Targeting a self/tumor antigen expressed in the prostate for adoptive T cell immunotherapy of prostate cancer

Cassie K. Chou

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Reading Committee:

Philip D. Greenberg, Chair

Daniel J. Campbell

Steven F. Ziegler

Program Authorized to Offer Degree:

Department of Immunology

University of Washington

Abstract

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Cassie K. Chou

Chair of Supervisory Committee:
Professor Philip D. Greenberg
Department of Medicine and Immunology

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 tumor-specific T cells remain major problems. Most identified tumor antigens are self-proteins, therefore T cells bearing high avidity TCRs for tumor antigens are often deleted in the thymus and any self/tumor-reactive T cells in the periphery are often eliminated or rendered dysfunction by peripheral tolerance mechanisms. The tumor microenvironment poses additional immunosuppressive obstacles, such as the expression of immunosuppressive cytokines and inhibitory ligands. Transforming growth factor beta (TGFβ) is a potent immunosuppressive cytokine that is required to prevent autoimmunity and is often present at high levels within tumor microenvironments. Programmed death ligand 1 (PD-L1) is an inhibitory ligand that is expressed by many cancers and it's receptor, PD-1, is expressed by many dysfunctional tumor-infiltrating T cells. One advantage of ACT is the ability to genetically engineer T cells to improve function, such as by transducing high affinity tumor-specific TCRs or disrupting inhibitory signaling pathways. In these studies, we used transgenic mouse models to (1) characterize prostate-specific T cell responses to

the normal prostate and (2) evaluate the effects of abrogation of TGFβ and/or PD-1 signaling in self/tumor specific CD8 T cells for use in ACT of prostate cancer.

First, we found that prostate-specific CD8 T cells were subjected to both central and peripheral deletion. Using an adoptive transfer model, we found that *in vivo* immunization and/or pre-activation of prostate-specific CD8 T cells resulted in prostate infiltration but no subsequent prostate damage. Second, we found that abrogation of TGFβ signaling increased persistence and augmented anti-tumor activity of self/tumor specific CD8 T cells in a murine model of autochthonous prostate cancer. However, over time, prostate infiltrating T cells became dysfunctional and expressed high levels of PD-1. Moreover, blockade of PD-1 signaling did not rescue or further sustain the function of these cells. These findings reveal that when targeting a tumor antigen that is also expressed as a self-protein, substantive obstacles in addition to TGFβ and PD-1 are operative within the tumor microenvironment.

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ACT	adoptive T cell therapy
CAR	chimeric antigen receptor
CFSE	Carboxyfluorescein succinimidyl ester
CFU	colony forming unit
Cre	Cre recombinase
DC	dendritic cell
dLCK	distal LCK promoter
Foxp3	forkhead box P3
IFN	interferon
IL-	interleukin
KO	knock-out
LM	Listeria Monocytogenes
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
OTI	transgenic CD8 T cells specific to immunodominant K^b OVA epitope
OTII	transgenic CD4 T cells specific to I-A^b OVA epitope
OVA	ovalbumin
PD-1	programmed cell death 1
PD-L1	programmed cell death ligand 1
PD-L2	programmed cell death ligand 2
POET	prostate ovalbumin expressing transgenic mice
SP	single positive
TCR	T cell receptor
TGFβ	transforming growth factor beta
TGFβRII	transforming growth factor beta receptor II
TRAMP	transgenic adenocarcinoma of the mouse prostate mouse
Treg	regulatory T cell
WT	wildtype
YFP	yellow fluorescent protein

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Chapter 1: Introduction

The Immune system

A healthy immune system is poised to defend against a variety of foreign pathogens while avoiding autoimmunity. This fundamental characteristic of the immune system requires the ability to distinguish between what is foreign, and presumably dangerous to the host, and what is self. Multiple mechanisms are in place to allow for the detection and eradication of foreign invaders while preventing damage to host tissue. T cells, especially the CD8 subset, play important roles in fighting infections and tumor surveillance. T cells develop in the thymus through an intricate process in which T cells bearing functional T cell receptors (TCRs), which can recognize either self or foreign antigens, are first positively selected. These T cells then undergo a second round of selection, during which T cells that recognize self-antigens are negatively selected, with the majority undergoing clonal deletion (1). Despite central tolerance mechanisms, some self-reactive T cells will exit the thymus into the periphery, with the potential to induce autoimmunity. Therefore, several peripheral tolerizing mechanisms are also in place to prevent functional activation and destruction of host tissue by self-reactive T cells (2).

The Immune system and cancer

In the early 1900s, Paul Ehrlich was one of the first people to propose that the immune system played a role in protecting the host from cancer. However, it wasn't until the late 1990s that appropriate mouse models became available and experiments were conducted to more definitively address the role of the immune system in cancer. Through these studies, it was found that the immune system possessed the ability to seek out and eliminate transformed cells in a process referred to as cancer immunosurveillance (3). Today, there is ample evidence that the immune system plays both a role in tumor suppression as well as tumor promotion (3, 4) and "avoiding immune destruction" has been added as an emerging hallmark of cancer to the classic six hallmarks of cancer (5). Furthermore, it was demonstrated by *Galon, et al.* in 2006, that infiltration of T cells into human colorectal tumors correlated with a better clinical

outcome (6). As we increase our understanding of how the immune system succeeds and fails to control tumors, better-targeted immunotherapies for the treatment of cancers can be developed and tested.

The recent approval by the FDA of two cancer immunotherapies, a vaccine (Sipuleucel-T) for treatment of prostate cancer (7), and an anti-CTLA-4 blocking antibody (ipilimumab) for treatment of metastatic melanoma (8), has highlighted the ability to modulate the immune system to attack tumors. One form of immunotherapy, adoptive T cell therapy (ACT) takes advantage of the specificity and killing ability of CD8 T cells for the treatment of malignancies. Typically, 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 (9). Although efficacy has clearly been demonstrated with this approach (10-13), difficulty sustaining both adequate numbers of tumor-reactive T cells and functional anti-tumor activity following transfer into the patient has often hindered success (14).

Obstacles to adoptive cell therapy of cancer

The tumor microenvironment itself is often immunosuppressive in nature, which can inhibit rather than stimulate potentially effective anti-tumor T cell responses (15). Tumor cells can secrete immunosuppressive cytokines, such as TGFβ, express inhibitory ligands, such as PD-L1 and recruit inhibitory cells, such as Foxp3$^+$ regulatory T cells and myeloid derived suppressor cells (MDSCs) which, along with the tumor cells, render tumor-infiltrating lymphocytes unresponsive or dysfunctional (15).

Additionally, 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, resulting in deletion of endogenous self/tumor specific T cells in the thymus that bear high affinity TCRs (16, 17). The ideal tumor antigen is uniquely expressed by the tumor cell and highly immunogenic. However, despite evidence that such tumor-specific antigens exist in murine and some human cancers, most are unique to an individual patient and it has been difficult to isolate T cells specific for these antigens (18). Most of the characterized human tumor antigens are self-antigens, which are often overexpressed by tumor cells, but are also detectable on some normal tissue. For T-cell mediated immunotherapy to

be successful, it is necessary to identify and understand the self-tolerizing mechanisms for the antigen to be targeted and develop methods to break tolerance without inducing life-threatening autoimmunity.

One advantage of ACT rather than *in vivo* augmentation of endogenous responses is the ability to genetically engineer T cells to improve function prior to infusion into the patient, such as by transducing high affinity tumor-specific TCRs, abrogating T cell intrinsic negative regulators, or disrupting inhibitory signaling pathways that may be engaged in the tumor microenvironment (16, 19).

Prostate Cancer

Prostate cancer is the second most commonly diagnosed cancer in men in the United States with an estimated 241,740 new cases in 2012 and the second leading cause of cancer death in men (20). If detected early, and disease remains local, the 5 year survival rate is close to 100%, however, for patients with late stage, metastatic disease the 5 year relative survival rate drops to 28.8% (8). The recent approval by the FDA of a vaccine (Sipuleucel-T) for treatment of prostate cancer (7) has highlighted the ability to modulate the immune system to attack prostate tumors. Prostate cancer is currently being pursued as a target for the expanding applicability of T cell mediated immunotherapy. In large part this reflects the identification of immunogenic prostate-restricted antigens, which are expressed in malignant and normal prostate tissues but not in other normal tissues that might be potential targets of toxicity, and that can elicit cytolytic T cell responses (21). Furthermore, CD8 T cells infiltrate human prostate cancer lesions, but appear deficient in the production of anti-tumor effector molecules and express the inhibitory receptors (22, 23).

Transforming growth factor beta: A potent immunosuppressive cytokine

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 (24-29). 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 (29). TGFβ signaling is anti-proliferative, causing G_1 cell cycle arrest in a variety of cell types, including epithelial and T cells (30, 31). Many tumors are able to evade the cytostatic and anti-proliferative effects of TGFβ due to acquired mutations in the TGFβ receptor and/or downstream Smad signaling proteins (29). Activated T cells, however, express higher levels of the TGFβ receptor and can produce TGFβ (32, 33). Molecular analysis of naïve CD8 T cells *in vitro* has revealed that TGFβ can suppress key molecules involved in the effector and cytolytic activities of T cells, including expression of IFNγ (34). Inhibition of TGFβ signaling through various mechanisms, such as TGFβ neutralizing antibodies or small molecule kinase inhibitors is being pursued in clinical trials (35), but significant therapeutic benefits have not yet been reported. This may partly reflect failure to achieve full blockade of TGFβ with these strategies, 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 and effectively abrogate the potentially profound immunosuppressive activity of TGFβ only in the T cells being used to target the tumor. In regards to the prostate, TGFβ is not only present and necessary for normal prostate homeostasis, but is found in increased levels in the malignant prostate (36, 37), which can therefore pose a substantive obstacle to T cell therapy of this tumor.

Other potential obstacles: PD-1 and regulatory T cells

In addition to TGFβ, there are numerous inhibitory molecules and cells that are present in the tumor microenvironment. Two such obstacles particularly relevant to prostate cancer are PD-1 and Tregs. PD-1 is a 288 amino acid type I transmembrane protein containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 is not expressed by naïve T cells but is upregulated upon T cell activation. PD-1 signaling is critical for both the induction and maintenance of peripheral tolerance. C57BL/6 mice deficient in *Pdcd1*, the gene encoding PD-1, develop mild glomerulonephritis and mice on autoimmunity prone backgrounds, such as the NOD mouse, develop more severe forms of

autoimmunity (38). PD-1 has also been identified as an inhibitory co-receptor up-regulated by T cells in many settings of chronic viral infection and has been reported to be expressed on human prostate tumor infiltrating CD8 T cells (23). 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). PD-L1 is up-regulated on many human tumors, including prostate cancer (39), and high PD-L1 expression in some tumor tissues correlates with a decrease in CD8 T cell infiltrates (38).

Tregs are essential for preventing fatal autoimmunity and are often found in increased numbers in tumors (40-42). Early studies showed that day 3 thymectomized mice, which fail to develop Tregs, develop autoimmune prostatitis (43). Increased numbers of Tregs and increased suppressive function of Tregs in peripheral blood and tumors of prostate cancer patients have also been reported (44, 45).

Questions addressed in this thesis work

The overall goal of this thesis work was to provide insights needed to develop effective CD8 T cell immunotherapy of prostate cancer. We used various transgenic mouse models and immunologic strategies to first characterize T cell responses to a self antigen expressed in the normal mouse prostate and second to evaluate the effects of abrogation of key prostate/tumor induced immunosuppressive elements for enhancement of CD8 T cell adoptive therapy in a clinically relevant model of prostate cancer.

The following specific aims were addressed:

Aim 1: Characterize T cell responses to a self-antigen expressed in the normal mouse prostate.

Aim 2: Determine if cell intrinsic abrogation of TGFβ signaling enhances persistence and/or anti-tumor activity of prostate self/tumor specific CD8 T cells in an autochthonous murine prostate cancer model

Aim 3: Determine whether blockade of PD-1 signaling or Foxp3 ablation further increases the therapeutic efficacy of adoptively transferred prostate self/tumor specific CD8 T cells rendered resistant to TGFβ signaling.

Chapter 2: Materials and Methods

Mice

POET mice (46) were obtained from T. Ratliff (Purdue University, West Lafayette, IN). TRAMP mice (47) were obtained from N. Greenberg (Fred Hutchinson Cancer Research Center, Seattle, WA). Foxp3^{DTR} mice (42) was a gift from A. Rudensky (Memorial Sloan Kettering, New York City, NY). TGFβRII$^{Flx/Flx}$ mice was a gift from D. Dichek (University of Washington, Seattle, WA) with permission from S. Karlsson (Lund University, Lund, Sweden) (24). Distal Lck-Cre (*d-lck*Cre) mice (48), and Rosa^{YFP} mice (49) and were gifts from P. Fink (University of Washington, Seattle, WA) with permission from N. Killeen (University of California, San Francisco, CA). OTI TCR transgenic mice (50), and OTII mice (51) were gifts from M. Bevan (University of Washington, Seattle, WA). PD-1$^{-/-}$ mice (52) was a gift from T. Gajewski (University of Chicago, Chicago, IL). *Rag1*$^{-/-}$, Ly5.1, Thy1.1 and C57BL/6 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 SV40 transgene and OVA expression (TRAMP$_{OVA}$) and mice expressing SV40 transgene only (TRAMP). All TRAMP$_{OVA}$ and TRAMP mice used were between 25-27 weeks of age, an age in which all mice have high grade neoplasia (53). For Foxp3 depletion studies, TRAMP and TRAMP$_{OVA}$ mice were bred to Foxp3^{DTR} mice to generate TRAMP$_{OVA}$ X Foxp3^{DTR} and TRAMP X Foxp3^{DTR} mice.

To generate OVA specific TGFβRII deficient mice (TGFβRII KO), mice expressing floxed TGFβRII genes (TGFβRII$^{Flx/Flx}$) were first bred to *d-lck*CRE mice or OTI$^{Ly5.1}$ mice. F1 offspring from those crosses 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$^{Ly5.1}$ x TGFβRII$^{Flx/Flx}$ x *d-lck*CRE). OTI$^{Ly5.1}$ x TGFβRII$^{Flx/Flx}$ littermates were used as WT donors. OTI x TGFβRII$^{Flx/Flx}$ x *d-lck*CRE mice were also bred to Rosa$^{YFP/YFP}$ mice, in which a stop cassette is flanked by LoxP sites flanked upstream of the enhanced yellow fluorescent protein (YFP) gene and is knocked in downstream of the

constitutively active Rosa26 promoter (49). In these mice, the expression of Cre results in excision of the stop cassette and expression of the YFP protein.

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

OTI peptide (SIINFEKL) and OTII peptide (ISQAVHAAHAEINEAGR) were synthesized by the Immune Monitoring Lab at Fred Hutchinson Cancer Research Center (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 were lysed with ammonium chloride potassium buffer (ACK). Cells were washed twice with complete RPMI media (RPMI 1640 supplemented with 2 μM glutamine, 100U/ml penicillin/streptomycin and 10% fetal calf serum). Thymuses were prepared as described above but were not lysed with ACK. Prostate draining lymph nodes (peri-aortic; PDN) were dissociated with microscope slides. Prostate lobes were microdissected and weighed. Individual lobes were divided in half. Half of the prostate was used for histology and the other half of the prostate was 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 donor mice. For naïve cell transfers, splenocytes were counted and washed twice with HBSS prior to injection into the lateral tail vein of recipient mice at a volume of 0.2mL. For cells requiring *in vitro* activation, single cell suspensions were generated and co-cultured with irradiated (3000 rads) congenic splenocytes pulsed with SIINFEKL peptide (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 into the lateral tail vein of mice at a volume of 0.2mL.

CFSE proliferation assays

Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen and reconstituted in DMSO. Splenocytes (10^7 cells/ml) were labeled with 10μM CFSE in serum-free HBSS for 10 minutes at 37°C. The reaction was quenched by the addition of RPMI + 20% FCS followed by additional washes with complete RPMI media. For *in vitro* CFSE proliferation assays, labeled cells were resuspended in complete RPMI media and stimulated with indicated concentration of peptide and analyzed 5 days later. For *in vivo* CFSE proliferation assays, cells were washed once more, resuspended in HBSS and injected *i.v.* at a final volume of 200μL/mouse.

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, CD44, Thy1.1, IFNγ, TNFα, CXCR3 and PD-1. Surface staining was done at 4°C in staining buffer. OVA tetramer was purchased from Beckman Coulter. Tetramer staining was done at 25°C. 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 of splenocytes, lymph nodes and prostates 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, University of Washington. Flow data analysis was done 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 Fred Hutchinson Cancer Research Center, Seattle, WA. Histologic sections were evaluated by a comparative pathologist who was 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, photographic images were captured with an Orca-ER digital camera and assembled into RGB images with Image J and Adobe Photoshop.

Listeria immunization

Attenuated (deficient in actin A and internalin B) *Listeria Monocytogenes* was engineered to express OVA (LM_{OVA}) and provided by Cerus Corporation (Concord, CA) (54). Bacteria were stored as glycerol stocks in the -80°C. To prepare working stocks, 5 mL of brain heart infusion (BHI) media was inoculated from glycerol stocks and cultures were grown overnight in a 37°C shaking incubator. Cultures were diluted 1:100 and incubated for 2 additional hours and the concentration of bacteria was measured using the 600_{OD} cell growth program on a spectrometer (0.1 OD = 10^8 CFU). Bacteria was

washed with ice cold PBS and resuspended in PBS. A dose of $3x10^7$ CFU/200μL was given to each mouse i.p.

Transplantable E.G7 Tumors

The E.G7 tumor line is a T-lineage lymphoma which expresses a transgene encoding OVA and G418 resistance cassette (55). E.G7 cells were maintained in complete RPMI and 2mg/mL of G418 for 3 days followed by overnight incubation in complete RPMI without G418 prior to implantation. Cells were washed 2x with HBSS and resuspended at a concentration of $1x10^6$ cells/200μL. E.G7 tumor cells were injected subcutaneously into shaved flanks of $B6_{OVA}$ mice. Ten days later, when tumors are established (>200mm^3), $3x10^6$ *in vitro* activated WT or TGFβRII KO effectors were injected i.v. into tumor bearing mice. Tumor growth was monitored 3x/week using calipers. Tumor volumes were approximated by (LxWxH)/2. Mice were euthanized when tumors reached 10% of body weight.

Antibody blockade treatment

Monoclonal αPD-1 (29F.1A12) (56), αPD-L1 (10F.9G2) (57) and αPD-L2, (3.2) (58) antibodies were provided by G. Freeman (Harvard Medical School, Boston, MA). To assure adequate blockade, the timing and dose of administration of these antibodies that was established for each individual antibody as described in (59) was used. 200μg 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.

