Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo

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The efficacy of chimeric antigen receptor (CAR) T cell therapy against poorly responding tumors can be enhanced by administering the cells in combination with immune checkpoint blockade inhibitors. Alternatively, the CAR construct has been engineered to coexpress factors that boost CAR-T cell function in the tumor microenvironment. We modified CAR-T cells to secrete PD-1-blocking single-chain variable fragments (scFv). These scFv-secreting CAR-T cells acted in both a paracrine and autocrine manner to improve the anti-tumor activity of CAR-T cells and bystander tumor-specific T cells in clinically relevant syngeneic and xenograft mouse models of PD-L1+ hematologic and solid tumors. The efficacy was similar to or better than that achieved by combination therapy with CAR-T cells and a checkpoint inhibitor. This approach may improve safety, as the secreted scFvs remained localized to the tumor, protecting CAR-T cells from PD-1 inhibition, which could potentially avoid toxicities associated with systemic checkpoint inhibition.

T cells can be directed to target tumor cells through expression of a CAR. CARs are synthetic receptors consisting of an extracellular antigen recognition domain, which is most commonly a scFv, but can also take the form of any antigen-binding peptide. This binding domain is then linked, with or without a hinge domain, to intracellular T cell activation and co-stimulation domains. Although CAR-T cell therapy has shown notable results in patients with B-cell acute lymphoblastic leukemia1, its efficacy in treating other hematological and solid tumors has been less impressive1.

These modest responses may relate to the tumor microenvironment (TME). When infused into patients, CAR-T cells often encounter an inhibitory TME, with cells and inhibitory ligands that can bind to inhibitory receptors on T cells and hinder T cell anti-tumor responses. For instance, in ovarian cancer, an immunosuppressive M2-polarized tumor-associated macrophages (TAMs)2 and regulatory T (Treg) cells3,4 have been found to populate the TME, and the presence of these cells correlates with reduced tumor-infiltrating lymphocytes5 and poor outcomes in patients2,3. Both TAMs and Treg cells suppress infiltrating T cells via contact- and cytokine-mediated mechanisms5,6. Furthermore, following activation, T cells secrete IFN-γ, an effector cytokine, which has been shown to dynamically upregulate programmed death ligand-1 (PD-L1) expression on ovarian cancer cells in both clinical7 and preclinical models8. PD-L1 binds to the inhibitory receptor programmed death 1 (PD-1) on T cells and suppresses T cell function9. Interruption of PD-1/PD-L1 ligation via CRISPR-mediated deletion of PD-L1 on ovarian cancer cells improved the efficacy of adoptively transferred second-generation CAR-T cells in preclinical models8. These factors may contribute to the lack of clinical efficacy of CAR-T cells for this solid tumor malignancy9.

Checkpoint blockade therapy, which uses antibodies to disrupt the interaction between inhibitory receptors on T cells, particularly CTLA-4 and PD-1, and their suppressive ligands on tumors cells, has yielded clinical responses in patients with a range of solid tumors10–13 and hematological malignancies14. Correlates for the efficacy of checkpoint blockade therapy include T cell activation markers, tumor cell expression of PD-L1, a pre-existing CD8+ T cell infiltrate in the tumor15,16 and tumor mutational burden15,17–20. Together, these results suggest that tumor-specific T cells are an integral mechanism of action of checkpoint blockade and that re-engagement of pre-existing tumor-specific T cells is critical to the success of this therapeutic modality.

We have previously described ‘armored’ CAR-T cells, which are CAR-T cells that are co-modified to express immunomodulatory ligands such as CD40L21 or to secrete cytokines such as IL-12 (refs. 8,22–25) or IL-18 (ref. 26) to enhance CAR-T cell function in the tumor microenvironment. Based on our previous findings, rather than combining CAR-T cells with existing systemic checkpoint blockade antibody treatment, as studied previously in preclinical models8,27,28, we sought to use our armored CAR-T cell platform to create a single therapy in which CAR-T cells secrete an immune checkpoint blockade scFv. Given that CAR-T cells traffic to the tumor, the PD-1-blocking...
scFvs would be delivered locally to the site of disease, thereby minimizing the toxicities associated with immune checkpoint blockade. We found that CAR-T cells that secreted a PD-1-blocking scFv enhanced the survival of PD-L1+ tumor-bearing mice in syngeneic and xenogeneic mouse models through both autocrine and paracrine mechanisms. This strategy has the potential to enhance the efficacy of CAR-T cell therapy in cancers with an immune-suppressive TME.

RESULTS Modification of mouse CAR-T cells to secrete an anti-mouse PD-1-blocking scFv
To test our approach in an immunocompetent syngeneic mouse model, we generated retroviral second-generation CAR constructs containing binding domains that recognize CD19 or the retained portion of MUC16 (MUC16<sup>ecto</sup>)<sup>29</sup> and mouse CD28 and CD3 zeta T cell signaling domain. These conventional CARs were labeled 19m28mZ or 4H11m28mZ, respectively. Armored mouse CAR constructs, labeled 19m28mZRMP1-14 or 4H11m28mZRMP1-14, used the same backbone, binding domain and mouse signaling domain as the second-generation mouse CAR and were also co-modified to include a c-myc-tagged scFv derived from variable heavy and light chains from the anti-mouse PD-1-blocking monoclonal antibody (mAb), RMP1-14 (ref. 30; Fig. 1a). We transduced primary mouse T cells to express the CAR constructs (Fig. 1b) and secrete RMP1-14 scFvs (25.6 kDa; Fig. 1c and Supplementary Fig. 1a).

We co-cultured scFv-secreting CAR-T cells with human CD19-expressing mouse lymphoma EL4 cells (hCD19 mPD-L1) or MUC16<sup>ecto</sup>-expressing ovarian tumor ID8 cells, which upregulate PD-L1 after exposure to IFN-γ (Fig. 1d). Conventional and RMP1-14 scFv-secreting CAR-T cells specifically lysed (Fig. 1e) and produced IFN-γ (Fig. 1f) when cultured with tumor targets.

We next evaluated the binding of secreted RMP1-14 scFvs to PD-1. There was a significant decrease in the amount of surface PD-1 on 19m28mZRMP1-14 T cells compared with cells modified to express 19m28mZ (P = 0.01), consistent with the scFvs binding in an autocrine manner to secrete CAR-T cells (Fig. 1g and Supplementary Fig. 1b). To determine whether scFvs bind to bystander PD-1-expressing cells, we co-cultured 4H11m28mZ T cells in the bottom well of a transwell plate with either 19m28mZ or 19m28mZRMP1-14 T cells on top. After 24 h, we detected lower levels of PD-1 on the surface of 4H11m28mZ cells cultured with 19m28mZRMP1-14 cells than on those cultured with 19m28mZ T cells (P = 0.04; Fig. 1h), consistent with the binding of secreted scFvs to bystander cells.

