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CAR T Cell-Packaged Oncolytic Vaccinia Virus Displays Enhanced Antitumor Efficacy

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Abstract

Background: Oncolytic vaccinia virus is a promising cancer therapeutic modality. However, the effectiveness of oncolytic viruses is limited by several factors. Systemic or intratumoral delivery of vaccinia viruses with the subsequent quick clearance of the viruses from the tumor site and the body by the strong immune responses induced by the virus are among the key challenges. In this study, we explored CAR T cell-packaged oncolytic vaccinia virus as a combinational therapy strategy in order to overcome current limitations for oncolytic virotherapy.

Materials and Methods: We generated human HER2-CAR T cells and infected the HER2-CAR T cells with a EphA2-CD3 T cell engager-armed oncolytic vaccinia virus and evaluated the virus infectivity and replication within the T cells by flow analysis and virus tittering. T cell activation and cytotoxicity were determined by ELISA and ^{51}Cr release assay.

Results: We demonstrated that oncolytic vaccinia virus infected human HER2-CAR T cells effectively and virus particle in the activated human T cells increased >1000 fold in 3 days. In addition, EphA2-CD3 T cell engager effectively activated HER2-CAR T cells in the presence of $HER2^{dim}EphA2^{high}$ NSCLC A549 cell lines, indicated by the elevated expression level of IFNγ and IL2. Importantly, *in vitro* studies showed that HER2-CAR T cell-packaged EphA2-TEA-VV displayed enhanced cytotoxicity against $HER2^{dim}EphA2^{high}$ NSCLC A549 cell lines compared to HER2-CAR T cells or EphA2-TEA-VV alone.

Conclusion: HER2-CAR T cell-packaged EphA2-TEA-VV is a promising therapeutic candidate with the ability to overcome the virus's high immunogenicity and tumor heterogeneity, resulting in enhanced antitumor effects.

Keywords: CAR T cell therapy; Oncolytic vaccinia virus; Immunotherapy; T cell engager

Introduction

Oncolytic viruses have emerged as a promising cancer therapeutic modality since they specifically infect, replicate in, and lyse tumor cells without damaging normal cells. The tumor selectivity pegs oncolytic viruses as ideal delivery tools for the development of novel cancer immunotherapies [1-6]. Various oncolytic virotherapies have been developed by arming the virus with therapeutics genes, such as GM-CSF, IL-12, CD40-L, 4-1 BB-L, OX-40L, IL-2, TNF-a, anti-CTLA-4 antibody or anti-PD1/PD-L1 antibody [7-13]. Oncolytic virus has also been engineered to express Tumor Associate Antigens (TAA) in order to induce TAA-specific immune responses, providing long-term protection against tumor relapse [14-16]. In addition, oncolytic vaccinia virus has also been armed by T-cell engager (TEA-VV), directing endogenous T cells to recognize and kill the tumor cells regardless of the oncolytic virus infection of the tumor cells, increasing the efficiency of oncolytic virotherapy [17-21].

Although the anti-tumor efficacy of oncolytic viruses has been proved by the animal and human studies, it has been realized that the effectiveness of oncolytic viruses is significantly hindered by several factors [22]. Rapid and efficient clearance of the virus by host immune system is among the key challenges. Immune factors such as antibodies neutralize the virus by binding to it directly and preventing a successful infection of the cells or by marking it for destruction either by complement or by immune cells [23-29]. With each subsequent administration of the oncolytic virus, the immune responses induced by the virus become faster and stronger, preventing the virus reaching the tumor cells. Intratumoral or regional injection of the oncolytic virus can certainly overcome this limitation by delivering all the viral particles directly to tumor or the surrounding tissues. However, this approach may be only suitable for some surficial tumors and does not take into the account cases in which the tumors are located at deep in the tissues or have metastasized to other locations. Thus, systemic administration of the virus is desirable since it potentially allows the virus to reach all the tumor cells.

Various strategies have been developed to overcome the anti-virus neutralization antibody and deliver the virus systemically. For example, Cytokine-Induced Killer (CIK) cells have been utilized to package the oncolytic vaccinia virus and deliver the virus to the tumor cells via systemic injection route [30-32]. The studies have shown that systemic administration of CIK-packaged WR strain double deleted

