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14. ABSTRACT

The goal of this project is to characterize T cell tolerance to prostate tumor antigens and to identify the role of costimulatory receptors in overcoming this tolerance. Identification of these processes will assist in the development of novel therapeutic approaches for treating prostate cancer. We use the TRAMP model, a transgenic mouse line that develops primary prostatic tumors due to expression of the SV40 T antigen (TAg) under the transcriptional control of a prostate-specific promoter. In this final summary, we report that subsequent to adoptive transfer of naïve TAg-specific T cells into TRAMP mice, there is rapid expansion and contraction of the tumor-specific T cells, followed by accumulation of a population of T cells that persist in the prostate as tolerant and suppressive. Co-transfer of TAg-specific CD4+ T cells partially rescues the tolerant, suppressive phenotype of prostate-tumor-specific T cells, although over time, tolerance of the CD8+ T cells ensues. In contrast, transfer of CD4+ T cells does not reverse tolerance of the previously-tolerized CD8+ cells. The suppressive nature of these CD8+ T cells was also studied and we present preliminary data on the characterization of these novel suppressor cells. These data demonstrate the critical balance between T cell activation and tolerance and support a mechanism by which tumor growth may induce tolerance and suppressor activity in T cells previously primed to tumor-specific antigens. A greater understanding of how tolerance of these tumor specific T cells can be reversed willcertainly lead to more potent anti-tumor immunotherapies.

15. SUBJECT TERMS
Immunology, T Cell, Tolerance, Immunotherapy, Mouse Model

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Introduction

It is well-appreciated that growing tumors suppress the anti-tumor response by at least 2 mechanisms-generalized immunosuppression and antigen-induced tolerance. The goal of this research project is to test the hypothesis that modulating costimulatory receptors expressed by T cells can reverse tolerance to prostate tumor antigens and elicit a more potent anti-tumor immune response. We use a transgenic mouse model of human prostate cancer, the **TR**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate (TRAMP) model, to study T cell responses to prostatic tumors. In TRAMP mice, primary tumors develop as a consequence of prostate-specific expression of a transforming antigen, the SV40 T antigen (TAg) (1). In this model system, TAg serves as a surrogate tumor antigen. In combination with TRAMP mice, we use two other mouse lines which each bear T cell receptor transgenes that encode either MHC class I-restricted (TcR-I) (2) or class II-restricted (TcR-II) antigen receptors (3). Our goal is to use these murine lines to understand how T cells develop tolerance to tumor antigens and to test whether modulation of costimulatory receptors is sufficient to overcome tolerance to tumors by understanding these basic immunologic processes.

Body

Task 1: *To determine the Developmental Stage at Which TRAMP Mice Become Tolerant to TAg*:

We initially reported (2002) that SV40 TAg expression has a diverse expression pattern in TRAMP mice. mRNA for TAg was detected in the thymus as early as 7.5 weeks of age. This is consistent with another report suggesting central tolerance to TAg in TRAMP mice (4). In addition, we observed TAg message in the prostate as early as 1 week of age, which was the earliest time point prostate dissection was possible. Taken together, these findings suggested that TRAMP mice may have both central and peripheral mechanisms to generate tolerance to a tumor antigen. Thus, we next sought to test T cell tolerance using a tumor cell-based vaccination approach.

As previously reported (2003), we attempted to vaccinate TRAMP mice with a syngeneic tumor cell-based vaccine that expressed the full-length SV40 TAg. Despite confirmation of TAg expression by the cell lines, these cells were unable to elicit a TAg-specific T cell response in wild-type mice, nor were they able to stimulate TcR-I cell *in vitro*. As a result, we were unable to use this cell line as a vaccine to test TRAMP tolerance to TAg. Similarly, the TcR-I peptide emulsified in adjuvant was only a weak stimulator of T cell responses in wild-type mice. Therefore, we focused our efforts on the adoptive transfer studies which are presented in Aim 3.

Task 2: *To test the hypothesis that blockade of CTLA-4/B7 interactions, alone, or in combination with modulation of the costimulatory receptors CD40 and 4-1BB, can reverse tolerance to TAg in TRAMP mice*

As described in the 2003 Annual Report, we did not pursue this Specific Aim due to the difficulties in generating a TAg-specific vaccine, as described in Task 1, above. Our effort focused on the adoptive transfer model system, which has generated significant and highly relevant data.

Task 3: *To use an adoptive transfer system where transgenic T cells that recognize MHC class I- and class II-restricted TAg epitopes can be monitored to test the hypothesis that a developing prostatic tumor can tolerize naïve TAA-specific T cells.*

Many studies suggest that as a tumor develops, T cell tolerance to TAA's ensues. Most of these studies have employed transplantable tumor lines that express xenogeneic antigens that are thus highly stimulatory to the immune system. The TRAMP model presents a novel model where primary tumors develop under the developmentally regulated expression of a tissue-restricted promoter. We proposed to study T cell tolerance using the TcR-I and TcR-II transgenic lines which bear transgenes encoding TcR genes that recognize MHC class I- and class II-restricted epitopes of TAg, respectively.

The TcR-I mouse strain was bred to transgene homozygosity on the C3H background. Lymph node cells (LNCs) from these mice were used as donor cells for transfer in TRAMP x C3H (TRAMP/F_1) mice. Similar transfers were performed using wild-type C57BL/6 x C3H (WT/F_1) as recipients or using WT C3H cells as donor cells. Donor LNC were labeled with CFSE, a fluorescent dye that distributes evenly among daughter cells as the cells divide and therefore a linear reduction of fluorescence is observed at each mitotic division.

In the previous reports (2004, 2005), we presented data that characterize TcR-I responses in TRAMP mice. TcR-I cells undergo an initial expansion followed by an apoptotic contraction that results in deletion from the peripheral lymphoid tissues and trafficking of a fraction of cells to the prostate. During activation in the lymph node, the T cells transiently express activation markers (CD26, CD69) and have persistent expression of CD44, and indicator of having encountered antigen. TheTcR-I cells are TUNEL (+), inidicating they are undergoing apoptosis. The residual, tumor-infiltrating cells persist as tolerant in the TRAMP prostate. These cells do not secrete GranzymeB or interferon-γ, and are refractory to proliferation signals.

We further demonstrated that a dendritic cell (DC) vaccine can prime TcR-I cells in TRAMP mice, prevent tolerization for up to 3 weeks, and reduce tumor burden, as indicated by prostate size. However, over time, persistence of TAg expression tolerizes TcR-I cells and tumor growth is restored. These data demonstrate that, despite initial successful priming, TcR-I cells become tolerized due to progressive tumor growth. These findings support our hypothesis and have critical implications when considering tumor vaccines. These findings were submitted to the *Journal of Immunology* and revisions are currently under secondary review. The manuscript draft is attached.

