Therapeutics, Targets, and Chemical Biology

Cancer Research

A Chimeric Switch-Receptor Targeting PD1 Augments the Efficacy of Second-Generation CAR T Cells in Advanced Solid Tumors

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Abstract

Chimeric antigen receptor (CAR)–modified adoptive T-cell therapy has been successfully applied to the treatment of hematologic malignancies, but faces many challenges in solid tumors. One major obstacle is the immune-suppressive effects induced in both naturally occurring and genetically modified tumor-infiltrating lymphocytes (TIL) by inhibitory receptors (IR), namely PD1. We hypothesized that interfering with PD1 signaling would augment CAR T-cell activity against solid tumors. To address this possibility, we introduced a genetically engineered switch receptor construct, comprising the truncated extracellular domain of PD1 and the transmembrane and cytoplasmic signaling domains of CD28, into CAR T cells. We tested the effect of this supplement, "PD1CD28," on human CAR T cells targeting aggressive models of human solid tumors expressing relevant tumor antigens. Treatment of mice bearing large, established solid tumors with PD1CD28 CAR T cells led to significant regression in tumor volume due to enhanced CAR TIL infiltrate, decreased susceptibility to tumor-induced hypofunction, and attenuation of IR expression compared with treatments with CAR T cells alone or PD1 antibodies. Taken together, our findings suggest that the application of PD1CD28 to boost CAR T-cell activity is efficacious against solid tumors via a variety of mechanisms, prompting clinical investigation of this potentially promising treatment modality. *Cancer Res; 76(6); 1578–90. ©2016 AACR.*

Introduction

Adoptive T-cell transfer (ATC) for cancer has demonstrated success in malignant melanoma and hematologic malignancies (1, 2). T cells were originally derived from tumor-infiltrating lymphocytes (TIL). More recently, engineering T cells with chimeric antigen receptors (CAR) or tumor-reactive T-cell receptor (TCR) clones has been used to produce tumor-reactive T cells. TCR engineering allows for the generation of tumor-reactive T cells that are able to process tumor-associated antigens (TAA) but require presentation in the MHC:antigen complex (3). CARs, on the other hand, confer high-affinity, high-specificity, MHC-independent recognition of surface TAAs with potent T-cell activation via genetic engineering and the combination of various costimulatory domains (4). Though CAR T cells have demonstrated significant responses in patients with treatment-refractory hematologic malignancies (5), they have resulted in, at best, only modest results in solid tumors. This is likely due to a host of hurdles encountered in the tumor microenvironment (TME) of solid tumors (6–12), including intrinsic inhibitory pathways mediated by upregulated inhibitory receptors (IR) reacting with their cognate ligands within the tumor (12).

One of the most extensively studied T-cell IRs is programmed death-1 (PD1; CD279). PD1 is a cell surface receptor that belongs to the immunoglobulin superfamily and is expressed on T cells and pro-B cells (13). Its expression is upregulated after antigen- and ligand-receptor engagement (14), and its currently known ligands are PDL1 (also known as B7-H1 or CD274) and PDL2 (also known as B7-DC or CD273). In the nonmalignant context, PD1 is responsible for preventing T-cell–mediated autoimmunity (15). In various cancers, however, PDL1 is upregulated on the surface of solid tumors, often in response to cytokines secreted by T cells that are tumor-reactive, and serves as a method of immune escape (10). In some studies, expression levels of PDL1 have been shown to correlate with the degree of tumor immune infiltration (16), decreased function of T-cell infiltrates (17), tumor aggressiveness (18), and overall patient prognosis (19). PD1 blockade is being tested as a novel immunotherapeutic in different cancers and has demonstrated durable clinical responses in a subpopulation of patients (20).

Our recent description of solid tumor-induced hypofunction of CAR T cells demonstrated the contribution of PD1 upregulation on tumor-infiltrating CAR T cells (21), and supports the strategy

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of combining adoptive transfer of genetically redirected human T cells with blockade of inhibitory signals triggered by IRs. Herein, we demonstrated that combining CAR-based ATC with IR interference is superior in tumor control than either alone.

We first demonstrated this by using anti-PD1 antibodies in combination with CAR T cells, followed by a genetic approach described by others (22–24) in which T cells were transduced with both a CAR and a chimeric switch-receptor containing the extracellular domain of PD1 fused to the transmembrane and cytoplasmic domain of the costimulatory molecule CD28. We confirmed in our own tumor targets that when the PD1 portion of this switch-receptor engages its ligand, PDL1, it will transmit an activating signal (via the CD28 cytoplasmic domain) instead of the inhibitory signal normally transduced by the PD1 cytoplasmic domain. But more importantly, we demonstrated for the first time that PD1CD28 is able to augment human CAR T-cell control of large, established solid tumors. This is done using human T cells targeting human tumors bearing clinically relevant tumor antigens. Furthermore, we built upon prior work elucidating multiple mechanisms of PD1CD28's function and also showed that while PD1 blockade augments the antitumor efficacy of CAR T cells, the use of CAR T cells expressing PD1CD28 was far superior in controlling tumor burden.

Materials and Methods

Cell lines and cell culture conditions

A human mesothelioma cell line derived from a patient's tumor (March 2010) was used—EMP (parental). Because EMP did not have baseline expression of the TAA mesothelin, it was lentivirally transduced to express human mesothelin (EMMESO). GFP with firefly luciferase was lentivirally transduced into the lines to produce EMPffluc and EMMESOffluc.

Nalm6 is a B-cell precursor leukemia with high expression of CD19 (German DSMZ Cell Collection Cat#: ACC 128). Click beetle red (CBG) was lentivirally transduced into Nalm6 to produce Nalm6-CBG.

K562 is a chronic myelogenous leukemia (ATCC; Cat#: CCL-243). CD19 was lentivirally transduced into K562 to produce K562-CD19.

PC3 is a prostate cancer tumor line (ATCC; Cat#: CRL-1435). Prostate-specific cancer antigen (PSCA) was lentivirally transduced into PC3 to produce PC3-PSCA. CBG was lentivirally transduced into PC3-PSCA to produce PC3-PSCA-CBG.

Nalm6, K562, and PC3 cell lines were purchased from the ATCC and authenticated, and cultured as instructed.

All tumor cell lines used expressed low levels of PDL1 in the absence of IFNγ exposure. Thus, PDL1 was also lentivirally transduced into the aforementioned lines to produce versions that had high stable expression of PDL1.

Tumor cells and T cells were cultured in RPMI 1640 (Gibco 11875-085) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 1% L-glutamine.

Generation of CAR constructs and PD1CD28 switch-receptor

CARs specific for CD19 (CD19Z with CD3ζ signaling), mesothelin (SS1BBz with CD3ζ signaling and 41BB costimulation), and PSCA (PSCA-BBz with CD3ζ signaling and 41BB costimulation) were synthesized and/or amplified by PCR, based on sequencing information provided by the relevant publications (25–28), and subcloned into pGEM.64A RNA-based vector (29), pTRPE lentiviral vectors (25), and MSGV retroviral vectors, respectively (Supplementary Fig. S1; ref. 27).

The PD1CD28 switch-receptor was constructed by fusing a truncated extracellular PD1 (AA1-155) derived from PD1-cDNA (Origene) with the transmembrane and cytoplasmic domains of CD28 (AA141-220). We also constructed a mutated version of the switch-receptor where signaling was abrogated by modifying the CD28 signaling transduction proximal YMNM motif (mutated to FFFF) and distal proline-rich motifs PRRP (mutated to ARRA) and PYAP (mutated to AYAA). We also constructed a "tailless" version of PD1 where the truncated extracellular PD1 and the PD1 transmembrane domain were included, but the signaling domains (ITIM or ITSM) were excluded (Supplementary Fig. S2).

The PD1CD28 switch-receptor was subcloned into the viral vectors upstream of a T2A/F2A sequence that was followed by the SS1BBz or the PSCA-BBz CAR, respectively (Supplementary Fig. S2A and S2B).

Isolation, bead activation, transduction, and expansion of primary human T lymphocytes

Isolation, bead activation, transduction, and expansion of primary human T lymphocytes were conducted as previously described (21).

mRNA electroporation and retroviral/lentiviral transduction of human T cells undergoing CD3/CD28 Dynabead activation have been previously described (25, 27, 29).

In-vitro T-cell and *ex-vivo* TIL effector assays

T-cell and TIL effector assays assessing tumor lytic ability and cytokine secretion ability were conducted as previously described (21).

