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14. ABSTRACT
Advances in the treatment for renal cell carcinoma (RCC) have resulted in increased progression-free survival rate of many patients. However, the therapies are either toxic, or unable to achieve durable long-term complete responses. Carbonic anhydrase IX (CAIX), a tumor-associated antigen (TAA) overexpressed among serval solid tumor types, particularly in clear cell RCC, has been utilized in the design of chimeric antigen receptor (CAR) T cell therapies for metastatic clear cell RCC patients. Unlike the success shown in the CAR T therapies for hematological malignancies, the clinical development of CAIX CAR T therapy for RCC is limited by the "on-target off-tumor" toxicity and immunosuppressive signaling from the tumor microenvironment. We tested the approach for designing the masked CAIX CARs with the attempt to improve its tumor selectivity. The masked CAR can be successfully constructed and their expression was observed in T cells. However, even with incorporated masking peptides, this CAR lacked the masking effect. We also demonstrated the potential of anti-PD1 trap protein self-secreting CAR-T as a potential cancer immunotherapy therapy.

15. SUBJECT TERMS
Cancer immunotherapy, chimeric antigen receptor, masked CAR, adoptive cell therapy

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Advances in the treatment for renal cell carcinoma (RCC) have resulted in increased progression-free survival rate of many patients; however, the therapies are either toxic, or unable to achieve durable long-term complete responses. Carbonic anhydrase-IX (CAIX), a tumor-associated antigen (TAA) overexpressed among several solid tumor types, particularly in clear cell RCC, has been utilized in the design of chimeric antigen receptor (CAR) T cell therapy for metastatic clear cell RCC patients. Unlike the success shown in the CAR T therapies for hematologic malignancies, the clinical development of CAIX CAR T therapy for RCC is limited by the "on-target off-tumor" toxicity and immunosuppressive signaling from the tumor microenvironment. Recently, the masked CAR engineered by our research team has shed light on overcoming the "on-target off-tumor" toxicity by protease-mediated tumor selective activation. For the immunosuppressive tumor microenvironment, our armored CAR T cells with self-secretion of PD-1 antibody led to alleviated T cell hypofunction and improved CAR T cell treatment for human solid tumors in xenograft mouse model. In the proposed studies, we aim to combine the masking strategy and PD-1 antibody self-secretion strategy in CAIX CAR T cells to improve the safety and efficacy CAIX CAR T therapy for RCC. In order to achieve the goal, we set three major aims for this proposal listed as following:

Aim 1: To generate masked CAIX CAR T cells and investigate the functions in vitro. We aim to engineer the masked CAIX CAR T cells that only recognize CAIX in the presence of protease, and demonstrate the biological functions of masked CAR T cells by multiple standard in vitro assays.

Aim 2: To investigate the homing effect and antitumor activity of masked CAIX CAR T cells in a xenograft RCC mouse model. After confirming tumor selective activation and functions in vitro, we will conduct studies for the persistence and trafficking of the masked CAIX CAR T cells into tumor site in a xenograft RCC mouse model, followed by antitumor efficacy studies.

Aim 3: To investigate the potency of masked CAIX CAR T cells armored to express anti-PD-1 antibody for treating RCC. Based on the masked CAIX CAR T cells, we aim to further improve the antitumor potency by introducing PD-1 antibody self-secretion strategy, and study whether **α**PD-1 armored masked CAIX CAR T cells can sufficiently block T cell inhibition and exhibit enhanced therapeutic activity to suppress RCC in xenograft mouse model.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Cancer immunotherapy; adoptive T cell therapy; cellular immunotherapy; chimeric antigen receptor; armored chimeric antigen receptor; checkpoint inhibitor; carbonic anhydrase-IX

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

Specific Aim 1: To Design and test a novel masked CAR targeting RCC in vitro
- Major Task 1: To construct functional CAIX CARs
  - Subtask 1.1: construct lentiviral vectors encoding masked, unmasked or NSUB CAIX CARs
  - Subtask 1.2: test the expression of these CARs in T cells via lentiviral transduction
- Major Task 2: To evaluate the immune functions of CAIX CAR-T cells
  - Subtask 2.1: construct the targeting cell line K562.CAIX
  - Subtask 2.2: measure cytokine production of CAR-T cells upon co-culture with target cells
  - Subtask 3: measure degranulation of CAR-T cells upon co-culture with target cells
  - Subtask 4: evaluate the killing ability of CAR-T cells upon co-culture with target cells.

Specific Aim 2: To assess masked CAIX CAR therapy in mouse models of RCC
- Major Task 3: To examine the persistence and trafficking of CAIX CAR-T cells in vivo
  - Subtask 3.1: establish the xenografted mouse model of RCC
  - Subtask 3.2: generate luciferase-labeled CAIX CAR-T cells
  - Subtask 3.3: monitor the persistence of CAR-T cells using BLI
  - Subtask 3.4: evaluate the biodistribution of CAR-T cells using BLI
  - Subtask 3.5: study the tumor-directed trafficking via CFSE-labeled CAR-T cells
- Major Task 4: To evaluate the therapeutic activity of masked CAR-T therapy in vivo
  - Subtask 4.1: perform CAR-T therapy in mice using the SK-RC-52-based RCC model
  - Subtask 4.2: perform CAR-T therapy in mice using the K562.CAIX leukemia model

Specific Aim 3: To evaluate the potency of masked CAR-T cells armored to express anti-PD-1 antibody for treating RCC
- Major Task 5: To generate masked CAIX CAR-T cells capable of making bifunctional trap (αPD1+TGFβ)
  - Subtask 5.1: construct lentiviral vectors encoding CAR and bifunctional trap (αPD1+TGFβ)
  - Subtask 5.2: confirm the expression of CAR and bifunctional trap (αPD1+TGFβ)
  - Subtask 5.3: evaluate the effect of bifunctional trap (αPD1+TGFβ) on biological functions of CAR-T cells

What was accomplished under these goals?

Major activity: To construct functional CAIX CARs (Major Task 1; accomplished)

Specific objective: construct lentiviral vectors encoding masked, unmasked CAIX CARs (Subtask 1.1, accomplished)

Significant results:
The retroviral vector encoding the unmasked anti-CAIX CAR (unmasked CAR) was constructed base on the MP71 retroviral vector. The insert for unmasked CAR vector consisted of the following components in frame 5' end to 3' end: a NotI site, an anti-CAIX scFv heavy chain variable region, a GS liner, an anti CAIX scFv light chain variable region, a CD8α hinge and transmembrane region, CD28 and 4-1BB (CD137) cytoplasmic domains, a CD3ζ signaling domain and an EcoRI site. The anti-CAIX scFv DNA sequence in the Unmasked CAR was derived from the amino acid sequence of Girentuximab, a chimeric IgG1 monoclonal antibody to CAIX, and was codon-optimized for the optimal expression in human cells.