CD4$^+$ T Cell Responses to TRAMP Tumors

Since the last report, we have extended our studies by examining TcR-II responses to TRAMP antigens. TRAMP mice were transferred with 3 x 10^6 TcR-II cells that were previously labeled with CFSE. We monitored T cell trafficking and activation for 3 weeks post-transfer. Similar to TcR-I cells, TcR-II cells undergo and proliferative yet abortive response in the peripheral lymph nodes. Within 24 hours, TcR-II cells upregulate activation markers like CD25 and CD69 and commence proliferation, marked by dilution of CFSE. This continues for approximately 3 days, after which time activation markers are down-regulated but proliferation continues. TcR-II cells undergo 3-5 rounds of proliferation after which time they undergo apoptosis and disappear from the peripheral lymph nodes. By 10 days after transfer, most cells have undergone at least one mitotic division (figure 1). This is in contrast to TcR-I cells, which are almost absent from the peripheral lymphoid tissues by 6 days after transfer. By 3 weeks after transfer, most cells have been deleted from the lymph nodes and only a small residual population is left. In contrast, most cells have trafficked to the prostate, where they persist for extended periods (detected up to 35 days post-transfer) and are tolerant of TAg. This latter observation is similar to the trafficking of TcR-I cells.
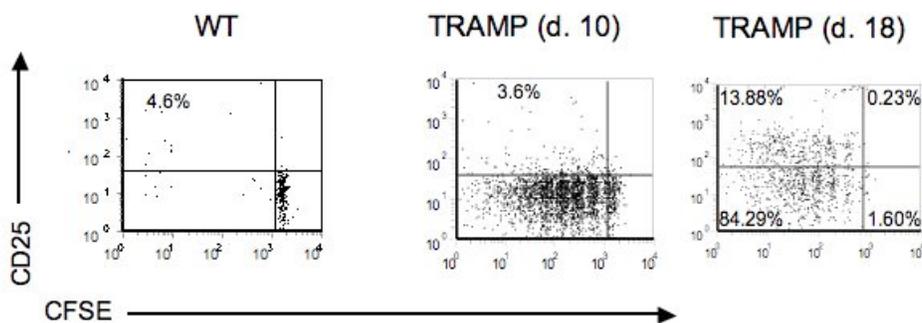
Figure 1: Wild-type (WT) or TRAMP mice were transferred with CFSE-labeled TcR-II cells. Mice were euthanized at various time points after transfer and lymph node cells were analyzed by flow cytometry. Cells were gated on Thy1.1 and analyzed for CFSE and activation markers.

Co-transfer of TcR-II Cells Delays Induction of TcR-I Cell Tolerance

In our previous report, we demonstrated that a DC vaccine delayed the onset of TcR-I tolerance. Given the transient activation of TcR-II cells, we tested whether co-transfer of TcR-I cells with TcR-II cells would prevent TcR-I cell tolerance. Mice were pre-transferred with TcR-II cells and 18-24 hours later, transferred with CFSE-labeled, Thy1.1$^+$ TcR-I cells. At various time points after transfer, TcR-I cells were purified from prostatic tissue using anti-Thy1.1-conjugated magnetic beads and subsequently used in functional assays to test antigen responsiveness. As demonstrated in Figure 2, at 10 days after transfer, TcR-II cells prevented tolerance induction in TcR-I cells. However, by 20 days after transfer, TcR-I cells from co-transferred TRAMP mice were as non-responsive as TcR-I cells from mice transferred with TcR-I cells alone. Similarly, TcR-II cells initially increased the frequency of TcR-I cells, but by 2 weeks after transfer, the effect was lost (Table 1).
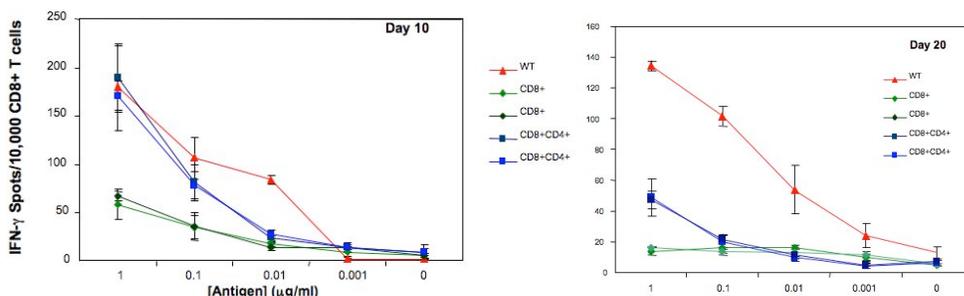
Figure 2: TRAMP mice were transferred with TcR-I cells (CD8+) or both TcR-I and TcR-II cells (CD8+CD4+) and euthanized at either 10 or 20 days post-transfer. TcR-I cells were enriched from the prostate using Thy1.1 magnetic beads and tested for IFN-γ production by ELISPoT.

|	day 5	day 10	day 20
TcR-I	213,000	95,000	62,000
TcR-1 + TcR-II	466,000	382,000	59,000

Table 1: Frequency of prostate-infiltrating TcR-I cells after transfer into TRAMP mice. Numbers are reported as the number of TcR-I cells per mouse after pooling up to 5 mice per group.

These findings are similar to our previously findings using the DC vaccine and suggest that despite an early rescue of tumor-specific CD8$^+$ T cell responsiveness in TRAMP mice, TcR-II cells cannot rescue these cells long-term. This may be due to progressive growth of the tumor or tolerization of the TcR-II cells, themselves.

TcR-II Cells Cannot Reverse TcR-I T Cell Tolerance

We next tested whether TcR-II cells could rescue TcR-I cells from tolerance. To do this, TRAMP mice were pre-transferred with TcR-I cells. 10 days later, TRAMP mice were transferred with TcR-II

cells and 10 days later, tested for TcR-I cell responsiveness. As demonstrated in Figure 3, although TcR-II cells could prevent tolerance induction when co-transferred with TcR-I cells, TcR-II cells could not reverse tolerance of pre-transferred TcR-I cells. These findings suggest that despite trafficking to the tumor, activated, tumor-specific CD4$^+$ T cells cannot reverse the tolerance of CD8 cells induced by the tumor. This finding is critical in understanding how to activate tolerant, tumor-specific T cells to generate a potent anti-tumor response. On-going studies are addressing the mechanisms by which the TRAMP tumor induces tolerance in TcR-I cells so as to identify ways to reverse TcR-I tolerance.
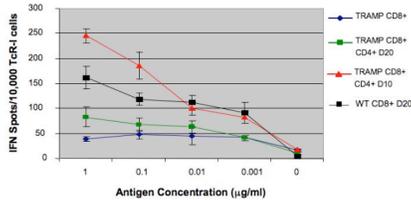

Figure 3: TRAMP or wild-type (WT) mice were transferred with TcR-I cells on day 0. On day 10, some TRAMP mice were transferred with TRAMP mice were transferred with TcR-II cells (TRAMP CD8+ CD4+ D20). As a positive control, a separate group of TRAMP mice were co-transferred with both TcR-I and TcR-II cells on day 10. All mice were euthanized on day 20 and TcR-I cells isolated by magnetic beads and tested for antigen

TRAMP Tumors Induce Suppressor Activity in CD8$^+$ T Cells

In the previous report, we presented preliminary data demonstrating a potential role for tolerant TcR-I cells as regulatory or suppressor T cells. We have continued these studies to attempt to identify the mechanism by which tolerized TcR-I cells exert their suppressive effects. Unfortunately, due to changes in personnel in the laboratory, these finding are still preliminary and therefore are only summarized below.

After purification from TRAMP prostates, (tolerant) TcR-I cells were co-cultured with naïve TcR-I cells and the proliferation of the naïve cells was measure by ^{3}H-thymidine incorporation. We have consistently observed a (tolerant) TcR-I cell number-dependent decrease in proliferation of naïve T cells (Figure 4). This suggests that these tolerant TcR-I cells are capable of suppressing T cell responses, similar to CD4$^+$CD25$^+$ regulatory T cells (Tregs).
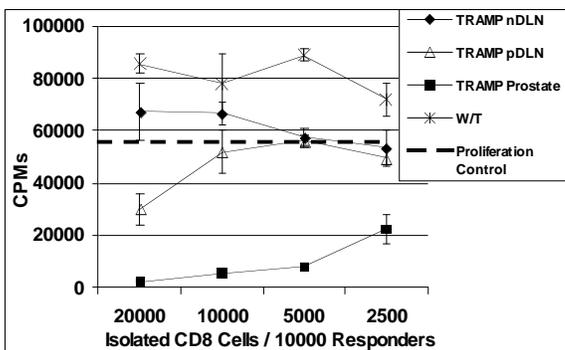
Figure 4: TcR-I cells isolated from TRAMP non-draining lymph nodes (nDLN), prostate-draining LN (pDLN), and prostate or TcR-I cells isolated from LN from wild-type (W/T) mice were cocultured with 10,000 naïve TcR-I cells in the presence of antigen. Proliferation was assayed after 72 hours by pulsing wells with ^{3}H-thymidine for 12 hours and assessing ^{3}H-thymidine incorporation.