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vaccinia virus (vvDD) successfully delivered the virus to tumor tissues and achieved regression of tumors in both immunocompetent and immunodeficient mouse models. Administration of CIK-packaged vvDD resulted in a prolonged persistence of the virus within the tumor tissues compared to administration of vvDD. And the vvDD-infection didn't affect CIK cell's ability of tumor homing and infiltrating. These results indicated the potential of immune cell-packaged oncolytic vaccinia virus for systemic therapy of solid tumors. Adoptive immune cellular therapy such as Chimeric Antigen Receptor (CAR) T cell therapy has demonstrated tremendous success in eradicating hematological malignancies resulting in FDA's approval of four CAR T cell therapies for leukemia, lymphoma, and multiple myeloma [33-37]. However, the clinical results of CAR T cell therapy for solid tumors so far have been suboptimal. The limitations for the CAR T cell therapy for solid tumors might be due to tumor heterogeneity/antigen loss variants (ALV), limited tumor infiltration due to surrounding matrix, and the immune suppressive environment [38-43]. In this study, we developed a HER2-CAR T cell-packaged EphA2-TEA-VV strategy and evaluated VV's infectivity of human T cells and its efficacy against tumor cells *in vitro*.

Materials and Methods

Cell line and blood donors

The breast cancer cell line SK-BR-3 that expresses HER2 and non-small-cell lung cancer cell (NSCLC) A549 that is $HER2^{dim}EphA2^{high}$ [44,45] were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with 10% Fetal bovine serum (FBS, HyClone, Logan, UT), supplemented with 2mmol/l GlutaMAX-I, 1.5g/l sodium bicarbonate, 0.1mmol/l nonessential amino acids, and 1.0mmol/l sodium pyruvate (Invitrogen). Human Peripheral Blood Mononuclear Cells (PBMC) were used in this study and purchased from Gulf Coase Regional Blood Center in Houston, Texas. The individually identifiable private information was not collected for the study. Human PBMCs were maintained in RPMI 1640 with 10% FBS containing 2mmol/l GlutaMAX-I.

Production of retroviral vectors

The HER2-specific CAR vector was a gift from Dr. Stephen Gottschalk [40,41]. The HER2-specific CAR contains the HER2-specific single chain variable fragment derived from the anti-HER2 antibody FRP5 clone, a hinger region from human IgG1, a human CD28 transmembrane domain and a CD28.ζ signaling domain. To produce HER2-specifc retroviral supernatant, 293T cells were co-transfected with HER2-specific CAR vector, Peg-Pam-e plasmid containing the sequence for MoMLV gag-pol, and plasmid pMEVSVg encoding the sequence for vesicular stomatitis virus G, using GeneJuice transfection reagent (EMD Biosciences, San Diego, CA) following the manufacturer's instructions. The 293T cell culture supernatants that contains the HER2-CAR retroviral particles were collected at 48 and 72 hours post plasmid transfection and subjected to retroviral transduction of human T cells.

Retroviral transduction of human T cells

Human PBMC were activated with anti-CD3 (OKT clone) and anti-CD28 antibodies followed by retroviral transduction as previously described [40,41]. Briefly, human PBMC were washed with phosphate-buffered saline (PBS; Sigma, St Louis, MO) for three times and seed in 24-well plate that was pre-coated with anti-CD3 antibody OKT3 (OrthoBiotech, Raritan, NJ) and anti-CD28 antibodie (BD Biosciences, Palo Alto, CA) at a final concentration of 1μg/ml for each antibody. 24 hours later, the cells were harvested for retroviral transduction. A non-tissue culture treated 24-well plate was pre-coated with a recombinant fibronectin fragment (FN CH-296; Retronectin; Takara Bio USA, Madison, WI) following the manufacturer's instructions. Wells were then washed with PBS and incubated twice for 30 minutes with the 293T cell culture supernatants that contains the HER2-specifc retroviral particles. And then $3x10^5$ anti-CD3/CD28 antibody activated human T cells were added to each well in the presence of 100 U IL-2/ml. 48 hours later, cells were harvested and cultured in the presence of 100 U/IL2/ml for 5 days prior to use.

VV transduction of human T cells

Double-deleted VVs (vvDD, Western Reserve strain) expressing EphA2-scFv-CD3-scFv T cell engager (EphA2-TEA-VV) was generated as previously described [20]. The EphA2-TEA-VV also expresses Red Fluorescent Protein (RFP) allowing flow analysis of the VV infectivity. To infect the human T cells, HER2-CAR T cells or control T cells were infected with EphA2-TEA-VV or control VVs at increasing MOIs (i.e. 0.1, 1, or 5) in minimum essential medium with 2.5% FBS for 2 hours at 37°C. Then the medium was replaced with fresh RPMI medium with 10% FBS, and T cells were incubated until harvesting at 1, 2, 3, 4, 5, and 7 days after infection. After three freeze-thaw cycles, virus was quantified by plaque tittering on CV-1 cells as described previously.

