Enhancement of PSMA-Directed CAR Adoptive Immunotherapy by PD-1/PD-L1 Blockade

Inna Serganova,1,4,5 Ekaterina Moroz,1,4,5 Ivan Cohen,2,5 Maxim Moroz,3,4 Mayuresh Mane,1,4 Juan Zurita,3,4 Larissa Shenker,3,4 Vladimir Ponomarev,3,4 and Ronald Blasberg1,3,4

1Department of Neurology, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA; 2Gerstner Sloan Kettering Graduate School of Biomedical Sciences, New York, NY 10065, USA; 3Department of Radiology, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA; 4Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in American men.1,2 Although hormonal and radiation therapy can be very effective for local disease, patients usually become refractory to hormonal therapy (castration resistant) within 1–3 years. This is usually associated with the transition to a more aggressive form of the disease, leading to the development of bone and organ metastases. The addition of combination chemotherapy in the late stages of the disease has limited benefit, increasing survival for only several months.3 Since most conventional treatments eventually fail, additional therapeutic strategies have been developed.4 These therapeutic strategies have employed oncolytic viruses,5 vaccines,6 adjuvant immune modulation therapies (checkpoint inhibitors) (Slovin et al., 2012, J. Clin. Oncol, abstract), and adoptive immune cell (T cell) therapies.7

During the past 15 years, genetic engineering has been applied to more effectively direct T cells to tumor-expressing antigens, through the expression of specific chimeric antigen receptors (CARs) on an individual patient’s T cells.8–10 CARs consist of a tumor antigen-binding domain that is fused to an intracellular signaling domain and costimulatory receptors capable of activating T cells.10–12 Therefore, antigen-recognition is not MHC-restricted, as is the case for T cell receptor (TCR)-mediated antigen recognition. In vivo efficacy of CAR-modified effector human and murine T cells has been demonstrated,13–22 and prostate-specific membrane antigen (PSMA) is a promising molecular marker for targeted therapy of prostate cancer. PSMA is a glycosylated type-II membrane protein that is upregulated during malignant transformation in more aggressive prostate cancer, resulting in abnormally high levels of it on the cell surface.23 We24,25 and others21,26–28 (Slovin et al., 2012, J. Clin. Oncol, abstract) have participated in developing imaging approaches to visualize anti-hPSMA CAR T cells and optimization of the structure of CARs to achieve more profound effects in the targeting of tumors bearing the corresponding antigen (hPSMA).

CAR T cell therapy in solid tumors has not achieved the clinical success that has been observed in hematologic malignancies.29–31 One...
The failure of a substantial CAR-mediated T cell response in solid tumors relates to a number of factors—including CAR T cell inactivation and possible exclusion from the tumor mass, the reciprocal interactions between tumor and stromal cells, and propensity of cancer like prostate to disseminate preferentially to bone. Thus, preclinical studies that incorporate imaging to monitor T cell trafficking and activation are necessary to adequately explore the biology and efficacy of different treatment strategies designed to enhance T cell targeting and penetration of solid tumors. Several strategies have been employed to optimize the migration, survival, and effector functions of adoptive cell therapy (ACT) using TILs (tumor-infiltrating lymphocytes). For example, (1) increasing CXCR2 expression resulted in the improvement of T cell migration to tumors; (2) genetic manipulation of IL-12 production by transferred T cells extends T cell survival; and (3) the generation of novel CAR-based engineered T cells to improve tumor recognition and T cell activation, as well as the delivery of CAR T cells through specific polymer implants or local injection. Recently, it was demonstrated that CAR T cell therapy and programmed cell death protein 1 (PD-1) checkpoint blockade are a rational combination in a solid tumor model.

To generate an appropriate murine model for the anti-hPSMA CAR T cell therapy, wild-type (WT) Myc-CaP cancer cells were stably transduced with a hPSMA-containing retroviral vector and sorted for our initial studies. Myc-CaP tumors are composed of sheets and indistinct lobules/nests of polygonal to oval cells separated by fine fibrovascular straie, as described previously. Tumor cells have abundant amphophilic cytoplasm and large, round to polygonal nuclei. Necrosis is rare in small tumors; larger tumors have more extensive necrosis. The percentage of necrosis varied from 2% to 25%, depending on the overall tumor mass (Figure 1).

**RESULTS**

Characterization of a New Murine Tumor Model for hPSMA-Directed CAR T Cell Therapy

Several prostate tumor models, both human and murine origins, have been described and used to study the targeting of hPSMA-CAR T cells, but some limitations have been identified. We focused on murine cell lines, with prospective to utilize them in a syngeneic mouse model using immunocompetent mice. We chose a well established and studied murine prostate cancer cell line, Myc-CaP, for our initial studies. Myc-CaP tumors are positive for PD-L1 expression. The possibility that PD-L1/PD1-mediated T cell inhibition might be involved in immune evasion in prostate cancer. Although only 1% PD-L1 cells were detected in prostate tumor samples, immune cells (PD-1- and PD-L1-positive) were observed to surround prostate tumor nodules. More recently, a novel monoclonal rabbit antibody to PD-L1 revealed that 62% human prostate tumors stain positive for PD-L1 expression. The possibility that PD-L1/PD1-mediated T cell inhibition might be involved in immune evasion in prostate cancer is being explored in several clinical trials. Patients with metastatic castration-resistant prostate cancer (mCRPC) are being treated with anti-PD-1 antibody in two phase II clinical trials, involving pembrolizumab (Keytruda) and CT-011 (anti-PD-1 antibody; CureTech).

The goal of this study was to study the efficacy of human hPSMA CAR-directed T cell therapy in an appropriate animal model. We show that anti-hPSMA CAR-directed T cell therapy of Myc-CaP:PSMA(+) tumors alone was unsuccessful, whereas the combination of anti-hPSMA CAR-directed T cells plus anti-hPD1 mAb immune modulation therapy provided a partial, short-duration and sub-optimal response.
anti-hPSMA CAR Plg28x vector and with SFG-tdRFP/CBLR luc vector. Reproducible levels of hPSMA CAR expression on transduced human T cells were observed (Figure S1B). Cytotoxicity studies revealed that hPSMA targeted CAR T cells have a 13.8 ± 5.9% cytolytic efficacy against Myc-CaPPhPSMA(+) cells, when the ratio of the effector to the target is 40 to 1. However, the same hPSMA-targeted CAR T cells showed 2-fold higher cytotoxic activity toward a human prostate cancer cell line PC3/PSMA-IRESPuromycin (PC3-PIP) (Figure S1C).

