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Cell-Intrinsic Abrogation of TGF-β Signaling Delays but Does Not Prevent Dysfunction of Self/Tumor-Specific CD8 T Cells in a Murine Model of Autochthonous Prostate Cancer

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Adoptive T cell therapy (ACT) for the treatment of established cancers is actively being pursued in clinical trials. However, poor in vivo persistence and maintenance of antitumor activity of transferred T cells remain major problems. TGF-β is a potent immunosuppressive cytokine that is often expressed at high levels within the tumor microenvironment, potentially limiting T cell-mediated antitumor activity. In this study, we used a model of autochthonous murine prostate cancer to evaluate the effect of cell-intrinsic abrogation of TGF-β signaling in self/tumor-specific CD8 T cells used in ACT to target the tumor in situ. We found that persistence and antitumor activity of adoptively transferred effector T cells deficient in TGF-β signaling were significantly improved in the cancerous prostate. However, over time, despite persistence in peripheral lymphoid organs, the numbers of transferred cells in the prostate decreased and the residual prostate-infiltrating T cells were no longer functional. These findings reveal that TGF-β negatively regulates the accumulation and effector function of transferred self/tumor-specific CD8 T cells and highlight that, when targeting a tumor Ag that is also expressed as a self-protein, additional substantive obstacles are operative within the tumor microenvironment, potentially hampering the success of ACT for solid tumors. The Journal of Immunology, 2012, 189: 3936–3946.

The recent U.S. Food and Drug Administration approval of two cancer immunotherapies, a vaccine (sipuleucel-T) for treatment of prostate cancer (1) and an anti-CTLA-4 blocking Ab (ipilimumab) for treatment of metastatic melanoma (2), has highlighted the ability to modulate the immune system to attack tumors. An alternative therapeutic strategy, which is being actively pursued in multiple clinical settings, is adoptive T cell therapy (ACT), in which tumor-reactive T cells are generated and/or expanded ex vivo from T cells isolated from the blood or tumor of cancer patients and then infused back into the patient (3). Although efficacy has clearly been demonstrated (4–6), the difficulty in sustaining adequate numbers and function of tumor-reactive T cells following transfer into patients has hindered success (7). This in part reflects immunosuppressive tumor microenvironments, which can inhibit rather than stimulate potentially effective antitumor T cell responses (8). Tumor cells can express inhibitory ligands for T cells and recruit inhibitory cells, and both can secrete immunosuppressive cytokines that render tumor-infiltrating lymphocytes unresponsive or dysfunctional (8). Furthermore, T cells isolated directly from the patient for use in ACT are often of only low avidity, because most of the identified tumor Ags are self-proteins, and endogenous self/tumor-specific T cells that bear high-affinity TCRs are deleted in the thymus (9, 10). However, one potential advantage of ACT over in vivo augmentation of endogenous responses is the ability to genetically engineer T cells to improve function prior to infusion, such as by expressing high-affinity tumor-specific TCRs, abrogating T cell-intrinsic negative regulators, or disrupting inhibitory signaling pathways that may be engaged in the tumor microenvironment (9, 11).

TGF-β is a pleiotropic cytokine that plays important roles in maintaining normal tissue homeostasis and inhibiting autoimmune responses, and depending on the context it can promote or suppress tumor growth (12–17). The bioactive form of TGF-β binds to the TGF-β type I and TGF-β type II serine/threonine kinase receptor complexes, resulting in receptor-mediated phosphorylation of downstream transcription factors Smad2 and Smad3 (17). TGF-β signaling is antiproliferative, causing G1 cell cycle arrest in a variety of cell types, including epithelial and T cells (18, 19). Many tumors evade the cytostatic and antiproliferative effects of TGF-β by acquiring mutations in the TGF-β receptor and/or downstream Smad signaling proteins (17). Activated T cells, however, express higher levels of the TGF-β receptor and can produce TGF-β (20, 21). Molecular analysis of naive CD8 T cells in vitro has revealed that TGF-β suppresses key molecules involved in the effector and cytolytic activities of T cells, including expression of IFN-γ (22).
Inhibition of TGF-β signaling by mechanisms such as neutralizing Abs or kinase inhibitors is being pursued in clinical trials (23), but significant therapeutic benefits have not yet been reported. This may partly reflect failure to achieve full blockade of TGF-β, particularly in tumor tissues. Moreover, administering these agents at doses high enough to sustain full blockade may be too toxic. In the context of ACT, it would be possible to selectively abrogate the potentially profound immunosuppressive activity of TGF-β only in the T cells being used to target the tumor.

Prostate cancer is currently being pursued as a target for expanding applicability of T cell-mediated immunotherapy. In large part this reflects identification of immunogenic prostate-restricted Ags that are expressed in malignant and normal prostate tissues but not other tissues that might be potential targets of toxicity, and that can elicit cytolytic T cell responses (24). However, TGF-β is present and necessary for normal prostate homeostasis, and it is found in increased levels in the malignant prostate (25, 26), which can pose a substantive obstacle to T cell therapy of this tumor. Expression of a dominant-negative form of TGF-βRII or abrogation of TGF-β production exclusively in T cells of mice that develop autochthonous prostate cancer can delay tumor growth (21, 27), suggesting that TGF-β interferes with the development and/or expression of an endogenous response. Studies in transplantable tumor models also demonstrated that TGF-β signaling blockade improves the therapeutic efficacy of tumor-reactive T cells (28–30).

