Am J Cancer Res 2020;10(2):688-703
www.ajcr.us /ISSN:2156-6976/ajcr0104768

Original Article

Overcome trastuzumab resistance of breast cancer using anti-HER2 chimeric antigen receptor T cells and PD1 blockade

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Received November 11, 2017; Accepted January 2, 2020; Epub February 1, 2020; Published February 15, 2020

Abstract: Trastuzumab-resistance is still a major challenge in treating patients with HER2 positive breast cancer. In this study, we tried to overcome transtuzumab-resistance by examining the therapeutic efficacy of third generation anti-HER2 chimeric antigen receptor (CAR)-T cells alone and in combination with PD1 blockade against HER2 positive and trastuzumab-resistance breast cancer cells in vitro and xenograft model. The anti-HER2 CAR-T cells were generated by infecting CD3/CD28 activated peripheral blood mononuclear cells with lentivirus expressing third generation anti-HER2 CAR. Anti-HER2 CAR-T cells were specifically targeted to HER2 positive BT474 and trastuzumab resistant HCC1954 cells compared with HER2 negative breast cancer cells. Results from ELISA revealed that the secretion of IL-2 and IFN-γ was increased in anti-HER2 CAR-T cells after being co-cultured with HCC1954 cells, and was further increased with the addition of anti-PD1 antibody in the co-culture system. Furthermore, data from lactate dehydrogenase assay showed that anti-HER2 CAR-T cells displayed a potent cytotoxicity against HCC1954 and BT474 cells. Addition of anti-PD1 antibody further enhanced the cytotoxicity of anti-HER2 CAR-T cells against HCC1954 cells. Lastly, injection of anti-HER2 CAR-T cells significantly reduced the growth of HCC1954 xenograft tumors. Combining anti-HER2 CAR-T cells with anti-PD1 antibody further impaired the growth of HCC1954 tumors. The present results indicate that anti-HER2 CAR-T cells have therapeutic efficacy against trastuzumab resistant breast tumors and addition of the PD1 antibody can further enhance the therapeutic effect of anti-HER2 CAR-T cells. Thus, third generation anti-HER2 CAR-T cells along with PD1 blockade is a potential therapy to overcome trastuzumab resistance of breast cancer.

Keywords: CAR-T cells, anti-PD1 antibody, HER positive breast cancer, trastuzumab resistance

Introduction

Breast cancer is the leading cause of cancer death for women in the world [1]. Breast cancer can be classified into three main types in clinic that include luminal, HER2 positive, and triple negative breast cancer [2] based on the expression of three markers: estrogen receptors (ER) [3], progesterone receptor (PR) [4], and human epidermal growth factor receptor 2 (HER2). About 15-25% of the breast cancer are HER2 positive [5].

Current standard therapy for HER2 positive breast cancer includes chemotherapy in combination with trastuzumab/herceptin, a humanized monoclonal antibody that binds to the extracellular domain of HER2 [6]. Herceptin inhibits the cell growth and proliferation, and kills HER2 positive tumor cells through antibody-dependent cellular cytotoxicity (ADCC) by the immune cells present in the tumor microenvironment[6]. However, the efficiency of this targeted therapy is diminished due to the primary and acquired resistance of the HER2 positive tumors in response to herceptin treatment [7, 8]. There are multiple mechanisms contributing to herceptin resistance that include activation of the HER2 downstream signaling pathways and parallel receptor tyrosine kinase pathways

[9, 10], all of which provide potential targets to combat herceptin resistance.

In the past five years, chimeric antigen receptor (CAR)-T cell immunotherapy has achieved significant success in the treatment of recurred and drug resistant $CD19^+$ leukemia and lymphomas [11]. The CAR-T immunotherapy utilizes genetically engineered T cells to express CAR that can recognize a specific antigen on the cell surface. The first-generation CAR is composed of the antigen recognition domain of single chain variable fragment (scFv) in the antibody and the essential T cell receptor (TCR) activating signal chain CD3ζ [12]. The second generation CAR is modified to contain one TCR co-stimulatory molecule (e.g., CD28), and the third generation CAR consists of two co-stimulatory molecules (e.g., CD28 and 4-1BB/CD137) [13]. It has been well documented that 3rd generation CAR-T has better overall peak expansion, long term persistence [14, 15] and efficacy [16] in vivo compared to 2nd generation CAR-T. The binding of specific scFv in CAR to its antigen directly triggers an immune response of the CAR-T cells in a non-major histocompatibility complex (MHC)-restricted manner. CAR-T therapy is superior to traditional autologous T cell therapies during which tumor cells can evade the immune system by down-regulating the expression of MHC [17]. Because HER2 is a surface antigen that is over-expressed in HER2 positive breast cancer, a CAR designed to target HER2 could be the potential solution to overcome trastuzumab resistance.

While CAR-T cells have demonstrated potent anti-tumor capacity in $CD19^+$ leukemia and lymphoma, efficacy in other liquid tumors and many solid tumors has been less impressive [18]. One reason is that CAR-T cells are immune-suppressed by the PD1 checkpoint pathway activated upon binding to its ligand present in both tumor cells and surrounding tissues (e.g. stroma or tumor vasculature) [19, 20]. PD1 is a critical negative regulator of T cell fate and function. PD1 is transiently up-regulated in T cells following T cell activation and a marker of T cell exhaustion, which is a hypo-functional cell state found during chronic viral infections and among tumor infiltrating lymphocytes [21]. Notably, expression of the PD1 ligands PDL1 and PDL2 is correlated with poor prognosis in multiple tumors [22]. Anti-PD1/anti-PDL1 blocking antibodies have been shown to induce potent anti-tumor immune responses in patients with diverse malignancies [23], demonstrating the critical roles of PD1/PDL1 in suppressing T cell immunity by tumors.

Recent studies have begun to understand the role of PD1/PDL1 in regulating CAR-T cell function. John et al. demonstrated that antibody-mediated PD1 blockade enhanced CAR-T cell function in a syngeneic mouse model [24]. However, at least part of this effect was mediated through inhibition of myeloid-derived suppressor cells (MDSCs) that express PD1, rather than direct impact on CAR-T cells. Fedorov et al. have shown that recruitment of the PD1 intracellular domain through synthetic inhibitory CARs (iCARs) can suppress activity of CAR-T cells [25], suggesting that PD1 ligation might inhibit CAR function. Notably, both of these studies employed second generation CAR (CD28ζ or CD137ζ) rather than third generation CAR (CD28/CD137ζ). Thus, the cell autonomous effect of endogenous PD1/PDL1 ligation on human third generation CAR-T cells in vivo remains to be determined.

Considering no current clinical trial has been conducted using combined 3rd generation anti-HER2 CAR-T and anti-PD1 antibody, we generated genetically-modified T-cells expressing 3rd generation HER2 CAR with CD28 and CD137 as co-stimulatory molecules, and tested their efficacy alone and in combination with PD1 blocking antibody against HER2 positive and trastuzumab resistant breast cancer cells in vitro and in vivo in our present study. Our result shows anti-HER2 CAR-T cells combined with PD1 blockade is a potential therapy to overcome trastuzumab resistance of HER2 positive breast cancer.

Materials and methods

Cell lines and media

HER2-negative MB468, HER2-positive BT474 and HCC1954 human breast cancer cell lines, and 293T-17 cells were purchased from American Type Culture Collection (ATCC). HCC1954 is a de novo trastuzumab-resistant cell line whereas BT474 is a trastuzumab-sensitive cell line [26, 27]. MB468, BT474 and 293T-17 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, California, USA), and HCC1954 cells were cultured in RPMI 1640 (Gibco). All media were supplemented with 10% fetal bovine serum (FBS) (Gibco).

Recombinant lentivirus production and generation of anti-HER2 CAR-T cells

Lentiviral vector expressing the third generation anti-HER2 CAR (pLVX-EF1α-CAR-IRES-ZsGreen1) was generated as described [28]. The recombinant lentivirus was produced by co-transfecting 293T-17 cells using polyethyleneimine (PEI) with pLVX-EF1α-CAR-IRES-ZsGreen1 and packaging plasmids pSPAX2 and pMD2.G. Virus supernatants were concentrated and purified using our optimized methods as described previously [28]. The titers of the concentrated virus particles used in this study were determined by infecting 293T-17 cells with the concentrated virus stocks followed by flow cytometry analysis, which were between 10^8-10^9 TU/ml.

