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Cell intrinsic abrogation of TGFβ 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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Abstract

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 anti-tumor activity of transferred T cells remain major problems. Transforming growth factor beta (TGFβ) is a potent immunosuppressive cytokine that is often expressed at high levels within the tumor microenvironment, potentially limiting T cell mediated anti-tumor activity. Here, we used a model of autochthonous murine prostate cancer to evaluate the effect of cell intrinsic abrogation of TGFβ signaling in self/tumor specific CD8 T cells used in ACT to target the tumor *in situ*. We found that persistence and anti-tumor activity of adoptively transferred effector T cells deficient in TGFβ signaling was 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 TGFβ negatively regulates the accumulation and effector function of transferred self/tumor specific CD8 T cells and highlight that, when targeting a tumor antigen 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.

Introduction

The recent FDA approval of two cancer immunotherapies, a vaccine (Sipuleucel-T) for treatment of prostate cancer (1), and an anti-CTLA-4 blocking antibody (ipilimumab) for treatment of metastatic melanoma (2), has highlighted the ability to modulate the immune

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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), difficulty 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 anti-tumor 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 (TILs) unresponsive or dysfunctional (8). Furthermore, T cells isolated directly from the patient for use in ACT are often of only low avidity, since most of the identified tumor antigens 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).

Transforming growth factor beta (TGFβ) is a pleiotropic cytokine that plays important roles in maintaining normal tissue homeostasis and inhibiting autoimmune responses, and depending on the context can promote or suppress tumor growth (12–17). The bioactive form of TGFβ binds to the TGFβ-type I and TGFβ-type II serine/threonine kinase receptor complexes, resulting in receptor mediated phosphorylation of downstream transcription factors Smad 2 and Smad 3 (17). TGFβ signaling is anti-proliferative, causing G_1 cell cycle arrest in a variety of cell types, including epithelial and T cells (18, 19). Many tumors evade the cytostatic and anti-proliferative effects of TGFβ by acquiring mutations in the TGFβ receptor and/or downstream Smad signaling proteins (17). Activated T cells however, express higher levels of the TGFβ receptor and can produce TGFβ (20, 21). Molecular analysis of naïve CD8 T cells *in vitro* has revealed that TGFβ suppresses key molecules involved in the effector and cytolytic activities of T cells, including expression of IFNγ (22). Inhibition of TGFβ signaling by mechanisms such as neutralizing antibodies 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 TGFβ, 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 TGFβ 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 antigens 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, TGFβ is present and necessary for normal prostate homeostasis, and 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 TGFβRII (DNR-TGFβRII) or abrogation of TGFβ production exclusively in T cells of mice that develop autochthonous prostate cancer can delay tumor growth (21, 27), suggesting TGFβ interferes with the development and/or expression of an endogenous response. Studies in transplantable tumor models also demonstrated that TGFβ 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, while 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 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 antigen 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 weeks of age and progression through distinct histological stages of adenocarcinoma (33, 34). We crossed these mice with the Probasin Ovalbumin Expressing Transgenic ($POET_1$) mice, which express a membrane bound form of the model antigen ovalbumin (OVA) driven by the prostate-specific ARR_2PB rat probasin promoter (35). TRAMPx$POET_1$ mice, denoted $TRAMP_{OVA}$, express a targetable self/tumor antigen (OVA) in the context of a spontaneously arising prostate cancer. The use of OVA as a model self/tumor antigen 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 prostatic self-antigen with T cells in which TGFβ 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 (FHCRC), Seattle, WA). POET mice (35) and were obtained from T. Ratliff (Purdue University, West Lafayette, IN). $TGF\beta RII^{Flx/Flx}$ mice were provided by D. Dichek (University of Washington (UW), Seattle, WA) with permission from S. Karlsson (Lund University, Lund, Sweden) (12). Distal Lck-Cre (*d-lck*Cre) mice (37), which express Cre recombinase under control of the distal Lck promoter, were provided by P. Fink (UW, 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 ovalbumin (OVA), were a gift from M. Bevan (UW, Seattle, WA). Ly5.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). To generate prostate cancer mice expressing a targetable self/tumor antigen, $TRAMP^{+/+}$ male mice were crossed to female $POET_1{}^{+/-}$ to generate to generate F_1 mice hemizygous for the SV40 transgene and OVA expression ($TRAMP_{OVA}$), and littermates expressing SV40 transgene only (TRAMP). All $TRAMP_{OVA}$ and TRAMP mice used were between 25–27 weeks of age, at which time all mice have high grade neoplasia (34). To generate OVA specific TGFβRII deficient mice (TGFβRII KO), mice expressing floxed TGFβRII genes ($TGF\beta RII^{Flx/Flx}$) were first bred to *d-lck*CRE mice or $OTI^{Ly5.1}$ mice. The F1 offspring were bred together to produce mice harboring $OTI^{Ly5.1}$ CD8 T cells with a conditional deletion of TGFβRII in mature CD8 T cells ($OTI^{Ly5.1\times}TGF\beta RII^{Flx/Flx}\times$ *d-lck*CRE). $OTI^{Ly5.1\times}TGF\beta RII^{Flx/Flx}$ littermates were used as WT donors. All mice were maintained under specific pathogen-free conditions at the UW under the guidelines of the Institutional Animal Use and Care Committee.

Peptide

SIINFEKL peptide was synthesized by the Immune Monitoring Lab at FHCRC (Seattle, WA). Peptide was reconstituted in 100% DMSO at 10mg/ml and stored at −20°C.

Cell isolation

Mice were euthanized by cervical dislocation. Spleens were mechanically disrupted with the back of a 3mL syringe, filtered through a 70 μm strainer and red blood cells lysed with ammonium chloride potassium buffer. Cells were washed twice with complete RPMI media (RPMI 1640 supplemented with 2 μM glutamine, 100U/ml penicillin/streptomycin and 10% fetal calf serum). Prostate draining lymph nodes (peri-aortic; PDN) 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 hour 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 $OTI^{Ly5.1}$ TGFβRII KO mice. CD4 and B cells were depleted using αCD4 and αB220 DynaBeads (Invitrogen). Remaining cells were co-cultured with irradiated (3000 rads) congenic splenocytes pulsed with SIINFEKL (10^{-1} μg/ml) at a 1:5 ratio and 25 U/mL human recombinant interleukin 2 (IL2, 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 percent of $7AAD^{-}CD8^{+}V\alpha2^{+}V\beta5^{+}$ cells by flow cytometry. Cells were washed twice with HBSS prior to injection of $5–7\times10^{6}$ OTI cells into the lateral tail vein of mice at a volume of 0.2mL.