Antibodies

For details, see Supplementary Methods.

In-vivo xenograft experiments

Using different tumor cells injected subcutaneously (5×10^6 EMMESO, 1×10^6 PC3-PSCA-PDL1, or 1×10^6 PC3-PSCA per mouse), *in-vivo* experiments were conducted as previously described (21).

Groups contained 10 mice each. The *in-vivo* experiments were repeated three times in independent fashion.

Animals

For details, see Supplementary Methods.

Statistical analysis

For details, see Supplementary Methods.

Results

Human T cells electroporated with mCD19Z CAR and PD1CD28 demonstrate enhanced killing and cytokine secretion

Activated human T cells were successfully electroporated with mRNAs encoding: (1) CD19Z CAR alone, (2) CD19Z CAR plus an intact PD1 (inhibitory) construct, or (3) the CD19Z CAR plus PD1CD28 as measured by FACS (Fig. 1A).

The T cells demonstrated dose-dependent killing when cocultured with Nalm6 cells at E:T ratios of 0.5:1 to 15:1 (Fig. 1B, left

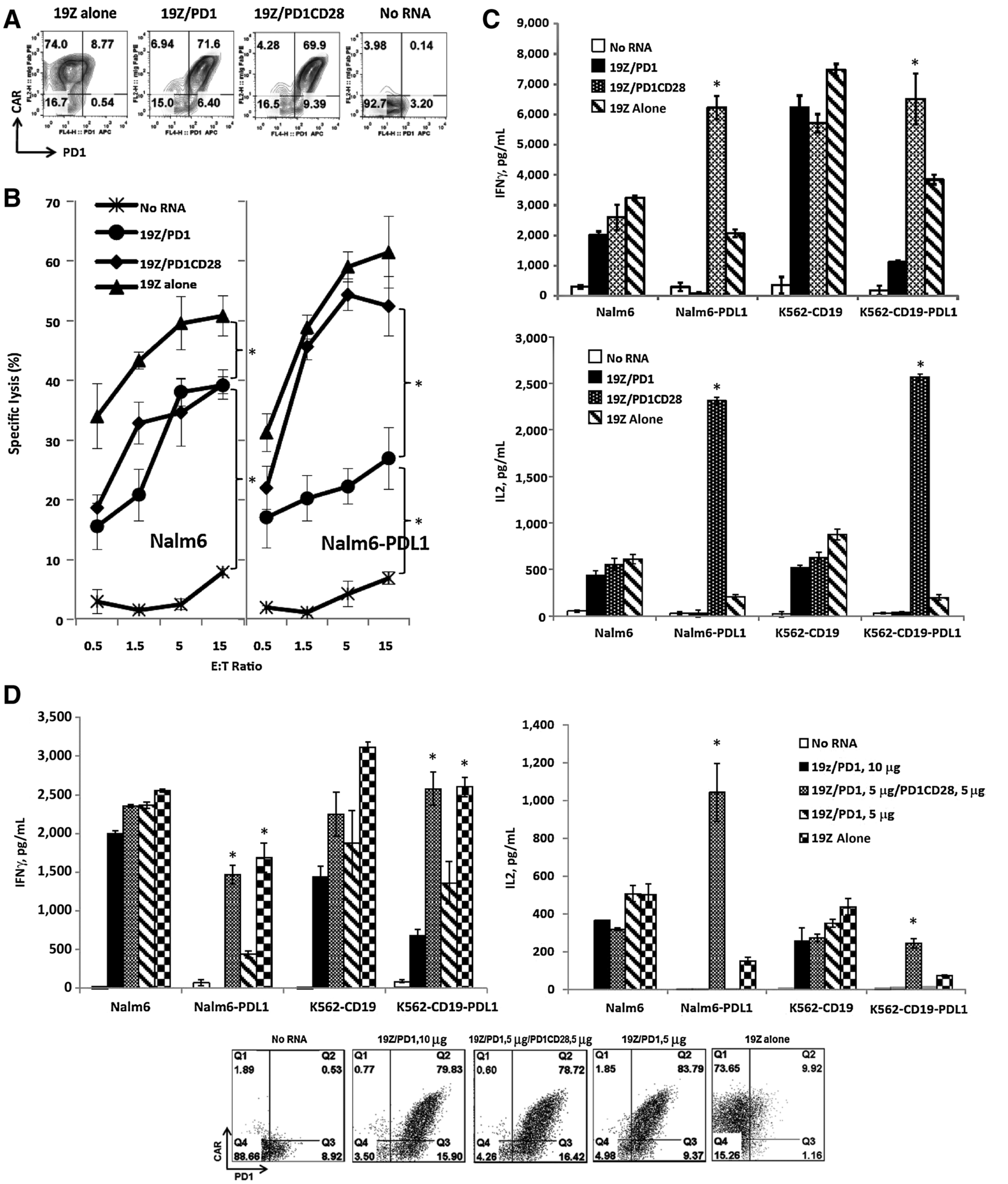


Figure 1.

Increased cytokine production of T cells coexpressing 19Z CAR and PD1CD28 switch-receptor via mRNA electroporation. A, FACS analysis of T cells 1 day after electroporation with no mRNA or mRNA for CD19-ζ alone (19Z alone), coelectroporated with CD19-ζ and PD1 (19Z/PD1) or CD19-ζ and PD1CD28 switch-receptor (19Z/PD1CD28). The CAR expression was detected using an anti-mouse IgG Fab antibody, PD1 or PD1CD28 were detected with anti-PD1 antibody.
B, T cells were tested for their cytolytic activity at indicated E:T ratios for 8 hours against Nalm6 (left) or Nalm6-PDL1 (right). The results shown are the averages of three independent experiments. C, the T cells were also cocultured with indicated tumor cell lines for 24 hours for ELISA cytokine secretion measurement in culture supernatants. Bar graphs show results from a representative experiment (values represent the average ± SE of triplicates) for IFNγ (top) and IL2 (bottom). D, T cells were coelectroporated with 10 μg 19Z mRNA and 5 μg PD1 mRNA (19Z/PD1, 5 μg), with 5 μg PD1CD28 mRNA (19Z/PD1, 5 μg/PD1CD28), or 5 μg PD1 (19Z/PD1, 10 μg) as indicated. 19z alone and no RNA served as controls. One day after the electroporation, the T cells were analyzed by FACS to confirm expression (dot plots) and were cocultured with indicated tumor cell lines for 24 hours. Cytokine secretion was measured by ELISA analysis of culture supernatants.
Bar graph shows results from a representative experiment (values represent the average ± SD of triplicates) for IFNγ (left) and IL2 (right).

column). Killing by 19Z/PD1 and 19Z/PD1CD28 was similar, but slightly lower at each ratio compared with 19Z T cells. However, when the cells were cocultured with Nalm6-PDL1 cells, the killing efficacy of the 19Z/PD1 T cells was diminished, while the killing efficacy of the 19Z/PD1CD28 T cells was increased (Fig. 1B, right column).

The amount of cytokine produced by CAR T cells was similar after coculture with Nalm6 at 1:1 E:T ratio for 24 hours; however, when cocultured with Nalm6-PDL1 cells, 19Z/PD1CD28 CAR T cells generated significantly higher amounts of IFNγ and IL2 (Fig. 1C, left columns; $P < 0.01$) when compared with 19Z and 19Z/PD1 T cells. We extended our findings to K562-19 and K562-19-PDL1 cell lines (Fig. 1C, right columns).

The levels of endogenous PD1 on the T cells used in the experiments above were relatively low. To determine if PD1 upregulation (as seen in hypofunctional TILs) would affect the efficacy of the switch-receptor, we electroporated T cells with PD1 or PD1 plus PD1CD28 (Fig. 1D, dot plots), and cocultured them with PDL1-expressing target cells. As expected, lower levels of IFNγ and IL2 were produced by 19Z/PD1 T cells (Fig. 1D, striped and solid bars) compared with 19Z T cells (Fig. 1D, checkered bars). However, 19Z/PD1/PD1CD28-electroporated T cells continued to produce IFNγ and IL2 (Fig. 1D, gray bars), indicating that the PD1CD28 switch-receptor augmented T-cell cytokine production even in the presence of high levels of inhibitory PD1.

Overall, these data indicate that in the CD19Z mRNA CAR system, addition of the PD1CD28 switch-receptor can convert inhibitory signals, as induced by PDL1, into stimulatory signals, therefore resulting in increased tumor killing and cytokine production.