Flow cytometry

To determine the HER2-specfic CAR expression on the surface of human T cells, the T cells were first incubated with a recombinant HER2-Fc fusion protein (R&D Systems, Minneapolis, MN) for 30 minutes followed by staining with a goat anti-Fc FITC secondary antibody (Chemicon, Temecula, CA). T cells were washed three times with PBS containing 2% FBS and 0.1% sodium azide (Sigma; FACS buffer) prior to addition of HER2-Fc or antibodies. To determine the VV infectivity, human T cells were infected with EphA2-TEA-VV expressing RFP and the RFP expression in T cells was used to determine the VV infectivity. The cells were analyzed using a FACScalibur instrument (Becton Dickinson, Mountain View, CA) and CellQuest software (Becton Dickinson).

Chromium-51 release assay

Chromium-51 release assay was performed as previously described. Briefly, tumor cells were labeled with ^{51}Cr as described and mixed with decreasing numbers of HER2-specific CAR T cells to give T cells to tumor cells ratios of 40:1, 20:1, 10:1, 5:1, and 0:1. ^{51}Cr labeled tumor cells were incubated in cell culture medium alone or in 1% Triton X-100 as negative or positive control to determine spontaneous or maximum ^{51}Cr release respectively. ^{51}Cr labeled tumor cells and HER2-CAR T cells were co-cultured for 4 hours, and supernatants were collected and assessed as described.

Co-culture of tumor cells and HER2-CAR T cells and analysis of cytokine production

HER2-specfic CAR T cells from healthy donors were co-cultured

with HER2-positive tumor cell lines at a 2:1 T cell to tumor cell ratio in 24-well cell culture plate in the presence of 100 U IL-2/ml. After 48 hours incubation, culture supernatants were harvested and subjected to enzyme-linked immunosorbent assay (ELISA) to measure the presence of IFN-γ and IL-2 following the manufacturer's instruction (R&D Systems).

Statistical analysis

All *in vitro* experiments were performed in duplicate or in triplicate. The data were presented as mean ± SD. The differences between means were tested by student t-test. The significance level used was $P < 0.05$.

Results

Generation of HER2-specific CAR modified human T cells

We generated a second-generation CAR specific for human HER2 using the single chain variable fragment scFv derived from FRP5 antibody (HER2-CAR). Human T cells were activated by anti-CD3/CD28 antibodies and transduced with a retroviral vector encoding HER2-CAR to generate HER2-specific T cells (HER2-T cells). Seven days after transduction, the T cells were cultured with HER2-Fc fusion protein for 30 minutes followed by staining with FITC conjugated anti-Fc antibody. HER2-specific CAR expression was then measured by flow cytometry. Over 90% of the T cells were HER2-CAR positive (Figure 1A), indicating the susceptibility of anti-CD3/CD28 antibodies activated human T cell to retroviral infection.

We next investigated the cytotoxicity of HER2-specific T cells. SK-BR-5 is a HER2 overexpressed human breast tumor cell line and has been widely used by other investigators for the development of HER2-targeted therapies. A standard ^{51}Cr release cytotoxicity assay was conducted to determine the cytotoxicity of HER2-speicifc T cells against SK-BR-5. HER2-specific T cells as well as non-transduced (NT) T cells were incubated with SK-BR-5. The results suggested that HER2-CAR T cells induced SK-BR-5 tumor cell lysis effectively, whereas non-transduced T cells (NT-T) didn't kill SK-BR-5 cell lines due to the lack of HER2 CAR expression on the surface of the T cells (Figure 1B). These results indicated that the T cell-mediated killing of HER2-positive breast tumor cell line depended on the expression of HER2-specific CARs on the T cells.

Oncolytic VV infected HER2-CAR T cells effectively

We next investigated EphA2-TEA-VV's infectivity in HER2-CAR T cells. EphA2-TEA-VV was produced as described previously. EphA2-TEA-VV also encodes RFP, allowing us to identify the virus infected cells. Human PBMCs were activated by anti-CD3/CD28 antibodies followed by transduction of retroviral vector encoding HER2-specific CAR. The HER2-specfic T cells were cultured in the presence of 100U IL2/ml for 7 days prior to the VV infection. Non-transduced T cells and CIKs were produced as control. CIK cells are cytokine-induced cell population with phenotypic markers of NK and T-cells. Human PBMC was activated by interferon-γ and anti-CD3 antibody and expansion in IL-2 as described previously. HER2-CAR T cells (CAR T), CIK and non-transduced T cells (NT-T) were infected with EphA2-TEA-VV at increasing MOI of 0, 0.1, 1, to 5. At different time point, the cells were harvested and subjected to flow analysis of EphA2-TEA-VV's infectivity. The results showed that EphA2-TEA-VV infected HER2-CAR T cells (CAR T), CIK, or NT-T cells effectively. We observed that 27.79% - 36.56% of HER2-CAR T cells (CAR T), CIK, or NT-T cells were infected with EphA2-TEA-VV at day 7 (Figure 2A). Comparison of MOIs revealed that EphA2-