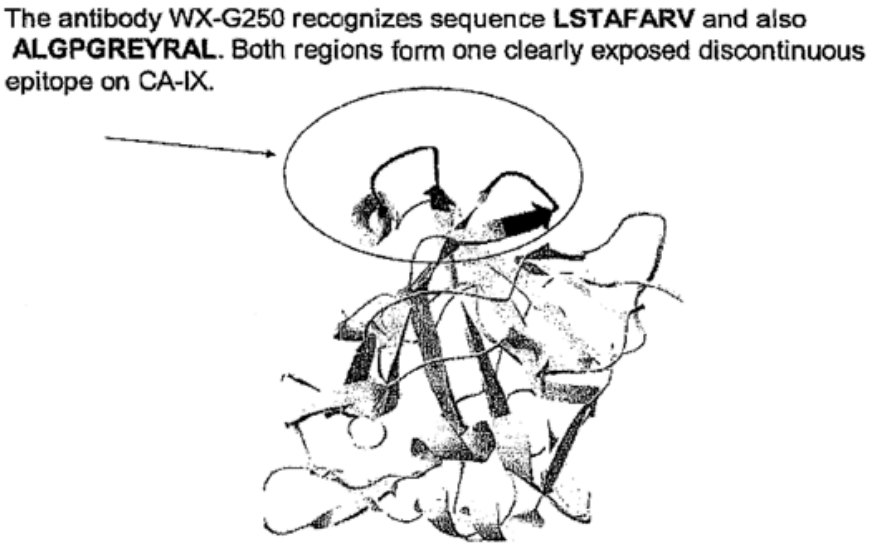

Figure 1. The localization of the epitope sequences LSTAFARV and ALGPGREYRAL in CAIX. Both regions form a clearly exposed discontinuous epitope on CAIX.

For the masked CAR, a masking peptide along with a cleavable linker sequence was introduced upstream of the scFv domain, using the amino acid sequences of the binding epitope for Girentuximab determined by conformational epitope mapping of CAIX (Figure 1.). Here we applied two masking peptide candidate sequences, LSTAFARV and ALGPGREYRAL to generate Masked CAR 1 and Masked CAR 2 (Figure 2.). Theoretically, the masking peptides can bind to the antigen recognition domain of the scFv, thereby blocking the binding between CAR and CAIX. The cleavable linker sequence was selected by using cellular libraries of peptide substrates (CLiPS) for its responsiveness to multiple tumor-associated proteases, including urokinase-type plasminogen activator (uPA), membrane-type serine protease 1 (MT-SP1/matriptase), and legumain, which are upregulated in the tumor microenvironment.

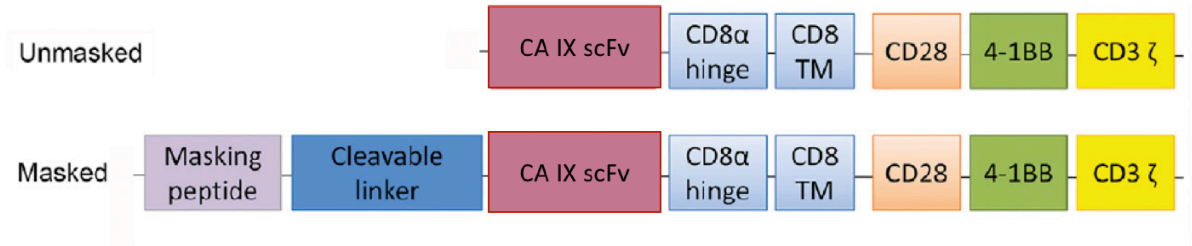

Figure 2. Schematic representation of various CAIX CAR constructs. The scFv sequence is derived from the monoclonal antibody Girentuximab. The scFv is fused in frame with the CD8α hinge and transmembrane domain, followed by the CD28/41BB/CD3ζ signaling domains, and then cloned into the retroviral vector to yield the unmasked CAR. The masking peptide and protease-sensitive linker are inserted upstream of scFv in the Unmasked CAR to generate Masked CAR constructs.

Specific objective: test the expression of these CARs in T cells via lentiviral transduction (Subtask 1.2, accomplished)

Significant results:

Retroviral vectors were prepared by transient transfection of 293T cells using a standard calcium phosphate precipitation protocol. 293T cells were transfected with the retroviral backbone plasmids, along with the envelope plasmid RD114 and the packaging plasmid encoding gag-pol. The viral supernatant was harvested and filtered 48 h post transfection. PBMCs were thawed in TCM medium and rested overnight. Before retroviral transduction, PBMCs were activated for 48 h by culturing with Dynabeads™ Human T-Activator CD3/CD28 (bead-to-cell ratio 1:1) and recombinant human IL-2. To detect CAIX CAR expression on the cell surface, cells were stained with biotin conjugated anti-mouse F(ab')2 antibody followed by APC-conjugated streptavidin, and analyzed by flow cytometry on day 2 and day 8 post transduction.

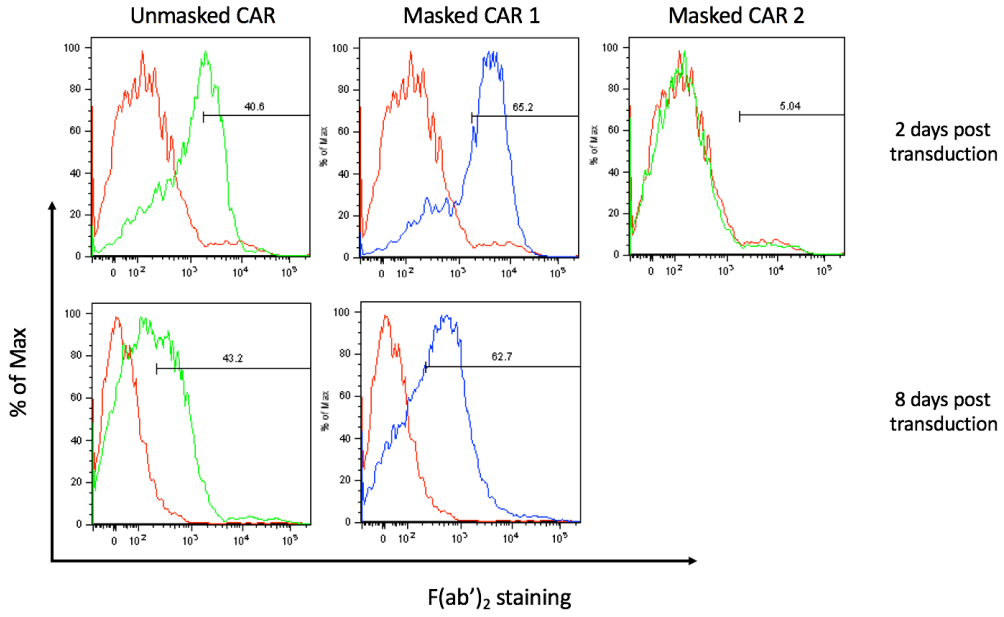

Figure 3. Expression of three CARs in human T cells. The CAR T cells were stained with biotin conjugated anti-mouse F(ab')2 antibody followed by APC-conjugated streptavidin to detect CAR expression on the cell surface. A viable CD3+ lymphocyte gating strategy was used. Nontransduced T cells were used as a control.