Many tumor therapy studies have been performed using transplantable tumor cell lines, and such models, although advancing the discovery and testing of tumor therapies, have limitations. Injection of a large number of tumor cells is often necessary for successful implantation, with many cells dying rapidly after injection, which can induce an immune response prior to establishment of the tumor (31). More importantly, these tumors do not develop in the same organ-specific environment of tumors that develop and grow in situ. Autochthonous tumor models, in which the tumor develops “spontaneously” usually from enforced expression of a driver oncogene, also have some limitations, but they do allow study of tumors derived from the organ of origin that develop over months in the context of a normal host immune system. Therefore, we used the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse, which expresses the SV40 T Ag under the prostate-specific probasin promoter, resulting in spontaneously arising prostate adenocarcinoma (32). The pathogenesis of prostate cancer in these mice has been well studied and models many aspects of human prostate cancer, including development of prostate intraepithelial neoplasia by 12 wk age and progression through distinct histological stages of adenocarcinoma (33, 34). We crossed these mice with the prostate OVA-expressing transgenic (POET1) mice, which express a membrane-bound form of the model Ag OVA driven by the prostate-specific ARPB rat probasin promoter (35), TRAMP × POET1 mice, denoted TRAMPova, express a targetable self/tumor Ag (OVA) in the context of a spontaneously arising prostate cancer. The use of OVA as a model self/tumor Ag allowed analysis of the efficacy in ACT of high-affinity OVA-specific CD8 T cells, derived from OTI TCR transgenic mice (36), and of targeting a prostastic self-Ag with T cells in which TGF-β signaling has been abrogated to overcome a potentially substantive obstacle to antitumor activity in the environment of a cancerous prostate gland.

Materials and Methods

Mice

TRAMP mice (32) and were obtained from N. Greenberg (Fred Hutchinson Cancer Research Center, Seattle, WA). POET1 mice (35) were obtained from T. Ratliff (Purdue University, West Lafayette, IN). TGF-βRIIfl/fl mice were provided by D. Dichek (University of Washington, Seattle, WA) with permission from S. Karlsson (Lund University, Lund, Sweden) (12). Distal Lck-Cre mice (37), which express Cre recombinase under control of the distal Lck promoter, were provided by P. Fink (University of Washington, Seattle, WA) with permission from N. Killeen (University of California, San Francisco, CA). OTI TCR transgenic mice (36) containing CD8 T cells specific for the immunodominant epitope (SIINFEKL) of OVA were a gift from M. Bevan (University of Washington, Seattle, WA). Ly5.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). To generate prostate cancer mice expressing a targetable self/tumor Ag, TRAMPova male mice were crossed to female POET1/6 mice to generate F1 mice hemizygous for the SV40 transgene and OVA expression (TRAMPova) and littermates expressing SV40 transgene only (TRAMP). All TRAMPova and TRAMP mice used were between 25 and 27 wk age, at which time all mice have high-grade neoplasia (23). To generate high-grade knockout mice, TGF-βRII-deficient mice (TGF-βRII knockout [KO]), mice expressing floxed TGF-βRII genes (TGF-βRIIflox/flox) were first bred to distal Lck-Cre mice or OTI Ly5.1 mice. The F1 offspring were bred together to produce mice harboring OTI Ly5.1 CDB T cells with a conditional deletion of TGF-βRII in mature CD8 T cells (OTI Ly5.1 × TGF-βRIInew × distal Lck-Cre). OTI Ly5.1 × TGF-βRIIflox/flox littermates were used as wild-type (WT) donors. All mice were maintained under specific pathogen-free conditions at the University of Washington under the guidelines of the Institutional Animal Use and Care Committee.

Peptide

SIINFEKL peptide was synthesized by the Immune Monitoring Laboratory at the Fred Hutchinson Cancer Research Center (Seattle, WA). Peptide was reconstituted in 100% DMSO at 10 mg/ml and stored at −20°C.

Cell isolation

Mice were euthanized by cervical dislocation. Spleens were mechanically disrupted with the back of a 3-ml syringe, filtered through a 70-μm strainer, and RBCs were lysed with ammonium chloride potassium buffer. Cells were washed twice with complete RPMI media (RPMI 1640 supplemented with 2 μM glutamine, 100 U/ml penicillin/streptomycin, and 10% FCS). Prostate draining lymph nodes (PDN; periarterial) were dissociated with microscope slides. Prostate lobes were microdissected and weighed. Individual lobes were divided in half, with half used for histology and half digested with collagenase D (Roche) and DNase I (Fermenta) for 1 h at 37°C. Digested tissue was mechanically disrupted through a 40-μm strainer.

In vitro activation and adoptive transfer

Single-cell suspensions were generated from spleens of OTI Ly5.1 WT and OTI Ly5.1 TGF-βRII KO mice. CD4 and B cells were depleted using anti-CD4 and anti-B220 Dynabeads (Invitrogen). Remaining cells were cocultured with irradiated (3000 rads) congenic splenocytes pulsed with SIINFEKL (10−7 μM) at a 1:5 ratio and 25 U/ml human rIL-2 (National Institute of Allergy and Infectious Diseases) in complete RPMI media. On day 5, OTI cells, which express the TCR chains Vα2 and Vβ5, were quantitated based on cell count and percentage of 7-aminocanthomycin DCD8×Vα2×Vβ57 cells by flow cytometry. Cells were washed twice with HBSS prior to injection of 5–10×106 OTI cells into the lateral tail vein of mice at a volume of 0.2 ml.

