Bortezomib improves adoptive carbonic anhydrase IX-specific chimeric antigen receptor-modified NK92 cell therapy in mouse models of human renal cell carcinoma

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Abstract. Adoptive chimeric antigen receptor (CAR) T or NK cells offer new options for cancer treatment. Clinical results indicate that CAR-modified T cell (CAR-T) therapy has curative therapeutic efficacy for hematological malignancies. However, the efficacy of the therapy in most solid tumors, including advanced renal cell carcinoma (RCC), remains highly limited. New regimens, including combination of CAR-T cells with chemical drugs, must be studied to enhance the therapeutic efficacy of CAR-T or NK cells for solid tumors. In the present study, a carbonic anhydrase IX (CAIX)-specific third-generation CAR was transduced into NK92 cells by lentiviral vectors. The immune effects, including cytokine release and cytotoxicity, of the CAR-NK92 cells against CAIX-positive RCC cells were evaluated *in vitro*. Combination therapeutic effects of bortezomib and CAR-NK92 cells were analyzed in a mouse model with human RCC xenografts. The results revealed that CAIX-specific CAR-NK92 cells specifically recognized *in vitro* cultured CAIX-positive RCC cells and released cytokines, including IFN-γ, perforin and granzyme B, and exhibited specific cytotoxicity. The cytotoxicity of the CAR-NK92 cells was enhanced after treating RCC cells with bortezomib *in vitro*. The suppressive efficacy of bortezomib combined with CAR-NK92 cells against established CAIX-positive tumor xenografts was more significant than that of the monotherapy with either CAR-NK92 cells or bortezomib. Therefore, bortezomib can enhance the effects of the CAR-NK92 cells against RCC *in vitro* and *in vivo*. This study provided an experimental basis for the novel clinical regimen of CAIX-specific CAR-modified NK or T cells for the treatment of RCC.

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Introduction

Treatment options remain very limited for patients with advanced renal cell carcinoma (RCC). Metastasis and recurrence occur in 20-30% of patients who have received radical resection. This disease is resistant to chemotherapy and/or radiotherapy, and the median survival is only 7-10 months (1). In recent years, small-molecule targeted therapy, including tyrosine kinase inhibitors (TKIs), have become the first-line treatment for advanced RCC, though prognosis remains poor (2). However, RCC appears to be sensitive to immunotherapy (3,4).

The chimeric antigen receptor (CAR) modified T-cell (CAR-T) therapy is a newly developed adoptive treatment of cancers. A CAR is a recombinant receptor construct composed of an extracellular single-chain variable fragment (scFv) derived from an antibody, a hinge and transmembrane domain, a costimulation signaling domain (CD28 or CD137) and a CD3ζ signaling domain to provide T-cell activation signals (5). The CAR thereby redirects T-cell specificity to the tumor in a major histocompatibility complex (MHC)-independent manner (6). Currently, CAR-T therapy has shown potential for curative therapeutic efficacy in patients with hematological malignancies, particularly for treating B-cell malignancies with CD19-specific CAR-T cells (7,8). Associated CAR-T products, CTL019 (Novartis, Basel, Switzerland) and Yescarta (KTE-C19; Gilead, Foster City, CA, USA), have been approved in the past few months to treat patients with

B-cell malignancies. However, the therapeutic efficacy of CAR-T cells in most solid tumors, including RCC, remains less impressive (9). Weijtens *et al* designed a first-generation CAR (scFv-FcRγ) directed against carbonic anhydrase IX (CAIX), an RCC antigen, and used the CAR-modified T cells to treat patients with CAIX-expressing metastatic RCC (10,11). Although the blood cytokine profiles mirrored CAR-T cell presence and *in vivo* activity, no clinically objective responses were observed in any of the 12 patients.

Natural killer (NK) cells are effector cells of the innate immune system that are capable of killing tumor cells, as well as producing cytokines without previous stimulation (12). The efficacy of autologous NK cells in the immunotherapy of solid tumors was evaluated in various clinical settings (13-15). Since autologous NK cells may be functionally inhibited by the interaction between self-HLA class I molecules on tumor cells and inhibitory NK receptors, and because allogeneic NK cells do not attack non-hematopoietic tissues in the recipient, the adoptive transfer of allogeneic NK cells may represent a better approach (12). Over the past decade, adoptive transfer of *ex vivo*-activated or *ex vivo*-expanded allogeneic NK cells has emerged as a promising immunotherapeutic strategy for cancers (16-18). Given the potent cytolytic function of these cells and the shorter lifespan, mature NK cells are considered as attractive candidate effector cells to express CARs for the therapy of patients with cancers. Primary human NK cells have been successfully engineered to express CARs against a number of targets, including CD244 (19), GD2 (20) and EGFR (21).

NK92, a human NK-like cell line, was derived from the peripheral blood of a female patient with non-Hodgkin's lymphoma and is an IL-2 dependent immortalized cell line (22,23). The general safety of infused NK92 cells has been established in phase I clinical trials with clinical responses observed in 11 treated renal cancer patients (24). The adoptive transfer of CAR-NK92 cells has several theoretical advantages over the use of patient- or donor-derived T cells or NK cells. The advantages are primarily related to the lack of expression of inhibitory killer Ig-like receptors (iKIRs), presumed lack of immunogenicity and graft-vs.-host disease (GVHD), ease of expansion and availability as an 'off-the-shelf' product, which can greatly reduce the treatment cycle and cost of treatment.

However, NK92 also has obvious disadvantages, such as tumorigenicity and the potential susceptibility to the EB virus. Therefore, as a safety consideration, NK92 must be irradiated before its clinical use. Researchers, such as Tam *et al* and Tonn *et al*, have studied the safety of NK92 cells for adoptive immunotherapy (25,26). Their studies indicated that after the proper amount of γ-ray irradiation, NK92 cells could not only be safe for clinical application but could also maintain their cytotoxicity for a certain period. Currently, NK92 cells have been successfully engineered to express CARs against a number of targets, including CD19 (27), CD20 (28), Her2 (29,30), GD2 (31), EpCAM (32), CS1 (33), CD138 (34), EGFR (35-37), CD3 (38) and CD5 (39). CAR-transduced NK (CAR-NK) cells exhibit efficient *in vitro* and *in vivo* tumor cell killing ability, although no clinical data from CAR-NK cell therapy have been reported to date (40).

