Safety and Efficacy of an Immune Cell-Specific Chimeric Promoter in Regulating Anti-PD-1 Antibody Expression in CAR T Cells

Yuan Fang,[1] Yajun Zhang,[2] Chuanxin Guo,[3] Chumeng Chen,[1] Haixia Gao,[3] Xiumei Zhou,[1] Tao Liu,[3] and Qijun Qian[1,3,4]

[1]College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, China; [2]Eastern Hepatobiliary Surgery Hospital, The Second Military University, Shanghai 201805, China; [3]Shanghai Cell Therapy Research Institute, Shanghai Cell Therapy Group, Shanghai 201805, China; [4]Shanghai Mengchao Cancer Hospital, Shanghai Cell Therapy Group, Shanghai 201805, China

T cells modified to co-express cytokine or other factors with chimeric antigen receptor (CAR) can induce substantial and persistent increases in antitumor capacity *in vivo*. However, the uncontrolled expression of cytokines or factors can lead to the overactivation of immune cells, causing severe adverse events such as cytokine release syndrome (CRS) and neurotoxicity by CAR T cells with excessive growth potential. Conventional promoters are unregulated, and their expression is unlimited in T cells. In this study, by connecting the cytomegalovirus (CMV) enhancer, core interferon gamma (IFN-γ) promoter, and a T-lymphotropic virus long terminal repeat sequence (TLTR), we constructed and screened the chimeric promoter CIFT, which was highly expressed in some cell lines secreting IFN-γ and silenced in others. We placed this promoter upstream of the anti-programmed cell death protein 1 (anti-PD-1) antibody gene, and this construct was co-transfected with the CAR construct into T cells. *In vitro* or *in vivo*, CAR T cells showed increased secretion of anti-PD-1 antibody under control of the chimeric promoter CIFT. pS-CIFT-αPD-1/CAR T also had similar or lower PD-1 expression, higher levels of T cell activation, more release of IFN-γ, and better antitumor activity specifically against mesothelin-positive and PD-1 ligand 1 (PD-L1)-positive cell lines. The chimeric promoter may be a promising strategy to manipulate the content of immune checkpoint inhibitors or other proteins in future therapeutic approaches for cancer treatment.

INTRODUCTION

Chimeric antigen receptor (CAR) T cell therapy, one of the most significant and promising autologous immunotherapies, generates potent antitumor responses in patients with refractory or relapsed B cell malignancies.[1–4] However, in contrast to the success in treating leukemia, CAR T cells are not efficient in treating patients with advanced solid malignancies and other hematological malignancies.[5–7] The ineffective treatment of solid tumors is partially due to the immunosuppressive tumor microenvironment[8] and CAR T cell exhaustion.[9,10] Upregulating immune checkpoint blockade in-hibitors, such as anti-programmed cell death protein 1 (PD-1) single-chain variable fragment (scFv) secreted by CAR T cells, leads to notable improvement in impeding T cell exhaustion and normalization of the antitumor response.[11–15] The additional expression of cytokine-encoding genes in CAR T cells also effectively enhances their persistent and antitumor effects *in vivo*.[16–20] Although immune checkpoint blockade inhibitors or cytokines can improve the effector response and persistence of T cells *in vivo*, releasing supercharged T cells can lead to overactive immune cells, causing severe immune-related adverse events such as cytokine release syndrome (CRS) and neurotoxicity.[21–23]

In this study, using CAR T cells equipped with an autocrine anti-PD-1 scFv model, we designed and screened a T cell-specific and highly active artificial chimeric promoter, which was only active after antigen engagement, to control the secretion of anti-PD-1 antibodies at the tumor site, thereby counteracting the inhibitory effect of PD-1 signaling in CAR T cells and their endogenous counterparts.

Currently, the elongation factor 1 alpha (EF-1α) promoter is always selected for CAR contruction,[24] as it often leads to strong, stable expression uncorrelated with tissue specificity and no relationship to the T cell activation state. In T cells, the promoters of cytokine genes, such as interferon gamma (IFN-γ), interleukin 2 (IL-2), IL-3, tumor necrosis factor alpha (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF), are mainly induced by immune and proinflammatory signals. IFN-γ is primarily secreted by activated lymphocytes, especially by activated T cells, in a strictly controlled and restricted way.[25,26] Therefore, we developed a novel chimeric
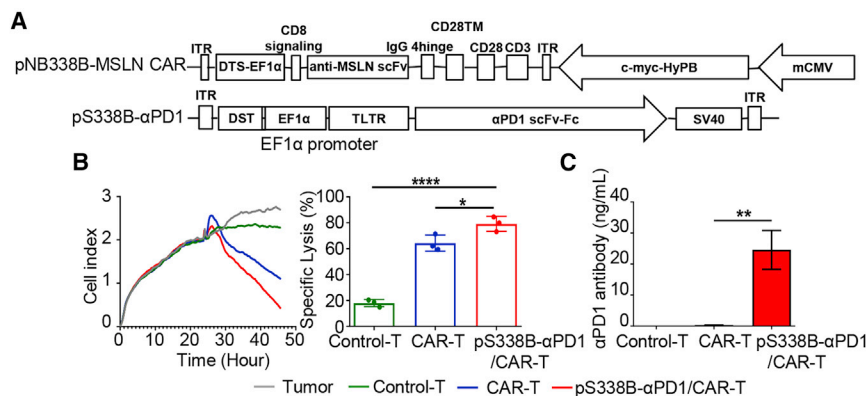
Figure 1. CAR T Cells Secreting Anti-PD-1 Antibody Have Enhanced Antitumor Function

(A) Schematic of PB vectors encoding the MSLN-targeted CAR, pNB338B-MSLN CAR, or an anti-PD-1 scFv of nivolumab with Fc fragment of human IgG4 and pS338B-αPD-1. (B) RTCA demonstrating the MSLN-specific cytotoxicity of MSLN CAR T or pS338B-αPD-1/CAR T cells after 24 h of co-culture with targets at an E:T ratio of 1:4. Not transfected T cells (NT) PBMCs served as controls (n = 3, three donors). (C) ELISA detecting expression of secreted anti-PD-1 antibody in the supernatant by control T, MSLN CAR T, or pS338B-αPD-1/CAR T cells after co-culturing with tumor cells for 24 h (n = 3, three donors). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are expressed as the mean ± SEM.

promoter construct capable of inducing enhanced anti-PD-1 antibody secretion in T cells after antigen stimulation. This new-generation chimeric promoter consisted of the cytomegalovirus (CMV) enhancer, the core IFN-γ promoter, and a human T-lymphotropic virus long terminal repeat (TLTR) sequence. The pS-CIFT-αPD-1/CAR T cells showed increased production of anti-PD-1 antibody. The pS-CIFT-αPD-1/CAR T cells showed antigen-dependent secretion of anti-PD-1 antibody, similar or lower PD-1 expression, greater T cell activation, more release of IFN-γ, and superior antitumor activity. Taken together, these results suggest that the chimeric promoter CIFT has the potential to manipulate the content of immune checkpoint inhibitors or other proteins in future therapeutic approaches for cancer treatment.