Human T cells retrovirally transduced with PSCA-BBZ CAR and PD1CD28 demonstrate CD28 signaling domain-dependent enhancement of cytokine secretion

To distinguish between a dominant-negative effect on PD1 offered by the switch-receptor versus its CD28 activating signal, PD1CD28 was compared with the mutated PD1CD28m. To generalize our findings and in anticipation of *in-vivo* studies, we conducted studies using T cells retrovirally transduced with PSCA-BBz CAR (Fig. 2A) targeting PC3-PSCA with and without PDL1 (Fig. 2B).

PSCA-BBz/PD1CD28 T cells generated more IFNγ and IL2 upon coculture with PC3-PSCA-PDL1 cells than PSCA-BBZ T cells ($P < 0.05$). However, mutated CD28 abrogated this effect, as the PSCA-BBZ/PD1CD28m T cells produced similar levels of IFNγ and IL2 as the PSCA-BBZ cells (Fig. 2C).

Human T cells lentivirally transduced with SS1BBZ CAR and PD1CD28 demonstrate enhanced tumor killing and cytokine secretion

We conducted similar studies using human T cells transduced with a second-generation ant-mesothelin CAR with (SS1BBz) and without the PD1CD28 switch-receptor (SS1BBZ/PD1CD28; Fig. 3A, dot plot) and cocultured them with mesothelin and PDL1-expressing tumor lines (Fig. 3A, histograms). Similarly as above, when exposed to EMMESO cells, both types of T cells released equivalent amounts of cytokines (Fig. 3B and C, left columns). In contrast, when exposed to EMMESO-PDL1 cells, the SS1BBZ/PD1CD28 T cells exhibited much greater secretion of IFNγ ($P < 0.05$; Fig. 3B, right columns) and especially IL2 ($P < 0.01$; Fig. 3C, right columns) at all ratios compared with

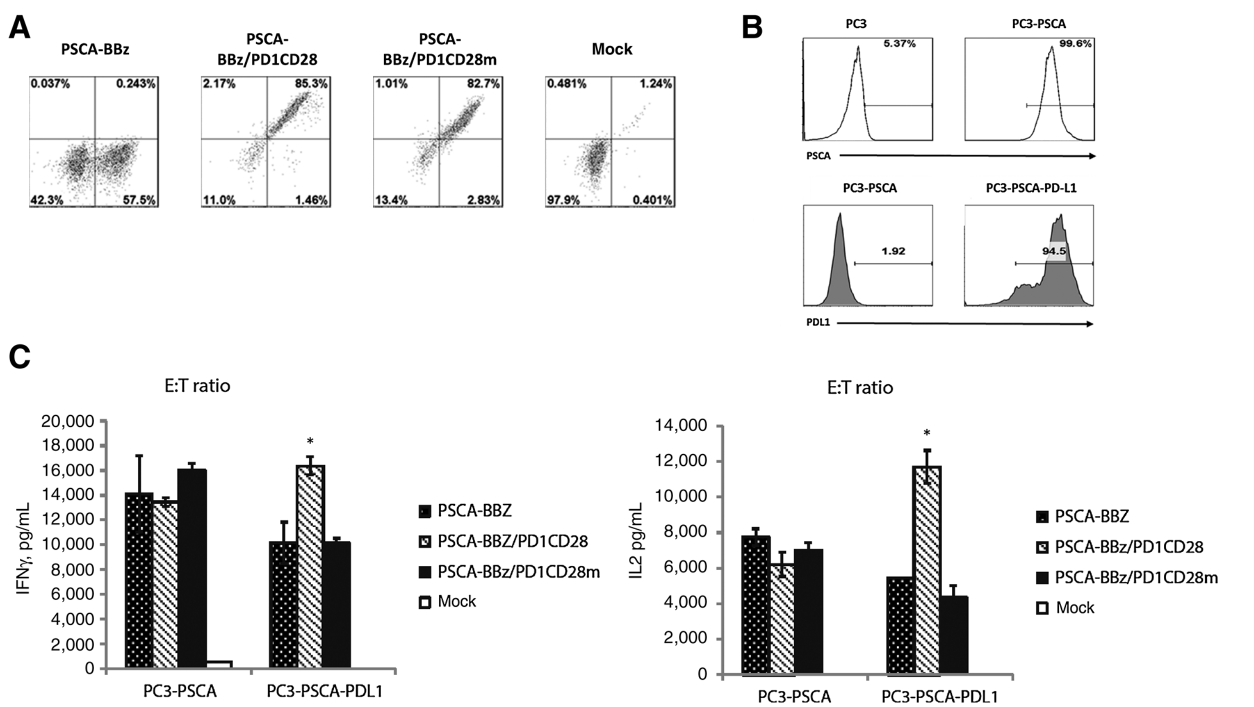


Figure 2.

PD1CD28-induced enhanced cytokine secretion is dependent on CD28 signaling upon PDL1 binding. T cells were retrovirally transduced with either PSCA-BBz alone, PSCA-BBz with PD1CD28 switch-receptor (PSCA-BBz/PD1CD28), or PSCA-BBz with mutated PD1CD28 switch-receptor (PSCA-BBz/PD1CD28m). After confirming successful expression of the transgenes (A), T cells were cocultured with PC3-PSCA or PC3-PSCA-PDL1 target cells (B) at an E:T ratio of 10:1 for 24 hours. C, levels of secreted IFNγ and IL2 were measured by ELISA. Mock-transduced T cells (mock) served as control.

SS1BBZ cells. SS1BBZ/PD1CD28 T cells demonstrated similar 18-hour lytic activity against EMMESO compared with SS1BBZ T cells at all E:T ratios (Fig. 3D, top graph). When cocultured with EMMESO-PDL1 target cells, SS1BBZ/PD1CD28 T cells consistently demonstrated superior tumor killing compared with SS1BBZ T cells at all E:T ratios (66% vs. 37% at 10:1, $P < 0.05$; 51% vs. 22% at 5:1, $P < 0.05$, 22% vs. 0% at 1:1, $P < 0.01$; Fig. 3D, bottom graph; Supplementary Fig. S3).

PD1CD28 augments CAR T-cell antitumor activity beyond PD1 antibody blockade in animal models of solid tumor growth

We evaluated the effect of PD1CD28 in two independent *in vivo* model systems, the EMMESO mesothelioma tumor model using SS1BBz CAR T cells and the PC3 prostate cancer model using PSCA BBz CAR T cells (both CAR T cell types are currently or soon will be in clinical trials). To further explore the mechanism, we also studied the effect of an anti-PD1 antibody (pembrolizumab) in the EMMESO model, and PD1CD28m in the PC3 model.

EMMESO flank tumor-bearing NSG mice were treated with a single dose of 1×10^7 mock-transduced (mock) or SS1BBZ CAR T cells i.v. ± i.p. administration of pembrolizumab (10 mg/kg every 5 days; Fig. 4A). Mice injected with mock T cells or with pembrolizumab alone grew at similar rates. Treatment with pembrolizumab + mock T cells did not result in significant reduction of tumor volume. Treatment with SS1BBZ CAR resulted in a marked and significant slowing of tumor growth (1,340 mm^3 in mock vs. 562 mm^3 in SS1BBZ at day 34, $P < 0.01$).

The addition of pembrolizumab treatment to the SS1BBZ CAR group resulted in a modest tumor inhibition (422 vs. 552 mm^3, $P < 0.05$). Notably, the strongest antitumor effect was seen in the SS1BBZ/PD1CD28-treated group (552 mm^3 in SS1BBz vs. 147 mm^3 in SS1BBZ/PD1CD28, $P < 0.05$) where some tumors actually regressed.

