Original Article

Antitumor effects and persistence of a novel HER2 CAR T cells directed to gastric cancer in preclinical models

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Abstract: New immunotherapeutic approaches are urgently needed for gastric cancer due to its poor survival and unsatisfactory treatment. Here we applied the humanized chA21 scFv based chimeric antigen receptor (CAR) modified T cells approach to the HER2 overexpressing gastric cancer treatment. The chA21-4-1BBz CAR T cells specifically exerted Th1 skewed cytokine response and efficient cytolysis of HER2 overexpressing human gastric cancer cells in vitro. Both the cytokine production and cytotoxicity levels were correlated with the level of HER2 surface expression by tumor cells. In established subcutaneous xenograft and peritoneal metastasis models, chA21-4-1BBz CAR T cells dramatically facilitated regression of HER2 overexpressing tumor and prolonged survival of tumor-bearing mice, whereas spared the progression of HER2 low-expressing tumor. Additionally, the capability of these CAR T cells to persist in circulation, as well as specifically home to, and accumulate in tumor sites were identified. Taken together, these results provide the basis for the future clinical investigation of the humanized chA21 scFv based, 4-1BB costimulated CAR T cells for the treatment of gastric cancer, and other HER2-expressing solid tumors.

Keywords: Chimeric antigen receptor, HER2, gastric cancer, T cells, immunotherapy

Introduction

Gastric cancer (GC) is the fifth most frequently diagnosed cancer and the third leading cause of death from cancer in the world, and its estimated incidence and mortality rates is highest in East Asia [1]. Despite the development of multimodality therapies such as surgery, chemotherapy and radiation therapy, the mortality rates of metastatic GC remain dismal. The identification of human epidermal growth factor receptor 2 (HER2) overexpression in GC represented a significant step towards unraveling the molecular complexity of this disease [2, 3]. The reported rates of HER2 overexpression in GC patients range from 9% to 23% [4]. The ToGA study, a randomized, prospective, multi-center phase III trial, established trastuzumab as the first targeted therapeutic for patients with HER2-positive advanced or metastatic GC [5]. Nevertheless, benefits from trastuzumab remain unsatisfactory in survival and some drawbacks are arising, such as cost, need for continuous treatment, increased risk of cardiac toxicity, what’s more, the development of resistance. Therefore, novel and powerful therapeutic strategies for treatment of GC are urgently needed, which prompted us to study active immunotherapeutic strategies targeting HER2.

Among the immunotherapies that have been incorporated into cancer treatment, adoptive transfer of T cells expressing chimeric antigen receptor (CAR) has been at the forefront for the last decade [6, 7]. Introduction of CAR into T cells enables these effector cells to recognize tumor associated antigen (TAA) via the single-chain variable fragment (scFv) and activate T cells through the cytoplasmic signaling domains, releasing perforin, granzyme and various cytokines to exert potent anti-tumor effect. Thus, CAR T cells function in non-MHC restricted manner, which cleverly combine the potent tumor-killing capacity of cytotoxic T cells and
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the specific antigen recognition of antibody together [8]. The CAR T cells approach first showed compelling success in eradicating hematological tumors, and later also particular relevance and encouraging results for solid tumors in both preclinical studies and early phase clinical trials [6, 9]. They showed CAR T cells can actively and specifically home to tumor sites via the antigen recognition and also expand and persist in vivo over a long term [10, 11]. Compared with monoclonal antibody (mAb) therapy, CAR T cells approach is more effective in generating durable tumor response and also providing stronger penetrability in solid tumors with lower risk of resistance.

Based on our previous study [12], we move forward to evaluate the anti-tumor activity, persistence, and application feasibility of chA21 scFv-based CAR T cells in HER2 overexpressing gastric cancer, attempting to establish an alternative targeted therapy for GC patients in preclinical settings. In the present study, our HER2 CAR was incorporated a costimulatory domain 4-1BB (CD137), which may be superior to CD28 for persistence and antitumor activities [8, 13-16]. Our results showed that the chA21-4-1BBz CAR T cells specifically reacted against HER2 positive tumor cells in vitro; and these CAR T cells persisted in circulation, specifically homed to, and accumulated in tumor, which caused sustained inhibition of HER2 overexpressing tumor in established human gastric cancer xenograft models.

