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*J Immunol* 2007; 178:1268-1276; doi: 10.4049/jimmunol.178.3.1268
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Tolerization of Tumor-Specific T Cells Despite Efficient Initial Priming in a Primary Murine Model of Prostate Cancer

Michael J. Anderson,*† Kimberly Shafer-Weaver,*‡ Norman M. Greenberg,*§ and Arthur A. Hurwitz*²

In this report, we studied T cell responses to a prostate cancer Ag by adoptively transferring tumor Ag-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 IFN-γ and granzyme B. We next sought to determine whether providing an ex vivo-matured, peptide-pulsed dendritic cell (DC) vaccine could overcome the tolerizing mechanisms of tumor-bearing transgenic adenocarcinoma of the mouse prostate model mice. We demonstrate that tumor Ag-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 antitumor 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 antitumor immune response, two obstacles must be considered: to maintain tumor Ag 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 microenvironment. *The Journal of Immunology, 2007, 178: 1268–1276.

The context in which an APC presents Ag plays a key role in determining the fate of T cells. Dendritic cells (DCs) can be extremely effective in priming naive T cells upon TCR engagement of cognate Ag MHC (1). Depending on the maturation state of the DC, naive 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 costimulatory molecules, whereas immunogenic DCs have encountered maturation stimuli to up-regulate expression of MHC and costimulatory molecules and increase IL-12 secretion (2, 3). Tolerogenic DCs 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 antitumor 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 (LN), as well as to present this Ag to naive T cells (14, 15). If the presentation of tumor Ag is in the context of minimal costimulation 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 before 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 from tolerance induction is critical for successful immunotherapeutic approaches to cancer.

To address these issues, we used 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 TAgS 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 a tumor Ag in this model. It was previously demonstrated that TRAMP mice develop tolerance to TAg (22, 23). TAg mRNA expression (M. J. Anderson and A. A. Hurwitz, unpublished data) 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-2Kb-restricted TAg₅₆₀–₅₆₈ epitope. Using these two mouse models, we studied the fate of naive, tumor-specific CD8⁺ T cells after exposure to endogenous tumor Ag in TRAMP mice. In this study, we demonstrate that, upon transfer into male TRAMP mice, tumor-specific CD8⁺ TCR-I T cells underwent up to six 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 the transferred T cells undergoing apoptosis 2–5 days after 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 h after AT resulted in enhanced expansion of tumor-specific T cells, sustained up-regulation of activation markers and IFN-γ 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). In brief, the small and large TAg 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 nontransgenic C3H/HeN mice (National Cancer Institute). All (TRAMP × C3H)F1 mice used were male and between 10 and 12 wk of age. B6C3F1, nontransgenic control mice (C57BL/6 × C3H/HeN) were purchased from the National Cancer Institute. The TCR-transgenic mouse strain TCR-I, homozygous for a TCR gene that recognizes the H-2Kk-p560–568(SEFLLEKRI) peptide. On day 8, nonadherent cells were collected, washed in serum-free medium for 2–3 h at 37°C. Variance numbers of effector cells and 5×10^6 Ag-specific TCR-I T cells were transferred i.v. into recipient mice.

**DC preparation and vaccination**

DCs were prepared from B6C3F1, bone marrow. Femurs and tibias 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 min on ice: Thy1.1 PE, CD8 Alexa Fluor 405 (Caltag Laboratories), CD69 PE-Cy7, CD44 allopredocyanin, and CD25 allopredocyanin-Cy7. Unless indicated, all Abs were purchased from BD Pharmingen. Cells were analyzed on a BD Biosciences LSR II flow cytometer and data were interpolated using FCS Express analysis software (De Novo Software). Total cell counts for LNs (and spleen; data not shown) were not affected by transfer or vaccination. Thus, data are presented as percent 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 min on ice, fixed in 4% paraformaldehyde, and then labeled with the Beckman Coulter Mestain Apoptosis kit using biotinylated dUTP. Cells were then incubated with streptavidin-conjugated APC (BD Pharmingen) for 30 min at room temperature. Cells were analyzed as described above.

