The efficacy of third generation anti-HER2 chimeric antigen receptor T cells in combination with PD1 blockade against malignant glioblastoma cells

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Abstract. Without effective treatment, glioblastoma is one of the deadliest cancers worldwide. The aim of the present study was to explore whether combinational immunotherapy is effective for treating malignant glioblastoma *in vitro*. The therapeutic efficacy of third generation anti-human epidermal growth factor receptor 2 (HER2) chimeric antigen receptor (CAR)-T cells alone and in combination with PD1 blockade was investigated for the treatment of malignant glioblastoma cells *in vitro*. Anti-HER2 CAR-T cells were prepared by transducing activated primary human T cells with lentiviruses which expressed third generation anti-HER2 CAR. The CAR-positive cell ratio was detected using flow cytometry. The expression level of CAR was detected by western blot analysis. The binding of anti-HER2 CAR-T cells to HER2$^+$ U251 glioblastoma cells was examined under a fluorescence microscope. The cytokine secretion of CAR-T cells induced by target cells was analyzed via ELISA. The cytotoxicity of anti-HER2 CAR-T cells alone or in combination with anti-programmed death-1 (PD1) antibody against HER2$^+$/PDL1$^+$ U251 cells was examined using an LDH assay. The CAR-positive cell ratio and expression level of CAR in prepared CAR-T cells were both high enough. Anti-HER2 CAR-T cells could specifically bind to U251 cells. The IL-2 and IFN-γ secretion of CAR-T cells increased after being co-cultured with U251 cells, and further increased in the presence of anti-PD1 antibody. Anti-HER2 CAR-T cells displayed a potent cytotoxicity against U251 cells. In addition, the presence of anti-PD1 antibody further enhanced the efficacy of anti-HER2 CAR-T cells against U251 cells. The present results indicated that blocking PD1 immuno-suppression can increase the activation of CAR-T cells after they are activated by a targeting antigen. Third generation anti-HER2 CAR-T cells along with PD1 blockade have a great therapeutic potential for combatting malignant glioblastoma.

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Abbreviations: CAR, chimeric antigen receptor; PD1, programmed death-1; ATCC, American Tissue Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBMCs, peripheral blood mononuclear cells; IL-2, interleukin-2; IFN-γ, interferon-γ; MOI, multiplicity of infection; LDH, lactate dehydrogenase; 7-AAD, 7-aminoactinomycin D; TIL, tumor-infiltrating lymphocyte

Key words: HER2, chimeric antigen receptor, T cells, PD1, glioblastoma

Introduction

Glioblastoma is the deadliest primary central nervous system tumor and has a prevalence of 3.19% in the United States (1). In the past 15 years, treatment of glioblastoma has included maximal safe surgical resection combined with radiotherapy and temozolomide chemotherapy (2). Despite this, the overall five-year survival is still less than 5%, with an average survival of just 14 months following initial diagnosis (3). Immunotherapy is an alternative approach which has the potential to overcome the limitations of the current standard therapies. In the past five years, chimeric antigen receptor (CAR)-T immunotherapy has had substantial success in the treatment of chronic lymphocytic leukemia and acute lymphoblastic leukemia patients who have relapsed from chemotherapy treatment (4-6).

The CAR-T immunotherapy method uses genetically engineered T cells to express CAR. First generation CAR is composed of an antigen recognition domain (scFv) and the essential activating signal CD3ζ, while second generation CAR includes one co-stimulatory molecule and the third generation includes two co-stimulatory molecules (7).

The antigen recognition domain confers a specificity of CAR-T cells for tumor-associated antigens. Thus, finding a suitable tumor antigen is important for the use of CAR-T cells to treat glioblastoma. Human epidermal growth factor receptor 2 (HER2), a member of the EGFR family, encodes a 185-kDa transmembrane protein via tyrosine protein kinase activity. The gene amplification and protein overexpression of HER2 in human tumors have been associated with more aggressive cancers (8,9). The HER2 antigen is overexpressed in approximately 25-30% of patients living with breast cancer, 30-40% of patients with primary renal cell carcinoma and 30-35% of patients with lung adenocarcinoma (10-12). HER2 expression is absent in the adult central nervous system (13). CAR-T cells targeting HER2 have been demonstrated to be safe and effective in clinical trials (14,15).

According to previous studies, HER2 is expressed in as many as 80% of glioblastoma cases (16,17). Furthermore, overexpression of HER2 has been revealed to be correlated with the malignancy of glioma (18) and with high-grade glioma (19) as well as with early mortality (9). These data indicate that HER2 is an attractive therapeutic target for glioblastoma. In the preclinical model of glioblastoma, CAR-T cells being studied for glioma-targeted antigens IL-13Rα2, EphA2, HER2 (using a second generation anti-HER2 CAR) and EGFRvIII (20) demonstrated strong anti-tumor activity. In addition to careful selection of the tumor-associated antigen, the optimal design of the CAR architecture (such as using a new generation anti-HER2 CAR) is an important prerequisite for achieving significant responses in CAR-mediated immunotherapy.

While CAR-T cells have exhibited significant responses in intractable hematological malignancies, they have low therapeutic effects for solid tumors. This may be due to the many obstacles in the tumor microenvironment of solid tumors (21-26), such as the intrinsic inhibition pathway mediated by T cell surface inhibitory receptors binding to their tumor cell surface ligands (26). One of the most frequently studied T cell inhibitory receptors is programmed death-1 (PD1/CD279), which is upregulated following the engagement of T cell receptor with its ligand. Currently, the known ligand of PD1 in various cancers is programmed cell death 1 ligand 1 (PDL1/CD274) (27). However, PDL1 on the cell surface of solid tumors is typically upregulated in response to cytokines (such as IL-2 and IFN-γ) which have been secreted by T cells, thus serving as tumor immune evasion (24).