Materials and methods

Cells

The human GC cell lines NCI-N87, HGC-27, MKN-45, BGC-823, MKN-28 and 293 T cells were purchased from the China Infrastructure of Cell Line Resource; the ovarian cancer cell line SKOV3 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All tumor cell lines, 293T and PBMCs were cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum and 2 mM glutamine (all from Life Technologies).

CAR construction and lentivirus production

The chA21-4-1BBz CAR sequences were preceded in frame by a green fluorescent protein (GFP) sequence followed by the 2A ribosomal skipping sequence was generated through gene splicing by multistep overlap extension PCR (OE-PCR) as described in previous study [12]. The CAR construct comprise the chA21 scFv linked to a CD8α hinge and transmembrane region, followed by an 4-1BB intracellular domain and a CD3ζ signaling motif. The CAR construct was then subcloned into pSin lentiviral backbone to create the pSin-chA21-4-1BBz plasmid. Lentiviral particles were produced by transfecting 293 T cells with the lentiviral expression plasmid and the packaging plasmids. Briefly, 293 T cells were seeded into a 75-cm² flask, and Lipofectamine 2000 (Invitrogen) was used as the transfection reagent at a ratio of 1 μg of DNA to 1.5 μl of Lipofectamine, according to the manufacturer’s instructions. The following amounts of DNA per 75-cm² flask were used: 7.5 ug of chA21-4-1BBz CAR transgene plasmid, 3.5 ug pVSV-G (VSV glycoprotein expression plasmid), 9 ug pRSV.REV (Rev expression plasmid) and 9 ug pMDLg/p.RRE (Gag/Pol expression plasmid). Supernatants were collected 24 hours and 48 hours after transfection, concentrated 10-fold by ultracentrifugation for 3 hours at 25,000 rpm with a Beckman SW32Ti rotor (Beckman Coulter). Titration of the concentrated lentiviral vectors were determined by transfecting 293 T cells with a series of 3-fold lentiviral dilutions.

Generation of CAR engineered T cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and then activated with anti-CD3/CD28 beads (Miltenyi Biotec). Twenty-four hours after activation, PBMCs were transduced with lentiviral vectors at a multiplicity of infection (MOI) of 5 and expanded for approximately 2 weeks. PBMCs were cultured in complete RPMI 1640 medium with addition of 100 IU/ml human recombinant interleukin 2 (IL-2) (PeproTech, Suzhou, China). T cells were rested without IL-2 support for 1-2 days before functional assays.

Flow cytometric analysis

All anti-human CD45, CD3, CD45RO, CD62L antibodies were purchased from BioLegend (San Diego, CA, USA). All anti-human CD45, CD3, CD45RO, CD62L antibodies were purchased from BioLegend (San Diego, CA, USA). Cell surface expression of HER2 was detected by fluorescein isothiocyanate anti-HER2 antibody (clone 24D2; Bio-
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Legend) and matched isotype control antibodies. CAR expression was detected by R-Phycoerythrin-AffiniPure F(ab’)2 antigen-binding fragment goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Flow cytometric data were analyzed using FlowJo version 7.2.5 software (TreeStar, Ashland, OR, USA).

Cytokine release assays

Cytokine release assays were performed by coculture of $1 \times 10^5$ T cells with $1 \times 10^5$ target cells per well in triplicate in 96-well flat-bottom plates. After 24 hours incubation, cell-free supernatants were harvested and assayed for the presence of IFN-γ, IL-2, TNF-α, IL-4, IL-6, IL-10, IF-17A cytokines by flow cytometry using the Multi-Analyte Flow Assay Kit (BioLegend LEGENDplex™). The assay was performed according to manufacturer’s instructions and analyzed on LEGENDplex™ software.

Cytotoxicity assays

The cytolytic activity of T cells was measured by lactate dehydrogenase (LDH) release assay kit (Biyotime, China). T cells were coincubated with target cells at an effector: target cell ratio (E/T ratio) of 1:1, 3:1, 10:1, 30:1 in triplicate in 96-well plates for 24 hours. The LDH concentrations and the absorbance values were obtained in culture supernatants according to the manufacturer’s instructions. The percentage of specific cytotoxicity was calculated using the following formula: % Cytotoxicity = (Experimental LDH release-Spontaneous LDH release)/(Maximal LDH release-Spontaneous LDH release) × 100. The experimental LDH release is the LDH released on coculture of effector and target cells, whereas the spontaneous release is the LDH released from tumor cells in the absence of effector cells. The maximal LDH release represents the release after addition of Triton X-100 (100% LDH release) to cells.

