaxa Nucleofector system (Amaxa Biosystems, Köln, Germany). In brief, $5x10^6$ NK-92 cells were placed in a 0.1 ml electroporation solution with 4 μg of the PCDNA3.1 plasmid DNA encoding the scFv-erbB2-CD28-ζ chimeric receptor. The appropriate Nucleofector Program A-024 was selected. Following electroporation, the cells were incubated in 2 ml Amaxa recovery medium with 1000 IU/ml recombinant human IL-2 for 24 h before being used in the experiments.

Analysis of chimeric antigen receptor expression. For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from the NK-92-scFv-erbB2-CD28-ζ cells using the TRIzol method. RT-PCR was performed with the SuperScript III One-Step RT-PCR System (Invitrogen) using the sense primer P1 (5'-CTTAAGCCTATGCAGGTCCAACT GCA-3') and antsense primer P2 (5'-CTGAATTCCTAGCGAG GGGGCA-3').

A total of $5x10^6$ NK-92-scFv-erbB2-CD28-ζ or NK-92 cells were harvested by centrifugation, washed with PBS twice, and then stained with FITC-labeled c-myc tag Ab (SH1-26E7.1.3; Miltenyi Biotec, Gladbach, Germany) for 30 min at 4˚C for immunofluorescence analysis. Background fluorescence was determined by staining cells with an isotype control antibody (Ab) followed by a secondary PE-conjugated anti-mouse Ig mAb. Fluorescence was analyzed with a FACScan (FACS Calibur; Becton, Dickinson and Co., USA).

Phenotyping analysis of cell surface marker expression on NK-92 cells. The phenotyping of cell surface marker expression on NK-92-scFv-erbB2-CD28-ζ and parental NK-92 cells was determined using FACScan by staining cells with PE-labeled Abs specific for CD27 (O323), NKG2D (1D11), CD158d (mAb33) and Alexa Fluor 647-conjugated Ab specific for CD85 (GHI/75) (all from BioLegend, USA).

Cytotoxicity assay. The ability of gene-modified NK-92 cells to specifically kill tumor targets was assessed in a 4 h ^{51}Cr-release assay. In brief, NK-92 cells were incubated with $1x10^5$ ^{51}Cr-labeled MDA-MB-453, MDA-MB-231, SKBr3, MCF7 and K562 cells at various E/T ratios in triplicate wells of a 96-well round-bottom plate (in 200 μl of complete DMEM). The percentage of specific release of ^{51}Cr into the supernatant was assessed as previously described (22).

Mouse models with modified NK-92 cells and in vivo antitumor activity. BALB/C nude mice (6-12 weeks of age) were bred under specific pathogen-free conditions at the Experimental Animal Center of the General Hospital of PLA.

Two mouse models were developed to assess the ability of genetically modified NK-92 cells expressing the anti-erbB2-CD28-ζ receptor to inhibit tumor growth. In the first mouse model, 28 BALB/C mice were randomly divided into 4 groups with 7 mice in each group. One type of tumor cells (MDA-MB-453 or MDA-MB-231 cells, $2x10^6$) were injected into the flanks of BALB/C mice, and one type of NK-92 cells (NK-92-scFv-erbB2-CD28-ζ or parental NK-92 cells, $1x10^7$, E/T ratio of 5:1) suspended in 0.1 ml of PBS were injected into the caudal vein simultaneously. The tumor formation rates for each group were analyzed on day 10 (Fig. 1).

In the second model, either MDA-MB-453 or MDA-MB-231 cells ($2x10^6$) were respectively injected into the flanks of BALB/C mice to establish the mouse models bearing transplantated tumors. Thirty mice bearing MDA-MB-453 and 30 mice bearing MDA-MB-231 were then randomly divided into 6 groups of 10 animals in each group. Mice were treated on