Bioluminescence Imaging of Anti-hPSMA CAR T Cell Targeting Myc-CaP:hPSMA(+) Tumors
Using two distinct luciferase reporter systems, we were able to image the location of Myc-CaP tumors with the Renilla luciferase (Rluc) reporter and image the trafficking of anti-hPSMA CAR T cells with the tdRFP/CBLR luc reporter. First, we assessed the capacity of systemically administered anti-hPSMA CAR T cells to traffic and accumulate within established subcutaneous Myc-CaP:hPSMA(+) tumors. When tumors reached ~50–100 mm$^3$ size, mice were injected intravenously with $2 \times 10^6$ anti-hPSMA CAR T cells (~87% of T cells had anti-hPSMA CAR expression, and ~67% of CAR-positive T cells were also positive for the tdRFP/CBLR fusion reporter). Bioluminescence imaging (BLI) following i.v. injection of anti-hPSMA CAR T cells was performed on 0, 1, 5, 9, and 12 days after administration. Initially, CAR T cells sequestered in the lungs for up to 9–10 days, as visualized by BLI (Figure S2A). Their presence in lungs was confirmed by CD3$^+$ immunohistochemistry (IHC) staining (Figure S2B). A minimal BLI signal was observed within Myc-CaPPhPSMA(+) tumors during the first 48 hr, suggesting that some CAR T cells trafficked to the tumor. However, only a minimal (non-statistically-significant) response of anti-hPSMA CAR T cell therapy was observed on tumor growth/volume, compared with control (no CAR-T cells treated) tumors (Figure S2C).

To further evaluate in vivo therapeutic efficacy of anti-hPSMA CAR-transduced T cells, we performed “pre-targeting” experiments using the Winn assay (Figure 2). Anti-hPSMA CAR T cells alone or mixed with Myc-CaPPhPSMA(+) or Myc-CaPPhPSMA(−) target cells were prepared just prior to subcutaneous inoculation into NOD.SCID II2rg−/− (NSG) mice; the inoculum included a 1:10 tumor-to-T cell ratio, mixed in growth media and Matrigel Matrix (1:1) at 4°C. The location of the tumor was identified using Renilla Luciferase BLI (Figure 2A). We also monitored tumor growth by BLI (Figure 2C) (in addition to caliper measurements; Figure 2D) until the point where the tumors reached a size where the BLI Renilla Luciferase signal was saturated and tumors showed evidence of necrosis (day 15; Figure 2C).

Progressive tumor growth was observed in the two “control groups” of mice: (1) mice injected with Myc-CaPPhPSMA(+) tumors alone (without CAR T cells) and (2) Myc-CaPPhPSMA(−) tumors (without hPSMA expression) but mixed with anti-hPSMA CAR T cells (Figures 2A, 2C, 2D). A marked reduction in tumor growth rate was observed in the “test group” of mice: tumors that developed from the mixture of Myc-CaPPhPSMA(+) cells and anti-hPSMA CAR T cells (Figures 2A, 2C, 2D). By day 26, the “test group” tumor size was 224 ± 240 mm$^3$, whereas the size of control tumors without anti-hPSMA CAR T cell inclusion was significantly larger, 1,676 ± 406 mm$^3$ (p < 0.05). The “pre-targeting” Winn assay experiments clearly demonstrate that anti-hPSMA CAR T cells can inhibit Myc-CaPPhPSMA(+) tumor growth and that this inhibition is hPSMA dependent.

The persistence of anti-hPSMA-targeted CAR T cells in the same Winn assay described above; T cells were monitored by the CBLR luciferin BLI signal generated from the reporter-transduced CAR T cells (Figures 2B and 2E). We observed that anti-hPSMA CAR T cells injected alone in Matrigel matrix (control, in the absence of any tumor cells) were localized at the site of injection and were visualized over 8 days. However, the presence Myc-CaP tumor cells (with or without human PSMA/antigen) notably influenced the BLI signal intensity at the injection site. Since the intensity of the BLI signal has been used as indicator of the injected CAR T cells, the difference in BLI signal suggested that the survival of CAR T cells was different in the three study groups. The BLI signal from CAR T cells was visualized longest (~8 days) when they were injected alone (non-tumor group), less in the hPSMA(−) tumor group (~6 days), and least in the hPSMA(+) tumor group (~4 days). These data suggest that BLI detection and the survival of CAR T cells was shortened by the presence of hPSMA(+) on tumor cells. Interestingly, this group demonstrated a better treatment response (Figure 2D).

Metabolism of hPSMA CAR T Cells
It is known that T cells undergo a transition from a quiescent to a highly active effector phenotype upon stimulation/activation and transduction with the anti-hPSMA CAR vector. This transition also involves a significant shift from oxidative to glycolytic metabolism. To study the metabolic status of T cells during activation, transduction, and proliferation, we measured the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) of these cells. The rate of glycolysis (ECAR), as well as basal and maximal OCRs (an indicator of mitochondrial respiration), was assessed on days 2, 8, 10, and 15 of CAR T cell preparation (Figure 3). Human T cells from three donors were isolated from buffy coat using ficoll separation. Forty-eight hours after phytohemagglutinin (PHA) stimulation, cells were transduced with a retroviral vector bearing a CAR targeting hPSMA. We obtained a measure of ECAR in naive T cells, T cells stimulated by PHA and hPSMA CAR-transduced T cells (Figure 3A). Naive non-stimulated T cells demonstrated low levels of glycolysis, whereas non-specific PHA stimulation of T cells results in a significant (p < 0.05) increase in glycolysis, that is maintained at high levels over the course of T cell transduction and expansion (Figure 3A). The initial increase in a basal mitochondrial respiration after non-specific PHA stimulation is followed by a progressive decline in mitochondrial function during subsequent procedures (Figure 3B). FCCP (carboxyl cyanide p-trifluoromethoxy phenylhydrazozone) was added to uncouple oxidative phosphorylation from the electron transport chain to measure the maximum respiratory capacity. 56

Molecular Therapy: Oncolytics Vol. 4 March 2017 43
The maximum respiratory rate (OCR) was significantly higher at day 2 after PHA stimulation but declined by day 8 (Figure 3C). Similar results were obtained for CAR-transduced T cells, showing low mitochondrial function following anti-hPSMA CAR transduction (Figures 3B and 3C).

