

PD-L1 chimeric costimulatory receptor improves the efficacy of CAR-T cells for PD-L1-positive solid tumors and reduces toxicity *in vivo*

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Abstract

Background: On-target off-tumor toxicity impedes the clinical application of chimeric antigen receptor-modified T cells (CAR-T cells) in the treatment of solid tumors. The combinatorial antigen recognition strategy can improve the therapeutic safety of CAR-T cells by targeting two different tumor-associated antigens (TAAs) using a CAR and a chimeric costimulatory receptor (CCR). Although programmed death-ligand 1 (PD-L1, also known as B7-H1) is expressed on multiple tumors, the potential of PD-L1 as a universal target for designing CCR remains unknown.

Methods: A first-generation CD19 or HER2 CAR and a PD-L1 CCR containing the CD28 signaling domain were constructed and delivered into Jurkat T cells or primary T cells by a pseudotyped lentivirus. The release of cytokines, including IL-2, IFN- γ and TNF- α , was quantified using enzyme-linked immunosorbent assay (ELISA) kits or a cytometric bead array (CBA). The *in vitro* cytotoxicity of CAR-T cells was detected with a luciferase-based killing assay. The *in vitro* proliferation of CAR-T cells was assessed by flow cytometry. The therapeutic safety and efficacy of CAR-T cells was evaluated using a subcutaneous dual-tumor model *in vivo*.

Results: Jurkat T cells or primary T cells expressing both the CD19/HER2 CAR and PD-L1 CCR produced higher levels of cytokines in the presence of CD19/HER2 and PD-L1 than in the presence of HER2/CD19. Compared to HER2-z-engineered T cells, HER2-z-PD-L1-28-engineered T cells had higher *in vitro* cytotoxicity potential against PD-L1-positive tumor cells. CD19/HER2-z-PD-L1-28-engineered T cells showed higher proliferation potential in the presence of CD19/HER2 and PD-L1 than in the absence of PD-L1. CD19/HER2-z-PD-L1-28-engineered T cells preferably destroyed xenograft tumors expressing CD19/HER2 and PD-L1 *in vivo* and did not significantly affect CD19/HER2-expressing tumors. The PD-L1 CCR improved the antitumor efficacy of low-affinity HER2 CAR-T cells against PD-L1-positive tumors expressing high levels of HER2.

Conclusion: Our findings confirmed that PD-L1 can be used as a universal target antigen for designing CCR, improving the efficacy of CAR-T cells in the treatment of PD-L1-positive solid tumors but reducing toxicity within PD-L1-negative normal tissues expressing low levels of TAA *in vivo*.

Introduction

CD19-targeting chimeric antigen receptor T cells (CAR-T cells) have achieved great success in treating hematological malignancies, with higher response rates than ever seen before [1–3]. In 2017, Kymriah and Yescarta, two drugs involving CD19-targeted CAR-T cells, were approved by the US Food and Drug Administration (FDA) for treating relapsed and refractory B cell acute lymphoblastic leukemia and refractory large B cell lymphoma, respectively. Clinical trials have shown that B cell aplasia often occurs in patients treated with CD19 CAR-T cells because of high CD19 levels on normal B cells [1, 4, 5], demonstrating that CAR-T cells can cause severe “on-target off-tumor” toxicity. Renal cancer-targeted CAR-T cells engineered with a murine single-chain antibody fragment (scFv) directed against carbonic

anhydrase IX (CAIX), a target antigen shared by normal bile duct epithelial cells and renal cell carcinoma, resulted in liver toxicity without inducing cancer remission [6]. Similarly, trastuzumab-based HER2-targeted CAR-T cells led to a lethal cytokine storm, which was attributed to the recognition of low levels of HER2 on lung epithelial cells [7]. Therefore, it is necessary to improve the therapeutic safety of CAR-T immunotherapy to make it more broadly applicable. Many CAR-based strategies are currently being developed to discriminate normal tissues from tumor tissues. Logic-gated CARs with the capacity for combinatorial antigen recognition via both a suboptimal CAR and a chimeric costimulatory receptor (CCR) are codelivered into T cells: the CAR provides the first signal for suboptimal activation after encountering a single TAA, and the CCR engages with another distinct TAA to provide a costimulatory signal for optimal activation [8]. The pro-antibody-based masked CAR can selectively kill cancer cells within the matrix metalloproteinase (MMP)-enriched tumor microenvironment [9]. Synthetic Notch (synNotch) receptor-engineered T cells with combinatorial antigen-sensing circuits apply a synthetic Notch receptor to recognize one antigen and then trigger the expression of a CAR that binds to another antigen [10]. Recently, a split, universal, and programmable (SUPRA) CAR system was also developed to combat tumor relapse, mitigate T cell overactivation, and enhance the specificity of engineered T cells; this system consists of a zipCAR and a leucine zipper-fused zipFv [11]. Among these strategies, the feasibility of a logic-gated CAR was confirmed in many preclinical studies, including studies of HER2⁺MUC1⁺ breast cancer [12], PSCA⁺PSMA⁺ prostate cancer [8], GPC3⁺ASGR1⁺ hepatocellular carcinoma [13] and CEA⁺MSLN⁺ pancreatic cancer [14]; however, this strategy will impede the broad application of CAR-T cells because of tumor antigen heterogeneity. Many tumor antigens [e.g., EGFR, HER2, MSLN and MUC1] identified in solid tumors are not broadly expressed on different types of tumors or the same tumor cells, e.g., MSLN was found in 25 ~ 30% of breast cancers, 40 ~ 45% of colon cancers and 80 ~ 85% of pancreatic cancers [15], so simultaneously targeting two TAAs expressed on the same tumor cells with current logic-gated CAR is more difficult than using a CAR targeting a single tumor antigen. Thus, there is a need to choose a relatively universal target antigen to design a CCR, which can expand and accelerate the clinical application of logic-gated CARs.

PD-L1/B7-H1 is abundant in many human cancers, e.g., 100% of melanomas, 95.2% of lung cancers, 90% of ovarian cancers and 52.6% of colon cancers, but is expressed at very low levels in normal tissues [16] and represents an ideal and universal CCR directed against shared antigens. Tumor-associated PD-L1 inhibits the immune response by engaging with PD-1 expressed on T cells to transduce inhibitory signals, and PD-L1 or PD-1 blockade can restore the host antitumor immune response [17]. Therefore, we constructed a new logic-gated CAR composed of a TAA-targeted CAR (e.g., CD19, HER2) and a universal CCR for PD-L1 and tested the functionality of this logic-gated CAR design *in vitro* and *in vivo*. Herein, we demonstrated that the PD-L1 CCR provided an efficient costimulatory signal for CD19/HER2-targeted CAR-T cells when it bound to PD-L1 both on tumor cells *in vitro*, with increased cytokine levels and enhanced proliferation, and more importantly, in an *in vivo* dual-tumor xenograft mouse model of human cancers, revealing the potential of PD-L1 CCR for developing a safer and more effective therapeutic modality for PD-L1-positive malignant tumors.

Methods

Cell lines

The following cell lines were used: K562 (a chronic myelogenous leukemia cell line, ATCC #CCL-243), Jurkat, clone E6-1 (an acute T cell leukemia cell line, ATCC #TIB-152), A549 (a lung cancer cell line, ATCC #CCL-185), NCI-H292 (a lung cancer cell line, ATCC #CRL-1848), SKOV3 (an ovarian cancer cell line, HTB-77) and HEK293T (an embryonic kidney cell line, ATCC #CRL-3216). All cell lines were purchased from ATCC and maintained in RPMI 1640 (Corning #10-040-CVR) supplemented with 10% fetal bovine serum (FBS) (BI #04-001-1acs) and 1% penicillin-streptomycin (Corning #30-002-CI), except for HEK293T cells, which were cultured in DMEM (Corning #10-013-CV) supplemented with 10% FBS and 1% penicillin-streptomycin. All tumor cell lines used in our study expressed little or no PD-L1 (Additional file 2: Figure S2). Thus, pseudotyped viruses carrying the PD-L1 gene were delivered into tumor cells to produce lines that had high, stable expression of PD-L1, e.g., K562-PD-L1, A549-PD-L1, NCI-H292-PD-L1 and SKOV3-PD-L1. Furthermore, the firefly luciferase gene or CD19 was also delivered into tumor cells via pseudotyped lentiviruses to generate A549-Luc, A549-Luc-PD-L1, A549-Luc-CD19, A549-Luc-CD19-PD-L1, NCI-H292-Luc-CD19, NCI-H292-Luc-CD19-PD-L1, SKOV3-Luc and SKOV3-Luc-PD-L1. These cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

CAR and CCR designs

DNA encoding the human CD8 signal peptide (SP) (NP_001759.3 aa 1–21), human PD-L1-binding scFv, Flag tag (DYKDDDDK), the hinge spacer, transmembrane and signaling domain of human CD28 (NP_006130.1 aa 114–220), the human 4-1BB signaling domain (AAA53133.1 aa 209–255) and the human CD3 ζ cytosolic domain (NP_932170 aa 52–164) was cloned into the empty lentiviral transfer plasmid (pHAGE_EF1 α _MCS_IRES_ZsGreen) to generate the recombinant lentiviral transfer plasmid containing PD-L1 CAR (pHAGE_PD-L1-28BBz) (Additional file 1: Figure S1a). The expression cassette encoding human CD8 SP, anti-human CD19 scFv (FMC63 clone), human HER2 scFv (4D5 clone) or low-affinity HER2 scFv (4D5-5 clone), Myc tag (EQKLISEEDL), the hinge and transmembrane domain of CD8 (NP_001759.3 aa 138–206) and the CD3 ζ cytosolic domain was cloned into another empty lentiviral transfer plasmid (pKL_EF1 α _MCS_P2A_EGFP) to generate recombinant lentiviral transfer plasmids carrying CD19 CAR (pKL_CD19-z) or HER2 CAR (pKL_HER2-z, pKL_La-HER2-z) (Fig. 1a and Additional file 3: Figure S3). The artificial gene encoding both the CD19/HER2 CAR and the PD-L1 CCR is composed of the CD8 SP, PD-L1 scFv, Flag tag, and the hinge, transmembrane and signaling domain of CD28 (PD-L1-28). The CD19/HER2 CAR was linked to the PD-L1 CCR via the self-cleaving T2A peptide sequence. The expression cassette encoding both the CAR and CCR was also cloned into the empty pKL_EF1 α _MCS_P2A_EGFP plasmid to generate the recombinant pKL_CD19-z-PD-L1-28 and pKL_HER2-z-PD-L1-28 plasmids (Fig. 1a and Additional file 3: Figure S3). All genes were synthesized by Genaray Biotech (Shanghai) Co., Ltd. The lentiviral transfer plasmids also encoded enhanced green fluorescent protein (EGFP) or ZsGreen to evaluate the transduction efficiency of pseudoviruses carrying the CAR or CCR.

