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Partially Circumventing Peripheral Tolerance for Oncogene-Specific Prostate Cancer Immunotherapy

Yilin C. Neeley,[1] Mohamed S. Arredouani,[2] Brent Hollenbeck,[1] Marvin H. Eng,[1] Mark A. Rubin,[1] and Martin G. Sanda[2]*

[1]Departments of Urology, Surgery, and Pathology, University of Michigan, Ann Arbor, Michigan
[2]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

BACKGROUND. Failure of cancer immunotherapy is essentially due to immunological tolerance to tumor-associated antigens (TAAs), as these antigens are also expressed in healthy tissues.

METHODS. Here, we used transgenic adenocarcinoma of mouse prostate (TRAMP) mice, which develop lethal prostate cancer due to prostate-specific expression of SV40 T antigen (Tag), to evaluate effects of prostatic transformation on oncogene TAA-specific tolerance and to test the possibility of breaking such tolerance using a modified recombinant vaccinia virus.

RESULTS. We showed that Tag expression in TRAMP mice is uniquely extra-thymic, and levels of prostatic Tag expression positively correlate with malignant transformation of the prostate. Yet, young tumor-free TRAMP mice were tolerant to Tag antigen. We therefore attempted overcoming such peripheral oncogene-specific T cell tolerance through immunization with a vaccinia construct encoding Tag immunogenic epitopes. This vaccination modality showed that oncogene-specific tolerance was successfully overcome by effective in vivo priming of Tag-specific cytotoxic T cells (CTLs). However, this was restricted to young TRAMP mice. Tag-specific CTL from ''tumor naïve'' young TRAMP mice showed significant anti-tumor efficacy in vivo by eliminating established heterotopic prostate tumors and prolonging survival in SCID mice harboring Tag-expressing tumors. In contrast, older TRAMP mice with established prostate tumors exhibited oncogene-specific tolerance as evidenced by failure to generate Tag-specific CTL following Tag-specific immunization.

CONCLUSIONS. Peripheral tolerance can be overcome for effective anti-tumor therapy following oncogene-specific immunization. However, this ability to elicit oncogene-specific CTL is impeded in the tumor-bearing host, in the context of increased oncogene expression associated with tumor progression. *Prostate 68: 715–727, 2008.*

KEY WORDS: **prostate cancer; peripheral tolerance; recombinant vaccinia**

INTRODUCTION

Central immune tolerance plays a critical role in preventing autoimmune diseases by depleting potentially self-reactive lymphocytes from the T cell repertoire. However, as most tumor-associated antigens (TAAs) can also be found in normal tissues, T cell-mediated response to tumors is often impeded by T cell tolerance to TAAs, leading to malignant transformation and aggressive tumor growth [1–5]. Therefore, breaking tolerance to TAAs represents the principal challenge in developing effective anti-tumor immunotherapy [6].

Recent reports showed that immunization of animals using T cell-dependent tumor antigen-specific

Abbreviations: CTL, cytotoxic T lymphocyte; TAA, tumor-associated antigen; Tag, SV40 large T antigen; TRAMP, transgenic adenocarcinoma of mouse prostate; vac-mTag, safety-modified Tag-expressing vaccinia virus.

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Mark A. Rubin's present address is Department of Pathology, Cornell University School of Medicine, New York, NY.

*Correspondence to: Martin G. Sanda, MD, BIDMC/Division of Urology, Rabb 440, 330 Brookline Ave, Boston, MA 02115.
E-mail: msanda@bidmc.harvard.edu
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vaccines, such as recombinant vaccinia virus and whole-cell vaccines, has proved effective at eliciting a protective response in TAA tolerant transgenic hosts against autologous tumor challenge [7–11]. Likewise, although not being able to achieve a complete eradication of spontaneous tumors, some T cell-mediated vaccines sufficiently delay the onset of primary tumors [8,9]. For prostate cancer immunotherapy, irradiated whole cell-based tumor vaccines have shown some potential in controlling heterotopic tumor growth in non-transgenic mice [12]. Also, using prostate-specific antigen (PSA) transgenic mouse as a model, Wei et al. [13] have demonstrated that tumor-infiltrating lymphocytes (TILs) were able to lyse PSA-expressing tumor targets in vitro.