Limited Dissemination of Endogenous T Cells in PD-L1-Positive Myc-CaP Tumors

To assess the associations between PD-L1/PD-1 signaling and T cell targeting of Myc-CaP tumors, and the impact on tumor progression, we performed immunofluorescence staining for PD-L1 in (1) Myc-CaP:hPSMA(+) tumors growing in NOD.SCID mice and (2) Myc-CaP WT tumors growing in immune-competent FVB/N. Both tumors stained positive for PD-L1 (Figure 4A). The spatial distribution of CD3+ T cells in Myc-CaP WT tumors (growing in FVB/N mice) was also examined; a predominance of T cells was observed along the invasive tumor margin (stromal-tumor edge), with reduced numbers in the center of the tumor (Figures 4B and 4C). We then evaluated whether anti-murine programmed death-ligand 1 (mPD-1) mAb treatment increased the number of tumor-infiltrating lymphocytes in subcutaneous Myc-CaP tumors (FVB/N mice). We administrated 5 doses of 200 μg of anti-mPD1 mAb per mouse (on days 9, 11, 13, 15, and 17 after tumor cell injection) and observed a significant delay (p < 0.05) in tumor growth (Figure 5A), which continued even after mice stopped receiving anti-mPD1 therapy. Interestingly, tumors <50 mm3 responded to the anti-mPD1 mAb treatment, whereas larger tumors failed to respond to the treatment (Figure 5A).

The density of CD3+ T cells per square millimeter of tumor area (Figures 5B and 5D), T cell size (Figure 5D) and the correlation between these two parameters and T cells distribution along the tumor edge...
was evaluated (Figure 5C). A marked increase (~13-fold) in CD3+ T cell density was observed in central tumor areas following anti-mPD1 therapy (Figure 5D). We also noticed that T cell size increased in responder tumors from 40 ± 15 μm² (IgG treated) to 51 ± 23 μm² (anti-mPD1 treated) but not in the non-responding tumor (42 ± 15 μm²). A comparison of CD3+ T cell distribution over the tumor sections showed that CD3+ T cells predominantly localized along the rim of tumor nodules prior to and following anti-PD1 treatment, even though the restriction of CD3+ T cells from the center of the tumor nodules was reversed (Figure 5C). No significant difference was observed in CD31 blood vessel staining of the tumors (data not shown).

**Anti-hPD1 mAb Treatment Enhances Adoptive Anti-hPSMA CAR-Mediated T Cell Therapy**

Encouraged by the response of Myc-CaP WT tumors to anti-mPD1 mAb treatment in FVB/N mice, we evaluated the effect of anti-hPD1 treatment combined with anti-hPSMA CAR T cell adoptive therapy in the Myc-CaP:hPSMA(+) and Myc-CaP:hPSMA(-) tumor models. Anti-hPSMA CAR T cells (transduced with the CBRluc reporter) were monitored by BLI at different time points after i.v. administration (days 0, 1, and 6) (Figure 6B). Ten minutes following CAR T cell injection, the T cells were localized largely in the lungs of all animals, with a variable low intensity signal appearing in the area of the tumors (Figure 6B). Interestingly, the highest tumor-associated T cell BLI signal was observed in the Myc-CaP:hPSMA(-) tumors (anti-hPD1-treated control group), and the lowest signal was observed in the Myc-CaP:hPSMA(+) tumors (anti-hPD1-treated test group). Nevertheless, Myc-CaP:hPSMA(+) tumor-bearing mice receiving the combined treatment (both anti-hPSMA CAR T cells (10 × 10⁶) and anti-hPD-1 mAb; test group) had a significant (p = 0.03, p = 0.04) treatment response (tumor volume), when compared to the two control groups (Figures 6C and 6D). The absence of a treatment response in Myc-CaP:hPSMA(+) tumors (anti-hPD1 mAb-treated control group), despite the more robust T cell trafficking-to and BLI signal-in the tumor region is of particular interest.

A comparison of CAR T cell BLI signals from tumors and lungs during the first 24 hr showed that the BLI signal in the tumors was more stable (Figure S3A) compared with that in the lungs (Figure S3B), where a decline in signal intensity was observed in all treatment groups. By day 6 after anti-hPSMA CAR T cell injection, there was near total fading of the BLI signal from the lungs, whereas a longer-lasting BLI signal in the tumor areas was observed (Figure 6B). Following the cessation of anti-hPD1 treatment (day 20), we observed an increase in tumor growth of hPSMA(+) positive tumors treated with both anti-hPD1 mAb (five doses) and anti-hPSMA CAR T cells (one dose injection), comparable with other groups of mice (Figure 6C).
T cells were localized along the periphery of these tumors (Figure S4). In contrast, CD31 staining of the tumors following 6 days of combined treatment showed no difference in microvessel density compared to controls (Figures S4 and S5), although more CAR T cells were observed in the anti-IgG, hPSMA(+) and anti-hPD1, PSMA(−) group (hPSMA(+), anti-hPD1), whereas 29 and 26 TUNEL+ cells/mm² were detected for the test group and both control tumors showed considerably less necrosis (Figures S2).49 Namely, anti-hPSMA CAR T cell therapy alone was not effective against established subcutaneous Myc-CaP hPSMA(+) tumors. Nevertheless, a Winn assay55 demonstrated that anti-hPSMA CAR T cells can inhibit Myc-CaP:hPSMA(+) tumor growth in a scarce nutrient microenvironment, and that this inhibition is hPSMA dependent. However, Myc-CaP:hPSMA(+) tumors re-grew in 3 weeks after implantation (Figures 2C and 2D).

To evaluate the effectiveness of second-generation anti-hPSMA CAR T cells in a pre-clinical setting, we developed the Myc-CaP (hPSMA(+)) tumor model in immune deficient mice (NOD.SCID II2rg−/−, NSG). The Myc-CaP murine prostate cancer model was chosen to investigate hPSMA-directed adoptive CAR T cell therapy for the following reasons: (1) Myc-CaP s.c. tumors are slow-growing (allowing time for immune targeted therapy) and can be used in a syngeneic mouse model using immune competent mice; (2) moderate size tumors have little necrosis (reducing confounding factors in the interpretation of results); (3) a genetically modified Myc-CaP cell line with high cell-membrane localized PSMA expression was developed to allow direct comparisons between PSMA(+) and PSMA(−) tumors; (4) both PSMA(+) and PSMA(−) tumors had very similar growth and morphological characteristics (Figure 1).

This study focuses on the potential for enhancing the efficacy of hPSMA CAR-directed T cell therapy using an immune checkpoint inhibitor (PD-1L/PD1 blockade) just prior to i.v. administration of CAR T cells. Our initial efforts to evaluate the effectiveness of second-generation anti-hPSMA CAR T cells24,53 were comparable to that of others (Figure S2).49 Namely, anti-hPSMA CAR T cell therapy alone was not effective against established subcutaneous Myc-CaP hPSMA(+) tumors. Nevertheless, a Winn assay55 demonstrated that anti-hPSMA CAR T cells can inhibit Myc-CaP:hPSMA(+) tumor growth in a scarce nutrient microenvironment, and that this inhibition is hPSMA dependent. However, Myc-CaP:hPSMA(+) tumors re-grew in 3 weeks after implantation (Figures 2C and 2D).

