Increased CD8+ T cell function following castration and immunization is countered by parallel expansion of regulatory T cells

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ABSTRACT

Although androgen ablation therapy is effective in treating primary prostate cancers, a significant number of patients develop incurable castration-resistant disease. Recent studies have suggested a potential synergy between vaccination and androgen ablation, yet the enhanced T cell function is transient. Using a defined tumor antigen model, UV-8101-RE, we found that concomitant castration significantly increased the frequency and function of antigen specific CD8+ T cells early after immunization of wild-type mice. However, at a late time point after immunization, effector function was reduced to the same level as non-castrated mice, and was accompanied by a concomitant amplification in CD4+CD25+FoxP3+ regulatory T cells (Tregs) following immunization.

We investigated whether Treg expansion occurred following castration of prostate-tumor bearing mice. In the prostate-specific Pten/- mouse model of prostate cancer, we observed an accelerated Treg expansion in mice bearing the castration-resistant endogenous prostate tumor, which prevented effector responses to UV-8101-RE. Treg depletion together with castration elicited a strong CD8+ T cell response to UV-8101-RE in Pten/- mice, and rescued effector function in castrated and immunized wild-type mice.

In addition Treg expansion in Pten/- mice was prevented by in vivo IL-2 blockade suggesting that increased IL-2 generated by castration and immunization promotes Treg expansion. Our findings therefore suggest that although effector responses are augmented by castration, the concomitant expansion of Tregs is one mechanism responsible for only transient immune potentiation after androgen ablation.
INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in males of the Western world (1). Effective treatment of prostate cancer currently involves radical prostatectomy, radiation therapy and/or androgen ablation (2). Removal of androgen leads to decreased proliferation and programmed cell death in prostate epithelial cells. Castration-resistant tumor and metastatic disease which may arise years later is largely incurable.

Immune therapy may be a promising treatment modality for castration-resistant prostate cancer (3). However, the role of androgen in modulating immune function, and the consequence of androgen removal/blockade on adaptive immune responses is not completely understood. Androgen is generally regarded as immunosuppressive and hormone removal increases T cell function in autoimmune disease models (4, 5). These observations suggest that androgen removal may augment T cell responses following immunization against prostate tumor antigens, including self proteins. However, CD8+ T cell responses to a prostate self antigen expressed in murine prostate cancer cells were only accentuated if immunization was prior to castration (6). In contrast, the function of transgenic CD8+ T cells specific for Prostate Specific Antigen (PSA) was enhanced until 4 weeks post-castration (7). Furthermore, in a CD4+ transgenic T cell model, a synergy between androgen deprivation and vaccination alleviated systemic tolerance to a model prostate tumor antigen, and increased T cell proliferation early after castration (8). Thus, the ability of androgen ablation to augment immune responses to prostate tumor antigens may depend on the antigen in question, and the strength of the T cell response.

To determine the mechanism responsible for transiently increased effector function, we first investigated the effect of androgen ablation on CTL responses to a well-defined immuno-dominant tumor antigen in non-tumor bearing C57BL/6 mice. The ultraviolet light-induced murine sarcoma, UV-8101-RE expresses a mutant tumor-specific protein which induces a strong CD8+ T cell response (9). We immunized mice intra-prostatically with this antigen, and found that concomitant castration enhanced the proportion of antigen-specific CD8+ T cells in the spleen, and increased their function early after immunization which declined by 5 weeks post-
immunization. However, we also observed an increased proportion of CD4+CD25+FoxP3+ regulatory T cells (Tregs) in the castrated and immunized animals.

We previously showed that functional CD8+ T cells responding to unknown prostate tumor antigens transiently increased following castration of prostate-specific Pten-/- mice (10). In this study, we demonstrate that castration of Pten-/- mice also expanded Tregs. An increased frequency of functional CD8+ T cells was only detected when Tregs were depleted prior to castration and immunization with the model antigen UV-8101-RE. We further show that in vivo blockade of IL-2 prevents Treg expansion. Thus, androgen ablation amplifies both stimulatory and inhibitory arms of the immune system.
MATERIALS AND METHODS

Animals and cell lines

C57BL/6 mice (8-12 wk old, Jackson Laboratory, Bar Harbor, ME)) were maintained under pathogen-free conditions in accordance with Institutional Animal Care and Use Committee guidelines. Prostate-specific Pten-/- mice (12-16 wk old) on the syngeneic C57BL/6 background were bred as described previously (10, 11). UV-8101-RE (from Dr. Hans Schreiber) and TRAMP-C1 (from Dr. Owen Witte) cell lines were cultured as described previously (10). The authenticity of UV-8101-RE was determined by specific killing of the cell line by α-8101-specific T cells which also recognize the mutant peptide epitope. Expression of SV40 viral DNA sequences and H-2K\(^b\) were determined in TRAMP C-1 cells.