In transgenic adenocarcinoma of mouse prostate (TRAMP) mice, the oncogenic simian virus large T antigen (SV40 Tag) is expressed specifically in the prostate in an androgen-dependent fashion, leading to lethal prostate cancer in older mice [14,15]. The striking resemblance between the TRAMP mouse model and prostate cancer progression in humans provides a unique pre-clinical model for prostate cancer therapy. Although previous works [16,17] have reported that therapy with antibodies to CTLA-4 effectively inhibited the outgrowth of transplantable tumors as well as orthotopic prostate tumors in TRAMP mice, the lack of evidence of complete eradication of the Tag oncogene and the partial destruction of normal prostatic epithelium clearly elucidates the need for tumor antigen-specific immunotherapy. We have previously reported that a safety-modified recombinant vaccinia virus, vac-mTag, is able to elicit SV40 Tag-specific CTL activity and protect non-transgenic mice against Tag-expressing tumors as well as treat pre-established Tag-expressing microscopic tumors [18]. Here, we first determined whether extrathymic expression of the oncogenic Tag results in peripheral tolerance in TRAMP mice. Studies were then undertaken to evaluate the ability of vac-mTag to break tolerance in TRAMP mice by inducing Tag-specific CTL activity. Finally, we conducted experiments to assess the efficacy of adoptive transfer of vac-mTag-elicited Tag-specific CTLs in the treatment of heterotopic and orthotopic tumors in vivo.

MATERIALS AND METHODS

Cell Lines

B6wt19 (provided by S. Tevethia, Pennsylvania State University College of Medicine, Hershey, PA) and mKSA (provided by J. Butel, Baylor College of Medicine, Houston, TX) are SV40 Tag-expressing cell lines of H-2^b and H-2^d haplotype, respectively. RM-1 (provided by T. Thompson, Baylor College of Medicine, Houston, TX) is a prostate cancer cell line with H-2^b haplotype that lacks Tag. NK sensitive YAC-1 cell line was obtained from the American Type Culture Collection (Manassas, VA). All these cell lines were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 100 U of penicillin, 100 mg of streptomycin per ml, and 10% fetal bovine serum (Life Technologies, Inc.) at 37°C and 5% CO_2.

Mouse T cell lymphoma line E22 (H-2^b) and colon carcinoma cell line CT26.CL25 (H-2^d) (kindly provided by Dr. Nicholas Restifo at NCI) express β-galactosidase and were maintained in complete medium containing 400 μg/ml G418.

Animals

Six- to 8-week-old male C57BL/6 mice were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN). C57Bl/6-Prkdc (SCID) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The transgenic adenocarcinoma of the mouse prostate (TRAMP) mice were described previously [14] and maintained by our laboratory personnel. In this study, the transgenic mice were all hemizygous (rPB-Tag+/−) progeny of TRAMP 4741, a progeny of founder mouse 8247 which had been previously provided by Dr. Greenberg (Baylor College of Medicine, Houston, TX). The transgenic colony was maintained in the C57BL/6 strain, with transgene carriers mating to C57BL/6 breeders to consistently yield hemizygous progeny. Genotyping was performed via Tag-specific PCR [15]. All experiments were approved by the University of Michigan Committee on Use and Care of Animals and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Hematoxylin-Eosin Staining

Histological analysis was performed on normal prostate tissue and prostate tumors. Tissues collected at necropsy were fixed in 10% (vol/vol) buffered formalin, transferred to 70% (vol/vol) ethanol, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin/eosin.

Immunohistochemistry

TRAMP prostates were frozen upon necropsy in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA) and sectioned at 5 μm. Biotin-conjugated antibodies (BD PharMingen, San Diego, CA) against mouse CD3 (1:1,200), CD4 (1:800), or CD8 (1:1,200) were used to stain T lymphocytes. ExtrAvidin-peroxidase (Sigma, St. Louis, MO; 1:100) was used as secondary reagent. All slides were counterstained with hematoxylin.

Construction of Recombinant Vaccinia Encoding an SV40 Tag Fragment

The recombinant vaccinia encoding a SV40 Tag fragment (mTag) was constructed as described previously [18]. Briefly, vac-mTag was generated by homologous recombination in BSC-1 cells transfected with pSC65-mTag, with the recombinant BSC-1 plaques identified by immunocytostaining with Tag-specific monoclonal antibody Pab204 (provided by Dr. J. Pipas, University of Pittsburgh, PA) as previously described [18].

Reverse Transcription-Coupled PCR

Total cellular RNA was extracted from prostates of normal C57BL/6 or TRAMP mice using RNAzol B (Tel-Test, Friendswood, TX) and quantified by spectrophotometry. Reverse transcription was performed using the Promega Reverse Transcription System (Promega Corp., Madison, WI). PCR amplification was performed with 5′ (5′-TGGTTCTACAGGCTCTG-CTGAC-3′) and 3′ (5′-CCAATCTCTCTTTCCACT-CCAC-3′) Tag primers. Electrophoresis of PCR products was run on 1% agarose gels (Boehringer Mannheim, Indianapolis, IN), and visualized with ethidium bromide. β-actin or β-globin were used as internal controls for the loading.

Southern Blot Analysis

Plasmid rPB-Tag (kindly provided by Dr. Norman Greenberg, Baylor College of Medicine, Huston, TX) [14] was used as template for making a Tag cDNA probe by PCR. PCR amplification was performed with 5′ (5′-CCGGTCGACCGGAAGCTTCCACAAGTGCA-TTTA-3′) and 3′ (5′-CTCCTTTCAAGACCTAGAAGG-TCCA-3′) Tag primers and digoxigenin (DIG) coupled dUTP using DIG high prime DNA labeling and detection starter kit II (Boehringer Mannheim). Ten micrograms of RT-PCR product (above) was loaded into each lane for Southern blot analysis. The 442bp DIG-labeled PCR product was then hybridized to membrane blotted nucleic acids according to the manufacturer's protocol.

