

Bispecific cellular nanovesicles potentiate NK cell immunity against gastric cancer

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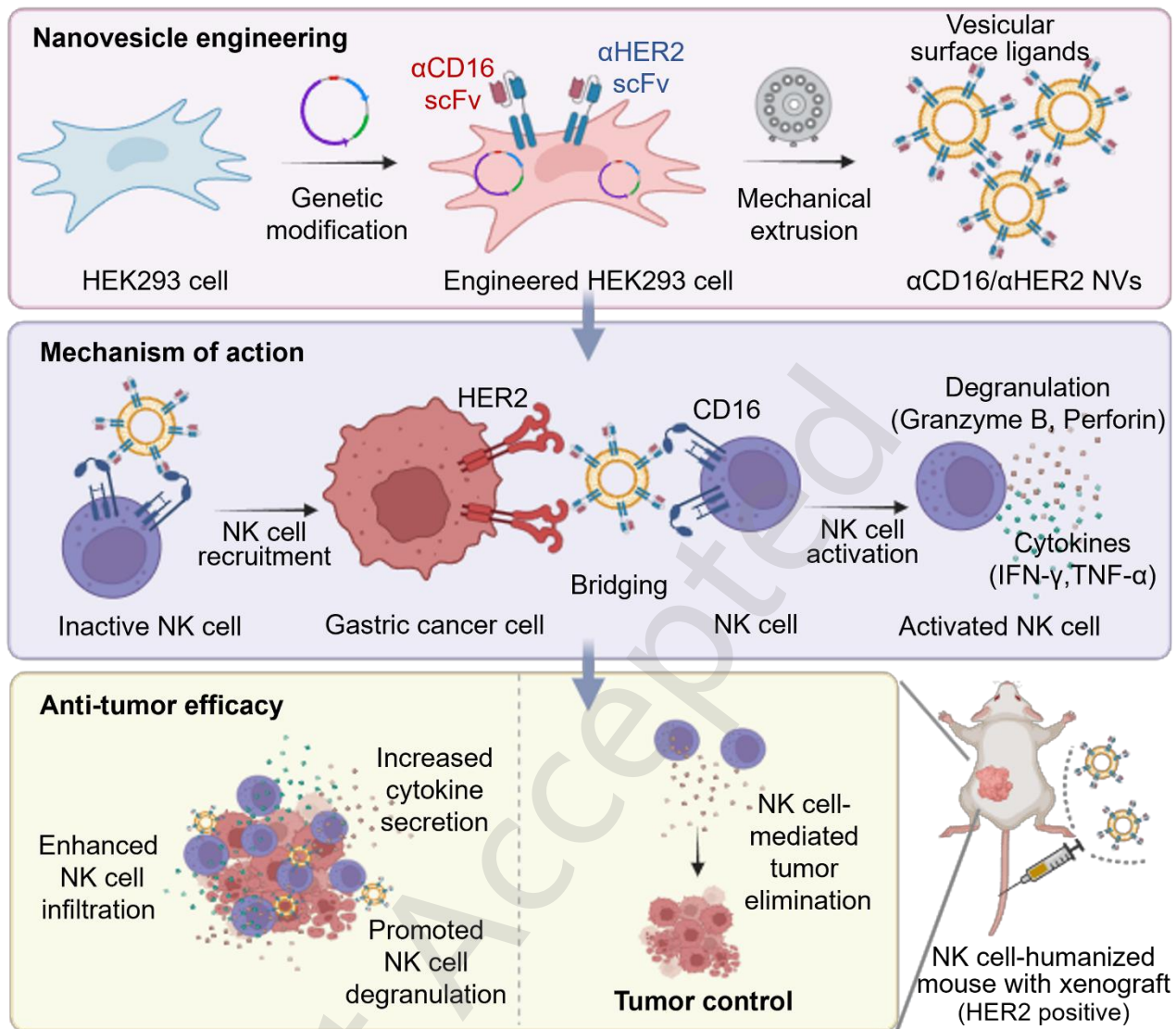
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Bispecific nanovesicles (NV) displaying anti-CD16 and anti-HER2 ligands were generated by mechanical extrusion of engineered HEK293 cells. These NVs activate and redirect CD16-expressing NK cells toward HER2-positive gastric cancer cells, thereby enhancing NK cell infiltration, cytokine secretion, and degranulation. This biomimetic platform significantly inhibits tumor growth and provides a promising strategy for potentiating endogenous NK cell immunity against HER2-positive solid tumors.

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ABSTRACT: Natural killer (NK) cells are potent immune modulators that can quickly lyse tumor cells without prior sensitization or recognition of specific tumor antigens. However, NK cells in tumor microenvironment are frequently dysfunctional. Despite advances in cytokine therapy and NK cell adoptive transfer, tumor-expressed ligands that interact with NK cell-expressed checkpoint receptors can greatly suppress NK cell-mediated tumor lysis, leading to suboptimal therapeutic outcomes. Here we explored a new approach of utilizing cellular nanovesicles (NVs) as artificial controllers of cellular immunity to redirect and activate NK cells for tumor eradication. Through this strategy, we engineered HEK293 cells-derived NVs genetically modified to display two distinct types of surface monoclonal antibodies (mAbs), namely human CD16 and epidermal growth factor receptor 2 (HER2) antibodies, generating dual α CD16/ α HER2 NVs that simultaneously target CD16-expressing NK cells and HER2-expressing gastric cancer cells. Following thorough characterization, the α CD16/ α HER2 NVs exhibited highly potent and specific anti-tumor activity both in vitro and in vivo. This preclinical work demonstrates the feasibility of using dual-targeting NVs to navigate functional NK cells to recognize and eliminate gastric cancers, presenting α CD16/ α HER2 NVs as an effective and versatile platform for cancer immunotherapy.

KEYWORDS: cellular nanovesicles, natural killer (NK) cells, immunotherapy, gastric cancer, genetic engineering

1 Introduction

Gastric cancer is one of the most important malignancies worldwide due to its high disease burden and lethality[1]. Among all cases, approximately 17-20% exhibit human epidermal growth factor receptor 2 (HER2) oncogene amplification and HER2 protein overexpression[2]. The combination of trastuzumab and chemotherapy remains the primary treatment regimen for HER2-positive gastric cancer. However, acquired resistance becomes a major limitation to its therapeutic efficacy [2, 3]. Therefore, new treatment strategies for HER2-positive gastric cancer need to be explored.

Immuno-oncology has recently emerged as a fast-growing and transformative field in cancer treatment[4]. Being a type of cytotoxic lymphocytes, natural killer (NK) cells play a critical role in the innate immune system, as their anti-tumor properties have been increasingly recognized and utilized in cancer immunotherapy [5]. NK cell function is regulated by a repertoire of inhibitory and activating surface receptors[5]. However, the detrimental imbalance between signals delivered by inhibitory and activating receptors limits the effectiveness of NK cells in eliminating tumor cells[6]. In addition, the immunosuppressive microenvironment of solid tumors causes the sparsely infiltrated intratumoral NK cells to become largely exhausted[7]. Therefore,

it is urgent to develop a practical strategy to redirect and activate endogenous NK cells for effective cancer eradication.

NK cell killing can occur through distinct mechanisms, including natural cytotoxicity mediated by NKG2D and natural cytotoxicity receptors, or antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by the potent activating receptor CD16 (Fc γ RIII) [5]. The therapeutic potential of targeting CD16 for cancer treatment has been demonstrated through monoclonal antibody (mAb) therapies[8]. Novel single-chain variable fragment (scFv) recombinant reagents termed bispecific antibodies (BsAbs) or bispecific engagers (BiKEs), which specifically target CD16 on effector NK cells and tumor-associated antigens on cancer cells, are currently under clinical development[9-11]. Despite promising preclinical results, BiKEs had a short blood half-life and limited NK cell activation capacity[12], necessitating further improvement.

Advances in nanomedicines have provided numerous opportunities for cancer immunotherapy[13]. Nanoparticles engineered to reshape the tumor microenvironment have shown promising results in enhancing immunotherapy by facilitating targeted delivery and strengthening immune modulation [14]. Extracellular vesicles (EVs) at nano-to-micro scale, whether naturally secreted or engineered from a variety of cells as pharmaceutically active carriers, have been exploited to fortify

cancer immunotherapy. For example, SMART-Exos are engineered exosomes that display HER2 or EGFR and CD3 antibodies on their surface to control T cell immunity in breast cancer [15, 16]. Compared with free bispecific antibodies, SMART-Exos have higher avidity due to their multivalent surface display, leading to better HER2-specific cytotoxicity and T cell activation at equivalent scFv or molar antibody concentration [16]. These results support nanoparticles can provide high-density multivalent antibody display on a natural lipid bilayer. This multivalent presentation better mimics physiological immunological synapses than monomeric BsAbs, potentially leading to stronger receptor clustering and activation signals [16]. Furthermore, NVs benefit from extended circulation due to their cell-derived membranes and can naturally exploit the enhanced permeability and retention (EPR) effect in solid tumors [17].

Cellular NVs are usually generated through mechanical extrusion of donor cells [18], and they exhibit biomimetic properties similar to EVs but with higher yield and lower cost [19]. Differing from conventionally engineered nanoparticles, NVs are composed of bilayer membranes enriched with various surface proteins, demonstrating excellent biocompatibility [20]. By leveraging these unique properties, NVs have been utilized as EV substitutes to deliver therapeutic cargos to diseased sites for cytotoxicity or immunity modulation [21, 22]. Hematopoietic stem cell (HSC)-derived NVs were engineered with aPD-L1 and aNKG2D gripper ligands and loaded with colony-stimulating factor (CSF), forming CSF@E-Hn. These vesicles target the bone marrow and bring tumor cells and NK cells together to activate NK-mediated tumor killing [23]. Additionally, this nanosystem promotes HSC differentiation, boosts memory T cells, preserves bone homeostasis, and prevents long-term relapse in hematologic malignancies [23]. These preclinical studies have encouraged the development of NV-based systems for immune regulation, drug delivery, and tumor therapy. However, whether this system might be effective in directing NK cells for targeted gastric cancer therapy remains unexplored, and whether the NV-based linker could modulate NK cell activation via the activating receptor CD16 has not yet been reported.

In this study, we genetically engineered NV by decorating two types of mAbs on the vesicular surfaces, which are respectively specific for human CD16 and HER2. It was hypothesized that the dual-targeting α CD16/ α HER2 NVs might recruit CD16-expressing human NK cells to HER2-positive gastric cancers, where they could induce potent and specific killing of gastric cancer cells in the presence of human peripheral blood mononuclear cells (PBMCs). Moreover, *in vivo* studies using NCG mice, triple-immunodeficient mouse models lacking T, B, NK cells, xenografted with human NK cell transplant were applied to examine the tumor inhibitory effect following systemic administration of α CD16/ α HER2 NVs and explore the underlying mechanism. Through this strategy, we aim to provide a dual-targeting platform to link NK cells for targeted and enhanced immunotherapy in HER2-positive gastric cancer.

2 Experimental

2.1 Chemicals and cell cultures

Bicinchoninic acid (BCA) protein assay kit and RIPA lysis buffer were purchased from Thermo Fisher Scientific (Massachusetts, USA). Lymphocyte separation medium was obtained from Solarbio (Beijing, China). Human IFN- γ and TNF- α ELISA kits were purchased from Boster (Wuhan, China). FITC-anti-HumanCD81, FITC-anti-CD63, FITC-anti-CD9, FITC-anti-HA, PE-anti-CD107a, PE-anti-HER2, PE-anti-CD3, APC-anti-CD56 and PE-Cy7-anti-CD16 antibodies were purchased from BioLegend (CA, USA). Hoechst 33342 was obtained from Beyotime (Shanghai, China). Anti-HA, anti-Alix, anti-HSP70, anti-CD9 and anti-calnexin Abs were purchased from Cell Signaling Technology (Boston, USA). CCK-8 kits were purchased from Vazyme (Nanjing, USA). CellTracker™ Red and carboxyfluorescein succinimidyl ester (CFSE) were purchased from Thermo Fisher Scientific (Massachusetts, USA). Lead sulfide (PbS) quantum dots (QDs) were purchased from Nirmidas Biotech (Mountain View, USA).

All cell lines used were purchased from the Chinese Academy of Science in Shanghai and maintained at the School of Medicine of Jiangsu University (China). HEK293 (RRID: CVCL_0045), SGC7901 (RRID: CVCL_0520), HGC27 (RRID: CVCL_1279) and AGS (RRID: CVCL_0139) cells were cultured in Dulbecco's modified Eagle's medium (BI, Israel). Cells were cultured with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Hyclone, USA) at 37°C in a humidified atmosphere with 5% CO₂. Normal PBMCs were obtained from healthy volunteers, and cancerous and the corresponding paracancerous tissues were acquired from patients with gastric cancer in the Affiliated Hospital of Jiangsu University (AHJU). Written informed consents were obtained from all patients. The study was approved by the Ethics Committee of the AHJU, and the research was conducted in accordance with the Declaration of Helsinki.

2.2 Animal experiments

Six-week-old female BALB/c nude mice and female NOD/ShiLtJGpt-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Gpt (NCG) mice were obtained from Nanjing GemPharmatech (Jiangsu, China). All animals were bred in a specific pathogen-free environment. The animal study protocol was approved by the Institutional Animal Care and Use Committee at Jiangsu University (approval no. UJS-IACUC-AP-2023030901). The animal experiments were performed in accordance with the relevant guidelines and regulations for working with live animals.

2.3 Dataset analysis

Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>) was used to analyze CD56 (also called NCAM1) gene expression in gastric cancer tissues and normal stomach tissues, and the overall survival of patients with gastric cancer was analyzed.