PD1CD28 potentiates CAR T-cell expansion in EMMESO tumors

At the end of our *in-vivo* study, TIL analysis revealed that of the tumor digests, mock T-cells made up <5%; SS1BBZ T cells, 47%; and SS1BBz+Ab T cells, 58%. SS1BBZ/PD1CD28-treated mice exhibited the greatest degree of T-cell infiltration in the tumor

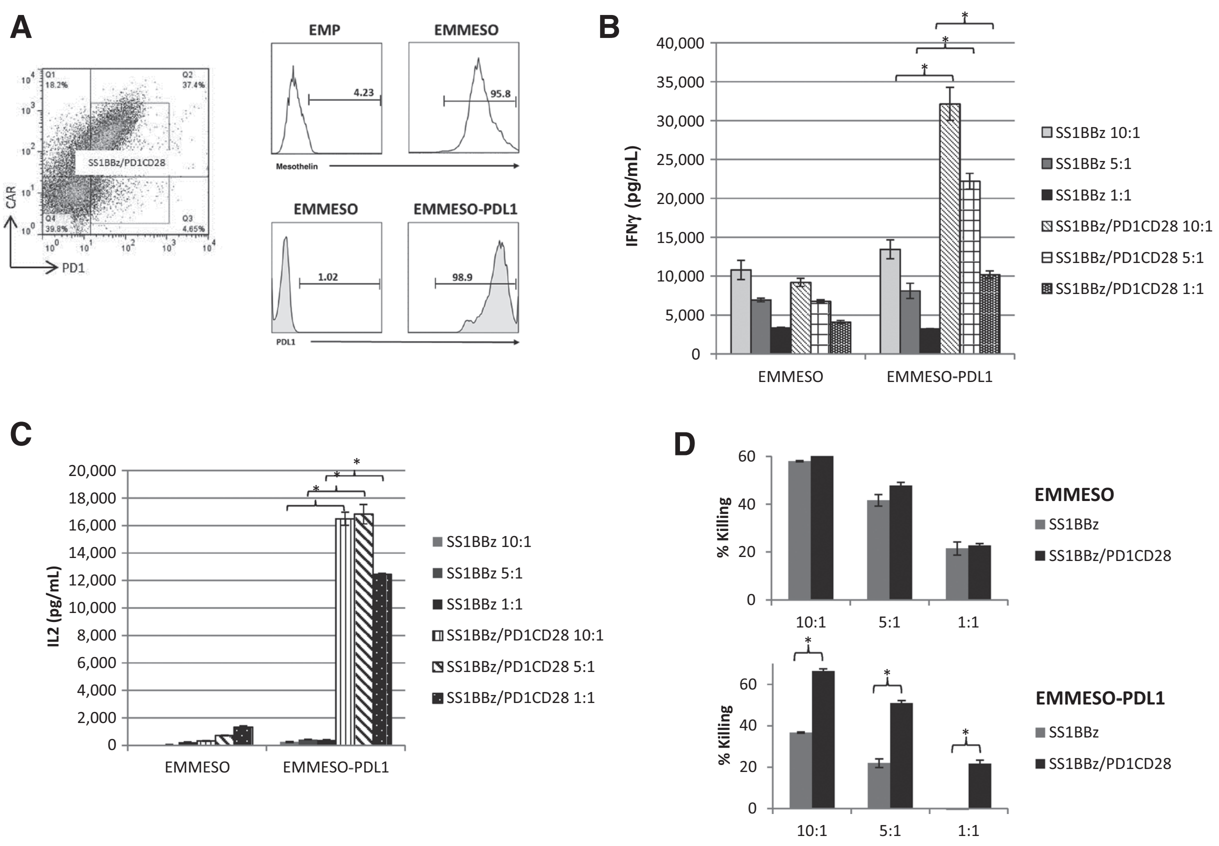


Figure 3.

Increased cytokine production by T cells coexpressing SS1BBz CAR and PD1CD28 switch-receptor. A, FACS analysis of CD4 and CD8 bulk T cells activated via anti-CD3/CD28 microbeads at a ratio of 3:1 bead:T cell and transduced with lentivirus led to successful coexpression of both SS1BBz CAR and PD1CD28 switch-receptor (~40%). The CAR expression was detected using an anti-mouse IgG Fab antibody, and PD1CD28 was detected with anti-PD1 antibody (dot plot). FACS analysis of EMP, EMMESO, and EMMESO-PDL1 tumor cells was performed to confirm high expression of mesothelin and PDL1 (histograms). B and C, T cells coexpressing SS1BBz and PD1CD28 (SS1BBz/PD1CD28) or SS1BBz alone (SS1BBz) were cocultured with EMMESO or EMMESO-PDL1, at different E:T ratios × 18 hours. ELISA was performed to measure the levels of IFNγ (B) and IL2 (C) present in the supernatants of the cocultures. D, percent-specific lysis of both EMMESO and EMMESO-PDL1 by SS1BBz and SS1BBz/PD1CD28 was also calculated after 18 hours of coculture. Bar charts show results from a representative experiment (values represent the average ± SE of triplicates).

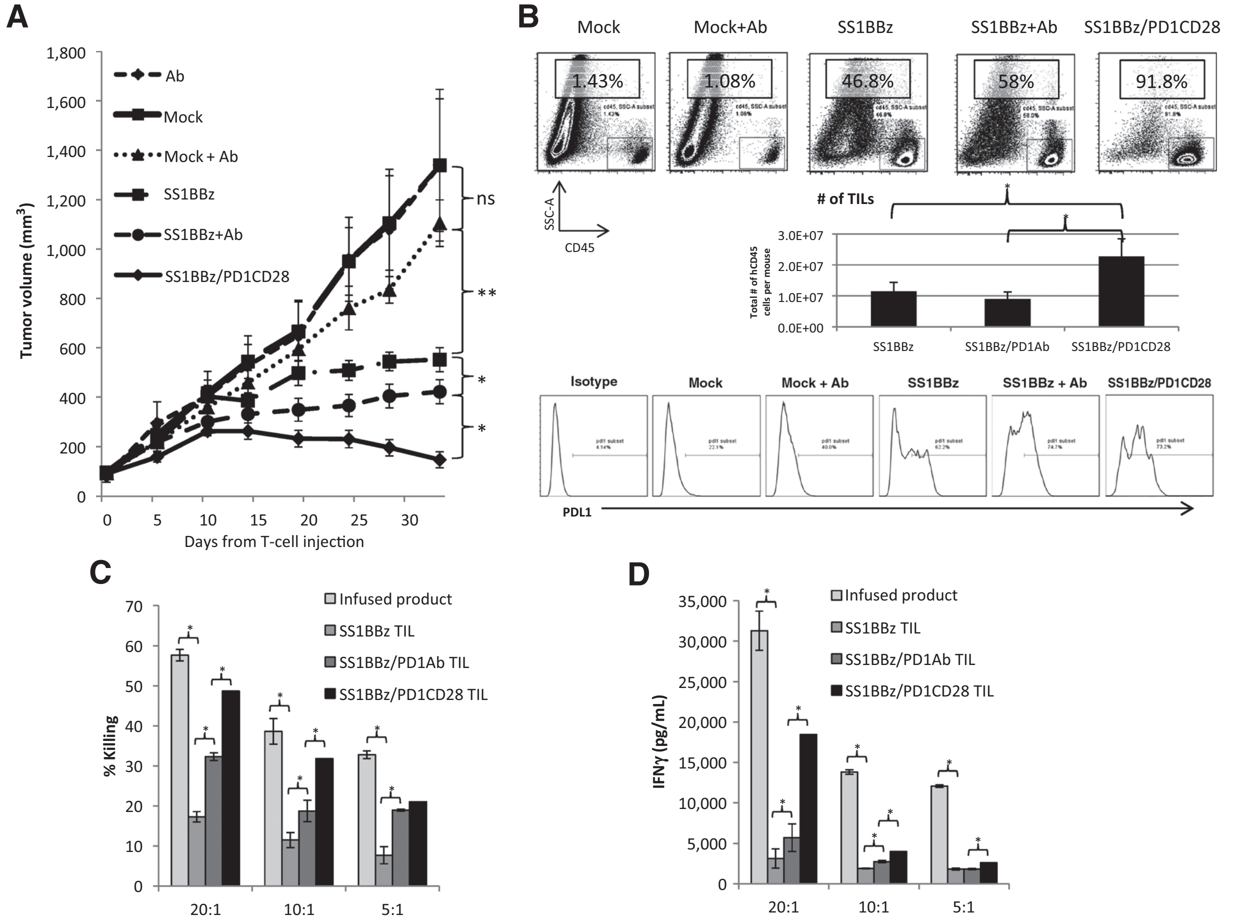


Figure 4.