Our study was approved by the ethical committee for human study of Wenzhou Medical University (Wenzhou, China). Peripheral blood materials were obtained from healthy donors with informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density-gradient centrifugation using a lymphocyte separation medium (Sigma, St Louis, MO, USA), and then activated using micro beads coated with 10 μg/ml of anti-CD2, anti-CD3 and anti-CD28 antibodies (Miltenyi Biotech, GmbH) in GT-T551 T cell medium (Takara Bio, Inc.) supplemented with 10% FBS (Gibco) and 300 IU/ml interleukin-2 (IL-2) (PEPROTECH, Rocky Hill, NJ, USA). After they were activated for two days, the T cells were transduced with pLVX-EF1α-CAR-IRES-ZsGreen1 lentivirus at a multiplicity of infection (MOI) value of 20 in the presence of 8 μg/mL polybrene (Sigma). For lentiviral transduction, the culture plate containing the activated T cells was centrifuged at 1,200×g for 2 hours and cultured for 14 hours subsequently. After that, CAR-T cells were replaced with fresh medium containing 300 IU/ml IL-2 and expanded for additional 5-7 days. The percentages of GFP-positive CAR-T cells used in this study were determined by flow cytometry to be about 30-40%.

Flow cytometry analysis for the expression of HER2, PDL1 and PD1

The expression of HER2 and PDL1 in the three breast cancer cell lines MB468, BT474 and HCC1954 was determined using APC-conjugated anti-human HER2/CD340 antibody (dilution 1:100) (BioLegend, San Diego, CA, USA) or PE-conjugated anti-human PDL1/CD274 antibody (dilution 1:100) (BD Biosciences, New Jersey, USA) respectively, with APC mouse IgG1κ (BioLegend) or PE mouse IgG2aκ (BD Biosciences) as isotype controls. After being co-cultured with $HER2^-$ MB468 cells, and $HER2^+$ BT474 and HCC1954 cells at the effector:target ratio of 4:1 for 48 h and 72 h, the expression of PD1 in anti-HER2 CAR-T cells was determined using PE-conjugated anti-human PD1 antibody (dilution 1:100) (BD Biosciences), with PE mouse IgG2aκ (BD Biosciences) as an isotype control. Flow cytometric data were analyzed by FlowJo VX software.

Examination of the targeting specificity of anti-HER2 CAR-T cells

HER2-negative MB468 cells and HER2-positive HCC1954, BT474 cells were seeded in a 96-well plate at the density of 1×10^4 cells/well. Six hours later, anti-HER2 CAR-T cells were added to each well at an effector:target ratio of 4:1. The interaction of transduced T cells with target cells was examined after they were co-cultured for 24 h using the Nikon Eclipse Ti-S fluorescence microscope.

ELISA analysis of cytokine secretion from anti-HER2 CAR-T cells

Non-transduced (blank) T cells or anti-HER2 CAR-T cells were co-cultured in the absence or presence of 20 μg/mL anti-PD1 antibody (Bio X Cell, New Hampshire, USA) with target cells at the effector:target ratio of 4:1 for 48 or 72 hours in a 96-well plate. IL-2 and IFN-γ secreted into the culture supernatants were analyzed by ELISA kits (R&D, Minneapolis, USA) following the manufacturer's instructions.

Detection of cytotoxicity for target cells

The blank T cells or anti-HER2 CAR-T cells were co-cultured in the absence or presence of 20 μg/mL anti-PD1 antibody with target cells at effector:target ratios of 2:1, 4:1, 8:1 and 16:1 for 18 hours in a 96-well plate. Lactate dehydrogenase (LDH) released from lysed target cells into culture supernatant was detected using cytotoxicity LDH detection kit (Genmed, Addlestone, UK) following the manufacturer's instruction. The relative activity of LDH present in culture media was used to assess the lysis degree of target cells to determine the effec-

tiveness of effector cells. Percentage of cytotoxicity was calculated according to OD values for different culture supernatant and cells using the following formula: Cytotoxicity (%) = (experimental-effector spontaneous-target spontaneous)/(target maximum-target spontaneous) × 100%.

Expansion of anti-HER2 CAR-T cells after being stimulated by target cells

The anti-HER2 CAR-T cells were co-cultured in the absence or presence of 20 μg/mL anti-PD1 antibody with target cells at an effector:target ratio of 4:1. The effector cells were counted respectively at 24 h, 48 h and 72 h after being co-cultured using hemocytometer with trypan blue exclusion.

Apoptosis of anti-HER2 CAR-T cells after being stimulated by target cells

The blank T or anti-HER2 CAR-T cells were co-cultured in the absence or presence of 20 μg/mL anti-PD1 antibody with target cells at an effector:target ratio of 4:1 for 72 h. T cells then were subjected to apoptosis analysis by flow cytometry using Annexin V and 7-AAD reagents (BioLegend).

Establishment of HCC1954 xenograft tumor model and anti-tumor treatments

Protocols for the animal study were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. All animal experiments were performed in accordance with the relevant guidelines and regulations. The trastuzumab-resistant breast cancer HCC1954 cells (2×10^6) in 0.1 mL PBS mixed with 0.1 mL matrigel (Corning, Bedford, MA, USA) were injected subcutaneously into the flanks of Balb/c female nude mice (5-week old) purchased from GemPharmatech (Nanjing, China) on day 0. Twelve days later, mice were randomized into 4 groups (6 mice/group): blank T group, blank T plus anti-PD1 group, CAR-T group, CAR-T plus anti-PD1 group. Blank T or anti-HER2 CAR-T cells (2×10^7/mouse) were injected via caudal vein twice (on days 12 and 19 post injection of HCC1954 cells), and anti-PD1 antibody (250 μg/mouse) was injected intraperitoneally on days 12, 16, 19, and 23. All the mice were injected intraperitoneally with 20000 IU IL-2/mouse on days 12, 16, 19, and 23. During anti-tumor treatment, tumor dimension was measured every 5 days using a digital caliper, and tumor volume was calculated using the equation V (mm^3) = $L\times W^2/2$, where L (length) is the largest diameter and W (width) is the smallest diameter. After all of the mice were euthanized on day 37 (25 days after anti-tumor treatment), all of the tumors were dissected out and weighted. The experiment was repeated three times.

Hematoxylin and Eosin (H&E) staining and immunohistochemistry of xenograft tumors

Dissected tumors were fixed and embedded. Tissues sections (three for each mouse) were stained using the Hematoxylin and Eosin Staining Kit (Solarbio, Beijing, China). For immunohistochemistry (IHC) assay, tissue sections (five for each mouse) were subjected to heat induced epitope retrieval twice in 10 mM citrate buffer, pH 6.0 in boiling water for 7 minutes followed by treatment with 3% hydrogen peroxide, and blocked with 5% goat serum (Solarbio), then incubated with rabbit anti-human cleaved caspase 3 monoclonal antibody (1:400) (Cell Signaling Technology, Danvers, MA, USA), or rabbit anti-human CD3ε mono-antibody (1:200) (Cell Signaling Technology), and followed by incubation with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Abcam). HRP was detected using the DAB substrate kit (Abcam, Cambridge, United Kingdom). Slides were counterstained with hematoxylin. For quantification of immunohistochemical staining, positively stained cells were counted in five random 400× microscopic fields for each tissue section. A total of three different sections were counted and the average percentage of positive cells per section with SEM was shown.

Statistical analysis

Probability (P) values were calculated using GraphPad Prism 5.0 software. All the experiments were repeated at least three times. The means of groups were compared via one-way analysis of variance/Newman-Keuls. Differences were considered statistically significant when $P<0.05$.