In the present study, a third generation anti-HER2 CAR (anti-HER2 scFv-CD28-CD137-CD3ζ) was used, which we previously designed and constructed, and which carried two co-stimulatory molecules (CD28 and CD137). The third generation anti-HER2 CAR has not been reported in clinical trials for treatment of malignant glioblastoma. Lentivirus-mediated T cell transduction was utilized to generate CAR-T cells. It was examined whether anti-HER2 CAR-T cells alone have specific and efficient cytotoxicity, as well as whether their combination with PD1 blockade enhances the therapeutic activity against $HER2^+/PDL1^+$ glioblastoma cells *in vitro*. The use of anti-HER2 CAR-T cells in combination with anti-PD1 antibody has not been reported in clinical trials for malignant glioma.

Materials and methods

Cell lines and media. 293T-17 cells [American Tissue Culture Collection (ATCC)], human malignant glioblastoma cells U87 (glioblastoma of unknown origin, HTB-14™) (ATCC) as well as U251 (China Center for Type Culture Collection) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) and supplemented with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc.). The U251 cell line was authenticated using STR analysis (Cell Bank, Type Culture Collection, Chinese Academy of Sciences).

Construction of anti-HER2 CAR and production of recombinant lentivirus. The third generation anti-HER2 CAR was developed by our research group and synthesized by the Beijing Genomics Institute in China. The recombinant lentivirus was produced using 293T-17 cells co-transfected with recombinant lentiviral vector pLVX-EF1α-CAR-IRES-ZsGreen1 as well as packaging plasmids psPAX2 and pMD2.G. Packaging, concentration and purification of the recombinant lentivirus were performed following protocols we had optimized (28).

Preparation of human peripheral T lymphocytes. Preparation of human peripheral T lymphocytes, including isolation, activation, and purification, was performed following the protocols we have previously described (28,29) but with some further optimization. The present study was approved by the Ethical Committee of Wenzhou Medical University (Wenzhou, China). Peripheral blood materials used in this study were obtained from healthy donors who provided informed consent. The total number of healthy donors used was three (including one male and two females), the age distribution was 24-28, collected from January 2017 to December 2018 at the First Affiliated Hospital of Wenzhou Medical University. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by density-gradient centrifugation (at 900 x g) utilizing a lymphocyte separation medium (Sigma-Aldrich; Merck KGaA). PBMCs were activated (stimulated) using anti-CD2-, anti-CD3- and anti-CD28-coated microbeads (Miltenyi Biotec GmbH) at a 1:1 bead to cell ratio for two days. Primary human peripheral T lymphocytes were cultured in GT-T551 medium (Takara Bio, Inc.) which had been supplemented with 10% FBS and 300 IU/ml Interleukin-2 (IL-2) (PeproTech, Inc.).

Transduction and expansion of T cells. The $CD3^+$ human peripheral T cells were transduced with recombinant lentivirus at a multiplicity of infection (MOI) value of 20, as previously described (29). For transduction, the cell plate was centrifuged at 1,200 x g for 2 h and cultured for 10-14 h, then replaced with fresh medium and cultured for expansion. After examining the proper GFP expression level (~40% GFP-positive cell ratio) under fluorescence microscope (Nikon Eclipse Ti), the transduced cells were subjected to flow cytometry and western blotting to evaluate the GFP-positive cell percentage and CAR expression level respectively.

Flow cytometric analysis. The expression of HER2 in glioblastoma cells U87 and U251 was determined using APC

anti-human CD340 (erbB2/HER2) antibody (cat. no. 324407), with APC mouse IgG1κ (both from BioLegend, Inc.) used as an isotype control. The expression of PDL1 in glioblastoma cells U87 and U251 was evaluated using PE anti-human CD274 (PDL1) antibody (BD Biosciences), while PE mouse IgG2aκ (BD Biosciences) was used as an isotype control. Transduction efficiency to T cells was measured by the percentage of transduced T cells expressing GFP (that is, CAR). Flow cytometric data were analyzed using FlowJo v10 software (FlowJo LLC).

Western blot analysis. Transduced human peripheral T cells were detected for the expression level of CAR by western blot analysis. T cells were lysed in modified RIPA lysis buffer [1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.25% Na-deoxycholate, 0.05% SDS, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA)]. The BCA assay kit (Beyotime Institute of Biotechnology) was used to determine the protein concentration. Lysates (10 μg per lane) were resolved by 10% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore). The membranes were blocked in TBST containing 5% non-fat dry milk, immunoblotted with anti-human CD3ζ antibody (cat. no. ab226475; dilution 1:1,000; Abcam) and anti-rabbit HRP-conjugated secondary antibody (cat. no. sc-2004; dilution 1:5,000; Santa Cruz Biotechnology, Inc.) and then developed utilizing ECL reagent (Beyotime Institute of Biotechnology). Light emission was detected using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.). Expression levels of CAR (containing exogenous CD3ζ) were detected, using endogenous CD3ζ as a loading control.

Examination of the targeting ability of CAR-T cells. In order to examine the targeting of anti-HER2 CAR-T cells on HER2-positive glioblastoma cells *in vitro*, HER2-positive U251cells (or HER2-negative control U87cells) were seeded in a 96-well plate at $1x10^4$ cells/well for 6 h. Anti-HER2 CAR-T cells were then added at an E:T (effector to target) ratio of 4:1 to co-culture for 24 h. The binding ability of transduced T cells to target cells as well as the status of target cells were observed using a fluorescence microscope.