Results of current clinical trials have demonstrated that mono-therapy with CAR-T cells is not an effective strategy for solid tumors. The combination of CAR-T cells with chemotherapeutic drugs may be a promising approach to enhance therapeutic efficacy (41). Bortezomib (Velcade; PS-341), which binds the catalytic site of the 26S proteasome, was the first proteasome inhibitor brought into clinical use, inhibiting the chymotrypsin-like and, to a lesser extent, the trypsin-like and postglutamyl peptide-hydrolyzing activities (42). The proteasome pathway plays an important role in cellular homeostasis by degrading misfolded or deleterious proteins to maintain normal cellular physiology (43-45). The proteasome system of malignant cells is usually overloaded by the accumulation of defective proteins (44,45). Bortezomib was the first FDA-approved proteasome inhibitor for the treatment of relapsed/refractory multiple myeloma disease due to its impressive clinical activity (44-46). However, the therapeutic efficacy in solid tumors, including RCC, was limited when bortezomib was used as a single agent (47,48). New data have revealed that bortezomib could be an attractive candidate for combination with antitumor natural killer cell and/or T-cell adoptive therapy due to its intrinsic ability to increase the levels of immunostimulatory cytokines and components of the Notch and NFκB signaling pathways in these lymphocytes (49,50).

In the present study, we constructed a third-generation CAR against CAIX, which consisted of an anti-CAIX scFv, CD8 hinge and transmembrane regions, and intracellular signal domains of CD28, CD137 and CD3ζ. The efficacy of combination therapy with the CAIX-specific CAR-modified NK-92 (CAIX-CAR-NK92) cells and bortezomib against RCC were investigated *in vitro* and in a mouse model of human RCC. Our results indicated that the combination of CAIX-CAR-NK92 cells with bortezomib is a promising strategy to treat RCC.

Materials and methods

Cell culture. NK92 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). NK92 cells and transduced NK92 cells were incubated in α-modification of Eagle's minimum essential medium (α-MEM; Gibco; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 2 mM L-glutamine, 0.2 mM Myo-inositol, 0.02 mM folic acid, 0.1 mM 2-mercaptoethanol, 400 IU/ml IL-2 (PeproTech, Inc., Rocky Hill, NJ, USA), 12.5% fetal bovine serum (FBS) and 12.5% horse serum (Gibco, Life Technologies; Thermo Fisher Scientific, Inc.), 1/100 penicillin/streptomycin (Gibco, Life Technologies; Thermo Fisher Scientific, Inc.). The human renal cancer cell line OSRC-2 was cultured in RPMI-1640 medium (Gibco, Life Technologies), and the human renal cancer cell lines ACHN, Ketr-3 and human embryonic kidney cells 293 were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Life Technologies; Thermo Fisher Scientific, Inc.). RPMI-1640 and DMEM medium were supplemented with 10% FBS and 1/100 penicillin/streptomycin when used. All of the cell lines were cultured at 37˚C in a humidified atmosphere with 5% CO_2. Cells in the logarithmic growth phase were used for all experiments.

Construction of CAIX-specific CAR and lentivirus preparation. The CAIX-CAR construct (LV5-scFv-CD8-CD28-CD137-CD3ζ) was composed of anti-CAIX single

chain variable fragment (scFv, G250), CD8 (138-208 aa), CD28 (180-220 aa), 4-1BB (214-255 aa) and CD3ζ (52-164 aa) domains. The encoding sequence of the CAR was inserted into a lentiviral vector designated LV5-EF1a-Puro (Shanghai GenePharma Co., Ltd., Shanghai, China). The methods for lentivirus preparation were the same as in our previous study (51).

Transduction of NK92 cells. For lentivirus infection, wells of 24-well plates were coated with RetroNectin (Takara Bio, Inc., Shiga, Otsu) (1 ml of 25 μg/ml in PBS) overnight at 4˚C. RetroNectin was later removed, and the wells were blocked (30 min, room temperature) with sterile-filtered PBS containing 2% BSA and washed with PBS. NK92 cells were adjusted to $2x10^5$ cells/ml using α-MEM with IL-2 and 2-mercaptoethanol. Next, the cells were loaded in the RetroNectin-precoated 24-well plates followed by the addition of lentivirus at an MOI of 50 in the presence of 5 μg/ml Polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and they were centrifuged (2 h, 380 x g, at 32˚C) and incubated for 24 h. Next, the cells were transferred to uncoated wells with fresh complete medium and cultured for 12 h. Following this step, the infection protocol was repeated. Starting from day 3 after the second infection, transfected NK92 cells were repeatedly selected by puromycin (Sigma-Aldrich; Merck KGaA) with a final concentration of 1 μg/ml every two weeks in order to establish a stable transfected NK92 cell line. Control lentivirus (empty vector with a puromycin select gene) transfected NK92 cells (Ctrl-NK92) were also selected with puromycin.

FACS analysis. For CAR detection, $0.5x10^6$ Ctrl-NK92 and CAR-NK92 cells were washed once with FACS buffer (2% FBS in PBS) and were blocked with 1:100 diluted goat serum in PBS at 4˚C for 1 h. The blocked cells were resuspended in 50 μl FACS buffer containing 2.5 μl Alexa Fluor 647-conjugated AffiniPure $F(ab')_2$ Fragment Goat Anti-mouse IgG, $F(ab')_2$ Fragment-Specific antibody (dilution 1:200; cat. no. 115-606-072; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and were incubated at 4˚C for 1 h. The cells were then washed, suspended and evaluated with a FACS machine (FACSCanto II; BD Biosciences, Franklin Lakes, NJ, USA). Unstained NK92 cells were used as the control. The data were analyzed with FlowJo software (7.6.1 version; FlowJo LLC, Ashland, OR, USA).

For the membrane surface expression of CAIX in renal cancer cell lines, $0.5x10^6$ cells were suspended in 50 μl FACS buffer containing 2 μl FITC-conjugated mouse anti-human CAIX antibody (dilution 1:10; cat. no. FAB2188F; R&D Systems, Inc., Minneapolis, MN, USA) and incubated at 4˚C for 30 min. A FITC-conjugated mouse IgG2A isotype antibody (dilution 1:10; cat. no. IC003F R&D Systems, Inc.) was used as the control. FACS machine detection and data analysis were aforementioned.