Results

Expression of HER2 and PDL1 in breast cancer cell lines

To test the efficacy of anti-HER2 CAR-T cells in combination with anti-PD1 antibody against $HER2^+/PDL1^+$ breast cancer, it is important to

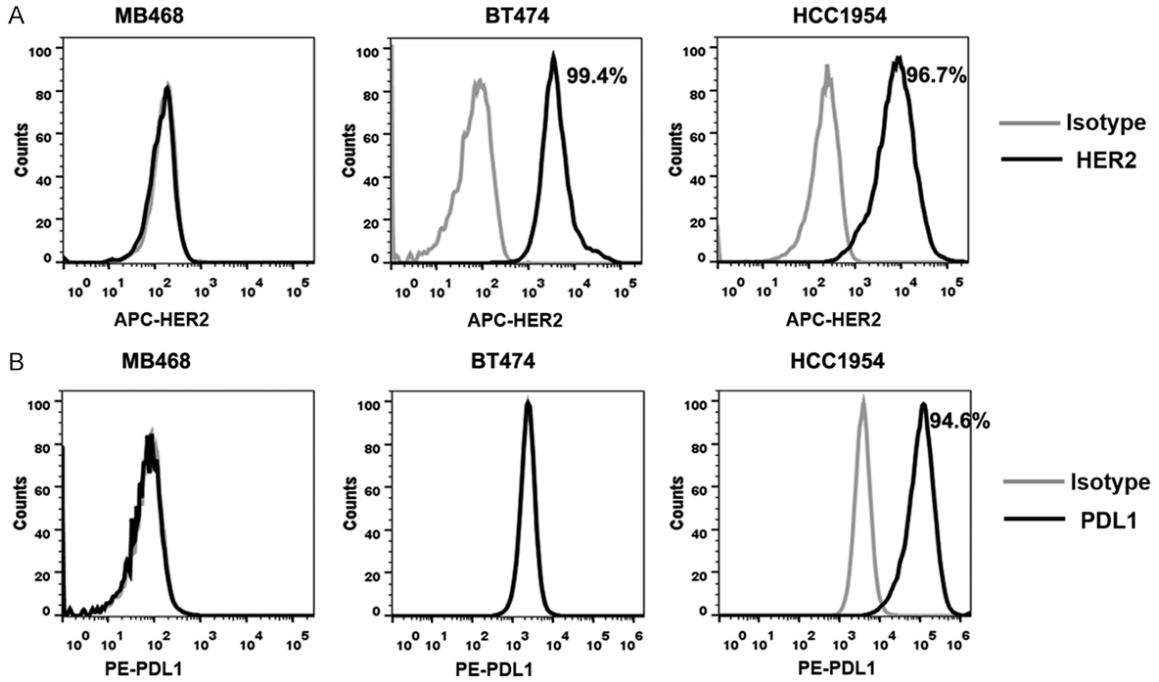

Figure 1. Expression of HER2 and PDL1 in breast cancer cell lines. A. The expression of HER2 in MB468, BT474 and HCC1954 cells. Cells were subjected to flow cytometry analysis using APC-anti-human HER2 antibody (black histogram) and APC-isotype control antibody (grey histogram). B. The expression of PDL1 in MB468, BT474 and HCC1954 cells. Cells were subjected to flow cytometry analysis using PE-anti-human PDL1 antibody (black histogram) and PE-isotype control antibody (grey histogram).

determine the expression level of HER2 and PDL1 in breast cancer cell lines. Flow cytometry was used to examine the expression of HER2 and PDL1 in MB468, BT474, and HCC1954 cell lines. The expression level of HER2 was similarly high in BT474 and HCC1954 cells (99.4% and 96.7% respectively) while it was undetectable in MB468 cells (**Figure 1A**). PDL1 was highly expressed in HCC1954 cells (94.6%) while it was undetectable in BT474 and MB468 cells (**Figure 1B**). HCC1954, BT474 and MB468 cell lines were set as $HER2^+/PDL1^+$ trastuzumab-resistant target cells, $HER2^+/PDL1^-$ trastuzumab-sensitive target cells and $HER2^-/PDL1^-$ control cells respectively.

Anti-HER2 CAR-T cells specifically target HER2-positive breast cancer cells

To examine the targeting specificity of anti-HER2 scFv on anti-HER2 CAR-T cell surface, anti-HER2 CAR-T cells were co-cultured with $HER2^-$ MB468 cells or $HER2^+$ BT474 and HCC1954 cells. When co-cultured with MB468 cells, anti-HER2 CAR-T cells were not aggregated around MB468 cells, dispersing in spaces (**Figure 2B**, **2C**). Most of the co-cultured MB468 cells were alive (**Figure 2B**). In contrast, when co-cultured with $HER2^+$ BT474 and HCC1954 cells, anti-HER2 CAR-T cells, which were shown as GFP^+ cells, were aggregated around BT474 (**Figure 2E**, **2F**) and HCC1954 (**Figure 2H**, **2I**) cells. In addition, viable BT474 and HCC1954 cells were significantly decreased (**Figure 2E**, 2H) compared with corresponding target cells that were not co-cultured with anti-HER2 CAR-T cells (**Figure 2D**, **2G**).

PD1 expression is enhanced in anti-HER2 CAR-T cells co-cultured with HER2-positive breast cancer cells

It is known that the expression of inhibitory receptors including PD1 is increased after T cells are activated [29, 30]. Thus, we examined the PD1 expression in anti-HER2 CAR-T cells after activation by HER2-positive breast cancer cells. Flow cytometry results showed that 18.5% of the anti-HER2 CAR-T cells were $PD1^+$ when co-cultured with $HER2^-$ MB468 cells (**Figure 3B**). In contrast, $PD1^+$ cells in anti-HER2 CAR-T cells were significantly increased to 48.7% and 44.7% when co-cultured with $HER2^+$

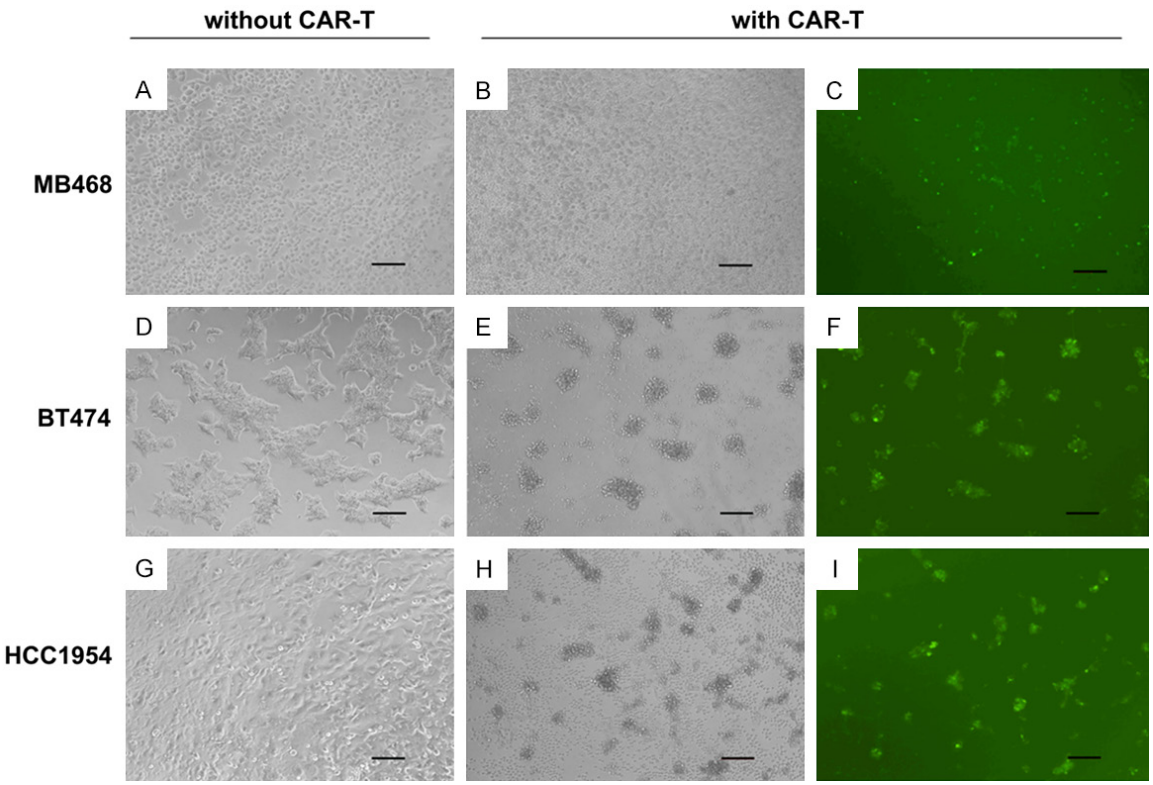

Figure 2. Anti-HER2 CAR-T cells specifically target HER2-positive breast cancer cells. The HER2$^-$ MB468 cells (A-C), and HER2$^+$ BT474 (D-F) and HCC1954 (G-I) cells were co-cultured with or without anti-HER2 CAR-T cells at an effector:target ratio of 4:1 for 24 h, and were examined under phase contrast (A, B, D, E, G, H) and fluorescent (C, F, I) microscope respectively. Scale bar: 100 μm.