RESULTS

Modification of CAR T Cells to Secrete Anti-PD-1 Antibody

Studies have indicated that nivolumab, an anti-PD-1 antibody, plays a critical role in blocking the PDCD1-mediated pathway, which can inhibit T cell exhaustion and normalize the antitumor response. To test the function of CAR T cells with autocrine anti-PD-1 antibody, peripheral blood mononuclear cells (PBMCs) isolated from healthy donors were electroporated with the *piggyBac* (PB) transposase-CAR vector and transposon-αPD-1 plasmids. We used a mesothelin CAR (pNB338B-MSLN3CAR)[27] and an anti-PD-1 scFv of nivolumab with a Fc fragment of human immunoglobulin G4 (IgG4) (pS338B-αPD-1) (Figure 1A). In cytotoxicity assays, single MSLN CAR T cells showed inferior cytotoxicity to anti-PD-1 antibody-secreting CAR T (pS338B-αPD-1/CAR T) cells (Figure 1B). However, we only detected a low concentration of anti-PD-1 antibody in the supernatant of the tumor co-cultures with pS338B-αPD-1/CAR T cells (Figure 1C).

Construction and Screening of an Efficient Chimeric Promoter in PBMCs

Thirteen chimeric promoters based on the promoter of pS338B-αPD-1, consisting of a DNA nuclear targeting sequence (DTS), an EF-1α promoter, and a TLTR sequence, were generated (Figure S1). All chimeric promoters were ligated upstream of the enhanced green fluorescent protein (EGFP) reporter gene on the same backbone. PBMCs were electroporated with the PB transposase-CAR vector and transposon-

EGFP plasmids to visually and quantitatively test the promoter activities. We classified 13 chimeric promoters by EGFP expression using flow cytometry. Two chimeric promoters, pS-IFPT-EGFP and pS-CIFPT-EGFP, expressed EGFP at a mean fluorescence intensity higher than that of pS338B-EGFP; the other promoters expressed lower levels of EGFP (Figure S2). We chose the better chimeric promoter to generate dual-luciferase reporter genes to confirm the properties of the best chimeric promoter constructs quantitatively (Figure 2). The results confirmed that the pS-CIFT-firefly luciferase (Fluc) vector showed the highest transfection efficiency in PBMCs (Figure 2).

The Chimeric Promoter Shows Enhanced Activity in Cells Secreting IFN-γ

To understand promoter differences between the CAR T cells that may influence their function, flow cytometry, enzyme-linked immunospot (ELISPOT), dual-luciferase reporter analyses, and fluorescent staining analyses were used to assess the function of the chimeric promoter. First, we confirmed the relationship between the activity of CIFT and IFN-γ release. The chimeric promoter (pS-CIFT-EGFP) had high expression of EGFP under high levels of secreted IFN-γ in the presence of CAR, and low expression of EGFP under low levels of secreted IFN-γ (Figure 3A). These results showed that the chimeric promoter CIFT regulates EGFP expression related to the release of IFN-γ.

Next, we classified five different cell lines by IFN-γ expression using ELISPOT. Four cell lines expressed IFN-γ at a low level, which we defined as IFN-γ negative (IFN-γ neg); the T cell was IFN-γ positive (IFN-γ pos) (Figures 3B and S3). EGFP was expressed at a higher level in all cells transfected with pS338B-EGFP. IFN-γ-pos cell lines expressed a high level of EGFP, whereas IFN-γ-neg cell lines expressed very low levels of EGFP and almost no EGFP when using a CIFT or IFPT chimeric promoter (Figures 3C and S4). These data indicated that the chimeric promoter CIFT was highly active in cells secreting IFN-γ and silent in other cells.

The Chimeric Promoter Leads to Anti-PD-1 Antibody Secretion and Inhibits T Cell Exhaustion *In Vitro*

To determine whether the chimeric promoter CIFT could improve the production of anti-PD-1 antibody, we first ligated the promoter
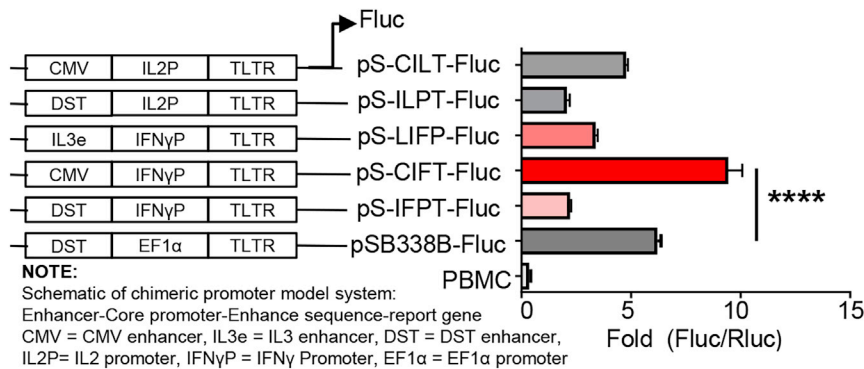
upstream of the anti-PD-1 antibody gene (pS-CIFT-αPD-1) (Figure 4A). PBMCs were electroporated with MSLN CAR and pS-CIFT-αPD-1 or pS338B-αPD-1. The transfection efficiency of the two groups was similar and slightly above 65% at 13 days post-transfection (Figure 4B). We further detected the production of IFN-γ and anti-PD-1 antibody in the supernatant for 216 h in 24-h intervals, secreted by 2×10^5 T cells. T cells were only stimulated with recombinant MSLN (rMSLN) antigen in the second 24 h. The IFN-γ activity was noted in activated CAR T cells, and no substantial release of IFN-γ was detected in resting CAR T cells (Figure 4C). Moreover, compared to pS338B-αPD-1/CAR T cells, pS-CIFT-αPD-1/CAR T cells secreted higher levels of anti-PD-1 antibody in an activated state, but lower levels in resting CAR T cells (Figure 4D). Next, we evaluated whether anti-PD-1 antibody contributed to blocking PD-1 expression on the surface of T cells. There were varying degrees of decrease in the amount of surface PD-1 on pS-CIFT-αPD-1/CAR T cells compared with T cells modified to express pS338B-αPD-1/CAR (Figure 4E). To further evaluate the effect of enhanced anti-PD-1 antibody on T cell function, we analyzed the related phenotype of T cell activation, exhaustion, and differentiation. pS-CIFT-αPD-1/CAR T cells expressed significantly higher levels of CD25 and CD69, but relatively stable percentages of TIM-3, LAG-3, and CD8 compared to pS338B-αPD-1/CAR T cells (Figure 4F). pS-CIFT-αPD-1/CAR T cells contained more durable effector function upon antigen stimulation (Figure 4F). These results confirmed that pS-CIFT-αPD-1/CAR T had increased anti-PD-1 antibody secretion with no increase in T cell exhaustion, led to anti-PD-1 antibody secretion related to T cell activation status, and sustained T cell activation. Furthermore, secreted anti-PD-1 antibody could significantly bind PD-1 on the surface of T cells.

The Chimeric Promoter Enhances Antitumor Ability

The antitumor activity of pS-CIFT-αPD-1/CAR T cells was evaluated against the human mesothelin-expressing ovarian tumor cell line SKOV3.[27] SKOV3 cells were artificially modified with PD-1 ligand 1 (PD-L1) (SKOV3-PD-L1) (Figure 5A). We co-cultured control T cells, pS338B-αPD-1/CAR T cells, or pS-CIFT-αPD-1/CAR T cells with SKOV3 cells at different effector-to-target (E:T) ratios. As shown in Figure S5, there was no apparent difference in cytotoxicity between pS338B-αPD-1/CAR T cells and pS-CIFT-αPD-1/CAR T cells when

effector T cells were co-cultured with target tumor cells at different ratios in a real-time cell analysis (RTCA) assay. Thus, we chose another method to detect the antitumor activity. The pS-CIFT-αPD-1/CAR T cells more efficiently controlled SKOV3-PD-L1 cell growth at a T cell-to-tumor cell ratio of 1:2 (Figures 5B, 5C, and S6). The antitumor activity of pS-CIFT-αPD-1/CAR T cells was also confirmed by the high production of IFN-γ in the culture supernatant (Figure 5E). We also assessed the concentration of anti-PD-1 antibody in the culture supernatant. There was a significant increase in anti-PD-1 antibody secretion in pS-CIFT-αPD-1/CAR T cells compared with pS338B-αPD-1/CAR T cells at a high T cell-to-tumor cell ratio of 1:2 (Figure 5D).