Western Blot Analysis

Tissues were rinsed with PBS, and homogenized with a manual homogenizer (Thomas, Philadelphia, PA), and then resuspended in protein lysis buffer (5 mM Tris, 120 mM NaCl, 0.5% IGEPAL, Sigma) with protease inhibitors phenylmeth-ylsulfonyl fluoride, leupeptin, and aprotinin (Boehringer Mannheim). The suspension was then sonicated at 23 kHz for 5 sec bursts (Microson Ultrasonic cell disrupter, Heat Systems, Inc., Farmingdale, NY) and centrifuged at 14,000*g* for 15 min at 4°C. Protein concentration in the supernatant was calculated using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Forty micrograms of protein were separated using an 8% Tris–Glycine gel run at 125 V for 2 hr and transferred to nitrocellulose at 25 V for 2 hr (Novex Xcell IITM system, Novex, San Diego, CA). After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 hr at room temperature, the membrane was incubated with a Rabbit-Polyclonal anti-Tag antibody (1:5,000; provided by Dr. Janet Butel, Baylor College of Medicine, Houston, TX) at 4°C in 1.5% milk in TBS-T overnight. Following three washes in TBS-T, the membrane was incubated with HRP-conjugated donkey anti-rabbit IgG antibody (1:5,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1.5 hr at room temperature, and visualized using ECL detection reagents (Amersham, Arlington Heights, IL).

SV40 Tag-Specific Cytotoxic T-Lymphocyte (CTL) Activity

Splenocytes from C57BL/6 or TRAMP mice were harvested 3 weeks after intravenous (i.v.) injection with either vac-mTag or a control vaccinia rVV-β-gal at 10^7 pfu/mouse. CTL activity was evaluated by ^{51}Cr release assay after 1 week of splenocyte stimulation with either mitomycin C (Sigma)-treated syngeneic Tag-expressing tumor cells or β-galactosidase peptide and 10 U/ml rIL-2 in vitro. ^{51}Cr-labeled target cells were incubated with splenocytes at ratios of 33, 11, and 3.7 for 4 hr, and lysates were harvested and analyzed as described previously [18]. Percent specific lysis was calculated from triplicate samples as follows: [(experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm)] × 100. Data included in this report represent all assays in which spontaneous release of labeled target cells was less than 20% of maximal release, and standard deviation of triplicate values were less than 15%. Similar experiments were repeated at least three times.

Heterotopic Transplantable TRAMP Prostate Tumor

TRAMP mouse #870 was an offspring of a heterozygous female TRAMP (#465) and a non-transgenic C57BL/6 male mouse. Its Tag-positive genotype was tested by Tag PCR. Primary prostate tumor was isolated aseptically from TRAMP mouse #870 at 31-week of age upon death and rinsed in sterile PBS. The Tumor was then cut into small chunks of 3–4 mm in diameter. C57BL/6, TRAMP, and SCID mice were anesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally (i.p.). A small cut was made on the skin of the recipient mouse on its right flank. A tumor chunk was inserted underneath the skin through the

cut. The incision was then stapled with a sterile AUTOCLIP applier (Becton Dickinson, Sparks, MD). Two weeks later, the clip was removed with an AUTOCLIP remover (Becton Dickinson, MD). Tumor growth was monitored three times a week. Western blot analysis was performed to confirm the expression of Tag by the 870 tumor chunks.

Adoptive Transfer

For immunotherapy of Tag-expressing heterotopic tumors in vivo, splenocytes from vac-mTag-immunized 2-month-old TRAMP mice or V69-immunized age-matching C57BL/6 mice were stimulated in vitro with mitomycin C-treated B6wt19 cells and 10 U/ml rIL-2 for 1 week. TRAMP mice-derived Tag-expressing prostate tumor chunks were implanted subcutaneously (s.c.) into SCID mice 2 days before adoptive transfer. Ten million CTLs were collected from culture and injected i.v. to each tumor-bearing SCID mouse followed by 5 days of rIL-2 therapy (30,000 IU/ml, i.p., twice a day). For therapy of orthotopic prostate tumors, splenocytes from vac-m Tag-immunized young tumor-free TRAMP mice and C57BL/6 mice, or control vaccinia V69-immunized C57BL/6 mice were stimulated in vitro with mitomycin C-treated B6wt19 cells and 10 U/ml rIL-2 for one week. Four- to 5-month-old TRAMP mice were given 10^7 Tag-specific CTLs each through tail vein injection followed by 5 days of rIL-2 therapy (30,000 IU/ml, i.p., twice a day). A second dose of CTL adoptive transfer was given 8–9 weeks after the first treatment. Tumor growth and related death in all recipient mice were monitored two to three times weekly by an individual who was blinded to the immunization status.