Xenograft model of gastric cancer

The animal studies were approved by the Animal Ethics Committee of Shandong University. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Beijing HFK Bioscience Co (Beijing, China). Six- to eight-week-old female mice were bred and maintained under pathogen-free conditions in-house according to protocols approved by the Shandong University Institutional Animal Care and Use Committee.

For the subcutaneous model of human GC, mice were subcutaneously (s.c.) inoculated with $3 \times 10^6$ NCI-N87 tumor cells on the left flank and $3 \times 10^6$ MKN-28 tumor cells on the right flank at the same time. Six tumor-bearing mice per group were randomized to two groups before treatment. When tumor volume reached more than 300 mm$^3$, $1 \times 10^7$ chA21-4-1BBz CAR T cells (50% CAR+) or Untransduced T (UT) cells were given twice on day 40 and 45 post-tumor inoculation to the two groups of mice by tail vein injection. Tumor dimensions were measured with calipers, and tumor volumes were calculated using the following formula: $V = \frac{1}{2} \times (\text{length} \times \text{width}^2)$, where length is the greatest longitudinal diameter and width is the greatest transverse diameter. On day 80 post-tumor inoculation, tumors were resected immediately after euthanasia for weight measurement and immunohistochemistry. For the peritoneal model of human GC, mice were intra-peritonally (i.p.) injected with $3 \times 10^6$ NCI-N87 tumor cells. Six tumor-bearing mice per group were randomized to two groups before treatment. $1 \times 10^7$ chA21-4-1BBz CAR T cells or UT T cells were given twice on day 7 and 10 post-tumor inoculation to the two groups of mice by intraperitoneal injection. Mice were monitored daily until death or day 90 post-tumor inoculation and then necropsied for exploration of ascites or nodular peritoneal tumors.

Immunohistochemistry analysis

The mice were killed by carbon dioxide (CO$_2$) inhalation, and the tumors were removed and frozen in Tissue-Tek O.C.T. compound at -80°C. A standard streptavidin horseradish immunoperoxidase method was used for human CD3 staining as described previously [12].

Statistical analysis

All data are reported as means ± SD. Data were analyzed using GraphPad Prism version 5.0. Statistical analysis was performed by analysis of variance for the tumor burden (tumor volume). Student’s t-test was used to evaluate differences in cytokine secretion and specific cytolyis. $P$-values <0.05 were considered significant.
Results

**HER2-specific chA21-4-1BBz CAR construct and expression on primary human T cells**

Bicistronic HER2 CAR vector incorporated an upstream green fluorescent protein (GFP) sequence to confirm engineered T cell detection. The humanized scFv chA21 has a high binding affinity for HER2 \((K_d=11 \text{ nm})\) [17], linked the CD8α hinge and transmembrane (TM) domain followed by the 4-1BB and CD3z intracellular signaling domains, and named chA21-4-1BBz CAR (Figure 1A).

The activated primary human PBMCs were efficiently transduced with CAR lentiviral vector with transduction efficiencies reproducibly above 50%. Bicistronic expression vectors incorporating a 2A peptide sequences permitted dual expression analysis of GFP and the chA21-4-1BBz CAR. Engineered HER2 CAR and GFP displayed a linear coexpression pattern on CD3+ T cells (Figure 1B). More than 70% CAR transduced T cells expressed the CD45RO+ CD62L+ central memory T phenotype, consistent with what we observed in previous study (data not shown) [12]. Studies have proved that adoptive cell transfer of less differentiated T cell subsets exhibited superior in vivo expansion, persistence, and antitumor capacities [18, 19]. With continuous human recombinant IL-2 supplement, chA21-4-1BBz CAR T cells and untransduced T cells (UT T cells) both expanded, to a similar extent (55-60 fold) after 14 days culture (Figure 1C), then we cut off IL-2 supplement to let T cells fully rest down prior to functional assays.

**chA21-4-1BBz CAR T cells mediated antigen specific recognition and expansion**

HER2 surface expression on a panel of GC cell lines was analyzed by flow cytometry. The majority of lines including NCI-N87, HGC-27, MKN-45 and BGC-823 expressed a high to moderate level of HER2, however lower level of expression was detected on MKN-28 cells (Figure 2A). The panel of GC cell lines with varying levels of HER2 expression was assembled for functional assays, meanwhile, the HER2 overexpressing ovarian cancer cell line SKOV3 was used as a known positive comparison.