2.4 Phenotype analysis of NK cells by single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) data were obtained from the Genome Sequence Archive in the National Genomics Data Center, China National Center for Bioinformatics, and Beijing Institute of Genomics, Chinese Academy of Sciences database under accession code GSA-Human: HRA003650 (<https://ngdc.cncb.ac.cn/gsa-human/>) [24]. Cell Ranger software

(v7.0.0, 10X Genomics) was then used to analyze the sequencing data, and gene expression information was obtained for each cell. Cell Ranger used the STAR aligner to perform splicing-aware alignment of reads to the genome and Cell Ranger outputs were loaded onto Seurat (v4.1.1) software for dimensionality reduction, clustering, and analysis of scRNA-seq data. Overall, 80,111 cells passed the quality control threshold, and genes expressed in fewer than three cells were excluded. Cells were retained if they had the number of genes expressed per cell between 500 and 5,000, with a unique molecular identifier (UMI) count >500 and the rate of mitochondrial gene expression <25%. Doublet Finder (v2.0.3) was used to remove multiplet cells from sequencing data. To visualize data, the LogNormalize method of the 'Normalization' function in Seurat software was utilized to calculate the expression levels of the genes. Principal component analysis was performed using the normalized expression levels, and the top 10 principal components were used to carry out clustering and *t*-distributed stochastic neighbor embedding (t-SNE) analysis. Due to the batch effect among samples, Harmony (v0.1.0) was used for batch effect correction. Normalization, clustering, differential gene expression analyses, and visualization were performed using the R package Seurat 3.1. Clustering of cells was performed using the implemented community identification method in the 'Find Clusters' function and visualized using the 'Run t-SNE' function. The specific marker genes of cell clusters were identified using the Seurat 'Find Markers' function. Specifically, the differentially expressed genes (DEGs) for a specific cluster were identified by comparing cells from that cluster to the other cells using the Wilcoxon rank-sum test. Bonferroni-corrected *p* values <0.05 were used as cutoffs for identifying statistically significant DEGs. Marker genes were selected as the genes with average expression in the cluster that was more than 2-fold higher than their expression in other clusters. The canonical marker genes and top-ranked DEGs were used to annotate the cell types for each cluster.

2.5 Molecular cloning and expression

Synthetic genes encoding the anti-HER2 and anti-CD16 single-chain variable fragments (scFvs) were purchased from Tianyi Huiyuan (Beijing, China), which were derived from anti-HER2 trastuzumab and anti-CD16 NM3E2 Abs, respectively. The anti-CD16 scFv was derived from a human phage-display library[25]. The anti-CD16 scFv had an N-terminal light chain variable region (VL α CD16) and C-terminal heavy chain variable region (VH α CD16) of the anti-CD16 NM3E2 Ab connected by a flexible (GGGGS)₃ linker. Simultaneously, the anti-HER2 scFv contained an N-terminal heavy chain variable region (VH α HER2) and C-terminal light chain variable region (VL α HER2) of trastuzumab separated by a flexible (GGGGS)₃ linker. A 20-amino acid peptide linker (GGGGS)₄ was placed between the anti-CD16 and anti-HER2 scFvs. To generate α CD16/ α HER2 NVs, gene fragments were cloned into pLenti-C-mGFP-P2A-puro vector (OriGene, USA) between BamHI and NotI restriction enzyme sites, resulting in the fusion proteins of scFv antibody-transmembrane domain (TM) of lysosomal-associated membrane protein 2 (LAMP2B). A hemagglutinin (HA) tag was located at the N-terminus of the designed fusion proteins. The generated expression constructs (Fig. S1) were confirmed by DNA sequencing conducted by Tianyi Huiyuan

(Beijing, China). The endotoxin-free plasmids were prepared using PureLink™ HiPure Plasmid Miniprep Kits (Thermo Fisher Scientific, MA), followed by transfection into HEK293 cells using Lipofectamine 2000 transfection kits (Thermo Fisher Scientific, MA) according to the manufacturer's instruction.

2.6 Generation and characterization of NVs

HEK293 cells were transfected with above mentioned plasmids. The cells were then collected and washed with phosphate-buffered saline (PBS) via centrifugation before the supernatant was discarded. The cell pellet was gently resuspended in PBS at a concentration of 5×10^6 cells/mL. The cell suspension was sequentially extruded through 1- μ m, 400-nm, and 100-nm polycarbonate membrane filters by using a mini extruder (Avanti Polar Lipids, USA), to yield a homogenous population of vesicles[26]. Then, the extruded samples were ultracentrifuged at $100,000 \times g$ for 30 min at 4°C, and resuspended in PBS, used immediately or within 1 week when stored at -80°C. For characterization, the protein concentration of NV was determined by using BCA protein assay kit (Vazyme, China). To confirm the successful generation and purification of NVs, biomarkers including Alix, HSP70, CD9, calnexin and HA-tag were determined by Western blot. The morphology, size distribution and zeta potential of NVs were identified using transmission electron microscopy (TEM) (Philips FEI Tecnai 12, Netherlands) and nanosight tracking analysis (NTA) (NanoSight, UK).

2.7 Immunofluorescence analysis

For NV binding to NK cells, NVs (100 μ g/mL) were added to NK cell suspensions (1×10^6 /mL) in PBS and incubated for 1 h at 4°C. Samples were washed twice with PBS when centrifuged at the speed of $800 \times g$ for 10 min at 4°C. Then, NK cells were incubated with FITC-labeled anti-HA Ab for 30 min at 4°C. After two washes using fresh PBS, NK cells were fixed in 4% paraformaldehyde. The samples were stained with Hoechst 33342 (1:500) (Sigma, USA) before PBS washes and observed by an optical microscope (Nikon, Japan). For analysis of cell crosslinking, gastric cancer cells and NK cells were stained with CellTracker™ Red and CFSE, respectively, by following the manufacturer's protocols. In another experiment, α CD16/ α HER2 NVs were pre-incubated with HER2 protein (MCE, USA) or CD16a protein (MCE, USA) in 100 μ L PBS for 30 min at 4°C. The samples were washed with PBS at $100,000 \times g$ for 30 min at 4°C and resuspended in fresh PBS. CFSE-labeled NK cells were then incubated with α CD16/ α HER2 NVs (100 μ g/mL), α CD16/ α HER2 NVs (100 μ g/mL) with HER2 protein, α CD16/ α HER2 NVs (100 μ g/mL) with CD16 protein, or empty vector NVs (control) (100 μ g/mL) in 100 μ L of PBS for 30 min at 4°C. In separate tubes, NK cells were incubated with PBS as control. After washing with 1 mL of ice-cold PBS, the NK cells (4×10^3) were resuspended with 100 μ L of RPMI 1640 medium with 10% FBS and then mixed with gastric cancer cells (2×10^3) in the same medium (100 μ L). The cell mixtures were added in 96-well cell culture plates and incubated at 37°C with 5% CO₂. After 4 h, wells were gently washed with PBS three times and imaged using an optical microscope (Nikon, Japan).

2.8 Analysis of NV binding to gastric cancer cells by flow

cytometry

For NV binding to gastric cancer cell lines (SGC7901, HGC27, AGS cells), cells ($1 \times 10^6/\text{mL}$) were incubated with NV (100 $\mu\text{g}/\text{mL}$) in PBS for 1 h at 4°C . Following three washes with PBS, cells were incubated with the FITC-labeled anti-HA Ab for 30 min at 4°C . After washing three times with PBS, cells were analyzed by flow cytometry (Beckman Coulter, USA).

2.9 In vitro cytotoxicity

For cytotoxicity with effector cells, assays were performed using non-activated PBMCs as effector cells and gastric cancer cells as target cells. To assess the effect of various NV concentrations on cytotoxicity, target cells (1×10^4) were mixed with PBMCs (1×10^5) and incubated with various concentrations of $\alpha\text{CD16}/\alpha\text{HER2}$ NVs, control NVs or PBS for 24 h. To determine the optimal ratio of effector-to-target cell ratios (i.e., E:T) for cytotoxicity, $\alpha\text{CD16}/\alpha\text{HER2}$ NVs (100 $\mu\text{g}/\text{mL}$), control NVs (100 $\mu\text{g}/\text{mL}$) or PBS solutions were added to 96-well plates containing cells at various ratios of E:T. Cells were washed with PBS to remove PBMC suspensions and then incubated with CCK-8 solution for 2 h at 37°C . Absorbance of each well was measured at 450 nm by using an enzyme-linked immunosorbent plate assay reader (FLX800, USA). To further validate cytotoxicity of $\alpha\text{CD16}/\alpha\text{HER2}$ NVs, non-activated PBMCs and gastric cancer cells were mixed and pre-incubated with PBS, CD16-blocking Abs (10 $\mu\text{g}/\text{mL}$, Invitrogen, USA) or HER2-blocking Abs (10 $\mu\text{g}/\text{mL}$, Invitrogen) for 30 min at 37°C . Then, control NVs (100 $\mu\text{g}/\text{mL}$) or $\alpha\text{CD16}/\alpha\text{HER2}$ NVs (100 $\mu\text{g}/\text{mL}$) were added into the mixture for 24 h, respectively. Cells were washed with PBS to remove PBMC suspensions and then stained with crystal violet. Images were captured using an optical microscope (Nikon, Japan).

2.10 In vitro NK cell activation

Purified NK cells (1×10^5) were incubated with target cells (1×10^4) in the presence of $\alpha\text{CD16}/\alpha\text{HER2}$ NVs (100 $\mu\text{g}/\text{mL}$), control NVs (100 $\mu\text{g}/\text{mL}$) or PBS for 24 h at 37°C . The levels of secreted cytokines IFN- γ and TNF- α in the collected culture media were measured using ELISA kits (Boster, China). The expression of CD107a in NK cells was analyzed by flow cytometry. Data were shown as mean $\pm$ standard deviation (SD) ($n=3$).

2.11 In vivo biodistribution of NVs

In HGC27 cell-xenografted BALB/c nude mice, *in vivo* biodistributions of $\alpha\text{CD16}/\alpha\text{HER2}$ NVs and control NVs were assessed and compared. $\alpha\text{CD16}/\alpha\text{HER2}$ NVs or control NVs (6.4 mg/mL) were incubated with 0.5 mg/mL of PbS QDs at 37°C , and the mixture was transferred to the ultrafiltration tube of 100 kDa molecular weight cut-off (MWCO) and centrifuged at $1500 \times g$ for 30 min, followed by PBS washing three times. The obtained NVs were suspended in 200 μL PBS and passed through a 0.45 μm polyethersulfone filter for next use. Then, PbS QDs-labeled $\alpha\text{CD16}/\alpha\text{HER2}$ NVs and control NVs were injected via the tail vein, and images were acquired at different time points (0.5, 2, 12, 24, 48 h post injection or p.i.). At the end of the experiments, mice were sacrificed, and major organs were extracted and measured by DeepVision NIR-II *in vivo* imaging system ($\lambda_{\text{ex}} = 808 \text{ nm}$; $\lambda_{\text{em}} = 1300 \text{ nm}$). ImageJ software 1.8.0 (National Institutes of Health, USA) was used for image analysis.

2.12 In vivo therapeutic efficacy

HER2-positive human gastric cancer cell line HGC27 was used to evaluate the *in vivo* efficacy of $\alpha\text{CD16}/\alpha\text{HER2}$ NVs. 5×10^6 HGC27 cells in 200 μL PBS solutions were subcutaneously injected into the right flank of each NCG mouse. When the tumor size reached approximately 50 mm^3 , mice were randomly divided into three groups ($n=5$); that is, PBS (100 μL), control NVs (5 mg/kg mouse body weight), and $\alpha\text{CD16}/\alpha\text{HER2}$ NVs (5 mg/kg mouse body weight). 1.5×10^6 NK cells were intravenously administered three times at a six-day interval and 20,000 IU human recombinant IL-2 (rhIL-2, R&D Systems, MN, USA) was intraperitoneally injected every three days for a total of five times. Two days following the first human NK cells injection, mice were intravenously administered with $\alpha\text{CD16}/\alpha\text{HER2}$ NVs or control NVs or PBS every other day for a total of six times. Tumor volumes and body weights were measured and recorded every two days. Tumor volumes were calculated as $V = \frac{\text{length} \times \text{width}^2}{2}$. On the 14th day of treatment, mice from each group were sacrificed and tumors were resected and weighed. Major organs (lung, heart, spleen, liver, and kidney) and blood were also extracted for image recording. To evaluate the hepatic and renal functions after different treatments, the levels of alanine transaminase (ALT), aspartate transaminase (AST), creatinine (CREA), blood urea (Urea) and uric acid (UA) in the plasma were determined for the routine blood biochemical analysis. Meanwhile, major organs were fixed with 4% paraformaldehyde for 48 h and further processed and stained with hematoxylin and eosin (H&E) for histopathological analysis. Images of tissue sections were examined by fully automatic digital slice scanner (Pannoramic MIDI, 3DHISTECH, Hungary).

To detect NK cell infiltration and activity, tumor tissues were cut into small pieces and subjected to mechanical disruption and separation, followed by passing through 70 μm strainers and treating with the erythrocyte lysis solution (Solarbio, China). The resulting single-cell suspensions were stained with PE-anti-CD3 and APC-anti-CD56 Abs and analyzed by flow cytometry. The levels of secreted cytokines IFN- γ and TNF- α in the collected peripheral blood of mice were measured using ELISA kits (Boster, China) following the manufacturer's instructions. The expressions of HA, CD56, granzyme B, and perforin were assessed by staining with each corresponding Ab for immunofluorescence analysis. Images of tissue sections were examined by an optical microscope (Nikon, Japan).