In-vivo and *ex-vivo* antitumor function of SS1BBz T cells augmented by anti-PD1 antibody blockade and PD1CD28 modification. When EMMESO tumors injected in the flank of mice ($n = 10$/group; 2×10^6 cells/mouse, s.c.) reached an average volume of approximately 100 mm^3, mice were randomly assigned to six groups and were injected with either anti-PD1 Ab alone (Ab), 1×10^7 mock transduced T cells (mock), 1×10^7 mock T cells and anti-PD1 Ab (mock + Ab), 1×10^7 SS1BBz T cells (SS1BBz), 1×10^7 SS1BBz T cells and anti-PD1 Ab (SS1BBz + Ab), or 1×10^7 SS1BBz T cells modified with PD1CD28 switch-receptor (SS1BBz/PD1CD28). T cells were injected once intravenously, and antibody was injected at a dose of 10 mg/kg/mouse every 5 days intraperitoneally. Flank tumors were measured by calipers every 5 days. Values represent the average flank tumor volume ± SE of measurements of 10 mice/group. A, approximately 35 days after treatment initiation, flank tumors were harvested, digested, and processed into single-cell suspension. A portion of the cells was stained with CD45 antibody to assess T-cell infiltration via FACS analysis. Dot plots are FACS analysis from a representative experiment. Bar graphs represent average absolute number of CD45$^+$ cells in tumors per mouse ± SE. Histograms represent PDL1 staining on the live, CD45$^-$ events in the tumor digest from a representative experiment. B, the rest of the cells in each group were pooled and were subjected to CD45-positive isolation via magnetic beads and were cocultured at 20:1, 10:1, and 5:1 E:T ratios with EMMESO tumor targets × 18 hours. C and D, specific lysis (C) and IFNγ measurement by ELISA (D) of TILs from each group were measured in comparison with uninjected cryopreserved control SS1BBz T cells (infused product). Bar graph values represent the average values ± SE of measurements from triplicates.

(92%; Fig. 4B, dot plots). To address the potential confounding variable of different tumor sizes, absolute numbers of TILs were calculated and confirmed that a greater number of TILs was present in the SS1BBZ/PD1CD28-treated mice (Fig. 4B, bar graph). Analysis of PDL1 expression on tumor cells revealed upregulation of PDL1 expression with treatment (Fig. 4B, histograms).

PD1CD28 preserves tumor lytic activity and cytokine secretion of CAR TILs

TILs and T cells frozen at time of injection (infused product) were exposed to freshly cultured tumor cells *ex vivo* at varying E:T ratios to assess killing and cytokine release. Freshly isolated TILs show marked decrements in tumor lytic activity and IFNγ release (Fig. 4C and D, infused product vs. SS1BBZ TIL bars). However, compared with the SS1BBz TILs, the SS1BBz+Ab TILs showed significantly enhanced TIL function ($P < 0.05$). Importantly, SS1BBZ/PD1CD28 TILs exhibited significantly ($P < 0.01$) greater lytic and cytokine-producing ability than either of the other types of TILs (Fig. 4C and D; Supplementary Fig. S4)

PD1CD28 attenuates upregulation of IRs on EMMESO-infiltrated TILs

Compared with infused T cells, we observed significant upregulation of PD1 and LAG3 expression on SS1BBZ TILs. The percentage of CD8 T cells expressing PD1 increased from 0.06% to 41% (Fig. 5, 1st row/1st dot plot versus 2nd row/1st dot plot). SS1BBZ+Ab TILs had no detectable PD1 staining,

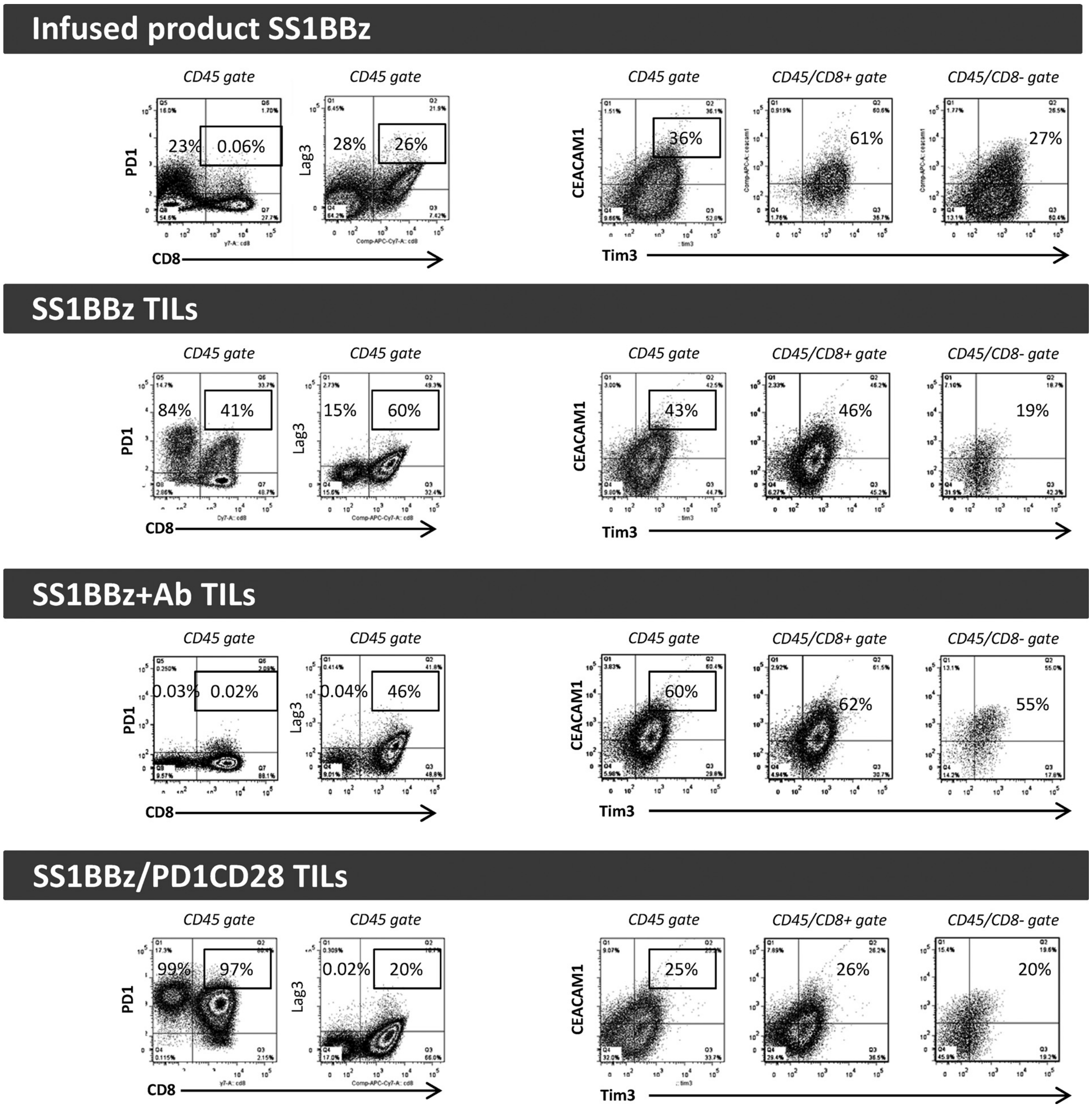


Figure 5.

PD1CD28 leads to reduced upregulation of IRs on SS1BBz TILS. Single-cell suspension from digested tumors was subjected to FACS analysis to measure expression of PD1, Lag3, Tim3, and CEACAM1. The first three dot plots of each row represent analyses of cells in the $CD45^+$ gate. The fourth and fifth dot plots of each row represent analyses of cells in the $CD45^+/CD8^+$ and $CD45^+/CD8^-$ gates, respectively. The highlighted percentages represent frequency of $PD1^+$ events among $CD8^+$ events (the first two dot plots) and the frequency of Tim3/CEACAM1 double-positive events (the last three dot plots). Each row demonstrates representative analysis of TILs from three independent animal experiments. Infused product SS1BBz T cells were used as control comparisons for baseline levels of IRs prior to adoptive transfer into mice.

confirming adequate exposure of T cells to the pembrolizumab throughout the experiment (Fig. 5; 3rd row/1st dot plot). Almost all SS1BBZ/PD1CD28 TILs expressed PD1, reflecting both PD1 upregulation and enrichment of gene-modified T cells (Fig. 5, 4th row/1st dot plot). The percentage of CD8 T cells expressing LAG3 increased from 26% to 60% (Fig. 5, 1st row/2nd dot plot vs. 2nd row/2nd dot plot), but decreased to 46% when SS1BBZ TILs were combined with pembrolizumab (Fig. 5, 3rd row/2nd dot plot), and decreased even further to 20% in the SS1BBZ/PD1CD28 TILs (Fig. 5, 4th row/2nd dot plot). The percentage of infused product SS1BBZ T cells coexpressing TIM3 and CEACAM1 was 36%, but increased to 43% in the SS1BBZ TILs (Fig. 5, 1st row/3rd dot plot vs. 2nd row/3rd dot plot). It was further increased to 60% in SS1BBZ+Ab TILs (Fig. 5, 3rd row/3rd dot plot). SS1BBZ/PD1CD28 TILs had the lowest TIM3/CEACAM1 expression, at 25% (Fig. 5, 4th row/3rd dot plot).