Chimeric Promoter Enhances Antitumor Ability *In Vivo*

To evaluate the antitumor effects of pS-CIFT-αPD-1/CAR T cells versus pS338B-αPD-1/CAR T cells *in vivo*, we implanted Fluc-transduced SKOV3-PD-L1 tumor cells into NSG mice (Figure 6A). At 7 days xenotransplantation, mice were infused with control T cells, MSLN CAR T cells, pS338B-αPD-1/CAR T cells, or pS-CIFT-αPD-1/CAR T cells when the tumor volume was about 100 mm^3 (Figure 6B). pS-CIFT-αPD-1/CAR T cells effectively eliminated SKOV3-PD-L1 tumor cells and prolonged the survival of mice (Figure 6B). At 64 days after treatment, all mice infused with control T cells were dead, while pS338B-αPD-1/CAR T cell- or pS-CIFT-αPD-1/CAR T cell-treated mice were still alive (Figure 6C). To further assess the function of pS-CIFT-αPD-1/CAR T cells exposed to a specific antigen *in vivo*, the peripheral blood cells from treated mice were tested. At 30 days after infusion, there were more circulating pS-CIFT-αPD-1/CAR T cells than control T cells, CAR T cells, or pS338B-αPD-1/CAR T cells in PBMCs (Figure 6D). We analyzed the expression of anti-PD-1 antibody bi-weekly after treatment. As shown in Figure 6E, both pS338B-αPD-1/CAR T cells and pS-CIFT-αPD-1/CAR T cells effectively expressed anti-PD-1 antibody, which confirmed the *in vitro* results when pS338B-αPD-1/CAR T cells and pS-CIFT-αPD-1/CAR T cells were co-cultured with SKOV3-PD-L1. As shown in Figure 6F, 30 days after infusion, pS-CIFT-αPD-1/CAR T cells released more IFN-γ and controlled the release of IL-6 in the serum.

DISCUSSION

In this study, we analyzed the novel chimeric promoter CIFT regulation in anti-PD-1 scFv secretion of CAR T cells. Using this optimized methodology for secreted anti-PD-1 antibody might lead to local enhancement of anti-PD-1 scFv concentration in the tumor
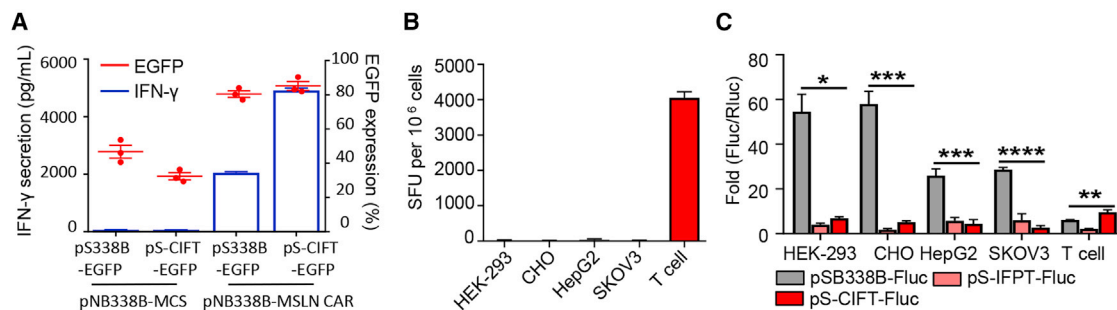
Figure 3. Function of the Chimeric Promoter

(A) Representative analysis of IFN-γ secretion and EGFP expression in T cells co-transfected the pS-CIFT-EGFP vectors with a control vector, pNB338B-MCS, or a CAR vector, pNB338B-MSLN CAR, with pS338B-EGFP having served as the control (n = 3, three donors). (B) ELISPOT analysis of IFN-γ release in HEK293, CHO, Hep G2, SKOV3, and T cells. Positive responses were represented by spot forming units (SFU). Data shown are representative of three independent experiments. (C) Dual-luciferase reporter analysis of chimeric promoter activities in HEK293, CHO, Hep G2, SKOV3, and T cells. Data shown are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are represented as the mean ± SEM.

microenvironment, accelerate T cell activation, and prevent exhaustion, thereby increasing the antitumor efficiency of the engineered T cells. Furthermore, the CIFT chimeric promoter was specific to the immune cells, was significantly related to the T cell activation status, and occurred during the expansion culture period after the specific antigen stimulated CAR T cells. These effects may be a consequence of the pivotal role of inducible anti-PD-1 antibody secretion in CAR T autocrine secretion of anti-PD-1 antibody immunotherapy.

The promoter CIFT safely induced enhancement of anti-PD-1 antibody secretion. Previous studies have shown that CAR T cells combined with an immune checkpoint inhibitor, such as exogenously added monoclonal antibody against PD-1/PD-L1 or autonomously produced by CAR T cells, play a significant role in solid and hematologic malignancies.[28,29] Interestingly, PD-1-blocking scFv-secreting CAR T cells have appreciable antitumor efficacy *in vivo*.[30] In mainstream research, the EF-1α promoter is frequently used in CAR T cell therapies, because the EF-1α promoter can evoke a more stable and higher expression in primary CD4 and CD8 T cells, in contrast to several promoters.[24,31] However, when using the EF-1α promoter, there is no mention of control over the activity of CAR T cells secreting anti-PD-1 antibody after tumor elimination. Preclinical studies have previously demonstrated that expression of the IFN-γ promoter requires activation with ionomycin and phorbol myristate acetate.[32] Similarly, we found that the CIFT promoter was also capable of effectively enhancing the expression of anti-PD-1 antibody in CAR T cells *in vitro* only upon antigen engagement. Furthermore, we confirmed that the regulation of anti-PD-1 antibody expression driven by the CIFT chimeric promoter was specific to cells secreting IFN-γ, averting the risk of unintentionally transfecting the CAR gene into unrelated cells.[33] The above-mentioned results reveal that the CIFT promoter may play a pivotal role in the safe and inducible CAR T cell autocrine secretion of an anti-PD-1 antibody in immunotherapy strategies.

The sufficient anti-PD-1 antibody alleviates T cell activation and exhaustion. The PD-1/PD-L1 axis is related to tumor proliferation,

metastasis, and poor prognosis. Therefore, effectively blocking PD-1/PD-L1 is an important approach for treating tumors and restoring tumor-specific T cell immune function.[34–38] Studies have also shown that autocrine PD-1 antibodies of CAR T cells can effectively bind and block the PD-1 receptor on the surface of T cells and reduce the proportion of PD-1-positive cells.[39] We demonstrated that PD-1 antibodies secreted by pS-CIFT-αPD-1/CAR T cells could block the PD-1 receptor on the surface of T cells. Interestingly, PD-1-responsive T cells tend to be subsets of cells with similar molecular characteristics, such as memory T cells or stem cells.[40] When using the CIFT promoter, we determined that CAR T cells secreted an increased level of PD-1 antibody but no increase in T cell exhaustion. The increased anti-PD-1 antibody could effectively induce the response of T cells, such as enhancing the CD25- and CD69-positive subsets, thereby "resurrecting" T cells and reversing the state of T cell depletion, leading to dysfunction.[41] Moreover, when using the CIFT promoter, we determined that memory T cells quickly recovered effector function upon secondary antigen stimulation and induced more durable responses, thus rapidly killing and removing tumor cells.[42,43]

Our results demonstrated that the CIFT promoter could be used to control the expression of exogenous genes in CAR T cells. Immunotherapy is evolving rapidly. We think that this promoter will be safely and widely used for the expression of exogenous protein genes in T cells.