Flow cytometry

All single cell suspensions were washed with staining buffer (PBS + 1% fetal calf serum) prior to phenotypic and functional characterization. The following antibodies were purchased from eBiosciences: CD8α, Ly5.1, IFNγ, TNFα, and PD-1. Surface staining was done at 4°C in staining buffer. Ki-67 (BD Biosciences) and Bim (Cell Signaling) staining was performed using the eBiocience fixation/permeabilization buffer kit per manufacturer's instructions. Briefly, following surface staining with CD8 and Ly5.1 antibodies, cells were fixed, permeabilized and stained with antibody to Ki-67 and Bim. A secondary PE-anti-rabbit Fab_2 fragment (Invitrogen) was used to detect Bim. Intracellular cytokine staining was performed using the Cyofix/Cytoperm Plus kit (BD Biosciences) per manufacturer's instructions. Briefly, single cell suspensions from spleen, lymph node, and prostate were stimulated directly *ex vivo* for 5 hours with 10^{-1} 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 antibodies to IFNγ and TNFα. Flow cytometric analysis was performed using FACSCanto and LSRII at the Cell Analysis Facility, Department of Immunology, UW. Flow data was analyzed with FlowJo8.8.7 (Tree Star, Inc, Ashland, OR).

Prostate histology and immunohistochemistry

For hematoxylin and eosin (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 FHCRC, Seattle, WA. Histologic sections were evaluated by a comparative medicine pathologist blinded to group assignments. Images were captured using a Nikon Eclipse 80*i* microscope with DS-Fi1 digital camera and NIS Elements software.

For immunofluorescence staining, microdissected prostate lobes were frozen in optimal cutting temperature (Sakura). Seven μM frozen prostate sections were cut on a cryostat. Sections were fixed with ice-cold acetone and blocked with PBS + 1% goat serum prior to staining. Primary antibodies included: Ly5.1-PE (eBioscience), rat anti-mouse PD-L1 (eBioscience), MHC Class I-PE (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 captured with an Orca-ER digital camera and assembled into RGB images with Image J and Adobe Photoshop.

Antibody blockade treatment

Monoclonal αPD-1 (29F.1A12) (38), αPD-L1 (10F.9G2) (39) and αPD-L2, (3.2) (40) antibodies were provided by G. Freeman (Harvard Medical School, Boston, MA). To assure adequate blockade, the timing and dose of administration of these antibodies established for each individual antibody (41) was used. 200 μg of each blocking antibody was injected *i.p.* into recipient mice starting on the day of T cell transfer and continued every 3 days 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 unpaired two-tailed Student *t* tests. A *p* value of <0.05 was considered statistically significant.

Results

Abrogation of TGFβ signaling increases the accumulation of transferred prostate self/tumor antigen-specific CD8 T cells

To investigate the T cell intrinsic role of TGFβ in the setting of ACT of prostate cancer, we transferred 5–7×10^6 *in vitro* activated OTI WT and TGFβRII KO CD8 T cells into tumor bearing 25–27 week old $TRAMP_{OVA}$ and TRAMP males. We first assessed if abrogating TGFβ signaling affected expansion of the transferred cells and found a significantly increased accumulation of TGFβRII KO cells compared to WT cells in the spleen, PDN and prostate of $TRAMP_{OVA}$ mice 1 week post transfer (Fig. 1*A*). To account for potential differences in prostate size, cells/gram of prostate tissue was also calculated, and a similar increase of TGFβRII KO cells was observed. To determine if the preferential accumulation of TGFβRII KO cells was antigen-specific, WT and TGFβRII KO cells were also transferred into TRAMP hosts (which do not express OVA in the prostate). Significantly less TGFβRII KO cells were detected in the PDN and prostate of TRAMP mice compared to $TRAMP_{OVA}$ mice (Fig. 1*A*), and there was no significant difference between the numbers of WT cells in $TRAMP_{OVA}$ compared to TRAMP mice or between the numbers of WT and TGFβRII KO cells in any of the tissues examined in TRAMP mice. This data suggests cell intrinsic TGFβ signaling negatively impacts the accumulation of prostate self/tumor antigen-specific CD8 T cells in the context of responding to self-antigen.

The increased accumulation of TGFβRII KO cells could be a result of increased proliferation, as TGFβ signaling can inhibit cellular proliferation (17). Intracellular staining of WT and TGFβRII KO cells directly *ex vivo* for the proliferation marker, Ki-67 revealed that significantly increased numbers of TGFβRII KO cells expressing Ki-67^+ in the spleen, PDN and prostate of $TRAMP_{OVA}$ mice (Fig. 1*B*). The enhanced proliferation was largely antigen-specific, as Ki-67 expression was greatly reduced in all transferred cells isolated from TRAMP mice, indicating that antigen exposure induced transferred cells to remain cycling for at least 1 week (Fig. 1*C*). The increased percentage of Ki-67^+ WT cells in

$TRAMP_{OVA}$ mice compared to TRAMP mice despite the failure to accumulate suggested that WT cells in $TRAMP_{OVA}$ mice may have a higher rate of apoptosis. TGFβ signaling upregulates the BH-3 only pro-apoptotic protein Bcl-2-interacting mediator of cell death (Bim) (42, 43), and a higher percentage of TGFβRII KO cells were Bim^{low} compared to WT cells in all organs examined in $TRAMP_{OVA}$ mice, especially in the proliferating ($Ki\text{-}67^{+}$) population (Fig. 1*D*), whereas no differences between WT and TGFβRII KO cells were observed in TRAMP mice. These results suggest abrogation of TGFβ signaling increases the accumulation of prostate self/tumor antigen-specific CD8 T cells in part through increased proliferation and in part through reduced apoptosis by decreasing expression of pro-apoptotic proteins.

Abrogation of TGFβ signaling increases the effector function of transferred prostate tumor/self antigen-specific CD8 T cells

The ability of tumor-specific CD8 T cells to produce effector cytokines is critical for tumor regression (44, 45). Therefore, transferred T cells were harvested at 1 week post transfer, stimulated for 5 hours *ex vivo* with SIINFEKL peptide, and analyzed for cytokine production by intracellular staining. Abrogation of TGFβ signaling significantly increased the percentage and number of transferred cells capable of co-producing IFNγ and TNFα in the prostate and PDN (Fig. 2*A–C*). However, TGFβRII KO cells in the prostate of $TRAMP_{OVA}$ mice exhibited attenuated cytokine production compared to TGFβRII KO cells in the spleen, suggesting an additional TGFβ independent, organ-specific suppression of cellular function in the prostate (p=0.0018).

This functional impairment in the prostate was antigen-specific, as there was no significant difference in cytokine production between transferred WT and TGFβRII KO cells in any of the organs examined in TRAMP mice. However, decreased percentages of WT and TGFβRII KO cells from $TRAMP_{OVA}$ PDN compared to TRAMP produced cytokines (for WT p=0.0018, for KO p=0.0020) and significantly decreased percentage of TGFβRII KO cells from the prostate of $TRAMP_{OVA}$ compared to TRAMP mice co-produced IFNγ and TNFα (p=0.006). These results indicate that at least a component of the functional defect in cytokine production is antigen-specific, that abrogation of TGFβ signaling partially rescues the defect, and that the observed dysfunction of prostate self/tumor antigen T cells is organ-specific and rapidly induced.

