

Immunity to Murine Prostatic Tumors: Continuous Provision of T-Cell Help Prevents CD8 T-Cell Tolerance and Activates Tumor-Infiltrating Dendritic Cells

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Abstract

We reported previously that tumor-specific CD8⁺ T cells (TcR-I) become tolerant in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. In this study, we show that CD4⁺ TcR transgenic (TcR-II) T cells transferred into TRAMP mice became activated in lymph nodes, trafficked to the prostate, and initially functioned as T_H1 cells. Although a single cotransfer of TcR-II cells delayed TcR-I cell tolerization, repeated transfer of TcR-II cells was required to prevent TcR-I cell tolerization and significantly slowed progression of TRAMP prostate tumors. After transfer of TcR-II cells, dendritic cells within the tumor expressed higher levels of costimulatory molecules and displayed an enhanced ability to stimulate proliferation of naive T cells. Blockade of CD40-CD40L interactions during TcR-II transfer resulted in a profound reduction in dendritic cell stimulatory capacity and a partial loss of TcR-I effector functions and tumor immunity. These data show that sustained provision of activated tumor-specific CD4⁺ T cells alters the immunosuppressive tumor microenvironment, ultimately leading to the control of tumor growth. These findings will assist in the design of more effective immunotherapeutic approaches for cancer. [Cancer Res 2009;69(15):6256–64]

Introduction

Traditionally, cancer-specific immunity has been aimed at activating CD8⁺ CTLs based on the observation that most tumors express MHC class I. However, immunotherapy using vaccines composed of MHC I peptides or adoptive transfer of CD8⁺ T cells alone has resulted in limited clinical success (1–3). Tolerization of tumor-specific T cells diminishes the repertoire of immune responses and in turn impairs the ability to elicit effective antitumor immunity. Although adoptive immunotherapy strategies may initially bypass this problem, transferred effector T cells can undergo tolerization (4–6). The limited responses observed in adoptive T-cell therapy trials may be due, in part, to a lack of help from CD4⁺ T cells (7, 8). Some reports suggest that CD4⁺ T cells enhance the infiltration CD8⁺ T cells into tumors and are important

in enhancing the immunostimulatory capacity of the tumor environment (9, 10).

It is well established that CD4⁺ T cells are critical to establish effective CD8⁺ T cells (11–13) and long-term maintenance of antigen-activated CD8⁺ T cells (14–16). CD4⁺ helper T cells can produce cytokines such as interleukin (IL)-2 and IFN- γ that are important for CTL differentiation, expansion, and survival as well as for the activation and regulation of macrophage and dendritic cell responsiveness. Furthermore, CD4⁺ T lymphocytes play a pivotal role by activating antigen-presenting cells (APCs) through CD40/CD154 interactions, inducing IL-12 production and the up-regulation of costimulatory molecules (17–20).

To study T-cell responses to tumor antigens, our laboratory uses the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (21). Male TRAMP mice selectively express the SV40 T antigens (TAg) in the prostate. We reported previously that naive TAg-specific CD8⁺ (TcR-I) T cells are tolerized following encounter with their cognate antigen in TRAMP mice. Although a peptide-pulsed dendritic cell vaccine enhanced TcR-I priming, these stimulatory effects were not durable in the tumor microenvironment, as TcR-I cells that persisted in the TRAMP prostate lost their CTL effector functions (22). This model is a rigorous test of T-cell priming against tumor antigens, as the continued transformation of prostatic epithelium presents a major obstacle for eliciting sustained T-cell responses against tumor antigens, in this case TAg.

The current study was aimed at testing whether adoptive transfer of tumor-specific CD4⁺ T cells (TcR-II) could prevent tolerization of TcR-I cells and help generate a more potent antitumor response. We show that cotransfer of TcR-II cells with TcR-I cells delayed TcR-I T cells tolerance induction, but ultimately this protection did not withstand the highly immunosuppressive tumor microenvironment. However, repeated administration of TcR-II cells prolonged CTL activity of TcR-I cells in the tumor microenvironment that resulted in reduction of prostate tumor burden in TRAMP mice. Transfer of TcR-II cells activated tumor-resident dendritic cells and enhanced their ability to stimulate proliferation of naive T cells *in vitro*. Blockade of CD40-CD40L interactions resulted in a loss of dendritic cell stimulatory capacity. These findings show that tumor-specific CD4⁺ T-cell-mediated help can enhance immunity to tumors by both activating tumor-resident APCs *in situ* and preventing the tolerization of CD8⁺ T cells.

Materials and Methods

Mice. TRAMP mice backcrossed once to the C3H/HeN background (21) and C57BL/6 \times C3H/HeN [wild-type (WT)] mice (purchased from the