FACS Analysis

Splenocytes from either vac-mTag- or V69-immunized C57BL/6 and TRAMP mice were cultured with mitomycin C-treated B6wt19 tumor cells and 10 U/ml rIL-2 for 7 days in vitro. Single cell suspension was obtained from cell culture and washed twice with PBS supplemented with 0.1% bovine serum albumin and 0.1% sodium azide. One million cells were incubated with 0.5 μg of a FITC-conjugated anti-CD8 antibody and PE-conjugated anti-CD25 antibody (BD PharMingen) in a final volume of 50 μl at 4°C for 30–40 min. A total of 10,000 viable cells were analyzed per sample in a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA).

Statistical Analysis

Statistical significance for survival data was evaluated by Kaplan–Meier plots and logrank analysis was performed with Statistica software (StatSoft, Inc., Tulsa, OK).

RESULTS

Characterization of SV40 Tag Expression Pattern in TRAMP Mice

We first sought to characterize patterns of SV40 Tag expression as TRAMP mice age and undergo malignant prostate transformation. To this aim, protein lysates were prepared from prostates of both non-transgenic B6 and TRAMP mice. Western blot analysis of Tag using mouse prostate tissue lysates showed that TRAMP mice (748 and 3512) with palpable prostate tumors exhibit significantly higher levels of Tag expression compared to cancer-free TRAMP mice (2-month-old mice 3191 and 3192; Fig. 1A). Low levels of constitutive Tag transcripts were detectable by RT-PCR followed by Southern blot in both 6-month-old cancer-bearing and 2-month-old cancer-free TRAMP mice (Fig. 1A). These results suggest that malignant prostate transformation is associated with increased level of Tag oncogene expression in TRAMP prostates.

We then evaluated relative central (thymic) and peripheral (prostatic) expression of SV40 Tag in TRAMP mice. Total RNA was extracted from the thymus and prostates of both young and old TRAMP and non-transgenic C57BL/6 mice. SV40 Tag transcripts were not detected by RT-PCR in neither TRAMP nor B6 thymi, irrespective of their age (Fig. 1B). In contrast, prostates from both 2- and 6-month-old TRAMP mice expressed Tag whereas age-matched C57BL/6 control mice did not (Fig. 1B). This result is consistent with a previous report where Tag transcript was only detectable in dorsal and ventral prostate tissue in TRAMP mice [14]. Therefore, the tissue distribution of the androgen-responsive rat probasin promoter (rPB)-controlled SV40 Tag in the TRAMP mice is restricted to the prostates.

Evidence for Age-Independent Peripheral Tolerance to Tag in TRAMP Mice

To more directly evaluate tolerance to Tag oncogene in TRAMP mice, we compared engraftment of Tag-expressing prostate tumors in control C57BL/6, TRAMP, and B6 SCID mice. While tumor engraftment failed in 7 out of 8 C57BL/6 mice, 12 out of 13 SCID mice developed palpable subcutaneous tumors (Table I). Surprisingly, all 5 old (>4-month-old) as well as 12 juvenile (<2-month-old) TRAMP mice developed subcutaneous tumors (Table I), suggesting that TRAMP mice develop tolerance to Tag oncogene regardless of their age and the status of prostate transformation.