BT474 and HCC1954 cells respectively (P<0.05) (**Figure 3B**).

HER2-positive breast cancer cells induce cytokine production of anti-HER2 CAR-T cells

To determine whether anti-HER2 CAR-T cells were activated when co-cultured with target cells, we examined cytokine secretion of anti-HER2 CAR-T cells by ELISA. The results showed that the secretion of IL-2 or IFN-γ was significantly increased in anti-HER2 CAR-T cells compared with blank T cells after being co-cultured with HER2$^+$ HCC1954 and BT474 cells for 48 and 72 hours (P<0.01 or P<0.001) (**Figure 4A**, **4B**). In addition, the addition of anti-PD1 antibody to the HCC1954 co-culture system for 72 hours further enhanced the production of IL-2 or IFN-γ compared with anti-HER2 CAR-T cells alone (P<0.01 or P<0.001) (**Figure 4A**, **4B**). However, the addition of anti-PD1 antibody to the BT474 (PDL1-negative) co-culture system had no effects on the production of IL-2 or IFN-γ compared with anti-HER2 CAR-T cells alone (**Figure 4A**, **4B**).

Anti-HER2 CAR-T cells evoke significant cytotoxicity against HER2$^+$ breast cancer cells

To evaluate the efficacy of CAR-T cells alone or in combination with PD1 blockade on target cells in vitro, anti-HER2 CAR-T cells were co-cultured with HER2$^-$/PDL1$^-$ (MB468), HER2$^+$/PDL1$^-$ (BT474) and HER2$^+$/PDL1$^+$ (HCC1954) breast cancer cells at effector: target ratios of 2:1, 4:1, 8:1 and 16:1 in the absence and presence of anti-PD1 antibody for 18 hours followed by LDH assay for cytotoxicity (**Figure 5**). As a negative control, blank T or anti-HER2 CAR-T cells at all of the effector:target ratios had similarly low cytotoxicity against MB468 cells regardless of anti-PD1 antibody addition (**Figure 5A**). In contrast, anti-HER2 CAR-T cells exerted significant increase in cytotoxicity against BT474 cells with the increased effector:target ratios (**P<0.01, ***P<0.001) (**Figure 5B**). However, addition of anti-PD1 antibody to anti-HER2 CAR-T cells did not further enhance cytotoxicity against BT474 (**Figure 5B**), which is consistent with the lack of PDL1 expression in BT474 cells (**Figure 1B**). Similar to the effect of

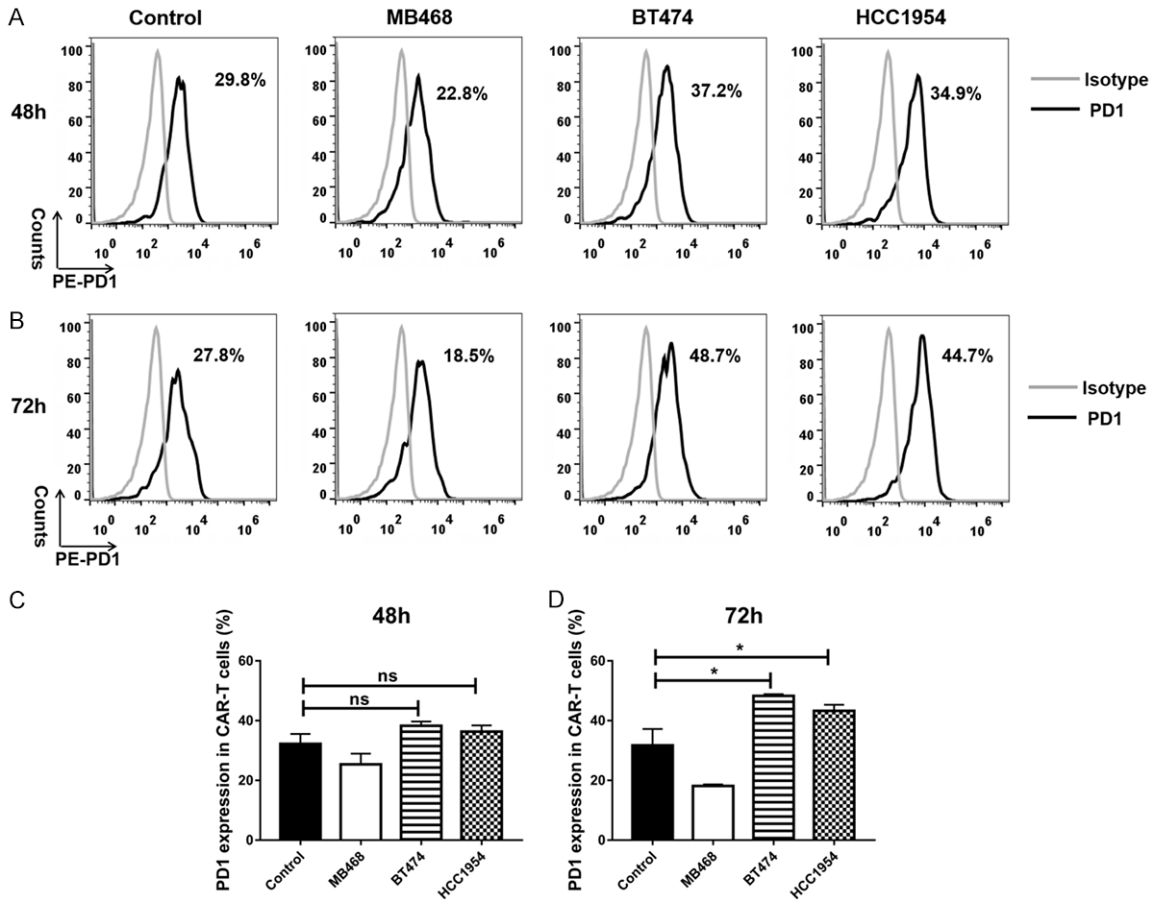

Figure 3. PD1 expression is increased in anti-HER2 CAR-T cells stimulated by HER2-positive breast cancer cells. Anti-HER2 CAR-T cells were co-cultured with mock control, $HER2^-$ MB468 cells, and $HER2^+$ BT474 and HCC1954 cells at an effector:target ratio of 4:1 for 48 h and 72 h. Then, anti-HER2 CAR-T cells were subjected to flow cytometry analysis using PE-anti-human PD1 antibody (black histogram) and PE-isotype control antibody (grey histogram). Representative flow cytometry images of PD1 expression in CAR-T cells at 48 h (A) and 72 h (B) are shown. Mean values at 48 h (C) and 72 h (D) from three repeated experiments were analyzed, *P<0.05.

anti-HER2 CAR-T cells on BT474 cells, anti-HER2 CAR-T cells also caused dramatic increase in cytotoxicity against HCC1954 cells with the increased effector:target ratios (*P< 0.05, ***P<0.001) (**Figure 5C**). Importantly, addition of anti-PD1 antibody to anti-HER2 CAR-T cells further enhanced cytotoxicity against HCC1954 (##P<0.01, ###P<0.001) (**Figure 5C**), which is consistent with the high PDL1 expression in HCC1954 cells (**Figure 1B**). As expected, blank T cells alone or blank T cells plus anti-PD1 antibody had minimal effects on the cytotoxicity against BT474 and HCC1954 cells (**Figure 5B** and **5C**). At effector:target ratio of 16:1, the cytotoxicity of anti-HER2 CAR-T cells was significantly higher in co-culture system with BT474 or HCC1954 cells than with MB468 cells in the absence or presence of anti-PD1 antibody (***P<0.001) (**Figure 5D**). At the effector: target ratio of 16:1, anti-HER2 CAR-T cells exerted lower cytotoxicity against HCC1954 cells compared with BT474 cells in the absence of anti-PD1 antibody (**P<0.01) whereas anti-HER2 CAR-T cells had the same cytotoxicity against these two cell lines in the presence of anti-PD1 antibody (**Figure 5D**).