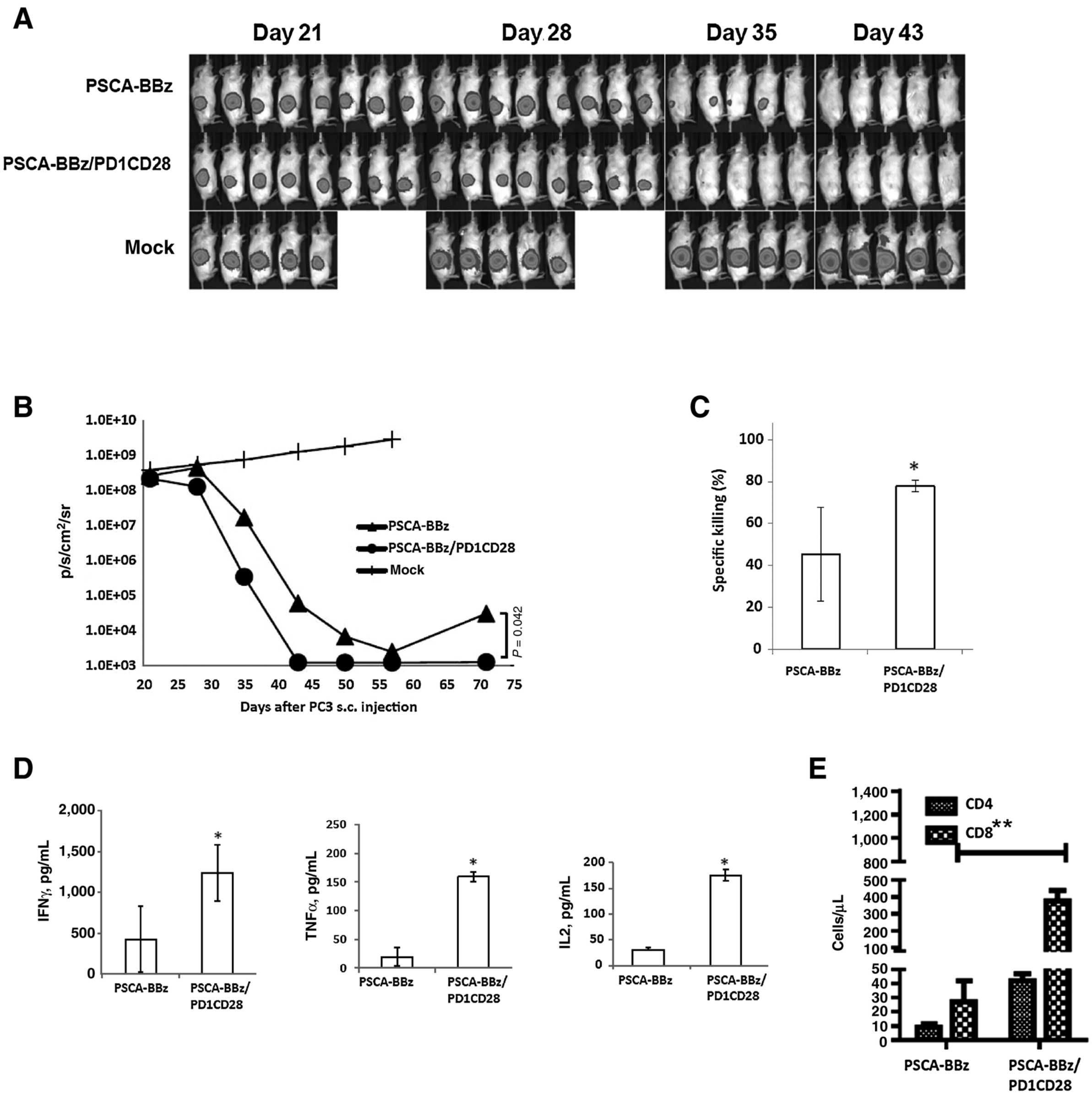


Figure 6.

PD1CD28 improves the therapeutic effect of PSCA-BBz T cells against advanced vascularized PC3-PSCA tumors in mice. T cells modified with PSCA-BBz alone or PSCA-BBz with PD1-CD28 (PSCA-BBz/PD1CD28) by retroviral transduction were tested in PC3-PSCA-CBG engrafted NSG mice. Mice ($n = 5$–8) were implanted with PC3-PSCA-CBG tumor cells (1×10^6 cells/mouse, s.c.) on the right flank on day 0. The mice were treated with 2×10^6 T cells (i.v.) at day 23 after tumor inoculation. Mock transduced T cells (mock) served as control. A and B, animals were imaged at the indicated times after tumor inoculation. Thirty-four days after tumor inoculation, three mice from each treatment group were sacrificed, and TILs were isolated. C, an aliquot of freshly purified TILs was used in a killing assay using PC3-PSCA-CBG as target cells at E:T ratio of 5:1. D, levels of secreted IFNγ, TNFα, and IL2 were measured in the supernatants by ELISA. E, thirty-five days after tumor inoculation, blood drawn from the remaining mice (5 mice/group) was subjected to FACS True-Count staining to detect human CD4 and CD8 T cells.

PD1CD28 augments *in-vivo* tumor control of PC3 flank tumors

To ascertain the generalizability of these findings, we also assessed PD1CD28 efficacy in mice bearing PSCA-expressing PC3 tumors. Tumor-bearing NSG mice were treated i.v. with 2×10^6 PSCA-BBZ±PD1CD28 CAR T cells or mock T cells. Tumors were tracked with bioluminescence imaging (Fig. 6A and B). The greatest antitumor effects were seen with PSCA-BBZ/PD1CD28 T-cell administration (day 35, $P = 0.05$; day 43, $P = 0.05$; day 70, $P = 0.042$; Fig. 6B). Although a few tumors escaped by day 70 in the PSCA-BBZ group, all remaining animals in the PSCA-BBZ/PD1CD28 group remained cured ($P < 0.05$; Fig. 6B).

PSCA-BBZ/PD1CD28 TILs demonstrated greater infiltration, *ex-vivo* killing ability, and cytokine secretion than PSCA-BBZ CAR TILs

When TILs were harvested from flank tumors at the end of the experiment, and cocultured with fresh PC3-PSCA tumors cells at a

5:1 E:T ratio, PSCA-BBZ/PD1CD28 TILs demonstrated greater tumor lysis than PSCA CAR TILs ($P = 0.05$; Fig. 6C).

Compared with PSCA-BBZ TILs, PSCA-BBZ/PD1CD28 TILs showed significantly greater ($P < 0.05$) secretion of IL2, TNFα, and IFNγ after overnight TIL coculture with PC3-PSCA cells (Fig. 6D).