Like Tregs, tolerant TcR-I cells express markers of activation (CD25, CTLA-4, CD69, CD122, and CD103, an integrin-binding protein associated with Tregs) as well as FoxP3, a transcription factor associated with Treg activity. Interestingly, only a small fraction (10-15%) of the CD8$^+$ cells express FoxP3. Our preliminary studies suggest the following:

-Co-culture with naïve T cells of a different antigenic specificity can result in suppression, even in the absence of TcR-I cell stimulation.
-Culture of naïve T cells in the presence of supernatant from stimulated tolerant TcR-I cells can suppress proliferation, suggesting that the suppressive activity may be due, at least in-part, to a soluble factor produced by TcR-I cells

-Culture of naïve, TcR-II CD4$^+$ T cells with tolerant TcR-I cells does not suppress the proliferation of the CD4$^+$ cells, suggesting a specificity of suppression for CD8$^+$ cells.

Currently, we are working to confirm and extend these preliminary findings. Clearly, the identification of this novel mechanism by which tolerant CD8$^+$ T cells can adversely affect T cell responses, including anti-tumor T cell responses, will have critical importance for on-going and future clinical studies as they imply that persistence of tolerant T cells will suppress any future attempts at eliciting anti-tumor immune responses.

Task 4: *To determine whether tolerance of TAA-specific T cells is associated with defects in T cell signaling pathways*

As described in the 2002 Annual Report, this Specific Aim was eliminated at the suggestion of the reviewers. It was believed that the proposal was over-ambitious and this Aim would be beyond the scope of the funding period.

<u>Key</u> <u>Research</u> <u>Accomplishments</u>

Establishment of adoptive transfer model

Characterization of TcR-I cells transferred into TRAMP mice

Identification of DC vaccine as effective in preventing deletion of TcR-I cells and promoting survival and expansion of TcR-I cells in prostate

Identification of TcR-II cells as effective in preventing initial deletion and tolerization of TcR-I cells in co-transfer studies

Identification and partial characterization of suppressor activity of tolerant TcR-I cells

<u>Reportable</u> <u>Outcomes:</u>

One manuscript was accepted for publication in *The Journal of Immunology* and is attached.

<u>Conclusions:</u>

Our long-term goal is to understand the role of costimulatory receptors in regulating T cell tolerance to tumor antigens. Our early data suggest that TRAMP mice may exhibit both central and peripheral tolerance to TAg, a surrogate tumor antigen. We have focused our research on using the TRAMP mouse as a recipient for TAg-specific T cells.

Our findings suggest that prostate-specific T cells undergo an initial proliferative response after antigen encounter. This is followed by deletion from the peripheral lymphoid organs and the prostate, the site of antigen expression. However, sensitization with an antigen-pulsed DC vaccine, or provision of a naïve CD4$^+$, tumor-specific T cell, prevents deletion of prostate–specific T cells and temporarily prevents tolerance induction. The T cells that persist on the prostate exhibit a potent suppressive activity that may be a critical factor to overcome when attempting to elicit anti-tumor immune responses.

Our on-going studies are characterizing both the deletional tolerance process as well as the mechanism by which the DC vaccine rescues T cells. In addition, we are studying the mechanisms

by which these tolerant, CD8^{+} cells exert their suppressor function. Finally, we are continuing to pursue studies that characterize T cell tolerance in TRAMP mice by developing novel vaccination approaches to elicit anti-TAg T cell responses. **We have requested an extension to expend the final funds of this proposal to complete Aim 3. This request is pending approval of the final report.**

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Arthur A. Hurwitz, PI (only personnel supported: 2002-2003)
Michael J. Anderson, Graduate Student
Jami Willette-Brown, Technician
Kim Shafer-Weaver, Graduate Student

Bibliography:

The work supported by this grant was presented at the following Meetings:

2004: American Association of Immunologists Annual Meeting, Washington, DC
2004: Keystone Conference on Tumor Immunology, Keystone, CO
2006: Keystone Conference on Tumor Immunology, Breckenridge, CO (abstract follows)

Tumor-Specific CD8^{+} T Cells Acquire Suppressor Function Upon Transfer into a Prostate Tumor-Bearing Host

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We have utilized a primary prostate tumor model to study the fate and function of naive, tumor-specific CD8^{+} T cells upon encounter with tumor Ag. **TR**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate (TRAMP) mice carry the SV40 T antigen (TAg) transgene under the control of a prostate-specific promoter; thus, as prostate tumors develop, TAg serves as both a surrogate tumor- and self-Ag. Using a model where we adoptively transfer TAg-specific, CD8^{+} T cells into TRAMP mice, we have demonstrated that T cells initially undergo an abortive proliferation that results in deletion from the lymphoid organs and a paradoxical accumulation in the prostate. Surviving CD8^{+} tumor-specific T cells are tolerant of TAg. Interestingly, administration of a dendritic cell (DC) vaccine at the time of transfer can prevent tolerance induction and slow tumor growth in the short-term, but over time (2-3 weeks), T cells become gradually tolerized and tumor growth is restored. In the present study, we have observed that not only are adoptively transferred, tumor-specific T cells tolerant, but they exert suppressive activity over naïve T cell effector responses. Administration of the DC vaccine that prevents tolerance induction also prevents acquisition of suppressor function. Our preliminary data suggest that the induction of tolerance is independent of CD4^{+}25^{+} Treg cells, whereas acquisition of

suppressor function require CD4$^+$25$^+$ Treg cells. These results demonstrate the complex role that DCs, regulatory T cells, and the tumor microenvironment play in determining the fate of naïve, tumor-specific T cells. If insufficient stimuli are present during T cell priming, as may be the case during tumor development, tumor-specific T cells can become tolerant and acquire suppressor function. When trying to initiate a productive anti-tumor response, tumor-specific T cells must not only be efficiently primed to overcome endogenous tumor-Ag presented in a tolerizing manner but must be protected from the suppressive mechanisms of both regulatory T cells and the tumor micro-environment.

Tolerization of Tumor-Specific T Cells Despite Efficient Initial Priming in a Primary Murine Model of Prostate Cancer

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Abstract

In this report, we studied T cell responses to a prostate cancer antigen (Ag) by adoptively transferring tumor antigen-specific T cells into prostate tumor-bearing mice. Our findings demonstrate that CD8^{+} T cells initially encountered tumor-Ag in the lymph node and underwent an abortive proliferative response. Upon isolation from the tumor, the residual tumor-specific T cells were functionally tolerant of tumor-Ag as measured by their inability to degranulate and secrete IFNγ and granzyme B. We next sought to determine whether providing an *ex vivo* matured, peptide-pulsed DC vaccine could overcome the tolerizing mechanisms of tumor bearing TRAMP mice. We demonstrate that tumor antigen-specific T cells were protected from tolerance following provision of the DC vaccine. Concurrently, there was a reduction in prostate tumor size. However, even when activated DCs initially present tumor-Ag, T cells persisting within the tolerogenic tumor environment gradually lost Ag-reactivity. These results suggest that even though a productive anti-tumor response can be initiated by a DC vaccine, the tolerizing environment created by the tumor still exerts suppressive effects on the T cells. Furthermore, our results demonstrate that when trying to elicit an effective anti-tumor immune response, two obstacles must be considered: to maintain tumor antigen responsiveness, T cells must be efficiently primed to overcome tumor-Ag presented in a tolerizing manner and protected from the suppressive mechanisms of the tumor micro-environment.