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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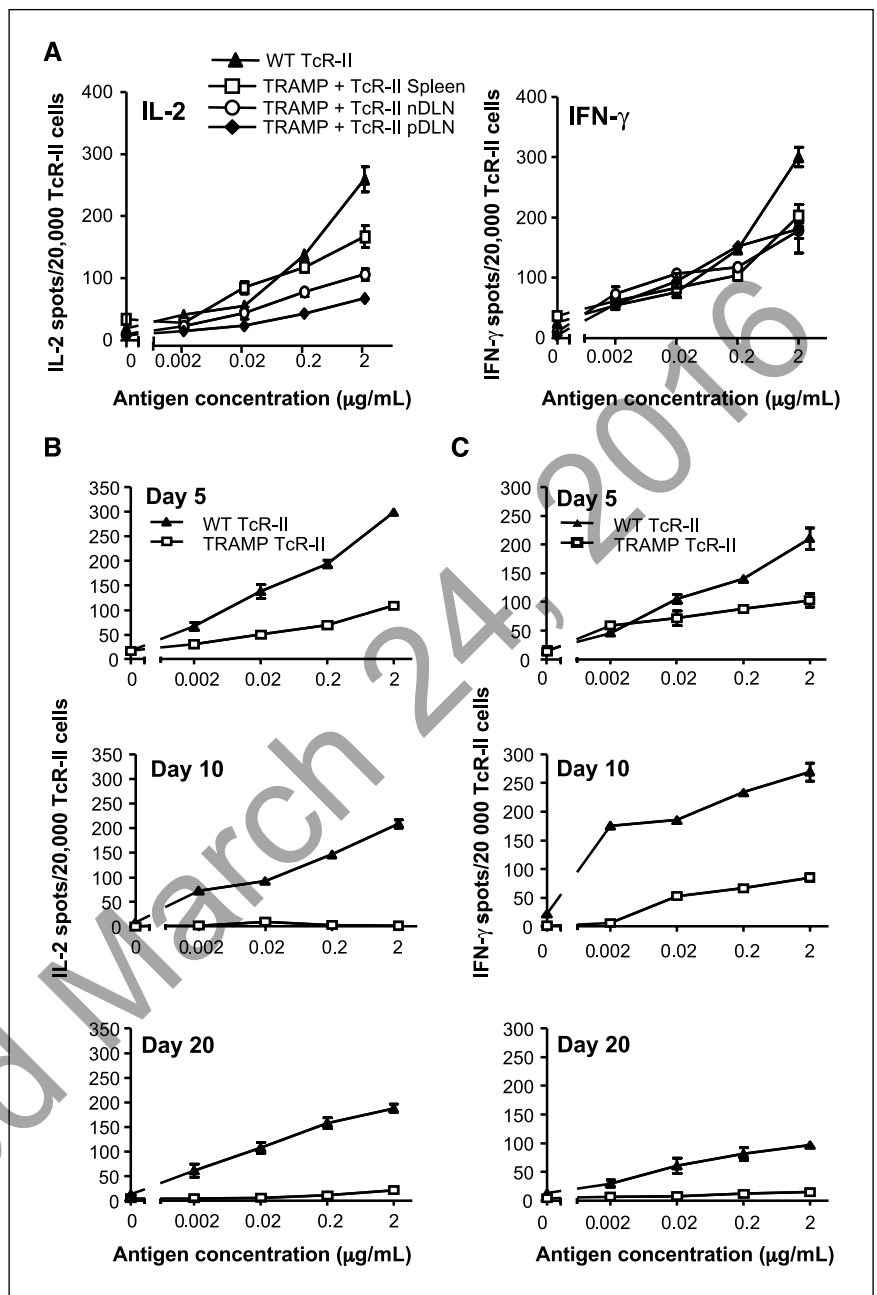


Figure 1. Progressive loss of TcR-II cell proliferation and cytokine secretion. *A*, TcR-II T cells isolated from TRAMP or WT mice 3 d after transfer were stimulated with increasing concentrations of antigen (TAG₃₆₂₋₃₈₄) and tested for IL-2 (*left*) and IFN- γ (*right*). Mean \pm SE. Representative of three separate experiments. Frequency of TcR-II T cells isolated from TRAMP prostates or WT mice that secrete IL-2 (*B*) or IFN- γ (*C*) in response to stimulation with TAG₃₆₂₋₃₈₄ was tested at the indicated time after transfer. *B*, WT versus TRAMP: $P < 0.01$ (day 5) and $P < 0.001$ (days 10 and 20). *C*, WT versus TRAMP mice: $P < 0.001$ on days 10 and 20. P values represent significant differences between experimental groups for data across all antigen concentrations ≥ 0.002 $\mu\text{g/mL}$. Mean \pm SE. Representative of three separate experiments. *nDLN*, nondraining lymph nodes, *pDLN*, prostate-draining lymph nodes.

Charles River Labs) were used at ages 10 to 12 weeks. The CD4⁺ TCR-transgenic mouse strain (TcR-II) bears a TCR gene that recognizes the I-A^k-restricted TAG₃₆₂₋₃₈₄ epitope (23). TcR-II mice were backcrossed and maintained on a C3H \times RAG^{-/-} background (23). The CD8⁺ TCR-transgenic mouse strain (TcR-I), which bears a TCR gene that recognizes the H-2K^k-restricted epitope TAG₅₆₀₋₅₆₈, was backcrossed and maintained on a C3H \times RAG^{-/-} background (24). TcR-I and TcR-II mice were bred one generation to Thy1.1⁺ \times RAG^{-/-} mice (*B6.PL-Thy1^o/Cy*; The Jackson Laboratory). National Cancer Institute-Frederick is accredited by Association for Assessment and Accreditation of Laboratory Animal Care-International and follows the USPHS Policy for the Care and Use of Laboratory Animals.

Peptides. TAG₃₆₂₋₃₈₄ (TNRFNDLLDRMDIMFGSTGSADI) and TAG₅₆₀₋₅₆₈ (SEFLLEKRI) peptides were purchased from New England Peptide. The peptides were dissolved at 2 mg/mL, filter sterilized, and stored at -20°C .

Adoptive transfer of transgenic lymphocytes. TcR-II and TcR-I mice were euthanized by CO₂ inhalation and the lymph nodes were aseptically

removed and minced into a single-cell suspension. Lymph node cells of TcR-I donor mice were $>95\%$ CD8⁺ (100% of Thy1.1⁺, CD8⁺ cells were tetramer⁺). Lymph node cells of TcR-II donor mice were $>90\%$ CD4⁺. Cells were labeled with 5 $\mu\text{mol/L}$ CFSE before adoptive transfer. Cell numbers were adjusted to 2×10^6 TcR-II cells or 3×10^6 Ag-specific TcR-I T cells and transferred intravenously. For cotransfer studies, TcR-II (Thy1.1⁻) T cells were transferred ~ 12 h before transfer of Thy1.1⁺, TcR-I T cells. To a portion of the cotransferred TRAMP mice (TcR-I + TcR-II), additional TcR-II T cells were transferred every 5 days for up to 15 days (TcR-I + Multi TcR-II). In some experiments, the drug FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol; Cayman Chemical) was supplied in the drinking water at 3.3 $\mu\text{g/mL}$ for an estimated daily dose of 0.5 mg/mouse. FTY720-containing drinking water was administered beginning 1 day before cell transfer and changed every 3 days.

Cell isolation. Single-cell suspensions were obtained from prostate-draining lymph nodes (iliac lymph nodes), nondraining lymph nodes

(inguinal lymph nodes), and prostates. Thy1.1⁺, TcR T cells were isolated using Thy1.1-specific antibodies and magnetic beads as described previously (22). Dendritic cells were isolated from single-cell suspensions of the prostate using the Miltenyi MACS cell separation system and the Pan-DC magnetic beads, which consist of anti-CD11c and anti-mPDCA-1 Ab. Cell separations were completed according to the manufacturer's instruction and consistently yielded purity of >90% CD11c⁺ cells.