As revealed by the cytokine release assays, chA21-4-1BBz CAR T cells specifically produced
Figure 2. chA21-4-1BBz CAR T cells mediated antigen specific recognition and expansion. A. Surface HER2 expression by a panel of human gastric cancer cell lines (NCI-N87, HGC-27, MKN-45, BGC-823, MKN-28) and ovarian cancer cell line SKOV3 was analyzed by flow cytometry by staining with anti-HER2 monoclonal antibody (black thick lines) and control isotype antibody (filled gray histograms). Mean fluorescence intensity (MFI) of each analysis was annotated. Experiments were repeated three times with similar results and the figure above shows a representative example. B. chA21-4-1BBz CAR T cells secreted high levels of IFN-γ, IL-2, TNF-α but little to no amounts of IL-4, IL-6, IL-10, IL-17A cytokines following a 24 hour incubation with HER2 overexpressing tumor cell lines at effector: target cell ratio (E/T ratio) of 1:1. Cell-free supernatants were harvested and detected for multiple cytokines by cytometric bead array. Supernatants collected from T cells alone were used as negative controls. Mean cytokines concentrations ± SD (in pg/ml) from three repeated experiments with triplicate cultures were shown. C. Direct correlation of
great amounts of IFN-γ, IL-2 and TNF-α cytokines, while little to no IL-4, IL-6, IL-10, IL-17A cytokines when stimulated with HER2 overexpressing tumor cell lines SKOV3 and NCI-N87, suggesting a preferential Th1-cytokine response (Figure 2B). Nevertheless, chA21-4-1BBz CAR T cells secreted much lower levels of cytokines when incubated with HGC-27, MKN-45, BGC-823 cells; and almost undetectable reactivity when in coculture with MKN-28 cells. Generally, the amount of cytokines secreted positively correlated with the level of HER2 surface expression by target cells (Figure 2C). As expected, UT T cells did not secret cytokines after HER2 positive tumor cells stimulation.

Figure 3. chA21-4-1BBz CAR T cells redirected antigen-specific lysis toward human HER2 overexpressing tumor cells. A. Antigen-specific killing of HER2 overexpressing tumor cells by chA21-4-1BBz CAR T cells was determined by LDH release assay. chA21-4-1BBz CAR T cells showed graded levels of cytolytic activity in response to HER2 overexpressing tumor cells when set in different E/T ratio. UT T cells served as controls. Mean and SD were shown from three repeated experiments with triplicate cultures. B. In the coculture system of chA21-4-1BBz CAR T cells with NCI-N87 cells, formation of T cell clusters and lysis of NCI-N87 cells were visible.

We next evaluated the T cells expansion capacity with stimulation of specific tumor antigen. Rested CAR and UT T cells were further cocultured with NCI-N87 or MKN-28 cells respectively, and a repeated tumor stimulation was performed 4 days later. We observed that chA21-4-1BBz CAR T cells underwent numerical expansion when stimulated with NCI-N87 cells, while no substantial cell expansion when with MKN-28 cells. The evident increased expansion of CAR T cells compared with UT T cells in response to antigen stimulation was in agreement with the cytokine secretion activity, jointly confirming the antigen specificity for CAR T cells expansion and activity (Figure 2D).

IFN-γ, IL-2, TNF-α concentration (pg/ml) produced by chA21-4-1BBz CAR T cells with HER2 expression level (mean fluorescence intensity, MFI) of coincubated tumor cells were demonstrated. R² shown in each Scatter gram were determined by linear regression analysis. D. Viable cell counting every other day showed effective expansion of rested chA21-4-1BBz CAR T cells when stimulated twice with NCI-N87 cells but not with MKN-28 cells. Rested UT T cells showed no substantial cell expansion with stimulation of either tumor cell lines. Mean and SD were shown from three repeated experiments for T cells from different donors.
**cha21-4-1BBz CAR T cells redirected antigen-specific lysis toward human HER2 overexpressing tumor cells**