T cells deficient in TGFβ signaling mediate increased cellular infiltration and focal epithelial disruption in the prostates of $TRAMP_{OVA}$ mice

We examined tissue sections of the prostate to determine if the increased numbers and effector function of TGFβRII KO cells compared to WT cells led to increased destruction/damage to the prostate tumors. Mice were euthanized at 1 week post-transfer, and the prostate lobes micro-dissected and either processed for H&E staining or frozen for immunofluorescence staining. The prostates of $TRAMP_{OVA}$ 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. 2*D*). In contrast, prostates from $TRAMP_{OVA}$ mice receiving TGFβRII 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. 2*D*). The infiltrates contained adoptively transferred T cells, as immuno-histochemical staining of frozen prostate sections revealed increased $Ly5.1^{+}$ cells in prostate glands of mice receiving TGFβRII KO cells compared to WT cells (Fig. 2*E*).

Despite evidence of increased anti-tumor activity in $TRAMP_{OVA}$ mice treated with TGFβRII KO cells, prostatic inflammation was not sustained

To determine if transfer of WT or TGFβRII KO cells affected tumor burden, prostates of treated mice were harvested 3 weeks post 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 $TRAMP_{OVA}$ mice receiving TGFβRII KO cells compared to mice receiving WT cells (Fig. 3*A*). However, histology specimens obtained 3 weeks post 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 $TRAMP_{OVA}$ mice receiving WT or TGFβRII KO cells (Fig. 3*B*). Despite the decrease in prostate weight, neoplasia was still present in mice treated with TGFβRII KO cells. Thus, the increased infiltration of TGFβRII KO cells and anti-tumor activity observed at 1 week post-transfer in $TRAMP_{OVA}$ prostates was transient, and not sufficient for persistent therapeutic efficacy.

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

The limited efficacy suggested transferred T cells did not persist and/or became dysfunctional, obstacles also encountered in human ACT (7). To determine if the enhanced accumulation and function of TGFβRII KO cells evident at week 1 was maintained, mice were examined at week 3 post-T cell transfer. No significant differences in accumulation, proliferation or effector functions were observed between WT and TGFβRII KO cells in the prostate (Fig. 4). In contrast, increased numbers of TGFβRII KO cells compared to WT cells were still demonstrable in the spleen and PDN of $TRAMP_{OVA}$ mice (Fig. 4*A*), and there was no significant change in the number of TGFβRII KO cells in the spleen and PDN of $TRAMP_{OVA}$ mice at week 3 compared to week 1 ($spleen_{wk\ 1}$: 5.4×10^5 cells, $spleen_{wk\ 3}$: 2.8×10^6 cells, $p = 0.1682$; and $PDN_{wk\ 1}$: 2×10^5 cells, $PDN_{wk\ 3}$: 8.1×10^5 cells, $p = 0.1765$). Analysis of proliferation by staining for Ki-67 revealed that only in the PDN did a higher percentage of TGFβRII KO cells express Ki-67 compared to WT cells or to TGFβRII KO cells in TRAMP hosts (Fig. 4*B*). Similar to week 1, a higher percentage of TGFβRII KO cells were $Ki\text{-}67^+\ Bim^{low}$ compared to WT cells in $TRAMP_{OVA}$ mice, but a higher fraction of TGFβRII KO cells were now Bim^{high} compared to week 1 (Fig. 4*C*). Thus, TGFβ signaling prevents accumulation of prostate-specific cells in peripheral lymphoid organs, but additional factors beyond TGFβ signaling appear to contribute to the lack of persistence of prostate infiltrating cells.

By week 3 post transfer, prostate-infiltrating TGFβRII KO cells were also severely attenuated in effector cytokine production and increased dual-cytokine producing TGFβRII KO cells compared to WT cells were no longer detected in the prostate (Fig. 4*D–F*). Increased numbers of $IFN\gamma^+TNF\alpha^+$ TGFβRII KO cells were still present in the spleen and PDN compared to WT cells (we were unable to recover sufficient numbers of WT cells from the PDN of $TRAMP_{OVA}$ mice at 3 weeks to analyze cytokine production) (Fig. 4*F*). WT and TGFβRII KO cells were also transferred into TRAMP mice and analyzed for cytokine production, revealing 2 important findings. First, similar to week 1 post-transfer, the majority of transferred cells recovered from TRAMP mice at week 3 post-transfer produced both cytokines (Fig. 2*B*, Fig. 4*E*), suggesting the decreased cytokine production by transferred cells in $TRAMP_{OVA}$ mice was due to persistent cognate antigen recognition. Second, both TGFβRII KO cells and WT cells isolated from the prostate showed a decreased ability to produce cytokines compared to transferred cells isolated from the spleen (TGFβRII KO cells $p = 0.1446$; WT cells $p = 0.0370$), suggesting factors within the prostate tumor microenvironment impacts the activity of these cells in an antigen-independent manner. Thus, despite persistence of TGFβRII KO cells in the periphery of $TRAMP_{OVA}$

mice, by week 3 TGFβRII KO cells no longer accumulate in the prostate and are severely attenuated in effector function.

$TRAMP_{OVA}$ 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 down-regulate 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 up-regulated in TRAMP prostate tumors (48). To determine if TRAMP prostate tumors maintained MHC Class I expression following cell transfer, we stained frozen prostate sections before and after transfer of WT or TGFβRII KO cells with anti-MHC Class I antibody, and found sustained Class I expression with no detectable change in $TRAMP_{OVA}$ prostates following therapy (Supplementary Figure 1).

Persisting transferred TGFβRII KO T cells express PD-1 and $TRAMP_{OVA}$ prostates express the ligand, PD-L1

The failure of prostate-infiltrating TGFβRII 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 the transferred T cells might become functionally exhausted. Chronic antigen exposure can lead to T cell exhaustion (49, 50), which is characterized by a progressive hierarchical loss of CD8 T cell functions. Generally, the ability to produce IL2, maintain a high proliferative capacity, and kill targets *ex vivo* are lost first, followed by loss of TNFα production and partial loss of IFNγ production, then complete loss of IFNγ production, and eventually cell death (51, 52). Programmed death 1 (PD-1), an inhibitory co-receptor up-regulated in many settings of T cell exhaustion, has been reported to be expressed on human prostate tumor infiltrating CD8 T cells (53). At 1 week post-transfer, WT cells expressed higher levels of PD-1 in the PDN and prostate of $TRAMP_{OVA}$ mice than TRAMP mice (Fig. 5*A*). Abrogation of TGFβ signaling resulted in lower PD-1 expression at 1 week on transferred cells in the prostate and PDN of $TRAMP_{OVA}$ mice. However, at week 3 post-transfer, both TGFβRII KO and WT cells expressed high levels of PD-1 in the PDN and prostate of $TRAMP_{OVA}$ mice. This pattern of PD-1 expression correlated with the severity of the observed functional defect, suggesting PD-1 signaling may be inhibiting anti-tumor activity in the prostate, and that the defects in the prostate and PDN may reflect in part consequences of continued antigen recognition. There are currently 2 known ligands for PD-1, PD-1 ligand 1 (PD-L1; B7-H1) and PD-1 ligand 2 (PD-L2; B7-DC) (54). PD-L1 is up-regulated 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 $TRAMP_{OVA}$ prostates 3 weeks post-transfer of TGFβRII KO cells revealed PD-L1 was expressed on prostate epithelium (Fig. 5*B*).