Flow cytometry. Cell suspensions were blocked using supernatant from the 2.4G2 hybridoma, washed, and incubated with antibodies for 30 min on ice. For lymphocyte staining, the following panel of antibodies was used: Thy1.1-PE, CD8-Alexa Fluor 405, CD4-PerCy5, CD69-PE-Cy7, CD44-APC, CD25-APC-Cy7, CD62L-FITC, and CCR7-APC. For APC staining, the following panel of antibodies was used: CD11c-APC, B220-PerCP, CD40-PE, CD80-FITC, CD86-PE, and CD19-PerCy-7 (BD Pharmingen). Cells were analyzed on a BD LSR II flow cytometer and data were interpolated using FCS Express analysis software (De Novo Software). Expression of activation markers was determined for cells within the Thy1.1⁺ populations. Total cell counts for lymph nodes and spleens were not affected by transfer.

In vitro proliferation assays. Positively selected TcR T cells were used as responder cells in a proliferation assay; 2×10^4 T cells were stimulated with antigen and 1.5×10^5 irradiated splenocytes were isolated from WT mice. To measure stimulation by prostate-derived dendritic cells, 2×10^4 naive TcR-I T cells and 2×10^4 purified dendritic cells were cultured in the presence of TAG₅₆₀₋₅₆₈. After 72 h of culture, wells were pulsed with 1 μ Ci [³H]thymidine (Amersham) for 16 h. The cells were then harvested using a Cell Harvester (Tomtech) and radioactivity was measured in a Liquid Scintillation Counter (Trilux MicroBeta, Wallac).

ELISPOT assays. ELISPOT assays for measuring secretion of IL-2, IFN- γ , and granzyme B (GrB) were done as described previously (22). Briefly, multiscreen IP plates (Millipore) were coated with 100 μ L capture antibody (R&D Systems) overnight at 4°C. Plates were washed and then blocked with complete medium for 2 h at 37°C. Thy1.1⁺ T cells were purified as described above and 2×10^4 purified TcR-II or 1×10^4 TcR-I T cells, 7.5×10^5 WT splenocytes, and increasing concentrations of peptide were added to a final volume of 100 μ L/well and incubated for 36 h at 37°C. For the GrB ELISPOT assays, 2×10^3 isolated TcR-I T cells and 5×10^4 BW cells (an AKR-derived murine thymoma cell line that expresses H-2K^k) were added per well. BW cells were prepulsed with increasing concentrations of TAG₅₆₀₋₅₆₈. Plates were incubated with responder T cells for 4 h at 37°C. After incubation, plates were washed and incubated overnight at 4°C with 100 μ L biotinylated detecting antibody. Plates were washed, and 100 μ L streptavidin-conjugated alkaline phosphatase (Mabtech) was added to each well. Plates were incubated at room temperature for 2 h and washed, and spots were developed with 100 μ L Vector Blue substrate (Vector Laboratories) for 5 to 10 min in the dark. Spots were counted with an ImmunoSpot analyzer (Cellular Technology).

Statistical analysis. Data in this study were analyzed using descriptive and graphical techniques, univariate ANOVA, and standard posteriori (post hoc) tests for multiple comparisons (e.g., Tukey's and Dunnett's tests). CD4, IL-2, IFN- γ , and GrB data were transformed to their common logarithms to satisfy homogeneity of variance and normality requirements in the ANOVAs. Treatment conditions \times concentration levels were routinely tested for significant interaction effects, and appropriate post hoc comparisons applied as necessary. Graphical data are expressed as mean \pm SE unless otherwise indicated. All tests were two-sided; probability values < 0.05 were considered significant.

Results

TcR-II cells transferred into TRAMP mice proliferate in the lymph nodes and traffic to the prostate, where they become tolerized. We first sought to characterize the fate of CD4⁺ TcR-II T cells transferred into TRAMP mice. Naive, TcR-II T cells were transferred into 12-week-old male TRAMP or nontransgenic, WT mice. On days 3, 10, and 18 after transfer, Thy1.1⁺, CD4⁺ cells in the nondraining lymph nodes (inguinal lymph nodes), prostate-

draining lymph nodes (iliac lymph nodes), and prostate tissue were analyzed. TcR-II cells transferred into WT mice did not undergo proliferation as observed by the absence of CFSE dilution (data not shown). In contrast, TcR-II T cells transferred into TRAMP mice underwent multiple rounds of cell division within lymph nodes with maximal expansion occurring between 2 and 7 days after transfer and cells appreciably accumulating in the prostate 7 to 10 days after transfer (Supplementary Fig. S1). Interestingly, these observed kinetics were slower than those observed for TcR-I T cells, which fully diluted CFSE beyond detectable levels and were absent from secondary lymphoid tissues by 3 to 5 days after transfer and accumulated in the prostate as early as 3 days after transfer (22). Taken together, these data show that tumor antigen is presented to TcR-II T cells in the lymph nodes of TRAMP mice, which results in TcR-II T-cell proliferation and eventually trafficking to the prostate.

TcR-II T cells transiently exhibit effector functions after transfer into TRAMP mice. To determine whether TcR-II cells transferred into TRAMP mice gained effector functions, TcR-II T cells were isolated from the lymph nodes and prostate of TRAMP mice and tested for their ability to secrete IL-2 and IFN- γ in response to antigen. On day 3 after transfer, TcR-II cells isolated from the nondraining lymph nodes, prostate-draining lymph nodes, and spleen of TRAMP mice secreted IL-2 and IFN- γ in