For intracellular cytokine staining assays, LNs were harvested as described above, and cells were incubated in complete DMEM containing 1 µg/ml TAg560–568 and 1 µl/ml GolgiPlug (BD Pharmingen) for 12 h at 37°C. Cells were washed, and Fc receptors were blocked and then incubated with Thy1.1 PE and CD8 CyChrome for 30 min on ice. Intracellular IFN-γ staining was performed using the Cytotox/Cytoperm kit and anti-IFN-γ APC (all from BD Pharmingen). Cells were analyzed as described 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, and 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) to enrich for positively selected cells. Positive selected cells were purified to greater than 90% using biotinylated anti-Thy1.1 Ab. To measure T cell responses, 5×10^3 purified T cells (normalized for input of tetramer+ cells), 7.5×10^5 C3H splenocytes, and increasing concentrations of TAg560–568 were added to a final volume of 100 µl/well and were incubated for 36 h at 37°C. After incubation, plates were washed and incubated with 50 µl of 5-bromo-4-chloro-3-indolyl phosphate/NBT phosphate substrate (Kirkegaard & Perry Laboratories) for 5 min. Plates were rinsed and air dried, and spots were counted with an ImmunoSpot Analyzer (Cellular Technology).

**ELISPOT assays**

For IFN-γ ELISPOT assays, multiscreen plates (Millipore) 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 h 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, 5×10^4 purified T cells (normalized for input of tetramer+ cells), 7.5×10^5 C3H splenocytes, and increasing concentrations of TAg560–568 were added to a final volume of 100 µl/well and were incubated for 36 h 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 h at 37°C. Plates were rinsed and 50 µl of streptavidin-conjugated alkaline phosphatase (Rockland), diluted 1/2000 in PBS, was added to each well. Plates were incubated at room temperature for 45 min, rinsed, and developed with 50 µl of 5-bromo-4-chloro-3-indolyl phosphate/NBT phosphate substrate. Plates were incubated at room temperature for 2 h and washed, and spots were developed with 100 µl of Vector Blue substrate (Alkaline Phosphatase Substrate Kit II; Vector Laboratories) for 5 min in the dark. Plates were then rinsed and air dried. Spots were counted with an ImmunoSpot analyzer (Cellular Technology).

**CD107a degranulation assay**

A total of 1×10^5 purified T cells were mixed with 1×10^5 PKH26-labeled (Sigma-Aldrich), peptide-pulsed BW cells in a 5-m M polypropylene tube in a total volume of 400 µl. Unpulsed BW cells were used as a
specificity control. Four microliters of anti-mouse CD107a-FITC was added to each tube and incubated for 2 h at 37°C. After incubation, cells were washed and stained with anti-mouse CD8-allophycocyanin (BD Pharmingen) for 30 min on ice. Cells were washed and analyzed via flow cytometry as described above.

**Statistical analysis**

Student’s t test was used to compare proliferative responses (see Figs. 3 and 4). An unpaired t test was used to compare the prostate wet weights (see Fig. 7).

**Results**

*Tumor-specific T cells undergo transient activation and deletion in the LNs*

To study the fate of naive, tumor-specific T cells in a primary model of prostate cancer, CFSE-loaded TAg560–568-specific TCR-I T cells were transferred into 12-wk-old male TRAMP mice. pDLN and nDLN were harvested from recipient mice days 1–6 after 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 WT mice, where no proliferation or activation was seen (dot plot shows one time point, data from days 1 to 6 were identical). B, Percentage of Thy1.1+CD8+ within total lymphocytes in the LNs indicates that TCR-I T cells underwent expansion and 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 forward side scatter vs side scatter gate demonstrates increased T cell infiltration over time. E, Percentage of infiltrating T cells with in the prostate expressing the indicated activation marker. B, C, and E, Data are mean ± SD of three mice. Each experiment was repeated at least three times. These results are representative of at least three similar experiments.