PD-1-blocking scFvs are secreted by CAR-T cells in vivo
We next validated the presence of PD-1-blocking scFvs secreted by CAR-T cells in the TME. Tumor-bearing mice with palpable ascites were injected intraperitoneally (i.p.) with CAR-T cells (Fig. 2a). Ascites from these mice was harvested 48 h later and RMP1-14 scFvs secreted from 4H11m28mZRMP1-14 T cells was detected by immunoprecipitation with an anti-myc-tag antibody and western blot analysis (Fig. 2b and Supplementary Fig. 2), as well as by anti-myc-tag Luminex (P = 0.0004; Fig. 2c).

We next tested the efficacy of scFv-secreting CAR-T cells in a syngeneic, immune-competent mouse model of metastatic ovarian carcinoma. C57BL/6 mice were injected i.p. with ID8 cells and treated 7 d later with CAR-T cells. 4H11m28mZRMP1-14 T cells showed enhanced survival as compared with mice treated with 4H11m28mZ (P = 0.004) and 19m28mZRMP1-14 T cell controls (P = 0.0006). Treatment with 4H11m28mZRMP1-14 T cells or 4H11m28mZ T cells + RMP1-14 antibody revealed a comparable survival benefit (P = 0.051; Fig. 2d). Tumor-bearing mice treated with RMP1-14 mAb alone had no survival benefit over mice treated with control CAR-T cells.

PD-1 binding to PD-L1 can result in T cell exhaustion, anergy and/or apoptosis. We found that long-term surviving mice treated with 4H11m28mZ/RMP1-14 T cells or 4H11m28mZ T cells + RMP1-14 antibody had detectable CAR-T cells by PCR in the bone marrow over 120 d post-tumor inoculation (Fig. 2e). In addition, 4H11m28mZRMP1-14 treated mice were able to mount an anti-tumor response when re-challenged with the initial dose of ID8 tumor cells (P < 0.0001; Fig. 2f), as compared with naive untreated mice.

We hypothesized that CAR-T cells secreting PD-1-blocking scFvs could re-invigorate endogenous tumor-specific T cells. To test this, we injected C57BL/6 mice subcutaneously (s.c.) with the immunogenic melanoma cell line B16-F10. These mice mount an endogenous response to the tumor but cannot eradicate it<sup>30</sup>. Endogenous bystander tumor-infiltrating lymphocytes isolated from mice treated intratumorally with 4H11m28mZ/RMP1-14 CAR-T cells expressed significantly higher levels of CD80, CD107α, IFN-γ and granzyme B than those treated with 4H11m28mZ (P < 0.05; Fig. 2g and Supplementary Fig. 3).

Selection of human PD-1-blocking scFv E27
The human PD-1-blocking scFvs E23, E26 and E27 were isolated from a human scFv phage display library (Eureka Therapeutics). The dissociation constants (K<sub>D</sub>) for the three lead candidates, E23, E26 and E27, binding to PD-1 were 8.3, 6.3 and 3.6 nM, respectively. The E23, E26 and E27 antibodies were able to block PD-1 from binding to PD-L1 in a dose-dependent manner (Fig. 3a). We collected supernatant from equivalent numbers of transduced viral producer cells and detected anti-PD1 secretable scFvs (Fig. 3b,c). The most intense band was observed for clone E27, indicating that the scFv was the most stable in supernatant. For this reason, we used E27 in subsequent studies (Fig. 3c and Supplementary Fig. 4a).

Modification of human CAR-T cells to secrete an anti-human PD-1-blocking scFv
We generated human CAR constructs encoding the CD19- or MUC16<sup>ecto</sup>-targeted CAR and the His/HA-tagged E27 scFv (1928z-E27 and 4H1128z-E27, respectively; Fig. 3d). Transduced T cells expressed CAR on the surface (Fig. 3e) and secreted E27 scFv (Fig. 3f and Supplementary Fig. 4b). Binding of E27 to PD-1 was demonstrated by HA-tag detection on 293Glv9-PD1 cells after incubation in supernatant from 1928z-E27 T cells (Fig. 3g and Supplementary Fig. 4c). Furthermore, we detected less PD-1 on 1928z-E27 and 4H1128z-E27 T cells than on cells modified to express 1928z or 4H1128z, consistent with the binding of E27 scFv to T cell PD-1 (Fig. 3h and Supplementary Fig. 4d). We corroborated antigen-dependent lysis by CAR-T cells in the context of tumor cells (Fig. 3i).