Flow cytometry

All single-cell suspensions were washed with staining buffer (PBS plus 1% FCS) prior to phenotypic and functional characterization. The following Abs were purchased from eBioscience: CD8α, Ly5.1, IFN-γ, TNF-α, and programmed death (PD)-1. Surface staining was done at 4°C in staining buffer. Ki-67 (BD Biosciences) and Bcl-2 interacting mediator of cell death (Bim; Cell Signaling Technology) staining was performed using the eBioscience fixation/permeabilization buffer kit per the manufacturer’s instructions. Briefly, following surface staining with CD8 and Ly5.1 Abs, cells were fixed, permeabilized, and stained with Ab to Ki-67 and Bim. A secondary PE-anti-rabbit F(ab’)2 fragment (Invitrogen) was used to detect Bim. Intracellular cytokine staining was performed using the Cytofix/Cytoperm Plus kit (BD Biosciences) per the manufacturer’s instructions. Briefly, single-cell suspensions from spleen, lymph node, and prostate were stimulated directly ex vivo for 5 h with 10−3 μg/ml SIINFEKL peptide and congenic (Ly5.2+) splenocytes in the presence of brefeldin A. Following surface staining with CD8 and Ly5.1, cells were fixed, permeabilized, and stained with Abs to IFN-γ and TNF-α. Flow cytometric analysis was performed using FACSCanto and LSRII at the Cell Analysis Facility, Department of Immunology, University of Washington (Seattle, WA).
Flow data were analyzed with FlowJo version 8.8.7 (Tree Star, Ashland, OR).

Prostate histology and immunohistochemistry

For H&E staining, microdissected prostate lobes were fixed in 4% paraformaldehyde then stored in 70% ethanol until processed by the Experimental Histopathology Core at the Fred Hutchinson Cancer Research Center (Seattle, WA). Histologic sections were evaluated by a comparative medicine pathologist blinded to group assignments. Images were captured using a Nikon Eclipse 80i microscope with a DS-FIi digital camera and NIS-Elements software.

For immunofluorescence staining, microdissected prostate lobes were frozen in OCT compound (Sakura Finetek). Seven-micrometer frozen prostate sections were cut on a cryostat. Sections were fixed with ice-cold acetone and blocked with PBS plus 1% goat serum prior to staining. Primary Abs included Ly5.1-PE (eBioscience), rat anti-mouse PD ligand (PD-L)1 (eBioscience), and rat IgG2a isotype control (eBioscience). When required, secondary goat anti-rat Alexa Fluor 488 (Invitrogen) was used. All slides were counterstained with DAPI (Invitrogen). Slides were analyzed on a Leica fluorescence microscope, and photographic images were captured with an Orca-ER digital camera and assembled into RGB images with ImageJ and Adobe Photoshop.

Ab blockade treatment

Monoclonal anti-PD-1 (29F.1A12) (38), anti–PD-L1 (10F.9G2) (39), and anti–PD-L2 (3.2) (40) Abs were provided by G. Freeman (Harvard Medical School, Boston, MA). To assure adequate blockade, the timing and dose of administration of these Abs established for each individual Ab (41) were used. Each blocking Ab (200 μg) was injected i.p. into recipient mice starting on the day of T cell transfer and continued every 3 d until mice were euthanized.

Statistical analysis

Bar graphs are displayed as mean ± SEM. Statistical analyses were performed with Prism version 5.0 (GraphPad Software) using an unpaired two-tailed Student t test. A p value <0.05 was considered statistically significant.

Results

Abrogation of TGF-β signaling increases the accumulation of transferred prostate self/tumor Ag-specific CD8 T cells

To investigate the T cell-intrinsic role of TGF-β in the setting of ACT of prostate cancer, we transferred 5–7 × 10^6 in vitro-activated OTI WT and TGF-βRII KO CD8 T cells into tumor-bearing 25- to 27-wk-old TRAMP OVA and TRAMP males. We first assessed whether abrogating TGF-β signaling affected expansion of the transferred cells and found a significantly increased accumulation of TGF-βRII KO cells compared with WT cells in the spleen, PDN, and prostate of TRAMP OVA mice 1 wk after transfer (Fig. 1A). To account for potential differences in prostate size, cells per gram prostate tissue was also calculated, and a similar
increase of TGF-βRII KO cells was observed. To determine whether the preferential accumulation of TGF-βRII KO cells was Ag-specific, WT and TGF-βRII KO cells were also transferred into TRAMP hosts (which do not express OVA in the prostate). Significantly fewer TGF-βRII KO cells were detected in the PDN and prostate of TRAMP mice compared with TRAMP<sub>OVA</sub> mice (Fig. 1A), and there was no significant difference between the numbers of WT cells in TRAMP<sub>OVA</sub> compared with TRAMP mice or between the numbers of WT and TGF-βRII KO cells in any of the tissues examined in TRAMP mice. These data suggest that cell-intrinsic TGF-β signaling negatively impacts the accumulation of prostate self/tumor Ag-specific CD8<sup>+</sup> T cells in the context of responding to self-Ag.