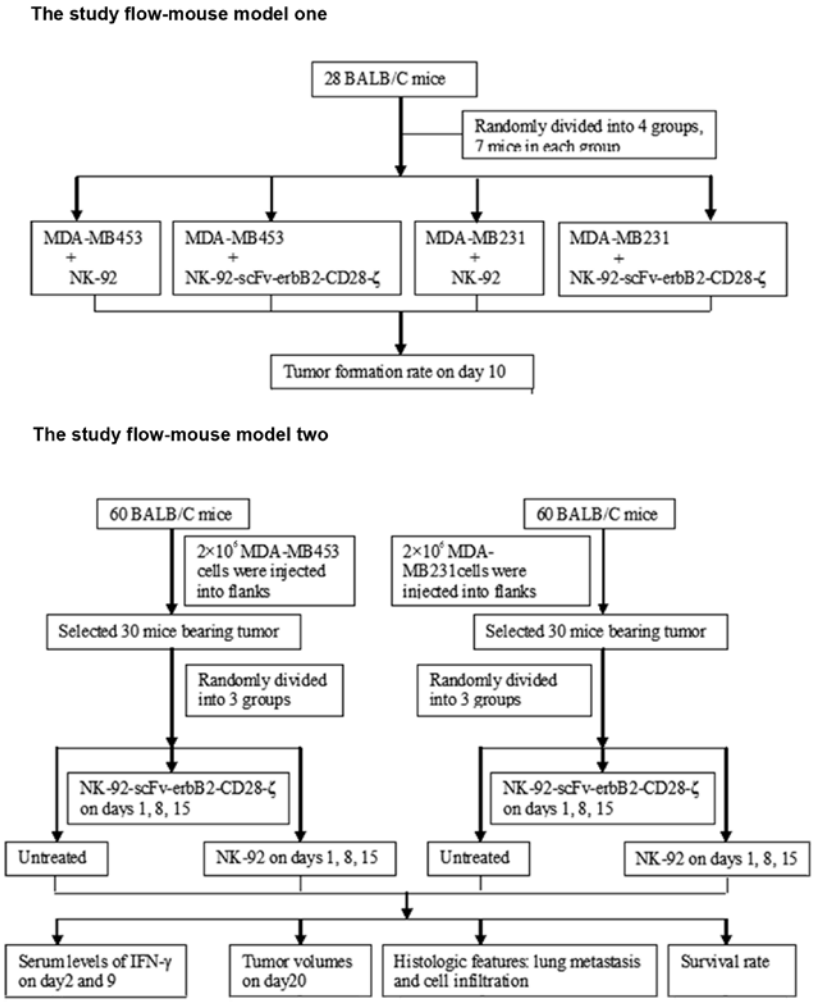

Figure 1. The flowchart of the present study: Mouse Model One and Two.

days 1, 8 and 15 with either $1x10^7$ NK-92-scFv-erbB2-CD28-ζ or parental NK-92 cells delivered i.v. (caudal vein) (Fig. 1). Serum levels of interferon-γ (IFN-γ) on day 2 and 9 were determined by ELISA. Tumor volumes on day 20 were calculated using the formula: π x length x $(\text{width})^2 \div 6$.

Histologic procedures. For each animal, upon euthanasia, the hypodermic transplantation block and lung were excised, fixed in formalin, embedded in paraffin, and serially sectioned at a 2-μm thickness. Routine H&E staining was performed at an interval of every 10 sections. Immunohistochemichal assay (Dako REAL EnVision Detection System, K8010; Dako, Glostrup, Denmark) was used to stain the tissues with CD3 (F7.2.38; Santa Cruz Biotechnology, USA), CD56 (IS628, clone 123C3; Dako), NKG2D (14F-2; Santa Cruz), CD16 (2Q1240; Santa Cruz), for analysis of the infiltration of NK-92 cells in the tumor in the unstained paraffin sections.

Statistical analysis. The Mann-Whitney U test, Chi-square test and one-way ANOVA in SPSS 10.0 were used for the statistical analysis. The Kaplan-Meier test was used to analyze survival rates. Values of $P<0.05$ were considered to indicate statistically significant differences.

Results

Expression of the chimeric anti-erbB2 receptor in the NK-92 cells. A 1803-bp long chimeric anti-erbB2 scFv-CD28-ζ receptor was generated by oligo chemic synthesis and PCR amplification (Fig. 2A). The expression of anti-erbB2 scFv-CD28-ζ mRNA was confirmed by RT-PCR (Fig. 2B). A high level of the anti-erbB2 receptor was observed in the NK-92 cells following staining with a c-myc tag mAb specifically recognizing a c-myc tag epitope incorporated into the extracellular domain of the chimeric receptor (Fig. 2C). Cell viability ranged between 60 and 90% following electroporation.