Western blot analysis. In total, $1x10^6$ cells were lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich; Merck KGaA). The protein concentration was determined with the BCA method. The mass of protein loaded per lane was 60 μg. Total proteins were separated on a 10% SDS polyacrylamide gel (SDS-PAGE). The proteins were subsequently transferred to nitrocellulose membranes and incubated with a primary rabbit anti-human CD3ζ antibody (dilution 1:1,000; cat. no. ab40804; Abcam, Cambridge, MA, USA) or rabbit anti-human GAPDH antibody (dilution 1:1,000; cat. no. GTX627408; GeneTex, Inc., Irvine, CA, USA) overnight at 4˚C. The membranes were washed and incubated with a goat anti-rabbit IgG (H+L) secondary antibody (dilution 1:10,000; cat. no. VA001; Vicmed Life Sciences, Xuzhou, China) at room temperature for 1 h. The blots were visualized using an enhanced chemiluminescence (ECL) detection kit (Beyotime Biotechnology).

Cytokine release assay. Effector cells were co-cultured with $1x10^4$ target cells at an effector-to-target (E:T) ratio of 1:1 in a final volume of 200 μl of α-MEM complete media in triplicate sets of wells in round-bottom 96-well culture plates. After 24 h, supernatants were assayed for the presence of IFN-γ, perforin and granzyme B by ELISA using human IFN-γ ELISA kits and human perforin ELISA kits (both from Dakewe Biotech Co., Ltd., Shenzhen, China) and human granzyme B ELISA kits (BioLegend, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Cytotoxicity assay. The LDH release assays were performed with the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Briefly, effector cells were incubated with $1x10^4$ target cells at effector-to-target (E:T) ratios of 30:1, 10:1, 3:1, or 1:1 in a final volume of 100 μl of RPMI-1640 medium with 5% FBS in round-bottom 96-well culture plates at 37˚C for 4 h. The test was performed in three replicates for each E/T ratio. The CAR-NK92 cells used in these assays were compared with empty vector-transduced NK92 cells (Ctrl-NK92) in the assay. Target cell cytotoxicity was calculated using the following formula: % Cytotoxicity=100x[(experimental-effector spontaneous-target spontaneous)/(target maximum-target spontaneous)].

In vivo efficacy studies. All protocols for the animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Jiangsu Provincial Academy of Chinese Medicine (SCXK2012-005). Six-week-old female NOD/SCID mice were purchased from Beijing Huafukang Bioscience, Co., Inc. (Beijing, China). The housing conditions of the mice included a constant temperature of 28˚C and ventilation was required 10-15 times per hour. Every day 10 h of illumination and 14 h of darkness were maintained and food and water were sterilized by high pressure steam. Each mouse was inoculated subcutaneously with $3x10^6$ Ketr-3^{luc+} cells (Ketr-3 cells expressing luciferase) in the right flank on day 0. Next, the mice were randomly assigned to six groups, including the untreated group, the bortezomib group, the Ctrl-NK92 group, the CAR-NK92 group and the CAR-NK92+bortezomib group. Bortezomib (5 μg/mouse in DMSO; Selleck Chemicals, Houston, TX, USA) was injected 5 days after the tumor injection (day 5), and 24 h later, the mice in the associated groups were infused with $2.5x10^6$ Ctrl-NK92 cells or CAR-NK92 cells. From the day of the NK92 cell infusion, all mice were administered 2000 IU of IL-2 daily

by intraperitoneal (i.p.) injection for 60 days. The bortezomib/NK92 cell treatment cycle was repeated once a week until the experiment was terminated. During the treatment, the mice were monitored frequently for subcutaneous xenograft progression. The tumors were assessed with a caliper at their greatest length and width twice a week to estimate the tumor volume after the tumors had grown to a palpable size. The tumor volume was calculated using the following formula: Tumor volume = length x $(width)^2/2$.

On day 72, the experiment was terminated. Before the mice were humanely sacrificed by cervical dislocations, the mice bearing Ketr-3^{luc+} tumors were infused intraperitoneally with D-luciferin (150 mg/kg; Berthod Technologies GmbH & Co. KG, Bad Wildbad, Germany), anesthetized with 1% pentobarbital (50 mg/kg; Beyotime Institute of Biotechnology) by intraperitoneal (i.p.) injection, and imaged using an *in vivo* imaging system (Berthold Technologies GmbH & Co. KG).

Immunohistochemical analysis. The tumor tissues were fixed in 4% paraformaldehyde, decalcified in saturated EDTA, and embedded in paraffin. The embedded tumor tissues were cut into 3-5-μm thick sections. The sections were stained with rabbit anti-human CD3ζ monoclonal antibody (dilution 1:500; cat. no. ab40804; Abcam), and HRP-conjugated anti-rabbit IgG (cat. no. SPN9001; ZSGB-BIO; OriGene Technologies, Inc. Rockville, MD, USA) was used as a secondary antibody followed by a peroxidase enzymatic reaction. Tissues were visualized with diaminobenzidine (DAB; ZSGB-BIO; OriGene Technologies, Inc.) and counterstained with hematoxylin. For the quantification of infiltrated NK92 cells into the tumors, NK92 cells were counted in 10 randomly selected intratumoral fields of each slide at an x200 magnification.

Statistical analysis. All statistical analyses were performed with Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis for the comparison of two groups was performed using unpaired t-tests. For comparison of more than two groups, one-way analysis of variance (ANOVA) was used, and multiple comparisons between the groups were performed using the S-N-K tests. For the bioluminescence results, the signal intensity was log-transformed and compared by two-tailed Student's t-test. P-values <0.05 were considered indicate a statistically significant difference.

Results

Construction and expression of CAIX-specific CAR. To generate a third-generation CAR specific to CAIX, a single chain variable fragment (scFv) from an anti-CAIX monoclonal antibody (G250) was constructed by linking the heavy chain variable region and light chain variable region with a 218 linker (GSTSGSGKPGSGEGSTKG). This anti-CAIX scFv was fused with the CD8 hinge and transmembrane domains, and CD28, 4-1BB costimulatory signaling domains, followed by a CD3ζ activation domain to create a CAIX-specific CAR (CAIX-CAR, Fig. 1A). We prepared the CAIX-CAR-modified NK92 cells (CAIX-CAR-NK92) and control NK92 cells (Ctrl-NK92) by infecting human NK92 cells with a lentivirus containing the encoding sequence of CAIX-CAR and a control lentivirus (empty vector), respectively.

Since the lentivirus vector contains a puromycin selection marker, we generated stable CAIX-CAR-NK92 and Ctrl-NK92 cell lines through repeated selection of the infected NK92 cells with puromycin. After selection for two weeks, the surface expression of CAIX-CAR of the infected NK92 cells was detected by FACS analysis with an Alexa Fluor 647-conjugated goat $F(ab')_2$ antibody reacting with the $F(ab')_2$/Fab portion of mouse IgG. Uninfected NK92 cells were used as the negative control (Fig. 1B). To further examine CAIX-CAR expression in NK92 cells, we performed western blot analysis using a rabbit anti-human CD3ζ monoclonal antibody that recognized the ζ chain portion of human CD3. As expected, CAIX-CAR was only detected in CAIX-CAR-NK92 cells (Fig. 1C).