The increased accumulation of TGF-βRII KO cells could be a result of increased proliferation, as TGF-β signaling can inhibit cellular proliferation (17). Intracellular staining of WT and TGF-βRII KO cells directly ex vivo for the proliferation marker, Ki-67, revealed significantly increased numbers of TGF-βRII KO cells expressing Ki-67<sup>+ </sup>in the spleen, PDN, and prostate of TRAMP<sub>OVA</sub> mice (Fig. 1B). The enhanced proliferation was largely Ag-specific, as Ki-67 expression was greatly reduced in all transferred cells isolated from TRAMP mice, indicating that Ag exposure induced transferred cells to remain cycling for at least 1 wk (Fig. 1C). The increased percentage of Ki-67<sup>+ </sup>WT cells in TRAMP<sub>OVA</sub> mice compared with TRAMP mice, despite the failure to accumulate, suggested that WT cells in TRAMP<sub>OVA</sub> mice may have a higher rate of apoptosis. TGF-β signaling upregulates the BH3–only proapoptotic protein Bim (42, 43), and a higher percentage of TGF-βRII KO cells were Bim<sup>low</sup> compared with WT cells in all organs examined in TRAMP<sub>OVA</sub> mice, especially in the proliferating (Ki-67<sup>+</sup>) population (Fig. 1D), whereas no differences between WT and TGF-βRII KO cells were observed in TRAMP mice. These results suggest that abrogation of TGF-β signaling increases the accumulation of prostate self/tumor Ag-specific CD8<sup>+</sup> T cells in part through increased proliferation and in part through reduced apoptosis by decreasing expression of proapoptotic proteins.

Abrogation of TGF-β signaling increases the effector function of transferred prostate self/tumor-Ag–specific CD8<sup>+</sup> T cells

The ability of tumor-specific CD8<sup>+</sup> T cells to produce effector cytokines is critical for tumor regression (44, 45). Therefore, transferred T cells were harvested at 1 wk after transfer, stimulated for 5 h ex vivo with SIINFEKL peptide, and analyzed for cytokine production by intracellular staining. Abrogation of TGF-β signaling significantly increased the percentage and number of transferred cells capable of coproducing IFN-γ and TNF-α in the prostate and PDN (Fig. 2A–C). However, TGF-βRII KO cells in the prostate of TRAMP<sub>OVA</sub> mice exhibited attenuated cytokine production compared with TGF-βRII KO cells in the spleen, suggesting an additional TGF-β–independent, organ-specific suppression of cellular function in the prostate (p = 0.0018).

This functional impairment in the prostate was Ag-specific, as there was no significant difference in cytokine production between transferred WT and TGF-βRII KO cells in any of the organs examined in TRAMP mice. However, decreased percentages of WT and TGF-βRII KO cells from TRAMP<sub>OVA</sub> PDN compared with TRAMP produced cytokines (for WT, p = 0.0018; for KO, p = 0.0020), and a significantly decreased percentage of TGF-βRII KO cells from the prostate of TRAMP<sub>OVA</sub> compared with TRAMP mice coproduced IFN-γ and TNF-α (p = 0.006). These results indicate that at least a component of the functional defect in cytokine production is Ag-specific, that abrogation of TGF-β signaling partially rescues the defect, and that the observed dysfunction of prostate self/tumor Ag T cells is organ-specific and rapidly induced.

**T cells deficient in TGF-β signaling mediate increased cellular infiltration and focal epithelial disruption in the prostates of TRAMP<sub>OVA</sub> mice**

We examined tissue sections of the prostate to determine whether the increased numbers and effector function of TGF-βRII KO cells compared with WT cells led to increased destruction/damage to the prostate tumors. Mice were euthanized at 1 wk after transfer, and the prostate lobes were microdissected and either processed for H&E staining or frozen for immunofluorescence staining. The prostates of TRAMP<sub>OVA</sub> mice that received WT cells showed intact glandular and tumor epithelium with few apoptotic bodies and little evidence of cellular infiltrates in the epithelium or the fibromuscular stroma (Fig. 2D). In contrast, prostates from TRAMP<sub>OVA</sub> mice receiving TGF-βRII KO cells had increased cellular infiltrates in the fibromuscular stroma, including both the interstitium and smooth muscle layer surrounding the glands, and evidence of epithelial disruption with areas of focal necrosis within the gland (Fig. 2D). The infiltrates contained adoptively transferred T cells, as immunohistochemical staining of frozen prostate sections revealed increased Ly5.1<sup>+</sup> cells in prostate glands of mice receiving TGF-βRII KO cells compared with WT cells (Fig. 2E).

**Despite evidence of increased antitumor activity in TRAMP<sub>OVA</sub> mice treated with TGF-βRII KO cells, prostatic inflammation was not sustained**

To determine whether transfer of WT or TGF-βRII KO cells affected tumor burden, prostates of treated mice were harvested 3 wk after T cell transfer and weighed, with prostate weight used as a surrogate for tumor burden, as described (33). There was a small, but statistically significant, decrease in the prostate weight of TRAMP<sub>OVA</sub> mice receiving TGF-βRII KO cells compared with mice receiving WT cells (Fig. 3A). However, histology specimens obtained 3 wk after transfer showed few cellular infiltrates in the interstitium, no significant infiltration of mononuclear cells in the smooth muscle or gland, and no epithelial destruction in TRAMP<sub>OVA</sub> mice receiving WT or TGF-βRII KO cells (Fig. 3B). Despite the decrease in prostate weight, neoplasia was still present in mice treated with TGF-βRII KO cells. Thus, the increased infiltration of TGF-βRII KO cells and antitumor activity observed at 1 wk after transfer in TRAMP<sub>OVA</sub> prostates were transient and not sufficient for persistent therapeutic efficacy.