Phenotypic characterization of the genetically modified NK-92 cells. We investigated whether expression of the chimeric scFv receptor had an effect on the NK-92 cell phenotype. Flow cytometry was used to compare the expression of a number of molecules expressed by the anti-erbB2-NK-92 and parental NK-92 cells, including activation and inhibition receptors. Based on independently performed experiments, no difference in the expression of NK-92 cell markers CD27, NKG2D, CD158d or CD85 was noted between the parental NK-92 and the anti-erbB2-NK-92 cells (Fig. 3). These data indicate that

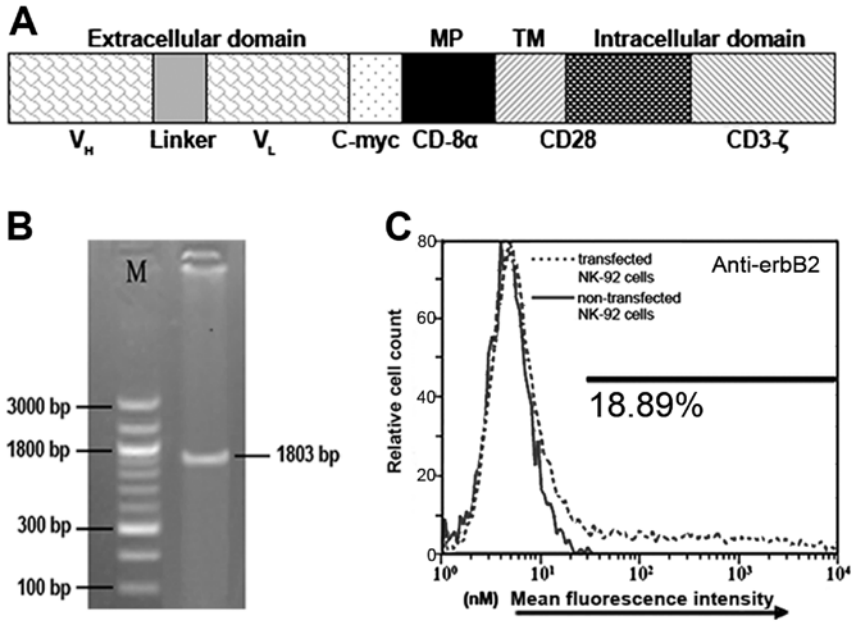

Figure 2. Genetic modification of NK-92 cells with the anti-erbB2 chimeric receptor. (A) Schematic image of the chimeric receptor anti-erbB2 scFv-CD28-ζ. The chimeric receptor consisted of the VH and VL regions of the anti-erbB2 mAb joined by a flexible linker, C-myc tag, a CD8-α membrane-proximal hinge region (MP), and the transmembrane (TM) and cytoplasmic regions of the human CD28 signaling chain fused to the intracellular domain of human TCR-ζ. (B) RT-PCR analysis with primers specific for the chimeric receptor sequence of mRNA from NK-92-anti-erbB2 scFv-CD28-ζ cells. The position of the anti-erbB2 scFv-CD28-ζ DNA fragment is indicated. (C) The expression of the chimeric scFv anti-erbB2 receptor in NK-92 cells was analyzed following staining with an FITC-labeled c-myc tag Ab. The dotted line represents transfected NK-92 cells; solid line represents non-transfected NK-92 cells.

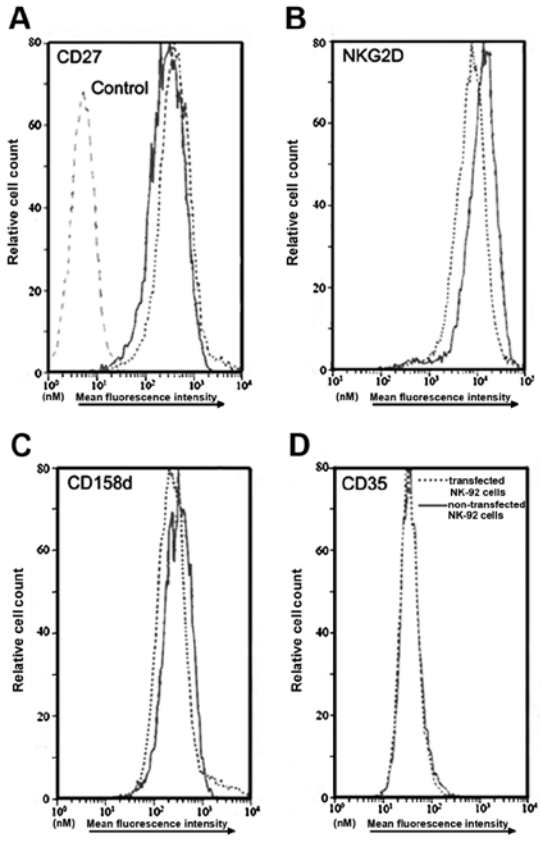

Figure 3. Phenotypic characterization of gene-modified NK-92 cells. The surface expression of various NK-92 cell activation/inhibition receptors was analyzed by flow cytometry following staining with appropriate Abs. There was no significant difference in expression of the following molecules between anti-erbB2-transfected or parental NK-92 cells: (A) CD27, (B) NKG2D, (C) CD158d or (D) CD85. The dotted line represents transfected NK-92 cells; solid line represents non-transfected NK-92 cells.

transfection of NK-92 cells with the scFv chimeric receptor did not phenotypically alter the expression of a number of important NK-92 cell-associated markers.