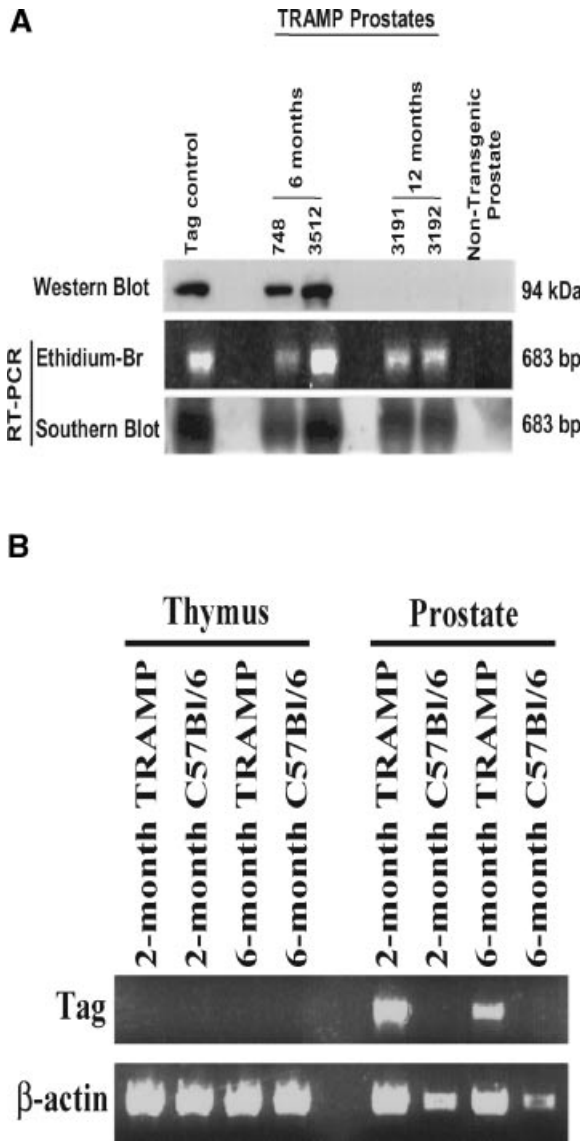


Fig. 1. Prostatic and thymic expression of Tag oncogene in aging TRAMP mice. **A**: Prostate tissue lysates of 6-month-old tumor bearing TRAMP mice (748 and 3512) and 2-month-old tumor-free TRAMP mice (3191 and 3192) were assessed for Tag protein expression by Western blot (**upper panel**). Western blot was performed using rabbit anti-SV40 Tag polyclonal antibody (1:5,000). Forty micrograms of total protein was loaded in each lane. Total RNA extracted from the same TRAMP prostates were analyzed for Tag gene expression by RT-PCR (**lower panel**). Southern blot was performed following RT-PCR using Tag cDNA as probe. Ten micrograms of RT-PCR product was loaded in each lane for the Southern blot. Tag expressing mKSA tumor and non-transgenic prostate were used as positive and negative controls, respectively, for both Western blot and RT-PCR. **B**: RT-PCR was performed to detect Tag transcripts in prostates and thymus of 2- and 6-month-old TRAMP and non-transgenic C57BL/6 mice (**upper panel**). The quality of the total RNA from tissue was evaluated by β-actin primers in a parallel RT-PCR reaction (**lower panel**). One representative out of three separate experiments is shown.

vac-mTag Does Not Induce Prostate Inflammation

Having established the correlation between prostatic expression of Tag oncogene and tumor growth, and the tolerance to Tag oncogene in TRAMP mice, we subsequently addressed the possibility of breaking tolerance to Tag by immunizing mice with a safety modified recombinant vaccinia virus, vac-mTag. A unique safety modified fragment of Tag cDNA encoding the five H-2^b-restricted immunogenic epitopes was introduced into the vaccinia virus through homologous recombination to generate vac-mTag [18]. Because previous reports regarding inflammatory damage that can take place in the prostate following immunization against prostate-specific antigens remain controversial [19–22], we first examined mouse prostates for prostatic lymphocyte infiltration and autoimmune prostatitis following immunization with vac-mTag. Histopathologic analysis showed no significant inflammation in the prostates of 2-month-old TRAMP mice after immunization with vac-mTag (Fig. 2B) as compared to vac-mTag immunized C57BL/6 prostates (Fig. 2A) or control vaccinia immunized TRAMP prostates of the same age (Fig. 2C). In addition, although histochemistry of the prostates from 6-month-old TRAMP mice showed high grade, poorly differentiated unstructured prostate carcinoma (Fig. 2F), immunization with vac-mTag did not cause any significant inflammatory infiltrates (Fig. 2E), similar to what we observed in prostates of vac-mTag immunized C57BL/6 mice of the same age (Fig. 2D). Further evidence was obtained by immunohistostaining with anti-CD3, -CD4, or -CD8 antibodies, pointing to the absence of any significant T lymphocyte infiltrates in the TRAMP prostates following vac-mTag immunization (data not shown).

The Ability of vac-mTag to Elicit Tag-Specific CTL Is Limited to Tumor-Free Mice

We next tested the interference of prostate malignant transformation with the ability to induce Tag-specific CTL activity in TRAMP mice by Tag-specific immunization. Immunization with vac-mTag was able to generate a Tag-specific MHC-restricted CTL response in control C57BL/6 mice, whereas tolerance against autologous Tag oncogene was evident in 5-month-old TRAMP mice, as judged by absence of Tag-specific CTL activity (Fig. 3). Interestingly enough, immunization of young tumor-free TRAMP mice with vac-mTag prior to malignant transformation was successful in circumventing Tag-specific tolerance. The cytolytic activity against Tag-expressing syngeneic tumor target (B6wt19, H-2^b) in vac-mTag immunized young TRAMP was significant and comparable to that achieved by vac-mTag immunized C57BL/6 mice (Fig. 3). No significant CTL activity was shown against

TABLE I. TRAMP Mice Are Tolerant to Engraftment of Tag-Expressing Prostate Tumor Chunks

			TRAMP mice	
	Non-transgenic mice	SCID mice	Juvenile (≤2 months)	Mature (>4 months)
Proportion engraftment	1/8	12/13	12/12	5/5

C57Bl/6, SCID, and TRAMP mice of different ages were implanted with chunks of a TRAMP prostate tumor. They were followed for palpable subcutaneous tumor growth and progression. Numbers indicate proportion of engrafted mice that developed subcutaneous tumors.