Detection of cytokine secretion of CAR-T cells. Anti-HER2 CAR-T cells (effector cells) (or in combination with 20 μg/ml anti-PD1 antibody) were co-cultured with $HER2^+$ glioblastoma cells U251 (target cells) at an E:T ratio of 4:1, for 24, 48 or 72 h in a 96-well plate. Anti-PD1 antibody (clone RMP1-14) was obtained from Bio X Cell. ELISA kits (R&D Systems, Inc.) for detecting IL-2 and IFN-γ were utilized to analyze supernatants of cells according to the manufacturer's instructions. Supernatants from co-culture of anti-HER2 CAR-T cells with $HER2^-$ glioblastoma cells U87 as well as from co-culture of (untransduced) blank T cells (or in combination with anti-PD1 antibody) with $HER2^+$ glioblastoma cells U251 were the negative controls.

Cytotoxicity assay. Anti-HER2 CAR-T cells (effector cells) (or in combination with 20 μg/ml anti-PD1 antibody) were co-cultured with $HER2^+$ glioblastoma cells U251 (target cells) at E:T ratios of 2:1, 4:1, 8:1 and 16:1 for 18 h in a 96-well plate. The co-cultures of anti-HER2 CAR-T cells with $HER2^-$ glioblastoma cells U87 and the blank T cells (or in combination with anti-PD1 antibody) with $HER2^+$ glioblastoma cells U251 were used as negative controls. Specific lactate dehydrogenase (LDH) which had been released from target cells in cell-free supernatant was detected using a cytotoxicity LDH detection kit (Genmed) according to the manufacturer's instructions. The amount of LDH released was used to assess the lysis of target cells, which may be translated into the effectiveness of effector cells. Percent cytotoxicity was calculated according to OD values utilizing the following formula: Cytotoxicity (%)=(Experimental-Effector spontaneous-Target spontaneous)/(Target maximum-Target spontaneous) x100%.

Statistical analysis. Probability (P)-values were calculated with GraphPad Prism 5.0 software (GraphPad Software). All experiments were repeated at least three times. Group means were compared via one-way analysis of variance/Newman-Keuls. Differences of $P<0.05$ were considered to indicate a statistically significant difference.

Results

Expression of HER2 and PDL1 in glioblastoma cells. In order to assess the efficacy of anti-HER2 CAR-T cells in combination with anti-PD1 antibody against tumor cells, it is crucial to determine the expression level of HER2 and PDL1 in tumor cells. Flow cytometry was used to detect HER2 and PDL1 expression. The expression levels of HER2 in glioblastoma cells U251 and U87 were 52.6 and 0.55% respectively (Fig. 1A). The expression levels of PDL1 in glioblastoma cells U251 and U87 were 90.9 and 9.4% respectively (Fig. 1B). Glioblastoma cell line U251 was set as a $HER2^+/PDL1^+$ positive target cell, while glioblastoma cell U87 was set as a $HER2^-/PDL1^-$ negative control cell.

Preparation of the third generation anti-HER2 CAR-T cells. The structure of anti-HER2 CAR constructed by our research group is presented in Fig. 2A. Human peripheral T cells were separated and activated. Once human peripheral T cells had been transduced with recombinant lentivirus at a multiplicity of infection (MOI) of 20 for five days, they were observed under a fluorescence microscope in visible light (Fig. 2B-a) and fluorescence (Fig. 2B-b) to evaluate the percentage of GFP-positive cells. Percentages of viable cells (7-AAD negative cells) and GFP-positive cells (that is, transduction efficiency) were analyzed using flow cytometry (Fig. 2C). Transduction efficiency was 38.2% (Fig. 2C). The expression levels of CAR (containing exogenous CD3ζ) were detected utilizing western blotting, using endogenous CD3ζ as a loading control. CAR-T cells expressed not only endogenous CD3ζ (16 kDa) but also the expected exogenous CD3ζ included in CAR (58 kDa) (Fig. 2D).

Targeting of anti-HER2 CAR-T cells to HER2-positive glioblastoma cells. In order to examine the targeting of anti-HER2 scFv on the anti-HER2 CAR-T cell surface, anti-HER2 CAR-T cells were co-cultured with HER2-positive U251 cells for 24 h. The binding ability of anti-HER2 CAR-T cells to U251 cells was examined under a fluorescence microscope in both visible light and fluorescence. CAR-T cells