Introduction

The context in which an APC presents antigen plays a key role in determining the fate of T cells. Dendritic cells (DCs) can be extremely effective in priming naïve T cells upon T cell receptor (TCR) engagement of cognate Ag-MHC complexes (1). Depending on the maturation state of the DC, naïve T cells can differentiate into efficient CTLs or undergo tolerance and/or deletion. Evidence suggests that "tolerogenic" DCs correspond to resting DCs, expressing low levels of both MHC and co-stimulatory molecules, while immunogenic DCs have encountered maturation stimuli to up-regulate expression of MHC and co-stimulatory molecules, and increased IL-12 secretion (2, 3). Tolerogenic DC may also arise to due interactions with regulatory cells (4, 5). Various stimuli, such as pathogen by-products that stimulate TLRs (6, 7), activated CD4^{+} T cells (8), and even CD8^{+} T cells (9) can provide the appropriate signals to activate tolerogenic DCs.

When trying to elicit an anti-tumor response, several regulatory mechanisms may exist. Among these are the secretion of immunosuppressive factors from the tumor (10) and the existence of regulatory T cells that can suppress productive T cell responses (11, 12). Another problem is that as tumors develop, the inflammatory stimuli present may be insufficient to properly activate DCs and they function in a "tolerogenic" state (13). DCs have been shown to capture Ag, traffic to the lymph nodes (LNs), and present this Ag to naïve T cells (14, 15). If the presentation of tumor-Ag is in the context of minimal co-stimulation and cytokine help, T cells may become tolerant of the tumor Ag (16, 17). When encountering Ags presented in this manner, T cells initially undergo proliferation and transient activation prior to the induction of tolerance, with the majority of cells undergoing deletion (18, 19). Understanding these tolerance mechanisms and investigating experimental approaches to protect tumor-specific T cells them from tolerance induction is critical for successful immunotherapeutic approaches to cancer.

To address these issues, we utilized both the TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model (20) and a tumor-specific TCR transgenic mouse strain (TcR-I) (21). Male TRAMP mice express the large and small T antigens (TAg) from SV40 as a transgene under the transcriptional control of the prostate-specific promoter, probasin. All male TRAMP mice 'spontaneously' develop prostate cancer. TAg serves both as a self Ag and tumor Ag in this model. It was previously demonstrated that TRAMP mice develop tolerance to TAg (22, 23). TAg mRNA expression (unpublished data, MJA and AAH) and TAg protein have been detected in the thymus of TRAMP mice, leading to thymic deletion of TAg-specific T cells (23). TcR-I mice express a transgenic TCR that recognizes the H-2K^k restricted TAg$_{560-568}$ epitope. Using these two mouse models, we studied the fate of naïve, tumor-specific CD8^{+} T cells after exposure to endogenous tumor-Ag in TRAMP mice.

In this report, we demonstrate that upon transfer into male TRAMP mice, tumor-specific CD8^{+} TcR-I T cells underwent up to 6 rounds of proliferation in the LNs and displayed transient up-regulation of activation markers. A substantial fraction of TcR-I T cells subsequently underwent deletion, with a majority of transferred T cells undergoing apoptosis 2-5 days post Ag-encounter. The remaining T cells were confined to the prostatic tissue and were tolerant to tumor-Ag. In contrast, the provision of an *ex vivo* matured, Ag-pulsed DC vaccine 18 hours post-AT resulted in enhanced expansion of tumor-specific T cells, sustained up-regulation of activation markers and IFNγ production, and acquisition of effector function. A concomitant decrease in genitourinary tract weight, reflective of diminished tumor burden, was also observed. However, properly primed T cells eventually undergo a gradual induction of tolerance due to persistence in a tolerogenic prostate tumor.

Materials and Methods
Mice

TRAMP mice have been previously described (20). Briefly, the small and large T antigen genes derived from SV40 have been placed under the control of the androgen driven, prostate-specific probasin promoter. TRAMP mice homozygous for the TAg transgene were maintained on a pure C57BL/6 background. To obtain experimental mice, TRAMP mice were bred one generation to non-transgenic C3H/HeN mice (purchased from the National Cancer Institute, Frederick, MD). All TRAMP x C3H F1 mice used were male and between 10 and 12 weeks of age. B6C3F1 non-transgenic control mice [C57BL/6 x C3H/HeN] were purchased from the National Cancer Institute, Frederick, MD. The TCR transgenic mouse strain TcR-I, homozygous for a TCR gene that recognizes the H-2K^k restricted epitope TAg$_{(560-568)}$, were backcrossed and maintained on a pure C3H background (21). TcR-I mice were bred one generation to non-transgenic, Thy1.1$^+$ C57BL/6 mice (*B6.PL-Thy1^a/Cy*, purchased from The Jackson Laboratory). All mice were housed under specific pathogen-free conditions in the National Cancer Institute's animal facility. Mice were treated in accordance with NIH guidelines under protocols approved by the National Cancer Institute's Animal Care and Use Committee.

Peptide

TAg$_{(560-568)}$ (SEFLLEKRI) peptide was synthesized by New England Peptide using FMOC chemistry and the purity was over 90% based on HPLC assay. Peptide was dissolved in water at 1 mg/ml, sterile filtered, and aliquotted for storage at −20° C.

Adoptive Transfer of Transgenic Lymphocytes

Thy1.1$^+$ TcR-I mice were euthanized by CO_2 inhalation and the inguinal, axillary, brachial, iliac and mesenteric LNs were aseptically removed and minced into a single cell suspension. LN cells of donor mice were 50% CD8$^+$. All CD8$^+$Thy1.1$^+$ T cells were tetramer positive. When indicated, cells were labeled with 5 μM CFSE in 1 mL DMEM plus 10% FBS for 15 minutes at room temperature. CFSE was quenched with an equal volume of FBS and cells were washed with serum-free media prior to adoptive transfer. Cell numbers were adjusted so 3x10^6 Ag-specific TcR-I T cells were transferred intravenously into recipient mice.

DC Preparation and Vaccination

Dendritic cells were prepared from B6C3F1 bone marrow. Femurs and tibias were removed and bone marrow was flushed with a 25-gauge needle. After red blood cell lysis, bone marrow cells were plated in a 6 well plate in complete RPMI supplemented with 10% supernatant from a GM-CSF secreting cell line (24). Non-adherent cells were washed away and discarded on day 2, cells were re-fed on day 4, and on day 7, non-adherent cells were collected and re-plated overnight with TAg$_{(560-568)}$ or an irrelevant control peptide. On day 8, non-adherent cells were collected, washed in serum free media, and injected sub-cutaneously at 1 x 10^6 cells per mouse. The cultures were routinely greater than 70% CD11c+ and were of comparable potency to dendritic cells generated in supernatants supplemented with GM-CSF and IL-4.

Flow Cytometry

Prostate draining LNs (pDLN = iliac LN), non-draining LNs (nDLN = inguinal LN), and prostates were harvested. LNs were minced between two microscope slides to obtain a single cell suspension. Prostates were micro-dissected and digested in dissociation solution (DMEM + collagenase + DNase) for 2-3 hours at 37° C. The resulting cell suspension was passed over a histopaque gradient, centrifuged at 950 x g, and cells from the interface were removed and used for flow cytometric analysis. Fc receptors were blocked with supernatant from the 2.4G2 hybridoma. Cells were washed and incubated with the following Abs for 30 minutes on ice: Thy1.1 PE, CD8 AlexaFluor 405 (Caltag), CD69 PE-Cy7, CD44 APC, and CD25 APC-Cy7. Unless indicated, all Abs were purchased from BD-Pharmingen. Cells were analyzed on a BD LSR II flow cytometer and data interpolated using FCS Express analysis software. Total cell counts for lymph node (and spleen, data not shown) were not affected by transfer or vaccination. Thus, data is presented as % positive for the indicated phenotypic

marker.