Lentivirus Production

Transient lentiviral supernatant was produced as described below. Lentiviral vectors were prepared by transient transfection of HEK293T cells using TurboFect transfection reagent (Thermo Scientific #R0531). HEK293T cells (6×10^6) cultured in 10-cm tissue culture dishes were transfected with the lentiviral transfer plasmid (3 μ g), the VSV-G envelope plasmid PMD2.G (Addgene #12259) (3 μ g) and the packaging plasmid psPAX2 encoding gag-pol (Addgene #12260) (9 μ g). The lentiviral supernatant was harvested 48 h after transfection and filtered through a 0.45- μ m filter (PALL #4614). Lentiviral particles were further concentrated by ultracentrifugation for 2 h at 28000 rpm with a Beckman SW28 rotor (Beckman) before use.

T cell transduction and expansion

Frozen human peripheral blood mononuclear cells (PBMCs) were obtained from Shanghai Public Health Clinical Center. PBMCs were thawed in T cell growth medium (TCM), consisting of X-VIVO 15 medium (Lonza #BE02-060F), human IL-7 (R&D systems #P13232), human IL-15 (R&D systems #P40933) and human IL-21 (Novoprotein #GMP-CC45), and then rested for 4 ~ 6 h. The thawed PBMCs were further sorted into CD4⁺ and CD8⁺ T cells with an EasySep™ Human CD4⁺ T Cell Enrichment Kit (STEMCELLS #19052) and EasySep™ Human CD8⁺ T Cell Enrichment Kit (STEMCELLS #19053), respectively. Before transduction, PBMCs or sorted CD4⁺/CD8⁺ T cells were stimulated for 24 ~ 36 h with anti-hCD3 and anti-hCD28-coated immunobeads at a bead-to-cell ratio of 1:1 in TCM. The activated T cells were incubated with freshly concentrated lentiviral vectors in NovoNectin (Novoprotein # GMP-CH38)-coated 48-well flat plates at 32°C and centrifuged at 1000 \times g for 1.5 h. The culture medium was changed to fresh TCM overnight. The immunobeads were removed 6 ~ 7 days post-transduction, and the T cells were expanded until they were rested and could be used in further assays. During *ex vivo* expansion, the TCM was replenished, and the cell density was adjusted to 0.5 ~ 2 $\times 10^6$ /mL every 3 days.

Generation Of Car-engineered Jurkat T Cells

Jurkat T cells were transduced with lentiviral particles carrying PD-L1-28BBz, CD19-z or CD19-z-PD-L1-28. After transduction, Jurkat T cells expressing EGFP/ZsGreen were sorted by flow cytometry (BD FACS Aria II). The expanded positive Jurkat T cells were then used in further assays.

Cell Aggregation Assay

To determine whether the PD-L1 CAR-engineered Jurkat T cells bound to PD-L1 presented on the tumor cell surface to promote cell aggregation, PD-L1-positive K562 tumor cells labeled with Cell Proliferation Dye eFluor 670 (Invitrogen #65-0840) were mixed with PD-L1 CAR-modified Jurkat T cells or untransduced Jurkat T cells labeled with CellTrace CFSE (Invitrogen #C34554) at an effector: target (E: T) ratio of 1:1 in a 1.5 mL eppendorf tube at room temperature (RT) for 1 h. The proportion of cells forming heterologous cell aggregates (eFluor 670⁺CFSE⁺) was assessed by flow cytometry (BD LSRFortessa).

Surface Immunostaining And Flow Cytometry

For tumor cells expressing CD19 and/or PD-L1, 5×10^5 tumor cells were harvested and washed twice with FACS buffer (1 × PBS containing 2% FBS). Then, tumor cells were stained with 0.5 μL of APC/Cy7-conjugated mouse anti-human CD19 (BD Pharmingen #557791) or 0.5 μL of APC-conjugated mouse anti-human PD-L1 (eBioscience #17-5983-73) at 4 °C for 30 min, washed with FACS buffer twice, and resuspended in FACS buffer for assessment. Additionally, 5×10^5 washed tumor cells were incubated with 2 μg/mL of trastuzumab prepared in our laboratory at 4°C for 30 min, washed twice with FACS buffer, and further stained with 0.5 μL of PE-conjugated anti-human IgG Fc (Biolegend #409304) at 4°C for 30 min, washed with FACS buffer twice, and then resuspended in FACS buffer to detect HER2. To detect the HER2 CAR, PD-L1 CAR or PD-L1 CCR presented on the T cell surface, T cells were stained with Alexa Fluor 647-conjugated anti-Myc tag (CST #2233S) or PE-conjugated anti-DYKDDDDK (Biolegend #637310). T cells (5×10^5) were harvested and washed twice with FACS buffer. For Myc or Flag tag staining, T cells were stained with 0.5 μL of Alexa Fluor 647-conjugated anti-Myc tag or PE-anti-DYKDDDDK at 4 °C for 30 min, washed twice with FACS buffer, and then resuspended in FACS buffer for detection. Fluorescence was assayed using a BD LSRFortessa, and all FACS data were analyzed with FlowJo V10 software.

***In vitro* stimulation of CAR-T cells and quantitation of cytokines**

CAR-T cells (1×10^5) were cocultured with K562 cells at an E:T ratio of 1:1. After mixing CAR-T cells and K562 cells in a 96-well round-bottom plate, the plate was centrifuged for 1 min at $400 \times g$ to force cell interactions. For adherent tumor cells, 1×10^5 CAR-T cells were cocultured with tumor cells at an E:T ratio of 3:1 in a 96-well flat-bottom plate. The coculture supernatant was collected after 24 h and stored at -20 °C for further quantitation. IL-2, IFN-γ or TNF-α levels in the culture supernatant were determined using the Human IL-2 ELISA Set (BD Biosciences #555190), Human IFN-γ ELISA Set (BD Biosciences #555142) or BD CBA Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences #560484).

***In vitro* evaluation of CAR-T cell cytotoxicity**

Tumor cell lysis by CAR-T cells was assessed by using an 18-h luciferase-based killing assay. Briefly, tumor cells expressing target antigen and luciferase were plated in triplicate in a 96-well black flat-bottom plate (Greiner #655090) at a density of 1×10^4 cells per well and grown for 18 ~ 24 h. CAR-T cells were added to the plate at E:T ratios of 0.5:1, 1:1, 2:1 and 4:1 and then cultured for another 18 h. The culture supernatant was removed, and the viability of the tumor cells was assessed by quantifying the firefly luciferase fluorescence intensity with a GloMax® 96 reader (Promega #E6521) using a Luciferase Assay System (Promega #E1501). The formula used to calculate the percent normalized cytotoxicity is as follows:

$100 - (\text{luciferase fluorescence intensity in untransduced T cell well} - \text{luciferase fluorescence intensity in assay well}) / (\text{luciferase fluorescence intensity in untransduced T cell well}) \times 100.$

Detection of the *in vitro* proliferation of CAR-T cells

For *in vitro* proliferation assays, primary T cells expressing CD19-z, CD19-z-PD-L1-28, HER2-z or HER2-z-PD-L1-28 were washed with 1 × PBS and then labeled with Cell Proliferation Dye eFluor 670 at a final concentration of 5 μM, according to the manufacturer's instructions. Tumor cells expressing CD19/HER2 or PD-L1 were treated with 50 μg/mL mitomycin C (Biotech well #WF0197) to result in replication-defective tumor cells. Then, these labeled CAR-T cells were cocultured with pretreated tumor cells at an E:T ratio of 2:1 in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin, and the mixed cells were collected for flow cytometry analysis. Finally, the proliferation of CAR-T cells was assayed by monitoring the dilution of the cell proliferation dye after four days of coculture.

In vivo dual-antigen recognition of PD-L1 CCR-engineered CAR-T cells

The animal protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Public Health Clinical Center. Female NOD-*Prkdc^{scid} Il2rg^{tm1}*/Bcgen (B-NDG) (Biocytogen) mice aged 6 ~ 8 weeks were subcutaneously inoculated with four xenograft tumors in the right flank: 1) 5 × 10⁶ CD19⁺ and 5 × 10⁶ CD19⁺PD-L1⁺ A549 cells; 2) 2 × 10⁶ CD19⁺ and 2 × 10⁶ CD19⁺PD-L1⁺ NCI-H292 cells; 3) 2 × 10⁶ HER2⁺ and 2 × 10⁶ HER2⁺PD-L1⁺ NCI-H292 tumor cells; and 4) 1 × 10⁶ HER2⁺ and 1 × 10⁶ HER2⁺PD-L1⁺ SKOV3 cells. Five or ten days after tumor inoculation, 2 × 10⁶ CD4⁺ and CD8⁺ T cells were injected intravenously into the A549 tumor-bearing mice, while 4 × 10⁶ CD4⁺ and CD8⁺ T cells were injected into the NCI-H292/SKOV3 tumor-bearing mice. These T cells were either untransduced or engineered with CD19/HER2-z or CD19/HER2-z-PD-L1-28. Tumor size was monitored by calipers every 3, 5 or 10 days after T cell transfer, and tumor volume was calculated with the following formula: $V = (\text{length} \times \text{width}^2)/2$. Mice were considered dead when the tumor size reached the euthanasia criteria.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM) unless otherwise described. Statistical differences were determined by a paired Student's t-test (two-tailed) unless otherwise noted. The statistical significance of differences among three or more groups was analyzed by one-way ANOVA with Tukey's test for further multiple comparisons. A *P*-value < 0.05 was considered statistically significant. All statistical analyses were performed with Prism 7.0 (GraphPad), and statistical significance was reported as **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, and *****P* ≤ 0.0001.