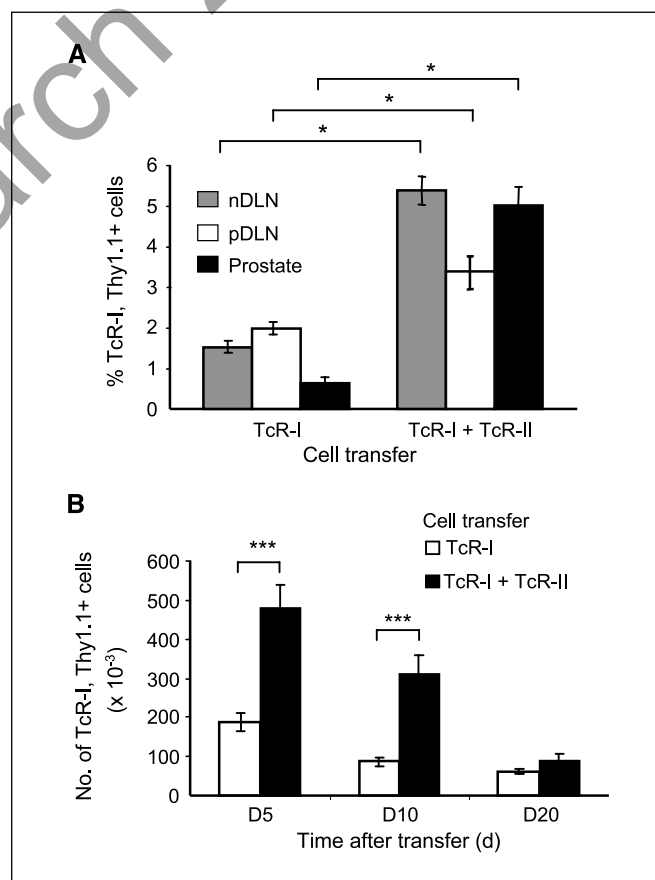
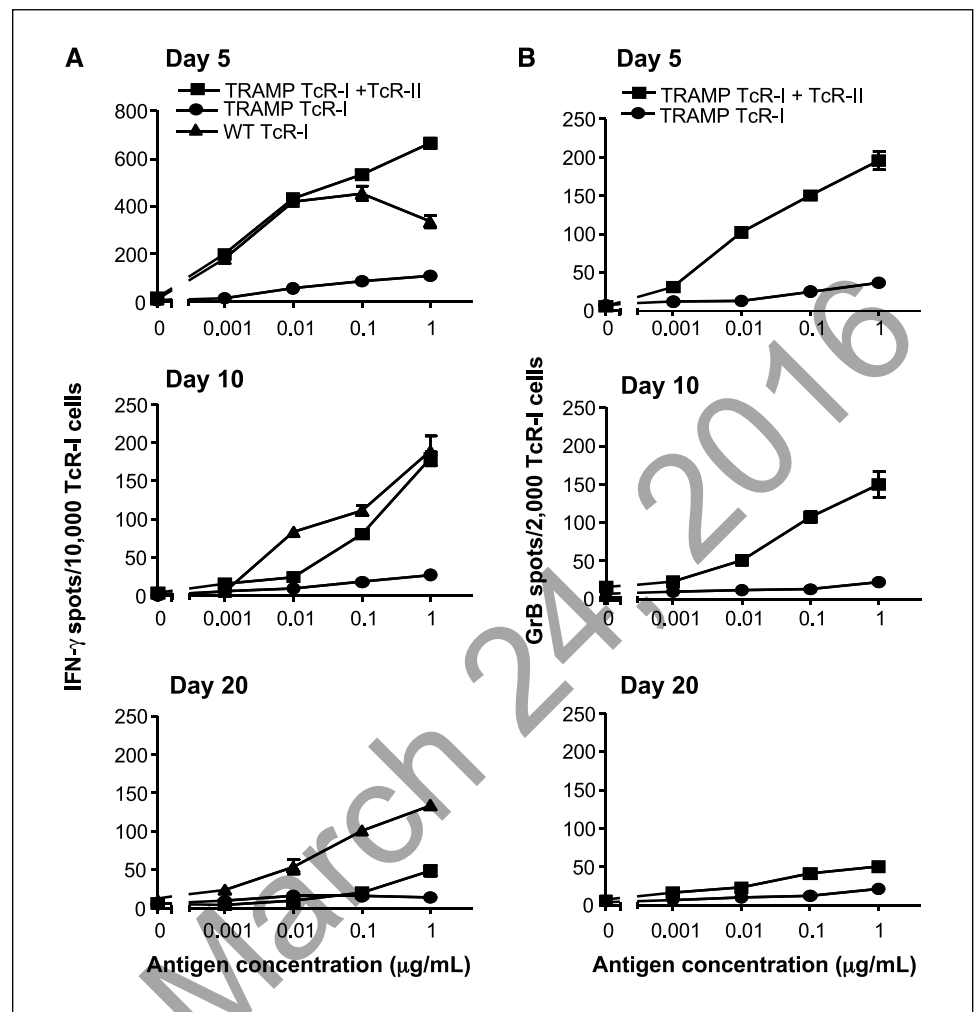


Figure 2. TcR-II cells enhance TcR-I cell priming and trafficking to the tumor. The percentage (A) and total number (B) of TcR-I cells present in nondraining lymph nodes, prostate-draining lymph nodes, or prostates of TRAMP mice at various times after transfer of the indicated cell populations. The percentage of TcR-I cells was determined by gating on the Thy1.1⁺, CD8⁺ population. Mean \pm SE. Representative of three (A) or five (B) separate experiments. *, $P < 0.05$; ***, $P < 0.001$.

Figure 3. TcR-II cell enhancement of antitumor effector functions is not sustained in the tumor microenvironment. The frequency of IFN- γ -secreting (A) and GrB-secreting (B) TcR-I cells from TRAMP mice receiving TcR-I cells alone or as cotransfer (TcR-I + TcR-II) was determined by ELISPOT assay. The number of IFN- γ -secreting TcR-I cells from the cotransfer group is significantly greater at days 5 and 10 after transfer ($P < 0.001$ for data across all antigen concentrations ≥ 0.001 $\mu\text{g/mL}$). The number of GrB-secreting TcR-I cells from the cotransfer group is significantly greater than TcR-I alone at all time points ($P < 0.001$ for data across all antigen concentrations ≥ 0.01 $\mu\text{g/mL}$). Mean \pm SE. Representative of five separate experiments.



response to restimulation with antigen (Fig. 1A). Similarly, they also proliferated in response to antigen (data not shown). TcR-II cells infiltrating the TRAMP prostate 5 days after transfer also secreted IL-2 and IFN- γ in response to antigen (Fig. 1B and C). However, by day 10 after transfer, prostate-infiltrating TcR-II T cells lost the ability to secrete IL-2 (Fig. 1B) while maintaining the ability to secrete IFN- γ (Fig. 1C); by 20 days after transfer, TcR-II cells completely lost all effector functions (Fig. 1B and C). Taken together, these data show that TcR-II cells initially acquire effector functions in lymph nodes of TRAMP mice, but similar to TcR-I, they become unresponsive as a result of persistence in the tumor microenvironment.