**Increased accumulation of TGF-βRII KO prostate-specific T cells is sustained in the peripheral lymphoid organs but not in the prostate**

The limited efficacy suggested that transferred T cells did not persist and/or became dysfunctional, obstacles also encountered in human ACT (7). To determine whether the enhanced accumulation and function of TGF-βRII KO cells evident at week 1 was maintained, mice were examined at week 3 after T cell transfer. No significant differences in accumulation, proliferation, or effector functions were observed between WT and TGF-βRII KO cells in the prostate (Fig. 4). In contrast, increased numbers of TGF-βRII KO cells compared with WT cells were still demonstrable in the spleen and PDN of TRAMP<sub>OVA</sub> mice (Fig. 4A), and there was no significant change in the number of TGF-βRII KO cells in the spleen and PDN of TRAMP<sub>OVA</sub> mice at week 3 compared with week 1 (spleen week 1, 5.4 × 10<sup>5</sup> cells, and spleen week 3, 2.8 × 10<sup>5</sup> cells, p = 0.1682; and PDN week 1, 2.1 × 10<sup>5</sup> cells, and PDN week 3, 8.1 × 10<sup>5</sup> cells, p = 0.1765). Analysis of proliferation by staining for Ki-67 revealed that only in the PDN did a higher percentage of TGF-βRII KO cells express Ki-67 compared with WT cells or to TGF-βRII KO cells in TRAMP
hosts (Fig. 4B). Similar to week 1, a higher percentage of TGF-βRII KO cells was Ki-67+Bimlow compared with WT cells in TRAMPOVA mice, but a higher fraction of TGF-βRII KO cells was now Bim high compared with week 1 (Fig. 4C). Thus, TGF-β signaling prevents accumulation of prostate-specific cells in peripheral lymphoid organs, but additional factors beyond TGF-β signaling appear to contribute to the lack of persistence of prostate-infiltrating cells.

By week 3 after transfer, prostate-infiltrating TGF-βRII KO cells were also severely attenuated in effector cytokine production, and

FIGURE 2. Transferred TGF-βRII KO CD8 effector T cells exhibit enhanced effector function, show increased cellular infiltration, and mediate epithelial damage in the prostate. Mice were euthanized 1 wk after adoptive T cell transfer (same experimental protocol as Fig. 1). (A–C) Intracellular IFN-γ and TNF-α expression by transferred WT and TGF-βRII KO cells from spleen, PDN, and prostate of TRAMP OVA mice following 5 h ex vivo stimulation with SIINFEKL peptide. Plots are gated on CD8+Ly5.1+ cells. (A) Representative flow plots of cytokine production by transferred WT and TGF-βRII KO cells. Numbers represent percentages of gated cells in each quadrant. (B) Percentage of transferred WT and TGF-βRII KO cells exhibiting the ability to coproduce both TNF-α and IFN-γ. No significant differences were found between WT and TGF-βRII KO cells from each organ in TRAMP mice. (C) Numbers of cytokine-producing WT and TGF-βRII KO cells in TRAMP OVA mice. (A–C) Data represent pooled results from at least three independent experiments (n = 2–3 mice/group for TRAMP OVA hosts and n = 1–2 mice/group for TRAMP hosts). Bar graphs include means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired Student t test). (D and E) Prostate lobes from TRAMP OVA mice receiving either WT or TGF-βRII KO cells were microdissected and processed for histological analysis. (D) TRAMP OVA prostate lobes were processed and stained with H&E. Original magnification ×10 and ×20 (as shown). The presence of neoplasia in the glands (G), cellular infiltrates in the surrounding fibromuscular stroma (S), and interstitium (I) of TRAMP OVA mice receiving TGF-βRII KO cells is evident at original magnification ×10. Black arrowheads point to apoptotic cells, and yellow arrows point to lymphoid cells at original magnification ×20. (E) Frozen sections of TRAMP OVA prostate lobes were stained with DAPI (blue) and Ly5.1 (red). Original magnification ×20. (D and E) Histology slides show one representative mouse from each experimental group (n = 3–5 mice/group) from at least two independent experiments.
TRAMPOVA prostates at 3 wk after T cell transfer show absence of cellular was determined with an unpaired Student $t$ test. Dissected and analyzed 3 wk after transfer of WT and TGF-βRII KO cells, breeding TRAMP mice onto a PD-L1 $^2$ background was reported to not prevent tolerization of prostate-specific antigen expression (48). TRAMPOVA prostate tumors express MHC class I and maintain expression following adoptive transfer.