For TUNEL assays, LNs were harvested and processed as described above. Cells were stained with Thy1.1-PE and CD8-CyChrome for 30 minutes on ice, fixed in 4% PFA, then labeled with the Beckman Coulter Mebstain Apoptosis kit using biotinylated dUTP. Cells were then incubated with streptavidin conjugated APC (BD Pharmingen) for 30 minutes at room temperature. Cells were analyzed as above.

For intracellular cytokine staining assays, LNs were harvested as above and cells were incubated in complete DMEM containing 1 μg/ml of TAg$_{(560-568)}$ and 1 μl/ml GolgiPlug (BD Pharmingen) for 12 hours at 37° C. Cells were washed, Fc receptors blocked, and then incubated with Thy1.1 PE and CD8 CyChrome for 30 minutes on ice. Intracellular IFNγ staining was performed using the Cytofix/Cytoperm kit and anti-IFNγ APC, all from BD Pharmingen. Cells were analyzed as above.

Proliferation Assays

For CD11c$^+$ cell isolation, mice were euthanized and prostates and LNs were removed and digested as mentioned above. Cells were washed in IMag buffer (PBS, 0.5% BSA, 2 mM EDTA, BD Biosciences), incubated with biotinylated anti-CD11c Ab followed by streptavidin conjugated magnetic beads (BD Pharmingen) and passed before an IMagnet (BD Biosciences). Positively selected cells, the purity of which was routinely greater than 70%, were used as stimulator cells in a proliferation assay. To measure priming by CD11c$^+$ cells, 2 x 10^4 naïve, TcR-I T cells and 2 x 10^4 purified CD11c$^+$ cells were added to a final volume of 200 μl per well. Cells were incubated for 72 hours, then pulsed over night with 1 μCi / well of ^{3}H-thymidine. As a positive control, peptide-pulsed, *ex vivo* matured DCs were used. When indicated, 100 ng/ml of TAg$_{(560-568)}$ was added.

ELISpot Assays

For IFNγ ELISPOTs, multiscreen plates (Millipore, Bedford, MA) were coated with 10 µg/ml anti-mouse IFN-γ (clone R4-6A2, BD Biosciences) in PBS (50 µl/well) overnight at 4°C. Plates were washed and blocked with complete medium for 2 hours at 37 °C. T cells were purified using the same protocol described above to purify CD11c$^+$ cells using a biotinylated anti-Thy1.1 Ab. To measure T cell responses, 5x10^3 purified T cells (normalized for input of tetramer$^+$ cells), 7.5x10^5 C3H splenocytes and increasing concentrations of TAg$_{(560-568)}$ peptide were added to a final volume of 100 µl per well and incubated for 36 hours at 37 °C. After incubation, plates were washed and incubated with 50 µl of 0.5 µg/ml biotinylated rat anti-mouse IFN-γ (clone XMG1.2, BD Biosciences) in PBS/0.5% BSA for 2 hours at 37°C. Plates were rinsed and 50 µl of streptavidin-conjugated alkaline phosphatase (Rockland, Gilbertsville, PA), diluted 1/2000 in PBS, was added to each well. Plates were incubated at room temperature for 45 minutes, rinsed, and developed with 50 µl BCIP/NBT phosphatase substrate (KPL Inc., Gaithersburg, Maryland) for 5 minutes. Plates were rinsed, air-dried, and spots were counted with an ImmunoSpot™ Analyzer (Cellular Technology Ltd., Cleveland, OH)

For GrB ELISPOTs, multiscreen plates (Millipore, Bedford, MA) were coated with 100 μl anti-mouse granzyme B capture Ab (ELISpot Development Module Mouse Granzyme B, R & D Systems, Minneapolis, MN), diluted 1:60 in PBS, overnight at 4°C. Plates were washed and blocked with complete medium for 2 hours at 37 °C. Varying numbers of effector cells and 5 x 10^4 BW cells (an AKR-derived murine thymoma cell line that expresses H-2K^k, available from ATCC) were added per well. Unpulsed BW cells were used as a specificity control. Plates were incubated for 4 hours at 37° C, washed, and incubated with 100 µl of anti-mouse granzyme B detection Ab (ELISpot Development Module Mouse Granzyme B, R&D Systems, Minneapolis, MN) in PBS/0.5% BSA overnight at 4°C. Plates were washed and 100 µl of streptavidin-conjugated alkaline phosphatase (MabTech USA, Mariemont, OH), diluted 1:1500 in PBS, was added to each well. Plates were incubated at room temperature for 2 hours, washed, and spots were developed with 100 µl Vector Blue substrate (Alkaline Phosphatase Substrate Kit III, Vector Laboratories, Burlingame, CA) for 5 minutes in the dark. Plates were then rinsed and air-dried. Spots were counted with an ImmunoSpot™ Analyzer

(Cellular Technology Ltd., Cleveland, OH)

CD107a Degranulation Assay

1×10^5 purified T cells were mixed with 1×10^5 PKH26 (Sigma) labeled, peptide-pulsed BW cells in a 5 ml polypropylene tube in a total volume of 400 μl. Unpulsed BW cells were used as a specificity control. 4 μl of anti-mouse CD107a-FITC was added to each tube and incubated for 2 hours at 37^0 C. After incubation, cells were washed and stained with anti-mouse CD8-APC (BD Pharmingen) for 30 minutes on ice. Cells were washed and analyzed via flow cytometry as above.

Statistical Analysis

A Student's T-test was used to compare proliferative responses in Figures 3 and 4. An unpaired T-test was used to compare the prostate wet weights in Figure 7.

Results

Tumor-specific T cells undergo transient activation and deletion in the lymph nodes

To study the fate of naïve, tumor-specific T cells in a primary model of prostate cancer, CFSE-loaded TAg$_{(560-568)}$-specific TcR-I T cells were transferred into 12-week-old male TRAMP mice. Prostate-draining and non-draining lymph nodes and prostate tissue were analyzed. Flow cytometric analyses of the three tissues revealed that tumor-specific T cells initially encountered tumor Ag and underwent cell division within the pDLNs, immediately followed by the nDLN (Figures 1A,B). Expansion of TcR-I cells in the spleen was comparable to that observed in the nDLN (data not shown). Cell expansion occurred from 1 to 3 days post-AT (Figure 1B). In contrast, no activation or proliferation of TcR-I cells was observed when transferred into non-transgenic 'wild-type' B6C3F1 mice (Figure 1A). Tumor-specific T cell numbers began to decrease in the LNs between days 3-6 post ATx. As seen in figure 1C, this decrease was accompanied by a marked increase in the apoptosis of T cells, as measured by TUNEL staining. By day 8 post ATx, no tumor antigen-specific T cells were detected in the lymph nodes of tumor-bearing mice. Taken together, these data show that tumor-Ag is presented in the lymph nodes of TRAMP mice and Ag-presentation results in a transient activation followed by an abortive proliferative response from the tumor-specific T cells, resulting in deletion of T cells from lymph nodes.

Concomitant with cell expansion was the transient up-regulation of T cell activation markers (figure 1A). Naïve TcR-I cells that were transferred into TRAMP mice were CD69$^-$, CD25$^-$, and CD44^{low}. Within 24 hours of Ag encounter in the LN, T cells up-regulated the IL-2Rα chain (CD25), the early activation and immunoregulatory molecule CD69, and the glycoprotein CD44, which serves as a marker for "antigen-experienced" T cells. However, by 72 hours post ATx, tumor antigen-specific T cells had almost completely lost CD25 and CD69 expression, indicating their activation state was not sustained. It remains possible that activated cells had left the lymphoid tissues and trafficked to the prostate (as described below), leaving only naïve cells in the lymph node, but we believe this to be unlikely due to the kinetics of activation and the appearance of TUNEL-positive cells.