TcR-II T-cell enhancement of TcR-I T-cell priming and effector function is not durable in the tumor microenvironment. Previously, we reported that administration of a peptide-pulsed dendritic cell vaccine was capable of efficiently priming CD8⁺ TcR-I cells in TRAMP mice (22). To determine whether TcR-II cells could enhance priming and prevent the tolerization of TcR-I cells, we cotransferred TRAMP mice with naive TcR-I (Thy1.1⁺) and TcR-II T (Thy1.1⁻) cells. The frequency of TcR-I cells present in the nondraining lymph nodes, prostate-draining lymph nodes, and the prostate 3 days after cotransfer was increased compared with TcR-I transfer alone (Fig. 2A) and persisted at elevated numbers in the prostate for up to 10 days when cotransferred with TcR-II cells

(Fig. 2B). However, by 3 weeks after transfer, the frequency of TcR-I cells in the prostate of cotransferred mice was indistinguishable from those of TRAMP mice given TcR-I cells alone.

Although it is evident from the data presented in Fig. 2 that TcR-II cells enhance expansion of TcR-I cells, to achieve a successful antitumor immune response, tumor-specific T cells that traffic to the tumor must retain responsiveness to tumor antigens. Therefore, TcR-I cells cotransferred with TcR-II cells were tested for their ability to secrete IFN- γ and GrB. As reported previously (22), TcR-I cells transferred alone were unresponsive to stimulation with antigen by 5 days after transfer. However, TcR-I cells from mice that also received TcR-II cells retained the capacity to secrete IFN- γ and GrB for up to 2 weeks after cotransfer, but by 3 weeks after transfer, these effector functions were lost (Fig. 3A and B). This loss of TcR-I T-cell effector functions corresponds to the time frame shortly after which TcR-II cells become tolerized in the tumor microenvironment.

Sustained provision of TcR-II T cells prevents tolerance induction of TcR-I cells. Our observation that cotransfer of TcR-I and TcR-II cells only delays TcR-I cell tolerization suggests that long-term prevention of TcR-I cell tolerance induction may require more than enhanced priming. To test whether sustained provision of TcR-II-mediated help could maintain TcR-I cell effector functions, cotransfer studies were done where TRAMP mice