Cytokine release of CAIX-CAR-NK92 cells in vitro. To identify the potential target cell lines of CAIX-CAR-NK92 cells for *in vitro* and *in vivo* experiments, CAIX expression on the surface of renal cancer cells was determined. As shown in Fig. 2A, CAIX was highly expressed on the surface of human renal cancer Ketr-3 and OSRC-2 cell lines, whereas its expression on the surface of the human renal cancer cell line ACHN was low, and there was no expression on the surface of 293 cells.

To examine whether CAR-NK92 cells specifically recognized target cells and elicited specific effector cell functions, Ctrl-NK92 and CAR-NK92 cells were co-cultured with Ketr-3, OSRC-2, ACHN or 293 cell lines for 24 h. Next, released cytokines, such as INF-γ, perforin, and granzyme B, which play an important role in mediating adoptive immunotherapy efficacy, were detected by ELISA in the cell culture supernatant. As revealed in Fig. 2B, compared with Ctrl-NK92 cells, CAR-NK92 cells mediated robust cytokine secretion when co-cultured with CAIX-positive tumor cell lines. However, the cytokine release of CAR-NK92 cells and Ctrl-NK92 cells were not significantly different for CAIX-negative 293 cells. These results indicated that CAR-NK92 cells specifically recognized CAIX-positive target cells and elicited effector cell functions.

Cytotoxicity of CAIX-CAR-NK92 cells against CAIX-positive renal cancer cells in vitro. To further confirm whether CAIX-CAR-NK92 cells specifically recognized CAIX-positive renal cancer cells and mediated specific cytotoxicity, dose-dependent lactate dehydrogenase (LDH) release assays were performed. CAIX-CAR-NK92 cells and Ctrl-NK92 cells were co-cultured with Ketr-3, OSRC-2, ACHN or 293 cells for 4 h. LDH released into the cell culture supernatant was detected. As revealed in Fig. 3, compared with the Ctrl-NK92 cells, CAIX-CAR-NK92 cells exhibited stronger killing activity against CAIX-positive cancer cells Ketr-3, OSRC-2 and ACHN. However, the difference in cytotoxicity between the Ctrl-NK92 and the CAIX-CAR-NK92 cells against 293 cells was not significant. Additionally, the cytotoxicity of CAIX-CAR-NK92 cells against CAIX-positive tumor cells was positively correlated with the E:T ratios (Fig. 3). These data revealed that CAIX-CAR NK92 cells mediated specific killing against CAIX-positive renal cancer cells.

Pretreatment with bortezomib increases the sensitivity of renal cancer cells to CAIX-CAR-NK92 and NK92 cells

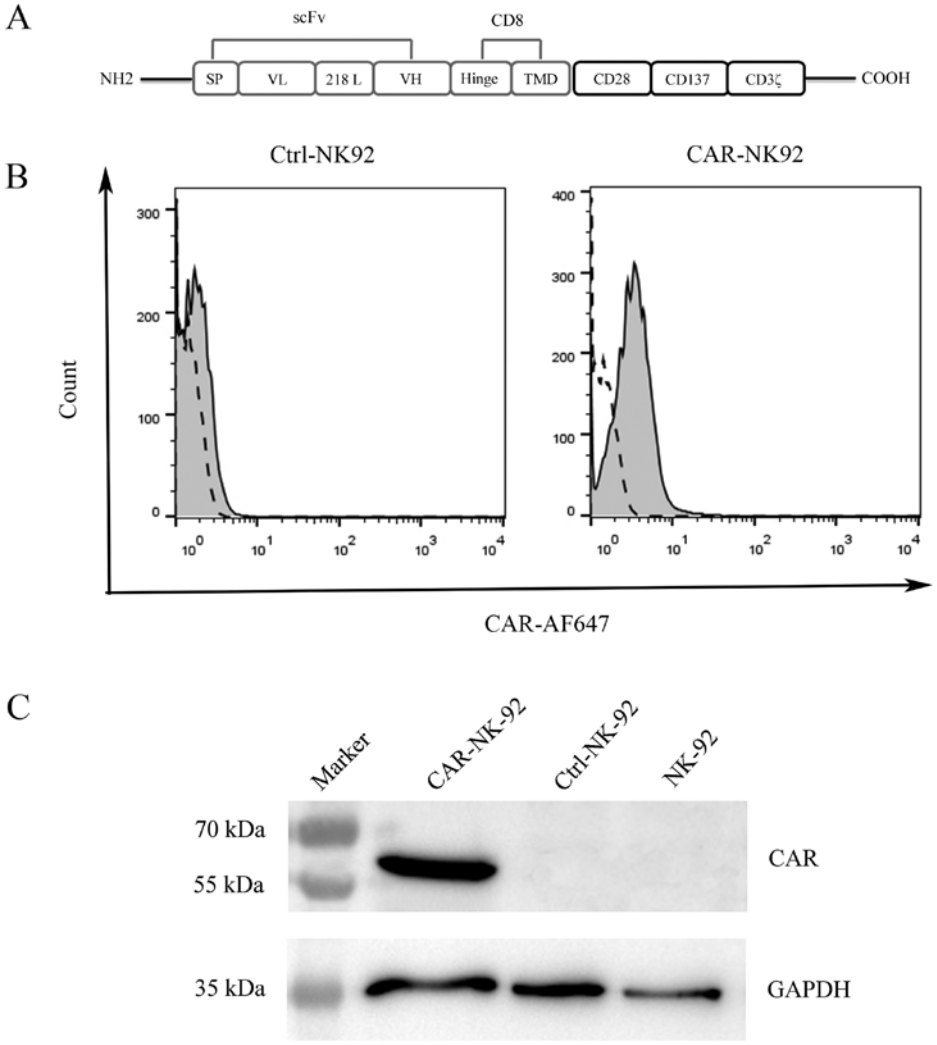

Figure 1. Generation and characterization of CAIX-specific CAR-NK92 cells. (A) Structure diagram of CAIX-specific CAR. SP, signal peptide; scFv, single-chain variable fragment; 218 L, 218 linker; TMD, transmembrane domain. (B) CAR-positive rate of lentivirus-transduced NK92 cells. NK92 cells were transduced with empty lentivirus vector (Ctrl-NK92) or lentivirus containing the CAIX-specific CAR encoding sequence (CAR-NK92) and were selected with puromycin. Cell surface expression of CAR was verified by FACS analysis. (C) Western blot analysis of the CAR expression in NK92 cells with a monoclonal anti-human CD3ζ antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also detected as an internal control. CAR, chimeric antigen receptor.