Blockade of PD-1 signaling does not further improve anti-tumor activity of TGFβRII deficient cells

PD-1 blockade has enhanced anti-tumor activity in transplantable tumor models (56, 57) and recently phase I human clinical trials of PD-1 blockade in cancer patients have demonstrated anti-tumor 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 $PD\text{-}L1^{-/-}$ background was reported to not prevent tolerization of prostate-specific CD8 T cells (62). Since PD-1 may signal through interactions with other known ligands, such as PD-L2, or unidentified ligands, we examined if blockade of PD-1 signaling in TGFβRII KO cells with a combination of PD-1, PD-L1 and PD-L2 blocking antibodies could promote

more persistent and effective anti-tumor activity. *In vitro* activated TGFβRII KO cells were transferred into $TRAMP_{OVA}$ hosts, and cohorts of mice received either 200μg of each blocking antibody or PBS *i.p.* every 3rd day, starting on the day of T cell transfer. Mice were euthanized 3 weeks 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 TGFβRII KO cells in the spleen, PDN or prostate in mice that received the blocking antibody cocktail or control PBS (Fig. 5*C–D*). Prostates were also weighed and examined histologically, and no significant differences were detected (data not shown). As this could reflect limitations to these Abs effectively penetrating *in situ* tumor sites, we stained recovered TGFβRII KO cells with a secondary antibody to the IgG isotype of the blocking PD-1 antibody, 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 TGFβRII KO cells, and expression of PD-L1 on prostate tumor cells, antibody blockade of PD-1 signaling is not adequate to significantly synergize with the enhancement initially achieved by blockade of TGFβ signaling, implying additional inhibitory pathways are operative in the environment of prostate cancers.

Discussion

ACT is being actively pursued in clinical trials to treat malignancies, with successes reported in some cancers (4–6), but, even for tumors with identifiable tumor target antigens, substantive obstacles to broad applicability and the achievement of predictable and reproducible benefits remain. In this study we investigated if cell intrinsic abrogation of TGFβRII signaling in self/tumor antigen 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 $TRAMP_{OVA}$ mice receiving TGFβRII KO cells compared to mice receiving WT cells at 3 weeks post transfer was consistent with enhanced anti-tumor activity. However, unlike some transplantable models in which TGFβR blockade in tumor-reactive T cells resulted in complete elimination of the tumor (29, 30), anti-tumor activity in the TRAMP model was not sustained, suggesting additional barriers are present for targeting a tumor *in situ*.

Lack of persistence and failure to maintain *in vivo* anti-tumor activity following T cell transfer are frequent problems in clinical ACT targeting established tumors (7). We demonstrated that abrogation of TGFβ 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, antibody blockade of PD-1 signaling failed to significantly synergize with abrogation of TGFβ signaling, with no evidence of maintenance or restoration of anti-tumor activity detectable at 3 weeks post-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 $PD\text{-}1^{int}$ but not $PD\text{-}1^{hi}$ LCMV-specific CD8 T cells (63). It appears likely that the transferred cells in our model resemble the $PD\text{-}1^{hi}$ 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 weeks post-transfer on TGFβRII KO cells in the prostate and PDN but not the spleen of $TRAMP_{OVA}$ mice compared to TRAMP mice (data not shown). Temporary restoration of cytotoxicity of endogenous prostate-specific CD8 T cells following αLAG3 treatment and vaccination has been reported

(65). However, whether blockade can augment the benefits of TGFβR disruption and/or synergize with other blocking reagents for treatment of *in situ* tumors remains unknown.

The context in which a T cell encounters antigen 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, since a self-antigen is being targeted, transferred cells are likely encountering antigen not only on tumor cells but also normal prostate cells and/or dendritic cells (DCs) presenting the peptide in a tolerogenic context. Chronic antigen 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 TGFβ blockade can lead to increased numbers of LCMV-specific CD8 T cells and promote clearance of chronic LCMV, but, in experimental conditions in which the viral antigen is not cleared, the TGFβR-deficient T cells also become functionally exhausted (43). Second, tumor associated DCs (TADCs) have been identified in TRAMP prostate tumors and can directly suppress naïve prostate-specific CD8 T cells (68), therefore, it is possible continuous encounters by transferred self/tumor-specific effector T cells with TADCs in the prostate prevent sustained anti-tumor activity. DC 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 (Tregs). Similar to published studies (72), we found increased numbers of $CD4^+Foxp3^+$ cells in 25 week-old $TRAMP_{OVA}$ prostates compared to healthy age-matched male mice. To test if $Foxp3^+$ Tregs play a dominant role in suppressing adoptively transferred effectors, we bred $TRAMP_{OVA}$ mice to $Foxp3^{DTR}$ mice (73). In preliminary studies utilizing the $TRAMP_{OVA}$×$Foxp3^{DTR}$ mice, in which near complete ablation of $Foxp3^+$ T cells (>97%) can be achieved, no enhanced infiltration or cytokine production by transferred TGFβRII KO cells in the prostates of $TRAMP_{OVA}$ mice was observed (data not shown). Moreover, these Treg-depleted mice developed systemic autoimmunity, as previously reported (73), affirming the inherent difficulties associated with pursuing effective global depletion of Tregs as a therapeutic strategy for treating tumors.

Our findings have implications for human adoptive therapy. We found increased function of both WT and TGFβRII KO cells in the spleen and PDN compared to the prostate. The greater dysfunction at the site where the activity is actually required highlights the importance of analyzing intra-tumoral 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).

These studies are the first to assess the effect of cell-intrinsic abrogation of TGFβRII signaling in self/tumor specific CD8 T cells in the context of ACT for a spontaneous solid cancer. The initial increase in accumulation of TGFβRII KO prostate-specific T cells and delay in loss of anti-tumor activity in the prostate does offer a window of opportunity for additional interventional therapies that could potentially result in synergistic anti-tumor activity before T cells become functionally impaired. Adjunctive therapies, such as radiation or chemotherapy, can augment anti-tumor 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 TGFβRII to increase therapeutic efficacy. Additionally, identifying and targeting tumor-specific antigens not expressed by normal cells may circumvent or delay functional exhaustion by reducing the extent of persistent

antigen stimulation. However, while some unique tumor-specific epitopes have been discovered in select tumors, tumor-specific antigens are often unique to each patient and the majority of antigens being targeted in clinical trials, including all known targetable prostate cancer antigens, are self-antigens (24, 78–80).