The finding that Myc-CaP tumors are PD-L1 positive in both an immunocompromised and immunocompetent mice raised the question whether the restriction of CD3+ T cells from the interior of Myc-CaP WT tumors in FVB/N mice was related to PD-L1/PD1 engagement (Figure 4B). A similar exclusion of CD3+ T cells was described in metastatic melanoma59 and other types of cancer.60,61 Given that PD-1 receptor expression is increased on activated T cells following engagement with PD-L1 ligand and high PD-L1/PD1 expression is associated with T cell exhaustion,59,62 we treated Myc-CaP WT tumors in FVB/N with anti-mPD1 mAb and observed a significant, but only a partial delay in tumor growth (Figure 5A).

Although based on a small number of animals (n = 5), we observed a ∼13-fold increase in intratumoral CD3+ T cell density following anti-PD1 inhibition (Figure 5B), consistent with releasing the PD-1 immune checkpoint and leading to T cell proliferation, intratumoral infiltration and increased effector function.63 We also observed that T cells changed their morphology and increased in size following

**DISCUSSION**

CAR T cell therapy for hematologic malignancies has shown some remarkable success in recent years.59,60,63,65 We focused on hPSMA targeting in prostate tumors using human anti-hPSMA CAR T cells, because of an existing clinical trial at our institution (MSKCC) (clinical trial #NCT01140373, https://clinicaltrials.gov) (Slovin et al., 2013,J. Clin. Oncol., abstract). It has been shown that adoptive transfer of T cells modified with the anti-hPSMA CAR could specifically mediate regression of pulmonary experimental lung metastasis in SCID mice.21,24,49 However, this approach was ineffective against established subcutaneous tumors,49 unless the CAR T cells were injected locally.49

H&E staining of one tumor from each treatment group shows different levels of necrosis 24 hr after initiation of treatment (Figure 7). hPSMA(+) tumors treated with both anti-hPD1 mAb and anti-hPSMA CAR T cells (test) showed the most necrosis (Figure 7A), whereas both control tumors showed considerably less necrosis (Figures 7B and 7C). The density of TUNEL positive cells showed a similar pattern; 54 TUNEL (+) cells/mm² were detected for the test group (hPSMA(+), anti-hPD1), whereas 29 and 26 TUNEL+ cells were observed in the anti-IgG, hPSMA(+) and anti-hPD1, PSMA(−) control groups, respectively (Figure 7, far right column). The distribution and density of anti-hPSMA CAR T cells was higher in the center of a hPSMA(+) tumor treated with anti-hPD1 mAb at day 6 compared to controls (Figures S4 and S5), although more CAR T cells were localized along the periphery of these tumors (Figure S4). In contrast, CD31 staining of the tumors following 6 days of combined treatment showed no difference in microvessel density (Figure S5).

**Figure 4. Limited Distribution of Endogenous T Cells in PD-L1-Positive Myc-CaP Tumors**

(A) PD-L1 expression in Myc-CaP:hPSMA(+) tumors growing in NOD.SCID II2rg−/− (NSG) mice on day 27 and MyC-CaP:WT in FVB/N mice on day 20 was studied by immunofluorescence imaging (IF). (B) CD3+ T cells are excluded from the core of Myc-CaP:WT tumor cell nests, and are present at high density in the tumor periphery; scale bar, 200 μm for the large image and 50 μm for the insets.
anti-mPD1 mAb treatment, consistent with cell cycle activation and proliferation. Although this suggests that anti-mPD1 mAb treatment resulted in increased CD3+ T cell activation, proliferation, and effector functions, the response was limited and indicates other factors are involved.

Nevertheless, the encouraging response of wild-type Myc-CaP tumors to anti-mPD1 mAb treatment in immune competent FVB/N mice led us to evaluate the effect of anti-hPD1 mAb treatment in combination with hPSMA-targeted CAR T cells in the Myc-CaP:hPSMA(+) tumor model. In these adoptive CAR T cell experiments, we first showed that combined treatment with anti-hPD1 antibody significantly inhibited Myc-CaP:hPSMA(+) tumor growth (Figure 6C) and that the antitumor response mediated by this combined therapy was both anti-hPD1 and hPSMA specific. Namely, the IgG-treated control group did not show an inhibitory effect, nor did tumors that were hPSMA(−). The blocking of hPD-1 enhanced the anti-tumor effect of hPSMA-targeted CAR T cells (Figure 6C), even in the presence of murine PD-L1 in the target tumors (Figure 4). The treatment response was confirmed by H&E and TUNEL staining of the tumor 24 hr after initiation of treatment (Figure 7A). These results suggest that there is a direct impact of PD-1 blockade on CAR T cell cytolytic function in these tumors.

Anti-PD1 mAb treatment of Myc-CaP WT tumors growing in immune-competent FVB/N mice had a more profound and prolonged effect, even following treatment withdrawal (Figure 5A). This effect was greater than a single injection of anti-hPSMA CAR T cells and five doses of anti-hPD1 mAb treatment of Myc-CaP:hPSMA(+) tumors growing in NSG mice (tumor growth was delayed for only 10 days). We suggest that anti-hPD1 antibody treatment combined with anti-hPSMA CAR human T cells had enhanced killing capacity.
and cytokine function against Myc-CaP:hPSMA(+) tumors, although the treatment response was comparatively short in duration. Recent literature40,66 demonstrates that adoptive immunotherapy using genetically modified T cells in combination with PD-1 checkpoint blockade can enhance the antitumor effects of CAR T cells against established subcutaneous tumors in an immune compromised host. Nevertheless, our results indicate that other immune modulation mechanisms exist and restrict CAR T cell targeting, function, and persistence in hPSMA expressing Myc-CaP tumors.

Rapid destruction of i.v. administered CAR T cells is well known.49,67 Nevertheless, the persistence of CAR T cells is well documented in the treatment of many “liquid” tumors,58–70 whereas the persistence of CAR T cells is not a common finding in the treatment of solid tumors.71 Previous studies have shown that the therapeutic efficacy of adoptive cell transfer is correlated with the ability of T cells to proliferate and survive in vivo.72 To address this issue, we used bioluminescence reporter-gene imaging (BLI) and immunofluorescence staining to track adoptively administered CAR T cells. In our BLI studies, there was a clear difference in anti-hPSMA CAR T cell trafficking to and initial persistence in Myc-CaP tumors (with and without the presence of the target antigen - hPSMA), even during the first minutes after T cells injection (Figures 6A and 6B). This difference in BLI signal was present over days 0, 1, and 6 following CAR T cells injection (Figure 6B). The seemingly contradictory T cell BLI and treatment response observations (Figures 2B, 2E, 6B, and 6C) and inconsistencies between T cell BLI (Figure 6) and CD3+ staining for T lymphocytes (Figure S5), can be explained in several ways.