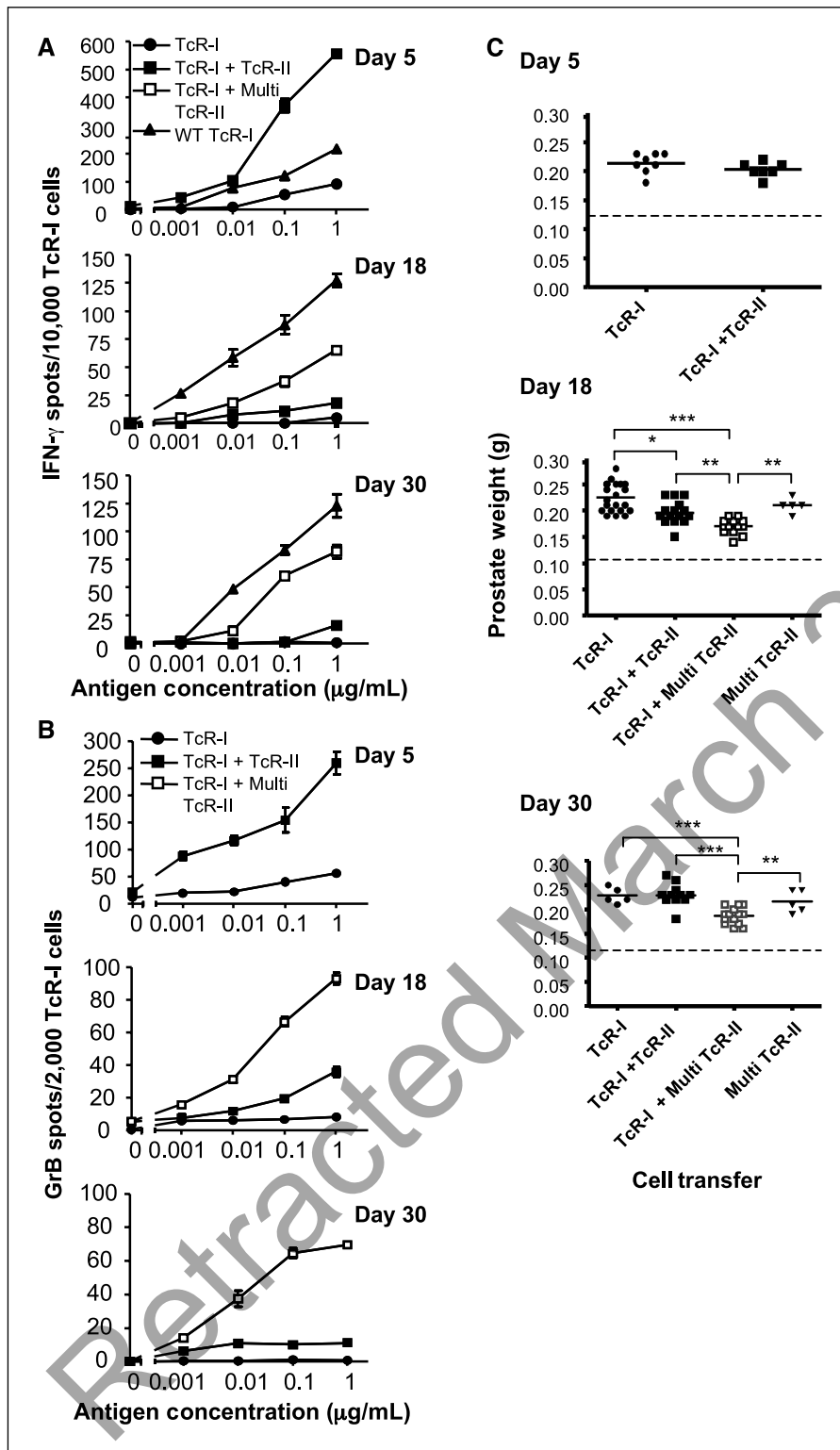


Figure 4. Continuous provision of TcR-II cells prevents the induction of tolerance of TcR-I cells. **A**, frequency of IFN- γ -secreting (A) or GrB-secreting (B) TcR-I cells isolated from TRAMP mice was determined by ELISPOT assay. Mice receiving TcR-I with multiple TcR-II cell transfers (TcR-I + Multi TcR-II) versus single cotransfer (TcR-I + cR-II) or TcR-I alone produced significantly greater IFN- γ ($P < 0.05$, all time points) and GrB ($P < 0.001$, day 5; $P < 0.05$, days 18 and 30). Mean \pm SE. Representative of three separate experiments. **C**, to estimate tumor burden, the prostatic complex was dissected and weighed. Dashed line, mean wet weight of WT prostate. In nontransferred TRAMP mice, the average prostatic weight was 0.25 ± 0.02 g. Dots, individual mouse; horizontal bars, group mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

received an infusion of TcR-II T cells every 5 days for 15 days. Based on our previous data, transferring TcR-II cells every 5 days should result in a consistent influx of functional, tumor-specific T helper cells into the prostate. In contrast to mice receiving a single transfer of TcR-II cells, TcR-I cells isolated from mice receiving multiple transfers of TcR-II T cells had a higher frequency

of antigen responsiveness up to 30 days after transfer, as measured by IFN- γ (Fig. 4A) and GrB secretion (Fig. 4B), and they maintained their capacity to secrete both effector molecules for at least 2 weeks after the last transfer of TcR-II T cells.

We next assessed whether the sustained CTL function of TcR-I cells conferred antitumor immunity in TRAMP mice using

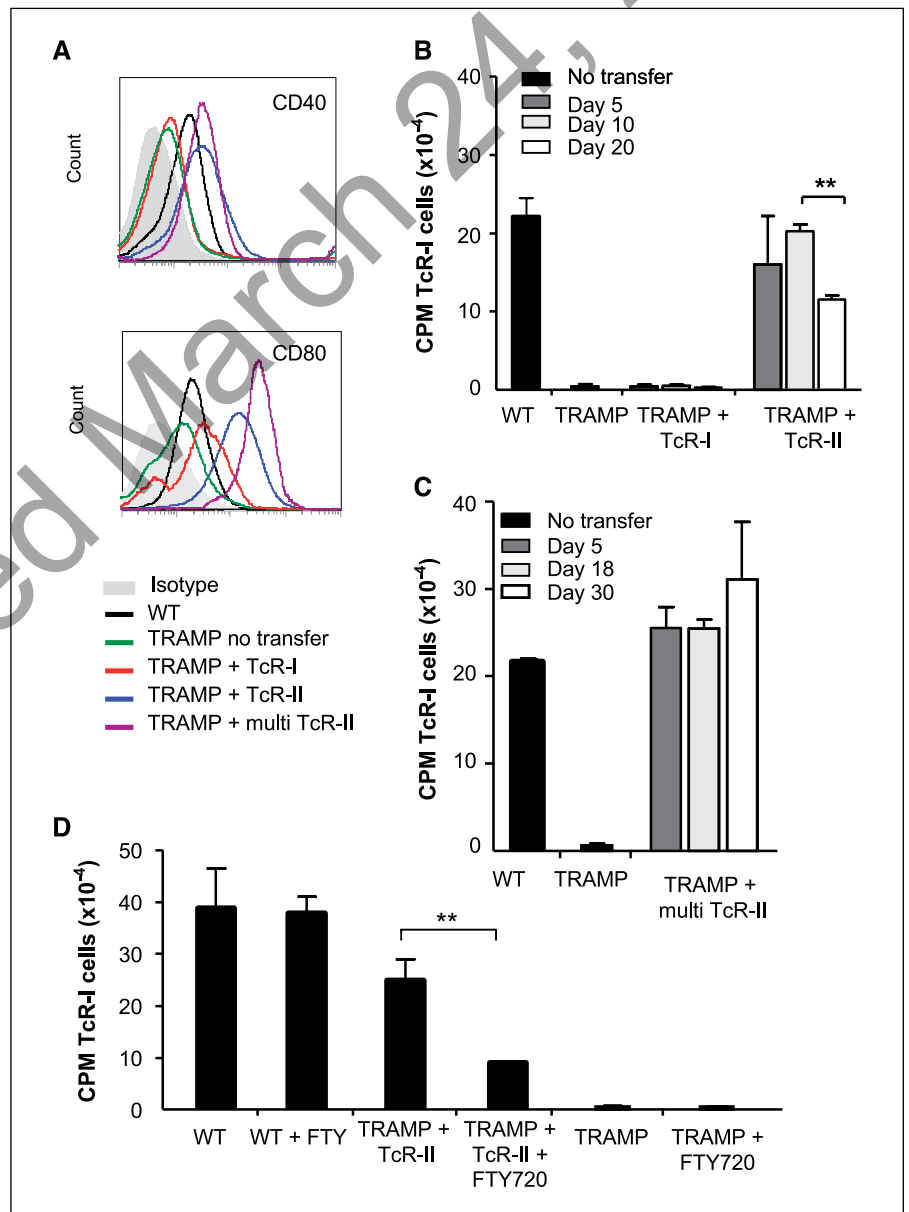
prostatic weight as an estimation of tumor progression (22, 25). Five days after transfer, no significant reduction in prostate weight was observed following the single TcR-II cell transfer (Fig. 4C). Eighteen days after transfer, both single and multiple transfers of TcR-II cells with TcR-I cells resulted in a reduction in tumor burden. However, by 30 days after TcR-I cell transfer, only TRAMP mice that received multiple transfers of TcR-II cells with TcR-I cells had significant reduction in prostate tumor burden (Fig. 4C). In contrast, at all time points, transfer of only TcR-I cells or only TcR-II cells had no significant effect on prostate weight. These findings are consistent with the sustained enhancement of TcR-I effector function in the presence of more durable antigen-specific CD4⁺ T-cell help.