2.13 Statistical analysis

Data were expressed as mean $\pm$ SD. Differences between two groups were analyzed using Student's t-test. Differences among three groups were analyzed by one-way ANOVA. The Kruskal-Wallis H test was applied to analyze the differences between *in vivo* tumor growths. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, USA) and Origin 2025 (USA). Significance was defined as: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3 Results

3.1 The immunosuppressive state of NK cells in gastric cancer

To investigate the status of NK cells in gastric cancer, we first analyzed the expression of CD56 (one of the classical markers of NK cells) through the GEPIA database. The level of CD56 mRNA expression was substantially lower in gastric cancer

tissues (C) than in normal gastric tissues (N) (Fig. 1(a)). and paracancerous
Meanwhile, CD3⁺CD56⁺ NK cells from cancerous tissues (C)

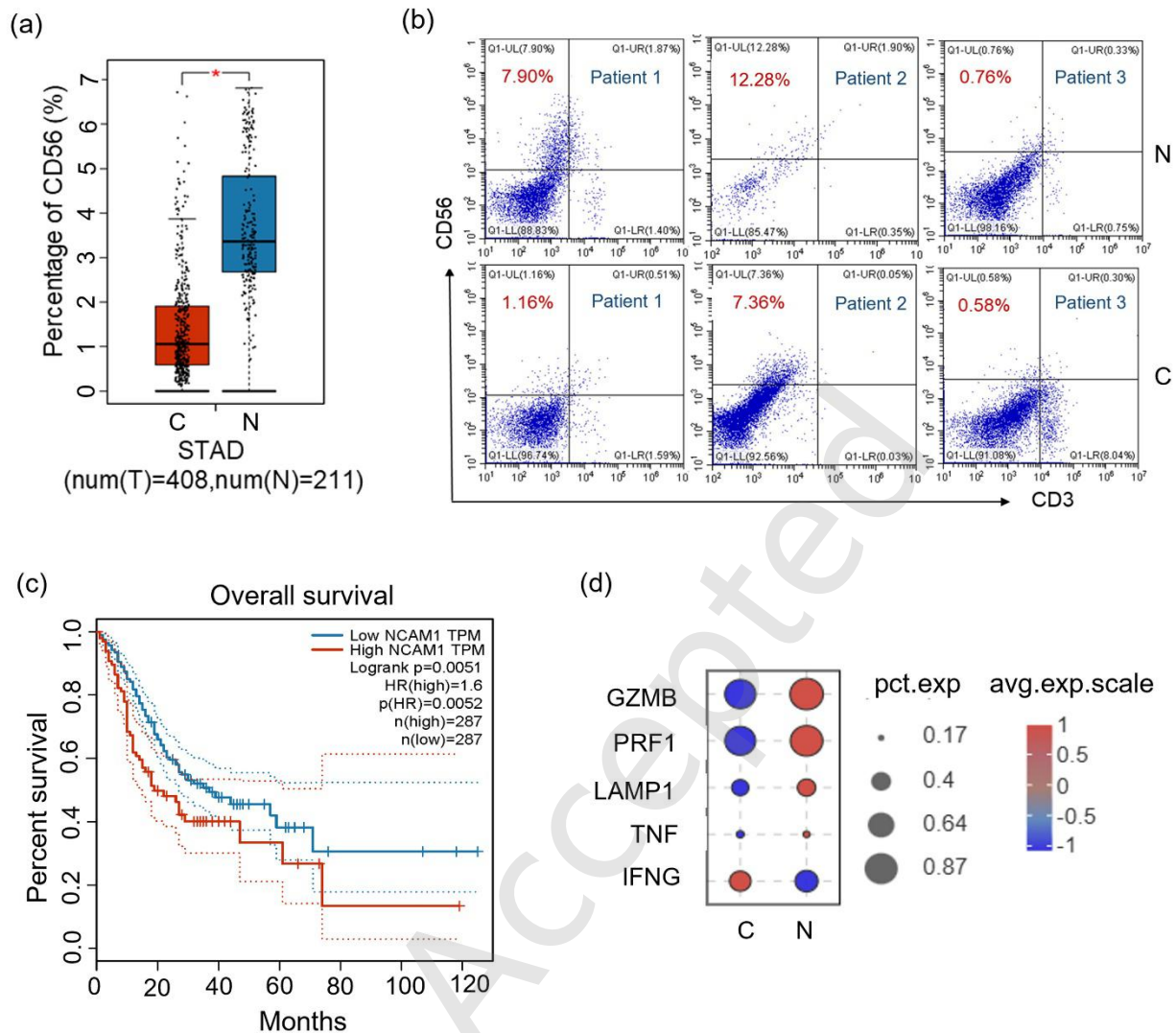


Figure 1 NK cells in human gastric cancer and adjacent paracancerous tissues. (a) The mRNA expression of CD56 gene in cancerous (C) and non-cancerous (N) tissues from patients with gastric cancer using Gene Expression Profiling Interactive Analysis (GEPIA). (b) Representative dot plots of CD3⁺CD56⁺ NK cells in the single-cell suspensions from human gastric cancer tissues and the corresponding non-cancerous tissues. (c) Overall survival curves of the patients with gastric cancer in GEPIA, stratified by the mRNA expression levels of CD56 (also known as NCAM1). The red line shows the survival curve of patients exhibiting high NCAM1 expression levels in tumor samples (top 30% of all samples); the blue line shows the survival curve of the remaining patients ($p=0.0052$). (d) Bubble heatmap showing the genes encoding functional molecules in gastric cancerous (C) and non-cancerous (N) tissues.

tissues (N) of patients diagnosed with gastric cancer were analyzed by flow cytometry, which also showed a lower proportion of intratumoral NK cells compared with that in adjacent non-cancerous gastric tissues (Fig. 1(b)). These results indicated that the population of tumor-infiltrating NK cells was significantly reduced in human gastric cancer. Moreover, in the GEPIA database, patients with gastric cancer who had relatively high CD56 expression showed worse overall survival (Fig. 1(c)), which might be associated with impaired function of tumor-infiltrating NK cells.

To further examine the functional status of tumor-infiltrating NK cells, we performed single-cell sequencing in five pairs of gastric cancer tissues and adjacent paracancerous tissues from patients (Figs. S2(a)-S2(b)). NK cells in gastric cancer tissues (denoted as C) expressed lower levels of functional molecules, such as TNF- α , granzyme B, perforin, and CD107a (also called LAMP1), than those in non-cancerous tissues (denoted as N) (Fig. 1(d)). Simultaneously, tumor-infiltrating NK cells

expressed higher levels of inhibitory receptors than those in non-cancerous tissues (Fig. S2(c)), such as NKG2A (also called KLRC1), TIM-3 (also called HAVCR2), and PD-1 (also called PDCD1), suggesting an exhausted phenotype of NK cells in gastric cancer. These results corroborated an immunosuppressive state of intratumoral NK cells in human gastric cancer.

3.2 Production and characterization of α CD16/ α HER2 NVs

Given that NK cells are sparsely infiltrated and mostly exhausted in gastric cancer tissues, we sought to design and construct dual-targeting α CD16/ α HER2 NVs to redirect and further activate NK cells in the peripheral blood towards gastric cancer, so promoting their antitumor immunity. The plasmid co-expressing the scFvs of CD16 Ab and HER2 Ab with an HA tag was prepared and then transfected into HEK293 cells, while a control plasmid without such a fragment was also applied (Fig. S3(a)). Results from flow cytometry showed that the expression efficiency of the HA-fusion protein on the cell membrane was

more than 90% (Fig. S3(b)), indicating successful construction of engineered cells with high expression of the fusion protein.

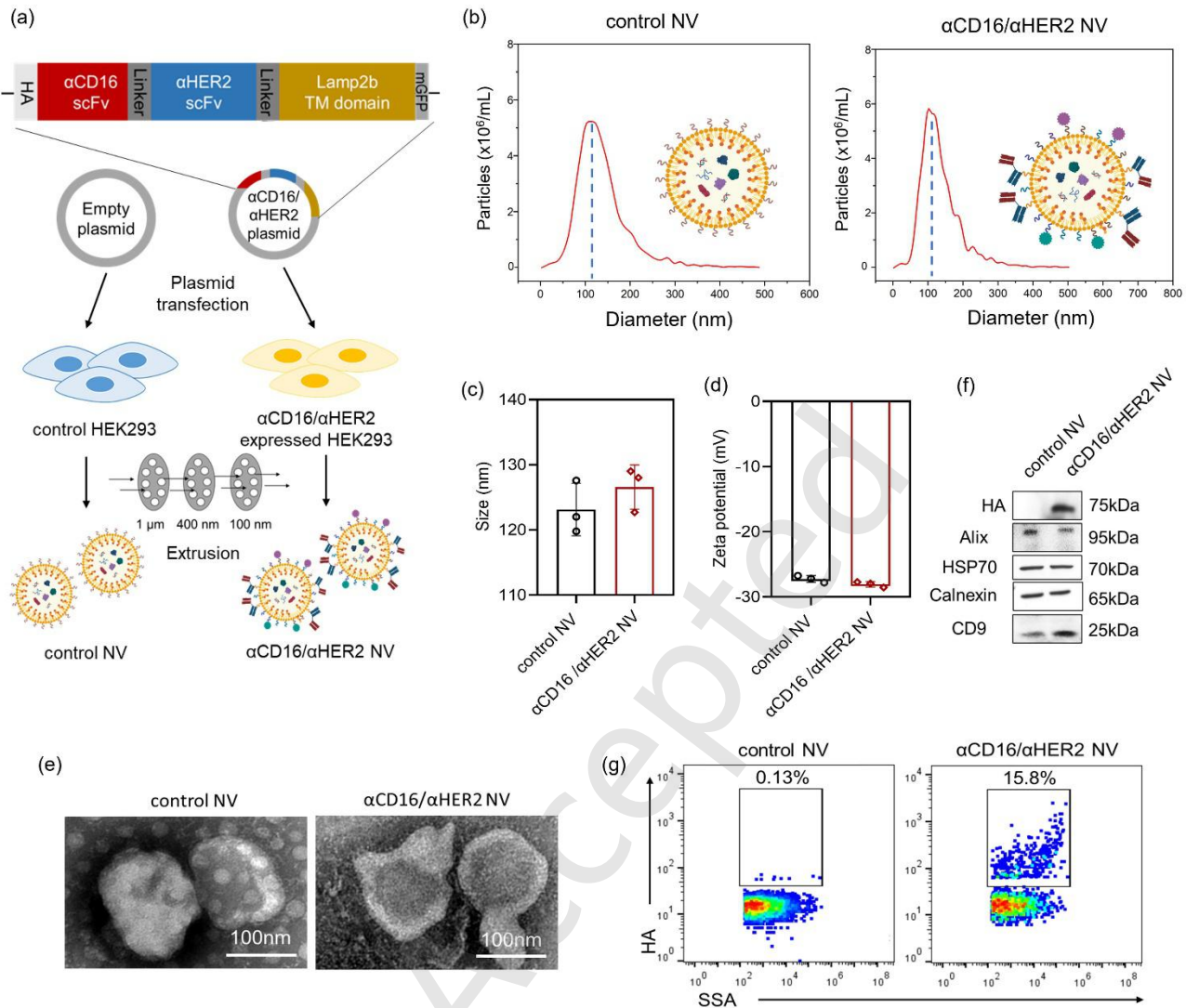


Figure 2 Characterization of αCD16/αHER2 NVs. (a) Flow chart showing production of control and αCD16/αHER2 NVs. (b, c) Size distribution and (d) zeta potential of control and αCD16/αHER2 NVs analyzed by NTA. (e) Morphology of control and αCD16/αHER2 NVs visualized by TEM. (f) Western blot images of control and αCD16/αHER2 NVs. (g) Surface expression of HA-tagged protein on control and αCD16/αHER2 NVs analyzed by nanoflow cytometry.

Next, the control and HA-tagged αCD16/αHER2 NVs were prepared from each type of cultured HEK293 cells through physical extrusion (Fig. 2(a)). NTA showed that the particle size distribution of control and αCD16/αHER2 NVs peaked at 123 ± 3.4 nm and 126 ± 3.0 nm ($p=0.321$), with average zeta potentials of -27.23 ± 0.8 mV and -28.06 ± 0.4 mV ($p=0.103$), respectively, showing no significant difference in their sizes or surface charges (Figs. 2(b)-2(d)). To assess the stability of αCD16/αHER2 NVs, we measured the particle size of the NVs in PBS buffer and PBS buffer with 20% FBS over one week. The results revealed that the particle size of both control and αCD16/αHER2 NVs remained relatively unchanged in PBS and PBS buffer with 20% FBS (Fig. S4), indicating their stability in vitro. TEM images revealed similar morphological characteristics for both particles, which had a round cup-like shape and a size of ~ 120 nm (Fig. 2(e)). Subsequently, Western blot images verified characteristic EV protein markers, including CD9, Alix, HSP70, and calnexin, which were expressed in both control and αCD16/αHER2 NVs (Fig. 2(f)). In parallel, results from nanoflow cytometry also confirmed the expression of EV protein markers, including CD63, CD81, and CD9 (Fig. S3(c)). For difference, the expression of HA-fusion protein was

observed in αCD16/αHER2 NVs but not in control NV (Fig. 2(f)), while 15.8% αCD16/αHER2 NVs were further identified as expressing the HA-fusion protein on the surface (Fig. 2(g)). Regarding batch-to-batch consistency, we monitored HA-tag expression on independently prepared NVs by nanoflow cytometry. An HA-positive proportion of $15.7 \pm 0.7\%$ was obtained from three independent preparations, showing less than 5% variation among batches and indicating good reproducibility of the current preparation process. Overall, these results demonstrated successful production of αCD16/αHER2 NVs with HA-fusion proteins expressed on their outer membrane.