MHC class I expression is necessary for target cell destruction, sustained infiltration, and retention of CD8 lymphocytes in tissues (46), and tumor cells can downregulate MHC class I expression as a form of immune evasion (47). Although MHC class I expression is not readily detectable on normal B6 prostate cells, it has been shown to be upregulated in TRAMP prostate tumors (48). To determine whether TRAMP prostate tumors maintained MHC class I expression following cell transfer, we stained frozen prostate sections before and after transfer of WT or TGF-βRII KO cells with anti-MHC class I Ab and found sustained class I expression with no detectable change in TRAMPOVA prostates following therapy (Supplemental Fig. 1).

Persisting transferred TGF-βRII KO cells express PD-1 and TRAMPOVA prostates express the ligand, PD-L1.

The failure of prostate-infiltrating TGF-βRII KO cells to mediate continued significant prostate tumor damage, in addition to the decrease in proliferation and attenuation of effector cytokine production observed by week 3, suggested that the transferred T cells might become functionally exhausted. Chronic Ag exposure can lead to T cell exhaustion (49, 50), which is characterized by a progressive hierarchical loss of CD8 T cell functions. Generally, the abilities to produce IL-2, maintain a high proliferative capacity, and kill targets ex vivo are lost first, followed by loss of TNF-α production and partial loss of IFN-γ production, then complete loss of IFN-γ production, and eventually cell death (51, 52). PD-1, an inhibitory coreceptor upregulated in many settings of T cell exhaustion, has been reported to be expressed on human prostate-tumor-infiltrating CD8 T cells (53). At 1 wk after transfer, WT cells expressed higher levels of PD-1 in the PDN and prostate of TRAMPOVA mice than in TRAMP mice (Fig. 5A). Abrogation of TGF-β signaling resulted in lower PD-1 expression at 1 wk on transferred cells in the prostate and PDN of TRAMPOVA mice. However, at week 3 after transfer, both TGF-βRII KO and WT cells expressed high levels of PD-1 in the PDN and prostate of TRAMPOVA mice. This pattern of PD-1 expression correlated with the severity of the observed functional defect, suggesting that PD-1 signaling may be inhibiting antitumor activity in the prostate, and that the defects in the prostate and PDN may reflect in part consequences of continued Ag recognition. There are currently two known ligands for PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC) (54). PD-L1 is upregulated on many human tumors, including prostate cancer (55), and high PD-L1 expression in some tumor tissues correlates with a decrease in CD8 T cell infiltrates (54). Analysis of frozen TRAMPOVA prostates 3 wk after transfer of TGF-βRII KO cells revealed that PD-L1 was expressed on prostate epithelium (Fig. 5B).

Blockade of PD-1 signaling does not further improve antitumor activity of TGF-βRII-deficient cells.

PD-1 blockade has enhanced antitumor activity in transplantable tumor models (56, 57), and recently phase I human clinical trials of PD-1 blockade in cancer patients have demonstrated antitumor activity for certain cancers (58–61). However, in the TRAMP model, despite increased PD-1 expression on prostate-specific CD8 T cells, breeding TRAMP mice onto a PD-L1$^{-/-}$ background was reported to not prevent tolerization of prostate-specific...
CD8 T cells (62). Because PD-1 may signal through interactions with other known ligands, such as PD-L2, or unidentified ligands, we examined whether blockade of PD-1 signaling in TGF-βRII KO cells with a combination of PD-1, PD-L1, and PD-L2 blocking Abs could promote more persistent and effective antitumor activity. In vitro-activated TGF-βRII KO cells were transferred into TRAMP OVA hosts, and cohorts of mice received either 200 μg each blocking Ab or PBS i.p. every third day, starting on the day of T cell transfer. Mice were euthanized 3 wk following treatment and assayed for T cell function and tumor burden. No significant differences were found between the numbers or function, as reflected by cytokine production, of TGF-βRII KO cells in the spleen, PDN, or prostate in mice that received the blocking Ab mixture or control PBS (Fig. 5C, 5D). Prostates were also weighed and examined histologically, and no significant differences were detected (data not shown). Because this could reflect limitations to these Abs effectively penetrating in situ tumor sites, we stained recovered TGF-βRII KO cells with a secondary Ab to the IgG isotype of the blocking PD-1 Ab and detected Ab bound to transferred T cells in the PDN and prostates of TRAMP OVA but not TRAMP mice (data not shown). These results suggest that, despite expression of PD-1 on transferred TGF-βRII KO cells, as