MATERIALS AND METHODS
Plasmids
Our laboratory produced the non-viral PB transposon control vector (pNB338B-MCS), the non-viral PB transposon targeting the mesothelin CAR vector (pNB338B-MSLN CAR), and the control vector encoding anti-PD-1 antibody (pS338B-αPD-1) (Shanghai Cell Therapy Group, Shanghai, China). The promoter of pS338B-αPD-1, which comprises the SV40 enhancer (DTS), EF-1α promoter, and a TLTR sequence, was modified to generate a series of artificial
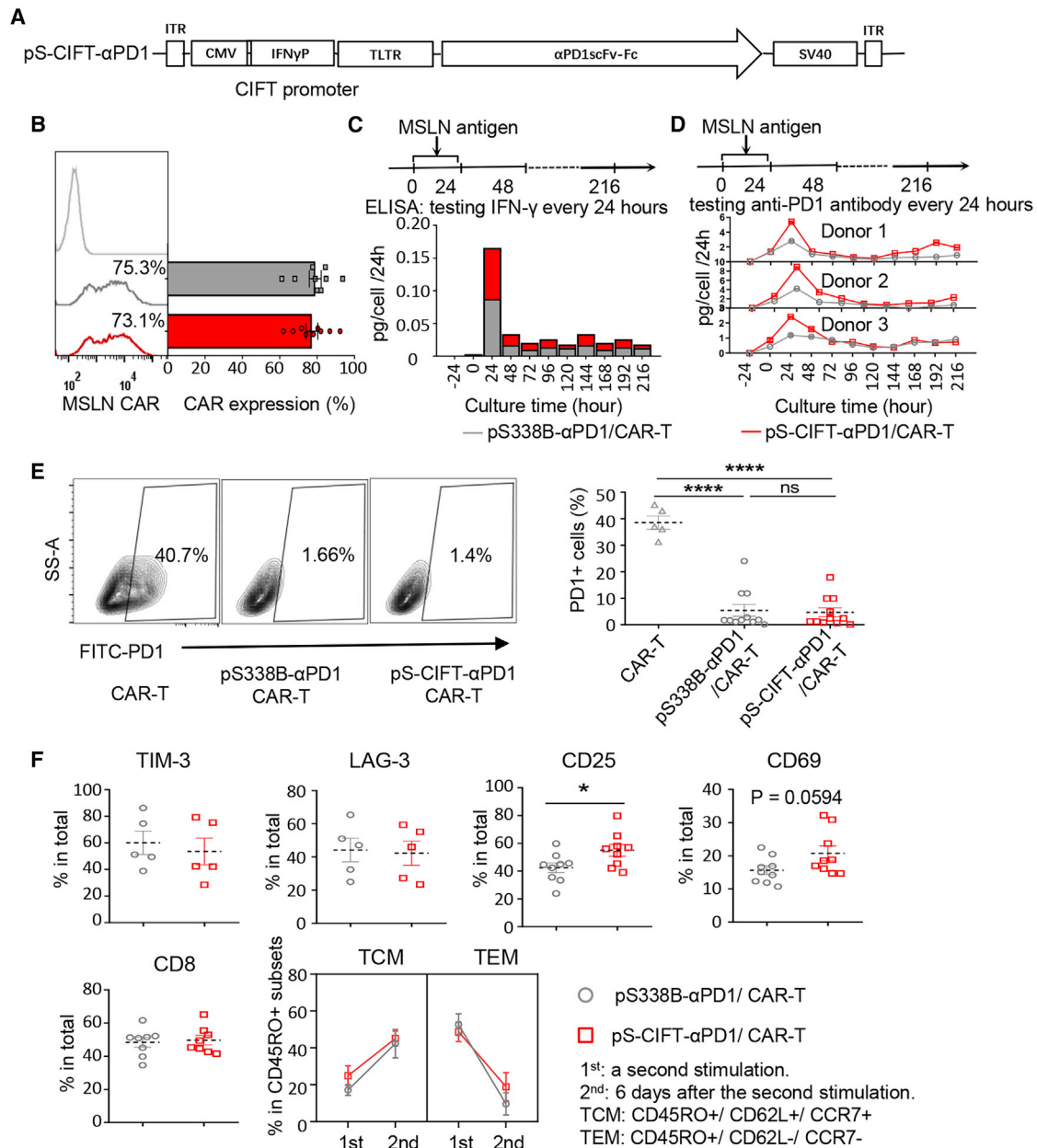
Figure 4. pS-CIFT-αPD-1/CAR T Cells Have a Sensitive Response to Targeted Antigen-Expressing Cells and Reduced Antigen-Induced Differentiation

(A) A schematic of PB vector encoding the anti-PD-1 antibody, pS-CIFT-αPD-1. (B) Left: representative histogram of CAR expression on day 13. Right: fluorescence-activated cell sorting (FACS) analysis of CAR expression on day 13 (n = 9, representative of nine donors). (C and D) IFN-γ release (n = 2, two donors) (C) and anti-PD-1 antibody secretion (n = 3, three donors) (D) by ELISA on a daily basis in CAR T cells before and after stimulation. (E) Proportion of PD-1+ T cells as assessed by FACS. (F) FACS analysis of phenotype of CD3+ T cells demonstrating different markers of T cell exhaustion, activation, and differentiation (n = 5, five donors). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are expressed as the mean ± SEM.

chimeric promoters containing different combinations of *cis*-regulatory elements, namely a core human cytokine gene promoter (core IFN-γ promoter or core IL-2 promoter), an efficient upstream enhancer (CMV, DTS, or IL-3 enhancer), and downstream introns (TLTR sequence or IFN-γ introns). pS338B-EGFP (or -Fluc) was generated by inserting the EGFP or Fluc into the pS338B-αPD-1 vec-