3.3 αCD16/αHER2 NVs interact efficiently with target cells in vitro and in vivo

To confirm the binding ability of αCD16/αHER2 NVs to target cells, the expression levels of CD16 on NK cells from the peripheral blood of patients with gastric cancer and HER2 on three human gastric cancer cell lines were identified by flow cytometry. As a result, CD16 was highly expressed on NK cells (Fig. S5(a)), and HER2 was differentially expressed among three gastric cancer cell lines, with the expression levels of HER2 ranked as SGC7901 (HER2⁻) < HGC27 (HER2⁺) < AGS

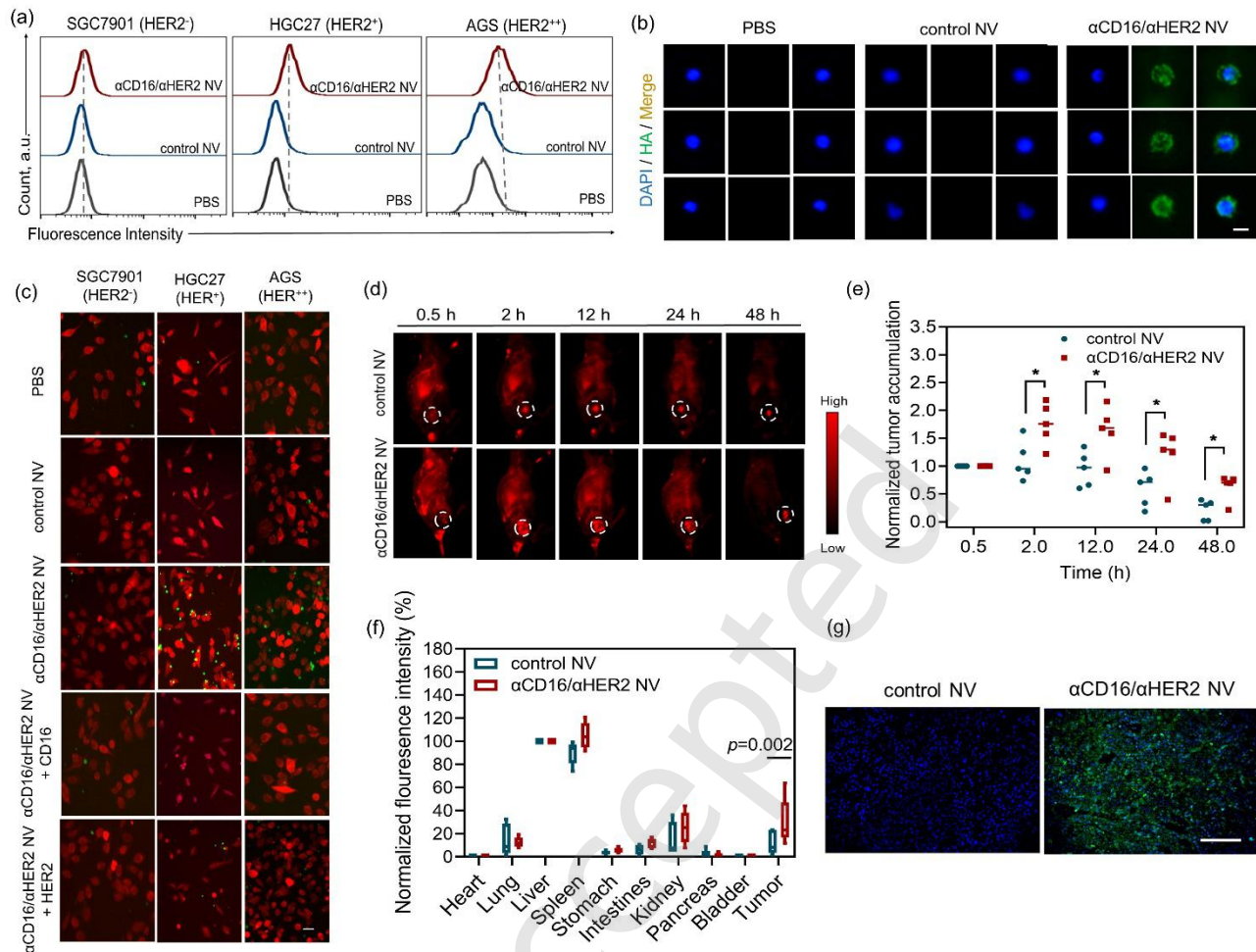
(HER2⁺⁺)

Figure 3 In vitro and in vivo binding of α CD16/ α HER2 NVs. (a) Flow cytometric analysis of α CD16/ α HER2 NV binding to human gastric cancer cell lines. (b) Microscopic images showing the binding of α CD16/ α HER2 NVs to NK cells. Scale bar = 10 μ m. (c) Representative fluorescent images showing the cross-linking of NK cells (green) and gastric cancer cells (red) by α CD16/ α HER2 NVs in contrast to other conditions as indicated. Scale bar = 20 μ m. (d) Representative fluorescence images of one mouse under different treatments over time, illustrating the circulation of fluorescently labelled NVs and their accumulation in the subcutaneous tumors (white circle). (e) Fluorescence intensities of tumors were recorded over time, normalized to the first fluorescence reading in each tumor and plotted versus time (n = 5). (f) At 48 h p.i., mice were sacrificed, and major organs and the tumors were harvested. The fluorescence intensity of each organ was recorded per tissue area and normalized to that of liver (n = 5). (g) The IHC images of HA in tumor tissues. Blue: DAPI; green: HA. Scale bar = 200 μ m. * p < 0.05.

(Fig. S5(b)). Flow cytometric analysis and fluorescent imaging results confirmed that α CD16/ α HER2 NVs could closely bind to both HER2-positive gastric cancer cells (Fig. 3(a)) and CD16⁺ NK cells (Fig. 3(b)), demonstrating that the dual scFv Abs on the NV surface allowed NVs to effectively bind to both CD16- and HER2-expressing cells. At the same time, α CD16/ α HER2 NVs exhibited more specific binding to target cells than control NVs (Figs. 3(a)-3(b)). Conversely, few α CD16/ α HER2 NVs displayed noticeable binding to SGC7901 cells (HER2⁻), revealing that the observed binding of α CD16/ α HER2 NVs to different gastric cancer cell lines was positively correlated with their HER2 expression levels.

To examine whether α CD16/ α HER2 NVs could recruit NK cells towards HER2-expressing cancer cells, the dual-targeting NV-mediated cell interaction was analyzed by fluorescence microscopy, in which fluorescently labeled gastric cancer cells (red) and NK cells (green) were spotted (Fig. 3(c)). In contrast to control NVs, α CD16/ α HER2 NVs resulted in crosslinking of NK cells with HGC27 (HER2⁺) or AGS (HER2⁺⁺) cells, but not with SGC7901 (HER2⁻) cells (Fig. 3(c)). These results

visualized the cell interactions mediated by α CD16/ α HER2 NVs between CD16⁺ NK cells and HER2-positive gastric cancer cells. In addition, HER2 or CD16 protein was pre-incubated with α CD16/ α HER2 NVs to functionally block one targeting arm. NVs with CD16 or HER2 pre-incubation showed weaker crosslinking of NK cells to gastric cancer cells compared with the unblocked α CD16/ α HER2 NVs (Fig. 3(c)). These results demonstrated the necessity of both target-binding arms on α CD16/ α HER2 NVs.

To further investigate their tumor-targeting ability and biodistribution in vivo, 5 mg/kg control NVs or α CD16/ α HER2 NVs labeled with fluorescent PbS QDs were injected intravenously into mice xenografted with HGC27 cells (HER2⁺) via tail vein. The NIR-II fluorescence intensities ($\lambda_{ex}/\lambda_{em}$ = 808/1300 nm) of subcutaneous tumors were recorded (Fig. 3(d)), normalized to the first fluorescence reading of each tumor and plotted over time as shown in Figure 3(e). As a result, the fluorescence intensities within subcutaneous tumors in the α CD16/ α HER2 NV group were substantially higher than those in the control NV group over time (Figs. 3(d)-3(e)). At 48 h p.i.,

mice were sacrificed, and the major organs and tumors were removed for ex vivo fluorescence imaging. In each mouse, the fluorescence intensity of each organ or tumor was normalized to

that of the liver (the largest organ in size with the highest uptake after

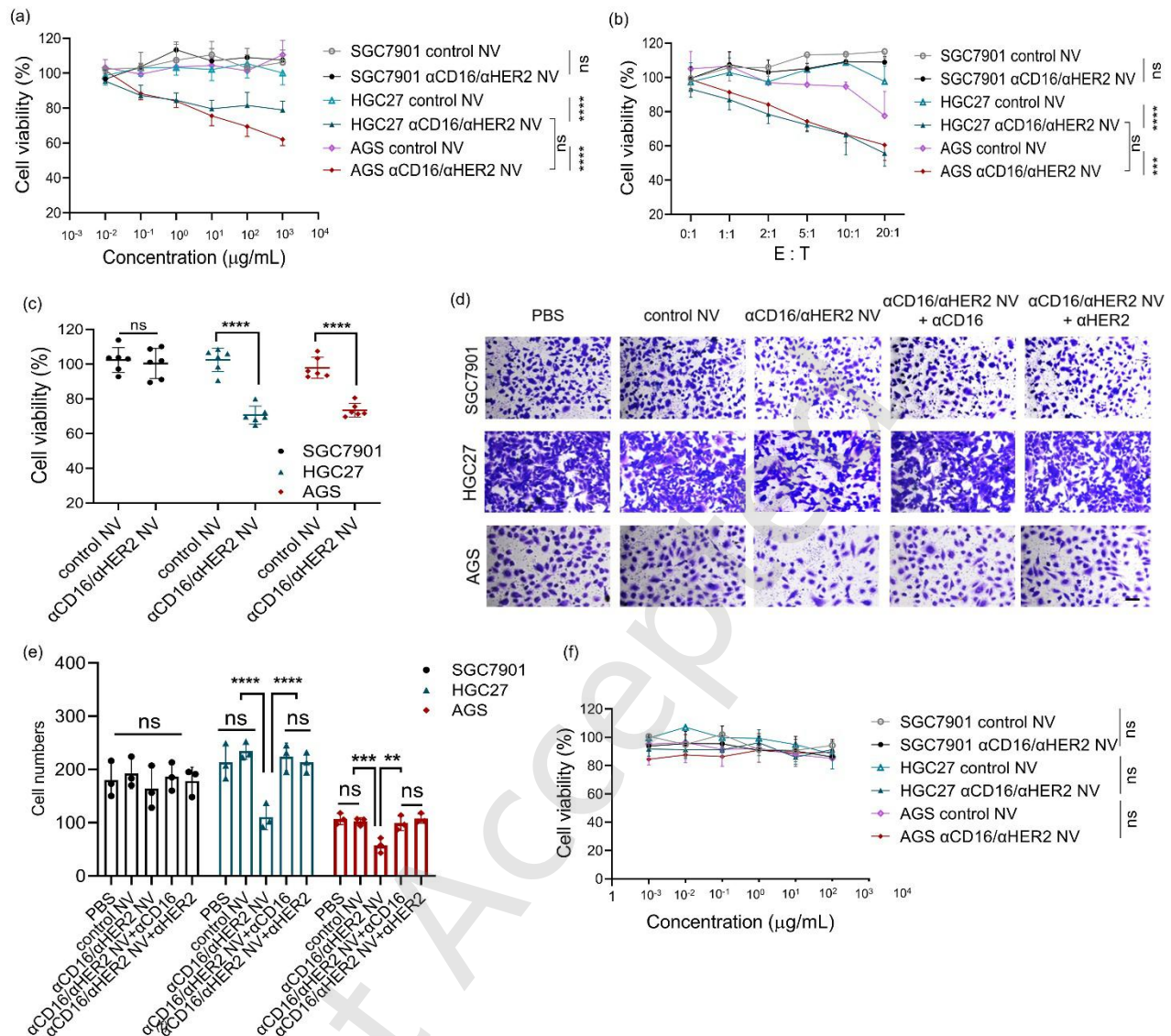


Figure 4 α CD16/ α HER2 NVs promoted anti-tumor immunity of NK cell in vitro. Human PBMCs (effector cells) were incubated with gastric cancer cells (target cells) at different E:T ratios in the presence of control or α CD16/ α HER2 NVs at varying concentrations. (a) Dose-dependent cytotoxicity of α CD16/ α HER2 NVs against three gastric cancer cell lines at an E:T ratio of 10:1. **** $p < 0.0001$. (b) Cytotoxicity of α CD16/ α HER2 NVs against three gastric cancer cell lines at various E:T ratios. *** $p < 0.001$. (c) Cytotoxicity of α CD16/ α HER2 NVs against gastric cancer cell lines in the presence of human PBMCs from six different individuals. **** $p < 0.0001$. (d) Representative images showing the cytotoxicity of α CD16/ α HER2 NVs against different gastric cancer cells and (e) quantitative cytotoxicity measures. ns, not significant; *** $p < 0.001$; **** $p < 0.0001$. (f) In vitro cytotoxicity of control and α CD16/ α HER2 NVs at various concentrations against gastric cancer cell lines in the absence of human PBMCs. Data were shown as mean $\pm$ SD ($n = 3$).

injection). As shown in Figure 3(f), the fluorescence signals were mainly distributed in the liver, spleen, kidney, lung, and tumor, and the fluorescence intensity of tumor in the α CD16/ α HER2 NV group was significantly higher than that in control NV group. In addition, immunohistochemical (IHC) analysis of HA-fused protein in tumor tissues revealed considerable accumulation of α CD16/ α HER2 NVs inside tumors (Fig. 3(g)). These results indicated that the dual-targeting NVs possessed an enhanced capacity for tumor accumulation in vivo, suggesting their potential for therapeutic cargo delivery.

To further delineate the biological characteristics of dual-targeting NVs, the pharmacokinetic patterns of α CD16/ α HER2 NVs and control NVs were also examined. Mouse blood samples were collected at different time points after intravenous injection of NVs, and their NIR-II fluorescence intensity was

plotted versus sampling time, being fit into an exponential function. The exponential decay constant was then obtained and converted into the circulation half-time ($t_{1/2}$). As shown in Figure S6, $t_{1/2}$ was calculated to be 7.84 ± 1.38 h for α CD16/ α HER2 NVs and 6.48 ± 0.89 h for control NV, with no statistical difference ($p > 0.05$).

3.4 α CD16/ α HER2 NVs enhance anti-tumor immunity of NK cells in vitro

In vitro cytotoxicity assays were performed using human PBMCs from healthy donors (effector cells, E) and HER2-expressing gastric cancer cell lines (target cells, T). In the presence of human PBMCs, no cytotoxicity was found in SGC7901 cells (HER2-) or when control NVs were used (Figs. 4(a)-4(b)). In contrast, α CD16/ α HER2 NVs resulted in selective

killing of HGC27 cells (HER2⁺) and AGS cells (HER2⁺⁺) in an effector-to-target (E:T) ratio- and dose-dependent manner.