neither a Tag-negative syngeneic tumor target (RM-1, H-2^b) nor a Tag-expressing tumor target of a different MHC haplotype (mKSA, H-2^d), suggesting that the CTL response induced by vac-mTag in 2-month-old TRAMP mice was Tag-specific and H-2^b restricted (Fig. 3). Tag specificity of potentiated tolerance in older TRAMP mice was evidenced by competent anti-β galactosidase (a heterologous antigen) responsiveness induced by rVV-β-gal immunization, excluding the possibility of global immunosuppression induced by tumors (Fig. 3). Taken together, these data demonstrate that the tolerance to autologous oncogene can be overcome via induction of oncogene-specific CTL by recombinant vaccinia immunization under conditions that minimize peripheral expression of the oncogene tolerogen by the host.

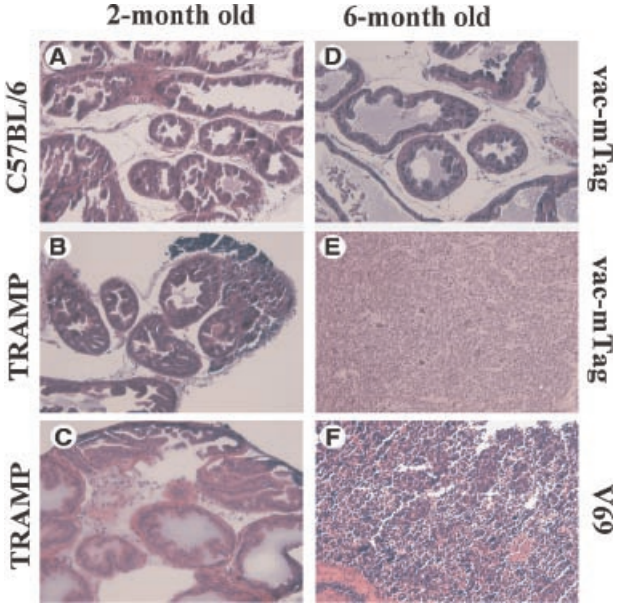


Fig. 2. Immunization of vac-mTag did not induce significant inflammation in TRAMP prostates. Two- and 6-month-old male C57BL/6 and TRAMP mice were immunized intravenously with vac-mTag and V69. Three weeks later the mice were euthanized, their prostates removed and fixed with 10% buffered formalin. Paraffin-embedded prostates were prepared and stained with hematoxylin-eosin. Magnification, 20×.

To assess the potential of vac-mTag induced Tag-specific CTL from young TRAMP mice in treating heterotopic Tag-expressing tumors in vivo, SCID mice were implanted subcutaneously with Tag-expressing 870 transplantable TRAMP prostate tumor. Two days later, CTLs induced by vac-mTag or V69 control vaccinia immunization of 2-month-old TRAMP mice or C57BL/6 mice were adoptively transferred to tumor-bearing SCID mice. As shown in Figure 4, Tag-specific CTLs from vac-mTag-immunized young TRAMP mice, but not CTLs from V69-immunized C57BL/6 mice, were able to both significantly delay the onset of the implanted Tag-expressing tumors in SCID mice (Fig. 4A) and prolong the survival of tumor bearing animals (Fig. 4B). Hence, Tag-specific CTLs induced with vac-mTag in young TRAMP mice were effective in treating Tag-expressing heterotopic tumors in vivo.

Vac-mTag-Induced Tag-Specific CTL From TRAMP Mice Fail to Mount a Response Against Orthotopic Tumors

We further sought to evaluate the efficacy of vac-mTag-induced Tag-specific CTLs in targeting pre-established Tag-expressing orthotopic prostate tumors in TRAMP mice. To this end, we adoptively transferred Tag-specific CTLs from immunized C57BL/6 and young tumor-free TRAMP mice to 4- to 5-month-old tumor-bearing TRAMP mice. Although CTLs from vac-mTag-immunized young TRAMP mice significantly improved the survival of SCID hosts with heterotopic tumors (Fig. 4), they were not as effective against orthotopic prostate tumors in older TRAMP mice (Fig. 5A). In contrast, Tag-specific CTLs from vac-mTag-immunized C57BL/6 mice generated an effective immune response against primary prostate tumors in almost 30% of the hosts (Fig. 5A). Therefore, we sought to reassess possible phenotypic differences between Tag-specific CTLs from C57BL/6 and TRAMP mice by evaluating CTL surface CD25 expression. CD25 expression on the $CD8^+$ T cell population of cultured splenocytes from vac-mTag-immunized C57BL/6 mice (Fig. 5B, upper panel) was significantly greater than that from vac-mTag-immunized TRAMP