**FIGURE 4.** TGF-βRII KO cells persist up to 3 wk in the peripheral lymphoid organs but lose function and no longer accumulate in the prostate of TRAMP OVA mice. Mice were euthanized and analyzed 3 wk after adoptive transfer (same experimental protocol as Fig. 1) (A) Numbers of adoptively transferred WT and TGF-βRII KO cells were quantitated in the spleen and PDN of TRAMP OVA mice. Total WT and TGF-βRII KO cells in the prostate are also expressed as cells per gram prostate. (B) Ki-67 expression in transferred cells at week 3 after transfer. No significant differences were detected between WT and TGF-βRII KO cells from each organ in TRAMP mice. (C) Representative flow plots of Ki-67 and Bim expression by transferred cells isolated from TRAMP OVA and TRAMP mice. Flow plots are gated on CD8^+Ly5.1^+ cells. Results are from two independent experiments. (D) Representative flow plots of cytokine production by WT and TGF-βRII KO cells (gated on CD8^+Ly5.1^+ cells). Numbers represent percentage of gated cells in each quadrant. (E) Percentage of transferred WT and TGF-βRII KO cells that coproduce TNF-α and IFN-γ 5 h after ex vivo peptide stimulation. No significant differences were detected between WT and TGF-βRII KO cells from each organ in TRAMP mice. (F) Numbers of transferred TGF-βRII KO cells in each tissue that produce TNF-α and IFN-γ. (A, B, and D–F) Results represent pooled data from at least three independent experiments (n = 1–3 mice/group/experiment). Bar graphs show means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired Student t test).
well as expression of PD-L1 on prostate tumor cells, Ab blockade of PD-1 signaling is not adequate to significantly synergize with abrogation of TGF-β signaling, with no evidence of maintenance or restoration of anti-tumor activity detectable at 3 wk after T cell transfer. Analysis of the successful PD-1 blockade studies performed in the setting of chronic lymphocytic choriomeningitis virus (LCMV) infection revealed that PD-L1 blockade selectively restored the function of LCMV-specific CD8 T cells (63). It appears likely that the transferred cells in our model resemble the PD-1 hi LCMV-specific CD8 T cell subset. The reason for lack of efficacy with this subset is not likely due to insufficient blockade but rather that additional inhibitory receptors, such as CTLA-4 (64), LAG3 (65), TIM-3 (56), and/or 2B4 (66), may be simultaneously expressed and limiting T cell function. In fact, we found LAG3 expressed at increased levels at 3 wk after transfer on TGF-βRII KO cells in the prostate and PDN but not in the spleen of TRAMPova mice compared with TRAMP mice (data not shown). Temporary restoration of cytotoxicity of endogenous prostate-specific CD8 T cells following anti-LAG3 treatment and vaccination has been reported (65). However, whether blockade can augment the benefits of TGF-βR disruption and/or synergize with other blocking reagents for treatment of in situ tumors remains unknown.

The context in which a T cell encounters Ag influences function and differentiation state (67). Thus, many additional events may be contributing to the failure of transferred effector cells to maintain function while targeting a prostate tumor. First, because a self-Ag is being targeted, transferred cells are likely encountering Ag not only on tumor cells but also normal prostate cells and/or dendritic cells presenting the peptide in a tolerogenic context. Chronic Ag stimulation alone can induce T cell exhaustion (49, 50), and in some settings this exhaustion is not rescued by PD-1 blockade (50), as may be occurring in the prostate. Studies in the chronic LCMV infection model have also demonstrated that cell-intrinsic TGF-β blockade can lead to increased numbers of LCMV-specific CD8 T cells and promote clearance of chronic LCMV; however, in experimental conditions in which the viral Ag is not cleared, the TGF-βR-deficient T cells also become functionally exhausted (43). Second, tumor-associated dendritic cells have been identified in TRAMP prostate tumors and can directly suppress naïve prostate-specific CD8 T cells (68). Therefore, it is possible that continuous

Discussion
ACT is being actively pursued in clinical trials to treat malignancies, with successes reported in some cancers (4–6); however, even for tumors with identifiable tumor target Ags, substantive obstacles to broad applicability and to the achievement of predictable and reproducible benefits remain. In this study we investigated whether cell-intrinsic abrogation of TGF-βRII signaling in self/tumor Ag-specific CD8 T cells could enhance the efficacy of in vitro-activated effector T cells in ACT of prostate cancer, using an autochthonous model of murine prostate cancer that replicates many characteristics of human disease. The small but significant decrease in the prostate weight of TRAMPova mice receiving TGF-βRII KO cells compared with mice receiving WT cells at 3 wk after transfer was consistent with enhanced antitumor activity. However, unlike some transplatable models in which TGF-βR blockade in tumor-reactive T cells resulted in complete elimination of the tumor (29, 30), antitumor activity in the TRAMP model was not sustained, suggesting that additional barriers are present for targeting a tumor in situ.

Lack of persistence and failure to maintain in vivo antitumor activity following T cell transfer are frequent problems in clinical ACT targeting established tumors (7). We demonstrated that abrogation of TGF-β signaling was adequate to numerically sustain transferred T cells in distal secondary lymphoid organs, but additional immunosuppressive factors operative within the prostate and possibly PDN eventually rendered cells remaining at these sites dysfunctional. Although transferred cells in the PDN and prostate upregulated the inhibitory receptor, PD-1, Ab blockade of PD-1 signaling failed to significantly synergize with abrogation of TGF-β signaling, with no evidence of maintenance or restoration of anti-tumor activity detectable at 3 wk after T cell transfer. Analysis of the successful PD-1 blockade studies performed in the setting of chronic lymphocytic choriomeningitis virus (LCMV) infection revealed that PD-L1 blockade selectively restored the function of PD-1hi but not PD-1im CD8 T cells (63). It appears likely that the transferred cells in our model resemble the PD-1hi LCMV-specific CD8 T cell subset. The reason for lack of efficacy with this subset is not likely due to insufficient blockade but rather that additional inhibitory receptors, such as CTLA-4 (64), LAG3 (65), TIM-3 (56), and/or 2B4 (66), may be simultaneously expressed and limiting T cell function. In fact, we found LAG3 expressed at increased levels at 3 wk after transfer on TGF-βRII KO cells in the prostate and PDN but not in the spleen of TRAMPova mice compared with TRAMP mice (data not shown). Temporary restoration of cytotoxicity of endogenous prostate-specific CD8 T cells following anti-LAG3 treatment and vaccination has been reported (65). However, whether blockade can augment the benefits of TGF-βR disruption and/or synergize with other blocking reagents for treatment of in situ tumors remains unknown.