We performed our experiments thinking that CBRLuciferase (CBRluc) BLI could be used as a readout of CAR T cell number and persistence. To explain the above observations, we assumed the anti-hPSMA CAR T cells were undergoing more rapid destruction in hPSMA(+) Myc-CaP tumors compared to hPSMA(-) tumors, since the T cells were bearing a second generation CAR. To confirm this hypothesis, we detected more apoptotic CAR T cells in co-culture experiments with hPSMA(+) than with hPSMA(-) Myc-Cap tumor cells (data not shown). Anti-hPSMA CAR T cells in the absence of the hPSMA are likely to remain in a non-activated (anergic) state and not be subject to PSMA antigen-induced activation leading to T cell death/destruction.73 Therefore, it was reasonable to consider that “quiescent T cells” could survive for a longer period of time and be visualized by BLI.

Figure 6. Anti-hPD1 mAb Treatment Enhances Adoptive Anti-hPSMA CAR-Directed T Cell Therapy

(A) Bioluminescence imaging of Myc-CaP:hPSMA(+) and Myc-CaP:hPSMA(-) tumors (transduced with the Rluc reporter) in NOD.SCID IL2rg(-/-) (NSG) mice to confirm tumor location. (B) Bioluminescence imaging of anti-hPSMA CAR T cells (transduced with the tdRFP/CBLuc reporter) 10 min after i.v. infusion and at 1 and 6 days. T cells trafficked largely to the lungs, although some localization to the tumor regions was visualized. (C) Plot of the mean tumor volume, comparing three treatment groups of mice (n = 5 per group). Treatments with anti-hPD1 mAb (test) or IgG mAb (control) are indicated by the black arrows; i.v. administration of anti-hPSMA CAR T cells is indicated by the red arrow. P value across groups is given for a one-tailed t test, *p < 0.05 versus control Myc-CaP:hPSMA(+) tumors and Myc-CaP:hPSMA(+) with IgG mAb.

(D) A comparison of tumor growth in individual mice after the initiation of treatment; three groups are compared: anti-hPD1 mAb treatment of hPSMA(+) tumors (test), non-specific IgG treatment of hPSMA(+) tumors (control), and anti-hPD1 mAb treatment of hPSMA(-) tumors (control, no PSMA target).
An additional and more plausible explanation is that following contact with tumor cell membrane-localized hPSMA, the anti-hPSMA CAR T cells undergo a transition from a quiescent to a highly active effector phenotype. This transition also leads to a significant shift in the metabolism of hPSMA CAR T cells, from a TCA cycle and oxidative phosphorylation OXYPHOS-based metabolism, to a more glycolysis-dependent metabolism to support macromolecule synthesis, proliferation, and effector function. These changes in the metabolism of CAR T cells transitioning from a quiescent to an activated state could directly affect CBRluc BLI signal intensity. Recently, it was observed that activated tumor-infiltrating T cells display a phenotype of metabolic insufficiency, characterized by a persistent loss of mitochondrial function and mass. Additionally, it was shown that chronic activation of T cells (associated with an anti-cancer response) represses oxidative metabolism, concomitant with a loss of mitochondrial mass and function, and that a significant decrease/loss of ATP was observed in activated CD8+ T cells.

In light of these observations and the data provided in Figure 3 showing lower mitochondrial activity in CAR T cells, we suggest that activated T cells can be associated with a low BLI readout (photons) using luciferin-luciferase-based reporter systems. The Click Beetle Red Luciferase (CBRluc) reporter is dependent on the presence of intra-cellular ATP, since this reporter belongs to the class of oxidative enzymes catalyzing the reaction of luciferin+ATP leading to formation of oxyluciferin, AMP and light. Therefore, the CBRluc reporter (and other ATP-dependent luciferases, including Firefly luciferase) is dependent on the presence of saturable amounts of ATP for reliable BLI measurements of reporter-cell number and persistence. Interestingly, several other investigators have found that Firefly luciferase is not an optimal reporter for tracking activated T cells (as well as unpublished conversations with other investigators).

There are several limitations to this study. First is the very short life of anti-hPSMA CAR T cells in the presence of hPSMA(+) Myc-CaP cells/tumors compared with hPSMA(-) cells/tumors, which leads to their longer persistence in the Winn assay (control group, hPSMA(-) tumors). Second is a modest cytotoxicity of CAR T cells (Figure S1) to murine cancer cells bearing hPSMA, which leads to Myc-CaP:hPSMA(+) tumor progression after 3 weeks in the Winn assay (Figures 2C and 2D) compared with total eradication of PC3/PIP tumors (a human prostate tumor genetically modified to overexpress hPSMA). The combination human CAR T cells (injected i.v.) and a murine prostate tumor transduced to express human PSMA may not be an optimal model to explore CAR T cell therapy. However, there is the potential for interaction between anti-hPD1 Abs with mPD-L1, since PD1 shares 64% of protein identity between murine and human species, and PD-L1 shares 77% identity. Murine PD-1 binds in vitro to both murine and human PD-L1, and human
PD-1 binds to the PD-L1 of each species. The structures of the murine PD-1 and human PD-L1 complexes have been published. Despite the high homology and the ability for binding between different species, interspecies differences are responsible for the variation in the affinity of human and mouse PD-1 for their ligands. Since human PD-1 is relatively "flexible," it does not appear to present any significant barriers to murine ligand binding.

Consistent with prior studies, we were able to enhance CAR T cells therapy when combined with PD-1 blockade. However, the treatment response was only partial, of short-duration and sub-optimal. Other second generation CARs have been shown to be as or more effective, including the CD28- and 4-1BB-based mesothelin-targeted CAR T cells that achieved tumor eradication, but only following direct intra-pleural administration.

We suggest that the limited response we observed also suggests that there are other mechanisms restricting CAR T cell trafficking and function in prostate solid tumors, which may extend to other solid tumors as well.

Conclusions

We show that anti-hPSMA-directed CAR T cell monotherapy of subcutaneous Myc-CaP:PSMA(+) tumors is ineffective, whereas the combination of anti-hPSMA-directed CAR T cells plus anti-hPD1 mAb immune modulation provides a short-duration, sub-optimal treatment response. These results also suggest that other immune modulation mechanisms need to be brought into play to further reverse the restriction to CAR T cell targeting and persistence in hPSMA expressing Myc-CaP tumors and to provide optimal CAR T cell therapy. The results also suggest that ATP-dependent Luciferin-luciferase bioluminescence reporters should be used with caution in the monitoring of T cell trafficking and persistence, particularly when T cells transition to an activated state.