Results

Characterization of the PD-L1 CAR and PD-L1 CCR in Jurkat T cells

The PD-L1/B7-H1 antigen is aberrantly expressed in many different types of cancer cells but minimally expressed in normal tissues [16], indicating its potential as an ideal target for designing CAR-T cells to combat lung cancer [18], gastric cancer [19] and other solid tumors [20]. Therefore, we tested whether we

can design a synthetic molecule incorporating a humanized scFv targeting PD-L1 that can be used for cell-based immunotherapy, especially in the context of a CAR and CCR. Initially, K562 and A549 tumor cells were transduced with lentiviral particles carrying a cassette encoding human PD-L1 to obtain PD-L1-positive K562 and A549 cells, namely, K562-PD-L1 and A549-PD-L1 cells, respectively, for further experiments (Additional file 1: Figure S1b). Moreover, we generated a PD-L1 scFv by fusing a variable region of the light chain to the heavy chain via a GS linker as a CD19 scFv-based CAR [21] based on sequences of PD-L1-neutralized antibodies (US8552154). We first tested the functionality of the PD-L1 scFv-based PD-L1 CAR. The PD-L1 CAR consists of the PD-L1 scFv, the CD28 hinge, transmembrane and intracellular signaling domains and the 4-1BB and CD3 ζ intracellular signaling domains (Additional file 1: Figure S1a). To determine the activity of the PD-L1 CAR, we first engineered Jurkat T cells to stably express the PD-L1 CAR ((Additional file 1: Figure S1c). Next, we mixed PD-L1 CAR-expressing Jurkat T cells (CFSE⁺) with K562-PD-L1 cells (eFluor 670⁺) at an E:T ratio of 1:1 for 1 h and evaluated cell aggregation. The eFluor 670⁺CFSE⁺ cell aggregates were found only in the coculture of engineered Jurkat T cells and K562-PD-L1 cells (6.52%); none were found in untransduced Jurkat T cells and K562-PD-L1 cells (Additional file 1: Figure S1d). Jurkat T cells can produce a large amount of IL-2 after T cell receptor (TCR) activation [22], so the secretion of IL-2 was used to evaluate T cell activation. PD-L1 CAR-expressing Jurkat T cells (PD-L1 CAR-Jurkat) were stimulated with K562-PD-L1 or A549-PD-L1 tumor cells, and PD-L1 CAR-Jurkat T cells produced a high level of IL-2 (Additional file 1: Figure S1e). In contrast, untransduced Jurkat T cells did not release any IL-2.

On-target off-tumor toxicity hinders the clinical application of CAR-T cells and is caused by the lack of tumor-specific antigens (TSAs). The combinatorial antigen recognition strategy, in which T cells are engineered with both a CAR that offers suboptimal activation upon engagement with a tumor antigen and a CCR that provides a costimulatory signal through binding to another antigen, can be used to reduce or avoid the side effects of CAR-T cells within solid tumors. Since PD-L1 is overexpressed in multiple types of cancers, we also tested whether PD-L1 can be used as a universal target antigen for designing a CCR to improve the therapeutic safety of CAR-T cells. Thus, the PD-L1 CCR was generated by fusing the PD-L1 scFv to the hinge, transmembrane and intracellular signaling domains of CD28, namely, PD-L1-28, to provide the costimulatory signal for T cell activation (Fig. 1a). We first created the CD19 CAR, composed of the CD19 scFv, the CD8 hinge and transmembrane domain, and the CD3 ζ intracellular signaling domain, to provide the initial suboptimal signal for T cell activation (Fig. 1a). To test the costimulatory activity of the PD-L1 CCR, we stably expressed both the CD19 CAR (CD19-z) and the PD-L1 CCR (PD-L1-28) in Jurkat T cells through transduction with a pseudolentivirus. CD19-z-PD-L1-28-expressing Jurkat T cells (CD19z.P28-Jurkat T cells) were cocultured with CD19⁺PD-L1⁺ K562 cells (Fig. 1b). CD19z.P28-Jurkat T cells activated by CD19⁺ K562 cells secreted the cytokine IL-2 at levels comparable to those of CD19-z-expressing Jurkat T cells (Fig. 1c). CD19z.P28-Jurkat T cells stimulated by CD19⁺PD-L1⁺ K562 cells produced a higher level of IL-2 than CD19⁺ K562 cells or CD19-z-engineered Jurkat T cells and were not stimulated by K562-PD-L1 cells (Fig. 1c).

Recent studies have proven that PD-L1 on host cells is critical for PD-L1 blockade therapy, regardless of whether tumor cells express PD-L1 [23, 24]. Therefore, we hypothesized that PD-L1 expressed on nontumor cells, including bone marrow-derived cells and stromal cells, in the tumor microenvironment engaged with the PD-L1 CCR presented on CAR-T cells to achieve optimal T cell activation to safely and efficiently kill PD-L1-negative cancer cells (Fig. 1d). Indeed, the addition of K562-PD-L1 or A549-PD-L1 to the coculture of CD19z.P28-Jurkat T cells and K562-CD19 or A549-CD19 cells augmented the production of IL-2 and inhibited the release of IL-2 from CD19-z-engineered Jurkat T cells (Fig. 1e).

The PD-L1 CCR has a strong costimulatory effect in CAR-T cells exposed to PD-L1-positive tumors

After confirming the activity of the PD-L1 CCR in Jurkat T cells, we tested whether our PD-L1 CCR works in human primary T cells. The expression cassette encoding CD19-z-PD-L1-28 was delivered into primary T cells to simultaneously express CD19 CAR (CD19-z) and PD-L1 CCR (PD-L1-28). To verify whether the PD-L1 CCR is active in primary T cells, CD19-z-PD-L1-28-expressing T cells (CD19z.P28-T cells) were stimulated with K562 cells expressing CD19 alone or both CD19 and PD-L1 (Fig. 2a). Only CD19z.P28-T cells showed strong activation and presented high levels of IL-2 when exposed to CD19⁺PD-L1⁺ K562 cells (Fig. 2b). Considering that CD4⁺ T cells are the major T cell subset that produces IL-2, we introduced both the CD19 CAR and the PD-L1 CCR into human primary CD4⁺ T cells to test the functionality of the PD-L1 CCR. Indeed, CD19-z-PD-L1-28-expressing CD4⁺ T cells (CD19z.P28-CD4⁺ T cells) produced higher levels of IL-2 in cocultures of CD19⁺PD-L1⁺ K562/A549 cells than in cocultures of CD19⁺ K562/A549 cells (Fig. 2d). Additionally, the production of IFN- γ was higher in CD19z.P28-CD4⁺ T cells activated by CD19⁺PD-L1⁺ K562/A549 cells than in those activated by CD19⁺ K562/A549 cells, while the difference was not statistically significant (Fig. 2d).

Considering that CD19 itself is not expressed in K562/A549 cells, we next investigated a tumor-associated antigen (TAA), HER2, which is overexpressed in various solid tumors (Additional file 2: Figure S2), to confirm the reliability and versatility of the PD-L1 CCR. The PD-L1 CCR and HER2 CAR could be expressed on T cells, including CD4⁺ and CD8⁺ T cells, through lentiviral particles carrying HER2-z or HER2-z-PD-L1 28 (Additional file 3: Figure S3b). HER2-z-PD-L1-28-expressing T cells (HRE2z.P28-T cells) were stimulated with SKOV3 cells (Fig. 2c). Only HER2z.P28-T cells produced high levels of IL-2 and IFN- γ when exposed to PD-L1⁺ SKOV3 cells (Fig. 2b). Similarly, HER2z.P28-CD4⁺ T cells secreted higher levels of IL-2 in the cocultures of PD-L1⁺ A549/NCI-H292/SKOV3 cells than in the cocultures of A549/NCI-H292/SKOV3 cells (Fig. 2e). As described above, the production of IFN- γ was also higher in HER2z.P28-CD4⁺ T cells activated by PD-L1⁺ SKOV3 cells than in those activated by SKOV3 cells (Fig. 2e).

The PD-L1 CCR transduces a costimulatory signal in CAR-T cells after exposure to PD-L1-positive host cells but not tumor cells

We also tested whether PD-L1 expressed on host cells activated PD-L1 CCR-expressing CAR-T cells in the presence of cognate antigens (Fig. 3a & Fig. 3d). PD-L1 presented on K562 cells, which mimicked host cells, augmented the release of IL-2 in the cocultures of CD19z.P28-CD4⁺ T cells and CD19⁺ K562

(Fig. 3b) or A549 cells (Fig. 3c). When CD19z.P28-CD4⁺ T cells engaged with PD-L1 expressed on K562 cells in the context of A549-CD19 cells, these engineered CD4⁺ T cells produced a large amount of IFN- γ (Fig. 3c). Additionally, K562-PD-L1 cells also boosted the production of IL-2, IFN- γ and TNF- α in cocultures of HER2z.P28-CD4⁺ T cells and A549 cells (Fig. 3e).

PD-L1 CCR enhances the cytolytic potency of CAR-T cells against PD-L1-positive tumor cells

Moreover, we tested whether the PD-L1 CCR could improve the cytotoxic capacity of CAR-T cells towards PD-L1-positive cancer cells. The luciferase-based killing assay was applied to evaluate tumor cell lysis. We first compared the cytotoxicity of CD19 CAR-CD8⁺ T cells and CD19z.P28-CD8⁺ T cells against PD-L1-expressing A549 or NCI-H292 cells. The *in vitro* cytotoxicity assays showed that both engineered CD8⁺ T cells efficiently eliminated CD19⁺PD-L1⁺ target cells (Fig. 3f, left panel). However, when using a constitutively expressed HER2 antigen, the PD-L1 CCR improved the cytotoxic capacity of HER2 CAR-T cells against PD-L1-positive tumor cells compared to that of HER2 CAR-T cells, which was independent of tumor cell type (Fig. 3f, right panel).

We then tested the cytolytic potential of CD19z.P28-CD8⁺ T cells against PD-L1^{+/-}CD19-positive A549/NCI-H292 cells. We found that CD19z.P28-CD8⁺ T cells efficiently killed CD19⁺ or CD19⁺PD-L1⁺ target cells, which was independent of PD-L1 CCR (Additional file 4: Figure S4a-b). When changing to another tumor antigen, HER2, tumor-derived PD-L1 slightly reduced the cytolytic potency of HER2z.P28-CD8⁺ T cells against PD-L1⁺ A549 (Additional file 4: Figure S4c), NCI-H292 (Additional file 4: Figure S4d) or SKOV3 (Additional file 4: Figure S4e) cells compared to that of PD-L1-negative tumor cells, which was also found in HER2-z-expressing CD8⁺ T cells. Overall, our study revealed that the PD-L1 CCR partially antagonized PD-L1 inhibition but showed limited selective tumor killing *in vitro*.