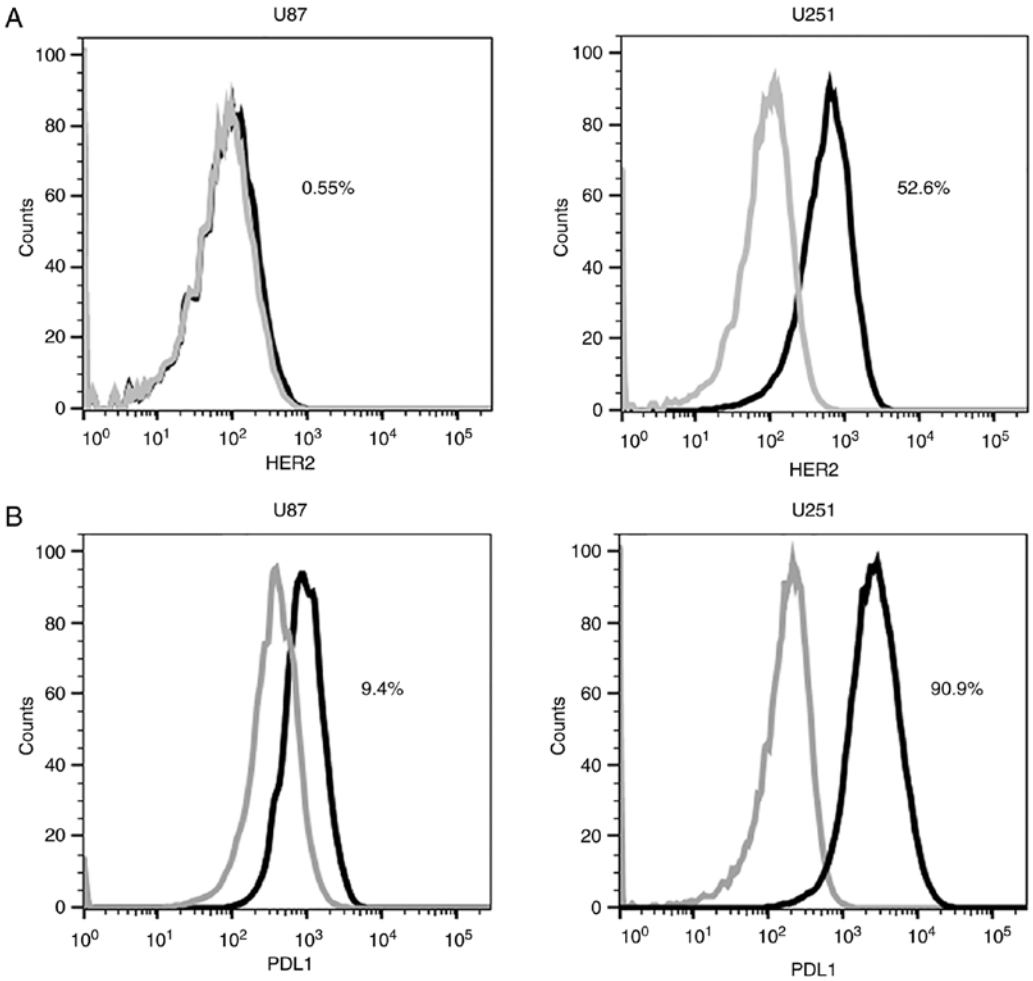

Figure 1. Expression of HER2 and PDL1 in glioblastoma cells. (A) The expression of HER2 in glioblastoma cells U87 and U251 was detected using flow cytometry (using anti-human APC-HER2). The values indicate the expression percentage of HER2. The grey histogram was an isotype control. (B) Expression of PDL1 in glioblastoma cells U87 and U251 was detected utilizing flow cytometry (using anti-human PE-PDL1). The values indicate the expression percentage of PDL1. The grey histogram was an isotype control. HER2, human epidermal growth factor 2; PDL1, programmed cell death 1 ligand 1.

which had been co-cultured with U251 cells formed clusters with U251 cells (Fig. 3C), while the number of viable U251 cells decreased and seemingly disappeared (Fig. 3B) when compared to the number of viable U251 cells not co-cultured with CAR-T cells (Fig. 3A). CAR-T cells did not combine with U87 cells, while most U87 cells remained alive (Fig. 3D-F).

Activation levels of anti-HER2 CAR-T cells induced by HER2-positive glioblastoma cells. In order to determine whether anti-HER2 CAR-T cells alone or in combination with anti-PD1 antibody were activated when co-cultured with target cells, cytokine secretion of anti-HER2 CAR-T cells was detected. The results demonstrated that IL-2 or IFN-γ secretion of the CAR-T group after being co-cultured with the U251 cells for 24, 48 or 72 h significantly increased when compared to the IL-2 or IFN-γ secretion of the blank T group which had been co-cultured with U251 cells for the same time ($P<0.01$ or $P<0.001$) (Fig. 4). Secreted IL-2 and IFN-γ from the CAR-T group, after being co-cultured with U251 cells for 24 h, increased 27-fold and 15-fold, respectively when compared to those of the blank T group. Secreted IL-2 and IFN-γ from the CAR-T group, after being co-cultured with U251 cells for 24 h, increased 5.4-fold and 12.5-fold respectively when compared to those of the CAR-T group which had been co-cultured with U87 cells for 24 h. Notably, following the addition of anti-PD1 antibody, CAR-T cells could significantly increase production of IL-2 and IFN-γ after being co-cultured with U251 cells for 72 h, when compared to those of CAR-T cells alone ($P<0.05$ and $P<0.01$) (Fig. 4). CAR-T cells in combination with anti-PD1 antibody could significantly increase production of IL-2 and IFN-γ after being co-cultured with U251 cells for 24, 48 or 72 h, when compared to those of blank T cells in combination with anti-PD1 antibody ($P<0.001$) (Fig. 4).

Cytotoxicity of anti-HER2 CAR-T cells alone or in combination with anti-PD1 antibody against HER2-positive glioblastoma cells. In order to evaluate the efficacy of effector cells alone or in combination with PD1 blockade on target cells *in vitro*, anti-HER2 CAR-T cells alone or those in combination with anti-PD1 antibody were co-cultured with HER2-positive glioblastoma cells U251 at E:T ratios of 2:1, 4:1, 8:1 and 16:1 for 18 h. The cytotoxicity (%) of CAR-T cells to lyse U251 cells was revealed to be significantly higher than that of blank T cells at every E:T ratio ($P<0.05$ and $P<0.001$) (Fig. 5A). Notably, the addition of anti-PD1 Ab significantly increased cytotoxicity (%) of CAR-T cells against U251 cells at the E:T