Foxp3 Depletion

Diphtheria toxin (DT) was purchased from Sigma-Aldrich as lyophilized powder and reconstituted in sterile PBS. Frozen stocks were stored in the -20°C. For Foxp3 depletion, 50μg/kg of DT toxin was administered to recipient mice i.p. on D-2 and D-1 prior to T cell transfer and D6 post T cell transfer.

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. For survival studies, the log-rank (Mantel-Cox) test was used.

Chapter 3: Characterization of the development and function of CD8 and CD4 T cell responses specific for a self-antigen expressed in the mouse prostate

Introduction:

A unique aspect of the immune system is the ability to distinguish between foreign and self-antigens. Multiple mechanisms are in place to allow for the detection and eradication of foreign invaders while preventing damage to host tissue. While these central (thymic) and peripheral tolerance mechanisms are essential for preventing autoimmunity, it poses significant obstacles for T cell mediated cancer immunotherapy, as most tumor antigens, and all currently identified prostate tumor antigens, are self-antigens. Therefore it is pertinent to characterize prostate-specific CD8 and CD4 responses to a self-antigen expressed in the prostate to determine potential obstacles to targeting such antigens for adoptive T cell therapy of prostate cancer.

To examine targeting prostate antigens, we used 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 (46). This promoter, which contains 2 copies of the androgen response binding domain, shows peak activity when mice are 7 weeks old and remains high until at least 35 weeks of age, with highest level of expression in the ventral lobe, 2nd highest in dorsal lobe, and 3rd highest in anterior lobe of the prostate (60). The use of OVA as a model self/tumor antigen allowed for analysis of the response of high affinity OVA specific CD8 T cells (OTI), derived from OTI TCR transgenic mice (50), which express a class I restricted transgenic TCR which recognizes the immunodominant epitope (SIINFEKL) of OVA, and OVA-specific CD4 T cells (OTII), derived from OTII TCR transgenic mice (51), which expresses a class II restricted TCR specific to OVA. Previous studies showed that OTI T cells are deleted in the thymus of $POET_1$ mice and adoptively transferred naïve OTIs are deleted in the periphery and do not induce prostate damage (46, 61). In our studies, we further characterized these mice in assessing both the response of naïve and pre-activated OTIs and OTIIs.

Results:

Prostate-specific CD8 T cells are subject to central and peripheral tolerance

To test whether OVA was expressed in the thymus and whether any OVA specific cells escape thymic deletion, POET mice were crossed to OTI mice (denoted as OTI_{OVA}). These mice were also crossed onto a *Rag1*$^{-/-}$ background to prevent expression of alternate endogenous Vα TCR chains. Evaluation of 9 week old OTI_{OVA} mice revealed that there was a slight decrease in the percentage of CD8 single positive (SP) cells in the thymus of OTI_{OVA} mice compared to age matched OTI mice (Figure 3.1A). To determine whether SP CD8 cells in the thymus expressed the OTI TCR chains, Vα2 and Vβ5, thymocytes were stained with the respective antibodies and expression of TCR chains was not detected, suggesting that OVA was expressed in the thymus and that the majority of $CD8^+V\alpha2^+V\beta5^+$ cells were centrally deleted (Fig. 3.1A). This is in congruent with the fact that T cells bearing TCRs of high affinity to self-peptide/MHC complexes in the thymus are clonally deleted (62). Despite central deletion of OTI cells in the thymus, a detectable population of CD8 T cells was present in the periphery. All CD8 T cells in the spleen of OTI_{OVA} mice expressed high levels of CD44, a marker of previous antigen experience, suggesting that these cells encountered antigen (Fig. 3.1B). Interestingly, unlike OTI mice in which the >85% of $CD8^+$ cells were OVA tetramer positive, less than 50% of $CD8^+$ cells in the spleen of OTI_{OVA} mice were tetramer positive (Fig. 3.1B), suggesting that TCR downregulation, which has been described as a form of peripheral tolerance, may have occurred following antigen encounter (63). We investigated whether TCR or CD8 co-receptor downregulation occurred in OTI cells and found that the majority of CD8 cells in the spleen of OTI_{OVA} mice were TCR negative (Fig. 3.1C) and also that the majority of TCR expressing cells in the spleen were CD8 negative (Fig. 3.1D). Interestingly, there was a population of TCR positive cells that expressed the CD4 co-receptor despite being on a Rag deficient background. However, since expression of the self-reactive TCR chains was forced in this transgenic system, it may also be an artifact of this artificial system. These data suggests that modulation of TCR and/or CD8 co-receptor expression may be a form of peripheral tolerance and prevent the accumulation of potentially pathogenic self-reactive OTI T cells in periphery of OTI_{OVA} mice.

Next, we examined the prostate for infiltration of OTI CD8 T cells and/or damage to the prostate epithelium. There were no detectable OTI T cells in the prostate by flow cytometry (data not shown) and H&E staining of prostate sections revealed no difference between prostates derived from OTI_{OVA} compared to OTI mice with no infiltration of mononuclear cells (Fig. 3.1E). These results suggest that central deletion of prostate-specific CD8 T cells is one form of tolerance in this mouse model and that although prostate-specific T cells exist in peripheral lymphoid organs, they do not infiltrate and damage the prostate.

Prostate-specific CD8 T cells proliferate to ex vivo peptide stimulation and respond to immunization.

The lack of prostatic damage in OTI_{OVA} mice in which all CD8 T cells in the periphery are presumably prostate-specific, led us to test whether prostate-specific cells in the periphery were functional. First, we tested whether OTI cells in the spleen produced the effector cytokines IFNγ and TNFα. Naïve CD8 T cells encountering antigen for the first time typically produce TNFα and very little IFNγ (Fig. 3.1F, left). In contrast, CD8 T cells from OTI_{OVA} mice had an effector-like cytokine profile in which both IFNγ and TNFα were produced (Fig. 3.1F, middle), further suggesting that these cells encountered antigen in the OTI_{OVA} mouse. The percentage of cells able to produce both cytokines was lower than primary effector and/or memory CD8 T cells (Fig. 3.1F, right), suggesting that there may be attenuation of effector cytokine production, which may be a form of tolerance in these mice. Tolerance can also be regulated at the level of proliferation (64), therefore we performed an *in vitro* CFSE proliferation assay to test whether CD8 T cells in the spleen of OTI_{OVA} mice maintained the ability to proliferate in response to cognate peptide. 75% of the $CD8^+$ cells from OTI_{OVA} mice diluted CFSE (Fig 3.1G). Unlike $CD8^+$ T cells from OTI mice, in which all T cells diluted CFSE, there was a persistent population of which did not dilute CFSE.

Immunization does not induce expansion of endogenous prostate-specific CD8 T cells

To determine whether endogenous prostate-specific cells could be elicited by immunization, we immunized $B6_{OVA}$ mice with recombinant LM_{OVA}. About 2% of CD8 T cells were detectable with OVA tetramer in the spleen of B6 mice following LM_{OVA} immunization (Fig. 3.1H). However, significantly fewer, if any, OVA specific cells were detected in $B6_{OVA}$ mice suggesting that in an immunocompetent mouse, prostate-specific CD8 T cells may also be subject to central and/or peripheral tolerance.

Naïve prostate-specific CD8 T cells are deleted in the periphery.

In the OTI_{OVA} mouse it is difficult to delineate whether functional defects of OTI cells in the periphery are due to intrinsic changes acquired by the T cells during thymic development, in which the self-antigen is expressed, or additional factors imposed in the periphery. To determine whether peripheral tolerizing factors exist in the $B6_{OVA}$ mice, we used an adoptive transfer system in which naïve prostate-specific OTI CD8 T cells, which have not encountered self-antigen during thymic development, were transferred into $B6_{OVA}$ mice. $4x10^6$ naïve $OTI^{Ly5.1}$ CD8 T cells were adoptively transferred into congenic $B6_{OVA}$ and B6 hosts. Mice were bled on a weekly basis to assess for the presence of transferred cells in the peripheral blood. There was a significant decrease in the percent of OTI CD8 cells in the peripheral blood of $B6_{OVA}$ mice compared to B6 mice (Fig 3.2A). By 4 weeks post transfer, a stable, antigen-experienced ($CD44^{hi}$) population remained (Fig 3.2B). To test whether remaining cells were functional, mice were immunized with either control recombinant LM or OVA expressing, LM_{OVA}. Both $OTI^{Ly5.1}$ cells in $B6_{OVA}$ and B6 mice expanded in the peripheral blood following immunization (Fig 3.2A). Mice were euthanized 6 days post immunization to assess for function and prostate damage. Significantly increased numbers of $OTI^{Ly5.1}$ cells were present in the spleen and PDN of $B6_{OVA}$ mice that received LM_{OVA} compared to mice that received LM, suggesting that persisting cells expand in response to antigen presented in an immunogenic context (Fig 3.2C). Decreased numbers of $OTI^{Ly5.1}$ cells were detected in the spleen of $B6_{OVA}$ mice compared to B6 mice, however a higher percentage of $OTI^{Ly5.1}$ cells infiltrated the prostate of $B6_{OVA}$ mice compared to B6 mice

(Fig 3.2C,D). Prostate sections were stained with H&E to determine whether prostate damage was elicited. Sections showed significant inflammatory infiltrates surrounding the glands in the prostates of $B6_{OVA}$ mice however, no epithelial disruption was observed. These results suggest that persisting non-deleted $OTI^{Ly5.1}$ cells are capable of responding to antigen when presented in an immunogenic context and infiltrate the prostate in an antigen dependent manner however, factors within the prostate microenvironment prevent observable tissue damage.

In vitro activated prostate-specific CD8 T cells initially infiltrate but are rapidly rendered dysfunctional and do not persist in the prostate.

It is possible that naïve $OTI^{Ly5.1}$ CD8 T cells are encountering OVA in a tolerogenic context and not developing into fully activated effector cells capable of cytolytic activity. To test whether activation of $OTI^{Ly5.1}$ CD8 T cells prior to adoptive transfer into a self-antigen environment resulted in prostate infiltration and damage, we *in vitro* activated $OTI^{Ly5.1}$ T cells by co-culturing naïve $OTI^{Ly5.1}$ T cells with SIINFEKL pulsed splenocytes and recombinant IL-2. Five days later, all $OTI^{Ly5.1}$ T cells were $CD44^{+}$, a marker of antigen-specific activation and had an effector cytokine profile (>99% cells expressed both $IFN\gamma^{+}TNF\alpha^{+}$ upon peptide stimulation) (Fig 3.3A). 10 $x10^{6}$ effector $OTI^{Ly5.1}$ CD8 T cells were adoptively transferred into 5 month old $B6_{OVA}$ and B6 males. Unlike transferred naïve OTI T cells, activated cells were not deleted in the peripheral blood (Fig 3.3B). To determine whether transferred cells remained functional and trafficked to the prostate, mice were euthanized at 1 week and 3 weeks post adoptive transfer. We found similar numbers of $OTI^{Ly5.1}$ CD8 T cells in the spleen and PDN of $B6_{OVA}$ and B6 mice and a slight increase in the percentage of $OTI^{Ly5.1}$ T cells in the prostate of $B6_{OVA}$ mice suggesting that at 1 week post transfer, no significant deletion of pre-activated $OTI^{Ly5.1}$ cells occurred and activated cells trafficked to the prostate (Fig. 3.3C, left). To test whether transferred cells remained functional, single cell suspensions from the spleen, PDN and prostate were stimulated directly *ex vivo* with cognate peptide and analyzed for IFNγ and TNFα production. $OTI^{Ly5.1}$ cells isolated from the spleen and PDN of $B6_{OVA}$ hosts were attenuated in effector cytokine production compared to $OTI^{Ly5.1}$ cells isolated from as early as 1 week post transfer (Fig. 3.3D, left).

Moreover, $OTI^{Ly5.1}$ CD8 T cells isolated from the prostate of $B6_{OVA}$ mice were further attenuated in cytokine production compared to $OTI^{Ly5.1}$ cells from the spleen or PDN of the same mouse (insufficient $OTI^{Ly5.1}$ CD8 T cells were isolated from the prostate of B6 mice to perform this assay) (Fig. 3.3D, left). By week 3 post transfer, <0.2% of live cells in the prostate consisted of $OTI^{Ly5.1}$ transferred cells and were insufficient to perform the intracellular cytokine assay (Fig. 3.3C-D, right). Also, $OTI^{Ly5.1}$ cells in the PDN of $B6_{OVA}$ mice were further attenuated in effector cytokine production (Fig. 3.3D, right). These data suggest that pre-activated prostate-specific $OTI^{Ly5.1}$ cells are not deleted in the periphery and traffic to the prostate but are rapidly rendered dysfunctional and do not persist in significant numbers in the prostate.

The PD-1 signaling axis is crucial in preventing autoimmunity in certain animal models (38). PD-1 signaling is important in regulating both the initial activation of self-reactive T cells as well as subsequent antigen re-encounters. Furthermore, inhibitory signals through the PD-1 receptor appear to have a greater effect on regulating effector function rather than proliferation of self-reactive T cells (38). These properties of PD-1 suggested that PD-1 signaling in transferred self-specific CD8 T cells might be inhibiting the function of prostate-antigen specific CD8 T cells in the $B6_{OVA}$. Indeed, PD-1 expression was increased on $OTI^{Ly5.1}$ cells isolated from $B6_{OVA}$ hosts compared to B6 hosts (Fig 3.3E).

Prostate-specific CD4 T cells do not encounter antigen and remain naïve in the periphery.

The detectable function of these self-reactive CD8 T cells suggested that CD4 T cell help might augment the activity in the prostate environment. We first tested whether OVA-specific CD4 T cells were subject to central deletion, we crossed $B6_{OVA}$ mice to OTII mice. At 20 weeks of age, there was a slight decrease in the percentage of CD4 SP cells in the thymus of $OTII_{OVA}$ compared to OTII mice and similar to findings in the OTI_{OVA} mice, there was an increase in the percentage of TCR negative CD4 SP cells (Fig. 3.3A) however, there was also a distinct population of CD4 SP cells with TCR expression levels similar to OTII mice (Fig 3.3A). There was a ~10-fold lower percentage of $CD4^{+}V\alpha2^{+}V\beta5^{+}$ cells in the spleen and PDN of $OTII_{OVA}$ and a large

population of $CD4^+$ TCR negative population (Fig 3.3A), suggesting that TCR downregulation may be a mechanism of peripheral tolerance. In contrast to the antigen-experienced phenotype of prostate-specific CD8 T cells in the periphery of OTI_{OVA} mice, CD4 T cells in the spleen and PDN did not upregulate CD44, suggesting that these cells maintained a naïve phenotype and did not encounter cognate antigen in the periphery. Furthermore, no CD4 cells were detected in the prostates of $OTII_{OVA}$ mice and no prostatic damage was observed (data not shown). These results suggest that despite expression of OVA in the thymus, a detectable population of OVA specific CD4 T cells exit the thymus and do not encounter antigen in the periphery.

Extensive studies have been performed on the differentiation of CD4 T cells and it has been shown that self-specific CD4 T cells can also develop into $Foxp3^+$ regulatory T cells (65). We tested whether there was an increased percentage of $Foxp3^+$ OTII cells in the periphery of $OTII_{OVA}$ mice. We found that a similar percentage of $CD4^+V\alpha2^+$ cells from $OTII_{OVA}$ mice expressed Foxp3 compared to OTII mice, suggesting that there was no preferential Foxp3 induction in peripheral $V\alpha2^+$ CD4 T cells in the $OTII_{OVA}$ mice (Fig. 3.4C). There was however an overall higher percentage of total CD4 T cells that expressed Foxp3 (in the spleen 11% vs.18% of CD4 cells and in the PDN, 7% vs. 17% expressed Foxp3). Because $OTII_{OVA}$ mice were not on a Rag deficient background, it is possible that alternate $V\alpha$ chains were expressed. Next, we tested whether OTII CD4 T cells in the spleen proliferated upon *ex vivo* peptide stimulation and found OTIIs from $OTII_{OVA}$ maintained the ability to proliferate (Fig 3.3D).

$OTII_{OVA}$ mice were also immunized with LM_{OVA} to determine whether *in vivo* activation of OTII cells led to prostate infiltration but there was no difference between the number of OTII cells in the spleen, PDNs or prostates of immunized compared to unimmunized $OTII_{OVA}$ or OTII mice, suggesting that OTII cells do not expand to LM_{OVA} immunization (data not shown).

Next, we tested if naïve OTII cells, which did not encounter antigen during thymic development, were capable of recognizing OVA in the periphery of $B6_{OVA}$ mice by adoptive transfer of naïve CFSE labeled congenic OTII cells from a $OTII^{Thy1.1}$ mice into $B6_{OVA}$ and B6 recipients. $B6_{OVA}$ and B6 recipients were euthanized on D27 and no CFSE dilution and no preferential deletion or accumulation of OTII cells in $B6_{OVA}$

compared to B6 mice was observed (Fig 3.3E). These data suggest that OTII cells do not encounter antigen in the periphery of $B6_{OVA}$ mice and are not subject to peripheral deletion.

Discussion:

The immune system employs multiple mechanisms of central and peripheral tolerance in order to prevent autoimmunity. In this chapter, we characterized CD8 and CD4 T cell responses to a self-antigen expressed in the murine prostate. We found that both CD8 and CD4 T cells are centrally deleted in the thymus but functional prostate-specific cells also existed in peripheral lymphoid organs and did not cause prostate inflammation. Provision of an immunogenic stimulus or pre-activation of CD8 prostate-specific T cells resulted in prostate inflammation but no damage to the epithelium was observed.

The lack of prostate inflammation in the OTI_{OVA} and $B6_{OVA}$ model following adoptive transfer of naïve CD8 OVA-specific T cells was similar findings in the K14-mOVA skin self tolerance model (66). In this model, OVA was expressed in the epithelial cells of the skin and adoptively transferred naïve OTI cells also acquired an antigen-experienced phenotype ($CD44^{hi}CD62L^{lo}$) but did not infiltrate the skin unless an additional inflammatory signal, such as tape stripping, was applied to the skin (66). Therefore in both the K14-mOVA skin model and $B6_{OVA}$ prostate model, provision of the antigen in an inflammatory setting was necessary to induce infiltration of tissue self-specific CD8 T cells. On the contrary, in the RIP-mOVA model, in which OVA is expressed in the pancreas under the rat insulin promoter adoptively transferred OTI cells were activated by cross-priming in draining lymph nodes and subsequently deleted but if a large number of cells, $5x10^6$ naïve OTI cells, are injected, the mice developed diabetes in 9 days (67). Furthermore, when a different founder of the $B6_{OVA}$ mouse, which expressed lower levels of the OVA transgene in the prostate, was bred to OTI mice, no thymic deletion was detected and significant inflammation but no tissue damage was observed in the prostate (46). Therefore, even when the same antigen is expressed under the same tissue promoter, there may be other variables, such as antigen expression levels,

that dictate the type of peripheral tolerance. These differences highlight the importance of characterizing each target antigen.