tor backbone. The vector pS-uIFp-EGFP was obtained from the pS338B-EGFP by replacing the promoter with a core IFN-γ promoter. The vector pS-uIFPT-EGFP has an additional TLTR sequence downstream of the core IFN-γ promoter in the pS-uIFp-EGFP. The vector pS-IFPT-EGFP (or -Fluc), whose promoter is called IFPT (comprising DTS, the core IFN-γ promoter, and a TLTR sequence),
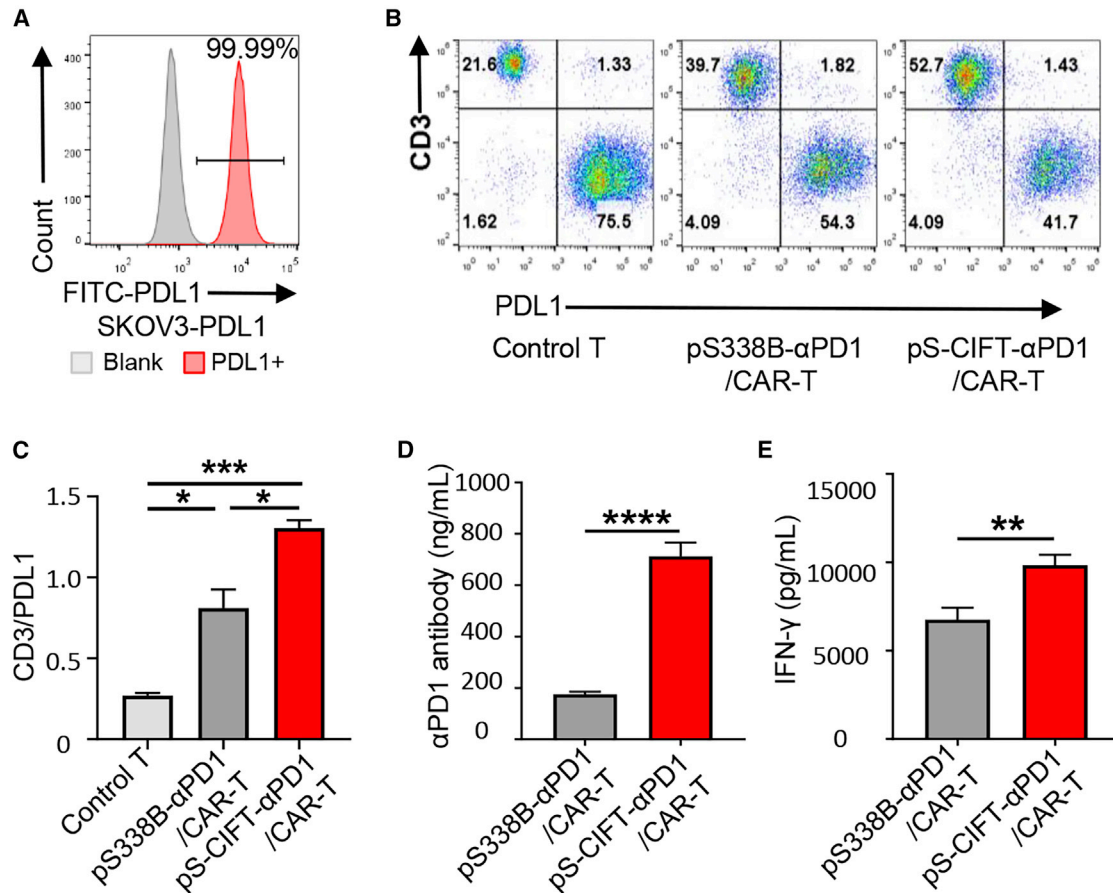
Figure 5. CAR T Cells Secreting Anti-PD-1 Antibody Have Enhanced Antitumor Function

(A) Representative histogram of PD-L1 expression of SKOV3. (B) Proportion of T cells (CD3+) and tumor cells (PDL1+) as assessed by FACS. T cells were co-cultured with tumor cells at a ratio of 1 to 2 for 24 hours. (C) analysis of 24-h cytotoxic assay demonstrating the antitumor effect of CAR T cells co-cultured with tumor cells at a ratio of 1 to 2. Data shown are representative of three independent experiments. (D and E) ELISA detecting expression of (D) secreted anti-PD-1 antibody and (E) released IFN-γ in the supernatant by control T, pS338B-αPD-1/CAR T, or pS-CIFT-αPD-1/CAR T cells after co-culture with tumor cells for 24 h. Data shown are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are expressed as the mean ± SEM.

was obtained from pS338B-EGFP (or -Fluc) by replacing the EF-1α promoter with the core IFN-γ promoter. The vector pS-CIFT-EGFP (or -Fluc), whose promoter is called CIFT (comprising the CMV enhancer, the core of the IFN-γ promoter, and a TLTR sequence) was generated by substituting the CMV enhancer for DTS into the pS-IFPT-EGFP vector backbone. The vector pS-LIFP-EGFP (or -Fluc), whose promoter is called LIFP (comprising DTS, a core IFN-γ promoter, and a TLTR sequence), was modified from pS-IFPT-EGFP with an IL-3 enhancer. The vector pS-uIFen-EGFP was modified from pS-uIFp-EGFP by inserting downstream an IFN-γ intron. The vector pS-IFen-EGFP was generated by substituting the IFN-γ introns for the TLTR sequence into the pS-IFPT-EGFP vector backbone. The vector pS-CIFen-EGFP was modified from pS-CIFT-EGFP with IFN-γ introns. The same approaches were used to construct the vectors having the core IL-2 promoter instead of the core IFN-γ promoter. The vector pS-CIFT-αPD-1 was originated

from pS-CIFT-EGFP by replacing the EGFP sequence with the anti-PD-1 scFv sequence.

Cell Lines

The Hep G2 (ATCC, USA) hepatocellular carcinoma cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% (v/v) fetal bovine serum (FBS, GIBCO, USA). The HEK293T human kidney epithelial cell line and SKOV3 (SKOV3 and SKOV3-PD-L1) ovarian carcinoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) containing 10% FBS. The Chinese hamster ovary (CHO) cell line was cultured in 44% DMEM and 44% RPMI 1640 containing 10% FBS, 1% L-glutamine, and 1% hypoxanthine-thymidine. All cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2. All tumor cells were detected by the PCR short tandem repeat (STR) method and screened for the presence of mycoplasma monthly.
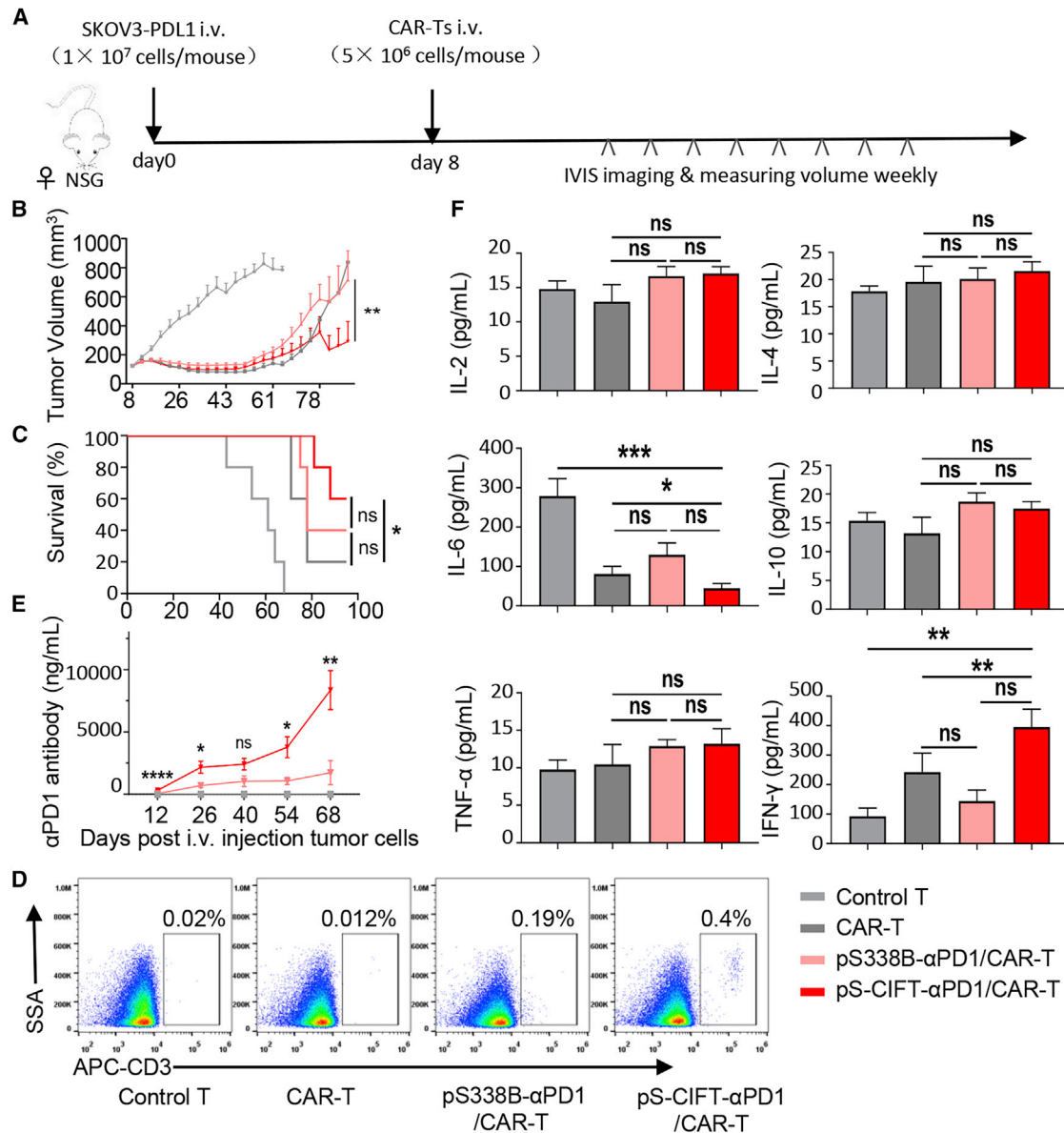
Figure 6. pS-CIFT-αPD-1/CAR T Cells Outperform Conventional CAR T Cells in Mice