Antigen-specific cytotoxicity mediated by anti-erbB2-NK-92 cells. To determine whether the genetically modified NK-92 cells expressing the anti-erbB2 chimeric receptor could augment cell death activity against erbB2-expressing targets, two human breast cancer cell lines were used. These cell lines either expressed erbB2 or were negative for erbB2, and underwent cytotoxicity assays with anti-erbB2-NK-92 cells or parental NK-92 cells. A significant increase in the level of cell death in the erbB2-expressing MDA-MB-453 and SKBr3 cells by anti-erbB2-NK-92 cells was observed when compared to the control parental NK-92 cells (MDA-MB-453: E/T 1:1, 9.52±0.30 vs. 5.35±0.23%, $P<0.05$; E/T 10:1, 55.92±0.41 vs. 10.38±0.28%, $P<0.05$; E/T 20:1, 82.10±0.29 vs. 13.15±0.30%, $P<0.05$) (Fig. 4A); (SKBr3: E/T 1:1, 9.66±0.24 vs. 4.65±0.15%, $P<0.05$; E/T 10:1, 50.52±0.27 vs. 10.54±0.22%, $P<0.05$; E/T 20:1, 72.10±0.29 vs. 13.82±0.20%, $P<0.05$) (Fig. 4C). This increased cell death was erbB2 antigen-specific as anti-erbB2-NK-92 and parental NK-92 cells mediated comparable lysis of erbB2 negative MDA-MB-231 (Fig. 4B) and MCF7 cells (Fig. 4D). In addition, expression of the scFv receptor had no impact on the endogenous cytotoxic ability of NK-92 cells. The cytotoxicity of the anti-erbB2-NK-92 and parental NK-92 cells to a NK cell-sensitive target cell line, K562, was comparable (Fig. 4E).

Antigen-specific inhibition of tumor growth mediated by anti-erbB2-NK-92 cells. The ability of genetically modified NK-92 cells expressing the anti-erbB2 chimeric receptor