MATERIALS AND METHODS

Cells and Culture Conditions

The Myc-CaP androgen-dependent prostate cancer cell line, derived from a c-myc transgenic mouse with prostate cancer, was provided by Dr. Charles Sawyers and was cultured in DMEM media supplemented with 10% FBS, 4 mM glutamine, and 5 mM glucose. Myc-CaP cancer cells were transduced with a newly generated vector SFG-hPSMA. A transgene containing human PSMA complementary DNA (cDNA) was amplified from total mRNA derived from human prostate cancer cell line LNCaP using 5′-ACATGTGGAATCTCCTTCACGAAAC-3′ and 5′-GGATCCTCGAGCTTAGGCTACTTCACTCAAAG-3′ primers set. Human PSMA cDNA was cloned into the SFG vector between the restriction site between the anti-hPSMA scFv and CD28 signaling motif in the SFG-P28z vector. For transduction we have used the PG13 producer cell lines, bearing anti-hPSMA CAR and SFG-tdRFP/CBRLuc vectors. Retroviral particles were obtained using the GPG29 producer cell line and were used to infect target cells. Cells were stably transduced by incubating 50% confluent cell cultures with virus-containing medium for 12 hr in presence of polybrene (8 μg/mL; Sigma-Aldrich). Cells were sorted using FACS (BD Biosciences) using GFP or tdRFP as fluorescence markers.

Generation of Genetically Modified T Cells

SFG-Pig28z- and SFG-tdRFP/CBRLuc- retroviral supernatants were produced as described above. Monocyte-depleted PBMCs were activated with anti-CD3/CD28 beads (Dynabeads; Thermo Fisher Scientific) in a 3:1 bead:cell ratio with 20 IU/mL IL-2 for 7 days. Activated T cells were then retrovirally transduced on days 3 and 4, supernatants from the different vectors were mixed on transduction days at a 1:1 ratio. Anti-CD3/CD28 beads were removed on day 7. Media and IL-2 were changed every 3 days. Transduction efficacy was confirmed by FACS after staining with anti-human IgG antibody (Southern Biotechnology Associates) for the detection of cells bearing anti-hPSMA vector and detection of tdRFP/CBRLuc. To assess CAR T cell function we decided to follow the clinical protocol of CAR T cell preparation. Two sets of CAR T cells (from different donors) were obtained for the current study. One set of CAR T cells was utilized for the first CAR T cell trafficking experiment (Figure S2) and a Wien assay. To perform anti-hPD1 mAb and anti-hPSMA CAR T cell treatment we obtained another set of CAR T cells. Transduction efficiencies varied from 87% to 99.8% for the anti-hPSMA marker after cell sorting, and between 67% and 34% for cells that were double-positive for both anti-hPSMA marker and tdRFP/CBRLuc. Cells were expanded over 18 days and cryopreserved using 2× cryopreserved medium composed of 7% Plasma-lyte, 10% DMSO (Mylan Institutional), 40% of albumin (human; GRIFOLS), and 33% (HESpan [hetastarch]).

T cell function studies were performed as described previously. Standard 51Cr release assays were performed to evaluate CAR T cell cytolytic ability. Target tumor cells were loaded with 100 μCi of 51Cr for 1 hr, and then 10,000 tumor cells were co-incubated with CAR T cells for 6 hr at effector-to-target (E:T) ratios ranging from 40:1 to 1:1. Supernatants were harvested and 51Cr release quantified using a Gamma Counter (Packard). Percent lysis was calculated as...
follows: percent lysis = (experimental lysis − spontaneous lysis)/(maximal lysis − spontaneous lysis) × 100%, where maximal lysis was induced by incubation in a 2% Triton X-100 solution.

**Metabolic Assessment of T Cells**

The metabolic profiles were determined at steps of T cell stimulation and transduction to assess glycolytic function and mitochondrial respiration of T cells during stimulation, transduction and expansion. We performed a series of real-time measurements of the extracellular acidification rate (ECAR) and OCR using a Seahorse XF96 Extracellular Flux Analyzer (Agilent). The XF Analyzer (Seahorse Biosciences) simultaneously measures energy producing pathways non-invasively in real-time. For the assessment of glycolysis, cells are initially incubated without glucose and ECAR is assessed. The first injection is a saturating concentration of glucose (25 mM), where glucose is taken up by the cells and catabolized through the glycolytic pathway to lactate, producing ATP and protons. The extrusion of protons into the surrounding medium produces a rapid increase in ECAR. In the mitochondrial respiration assay, basal OCR is measured under full media growing conditions followed by sequential addition of oligomycin, FCCP, and antimycin A/rotenone with a measurement of changes in OCR. The resulting profiles show the relative contribution of non-respiratory chain oxygen consumption, ATP-linked oxygen consumption and the maximal respiration after the addition of FCCP. Data presented here represent two independent experiments at different days and were normalized to 350,000 cells.

**In Vivo Studies**

NOD.SCID II2rg<sup>−/−</sup> (NSG) mice were obtained from Jackson Laboratories and FVB/N male mice were from Charles River Laboratories. All mice were 5–6 weeks of age and all mice groups consisted of n = 5 per group. The animal protocol was approved by the Memorial Sloan Kettering Institutional Animal Care and Use Committee. NOD.Cg-Prkd(scid)II2rg were injected subcutaneously with 1 × 10<sup>6</sup> Myc-CaP:hPSMA(+) or Myc-CaP:hPSMA(−) cells in 1:1 ratio of matrigel to media in the right flank subcutaneously. Myc-CaP wild-type cells (1 × 10<sup>6</sup>) were injected into FVB/N mice. We reduced the number of CAR T cells for injection from 20 × 10<sup>6</sup> per mouse (Figure S2) to 10 × 10<sup>6</sup>, in later experiments. Two independent Winn assay experiments (Figure 2) were performed. The first experiment included three mice per group, whereas the second experiment included five mice per group. The data are presented for the second and most representative experiment. For the combined treatment studies, anti-hPSMA CAR T cells were thawed and reconstituted in standard T cell media for 24 hr. Anti-hPD1 mAb or IgG-control mAb were administrated to three groups of mice 3 hours before i.v. infusion of CAR T cells, and then every other day (between days 10 and 20 of tumor growth). Three groups of mice consisted of Myc-CaP:hPSMA(+) IgG group n = 5; Myc-CaP:hPSMA(−) anti-PD1 group n = 5; and Myc-CaP:hPSMA(+) anti-PD1 groups n = 5 and n = 3. Myc-CaP:hPSMA(+) or Myc-CaP:hPSMA(−) tumors in NOD.Cg-Prkd(scid)II2rg mice were treated with anti-hPD-1 (human, J110) and isotype MOPC-21 (human, IgG control) antibodies. Myc-CaP wild-type tumors in FVB/N mice were treated with anti-mPD-1 (murine, clone RMP1-14), and isotype 2A3 (murine, IgG control) antibodies when tumors reached a size of ~50 mm<sup>3</sup>, which occurred within ~10 days after implantation of the tumor cells. All antibodies were obtained from BioXCell. Anti-mPD1 and anti-hPD1 antibody was administered by i.p. injection, at 200 µg per mouse every other day.