Cell surface expression level of CARs was detected for all three forms of CAIX CARs. The expression level of Unmasked CAR and Masked CAR 1 were about 40% and 60%, respectively, and the expression was stable for more than 1 week post transduction. In terms of cell proliferation, unmasked CAR and masked CAR 1 showed similar expansion as non-transduced T cells under the T cell culture conditions (Figure 4.). For masked CAR 2, we detected negligible expression even on day 2 post-transduction. Therefore, in subsequent studies, we only used unmasked CAR and masked CAR 1.

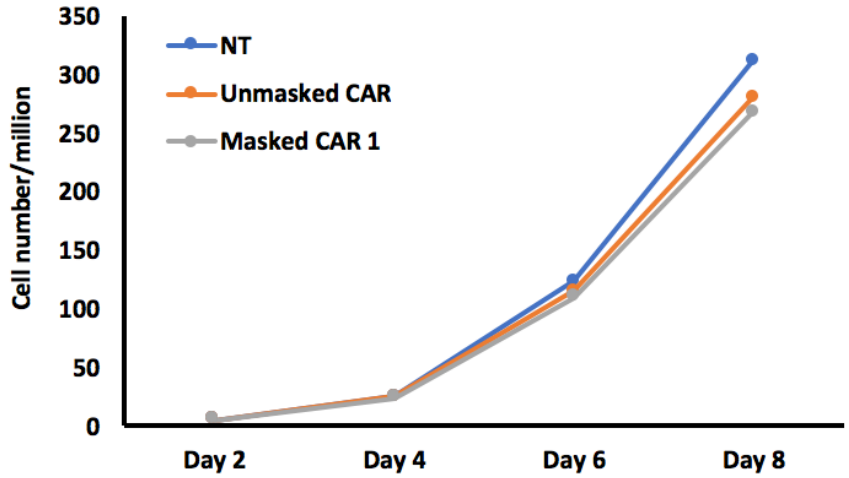

Figure 4. The proliferation of nontransduced T cells, Unmasked CAR T cells and Masked CAR 1 T cells. Cells were cultured in TCM with human IL-2 at the density of 1-2 million/mL. Cell numbers were counted on Day 2, 4, 6, 8 post transduction.

Major activity: To evaluate the immune functions of CAIX CAR-T cells (Major Task 2, accomplished)

Specific objective: construct the targeting cell line K562.CAIX (Subtask 2.1, accomplished)

Significant results:
In this project, the target cell lines for CAIX CAR T cells included SK-RC-52, K562-CAIX and H292-CAIX, among which SK-RC-52 has an intrinsic high expression level of CAIX, while K562-CAIX and H292-CAIX were generated by transduction of the parental K562 cells and H292 cells with lentiviral vector FUW-CAIX. The lentiviral vector encoding human CAIX (FUW-CAIX) was generated by insertion of the cDNA of PCR-amplified human CAIX into the pENTR plasmid and then the CAIX gene was cloned into lentiviral vector FUW via LR reaction

using the Gateway cloning kit.The transduced K562 cells and H292 cells were stained with anti-human CAIX antibody to determine the CAIX expression level. The results showed that CAIX expression level of K562-CAIX and H292-CAIX was higher than 90% after cell sorting and was stable after several freeze-and thaw cycles (Figure 5.).

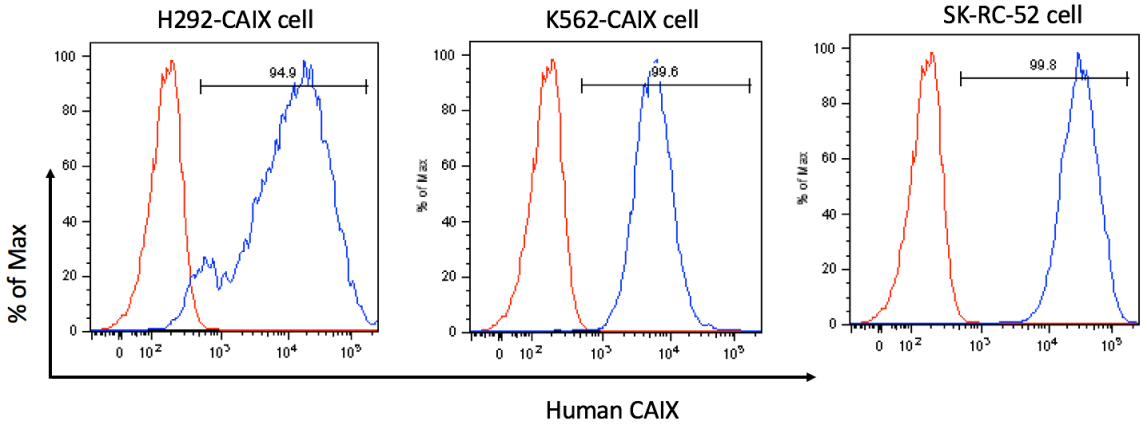

Figure 5. Expression of CAIX in H292-CAIX, K562-CAIX and SK-RC-52 cells. The cells were stained with PE-conjugated anti-human CAIX antibody. Nontransduced parent cell lines were used as control.

Specific objective: measure the binding of CAIX CARs to recombinant human CAIX (related to Subtask 2.2, accomplished)

To investigate the binding capability of masking peptide to anti-CAIX scFv in masked CAIX CAR, and whether the protease-sensitive linker could be cleaved to expose the binding site and restore CAR binding, we conducted recombinant human CAIX-Fc fusion protein (rhCAIX-Fc) binding with or without the presence of uPA. In the first study, CAR-T cells were incubated with rhCAIX-Fc followed by staining with anti-human IgG Fc antibody. Masked CAR 1 exhibited similar level of antigen binding (25%-30%) as unmasked CAR without the presence of uPA (Figure 6.).

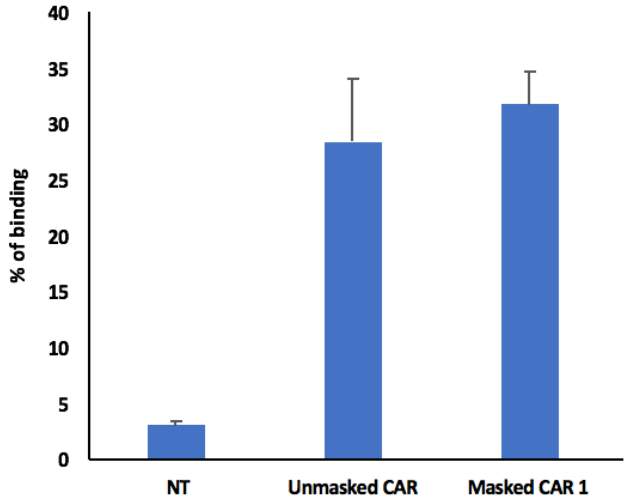

Figure 6. Binding ability of CARs to target antigen CAIX. The CAR T cells were incubated with recombinant human CAIX-Fc protein followed by staining with FITC-conjugated goat anti-human Fc antibody to assess the binding ability of CARs to their target antigen, human CAIX.

We further incubated CAR-T cells with rhCAIX-Fc in the presence of gradient concentrations of uPA. There was no significant difference in antigen binding between Unmasked CAR and Masked CAR 1 (Figure 7.). These results indicate that the blocking effect of masking peptide 1 is not as good as we expected.