**FIGURE 1.** Kinetics of cell proliferation, activation, apoptosis, and trafficking of CD8+ TCR-I cells following ATx into TRAMP mice. Twelve-week-old male TRAMP or WT mice were transferred with 3.0 × 106 CD8+, Thy1.1+, TAg560–568-specific TCR-I T cells. pDLN and nDLN were harvested from recipient mice days 1–6 after 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 WT mice, where no proliferation or activation was seen (dot plot shows one time point, data from days 1 to 6 were identical). B, Percentage of Thy1.1+CD8+ within total lymphocytes in the LNs indicates that TCR-I T cells underwent expansion and 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 forward side scatter vs side scatter gate demonstrates increased T cell infiltration over time. E, Percentage of infiltrating T cells with in the prostate expressing the indicated activation marker. B, C, and E, Data are mean ± SD of three mice. Each experiment was repeated at least three times. These results are representative of at least three similar experiments.
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Similarly, in WT mice, TCR-I cells were not detected in any nonlymphoid tissues, 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 LNs may have trafficked to and persisted in the prostate, and reacquired an activated profile. As demonstrated in Fig. 1D, tumor-specific T cells infiltrating the tumor.

As seen in other tumor models, tumor-specific T cells undergo proliferation and deletion in the LNs of tumor-bearing mice (18). Our data demonstrate that although tumor-specific T cells were no longer detectable in the lymphatic tissue 1 wk after ATx, not all tumor-specific T cells were undergoing apoptosis (Fig. 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 Fig. 1D, tumor-specific T cell infiltration into the prostate began as early as 2 days after ATx and plateaued at 5–6 days after 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 (Fig. 1E). Analysis of the lung and liver tissues failed to demonstrate any tumor Ag-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 LNs may have trafficked to and persisted in the prostate, and reacquired an activated profile. Similarly, in WT mice, TCR-I cells were not detected in any nonlymphoid tissues and at any point after transfer (data not shown).

72 h after ATx, tumor Ag-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 naive cells in the LNs, 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

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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 LNs or prostate of TRAMP mice based on Thy1.1 expression, and their Ag responsiveness was assayed by their ability to secrete IFN-γ. Thy1.1+ CD8+ tumor-specific T cells isolated from the nDLN and pDLN 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 afterward, TCR-I T cells isolated from the prostates were completely unresponsive to tumor Ag (Fig. 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 that T cells have reached the prostate, tolerance induction is complete, despite an alteration in the activation profile.

CD11c+ cells cross-present tumor Ag in the LNs

As demonstrated in Fig. 1B, tumor-specific T cells first begin to proliferate in the pDLN, but shortly thereafter, in the nDLN 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

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FIGURE 2. TCR-I cells were tolerated following transfer into TRAMP mice. Twelve-week-old male TRAMP or WT mice were transferred with 3.0 × 106 TCR-I T cells. Using magnetic beads, TCR-I T cells 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. As a control, TCR-I mice were transferred into and isolated from WT mice. Isolated T cells were assayed using an IFN-γ ELISPOT assay. Equivalent numbers of Thy1.1+ TCR-I cells were incubated with splenocytes and graded doses of Ag. Data are presented as mean ± SD of triplicate wells. These results are representative of at least three similar experiments.

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FIGURE 3. CD11c+ cells from TRAMP LNs and prostate can present tumor Ag to TCR-I cells. CD11c+ cells were isolated from draining LNs, pDLN, and prostates of 12-wk-old male TRAMP and WT mice as described. A, CD11c+ cells directly stimulated naive, 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 TAg560-568. Data are presented as mean ± SD of triplicate wells. These results are representative of two similar experiments.
least partly mediated by Ag-bearing DCs, pDLN, nDLN, and prostates were harvested from 12-wk-old TRAMP mice, and CD11c⁺ cells were isolated using magnetic beads. CD11c⁺ cells were then directly used as APC in a proliferation assay to stimulate naive, TCR-I cells. As seen in Fig. 3A, CD11c⁺ cells from both nDLN and pDLN could stimulate proliferation of TCR-I cells, although

FIGURE 4. Priming with a DC vaccine results in enhanced T cell expansion and decreased apoptosis. Twelve-week-old male TRAMP mice received $3 \times 10^6$ CFSE⁺, CD8⁺, and Thy1.1⁺ TCR-I T cells. Eighteen hours later, mice received a s.c injection of $1 \times 10^6$ peptide-pulsed DCs. Vaccine DLN (nDLN), pDLN, and prostates were harvested 3 days after DC vaccine. A, Percentage of TCR-I cells within the LNs and prostate. B, TUNEL staining of TCR-I cells. Dot plots 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. These results are representative of at least three similar experiments.