In conclusion, our results highlight some of the obstacles to ACT for solid tumors, and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ACT	adoptive T cell therapy
DNR-TGFβRII	dominant negative form of TGFβRII
LCMV	lymphocytic choriomeningitis virus
OVA	ovalbumin
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 2
TGFβ	transforming growth factor beta
TGFβRII	transforming growth factor beta receptor II
TRAMP	Transgenic Adenocarcinoma of the Mouse Prostate
PDN	prostate draining lymph node
POET	prostate ovalbumin expressing transgenic

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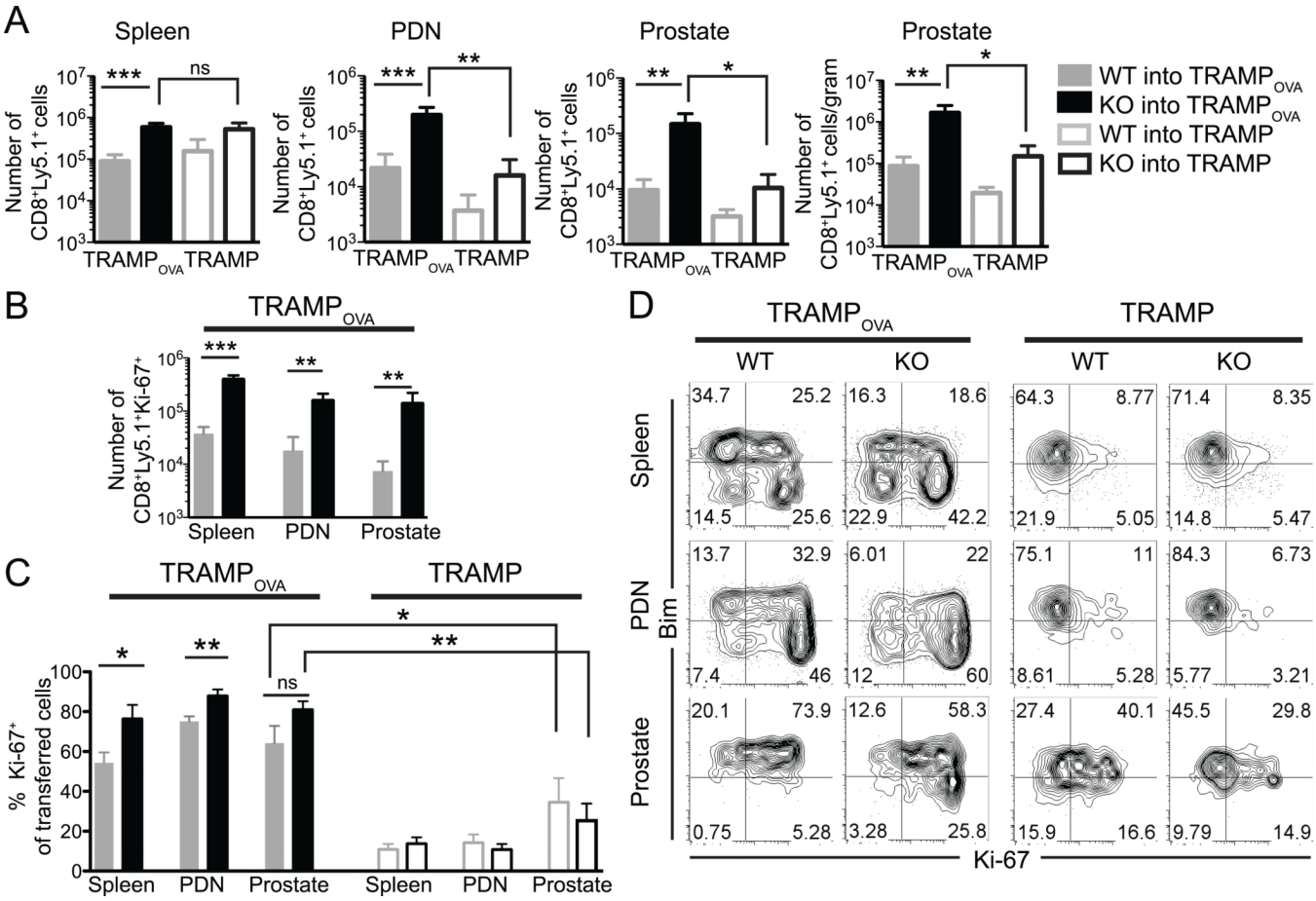

Figure 1. Increased accumulation of TGFβRII deficient prostate self/tumor antigen specific CD8 effector T cells in TRAMP$_{OVA}$ mice

(A) 5–7×10^6 effector WT or TGFβRII KO cells were transferred *i.v.* into 25–27 week old TRAMP$_{OVA}$ and TRAMP hosts. Mice were euthanized 1 week post transfer and spleen, PDN and prostates were analyzed. (A) Cell numbers were quantitated based on total cell counts and percent of CD8$^+$Ly5.1$^+$ cells from flow cytometric analysis. For the prostate, numbers of transferred cells/gram of tissue is also shown. No significant differences were detected between WT and TGFβRII KO cells from each organ in TRAMP mice. (B) Numbers of CD8$^+$Ly5.1$^+$Ki-67$^+$ WT and TGFβRII KO cells isolated from various organs in TRAMP$_{OVA}$ mice (C). Percent of transferred T cells expressing Ki-67 in the spleen, PDN and prostate of TRAMP$_{OVA}$ and TRAMP mice. No significant differences were detected between WT and TGFβRII KO cells from each organ in TRAMP mice. (A–C) Data represents pooled results from at least 3 independent experiments (n=2–3 mice/group/experiment for TRAMP$_{OVA}$ hosts and n=1–2 mice/group/experiment for TRAMP hosts). Bar graphs include mean ± SEM. **P<0.05*, ***P<0.01*, ****P<0.001* (unpaired Student's t test). (D) Representative flow plots of Ki-67 and Bim expression by transferred cells isolated from TRAMP$_{OVA}$ and TRAMP mice. Plots are gated on CD8$^+$Ly5.1$^+$ cells. Results from 2 independent experiments.