Surgery

Mice were injected i.p. with ketamine and xylazine (23.75 mg/mL ketamine + 1.25 mg/mL xylazine) (150 mg/kg). A lower midline incision was made and testes were removed. For immunization, 1x10\(^6\) live UV-8101-RE cells in 20 μl volume, containing bromphenyl blue for visualization were injected into both anterior and dorsal lobes of the prostate. All surgical and post-surgical care was in accordance with WFSM ACUC guidelines.

ELISpot assays

Rat anti-mouse IFN\(\gamma\) antibody (2.5 μg/ml, clone R4–6A2, BD Pharmingen, San Diego, CA) was used to coat 96-well filtration plates (Millipore, Bedford, MA). Splenocytes (1x10\(^6\)) were stimulated ex-vivo with UV-8101-RE cells at 1:1 ratio with irradiated (1200 rad) syngenic splenocytes as feeder cells for 36 hours in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 50mM 2-mercaptoethanol. After culture, plates were washed and incubated with biotinylated anti-IFN\(\gamma\) antibody (2.5 μg/ml, clone XMG 1.2, BD Pharmingen). Spots were developed with DAB substrate (Sigma, St Louis, MO). The plates were scanned with a plate scanner (Cellular Technology Ltd., Cleveland, OH) equipped with Image Acquisition 4.5 software, counted using ImageJ software (www.rsbweb.nih.gov/ij) and calculated as number of spot forming cells per spleen.
**Mixed lymphocyte tumor cell cultures (MLTC) and $^{51}$Cr-release assays**

MLTC and $^{51}$Cr release assays were conducted as described previously (9). The percentage of specific lysis was calculated by the formula: % cytolysis = $\frac{(\text{experimental release-\text{spontaneous release}})}{(\text{maximum release-\text{spontaneous release}})} \times 100$.

**Treg depletion**

Tregs were depleted by a single i.p. injection of 0.5 mg anti-CD25 antibody (12) (clone PC61, BioXCell, West Lebanon, NH), two days prior to castration and/or immunization.

**IL-2 neutralization**

To neutralize IL-2 function mice were given 1mg of anti-IL-2 mAb (clone S4B6, BioXCell, West Lebanon, NH) or isotype control i.p. two days before castration or sham-surgery, as previously described (13).

**Peptide production**

The mutant p68 RNA helicase peptide (SNFVFAGI) was produced by the WFSM Protein Analysis Core Laboratory essentially as described previously (9). Briefly, the peptide was synthesized on an Applied Biosystems (Foster City, CA) Model 430B automated peptide synthesizer, using the standard Applied Biosystems FastMoc solid phase peptide synthesis chemistry protocol. The peptide identity and purity was demonstrated by reverse phase HPLC, quantitative amino acid analysis, and MALDI-TOF mass spectrometry.

**Flow cytometry analysis**

Splenocytes were dissociated and stained with fluorochrome-conjugated antibodies specific for CD4 (clone RM4-5, FITC conjugated), CD8 (clone 53-6.7, FITC conjugated), CD62L (clone MEL-14, APC conjugated) from BD Pharmingen and CD44 (clone IM7, PE conjugated) and CD25 (clone PC61.5, PE conjugated) from eBioscience (San Diego, CA), all used at 1:100 dilution. Tregs were analyzed using the mouse Treg staining kit (eBioscience), and manufacturer's instructions. Cells were analyzed on a FACSCalibur™ equipped with Cellquest PRO® software (BD Biosciences).

**Tetramer production**

MHC monomer was constructed and purified as described (14), except a plasmid clone expressing H-2K$^b$ was used. The folding, purification, and biotinylation of K$^b$ + peptide complexes were performed as described (15).
To prepare tetramer, 20 μl of monomer (2mg/ml) was incubated at room temperature with 1/10 volume of 128 μl streptavidin-PE (0.5 mg/ml) (Jackson ImmunoResearch, West Grove, PA) or 95 μl streptavidin-PerCP-Cy5.5 (0.2mg/ml) (BD Pharmingen) added in 10 minute intervals, and incubated in the dark.