In contrast to OTI CD8 T cells, which proliferated in response to antigen recognition in $B6_{OVA}$ mice, OTII CD4 T cells remained naïve. Ignorance is one form of tolerance that has been observed in multiple self-antigen models (68-70). This may be the case for OTII CD4 T cells in the $B6_{OVA}$ model. In a similar prostate model, the Pro-HA model, in which the HA protein was expressed under a prostate-specific probasin promoter, HA-specific CD4 T cells were also not deleted in the thymus and adoptively transferred naïve HA-specific CD4 T cells did not become activated or proliferate (70). When Pro-HA mice were crossed to the prostate tumor TRAMP mice, HA-specific CD4 T cells became activated and infiltrated the prostate, suggesting that changes in prostate architecture and/or increase in antigen expression as a result of tumorigenesis may result in self/tumor antigen recognition by cognate CD4 T cells (70).

The difficulty in achieving damage to the normal prostate following adoptive transfer of effector prostate-specific CD8 T cells suggests that autoimmunity would not be a significant toxicity in the setting of treating prostate cancer with self-specific T cells. However, the profound self-tolerizing mechanisms may also prevent effective ant-tumor responses. Tumor settings are generally more inflammatory and express a higher antigen load, which may aid in attraction of effector self/tumor specific CD8 T cells, but can also pose additional substantive obstacles. Some key immunosuppressive mechanisms posed by tumors and especially in regards to prostate cancer, are the expression of the immunosuppressive cytokine TGFβ, PD-1 ligands and Foxp3 regulatory T cells. In the following chapter, we used an autochthonous murine prostate cancer model to test whether abrogation of these factors would enhance T cell adoptive immunotherapy of prostate cancer.

Figure 3.1: Characterization of CD8 T cell response to a self-antigen expressed in the prostate. (A-B) Thymuses and spleens were harvested from 9 week old male C57BL/6, OTI (B6), OTI (Rag) and OTI_{OVA} Rag mice and processed into single cell suspensions for flow cytometry. All numbers represent percent of cells within the gate. All flow plots were gated on live cells except where indicated. (A, *top*) Thymus single cell suspensions were stained with CD8 and CD4 antibodies. (A, *bottom*) Vα2 and Vβ5 TCR chain expression by single positive CD8 thymocytes. (B, *top*) Splenocytes were stained with CD4 and CD8 antibodies. (B, *bottom*) OVA tetramer and CD44 expression by $CD8^+$ splenocytes. (C-D) Splenocytes from OTI (B6), OTI (Rag) and OTI_{OVA} were stained with CD8, CD4, Vα2 and Vβ5 antibodies. TCR expression of $CD8^+$ splenocytes are shown in (C, bottom) and CD4 and CD8 expression of $V\alpha2^+V\beta5^+$ splenocytes are shown in (D, bottom). (A-D) C57BL/6, OTI (B6) and OTI_{OVA} (Rag) samples were stained and acquired in the same set of experiments with the same fluorescent antibodies and gating strategies. OTI Rag data is also shown as an additional control (since OTI_{OVA} mice were on a Rag deficient background) but were acquired independently and thus gates are slightly different. (E) Prostates from OTI and OTI_{OVA} mice were microdissected and processed for H&E staining. (F) Splenocytes from 3 month old OTI and OTI_{OVA} mice were incubated with SIINFEKL peptide and brefeldin A for 5 hours followed by intracellular cytokine staining for IFNγ and TNFα. Primary OTI effectors generated by incubation with SIINFEKL pulsed splenocytes and IL2 were used as a control for maximal cytokine production. Flow plots are gated on $CD8^+$ cells. (G) *In vitro* CFSE proliferation assay was performed using splenocytes from OTI and OTI_{OVA} Rag mice and SIINFEKL (black line) or irrelevant (gray shaded) peptide. (H) 23 week old male $B6_{OVA}$ and B6 mice were immunized with recombinant LM_{OVA} and euthanized 9 days post immunization. Single cell suspensions of spleens and PDNs were processed and stained for CD8 and OVA tetramer. Flow plots are gated on $CD8^+$ cells.

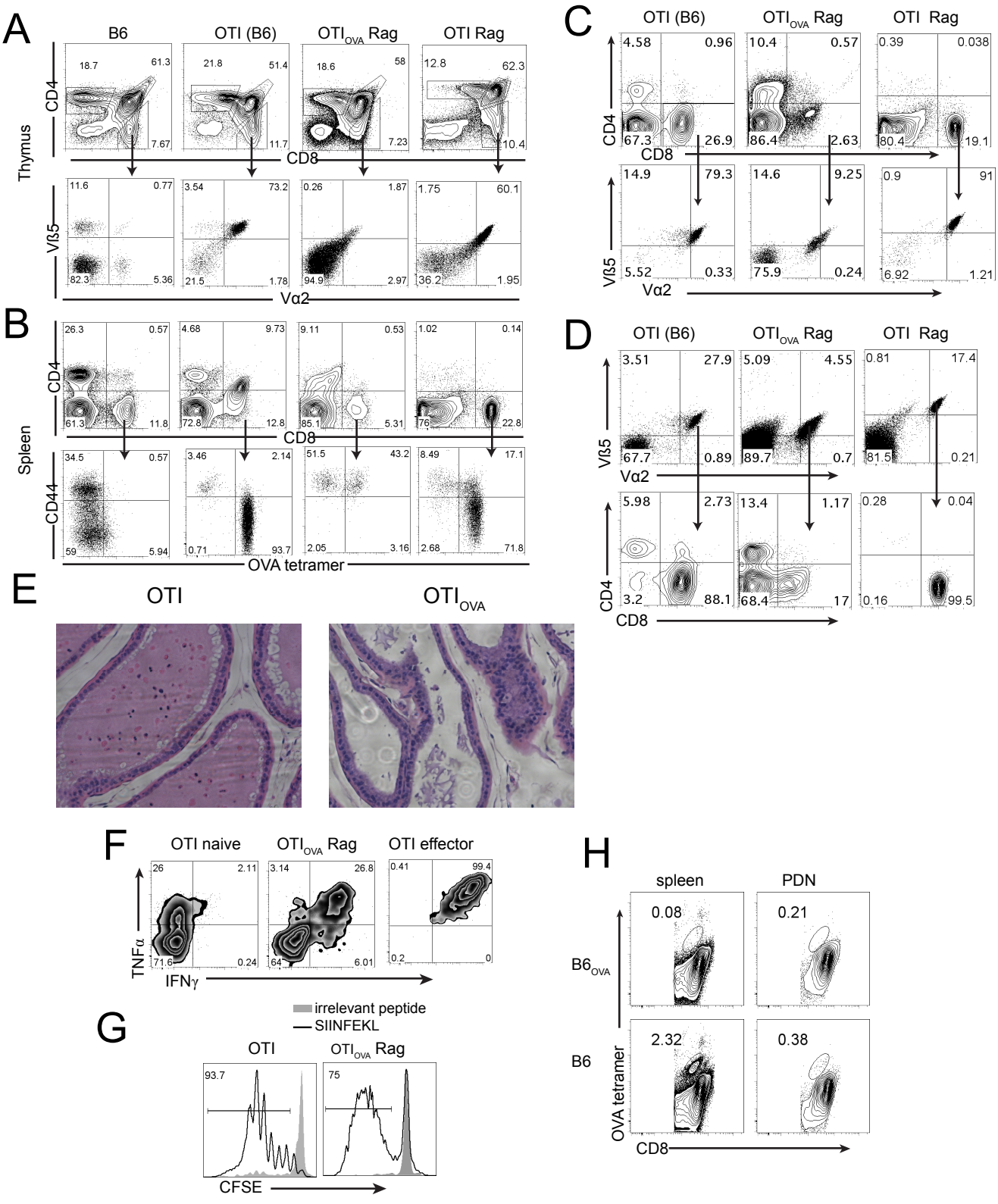

A
B6
OTI (B6)
OTI$_{OVA}$ Rag
OTI Rag
Thymus
CD4
CD8
Vβ5
Vα2
B
Spleen
CD4
CD8
CD44
OVA tetramer
C
OTI (B6)
OTI$_{OVA}$ Rag
OTI Rag
CD4
CD8
Vβ5
Vα2
D
OTI (B6)
OTI$_{OVA}$ Rag
OTI Rag
Vβ5
Vα2
CD4
CD8
E
OTI
OTI$_{OVA}$
F
OTI naive
OTI$_{OVA}$ Rag
OTI effector
TNFα
IFNγ
G
irrelevant peptide
SIINFEKL
OTI
OTI$_{OVA}$ Rag
CFSE
H
spleen
PDN
B6$_{OVA}$
B6
OVA tetramer
CD8

Figure 3.2: Naïve prostate-specific CD8 T cells expand and infiltrate the prostate following immunization but do not damage the prostate. $4x10^6$ naïve $OTI^{Ly.51}$ CD8 T cells were adoptively transferred into congenic ($Ly5.2^+$) $B6_{OVA}$ and B6 males. On D42 post adoptive transfer, a cohort of recipient mice were immunized with $3x10^7$ CFU of either LM_{OVA} or LM. Mice were euthanized D7 post immunization. (A) Peripheral blood was analyzed for presence of transferred cells on D7, D15, D27, D42 and D6 post LM immunization (D48). The percent of transferred cells ($Ly5.1^+$) out of the total CD8 population is shown. Student's t test was performed between the percent of transferred cells in $B6_{OVA}$ compared to B6 hosts. (B) Histogram shows CD44 staining of peripheral blood cells D27 post transfer. Gated on $CD8^+Ly5.1^+$ cells. (C) Numbers of transferred cells in the spleen and PDN of different cohorts as indicated on the graph. (D) Percent of transferred cells in the prostate of different cohorts as indicated on the graph. (A, C-D) Symbols show individual mice, bar shows mean $\pm$ SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (unpaired Student's t test). (E) H&E staining of prostates microdissected from $B6_{OVA}$ and B6 hosts following LM_{OVA} immunization shows the presence of mononuclear cells in the interstitial area of $B6_{OVA}$ prostates. (N=4 for $B6_{OVA}$ + LM_{OVA} N=2 for B6 + LM_{OVA} N=2 for $B6_{OVA}$ + LM).

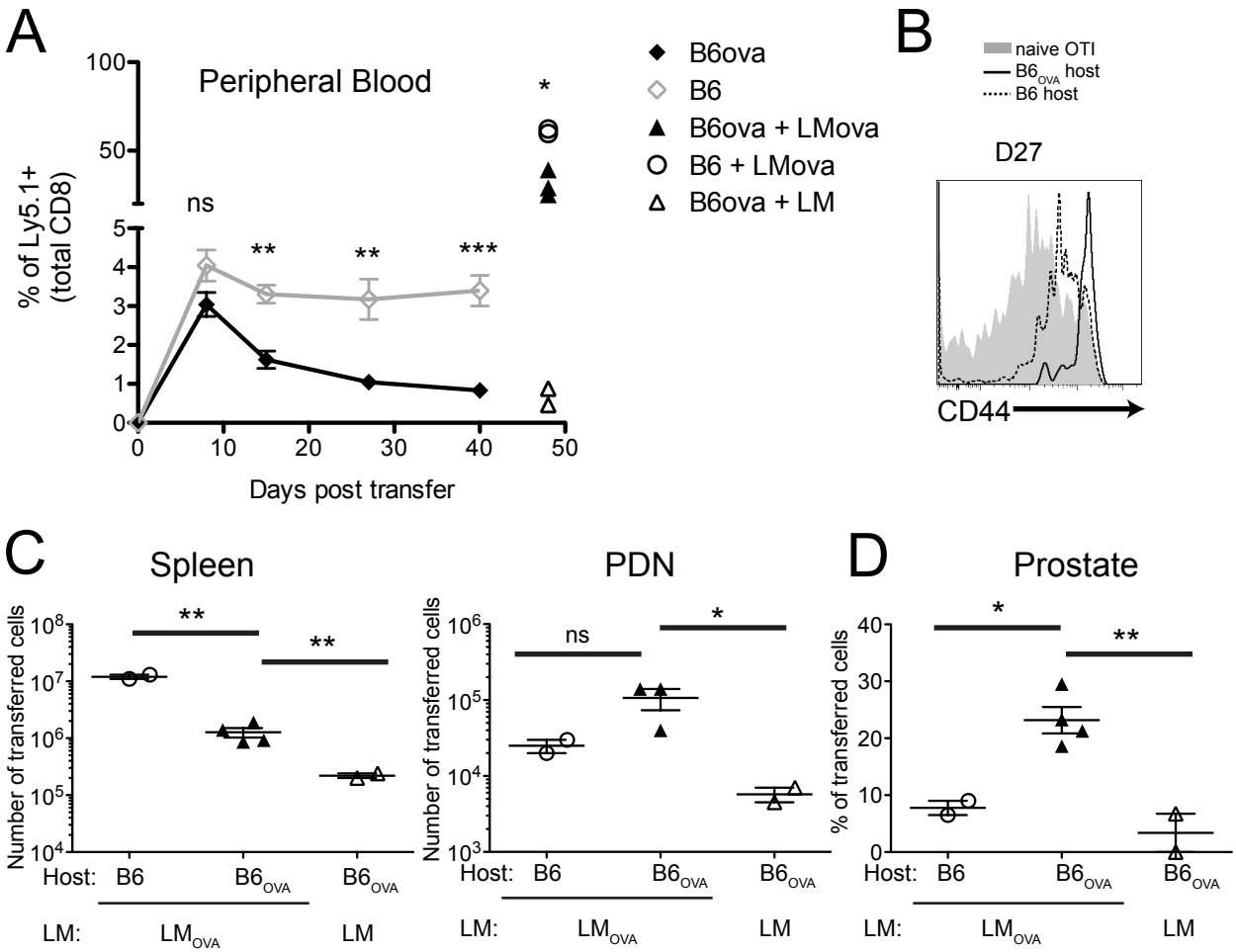

A
Peripheral Blood
% of Ly5.1+ (total CD8)
Days post transfer
ns
**
**
***
*
B6ova
B6
B6ova + LMova
B6 + LMova
B6ova + LM
B
naive OTI
B6OVA host
B6 host
D27
CD44
C
Spleen
PDN
Number of transferred cells
Host: B6 B6OVA B6OVA
LM: LMOVA LM
D
Prostate
% of transferred cells
E
B6 + LMova
POET + LMova

Figure 3.3 Adoptively transferred activated prostate-specific CD8 T cells infiltrate but do not persist in the prostate. (A) CD44 expression and intracellular cytokine staining for IFNγ and TNFα of D5 OTI effector cells. Plots gated on $CD8^{+}Ly5.^{1+}$ cells. (B-E)$10x10^{6}$ *in vitro* activated $OTI^{Ly5.1}$ CD8 cells were adoptively transferred into congenic ($Ly5.2^{+}$) $B6_{OVA}$ and B6 males. Cohorts of mice were euthanized 1 week and 3 weeks post transfer to assess for T cell function and prostate damage. (B) Percent of transferred $OTI^{Ly5.1}$ CD8 cells of total CD8 T cells in the peripheral blood at D7, D14 and D20 post transfer. (C) Numbers of transferred $OTI^{Ly5.1}$ CD8 cells in the spleen and PDN of $B6_{OVA}$ and B6 recipients at 1 week and 3 weeks post transfer. The percent of transferred $OTI^{Ly5.1}$ CD8 cells in the prostate is also shown. Gated on total live cells. Bars represent mean $\pm$ SEM. (D) Intracellular cytokine staining of transferred $OTI^{Ly5.1}$ CD8 cells isolated from $B6_{OVA}$ mice at week 1 and week 3 post transfer. Plots are gated on $CD8^{+}Ly.51^{+}$ cells. (E) PD-1 staining of $OTI^{Ly5.1}$ CD8 cells. (B-E) N=2 $B6_{OVA}$ and N=1 B6 for week 1 and N=3 $B6_{OVA}$ and N=2 B6 for week 3.