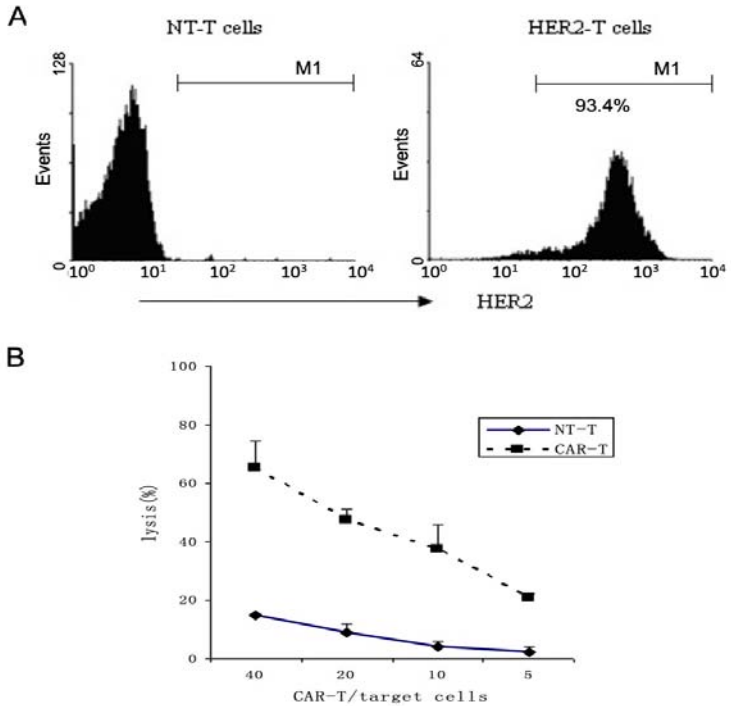

Figure 1: Construction of HER2-CAR T cells. OKT3/CD28 activated human T cells were transduced with the retrovirus encoding HER-2 specific CAR. The HER2-specific CAR expression on the surface of the T cells were measured by flow cytometry (A). HER2-positive SK-BR-5 breast tumor cells were labeled with ^{51}Cr and 51Crrelease assay was conducted to determine the tumor lysis by the HER2-CAR T cells (B).