**Tetramer enrichment**

Tetramer enrichment was performed as described previously (16). Dissociated splenocytes stained with PE and PerCP-Cy5.5 conjugated tetramers (4μg/ml each) were also co-stained with CD8-FITC and CD44-APC. PE-tetramer positive cells were enriched with anti-PE magnetic beads (BD Biosciences), and analyzed on a FACSCalibur®. Tetramer positive cells were defined as PE and PerCP-Cy5.5 double positive cells when gated on the CD8+CD44+ population. Nearly 100% of the tetramer positive cells were CD44hi.

**Intracellular cytokine staining (ICS)**

MLTC cell suspensions were re-stimulated with UV-8101-RE cells at the ratio of 1:1 for 4 hours in the presence of 10μg/ml brefeldin A (Sigma, St Louis, MO). TRAMP-C1 cells were used as negative control. Following surface CD8 staining, intracellular staining for IFN-γ (clone XMG 1.2, BD Pharmingen) was performed with the Cytofix/Cytoperm Kit (BD Biosciences).

**TGF-β ELISA**

Blood samples were collected from mice by cardiac puncture and the serum harvested by centrifugation. Total TGFβ levels were determined using a commercial ELISA kit (Promega, Madison, WI).

**Statistical analysis**

Statistical comparisons were performed using the unpaired Student’s t test with a two-sided α level of 0.05. Differences in values at p<0.05 were considered significant.
RESULTS

Precursor frequency and function of CD8+ T cells responding to a model tumor antigen increased following castration and immunization of non-tumor bearing mice

Previous studies reported that castration induces infiltration of T cells into the prostate in both normal (17) and prostate tumor bearing mice (10, 17). Using the prostate-specific Pten-/- mouse model of endogenous prostate cancer, we showed that the accumulation of functional T cells in the prostate glands is not long-lasting, evident at 2.5 weeks after castration but diminished at 5 weeks post-castration (10).

Antigens shed by dying prostate cancer cells after castration may stimulate CD8+ T cell responses. In an effort to track effector responses to an antigen located in the prostate, we utilized a defined tumor model system. The C57BL/6-derived murine sarcoma cell line UV-8101-RE expresses an H-2Kb-restricted immunodominant antigen which elicits a strong CTL response which rejects the tumor cells (9). To model T cell interactions to prostate antigens, normal male C57BL/6 mice were immunized with UV-8101-RE cells intra-prostatically in both anterior and dorso-lateral prostate lobes, and were either castrated or sham treated. Spleens and prostate draining lymph nodes (PDLNs) were harvested 2.5 weeks later.

In order to quantify the number of UV-8101-specific responding CD8+ T cells, we produced H-2Kb tetramers complexed with the mutant peptide. Primary ex vivo analysis of splenocytes detected an increased frequency of tetramer-positive CD8+ T cells in castrated and immunized mice, compared to sham castrated mice (Figure 1A). ELISpot analysis also detected increased IFN-γ secreting cells in the prostate draining lymph nodes (PDLNs) and spleens (Figure 1B), confirmed by in vitro analysis of cultured splenocytes (Figure 1C and 1D). Thus, castration increased the number and functional capacity of CD8+ T cells specific for UV-8101-RE.

Enhanced effector function declined 5 weeks post-castration and immunization of non-tumor bearing mice

To determine the longevity of effector responses after castration and immunization we evaluated mice 5 weeks following treatment. The frequency of tetramer positive cells was similar in castrated or sham castrated mice (Figure 2A). Both groups also had similar numbers of IFN-γ secreting cells in the spleens or PDLNs as
determined by ELISpot analysis (Figure 2B). MLTCs from mice that were castrated or sham castrated had low lytic activity (Figure 2C) and IFN-γ production (Figure 2D) after ex vivo re-stimulation at 5 weeks post-treatment. Thus, increased function after castration diminished rapidly.