PD-L1 CCR increases the proliferation potential of CAR-T cells

On the one hand, cancer cell-triggered expansion of CAR-T cells is indispensable for antitumor efficacy, but on the other hand, unmanageable T cell proliferation results in severe side effects. We next determined whether the expansion of PD-L1 CCR-engineered CAR-T cells relied on cancer cells expressing both tumor antigen and PD-L1. CAR-T cells were first labeled with cell proliferation dye to trace T cell expansion and then added to cultures of replication-defective cancer cells (Fig. 4a). Our study showed that HRE2z.P28-T cells had a higher proliferation capacity in the presence of PD-L1⁺ tumor cells (Fig. 4b), while HER2-z-expressing T cells presented lower proliferation activity. Additionally, CD19z.P28-CD4⁺ and CD8⁺ T cells also had a higher expansion capacity in the presence of CD19⁺PD-L1⁺ tumor cells, while CD19-z-expressing T cells presented lower proliferation potential (Fig. 4b & Fig. 4c). In summary, the proliferation of PD-L1 CCR-engineered CAR-T cells indeed required tumor antigen and PD-L1, whereas one tumor antigen alone did not completely trigger CAR-T cell proliferation.

Combinatorial antigen recognition controls tumor growth *in vivo*

PD-L1 CCR-engineered CAR-T cells showed high levels of cytokine production and proliferation when exposed to tumor cells expressing both tumor antigens (e.g., CD19 and HER2) and PD-L1. We sought to test whether PD-L1 CCR-engineered CAR-T cells could selectively inhibit tumors expressing both tumor antigen and PD-L1. For this purpose, we established a dual-tumor animal model with A549 or NCI-H292 tumor cells to evaluate the antitumor efficacy of CD19z.P28-T cells. Mice were subcutaneously implanted with various tumor cells, including A549-CD19, A549-CD9-PD-L1, NCI-H292-CD19 or NCI-H292-CD19-PD-L1 cells, in the right flank, and maintained for 5 or 10 days after inoculation. At day 5 or day 10, we injected various effector T cells, namely, CD4⁺ and CD8⁺ T cells carrying the CD19 CAR (CD19-z) or both the CD19 CAR and PD-L1 CCR (CD19-z-PD-L1-28), into these mice intravenously and monitored tumor growth with calipers for 25 days (Fig. 5a & Additional file 5: Figure S5a). Untransduced T cells were also used to treat a group of mice as a reference for tumor growth. We found that the size of CD19⁺PD-L1⁻ NCI-H292 tumors was decreased in mice that received CD19z.P28-T cells, but there were no significant differences compared to mice that received CD19-z-expressing T cells, confirming the inefficient tumoricidal effect of PD-L1 CCR-engineered CAR-T cells towards single antigen-positive tumors (Fig. 5b). In the CD19⁺PD-L1⁻ A549 tumor-bearing mice, we also did not observe any decrease in tumor size between the recipients of CD19-z or CD19z.P28-T cells-T cells (Additional file 5: Figure S5b). In contrast, in these mice bearing CD19⁺PD-L1⁺ tumor cells, especially NCI-H292 tumor cells expressing CD19 and PD-L1, CD19z.P28-T cells, robustly eradicated dual-antigen-expressing tumors (Fig. 5c, Fig. 5d & Additional file 5: Figure S5c).

Furthermore, we next tested whether PD-L1 CCR-engineered HER2 CAR-T cells could selectively inhibit tumors expressing both HER2 and PD-L1. To mimic normal tissues expressing low levels of TAAs, we chose NCI-H292 tumor cells expressing low levels of HER2 (Additional file 2: Figure S2) to perform an *in vivo* antitumor study. Mice were subcutaneously implanted with NCI-H292 or NCI-H292-PD-L1 cells in the right flank and maintained for 5 days after inoculation. At day 5, we injected various effector T cells, namely, CD4⁺ and CD8⁺ T cells carrying the HER2 CAR (HER2-z) or both the HER2 CAR and PD-L1 CCR (HER2-z-PD-L1-28), into these tumor-bearing mice intravenously and monitored tumor growth with calipers for 25 days (Fig. 5e). As expected, antitumor efficacy was not observed in NCI-H292 tumor-bearing mice treated with HER2 CAR-T cells and PD-L1 CCR-engineered HER2 CAR-T cells (Fig. 5f) but was found in PD-L1-expressing NCI-H292 tumor-bearing mice treated with HER2z.P28-T cells (Fig. 5g).

The PD-L1 CCR improves the antitumor efficacy of low-affinity HER2 CAR-T cells *in vivo*

A previous study showed that low-affinity HER2 CAR-T cells could control the growth of SKOV3 tumor cells expressing high levels of HER2 but spare normal tissues expressing physiological HER2 levels, revealing that the application of low-affinity CAR is an effective and safe strategy to broaden the use of CAR-T immunotherapy [25]. PD-L1, a main ligand of PD-1, is an immunosuppressive molecule that has been reported to be overexpressed in various solid tumors. Therefore, we further tested whether PD-L1 CCR-engineered affinity-tuned HER2 CAR-T cells could inhibit the growth of solid tumors overexpressing tumor antigen and PD-L1. Strikingly, low-affinity HER2-z-PD-L1-28-expressing T cells (La-HER2z. P28-T

cells) secreted higher levels of IL-2 and IFN- γ in cocultures of PD-L1⁺ SKOV3 cells than in cocultures of SKOV3 cells (Fig. 6a-b), and the PD-L1 CCR provided a strong costimulatory signal to antagonize PD-1/PD-L1 inhibition. Additionally, the PD-L1 CCR enhanced the cytolytic activity of low-affinity HER2 CAR-T cells against PD-L1^{+/-} SKOV3 cells (Fig. 6c). We next compared the antitumor efficacy of low-affinity HER2 CAR-T cells in the same B-NDG mouse with established SKOV3 tumors expressing PD-L1 (Fig. 6d). Mice were treated with either low-affinity HER2 CAR-T cells (La-HER2.z-T cells) or La-HER2z.P28-T cells exhibited complete remission of SKOV3 tumors and PD-L1-overexpressing SKOV3 tumors. In contrast, uncontrolled tumor growth of PD-L1-overexpressing SKOV3 cells was observed in mice treated with low-affinity HER2 CAR-T cells (Fig. 6e), indicating that PD-L1 impaired the antitumor activity of low-affinity HER2 CAR-T cells against HER2-overexpressing tumors. Unexpectedly, mice treated with La-HER2z.P28-T cells still exhibited nearly complete elimination of SKOV3 tumors expressing high levels of PD-L1 (Fig. 6f), implying that PD-L1 CCR-engineered low-affinity CAR-T cells could resist PD-L1 immunosuppression.

Discussion

There are still many challenges in the clinical use of CAR-T cells for solid tumors, although CAR-T cells have shown good responses in hematological malignancies [1–5]. Safe and effective CAR-T cells are required to solve the following challenges: tumor recognition, T cell trafficking, T cell persistence, the immunosuppressive tumor microenvironment and the control of engineered immune cells [26]. Among these challenges, the on-target off-tumor toxicity caused by the TAAs that are typically used as target antigens needs to be reduced or avoided by improving precise tumor recognition. Depletion of normal B cells often occurs in CD19 CAR-T cell clinical studies and is tolerable with intravenous immunoglobulin therapy (IVIG) because CD19 is expressed on normal B cells [27]. However, there have been several clinical trials involving TAA-targeting CAR-T cells within solid tumors, in which on-target off-tumor recognition of bystander tissue cells has led to life-threatening adverse effects caused by engineered T cells that attack essential normal tissues [6, 7]. Therefore, many researchers have attempted to improve the specificity of CAR-T cells; thus, the logic-gated CAR [8, 12–14], masked CAR [9], synNotch receptor [10, 28, 29] and SUPA CAR [11] were developed to reduce on-target off-tumor toxicity. Among these tools, logic-gated CAR-engineered T cells are fully activated only when both a suboptimal CAR and a CCR simultaneously recognize two TAAs on tumor cells, which markedly increases specificity and thus prevents nontumor cells from attacking. Nevertheless, recent reports involving logic-gated CARs have shown that two different target antigens, CAR-targeted antigens and CCR-targeted antigens, must be carefully chosen to treat different solid tumors [8, 12–14], which is time consuming and laborious.

PD-L1, a key immune checkpoint molecule, was found to be overexpressed in a broad range of cancers [30], including breast, colon, gastric, lung, esophageal, ovarian, pancreatic, renal cell, and urothelial cancers. In addition, the PD-L1 protein is also observed in activated cells [30], which is not feasible for designing PD-L1-directed CARs but suitable for a universal target antigen for costimulatory domain-containing CCRs without TCR signaling, such as T cells, B cells, dendritic cells (DCs), macrophages [23,

24, 31–33], natural killer (NK) cells [34], and activated vascular endothelial cells (VECs) [35]. First, we constructed a conventional PD-L1-targeted CAR with CD28, 4-1BB costimulatory domains and the CD3 ζ cytosolic domain and demonstrated the functional activity of the PD-L1 CAR in Jurkat T cells. We found that release of a significant amount of the cytokine IL-2 was observed only in PD-L1 CAR-expressing Jurkat T cells cocultured with tumor cells expressing PD-L1 and that the PD-L1 CAR specifically bound to PD-L1 expressed on the tumor cell surface, which revealed the feasibility of this PD-L1 scFv as an extracellular binding domain of a CAR or CCR. Furthermore, we confirmed that the CD28 costimulatory domain-containing PD-L1 CCR costimulated and promoted the activation of Jurkat T cells and primary T cells (CD4⁺ and CD8⁺ T cells). High levels of IL-2 secretion and enhanced proliferation were found in PD-L1 CCR-engineered CAR-T cells expressing a suboptimal CAR that simultaneously recognized both CAR-targeted antigen (e.g., CD19 and HER2) and PD-L1, which is consistent with a previous study that showed that the CD28 cytoplasmic domain induced a large amount of IL-2 that induced T cell expansion [36, 37]. The cytokine IL-2, a well-known T cell growth factor, plays an essential role in the immune response [38], and increased IL-2 production caused by a costimulatory signal was also observed in reports involving logic-gated CARs [8, 12–14], which led to enhanced T cell activation and proliferation. Additionally, PD-L1 has been reported to be aberrantly expressed by many solid tumor cells, and it is overexpressed on many nontumor cells, including DCs, macrophages and fibroblasts, within tumor microenvironment (TME) [39]. Our study showed that PD-L1 CCR-engineered CAR-T cells responded to PD-L1-expressing tumor or host cells when they simultaneously contacted the cognate antigen expressed on tumor cells, which indicates the potential of the PD-L1 CCR in broader clinical applications, especially in contexts with PD-L1-negative tumor cells but PD-L1-expressing TME-associated nontumor cells [23, 24]. In our study, we also found that the PD-L1 CCR (signal 2) did not promote dual-antigen tumor lysis of CAR-T cells with only the CD3 ζ signaling domain (signal 1), as shown in previous studies, and even slightly decreased cytotoxicity in HER2 CAR-T cells after exposure to PD-L1-expressing tumor cells. CD3 ζ signaling domain-containing CAR-T cells could effectively kill tumor cells expressing a single antigen or both antigens *in vitro* [7, 8, 40], which poses the potential risk of an on-target off-tumor effect in T cells engineered with a high-affinity antibody-based CAR, but we could minimize the initial signal for T cell activation to reduce this risk by choosing a lower affinity antibody as a suboptimal CAR [8]. However, when using the constitutively expressed HER2 tumor antigen, the PD-L1 CCR improved the cytolytic potential of HER2 CAR-T cells against PD-L1-positive tumor cells compared to that of CAR-T cells containing only the CD3 ζ signaling domain, revealing that the PD-L1 CCR partially antagonized PD-L1 inhibition to improve the tumor-lytic activity of CAR-T cells.