Consistent with cytokine production results, the cytotoxic capability of CAR T cells were also positively associated with the target tumor antigen levels. The cha21-4-1BBz CAR T cells exhibited direct and efficient lysis toward SKOV3 and NCI-N87 cells, decreased lyse toward MKN-45 cells, but no distinct lyse toward MKN-28 cells compared with the baseline killing activity of UT T cells (Figure 3A). CAR T cells showed graded levels of cytolytic activity in response to HER2 overexpressing tumor cells when set in different effector: target cell ratio (E/T ratio). The cell killing efficiency can achieve more than 80% at the indicated E/T ration of 30:1. Invariably, UT T cells didn’t exhibit distinct cell killing activity toward all tumor cell lines with whatever HER2 expression levels (Figure 3B). It was visible of the CAR T cells clusters formation and the NCI-N87 tumor cells lysis under the microscope, whereas the UT T cells did not proliferate well and died gradually (Figure 3B). These data support that the cha21-4-1BBz CAR T cells could maintain potent killing activity against tumor cells expressing high levels of HER2 while mostly spare cells with low or none levels of HER2 expression.

**cha21-4-1BBz CAR T cells specifically infiltrated, persisted, and delayed progression of HER2 overexpressing gastric tumor in vivo**

The antitumor efficacy of HER2 CAR was evaluated in a xenograft model of large, established cancer. NOD-SCID mice were inoculated subcutaneously (s.c.) with HER2 overexpressing NCI-N87 cells and HER2 low-expressing MKN-28 cells on opposite flanks. Tumor-bearing mice were intravenously (i.v.) treated with cha21-4-1BBz CAR T or UT T cells on day 40 and 45 post-tumor inoculation, when tumor volume reached more than 350 mm² (Figure 4A). NCI-N87 xenograft treated with CAR T cells experienced rapid tumor regression which was significantly better than UT T cells. By contrast, MKN-28 tumor grew progressively in mice receiving CAR or UT T cells (Figure 4B and 4C). Mice bearing NCI-N87 tumor treated with UT T cells had heavier tumor burden compared with mice treated with CAR T cells (648 ± 18 mg vs 268 ± 50 mg, P<0.001). While the tumor weight in MKN-28 xenograft mice treated with CAR T cells or UT T cells was not significantly different (718 ± 79 mg vs 678 ± 72 mg, P>0.05), confirming the efficient tumor-inhibiting function of CAR T cells toward HER2 overexpressing GC (Figure 4D). We can also conclude that cha21-4-1BBz CAR T cells selectively inhibited the progression of HER2 overexpressing but exactly spared the HER2 low-expressing xenograft in vivo. The selective tumor elimination further verified the antigen specificity and functional precision of adoptive CAR T cells therapy.

Four weeks after the second T cell dose, peripheral blood CD3⁺ T cell counts from mice injected with cha21-4-1BBz CAR T cells were significantly higher than in the UT T cells treated group (Figure 4E). Invariably, obvious accumulation of human CD3⁺ T cells was observed in regressing NCI-N87 lesions while very few human T cells in the UT T cells treated group of NCI-N87 lesions and both groups of MKN-28 lesions (Figure 4F), which was inversely correlated with the tumor burden of each group (Figure 4B). These results indicated that the CAR-based recognition can induce T cells homing to tumor sites and exerting anti-tumor function.