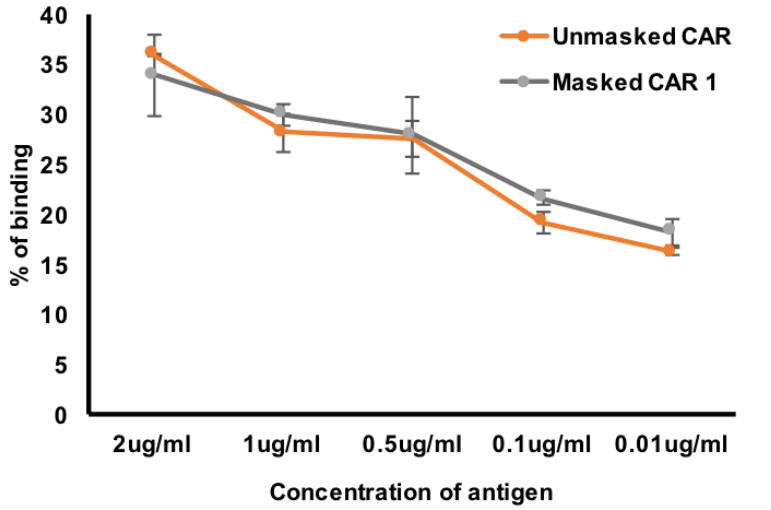

Figure 7. Binding of CARs to target antigen CAIX after protease treatment. Nontransduced Unmasked CAR, Masked CAR CAR-T cells were treated with uPA at various concentrations and then stained with recombinant human CAIX-Fc (rhCAIX-Fc) and goat anti-human Fc antibody to assess the effect of protease treatment on CAR binding to antigen.

Specific objective: evaluate the killing ability of CAR-T cells upon co-culture with target cells (Subtask 2.4; accomplished)

Significant results:
The target cell lysis capability of CAIX CAR T cells was measured by comparing death level of target cells after co-culture. The target cells were labeled with fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) before co-culture with CAR T cells for 24 h. The percentage of dead cells was analyzed by 7-AAD labeling and flow cytometric analysis.

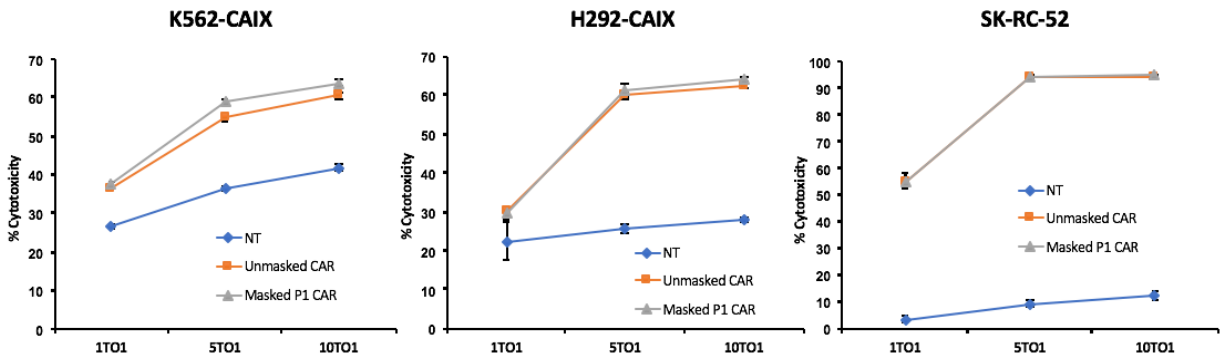

Figure 8. Cytotoxicity of CAR T cells against K562-CAIX, H292-CAIX and SK-RC-52 cells in vitro. Cells were cocultured for 24 h at 1:1, 5:1, or 10:1 effector-to-target ratios and cytotoxicity against target cells was measured by 7-AAD staining.

For three target cell lines, unmasked CAR and masked CAR 1 induced significantly higher target cell lysis compared with non-transduced T cells, but there was no difference between these two CARs in various effector-to-target ratios (Figure 8.). SK-RC-52 cell line and H292 cell, the parent cell line of H292-CAIX, have been well-documented to have protease secretion, therefore, we concluded that both unmasked CAR and masked CAR 1 could recognize target antigen CAIX and get stimulated in the presence of protease secreted by target cells. Normally, just like K562 cell line, K562-CAIX cell does not have protease secretion, but after 24 h co-culture with K562-CAIX, masked CAR 1 showed comparable cell lysis capability to unmasked CAR, indicating masked CAR 1 could recognize target antigen CAIX even without the presence of protease (Figure 8.). Consistent with the results of antigen binding study, blocking effect of masking peptide 1 was not observed in the specific cell lysis study.

Specific objective: evaluate the potency of CAR-T armored to express anti-PD-1 antibody (Major Tasks 5, partially accomplished)

The tumor microenvironment (TME) in solid tumor imposes immunosuppression on CAR-T cells, constituting a critical challenge to the success of CAR-T therapy in solid tumors. Besides the physical and metabolic barriers of, for example, low oxygen, low nutrient, or low pH, multiple mechanisms in TME act to inhibit CAR-T cell function and expansion. For instance, tumor cells have upregulated expression of immune checkpoint ligands, such as programmed cell death ligand 1 (PD-L1). When PD-L1 binds to its receptor programmed cell death protein 1 (PD-1) on CAR-T cells, the immune checkpoint interaction activates immunosuppressive cell signaling that causes CAR-T cell dysfunction and exhaustion, ultimately leading to the immune tolerance of tumor cells. Recent preclinical and clinical studies have shown that knockdown or knockout of the PD-1 gene in CAR-T cells, or combining immune checkpoint blockades with CAR-T cells, could significantly augment T cell immune response and enhance the antitumor efficacy of CAR-T therapy. Therefore, to provide a new approach for combinational CAR-T therapy, we previously developed CAR-T cells secreting checkpoint inhibitors to block the PD-1/PD-L1 interaction.

Compared with systemic administration of PD-1 antibody with CAR-T cells, anti-PD-1 self-secreting CAR-T cells have proven more functional and expandable, as well as more efficient in mediating tumor eradication.

Another well-defined immunosuppressive mechanism in TME comes from the soluble molecules secreted by tumor cells, stromal cells and suppressive immune cells. Among these molecules, transforming growth factor β (TGF-β) is particularly important in inhibiting T cell effector function and inducing T cell differentiation into the regulatory phenotype. Immunosuppression from TGF-β is potent and associated with immune checkpoint signaling pathways. Studies have found that active TGF-β signaling in TME might be responsible for the poor response rates observed in clinical trials of checkpoint inhibitors. Seeking to boost antitumor immunity, several studies have reported on checkpoint inhibitors with anti-TGF-β monoclonal antibodies or small- molecule inhibitors of the TGF-β receptor. In a recent study, Ravi *et al.* reported a bifunctional antibody-ligand trap protein comprising an anti-PD-L1 antibody fused with a TGFβRII ectodomain sequence. Through the dual-targeting effect, the trap protein simultaneously blocks immune checkpoints and inhibits TGF-β-mediated differentiation of Tregs, thereby offering a promising strategy for cancers that fail to respond to immune checkpoint inhibitors.