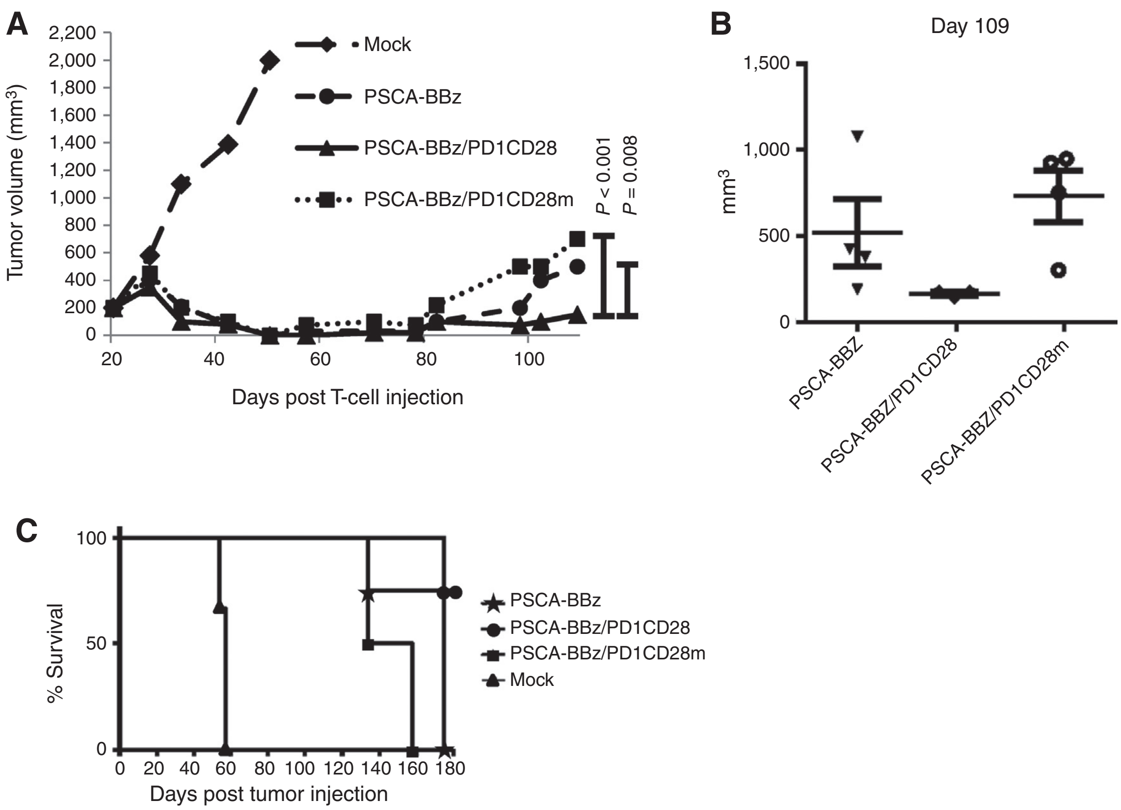
Analysis of blood from mice at 35 days after tumor inoculation using flow cytometry Tru-Count staining revealed greater number of CD8 T cells/ μl in the PSCA-BBZ/PD1CD28 group compared with the PSCA-BBZ group ($P < 0.01$; Fig. 6E).

The signaling motif of PD1CD28 is critical to augment PSCA-BBZ CAR T-cell control of PC3-PSCA tumor growth

After demonstrating the abrogation of PD1CD28-induced cytokine secretion by mutating the CD28 signaling motif (as described above), we compared the effects of PSCA-BBZ, PSCA-BBZ/PD1CD28, and PSCA-BBZ/PD1CD28m T cells at a dose of 2×10^6 T cells/mouse in PSCA-PC3-PDL1 flank tumor-bearing NSG mice. All T-cell types induced marked tumor regressions. However, at about 80 days after treatment, the tumors treated with the PSCA-BBZ T cells began to recur, and by day 109 reached an average size of approximately 600 mm^3 (Fig. 7A and B). The size of the tumors treated with PSCA-BBZ/PD1CD28 T cells was significantly smaller (180 mm^3; $P < 0.008$) than both other groups at day 109 (Fig. 7B). Interestingly, though the PSCA-BBZ/PD1CD28m-treated tumors initially regressed early in the study, they eventually rebounded and were just as large as the PSCA-BBZ–treated tumors by day 109 (Fig. 7A and B); we observed similar trends in terms of mortality in both groups (Fig. 7C).

Discussion

The potential efficacy of tumor immunotherapy utilizing the adoptive transfer of T cells has changed dramatically with the



	PSCA-BBz/PD1CD28	PSCA-BBz/PD1CD28m	Mock
PSCA-BBz	$P = 0.0379$	$P = 0.0725$	$P = 0.014$
PSCA-BBz/PD1CD28		$P = 0.0084$	$P = 0.014$
PSCA-BBz/PD1CD28m			$P = 0.014$

Figure 7.

The enhanced antitumor effect offered by PD1CD28 is dependent on CD28 signaling induced by PDL1 binding. T cells retrovirally transduced with either PSCA-BBz alone, PSCA-BBz with PD1CD28 switch-receptor (PSCA-BBz/PD1CD28), or PSCA-BBz together with mutated PD1CD28 switch-receptor in which the CD28 signaling transduction proximal YMNM motif and distal proline-rich motifs PRRP and PYAP were mutated to FFFF, ARRA, and AYAA, respectively (PSCA-BBz/PD1CD28m), were tested in PC3-PSCA-PDL1 tumor engrafted NSG mice. Mice ($n = 5$) were implanted in the flanks with PC3-PSCA-PD-L1 tumor cells (1×10^6 cells/mouse, s.c.) and were treated with T cells (i.v.) at day 23 after tumor inoculation. T cells were given as a single injection of 2×10^6/mouse. Injection of mock T cells served as control. A and B, tumor sizes were measured, and the tumor volume was calculated and plotted. C, the percentage survival per group was determined on a daily basis and is represented in a Kaplan–Meier survival curve.

introduction of CARs (30). However, the responses seen in hematologic malignancies have not yet been reflected in efforts against solid tumors (31). One primary reason is the tumor-induced hypofunction of TILs that has been described by multiple research groups in both humans and murine models (32–39). This hypofunction seems, in large part, due to the upregulation of IRs (40).

PDL1:PD1 interaction contributes to the suppression of effector T-cell function and clonal deletion with the goal of maintaining immune tolerance (41). However, tumors appear to take advantage of this pathway and express PDL1, presenting a substantial hurdle for adoptive T-cell immunotherapeutic strategies (42).

Using a unique *in-vivo* model of adoptively transferred human CAR T cells targeting human solid tumors, we have recently demonstrated tumor-induced CAR TIL hypofunction associated with PD1 upregulation similar to that described in naturally occurring TILs (21). Based on that observation, we set out to interrupt PD1 signaling in combination with adoptive CAR T-cell therapy. PD1 blockade using antibodies has already demonstrated promising responses in early clinical trials of melanoma, lung cancer, and other malignancies (20). The utility of checkpoint blockade in tumors, which lack sufficient infiltration of immune cells that bear tumor reactivity at baseline, is questionable. Thus, we and others have hypothesized that the combination of adoptively transferring genetically augmented tumor-reactive T cells with checkpoint interference could be a promising immunotherapeutic strategy for solid tumors. Specifically, it would provide PDL1-resistant, tumor-reactive T cells in cases where tumors express high levels of ligand but lack sufficient immune infiltration.

Prosser and colleagues initially introduced the PD1CD28 switch-receptor. Upon binding of PDL1, T cells showed increased levels of ERK phosphorylation, cytokine secretion, proliferation, and granzyme B expression (24). Subsequently, Ankri and colleagues were able to demonstrate enhanced antitumor function using PD1CD28 T cells in two somewhat artificial *in-vivo* models of very early melanoma formation—a chicken embryo chorioallantoic membrane model (CAM), and a WINN assay where T cells and tumors cells were mixed together and then injected into athymic nude-Foxn1nu mice (22). A primary goal of our study was to test the effects of PD1CD28 on CAR-engineered T cells injected to treat large, established, solid tumors that are more clinically relevant. We also wanted to compare the effects of PD1CD28 to PD1 antibody blockade (an immunotherapy strategy already in the clinic) and to dominant-negative receptor strategies.

In the context of different tumor cell lines, PD1CD28 expression enhanced cytokine secretion (particularly IL2) by T cells modified with CARs targeting mesothelin-expressing targets and CD19-expressing targets in a PDL1-dependent manner. SS1BBZ/PD1CD28 T cells secreted >30-fold more IL2 than SS1BBZ when cocultured with EMMESO-PDL1 cells. 19Z/PD1CD28 T cells secreted >10-fold more IL2 than CD19Z T cells when cocultured with Nalm6-PDL1 or K562-CD19-PDL1 cells.

PD1CD28 also demonstrated modestly increased killing of PDL1-transduced tumor targets in short-term *in-vitro* killing assays. More importantly, PD1CD28 led to significantly enhanced tumor control by CAR T cells (as measured by both bioluminescent imaging and caliper assessment) in two different xenograft models of established, large tumors (EMMESO, a human pleural mesothelioma cell line, and PC3-PSCA, a human prostate cancer cell line). By bioluminescence measurements, a faster onset of tumor regression and greater long-term tumor control were seen in mice treated with PSCA-BBZ/PD1CD28 T cells compared with those treated with of PSCA-BBZ T cells (Fig. 6). When PC3-PSCA flank tumors expressing high levels of PDL1 were targeted, PSCA-BBZ/PD1CD28 T cells demonstrated statistically significant augmentation of tumor control over PSCA-BBZ T cells (Fig. 7).