$HER2^+$ HCC1954 cells-induced expansion of anti-HER2 CAR-T cells can be further enhanced in the presence of anti-PD1 antibody

T cells can undergo dramatic expansion after activation by the antigen presented by MHC molecule [31]. We examined the proliferation of blank T or anti-HER2 CAR-T cells after they were co-cultured with $HER2^-/PDL1^-$ (MB468), and $HER2^+/PDL1^-$ (BT474) and $HER2^+/PDL1^+$ (HCC-1954) breast cancer cells at effector: target

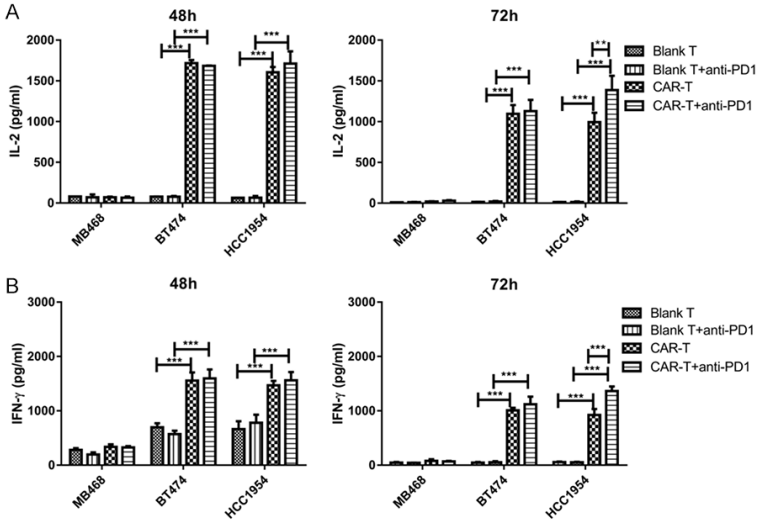

Figure 4. HER2$^+$ breast cancer cells induce cytokine secretion from anti-HER2 CAR T cells. Non-transduced (blank) T cells or anti-HER2 CAR-T cells were co-cultured in the absence or presence of anti-PD1 antibody with HER2$^-$ MB468 cells, and HER2$^+$ HCC1954 and BT474 cells at an effector:target ratio of 4:1 for 48 or 72 hours. IL-2 (A) and IFN-γ (B) concentrations in culture supernatants were analyzed using ELISA kits. Mean values from three different experiments were presented. **P<0.01; ***P<0.001. IFN-γ: interferon-γ; IL-2: interleukin-2.

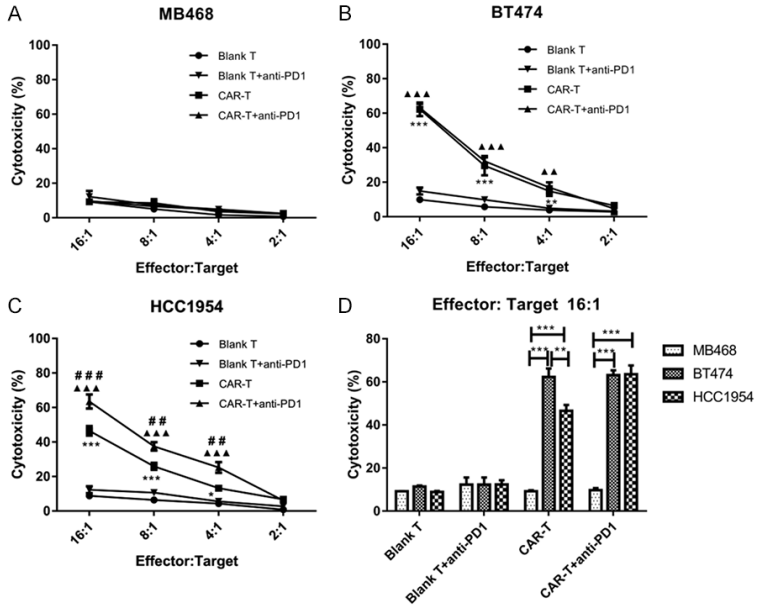

Figure 5. Anti-HER2 CAR-T cells evoke cytotoxicity against HER2$^+$ breast cancer cells. Blank T or anti-HER2 CAR-T cells were co-cultured with MB468, BT474 and HCC1954 cells in the absence or presence of anti-PD1 antibody at effector:target ratios of 2:1, 4:1, 8:1 or 16:1 for 18 hours. Cytotoxicity against breast cancer cells was determined by analyzing LDH released into culture supernatants using LDH detection kit. The cytotoxicity (%) was calculated as described in the Material and Method section. Mean values from three different experiments were presented. A. Anti-HER2 CAR-T cells cause minimal cytotoxicity against HER2$^-$ MB468 cells and addition of anti-PD1 antibody does not further enhance cytotoxicity against MB468 cells. B. Anti-HER2 CAR-T cells evoked cytotoxicity against HER2$^+$/PDL1$^-$ BT474 cells cannot be further enhanced with the addition of anti-PD1 antibody. C. Anti-HER2 CAR-T cells evoked cytotoxicity against HER2$^+$/PDL1$^+$ HCC1954 cells can be further enhanced with the addition of anti-PD1 antibody. CAR-T group was compared with blank T group, *P<0.05, **P<0.01, and ***P<0.001; or CAR-T plus anti-PD1 group was compared with blank T plus anti-PD1 group, ▲▲P<0.01 and ▲▲▲P<0.001; or CAR-T plus anti-PD1 group was compared with CAR-T group, ##P<0.01 and ###P<0.001. D. At the effector:target ratio of 16:1, the cytotoxicity (%) was compared among HER2$^-$/PDL1$^-$ (MB468), HER2$^+$/PDL1$^-$ (BT474), and HER2$^+$/PDL1$^+$ (HCC1954) breast cancer cells, **P<0.01 and ***P<0.001.

ratio of 4:1 in the absence and presence of anti-PD1 antibody for 24, 48, and 72 hours. As a negative control, anti-HER2 CAR-T cells, similar to blank T cells, proliferated slowly when co-cultured with MB468 cells regardless of the presence anti-PD1 antibody (**Figure 6A**). In contrast, the growth of anti-HER2 CAR-T cells was significantly higher than blank T cells when co-cultured with BT474 cells (***P<0.001) (**Figure 6B**). However, addition of anti-PD1 antibody to the BT474 co-culture did not further enhance the growth of either anti-HER2 CAR-T cells or blank T cells (**Figure 6B**). Similar to BT474 cells, HCC-1954 cells also dramatically increased the growth of anti-HER2 CAR-T in comparison with blank T cells (**P<0.01, ***P<0.001) (**Figure 6C**). Importantly, the addition of anti-PD1 antibody to the HCC19-54 co-culture system further enhanced the growth of anti-HER2 CAR-T cells (#P<0.05, ##P<0.01, ###P<0.001) but not the growth of blank T cells (**Figure 6C**), suggesting the