APCs within the tumor microenvironment are targets of TcR II T cells. To determine the effect of TcR-II cells on the tumor microenvironment, we assessed the immunostimulatory capabilities of APCs isolated from the prostate of TRAMP mice before and

after transfer of tumor specific T cells. We first examined the phenotype of APCs purified from the prostate of each treatment group (routinely >90% CD11c⁺). These prostate-resident CD11c⁺ dendritic cells from both WT and TRAMP mice were B220⁺/CD11b⁻/CD19⁻, which did not change as a function of T-cell transfer (data not shown). However, transfer of TcR-II cells enhanced expression of costimulatory molecules (Fig. 5A). Prostatic dendritic cells from TRAMP mice receiving TcR-I cells alone had minimal surface expression of CD40, whereas a single or a multiple transfer of TcR-II cells into TRAMP mice dramatically increased expression of both CD40 and CD80.

To assess the function of prostate-derived dendritic cells, we examined their ability to stimulate T-cell proliferation *in vitro*. Dendritic cells isolated from the prostates of untransferred TRAMP mice, or TRAMP mice that only received TcR-I T cells alone, were unable to stimulate naive T-cell proliferation (Fig. 5B). However, dendritic cells isolated from prostates of mice that had received a

Figure 5. APCs from prostate tumors have an increased stimulatory ability after TcR-II cell transfer. APCs were isolated from WT or TRAMP prostates at the indicated time after transfer of TcR cells. **A**, phenotype of isolated APCs day 20 after T-cell transfer (similar results were observed on days 5 and 10 after transfer). **B** and **C**, APCs isolated from nontransferred or mice receiving single transfers of TcR-I or TcR-II cells were analyzed for the ability to stimulate naive CD8⁺ T-cell proliferation *in vitro*. Mean ± SE. ** $P = 0.0017$ (**B**). Representative of at least three separate experiments. **D**, mice were treated with FTY720 before TcR cell transfer and APCs were isolated 7 days after transfer of TcR cells and analyzed for the ability to stimulate naive CD8⁺ T-cell proliferation *in vitro*. Average ± SE counts/min (CPM). **, $P < 0.01$.



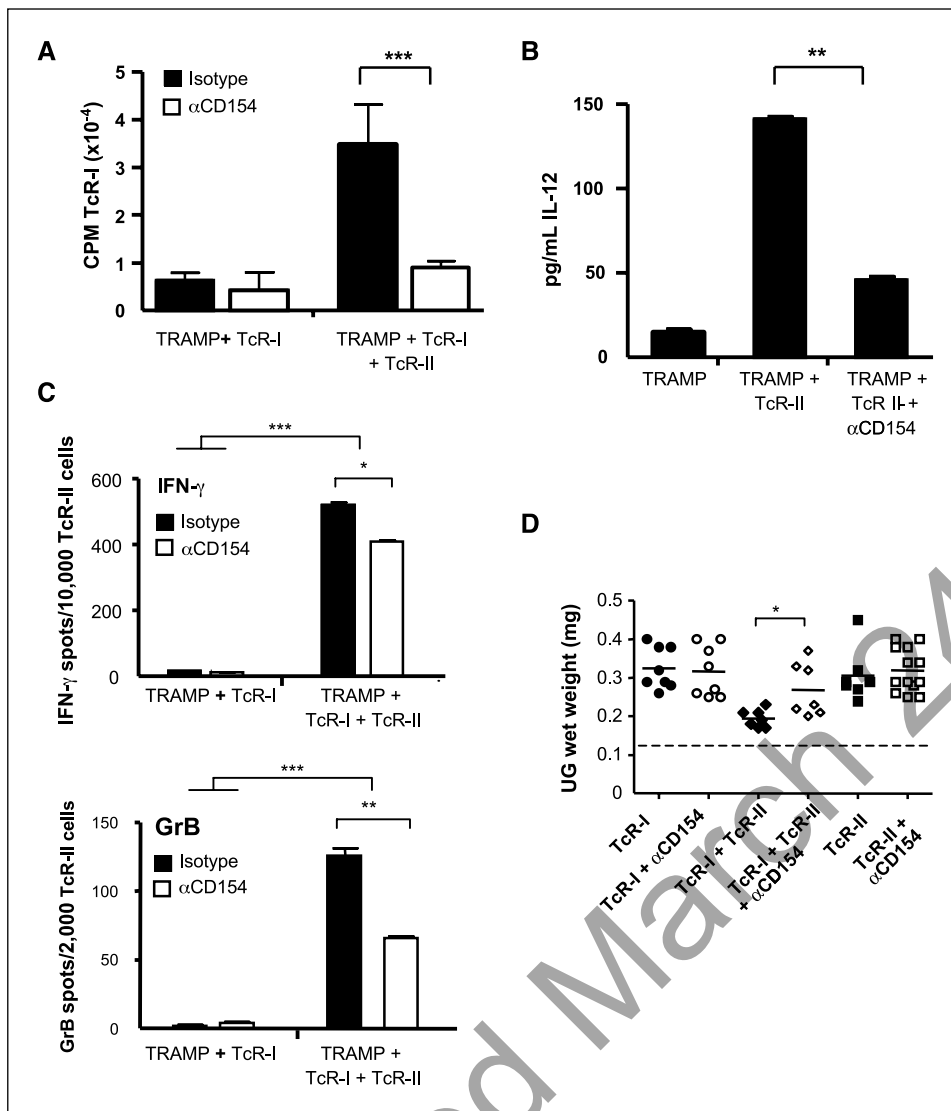


Figure 6. Blockade of CD40:CD154 reduced APC activation but only partially reduced TcR-I effector functions. APCs were isolated from the indicated treatment groups and used in stimulation assays as described in Fig. 5 (A) or cultured overnight and the supernatants tested for IL-12 expression (B). Mice were treated with anti-CD154 before T-cell transfer. T cells were purified from the indicated treatment groups 6 d after transfer and tested for the frequency of IFN- γ -expressing (top) and GrB-expressing (bottom) cells. Prostate weights were determined 12 d after T-cell transfers. Average \pm SE counts/min (CPM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

single transfer of naive TcR-II cells induced a strong proliferative response (Fig. 5B). The increased stimulatory capacity of dendritic cells from the single TcR-II T-cell transfer group was significantly decreased by 20 days after transfer (Fig. 5B), consistent with our observation that, by this time, TcR-II cells in the prostate were tolerized. In contrast, an increased stimulatory capacity of prostate-resident dendritic cells was sustained for up to 30 days following multiple transfer of TcR-II cells (Fig. 5C), showing that continued provision of T-cell help is needed to maintain dendritic cell stimulatory function.