Finally, we confirmed that PD-L1 CCR-engineered CAR-T cells effectively eradicated CD19⁺PD-L1⁺ tumors but reduced cytotoxicity against CD19⁺PD-L1⁻ tumors *in vivo*, while we also observed tumor regression in several CD19⁺PD-L1⁻ tumor-bearing mice after infusion of PD-L1 CCR-expressing CAR-T cells. PD-L1 expression can be induced by many cytokines [16, 33], of which IFN- γ produced by effector T cells is the most potent [41]. There is a possibility that PD-L1 may be induced in CD19⁺ tumors through PD-L1 CCR-expressing CAR-T cell-derived IFN- γ ; thus, a CAR using a low-affinity antibody must be combined with the PD-L1 CCR to obtain specific and optimal activation, which can be used to further reduce on-target off-

tumor toxicity. On the other hand, endogenous effector T cells and engineered T cells produce more IFN- γ within the tumor microenvironment when responding to tumor cells, which induces or increases PD-L1 expression, and it will be helpful for PD-L1 CCR-engineered CAR-T cells to obtain optimal activation. We also demonstrated the versatility of PD-L1 CCR in other CAR-T cells using the cognate antigen HER2 *in vivo*. PD-L1 CCR-engineered HER2 CAR-T cells controlled the growth of HER2⁺ PD-L1⁺ tumors but spared HER2⁺PD-L1⁻ tumors from killing.

Moreover, the use of affinity-tuned CAR-T cells is another safe strategy to promote the clinical application of CAR-T immunotherapy in solid tumors [42]. The low-affinity HER2/EGFR CAR-T cells could discriminate tumor antigen-overexpressing tumors from normal tissues expressing physiologic antigen levels and exhibited robust antitumor efficacy in the presence of high tumor antigen levels [42]. We also found that HER2-overexpressing SKOV3 tumors were completely inhibited in mice treated with low-affinity HER2 CAR-T cells, regardless of whether they expressed the PD-L1 CCR. However, PD-L1 has been reported to be overexpressed in different types of cancers, simultaneously inhibiting the immune response to cancers [16, 17]. We observed progressive growth of PD-L1-expressing SKOV3 tumors in mice treated with low-affinity HER2 CAR-T cells but almost complete regression of SKOV3 tumors expressing PD-L1 in mice treated with PD-L1 CCR-engineered low-affinity HER2 CAR-T cells, revealing that PD-L1 CCR-engineered low-affinity CAR-T cells may be a safe and effective therapeutic strategy for solid tumors in the future.

In summary, the PD-L1 CCR can kill two birds with one stone: its combinatorial antigen recognition improves the therapeutic safety of engineered T cells, as reported in our study, and its function as a chimeric switch receptor, PD-1:CD28 comprised of the extracellular domain of PD-1 and the transmembrane and signaling domain of CD28, to reverse the immunosuppressive effects of the PD-L1-PD-1 axis [43, 44] is consistent with our observation that PD-L1 CCR-engineered low-affinity CAR-T cells could resist PD-L1 inhibition.

There are some limitations of our study. Two tumor antigens, CD19 and HER2, were used to test the functionality of the PD-L1 CCR, and more cognate antigens (e.g., GPC3, MSLN and EGFR) are needed to confirm the versatility of the PD-L1 CCR. Engineered T cells expressing both a CAR and CCR show enhanced activation when two target antigens are present, but the partial signal mediated by CD3 ζ cytoplasmic signaling domain-containing receptors can generate sufficient *in vitro* T cell cytotoxicity to cause potential on-target off-tumor tissue damage [8, 12], which means that it is difficult to obtain a perfect logic-gated CAR with no activity towards either individual target antigen but maximal activity towards both target antigens. We can try our best to reduce or avoid these side effects by choosing synthetic receptors with appropriate affinity [8, 25] or selecting safer target antigens to expand the therapeutic window of the combinatorial antigen recognition strategy with the PD-L1 CCR.

Conclusion

In summary, the PD-L1 CCR, which includes the CD28 intracellular signaling domain, can be used to design logic-gated CARs. Combining a CAR-targeted cognate antigen and PD-L1 recognition produces

optimal T cell activation, while suboptimal activation is achieved by engaging with either individual target antigen, suggesting that the PD-L1 CCR may be a universal synthetic costimulatory and switch receptor for improving the therapeutic safety and efficacy of engineered T cells.

List Of Abbreviations

CAR-T cells: Chimeric antigen receptor T cells; TAAs: Tumor-associated antigens; CCR: Chimeric costimulatory receptor; PD-L1: Programmed death-ligand 1; ELISA: Enzyme-linked immunosorbent assay; CBA: Cytometric bead array; FDA: Food and Drug Administration; CAIX: Carbonic anhydrase IX; MMPs: Matrix metalloproteinases; SynNotch: Synthetic Notch; SUPRA: Split, universal, and programmable; RPMI 1640: Roswell Park Memorial Institute; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; ScFv: Single-chain variable fragment; SP: Signal peptide; PBMCs: Peripheral blood mononuclear cells; TCM: T cell growth medium; CFSE: Carboxyfluorescein diacetate succinimidyl ester; RT: Room temperature; FACS: Fluorescence-activated cell sorting; E:T ratio: Effector:target ratio; IACUC: Institutional Animal Care and Use Committee; SEM: Standard error of the mean; ANOVA: Analysis of variance; TSAs: Tumor-specific antigens; IVIG: Intravenous immunoglobulin; DCs: Dendritic cells; NK: Natural killer; TME: Tumor microenvironment

Declarations

Ethics approval and consent to participate

All study protocols were approved by the institutional review board at the Shanghai Public Health Clinical Center, and the involved healthy donors provided written informed consent. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Public Health Clinical Center.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JQX and XYZ designed this project and supervised the experiments. QBL performed all experiments and analyses. HH and MYY prepared the lentiviral transfer plasmids and performed *in vitro* experiments. XQD performed the *in vivo* experiments. JQX, XYZ, and QBL contributed to manuscript writing. All authors read and approved the final manuscript.

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Figures

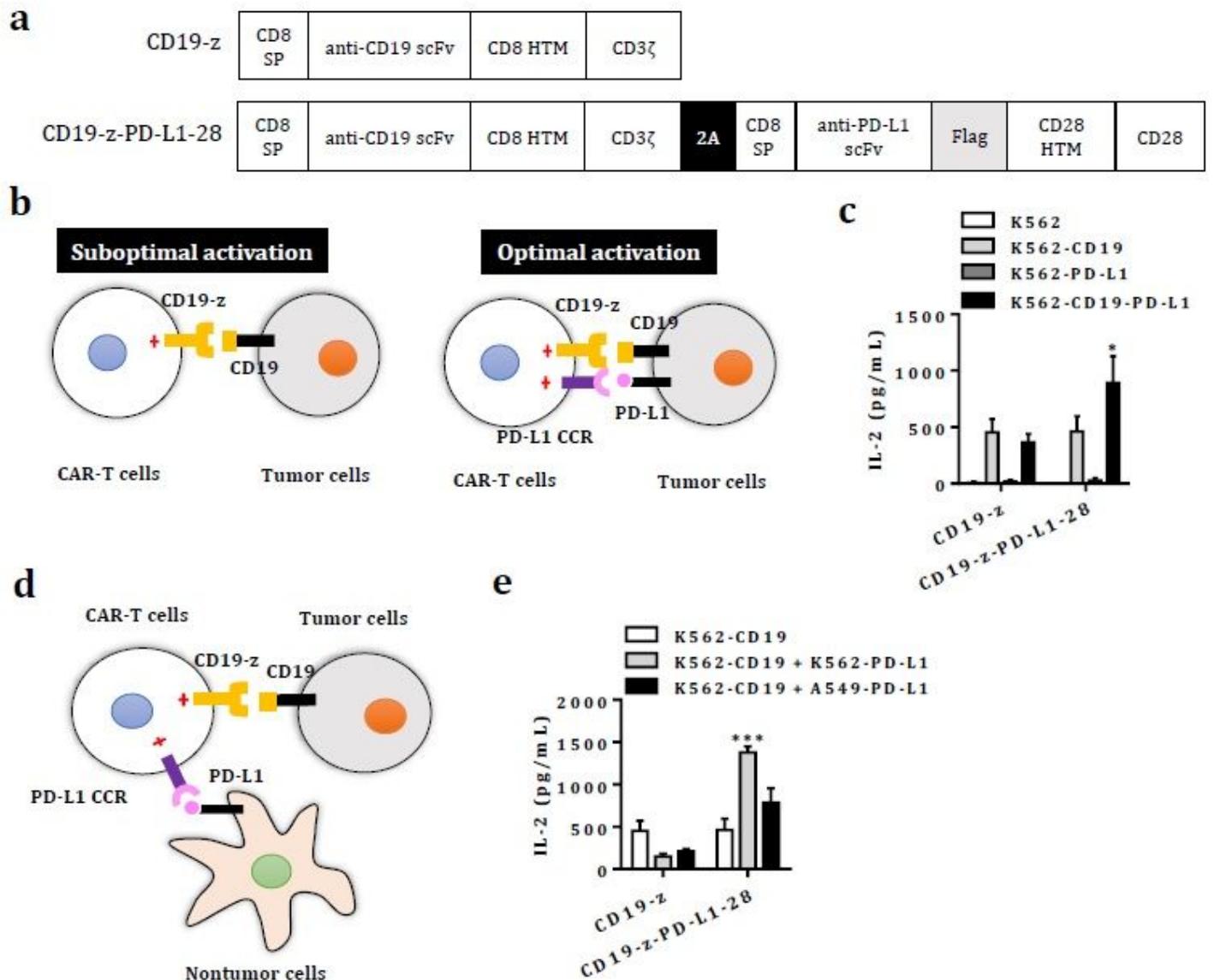


Figure 1

Design and characterization of the PD-L1 CCR in Jurkat T cells. **a** Schematic representation of the CD19 CAR and PD-L1 CCR constructs. The CD19 CAR (CD19-z) was generated by using the first generation of the CAR that contains the CD19-targeting scFv fused to the human CD8 hinger and transmembrane domain, followed by the CD3 ζ cytosolic signaling domain. CD19-z-PD-L1-28 was generated by linking CD19-z to the PD-L1 CCR, which was generated by fusing a humanized PD-L1-binding scFv to the hinger, transmembrane and intracellular signaling domains of human CD28, via the self-cleaving T2A peptide sequence. **b** The first generation of CAR-engineered T cells achieve suboptimal activation when exposed to a single target antigen but are fully activated by tumor cells expressing both target antigen and PD-L1 through coexpression of the PD-L1 CCR, providing a costimulatory signal. **c** The levels of IL-2 in the supernatant secreted by CD19-z- and CD19-z-PD-L1-28-engineered Jurkat T cells in the 24 h coculture system (E:T, 1:1) were measured by ELISA. The results are presented as the mean \pm SEM of seven independent experiments, * $P < 0.05$ with respect to coculture with CD19-expressing K562 cells, analyzed