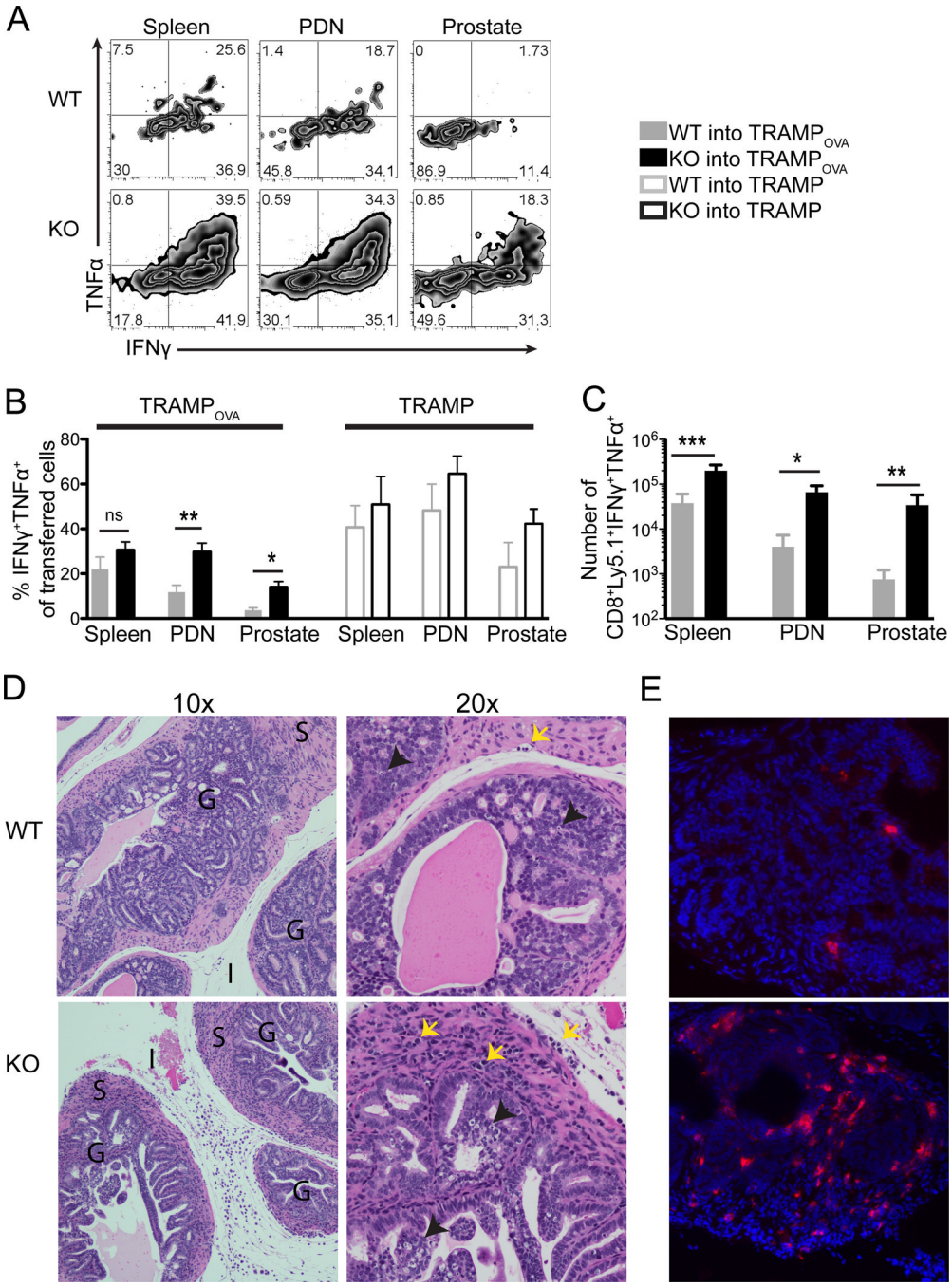

Figure 2. Transferred TGFβRII KO CD8 effector T cells exhibit enhanced effector function, show increased cellular infiltration and mediate epithelial damage in the prostate

Mice were euthanized 1 week post adoptive T cell transfer (same experimental protocol as Figure 1). (A–C) Intracellular IFNγ and TNFα expression by transferred WT and TGFβRII KO cells from spleen, PDN and prostate of TRAMP$_{OVA}$ mice following 5 hour *ex vivo* stimulation with SIINFEKEL peptide. Plots are gated on CD8$^+$Ly5.1$^+$ cells. (A) Representative flow plots of cytokine production by transferred WT and TGFβRII KO cells. Numbers represent percent of gated cells in each quadrant. (B) Percentage of transferred WT and TGFβRII KO cells exhibiting the ability to co-produce both TNFα and IFNγ. No significant differences between WT and TGFβRII KO cells from each organ in TRAMP

mice. (C) Numbers of cytokine producing WT and TGFβRII KO cells in $TRAMP_{OVA}$ mice. (A–C) Data represents pooled results from at least 3 independent experiments (n=2–3 mice/group for $TRAMP_{OVA}$ hosts and n=1–2 mice/group for TRAMP hosts). Bar graphs include mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (unpaired Student's t test). (D–E) Prostate lobes from $TRAMP_{OVA}$ mice receiving either WT or TGFβRII KO cells were micro-dissected and processed for histological analysis. (D) $TRAMP_{OVA}$ prostate lobes were processed and stained with hematoxylin & eosin. Two magnifications are shown, 10× and 20× objectives. The presence of neoplasia in the glands (G), cellular infiltrates in the surrounding fibromuscular stroma (S) and interstitium (I) of $TRAMP_{OVA}$ mice receiving TGFβRII KO cells is evident at 10×. Black arrowheads point to apoptotic cells and yellow arrows point to lymphoid cells at 20×. (E) Frozen sections of $TRAMP_{OVA}$ prostate lobes were stained with DAPI (blue) and Ly5.1 (red); 20×. (D–E) Histology slides show one representative mouse from each experimental group (n=3–5 mice/group) from at least 2 independent experiments.