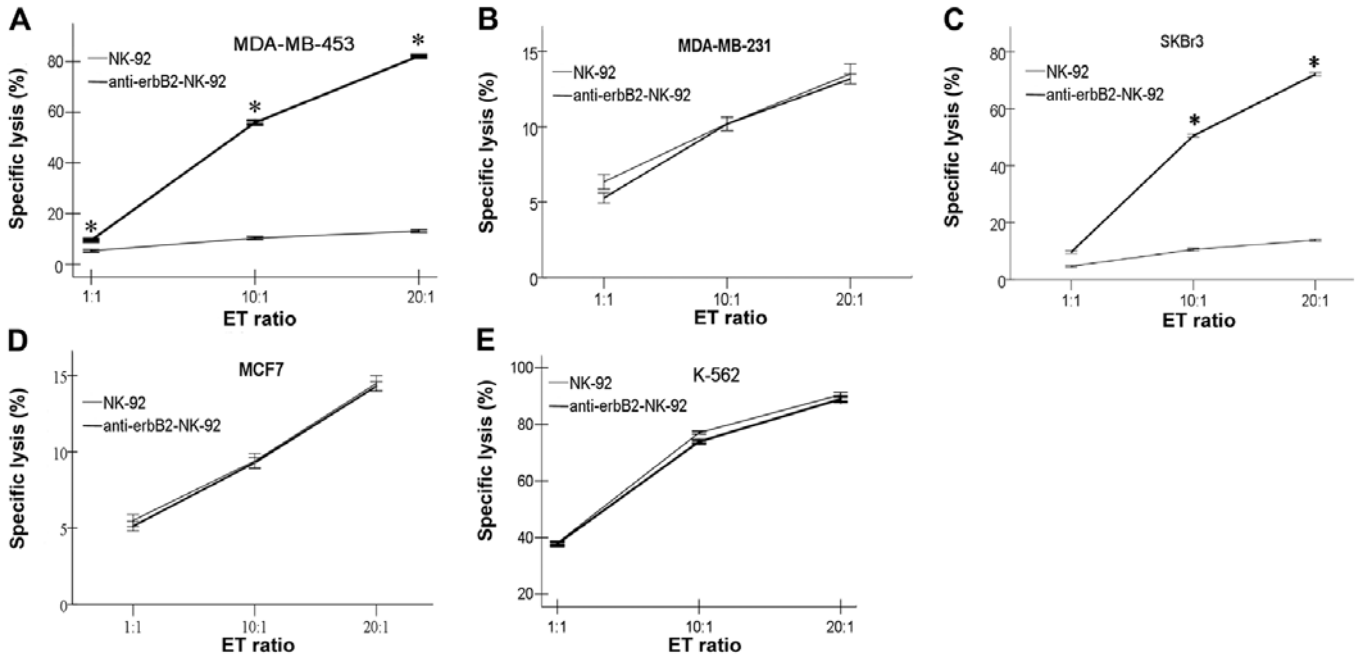

Figure 4. Enhanced cytotoxicity of the erbB2-expressing tumor cells to anti-erbB2-NK-92 cells. (A and C) Gene modification of NK-92 cells with the scFv chimeric anti-erbB2 receptor enhanced the killing of erbB2-expressing target cells compared with the parental NK-92 cells. (B and D) Anti-erbB2-NK-92 and parental NK-92 cells equivalently killed erbB2-negative target cells at a low level. (E) Anti-erbB2-NK-92 and parental NK-92 cells equivalently killed NK cell sensitive target K562 at a high level. (*P<0.05, as determined by Mann-Whitney U test). Results are expressed as average ± SEM of triplicates from three independent experiments.

to mediate antigen-specific inhibition of tumor growth was assessed *in vivo*. In the first experimental model, the tumor formation rate of mice in the MDA-MB-453 and genetically modified NK-92 cell group was lower than that in the MDA-MB-453 and parental NK-92 cell group (14.29 vs. 71.43%, P<0.05) on day 10. In contrast, the tumor formation rate in the MDA-MB-231 and genetically modified NK-92 cell group was similar to that of the MDA-MB-231 and parental NK-92 cell group (71.43 vs. 57.14%, P>0.05) (Fig. 5A). In the second experimental model, MDA-MB-453 and MDA-MB-231 cells were injected respectively into the flanks of BALB/C mice to establish the mouse models bearing transplanted tumors. The mice bearing tumors either remained untreated or were treated on days 1, 8, 15 with $1x10^7$ genetically modified NK-92 cells or parental NK-92 cells by i.v. (caudal vein). The serum IFN-γ level in the mice bearing MDA-MB-453 tumors that received anti-erbB2 gene-modified NK-92 cells was higher than the level in those mice that received parental NK-92 cells (day 2, 224.63±18.92 vs. 39.68±4.72 pg/ml, P<0.01; day 9, 254.34±10.59 vs. 59.90±5.57 pg/ml, P<0.01) (Fig. 5B and C). Mice bearing the MDA-MB-453 tumors that received anti-erbB2 gene-modified NK-92 cells, compared to those that received parental NK-92 cells, had significantly decreased tumor volume (0.05±0.02 vs. 0.59±0.12 cm^3, P<0.01) (Fig. 5D), lung metastasis (0 vs. 30%, P<0.05) (Figs. 5E and 6A and B) and increased survival (median survival 75 vs. 22.5 days, P<0.05) (Fig. 5F). Mice bearing the MDA-MB-453 tumors that received the genetically modified NK-92 cells had increased infiltration of the tumor tissue by lymphocytes (Fig. 6C and D) compared to the mice that received parental NK-92 cells as demonstrated by immunohistochemical analysis (Fig. 6E-H). These effects were antigen-specific as there were no significant differences in mice bearing the MDA-MB-231 tumors that received either anti-erbB2 NK-92 or parental NK-92 cells.

Discussion

Adoptive transfer of NK cells is gaining much attention in the immunotherapy for cancer. Recent results indicate an increase in survival in acute myeloid leukemia patients treated with alloreactive NK cells (23). In contrast, currently available therapies have no apparent effects on patients with acute lymphoid leukemia and most solid tumors. This is due in part to the nonspecific nature of these cancers and the HLA-mediated inhibitory signals induced by interaction with NK cell inhibitory receptors (5). Specifically, NK-92 cells show greater cytotoxic activity than other NK cells against many tumors, thus providing a basis for ongoing clinical development of NK-92 cells as an allogeneic cell therapy for adoptive cancer immunotherapy (24). An ideal adoptive therapy for cancer should consist of the development of robust effector cells with specific antitumor efficacy.