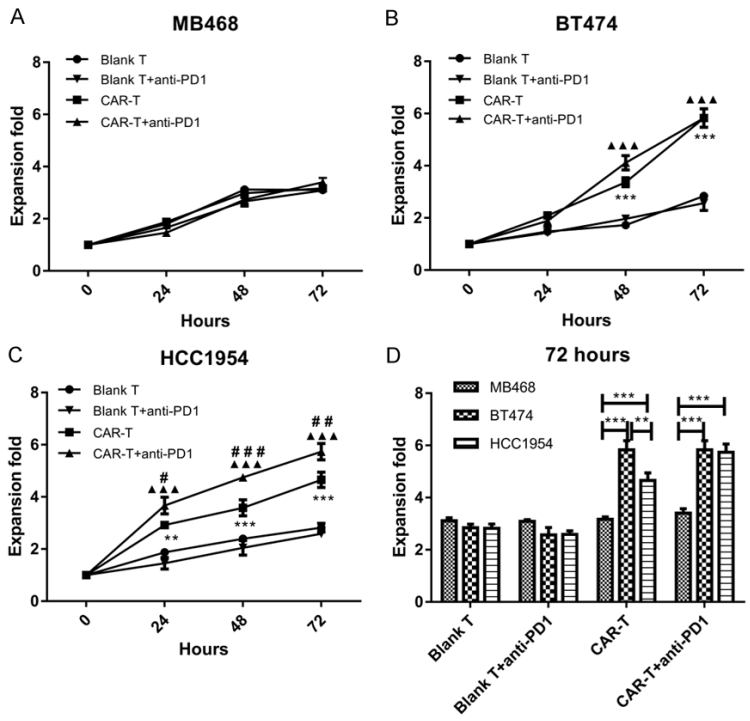

Figure 6. HER2 positive breast cancer cells promote the expansion of anti-HER2 CAR-T cells. A-C. Blank T cells or anti-HER2 CAR-T cells were co-cultured with the indicated target cells in the absence or presence of PD1 antibody at an effector:target ratio of 4:1 for 24, 48 or 72 hours. Exogenous IL-2 at 100 IU/ml was added to the T cell cultures every other day. Viable cells were counted by trypan blue exclusion each day. The mean values from three different experiments were presented. CAR-T group was compared with blank T group, **P<0.01 and ***P<0.001; or CAR-T plus anti-PD1 group was compared with blank T plus anti-PD1 group, ▲▲▲P<0.001; or CAR-T group plus anti-PD1 group was compared with CAR-T group, #P<0.05, ##P<0.01, and ###P<0.001. D. At 72 hours, the expansion of anti-HER2 CAR-T cells or anti-HER2 CAR-T cells with PD1 antibody addition was significantly higher in the presence of $HER2^+$ BT474 and HCC1954 cells compared with $HER2^-$ MB468 cells. ***P<0.001.

additive efficacy of combining CAR-T and PD1 blockade. At 72 hours, the expansion of anti-HER2 CAR-T cells in the absence or presence of anti-PD1 antibody was significantly higher in co-culture system with BT474 or HCC1954 cells than with MB468 cells (***P<0.001) (**Figure 6D**).

$HER2^+$ HCC1954 cells-enhanced apoptosis of anti-HER2 CAR-T cells can be inhibited in the presence of anti-PD1 antibody

In addition to cell proliferation, activated T cells also undergo apoptosis in part due to up-regulation of PD1 and its interaction with its ligand PDL1. To test whether anti-PD1 antibody can reduce apoptosis of anti-HER2 CAR-T cells after activation by $HER2^+$ target cells, we analyzed the apoptosis of anti-HER2 CAR-T cells alone or in combination with anti-PD1 antibody after being co-cultured with $HER2^-/PDL1^-$ (MB468), and $HER2^+/PDL1^-$ (BT474) and $HER2^+/PDL1^+$ (HCC1954) using Annexin V/7-ADD reagents (**Figure 7A** and **7B**). As a negative control, MB468 cells did not significantly increase apoptosis of anti-HER2 CAR-T cells nor blank T cells in the absence or presence of anti-PD1 antibody (**Figure 7B**). In contrast, both BT474 and HCC1954 cells significantly enhanced apoptosis of anti-HER2 CAR-T cells compared with blank T cells (P<0.001). However, anti-PD1 antibody reduced apoptosis of anti-HER2 CAR-T cells when co-cultured only with HCC1954 cells (P<0.01) but not with BT474 cells (**Figure 7B**).

Anti-HER2 CAR-T cells alone and in combination with anti-PD1 antibody inhibit the growth of HCC1954 xenograft tumor

Next, we tested whether anti-HER2 CAR-T cells alone and in combination with anti-PD1 antibody can inhibit the growth of xenograft tumors from HCC-1954, a trastuzumab resistant breast cancer cell line. As expected, HCC1954 tumors grew steadily when treated with blank T cells or blank T cells with anti-human PD1 antibody over the course of experiment (**Figure 8A**). In contrast, the growth rate (**Figure 8A**), size (**Figure 8B**), and weight (**Figure 8C**) of HCC1954 tumors were significantly inhibited when treated with anti-HER2 CAR-T cells compared with blank T cells. Importantly, addition of anti-PD1 antibody further enhanced the inhibition of HCC1954 tumor growth by the anti-HER2 CAR-T cell treatment (**Figure 8A-C**). Results of cleaved caspase 3 immunohistochemistry revealed that anti-HER2 CAR-T cells induced apoptosis of HCC-1954 tumor cells whereas anti-PD1 antibody combining with anti-HER2 CAR-T cells further enhanced apoptosis of tumor cells (**Figure 9B**,