**cha21-4-1BBz CAR T cells functioned in the gastric cancer peritoneal metastasis and prolonged survival**

Furthermore, an advanced intraperitoneal (i.p.) metastatic cancer model was established to evaluate the functional activity of HER2 CAR T cells against tumor in a more physiologically relevant compartment. NOD-SCID mice were inoculated i.p. with HER2 overexpressing NCI-N87 cells. On days 7 and 10 post-tumor inoculation, mice were i.p. treated with cha21-4-1BBz CAR T cells or UT T cells (Figure 5A). Noticeable abdomen distension and slower movement were observed within all six UT T treated mice 30 days post-tumor inoculation, while no observable ascites or tumor signs in all six mice treated with CAR T cells. During day 40-55 days post-tumor inoculation, all six mice in UT T cells treated group died and all the dead mice were found bloody ascites and multiple nodular peritoneal tumors under abdominal exploration (Figure 5B). However, only two mice of tumor-related mortality occurred in the CAR T cells treated group during a 90 days long term obser-
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Figure 4. chA21-4-1BBz CAR T cells specifically infiltrated, persisted, and delayed progression of HER2 overexpressing gastric tumor in vivo. A. Schematic diagram of subcutaneously transplanted human gastric cancer model established in NOD/SCID mice. $3 \times 10^6$ HER2 overexpressing NCI-N87 cells and $3 \times 10^6$ HER2 low-expressing MKN-28 cells were inoculated subcutaneously (s.c.) on opposite flanks. On days 40 and 45 post-tumor inoculation, mice were intravenously (i.v.) treated with $1 \times 10^7$ chA21-4-1BBz CAR or UT T cells by two different groups (n=6). B. On day 80 post-tumor inoculation, tumors were dissected immediately after euthanasia for weight measurement, and photograph of all six samples from four groups are shown. C. As measured by caliper-based sizing [$V=\frac{1}{2} \times (\text{length} \times \text{width}^2)$] for tumor volume every four days until euthanization on day 80, NCI-N87 tumor growth in the chA21-4-1BBz CAR T cells treated group regressed noticeably, while not in the UT T cells treated group. MKN28 tumor grew progressively in mice receiving CAR or UT T cells. Mean and SD of tumor volume (mm$^3$) were shown with n=6 for all groups. D. Tumor weight of all groups at 80 days post tumor implantation. Data are expressed as mean ± SD (mg) with n=6 for all groups. ****P<0.0001 (Student’s t-test). E. Peripheral blood from the tumor-bearing mice were collected 30 days after the last T cell dose and then quantified for persistent CD3$^+$ human T cells proportion by flow cytometry with staining anti-human CD3 monoclonal antibody (mAb). A certain amount of human T cells were found persistent in circulation of chA21-4-1BBz CAR T cells treated mice while not in UT T cells treated mice. F. The localization of human T cells in tumor xenograft was detected by immunohistochemical staining with anti-human CD3 mAb (brown). Human CD3$^+$ T cells was observed obviously in regressing NCI-N87 lesions compared with UT T cells treated group of NCI-N87 lesions and both groups of MKN-28 lesions. Representative sections are shown at 50 × original magnification.
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Discussion

In China, many GC patients present with inoperable or metastatic disease during their first visit and have an inauspicious prognosis with a median overall survival of less than 1 year [1]. The HER2 based targeted therapy has been proved to significantly improve the survival of patients with HER2 overexpressing GC. However, the effects are usually transitory because acquired resistance can arise. HER2 CAR T cells therapy that combines the anti-HER2 mAb specificity and efficient T cell effector function is expected to be more effective than mAb therapy. HER2 has been targeted by CAR T cells therapy in many tumor models, including breast cancer [16, 20, 21], ovarian cancer [22], biliary tract cancers and pancreatic cancer [23, 24], medulloblastoma [25], glioblastoma [26], and osteosarcoma [27]; some of the HER2 CAR T cells therapy have been evaluated in phase I/II clinical trials [24, 28]. However, this novel therapy for GC has not yet been broadly evaluated.

It has been proved that the incorporation of costimulatory molecules can lead to increased cytokine production and an enhanced ability of CAR T cells to proliferate and mediate tumor regression, and the majority of active clinical trials are testing second-generation CARs. CD28 and 4-1BB have been most commonly tested in clinical trials [11, 29]. Previously, we have demonstrated the anti-tumor activity of a CD28 costimulated HER2 CAR in breast cancer [12]. Here, we extend our work to GC, incorporating 4-1BB (CD137) costimulation signaling domain into our CAR construct, which appears superior to that of CD28 signaling domain and supports enhanced persistence of CAR T cells in multiple clinical trials. Recent studies have