(A) Schematic diagram of the experimental setup to detect anti-PD-1 antibody secretion *in vivo*; SKOV3-PD-L1-bearing mice were monitored until being subsequently treated with control T cells, CAR T cells, pS338B-αPD-1/CAR T cells, or pS-CIFT-αPD-1/CAR T cells. (B) Representative tumor volume 8 days after tumor cell infusion. (C) Kaplan-Meier analysis of survival of mice (using the Gehan-Breslow-Wilcoxon test). (D) T cells *in vivo* were analyzed by FACS 30 days after infusion. (E) *In vivo*, ELISA analysis of the secretion of anti-PD-1 antibody was detected by harvesting serum from tumor-bearing mice on a biweekly basis. A t test was conducted to compare pS338B-αPD-1/CAR T and pS-CIFT-αPD-1/CAR T cells. (F) T cells produce multiple cytokines in response to SKOV3-PD-L1 tumor cells *in vivo* (n = 5, five mice per group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are means ± SEM.

T Cell Isolation, Electroporation, and *Ex Vivo* CAR T Cell Expansion

Human PBMCs were obtained from healthy donor whole blood by Ficoll-Paque density gradient centrifugation. PBMCs were purchased from AllCells (USA) using a protocol approved by the Ethics Committee of the Chinese Peoples' Liberation Army General Hospital (S2019-063-01). In total, 5×10^6 PBMCs were collected and resus-pended in a P3 Primary Cell 4D-Nucleofector (Lonza, Switzerland) buffer, together with the PB transposase (i.e., pNB338B-MSLN CAR) and recombinant transposon plasmids (i.e., pS338B-EGFP or pS338B-αPD-1) using Lonza 4D-Nucleofector (Lonza) according to the manufacturer's instructions. After electroporation, T cells were expanded and activated with coated human recombinant mesothelin antigen/human anti-CD28 monoclonal antibodies (mAbs) for

4–5 days in Aim-V (Gibco, USA) media supplemented with 2% (v/v) FBS (Gibco, USA) and 500 IU/mL IL-2, and subsequently expanded by feeding them fresh Aim-V media supplemented with 2% FBS and 100 IU/mL IL-2.

Dual-Luciferase Reporter Assay

T cells were resuspended in the buffer and co-transfected with the firefly luciferase reporter gene (i.e., pS-AD-F-FLuc) and Renilla luciferase reporter gene (pS-AD-F-RLuc) using the human T cell Nucleofector kit (Lonza, Switzerland). At 24 h after electroporation, the cells were collected for luciferase activity measurements using the Dual-Luciferase reporter assay system (Promega, USA) following the manufacturer's protocol. The firefly luciferase activity was normalized to Renilla luciferase activity and expressed as a relative luciferase unit. Finally, the ratio of firefly luciferase activity/Renilla luciferase activity was the indicator of promoter activity. Data were collected using an EnVision multilabel reader (PerkinElmer, Seer Green, UK).

Human IFN-γ ELISPOT

IFN-γ release was measured with a human IFN-γ ELISPOT kit (Mabtech, Sweden). Plates were washed with phosphate-buffered saline (PBS) and blocked in medium containing 10% FBS. CHO, HEK293T, SKOV3, Hep G2, and activated T cells were seeded at a density of 1×10^5 cells per well and then incubated at 37°C in a humidified incubator containing 5% CO_2 for 12–24 h. Plates were rinsed with PBS and 7-B6-ALP (Mabtech) was added as recommended. After 2 h of incubation, plates were rinsed with PBS and BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitroblue tetrazolium)-plus (Mabtech) was added as recommended. Plates were rinsed with PBS and spots were imaged and enumerated using the AID vSpot Reader Spectrum (AID, Munich, Germany). Experiments were conducted according to the manufacturers' instructions.

Immunofluorescence Staining

CHO, HEK293T, SKOV3, Hep G2, and T cells were seeded at the recommended density of cells per well and grown in six-well plates for 24 h, and then co-transfected with the EGFP reporter gene (i.e., pS338B-EGFP) and mCherry reporter gene using ViaFect transfection reagent (Promega) as per the manufacturer's protocol. At 24–48 h after transfection, the cells were fixed in 4% paraformaldehyde and then incubated with 4′,6-diamidino-2-phenylindole (50 μg/mL; Sigma, USA) for 3 min to stain the nuclei in live cells. Finally, all samples were visualized and imaged using a fluorescence microscope (Olympus IX73; Olympus, Japan).

Flow Cytometry Assay

Tumor cells expressing PD-L1 were stained with fluorochrome-conjugated antibodies against PD-L1 (BD Biosciences, USA) markers. CAR T cell lines were stained with fluorochrome-conjugated antibodies against the following markers: CD3, CD8, PD-1, TIM-3, LAG-3, CD25, CD69, CD45RO, CD62L, and CCR7 (BD Biosciences, USA). For assessment of the active promoter, CAR T cells transfected with the EGFP receptor gene were directly measured using flow cytometry. CAR expression was detected using an indirect method

with biotinylated MSLN protein and streptavidin-coupled phycoerythrin (PE) antibody (BD Biosciences, USA). For the PD-1-blocking assay, CAR T cells were respectively stained with fluorochrome-conjugated antibodies against CD3, CD8, PD-1, TIM-3, LAG-3, CD25, CD69, CD45RO, CD62L, and CCR7 (BioLegend, USA). For assessment of CAR T cell cytotoxic activity, 2×10^5 CAR T cells (CD19 CAR T, pS338B-αPD-1/CAR T, or pS-CIFT-αPD-1/CAR T) and 2×10^5 tumor cells (SKOV3-PD-L1-EGFP or SKOV3-EGFP) were incubated for 24 h. The cell mixture was stained with anti-CD3 and anti-PD-L1 and was measured using flow cytometry. All samples were acquired on the Navios flow cytometer (Beckman Coulter, USA) and analyzed with FlowJo software vX.0.7, Kaluza v2.0 software, and GraphPad Prism 8 (GraphPad, USA).

Cytokine Release Assay

Culture supernatants (after T cells were co-cultured with tumor cells or specific antigen for 24 h) were collected to measure the release of IFN-γ, using specific ELISAs (R&D Systems, USA). Sera (obtained from mice) were collected to measure the release of IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α using the BD cytokine cytometric bead array (CBA) human Th1/Th2 cytokine kit II (BD Biosciences, USA) following the manufacturer's protocol. Data were collected using the Navios flow cytometer (Beckman Coulter, USA) and analyzed using the FCAP Array v3 software Analyze software. The conditioned media or sera were stored at −80°C in 1.5-mL tubes until use.