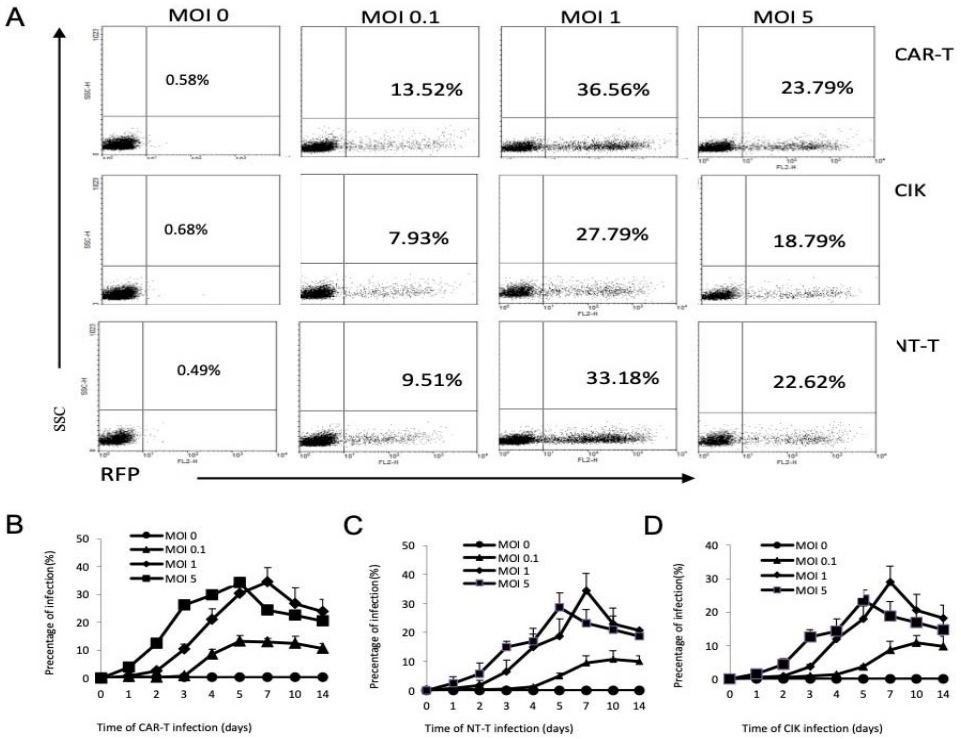

Figure 2: EphA2-TEA-VV effectively infected HER2-CAR T cells *in vitro*. HER2-CAR T cells (CAR T), CIK and non-transduced T cells (NT-T) were infected with EphA2-TEA-VV that encodes RFP at an MOI of 0.1 to 5 PFU/cell. CIK cells are cytokine-induced cell population with phenotypic markers of NK and T-cells. Human PBMC was activated interferon-γ and anti-CD3 antibody and expansion in interleukin-2, and has been described previously. (A) At day 7 post virus infection, the cells were collected and subjected to the flow analysis of viral-derived RFP expression by the cells. The viral-derived RFP expression by EphA2-TEA-VV loaded HER2-CAR T cells (B), non-transduced T cells (C), or CIK cells (D) at day 0, 1, 2, 3, 4, 7, 10, or 14 were analyzed and shown in a dot plot figure.

TEA-VV infection at MOI 1 resulted in an optimal infectivity in HER2-CAR T cells (CAR T), CIK, or NT-T cells (Figure 2A and 2B). Thus, we infected T cells with EphA2-TEA-VV or GFP-VV at MOI 1 in our following experiments. In addition, the cells were collected at different time point, and flow analysis demonstrated that the significant RFP expression was detected from day 3 to day 14 (Figure 2B). Taken together, these data suggested that EphA2-TEA-VV could infect CAR T cells, CIK, and NT-T cells effectively.

Oncolytic VV replicated in HER2-CAR T cells effectively

We investigated if the VVs can replicate effectively in CAR T cells. EphA2-TEA-VV or GFP-VV was transduced to HER2-CAR T cells at MOI 1. At different time point post infection, the T cells (CAR T) as well as cell culture medium (CM) were collected and subjected to the virus tittering in order to determine if the virus replicated effectively in CAR T cells or released in the culture medium. We observed that either EphA2-TEA-VV or GFP-VV particle in HER2-CAR T cells (Figure 3A) or NT-T cells (Figure 3B) increased > 1000-fold in 3 days. And up to 80% of the virus particles were located in T cells, while up to 20% of the virus particles were released to the culture medium of HER2-CAR T cells (Figure 3C) or NT-T cells (Figure 3D). These results indicated that oncolytic vaccinia virus replicated in HER2-CAR T cells effectively.

HER2-CAR T cell-packaged EphA2-TEA-VV displayed enhanced tumor killing ability

We next investigated if HER2-CAR T cell-packaged EphA2-TEA-VV displayed enhanced tumor killing ability since it co-targets HER2 and EphA2 in tumor cells. To do this, a $HER2^{dim}EphA2^{high}$ NSCLC A549 cell line was used as the target. A standard ^{51}Cr release assay was conducted to determine the cytotoxicity of HER2-CAR T cell-packaged EphA2-TEA-VV against A549. The results showed that HER2-CAR T cells that were infected with EphA2-TEA-VV at either MOI 0.1, 1, or 5-killed A549 tumor cells effectively. In contrast, no killing of A549 tumor cells by GFP-VV-loaded HER2-CAR T cells was observed (Figure 4A). This is likely due to the low HER2 expression density on the surface of A549 tumor cells and the suboptimal T cell function caused by the virus. The observed A549 killing by EphA2-TEA-VV loaded HER2-CAR T cells indicated that co-targeting HER2 and EphA2 on the surface of A549 resulted in the effective killing of A549 tumor cells. In addition, we compared the efficacy of EphA2-TEA-VV loaded HER2-CAR T cells (TEA-VV/T) with either VVs alone or HER2-CAR T cells alone and found that TEA-VV/T is superior to HER2-CAR T cells alone (T only), EphA2-TEA-VV alone (TEA-VV) or GFP-VV alone (Figure 4B). These results suggested that HER2-CAR T cell-packaged EphA2-TEA-VV represents a unique dual-targeting strategy with the ability of targeting both HER2 and EphA2 on the surface of the tumor cells, resulting in effective killing of tumor cells that express both HER2 and EphA2.