in vitro. Previous studies revealed that low-dose bortezomib sensitized human renal cancer cells to NK cells via the upregulation of DNAM-1 and NKG2D ligands (52,53), upregulation of DR4, and downregulation of HLA class I (54). To determine whether bortezomib treatment could increase the sensitivity of renal cancer cells to CAIX-CAR-NK92 or NK92 cell-mediated lysis, we treated Ketr-3 and OSRC-2 cells with 10 nM bortezomib for 24 h (55). Next, we detected the killing activity of the CAR-NK92 or NK92 cell lines respectively to the bortezomib-treated Ketr-3 and OSRC-2 cells. The results revealed that bortezomib treatment significantly increased the sensitivity of Ketr-3 and OSRC-2 cells to the CAIX-specific CAR-NK92 cells and NK92 cells and the synergistic efficacy was antigen independent (Fig. 4).

Combination with bortezomib improves the antitumor activity of CAIX-specific CAR-NK92 cells against RCC xenografts. To examine the efficacy of combination therapy with CAIX-CAR-NK92 cells and bortezomib against CAIX-positive RCC, we established a subcutaneous xenograft model in NOD/SCID mice using the human renal cancer line Ketr-3 cells expressing luciferase (Ketr-3^{luc+}). Next, the tumor-bearing mice were treated with CAR-NK92 cells by tail vein injection and bortezomib by peritoneal cavity injection. The treatment program is displayed in Fig. 5A.

As shown in Fig. 5, both the results of the tumor growth curve (Fig. 5B) and *in vivo* imaging (Fig. 5C and D) revealed that Ctrl-NK92 cells, CAR-NK92 cells or bortezomib treatment alone significantly reduced the growth of Ketr-3^{luc+} tumors compared to the untreated group. In addition, treatment with CAIX-CAR-NK92 cells significantly suppressed tumor growth compared with the Ctrl-NK92 cells. These results demonstrated that CAIX-CAR-NK92 cells can specifically kill CAIX-positive renal cancer cells *in vivo*. Furthermore, the combination of CAIX-CAR-NK92 cells and bortezomib significantly reduced the growth of Ketr-3^{luc+} tumors compared to bortezomib or CAIX-CAR-NK92 cells alone. The tumors of the mice that received CAIX-CAR-NK92 cells plus bortezomib treatment were almost eradicated. The values of the tumor volumes were concordant with those of the *in vivo* imaging.

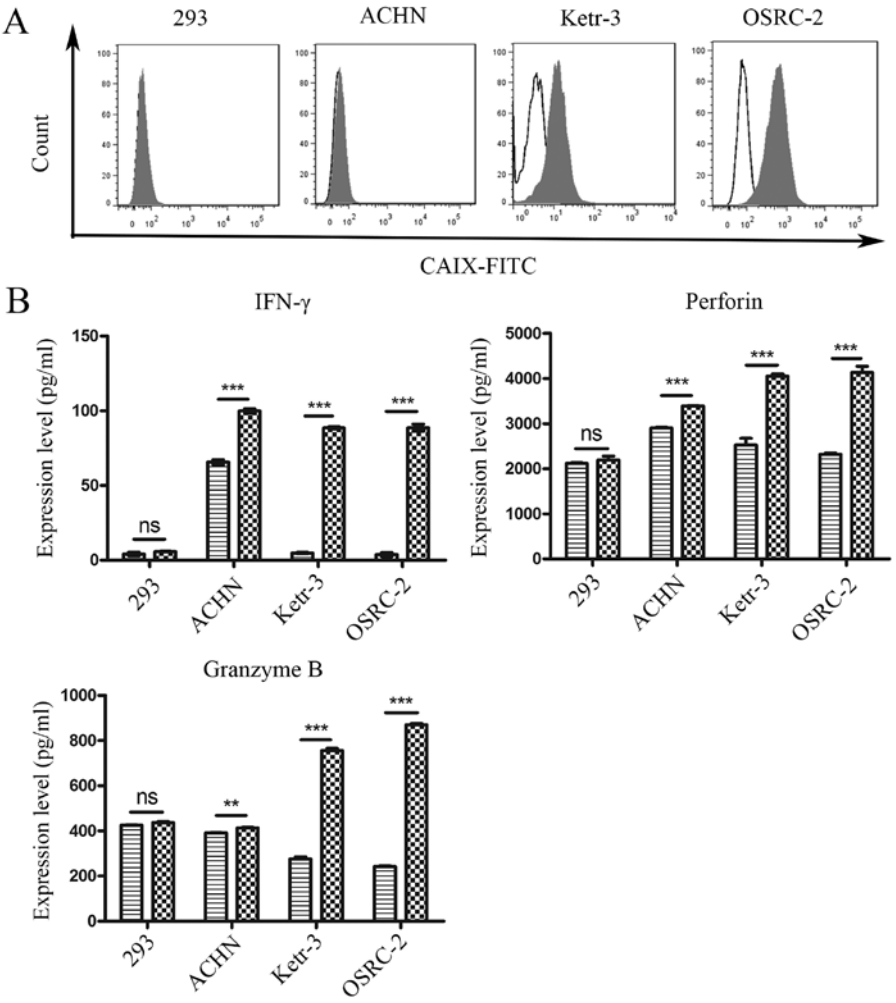

Figure 2. Specific cytokine release of CAIX-specific CAR-NK92 cells against CAIX$^+$ cells. (A) FACS was used to assess the surface expression of CAIX proteins in human renal cancer ACHN, Ketr-3, and OSRC-2 cell lines and in the 293 cells. (B) The levels of cytokines, released by Ctrl-NK92 and CAR-NK92 cells, were assessed by enzyme-linked immunosorbent assay (ELISA) after 24 h of incubation with CAIX$^-$ or CAIX$^+$ target cells at an effector-to-target (E/T) ratio of 1:1. **P<0.01; ***P<0.001. CAR, chimeric antigen receptor; ns, not significant.