Inspired by the findings from combination therapies that co-target immune checkpoints and TGF-β signaling pathway, we tested the development of anti-PD-1 self-secreting CAR-T cells with the capacity to release trap proteins. It consists of an anti-PD-1 single-chain fragment variant (scFv) fused with a TGFβRII ectodomain, and it is expected to enhance CAR-T therapy for RCC.

Generation and characterization of CAR-T cells and target cell lines

The schematic representation of the retroviral vector constructs used in this study is shown in Figure 9A. Based on the construct of the second-generation CAR (CAR) that contains an scFv, a hinge and transmembrane domain, an intracellular CD28 costimulatory domain and a CD3ζ activation domain, we generated the CAR with anti-PD1 scFv secretion (CAR-αPD1) by using a P2A element as the linker between the CAR sequence and the anti-PD1 scFv sequence. The feasibility and functionalities of CAR-αPD1 have been demonstrated by various *in vitro* and *in vivo* experiments in our previous study. In the present study, we further engineered the CAR with trap protein secretion (CAR-Trap) by fusing the anti-PD1 scFv sequence with a TGF-β binding sequence derived from TGF-βRII through a GS linker.

Human PBMCs were activated and transduced with each of the three CAR constructs. As shown in Figure 9B, CARs were expressed in primary lymphocytes at a similarly high level (>60%). During the two-week T cell expansion phase, CAR expression levels were stably maintained. After performing freeze-and-thaw, about 40% of CAR expression was maintained.

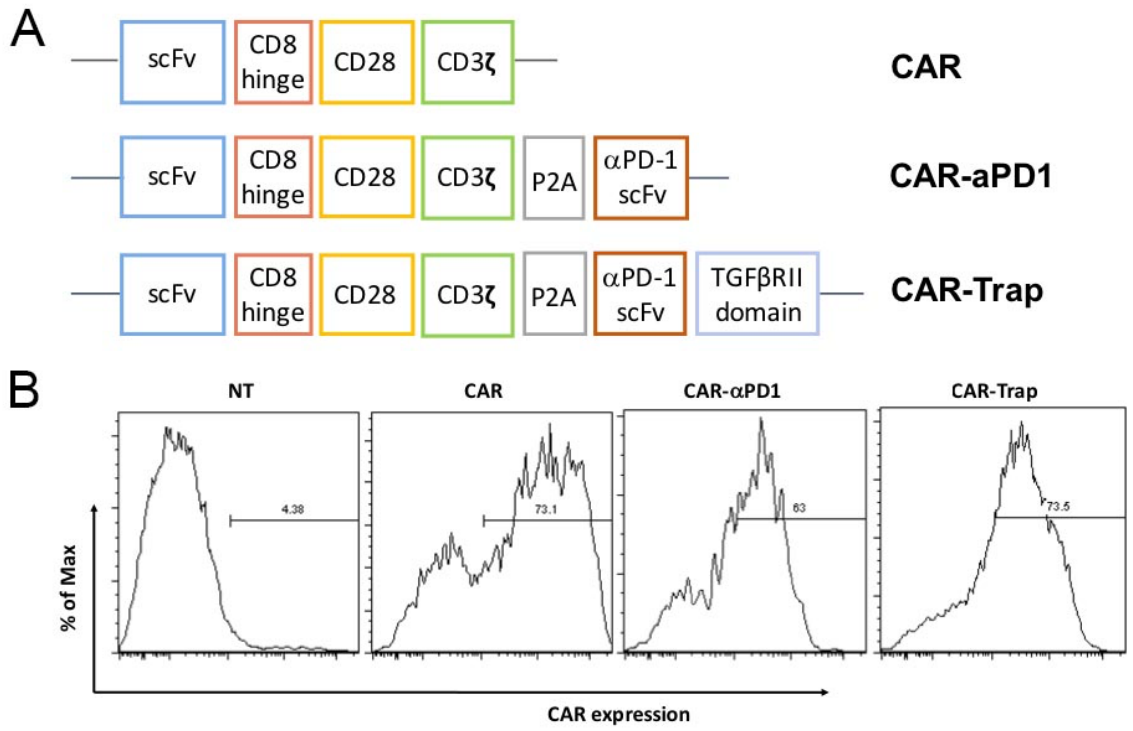

Figure 9: (A) Schematic representation of CAR constructs of the parental CAR (CAR), CAR with anti-PD-1 scFv secretion (CAR-αPD1) and CAR with trap protein secretion (CAR-Trap). (B) Expression of CARs in primary human T cells. CAR T cells were stained with biotin-conjugated rat anti-mouse F(ab')2 antibody, followed by APC-conjugated streptavidin, to detect CAR expression on the cell surface. NT indicates non-transduced T cells, which were used as a control.

Characterization of trap protein

To assess trap protein synthesized by engineered cells, 293T cells were transfected to produce trap protein labeled with a His-tag. 3 days post-transfection, the cell culture supernatant was harvested and used to incubate 293T cells engineered to express PD-1 on the cell surface. After 1 hour of incubation, trap protein binding to 293T-PD-1 was detected by either anti-His-tag antibody or anti-mouse F(ab')2 antibody (Fig. 10A, 10B).

Furthermore, trap protein was purified from cell culture supernatant of transfected 293T cells using a His-tag-labeled protein purification protocol. Western blotting analysis confirmed the secretion of trap protein with molecular weight of 56 kDa (Fig. 10C). The bifunctional binding activity of trap protein to PD-1 and TGF-β was confirmed by enzyme-linked immunosorbent assay (ELISA), wherein purified protein was added to recombinant human PD-1-Fc- or TGF-β-coated plates at different concentrations, and the binding was detected by anti-His-tag antibody (Fig 10D, 10E).