EphA2-TEA-VV activated HER2-CAR T cells in the presence of EphA2-positive A549 cells

We also investigated if EphA2-CD3 engager activated HER2-T cells in the presence of EphA2-positive A549 tumor cells. EphA2-TEA-VV loaded HER2-specfic CAR T cells (CAR T + EphA2-VV),

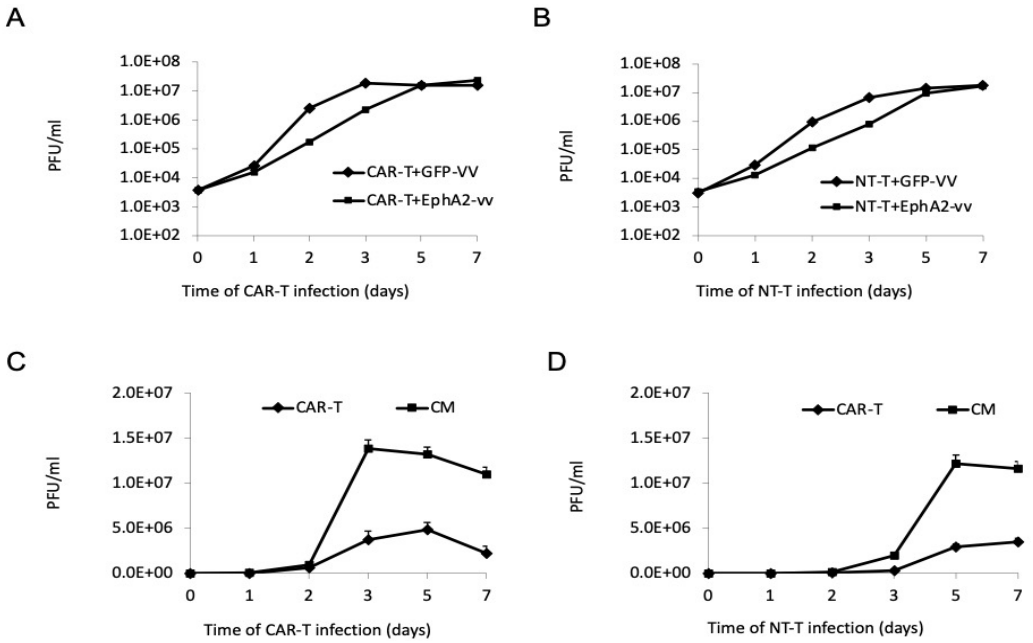

Figure 3: EphA2-TEA-VV replicated significantly in HER2-CAR T cells. HER2-CAR T cells non-transduced T cells were transduced with GFP-VV (CAR T + GFP-VV or NT-T + GFP-VV) or EphA2-TEA-VV (CAR T + EphA2-vv, or NT-T + EphA2-vv) at MOI 1. At day 0, 1, 2, 3, 5 or 7-post VV transduction, the EphA2-TEA-VV or GFP-VV loaded HER2-CAR T cells (A) or non-transduced T cells (B) were collected and subjected to the virus tittering using CV-1 plaque assay. To determine if the virus particles are in the cytoplasm or released to the cell culture medium, the cells (CAR T) or cell culture medium (CM) of EphA2-TEA-VV loaded HER2-CAR T cells (C) or non-transuded T cells (D) were collected and subjected to the virus tittering using CV-1 plaque assay.