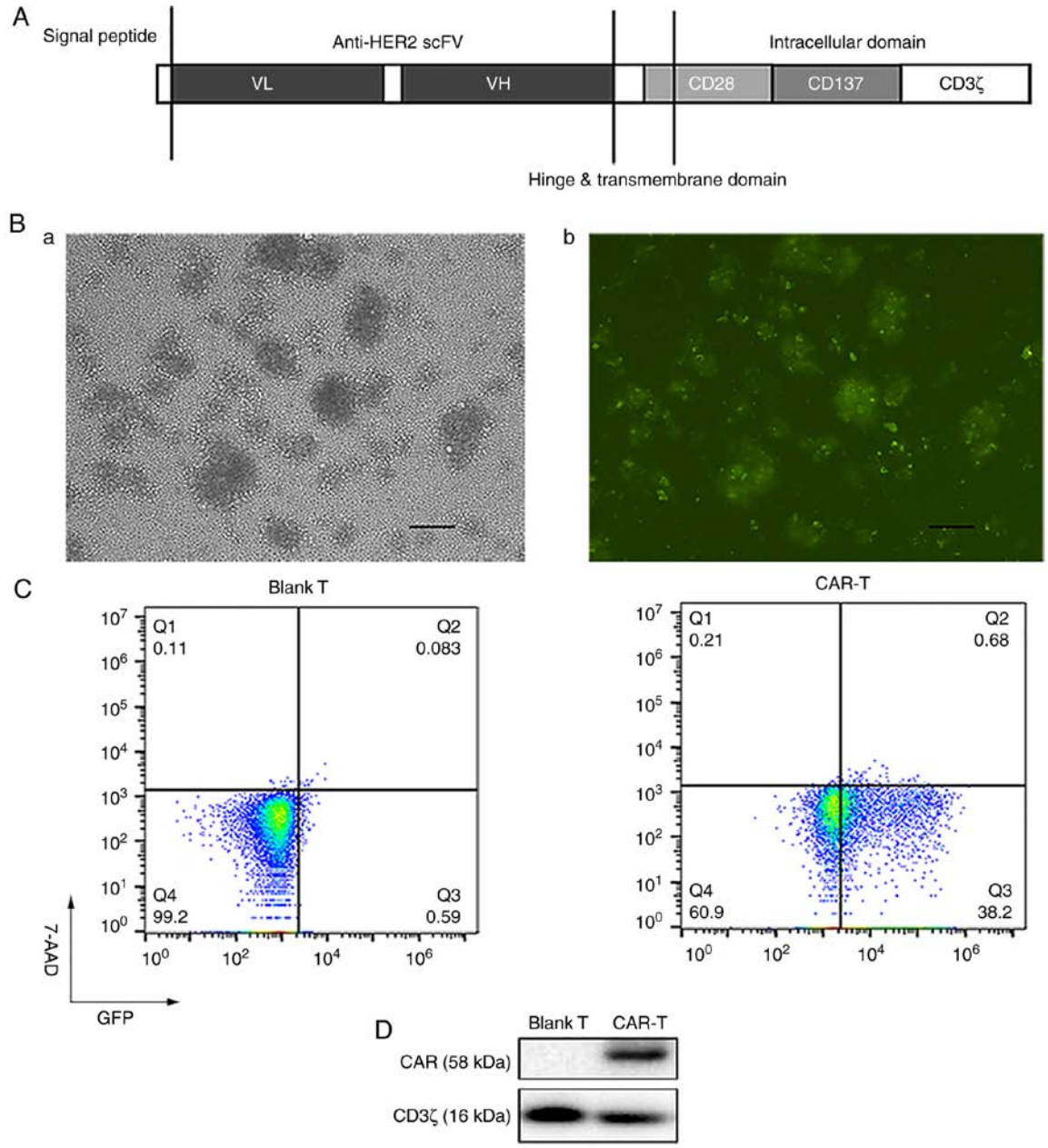

Figure 2. Preparation of the third generation anti-HER2 CAR-T cells. (A) Structure of anti-HER2 CAR. (B) After human peripheral T cells were transduced with recombinant lentivirus at MOI 20 for five days, T cells were observed under a fluorescence microscope in (a) visible light and (b) fluorescence to evaluate the percentage of GFP-positive cells (scale bar, 100 μm). (C) Percentages of viable cells (7-AAD negative cells) and GFP-positive cells (i.e., transduction efficiency) were analyzed using flow cytometry after the human peripheral T cells were transduced with recombinant lentivirus at MOI 20 for five days. (D) Recombinant lentivirus was transduced into human peripheral T cells. Expression levels of CAR (containing exogenous CD3ζ) were detected by western blotting, using endogenous CD3ζ as a loading control. HER2, human epidermal growth factor 2; CAR, chimeric antigen receptor; MOI, multiplicity of infection; scFv, single chain variable fragment; VL, light chain variable region; VH, heavy chain variable region; 7-AAD, 7-aminoactinomycin D; Blank T, T cells not transduced; CAR-T, T cells transduced with CAR.

ratios of 8:1 and 16:1 compared to that of CAR-T cells alone (P<0.05 and P<0.01) (Fig. 5A). CAR-T cells in combination with anti-PD1 antibody could significantly increase cytotoxicity (%) against U251 cells at the E:T ratios of 2:1, 4:1, 8:1 and 16:1, when compared to that of blank T cells in combination with anti-PD1 antibody (P<0.05, P<0.01 and P<0.001) (Fig. 5A). As a negative control, anti-HER2 CAR-T cells were co-cultured with HER2$^-$ glioblastoma cells U87 (Fig. 5B). At an E:T of 16:1, cytotoxicity (%) of CAR-T cells against U251 cells reached 46.65%, while cytotoxicity (%) against HER2-negtive U87 cells was much lower, at just 15.74% (Fig. 5C) (P<0.01). Furthermore, at the E:T ratio of 16:1, following the addition of anti-PD1 Ab, cytotoxicity (%) of CAR-T cells against U251 cells reached 63.08%, while cytotoxicity (%) against U87 cells remained low at 17.76%, meaning the former was significantly higher (Fig. 5C) (P<0.01).