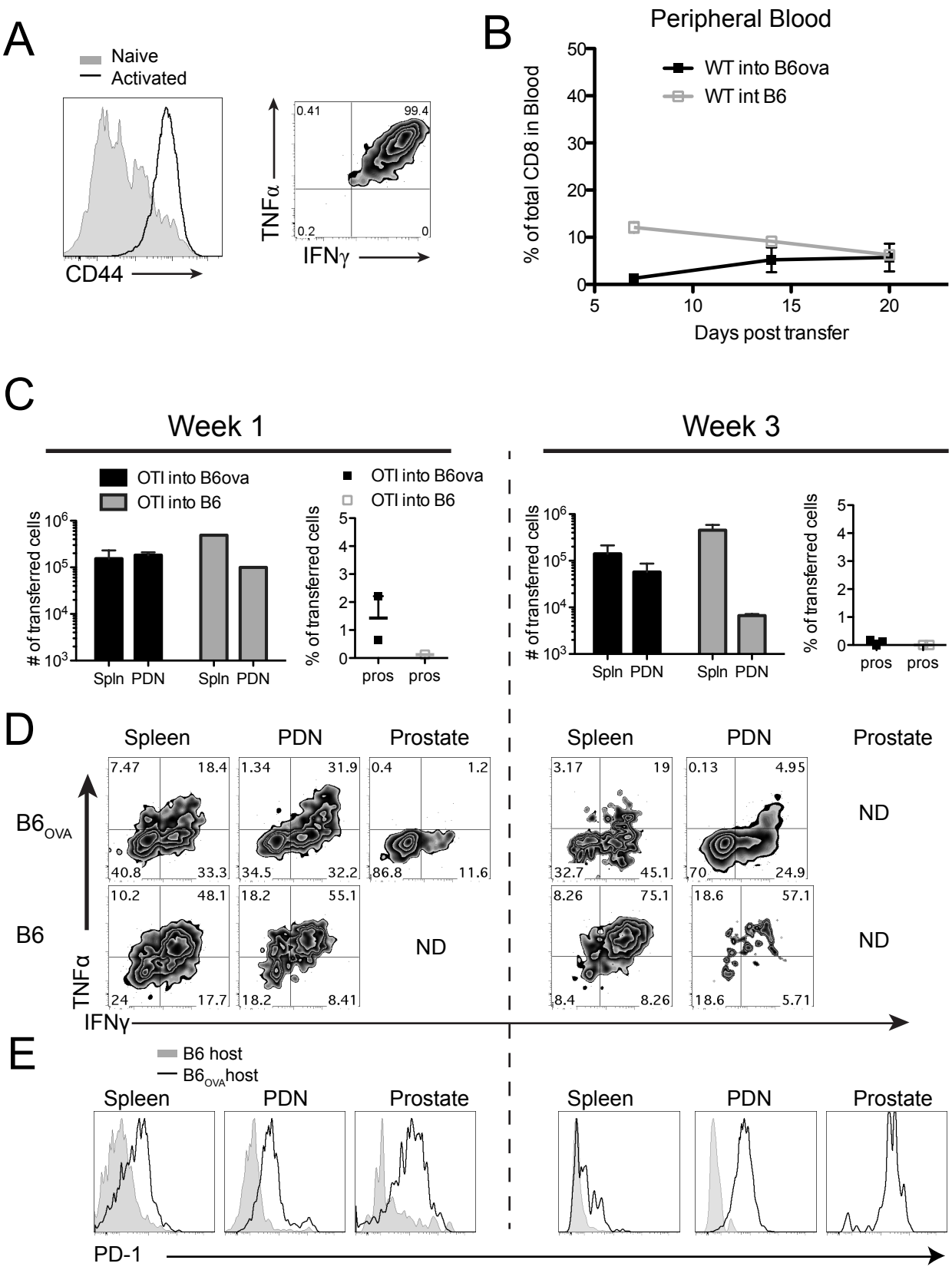
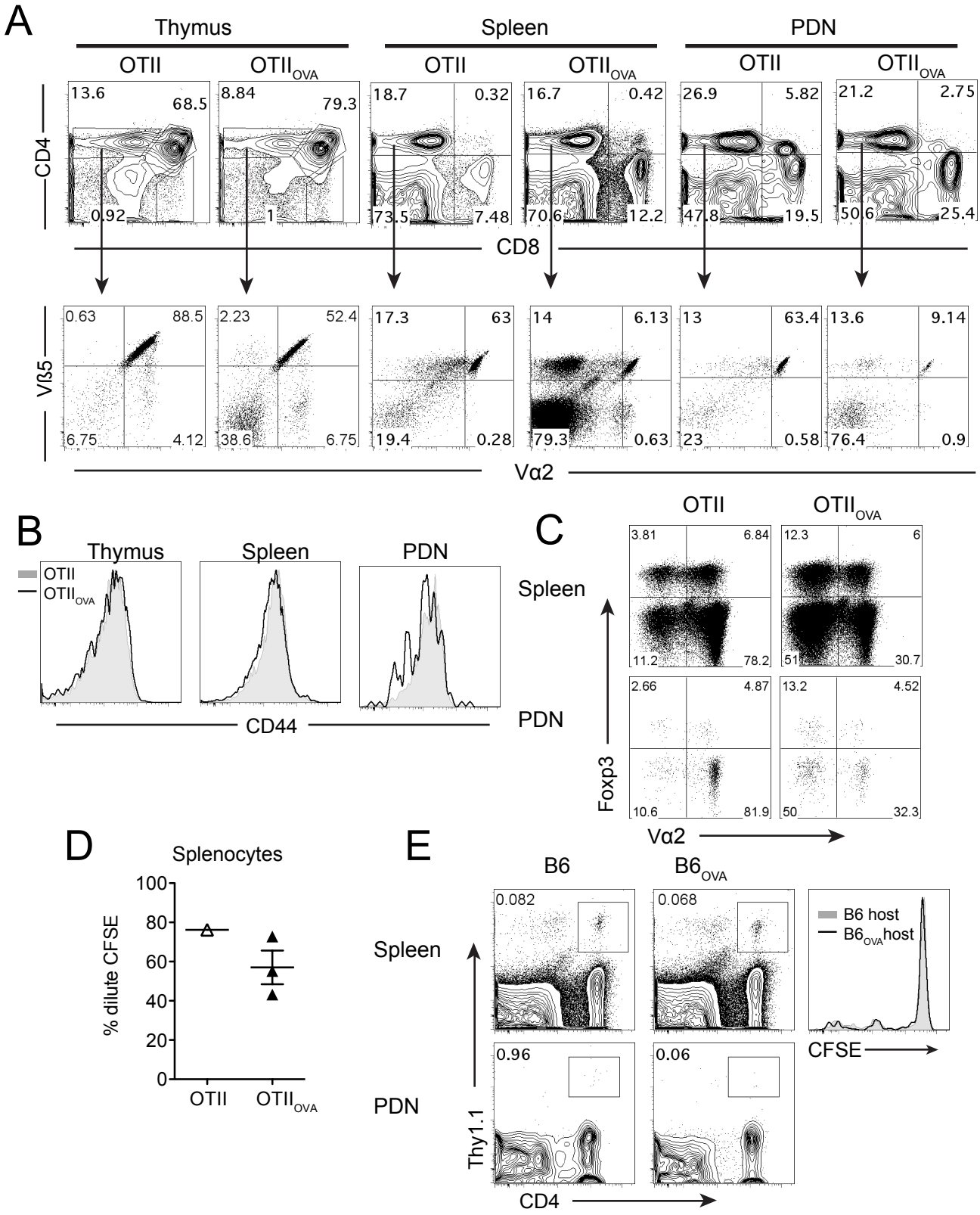

A
Naive
Activated
CD44
TNFα
IFNγ
0.41
99.4
0.2
0
B
Peripheral Blood
WT into B6ova
WT int B6
% of total CD8 in Blood
Days post transfer
C
Week 1
Week 3
OTI into B6ova
OTI into B6
# of transferred cells
% of transferred cells
Spln PDN Spln PDN
pros pros
D
Spleen
PDN
Prostate
B6OVA
B6
TNFα
IFNγ
7.47 18.4 40.8 33.3
1.34 31.9 34.5 32.2
0.4 1.2 86.8 11.6
3.17 19 32.7 45.1
0.13 4.95 70 24.9
10.2 48.1 24 17.7
18.2 55.1 18.2 8.41
8.26 75.1 8.4 8.26
18.6 57.1 18.6 5.71
ND
E
B6 host
B6OVA host
PD-1

Figure 3.4 Characterization of CD4 T cell response to a self-antigen expressed in the prostate. (A) Thymuses, spleens and PDNs were harvested from 20 week old male OTII and $OTII_{OVA}$ mice and processed into single cell suspensions for flow cytometry. All numbers represent percent of cells within the gate. All flow plots were gated on live cells except where indicated. (A, *top*) Single cell suspensions were stained with CD8 and CD4 antibodies. (A, *bottom*) Vα2 and Vβ5 TCR chain expression by single positive CD4 cells. (B) CD44 expression of $CD4^+V\alpha2^+V\beta5^+$ cells from the indicated organs. (C) Foxp3 expression in CD4 cells from the spleen and PDN of OTII and $OTII_{OVA}$ mice. Dot plots are gated on $CD4^+$ cells. (D) Splenocytes from OTII and $OTII_{OVA}$ mice were labeled with CFSE and stimulated with OTII peptide. Percentage of cells that diluted CFSE was analyzed on D5. Symbols represent individual mice. Bar shows mean $\pm$ SEM. (E) Naïve $CD4^+OTII^+Thy1.1^+$ splenocytes were labeled with CFSE and adoptively transferred into 12 week old $B6_{OVA}$ and B6 males. On D27 post transfer, mice were euthanized and the spleen and PDN were stained for the presence of transferred cells and CFSE dilution in the spleen.


A
Thymus
Spleen
PDN
OTII
OTII$_{OVA}$
CD4
CD8
Vβ5
Vα2
13.6
68.5
0.92
8.84
79.3
1
18.7
0.32
73.5
7.48
16.7
0.42
70.6
12.2
26.9
5.82
47.8
19.5
21.2
2.75
50.6
25.4
0.63
88.5
6.75
4.12
2.23
52.4
38.6
6.75
17.3
63
19.4
0.28
14
6.13
79.3
0.63
13
63.4
23
0.58
13.6
9.14
76.4
0.9
B
Thymus
Spleen
PDN
OTII
OTII$_{OVA}$
CD44
C
OTII
OTII$_{OVA}$
Spleen
PDN
Foxp3
Vα2
3.81
6.84
11.2
78.2
12.3
6
51
30.7
2.66
4.87
10.6
81.9
13.2
4.52
50
32.3
D
Splenocytes
% dilute CFSE
100
80
60
40
20
0
OTII
OTII$_{OVA}$
E
B6
B6$_{OVA}$
Spleen
PDN
Thy1.1
CD4
CFSE
0.082
0.068
0.96
0.06
B6 host
B6$_{OVA}$host

Chapter 4: Cell intrinsic abrogation of TGFβRII signaling in self/tumor specific CD8 T cells for adoptive cell therapy in a murine model of autochthonous prostate cancer.*

Introduction

Prostate cancer is an attractive candidate for developing T cell mediated immunotherapy, in part because there are already a number of well-defined prostate specific antigens, and potential induction of autoimmunity from targeting such antigens would not be life threatening. The ability to harness the specificity and cytolytic activities of CD8 T cells for eradicating cancer has been shown in numerous murine models but translation into the clinic has been more limited. Achieving persistent anti-tumor activity following transfer into the patient has proven difficult partly because most tumor antigens are self-proteins and are poorly immunogenic due to host mechanisms for tolerizing or deleting self-reactive cells. In addition, tumors can create a hostile, immunosuppressive microenvironment, rendering tumor infiltrating lymphocytes dysfunctional.

Many tumor therapy studies have been performed using transplantable tumor cell lines, and such models, while useful for advancing the discovery and testing of tumor therapies, have limitations. Injection of a large number of tumor cells is often necessary for successful tumor implantation, with many cells dying rapidly after injection, which can induce an immune response prior to establishment of the tumor (71). More importantly, these tumors do not develop in the same organ-specific environment of tumors that develop and grow *in situ*, which does not recapitulate the evolving tumor-immune interactions within a cancer patient. Autochthonous tumor models, in which the tumor develops "spontaneously" usually due to enforced expression of a driver oncogene, also have some limitations, but do allow for the study of tumors derived from

* This chapter contains modified figures and text from the following accepted manuscript: Chou, C.K., A. Schietinger, H. D. Liggitt, X. Tan, S. Funk, G. Freeman, T. Ratliff, N. Greenberg, P. D. Greenberg. 2012. 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. *J Immunol, in press*.

the organ of origin and develop over months in the context of a normal host immune system.

For our studies, 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 (47). 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 (53, 72). We crossed these mice with the $POET_1$ mice to generate TRAMPxPOET$_1$ mice, denoted $TRAMP_{OVA}$ mice, which now 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 (50).

Studies in the $B6_{OVA}$ modeled revealed the difficulty in eliciting destruction of normal prostate epithelium, suggesting that autoimmunity may not be pose to be a significant risk for ACT of prostate cancer. However, it could also mean that significant peripheral tolerance mechanisms will need to be overcome for successful targeting of the tumor. TGFβ is a potent immunosuppressive cytokine that is expressed by both the normal prostate to maintain epithelial homeostasis, but is also present in increased levels in the malignant prostate (36, 37). Expression of a dominant negative form of TGFβRII (DNR-TGFβRII) or abrogation of the cytokine TGFβ exclusively in T cells of mice that develop autochthonous prostate cancer can delay tumor growth (33, 73), suggesting TGFβ interferes with the development and/or expression of an endogenous response. Studies in transplantable tumor models have also demonstrated that TGFβ signaling blockade can improve the therapeutic efficacy of tumor-reactive T cells (74-76). In our studies, we investigated whether cell intrinsic abrogation of TGFβ signaling increased the persistence and therapeutic efficacy of prostate self/tumor specific CD8 T cells for ACT of prostate cancer.

Results:

Generation of conditional TGFβRII deficient prostate-specific CD8 T cells for adoptive therapy.

In order to address the cell intrinsic effects of TGFβ signaling abrogation, we used a conditional TGFβRII deficiency mouse model in which mice bearing the TGFβRII gene flanked by loxP sites (floxed) (24) were bred to mice expressing Cre-recombinase under the distal-Lck (dLck) promoter (48), which induces Cre expression in SP mature T cells. TGFβRII$^{Flox/Flox}$ x dLck-Cre mice were additionally crossed to OTI$^{Ly5.1}$ mice to generate mice bearing congenic OVA-specific CD8 T cells deficient in TGFβRII expression. For this chapter, OTI$^{Ly5.1}$ x TGFβRII$^{Flox/Flox}$ x dLck-Cre cells will be referred to as TGFβRII KO cells. To model adoptive therapy, WT and TGFβRII KO cells were *in vitro* activated by co-culture with SIINFEKL pulsed splenocytes (1:5 ratio) and 25U/mL IL-2. Five days later, the numbers of CD8$^+$Vα2$^+$Vβ5$^+$ cells were quantitated by cell count and flow cytometry and indicated numbers were adoptively transferred into recipient mice.

Abrogation of TGFβRII signaling in self/tumor antigen specific CD8 T cells increases the therapeutic efficacy for adoptive therapy of a transplantable tumor.

We first tested the therapeutic efficacy of the TGFβRII KO cells for treating tumors by using the OVA expressing E.G7 T cell lymphoma transplantable tumor model (55). To model targeting a self-antigen expressed by the tumor, we used B6$_{OVA}$ mice as hosts. 1x10^6 E.G7 tumor cells, were injected subcutaneously into the flanks of B6$_{OVA}$ mice. Ten days later, when tumors were established (>200mm^3), 3x10^6 effector WT or TGFβRII KO cells were adoptively transferred into tumor bearing B6$_{OVA}$ mice (Fig. 4.1A). Tumor regression was observed in 100% of mice treated with TGFβRII KO cells and the tumor was completely eradicated in 88% of these mice whereas only 40% of mice treated with WT cells eradicated the tumor (Fig. 4.1B). Tumor-bearing mice treated with TGFβRII KO cells also showed significantly increased survival (Fig 4.1C). These results demonstrate that cell intrinsic abrogation of TGFβRII signaling renders self/tumor antigen specific CD8 T cells capable of eradicating established transplantable solid

tumors. Next, we used the autochthonous murine prostate cancer TRAMP model to determine whether cell intrinsic abrogation of TGFβRII signaling enhances the efficacy of prostate-specific CD8 T cells for the treatment of tumors *in situ*.

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 adoptive T cell therapy of *in situ* prostate cancer, we adoptively transferred 5-7 x 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 abrogation of TGFβ signaling affected the expansion of the adoptively transferred cells and found that, compared to WT cells, there was a significantly increased accumulation of TGFβRII KO cells in the spleen, PDN and prostate of $TRAMP_{OVA}$ mice 1 week post transfer (Fig. 4.2A). 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. 4.2A), and there was no significant difference between the numbers of WT cells in $TRAMP_{OVA}$ compared to TRAMP mice, and no significant difference between the numbers of WT and TGFβRII KO cells in any of the tissues examined in TRAMP mice. These data suggest that 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 (29). Therefore, we performed intracellular staining of WT and TGFβRII KO cells directly *ex vivo* for the proliferation marker, Ki-67. For both WT and TGFβRII KO transferred cells, >50% of cells recovered from the spleen, PDN and prostates of $TRAMP_{OVA}$ mice expressed Ki-67, with a significantly greater percentage of Ki-67^+ TGFβRII KO cells compared to WT

cells in the spleen and PDN (Fig. 4.2B). In TRAMP mice, Ki-67 expression was greatly reduced in all transferred cells, indicating that antigen exposure induced transferred cells to remain cycling for at least 1 week (Fig. 4.2B). The increased percentage of TGFβRII KO cycling cells was also reflected as a significant increase in the numbers of Ki-67$^+$ TGFβRII KO cells in the spleen, PDN and prostate of TRAMP$_{OVA}$ mice (Fig. 4.2C) The increased percentage of Ki-67$^+$ WT cells in TRAMP$_{OVA}$ mice compared to TRAMP mice despite the failure to significantly accumulate in TRAMP$_{OVA}$ mice suggested that WT cells in TRAMP$_{OVA}$ mice may have not only a higher rate of proliferation but apoptosis as well. TGFβ signaling has been implicated to upregulate the BH-3 only pro-apoptotic protein Bcl-2-interacting mediator of cell death (Bim) (77, 78). Therefore, we stained WT and TGFβRII KO cells for total Bim protein. A higher percentage of TGFβRII KO cells were Bim^{low} compared to WT cells, especially in the proliferating (Ki-67$^+$) population, in all organs examined (Fig. 4.2D). TGFβRII KO and WT cells transferred into TRAMP mice were also analyzed, and in the absence of antigen no significant differences in BIM levels were observed in any of the organs examined. The results suggest that 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 (79, 80). 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. WT cells in the prostate exhibited severely attenuated IFNγ and TNFα production compared to WT cells in the PDN and spleen (Fig. 4.3A,B). Abrogation of TGFβ signaling significantly increased the percentage and number of transferred cells capable of co-producing IFNγ and TNFα in the prostate, as well as in the PDN (Fig. 4.3B,C). However, TGFβRII KO cells in the prostate of TRAMP$_{OVA}$ mice were attenuated in 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).

To determine if this functional impairment in the prostate was antigen-specific, WT and TGFβRII KO cells transferred into TRAMP hosts were also analyzed. We found no significant difference in cytokine production between WT and TGFβRII KO cells in any of the organs examined in TRAMP mice. However, significantly decreased percentages of WT and TGFβRII KO cells from $TRAMP_{OVA}$ PDN produced cytokines compared to TRAMP (for WT p=0.0018, for KO p=0.0020) and significantly decreased percentage of TGFβRII KO cells from the prostate of $TRAMP_{OVA}$ co-produced IFNγ and TNFα compared to cells from TRAMP mice (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 rapidly induced and organ-specific.

Increased cellular infiltrates and focal epithelial disruption in the prostates of $TRAMP_{OVA}$ mice receiving T cells deficient in TGFβ signaling.

Next 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. 4.2D). 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. 4.2D). To determine if the infiltrates included adoptively transferred T cells, frozen prostate sections were stained immuno-histochemically, and increased $Ly5.1^+$ cells were detected in prostate glands of mice receiving TGFβRII KO cells compared to WT cells (Fig. 4.2E).

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

To determine if adoptive transfer of WT or TGFβRII KO cells affected tumor burden, prostates of treated mice were harvested and weighed 3 weeks post T cell transfer, with prostate weight used as a surrogate for tumor burden, as described (72). 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. 4.4A). 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 T cells (Fig. 4.4B). Despite the decrease in prostate weight, evidence of neoplasia was still present in mice treated with TGFβRII KO cells. Thus, the initial increased infiltration of TGFβRII KO cells and anti-tumor activity observed at 1 week post transfer in TRAMP$_{OVA}$ prostates was transient, and was not sufficient for significant therapeutic efficacy.