ELISA

Culture supernatants (from the CAR T cell co-cultures with tumor cells or specific antigen) and sera from mice were obtained to measure the amount of human anti-PD-1 antibody and IFN-γ using the corresponding anti-PD-1 antibody and IFN-γ ELISA kits (R&D Systems, USA) according to the manufacturer's instructions. The conditioned media or sera were stored at −80°C in 1.5-mL tubes until subsequent use.

Cytotoxicity Assay

Cytotoxic responses to in vitro assays were detected by the CELLigence real-time cell analyzer System (ACEA Biosciences, USA) and flow cytometry. In the RTCA assay, tumor cells were seeded at a density of 1×10^4 cells per well and grown for 24 h. Next, the CAR and control T cells were added to the RTCA unit at different E:T ratios. The impedance signals were recorded using the instrument for 50 h with 5-min intervals. The supernatants were also collected for cytokine and anti-PD-1 antibody measurements. In the flow cytometry assay, tumor cells (transfected with EGFP reporter gene) were seeded at a density of 2×10^5 cells per well and grown on day 1. On day 2, the CAR and control T cells were added to the well at an E:T ratio of 1:1 for 24 h. On day 3, T cells and tumor cells were quantified by flow cytometry based on CD3 and PD-L1 expression, using the Navios flow cytometer (Beckman Coulter, USA) and analyzed using Kaluza Analysis (2.0) software or FlowJo X.

Animal Experiments

All mouse experiments were performed following the *Guide for the Care and Use of Laboratory Animals* approved by Zhejiang Sci-Tech

University (Hangzhou, China). Female NSG mice aged 6–8 weeks were purchased from Shanghai Runnuo Biological Technology (Shanghai, China). The NSG mice were inoculated subcutaneously with 1.0×10^7 SKOV3-PD-L1 tumor cells labeled with FLuc. Tumor engraftment was measured as tumor volume (V) using the formula V = $^1/_2$(length × width2), measured as bioluminescence signal intensity, and expressed as total flux (photons/s) using the *in vivo* imaging system (IVIS) (PerkinElmer, USA). At 7 days after tumor engraftment (the tumor volume reached 100 mm^3), mice were divided into four groups and inoculated with 5×10^6 control (Ctrl) T cells, CAR T cells, pS338B-αPD-1/CAR T cells, or pS-CIFT-αPD-1/CAR T cells. Mice were bled at specific intervals in 7–14 days to measure cytokines and anti-PD-1 antibody in the plasma and the frequency of CAR T cells. Mice were followed after CAR T cell treatment and euthanized when tumor growth caused discomfort as per the veterinarian's recommendation.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software version 8.0 (GraphPad). To calculate p values and test for significant differences as specified in the figure legends, a two-tailed Mann-Whitney test or two-tailed Wilcoxon matched-pairs signed-rank test was utilized. The log-rank (Mantel-Cox) test was used for comparison of the survival curve. A significant difference between groups was indicated by p $\leq$ 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.08.008.

REFERENCES

1. Kochenderfer, J.N., Dudley, M.E., Feldman, S.A., Wilson, W.H., Spaner, D.E., Maric, I., Stetler-Stevenson, M., Phan, G.Q., Hughes, M.S., Sherry, R.M., et al. (2012). B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood *119*, 2709–2720.

2. Ying, Z., Huang, X.F., Xiang, X., Liu, Y., Kang, X., Song, Y., Guo, X., Liu, H., Ding, N., Zhang, T., et al. (2019). A safe and potent anti-CD19 CAR T cell therapy. Nat. Med. *25*, 947–953.

3. Ghorashian, S., Kramer, A.M., Onuoha, S., Wright, G., Bartram, J., Richardson, R., Albon, S.J., Casanovas-Company, J., Castro, F., Popova, B., et al. (2019). Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR. Nat. Med. *25*, 1408–1414.

4. Ruella, M., Barrett, D.M., Kenderian, S.S., Shestova, O., Hofmann, T.J., Perazzelli, J., Klichinsky, M., Aikawa, V., Nazimuddin, F., Kozlowski, M., et al. (2016). Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies. J. Clin. Invest. *126*, 3814–3826.

5. Morgan, R.A., Yang, J.C., Kitano, M., Dudley, M.E., Laurencot, C.M., and Rosenberg, S.A. (2010). Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing *ERBB2*. Mol. Ther. *18*, 843–851.

6. Maus, M.V., Haas, A.R., Beatty, G.L., Albelda, S.M., Levine, B.L., Liu, X., Zhao, Y., Kalos, M., and June, C.H. (2013). T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. Cancer Immunol. Res. *1*, 26–31.

7. Khalil, D.N., Smith, E.L., Brentjens, R.J., and Wolchok, J.D. (2016). The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. Nat. Rev. Clin. Oncol. *13*, 394.

8. Tahmasebi, S., Elahi, R., and Esmaeilzadeh, A. (2019). Solid tumors challenges and new insights of CAR T cell engineering. Stem Cell Rev. Rep. *15*, 619–636.

9. Chen, J., López-Moyado, I.F., Seo, H., Lio, C.J., Hempleman, L.J., Sekiya, T., Yoshimura, A., Scott-Browne, J.P., and Rao, A. (2019). NR4A transcription factors limit CAR T cell function in solid tumours. Nature *567*, 530–534.

10. Lynn, R.C., Weber, E.W., Sotillo, E., Gennert, D., Xu, P., Good, Z., Anbunathan, H., Lattin, J., Jones, R., Tieu, V., et al. (2019). c-Jun overexpression in CAR T cells induces exhaustion resistance. Nature *576*, 293–300.

11. Li, S., Siriwon, N., Zhang, X., Yang, S., Jin, T., He, F., Kim, Y.J., Mac, J., Lu, Z., Wang, S., et al. (2017). Enhanced cancer immunotherapy by chimeric antigen receptor-modified T cells engineered to secrete checkpoint inhibitors. Clin. Cancer Res. *23*, 6982–6992.

12. Rosewell Shaw, A., Porter, C.E., Watanabe, N., Tanoue, K., Sikora, A., Gottschalk, S., Brenner, M.K., and Suzuki, M. (2017). Adenovirotherapy delivering cytokine and checkpoint inhibitor augments CAR T cells against metastatic head and neck cancer. Mol. Ther. *25*, 2440–2451.

13. John, L.B., Devaud, C., Duong, C.P., Yong, C.S., Beavis, P.A., Haynes, N.M., Chow, M.T., Smyth, M.J., Kershaw, M.H., and Darcy, P.K. (2013). Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. Clin. Cancer Res. *19*, 5636–5646.

14. Gargett, T., Yu, W., Dotti, G., Yvon, E.S., Christo, S.N., Hayball, J.D., Lewis, I.D., Brenner, M.K., and Brown, M.P. (2016). GD2-specific CAR T cells undergo potent activation and deletion following antigen encounter but can be protected from activation-induced cell death by PD-1 blockade. Mol. Ther. *24*, 1135–1149.

15. Chong, E.A., Melenhorst, J.J., Lacey, S.F., Ambrose, D.E., Gonzalez, V., Levine, B.L., June, C.H., and Schuster, S.J. (2017). PD-1 blockade modulates chimeric antigen receptor (CAR)-modified T cells: refueling the CAR. Blood *129*, 1039–1041.

16. Kagoya, Y., Tanaka, S., Guo, T., Anczurowski, M., Wang, C.H., Saso, K., Butler, M.O., Minden, M.D., and Hirano, N. (2018). A novel chimeric antigen receptor containing a JAK-STAT signaling domain mediates superior antitumor effects. Nat. Med. *24*, 352–359.