using Student's t-test. d PD-L1 CCR-engineered CAR-T cells can be stimulated with PD-L1-expressing host cells (e.g., macrophages, dendritic cells) when exposed to PD-L-negative tumor cells expressing CAR-targeted antigen. e The levels of IL-2 released by CD19-z- and CD19-z-PD-L1-28-engineered Jurkat T cells were measured by ELISA after 24 h of incubation at an E:T:host cell ratio of 3:1:1. The results are reported as the mean \pm SEM of three independent experiments, *** P < 0.001 with respect to coculture with CD19-expressing K562 cells, analyzed using Student's t-test.

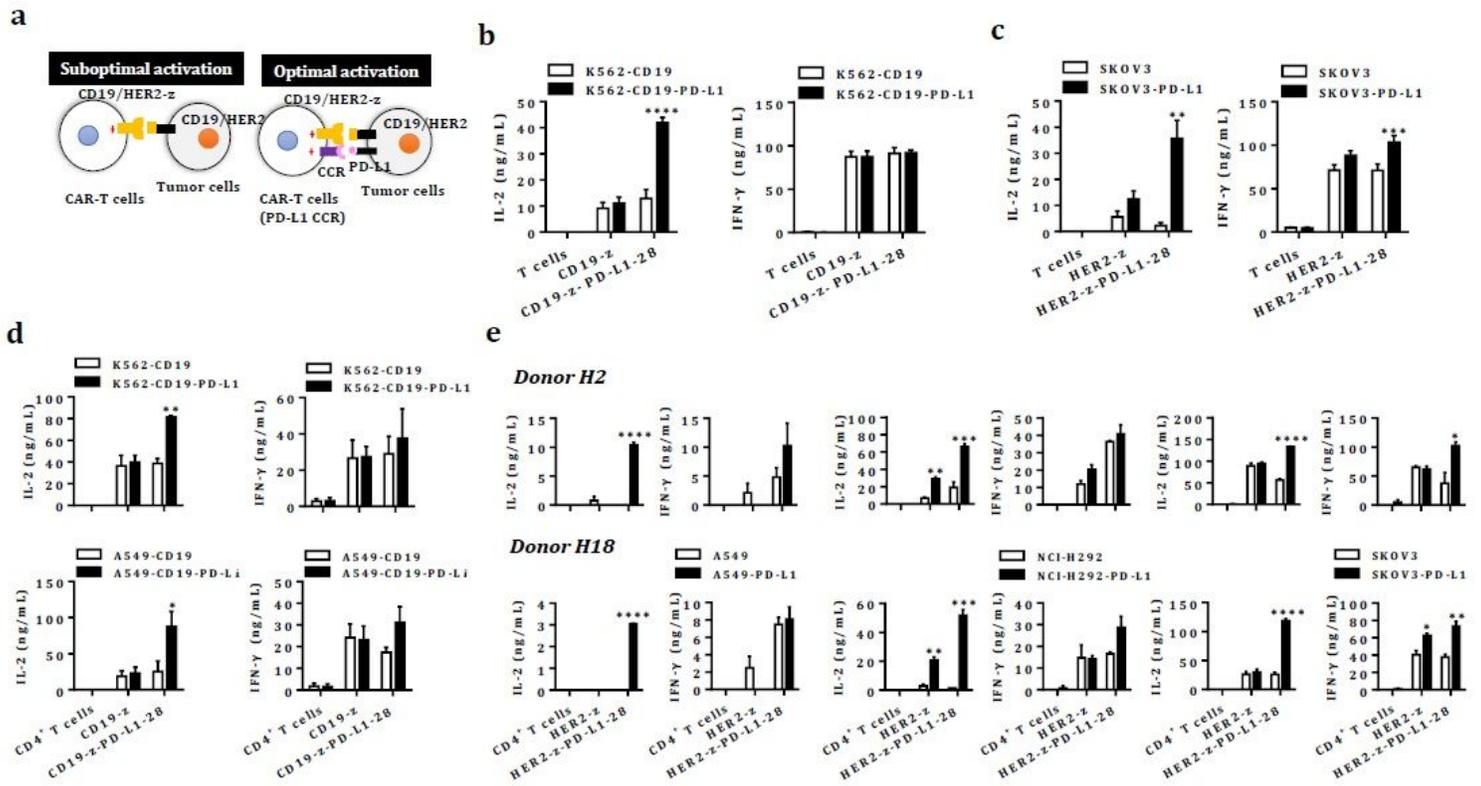


Figure 2

Characterization of the PD-L1 CCR in primary T cells. a CD19/HER2-z-expressing T cells received a suboptimal activation signal when exposed to a single antigen, but PD-L1 CCR-modified CD19/HER2-z-expressing T cells achieved optimal activation when engaged with both CAR-targeted antigen and PD-L1. b-c The levels of IL-2 and IFN- γ produced by untransduced, CD19-z-engineered or CD19-z-PD-L1-28-engineered T cells (b) and HER2-z-engineered or HER2-z-PD-L1-28-engineered T cells (c) were measured by ELISA after 24 h of cocultivation at an E:T ratio of 1:1 (b) or 3:1 (c). The results are reported as the mean \pm SEM for five healthy donors (b) or three healthy donors (c), ** P < 0.01, *** P < 0.001, **** P < 0.0001 with respect to coculture with CD19-positive K562 cells (b) or SKOV3 cells (c), analyzed using a paired Student's t-test. d The levels of IL-2 and IFN- γ secreted by untransduced, CD19-z-engineered or CD19-z-PD-L1-28-engineered CD4⁺ T cells were measured by ELISA after 24 h incubation at an E:T ratio of 1:1 (K562) or 3:1 (A549). The results are reported as the mean \pm SEM for three healthy donors, * P < 0.05, ** P < 0.01 with respect to coculture with CD19-positive K562/A549 cells, analyzed using a paired Student's t-test. e The levels of IL-2 and IFN- γ produced by untransduced, HER2-z-engineered or HER2-z-PD-L1-28-engineered CD4⁺ T cells were measured by ELISA after 24 h incubation at an E:T ratio of 3:1.

The results are reported as the mean \pm SEM for two healthy donors with technical triplicates, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ with respect to coculture with A549/NCI-H292/SKOV3 cells, analyzed using Student's t-test.