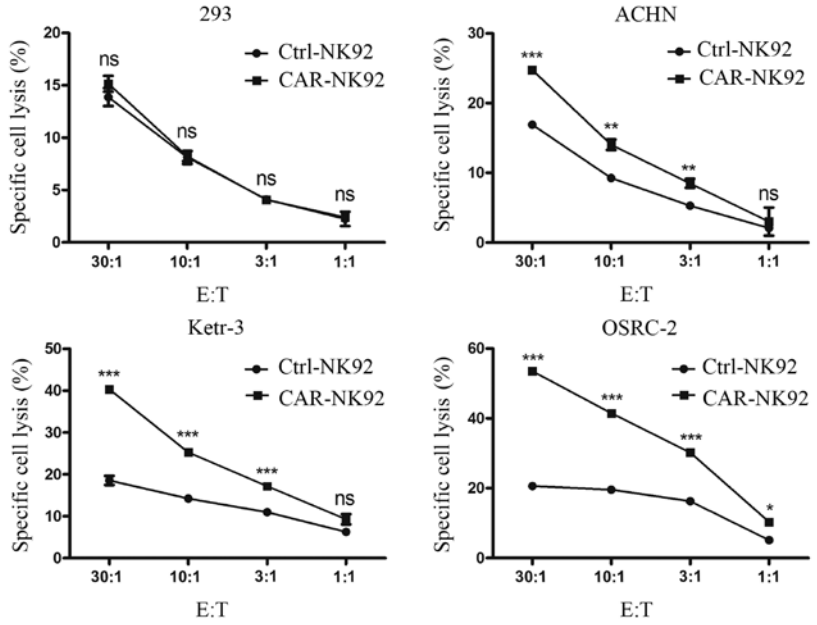

Figure 3. Specific cytotoxicity exhibited by CAIX-specific CAR-NK92 cells against CAIX$^+$ target cells. The cytotoxic activity of CAR-NK92 and Ctrl-NK92 cells against CAIX$^-$ or CAIX$^+$ cancer cells was determined using LDH release assay after a 4-h incubation with CAIX$^-$ or CAIX$^+$ target cells at an E/T ratio of 1:1, 3:1, 10:1 and 30:1. *P<0.05; **P<0.01; ***P<0.001. CAR, chimeric antigen receptor; LDH, lactate dehydrogenase; E/T, effector-to-target; ns, not significant.

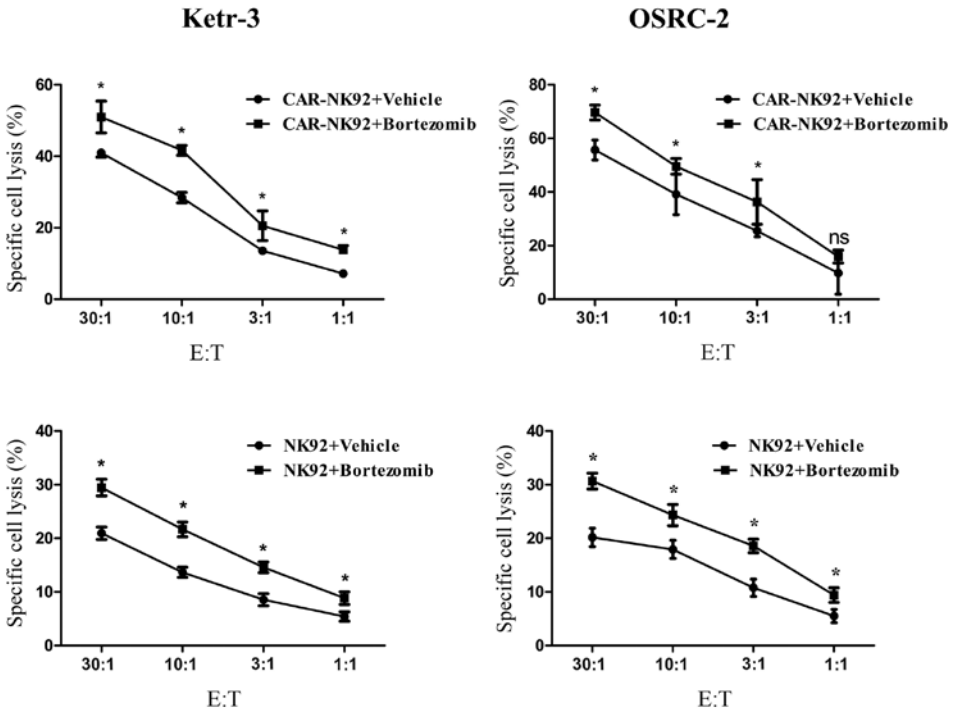

Figure 4. Bortezomib increases the sensitivity of renal cancer cells to CAIX-specific CAR-NK92 or NK92 cell-mediated killing. Ketr-3 and OSRC-2 cells were treated with 10 nM bortezomib or vehicle (DMSO) for 24 h and were then co-incubated with the CAR-NK92 or NK92 cells for 4 h. The cytotoxic activity of CAR-NK92 or NK92 cells was determined by LDH release assay. *P<0.05. CAR, chimeric antigen receptor; LDH, lactate dehydrogenase; ns, not significant.

Detection of NK92 cell infiltration in tumor tissues. Both our western blotting data (not shown) and a previous study revealed that wild-type NK92 cells expressed CD3ζ (28). Therefore, we used an anti-human CD3ζ antibody to detect infiltrated NK92 cells in tumor tissues by immunohistochemical (IHC) staining. As shown in Fig. 6A, CD3ζ-positive NK92 cells in the tumor tissue of Ctrl-NK92, CAR-NK92 and CAR-NK92+bortezomib groups were stained brown. However, no positive cells were observed in the tumor tissues of the untreated and bortezomib groups. The statistical results revealed that the number of NK92 cells in the tumors of the CAR-NK92 group was significantly greater than in the tumors of the Ctrl-NK92 group (P<0.001; Fig. 6B). The number of NK92 cells in the tumor tissues of the CAR-NK92+bortezomib group was larger than in the tumor tissues of the CAR-NK92 group. However, the difference was comparable (Fig. 6B). These findings indicated that CAIX-specific CAR-NK92 cells can traffic to tumor sites.

Discussion

NK cells are a type of immune effector cells that play important roles in immune surveillance. Numerous NK cell-based anticancer therapies, including CAR-NK92 cells, are currently under investigation. In the present study, for the first time to the best of our knowledge, we constructed a third-generation CAR against CAIX and developed the CAR-modified NK92 cell line. The CAR-NK92 cells exhibited robust CAIX-specific killing effects against CAIX-positive renal cancer cells. In addition, we reported, for the first time, that CAIX-CAR-NK92 cells exhibited synergistic therapeutic efficacy with bortezomib against RCC in a mouse model.