A novel way to enhance NK cell antitumor activity involves genetic modification with scFv chimeric receptors that can specifically recognize the TAA site. In the present study the Amaxa transfection system was used to genetically modify NK-92 cells with an erbB2-specific chimeric scFv receptor. This study demonstrated that NK-92 cells genetically engineered with the scFv anti-erbB2 receptor significantly enhanced the cytotoxicity of the NK-92-insensitive human breast cancer cell lines MDA-MB-453 and SKBr3, in an $erbB2^+$ antigen-specific manner. The expression of the chimeric antigen receptor did not

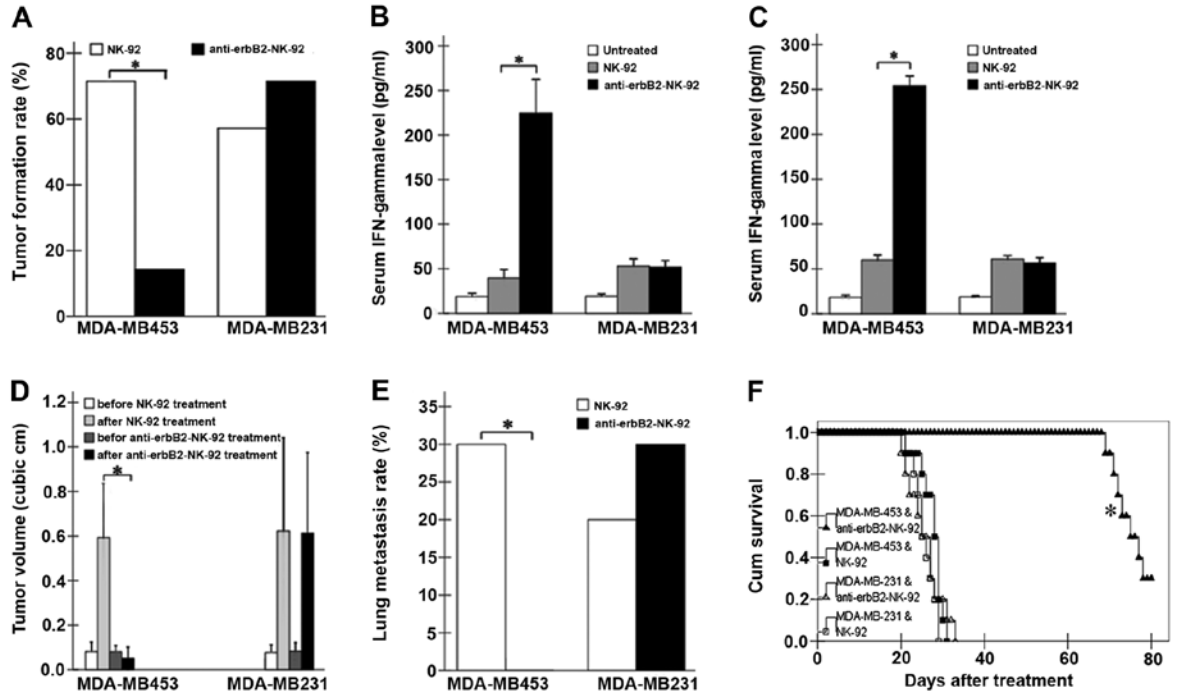

Figure 5. Specific inhibition of erbB2-expressing breast cancer cells by gene-modified NK-92 cells in tumor-bearing mice. (A) The tumor formation rate of mice in the MDA-MB-453 and transduced NK-92 cell group was lower than that in the MDA-MB-453 and parental NK-92 cell group on day 10, whereas the tumor formation rate was similar between the MDA-MB-231 and transduced NK-92 cell group and the MDA-MB-231 and parental NK-92 cell group (7 mice/group). (B) MDA-MB-453 and MDA-MB-231 cells were injected into the flanks of BALB/C mice to establish the mouse models bearing transplanted tumors. Mice bearing tumors were then treated on days 1, 8, 15 with $1x10^7$ gene-modified or parental NK-92 cells delivered i.v. (caudal vein). (B and C) Serum IFN-γ levels of mice with MDA-MB453 tumors that received anti-erbB2 gene-modified NK-92 cells were higher than those that received parental NK-92 cells. (B: day 2, 224.63±18.92 vs. 39.68±4.72 pg/ml, P<0.01; 10 mice/group) (C: day 9, 254.34±10.59 vs. 59.90±5.57 pg/ml, P<0.01; 10 mice/group). (D) Significantly decreased tumor volume (0.05±0.02 vs. 0.59±0.12 cm^3, P<0.01; 10 mice/group). (E) Decreased lung metastasis (0 vs. 30%, P<0.05; 10 mice/group). (F) Increased survival (10 mice/group) as assessed by Kaplan-Meier test, in mice bearing MDA-MB-453 tumors that received anti-erbB2 gene-modified NK-92 cells, compared to those receiving parental NK-92 cells. *P<0.05, as determined by Mann-Whitney U test.