Nonetheless, the cytotoxicity induced by dual-targeting NVs was not significantly different

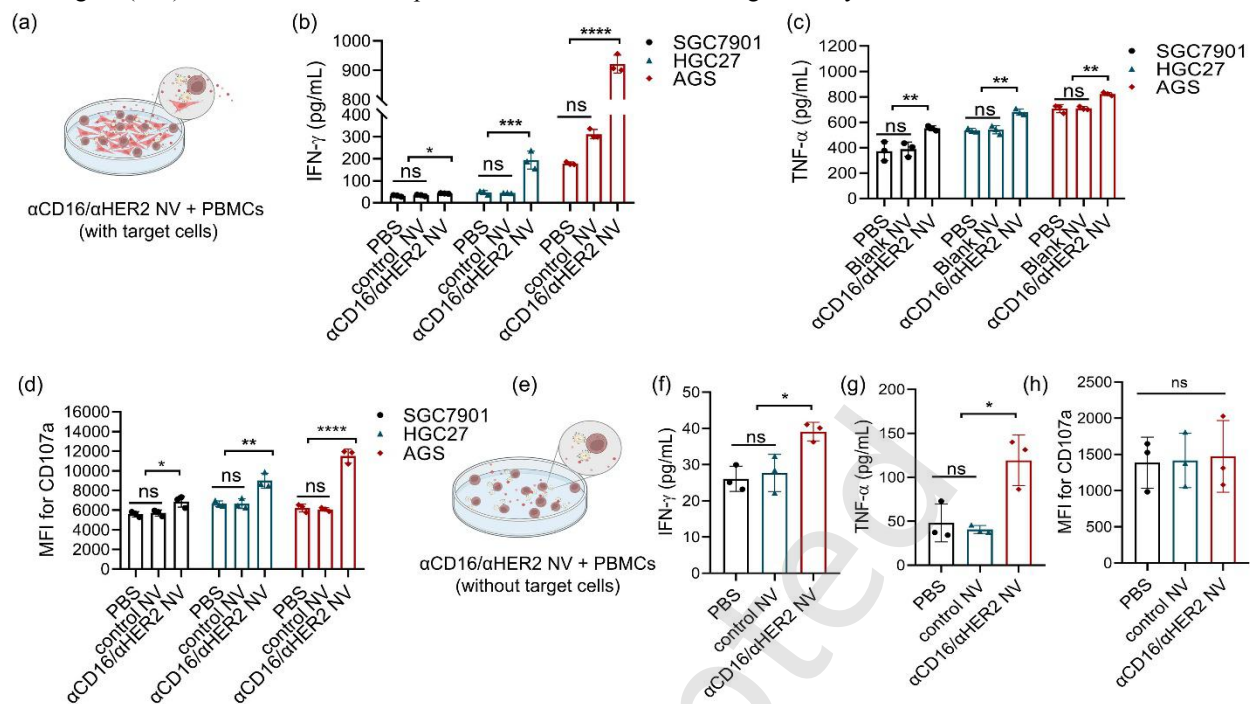


Figure 5 αCD16/αHER2 NVs activated NK cell in vitro. (a-d) Human NK cells were incubated with αCD16/αHER2 NVs in the presence of gastric cancer cell lines at an E:T ratio of 10:1. The levels of secreted IFN-γ (b) and TNF-α (c) cytokines were determined by ELISA. The mean fluorescent index (MFI) of CD107a (d) was analyzed by flow cytometry. ns, not significant; ** $p < 0.01$; *** $p < 0.001$. (e-h) NK cell activity induced by αCD16/αHER2 NVs in the absence of gastric cancer cells. The levels of secreted IFN-γ (f) and TNF-α (g) cytokines were determined by ELISA. The MFI of CD107a (h) was analyzed by flow cytometry. ns, not significant; * $p < 0.05$.

between HGC27 cells (HER2⁺) and AGS cells (HER2⁺⁺), thereby regardless of HER2 expression levels (Figs. 4(a)-4(b)).

To further validate the effectiveness of αCD16/αHER2 NVs, human PBMCs isolated from six healthy individuals were used. αCD16/αHER2 NVs showed potent efficacy in killing HGC27 and AGS cells, but not SGC7901 cells, in the presence of PBMCs from different donors (Fig. 4(c)). Similar results were shown by cell imaging (Figs. 4(d)-4(e)). In addition, the control NV group displayed no cytotoxic difference in either HER2-expressing cell types or HER2-negative SGC7901 cells compared with the PBS group (Figs. 4(d)-4(e)). To observe specific cytotoxicity of NK cells, purified NK cells were cocultured with AGS cells (HER2⁺⁺) in the presence of αCD16/αHER2 NVs. These results demonstrated that αCD16/αHER2 NVs directly induced cytotoxicity of NK cells to HER2-positive cells (Fig. S7). Additionally, blocking Abs against CD16 or HER2 were added in the presence of human PBMCs and gastric cancer cells. The results showed that the blocking Abs reduced the cytotoxicity of αCD16/αHER2 NVs (Figs. 4(d)-4(e)), supporting that the cytotoxicity of αCD16/αHER2 NVs depends on their binding to both CD16 and HER2. To further clarify whether the cytotoxicity was induced by direct NV toxicity, cancer cells were treated with αCD16/αHER2 NVs or control NVs in the absence of PBMCs. No killing effects were observed for αCD16/αHER2 NVs or control NVs on the three gastric cancer cell lines compared with PBS treated group (Fig. 4(f)). These results demonstrated the excellent potency and specificity of αCD16/αHER2 NVs in inducing anti-tumor immunity of NK cells against HER2-expressing cancer cells.

Next, the mechanism by which αCD16/αHER2 NVs induced

anti-tumor immunity was investigated. Increased IFN-γ and TNF-α secretion and elevated CD107a expression were detected in the presence of both HER2-expressing cells and αCD16/αHER2 NVs, denoting dual-targeting NV-mediated engagement of NK cells towards HER2-positive target cells. In contrast, αCD16/αHER2 NVs caused only minimal increase in IFN-γ and TNF-α secretion and CD107a upregulation of NK cells cocultured with SGC7901 (HER2⁻) cells, compared with the PBS or control NV group (Fig. 5(a)-5(d)). Moreover, pre-treatment of NK cells with αCD16/αHER2 NVs in the absence of gastric cancer cells revealed minimal effects on IFN-γ and TNF-α secretion and CD107a expression (Fig. 5(e)-5(f)). These results manifested an enhanced NK cell activity mediated by αCD16/αHER2 NVs in both HER2- and CD16-dependent manner.

3.5 In vivo efficacy of αCD16/αHER2 NVs

We next evaluated the in vivo efficacy of αCD16/αHER2 NVs in HER2⁺ human gastric cancer cells xenografted mouse model. Severely immunodeficient NCG mice bearing HGC27 tumors were engrafted with human NK cells every six days for a total of three times. Meanwhile, 20,000 IU human recombinant IL-2 was intraperitoneally injected every three days for a total of five times. When the tumor volume reached ~50 mm³ (n = 5 per group), mice were randomly divided into three groups and respectively administered with PBS, control NVs or αCD16/αHER2 NVs (5 mg protein/kg particles per mouse) via tail vein injection every other day for six cycles. The therapeutic regimen is illustrated in Figure 6(a). On day 14, we collected the peripheral blood from each mouse and detected the proportion of human-derived CD3⁺CD56⁺ NK cells. As a result,

approximately 50% human NK cells were present in the peripheral blood of mice after 3 weeks of NK cell injection in each group, with no significant differences observed among

groups treated with PBS, control NVs, or α CD16/ α HER2 NVs. This result suggested successful human immune reconstitution in the mouse model and ensured an

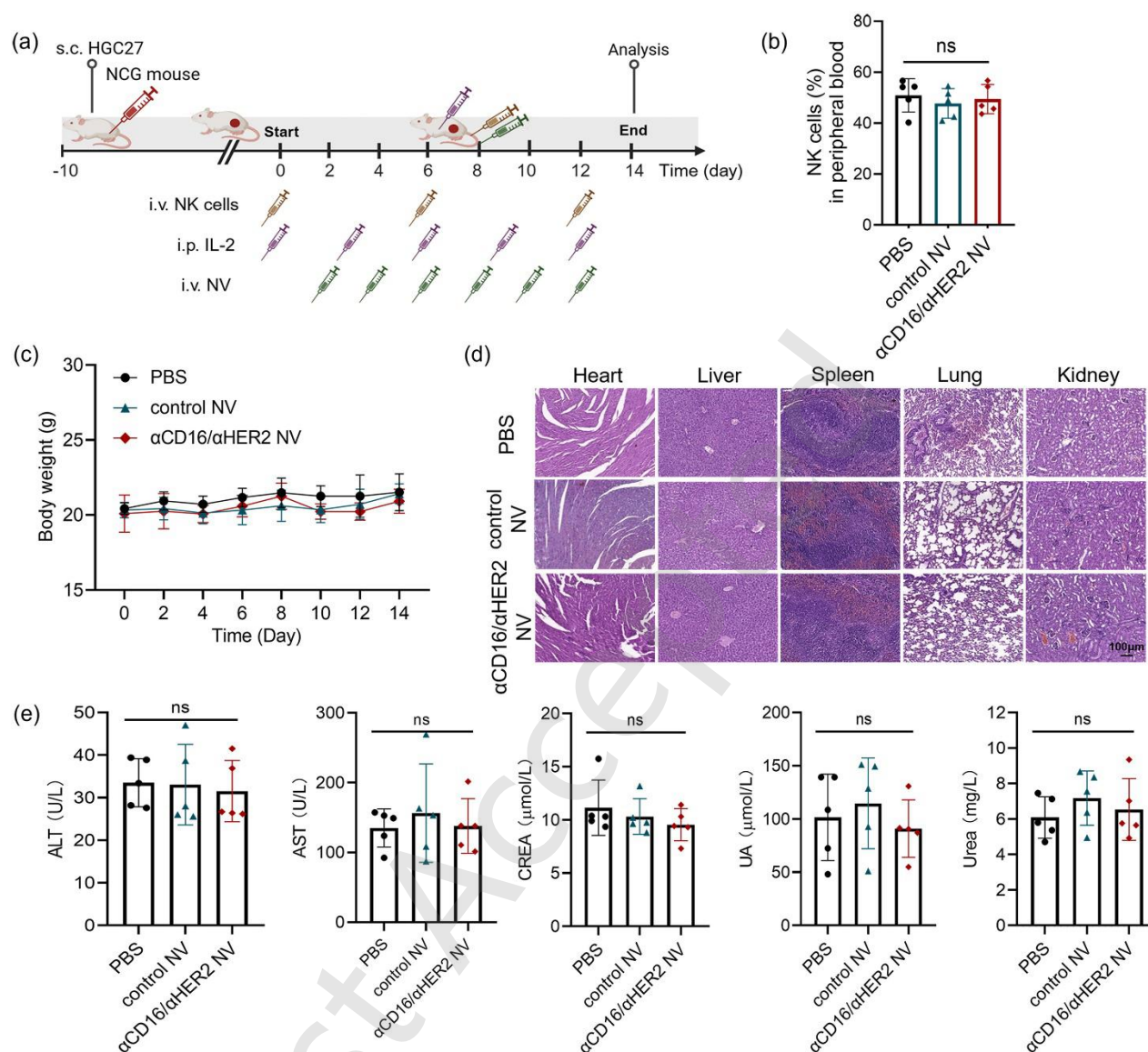


Figure 6 In vivo safety of α CD16/ α HER2 NVs. (a) Schematic illustration of the therapeutic regimen in HGC27 cells-xenografted NCG mice ($n=5$). i.v., intravenous; i.p., intraperitoneal; s.c., subcutaneous. (b) Quantification of human NK cells in the peripheral blood from each group of mice using flow cytometry ($n=5$). (c) Body weights of mice in the efficacy study were measured every two days. (d) Histological images of major organs. Scale bar = 100 μ m. (e) Concentrations of serum ALT, AST, Urea, CREA, and UA obtained from different groups. Data were shown as mean $\pm$ SD. ns, not significant.

adequate supply of NK cells to infiltrate tumor tissues (Figs. 6(b) and S7). Simultaneously, mouse body weight did not show statistical differences among groups treated with PBS, control NVs, or α CD16/ α HER2 NVs over time (Fig. 6(c)). No noticeable liver or kidney injury, as indicated by the plasma levels of ALT, AST, CREA, UA, and Urea, and no pathological changes in major organs after tissue extraction and H&E staining (Figs. 6(d)-6(e)) were found after PBS, control NV or α CD16/ α HER2 NV treatment. In addition, after high-dose (20 mg protein/kg particles per mouse) administration of α CD16/ α HER2 NVs or control NVs in healthy BALB/c mice, serum proinflammatory cytokines IL-6 and IL-1 β were not increased at 24 h compared with PBS group (Fig. S9). These results demonstrated a favorable safety of α CD16/ α HER2 NVs in vivo.