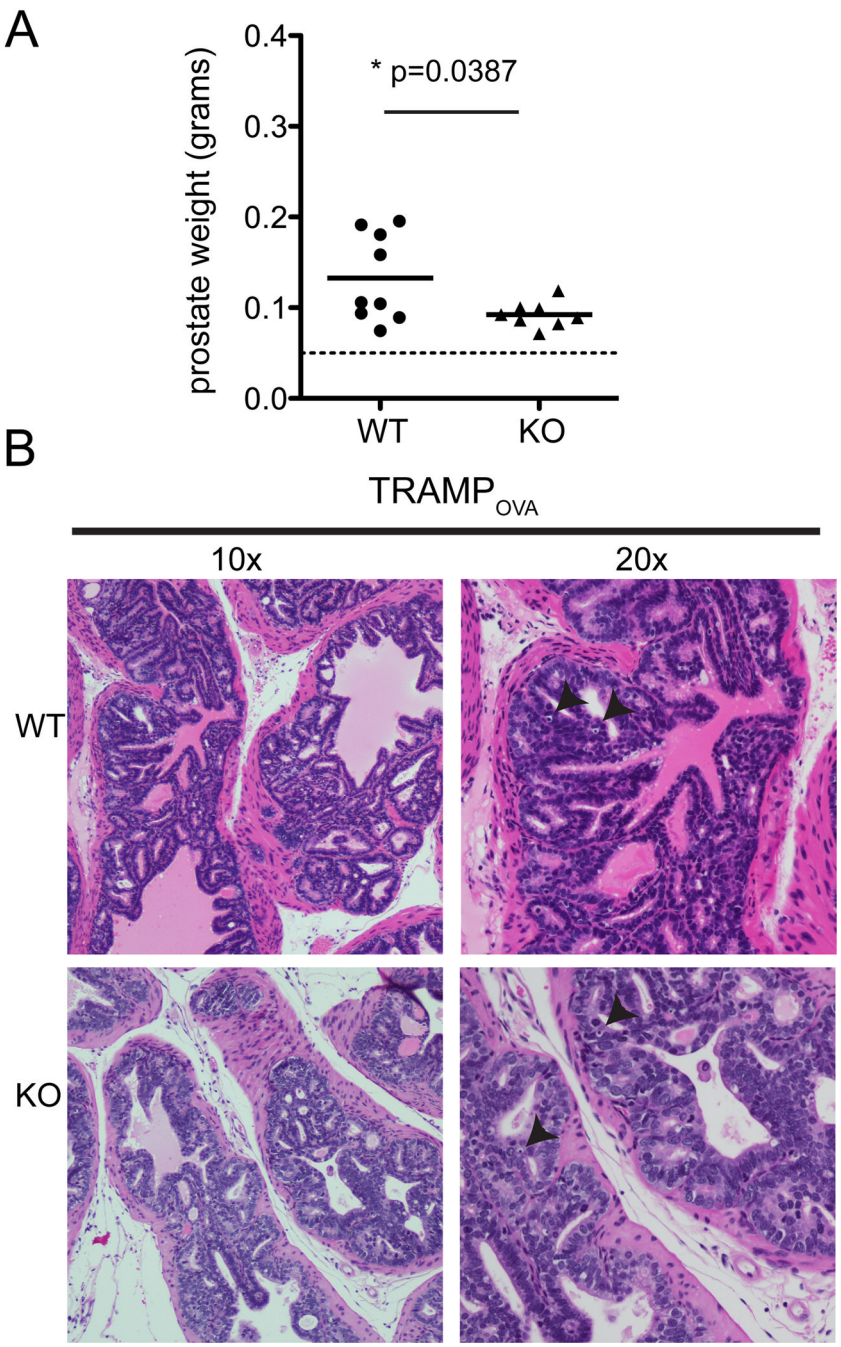

Figure 3. Cellular infiltration in the prostates of TRAMP$_{OVA}$ mice receiving TGFβRII KO cells was not sustained

Prostates were microdissected and analyzed 3 weeks post transfer of WT and TGFβRII KO T cells. (A) Prostate weights at 3 weeks post T cell transfer. Dashed line marks prostate weight of age-matched healthy C57BL/6 prostate. Symbols represent individual mice and bar shows mean weight. (unpaired Student's t test). (B) H&E staining of TRAMP$_{OVA}$ prostates at 3 weeks post T cell transfer show absence of cellular infiltrates and epithelial damage. Black arrowheads point to single, rare apoptotic cells.

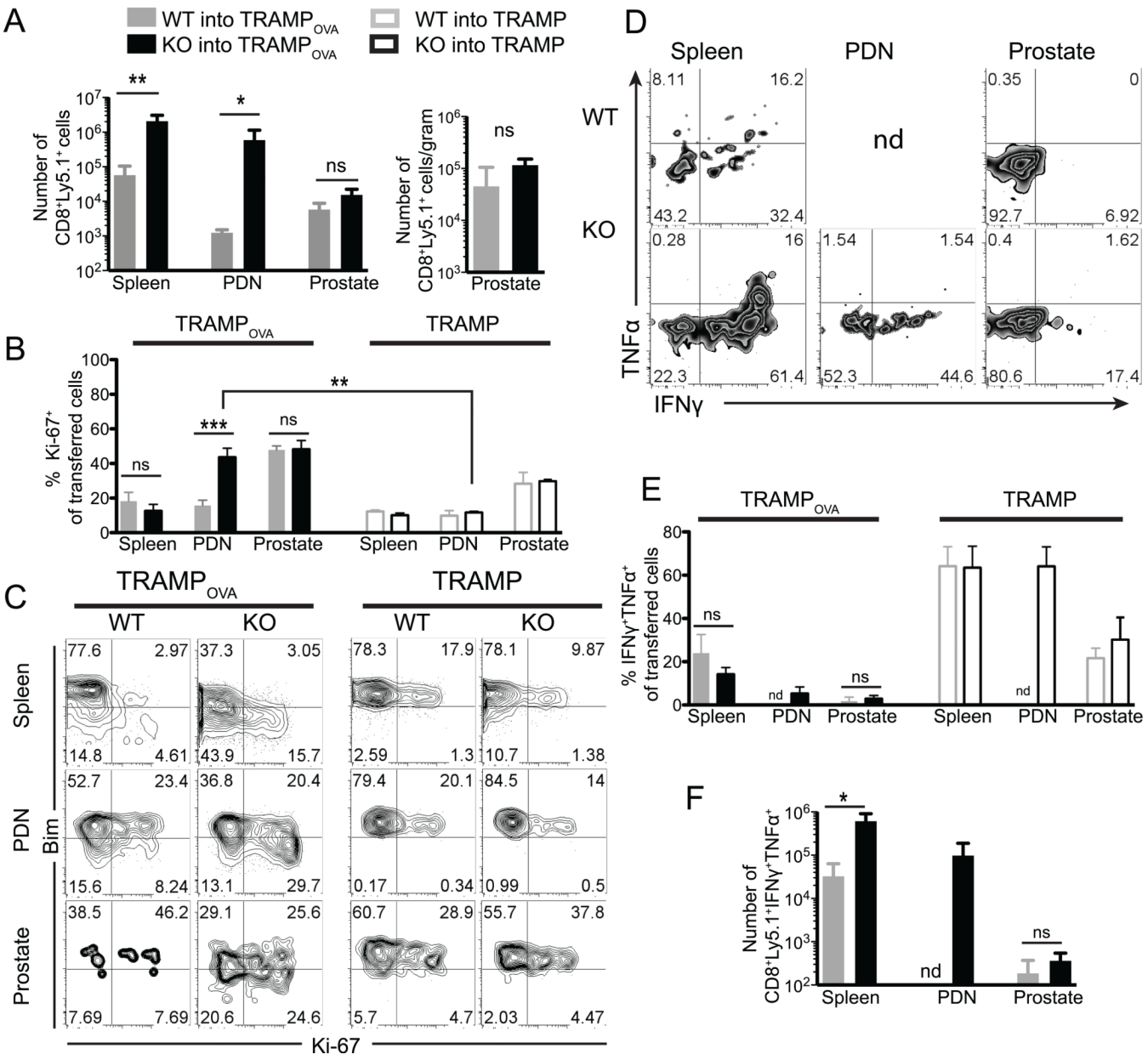

Figure 4. TGFβRII KO cells persist up to 3 weeks in the peripheral lymphoid organs but lose function and no longer accumulate in the prostate of TRAMP$_{OVA}$ mice