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

The lack of significant therapeutic efficacy suggested that transferred T cells did not persist and/or became dysfunctional, which are obstacles also encountered in human ACT (14). To determine if the enhanced accumulation and function of transferred TGFβRII KO cells evident at week 1 was maintained, multiple organs from treated TRAMP$_{OVA}$ mice were examined at 3 weeks post adoptive transfer. The increased numbers of TGFβRII KO cells compared to WT cells were still demonstrable in the spleen and PDN of TRAMP$_{OVA}$ mice at 3 weeks post-transfer (Fig. 4.5A) 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 post transfer compared to week 1 (spleen$_{wk\ 1}$: $5.4x10^5$ cells, spleen$_{wk\ 3}$: $2.8x10^6$ cells, $p = 0.1682$; and PDN$_{wk\ 1}$: $2x10^5$ cells, PDN$_{wk\ 3}$: $8.1x10^5$ cells, $p = 0.1765$). In the prostate, however, the initially increased accumulation of TGFβRII KO cells at week 1 was not sustained (Fig. 4.2A) and there was no longer a significant

difference between the number of TGFβRII KO and WT cells (Fig. 4.5A). Thus, additional factors beyond TGFβ signaling appear to contribute to the lack of persistence of prostate infiltrating cells.

To test if transferred cells continued to proliferate by week 3, transferred WT and TGFβRII KO cells were stained for Ki-67. Compared to week 1 post-transfer, a lower percentage of TGFβRII KO and WT cells in $TRAMP_{OVA}$ mice expressed Ki-67 at week 3 in all organs examined (Fig. 4.5B, Fig 4.2B). Furthermore, at week 3, 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. 4.5B). Thus, by week 3 post transfer, antigen-specific proliferation declined in TGFβRII KO cells, suggesting that factors in addition to TGFβ are likely preventing further expansion and accumulation of these cells in the prostate. Similar to week 1, a higher percentage of TGFβRII KO cells were Ki-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. 4.5C).

By week 3 post transfer, the enhanced effector cytokine production by TGFβRII KO cells compared to WT cells observed at 1 week in the prostate was also lost. The percentage of TGFβRII KO cells co-producing IFNγ and TNFα declined by 3 weeks compared to week 1 (Fig. 4.5D-E, Fig. 4.3A-B) and there was no significant difference between the percent of WT or TGFβRII KO cells able to produce these cytokines in the spleen and prostate. Despite increased numbers of $IFN\gamma^{+}TNF\alpha^{+}$ TGFβRII KO cells in the spleen compared to WT cells, there was no longer an increased accumulation of dual-cytokine producing cells in the prostate (Fig. 4.5F). We were unable to recover sufficient numbers of WT cells from the PDN of $TRAMP_{OVA}$ mice at 3 weeks to perform the intracellular cytokine assay. 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 were able to produce both cytokines (Fig. 4.3*B*, Fig. 4.5E) suggesting that 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 (for TGFβRII KO cells $p = 0.1446$; for WT cells $p = 0.0370$), suggesting that factors within the prostate tumor microenvironment may be affecting the activity of these cells in an antigen independent manner. Thus, despite the 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 (81) and tumor cells have been shown to down-regulate MHC Class I expression as a form of immune evasion (82). MHC Class I expression is not readily detectable on normal B6 prostate cells but has been shown to be up-regulated in TRAMP prostate tumors (83). To determine if TRAMP prostate tumors maintained MHC Class I expression following adoptive transfer, we stained frozen prostate sections with MHC Class I antibody and found continued Class I expression and no detectable change in Class I expression in $TRAMP_{OVA}$ prostates following treatment with WT or TGFβRII KO T cells (Fig. 4.6).

Another potential tumor evasion mechanism is downregulation and/or loss of target antigen expression. Multiple efforts to stain for OVA on frozen prostate sections proved unsuccessful. However, the sustained dysfunction of transferred cells 3 weeks post transfer in $TRAMP_{OVA}$ but not TRAMP prostates suggest that OVA expression was maintained.

Second infusion TGFβRII KO cells infiltrate the prostate and mediate some prostate damage.

The accumulation of TGFβRII KO cells in the spleen and PDN but not in the prostate suggested there might be a defect in continuous cellular infiltration into the prostate. To test whether intrinsic defects acquired by transferred cells or changes in the host prevented continuous infiltration into the prostate and destruction of prostate tumor cells, we did two consecutive adoptive transfers of differently marked TGFβRII

KO cells (1st batch expressed YFP ($4x10^6$) and the 2nd batch expressed Ly5.1 ($5x10^6$) at D0 and D21 into the same host $TRAMP_{OVA}$ mouse and assessed T cell function and prostate histology at D30 (Fig 4.7A). We found that at D30, 2nd transfer cells accumulated in the prostate, suggesting that cell intrinsic defects prevented continuous infiltration of transferred cells into the prostate. Moreover, these data suggest that OVA levels in the prostate 3 weeks post initial transfer were sufficient to attract TGFβRII KO cells. The effector cytokine profile of 2nd transfer cells in the PDN and prostate was more similar to the profile of TGFβRII KO cells 1 week post transfer (Fig 4.3A) than to 1st transfer cells (Fig 4.7C). Prostates were also stained with H&E and areas of necrosis was observed (Fig 4.7D), suggesting that OVA expression is maintained on tumor cells and the lack of tumor destruction as shown in Fig 4.4B was not likely due to antigen loss but rather cell intrinsic dysfunction of transferred cells. The ability of 2nd transfer cells to infiltrate the prostate and relative paucity of 1st transfer cells in the prostate despite detectable numbers in the spleen suggested that the 1st transfer cells may have acquired a defect in trafficking. Chemokine receptors expressed by T cells play a key role in the recruitment of effector CD8 T cells to peripheral tissue sites. It was demonstrated in an HSV infection model that CXCR3 expression by T cells was crucial for mobilization into virally infected vaginal mucosa (84). Furthermore, global defect in the CXCR3 gene resulted in accelerated disease in the TRAMP mouse (85), suggesting that CXCR3 plays a crucial role in controlling prostate cancer. CXCR3 expression levels were measured on transferred TGFβRII KO cells by flow cytometry. TGFβRII KO cells in the spleen and PDN of TRAMP hosts expressed high levels of CXCR3 (Fig 4.7E). 1st transfer cells were $CXCR3^{lo}$ in the spleen and $CXCR3^{lo\text{-}int}$ in the PDN while some 2nd transfer expressed higher levels of CXCR3 both in the spleen and PDN (Fig 4.7E). All transferred cells were $CXCR3^{lo}$ in the prostate, which may reflect ligand induced receptor endocytosis. These data suggest that CXCR3 downregulation may prevent continuous infiltration of prostate-specific cells into the prostate. Further studies to determine whether CXCR3 ligands, CXCL10 and CXCL9, are expressed in the prostate will be necessary.

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 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 may be functionally exhausted. Chronic antigen exposure has been shown to lead to T cell exhaustion (86, 87), 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* is lost first, followed by loss of TNFα production and partial loss of IFNγ production, then complete loss of IFNγ production, and eventually cell death (88, 89). Exhausted T cells also express inhibitory signaling receptors, including PD-1. Prostate tumor infiltrating cells in humans also expressed high levels of PD-1 (23). Analysis of WT cells at 1 week post-transfer into TRAMP$_{OVA}$ mice, revealed expression of higher levels of PD-1 in the PDN and prostate compared to WT cells transferred into TRAMP mice (Fig. 4.8A). 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 but not in the spleen of TRAMP$_{OVA}$ mice. This pattern of PD-1 expression correlated with the severity of the observed functional defect, suggesting that 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. PD-L1, one ligand for PD-1, is up-regulated on many human tumors, including prostate cancer (39). Analysis of frozen TRAMP$_{OVA}$ prostates 3 weeks post adoptive transfer of TGFβRII KO cells revealed that PD-L1 was expressed on prostate epithelium (Fig. 4.8B).

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

The crucial role of PD-1 signaling in mediating functional exhaustion has been most convincingly demonstrated in chronic lymphocytic choriomeningitis virus (LCMV) infection by blockade of PD-1 signaling, which partially restored cytokine production and

anti-viral activity in exhausted LCMV specific CD8 T cells (59). PD-1 blockade has also been shown to enhance anti-tumor activity in transplantable tumor models (90, 91). However, it was previously reported in the TRAMP model that, despite increased PD-1 expression on prostate specific CD8 T cells, breeding TRAMP mice onto a PD-L1$^{-/-}$ background did not prevent tolerization of prostate-specific CD8 T cells (92). Since PD-1 may signal through interactions with other known ligands, such as PD-L2, or other unidentified ligands, we examined if abrogation of PD-1 signaling in prostate-specific CD8 T cells could promote more persistent and effective anti-tumor activity.

We bred congenic OTI$^{Thy1.1}$ mice to PD-1 KO mice to generate OTI$^{Thy1.1}$xPD-1 KO mice (denoted as PD-1 KO). PD-1 KO and TGFβRII KO CD8 T cells were *in vitro* activated for 5 days and 5x10^6 PD-1 KO CD8 T cells and 2x10^6 TGFβRII KO CD8 T cells were cotransferred into TRAMP$_{OVA}$ and TRAMP males. Four weeks later, recipient mice were analyzed and a persistent population of TGFβRII KO cells was detected in the spleen and prostate whereas no PD-1 KO cells were detected in TRAMP$_{OVA}$ mice (Fig. 4.8C). PD-1 KO cells were still detectable in the spleens of TRAMP mice suggesting that the lack of persistence in TRAMP$_{OVA}$ mice is antigen-dependent and abrogation of PD-1 signaling alone does not enhance prostate-specific CD8 T cell persistence.

Next, we tested whether PD-1 blockade synergized with TGFβRII abrogation using a combination of PD-1, PD-L1 and PD-L2 blocking antibodies. *In vitro* activated TGFβRII KO cells were adoptively 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 the treatment regimen to assay 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 (Figure 4.8D-E). 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 were able to detect Ab bound to transferred T cells in the

PDN and prostates only in $TRAMP_{OVA}$ mice treated with αPD-1 antibodies but not TRAMP mice treated with αPD-1 antibodies or $TRAMP_{OVA}$ mice treated with PBS (Fig. 4.8F). 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 to maintain the function of the transferred cells, implying that additional inhibitory pathways and obstacles are operative in the environment of prostate cancers.

Depletion of Foxp3$^+$ regulatory T cells resulted in severe systemic autoimmunity.

Additional cell extrinsic factors may also be contributing to the immunosuppressive tumor environment, including $Foxp3^+$ regulatory T cells (Tregs). Similar to published studies (93), we found increased numbers of $CD4^+Foxp3^+$ cells in 25 week-old $TRAMP_{OVA}$ prostates compared to healthy age-matched male mice (Fig. 4.9A). The effect of Treg depletion in TRAMP mice had been addressed using anti-CD25 antibodies (93) and it was found that depletion with anti-CD25 antibodies did not prevent tolerance induction of endogenous SV40 specific CD8 T cells. Because activated T cells can also express high levels of CD25 and not all Tregs express CD25, we bred $TRAMP_{OVA}$ mice to $Foxp3^{DTR}$ mice (42) to test whether $Foxp3^+$ Tregs played a role in suppressing adoptively transferred effectors. We used a diphtheria toxin administration schedule shown in Fig. 4.9B. By D9 post initial DT administration, all endogenous CD4 and CD8 T cells in the peripheral blood were $CD44^{hi}$ suggesting that in the absence of $Foxp3^+$ Tregs, endogenous T cells became activated (Fig. 4.9C). 3 weeks post T cell transfer, the weights of 4 out of 7 prostates from depleted $TRAMP_{OVA}$ mice were comparable to the normal B6 prostate weight (Fig. 4.9D). However, Foxp3 depleted TRAMP mice, which did not express OVA, also had reduced prostate weights, suggesting that the decrease in tumor burden was not a direct result of transferred TGFβRII KO cells recognizing antigen in the prostate. Moreover, all Foxp3-depleted mice lost >10% of their original body weight, had splenomegaly, lymphadenopathy and showed overt signs of systemic autoimmunity, even though $Foxp3^+$ cells were present at the time of analysis (Fig. 4.9E). H&E staining of prostate sections from TGFβRII KO

treated mice with “normal” prostate weights all showed absence of tumor and significant stromal hyperplasia and mononuclear infiltrates, suggestive of chronic inflammation, regardless of OVA expression (Fig. 4.9F). Therefore, while temporary nearly complete ablation (>97%) of $Foxp3^+$ cells may lead to tumor regression in some mice, severe systemic autoimmunity precludes its feasibility for clinical use.

Prostate-specific CD4 T cells do not detect antigen in the $TRAMP_{OVA}$ model.

CD4 T cells can have direct and indirect effects on tumor eradication (94-96) and provision of CD4 T cells in conjunction with CD8 T cells has been shown to enhance CD8 T cell function in various tumor settings (97, 98). We first tested if OTIIs detect antigen and traffic to the prostate in the $TRAMP_{OVA}$ model by adoptive transfer of naïve CFSE labeled OTIIs into $TRAMP_{OVA}$ and TRAMP mice. Unfortunately, similar to experiments with $B6_{OVA}$ mice, OTIIs did not dilute CFSE and did not upregulate CD44 following transfer into $TRAMP_{OVA}$ mice (Fig. 4.10A, B). Pre-activation of OTIIs did not result in prostate infiltration and co-transfer experiments with OTIs did not reveal any synergistic enhancement of OTI activity (data not shown). These results suggest that in this model, OTII T cells do not appear to detect cognate antigen expressed by prostate epithelial cells.

Discussion:

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. WT cells specific for an antigen selectively expressed in normal and transformed prostate cells were rapidly rendered dysfunctional in the *in situ* prostate tumors of $TRAMP_{OVA}$ mice, whereas TGFβRII KO cells not only accumulated in greater numbers in the spleen, PDN and the prostate but retained the ability to produce effector cytokines and induced modest destruction of transformed prostatic epithelium. The retention of effector cytokine production in the PDN and prostate and evidence of some injury to the tumor affirmed that, similar to findings in transplantable models of prostate cancer (74-76),

TGFβ regulates the anti-tumor activity of transferred prostate self/tumor specific CD8 T cells targeting tumor spontaneously developing in the prostate. 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 is also consistent with enhanced anti-tumor activity. However, unlike in the transplantable E.G7 model, in which TGFβR blockade in tumor-reactive T cells resulted in complete elimination of the tumor, 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 is often a problem in clinical ACT targeting established tumors (14). We demonstrated that abrogation of TGFβ signaling was adequate to sustain transferred T cell numbers in distal secondary lymphoid organs, but additional immunosuppressive factors operative within the prostate and possibly PDN eventually rendered cells remaining at these sites dysfunctional. The upregulation of PD-1 receptor expression on the dysfunctional cells suggested that inhibitory signals through the PD-1 pathway could be preventing effective anti-tumor activity. However, antibody blockade of PD-1 signaling failed to significantly synergize with abrogation of TGFβ signaling, with no evidence of significant 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 LCMV infection revealed that PD-L1 blockade selectively restored the function of PD-1^{int} but not PD-1^{hi} LCMV-specific CD8 T cells (99). It appears likely that the transferred cells in our model fall into the same category as the PD-1^{hi} LCMV-specific CD8 T cell subset that were not rescuable by PD-1 blockade. 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 (100), LAG3 (101), TIM-3 (90) and/or 2B4 (102), may be simultaneously expressed and limiting T cell function.

The context in which a T cell encounters antigen influences its function and differentiation state (103). Thus, there are many additional events that 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, the transferred cells are likely encountering antigen not only on tumor cells but also on normal prostate cells

and/or dendritic cells (DCs) presenting the peptide in a tolerogenic context. Chronic antigen stimulation alone can induce T cell exhaustion (86, 87), and in some settings this exhaustion is not rescued by PD-1 blockade (87), as may be occurring in the prostate. Studies in a chronic viral infection model with LCMV has also demonstrated that cell intrinsic TGFβ blockade can lead to an increase in the number LCMV-specific CD8 T cells and promote clearance of chronic LCMV, but, in experimental conditions in which the virus and consequently viral antigen is not cleared, the TGFβR-deficient T cells also become functionally exhausted (78). Second, tumor associated DCs (TADCs) have been identified in TRAMP prostate tumors and can directly suppress naïve prostate-specific CD8 T cells (104), therefore, it is possible that in the setting of ACT, continuous encounters of transferred self/tumor specific effector T cells with TADCs in the prostate prevent sustained anti-tumor activity. Moreover, DC vaccines have been shown to transiently augment and/or restore the activity of prostate infiltrating T cells (105-107).

Despite the presence of increased numbers of regulatory T cells in TRAMP mice, our efforts to determine whether Foxp3$^+$ Tregs directly suppressed transferred effector cells were confounded by fatal, systemic autoimmunity. These findings affirm the inherent difficulties associated with pursuing effective global depletion of Tregs as a therapeutic strategy for treating tumors. Until agents that can specifically deplete Foxp3$^+$ Tregs within the tumor microenvironment are developed, it would be difficult to address whether effective ablation of this cell subset will result in increased intratumoral immunity and whether it would be feasible to translate into the clinic.

Our findings have implications for human adoptive therapy. We found increased accumulation of TGFβRII KO cells and increased effector function of both WT and TGFβRII KO cells at 1 week and 3 weeks post transfer 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. Thus, in clinical settings in which it is difficult to obtain tumor biopsies, analysis of cells of the same specificity in the blood may not accurately reflect the functional state of cells in the tumor. Evidence supporting this conclusion has also been provided in the study of

melanoma patients, in which tumor infiltrating lymphocytes in the metastatic lesions can exhibit an exhausted profile whereas T cells of the same specificity in the blood are functional (108).

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. Upon encounter with antigen, an increased expansion and accumulation of functional antigen-specific TGFβRII deficient cells is initially observed, but in settings in which the antigen is persistent, TGFβRII KO cells are eventually rendered dysfunctional at the site of antigen expression. Nevertheless, the initial increase in cell number and delay in loss of anti-tumor activity 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 (107, 109, 110). Studies from our lab recently demonstrated that lymphopenia-induced proliferation could transiently restore the function of tolerant T cells (111). Another recent study using the same conditional TGFβRII KO model showed that lymphopenia driven proliferation synergized with TGFβRII deficiency to drive autoimmunity caused by self-reactive T cells (112). These data together suggest that lymphodepletion of TRAMP mice may synergize with abrogation of TGFβ signaling to sustain anti-tumor activity and increase therapeutic efficacy.