In the same experiment, mice treated with α CD16/ α HER2

NVs displayed significant inhibition of tumor growth, whereas tumor growth in the PBS or control NV group increased (Fig. 7(a)-7(b)). To further detect NK cells in the tumor microenvironment, single-cell suspensions of tumor tissues were prepared for flow cytometric analysis. The results showed that the proportion of CD3⁺CD56⁺ NK cells in the α CD16/ α HER2 NV group was significantly higher than that in the PBS or control NV group (Figs. 7(c) and S10). The IHC analysis of tumor tissues extracted from different groups indicated that in comparison to levels of tumor-infiltrating NK cells in the PBS or control NV group, significantly higher NK cell infiltration was found in tumors extracted from α CD16/ α HER2 NVs-treated mice (Figs. 7(d)-7(e)). Next, immunofluorescence staining of NK cell degranulation markers, including human perforin and granzyme B, which are correlated with cytotoxicity of NK cells, was performed in tumor tissues. It was revealed that the

percentages of granzyme B- and perforin-positive cells were significantly increased in the α CD16/ α HER2 NV group compared with others (Figs. 7(f)-7(h)). Meanwhile, the levels of human cytokine IFN- γ and TNF- α produced by NK cells in the

mouse blood plasma collected at the end of experiment were significantly higher in the α CD16/ α HER2 NV group than in the other groups (Figs. 7(i)-7(k)). Thus, these results portrayed that effective dual targeting allowed α CD16/ α HER2 NVs to

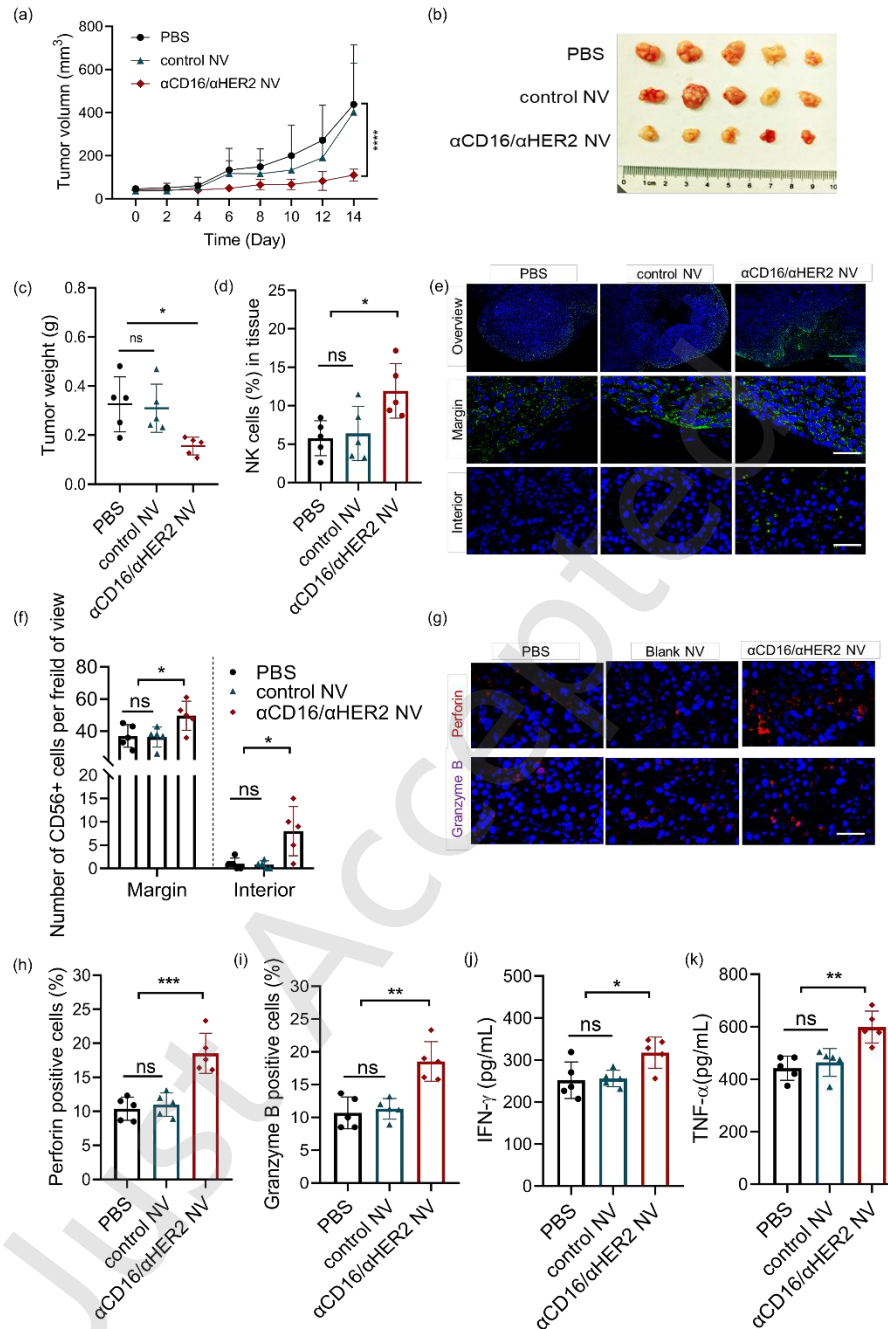


Figure 7 In vivo anti-tumor effects of α CD16/ α HER2 NVs. (a) Tumor growth curves in three groups subjected to different treatments. (b) Representative images and (c) weights of tumor tissues obtained from different groups. (d) Results from flow cytometry indicating the quantification of NK cells in tumors from each group ($n=5$). (e) Representative IHC images of the margin and interior regions of tumor dissections from mice treated with PBS, control NVs, and α CD16/ α HER2 NVs, respectively. Blue: DAPI; green: CD56. Scale bars = 200 μ m (green) and 50 μ m (white). (f) Analysis of CD56 expression in tumor samples shown in (e) ($n=5$). (g) Representative IHC images of perforin and granzyme B in tumor tissues after indicated administration (red: granzyme B or perforin; blue: DAPI). Scale bar = 50 μ m. (h, i) Statistical analysis of perforin (h) and granzyme B (i) in tumor samples shown in (g) ($n=5$). (j, k) Concentrations of serum IFN- γ (j) and TNF- α (k) from different groups. ns, not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data were shown as mean $\pm$ SD.

navigate NK cells for increased infiltration into tumors and enhanced anti-tumor activity in vivo, possibly through the secretion of immunostimulatory cytokines and cytotoxic proteins.

4 Discussion

In this work, we first transfected HEK293 cells to display dual scFv Abs on the cell surface and then prepared cell-derived NVs

through mechanical extrusion to impart α CD16/ α HER2 ligands on their vesicular surfaces. The dual-targeting NVs further enabled the activation and navigation of CD16-expressing NK cells to the proximity of HER2-positive gastric cancers for effective tumor elimination. In vivo studies using NCG mice bearing subcutaneous xenografts of human gastric cancer cells and engrafted with human NK cells indicated significant inhibition of tumor growth following intravenous administration of α CD16/ α HER2 NVs, owing to enhanced NK cell infiltration,

increased cytokine secretion and promoted degranulation. On top of it, we presented a novel linker platform of bispecific NVs to rejoin endogenous NK cells against HER2-positive solid tumors (e.g., gastric cancer) for precision immunotherapy.

Nanovesicles are widely used in liquid biopsy and vesicle-based therapy[27, 28]. So far, numerous studies have been performed by utilizing NV as transport vehicles for drug delivery[21], due to their targeting capabilities, low immunogenicity, and high structural elasticity, which permit them to cross challenging biological barriers and safely deliver cytotoxic proteins or specific RNA payloads directly into dense tumor masses[29]. Various cell types have been used to produce NVs, such as stem cells, tumor cells, and primary blood cells (e.g., anucleate erythrocytes) [30, 31]. Among them, HEK293 cell-derived NVs have attracted great attention due to their minimal toxicity and immunogenicity[32]. HEK293 cells derived from an adenovirus-transformed embryonic kidney cell line, albeit with oncogenic concerns, have been widely used in biomedical research because of their easy culture and reliable transfection efficiency[33]. Moreover, HEK293 cells are a standard and scalable platform widely accepted by regulatory bodies for producing biologics (such as adenoviral vectors and FDA-approved proteins)[34, 35]. In a preclinical study, doxorubicin-loaded HEK293 NVs exhibited approximately 10-fold higher delivery capacity than free doxorubicin, and reduced tumor growth to a similar extent as 20-fold higher doses of doxorubicin alone without systemic side effects[18]. HEK293 cell-derived CD64-NV-aPD-L1, which overexpressed CD64 on the NV membrane to capture the PD-L1 antibody, could simultaneously interrupt the immunosuppressive effect of PD-L1 and decrease the inhibition of Tregs, leading to suppression of tumor growth[36]. Consistently, in our study, HEK293 cell-derived NVs that overexpressed both α CD16 and α HER2 Abs on the vesicle surface effectively activated NK cells and reduced tumor growth, while no systemic toxicity was observed. In addition, after high-dose administration of HEK293 cell-derived α CD16/ α HER2 NVs or control NVs in mice, serum proinflammatory cytokines IL-6 and IL-1 β were not increased compared with the PBS group. These results demonstrated the safety of HEK293 cell-derived NVs in animal models. Certainly, for these advances to achieve widespread clinical implementation, whole cell-derived vesicles must meet stringent requirements for isolation, purification, and quality control. First, the field must standardize nanovesicle isolation protocols as isolation techniques may drastically alter surface marker expression and recovery yields[37]. Second, to mitigate allogeneic immunogenicity or contamination risks, manufacturing protocols must implement rigorous downstream purification to completely separate NVs from host cell proteins, DNA debris, or retrovirus-like particles[38]. Third, scale-up of mass production and the long-term pharmacokinetics of engineered NVs in diverse patient cohorts remain key areas for future clinical research[37].

A biomarker for a broad spectrum of human carcinomas, HER2 has been selected as a primary target of interest in many immunotherapeutic approaches[39]. Engineered nanoparticles for HER2 regulation can be manufactured by either surface modification, such as HER2-binding peptides (LTVSPWY)[40] or scFv[41], to enhance tumor cell enrichment, or encapsulation of therapeutic materials, such as HER2 siRNA, to inhibit dysregulated HER2 signaling in tumors[42]. In our study, HER2-targeting scFv was decorated on the outer membrane of α CD16/ α HER2 NVs for specific recognition and binding of HER2-expressing cells. α CD16/ α HER2 NVs exhibited enhanced accumulation in HER2-positive gastric cancer compared with control NVs in vivo. This may be driven by passive

extravasation via the EPR effect during initial entry, because the control NVs also exhibited a baseline signal in the tumor region. However, once inside the tumor microenvironment, the control NVs face rapid clearance or washout, whereas the α CD16/ α HER2 NVs achieve active retention via high-affinity binding to HER2-expressing gastric cancer cells. However, in the presence of PBMCs, the cytotoxicity of α CD16/ α HER2 NVs in AGS cells (HER2⁺) was comparable to that in HGC27 cells (HER2⁺), therefore irrespective of HER2 expression levels. These results suggested that overexpression of HER2-targeting scFv and NK cell-targeting α CD16 on NVs was sufficient to navigate NK cells to lyse HER2-expressing tumor cells.

NK cells are immune effectors that eliminate infected or aberrant cells by releasing cytotoxic proteins and cytokines. However, solid tumors are surrounded by an immunosuppressive microenvironment that impairs NK cell function[43]. Our results from scRNA-seq analysis indicated that cytotoxic proteins such as granzyme or perforin, as well as cytokines such as IFN- γ , were markedly reduced in tumor-infiltrating NK cells. The upregulation of inhibitory receptors on tumor-infiltrating NK cells and downregulation of activating receptor ligands on tumor cells lead to the dominance of inhibitory signals overriding activating receptor pathways, thereby attenuating NK cell-mediated anti-tumor responses[43]. Thus, it is necessary to activate NK cells by manipulating NK cell function via activating receptors for cancer treatment.

NK cells express several activating receptors that can be targetable to potentiate NK cell-mediated anti-tumor immunity, among which CD16 is the most common one[44]. NK cells mediate antibody-directed cellular cytotoxicity (ADCC) through the highly potent CD16-activating receptor. Signaling through CD16 induces a calcium flux and phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs), triggering the release of lytic granules and cytokines[45]. The classic 1633 bispecific killer cell engager (BiKE) and 161533 trispecific killer engager (TriKE), which contain scFv against CD16 and CD33 to create an immunologic synapse between NK cells and CD33⁺ myeloid targets, are currently in clinical trials for the treatment of refractory acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Their preclinical studies have shown that NK cells treated with BiKE or TriKE significantly enhanced degranulation and TNF- α and IFN- γ production induced by CD16 activation against endogenous CD33⁺ targets [9, 11]. Similarly, here we genetically engineered CD16 mAb-derived scFv onto α CD16/ α HER2 NVs to induce NK cell-mediated anti-tumor immunity. α CD16/ α HER2 NVs activated NK cells to release cytokines, including IFN- γ and TNF- α , and effector molecules, such as perforin and granzyme B, leading to inhibition of tumor growth. Although the comparison between α CD16/ α HER2 NVs and BiAbs against HER2 and CD16 were not performed in our system, a study has shown higher avidity, better HER2-specific cytotoxicity, and stronger T cell activation of bispecific EVs against HER2 and CD3 targets than BiAbs [16]. In addition, the advantages of extended circulation[17] and distinguished drug delivery abilities[22] make NVs more appealing. Thus, in our study, we established a bispecific nanoparticle platform that is promising for NK cell-mediated HER2-positive cancer treatment and further combination therapy.

Humanized mouse models, which refer to immunodeficient mice co-engrafted with human tumors and immune components, have been developed to test and validate a broad range of immunotherapeutic approaches[46]. The NCG mouse (NOD/ShiLtJGpt-Prkdcem26Cd52Il2rgem26Cd22/Gpt) is a strain of severe immunodeficiency obtained by using gene editing technology to knock out the Prkdc (inducing severe

combined immunodeficiency) and Il2rg (reducing host innate immunity) genes in NOD/Nju mice[47]. Being one of the most immunosuppressive mouse models to date, the NCG strain is highly suitable for non-clinical cell therapy development[48]. Mainly, there are two strategies to produce humanized mice; that is, engraftment of human CD34⁺ HSPCs and direct infusion of mature human immune cells. While the engraftment of HSPCs may result in limited and variable development of a human peripheral immune system in mice, direct infusion of PBMCs transiently sustains human innate immune cells, such as myeloid and NK cells, which persist for a few days before becoming undetectable in blood and tissues[47].

In our study, to ensure adequate NK cell infiltration into the tumors, we i.v. injected the peripheral blood-derived NK cells to construct a stable NK cell-humanized mouse model. To sustain the viability and growth of NK cells, the cytokine IL-2 was administered five times at four-day intervals[10]. Meanwhile, purified NK cells were injected three times at six-day intervals to ensure sufficient NK cell engraftment[21]. As a result, approximately half of human CD3⁺CD56⁺ NK cells injected were still detectable in the mouse blood even after three weeks p.i., with no noticeable toxicity or aberrant behavior observed in the host animals. Subsequently, bispecific α CD16/ α HER2 NVs were i.v. administered to the HGC27 cell (HER2⁺)-xenografted NK-humanized mouse model, demonstrating effective inhibitory effects on gastric tumor growth. This result validated the suitability of applying the NCG mouse model to assess endogenous NK cell functionality, as well as in vivo efficacy of bispecific α CD16/ α HER2 NVs in linking CD16-positive NK cells to eliminate HER2-positive gastric cancer.