To determine whether trafficking of TcR-II cells to the prostate is required for enhancing APC functions, we treated mice with FTY720, an immunomodulatory agent that inhibits the egress of activated lymphocytes from secondary lymphoid tissues. Following FTY720 treatment, the numbers of TcR-II T cells isolated from the prostate were profoundly reduced, with a concomitant increase in the number of TcR-II cells found in the lymph node (Supplementary Fig. S2). FTY720 treatment significantly reduced the stimulatory capacity of prostate-derived APCs isolated from TRAMP mice

receiving TcR-II cells (Fig. 5D). FTY720 treatment had no effect on the stimulatory capability of APCs isolated from WT animals or untransferred TRAMP animals. Similarly, FTY720 treatment had no effect on the *in vivo* stimulation of dendritic cells by anti-CD40 (Supplementary Fig. S3). Taken together, these data indicate that activation of tumor-specific helper T cells can enhance APC function, which is dependent on prostatic infiltration by the helper T cells.

We next sought to address the mechanism by which TcR-II cells activate prostate-resident dendritic cells. Mice were treated with an anti-CD154 antibody, which inhibits ligation of CD40 on APCs (19). TcR-II cell responsiveness was not significantly affected by CD154 blockade (Supplementary Fig. S4). In contrast, administration of anti-CD154 prevented the enhancement of dendritic cell stimulatory capacity and IL-12 production following TcR-II cell transfer (Fig. 6A and B). Surprisingly, whereas there was some reduction in TcR-I reactivity after CD154 blockade during cotransfer with TcR-II cells, prostate-infiltrating TcR-I cells still exhibited significant IFN- γ and GrB secretion (Fig. 6C). In parallel, blocking CD154

reduced the ability of TcR-II cells to help reduce tumor growth, although there were still mice that had reduced tumor burden (Fig. 6D). Taken together, these findings show that whereas the “licensing” of tumor-infiltrating APCs may be an important contributing factor to the enhancement of T-cell effector functions and tumor immunity, other mechanisms by which CD4⁺ T cells provide help to sustain antitumor immunity remain to be elucidated.

Discussion

Previously, we reported that in TRAMP mice, dendritic cells present tumor antigen to and prime CD8⁺ T cells in the prostate-draining lymph nodes (22) before their tolerization in the prostate. In the current study, we show that naive tumor-specific CD4⁺ TcR-II T cells also encounter tumor antigen in prostate-draining lymph nodes and become effector T-helper cells before infiltrating the prostate and subsequent tolerization. We further show that CD8⁺ tumor-specific T cells primed in the presence of activated CD4⁺ helper T cells had prolonged CTL effector functions in the tumor microenvironment. The extent of the response induced in the CD8⁺ T cells by CD4⁺ T cells is comparable with that induced by an *ex vivo*-matured, peptide-pulsed dendritic cell vaccine (22). However, over time, TcR-I cells cotransferred with TcR-II cells into TRAMP mice became tolerant in the tumor and the kinetics of TcR-I tolerization corresponded to tolerization of TcR-II cells in the TRAMP prostate. Thus, the transient helper activity of the cotransferred TcR-II cells was unable to overcome the profound immunosuppressive microenvironment of the TRAMP prostate and the TcR-I CTLs lost their antitumor potency. Ongoing studies are characterizing multiple mechanisms by which TRAMP tumors tolerize both TcR-I and TcR-II effector T cells.

In contrast to single cotransfer, the sustained provision of TcR-II-mediated help maintained TcR-I effector CTL functions that persisted for up to 30 days and resulted in a significant reduction in prostate tumor burden. These novel data show that activated helper T cells can prevent the tumor microenvironment from tolerizing TcR-I CTLs as long as the TcR-II cells retain their helper functions. Even more impressive are our data showing that given the trafficking patterns of the transferred cells, the TcR-II cells provide help and prevent tolerance within the tumor itself. The TRAMP prostate is a strongly immunosuppressive environment where transgenic expression of the oncogenic TAg protein leads to persistent tumor formation. Thus, overcoming tolerance via adoptive transfer of both CTL and helper T cells is an attractive approach. Our findings will have critical importance for cancer immunotherapy, as we show a clear-cut way to maintain effective tumor-specific CTLs in a highly immunosuppressive tumor microenvironment.

Although our data show that activated CD4⁺ tumor-specific T cells can provide help to CD8⁺ tumor-specific T cells and prevent their tolerization, the mechanism by which this occurs is only partially understood. Tumor microenvironments do not favor tumor-specific T-cell responsiveness (26) and many tumors produce factors that inhibit dendritic cell maturation (27–29) and thus inhibit their ability to sustain antitumor immunity. Results from our study show tumor-specific CD4⁺ T cells alter the tumor microenvironment and activate, or “license,” prostate-resident dendritic cells, which was dependent on TcR-II cells trafficking into the prostate and CD40:CD154 interactions. Staveley-O’Carroll and colleagues reported that activation of endogenous APC through CD40 ligation could prevent T-cell tolerization in a murine model of SV40 TAg-induced osteosarcomas (30). However, our data show that, even in the absence of activated tumor-infiltrating dendritic cells, TcR-I T cells maintained significant effector functions. Our ongoing studies are also testing the possibility that soluble factors secreted by TcR-II cells within the immunosuppressive tumor (e.g., IL-2 and IFN- γ) also contribute to sustaining CTL effector functions by TcR-I cells. Thus, to maintain tumor immunity, activated helper T cells may have a dual role of stimulating both effector CTLs and endogenous APCs.

Taken together, our findings show that sustained tumor-specific CD4⁺ T-cell help can elicit and maintain effective CTL effector functions. Thus, altering the tolerizing tumor microenvironment can maintain T-cell responses leading to sustained tumor immunity. By studying the ability of CD4⁺ T cells to enhance antitumor CTL activity as well as ways to reverse CD4 tolerance, we hope to elucidate novel approaches to stimulate a more potent antitumor immune response and eliminate development of T-cell tolerance to tumor antigens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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