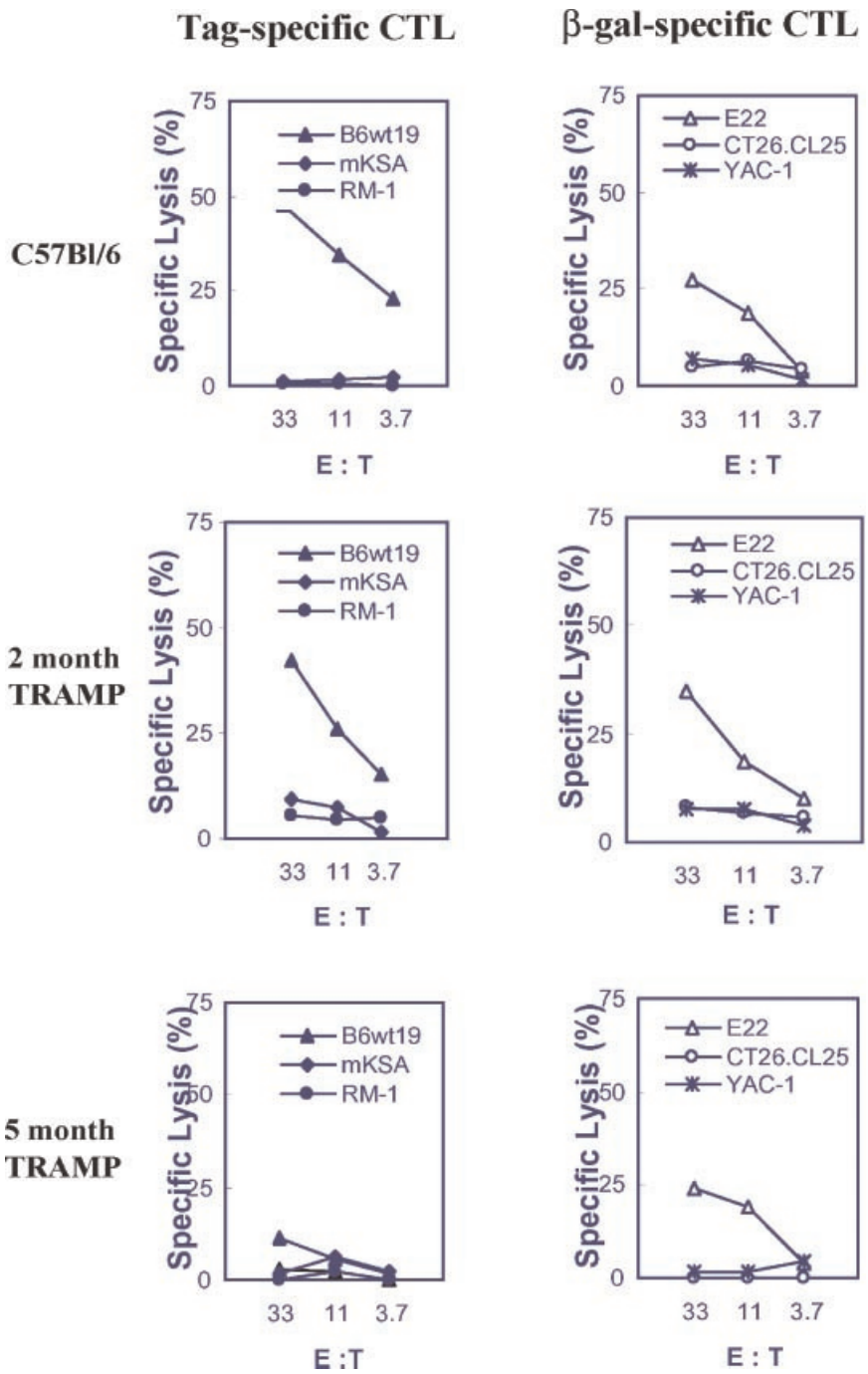


Fig. 3. The ability of vac-mTag immunization to break Tag-specific tolerance in TRAMP mice is impeded by malignant transformation and progressing prostatic tumors. TRAMP mice of 2- and 5-month of age and non-transgenic C57BL/6 mice were immunized with 10^7 pfu/mouse of vac-mTag or rVV-β-gal (control vaccinia) administered by tail vein injection. Three weeks post-immunization, splenocytes were stimulated in vitro with either mitomycin C-treated syngeneic Tag-expressing tumor cells for Tag-specific CTL, or with β-galactosidase peptide for β-galactosidase-specific CTL. Seven days later, chromium release assay was performed to assess cytolytic T cell activity. Tag-expressing B6wt19 (H-2^b), mKSA (H-2^d) cells, and Tag negative RM-1 cells (H-2^b) were used as targets for detecting Tag-specific CTL activity. To detect β-gal-specific CTL activity, we used β-gal expressing E22 (H-2^b) and CT26.CL25 (H-2^d), and β-gal negative NK sensitive YAC-1 cells. Spontaneous release of target cells never exceeded 20%. Individual conditions were performed in triplicate, and standard error at each measurement did not exceed 15%. Data representing one out of three similar experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