We transduced Raji and NALM6 tumor cells to express PD-L1 and co-cultured 1928z and 1928z-E27 T with either Raji-PD-L1 or NALM6-PD-L1 tumor at 1:1 tumor:CAR-T cell ratio. After 72 h of coculture, we used flow cytometry to detect the remaining tumor cells. 1928z-E27 T cells lysed more Raji-PDL1 and NALM6-PDL1 tumor cells than 1928z T cells (Fig. 4a,b). Using this assay, we found increased expansion of 1928z-E27 T cells following co-culture with Raji-PDL1 or NALM-PDL1 tumor compared with 1928z T cells (Fig. 4c), consistent with 1928z-E27 T cell response to PD-L1-mediated suppression. Following co-culture with Raji-PD-L1, 1928z-E27 cells showed lower levels of surface PD-1 detection than 1928z T cells, as determined by both the percentage of positive cells and the mean fluorescence intensity of the staining (Fig. 4d,e).
Figure 1 Mouse CAR-T cells can be co-modified to secrete the mouse PD-1-blocking scFv RMP1-14. (a) Schematic of the bi-cistronic vectors used for syngeneic mouse studies encoding the CD19-targeted 19m28mZ CAR or ovarian MUC16\textsuperscript{ecto}-targeted 4H11m28mZ CAR, linked with a P2A element to the secretable PD-1-blocking scFv RMP1-14. A c-myc-tag is included for detection of the scFv. (b) Representative flow cytometry plot demonstrating CAR expression following mouse T cell transduction, detected with fluorescently labeled CAR-specific idiotypic antibodies. Data shown are representative of 3 independent experiments. (c) Western blot on supernatant from equivalent numbers of viral packaging cells transduced to express the secretable scFvs with the CAR, detected with anti-myc-tag antibody. Data shown are representative of three independent experiments. (d) Flow cytometry histograms depicting expression of mouse PD-L1 on EL4 (hCD19 mPD-L1) or ID8 cells. Data shown are representative of three independent experiments. (e) Flow cytometry histograms depicting expression of mouse PD-L1 on EL4 (hCD19 mPD-L1) or ID8 cells. Data shown are representative of three independent experiments. (f) 4-h \textsuperscript{51}Cr release assay demonstrating lysis of tumor cells. Data shown are representative of three independent experiments. (f) All four CAR constructs produce antigen-specific IFN-\gamma after co-culture with tumor cells. Data shown are mean \pm s.e.m. from four independent experiments. (g) Quantification of PD-1 detection on CAR-T cells, as measured by flow cytometry. Data shown is mean \pm s.e.m. from four independent experiments, \( *P = 0.011 \) by two-tailed paired \( t \) test. (h) Experimental schematic and quantification of decreased PD-1 detection by flow cytometry on 4H11m28mZ T cells when cultured in a transwell plate with 19m28mZ or 19m28mZ/RMP1-14 T cells. Data shown are mean \pm s.e.m. from five separate donors, \( *P = 0.012 \) by two-tailed paired \( t \) test.
Figure 2 CAR-T cells secreting RMP1-14 scFv have enhanced anti-tumor function in syngeneic mouse tumor models. (a) Schematic diagram of experimental setup to detect scFv secretion in vivo. C57BL/6 mice were inoculated with ID8 tumor, monitored until development of ascites and subsequently treated i.p. with 4H11m28mZ or 4H11m28mZ/RMP1-14 T cells. In vivo secretion of RMP1-14 scFv was detected by harvesting ascites from tumor-bearing mice 48 h later. (b,c) The ascites was immunoprecipitated with an anti-myc-tag antibody and run on a western blot using an anti-myc tag antibody (b) or run on Luminex using anti-myc-tag beads (c, *P < 0.0004 using an two-tailed unpaired t test, mean ± s.e.m. = 2.3 ± 1.9 and 26 ± 1.2 for 4H11m28mZ and 4H11m28mZ/RMP1-14, respectively). Data shown are from two independent experiments. (d) C57BL/6 mice were injected i.p. with ID8 tumor cells and treated with CAR-T cells, 250 µg RMP1-14 mAb or a combination of both 7 d later. RMP1-14 mAb was given on days 3, 7 and 14 post-tumor inoculation (*P = 0.004 by Log-rank Mantel–Cox Test, with a 95% confidence interval (CI) of 0.4–0.9). Data shown are from two independent experiments. (e) PCR of bone marrow from mice surviving >120 d since tumor inoculation (d). CAR-T cells were detected in the bone marrow of long-term surviving mice treated with 4H11m28mZ T cells and RMP1-14 mAb or 4H11m28mZ/RMP1-14 T cells. Data shown are from two independent experiments. (f) C57BL/6 mice were inoculated with ID8 tumor and treated 7 d later with 4H11m28mZ or 4H11m28mZ/RMP1-14 T cells. Long-term surviving mice in the 4H11m28mZ/RMP1-14 T cell cohort were re-challenged with a second inoculation of ID8 cells and compared with naive untreated ID8-innoculated mice (*P = 0.007 by Log-rank Mantel-Cox Test, with a 95% CI of 0.2–0.3). (g) Quantification of flow cytometric analysis demonstrating that endogenous CAR-T cells extracted from C57BL/6 mice bearing B16-F10 mouse melanoma and treated with PD-1-blocking scFv CAR-T cells had enhanced activation and cytokines levels compared with mice treated with second-generation CAR-T cells (P values indicated on figure, two-tailed unpaired t test). Data shown are pooled from six mice and two independent experiments.

E27 binds to bystander cells
To demonstrate that untransduced bystander cells can benefit from the E27 scFv secreted from nearby CAR-T cells, we first co-cultured healthy human donor T cells with 1928z or 1928z-E27 CAR-T cells and stimulated the cells with anti-CD3 and anti-CD28 beads. After 4 d of co-culture, CAR⁺ and CAR⁻ T cells were sorted and western blot analysis demonstrated E27 binding to PD-1 on both CAR⁺ and CAR⁻ cells in the 1928z-E27/PD-1 T cell co-culture, but not the 1928z/PD-1 T-cell co-culture (Fig. 4f and Supplementary Fig. 5).

We cultured 1928z and 1928z-E27 T cells with 3T3-empty or 3T3-PDL1 cells and stimulated them with anti-CD3 and anti-CD28 beads.
Figure 3 Human CAR-T cells can be co-modified to secrete a PD-1-blocking scFv, E27. (a) Human PD-1-blocking mAb candidates E27, E26 and E23 were used in a competitive binding assay to detect the interruption of PD-1 binding to PD-L1 at varying concentrations, as compared with a human IgG1 isotype control mAb (control). Data shown represent the mean of three independent experiments. (b) Schematic representation of PD-1-blocking scFv designed from the E23, E26 and E27 mAbs. The signal peptide was linked to the variable heavy sequence, serine glycine linker and the variable light chain sequence. The His/HA tag was included for detection of the scFv. (c) Western blot on supernatant from equivalent numbers of 293-Glv9 packaging cells transduced to secrete scFvs with the 1928z CAR, stained with anti-HA antibody. Data shown are representative of two independent experiments. (d) Schematic of the bi-cistronic vector encoding the CD19-targeted 1928z CAR or ovarian MUC16-specific tandem 4H1128z CAR, linked with a P2A element to the secretable anti-human PD-1-blocking scFv E27. (e) Representative flow cytometry plot demonstrating CAR expression following donor human T cell transduction, detected with fluorescently labeled CAR-specific idiotypic antibodies. Data shown are representative of 3 independent experiments. (f) Western blot on supernatant from CAR-T cells stained with anti-HA mAb, demonstrating a ~30-kDa protein in the 1928z-E27 and 4H1128z-E27 T cells. Data shown are representative of two independent experiments. (g) Western blot analysis of 293Glv9-PD-1+ cells incubated in supernatant from 1928z and 1928z-E27 T cells, stained with anti-HA mAb, showing a ~30-kDa protein in the PD-1+ cells incubated with supernatant from 1928z-E27. Data shown are representative of two independent experiments. (h) Quantification of PD-1 detection by flow cytometry on 1928z-E27 and 4H1128z-E27 T cells, as compared to second-generation CAR-T cells. Data shown is mean ± s.e.m. from five independent donors. For comparison of 1928z to 1928z-E27, *P = 0.05; 4H1128z to 4H1128z-E27, **P = 0.006; both by a two-tailed paired t test. (i) 4-h 51Cr release assay demonstrating that all four CAR constructs had antigen-dependent lysis of tumor cells. Data shown are representative of three independent donors and experiments.
1928z T cells that were cultured with 3T3-empty cells showed enhanced proliferation compared with 1928z T cells cultured with 3T3-PDL1 cells (Fig. 4b). However, 1928z-E27 T cells showed similar expansion when cultured with 3T3-empty and 3T3-PDL1 cells (Fig. 4h). There was no significant difference between the expansion of 1928z and 1928z-E27 T cells when cultured with 3T3-empty cells (P = 0.063; Fig. 4h).