FIGURE 5. Priming with a DC vaccine results in up-regulation of activation markers and IFN-γ production. Twelve-week-old male TRAMP or nontransgenic, WT mice received $3 \times 10^6$ CFSE⁺, CD8⁺, and Thy1.1⁺ TCR-I T cells. Eighteen hours later, mice received peptide-pulsed DCs as previously described. Vaccine DLN were harvested 3 days after DC vaccine. Cells were analyzed for the expression of CD69, CD25, CD44, and IFN-γ. Dot plots represent cells within the CD8⁺Thy1.1⁺ gate. These results are representative of at least three similar experiments.
APC from the pDLN gave a significantly stronger response 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 Ag gradient consistent with Ag expression in the prostate and diminishing levels in the draining and nDLN.

To assess the relative potential to prime TCR-I cells, we pulsed the CD11c+ cells from TRAMP and WT LN tissues with exogenous TAg peptide and tested their ability to prime naive TCR-I cells (Fig. 3B). Interestingly, when Ag levels were not limiting, the LN-derived DC of TRAMP mice were comparable to the same cells isolated from the LNs of WT mice in their ability to elicit proliferation, which was ~50-fold greater than unpulsed TRAMP-derived cells. This may be due to the loss of Ag during the isolation process. This response was about half the response of Ag-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. The prostatic tissues of WT 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.

Provision of an ex vivo-matured, peptide-pulsed DC 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 h later, were given the DC vaccine. As demonstrated in Fig. 4A, administration of a DC vaccine shortly after transfer resulted in a robust expansion of tumor-specific T cells in the vaccine DLN (nDLN). This expansion was also reflected in a profound increase in the cells that accumulate in the prostate of vaccinated mice at later time points (Fig. 4B). Moreover, the vaccine also reduced the fraction of tumor-specific T cells from undergoing apoptosis (Fig. 4C). This
demonstrates that the 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 IFN-γ production of T cells following priming by the DC vaccine. As shown in Fig. 5, DC vaccination caused a profound increase in the frequency of tumor-specific T cells expressing CD69 and CD25 and producing IFN-γ (41, 65, 48%, respectively) compared with 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 Ag exposure rather than efficient activation. Interestingly, there remains a population of cells that do not express CD25 and IFN-γ (~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 IFN-γ expression in the LNs of tumor-bearing mice. However, to achieve a successful antitumor 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 Fig. 6, provision of a DC vaccine rescued tumor-specific T cells from tolerance induction up to 2 wk after vaccination. In mice that received the DC vaccine, TCR-I cells had the capacity to secrete IFN-γ (Fig. 6A) and granzyme B (Fig. 6B) and degranulate their lysosomes (as measured by cumulative CD107a expression, Fig. 6C). This is in contrast to tolerant TCR-I cells from TRAMP mice that received no vaccine and exhibited none of these functional indicators of T cell responsiveness. This prevention of tolerance induction persisted for up to 2 wk 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 (Fig. 6). This suggests that although the DC vaccine could initially enhance the priming of tumor-specific T cells, persistence within the tolerogenic tumor environment reversed tumor reactivity and resulted in tolerance to the tumor Ag.

To determine whether the ATx of tumor-specific T cells plus DC vaccine had an effect on tumor growth, we determined the weight of the prostate complex, consisting of the urethra, ampullary gland, and the dorsal, 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 between unmanipulated TRAMP mice and mice transferred with TCR-I cells (data not shown). As seen in Fig. 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 Fig. 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 wk after treatment, consistent with the loss of TCR-I cell reactivity 2 wk 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 antitumor effects, and retain their responsiveness to tumor Ag for up to 3 wk.

**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 pDLN. 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 LNs (days 4–6). Two to 6 days after transfer, T cells infiltrated the prostate tumor. Interestingly, after day 6, T cells were not detectable in the LNs and were only detectable within the prostate tissue. T cells that persisted in the prostate were tolerant of tumor Ag. We further demonstrate that provision of an Ag-pulsed DC vaccine primed T cells and prevented tolerance induction for up to 2 wk after T cell transfer. T cell responsiveness correlated with a decrease in prostate weight, indicative of a productive antitumor immune response.