Early in 1996, Weijtens *et al* constructed a first-generation CAR against CAIX, which was composed of a mouse anti-human CAIX scFv and the Fc(epsilon)RI signaling receptor gamma-chain (FcRγ) of mast cells, and they confirmed that introduction of this CAR into cytotoxic T lymphocytes (CTLs) rendered these lymphocytes specific for RCC (11). No clinical objective responses have been observed in the clinical trials of this CAR (10). The CAR that we constructed is a third-generation CAR with two costimulatory molecules, CD28 and CD137. According to previous studies, incorporation of the CD28 signaling domain can enhance the proliferation and cytotoxicity of CAR-T cells (56), and the CD137 signaling domain can enhance the persistence of CAR-T cells compared with first generation CARs containing only one costimulatory molecule (57).

In the *in vivo* studies, we confirmed that bortezomib and CAIX-CAR-NK92 cells exhibited synergistic effects by suppressing the growth of human RCC xenografts in NOD/SCID mice (Fig. 5). We then investigated the potential mechanisms. We revealed that the NK cells infiltrated into tumor tissue, and that NK cells in the tumors of mice that received treatment with CAR-NK92 cells and bortezomib were comparable with those in the tumors of mice that received treatment with CAR-NK92 alone (Fig. 6). Therefore, bortezomib promoted the antitumor effects of CAR-NK92 but not by enhancing the infiltrating ability of CAR-NK92 cells into tumors.

Bortezomib was reported to downregulate the cell-surface expression of human lymphocyte antigen (HLA) class I and to enhance natural killer cell-mediated lysis of myeloma (54). Bortezomib was also reported to enhance NK cell-mediated antitumor effects by upregulating the expression of MICA/B,

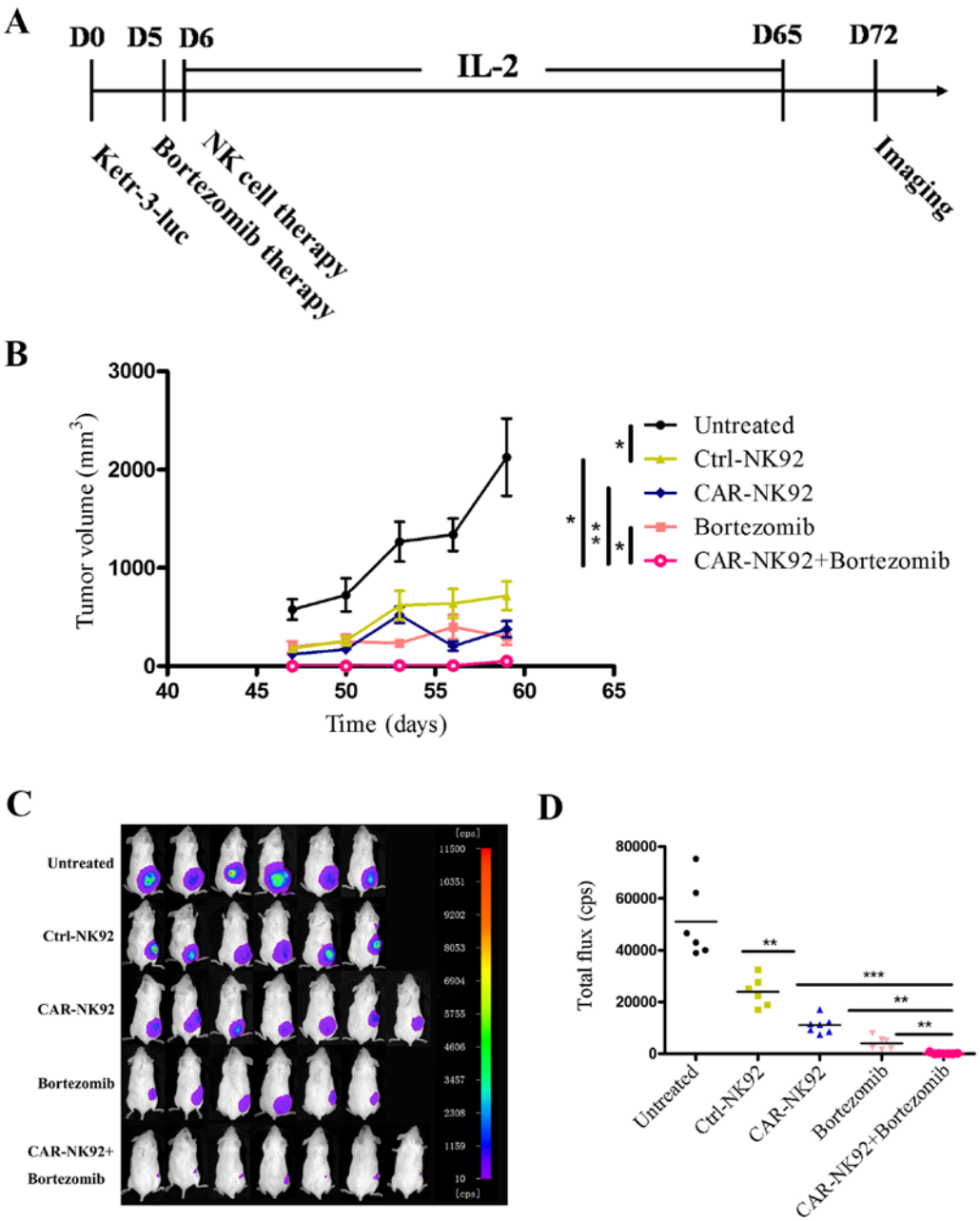

Figure 5. Therapeutic efficacy of CAIX-specific CAR-NK92 cells combined with bortezomib for human renal cancer xenografts established with Ketr-3^{Luc+} cells. (A) Schematic diagram revealing the treatment program of the mice. (B) The tumor growth curves during the experiment. (C) Luminescence images revealing the tumor size at the end of the treatment. (D) Quantitative results of the tumor luminescence intensity shown in C. *P<0.05; **P<0.01; ***P<0.001. CAR, chimeric antigen receptor.

the ligands of NKG2D, and DR5, the receptor of TRAIL in cancer cells (52,53). Bortezomib can also enhance NK cell-mediated antitumor effects by upregulating the expression of NK-cell activating receptors NKG2D, TRAIL, and DNAM-1 in NK cells treated with bortezomib (52,58,59). To investigate whether bortezomib enhanced the antitumor efficacy of CAIX-CAR-NK92 cells by changing the expression of these molecules in cancer cells or NK92 cells, we detected the expression changes of CA9, Fas, MicA/B, HLA-A2, and DR4 in the RCC cell lines Ketr-3, OSRC-2, ACHN, DNAM-1, NKG2D and NKp46 TRAIL in NK92 cells after treatment with bortezomib at concentrations of 0, 5, 10 and 20 nM for 24 h. However, in general, these molecules did not significantly change (data not shown). Therefore, the mechanism by which bortezomib enhanced the therapeutic efficacy of CAIX-CAR-NK92 cells may not be by changing the expression of these molecules in cancer cells or NK92 cells.