FIGURE 5. PD-1 and PD-L1 are expressed, respectively, by persisting transferred T cells and the prostate tumor in treated TRAMPova mice, but blockade of PD-1 signaling does not further increase accumulation or effector function of TGF-βRII KO cells at 3 wk after transfer. (A) PD-1 expression on WT and TGF-βRII KO cells at week 1 and week 3 after transfer. Histograms are gated on CD8+Ly5.1+ cells. The WT or TGF-βRII KO cells transferred into TRAMPova hosts are shown with a black line, and cells transferred into TRAMP hosts are in shaded gray. (B) Frozen sections of TGF-βRII KO cell-treated TRAMPova prostates 3 wk after transfer were stained with DAPI (blue) and anti–PD-L1 (green), and cells transferred into TRAMP hosts are in shaded gray. (C) Numbers of persisting transferred T cells in TRAMPova mice treated with Ab or PBS. (D) Percentage of transferred TGF-βRII KO cells coproducing TNF-α and IFN-γ following 5 h ex vivo peptide stimulation. All results represent pooled data from three independent experiments (n = 2–3 mice/group/experiment for mice treated with blocking Abs and n = 1–2 mice/group/experiment for control PBS treated). No significant differences between treated and untreated mice were detected (unpaired Student t test).

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encounters by transferred self/tumor-specific effector T cells with tumor-associated dendritic cells in the prostate prevent sustained antitumor activity. Dendritic cell vaccines may transiently augment and/or restore the activity of prostate-infiltrating T cells (69–71).

Additional cell-extrinsic factors may also contribute to the immunosuppressive tumor environment, including Foxp3+ regulatory T cells. Similar to published studies (72), we found increased numbers of CD4+Foxp3+ cells in 25-wk-old TRAMPOVA prostates compared with those in healthy age-matched male mice. To test whether Foxp3+ Tregs play a dominant role in suppressing adaptively transferred effectors, we bred TRAMPOVA mice to Foxp3DTR mice (73). In preliminary studies utilizing the TRAMPOVA × Foxp3DTR mice, in which near complete ablation of Foxp3+ T cells (>97%) can be achieved, no enhanced infiltration or cytokine production by transferred TGF-βRII KO cells in the prostates of TRAMPOVA mice was observed (data not shown). Moreover, these regulatory T cell-depleted mice developed systemic autoimmunity, as previously reported (73), affirming the inherent difficulties associated with pursuing effective global depletion of regulatory T cells as a therapeutic strategy for treating tumors.

Our findings have implications for human adoptive therapy. We found increased function of both WT and TGF-βRII KO cells in the spleen and PDN compared with the prostate. The greater dysfunction at the site where the activity is actually required highlights the importance of analyzing intratumoral T cells when assessing the function of T cells targeting an established tumor. Evidence supporting this conclusion has also been provided in studies of melanoma patients, in which tumor-infiltrating lymphocytes in metastatic lesions can exhibit an exhausted profile, whereas T cells of the same specificity in the blood are functional (74).

The initial increase in accumulation of TGF-βRII KO prostatic-specific T cells and delay in loss of antitumor activity in the prostate do offer a window of opportunity for additional interventional therapies that could potentially result in synergistic antitumor activity before T cells become functionally impaired. Adjunctive therapies, such as radiation or chemotherapy, can augment antitumor activity of prostate-specific T cells (71, 75, 76). We recently demonstrated that lymphopenia-induced proliferation could transiently restore the function of tolerant T cells (77). These data together suggest that lymphodepletion of TRAMP mice may synergize with abrogation of TGF-βRII to increase therapeutic efficacy. Additionally, identifying and targeting tumor-specific Ags not expressed by normal cells may circumvent or delay functional exhaustion by reducing the extent of persistent Ag stimulation. However, whereas some unique tumor-specific epitopes have been discovered in selected tumors, tumor-specific Ags are often unique to each patient, and most Ags being targeted in clinical trials, including all known targetable prostate cancer Ags, are self-Ags (24, 78–80).

In conclusion, our results highlight some of the obstacles to ACT for solid tumors, and they emphasize the need for testing potential ACT strategies in preclinical models that emulate the development and environment of tumors to identify and address potential pitfalls. The nature and relative importance of particular immunosuppressive mechanisms may vary with different tumor types, and a more complete analysis of the individual obstacles will likely be invaluable for designing combinatorial strategies to target selected tumors with T cells.

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Disclosures
G.J.F. has patents and receives patent royalties on the PD-1 pathway. The other authors have no financial conflicts of interest.

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Supplementary Figure 1: TRAMP prostate tumors express MHC Class I and maintain Class I expression following adoptive T cell transfer. Prostates were harvested from B6 mice and TRAMP$_{OVA}$ mice (pre-adoptive transfer and at week 1 and week 3 post adoptive transfer of TGFβRII KO or WT cells), and frozen in OCT. Frozen prostate sections were stained with DAPI (blue) and anti-MHC Class I mAb (red).