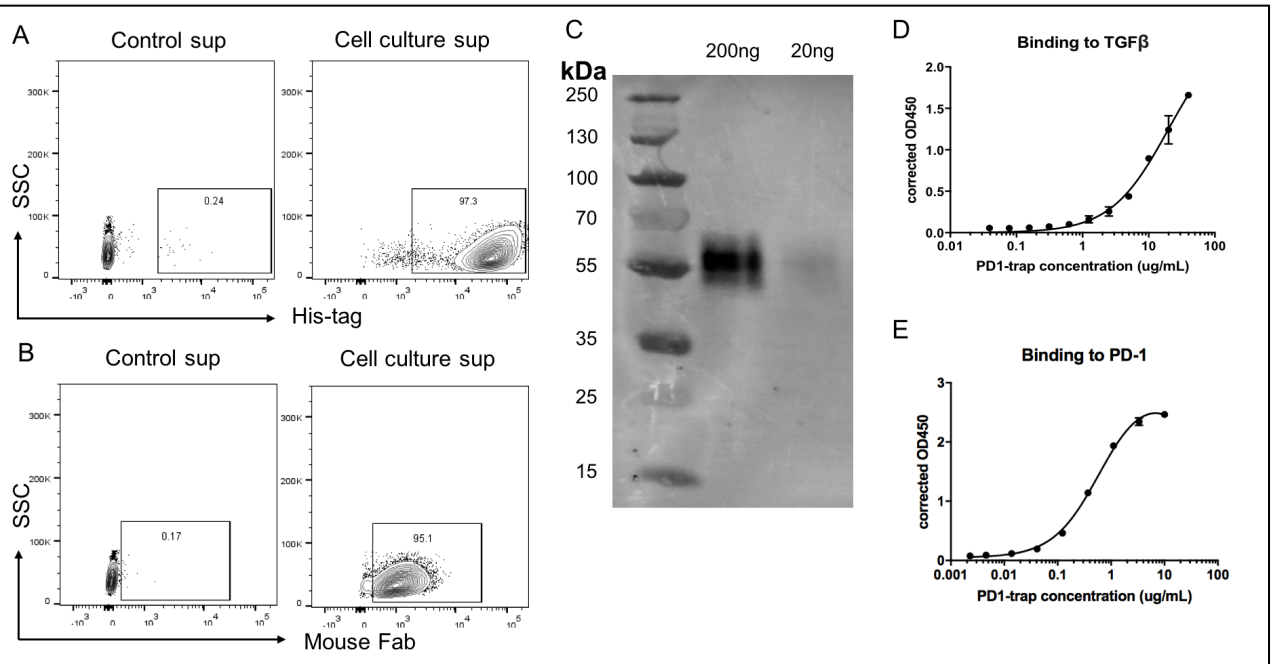

Figure 10: (A, B) Detection of trap protein binding to 293T-PD-1 cells by anti-His-tag antibody or anti-mouse Fab antibody. Cell culture supernatant was collected from 293T cells transfected with trap protein vector and used to incubate 293T cells with PD-1 expression at room temperature for 1 hour. Cell culture soup collected from wild-type 293T cells was used as control. (C) Trap protein purified from transfected 293T cells was analyzed by Western blot. (D, E) Binding ability of trap to PD-1 and TGF-β using sandwich ELISA, wherein purified trap protein was added to PD-1-Fc- or TGF-β-coated plates, followed by detection by anti-His-tag antibody conjugated with HRP.

Trap protein secretion improves cytokine expression and proliferation of CAR-T cells in response to antigen stimulation

To study how trap protein affects the response of CAR-T cells to target cells *in vitro*, we co-cultured CAR-T cells with target cells at a 1:1 cell-to-cell ratio for 16 hours in the presence of protein transport inhibitor. Compared with non-transduced control cells (NT), we found that CAR-T cells all responded to the stimulation from target cells with increased expression of the proinflammatory cytokines IFN-γ, TNF-α, and IL-2. However, the effects of protein drug secretion on the cytokines were quite different. For IFN-γ, CAR-αPD1 and CAR-Trap failed to exhibit a higher percentage of IFN-γ^+ T cells than CAR in either $CD4^+$ or $CD8^+$ T cell populations (Fig. 11A). For TNF-α, CAR-Trap showed more production than CAR in both $CD4^+$ and $CD8^+$ T cell populations, while CAR-αPD1 only had an effect similar to that of $CD4^+$ T cell population (Fig. 11B). When IL-2 was examined, no difference was detected with the exception of the CAR-αPD1 group, which exhibited a higher expression of the $CD4^+$ T cell population compared with the other two groups (Fig. 11C).

Granzyme B and CD107a expression were measured to assess the cytotoxic function of CAR-T cells upon antigen stimulation. Unlike the results of proinflammatory cytokine production, CAR-Trap showed a consistently enhanced expression of granzyme B and CD107a in its $CD8^+$ T cell population in all four groups (Fig. 11E, 11F). CAR-αPD1 had an improved production of granzyme B over that of CAR in the $CD8^+$ T cell population, but no significant improvement was observed in the production of CD107a (Fig. 11D, 11E).

Meanwhile, the expression of Ki67 in CAR-T cells was measured to elucidate the cell proliferative potential. Among three CAR-T groups, it was found that CAR-Trap had the highest positive rate of Ki67 in both $CD4^+$ or $CD8^+$ T cell populations, followed by CAR-αPD1 (Fig. 11D).

Taken together, these results clearly show that trap protein secretion differentially influenced T cell functional markers. Although trap protein did not profoundly affect pro-inflammatory cytokine expression, it did significantly enhance the expression of cell proliferation marker and cytotoxicity-related molecules, indicating that CAR-T cells with trap protein secretion can proliferate faster and exert a more potent target killing effect upon short-term stimulation.

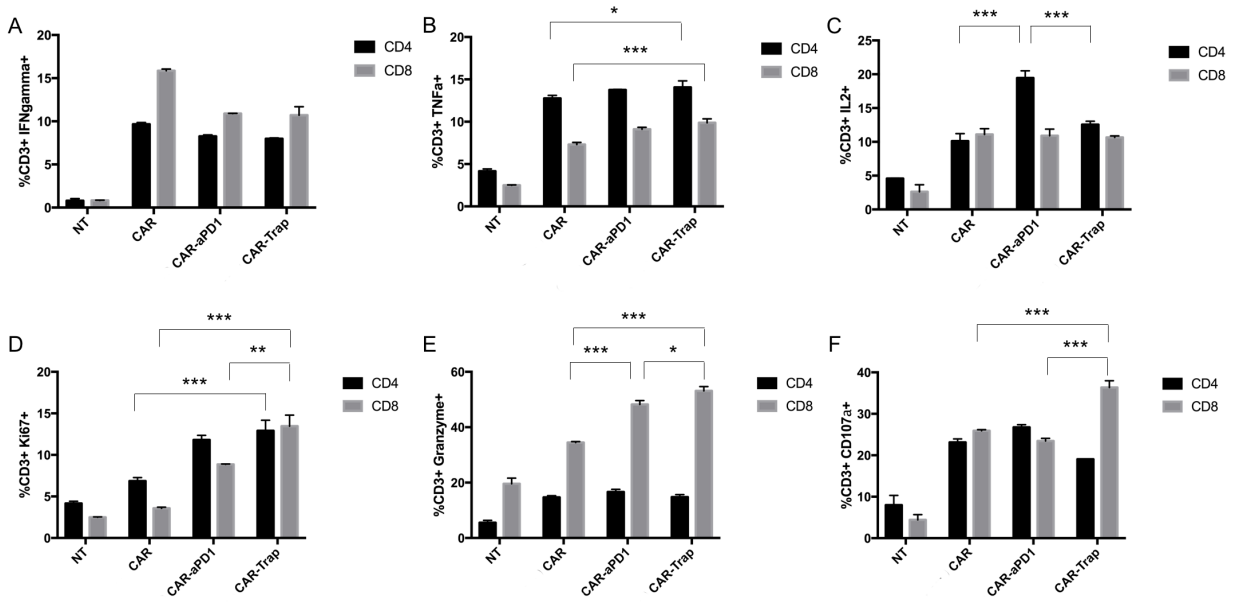

Figure 11: Reponses of CAR-T cells upon stimulation from target cells. CAR T cells were co-cultured with target cells for 16 hours in the presence of protein transport inhibitor Brefeldin A. (A) IFN-γ, (B) TNF-α, (C) Ki67, (D) IL2, (E) granzyme B, and (F) CD107a were measured by flow cytometry, and the percentages of marker- positive cells were shown in bar graphs. NT cells were used as a control (n=3, mean ± SD).