5 Conclusion

In summary, we engineered HEK293 cell-derived NVs genetically modified with human CD16 and HER2 antibodies, obtaining dual α CD16/ α HER2 NVs that simultaneously target CD16-expressing NK cells and HER2-expressing gastric cancer cells. The α CD16/ α HER2 NVs brought together tumor cells and NK cells, activating the latter to release cytotoxic proteins and cytokines for specific tumor elimination. This preclinical work demonstrates the feasibility of utilizing dual-targeting NVs to guide functional NK cells to recognize and eliminate gastric cancer, enabling them to be an innovative immunotherapeutic candidate for HER2-positive gastric cancer.

Electronic Supplementary Material: Supplementary material (the supplementary Methods and supplementary Figures) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94909052>

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and/or the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no competing interests in this work.

Author contribution statement

Yinghong Shi: Conceptualization, data curation, validation, writing manuscript, experimental design, funding acquisition. Wenting Zhang: Data curation, project administration, validation. Xinyang Zhang: Data curation, validation. Xu Zhang: Conceptualization, project administration. Xiaojun Shen: Conceptualization, project administration, investigation. Hui Qian: Conceptualization, project administration, investigation. Zhimin Tao: Conceptualization, project administration, investigation, writing manuscript, experimental design. All the authors have approved the final manuscript.

Informed consent

Written informed consent for participation was obtained from all patients.

Ethics statement

The animal experiments were carried out following the guidelines of the Institutional Animal Care and Use Committee at Jiangsu University and were approved by the Committee on Ethical Use of Animals of Jiangsu University (Ethical code: UJS-IACUC-AP-2023030901).

Use of AI statement

None.

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Electronic Supplementary Material

Bispecific cellular nanovesicles potentiate NK cell immunity against gastric cancer

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Materials and Methods

S1. Analysis of NK cells from human gastric cancer tissues

For analysis of tumor-infiltrating NK cells, the percentage of CD3⁺CD56⁺ NK cells was evaluated by flow cytometry. Cancerous and paracancerous tissues were dissected to collect single-cell suspensions with a GentleMACS Dissociator (Miltenyi Biotec, Germany). Briefly, we first removed fibrous and necrotic areas of samples and cut the remaining tissues into small pieces. Next, the small pieces were transferred into GentleMACS C Tubes containing the enzyme mixture. The C Tubes were carefully loaded onto the GentleMACS Dissociator to start the selected program. Then, a 70 µm mesh cell strainer was used to collect filtered cell suspensions into a 50 mL centrifuge tube. The cells were washed twice with fresh RPMI 1640 medium at 300×g for 10 min. The cells were stained with PE-anti-CD3 and APC-anti-CD56 antibodies for 30 min and washed with phosphate buffered saline (PBS) for 10 min. All samples were analyzed using a flow cytometer (Beckman Coulter, USA).

S2. Isolation of human PBMCs and NK cells

Human PBMCs were isolated by using Lymphocyte Separation Medium following the manufacturer's instruction. Briefly, blood was mixed with an equal volume of PBS and then added on top of two-thirds volume of Lymphocyte Separation Medium, followed by centrifugation at 400×g for 30 min without brake. The layer of PBMCs was transferred to a clean tube and washed three times with fresh PBS before use. NK cells were expanded and purified from PBMCs as previously described[31]. Briefly, PBMCs were first primed with rhIL-12 (10 ng/mL), rhIL-18 (50 ng/mL), and rhIL-15 (50 ng/mL) for 16 ± 2 h, and then cultured in complete L-15 medium (Lonza, Switzerland) containing 10% human AB serum (Sigma, USA) and 1% penicillin–streptomycin (Hyclone, USA) supplemented with rhIL-2 (1 ng/mL) to support survival. Half of the medium was replaced every 2 days with fresh cell medium supplemented with rhIL-2. After 14 days, NK cells were further purified from the culture using CD56 MicroBeads (Miltenyi Biotec, Germany). NK cells (CD3⁺CD56⁺ ≥ 85%) were used for the following experiments.

S3. Flow cytometric analysis of HER2 expression in gastric cancer cells

To evaluate the expression levels of HER2 on the cell surface, cultured SGC7901, HGC27, and AGS cells were stained with PE-anti-human HER2 antibody for 30 min at 4°C, respectively. After three washes with PBS, samples were then analyzed by flow cytometry (Beckman Coulter, USA). All data were then processed with FlowJo V10 software (Tree Star Inc., OR, USA).

S4. Effects of NVs on gastric cancer cell activity without effector cells

For cytotoxicity assays without effector cells, cancer cells (1×10⁴) were incubated with various concentrations of αCD16/αHER2 NVs,

control NVs or PBS for 24 h at 37°C with 5% CO₂. Before the measurement, the culture media of different groups were discarded, and fresh medium containing 10% (v/v) CCK-8 solution was added into 96-well plates for 2 h. Absorbance of each well was measured at 450 nm by using an enzyme-linked immunosorbent plate assay reader (FLX800, USA).

S5. Effects of NVs on NK cell activity without target cells

To examine the effect of NV on NK cell activation without gastric cancer cells, bead-purified NK cells were incubated with α CD16/ α HER2 NVs (100 μ g/mL), control NVs (100 μ g/mL), or PBS for 24 h at 37°C. The levels of secreted cytokines IFN- γ and TNF- α in the collected culture media were measured using enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instruction (Boster, China). Data were shown as mean $\pm$ SD (n=3).

S6. Western blot

Equal amounts (50 μ g) of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes were probed with specific primary antibodies and then with peroxidase-conjugated secondary antibodies. Protein bands were visualized with an enhanced chemiluminescence kit (Thermo Fisher Scientific, USA). The antibodies used were anti-HA, anti-Alix, anti-HSP70, anti-CD9, and anti-calnexin.

S7. Pharmacokinetics of NVs

In the HGC27 cell-xenografted BALB/c nude mouse model, the pharmacokinetics of α CD16/ α HER2 NVs and control NVs were assessed. First, α CD16/ α HER2 NVs or control NVs at 6.4 mg/mL were incubated with 0.5 mg/mL of lead sulfide (PbS) quantum dots (PbS QDs) (Nirmidas Biotech, Mountain View, USA) at 37°C, and the mixture was transferred to a 100 kDa MWCO ultrafiltration tube to be centrifuged under 1500 $\times$ g for 30 min, followed by washing three times with PBS. Finally, the obtained NVs were suspended in 200 μ L PBS and passed through a 0.45 μ m polyethersulfone filter for further use. Then, PbS QDs-labeled α CD16/ α HER2 NVs or control NVs were injected via the tail vein, and images of mouse blood were acquired at different time points (0.5, 1, 2, 4, 8, 12, 24, 48 h post injection or p.i.) by DeepVision NIR-II in vivo imaging system (λ_{ex} = 808 nm; λ_{em} = 1300 nm). ImageJ software 1.8.0 (National Institutes of Health, USA) was used for image assessment. The circulation half-life of α CD16/ α HER2 NVs in mice was determined by noncompartmental analysis using Origin.

S8. Enzyme-linked immunosorbent assay for serum cytokines

In BALB/c mice, serum pro-inflammatory cytokines were assessed by ELISA. α CD16/ α HER2 NVs (20 mg/kg particles per mouse) or control NVs (20 mg/kg particles per mouse) were injected via the tail vein, and blood from each group was collected at 24 h p.i. (n=6). The levels of serum cytokines IL-6 and IL-1 β in the collected peripheral blood of mice were measured using ELISA kits (Boster, China) following the manufacturer's instruction.

Figures

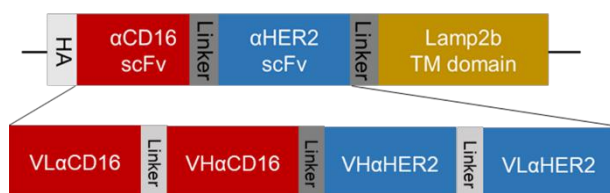


Figure S1 Molecular design of scFv antibody-LAMP2B TMD fusions for the generation of α CD16/ α HER2-expressing HEK293 cells. The fusion protein contains an N-terminal HA epitope tag and flexible linkers.

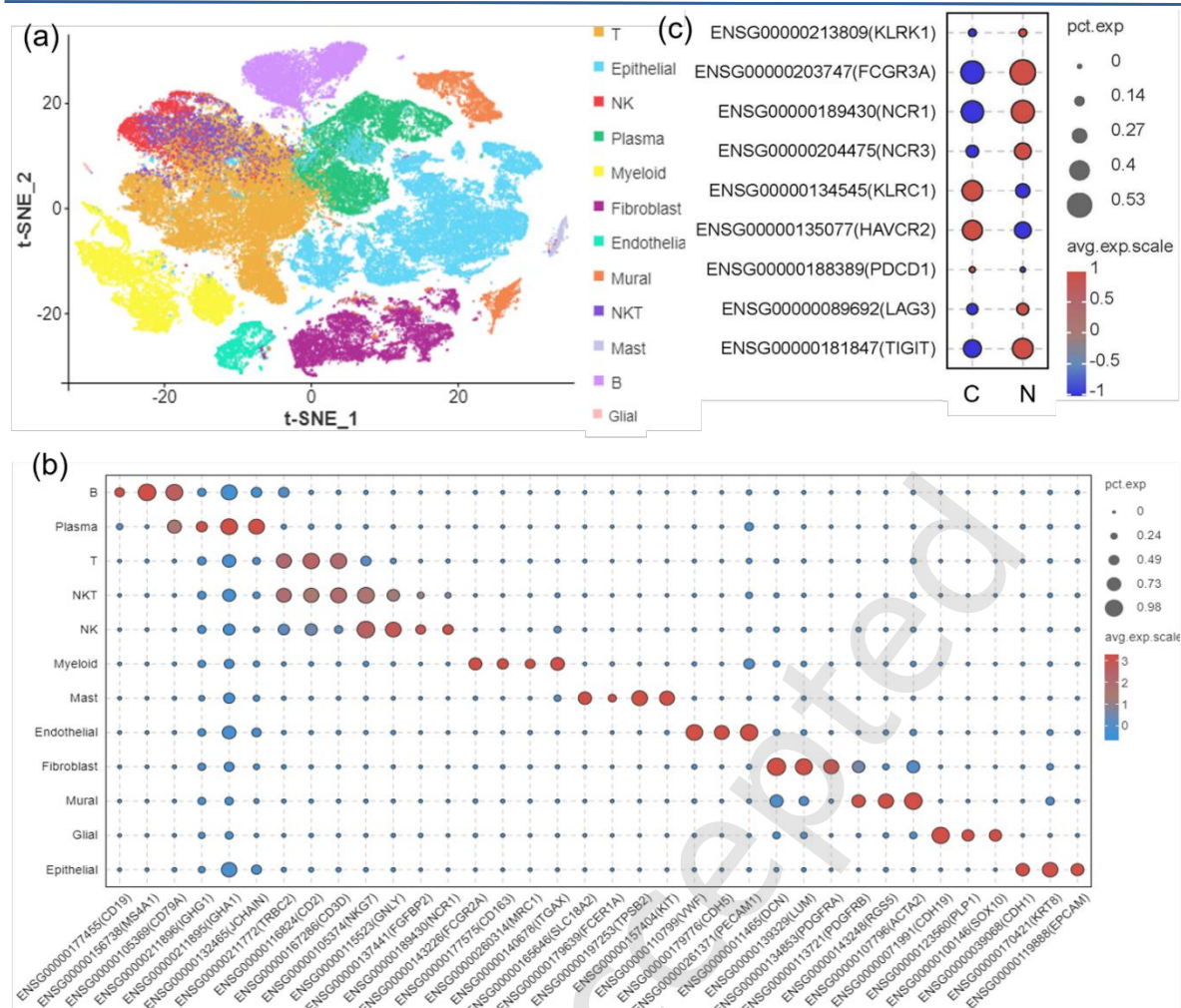


Figure S2 Overview of single cells isolated from five primary gastric cancer tissues and paired paracancerous samples. (a) t-Distributed Stochastic Neighbor Embedding (t-SNE) plot of the analyzed single cells. Each color represents one cluster. Annotated cell types are listed. (b) Bubble heatmap showing the expression of marker genes for the major cell types. (c) Bubble heatmap showing the genes of activating and inhibitory receptors in gastric cancer (C) and non-cancerous (N) tissues.