By day 12, 1928z T cells cultured with 3T3-PDL1 cells had significantly decreased cell numbers compared with those cultured with 3T3-empty cells. We anticipated that the population of CAR+ and CAR− cells would contract in this condition in a similar ratio and that the overall CAR+ percentage would remain the same relative to time 0. This is what we observed when we calculated the CAR+ cells relative to time 0 (Fig. 4i). However, because the 1928z-E27 cells cultured with 3T3-PDL1 cells expanded substantially by day 12, it may be expected that the total number of CAR+ cells would increase, thereby leading to an increased percentage of CAR+ cells. This would be the case if the PD-1-blocking scFvs bestowed a selective proliferative advantage only to the cell that was secreting it. However, the percentage of CAR+ 1928z-E27 T cells following expansion on 3T3-PDL1 cells was not increased on day 12 compared with day 0, indicating that the E27 scFv protects the expansion of transduced T cells as well as untransduced bystander cells in the context of PD-L1 (Fig. 4i).

**CAR-T cells secreting E27 scFv have enhanced anti-tumor function in vivo**

We determined the in vivo anti-tumor efficacy of E27-secreting CAR-T cells by inoculating SCID/Beige mice with Raji-PDL1 or NALM6-PDL1 tumor cells intravenously (i.v.). Infusion of 1928z-E27 T cells significantly enhanced the survival of both Raji-PDL1 (P = 0.03; Fig. 5a) and NALM6-PDL1 (P = 0.01; Fig. 5b) tumor-bearing mice compared with an infusion of 1928z T cells.

Next, we investigated the anti-tumor efficacy of E27-secreting CAR-T cells in a solid tumor model of peritoneal carcinomatosis. SKOV3-PDL1 tumor-bearing SCID/Beige mice treated with 4H1128z-E27 T cells showed enhanced survival compared with mice treated with 4H1128z T cells (P = 0.02; Fig. 5c). In addition, using this preclinical xenograft model of metastatic ovarian tumor, mice treated with E27 scFv-secreting 4H1128z T cells had enhanced survival benefit over mice treated with 4H1128z T cells + anti-human PD-1 mAb (P = 0.048; Fig. 5d).

To demonstrate in vivo the bystander effect of the E27-secreting CAR-T cells, we treated SKOV3-PD-1-bearing mice with a combination of antigen-irrelevant 1928z-E27 and tumor-specific 4H1128z CAR-T cells. In this experimental model, only the antigen-irrelevant CAR-T cells expressed the PD-1-blocking scFvs, and treatment with 1928z-E27 T cells alone had no survival benefit (Fig. 5e,f). However, when injected together, 1928z-E27 enhanced the anti-tumor function of second-generation 4H1128z cells, as compared with mice treated with just 4H1128z T cells (P = 0.03; Fig. 5f). This result suggests that the E27 scFv secreted by the 1928z-E27 cells binds to bystander tumor-specific cells in vivo.

**E27 scFv secreted by CAR-T cells is only detected in the local TME**

Using the SKOV3 solid tumor model of peritoneal carcinomatosis, we sought to determine the local and systemic levels of CAR-secreted scFvs. First, we used in vivo bioluminescence and fluorescence imaging to visualize the location of scFv or mAb, when administered i.p., over time. CAR constructs were generated with E27 scFv fused to Gaussia Luciferase (GLuc) enzyme, an ATP-independent signal that allows the imaging of E27 location in vivo after being produced by CAR-T cells. We conjugated a commercial anti-human PD-1 mAb to VivoTag 680 XL fluorochrome. CAR-T cells secreting E27-GLuc or CAR-T cells and fluorescently labeled mAb were i.p. injected into tumor-bearing mice and imaged over time (Fig. 6a). In as early as 3 h, the antibody could be found systemically in the mice, whereas the scFv was only detected in the peritoneal cavity. Following quantification, we did not observe a difference between whole body and local amounts of E27, as E27 remained localized at the tumor site. However, we found changes in the levels of the labeled commercial antibody, as it appeared to circulate out of the tumor area (Fig. 6b).

To further quantify the systemic levels of antibody or scFv over time, we used targeted mass spectrometry on a high-resolution quadrupole orbitrap instrument (Supplementary Fig. 6). We injected SCID/Beige mice i.p. with ovarian SKOV3 tumor cells and then i.p. injected them 7 d later with E27-secreting CAR-T cells or antibody and CAR-T cells. We harvested serum samples over time and carried out targeted parallel reaction monitoring spectrometry to quantify the amounts of E27 or commercial antibody over time. Two unique tryptic peptides from the complementarity-determining regions of the scFv and mAb that are not present in other mouse or human proteins were used. Commercial PD-1-blocking mAb was detected systemically in the serum and decreased over time, as expected (Fig. 6c). However, we did not detect E27 in the serum at any time point, indicating that it remained localized in the TME. A control sample consisting of isolated scFv was injected i.v. to demonstrate that our assay was capable of detecting E27 in the peripheral serum. It is important to note that the stability and half-life of an antibody and a scFv molecule differ, and this may affect our ability to detect scFv in serum. However, considering that we found that the scFv could be detected 30 min after systemic infusion (Fig. 6c) and that, in our system, the scFv is being produced constitutively by CAR T-cells in vivo, we should be able to detect CAR-T-cell-derived E27 if it is present in the periphery. Moreover, if the stability and half-life of the scFv does not allow it to leave the local TME and be detected in the periphery, this further supports our hypothesis that local checkpoint blockade using a scFv may be a safer alternative to antibodies. These results demonstrate that, unlike checkpoint blockade mAb, E27 secreted by CAR-T cells is not present systemically.

**DISCUSSION**

Using multiple in vivo models, we demonstrated that CAR-T cells that secrete PD-1-blocking scFv enhance the survival of mice in syngeneic and preclinical xenogeneic hematologic and solid tumor models. CAR-T cells that secrete PD-1-blocking scFv were equally effective or superior to therapy with CAR-T cells and PD-1-blocking mAb, indicating that localized delivery of PD-1 blockade is effective at enhancing CAR-T-cell anti-tumor function. PD-1 blockade on CAR-T cells results in long-term surviving mice that have detectable CAR-T cells in the bone marrow and can mount an anti-tumor response when re-challenged with tumor. PD-1-blocking scFv produced by CAR-T cells can enhance the function of tumor-specific bystander T cells in vivo in the TME. Finally, unlike systemic checkpoint blockade therapy, scFvs secreted by CAR-T cells remain locally in the TME.