Trap protein secretion attenuates TGF-β signaling, reduces the proportion of Treg, and improve the effector cytokine secretion of CAR-T cells

We next sought to study the capability of CAR-T cells with trap protein secretion to lyse target cells by co-culturing CAR-T cells with a series of target cells at various effector-to-target ratios for 24 hours. In the co-culture with various target cells, at different effector-to-target ratios, CAR-Trap exhibited a cell killing capability comparable to that of CAR and CAR-αPD1 (Fig. 12A). The antigen-specific cell lysis of CAR-T cells was so potent that saturation was observed at the effector-to-target ratio (Fig. 12A). The effect of trap protein secretion on cell killing capability was, however, not significant within 24 hours of co-culture.

In human $CD4^+$ T cells, antigen stimulation concomitant with TGF-β induces the expression of Foxp3 in naïve $CD4^+$ T cells and converts them to $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs. To confirm the ability of trap protein to block the TGF-β signaling pathway, TGF-β-induced phosphorylation of Smad2/3 and Foxp3 expression in human T cells was measured. When CAR-T cells were incubated with TGF-β at various concentrations for 24 hours, the phosphorylation of Smad2/3 was elevated in the NT, CAR and CAR-αPD1 groups in a dose-dependent manner, while that in the CAR-Trap group remained at the basal level (Fig. 12B, 12C). The expression of Foxp3 in the $CD4^+$ T cell population, representing the differentiation of Tregs, was measured with or without co-culture with target cells. We found that CAR and CAR-αPD1 exhibited a greater proportion of Tregs compared with NT, especially after being co-cultured with target cells. However, CAR-Trap contained fewer Tregs than CAR and CAR-αPD1, irrespective of co-culture with target cells (Fig. 12D). Notably, the proportion of Tregs in CAR-Trap T cells was lower than that in NT cells.

To evaluate the effect of trap protein on effector function, CAR-T cells were co-cultured with target cells for different durations. The conditioned supernatants were collected and processed for IFN-γ quantification by ELISA. Upon stimulation for 24 hours and 48 hours, CAR-αPD1 and CAR-Trap secreted a similar amount of IFN-γ, which was higher than the amount secreted by CAR (Fig. 12E). After 72 hours of antigen stimulation, CAR-Trap had a significantly higher secretion of IFN-γ than either CAR or CAR-αPD1 (Fig. 12E).

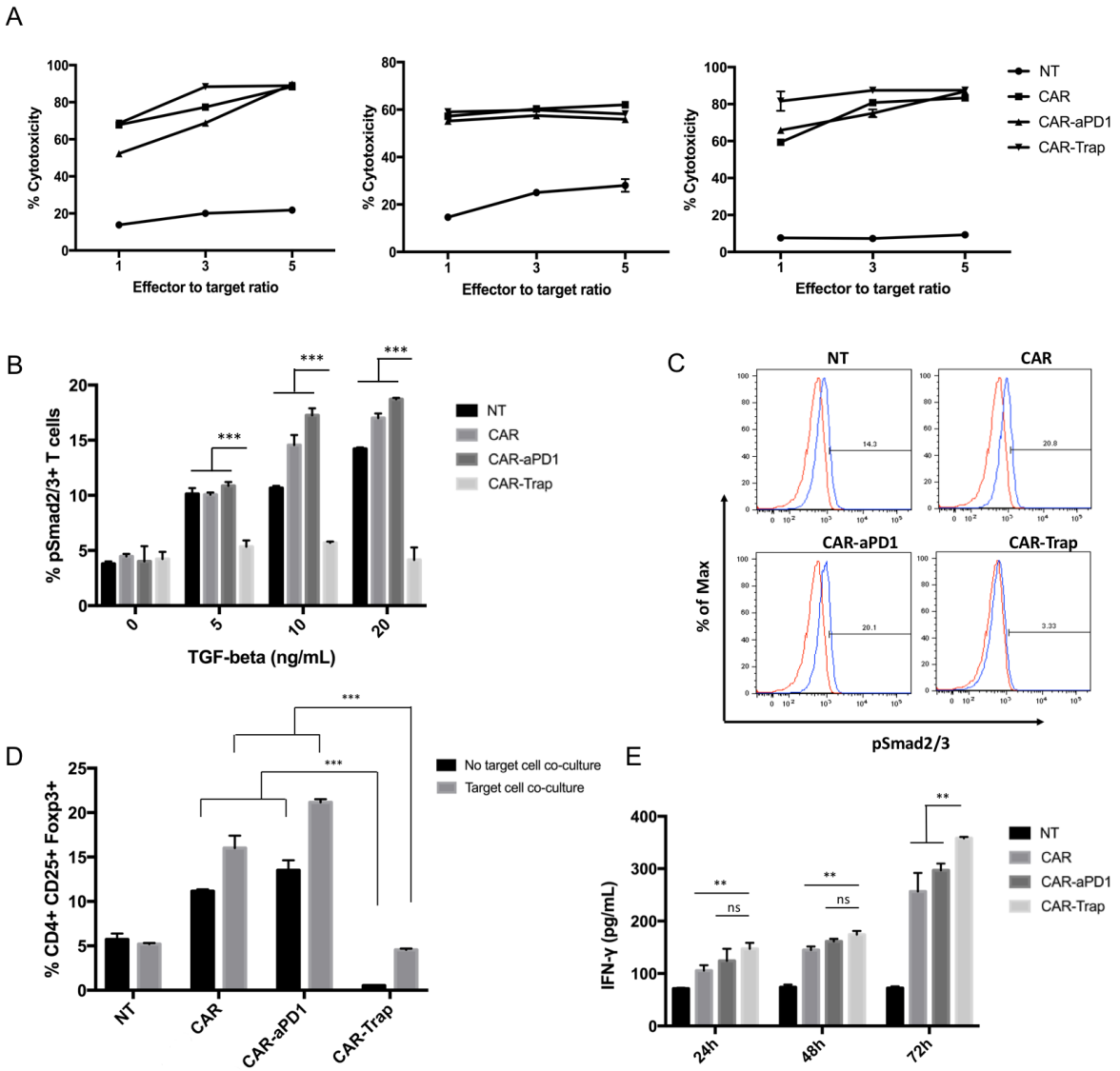

Figure 12. *In vitro* functionality of CAR-T cells. (A) Cytotoxicity of CAR-T cells against target cells. Three groups of CAR T cells were co-cultured for 24 hours with various target cells at 1:1, 3:1, and 5:1 effector-to-target ratios. NT cells were used as a control. (n=3, mean ± SD). (B) Percentages of Tregs ($CD4^+CD25^+Foxp3^+$) in different CAR-T cell groups with or without co-culture with target cells for 24 hours. The data from FACS were shown in bar graphs. NT cells were used as a control. (n=3, mean ± SD; ***P < 0.001). (C) CAR-T cells were co-cultured with target cells for various durations. IFN-γ secretion in supernatants was quantified by ELISA. (n=3, mean ± SD; ns, not significant; **P < 0.01). (D) Expression of phosphorylated Smad2/3 (pSmad2/3) in T cells after incubation in different concentrations of recombinant TGF-β for 24 hours. The percentages of $pSmad2/3^+$ T cells were shown in bar graphs. (n=3, mean ± SD; ***P < 0.001). (E) Representative FACS gating for $pSmad2/3^+$ T cells using NT cells without incubation as a negative control.