New data has revealed that besides the established role of bortezomib in sensitizing tumors to cell death, it may act as a multifaceted immunomodulatory drug and can sustain antitumor immune effector functions and address tumor-associated immunosuppression (49). Growing evidence indicates that bortezomib administered at 15-20 nM doses exhibited an intrinsic ability to increase the levels of immunostimulatory cytokines and components of the Notch and NFκB signaling pathways in lymphocytes, thereby amplifying their effector functions either directly or indirectly (50,60,61). Bortezomib also affected cytokine production in the tumor

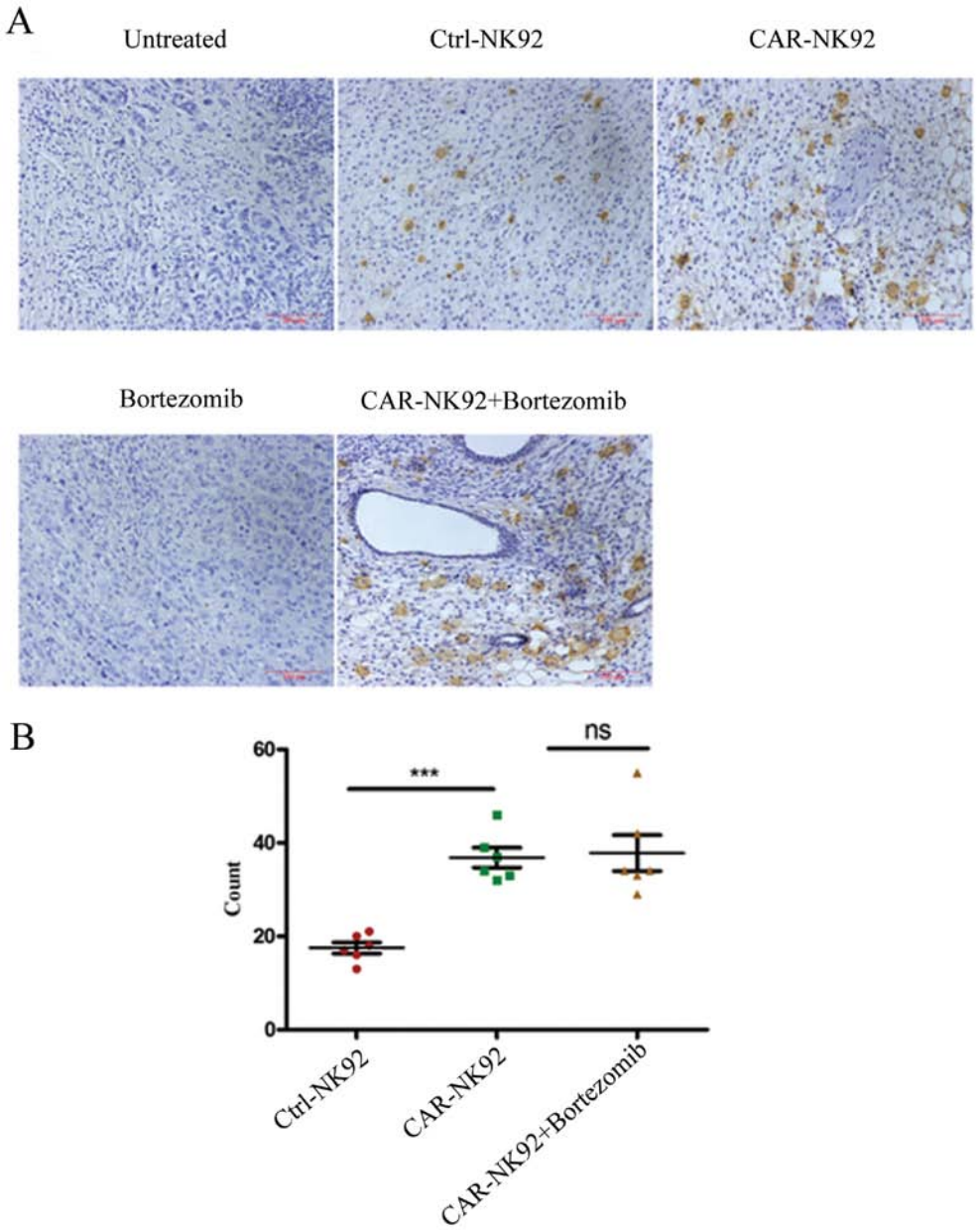

Figure 6. Tumor infiltration analysis of NK92 cells *in vivo*. (A) Immunohistochemical analysis of NK92 cells in established s.c. xenografts. NK92 cells were labeled with a rabbit anti-human CD3ζ monoclonal antibody and an HRP-conjugated anti-rabbit IgG antibody. The images were obtained at an x200 magnification. (B) The corresponding quantitative analysis results of NK92 cells shown in B. ***P<0.001. ns, not significant.

microenvironment, thereby polarizing the cytokine milieu in favor of antitumor immunity. Therefore, it is possible that bortezomib enhances the antitumor immunity of immune effector cells by overcoming tumor-induced suppression of numerous immune regulatory networks (62). However, in the present study, whether bortezomib enhanced the antitumor efficacy of CAIX-CAR-NK92 cells by these mechanisms requires further evaluation.

In conclusion, we constructed a third-generation CAR against the RCC antigen CAIX. Our results revealed that CAIX-specific CAR-NK92 cells have great potential to kill RCC cells, and combination with bortezomib enhanced the effects of CAR-NK92 cells against RCC *in vitro* and *in vivo*. The present study is based on the NK92 cell line. Future studies of this regimen can also be expanded to autologous or allogeneic primary NK or T cells. This study provides an experimental basis for the novel clinical regimen of CAIX-specific CAR-modified NK or T cells for the treatment of RCC.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

JZ and QF conceived and designed the study. QZ, JX, JD, HoL, HuL, HaL, ML, YM and ZW performed the experiments. QZ and JX wrote the paper. QZ, JX, JD, HoL and HuL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All protocols for the animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Jiangsu Provincial Academy of Chinese Medicine (SCXK2012-005).

Patient consent for publication

Not applicable.

Competing interests

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article and declare that they have no competing interests.

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