**Castration increased splenic Tregs following immunization of non-tumor bearing mice**

We investigated the mechanism for transient effector function after castration. Systemic Tregs can suppress CD8+ T cell responses to immunization (18-20). We quantified the proportion and number of Tregs in wild-type animals following immunization, and found similar levels at 2.5 weeks following castration and immunization, compared to sham-castrated and immunized mice (Figure 3A). Interestingly, we detected a modest but significant increase in the percentage of Tregs 5 weeks post-castration and immunization (Figure 3B, representative flow dot plots in Supplementary Figure S1). The numbers of Tregs were also increased since the spleens were larger after castration (data not shown) as previously reported (5, 21). The frequency and number of Tregs in PDLNs was similar in castrated and intact immunized mice (data not shown). In addition, Tregs expanded only after immunization, since castration alone did not increase Treg proportion or number in the spleen at either time point (Figure 3C) or in the PDLNs (data not shown), which is consistent with previous reports (5, 22).

If immunization was performed 5 weeks post-castration, lytic capacity (supplementary Figure S2A) and effector cytokine production (supplementary Figure S2B) of MLTC cells were not enhanced. Thus, reduced CD8+ effector function at 5 weeks post-castration may be due to inhibition by increased Tregs.

**Castration expanded Tregs in prostate-specific Pten-/- mice**

We previously reported that functional Granzyme B+CD8+ T cells were increased in cancerous glands of prostate-specific Pten-/- mice 2.5 weeks after castration, and declined by 5 weeks post-castration (10). To determine whether increased Tregs accounted for this decline, we measured systemic Treg levels in Pten-/- mice. The number of Tregs was significantly increased in PDLNs (Figure 4A, upper panels), and the number and frequency of Tregs increased in the spleen of castrated Pten-/- mice 2.5 weeks post-castration compared to
intact or sham-treated mice (Figure 4A, lower panels). Treg numbers remained elevated in the spleens of Pten-/mice 5 weeks post-castration (Figure 4B, lower panels). These data show that Treg expansion is induced by castration in tumor bearing mice, and may down-regulate the function of CD8+ T cells activated after castration (10).

**CD8+ T cell effector responses against UV-8101-RE were generated only with concomitant castration and Treg depletion of Pten-/ mice.**

To determine whether the increased Tregs would prevent generation of effector function to a defined tumor antigen, we castrated and immunized Pten-/ mice intra-prostatically with UV-8101-RE cells. A similar number of tetramer-positive cells was detected in the spleens of castrated or sham-treated mice 2.5 weeks post-treatment (Figure 5A), suggesting that castration did not increase the number of responding cells. Tetramer analysis was not performed on LNs due to insufficient numbers of cells for analysis of individual mice. In addition, ELISpot analysis detected similar numbers of functional cells in the PDLNs and spleens of both castrated and sham treated mice (Figure 5B). Thus, castration alone did not enhance CD8+ T cell function after immunization, in the presence of the endogenous tumor.

Treg depletion together with castration and immunization did not increase the proportion and number of splenic tetramer-positive CD8+ T cells at 2.5 weeks post-treatment (Figure 5A). However, the number of IFN-γ secreting cells was significantly higher both in the PDLNs and spleen (Figure 5B). The numbers of tetramer-positive cells (Figure 5C) and numbers of functional antigen-specific cells (Figure 5D) remained elevated in the PDLNs and spleens 5 weeks after Treg depletion, showing that enhancement of effector function by Treg depletion is long-lasting.

Treg depletion also enhanced effector responses 5 weeks after castration and immunization of wild-type mice (Supplementary Figure S3).
**Treg expansion in Pten-/- tumor bearing mice is prevented by in vivo IL-2 blockade**

Two key cytokines, TGFβ and IL-2 have been shown to play an important role in generation and expansion of Tregs. To explore the potential mechanism by which Tregs expand after castration in prostate tumor bearing mice, we measured the serum level of total TGFβ in Pten-/- mice 2.5 weeks after castration or sham-castration. Castration did not increase the concentration of serum TGFβ concentration compared to sham-treated animals, although the level was higher in Pten-/- mice than WT non-tumor-bearing mice (supplementary Figure S4).

IL-2 is the signature cytokine which expands and maintains Tregs (23, 24). Increased IL-2 levels resulting from effector CD8+ T cells responding to immunizations have been shown to also amplify Tregs (25). Notably, we found an increased population of CD25hi Treg cells in the PDLNs and spleens of castrated mice, suggesting that cell surface expression of CD25 was increased or a population of CD25hi cells was preferentially expanded (Figure 6A). We were unable to detect IL-2 in the serum of castrated or sham-treated Pten-/- mice (detection limit 3.1 pg/ml, data not shown).