Tumor-specific T cells traffic to and persist in the prostate

As seen in other tumor models, tumor-specific T cells undergo proliferation and deletion in the LNs on tumor-bearing mice (18). Our data demonstrate that although tumor-specific T cells were no longer detectable in the lymphatic tissue one week after ATx, not all tumor-specific T cells were undergoing apoptosis (figure 1C). This led us to examine the prostate to determine whether tumor-specific T cells were infiltrating the tumor.

An analysis of the prostatic tissue of adoptively-transferred TRAMP mice demonstrated that tumor-specific T cells were sequestered within the tumor. As shown in figure 1D, tumor-specific T cell infiltration into the prostate began as early as 2 days post-ATx and plateaud at 5-6 days post-ATx. Phenotypic analysis of T cells in the prostate showed that upon arrival in the prostate, tumor-specific T cells were both CD69 and CD25 low and by day 6, 70-80% of CD8$^+$ tumor-specific T cells expressed CD25 and CD69 (Figure 1E). Analysis of the lung and liver tissues failed to demonstrate any tumor antigen-specific T cell infiltration into these organs, confirming the specificity of the prostatic infiltration (data not shown) and suggesting that tumor-specific T cells that survived the initial encounter with Ag in the LN may have trafficked to and persisedt in the prostate and re-acquired an activated profile. Similarly, in wild-type mice, TcR-I cells are not detected in any non-lymphoid tissues and any point after transfer (data not shown).

Tumor-specific T cells isolated from the tumor are functionally tolerant of tumor-Ag.

The reacquisition of activation markers upon tumor infiltration suggests that tumor-specific T cells might be reactivated by appropriate stimuli and thus possess effector function. To determine whether CD8$^+$ tumor-specific T cells within the tumor could respond to their cognate Ag, TcR-I T cells

were isolated from the LN or prostate of TRAMP mice based on Thy1.1 expression and their Ag-responsiveness was assayed by their ability to secrete IFNγ. Thy1.1$^+$ CD8$^+$ tumor-specific T cells isolated from the nDLNs and pDLNs of tumor bearing mice on day 3 displayed diminished responsiveness to tumor Ag (data not shown). T cells isolated from prostates on day 5 were almost completely tolerant of TAg. By day 10 and after, TcR-I T cells isolated from the prostates were completely unresponsive to tumor-Ag (figure 2). These data demonstrate that programming of tolerance of tumor specific T cells is initiated upon Ag encounter in the LNs and by the time T cells have reached the prostate, tolerance induction is complete, despite an alteration in activation profile.

CD11c$^+$ cells cross-present tumor-Ag in the LNs

As demonstrated in figure 1B, tumor-specific T cells first began to proliferate in the prostate-draining LNs, but shortly thereafter, in the non-draining LNs as well. This suggests that tumor Ag was being presented to tumor-specific T cells within the LNs. Tumor Ag presentation could be mediated by either tumor cells within the LNs or by DC-mediated cross-presentation. To determine whether T cell expansion was at least partly mediated by antigen-bearing DCs, pDLNs, nDLNs, and prostates were harvested from 12-week-old TRAMP mice and CD11c$^+$ cells were isolated using magnetic beads. CD11c$^+$ cells were then directly used as APCs in a proliferation assay to stimulate naïve, TcR-I cells. As seen in figure 3A, CD11c$^+$ cells from both nDLN and pDLN could stimulate proliferation of TcR-I cells, although APC from the pDLN gave a <u>significantly stronger response</u> than those from the nDLN. However, a more robust proliferative response was noted when using CD11c$^+$ cells from the prostate of TRAMP mice. Similar results were found using IFN-γ production as a readout (data not shown). These findings suggest an antigen gradient consistent with antigen expression in the prostate and diminishing levels in the draining and non-draining LNs.

To assess the relative potential to prime TcR-I cells, we pulsed the CD11c$^+$ cells from TRAMP and wild-type LN tissues with exogenous TAg peptide and tested their ability to prime naïve TcR-I cells (Figure 3B). Interestingly, when antigen levels were not limiting, the LN-derived DC of TRAMP mice were comparable to the same cells isolated from the lymph nodes of wild-type mice in their ability to elicit proliferation, which was about 50-fold greater than unpulsed TRAMP-derived cells. This may be due to loss of antigen during the isolation process. This response was about half the response of antigen–pulsed bone-marrow-derived DC. However, TRAMP prostate-derived DC were considerably less potent at priming TcR-I cells than LN-derived DC, suggesting that chronic exposure to these APCs in the TRAMP prostate may be responsible for the induction of tolerance. <u>The prostatic tissues of wild-type mice do not contain sufficient numbers of DC to isolate using magnetic beads and thus could not be used for comparison to TRAMP prostate derived DC.</u>

Provision of an ex vivo-matured, peptide-pulsed dendritic cell vaccine can activate tumor-specific T cells and prevent tolerance induction

To determine whether the cellular context in which tumor-specific T cells first encounter tumor-Ag determines their fate, we tested whether *ex vivo*-generated, bone marrow-derived, peptide-pulsed DCs could effectively stimulate tumor-specific T cells in tumor-bearing TRAMP mice and prevent tolerance induction. TRAMP mice were transferred with TcR-I T cells and 18 hours later, were given the DC vaccine. As demonstrated in figure 4A, administration of a DC vaccine shortly after transfer resulted in a robust expansion of tumor-specific T cells in the vaccine DLN (inguinal LN). <u>This expansion was also reflected in a profound increase in the number of cells that accumulate in the</u>

prostate of vaccinated mice at later time points (figure 4B). Moreover, the vaccine also reduced the

fraction of tumor-specific T cells from undergoing apoptosis (figure 4C). This demonstrates that

provision of tumor-Ag presented by an activated APC can both significantly enhance T cell expansion

and reduce T cell death.

To determine whether *ex vivo*-matured, peptide-pulsed DCs could properly activate tumor-specific T cells in a tumor-bearing host, we investigated the phenotype and IFNγ production of T cells following priming by the DC vaccine. As shown in figure 5, DC vaccination caused a profound increase in the frequency of tumor-specific T cells expressing CD69 and CD25 and producing IFNγ (41%, 65%, 48%, respectively) compared to TcR-I cells from unvaccinated mice (5.6%, 4.9%, 2.2%, respectively). Not surprisingly, there was no notable change in CD44 expression, confirming its role as an indicator of antigen exposure rather than efficient activation. Interestingly, there remains a population of cells that do not express CD25 and IFN-γ (approximately 35% and 50%, respectively). These cells did not undergo a comparable proliferative response (as measured by CFSE dilution) and may be those TcR-I cells that did not encounter the DC vaccine but rather were primed by endogenous APC.

DC vaccination induces tumor-specific T cells with potent effector function
Our data show that an activated, peptide-pulsed DC vaccine induced an expansion of tumor-specific T cells, protection from apoptosis, and up-regulation of activation markers and IFNγ expression in the LNs of tumor-bearing mice. However, to achieve a successful anti-tumor immune response, tumor-specific T cells must both traffic to the tumor and retain responsiveness to Ag. We next tested whether the provision of a properly-matured DC vaccine could protect tumor-specific T cells from tolerance induction. TRAMP mice were transferred with TcR-I T cells and vaccinated as described above. At various time points after vaccination, TcR-I cells were isolated and tested *ex-vivo* for function. As seen in figure 6, provision of a DC vaccine rescued tumor-specific T cells from tolerance induction up to 2 weeks after vaccination. In mice that received the DC vaccine, TcR-I cells had the capacity to secrete IFN-γ (figure 6A) and granzyme B (figure 6B) and degranulate their lysosomes (as measured by cumulative CD107a expression, figure 6C). This is in contrast to tolerant TcR-I cells from TRAMP mice that received no vaccine and exhibited none of these three functional indicators of T cell responsiveness. This prevention of tolerance induction persisted for up to 2 weeks after DC vaccination. These findings demonstrate that by providing efficient priming via a DC vaccine, tumor-specific T cells can be properly programmed into efficient CTLs even in the presence of "tolerogenic" DCs.
By 21 days after transfer, T cell responses in DC-vaccinated mice were significantly decreased. This diminished reactivity was reflected in reduced IFN-γ and granzyme B production as well as reduced CD107a expression (figure 6). This suggests that although the DC vaccine could initially enhance priming of tumor-specific T cells, persistence within the tolerogenic tumor environment reversed tumor reactivity and resulted in tolerance to the tumor antigen.
To determine whether ATx of tumor-specific T cells plus DC vaccine had an effect on tumor

growth, we determined the weight of prostatic complex, consisting of the urethra, ampulary gland, and

the doral, lateral, anterior and ventral lobes of the prostate. Prostate weights can be used as an

indication of tumor growth in the TRAMP model (25). No difference in prostate weight was noted