Discussion

The anti-HER2 CAR developed by our group contains CD28-CD137-CD3ζ, which belongs to third-generation CAR (Fig. 2A). In a reported preclinical study for glioblastoma, the

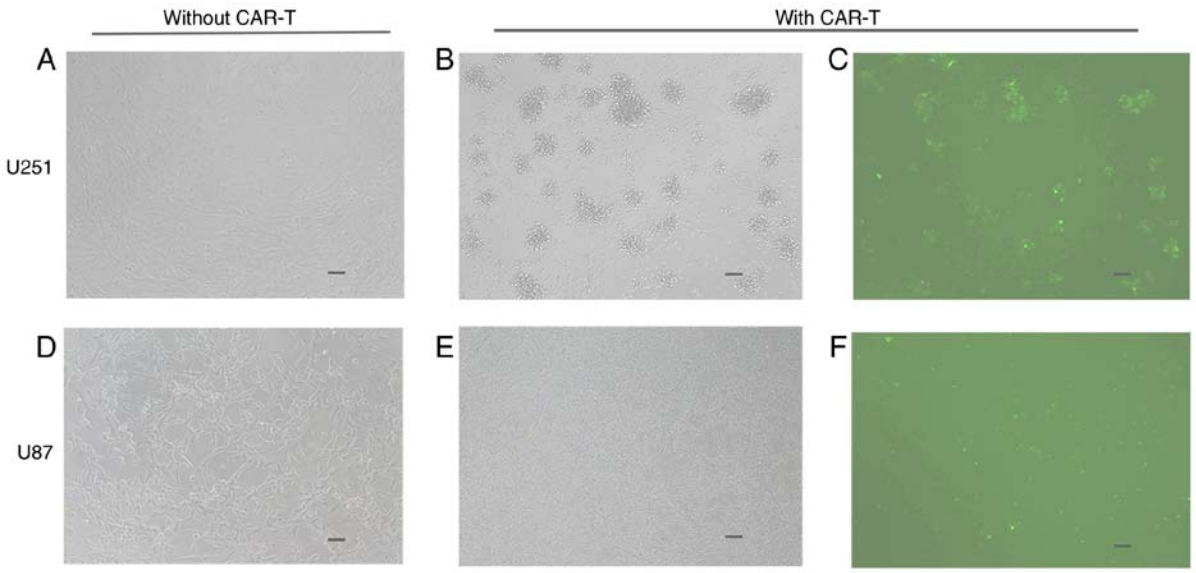

Figure 3. Targeting of anti-HER2 CAR-T cells to HER2-positive glioblastoma cells. HER2-positive U251 glioblastoma cells were co-cultured with anti-HER2 CAR-T cells at effector:target ratio 4:1 for 24 h, observed under a fluorescence microscope in (B) visible light and (C) fluorescence. U251 cells that were not co-cultured with CAR-T cells were examined in (A) visible light concurrently as a control of (B). (D-F) The HER2-negative U87 glioblastoma cells were considered as a negative control (scale bar, 100 μm). HER2, human epidermal growth factor 2; CAR, chimeric antigen receptor; CAR-T, T cells transduced with CAR.