T cell encounters with tumor Ag in the LNs have been demonstrated in many tumor models. Ag presentation has been shown to be mediated by APC (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 Ag and present it in the LNs. 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 deceptively primed by tumor cells that have metastasized to the LNs. However, based on the age of our experimental mice (10–12 wk 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 tolerate T cells in the LNs.

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 Ag gradient leading from the nDLN, through the pDLN, and into the prostate. This idea is supported by our observation that, when transferred into WT mice, TCR-I cells cannot be detected in any nonlymphoid tissues, even after Ag vaccination. In addition, it has been proposed that some self-Ags, including tumor Ags, 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, because 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 costimulation 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 CTLA4 (34). Second, after T cells are primed in the LNs, they traffic to and persist within the 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 Ag levels in TRAMP mice are insufficient to appropriately prime the transferred TCR-I cells. We believe this to be unlikely because provision of exogenous-soluble peptide Ag 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 nDLN and pDLN 3 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 IFN-γ. 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 suggest that, in TRAMP mice, there is competition between tolerogenic DCs and activated vaccine DCs that results in some T cells being professionally primed, whereas 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. (37) 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 APC in the tolerization of T cells.

Provision of a DC vaccine also led to a profound infiltration of the tumor, presumably reflective of enhanced expansion and reduced apoptosis in the LNs. Average cell recovery on day 6 after transfer was >10-fold greater in vaccinated mice compared with unvaccinated mice. The frequency of transferred cells diminished to a mere 2-fold difference 3 wk after transfer. It is unclear whether this decline in cell number is due to a loss of stimulation by the DC vaccine, the effects of trafficking to the immunosuppressive tumor bed, or a combination of both. Unfortunately, because 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 IFN-γ and granzyme B; 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 DC vaccine up to 10 wk of age may prevent T cell tolerance and reduce tumor incidence in TRAMP mice (40). CD4+ regulatory T cells (M. J. Anderson and A. A. Hurwitz, 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 antitumor 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 Ag after vaccination, but reactivity wanes, which presumably results in the loss of antitumor reactivity and increase in tumor size. It was somewhat surprising that the loss of tumor immunity was 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 antitumor 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 the 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 antitumor 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 microenvironment, T cells may eventually be rendered tolerant, allowing for uninhibited tumor growth.

Acknowledgments

We acknowledge the technical support of Dr. Anatoli Malyguine and the Laboratory of Cell-Mediated Immunity (National Cancer Institute-Frederick/SAIC). We appreciate the critical review of this manuscript by Drs. Protul Shrikant and Joost Oppenheim.
Disclosures
The authors have no financial conflict of interest.

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CORRECTIONS


The authors revised the Footnotes to include additional funding information. The corrected footnote is shown below.

1 This research was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, by the Department of Defense Congressionally Directed Medical Research Program/Prostate Cancer Research Program Award DAMD17-01-1-0085, and by the Prostate Cancer Foundation (CaP CURE). This work is in partial fulfillment of the degree of Doctor of Philosophy at State University of New York, Upstate Medical University for Michael J. Anderson.


The sixth author’s middle initial was omitted. The correct name is Rachel R. Caspi.


The institution for the 13th author and the institution for the 14th and 15th authors are reversed. Xia Bing is from the Departments of Internal Medicine and Geriatrics, The Zongnan University Hospital, Wuhan University, Wuhan, China. Qiao Li and Alfred E. Chang are from the Department of Surgery, University of Michigan Medical Center, Ann Arbor, MI 48109. The corrected author and affiliation lines are shown below.

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In Fig. 1F the data for WT and Daf-1$^{-/-}$ were reversed. The corrected figure is shown below.


In the title, “Nitrogen” is incorrect. The corrected title is shown below.

Cutting Edge: A Critical Role of Nitric Oxide in Preventing Inflammation upon Apoptotic Cell Clearance


In Footnotes, the current address for the first author is incorrect. The corrected footnote is shown below.

$^2$ Current address: Department of Immunology, Third Military Medical University, Chongqing 400038, People’s Republic of China.