Preclinical studies have previously demonstrated that CAR-T cells are susceptible to suppressed effector function mediated through the PD-1 receptor and subsequent combination with PD-1/PDL1-blocking antibodies, given exogenously or produced by CAR-T cells, results in enhanced CAR-T-cell-mediated anti-tumor response. However, these studies were limited to xenograft models that do not recapitulate the endogenous immunosuppressive TME, and bystander effects or...
Figure 4  Coexpression of CAR and E27 scFv protects proliferative and lytic capacity of T cells in the context of PD-L1⁺ tumor cells. (a) Representative flow cytometry dot plots demonstrating lysis of Raji-PDL1 tumor cells following 72 h co-culture. Data shown are representative of three independent donors and experiments. (b) 1928z-E27 T cells lysed significantly more Raji-PDL1 tumor cells than 1928z T cells. Data shown represent the mean ± s.e.m. from five independent experiments, * P = 0.05 for Raji experiment and ** P = 0.02 for NALM6 experiment, both by a two-tailed paired t test. (c) CAR-T cells expansion numbers following co-culture with Raji-PDL1 or NALM6-PD-L1 tumor cells as determined by flow cytometry. Data shown are the mean fold expansion ± s.e.m. from four independent experiments. * P = 0.03 by a one-tailed paired t test. (d) Expansion of 1928z-E27 T cells on 3T3-PDL1 cells was due to an increase in both CAR⁺ T cells and MFI of staining, both by two-tailed paired test. (e) Representative flow cytometry dot plots (d) and quantification (e) showing increased PD-1 detection on 1928z T cells compared with 1928z-E27 T cells following 7 d co-culture with Raji-PDL1 tumor cells. Data shown are the mean ± s.e.m. from three independent experiments. * P = 0.03 for percent positive CAR-T cell and MFI of staining, both by two-tailed paired t test. (f) 1928z and 1928z-E27 T cells were co-cultured with human T cells transduced to overexpress PD-1, stimulated with CD3/CD28 beads after 4d and sorted by flow cytometry to separate CAR⁺ and CAR⁻ cells. Western blot was performed on the sorted populations and probed with anti-HA mAb. Data shown are representative of three independent experiments and donors. (g) Representative example of 1928z and 1928z-E27 T cells fold expansion when cultured with 3T3-empty or 3T3-PDL1 cells and stimulated with anti-CD3 and anti-CD28 beads. Data shown are representative of three independent donors and experiments. (h) Cells were enumerated and re-plated on new 3T3 cells on days 3, 6, 9 and 12. 1928z T cells had reduced expansion when cultured with 3T3-PDL1 cells compared with 3T3-empty cells. 1928z-E27 cells had equivalent expansion when cultured with 3T3-empty or 3T3-PDL1 cells. Data shown are the mean fold expansion ± s.e.m. from four independent experiments, * P < 0.05 by two-tailed paired t test. (i) Expansion of 1928z-E27 T cells on 3T3-PDL1 cells was an increase in both CAR⁺ and CAR⁻ cells, comparing populations on day 0 and following expansion on 3T3-PDL1 cells on day 12. Data shown are representative of three independent experiments.
Figure 5 CAR-T cells that secrete E27 scFv have enhanced anti-tumor function in vivo. (a, b) SCID/Beige mice inoculated with Raji-PDL1 (a) or NALM6-PDL1 (b) tumor cells and treated with 1928z-E27 CAR-T cells have enhanced survival over mice treated with 1928z (P = 0.003 with 95% CI of 0.1–0.9 and P = 0.0004 with 95% CI of 0.02–0.3, respectively by log-rank Mantel–Cox test). Data shown are pooled from two independent experiments. (c) SCID/Beige mice treated with 4H1128z-E27 T cells had enhanced survival compared with mice treated with 4H1128z or antigen-irrelevant 1928z-E27 T cells in mice bearing SKOV3-PDL1 ovarian tumor cells. Data shown are from two independent experiments, P = 0.02 by log-rank Mantel–Cox test with a 95% CI of 0.2–1.5. (d) Survival curve showing mice treated with 4H1128z-E27 T cells had enhanced survival compared with mice treated with 4H1128z + anti-human PD-1 mAb. Data shown are from two independent experiments, P = 0.05 by log-rank Mantel–Cox test with a 95% CI of 0.4–3.1. (e) Schematic illustration of experiment to study bystander effect of scFv-secreting CAR-T cells in which SKOV3-PDL1 tumor-bearing mice were treated with a combination of E27-secreting 1928z T cells, which are antigen irrelevant in this model, together with ovarian-tumor specific 4H1128z T cells. PD-1-blocking scFv secreted by the 1928z-E27 T cells in vivo bound to PD-1 on 4H1128z T cells and enhanced tumor-specific function. (f) Mice treated 7 d after SKOV3-PDL1 inoculation with a mix of 1928z-E27 + 4H1128z T cells had enhanced survival compared with mice treated with 4H1128z (P = 0.007, 95% CI of 0.03–0.6) or 1928z-E27 (P < 0.0001, 95% CI of 0.02–0.2) T cells alone. Data shown are from two independent experiments.
localized delivery of antibody were not demonstrated. Specifically, a previous study,\textsuperscript{32} used CAR-T cells secreting an antibody (not scFv) and did not compare this to antibody alone or CAR-T cells and systemic antibody; as such, whether their approach is superior to established therapies is not clear. ScFvs are smaller, less stable molecules than antibodies and successful checkpoint blockade had not been demonstrated with them. Furthermore, the smaller size of scFvs enables the engineering of CAR constructs that are smaller, allowing for the possibility of tri-cistronic vectors with a combination of scFvs in the future.

Alternative methods to prevent PD-1-related dampening of CAR-T cell responses include coexpression of a chimeric switch receptor\textsuperscript{33}, with the extracellular domain of PD-1 being linked to activation signaling domains\textsuperscript{34}, or co-modifying CAR-T cells to express a dominant negative PD-1 receptor\textsuperscript{35}. Both of these methods have been shown to increase the levels of cytolysis, cytokine secretion and enhanced in vivo anti-tumor efficacy in response to PD-L1\textsuperscript{+} tumor targets compared with T cells expressing a CAR alone. However, this protective effect is limited to the CAR-T cells themselves. Endogenous tumor-infiltrating lymphocytes in the TME are still subject to PD-1-mediated suppression. Given that the PD-1-blocking scFvs secreted by CAR-T cells are able to bind to bystander cells, we hypothesize that local secretion of the PD-1-blocking scFvs described here may protect endogenous anti-tumor immune cells and reduce outgrowth of antigen-negative tumors.

Systemic administration of checkpoint blockade can result in immune-related adverse events (IRAEs)\textsuperscript{36}. IRAEs are frequent in patients treated with checkpoint blockade\textsuperscript{11,13,36}. Given that CAR-T cells traffic to and expand at the site of tumor, delivery of the PD-1-blocking scFvs was primarily localized to this area in our models. However, this protective effect is limited to the CAR-T cells themselves. Endogenous tumor-infiltrating lymphocytes in the TME are still subject to PD-1-mediated suppression. Given that the PD-1-blocking scFvs secreted by CAR-T cells are able to bind to bystander cells, we hypothesize that local secretion of the PD-1-blocking scFvs described here may protect endogenous anti-tumor immune cells and reduce outgrowth of antigen-negative tumors.