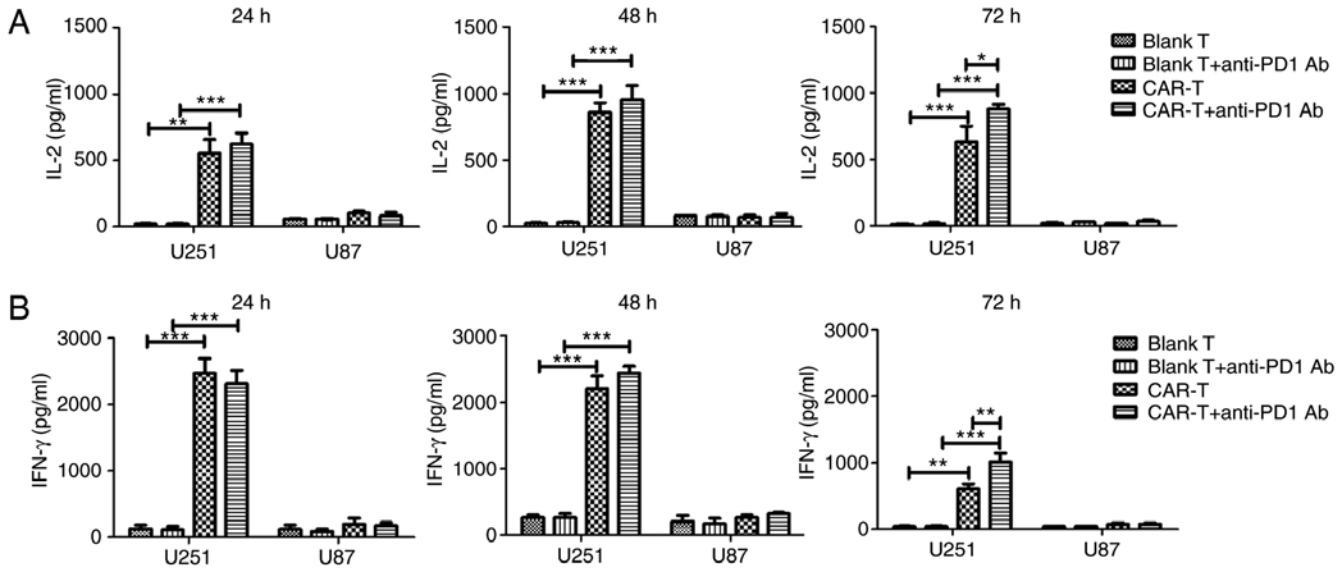

Figure 4. Cytokine secretion of anti-HER2 CAR-T cells. After anti-HER2 CAR-T cells (effector cells) (or in combination with anti-PD1 antibody) were co-cultured with HER2$^+$ glioblastoma cells U251 (target cells) at an effector to target ratio of 4:1 for 24, 48 or 72 h, supernatant was collected. ELISA kits for detecting (A) IL-2 and (B) IFN-γ were used to analyze supernatants. Supernatants from the co-culture of anti-HER2 CAR-T cells with HER2- glioblastoma cells U87, as well as from the co-culture of (untransduced) blank T cells (or in combination with anti-PD1 antibody) with HER2$^+$ glioblastoma cells U251 were used as negative controls. Three determinations were performed and the mean values are presented. For (A) IL-2 or (B) IFN-γ secretion, the CAR-T group was compared with the blank T group, or the CAR-T in combination with anti-PD1 Ab group was compared with blank T in combination with anti-PD1 Ab group, or the CAR-T in combination with anti-PD1 Ab group was compared with the CAR-T group. *P<0.05; **P<0.01; ***P<0.001. HER2, human epidermal growth factor 2; CAR, chimeric antigen receptor; CAR-T, T cells transduced with CAR; IL-2, interleukin-2; IFN-γ, interferon-γ.

anti-HER2 CAR utilized was second-generation (20). Most CAR-T clinical trials have used second-generation CAR for treating hematological malignancies. To date, there has been no successful clinical trial of anti-HER2 CAR-T cells which has utilized third-generation CAR (https://www.clinicaltrials.gov). In the present study, anti-HER2 CAR was transduced into T cells by lentiviral vector. To date, there has also been no successful clinical trial for anti-HER2 CAR-T cells which used lentiviral vectors (https://www.clinicaltrials.gov).

The present study demonstrated that third generation anti-HER2 CAR-T cells (with co-stimulatory molecules CD28 and 4-1BB/CD137) are able to eliminate HER2-positive malignant glioblastoma cells both specifically and efficiently. The specialty for binding of CAR to antigen can be affected by the affinity of the selected scFv. The present study used the anti-HER2 scFv from antibody 4D5 for CAR construction, and the CAR-T cells demonstrated HER2-specific tumor cell lysis *in vitro*. A functionality assay revealed that these redirected