Mice were euthanized and analyzed 3 weeks post adoptive transfer (same experimental protocol as Figure 1) (A) Numbers of adoptively transferred WT and TGFβRII KO cells were quantitated in the spleen and PDN of TRAMP$_{OVA}$ mice. Total WT and TGFβRII KO cells in the prostate are also expressed as cells per gram of prostate. (B) Ki-67 expression in transferred cells at week 3 post transfer. No significant differences were detected between WT and TGFβRII 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 from 2 independent experiments. (D) Representative flow plots of cytokine production by WT and TGFβRII KO cells (gated on CD8$^+$Ly5.1$^+$ cells). Numbers represent percent of gated cells in each quadrant. (E) Percentage of transferred WT and TGFβRII KO cells that co-produce TNFα and IFNγ 5

hour *ex vivo* peptide stimulation. No significant differences were detected between WT and TGFβRII KO cells from each organ in TRAMP mice. (F) Number of transferred TGFβRII KO cells in each tissue that produce TNFα and IFNγ. (A–B, D–F) Results represent pooled data from at least 3 independent experiments (n=1–3 mice/group/experiment). Bar graphs show mean ± SEM. **P<0.05*, ***P<0.01, ***P<0.001* (unpaired Student's t test).

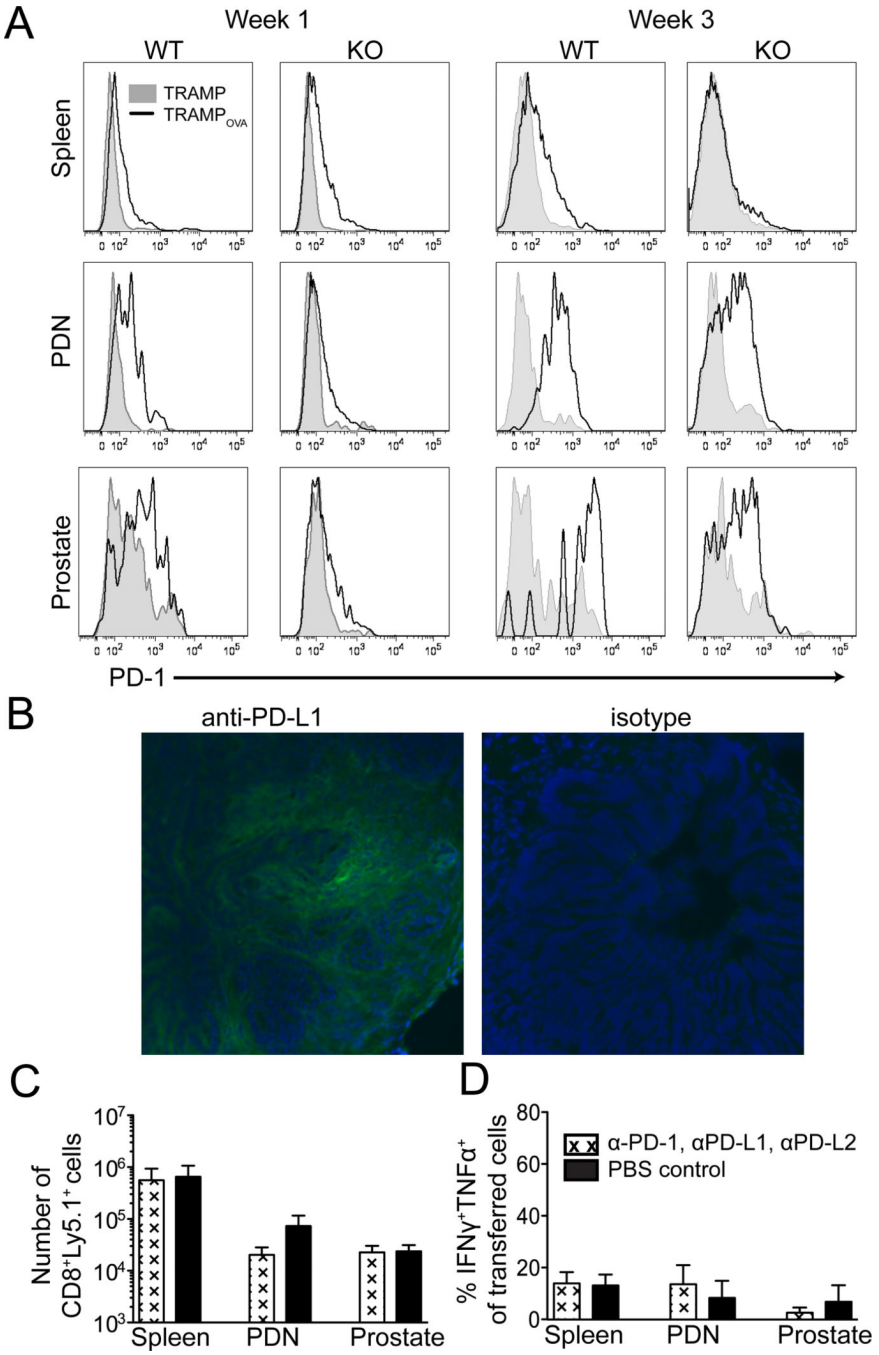

Figure 5. PD-1 and PD-L1 are expressed respectively by persisting transferred T cells and the prostate tumor in treated TRAMP$_{OVA}$ mice, but blockade of PD-1 signaling does not further increase accumulation or effector function of TGFβRII KO cells at 3 weeks post transfer

(A) PD-1 expression on WT and TGFβRII KO cells at week 1 and week 3 post transfer. Histograms are gated on CD8$^+$Ly5.1$^+$ cells. The WT or TGFβRII KO cells transferred into TRAMP$_{OVA}$ hosts shown with a black line, and cells transferred into TRAMP hosts in shaded grey. (B) PD-L1 expression of TGFβRII KO cell treated TRAMP$_{OVA}$ prostates 3 weeks post transfer. For PD-1 blocking experiments, blocking antibodies or PBS were administered *i.p.* every 3 days starting on the day of T cell transfer until mice were euthanized at 3 weeks post transfer. (C) Numbers of persisting transferred cells in

$TRAMP_{OVA}$ mice treated with antibody or PBS. (D) Percentage of transferred TGFβRII KO cells co-producing TNFα and IFNγ following 5 hour *ex vivo* peptide stimulation. All results represent pooled data from 3 independent experiments (n=2–3 mice/group/experiment for mice treated with blocking antibodies and n=1–2 mice/group/experiment for control PBS treated). No significant differences between treated and untreated mice were detected (unpaired Student's t test).