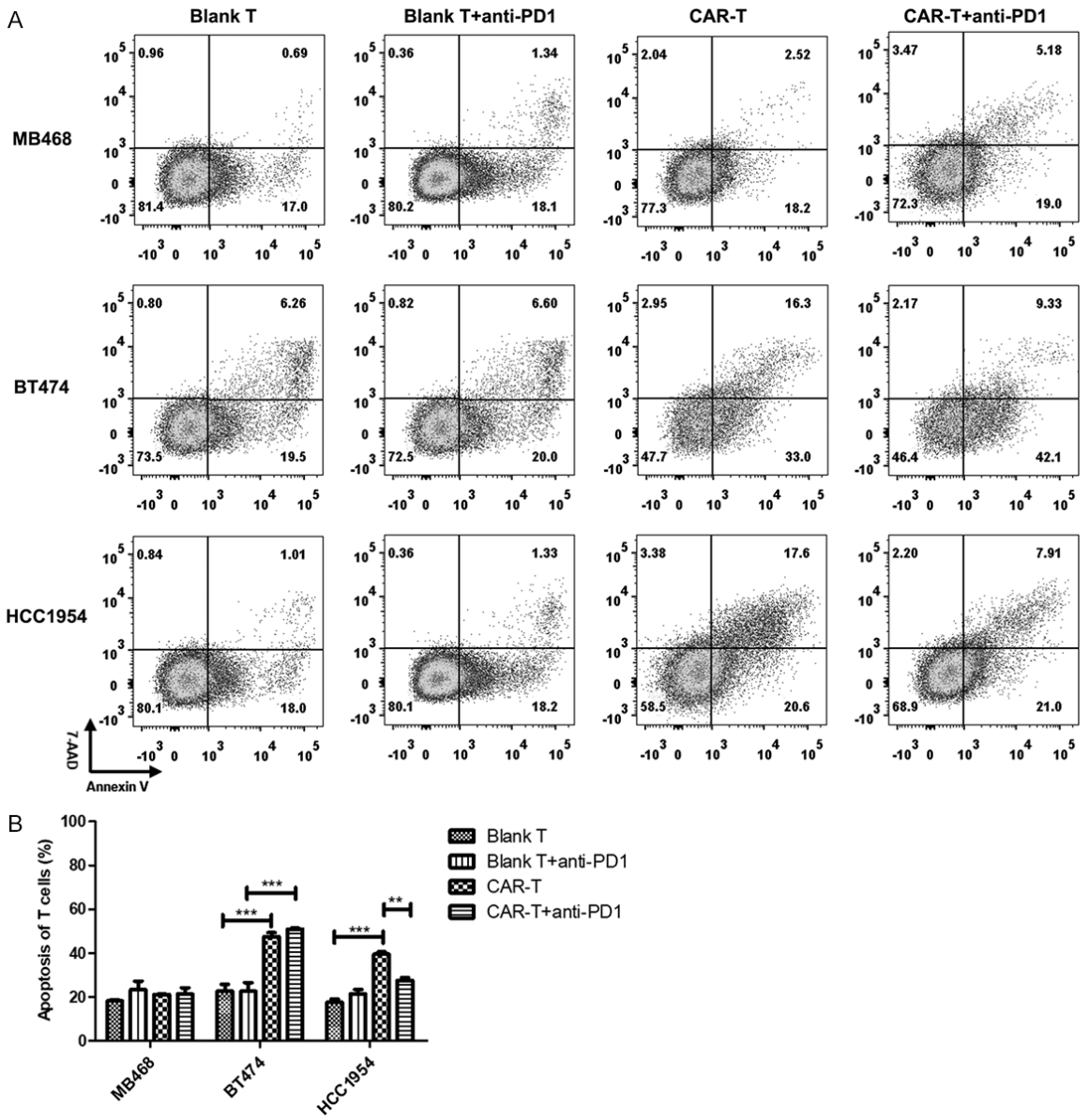

Figure 7. Anti-PD1 antibody decreases apoptosis of anti-HER2 CAR-T cells induced by HER2-positive breast cancer cells. (A) Blank T cells or anti-HER2 CAR-T cells were co-cultured in the absence or presence of anti-PD1 antibody with the indicated target cells at the effector:target ratio of 4:1 for 72 hours, then subjected to apoptosis analysis by flow cytometry analysis using Annexin V and 7-AAD reagents. Percentages of positive cells were shown in each quadrate. (B) Quantification of the flow cytometry analysis data from (A). The mean values from three different experiments were presented. CAR-T group was compared with blank T group, or CAR-T plus anti-PD1 group was compared with blank T plus anti-PD1 group, or CAR-T plus anti-PD1 group was compared with CAR-T group, **P<0.01 and ***P<0.001.

9C). Furthermore, results of human CD3 immunohistochemistry showed that significant numbers of anti-HER2 CAR-T cells were present in tumor stroma (~10%), and the addition of anti-PD1 antibody further enhanced the content of anti-HER2 CAR-T cells into tumor stroma (~20%). In contrast, there were few blank T cells present in tumor stroma regardless of anti-PD1 antibody treatment (**Figure 10A**, **10B**).

Discussion

The present study demonstrated that our third generation anti-HER2 CAR-T cells with CD28 and 4-1BB/CD137 as co-stimulatory molecules are able to eliminate HER2-positive breast cancer cells with both specificity and efficiency. The present study used the anti-HER2 scFv from specific anti-HER2 antibody 4D5-8 [32]

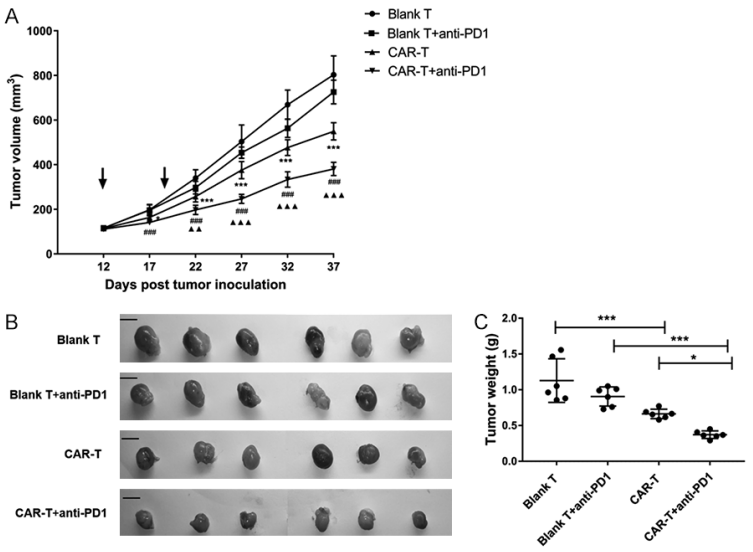

Figure 8. Anti-HER2 CAR-T cells alone and in combination with anti-PD1 antibody inhibit the growth of HCC1954 xenograft tumor. HCC1954 cells were injected subcutaneously into the flanks of 5-week old Balb/c nude mice. Twelve days later, mice were randomized into 4 groups (6 mice/group) and treated with blank T or anti-HER2 CAR-T cells (2×10^7/mouse) by intravenous injection twice (on days 12 and 19 post injection of HCC1954 cells, showed with arrows) and/or with anti-PD1 antibody (250 μg/mouse) by intraperitoneal injection on days 12, 16, 19, 23. Tumor growth was monitored by measuring the tumor dimension every five days, and tumor volume was calculated. A. The kinetics of HCC1954 tumor growth in response to treatments with anti-HER2 CAR-T cells and/or anti-PD1 antibody. CAR-T group was compared with blank T group, on day 17, *P<0.05 and ***P<0.001; CAR-T plus anti-PD1 group was compared with blank T plus anti-PD1 group, on day 17, ###P<0.001; CAR-T plus anti-PD1 group compared with CAR-T group, on day 22, ▲▲P<0.01 and ▲▲▲P<0.001. B. The sizes of tumors dissected from different treatment groups on day 37 post-injection of HCC1954 cells. Scale bar =1 cm. C. The weights of tumors dissected from different treatment groups on day 37 post-injection of HCC1954 cells, *P<0.05 and ***P<0.001.

for CAR construction. The efficiency of our anti-HER2 CAR-T cells came from our selection of two co-stimulatory molecules CD28 and 4-1BB/CD137 of CAR. 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) have been embedded in the CAR, and their roles in coordinating antitumor immunity are explored [14]. When compared with other co-stimulatory molecules, CD28 was more effective at enhancing IL-2 production, improving clonal expansion and maintaining the persistence of CAR-T cells. CAR with CD28/4-1BB/CD137 was more effective than with CD28 alone in eliciting cytotoxicity and IFN-γ production [33]. Our results have demonstrated the strong efficacy of CAR with CD28/CD137. Our data showed that the secretion of IL-2 and IFN-γ is significantly higher from our third generation anti-HER2 CAR-T cells compared with from blank T cells when co-cultured with HER2$^+$ breast cancer cells (**Figure 4**). The third generation anti-HER2 CAR-T cells displayed higher cytotoxicity and improved clonal expansion relative to blank T cells against HER2$^+$ breast cancer cells (**Figures 2**, **5**, **6**, **8**, **9**).

About 20% of breast cancers are HER2$^+$, with poor prognosis and survival rates [34]. Although specific HER2 targeted therapies including trastuzumab have great efficacy in improving overall survival rates for HER2$^+$ breast cancer, de novo and acquired resistance to HER2 targeting is still a major challenge in clinic. Results of our study demonstrated that the anti-HER2 CAR-T cells not only effectively killed trastuzumab-sensitive BT474 cells but also trastuzumab-resistant HCC1954, with no cytotoxicity against HER2 negative MB468 cells in vitro (**Figure 5**). Furthermore, anti-HER2 CAR-T cells impaired the growth of HCC1954 xenograft tumor (**Figures 8** and **9**). The mechanisms of trastuzumab resistance of HER2 breast cancer have been widely studied, which include activating mutations in HER2 [35], activation of HER2 downstream signaling pathways and other receptor tyrosine kinases in HER2$^+$ breast cancer [8]. Trastuzumab-resistant breast cancer in general still expresses HER2 on the cell surface. The anti-HER2 CAR in our study consists of the scFv fragment from trastuzumab [36]. Therefore, our anti-HER2 CAR-T cells should recognize and be effective for most trastuzumab-resistant breast cancers, except in the case of resistance involving p95HER2 in clinic [37].