Figure 5. chA21-4-1BBz CAR T cells functioned in the gastric cancer peritoneal metastasis and prolonged survival.
A. Schematic diagram of human gastric cancer peritoneal metastasis model established in NOD/SCID mice. $3 \times 10^6$ NCI-N87 cells were intraperitoneally injected (i.p.) to NOD-SCID mice, on day 7 and 10 post-tumor inoculation, mice were i.p. treated with $1 \times 10^7$ chA21-4-1BBz CAR or UT T cells by two different groups (n=6). B. Bloody ascites and multiple nodular peritoneal tumors were found in the UT T cells treated group under abdominal exploration while not noticeable in the CAR T cells treated group. C. Kaplan-Meier overall survival of tumor-bearing mice treated with either chA21-4-1BBz CAR T or UT T cells. A log-rank test verified significant differences between chA21-4-1BBz CAR T and UT T cells treated groups, $p$-value =0.0005.
demonstrated that 4-1BB but not CD28 costimulatory domain significantly enhanced persistence of CD19 CAR T cells in patients [15]. Mechanistically, 4-1BB costimulation enhances catabolic activity including fatty-acid oxidation and mitochondrial biogenesis, which support central memory cell differentiation and prolonged persistence. While the CD28 enhance glycolysis and yield more short-lived effector memory cell maturation. The choice of a CAR signaling domain can impact the subsequent fate of the T cells and the incorporation of 4-1BB in HER2 CARs may be superior to CD28 for persistence and antitumor activities [8, 13-16]. Besides, exploiting humanized scFv is of great significance for the safety and persistence of CAR T cells in clinical use. Humanized chA21 scFv was still utilized in our construct, which should be less immunogenic in humans and is therefore more desirable candidate for CAR therapy [30, 31]. Nevertheless, the toxicity and immunogenicity of chA21 scFv need to be further addressed in future clinical trials.

In vitro, results from our coculture assay showed that the chA21-4-1BBz CAR T cells exerted a Th1 skewed cytokines (IL-2, TNF-α, IFN-γ) response upon specific targets encounter, and both the cytokine production and cytotoxicity levels were correlated with the target HER2 levels. The cytokine secretion of CAR T cells after exposure to tumor cells is closely related to their killing activity [32]. IL-2 is pivotal for the proliferation and differentiation of CAR T cells to become effector T cells. The high levels of TNF-α may induce tumor cell apoptosis and a temporary tumor vasculature shut down that may limit early infiltration by endogenous T cells [33]. IFN-γ produced by CAR T cells contributed to exerting immune surveillance of tumors, which can directly inhibit proliferation and induce apoptosis of malignancies in vitro and in vivo through elusive mechanisms [34]. Little to no amounts of Th2 cytokines IL-4, IL-6 and IL-10 were produced by T cells when co-cultured with those HER2 expressing tumor cell lines. IL-17 was not produced by our CAR T cells, as inducible T cell costimulator (ICOS)-costimulated CAR T cells secrete high levels of IL-17, but 4-1BB or CD28-based CAR T cells do not [35]. Further, the chA21-4-1BBz CAR T cells lysed HER2 overexpressing GC cells with high efficiency, with more than 80% killing efficiency after 24 hours cocubation at the indicated E/T ratio of 30:1, and even a high level of 40% at 1:1 E/T ratio. At the time of preparation of this manuscript, Song et al. also evaluated in vitro the efficacy of a 4-1BB costimulated HER2-directed CAR T cells against gastric cancer [36]. Likewise, they observed elevated cytokines release as well as potent cytotoxic activity by HER2 CAR T cells, when cocultured with HER2+ tumor cells. Moreover, they verified these results in patient-derived HER2+ GC cells.

In vivo, we revealed first by the s.c. xenograft model that systemically delivered chA21-4-1BBz CAR T cells provide significant growth control of HER2 overexpressing tumors, whereas the progression of HER2 low-expressing tumors were almost unabated. This s.c. model is generally accepted to represent a test of T cell therapy more stringent than other models involving injection of T cells directly into the tumors. Consistent with the previous studies [10, 12], tumor response was associated with enhanced T cell persistence and tumor localization in vivo based on the analysis using flow cytometry and immunohistochemical analysis of tumor tissue. Tumor regression and T cell persistence were attainable via systemic T cell delivery, suggesting the capacity of transferred HER2 CAR T cells to circulate, home to tumor, expand, and perform antitumor functions. Song and colleague's results from their s.c. xenotransplanted model also agree with our findings of inhibited HER2 overexpressing tumor growth, persistent CAR T cells in the blood and obvious accumulation of CD3+ cells in tumors [36]. Better yet, in our s.c. human GC model, we set HER2 low-expressing MKN-28 tumor as self-control for better parallel HER2 overexpressing tumor growth, in consideration of the “on target, off tumor” effect of HER2 CAR T cells [37]. MKN28 cells may represent normal tissues with very low level of HER2 expression, which did not trigger remarkable antitumor activity both in vitro and in vivo mouse model. However, the low level of HER2 expression in vital normal tissue, such as lung epithelial cells, still raise safety concerns related to cytokine release syndrome (CRS) from “on target, off tumor” recognition of HER2 [32, 37]. Researchers have always been exploring and developing new approaches to prevent or mitigate toxicity [9, 38]. Briefly, affinity-tuned HER2 CAR, dual targeting CAR, truncated tag CAR, switchable CAR and inhibitory CAR are currently
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being explored [38, 39], which provide multiple safe platforms for HER2 CAR based therapies. We will further explore this problem in our follow-up study.