To determine whether Treg expansion was prevented by IL-2 blockade, we administered IL-2 neutralizing antibody or isotype control two days before castration of Pten-/- mice. We analyzed the percentage and number of Tregs in the PDLNs and spleen 2.5 weeks after castration. Indeed, IL-2 neutralization significantly reduced the percentage and number of Tregs in the spleen (Figure 6B). The Treg proportion in the PDLNs was also decreased, but the total number remained the same since the LNs were enlarged. Finally, IL-2 neutralization reduced CD25hiFoxp3+ cells in the spleen and PDLNs (Figure 6C, p=0.07 for numbers in PDLNs), suggesting that Tregs expanded in response to IL-2 generated after castration.
DISCUSSION

Some studies suggest that androgen is immune suppressive and can increase the proportion of systemic Tregs, and thus dampen or ameliorate autoimmune reactions (4, 5, 26). Other reports showed that effector cell function was increased in prostate tumors early after castration, and suggested that hormone blockade or surgical castration were immune stimulatory (17, 27). However, immune augmentation must be short lived, since castration-resistant disease recurs in many patients.

To separate the effect of androgen ablation from tumor-induced immune suppression, we evaluated CD8+ T cell responses to a defined antigen in tumor-free mice. The mutant epitope in UV-8101-RE induces strong CD8+ T cell responses, can be detected with specific probes, and is not expressed in the prostate. Thus, any responses to this epitope were the result of immunization alone, and not complicated by shedding of the antigen from dying prostate cells after castration. The increase in CD8+ T cell precursor frequency and function at 2.5 weeks after castration and immunization of wild-type mice was abrogated by 5 weeks post-castration, and paralleled by an increase in systemic Tregs. The correlation between Treg expansion and reduced CD8+ T cell function was supported by the observation that Treg depletion restored CD8+ T cell function. Administration of anti-CD25 antibody to deplete Tregs can also deplete CD8+ effector T cells (28, 29). Our results strengthen the notion that the timing of Treg depletion is critical to maintaining CD8+ T cell function.

The increase in systemic Tregs after castration was also observed in prostate-specific Pten-/- mice. Despite the presence of the endogenous castration-resistant prostate tumor, effector T cell responses to UV-8101-RE were also generated in Pten-/- mice when Tregs were depleted along with castration. The response was maintained at 5 weeks post-castration, showing that effector responses generated after castration in the absence of Tregs are long-lived.

Activation of CD8+ T cells in response to immunization, or castration of tumor bearing mice which is a form of immunization, induces production of interleukin-2 (IL-2) by activated cells, which in turn expands the pool of precursors by engaging IL-2R on responder cells (30). CD25, the high-affinity receptor for IL-2, is constitutively expressed by Tregs and is crucial for their development and suppressive function (23, 24). Expansion of Tregs by systemic IL-2 following immunization is observed in a variety of models (31-38),
demonstrating a direct link between T cell activation and amplification of inhibitory mechanisms (25). Our results support these observations, since Treg expansion was not observed in castrated and unimmunized wild-type mice.

In *Pten*-/⁻ mice, Treg expansion after castration was abrogated by neutralization of IL-2, showing that Treg expansion was driven by IL-2. Serum IL-2 was undetectable by ELISA in castrated or sham-castrated *Pten*-/⁻ mice and suggested that IL-2 generated by effector cells may be quickly consumed by Tregs. The critical role of TGFβ in generation and function of CD4+CD25+ Foxp3+ Tregs has been well established (39-41). Primary prostate cancers produce TGF-β (42-44) and the residual tumor remaining after castration can exert immune suppressive effects by secreting this cytokine. We detected TGF-β message in the tumors early and late after castration (data not shown), and similar levels of TGF-β was present in the serum of both intact and castrated *Pten*-/⁻ mice. The increase in systemic Tregs after castration of *Pten*-/⁻ mice was accelerated compared to wild-type mice, occurring 2.5 weeks after castration. Tumor-produced TGF-β may synergize with IL-2 generated after castration, resulting in early expansion of Tregs (45).

In summary, we have identified a dual effect of androgen ablation on immune function, which amplified both effector and inhibitory arms of the immune system. This dual function was also implied by a recent study of human prostate cancer samples (46). Therapeutic vaccination for treatment of castration-resistant prostate cancers has a small window of opportunity following androgen ablation which may be widened by the addition of Treg depletion to the regimen.