**Bioluminescence Imaging and Histology**

BLI was performed with an IVIS SPECTRUM Imaging System (Perkin Elmer) and analyzed as described previously. Tumor and lung tissue processing and staining was performed at Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center. The stained sections were digitized and scanned using a Panoramic Viewer (3DHistech) and analyzed with MetaMorph Image Analysis Software (Molecular Devices). A fluorescence threshold was used to include only cell-specific signals and exclude background. Size and morphology filters have been applied to ensure only cells are counted (not cellular debris), and the number of immune cells were recorded. Cell sizes were obtained by applying the above thresholds and obtaining the area of each identified T cell. The data (area of individual T cells) were combined for group statistics (n = 3,621 T cells for the control group; n = 14,531 T cells for anti-PD1 group). Detailed information for CD31/PDL1, CD3<sup>+</sup>, and TUNEL staining of tissues is provided in the Supplemental Material and Methods.

**Statistical Analysis**

Results are presented as mean ± SD. Statistical significance was determined by a two-tailed Student’s t test. p < 0.05 was considered significant. All data presented for T cells assessment using IFC staining were analyzed using GraphPad Prism (version 6.0; GraphPad Software) and are presented as mean ± SD. Results were analyzed using the unpaired Student’s t test, and statistical significance was defined as p < 0.05.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Materials and Methods and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.omto.2016.11.005.

**AUTHOR CONTRIBUTIONS**

Conception/design, I.S., E.M., V.P., and R.B.; Development of methodology, I.C., M. Moroz, J.Z., I.S., and I.S.; Acquisition of data, E.M., I.S., M. Mane, and M. Moroz; Analysis and interpretation of data, E.M., I.S., I.C., M. Mane, M. Moroz, V.P., and R.B.; Writing, review, and/or revision of the manuscript, I.S., I.C., V.P., and R.B.; Administrative, technical, or material support, V.P. and R.B.; Study supervision, I.S., V.P., and R.B.; Pathological diagnosis, analysis, and interpretation of the immunohistochemical data, I.S. and I.C.; and Carrying out experiments and analyzing data, E.M., I.S., M. Moroz, and I.C.

**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.
ACKNOWLEDGMENTS

We thank Nisargbhai S. Shah for a technical assistance and Drs. Taha Merghoub and Susan Slvin for valuable discussions. We thank Molecular Cytology Core Facility of MSKCC, especially Drs. K. Manova-Todorova and D. Yarlin for their scientific and technical advice during staining of CAR T cells in tumors. This work was supported by the MSKCC Center for Molecular Imaging in Cancer (P50CA86438) and NIH Grants R01 CA163980, R01 CA172846, R01 CA161138, and P30 CA008748 (MSK Cancer Center Support Grant/Core Grant).

REFERENCES

1. Hsing, A.W., Tsao, L., and Devesa, S.S. (2000). International trends and patterns of prostate cancer incidence and mortality. Int. J. Cancer 85, 60–67.

2. Siegel, R.L., Miller, K.D., and Jemal, A. (2015). Cancer statistics, 2015. CA Cancer J. Clin. 65, 5–29.

3. Wang, G., Chopra, R.K., Royal, R.E., Yang, J.C., Rosenberg, S.A., and Hwu, P. (1998). A T cell-independent antitumor response in mice with bone marrow cells retrovirally transduced with an antibody/Fc-chimeric chain chimeric receptor gene recognizing a human ovarian cancer antigen. Nat. Med. 4, 168–172.

4. Chekhanskaa, A.A., Rao, T.D., Nikhamin, Y., Park, K.J., Levine, D.A., Spriggs, D.R., and Brentjens, R.J. (2010). Successful eradication of established peritoneal ovarian tumors in SCID-Beige mice following adoptive transfer of T cells genetically targeted to the MUC16 antigen. Clin. Cancer Res. 16, 3594–3606.

5. Brentjens, R.J., Latouche, J.B., Santos, E., Marti, F., Gong, M.C., Lyddane, C., King, P.D., Larson, S., Weiss, M., Riviere, L., and Sadailain, M. (2003). Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. Nat. Med. 9, 279–286.

6. Gade, T.P., Hassen, W., Santos, E., Gunset, G., Saudement, A., Gong, M.C., Brentjens, R., Zhong, X.S., Stephan, M., Steffans, J., et al. (2005). Targeted elimination of prostate cancer by genetically directed human T lymphocytes. Cancer Res. 65, 9080–9088.

7. Asdusmilli, P.S., Cherkassky, L., Villena-Vargas, J., Colovos, C., Servais, E., Plotkin, J., Jones, D.R., and Sadailain, M. (2014). Regional delivery of mesothelin-targeted CAR T cell therapy generates potent and long-lasting CD4-dependent tumor immunity. Sci. Transl. Med. 6, 261ra151.

8. Hwu, P., Yang, J.C., Cowherd, R., Treisman, J., Steffans, J., Eshhar, Z., and Brentjens, R.J. (2010). Successful eradication of established peritoneal ovarian tumors in SCID-Beige mice following adoptive transfer of T cells genetically targeted to the MUC16 antigen. Clin. Cancer Res. 16, 3594–3606.

9. Darcy, P.K., Haynes, N.M., Snook, M.B., Trapani, J.A., Cerruti, L., Jane, S.M., and Haynes, N.M., Trapani, J.A., Teng, M.W., Jackson, J.T., Cerruti, L., Jane, S.M., McGuinness, R.P., Ge, Y., Patel, S.D., Kashmiri, S.V., Lee, H.S., Hand, P.H., Schlam, J., Finer, M.H., and McArthur, J.G. (1999). Anti-tumor activity of human T cells expressing the CC49-zeta chimeric immune receptor. Hum. Gene Ther. 10, 165–173.

10. Hwu, P., Yang, J.C., Cowherd, R., Treisman, J., Shafer, G.E., Eshhar, Z., and Rosenberg, S.A. (1995). In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. Cancer Res. 55, 3369–3373.

11. Wang, G., Chopra, R.K., Royal, R.E., Yang, J.C., Rosenberg, S.A., and Hwu, P. (1998). A T cell-independent antitumor response in mice with bone marrow cells retrovirally transduced with an antibody/Fc-chimeric chain chimeric receptor gene recognizing a human ovarian cancer antigen. Nat. Med. 4, 168–172.

12. Chekhanskaa, A.A., Rao, T.D., Nikhamin, Y., Park, K.J., Levine, D.A., Spriggs, D.R., and Brentjens, R.J. (2010). Successful eradication of established peritoneal ovarian tumors in SCID-Beige mice following adoptive transfer of T cells genetically targeted to the MUC16 antigen. Clin. Cancer Res. 16, 3594–3606.