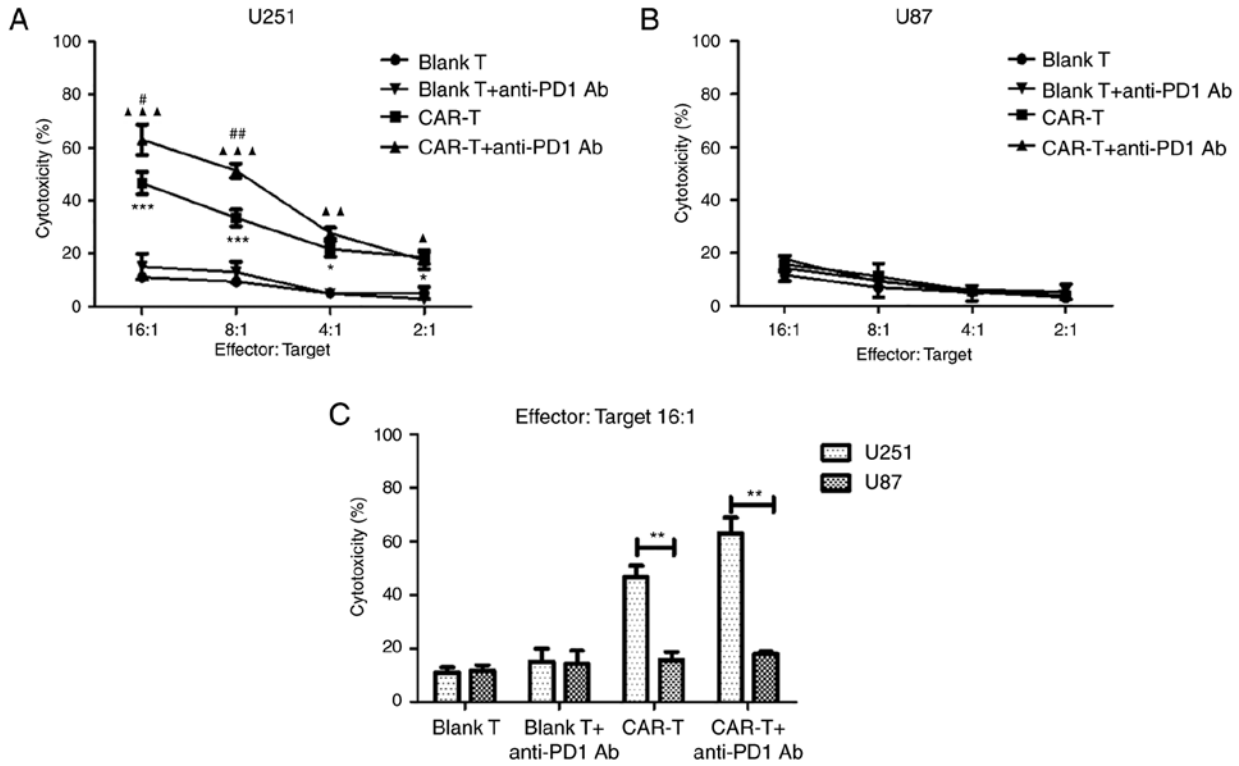

Figure 5. The cytotoxicity of anti-HER2 CAR-T cells (or in combination with anti-PD1 antibody) against HER2-positive glioblastoma cells. (A) After anti-HER2 CAR-T cells (effector cells) were co-cultured with HER2$^+$ glioblastoma cells U251 (target cells) (or in combination with anti-PD1 antibody) at different ratios of E:T for 18 h, supernatant was detected using a cytotoxicity LDH detection kit for LDH released from lysed target cells. Cytotoxicity (%) was calculated. At E:T ratios of 2:1, 4:1, 8:1 or 16:1 respectively, the cytotoxicity (%) of the CAR-T group was compared with the Blank T group, *P<0.05, ***P<0.001; or the CAR-T in combination with anti-PD1 Ab group was compared with blank T in combination with anti-PD1 Ab group, $^{\blacktriangle}$P<0.05, $^{\blacktriangle\blacktriangle}$P<0.01, $^{\blacktriangle\blacktriangle\blacktriangle}$P<0.001; or the CAR-T in combination with anti-PD1 Ab group was compared with the CAR-T group, $^{\#}$P<0.05, $^{\#\#}$P<0.01. (B) As a negative control, anti-HER2 CAR-T cells were co-cultured with HER2- glioblastoma cells U87. (C) At E:T ratio 16:1, the cytotoxicity (%) of the U251 group was compared with the U87 group, **P<0.01. Three determinations were performed and the mean values are presented. HER2, human epidermal growth factor 2; CAR, chimeric antigen receptor; CAR-T, T cells transduced with CAR; PD1, programmed death 1; E:T, effector to target; LDH, lactate dehydrogenase.

T cells could secret both cytokine IL-2 and IFN-γ as well as exert efficient cytotoxic T cell response in an HER2-specific manner. Major progress has been made since the introduction of co-stimulatory signaling into the architecture of CAR. Given the in-depth understanding of co-stimulatory signaling in T cell immune response, several co-stimulatory molecules (including CD28, 4-1BB/CD137, CD27, OX40) were embedded in the CAR, following which their roles in coordinating anti-tumor immunity were explored (30). When compared with other co-stimulatory molecules, CD28 was more effective at enhancing IL-2 production, improving clonal expansion and maintaining persistence of CAR-T cells. CD28 used with 4-1BB/CD137 signaling is more effective than CD28 alone in terms of cytotoxicity and IFN-γ production (31). The inclusion of the CD137 co-stimulatory molecule in CAR appears more key than CD28. Finney *et al* demonstrated that CD137 in the CAR induced a maximal effect on target cell lysis (32).

The present results revealed that the presence of anti-PD1 antibody is critical for enhancing T cell response to tumor cells. CAR-T cells, in combination with anti-PD1 antibody, could release more IL-2 and IFN-γ after targeting tumor cells, and were more efficient for eliminating tumor cells when compared with CAR-T cells alone. The main function of PD1 is to reduce the sensitivity of cancer patients to T cell-mediated anti-tumor immune response. Blocking PD1 signaling can rescue exhausted T lymphocytes and is an effective treatment for cancer. In recent years, checkpoint inhibitors for the PD1/PDLl checkpoint pathway have demonstrated significant antitumor effects in advanced melanoma, classic Hodgkin's lymphoma, non-small cell lung cancer (NSCLC) and other conditions (33-36). Research has suggested that CAR-T cell therapy (including targeting CD19, PSMA) in combination with PD1 blockade is an ideal therapy combination for the treatment of solid tumors (37,38).

Liu *et al* confirmed that treatment of mice that have large, established solid tumors with PD1-CD28 CAR-T cells led to a significant regression in tumor volume due to to enhanced CAR tumor-infiltrating lymphocyte (TIL) infiltrate, decreased susceptibility to tumor-induced hypofunction and attenuation of inhibitory receptor expression when compared to treatments with CAR-T cells alone or PD1 antibodies (39). Cherkassky *et al* have reported that anti-MSLN CAR-T cells used in combination with PD1 blockade could enhance the function of CAR-T cells in PDL1$^+$ tumor tissues, and may lead to a longer, tumor-free survival period for cancer patients (40).

Future research will verify the *in vivo* effect of third generation anti-HER2 CAR-T cells in combination with anti-PD1 antibody via a glioblastoma animal model and thus ascertain whether this therapy can prolong the survival of a glioblastoma animal model or even achieve tumor-free survival.

In summary, the present study demonstrated that third generation anti-HER2 CAR-T cells are able to eliminate

malignant glioblastoma cells specifically and efficiently. Blocking PD1 immuno-suppression can increase activation of CAR-T cells after activation by targeting antigens. This study revealed that, when used in combination with anti-PD1 antibody, anti-HER2 CAR-T cells have a greater therapeutic activity against $HER2^+/PDL1^+$ malignant glioblastoma cells when compared with anti-HER2 CAR-T cells alone.

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Availability of data and materials

Datasets used and analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

WY and LS undertook the research, analyzed the data and wrote the paper. SB, PL and JC helped to perform the research and experiments. HL and HG designed the research, wrote and revised the paper. All of the authors read and approved the final manuscript and agree to be for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Wenzhou Medical University (Wenzhou, China). Peripheral blood materials used in this study were obtained from healthy donors who provided informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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