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Figure 6 The PD-1-blocking E27 scFv secreted by CAR-T cells is only detected in the local TME. (a) Imaging and quantification over time of E27 scFv tagged with Gluc or fluorescently labeled anti-human PD-1 mAb in SKOV3-PDL1 tumor-bearing SCID/Beige mice treated i.p. with scFv-secreting CAR-T cells or CAR-T cells with anti-PD-1 mAb (three animals per group). (b) Schematic representation and quantitation of areas used for complete versus local detection of scFv or antibody. *P < 0.02 at all time points tested for antibody using multiple t-test with three animals per group. (c) Quantification using unique peptide sequences by liquid chromatography-tandem mass spectrometry (LC-MS/MS) of serum levels over time of E27 scFv and anti-human PD-1 mAb in SKOV3-PDL1 tumor-bearing SCID/Beige mice treated i.p. with scFv-secreting CAR-T cells or CAR-T cells with anti-PD-1 mAb (five animals per group). Systemic infusion of isolated E27 scFv was used as a positive control.
Our results validate the idea that CAR-T cells can be used to deliver immune modulatory scFvs to the TME in the absence of systemic blockade. With the rapid development of human phage display libraries, it is feasible to expand this immune-modulating CAR-T cell approach by using scFvs targeting other molecules, such as LAG-3 (refs. 37,38), TIM-3 (ref. 39) or CLTA-4 (ref. 40), as well as combination strategies. Armored CAR-T cells for targeted delivery of immune modulatory scFvs to the TME may improve clinical outcomes of CAR T cell and checkpoint blockade therapies.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
S.R., O.Y., H.I.J. and R.J.B. designed the experiments, interpreted the results and wrote the manuscript. T.J.P., D.G.V., D.J.D., M.S., M.M.M., Z.L., P.W., S.Y. J.I.X., X.M., R.C.H. and C.L. designed, performed and/or analyzed the experiments. V.E.S. performed the statistical analysis.

COMPETING INTERESTS
R.J.B. is a co-founder and receives royalties from Juno Therapeutics. R.J.B., S.R., H.I.J., O.Y. and C.L. have submitted a patent related to this work.

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Selection of PD-1 blocking scFv. The RMP1-14 scFv was engineered from the RMP1-14 hybridoma. The PD-1 blocking scFv, termed E27, was identified from a human antibody scFv phage library (Eureka Therapeutics). The scFv library was screened for binding to human PD-1. Briefly, biotinylated PD-1-Fc fusion protein was mixed with the human scFv phage library. The antigen-scFv complexes were pulled down by streptavidin-conjugated beads and bound clones were eluted and transformed into bacteria. The binding and affinity of scFv to PD-1 was determined using ForteBio Octet QK (Pall Life Sciences). The ability of the scFv to block PD-1 from interacting with PD-L1 was determined using an ELISA assay. Briefly, PD-L1-Fc (R&D Systems), was loaded onto a plate. PD-1-Fc was mixed with dilutions of antibody derived from the E27 scFv clones or human IgG1 isotype control mAb (Eureka Therapeutics) and then added to the PD-L1-Fc-coated plate. Binding of the clones was determined by a standard ELISA method against biotinylated PD-1. Streptavidin-conjugated HRP (Vector Laboratories) was used to detect binding on an Epoch-2 microplate reader (BioTek Instruments).

Generation of retroviral vectors. The RMP1-14 scFv was cloned into the SFG gammaretroviral vector encoding CD19-directed or MUC16*CD3*-directed CARs with mouse signaling domains. The E27 scFv was cloned into the SFG-retroviral vector encoding the CD19-targeted CAR, termed 1928z, to generate SFG-1928z-E27 or DNA encoding the MUC16*CD3*-targeted CAR and E27 scFv, termed SFG-4H1128z-E27.

Cell culture. Retroviral producer cells were maintained in DMEM and human T cells were maintained in RPMI-1640 medium. Tumors and mouse T cells were maintained in RPMI-1640 medium supplemented with nonessential amino acids, sodium pyruvate, HEPEs (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer, and 2-Mercaptoethanol (all from Invitrogen). All media was supplemented with 10% FBS (Atlanta Biological Flowery Branch), 2 mM l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). Retroviral producer cell lines were generated by using CaPO4 (Promega) to transiently transfect H29 cells with retroviral constructs encoding the CAR or the CAR and the PD-1-blocking E27 scFv. Supernatant from the H29 cells was used to transduce Phoenix-ECO or 293Gly9 cells to produce stable retroviral producer cell lines. DNA encoding mouse and human PD-L1 and PD-1 was purchased (Integrated DNA Technologies) and separately cloned into the SFG retroviral backbone (SFG-hPD-L1, SFG-hPD-1). This was transiently transduced into H29 cells using CaPO4 transfection (Promega) and the H29 supernatant was used to transduce tumor or 293Gly9 packaging cells. Tumor cells lines were sorted by FACS based on high expression of PD-L1, following staining with PE-conjugated anti-PD-L1 (BD Biosciences, clone MH5). ID8 and SKOV3 cells were validated by karotyping and all cells were routinely checked for mycoplasma.

T cell isolation and modification. Human T cells were activated and transduced as described previously. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor peripheral blood or leukopacks (New York Blood Center). All experiments were performed in compliance with all relevant ethical regulations and in accordance with IRB 095091. PBMCs were activated with 2 µg/ml phytohemagglutinin and 100 IU/ml of IL-2 for 2 d before transduction. Mouse T cells were mechanically isolated from spleens and activated using IL-2 and 4 µg/ml of concanavalin A (Millipore Sigma). Transduction was achieved by centrifugation of activated PBMCs and retroviral supernatant on RetroNectin-coated plates on 3 consecutive days (TakaraBio).

Flow cytometry. Flow cytometry was used to determine the transduction efficiency of transduced cells following staining with Alexa-Fluor 647 conjugated anti-idiotypic antibodies that detect the CD19-targeted CAR (clone 19E3), 1928z, as well as the ovarian tumor-antigen targeted CAR (clone 22G3), 4H1128z (both generated at the Memorial Sloan Kettering Cancer Center Antibody and Bioresource Core Facility). The following antibodies were used in flow cytometry experiments: FITC conjugated anti-PD-1 (BD Biosciences, clone MH5, Cat557860 CD279 Mouse anti-Human, FITC), anti-mouse PD-1 (BD Biosciences, clone J43, Cat561788 CD279 Hamster anti-Mouse, PE), anti-mouse PD-L1 from BD Biosciences (clone MIH5, Cat558091), Mouse Anti-Human CD19 mAb (clone SJ25-C1), FITC conjugate, CatMHCD1901 from Invitrogen and Anti-Human CD3 eF640450, Cat48-0037-42 from eBioscience.