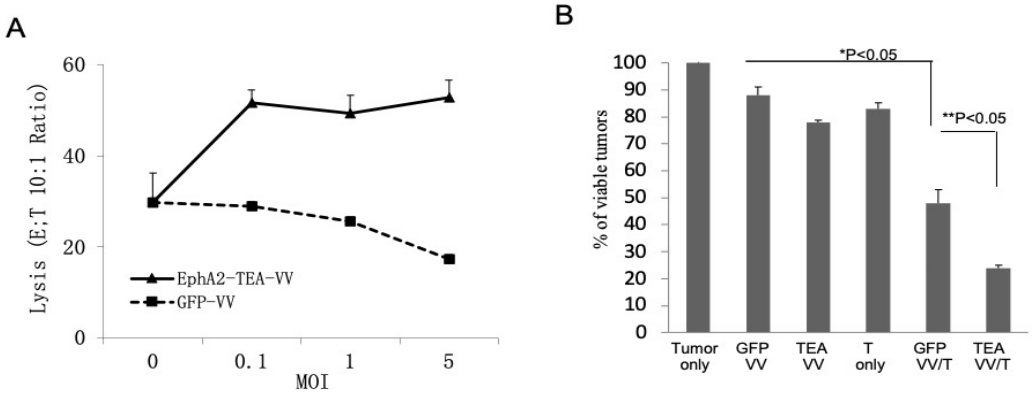

Figure 4: HER2-CAR T cell-packaged EphA2-TEA-VV displayed enhanced anti-tumor activity *in vitro*. A549 cells were labeled with ^{51}Cr and mixed with decreasing numbers of T cells to give T (effector) cells to tumor (target) cells (E:T) ratios of 40:1, 20:1, 10:1, 5:1, and 0:1. ^{51}Cr labeled A549 tumor cells and T cells were co-cultured for 4 hours, and supernatants were collected and assessed as described. (A) Data was analyzed for the co-culture of A549 with EphA2-TEA-VV loaded HER2-CAR T cells (EphA2-TEA-VV) or GFP-VV loaded HER2-CAR T cells (GFP-VV). *P value <0.05: A549 cell lysis rate by HER2-CAR T cells loaded with EphA2-TEA-VV at MOI of 0.1, 1, or 5 (EphA2-TEA-VV) *vs.* that of HER2-CAR T cells loaded with GFP-VV at MOI of 0.1, 1, or 5 (GFP-VV). (B) Data was analyzed for the culture of A549 tumor cells alone (Tumor only), GFP-VV infected A549 tumor cells (GFP-VV), EphA2-TEA-VV infected A549 tumor cells (TEA-VV), non-transduced T cells (T only), A549 tumor cells with GFP-VV loaded HER2-CAR T cells (GFP-VV/T), and A549 tumor cells with EphA2-TEA-VV loaded HER2-CAR T cells (TEA-VV/T). *P value <0.05: GFP-VV, TEA-VV, or T only *vs.* GFP-VV/T. **P value <0.05: TEA-VV/T *vs.* GFP-VV/T.

GFP-VV loaded HER2-specfic CAR T cells (CAR T + GFP-VV), HER2-specific CAR T cells (CAR T), or non-transduced T cells (NT-T) from healthy donors were co-cultured with A549 tumor cells. After 48 hours incubation, interferon (IFN) - γ and interleukin (IL)-2 production in the cell culture medium was determined by enzyme-linked immunosorbent assay (ELISA). EphA2-TEA-VV loaded HER2-specific T cells produced elevated level of IFN-γ and IL-2 compared to GFP-VV loaded HER2-specific T cells, indicating that elevated cytokine production was dependent on stimulation of EphA2 antigen. These results showed that tumor antigen dual targeting strategy could enhance T cell activation.

Discussion

In this study, we developed the HER2-CAR T cell-packaged EphA2-TEA-VV and assessed its activity *in vitro*. We first generated a second generation of anti-HER2 antibody FRP5 based HER2-CAR and constructed the HER2-CAR T cells. *In vitro* cell culture assays demonstrated the HER2-CAR T cells killed HER2-positive SK-BR-3

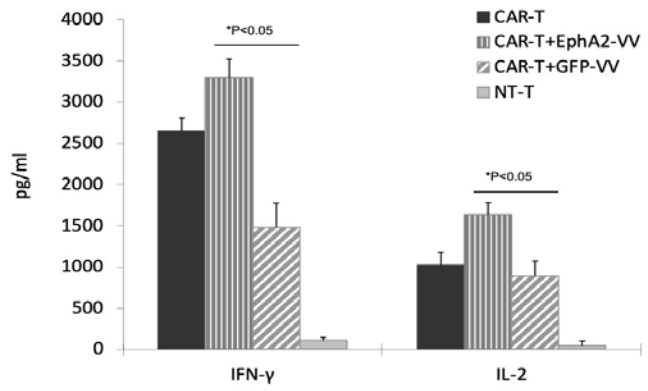

Figure 5: EphA2-TEA-VV activated HER2-CAR T cells in the presence of EphA2-positive A549 cells *in vitro*. 2x10^5 of EphA2-TEA-VV loaded HER2-specfic CAR T cells (CAR T + EphA2-VV), GFP-VV loaded HER2-specfic CAR T cells (CAR T + GFP-VV), HER2-specific CAR T cells (CAR T), or non-transduced T cells (NT-T) from healthy donors were co-cultured with 1x105 of HER2- and EphA2- double positive tumor cell A549 at a 2:1 T cell to tumor cell ratio in 24-well cell culture plate in the presence of 100 U of IL2/ml. The cells were cultured in triplicate. After 48 hours incubation, culture supernatants were collected and subjected to enzyme-linked immunosorbent assay (ELISA) to measure the presence of IFN-γ and IL-2 following the manufacturer's instructions (R&D Systems). The data were presented as average + SD. *P value <0.05: IFN-r and IL2 production of EphA2-TEA-VV loaded HER2-CAR T cells (CAR T + EphA2-VV) *vs.* that of GFP-VV loaded HER2-CAR T cells (CAR T + GFP-VV).