There are a number of likely mechanisms for this enhanced effect, many of which we are demonstrating for the first time. First, the PD1CD28 switch-receptor led to a significant increase in frequency of viable TILs as was observed in both the peripheral blood of mice bearing PC3-PSCA flank tumors 15 days after T-cell transfer, and in the tumor digests from mice bearing EMMESO flank tumors 34 days after T-cell transfer. Although not known for certain, we speculate this was due to a combination of enhanced survival and enhanced proliferation due to the higher levels of IL2 that were likely present at the tumor site. This enhanced antitumor effect was likely not seen to the same degree in *in-vitro* testing due to the relatively short period of time of assessment (18–24 hours). Second, the CAR/PD1CD28 TILs were able to retain more antitumor function and cytokine secretion ability compared with CAR TIL counterparts as measured by *ex-vivo* killing of freshly plated tumor targets. PD1CD28-expressing CAR TILs demonstrated greater killing of tumor cells and enhanced ability to secrete cytokines in response to both PC3-PSCA cells and EMMESO cells compared with nonexpressing CAR TILs upon fresh isolation from flank tumors. Furthermore, SS1BBZ/PD1CD28 TILs had greater *ex-vivo* antitumor function than SS1BBZ TILs from mice in the SS1BBZ+Ab group.

We hypothesized that there were at least two ways in which our switch-receptor is able to exert its effects: (i) the receptor functions as a dominant-negative receptor, engaging the PDL1 present on tumor and myeloid cells and sequestering it from the intact inhibitory PD1 receptor on the T cells; and (ii) the switch-receptor was actively signaling through the CD28 cytoplasmic domain after engagement with PDL1.

A number of pieces of data suggest that active signaling played the more important role. First, PD1CD28 augmented CAR T-cell antitumor function to a greater degree than pembrolizumab. Intraperitoneal injections of pembrolizumab every 5 days were able to augment tumor control by SS1BBZ CAR T cells by approximately 24% reduction in tumor size, whereas modification with PD1CD28 demonstrated a much greater augmentation in efficacy (~72% reduction in tumor size.) Second, to more carefully look at the role of signaling, we constructed a mutated, signal-dead version of PD1CD28 (PD1CD28m)—essentially a dominant-negative receptor. Intravenous injection of a single low dose (2×10^6 T cells/mouse) of PSCA-BBZ/PD1CD28m CAR T cells resulted in the same antitumor activity (as measured by tumor volume, bioluminescence, and survival) as the PSCA-BBZ CAR T cells when tested in NSG mice bearing PC3-PSCA-PDL1 flank tumors. This was in contrast with the significantly greater tumor control seen in mice injected with active PSCA-BBZ/PD1CD28 CAR T cells. This supports the conclusion that PD1CD28 is primarily exerting its enhancing effect through the CD28 costimulatory signal. When T cells were injected at a higher dose (10×10^6 T cells/mouse), we actually saw a decrease in the antitumor activity of PSCA-BBZ CAR T cells expressing PD1CD28m as measured by tumor volume and survival (data not shown). One explanation for this observation is that PD1 binding to PDL1 interferes with the antitumor activity of

CAR T cells independently of PD1 signaling via its cytoplasmic motifs, which is supported by data recently published (43). Yokosuka and colleagues demonstrated that PD1 can form microclusters that interfere with TCR synapse formation, independent of PD1's signaling via tyrosine motifs. Consistent with this mechanism, we have preliminary data showing that SS1BBZ T cells expressing a truncated, signal-dead PD1 (PD1tailless) injected into NSG mice bearing EMMESO flank tumors demonstrated significantly worse tumor control compared with SS1BBZ T cells (Supplementary Fig. S5). Studies are currently under way to test whether the effect demonstrated by Yokosuka and colleagues also takes place in CAR synapse formation.

We also identified another potential mechanism for enhanced T-cell function in PD1CD28-expressing cells. We conducted detailed FACS analysis to assess whether interference of PD1 signaling affected the expression pattern of other known IRs. This effort was in light of a growing body of literature describing the coexpression of multiple IRs in hypofunctional T cells (44–48), as well as our own published data demonstrating upregulation of PD1, TIM3, and LAG3 on human CAR TILs in human solid tumor (21). Analysis of TILs from our SS1BBZ/EMMESO experiment revealed upregulation of PD1, TIM3, and LAG3 on SS1BBZ TILs compared with the infused SS1BBZ cryopreserved T cells. We also found that CEACAM1, a cell adhesion molecule shown to endow TIM3 with inhibitory function (49), was coexpressed with TIM3 to a much greater extent on TILs than infused T cells. Pembrolizumab decreased the percentage of $LAG3^+$ CD8 TILs; however, there was a compensatory upregulation of $TIM3^+$/$CEACAM1^+$ TILs with antibody blockade. In contrast, modifying SS1BBZ CAR T cells with PD1CD28 led to reduction in both LAG3 expression and TIM3/CEACAM1 coexpression. This phenomenon, i.e., PD1CD28 allowing adoptively transferred T cells to circumvent inhibition by IRs other than PD1, was also demonstrated in a murine study looking at CTLA-4 (50). Further investigations to understand the underlying mechanisms are planned, but one leading hypothesis is that the PD1CD28 modified TILs that are exposed to significantly higher levels of IL2 represent "younger" T cells whose chronicity of activation and exposure to the TME is substantially less than their unmodified counterparts.

An additional theoretical advantage of PD1CD28 is the ability to introduce third-generation CAR signaling in a more targeted and safe fashion. In lieu of reports of toxicity using T cells bearing third-generation CARs with multiple costimulatory domains (i.e., CD3ζ, 4-1BB, and CD28; ref. 51), the majority of clinical trials testing adoptively transferred CAR T cells are using second-generation chimeric constructs that signal via CD3ζ plus 4-1BB or CD28, not both. However, thus far, the transfer of T cells bearing second-generation CARs has led to mixed results in trials involving solid tumor (52). Utilizing T cells modified with both CARs engineered with second-generation signaling and chimeric switch-receptors that provide an additional costimulatory signal, but only when triggered by checkpoint ligands expressed in the tumor microenvironment (EMMESO upregulates PDL1 in response to T-cell activity *in vivo*), would offer the maximum T-cell activation signal but only in the locale of the tumor, potentially avoiding systemic toxicity.

One deliberate decision we made in our study was to exclusively study human T cells. We chose this approach due to the large differences that we and others have observed in the behavior of murine versus human adoptively transferred T cells. Compared with transduced human T cells, transduced murine T cells (i) are much more sensitive to activation-induced cell death, (ii) have a much lower *in-vitro* and *in-vivo* proliferative potential, (iii) often require IL2 *in vivo*, and (iv) have much shorter persistence after injection into mice (in our experience only 7–10 days). Given the translational intent of our study, it seemed critical to study the behavior of the chimeric switch-receptor in a model where we have shown that T-cells proliferate, persist, and undergo hypofunction like naturally occurring TILs, thus requiring us to use human T cells. As a result of this approach, we acknowledge that a potential criticism of our study is that our immunodeficient mice cannot take into account the contributions of other potentially important cell types. As examples, endogenous myeloid derived suppressor cells (MDSC) might have high levels of PDL1 that could interact with T cells (53), or the increased levels of IL2 secretion by cells expressing the switch-receptor might increase the frequency of T-regulatory cells (54). These questions have been addressed to some extent in a recent study in which a murine version of similar switch-receptor was transduced into mouse OT1 cells and showed increased efficacy (23). The actual effects of MDSC and regulatory T cells on the switch-receptor–transduced human T cells in patients will need to await clinical trials.

In summary, this study demonstrates the ability to augment CAR T-cells targeting advanced solid tumors by coexpressing a chimeric PD1CD28 switch-receptor. More so, we have significantly built on prior PD1CD28 studies by (i) extending studies to human T cells, (ii) evaluating human T cells in large, established human tumors bearing clinically relevant tumor antigens, (iii) elucidating multiple new mechanistic pathways through which the switch-receptor augments human CAR T cells, and (iv) demonstrating a more potent effect of PD1CD28 on CAR T cells than currently available antibody-based PD1 blockade. Finally, the PD1CD28 switch-receptor offers a potential way to deliver second-generation CAR T cells with more potent third-generation activation turned on specifically within the immunosuppressive TME.

Disclosure of Potential Conflicts of Interest

C.H. June reports receiving commercial research grant from Novartis and has ownership interest (including patents) in the University of Pennsylvania. Y. Zhao reports receiving commercial research grant from Novartis and has ownership interest (including patents) in intellectual property and patents in the field of cell and gene therapy. No potential conflicts of interest were disclosed by the other authors.

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