For analysis of syngeneic bystander in vivo experiment, the following antibodies and reagents were used for flow cytometry: CD45-PE (clone 30-F11, Cat103106), CD3-APC/Cy7 (clone 17A2, Cat100222), CD80-PE (clone 16-10A1, Cat104708), IFN-γ-FITC (clone XM1G2, Cat11-7311-41 from eBioclone), CD107α (LAMP-1)-PE-Cy7 (clone 1D4B, Cat121619), Granzyme B-FITC (clone GB11, Cat151403) and Zombie UV Fixable Viability Kit (Cat423107) were purchased from BioLegend. After 20 min incubation with Zombie UV Fixable stain at room temperature, all samples were washed with ice-cold BD FACS Buffer, and stained with the appropriate surface antibodies. Intracellular staining for IFN-γ and Granzyme B was performed according to Foxp3/Transcription Factor Staining Buffer set (eBioscience). Data acquisition was performed on FACSCalibur (BD Biosciences) and analyzed via FlowJo. For analysis intratumoral CD3+ CAR T cell levels were assessed with phenotypic criteria of CD45+CD3+CAR+, and total CD45+ cells were used as a common denominator.

Western blot analysis. Supernatant was collected and filtered from either 293Gly9 packaging cells or transduced T cells as indicated. To demonstrate E27 binding to PD-1, 293Gly9-PD-L1 packaging cells were incubated for 24 h in filtered supernatant from 1928z or 1928z-E27 transduced T cells. To demonstrate that E27 binds to untransduced, bystander cells, 1928z and 1928z-E27 T cells were co-cultured with human T cells transduced to overexpress PD-1, after 4 d stimulation with CD3/CD28 beads, the cells were sorted by flow cytometry following staining with 19E3 mAb to separate CAR+ and CAR- cells. Supernatant or whole cell lysates was loaded onto mini protein TGX gels (BioRad) and then transferred to Immobilon PVDF membranes (Bio-Rad). The was probed with mouse-anti-HA antibody (Cell Signaling,6E2, Cat23675s) and then goat anti-mouse HRP conjugated antibody (Millipore, GT X MS AP127 Cat60112) for E27. RPM1-14 was detected using a HRP-conjugated mouse-anti-myc tag antibody (Cell Signaling, Cat2040S). Detection of antibody was achieved with Pierce ECL western blot substrate (Thermo Scientific). RPM1-14 scFv co-immunoprecipitation was performed using the Peirce c-Myc Tag IP/Co-IP Kit (Thermo Scientific) according to the manufacturer's instructions and detected by western blot as described above.

NIH/3T3 expansion assay. NIH/3T3 (3T3-Empty) or NIH/3T3 cells transduced to express human PD-L1 (3T3-PDL1) were plated and transduced T cells were added and incubated for 24 h. CD3/CD28 beads (Invitrogen) were then added at a 1:2 bead:T cell ratio. Every 3 d, the T cells were moved to fresh 3T3 coated plates to ensure continuous stimulation with PD-L1. At 6 d, the T cells were debedded before being plated on fresh 3T3 cells, and left without beads for 24 h. The cells were then re-stimulated with CD3/CD28 beads at the same ratio (second stimulation). Trypan blue exclusion was used to enumerate cells following removal from 3T3 cells.

Tumor expansion assay. Transduced T cells were co-cultured with Raji-PDL1.1eGFP or NALM6-PDL1.1eGFP tumor cells at 1:1 tumor:CAR+ T-cell ratio. Following 72 h co-culture, flow cytometry was used to detect tumor and T cells following staining with anti-human CD19-FITC (Invitrogen). Cells were enumerated using 123 count ebeads (eBiosciences) according to the manufacturer's instructions.

In vivo experiments. All experiments were performed in compliance with all relevant ethical regulations and in accordance with an Institutional Animal Care and Use Committee-approved protocol (protocol 00-05-065).

For syngeneic survival experiments, C57BL/6 mice (Jackson Laboratory) of 6–8 weeks were inoculated i.p. with 10 x 10^6 ID8 tumor cells and treated 7 d later with 2 x 10^6 CAR-T cells, as indicated, with or without 250 µg RPM1-14 antibody (Bio X Cell). Mice were given RPM1-14 antibody i.p. on days 7, 10 and 14 post tumor inoculation, for a total of three doses.

For syngeneic bystander experiments, 6–8-week-old female C57BL/6 mice (Jackson Lab) were injected subcutaneously with 10^6 B16-F10 mouse.
melanomas were transduced to express the MUC16 cell surface antigen. On day 10 after tumor implantation, 2 × 10^6 CAR-T cells were injected intratumorally. The mice were sacrificed 48 h after T cell injection. Tumor cells were mechanically dissociated and digested in collagenase-based buffer. The samples were subsequently stained for flow cytometric analysis, as described above.

Fox Chase CB17 (CB17.Cg-Pkd1<sup>+/−</sup>Prkdc<sup>−/−</sup>/Crl), SCID/Beige mice (Charles River Laboratories) of 6–8 weeks of age were inoculated i.v. with 1 × 10^6 Raji-PDL1 or NALM6-PDL1 tumor cells. The following day, mice received one systemic infusion of 1 × 10^7 CAR <sup>T</sup> cells. For xenograft solid tumor studies, SCID/Beige mice were inoculated i.p. with 1 × 10^3 SKOV3-PDL1 cells and treated 7 d later with 5 × 10^6 total CAR <sup>T</sup> cells, as indicated, with or without anti-human PD-1 blocking antibody (clone EH12.2H7, BioLegend). Mice were given 250 μg of antibody i.p. on days 7, 10 and 14 post tumor inoculation, for a total of three doses.

The PCR primers used to detect CAR isolated from splenocytes or bone marrow span mouse CD3 and the gamma-retroviral SFG backbone and are as follows: 5′-AGAACCTAGAACCTCGCTGGAAAG 3′ and 5′-GTGCATTGTATACGGCTTCCTGGGGGT 3′.

Bioluminescent imaging of scFv-GluC or anti-human PD-1 antibody (clone EH12.2H7, BioLegend) labeled with VivoTag 680 XL Fluorochrome (PerkinElmer) in SCID/Beige mice i.p. inoculated with SKOV3-PDL1 and treated 7 d later i.p. with 5 × 10^7 scFv-secreting CAR-T cells or CAR-T cells with 250 μg of anti-human PD-1 antibody (clone EH12.2H7, BioLegend), was performed using Xenogen IVIS imaging system with Living Image software (Xenogen Biosciences). Image acquisition was done on a 25-cm field of view at medium binning level at various exposure times.