17. Wang, Y., Jiang, H., Luo, H., Sun, Y., Shi, B., Sun, R., and Li, Z. (2019). An IL-4/21 inverted cytokine receptor improving CAR-T cell potency in immunosuppressive solid-tumor microenvironment. Front. Immunol. *10*, 1691.

18. Alizadeh, D., Wong, R.A., Yang, X., Wang, D., Pecoraro, J.R., Kuo, C.F., Aguilar, B., Qi, Y., Ann, D.K., Starr, R., et al. (2019). IL15 enhances CAR-T cell antitumor activity by reducing mTORC1 activity and preserving their stem cell memory phenotype. Cancer Immunol. Res. *7*, 759–772.

19. Chmielewski, M., and Abken, H. (2017). CAR T cells releasing IL-18 convert to T-bet^{high} FoxO1^{low} effectors that exhibit augmented activity against advanced solid tumors. Cell Rep. *21*, 3205–3219.

20. Jin, L., Tao, H., Karachi, A., Long, Y., Hou, A.Y., Na, M., Dyson, K.A., Grippin, A.J., Deleyrolle, L.P., Zhang, W., et al. (2019). CXCR1- or CXCR2-modified CAR T cells co-opt IL-8 for maximal antitumor efficacy in solid tumors. Nat. Commun. *10*, 4016.

21. Liu, Y., Fang, Y., Chen, X., Wang, Z., Liang, X., Zhang, T., Liu, M., Zhou, N., Lv, J., Tang, K., et al. (2020). Gasdermin E-mediated target cell pyroptosis by CAR T cells triggers cytokine release syndrome. Sci. Immunol. 5, eaax7969.

22. Zhang, L., Morgan, R.A., Beane, J.D., Zheng, Z., Dudley, M.E., Kassim, S.H., Nahvi, A.V., Ngo, L.T., Sherry, R.M., Phan, G.Q., et al. (2015). Tumor-infiltrating lympho-cytes genetically engineered with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma. Clin. Cancer Res. 21, 2278–2288.

23. Martins, F., Sofiya, L., Sykiotis, G.P., Lamine, F., Maillard, M., Fraga, M., Shabafrouz, K., Ribi, C., Cairoli, A., Guex-Crosier, Y., et al. (2019). Adverse effects of immune-checkpoint inhibitors: epidemiology, management and surveillance. Nat. Rev. Clin. Oncol. 16, 563–580.

24. Milone, M.C., Fish, J.D., Carpenito, C., Carroll, R.G., Binder, G.K., Teachey, D., Samanta, M., Lakhal, M., Gloss, B., Danet-Desnoyers, G., et al. (2009). Chimeric re-ceptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol. Ther. 17, 1453–1464.

25. Chrivia, J.C., Wedrychowicz, T., Young, H.A., and Hardy, K.J. (1990). A model of hu-man cytokine regulation based on transfection of gamma interferon gene fragments directly into isolated peripheral blood T lymphocytes. J. Exp. Med. 172, 661–664.

26. Ye, J., Ghosh, P., Cippitelli, M., Subleski, J., Hardy, K.J., Ortaldo, J.R., and Young, H.A. (1994). Characterization of a silencer regulatory element in the human interferon-γ promoter. J. Biol. Chem. 269, 25728–25734.

27. Zhang, Z., Jiang, D., Yang, H., He, Z., Liu, X., Qin, W., Li, L., Wang, C., Li, Y., Li, H., et al. (2019). Modified CAR T cells targeting membrane-proximal epitope of meso-thelin enhances the antitumor function against large solid tumor. Cell Death Dis. 10, 476.

28. Suarez, E.R., Chang, K., Sun, J., Sui, J., Freeman, G.J., Signoretti, S., Zhu, Q., and Marasco, W.A. (2016). Chimeric antigen receptor T cells secreting anti-PD-L1 anti-bodies more effectively regress renal cell carcinoma in a humanized mouse model. Oncotarget 7, 34341–34355.

29. Cherkassky, L., Morello, A., Villena-Vargas, J., Feng, Y., Dimitrov, D.S., Jones, D.R., Sadelain, M., and Adusumilli, P.S. (2016). Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. J. Clin. Invest. 126, 3130–3144.

30. Rafiq, S., Yeku, O.O., Jackson, H.J., Purdon, T.J., van Leeuwen, D.G., Drakes, D.J., Song, M., Miele, M.M., Li, Z., Wang, P., et al. (2018). Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. Nat. Biotechnol. 36, 847–856.

31. Frigault, M.J., Lee, J., Basil, M.C., Carpenito, C., Motohashi, S., Scholler, J., Kawalekar, O.U., Guedan, S., McGettigan, S.E., Posey, A.D., Jr., et al. (2015). Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. Cancer Immunol. Res. 3, 356–367.

32. Penix, L., Weaver, W.M., Pang, Y., Young, H.A., and Wilson, C.B. (1993). Two essen-tial regulatory elements in the human interferon gamma promoter confer activation specific expression in T cells. J. Exp. Med. 178, 1483–1496.

33. Ruella, M., Xu, J., Barrett, D.M., Fraietta, J.A., Reich, T.J., Ambrose, D.E., Klichinsky, M., Shestova, O., Patel, P.R., Kulikovskaya, I., et al. (2018). Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nat. Med. 24, 1499–1503.

34. Fife, B.T., and Pauken, K.E. (2011). The role of the PD-1 pathway in autoimmunity and peripheral tolerance. Ann. N Y Acad. Sci. 1217, 45–59.

35. Daskivich, T.J., and Belldegrun, A. (2015). Words of wisdom. Re: safety, activity, and immune correlates of anti-PD-1 antibody in cancer. Eur. Urol. 67, 816–817.

36. Beatty, G.L., O'Hara, M.H., Lacey, S.F., Torigian, D.A., Nazimuddin, F., Chen, F., Kulikovskaya, I.M., Soulen, M.C., McGarvey, M., Nelson, A.M., et al. (2018). Activity of mesothelin-specific chimeric antigen receptor T cells against pancreatic carcinoma metastases in a phase 1 trial. Gastroenterology 155, 29–32.

37. Horita, S., Nomura, Y., Sato, Y., Shimamura, T., Iwata, S., and Nomura, N. (2016). High-resolution crystal structure of the therapeutic antibody pembrolizumab bound to the human PD-1. Sci. Rep. 6, 35297.

38. Tan, S., Zhang, H., Chai, Y., Song, H., Tong, Z., Wang, Q., Qi, J., Wong, G., Zhu, X., Liu, W.J., et al. (2017). An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. Nat. Commun. 8, 14369.

39. Prosser, M.E., Brown, C.E., Shami, A.F., Forman, S.J., and Jensen, M.C. (2012). Tumor PD-L1 co-stimulates primary human CD8+ cytotoxic T cells modified to ex-press a PD1:CD28 chimeric receptor. Mol. Immunol. 51, 263–272.

40. Im, S.J., Hashimoto, M., Gerner, M.Y., Lee, J., Kissick, H.T., Burger, M.C., Shan, Q., Hale, J.S., Lee, J., Nasti, T.H., et al. (2016). Defining CD8+ T cells that provide the pro-liferative burst after PD-1 therapy. Nature 537, 417–421.

41. Zarour, H.M. (2016). Reversing T-cell dysfunction and exhaustion in cancer. Clin. Cancer Res. 22, 1856–1864.

42. Groom, J.R. (2019). Regulators of T-cell fate: Integration of cell migration, differen-tiation and function. Immunol. Rev. 289, 101–114.

43. Masopust, D., and Schenkel, J.M. (2013). The integration of T cell migration, differ-entiation and function. Nat. Rev. Immunol. 13, 309–320.