<u>between unmanipulated TRAMP mice and mice transferred with TcR-I cells (data not shown).</u> As seen in figure 7, TRAMP mice that received the DC vaccine had significantly lower prostate weights on days 12 and 21 than did mice that received ATx alone. These findings correlate with the data presented in figure 6, showing an initial robust response to tumor Ag followed by a subsequent loss of reactivity to tumor-Ag. However, prostate weights began to equilibrate among groups 5 weeks after treatment, consistent with the loss of TcR-I cell reactivity 2 weeks earlier. Taken together, our findings suggest that activated DCs can efficiently program tumor-specific T cells into effector cells even when tumor-Ag is also being presented by tolerogenic DCs. Once properly activated, tumor-specific T cells traffic to the prostate, exert anti-tumor effects, and retain their responsiveness to tumor Ag for up to 3 weeks.

Discussion

In the current study, we demonstrate that after adoptive transfer into TRAMP mice, prostate tumor-specific T cells first encountered tumor Ag in the pDLNs. Ag presentation was at least in-part mediated by CD11c$^+$ cells. T cell encounter with Ag in the LNs initially led to their expansion and transient activation (days 1-3), but a significant fraction of tumor-specific T cells underwent deletion in the lymph nodes (days 4-6). Two to six days after transfer, T cells infiltrated the prostate tumor. Interestingly, after day 6, T cells were not detectable in the lymph nodes, and were only detectable within the prostate tissue. T cells that persisted in the prostate were tolerant of tumor antigen. We further demonstrate that provision of an antigen-pulsed DC vaccine primed T cells and prevented tolerance induction for up to two weeks after T cell transfer. T cell responsiveness correlated with a decrease in prostate weight, indicative of a productive anti-tumor immune response.

T cell encounters with tumor Ag in the LN have been demonstrated in many tumor models. Ag presentation has been shown to be mediated by APCs (26, 27) and tumor cells (28). In our model, Ag presentation is at least in-part mediated by CD11c$^+$ cells, most likely DCs that have captured antigen and present it in the LN. Once T cells reach the prostate, chronic exposure of tumor-reactive T cells to CD11c$^+$ cells that do not properly prime T cells may result in complete T cell tolerance. T cells could also be defectively primed by tumor cells that have metastasized to the LNs. However, based on the age of our experimental mice (10 – 12 weeks old, where no evidence for LN metastasis exists) and the fact that T cells were observed to encounter tumor Ag within the nDLN and the spleen, it is unlikely that tumor metastases directly tolerize T cells in the LN.

The mechanism by which T cells traffic to and persist in the prostate is unclear. One possibility is that the prostate tumor may express proinflammatory cytokines or chemokines that attract and retain tumor-specific T cells. It was previously demonstrated that expression of chemokines within tumors can enhance T cell infiltration (29). In addition, previous reports suggest that T cell recognition of prostate Ags is dependent on tumor formation (30), implying that a unique environment develops within the prostate tumor. Another possible explanation for T cell trafficking to the prostate is that the prostate could create an "antigen gradient" leading from the nDLNs through the pDLNs and into the prostate. This idea is supported by our observation that when transferred into wild-type mice, TcR-I cells cannot be detected in any non-lymphiooid tissues, even after antigen vaccination. In addition, it has been proposed that some self-antigens, including tumor antigens, may be directly chemotactic (31, 32). This possibility is currently being explored.

Our data clearly demonstrate that upon isolation from the prostate tissue, tumor-specific T cells are functionally tolerant of tumor Ag. The cells retain the capacity for stimulation as they are responsive to PMA/Ionomycin stimulation (data not shown). There are at least two possible explanations for these findings. First, when presenting tumor-Ag to tumor-specific T cells, CD11c$^+$ cells are in a resting state and provide deficient co-stimulation and possibly little or no cytokine help. Tumor-induced suppression of DCs was previously demonstrated in prostate tumor bearing mice (33). Low levels of costimulatory molecule expression could tilt the balance toward tolerance induction through increased interaction with inhibitory molecules. This is supported by the observation that resting DCs can induce tolerance in CD8$^+$ T cells through interaction with the inhibitory molecules PD-1 and CTLA-4 (34). Second, after T cells are primed in the LNs, they traffic to and persist within the tumor. Tumor microenvironments are highly immunosuppressive with high levels of TGFβ (10) and CD4$^+$ regulatory T cells (12). The role of TGFβ in T cell tolerance is supported by recent reports which demonstrate that T cells rendered resistant to TGFβ signaling are not tolerized by the transplantable TRAMP prostate tumor cell line, TRAMP C2 (35). An alternative possibility is that antigen levels in TRAMP mice are insufficient to appropriately prime the transferred TcR-I cells. We believe this to be unlikely since provision of exogenous soluble peptide antigen to transferred TRAMP mice does not alter TcR-I cell expansion or reactivity (data not shown).

It is interesting to note that T cells isolated from nDLNs and pDLNs three days after ATx are incompletely tolerized (data not shown). It remains a possibility that in the TRAMP model, tumor-

specific T cells are programmed for deletion in the LNs and for tolerance in the prostate. Based on previous reports that demonstrate that low Ag levels lead to T cell deletion and high Ag levels lead to T cell tolerance (36), it could be argued that in the LNs of TRAMP mice, where Ag levels are low, T cells are programmed for deletion. Once T cells have reached the prostate, where Ag is constitutively being produced in high levels by the prostatic epithelium, T cell signaling could change and cells could be programmed for tolerance.

Provision of an ex vivo-matured, peptide-pulsed DC vaccine overcame deficient priming in the LNs and led to enhanced T cell proliferation, decreased T cell apoptosis, and increased expression of CD25, CD69, and IFNγ. It is interesting to note that the activation of these T cells was not as efficient as T cells transferred into WT mice that received the DC vaccine, where priming only derives from the DC vaccine. These findings suggests that in TRAMP mice, there is competition between tolerogenic DCs and activated vaccine DCs that results in some T cells being professionally primed while others are defectively primed by endogenous APCs and are still programmed for deletion. In a murine model of TAg-induced osteosarcomas, Staveley-O'Carroll *et al* demonstrated that activation of endogenous APC through CD40 ligation can prevent tolerance to transgenic expression of TAg in the liver, underscoring the critical role of endogenous APCs in tolerization of T cells (37).

Provision of a DC vaccine also led to a profound infiltration of the tumor, presumably reflective of enhanced expansion and reduced apoptosis in the lymph nodes. Average cell recovery on day 6 after transfer was more than 10-fold greater in vaccinated mice compared to unvaccinated mice. The frequency of transferred cells diminished to a mere 2-fold difference 3 weeks after transfer. It is unclear whether this decline in cell number is due to a loss of stimulation by the DC vaccine or the effects of trafficking to the immunosuppressive tumor bed, or a combination of both. Unfortunately, since all residual T cells were found in the prostate, administration of a booster vaccine to rescue these cells is not practical in this model.