Targeted LC-MS/MS (PRM) analysis of scFv and mAb in serum. To quantitate the levels of the E27 scFv and anti-human PD-1 mAb (clone EH12.2H7, Bioregen) proteins in serum a bioinformatics search was used to identify peptide sequences unique to the therapeutic proteins but not found in other protein human or mouse proteome. We then quantitated those peptides using targeted parallel reaction monitoring (PRM) on a high resolution and accurate mass quadrupole-orbitrap mass spectrometer. To accomplish this goal, we first used Skyline 4.1, a freely available software tool (online at http://skyline.maccosslab.org). Tryptic peptides that contained cysteine or methionine residues were excluded and peptides that were from the complementarity-determining region of the therapeutics were prioritized. Two tryptic peptides, one from the scFv (FSGSNSGNTATLISR) and one from the anti-PD-1 mAb (FGSNLESGIPAR), were identified by searching (M+2H)<sup>2+</sup> = m/z 624.3226 and (M+2H)<sup>2+</sup> = m/z 806.8999, respectively, with 10 p.p.m. mass tolerance and retention time within 60 s. Peaks of y-type fragment ions from both peptides were identified by searching corresponding m/z values with the same criteria and retention time based on the analysis of the recombinant scFv or antibody. ICIS integration algorithm was used to detect and integrate the area of each peak. The summed areas of all peaks (6 y-type ions) associated with EH12/2H7 peptide and E27 scFv peptide (FSGSNSGNTALLTISR) and E27 scFv peptide (FSGSNLESGIPAR) were identified by searching (M+2H)<sup>2+</sup> = m/z 624.3226 and (M+2H)<sup>2+</sup> = m/z 806.8999, respectively, with 10 p.p.m. mass tolerance and retention time within 60 s. Peaks of y-type fragment ions from both peptides were identified by searching corresponding m/z values with the same criteria and retention time based on the analysis of the recombinant scFv or antibody. ICIS integration algorithm was used to detect and integrate the area of each peak. The summed areas of all peaks (6 y-type ions) associated with EH12/2H7 peptide and E27 scFv peptide from each time point (6, 24 and 48 h) were calculated separately then plotted by time point using Prism. Chromatograms of untreated and scFv i.v. samples were integrated and plotted using the same method.

Statistical analysis. Log-rank, unpaired or paired t tests were performed using GraphPad Prism 7 software where appropriate.

Life Sciences Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper (and its supplementary information files).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- n/a
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection
- Flow cytometric data was collected using Kaluza 1.2 for Gallios Acquisition Software.

Data analysis
- GraphPad Prism 7 is a graphing software that was used for figures and statistical analysis. FlowJo vX.07 was utilized to analyze flow cytometry results. Skyline 4.1 was utilized for the LC-MS/MS experiments

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Not applicable.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
No statistical methods were used to predetermine the sample size. Sample sizes for experiments were estimated based on previous experience with similar setups that showed significance. For in vitro mouse T cell experiments, experiments were repeated at least 3 separate times to control for technical replicates. For in vitro human T cell experiments, experiments were done with at least 3 different donors to account for biological replicates and repeated at least twice to account for technical replicates. The number of animals used in survival studies were based on past experience and as the variability is larger in survival data, the minimum number of mice required to obtain informative results were used.

**Data exclusions**
No data was excluded.

**Replication**
All attempts at replication were successful.

**Randomization**
Samples and organisms were randomly allocated to groups.

**Blinding**
Investigators were not blinded to study to account for appropriate handling and data acquisition.

Reporting for specific materials, systems and methods

### Materials & experimental systems

| n/a | Involved in the study |
|-----|----------------------|
| ☑   | Unique biological materials |
| ☑   | Anti-body |
| ☑   | Eukaryotic cell lines |
| ☑   | Cellekology |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |

### Methods

| n/a | Involved in the study |
|-----|----------------------|
| ☑   | Flow cytometry |
| ☑   | ChIP-seq |
| ☑   | MRI-based neuroimaging |

**Antibodies used**

Flow cytometry was used to determine the transduction efficiency of transduced cells following staining with Alexa-Fluor 647 conjugated anti-idiotypic antibodies that detect the CD19-targeted CAR (clone 19E3), 1928z, as well as the ovarian tumor-antigen targeted CAR (clone 22G3), 4H1128z (both generated at the Memorial Sloan Kettering Cancer Center Antibody and Bioresource Core Facility). The following antibodies were used in flow cytometry experiments: FITC conjugated anti-PD-1 (BD Biosciences, clone MH4, Cat#557860), CD279 Mouse anti-Human, FITC, anti-mouse PD-1 (BD Biosciences, clone J43, Cat#561788), CD279 Hamster anti-Mouse, PE, anti-mouse PD-L1 from BD Biosciences (clone M558091), Mouse Anti-Human CD19 mAb (clone SJ25-C1), FITC conjugate, Cat#MHCD1901 from Invitrogen and Anti-Human CD3 eFluor450, Cat#48-0037-42 from eBioscience.

For analysis of syngeneic bystander in vivo experiment, the following antibodies and reagents were used for flow cytometry: CD45-PE (clone 30-F11, Cat#103106), CD3-APC/Cy7 (clone 17A2, Cat#100222), CD80-PE (clone 16-10A1, Cat#104708), IFN-γ-FITC (clone XMG1.2, Cat#11-7311-41 from eBioscience), CD107a (LAMP-1)-PE-Cy7 (clone 1D4B, Cat#121619), Granzyme B-FITC (clone GB11, Cat#515403) and Zombie UV Fixable Viability Kit (Cat#423107) were purchased from BioLegend.

For Western blot analysis, the membrane was probed with mouse-anti-HA antibody (Cell Signaling,6E2, Cat#2367S) and then goat anti-mouse HRP conjugated antibody (Millipore, GT X MS AP127 Cat#600112) for E27. RMP1-14 was detected using a HRP-conjugated mouse-anti-myc tag antibody (Cell Signaling, Cat#2040S).

**Validation**

All flow cytometric antibodies were validated by titration using known negative and positive cell line. For Western blots, antibodies were validated by including positive control lysates.
### Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | All cell lines were sourced from the ATCC. |
|---------------------|------------------------------------------|
| Authentication      | ID8 and SKOV3 cell lines were authenticated by karyotyping. |
| Mycoplasma contamination | All cell lines tested negative for mycoplasma. |
| Commonly misidentified lines | ID8 and SKOV3 are commonly misidentified lines. However, we authenticated these lines by karyotyping. |

### Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research**

| Laboratory animals | 6-8 week old female C57BL/6 mice (The Jackson Laboratory) or Fox Chase CB17 (CB17.Cg-PrkdcscidLystbg-J/Crl, SCID/Beige mice (Charles River Laboratories) were used where indicated. |
|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Wild animals        | This study did not involve wild animals |
| Field-collected samples | This study did not involve samples collected in the field. |

### Human research participants

**Policy information about studies involving human research participants**

| Population characteristics | Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above." |
|-----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Recruitment                 | Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results. |

### Flow Cytometry

**Plots**

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

| Sample preparation | Please refer to page 14 and 15 of materials and methods in the manuscript. |
|--------------------|--------------------------------------------------------------------------|
| Instrument         | Please refer to page 14 and 15 of materials and methods in the manuscript. |
| Software           | Please refer to page 14 and 15 of materials and methods in the manuscript. |
| Cell population abundance | Please refer to materials and methods in the manuscript. |
| Gating strategy    | Please refer to materials and methods in the manuscript and supplemental figures. |

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.