human breast tumor cell line effectively. We then assessed the EphA2-TEA-VV's infectivity in HER2-CAR T cells and more than 30% of the T cells were infected with VVs at 3 days post infection. We found the VVs replicated effectively in the T cell culture. The virus particles increased > 1000-fold in 3 days. And the majority of the virus was found in the T cells, while less than 20% of the viruses were released to the cell culture medium. The co-culture of HER2^{dim}EphA2^{high} NSCLC line A549 and the EphA2-TEA-VV loaded HER2-CAR T cells suggested that HER2-CAR T cell-packaged EphA2-TEA-VV displayed enhanced tumor killing ability compared to HER2-CAR T cells or EphA2-TEA-VV alone. In addition, EphA2-TEA-VV activated HER2-CAR T cells in the presence of EphA2-positive tumor cells, indicated by the elevated expression level of cytokines such as IFNγ and IL-2 by HER2-CAR T cells.

While some patients with HER2-positive breast tumors have benefited from anti-HER2 monoclonal antibody therapy, one arising challenge in the treatment of breast cancer is that some patients with HER2-positive breast tumors are resistant to the treatment of trastuzumab, an anti-HER2 monoclonal Ab [46-49]. Studies have shown that the resistance to trastuzumab is largely due to the elevated level of EphA2 in the breast tumors [50-52]. The elevated level of EphA2 in breast tumors is also correlated with the decrease in the overall and disease-free survival of the patients with HER2-positive breast tumors. Studies also showed that inhibiting EphA2 using anti-EphA2 antibody successfully restored the efficacy of trastuzumab treatment for HER2-positive breast tumor in mouse models. Thus, co-targeting EphA2 and HER2 provides a potentially effective strategy to treat the patients with trastuzumab-resistant breast tumors. In addition, both EphA2 and HER2 are overexpressed in other types of cancers at various level. For example, NSCLC has been reported to express both EphA2 and HER2. In this study, we have assessed the efficacy of our strategy to treat a HER2^{dim}EphA2^{high} NSCLC line A549 cells. Our results demonstrated that co-targeting EphA2 and HER2 enhanced anti-tumor activity compared to targeting EphA2 by EphA2-TEA-VV alone or targeting HER2 by HER2-CAR T cells alone. Thus, such a strategy that co-targets both EphA2 and HER2 might be suitable to the treatment of the patients with EphA2 and HER2 double positive tumors by overcoming the existing or potential resistance to HER2 targeted therapies.

In addition, such a bispecific therapeutic strategy that co-targets two TAAs has the potential to overcome the limitation of the therapies that target single target and have been proved to induce antigen loss variant (ALV). For example, clinical studies of CD19-CAR T cell therapies in B-ALL reported CD19 ALV in 30-95% of relapses [53]. The reported CD19 ALV is resulted from splice mutations and retained intracellular CD19 [54-57]. To overcome this limitation, current efforts have been focused on the development of bispecific CAR T cells with dual targeting of CD19 and CD22 [58-61]. Similar cases with ALV associated treatment resistance have been reported with a monospecific CD22-CAR or BCMA-CAR T cell therapy [62,63]. In addition, several reports have suggested that effective immunotherapies are often correlated with high target antigen expression density [64-68]. Thus, it remains an important unmet goal to develop novel strategies to overcome the resistance caused by monotherapy-induced ALV or the low target antigen expression density.

Conclusion

In conclusion, we designed and assessed the HER2-CAR T cell-packaged EphA2-TEA-VV and provided the preclinical evidence for the therapeutic potential of such a combinational strategy for patients with trastuzumab-resistant breast tumors or other cancers (i.e. EphA2- and HER2- positive NSCLC). In addition, our CAR T cell-packaged T-cell engager armed VV strategy may be applicable to a broad range of CAR T cells and TEA-VVs currently under investigation.

Declaration

Author's contributions: Conceptualization: X.W; Methodology: K.S and X.W; Validation: X.W; Formal analysis: K.S, X.W; Investigation: K.S, X.W; Resources: X.W; Data curation: X.W; Writing-original draft preparation: K.S; Writing-review and editing: K.S; Visualization: K.S, X.W; Supervision: X.W; Project administration: X.W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest and current contact information: The authors declare no conflict of interest. Kevin Song's current contact in-formation is Debakey High School, Houston, TX, USA; Xingbing Wang's current contact information is The First Affiliated Hospital of USTC, P.R. China, Department of Hematology, 17 Lujiang Road, Hefei, Anhui.

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