When isolated from the tumor, T cells from DC-vaccinated mice had the capacity to degranulate and to secrete both IFNγ and GrB; however, Ag reactivity was lost over time. These data are consistent with the findings in other models that effector T cells can be tolerized when faced with persistent Ag (36, 38, 39). They are also consistent with another recent study demonstrating that administration of a peptide-pulsed dendritic cell vaccine up to 10 weeks of age may prevent T cell tolerance and reduce tumor incidence in TRAMP mice (40). CD4$^+$ Treg cells (MJA and AAH, unpublished observations) and immunosuppressive cytokines (35) such as TGFβ that are present within TRAMP prostate tumors may contribute to the tolerization of the vaccine-activated anti-tumor T cells. These possibilities are also being explored.

The DC vaccine also led to a transient reduction in prostate weight. This is consistent with the observation that T cells are initially responsive to tumor antigen after vaccination, but reactivity wanes which presumably results in lost of anti-tumor reactivity and increase in tumor size. It was somewhat surprising that the loss tumor immunity and rapidly followed by a concurrent increase in prostate size. However, these findings indicate that maintenance of T cell responsiveness is critical for maintaining tumor immunity.

Our findings imply that even when an appropriate priming event takes place *in situ* and a potent anti-tumor response is generated, the effector cell population can revert to a tolerant state. This striking observation demonstrates a critical parameter that must be considered when attempting immunotherapy of tumors. Both the adoptive transfer of previously activated T cells and the activation of endogenous tumor-specific T cells via vaccination may initially demonstrate potent anti-tumor activity. This may be reflective of a common observation in clinical trials, presenting as partial or incomplete response among immunotherapy study participants. However, unless steps are taken to ensure sustained stimulation of T cells to negate the effects of the tumor micro-environment, T cells may eventually be rendered tolerant, allowing for uninhibited tumor growth.

Acknowledgements

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Figure Legends

FIGURE 1. Kinetics of cell proliferation, activation, apoptosis, and trafficking of $CD8^+$ TcR-I cells following ATx into TRAMP mice. 12 week-old male TRAMP or W/T mice were transferred with 3.0 x 10^6 $CD8^+$, $Thy1.1^+$, $TAg_{(560-568)}$ specific TcR-I T cells. pDLNs and nDLNs were harvested from recipient mice days 1-6 post ATx. (A) Phenotypic analysis of transferred cells demonstrates TcR-I cells encountered Ag and began to divide within LNs of tumor bearing mice. As a control, TcR-I cells were transferred into W/T mice, where no proliferation or activation was seen (dot plot shows one time point, data from days 1-6 were identical). (B) Percentage of $Thy1.1^+$, $CD8^+$ within total lymphocytes in the LNs indicates that TcR-I T cells underwent expansion then retraction in cell numbers. (C) Analysis of TcR-I cells undergoing apoptosis based on TUNEL staining indicates a significant percentage of transferred cells were being deleted. (D) Prostatic tissues were harvested from recipient mice at the indicated times after transfer. Percentage of $Thy1.1^+$, $CD8^+$ cells within a lymphocyte FSC vs. SSC gate demonstrates increased T cell infiltration over time. (E) Percentage of infiltrating T cells with in the prostate expressing the indicated activation marker. For B, C, and E data are mean +/- SD of three mice. Each experiment was repeated at least three times. <u>These results are representative of at least 3 similar experiments.</u>

FIGURE 2. TcR-I cells are tolerized following transfer into TRAMP mice. 12 week-old male TRAMP or W/T mice were transferred with 3.0 x 10^6 TcR-I T cells. Using magnetic beads, TcR-I T cells <u>were isolated from pooled groups of prostate tissue (TRAMP Prostate 1 and TRAMP Prostate 2) on day 5 (A) and day 10 (B) after transfer.</u> As a control, TcR-I mice were transferred into and isolated from W/T mice. Isolated T cells were assayed using an IFNγ ELISpot assay. Equivalent numbers of $Thy1.1^+$ $tetramer^+$ cells were incubated with splenocytes and graded doses of antigen. Data are presented as mean +/- SD of triplicate wells. <u>These results are representative of at least 3 similar experiments.</u>

FIGURE 3. $CD11c^+$ cells from TRAMP LN and prostate can present tumor-Ag to TcR-I cells. $CD11c^+$ cells were isolated from DLNs, pDLNs, and prostates of 12 week-old male TRAMP and W/T mice as described. (A) $CD11c^+$ cells directly stimulated naïve, TcR-I T cells in a proliferation assay. Data are presented as mean +/- SD of triplicate wells. (B) To assess priming potential, $CD11c^+$ cells were used as above with the addition of 100 ng/ml $TAg_{(560-568)}$. Data are presented as mean +/- SD of triplicate wells. <u>These results are representative of 2 similar experiments.</u>

FIGURE 4. Priming with a DC vaccine results in enhanced T cell expansion and decreased apoptosis. 12 week-old male TRAMP mice received 3x10^6 $CFSE^+$, $CD8^+$, $Thy1.1^+$ TcR-I T cells. 18 hours later, mice received a s.c injection of 1 x 10^6 peptide pulsed DCs. Vaccine DLNs (vDLNs = inguinal LNs), pDLNs, and prostates were harvested 3 days post-DC vaccine. (A) Percentage of TcR-I cells within the LNs and prostate. (B) TUNEL staining of TCR-I cells. Dotplots were gated on $CD8^+$, $Thy1.1^+$ cells. (C) Average recovery of $Thy1.1^+$ T cells from the prostates of TRAMP mice that received TcR-I cells alone or with the DC vaccine. <u>These results are representative of at least 3 similar experiments.</u>

FIGURE 5. Priming with a DC vaccine results in up-regulation of activation markers and IFNγ production. 12 week-old male TRAMP or non-transgenic, wild-type (W/T) mice received 3x10^6 CFSE$^+$, CD8$^+$, Thy1.1$^+$ TcR-I T cells. 18 hours later mice received peptide pulsed DCs as previously described. vDLNs were harvested 3 days post-DC vaccine. Cells were analyzed for the expression of CD69, CD25, CD44, and IFNγ. Dotplots represent cells within the CD8$^+$, Thy1.1$^+$ gate. These results are representative of at least 3 similar experiments.

FIGURE 6. TcR-I T cells primed with a DC vaccine are protected from tolerance and acquire cytolytic function. 12 week-old male TRAMP mice or W/T mice received 3x10^6 CD8$^+$, Thy1.1$^+$ TcR-I T cells. 18 hours later, mice received peptide pulsed DCs as previously described. Prostates were harvested on the indicated day post-vaccine and TcR-I cells were isolated by magnetic beads as described. (A) T cells were directly used as responder cells in an IFNγ ELISpot assay. (B) T cells were directly used as responder cells in a Granzyme B ELISpot assay. (C) T cells were assayed for their ability to degranulate in response to the cognate TAg epitope, based on CD107a expression. Data in A and B are presented as mean +/- SD of triplicate wells. These results are representative of at least 3 similar experiments.

FIGURE 7. Vaccination with a peptide-pulsed DC vaccine results in decreased prostate tumor burden. 12 week-old male TRAMP mice received 3x10^6 CFSE$^+$, CD8$^+$, Thy1.1$^+$ TcR-I T cells. 18 hours later mice received peptide-pulsed DCs as previously described. The genitourinary (GU) tract prostatic complex was harvested on the indicated day post-vaccine and seminal vesicles, bladder, and fat tissue was removed. The remaining prostate tissue, consisting of the urethra, ampulary gland, and the dorsal, lateral, anterior and ventral lobes of the prostate, were excised and weighed. The wet weight of the prostate is expressed as the mean +/- SD of at least five mice (except for DC vaccine on day 5, n = 2). These results are representative of 2 similar experiments.