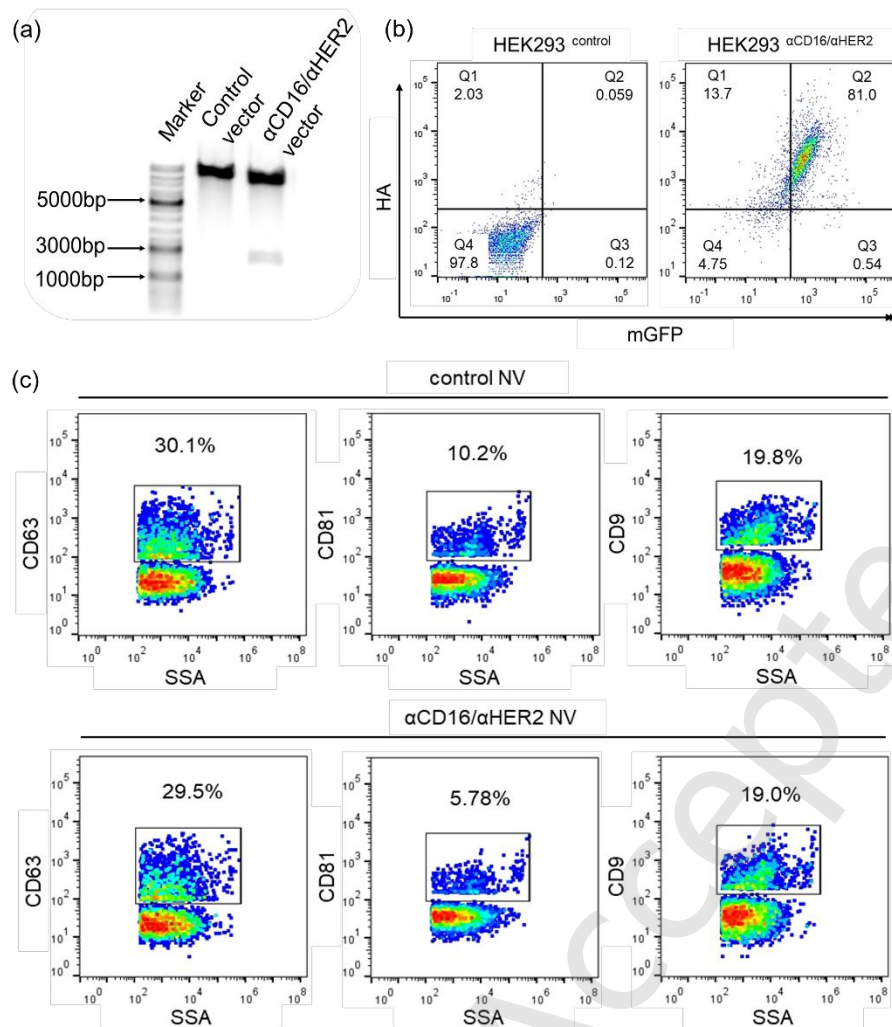


Figure S3 Characterization of α CD16/ α HER2 NV. (a) Gel analysis of plasmids expressing HA-tagged fusion proteins. (b) Expression of HA-tagged fusion protein on HEK293 cells analyzed by flow cytometry. (c) Expression of CD63, CD81, and CD9 on control and α CD16/ α HER2 NVs analyzed by nanoflow cytometry.

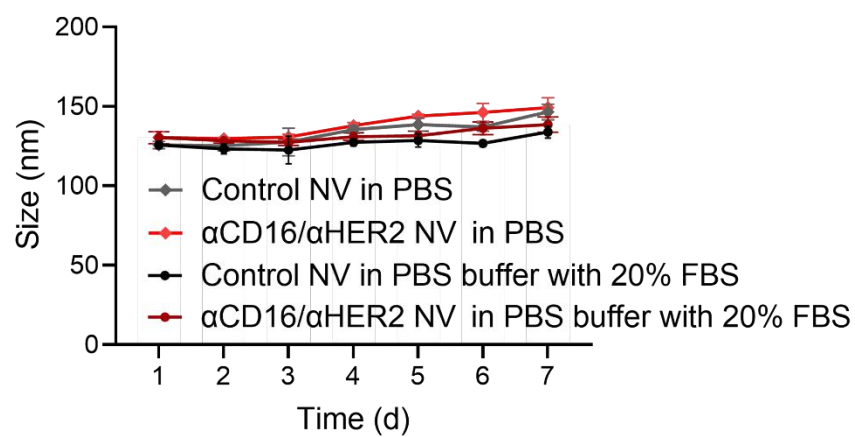


Figure S4 Stability of α CD16/ α HER2 NVs. Particle sizes of NVs in PBS buffer and PBS buffer with 20% FBS were measured by NTA (n=3).

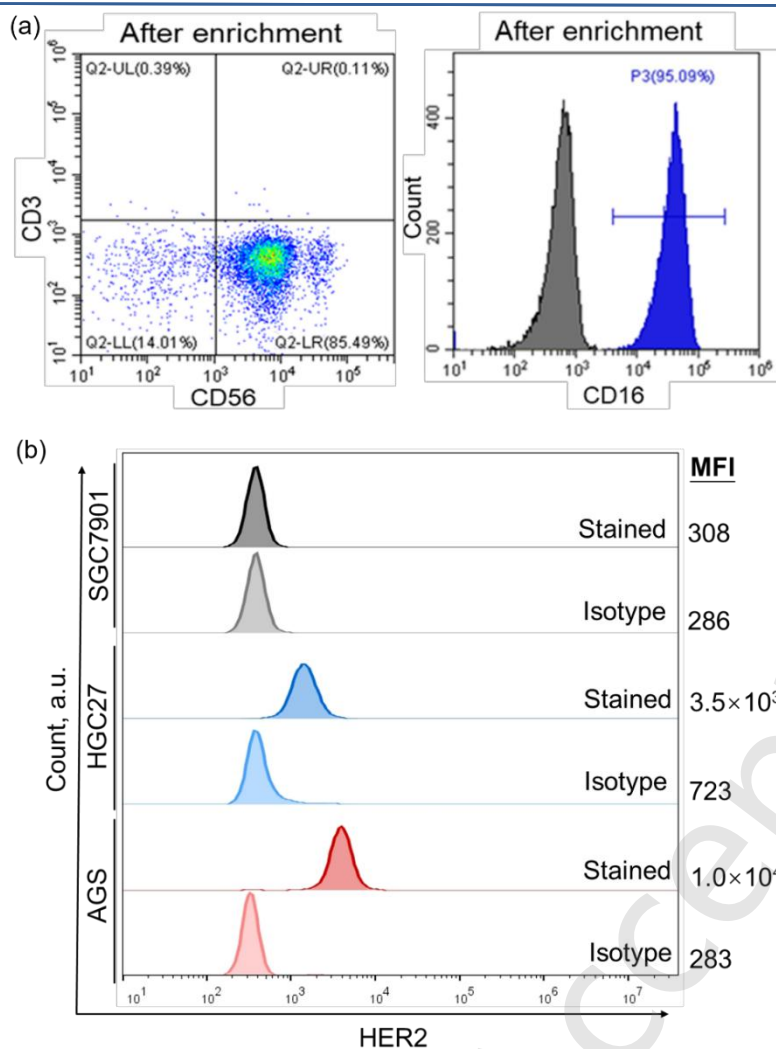


Figure S5 (a) Characterization of NK cells isolated from the peripheral blood of patients with gastric cancer. (b) Flow cytometric analysis of HER2 expression on three human gastric cancer cell lines, including SGC7901, HGC27, and AGS cells. Equal numbers of cells were incubated with PE-anti-human HER2 antibody or isotype antibody in PBS with 1% FBS at 4°C for 30 min.

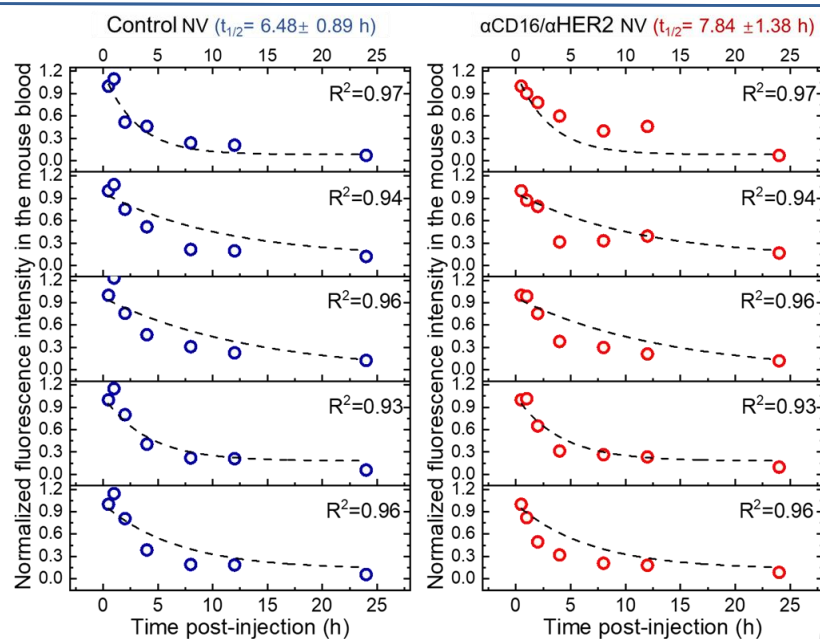


Figure S6 Pharmacokinetics of α CD16/ α HER2 NVs in mice. Fluorescently labeled NVs were injected into mice bearing subcutaneous tumors of HGC27 cells (HER2⁺). The fluorescence intensity of mouse blood was plotted after i.v. injection of control NVs and α CD16/ α HER2 NVs versus the sampling time, fitting into an exponential function, so the exponential decay constant was acquired for conversion into the circulation half-time ($t_{1/2}$). Data were shown as mean $\pm$ SD (n=5). The $t_{1/2}$ values of control NVs and α CD16/ α HER2 NVs are (6.48 $\pm$ 0.89) h and (7.84 $\pm$ 1.38) h, respectively.

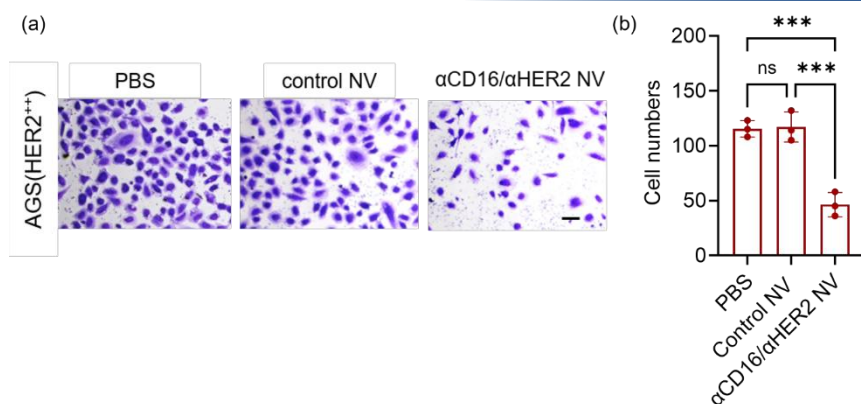


Figure S7 Human NK cells (effector cells) were incubated with gastric cancer AGS cells (target cells) at an E:T ratio of 5:1 in the presence of control or αCD16/αHER2 NVs. (a) Representative images showing cytotoxicity of αCD16/αHER2 NVs and (b) the quantitative cytotoxicity measures. ns, not significant; *** $p < 0.001$; **** $p < 0.0001$. Data were shown as mean $\pm$ SD (n=3).

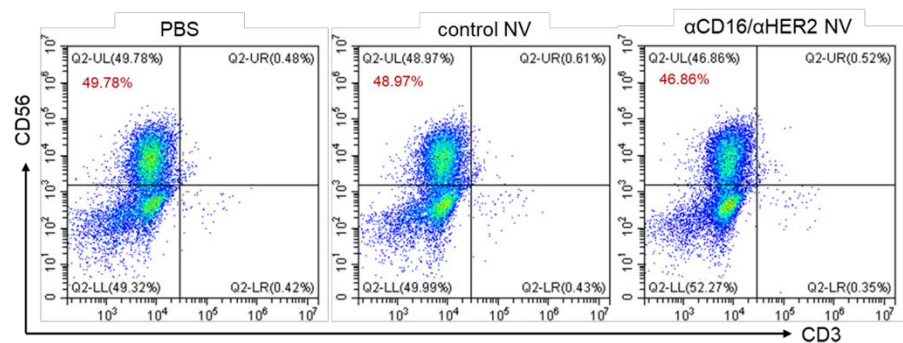


Figure S8 Presence of NK cells purified from human peripheral blood in NCG mice. Representative dot plots showed NK cell (CD3⁺CD56⁺) populations in different groups.

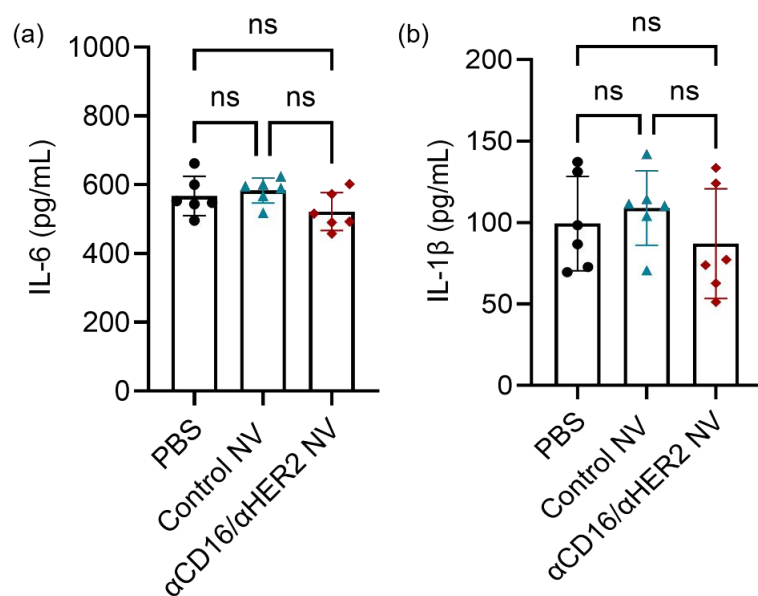


Figure S9. Concentrations of serum IL-6 (a) and IL-1 β (b) from different groups in healthy BALB/c mice. ns, not significant. Data were shown as mean $\pm$ SD (n=6).

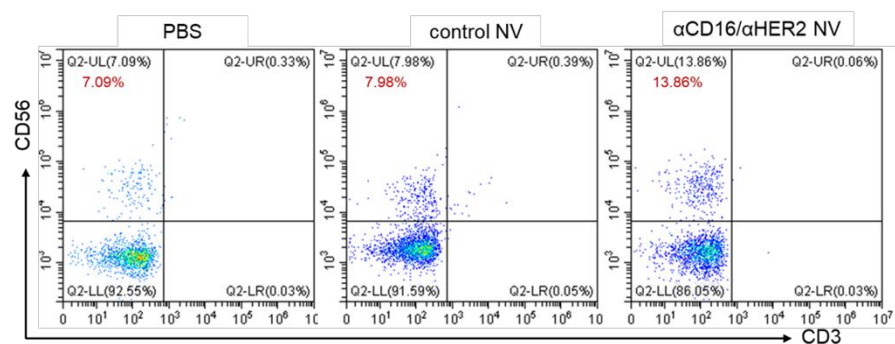


Figure S10 Representative dot plots of NK cell infiltration into tumor tissues from different groups.