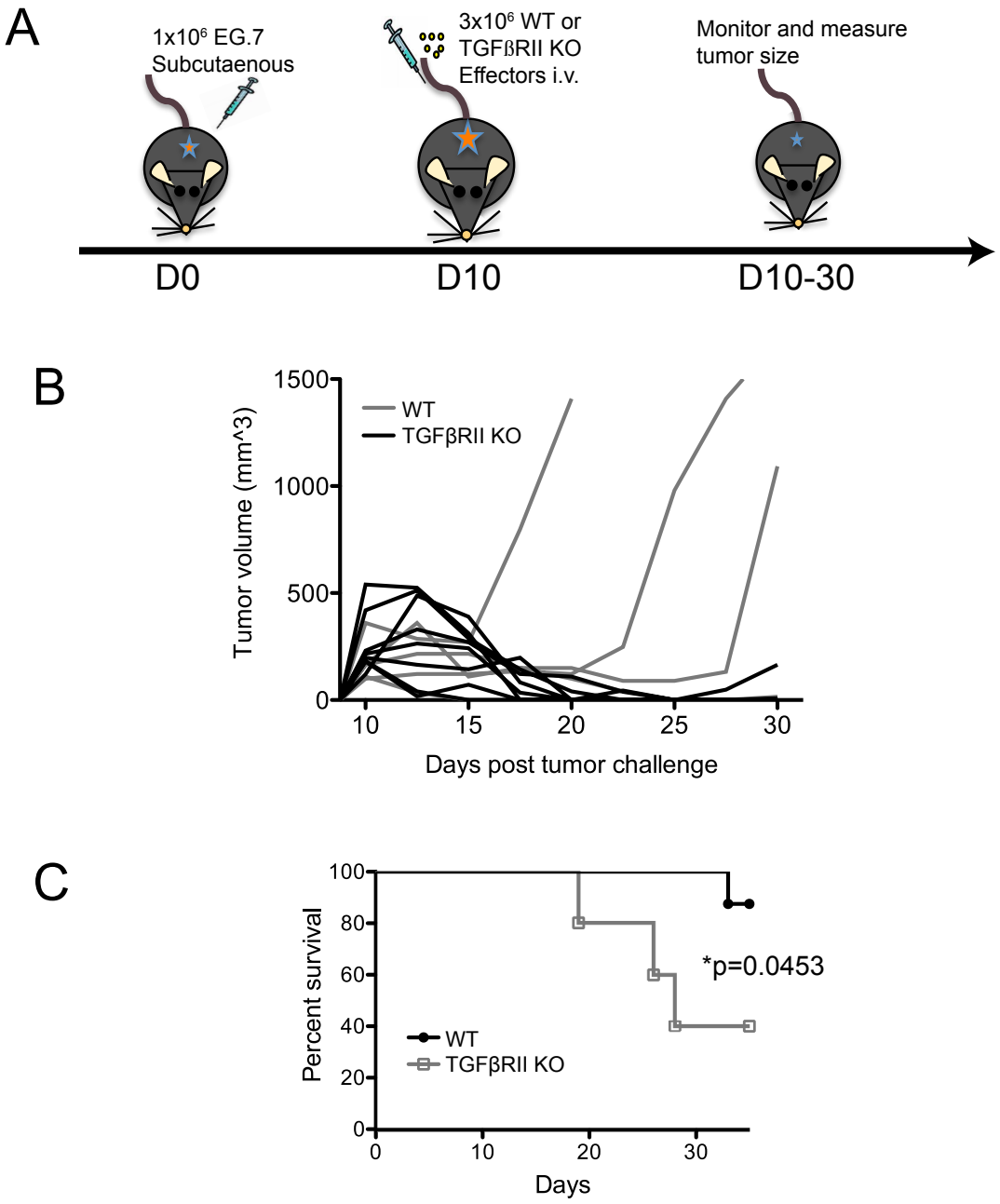


Figure 4.1: Abrogation of TGFβRII signaling in self/tumor specific CD8 T cells increases therapeutic efficacy in adoptive therapy of established E.G7 tumors. (A) Experimental protocol (B) Tumor growth was monitored 3x/week using calipers and tumor volumes were approximated by (LxWxH)/2. Lines show individual mice. (B) Survival of $B6_{OVA}$ tumor bearing mice treated with WT or TGFβRII KO T cells. (p=0.0453; Survival log-rank (Mantel-Cox) Test. Data represents pooled data from 3 independent experiments (n= 8 mice total for TGFβRII KO treated and n=5 mice total for WT treated)

Figure 4.2: Increased accumulation of TGFβRII deficient prostate self/tumor antigen specific CD8 effector T cells in TRAMP$_{OVA}$ mice. (A) 5-7x10^6 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 single cell suspensions were made and analyzed from the spleen, PDN and prostates. (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) Intracellular Ki-67 staining of isolated cells to determine proliferating population. Bars represent percent of transferred cells expressing Ki-67 in the spleen, PDN and prostate. No significant differences were detected between WT and TGFβRII KO cells from each organ in TRAMP mice. (C) Numbers of CD8$^+$Ly5.1$^+$Ki-67$^+$ WT and TGFβRII KO cells isolated from various organs in TRAMP$_{OVA}$ mice. (D) Ki-67 and BIM staining of CD8$^+$Ly5.1$^+$ isolated from indicated organs and mice 1 week post transfer. Representative flow plots are shown. Results are from 2 independent experiments (A-C) Data represents pooled data 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 $\pm$ SEM. **P<0.05*, ***P<0.01,* ****P<0.001* (unpaired Student's t test).

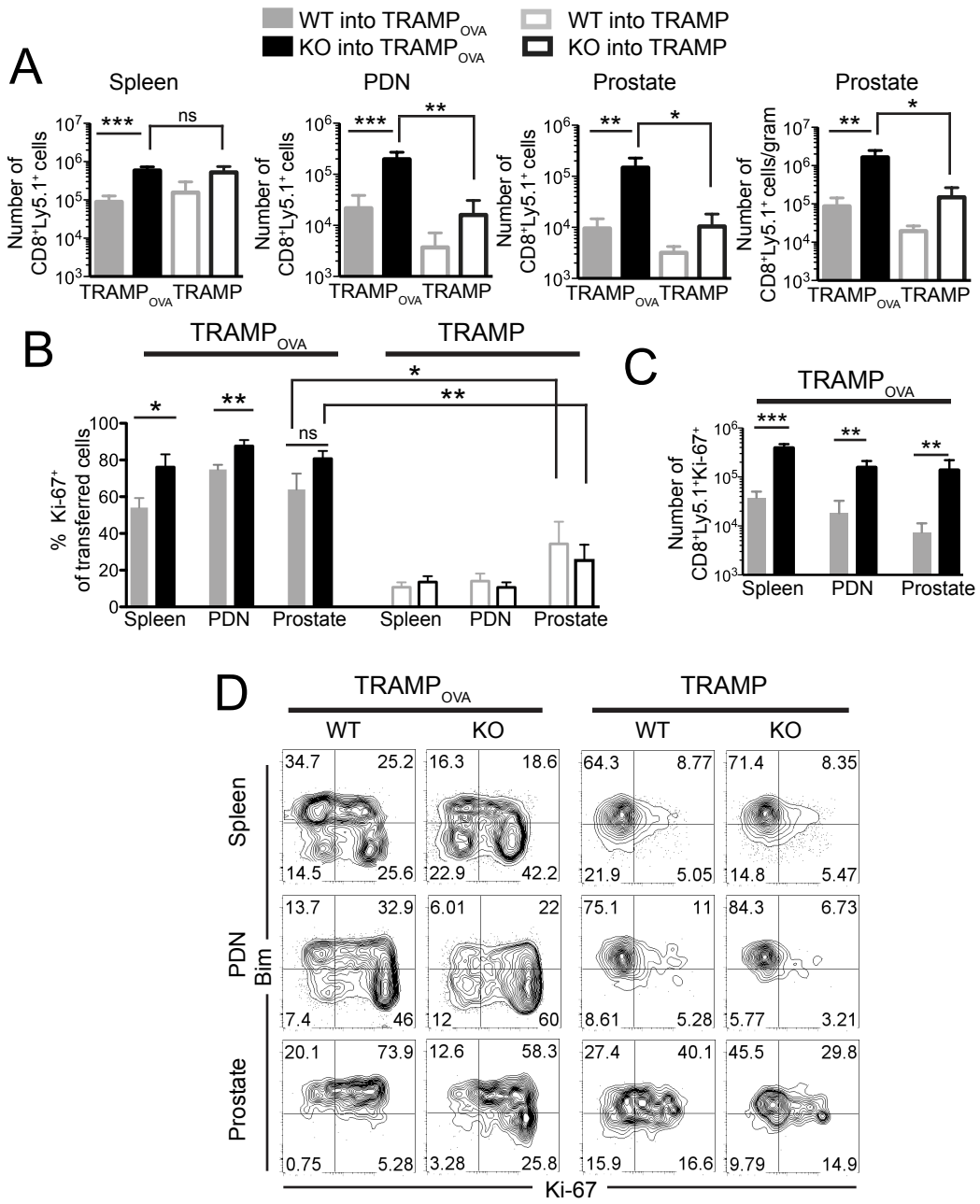
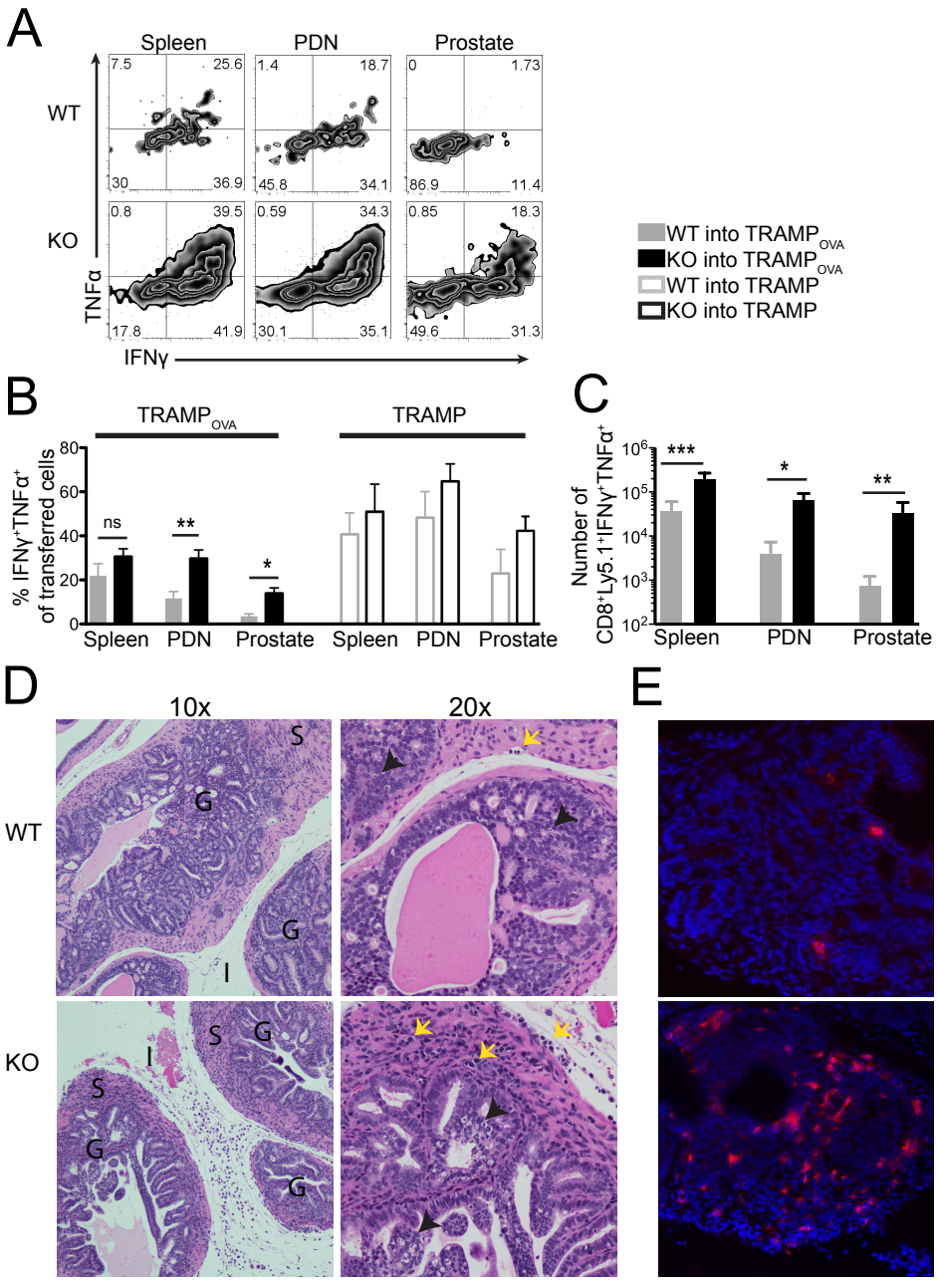

A
WT into TRAMP$_{OVA}$
KO into TRAMP$_{OVA}$
WT into TRAMP
KO into TRAMP
Spleen
PDN
Prostate
Prostate
Number of CD8$^+$Ly5.1$^+$ cells
Number of CD8$^+$Ly5.1$^+$ cells/gram
TRAMP$_{OVA}$
TRAMP
***
**
*
ns
B
% Ki-67$^+$ of transferred cells
Spleen
PDN
Prostate
C
Number of CD8$^+$Ly5.1$^+$Ki-67$^+$
D
WT
KO
Spleen
PDN
Bim
Prostate
Ki-67
34.7
25.2
14.5
25.6
16.3
18.6
22.9
42.2
64.3
8.77
21.9
5.05
71.4
8.35
14.8
5.47
13.7
32.9
7.4
46
6.01
22
12
60
75.1
11
8.61
5.28
84.3
6.73
5.77
3.21
20.1
73.9
0.75
5.28
12.6
58.3
3.28
25.8
27.4
40.1
15.9
16.6
45.5
29.8
9.79
14.9

Figure 4.3: Transferred TGFβRII KO CD8 effector T cells exhibit enhanced effector function and induce increased cellular infiltration and 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 SIINFEKL 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 H&E. Two magnifications are shown, 10x and 20x 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 10x. Black arrowheads point to apoptotic cells and yellow arrows point to lymphoid cells at 20x. (E) $TRAMP_{OVA}$ prostate lobes were frozen in OCT, sectioned on a cryostat and stained with DAPI (blue) and Ly5.1 (red); 20x. (D-E) Histology slides show one representative mouse from each experimental group.


A
Spleen
PDN
Prostate
WT
KO
TNFα
IFNγ
7.5
25.6
30
36.9
1.4
18.7
45.8
34.1
0
1.73
86.9
11.4
0.8
39.5
17.8
41.9
0.59
34.3
30.1
35.1
0.85
18.3
49.6
31.3
WT into TRAMP$_{OVA}$
KO into TRAMP$_{OVA}$
WT into TRAMP
KO into TRAMP
B
TRAMP$_{OVA}$
TRAMP
% IFNγ$^+$TNFα$^+$ of transferred cells
ns
**
*
Spleen
PDN
Prostate
C
Number of CD8$^+$Ly5.1$^+$IFNγ$^+$TNFα$^+$
***
*
**
D
10x
20x
WT
KO
S
G
I
E

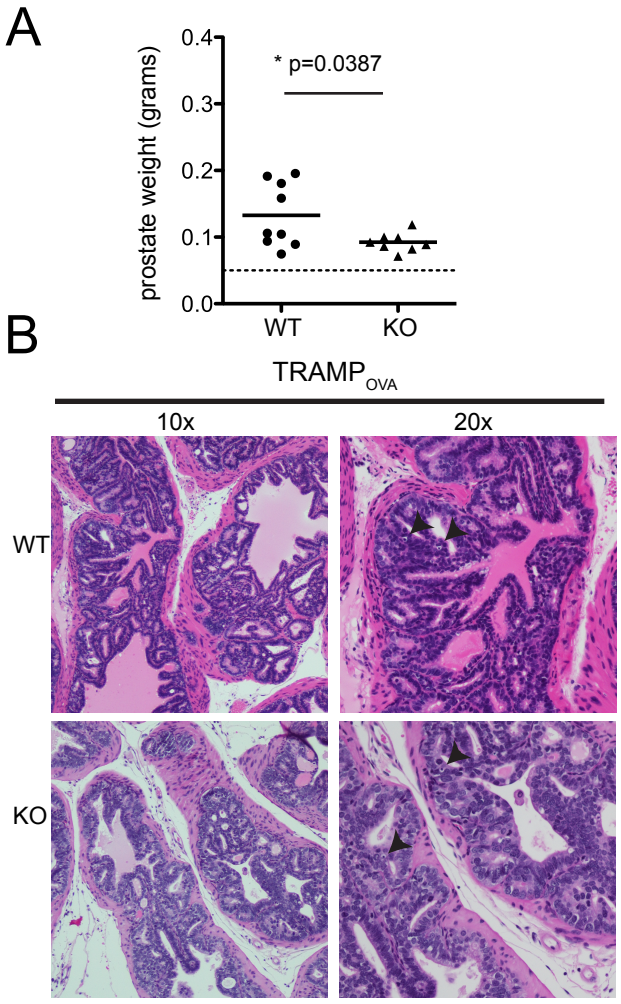
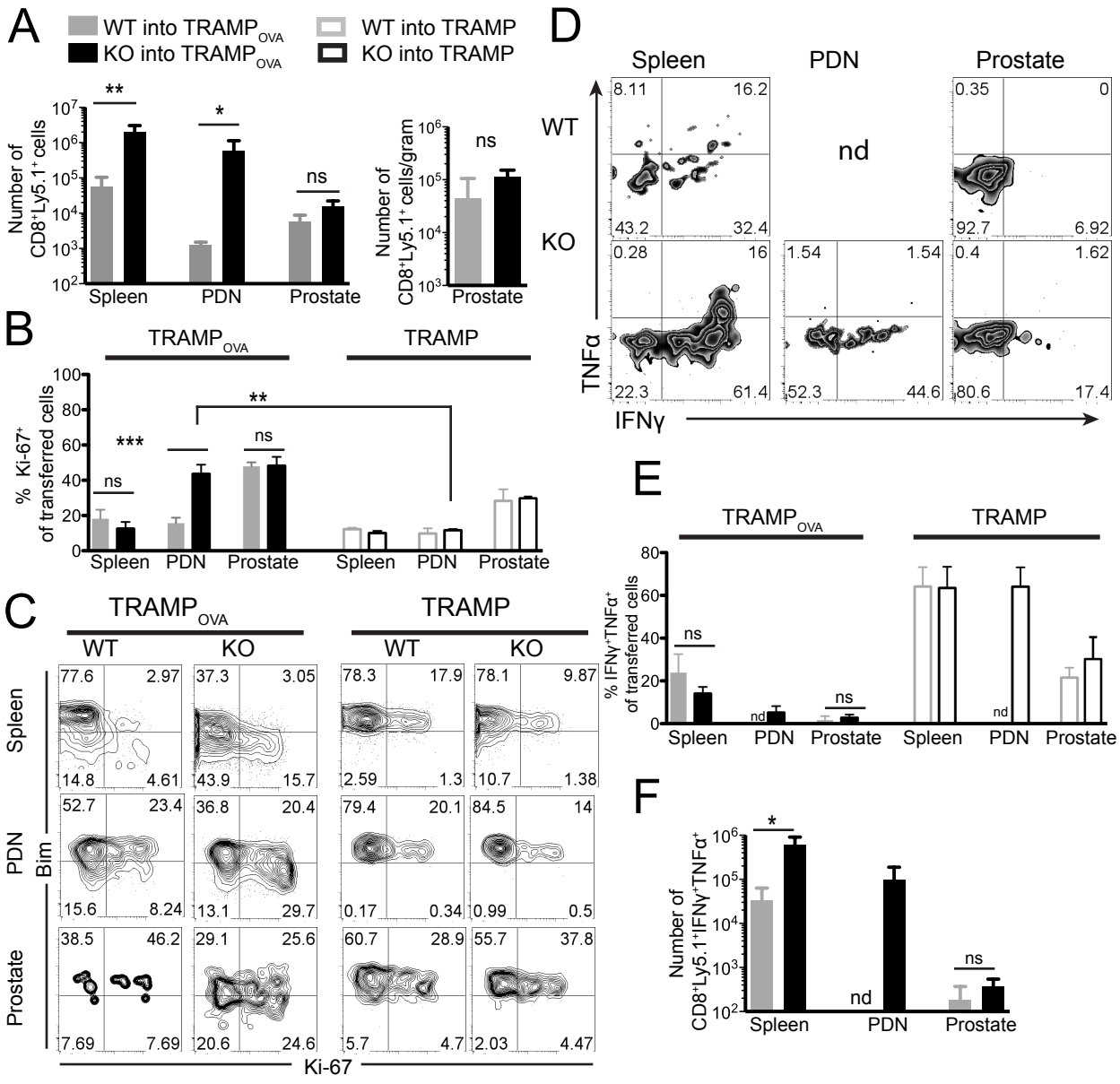