Trap protein secretion rescues CAR-T cell from exhaustion by limiting the up-regulation of immune checkpoint molecules

Importantly, trap protein blocks the binding between PD-1 and PD-L1, concomitant with the blocking of TGF-β signaling, as a step to remediate T cell exhaustion. Since PD-1/PD-L1 binding induces increased expression of PD-1 on the T cell surface, we investigated the effect of trap protein on PD-1 expression by co-culturing CAR-T cells with target cells. After 24 hours, all CAR-T groups exhibited upregulated PD-1 expression compared with NT. In contrast, PD-1 expression was significantly lower in the CAR-αPD1 and CAR-Trap groups compared with that in the CAR group, indicating that the secreted protein drugs, anti-PD1 scFv and trap protein, played a role in limiting the upregulation of PD-1 expression. No significant difference was observed between CAR-αPD1 and CAR-Trap (Fig. 13A). When different T cell subtypes were examined, similar results were observed in both $CD4^+$ T cells and $CD8^+$ T cells (Fig. 13B).

In addition to PD-1, other immune checkpoint molecules are expressed on the T cell surface, such as lymphocyte-activation gene 3 (LAG3), T cell immunoglobulin domain and mucin domain-containing protein 3 (TIM3) and PD-L1, contributing to T cell exhaustion upon antigen stimulation as a complex interaction network. To further evaluate the capability of trap protein to rescue CAR-T cells under these conditions, we tested the expression of LAG3, TIM3 and PD-L1 in CAR-T cells after co-culture with target cells for 24 hours. Again, CAR-αPD1 and CAR-Trap had less LAG3 and PD-L1 upregulation than that exhibited by CAR (Fig. 13C, 13E, 13F). In the analysis for TIM3, CAR-αPD1 and CAR-Trap showed a trend toward the inhibition of TIM3 upregulation, but it did not reach statistical significance (Fig. 13D).

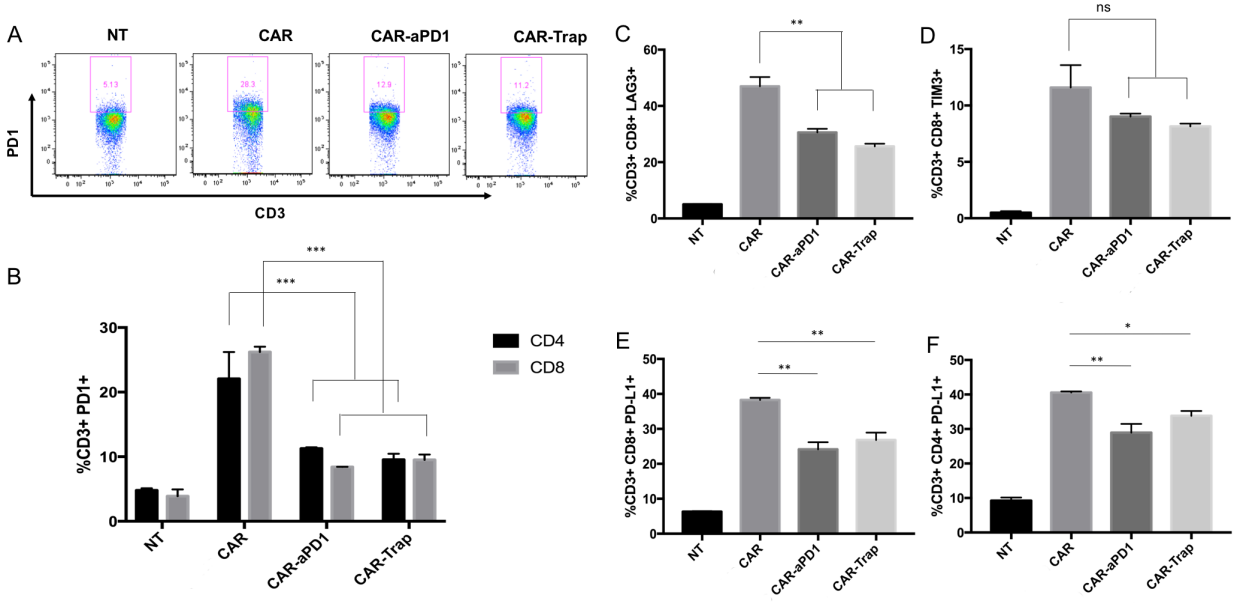

Figure 13. Expression of exhaustion markers in CAR-T cells. (A) $CD3^+$ T cells were shown in each panel. PD-1^+CD8^+ T cells were gated, and their percentage over total $CD3^+$ T cells was shown in each scatter plot. (B) The percentages of PD-1^+CD4^+ and PD-1^+CD8^+ T cells over total $CD4^+$ and $CD8^+$ T cells were shown in bar graphs. (C, D) LAG3 expression and TIM3 expression were measured by flow cytometry. The percentages of $LAG3^+CD8^+$ and $TIM3^+CD8^+$ T cells over total $CD8^+$ T cells were shown in bar graphs. (E, F) PD-L1 expression was measured by flow cytometry.

What opportunities for training and professional development has the project provided?

Two graduate students have been the major researchers for the project. This project provides an excellent training for them. They have learned skills related to molecular biology, viral vector engineering, T cell engineering, etc. They have been closely mentored by the PI on both research activities and soft skills related to abilities to identify problems, trouble-shooting, and critical thinking.

How were the results disseminated to communities of interest?

Nothing to Report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

The original proposal for Specific Aim 3 (Major tasks 5 &6) was to test anti-PD1-secreting CAR-T. During the course of studies, we further hypothesized that CAR-T cells that secrete biospecific trap protein co-targeting PD-1 and TGF-b could be an additional design that might be better overcome tumor microenvironment. Thus, we tested both designs.

Actual or anticipated problems or delays and actions or plans to resolve them

The masking peptides that were designed in the application did not result in masking effect by the in vitro studies. Thus, Major tasks 3 & 4 of in vivo studies could not be performed.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

Nothing to report

  Books or other non-periodical, one-time publications.

Nothing to report

  Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Xianhui Chen
Project Role:	Graduate Student
Nearest person month worked:	12
Contribution to Project:	Ms. Chen has performed work for the Major Tasks 1-6.
Name:	Yun Qu
Project Role:	Graduate Student
Nearest person month worked:	12
Contribution to Project:	Ms. Chen has performed work for the Major Tasks 1-2.
Name:	Pin Wang
Project Role:	PI
Nearest person month worked:	12
Contribution to Project:	Dr. Wang has directed the team to perform research outlined in the proposal.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES: *N/A*