Moreover, we also validated the therapeutic effects of chA21-4-1BBz CAR T cells in mice with peritoneal xenografts that exhibited the clinically relevant features of spreading to peritoneal cavity and forming bloody ascites. Tumor growth in the i.p. metastatic GC model was significantly delayed and mice survival was extended by i.p. administration of two doses of CAR T cells. Notably, i.p. transfer of UT T cells had no impact on tumor progression and survival, and 100% (6/6) control mice developed bloody ascites. To our best knowledge, we are the first to provide preclinical evidence for effectively targeting gastric cancer metastasis to the peritoneal cavity with local i.p. delivery of HER2 CAR T cells. There's growing understanding that the administration way of CAR T cells is of great importance for its safety and efficacy. Katz et al. demonstrated that regional i.p. delivery of anti-CEA CAR T cells resulted in superior tumor killing compared with systemic delivery for peritoneal metastasis of colorectal cancer (CRC) [40]. Most recently, by using human xenograft models of breast cancer metastasis to the brain, Priceman et al. demonstrated potent therapeutic efficacy of local intratumoral and regional intraventricular delivery of HER2 CAR T cells [16]. Regional delivery of CAR T cells may bypass trafficking restrictions and efficiently redistribute to tumor locations, potentially minimize its systemic distribution and circumvent T cells systemic targeting of normal tissues, further minimizing the risk of “on target, off tumor” toxicity.

It is well to be mentioned that Song et al. investigated the response of HER2 CAR T cells towards patient-derived gastric cancer stem-like cells (GCSCs) [36]. They found that HER2 CAR T cells efficiently phagocytized and degraded the tumor spheres in vitro, and restrained the tumorigenicity of GCSCs in nude mice. In the light of already demonstrated role of HER2 in maintaining the CSCs subpopulation of GC cells [41], these findings put forward an idea that HER2 CAR T cells are potentially effective in preventing the recurrence and metastasis of HER2\(^+\) GC and improving the treatment outcomes with eradication of CSCs.

This year marks a major milestone in the development of CAR T cells therapy, with Kymriah (tisagenlecleucel) firstly approved by the U.S. Food and Drug Administration (FDA) on August 30, 2017 for certain pediatric and young adult patients with a form of acute lymphoblastic leukemia (ALL), and followed by Yescarta (axicabtagene ciloleucel) approved on October 18, 2017 for adult patients with large B-cell lymphoma after at least two other kinds of treatment failed. During the last decade, the therapeutic efficacy and safety of CAR T cells therapy has been clinically validated in hematological malignancies but far less satisfactory outcomes in solid tumors due to many obstacles, such as inefficient homing and penetration to primary lesions, lack of specific targetable tumor antigens, inhibitory effect by the immunosuppressive microenvironment, “on target, off tumor” toxicity and other side-effects [42]. Li and Zhao have recently reviewed the current primary challenges and strategies to design safe and effective CAR T cells [43], including using novel cutting-edge technologies for CAR and vector designs to increase both the safety and efficacy [44], further T cell modification to overcome the tumor-associated immune suppression and using gene editing technologies to generate universal CAR T cells [45, 46]. All these CAR modifications and new technologies promote the development and evolution of CAR T cells therapy and will broaden the clinical experience of CAR T cells therapy from hematological malignancies to solid tumors. Meanwhile, lots of clinical trials of CAR T therapy in solid tumors are in progress and some trials have produced inspiring outcomes [47].

In conclusion, the functional characterization of our humanized chA21 scFv based, 4-1BB costimulated HER2 CAR provide the basis for the clinical investigation of the adoptive CAR T cells therapy for a wide spectrum of HER2-expressing cancers. Multiple strategies of improving safety of HER2 CAR are under active investigation and need to be tested in future clinical trials.

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Disclosure of conflict of interest

None.

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