Figure 4.4: Cellular infiltration in the prostates of TRAMP$_{OVA}$ mice receiving TGFβRII KO cells is not sustained. Prostates were microdissected and analyzed 3 weeks post transfer of WT and TGFβRII KO T cells. (A) Prostate weights at 3 weeks post T cell transfer. Dashed line marks prostate weight 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 4.5: 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. Same experimental protocol as Figure 1 except mice were euthanized and analyzed 3 weeks post adoptive transfer. (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) Ki-67 and BIM staining of $CD8^+Ly5.1^+$ isolated from indicated organs and mice 3 weeks post transfer. Representative flow plots are shown. Results are from 2 independent experiments (D) Representative flow plots of cytokine production by WT and TGFβRII KO cells. Numbers represent percent of gated cells in each quadrant. Plots gated on $CD8^+Ly5.1^+$ cells. (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-E) 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).


A
WT into TRAMP$_{OVA}$
KO into TRAMP$_{OVA}$
WT into TRAMP
KO into TRAMP
Number of CD8$^+$Ly5.1$^+$ cells
Number of CD8$^+$Ly5.1$^+$ cells/gram
Spleen
PDN
Prostate
**
*
ns
ns
B
TRAMP$_{OVA}$
TRAMP
% Ki-67$^+$ of transferred cells
***
**
ns
ns
C
TRAMP$_{OVA}$
TRAMP
WT
KO
Spleen
PDN
Prostate
Bim
Ki-67
77.6
2.97
14.8
4.61
37.3
3.05
43.9
15.7
78.3
17.9
2.59
1.3
78.1
9.87
10.7
1.38
52.7
23.4
15.6
8.24
36.8
20.4
13.1
29.7
79.4
20.1
0.17
0.34
84.5
14
0.99
0.5
38.5
46.2
7.69
7.69
29.1
25.6
20.6
24.6
60.7
28.9
5.7
4.7
55.7
37.8
2.03
4.47
D
Spleen
PDN
Prostate
WT
KO
TNFα
IFNγ
8.11
16.2
43.2
32.4
nd
0.35
0
92.7
6.92
0.28
16
22.3
61.4
1.54
1.54
52.3
44.6
0.4
1.62
80.6
17.4
E
TRAMP$_{OVA}$
TRAMP
% IFNγ$^+$TNFα$^+$ of transferred cells
ns
nd
ns
nd
Spleen
PDN
Prostate
F
Number of CD8$^+$Ly5.1$^+$IFNγ$^+$TNFα$^+$
*
nd
ns
Spleen
PDN
Prostate

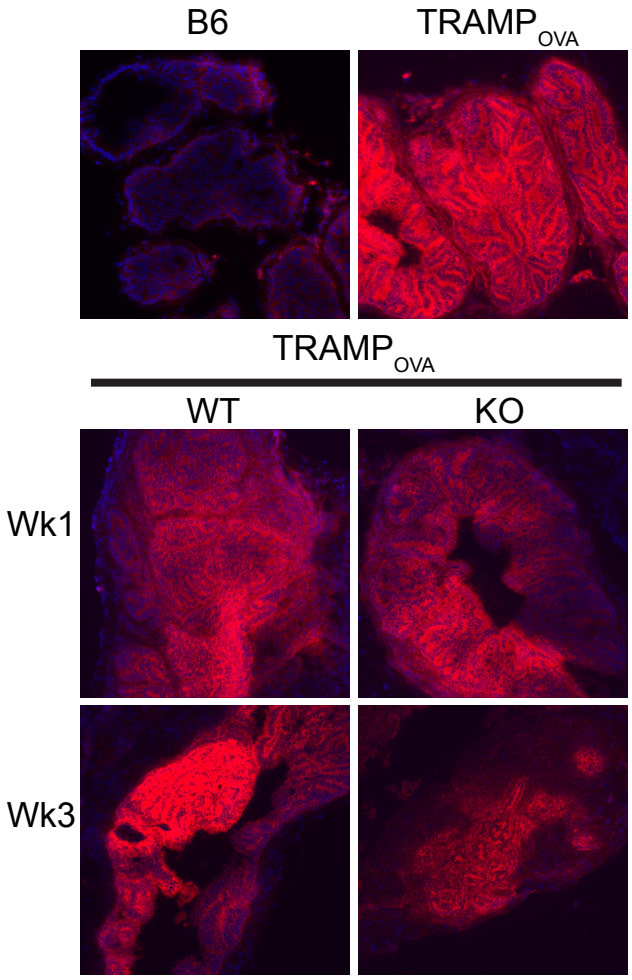


Figure 4.6: TRAMP prostate tumors express MHC Class I and maintain Class I expression following adoptive T cell transfer. Prostates were harvested from B6 mice and TRAMP$_{OVA}$ mice (pre-adoptive transfer and at week 1 and week 3 post adoptive transfer of TGFβRII KO or WT cells), and frozen in OCT. Frozen prostate sections were stained with DAPI (blue) and anti-MHC Class I mAb (red).

Figure 4.7: Second transfer cells infiltrate and damage the prostate. (A) Experimental protocol. (B) On D30 post 1st transfer, mice were euthanized and the spleen, PDN and prostate harvested. The number of $CD8^+YFP^+$ (1st transfer) and $CD8^+Ly5.1^+$ (2nd transfer) cells were enumerated. Bar graphs show mean ± SEM. ***P<0.01, ***P<0.001* (unpaired Student's t test). (C) Intracellular cytokine assay showing effector cytokine production by 1st transfer and 2nd transfer cells in the PDN and prostate of the same $TRAMP_{OVA}$. Representative flow plots are shown. (D) H&E staining of prostate from $TRAMP_{OVA}$ mice receiving 1st and 2nd transfer cells show the presence of apoptotic epithelial cells (red arrows), 40x. (E) CXCR3 expression levels of 1st and 2nd transfer cells in the spleen, PDN and prostate. (B-E) N=3 mice for 1st + 2nd transfer into $TRAMP_{OVA}$, n=1 mice each for 2nd transfer only into $TRAMP_{OVA}$ and TRAMP.

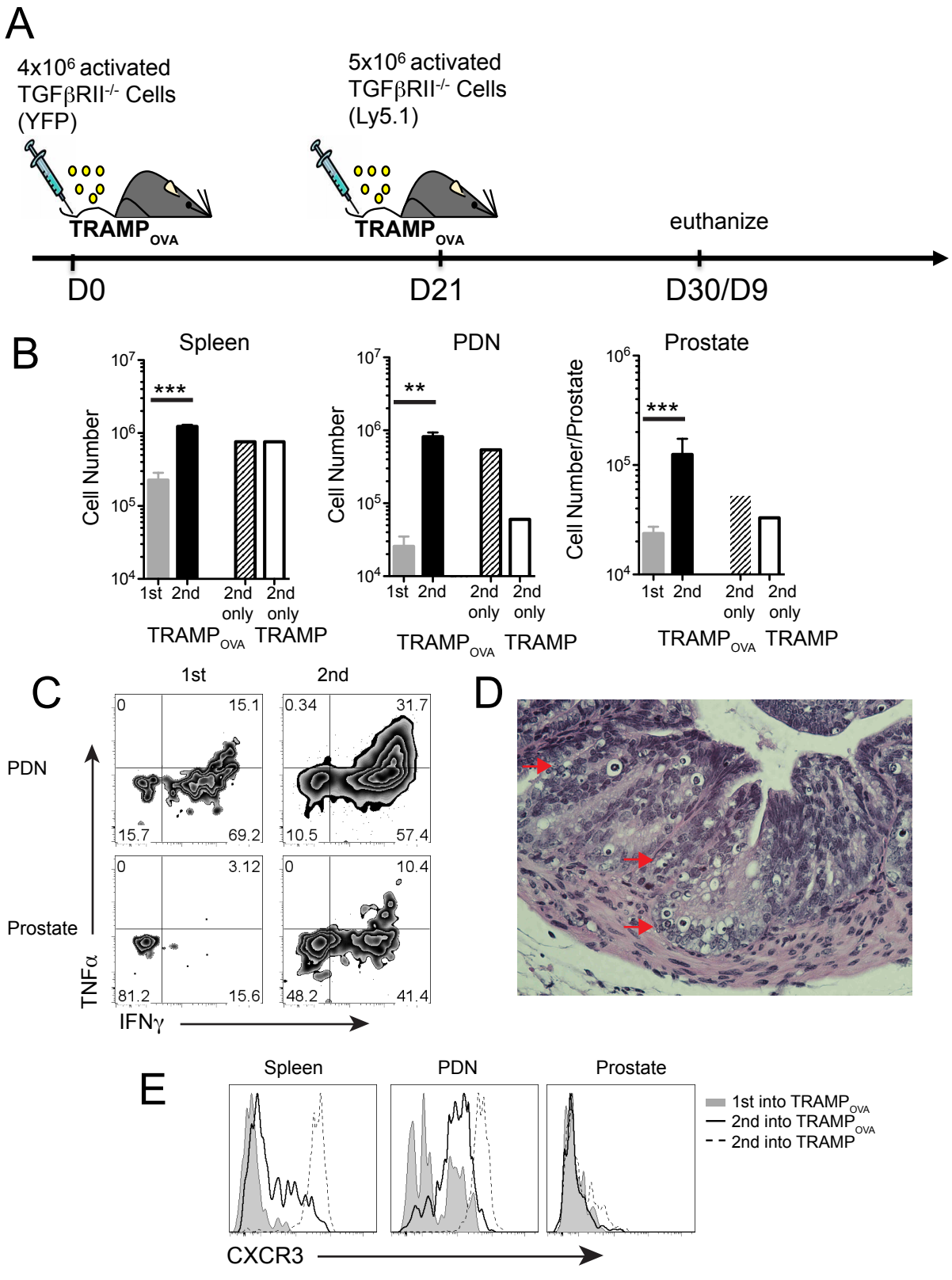

A
4x10^6 activated TGFβRII-/- Cells (YFP)
5x10^6 activated TGFβRII-/- Cells (Ly5.1)
TRAMP_OVA
TRAMP_OVA
euthanize
D0
D21
D30/D9
B
Spleen
PDN
Prostate
Cell Number
Cell Number/Prostate
***
**
***
1st 2nd 2nd only 2nd only
TRAMP_OVA TRAMP
C
1st
2nd
PDN
Prostate
TNFα
IFNγ
0 15.1 15.7 69.2
0.34 31.7 10.5 57.4
0 3.12 81.2 15.6
0 10.4 48.2 41.4
D
E
Spleen
PDN
Prostate
CXCR3
1st into TRAMP_OVA
2nd into TRAMP_OVA
2nd into TRAMP

Figure 4.8: Blockade of PD-1 signaling does not further increase accumulation or effector function of TGFβRII KO cells. (A) PD-1 expression on WT and TGFβRII KO cells at week 1 and week 3 post adoptive 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. (C) $5x10^6$ PD-1 KO (Thy1.1) and $2x10^6$ TGFβRII KO (Ly 5.1) were co-transferred into congenic (Thy1.2 Ly5.2) $TRAMP_{OVA}$ and TRAMP hosts. Mice were euthanized 4 weeks post transfer and the spleen and prostate were analyzed for the presence of transferred cells. Flow plots are gated on $CD8^+$ cells. (D-E) 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. (D) Numbers of persisting transferred cells at 3 weeks post adoptive transfer in $TRAMP_{OVA}$ mice treated with antibody or PBS. (E) Percentage of transferred TGFβRII KO cells co-producing TNFα and IFNγ following 5 hour *ex vivo* peptide stimulation. (D-E) 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). (F) Single cell suspensions of spleen, PDN and prostate from indicated groups of mice were stained with αrat-IgG, the isotype of the PD-1 blocking antibody. Histograms are gated on $CD8^+Ly5.1^+$ cells.

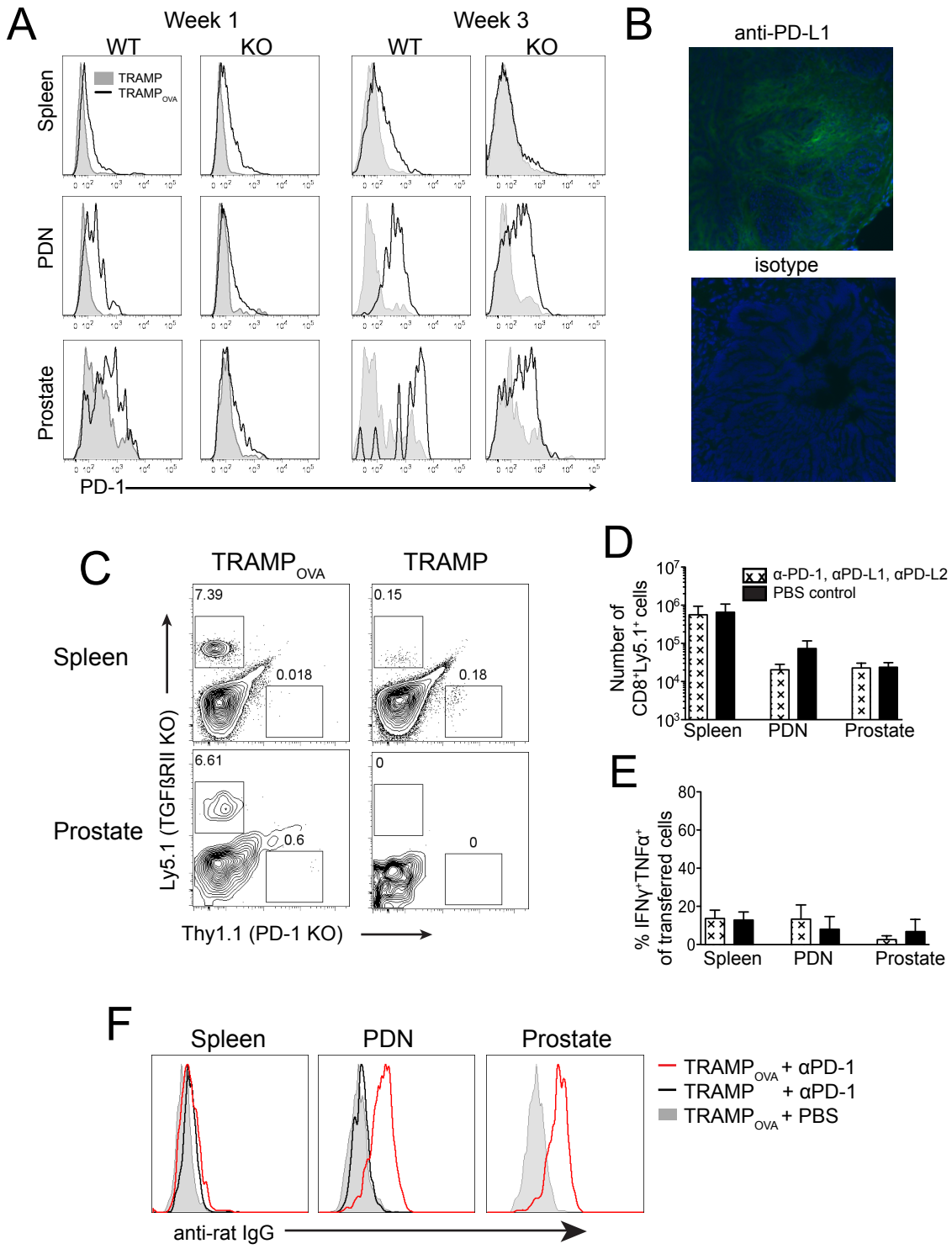
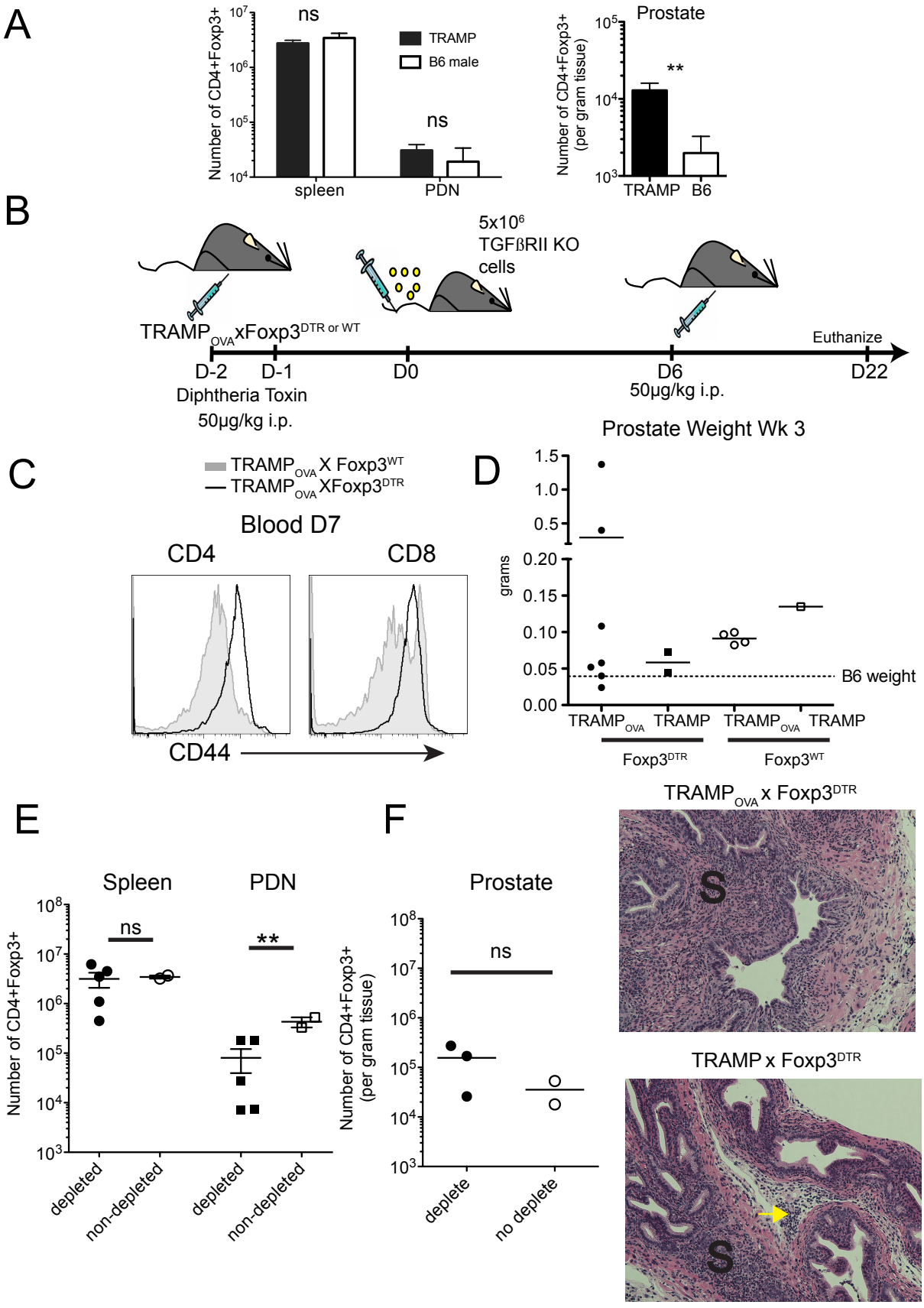