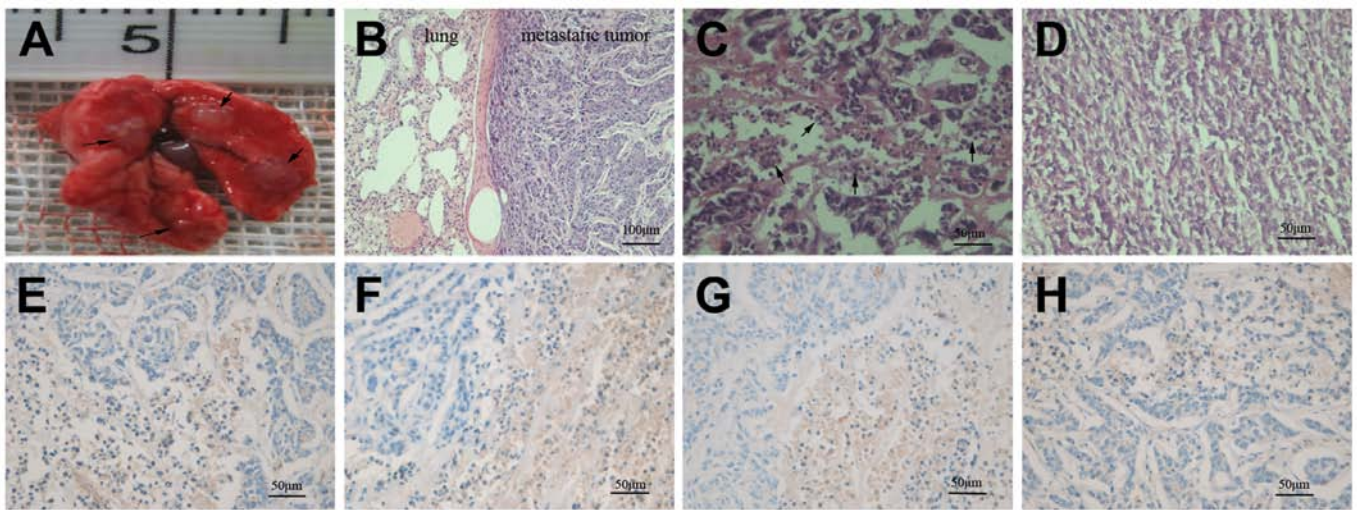

Figure 6. Representative histologic features (H&E staining and immunohistochemisty) of tumors from mice receiving different adoptive NK-92 cells. (A and B) Mice bearing MDA-MB-453 tumor suffered lung metastasis after receiving parental NK-92 cells. (C) Lymphocytes infiltrated tumor tissues that received anti-erbB2 gene-modified NK-92 cells. (D) Tumors of mice that received parental NK-92 cells following immunohistochemical staining with an antibody specific for CD3 (E, negative), CD56 (F, expressing), NKG2D (G, expressing), and CD16 (H, negative).

change the activity of NK-92 cells against the erbB2-negative human breast cancer cell lines MDA-MB-231 and MCF7. In contrast, both MDA-MB-453 and SKBr3 cells were effectively lysed by the anti-erbB2-NK-92 cells. There was no difference in the expression of several activation and inhibition receptors between the anti-erbB2-transfected and the parental NK-92-transfected cells, demonstrating that the enhancement of cytotoxicity was not associated with these inherent receptors, but with the TAA-specific chimeric receptor. Furthermore, it was demonstrated that adoptive transfer of anti-erbB2 receptor-

modified NK-92 cells could specifically not only decrease the tumor formation rate, tumor size and lung metastasis rate, but also increased serum IFN-γ levels and enhanced the survival rate of mice with erbB2-expressing tumors.