Immune checkpoint pathways including PD1/PDL1 mediate the inhibition of T cell function or

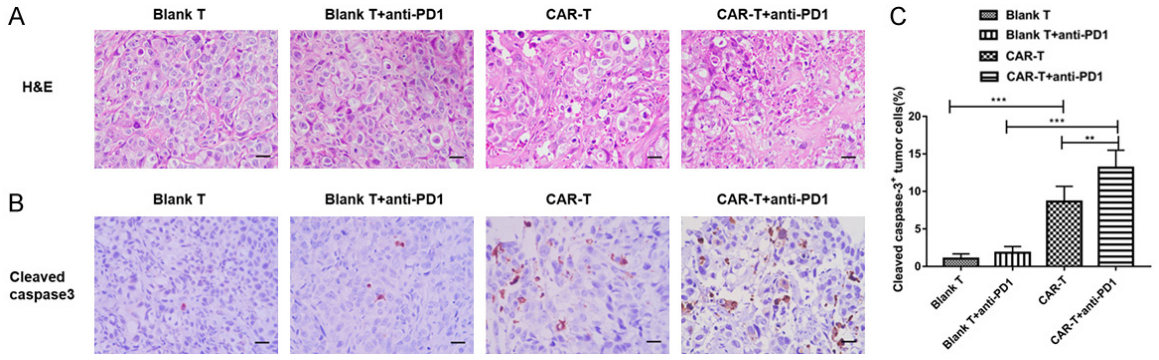

Figure 9. Anti-HER2 CAR-T cells alone and in combination with anti-PD1 antibody induces apoptosis of HCC1954 tumor cells. Tumors dissected from mice treated with blank T cells alone, blank T cells plus anti-PD1 antibody, anti-HER2-CAR-T cells alone, and anti-HER2 CAR-T cells plus anti-PD1 antibody were sectioned, and stained with Hematoxylin and Eosin (H&E) (A) and anti-human cleaved caspase 3 antibody (B). Scale bar =50 μm. (C) Quantification of cleaved caspase 3 immunohistochemistry result. Cleaved caspase 3^+ tumor cells and total cells were counted from five random 400× microscopic fields for each section. A total of three different sections were counted, and the average percentage of cleaved caspase 3^+ tumor cells per section is shown. **P<0.01 and ***P<0.001.

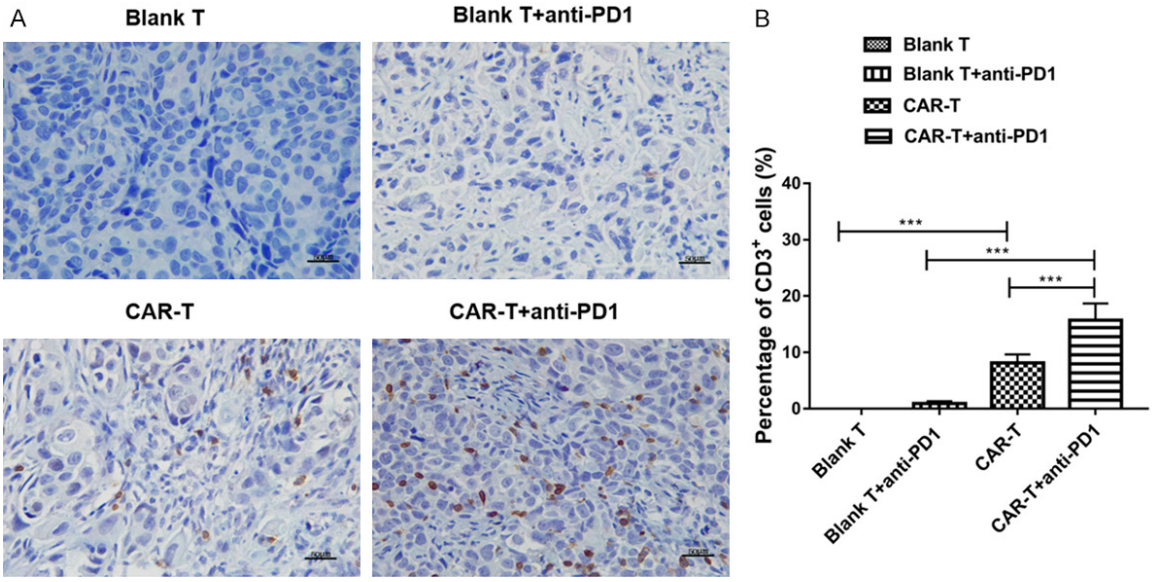

Figure 10. Anti-HER2 CAR-T cell content in HCC1954 xenograft tumor stroma. A. Tumors dissected from mice of blank T group, blank T plus anti-PD1 group, CAR-T group, and CAR-T plus anti-PD1 group were subjected to immunohistochemistry with anti-human CD3ε antibody. Scale bar =50 μm. B. Quantification of human CD3 IHC result. Human $CD3\varepsilon^+$ cells and total cells were counted. Three sections for each tumor, five random 400× microscopic fields for each section were counted, and the average percentages of human $CD3^+$ were compared among different groups, ***P<0.001.

depletion of T cells, which can contribute to the failure of CAR-T treatment [38]. Under the stimulation of tumor specific antigen, PD1 expression in CAR-T cells is increased significantly, resulting in the weakening of its anti-tumor immune response [38, 39]. Our result also showed that PD1 expression increased in anti-HER2 CAR-T cells after being co-cultured with $HER2^+$ breast cancer cells (**Figure 3**). Addition of the anti-PD1 antibody, pembrolizumab, has been used to enhance function and expansion of CAR-T cells. In a study of anti-GD2 CAR-T cells in the treatment of metastatic melanoma, Gargett et al. found that the proportion of activated anti-GD2 CAR-T cells decreased due to activating-induced cell death (AICD) after specific antigen stimulation. Although the application of pembrolizumab did not affect AICD, it

can restore the activity of CAR-T cells to the level before antigen stimulation, while promoting the secretion of cytokines [38]. Chong et al. reported that the proliferative activity of anti-CD19 CAR-T cells was increased greatly in patients with refractory diffuse large B-cell lymphoma after the addition of pembrolizumab [40]. Our result has demonstrated that addition of anti-PD1 antibody can enhance the efficacy of anti-HER2 CAR-T against trastuzumab-resistant breast cancer or $HER2^+$ solid tumors. Our study further supports the importance of blocking the T cells immune checkpoint pathways including PD1/PDL1 in enhancing the efficacies of CAR-T cells. Anti-HER2 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 anti-HER2 CAR-T cells alone (**Figures 4**, **5**). Anti-PD1 antibody increases the efficacy of the anti-HER2 CAR-T against HCC1954 xenograft tumor (**Figure 8**) likely through enhancing cytokine secretion (**Figure 4**), cytotoxicity (**Figure 5**), survival (**Figure 7**), and intratumoral content (**Figure 10**) of CAR-T cells. A published study has shown that anti-PD1 monoclonal antibody displayed efficacy against melanoma by increasing intratumoral recruitment of $CD8^+$ effector T cells [41]. Although various reports show that anti-HER2 CAR-T cells are effective in inhibiting the growth of different $HER2^+$ solid tumors including colon [42], breast [43], sarcoma [44, 45], brain [46], and gastric [47, 48] cancers, combining PD1 blockade has been lacking especially in preclinical studies and clinical trials using anit-HER2 CAR-T cells [49]. Interestingly, up-regulation of PDL1 in $HER2^+$ breast cancer cells has been recently implicated as a potentially mechanism for trastuzumab resistance [50]. Therefore, anti-HER2 CAR-T in combination with anti-PD1 or anti-PDL1 antibody should be considered as an effective therapy to overcome trastuzumab resistance of $HER2^+$ breast cancer.

The original application of trastuzumab-derived CAR-T cells in treating a colon cancer patient did cause the death of a patient due to the use of a very high number of infused anti-HER2 CAR-T cells (10^{10}/dose) [51]. The cause of death for the patient is likely due to the strong cytokine-release syndrome (CRS), which the medical team was not prepared to deal with at the time in 2010 [17]. Since then, various strategies have been used to combat the effects of CRS caused by CAR-T cells in clinical practice. Importantly, several clinical studies showed that infusing a lower number ($<10^8/m^2$) of anti-HER2 CAR-T cells has efficacy against glioblastoma [46] and sarcoma [45] without apparent toxicity. Therefore, utilizing a lower number of our anti-HER2 CAR-T cells together with PD1 antibody may be useful to overcome trastuzumab resistance of breast cancer in clinic.

In conclusion, our study demonstrated that third generation anti-HER2 CAR-T cells can eliminate $HER2^+$ trastuzumab-resistant breast cancer cells in vitro and in vivo with specificity and efficiency. When combined with anti-PD1 antibody, CAR-T cells have an even stronger therapeutic effect on $HER2^+/PDL1^+$ trastuzumab-resistant breast cancer cells in vitro and in vivo.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (81572980, 81972463, 81772819), Natural Science Foundation of Zhejiang Province, China (LY17H160058, LY18H080003, LY20C080001), Wenzhou Municipal Science and Technology Bureau of China (H20170001), Wenzhou Medical University Startup funds, and in part supported by the Key Discipline of Zhejiang Province in Medical Technology (First Class, Category A).

Disclosure of conflict of interest

None.

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