A
Week 1
Week 3
WT
KO
WT
KO
Spleen
PDN
Prostate
TRAMP
TRAMP$_{OVA}$
PD-1
B
anti-PD-L1
isotype
C
TRAMP$_{OVA}$
TRAMP
Spleen
Prostate
7.39
0.018
0.15
0.18
6.61
0.6
0
0
Ly5.1 (TGFßRII KO)
Thy1.1 (PD-1 KO)
D
α-PD-1, αPD-L1, αPD-L2
PBS control
Number of CD8$^+$Ly5.1$^+$ cells
10^7
10^6
10^5
10^4
10^3
Spleen
PDN
Prostate
E
% IFNγ$^+$TNFα$^+$ of transferred cells
80
60
40
20
0
Spleen
PDN
Prostate
F
Spleen
PDN
Prostate
TRAMP$_{OVA}$ + αPD-1
TRAMP + αPD-1
TRAMP$_{OVA}$ + PBS
anti-rat IgG

Figure 4.9: Depletion of Foxp3^{+} regulatory T cells results in tumor regression and systemic autoimmunity. (A) Numbers of CD4^{+}Foxp3^{+} cells are present in the spleen, PDN and prostate of 25 week old TRAMP (n=11) and B6 (n=5) males. Bar graph $\pm$ SEM. ns = not significant, ***P<0.01* (unpaired Student's t test). (B) Experimental schema for Foxp3 ablation studies. (C) CD44 expression by endogenous CD4 and CD8 T cells from the peripheral blood of TRAMP$_{OVA}$ x Foxp3^{DTR} depleted mice on D7. (C) TRAMP$_{OVA}$ x Foxp3^{DTR}, TRAMP x Foxp3^{DTR}, TRAMP$_{OVA}$ x Foxp3^{WT} (control non-depleted) and TRAMP$_{OVA}$ x Foxp3^{WT} (control non-depleted) were treated as indicated in (B) and prostates were microdissected and weighed 3 weeks post T cell transfer. Symbols represent individual mice. Dashed line is weight of age matched C57BL/6 prostate. Results are compiled from 2 independent experiments. (E) Number of CD4^{+}Foxp3^{+} cells week 3 post TGFβRII KO cells transfer in DT toxin treated TRAMP$_{OVA}$ x Foxp3^{DTR} (depleted) and TRAMP$_{OVA}$ (non-depleted) mice. Symbols show individual mice, bar is mean $\pm$ SEM. ns = not significant, ***P<0.01* (unpaired Student's t test). (F) H&E staining of prostate sections from TRAMP$_{OVA}$ x Foxp3^{DTR} and TRAMPx Foxp3^{DTR} mice 3 weeks post T cell transfer show single layer of epithelium surrounding glands with hyperplasia and hypercellularity in the fibromuscular stroma (S) and the presence of mononuclear cells (yellow arrow) in the interstitium, 20x.


A
Number of CD4+Foxp3+
ns
TRAMP
B6 male
ns
spleen
PDN
Prostate
Number of CD4+Foxp3+ (per gram tissue)
**
TRAMP
B6
B
5x10^6 TGFβRII KO cells
TRAMP_OVA xFoxp3^DTR or WT
Euthanize
D-2
D-1
D0
D6
D22
Diphtheria Toxin 50µg/kg i.p.
50µg/kg i.p.
C
TRAMP_OVA X Foxp3^WT
TRAMP_OVA XFoxp3^DTR
Blood D7
CD4
CD8
CD44
D
Prostate Weight Wk 3
grams
B6 weight
TRAMP_OVA
TRAMP
TRAMP_OVA
TRAMP
Foxp3^DTR
Foxp3^WT
E
Spleen
PDN
Number of CD4+Foxp3+
ns
**
depleted
non-depleted
depleted
non-depleted
F
Prostate
Number of CD4+Foxp3+ (per gram tissue)
ns
deplete
no deplete
TRAMP_OVA x Foxp3^DTR
S
TRAMP x Foxp3^DTR
S

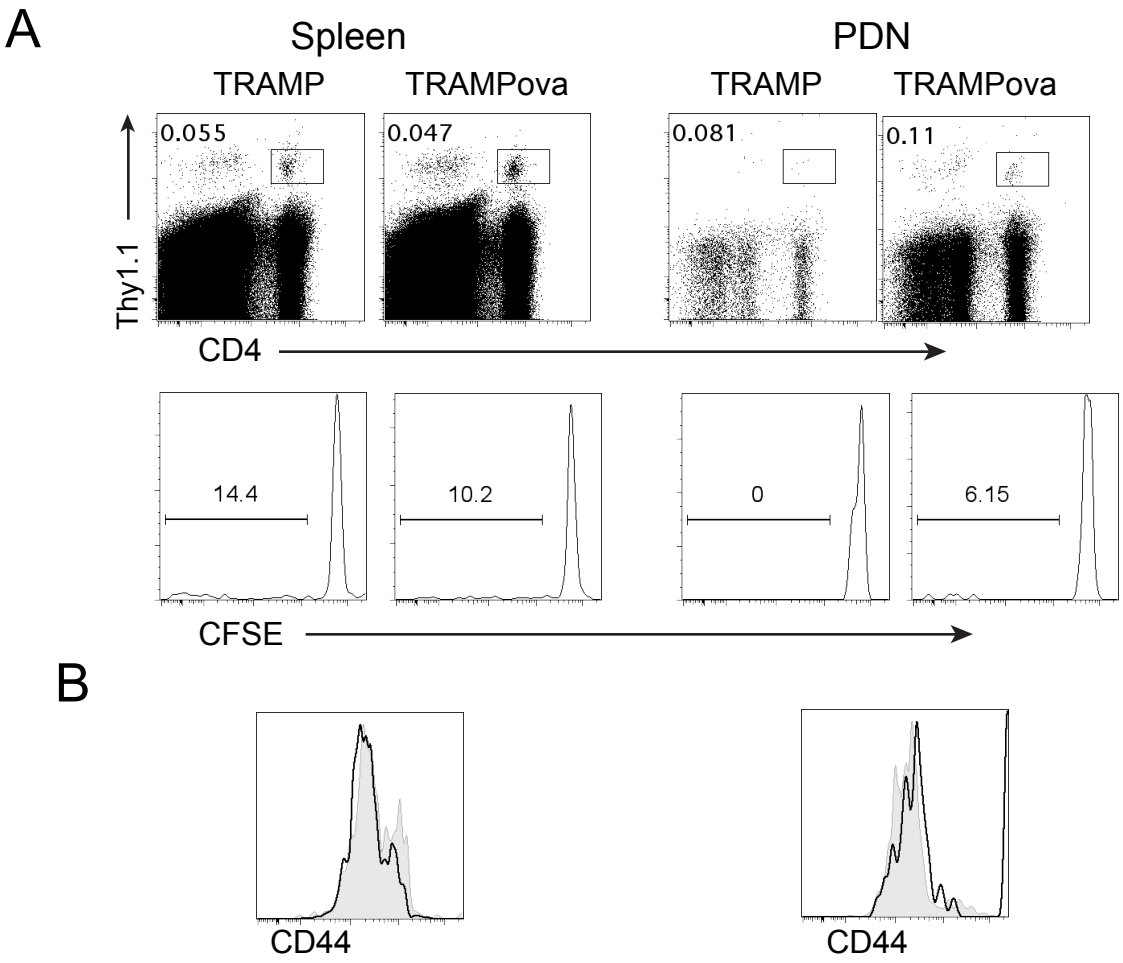


Figure 4.10: OTII T cells do not detect antigen in TRAMP$_{OVA}$ mice. CFSE labeled naïve OTII$^{Thy1.1}$ cells were adoptively transferred into 25 week old male TRAMP$_{OVA}$ and TRAMP mice. (A) Mice were euthanized 12 days post T cell transfer and stained for CD4 and Thy1.1 to detect donor cells in the spleen and PDN. Histograms show CFSE expression of CD4^{+}Thy1.1^{+} cells. (B) CD44 expression of transferred donor cells 12 days post transfer.

Chapter 5: Conclusions

The tumor microenvironment is comprised of an intricate network of blood vessels, tumor cells, stromal cells, various soluble factors and infiltrating immune cells. Studies in the past few decades have greatly increased our understanding of the role of the immune system in regulating cancer and have highlighted the potential and promise of harnessing the anti-tumor activity of the immune system for the development of immunotherapies in the treatment of cancer. The recognition that T cells can seek out and kill tumor cells formed the basic principle for T cell mediated ACT. ACT is actively being pursued in clinical trials for the treatment of malignancies, with successes reported in some cancers (10-12), but even in the context of a tumor with an identifiable tumor target antigen substantive obstacles to broad applicability and the achievement of predictable and reproducible benefits remain.

In our studies, we used various transgenic mouse models to characterize and evaluate T cell responses to a self-antigen expressed in normal and cancerous prostate tissue. We generated an autochthonous murine prostate cancer model, which expressed a targetable self/tumor antigen in order to identify and evaluate abrogation of potential obstacles to CD8 T cell mediated ACT of prostate cancer. We found that in our model, the majority of prostate-specific CD8 T cells are deleted in the thymus but functional prostate-specific cells can exist in the periphery. Through adoptive transfer experiments we showed that activation of prostate-specific T cells in an immunogenic context was required for infiltration into the prostate however, mechanisms within the prostate microenvironment prevented observable tissue damage. Using a transplantable tumor model, we found that abrogation of TGFβRII signaling increased the therapeutic efficacy of self/tumor specific CD8 T cells leading to complete tumor eradication. Extending our findings into an autochthonous prostate tumor model, we found that although TGFβRII deficient cells accumulated in greater numbers in the tumor and maintained effector function longer than WT cells, TGFβRII deficient cells were eventually rendered dysfunctional. Additionally, despite expression of PD-1 on transferred T cells and PD-L1 on tumor cells, PD-1 blockade did not prevent this loss of function. Therefore, we concluded that TGFβ negatively regulates the accumulation and effector function of transferred self/tumor specific CD8 T cells however, when targeting

a tumor antigen that is also expressed as a self-protein, substantive obstacles in addition to TGFβ and PD-1 are present within the tumor microenvironment, potentially hampering the success of ACT for solid tumors.

Clinical trial data from two large, multi-center anti-PD-1 antibody blockade (113) and anti-PD-L1 antibody blockade (114) trials were published in June 2012. The patients treated had a variety of cancers and objective responses were observed in melanoma, non-small-cell lung cancer and renal cancer patients, with an average of 1 out of 4 to 1 out of 5 total patients responding to treatment (17 prostate cancer patients were treated and no responses were observed). Data from these trials show the promise of PD-1 blockade however, it will now be pertinent to determine why the therapy was successful in some patients and failed in others.

One of the main challenges for ACT is choosing the right tumor antigen to target. Our studies showed that when targeting a tumor antigen that is also expressed as a self-antigen, profound tolerizing mechanisms exist. Identifying and targeting tumor-specific antigens that are 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 (18, 21, 115, 116). Furthermore, even “unique” tumor antigens can resemble “normal” self antigens and lead to dysfunction of cognate T cells (117, 118). CD8 TCR transgenic mice to the SV40 antigen expressed in TRAMP mice are also available and it would be useful to test whether SV40-specific T cells are subject to the same obstacles as self-antigen specific CD8 T cells. An alternative approach is the use of chimeric antigen receptor (CAR) engineered T cells (119). Recent clinical trials utilizing a CD19 CAR have demonstrated success in some patients with chronic lymphocytic leukemia (10, 13). A CAR is composed of an extracellular target binding domain, a transmembrane domain and an intracellular signaling domain. The extracellular domain is typically derived from antibodies and the intracellular domain usually consists of the zeta chain of the TCR complex (120). Advantages of utilizing CARs include, but are not limited to, HLA independence, no risk of receptor mispairing,

and the ability to modulate the intracellular signaling domain to incorporate co-stimulatory/activating molecules such as CD28 or CD137. One of the main limitations of CARs is that only proteins that are expressed on the tumor cell surface and that elicit an antibody response can be targeted.

In conclusion, our work highlights some of the obstacles to ACT for solid tumors, and emphasizes the need for testing potential ACT strategies in preclinical models that emulate tumor development and its environment to identify and address potential pitfalls. Combinatorial therapies are likely necessary for successful tumor eradication and the increased persistence and delayed loss of function in self/tumor specific TGFβRII deficient cells offers a window of opportunity for additional potentially synergistic therapies.

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Curriculum Vitae

Cassie K. Chou

Education

2005-present University of Washington, Seattle, WA
School of Medicine, Medical Scientist Training Program

2007-2012 University of Washington, Seattle, WA
Ph.D., School of Medicine, Department of Immunology

2000-2004 University of California, Berkeley, Berkeley, CA
B.A., Molecular and Cellular Biology, emphasis in Immunology
Honors in Molecular and Cellular Biology
High Distinction in General Scholarship

Research Experience

2007-2012 University of Washington, Seattle, WA
Dr. Philip Greenberg, Department of Immunology
Graduate Student

2004-2005 National Institutes of Health, Bethesda, MD
Dr. Joan Marini, National Institute of Child Health and Human Development
Post-baccalaureate Research Student

2001-2004 University of California, Berkeley, Berkeley, CA
Dr. Fenyong Liu, School of Public Health
Undergraduate Research Assistant

Publications

Chou, C.K., A. Schietinger, H. D. Liggitt, X. Tan, S. Funk, G. Freeman, T. Ratliff, N. Greenberg, P. D. Greenberg. 2012. 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. *J Immunol, in press*.

Dunn W., **Chou C.**, Li H., Hai R., Patterson D., Stolc V., Zhu H., Liu F. 2003. Functional profiling of a human cytomegalovirus genome. *Proc Natl Acad Sci U S A* 100(24):14223-8.

Awards

2010 Keystone Symposia Scholarship
2010 Graduate School Fund for Excellence and Innovation Student Travel Award
2004 Outstanding Undergraduate in Immunology

Research Support

2009-2012 Department of Defense – Prostate Cancer Training Award
2008-2009 Cancer Research Institute Pre-doctoral Fellowship
2007-2008 Medical Scientist Training Program Grant
2004-2005 NIH Post-baccalaureate Intramural Research Training Award

Oral Presentations

Abrogation of TGFβ signaling leads to increased persistence of prostate self/tumor antigen specific CD8 T cells but does not completely prevent tolerization. Keystone Symposia: Molecular and Cellular Biology of Immune Escape in Cancer, Keystone, CO, February 8, 2010.

Posters

Abrogation of TGF-Beta Signaling in Prostate-Specific CD8 T Cells for Adoptive Immunotherapy of Prostate Cancer. Innovative Minds in Prostate Cancer Today (IMPact) Conference, Orlando, FL, March 11, 2009.

Abrogation of TGFβ signaling leads to increased persistence of prostate self/tumor antigen specific CD8 T cells but does not completely prevent tolerization. Keystone Symposia: Molecular and Cellular Biology of Immune Escape in Cancer, Keystone, CO, February 8, 2010.

Characterization of T cell responses specific for a self-antigen expressed in the mouse prostate. American Association of Immunologists (AAI), Seattle, WA, May 2009.

Characterization of the Development and Function of CD8 and CD4 T Cell Responses Specific For a Self-Antigen Expressed in the Mouse Prostate
Keystone Symposia: Mobilizing Cellular Immunity for Cancer, Snowbird, Utah, Jan. 12, 2009.