The expression of erbB2 on the target cell surface was sufficient to enable recognition and lysis by NK-92-scFv anti-erbB2-CD28-ζ. MDA-MB-453 cells were resistant to parental NK-92 cells, however could be lysed effectively by NK-92-scFv anti-erbB2-CD28-ζ cells due to the expression of the erbB2 antigen. In normal adult human tissue, erbB2 expression is limited to epithelial cells in the reproductive tract, digestive tract, breast, kidney and skin, and is lower in tumors suitable for erbB2-directed therapy (25). This specific selection is the basis for antitumor strategies using erbB2-directed therapy.

The NK cell is usually described as an essential component of the innate immune system that plays an important role in the first phase of host defense against tumor development. NK cells with normal function naturally avoid malignant transformation. In the present study, genetically modified NK-92 cells significantly inhibited erbB2-expressing MDA-MB-453 cells from tumor formation in mice, indicating that genetically modified NK-92 cells are useful for the prevention of specific cancers. Several studies have shown that gene modification of various mouse and human NK cell lines with scFv chimeric receptors can specifically enhance their antitumor activity both *in vitro* and *in vivo* (15,17,26-28). In these studies however, the effector cells were regionally administered, which differs from the vein administration method currently used in clinical settings. In the present study, both parental and genetically modified NK-92 cells were injected into the mice through the caudal vein. It was demonstrated that genetically modified NK-92 cells specifically infiltrated the erbB2-expressing tumors and increased serum IFN-γ levels and inhibited tumor growth of erbB2-expressing cells.

In patients with malignant disorders, NK cell function can be impaired, resulting in a reduced proliferative response and reduced cytotoxic activity (29). Consequently, it may not be possible to generate suitable autologous effector cells in all cases where this is the case. Therefore, clinically applicable human cell lines such as NK-92 may provide a valuable alternative. Furthermore, in contrast to retroviral transduction of primary cells, individual clones of genetically modified NK-92 cells with molecularly defined retroviral insertion sites can be generated, thus limiting the risk of insertion mutagenesis and oncogenic activation (30). Remaining safety concerns could be relieved by inclusion of suicide gene constructs (31,32), or irradiation of the cells before *in vivo* application to block proliferation. This has previously been shown not to affect cell death activity of unmodified and retroviral NK-92 cells (10).

NK cell activation not only leads to a dramatic increase in cytolytic activity (33), but also produces a large number of cytokines that modulate antitumor immune responses. Pivotal among these cytokines is IFN-γ. IFN-γ is a pleiotropic cytokine capable of influencing both the innate and adaptive immune systems (34). The production of IFN-γ by active NK cells is critical for IL-12 antitumor therapy (35). Rapid NK cell activation is linked to the activation of professional antigen presenting cells (APCs), which produce IL-12. In this study, mouse endogenous NK cells and adoptive parental NK-92 cells could not be sufficiently activated by APCs nor tumor cells, whereas anti-erbB2 genetically modified NK-92 cells could specifically recognize erbB2-expressing tumor cells and were therefore directly activated to produce large amounts of IFN-γ and achieve an effective antitumor immune reaction.

To achieve high efficient receptor expression in this study a non-viral vector system was used. This is suitable for use in the laboratory or in the clinical setting compared with viral-based systems, due to the attractive aspects such as safety and high efficiency. Moreover, expression levels using this method were transient, which provides additional safety by reducing the risk of long-term autoimmunity associated with prolonged presence of potentially autoreactive cells (36). Recent studies using lentivirus transduction demonstrated long-term and high-level expression of GFP in mouse NK cells *in vitro* without affecting NK cell phenotype and function (29). However, there remains a risk in using viral-based transduction *in vivo*, highlighting the need for future experiments investigating whether the use of lentiviral vectors can increase antitumor effects in humans and avoid associated autoimmunity.

One of the limitations in this study is that BALB/C mice possess endogenous NK cells, which may have competed for important growth factors and impacted both the persistence and activity of genetically modified NK-92 cells. In future studies, it would be advantageous to investigate the role of genetically modified NK-92 cells in mice that lack both T lymphocytes and NK cells.

In conclusion, this study reported the generation of a eukaryotic expression vector encoding chimeric antigen receptor scFv anti-erbB2-CD28-ζ. This study achieved a high level of expression of the receptor in NK-92 cells, and demonstrated that adoptive transfer of these cells could mediate antigen-specific tumor inhibition *in vivo*. These genetically modified NK-92 cells were specifically reactive against erbB2-expressing tumor cells and were able to infiltrate tumor tissues specifically to inhibit tumor growth. Overall, the results of this study highlight that use of genetically modified NK-92 cells is a novel and exciting prospect for cancer immunotherapy.

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