13. Brentjens, R.J., Latouche, J.B., Santos, E., Marti, F., Gong, M.C., Lyddane, C., King, P.D., Larson, S., Weiss, M., Riviere, L., and Sadailain, M. (2003). Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. Nat. Med. 9, 279–286.

14. Gade, T.P., Hassen, W., Santos, E., Gunset, G., Saudement, A., Gong, M.C., Brentjens, R., Zhong, X.S., Stephan, M., Steffans, J., et al. (2005). Targeted elimination of prostate cancer by genetically directed human T lymphocytes. Cancer Res. 65, 9080–9088.

15. Asdusmilli, P.S., Cherkassky, L., Villena-Vargas, J., Colovos, C., Servais, E., Plotkin, J., Jones, D.R., and Sadailain, M. (2014). Regional delivery of mesothelin-targeted CAR T cell therapy generates potent and long-lasting CD4-dependent tumor immunity. Sci. Transl. Med. 6, 261ra151.

16. Hwu, P., Yang, J.C., Cowherd, R., Treisman, J., Steffans, J., Eshhar, Z., and Brentjens, R.J. (2010). Successful eradication of established peritoneal ovarian tumors in SCID-Beige mice following adoptive transfer of T cells genetically targeted to the MUC16 antigen. Clin. Cancer Res. 16, 3594–3606.

17. Darcy, P.K., Haynes, N.M., Snook, M.B., Trapani, J.A., Cerruti, L., Jane, S.M., and Haynes, N.M., Trapani, J.A., Teng, M.W., Jackson, J.T., Cerruti, L., Jane, S.M., McGuinness, R.P., Ge, Y., Patel, S.D., Kashmiri, S.V., Lee, H.S., Hand, P.H., Schlam, J., Finer, M.H., and McArthur, J.G. (1999). Anti-tumor activity of human T cells expressing the CC49-zeta chimeric immune receptor. Hum. Gene Ther. 10, 165–173.

18. Hwu, P., Yang, J.C., Cowherd, R., Treisman, J., Shafer, G.E., Eshhar, Z., and Rosenberg, S.A. (1995). In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. Cancer Res. 55, 3369–3373.
Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. Proc. Natl. Acad. Sci. USA 92, 6733–6737.

June, C.H., Ledbetter, J.A., Gillespie, M.M., Lindsten, T., and Thompson, C.B. (1987). Cancer immunotherapy strategies based on overcoming barriers within the tumor microenvironment. Curr. Opin. Immunol. 25, 268–276.

36. Fearon, D.T. (2014). The carcinoma-associated fibroblast expressing fibroblast activation protein and escape from immune surveillance. Cancer Immunol. Res. 2, 187–193.

37. Peng, W., Ye, Y., Rabinovich, B.A., Liu, C., Lou, Y., Zhang, M., Whittington, M., Yang, Y., Overwijk, W.W., Lizée, G., and Hwu, P. (2010). Transduction of tumor-specific T cells with CXCRI2 chemokine receptor improves migration to tumor and antitumor immune responses. Clin Cancer Res. 16, 5458–5468.

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

39. Kochenderfer, J.N., Dudley, M.E., Kassim, S.H., Nahvi, A., Ngyo, L.T., Sherry, R.M., et al. (2015). Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. J. Clin. Oncol. 33, 540–549.

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

41. Stephan, S.B., Taber, A.M., Ilieaeva, I., Pegues, E.P., Sentman, C.L., and Stephan, M.T. (2015). Biopolymer implants enhance the efficacy of adoptive T-cell therapy. Nat. Biotechnol. 33, 97–101.

42. Roff, T.J., Sheinin, Y., Lobse, C.M., Kuntz, S.M., Frigola, X., Inman, B.A., Krambeck, A.E., McKenney, M.M., Ekenes, R.J., Blute, M.L., et al. (2007). B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy. Cancer Res. 67, 7093–7099.

43. Martin, A.M., Nirschl, T.R., Nirschl, C.J., Francica, B.J., Kochel, C.M., van Bokhoven, A., Meeker, A.K., Lucia, M.S., Anders, R.A., DeMarzo, A.M., and Drake, C.G. (2015). Paucity of PD-L1 expression in prostate cancer: innate and adaptive immune resistance. Prostate Cancer Prostatic Dis. 18, 325–332.

44. Ebelt, K., Babarya, G., Frankenberger, B., Stief, C.G., Eiseenmenger, W., Kirchner, T., Schendel, D.J., and Noessner, E. (2009). Prostate cancer lesions are surrounded by FOXP3+PD-1+ and B7-H1+ lymphocyte clusters. Eur. J. Cancer 45, 1664–1672.

45. Gevensleben, H., Dietrich, D., Golletz, C., Stein, R., Jung, M., Whittington, M., Yang, Y., Overwijk, W.W., Lizée, G., and Hwu, P. (2010). Transduction of tumor-specific T cells with CXCRI2 chemokine receptor improves migration to tumor and antitumor immune responses. Clin Cancer Res. 16, 5458–5468.

46. Ponomarev, V., Dubrovin, M., Sernagan, I., Beresten, T., Vider, J., Shavrin, A., Ageyeva, L., Balatoni, J., Blasberg, R., and Tjuvajev, J.G. (2003). Cytoplasmically targeted HSV1-tk/GFP reporter gene mutants for optimization of noninvasive molecular-geographic imaging. Neoplasia 5, 245–254.

47. Maher, J., Brentjens, R.J., Sunet, G., Rivière, I., and Sadelain, M. (2002). Human T lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRbeta/CD28 receptor. Nat. Biotechnol. 20, 70–77.

48. Huang, R., Vider, J., Sernagan, I., and Blasberg, R.G. (2011). ATB-binding cassette transporters modulate both coelenterazine- and D-luciferin-based bioluminescence imaging. Mol. Imaging 10, 215–226.

49. Genovesi, E.V., Pettey, C.L., and Collins, J.J. (1984). Use of adoptive transfer and Winn assay procedures in the further analysis of antiviral acquired immunity in mice protected against Friend leukemia virus-induced disease by passive serum therapy. Cancer Res. 44, 1489–1498.

50. Morán, M., Rivero, H., Sánchez-Aragó, M., Blázquez, A., Merinero, B., Ugale, C., Arasa, J., Cuerva, J.M., and Martín, M.A. (2010). Mitochondrial bioenergetics and dynamics interplay in complex I-deficient fibroblasts. Biochim. Biophys. Acta 1802, 443–453.

51. Rivière, I., Brose, K., and Mulligan, R.C. (1995). Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. Proc. Natl. Acad. Sci. USA 92, 6733–6737.