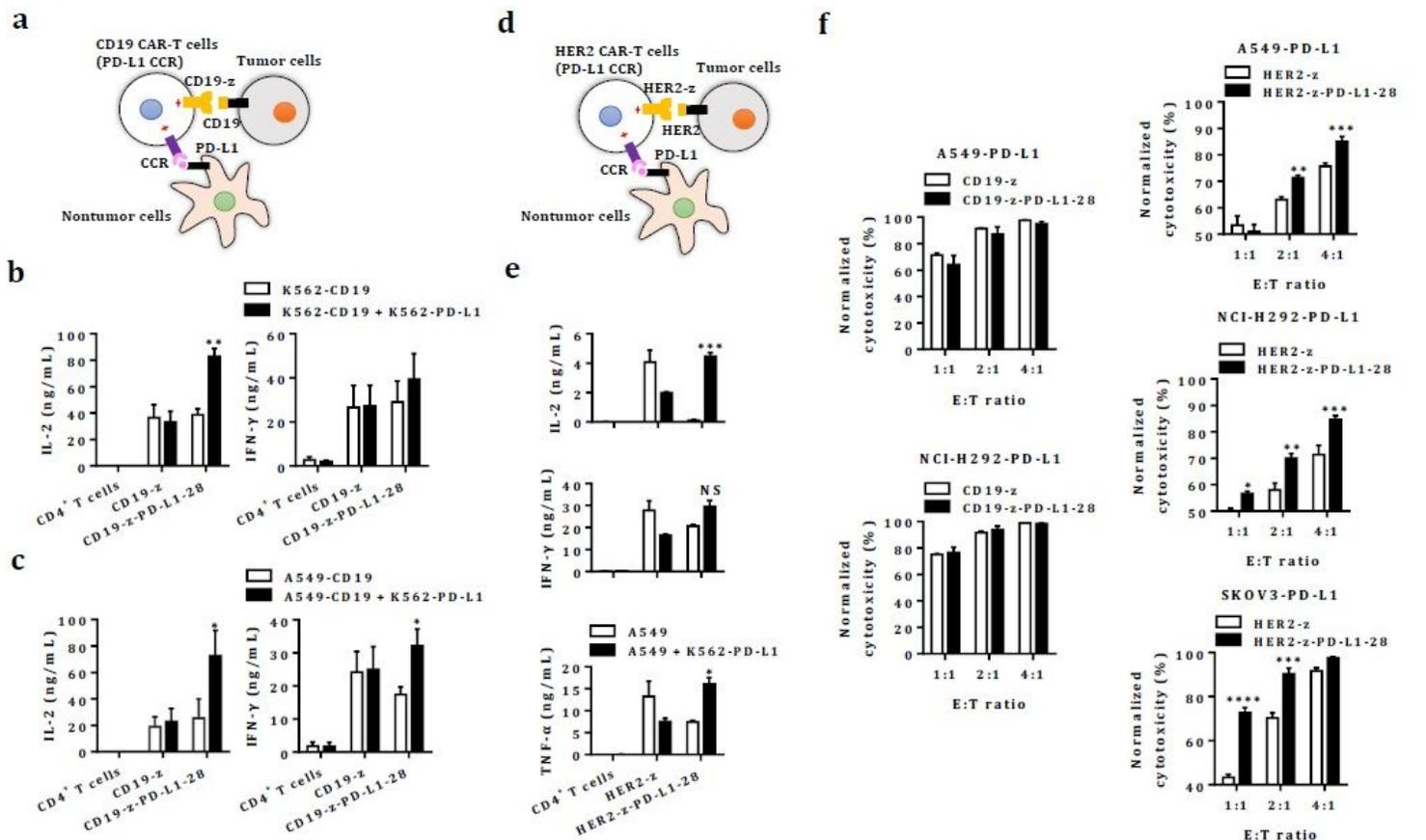


Figure 3

PD-L1 CCR-engineered CAR-T cells also achieved an optimal activation signal when stimulated with PD-L1-expressing host cells and PD-L1-negative tumor cells expressing CAR-targeted antigen. a-c The levels of IL-2 and IFN- γ released by untransduced, CD19-z-engineered and CD19-z-PD-L1-28-engineered CD4⁺ T cells were measured by ELISA after 24 h incubation at an E:T:host cell ratio of 1:1:1 (b, K562) or 3:1:1 (c, A549). The results are reported as the mean \pm SEM for three healthy donors, * $P < 0.05$, ** $P < 0.01$ with respect to coculture with CD19-expressing K562 (b) or A549 (c) cells, analyzed using a paired Student's t-test. d-e The levels of IL-2, IFN- γ , and TNF- α secreted by untransduced, HER2-z-engineered and HER2-z-PD-L1-28-engineered CD4⁺ T cells were measured by CBA after 24 h incubation at an E:T:host cell ratio of 3:1:1. The results are reported as the mean \pm SEM with technical triplicates, NS, no significance, * $P < 0.05$, *** $P < 0.001$ with respect to coculture with A549 cells, analyzed using Student's t-test. f The cytotoxicity of CAR-CD8⁺ T cells expressing or not expressing the PD-L1 CCR in vitro. CD19 CAR-T cells expressing or not expressing the PD-L1 CCR were tested for their cytotoxic activity at the indicated E:T ratio for 18 h against PD-L1-expressing CD19-positive A549 or NCI-H292 cells (left panel). HER2 CAR-T cells expressing or not expressing the PD-L1 CCR were tested for their cytolytic potency at the indicated E:T ratio for 18 h against PD-L1-expressing A549 cells, NCI-H292 cells or SKOV3 cells. The results shown

are the mean \pm SEM for three healthy donors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ with respect to coculture with CAR-T cells without the PD-L1 CCR, analyzed using a paired Student's t-test.

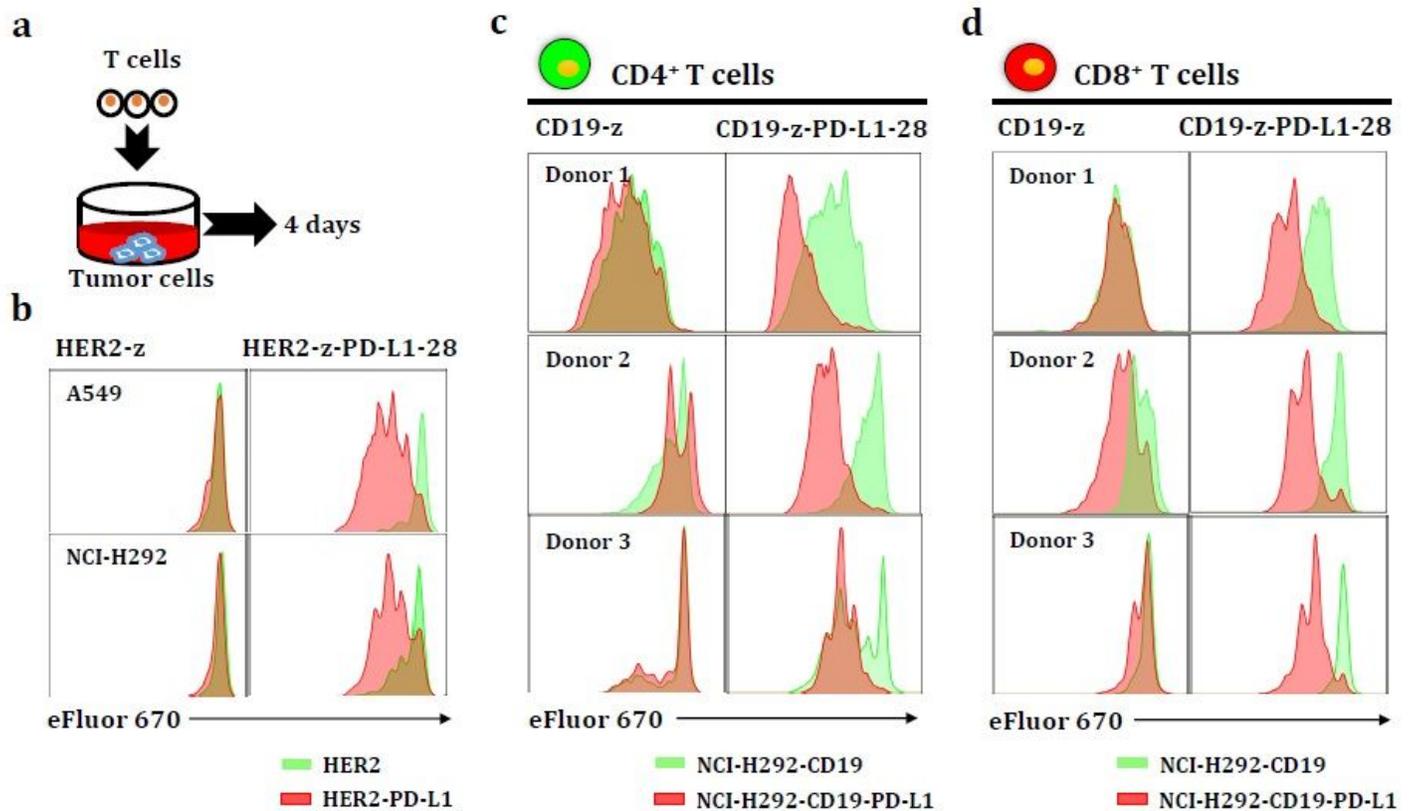


Figure 4

Combinatorial antigen-dependent PD-L1 CCR-engineered CAR-T cell proliferation in vitro. **a** Scheme of in vitro CAR-T cell proliferation. Primary T cells expressing the CD19/HER2 CAR or PD-L1 CCR or untransduced T cells were labeled with Cell Proliferation Dye eFluor 670 according to the manufacturer's instructions. A549, NCI-H292, or CD19-overexpressing NCI-H292 cells expressing PD-L1 or not were pretreated with mitomycin C to obtain replication-incompetent target cells. These labeled T cells were cocultured with mitomycin C-treated target cells at a E:T ratio of 2:1 in RPMI 1640 medium supplemented with 10% FBS without cytokines. Finally, the proliferation of T cells was assayed by monitoring the dilution of the cell proliferation dye after incubation for 4 days. **b** The results showed that PD-L1 CCR-engineered HER2 CAR-T cells have enhanced proliferation activity in the presence of PD-L1-expressing tumor cells, and HER2 CAR-T cells containing only the CD3 ζ signaling domain presented lower proliferation potential. **c-d** The results showed that PD-L1 CCR-modified CD19 CAR-CD4⁺ (**c**) and CD8⁺ (**d**) T cells have enhanced proliferation activity in the presence of tumor cells expressing both CD19 and PD-L1, while CAR-T cells containing only the CD3 ζ signaling domain presented a lower proliferation capacity, indicating that suboptimal CD3 ζ signaling is insufficient to trigger optimal T cell activation (n=3).