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REFERENCES


FIGURE LEGENDS

Figure 1: Enhanced antigen-specific effector responses 2.5 weeks after castration and immunization.

A, Splenocytes were stained with CD8, CD44, PE-Tetramer and Cy5.5-Tetramer. Cells enriched with anti-PE beads were analyzed. The representative dot plot (left panel) shows PE and Cy5.5 tetramer double positive cells gated on the CD8+CD44+ population. The number of tetramer positive cells was quantified (bar graphs; ■ Cx + Immun, □ Sx + Immun). The experiment was repeated once. B, IFNγ secreting cells in response to ex vivo UV-8101-RE stimulation were identified using ELISPOT assay with PDLNs (left panel) and spleen cells (right panel) from both groups. The average of triplicate wells in one representative experiment is shown. C, Lytic capacity of MLTC cells, 2.5 weeks after immunization, was determined in a 51Cr-release assay. Targets are UV-8101-RE (●) and TRAMP C-1 (▲). Line graphs show one representative animal from each group and bar graphs show the average of all animals in each group at an effector:target (E:T) ration of 50:1. D, Effector cytokine production was determined by ICS of MLTC cells. Flow diagram (left panel) shows representative samples of CD8+ cells which express IFNγ after ex vivo re-stimulation. The percentage of CD8+ T cells which express IFNγ was calculated (right panel). Data are mean values ± SD. * p<0.05, ** p<0.01 for Sx + immunization versus Cx + immunization. One representative experiment with 3-5 animals per group is shown. Each experiment was repeated at least once.

Figure 2: Enhancement of CD8+ effector function by castration was diminished 5 weeks after immunization.

A, The representative dot plot (left panel) shows PE and Cy5.5 tetramer double positive cells gated on the CD8+CD44+ population as described in Figure 1. The number of tetramer positive cells was quantified and represents the average of 5 animals in one experiment (bar graphs; ■ Cx + Immun, □ Sx + Immun). B, IFNγ secreting cells in response to ex vivo UV-8101-RE stimulation were identified using an ELISPOT assay with PDLNs (left panel) and spleen cells (right panel) from both groups. The average of triplicate wells in one representative experiment is shown. C, Lytic capacity of MLTC cells cultured for 6 days was determined by
$^{51}$Cr-release assay (♦ UV-8101-RE, ▲, TRAMP C-1). Line graphs show one representative animal from each group and the bar graph shows the average of all animals in each group at an E:T ratio of 50:1. D, IFN$\gamma$ expression by MLTC cells was assessed by an ICS assay (left panel). Percentages of IFN$\gamma$ expressing cells gated on CD8$^+$ T cells were calculated (right panel). Data are presented as mean values ± SD and one representative experiment of three independent experiments with 3-5 mice per group is shown.

Figure 3: Castration increased splenic CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells by 5 weeks post-immunization of wild-type mice.

A, Splenocytes from mice isolated at 2.5 weeks or B, 5 weeks post-immunization and castration or sham treatment were analyzed by flow cytometry. The number of CD4$^+$CD25$^+$FoxP3$^+$ cells was calculated by multiplying the percentage of CD4$^+$CD25$^+$FoxP3$^+$ positive cells times the total number of isolated splenocytes, and represents the average of 3 animals in the group. *: p <0.01 versus immunization only group. Data are from one representative experiment of three independent repeats. Mice were from the experiments shown in Figures 1 and 2. C, Mice were castrated and analyzed 2.5 weeks or 5 weeks later, as described above. Pooled data of two independent experiments are shown with 4-5 mice per group in each experiment.

Figure 4: Castration amplified CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in the spleen and prostate draining LNs early after castration in prostate tumor bearing Pten$^{-/-}$ mice.

A, PDLNs and spleens were harvested from Pten$^{-/-}$ mice that were either untreated or 2.5 weeks post-castration or sham-surgery. Cells were analyzed and the percentage and number of CD4$^+$CD25$^+$FoxP3$^+$ Tregs was quantified as described in Figure 3. B, PDLN cells and spleens from Pten$^{-/-}$ mice isolated at 5 weeks post-castration or sham-surgery were analyzed for the percentage and number of CD4$^+$CD25$^+$FoxP3$^+$ Tregs. *: p <0.05, **: p<0.01 for Cx group versus Sx group or Pre-Cx group. Pooled data of two independent experiments are shown with 3-5 mice per group in each experiment.
Figure 5: Effector responses to a tumor antigen were detected only after castration with concomitant Treg depletion in prostate tumor bearing mice.

A, Pten-/- mice were analyzed 2.5 weeks after immunization and castration (Cx), or sham treatment (Sx). Splenic tetramer-positive cells were enriched by magnetic bead selection followed by flow cytometry analysis. The number of tetramer-positive cells per spleen was calculated (bar graph, □ Sx + Immun, ■ Cx + Immun, ■ Cx + PC61 + Immun). *: p<0.05 for Cx + PC61 + Immun vs. Sx + Immun. B, IFNγ secreting splenocytes or PDLN cells in response to ex vivo UV-8101-RE stimulation were quantified using an ELISPOT assay for all three groups. C, The number of tetramer-positive cells per spleen 5 weeks after immunization and castration or sham surgery of Pten-/- mice is shown. D, IFNγ secreting cells in spleen or PDLNs following ex vivo UV-8101-RE stimulation were quantified using ELISPOT assays for all three groups. *: p<0.05, **: p<0.001 for Cx + PC61 + Immun group vs. Sx + Immun group or Cx + Immun group. Each group contained 3-5 animals, and the experiment was repeated once.

Figure 6: Treg expansion after castration in Pten-/- mice was mediated by IL-2.

A, Dot plots show CD25+Foxp3+ cells gated on CD4+ cells from PDLNs or spleen of one representative mouse in Sx or Cx groups for 2.5 wk post-castration. The rectangles designate the CD25hi population. The percentage and number of CD4+CD25hiFoxp3+ cells was calculated (bar graphs; ■ Cx 2.5 wk, □ Sx 2.5 wk). *: p<0.05, **: p<0.01 for Cx 2.5 wk compared to Sx 2.5 wk group. B, Pten-/- mice were injected with anti-IL-2 or isotype control monoclonal antibody two days before castration. The proportion and number of CD4+CD25+Foxp3+ was quantified PDLNs and spleens 2.5 weeks after treatment (bar graphs; ■ Cx + anti-IL-2, □ Cx + isotype). **: p<0.01 for Cx + anti-IL-2 compared to Cx + isotype group. Each group contained 3 mice, and the experiment was repeated once. C, Representative dot plots of CD25+Foxp3+ staining gated on CD4+ cells from PDLNs or spleens are shown for one mouse in the Cx + anti-IL-2 or Cx + isotype group. The percentage and number of CD4+CD25hiFoxp3+ cells was quantified (bar graphs; ■ Cx + anti-IL-2, □ Cx + isotype). Mice were from the same experiment in B. *: p<0.05, **: p<0.001 for Cx + anti-IL-2 compared to Cx + isotype group.
A 2.5 wk

Tetramer-Cy5.5

Sx+Immun  Cx+Immun

Tetramer-PE

# Tetramer+ cells / spleen

Sx+ Immun  Cx+ Immun

B

PDLNs

IFNγ secreting cells / PDLNs

Spleen

IFNγ secreting cells / spleen

Sx+ Immun  Cx+ Immun

C

Sx+Immun

Cx+Immun

% Cytolysis

E : T Ratio

UV-8101-RE  TRAMP-C1

% Cytolysis

Sx+ Immun  Cx+ Immun

D

Sx+Immun  Cx+Immun

IFNγ  CD8

% CD8+IFNγ+ / CD8+

Sx+ Immun  Cx+ Immun

Tang et al. Figure 1
Figure 2

(A) 

5 wk

Sx+Immun  Cx+Immun

Tetramer-Cy5.5

# Tetramer+ cells / spleen

(B) 

PDLNs

Spleen

IFNγ secreting cells / PDLNs

IFNγ secreting cells / spleen

(Sx+Immun) (Cx+Immun)

(C) 

Sx+Immun

Cx+Immun

% Cytolysis

E : T Ratio

100:1  25:1  7:1  2:1

(D) 

Sx+Immun  Cx+Immun

IFNγ

CD8

% CD8+IFNγ+/CD8+

(Sx+Immun) (Cx+Immun)
Tang et al. Figure 3
Tang et al. Figure 4