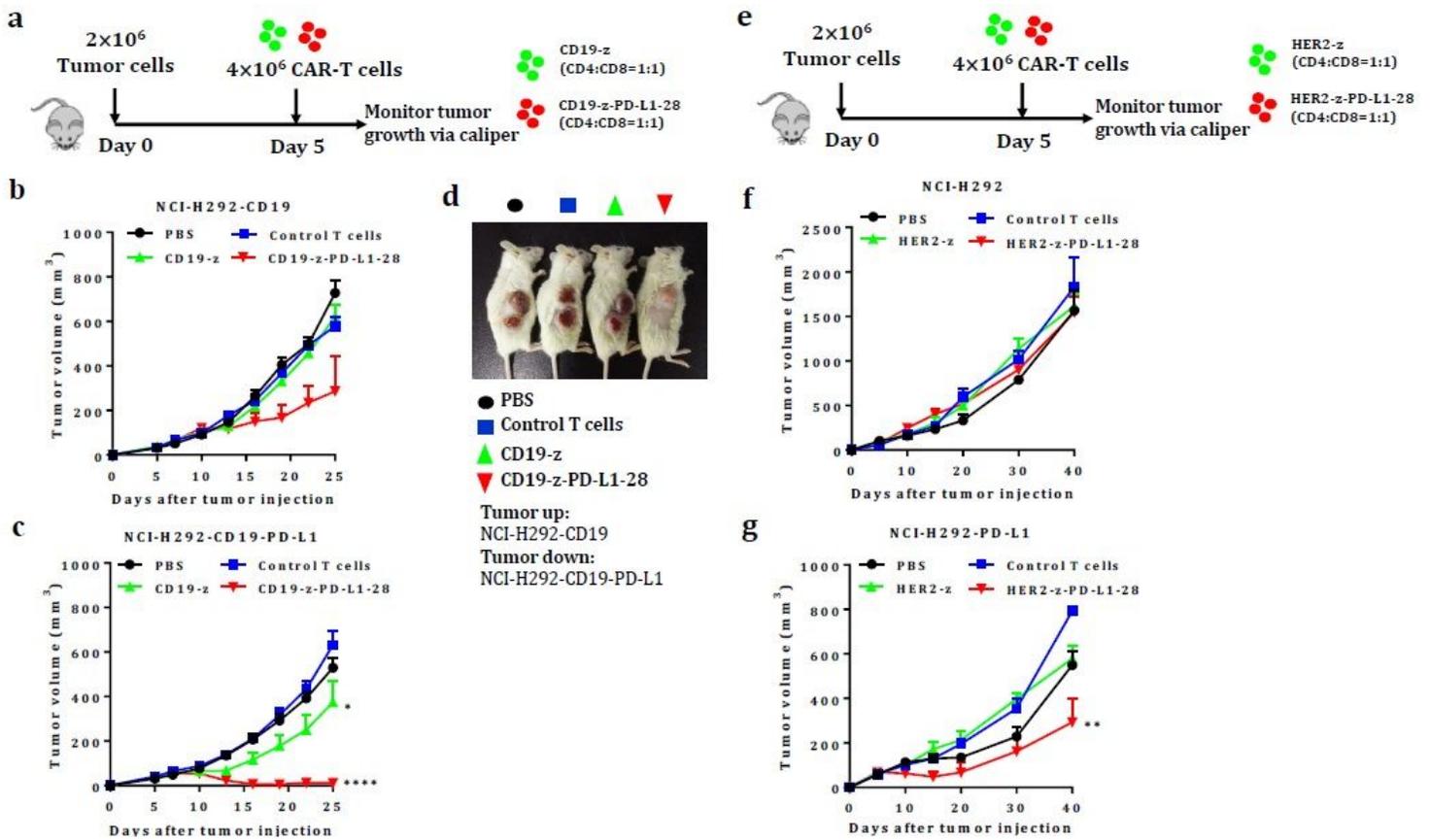


Figure 5

Tumoricidal effects of PD-L1 CCR-engineered CAR-T cells in vivo. a Schematic diagram of the mouse treatment strategy. A total of 2×10^6 NCI-H292-CD19 and 2×10^6 NCI-H292-CD19-PD-L1 tumor cells were injected subcutaneously into the same right flank of B-NDG mice. A total of 2×10^6 CD4⁺ T cells and 2×10^6 CD8⁺ T cells expressing CD19-z or both CD19-z and the PD-L1 CCR were injected intravenously into these tumor-bearing mice. Tumor volume was monitored over 20 days after intravenous injection of engineered T cells or untransduced T cells (control T cells). b-c Graphs presenting the tumor growth of CD19⁺ and CD19⁺PD-L1⁺ cells in mice treated with PBS, control T cells, CD19-z-engineered T cells and CD19-z-PD-L1-28-engineered T cells (n = 5 mice, error bars are the SEM, significance determined by one-way ANOVA, * P < 0.05, **** P < 0.0001 with respect to the control T cell treatment group). d Image showing that CD19⁺ (up) and CD19⁺PD-L1⁺ (down) NCI-H292 tumor-bearing mice were treated with PBS, control T cells, CD19-z-expressing T cells and CD19-z-PD-L1-28-engineered T cells at 1 month after tumor cell injection. PD-L1 CCR-engineered CD19 CAR-T cells eliminated the dual-antigen-positive tumor exclusively while sparing the CD19-only tumor. e NCI-H292 (2×10^6) and NCI-H292-PD-L1 (2×10^6) cells were injected subcutaneously into the same right flank of B-NDG mice. A total of 2×10^6 CD4⁺ T cells and 2×10^6 CD8⁺ T cells expressing HER2-z or both HER2-z and the PD-L1 CCR were injected intravenously into these tumor-bearing mice. Tumor volume was monitored over 35 days after intravenous injection of engineered T cells or control T cells. Where indicated, 2×10^6 CD4⁺ T cells and 2×10^6 CD8⁺ T cells transduced with HER2-z or HER2-z-PD-L1-28 were infused intravenously 5 days later. f-g Graphs presenting the tumor size of HER2⁺ and HER2⁺PD-L1⁺ cells in mice treated with PBS, control T cells,

HER2-z-expressing T cells and HER2-z-PD-L1-28-engineered T cells (n = 5 mice, error bars are the SEM, significance determined by one-way ANOVA, ** P < 0.01 with respect to the control T cell treatment group).

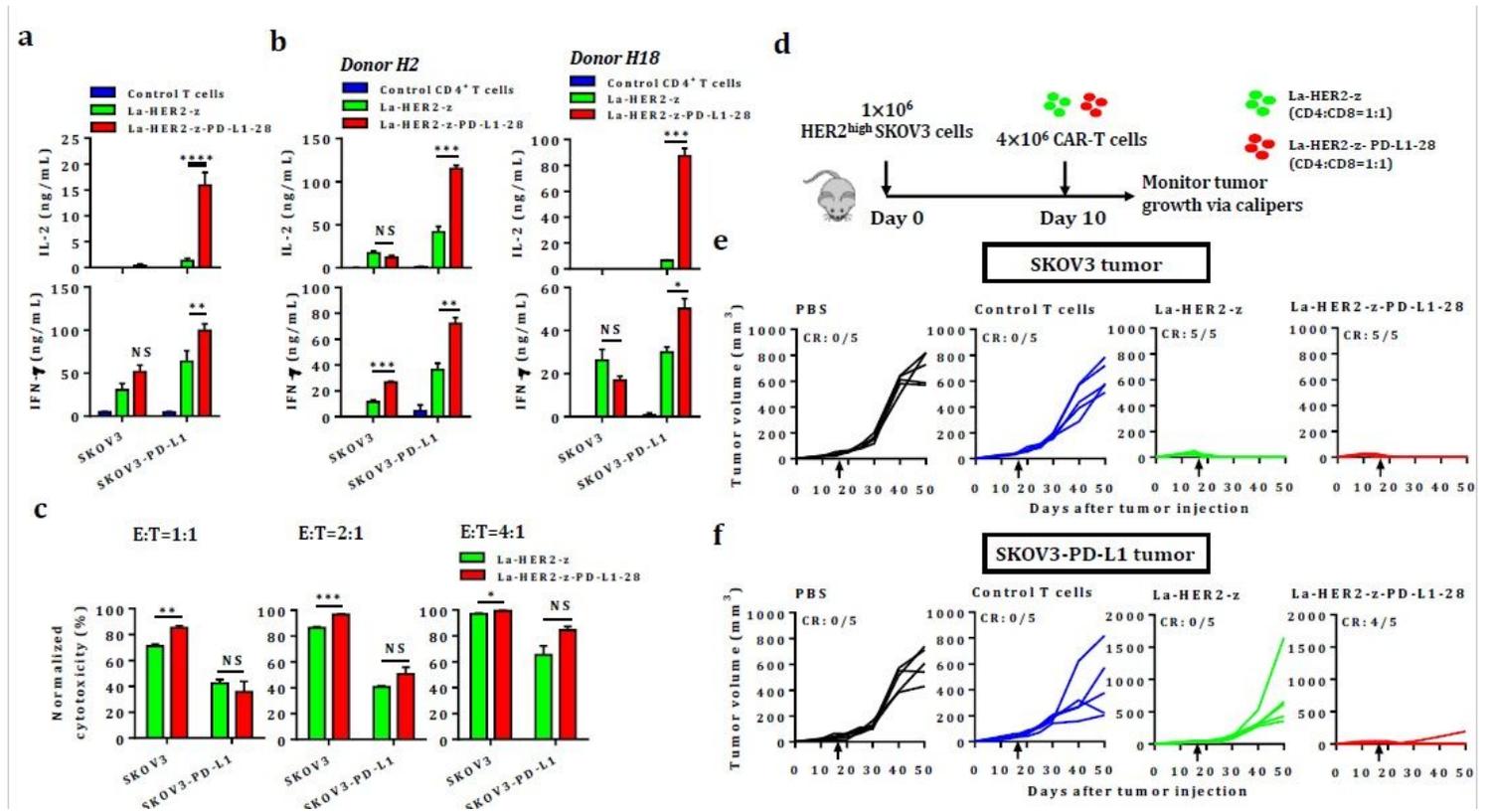


Figure 6

The antitumor activity of PD-L1 CCR-engineered low-affinity HER2 CAR-T cells. a Cytokine secretion was assayed in supernatants from cocultures of low-affinity HER2 CAR-T cells with or without the PD-L1 CCR. The primary T cells were transduced with lentiviral vectors containing low-affinity HER2 CAR or PD-L1 CCR. The resting CAR-T cells were cocultured with PD-L1-/+ SKOV3 tumor cells for 24 h, and the supernatant was collected and assayed by ELISA. Bar charts show data from three healthy donors, which are represented as the mean \pm SEM, for IL-2 (upper panel) and IFN- γ (lower panel) (NS, no significance; ** P < 0.01; **** P < 0.0001, analyzed using a paired Student's t-test). b Cytokine release was assayed in supernatants from CAR-CD4⁺ T cells. The primary CD4⁺ T cells were transduced with low-affinity HER2 CAR or PD-L1 CCR via lentiviral particles, as described above. The resting CAR-CD4⁺ T cells were cocultured with PD-L1-positive/negative SKOV3 tumor cells for 24 h, and the supernatant was collected and assayed by ELISA, as described above. Bar charts show data from a donor (values represent the mean \pm SEM of technical triplicates) for IL-2 (upper panel) and IFN- γ (lower panel) (NS, no significance; * P < 0.05; ** P < 0.01; *** P < 0.001; analyzed using Student's t-test). c Tumor lysis of PD-L1-/+ SKOV3 target cells by HER2 CAR-T cells, using untransduced T cells as a negative control for normalization, was measured. The primary CD8⁺ T cells were transduced with low-affinity HER2 CAR or PD-L1 CCR via lentiviral particles as indicated. The resting CAR-CD8⁺ T cells were cocultured with SKOV3 or SKOV3-PD-L1 target cells at the indicated E:T ratio, and normalized cytotoxicity was calculated after 18 h of

incubation. Bar charts show data from a representative experiment (values are expressed as the mean \pm SEM of technical triplicates) (NS, no significance; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, analyzed using Student's t-test). d-f The antitumor efficacy of low-affinity HER2 CAR-T cells with or without the PD-L1 CCR in vivo. d Schematic diagram of the in vivo CAR-T cell administration protocol. PD-L1-negative/positive SKOV3 tumor cells were injected subcutaneously into the same flank of B-NDG mice on day 0. Ten days post-inoculation, the mice were randomized into four groups (n=5 mice/group) and treated with 4 million control T cells, low-affinity HER2 CAR-T cells (CD4+: CD8+ = 1: 1), PD-L1 CCR-engineered low-affinity HER2 CAR-T cells (CD4+: CD8+ = 1: 1) or an equal volume of PBS. Tumor size was measured with calipers every 5 or 10 days. e Tumor growth curve for mice implanted with SKOV3 cells. The low-affinity HER2 CAR-T cells with or without the PD-L1 CCR eliminated tumors exclusively, but PBS and control T cells failed to inhibit tumor growth. f Tumor size measurement for mice engrafted with PD-L1-positive SKOV3 tumor cells. Only PD-L1 CCR-engineered low-affinity HER2 CAR-T cells showed high antitumor activity against PD-L1-positive SKOV3 tumors. However, mice treated with PBS, control T cells, or low-affinity HER2 CAR-T cells did not show tumor growth inhibition.

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