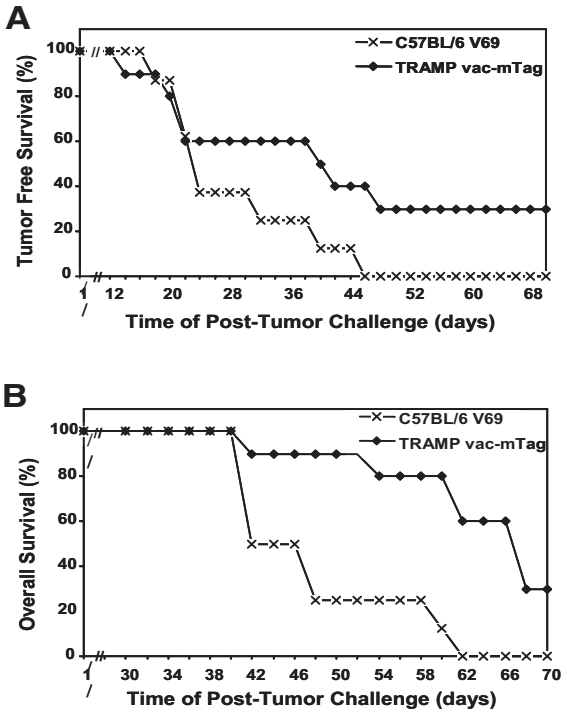
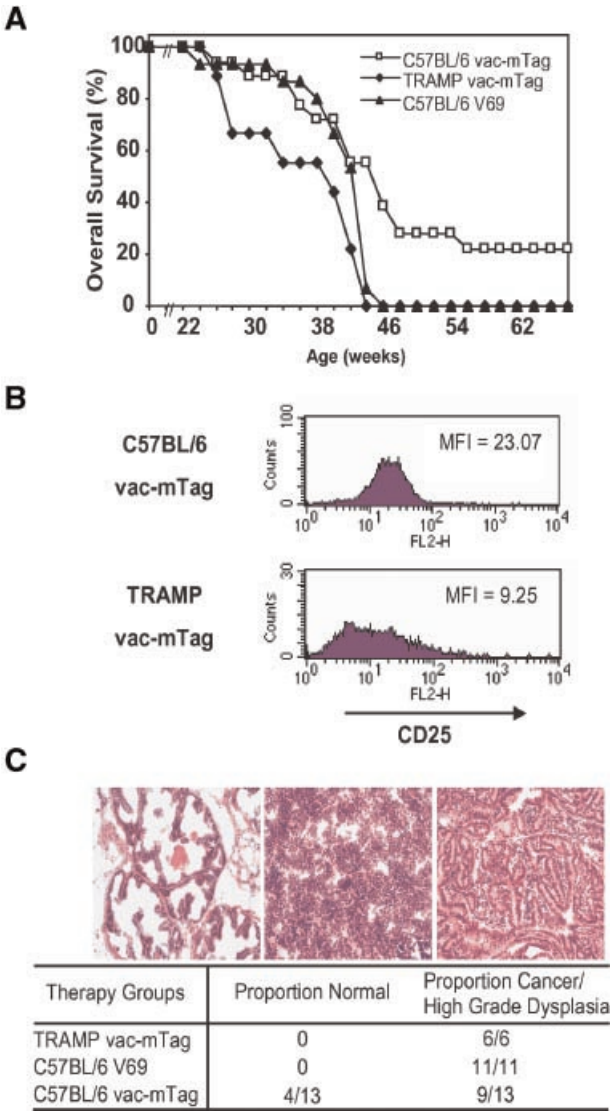


Fig. 4. Tag-specific CTL from in vivo primed TRAMP mice show Tag-specific anti-tumor efficacy in vivo. Two-month-old TRAMP or C57BL/6 mice were immunized with vac-mTag or V69, respectively. Three weeks later, their splenocytes were stimulated in vitro with mitomycin C-treated B6wt19 cells to generate Tag-specific CTLs for adoptive transfer. Two days before adoptive transfer, prostate tumor chunks of TRAMP mice were implanted s.c. into SCID mice. 10^7 Tag-specific CTLs were then adoptively transferred into these SCID mice via tail vein injection. Tumor free survival (time to palpable tumor; **A**), and overall survival (time to tumor-related death; **B**) of these tumor-bearing SCID mice were monitored three times a week ($n = 20$ mice/group; $P = 0.0037$).



Therapy Groups	Proportion Normal	Proportion Cancer/ High Grade Dysplasia
TRAMP vac-mTag	0	6/6
C57BL/6 V69	0	11/11
C57BL/6 vac-mTag	4/13	9/13

Fig. 5. Vac-mTag induced Tag-specific CTL from non-transgenic mice, but not from TRAMP mice, eradicated Tag-expressing orthotopic tumors in tolerant TRAMP hosts. 10^7 vac-mTag- or V69-induced Tag-specific CTLs were adoptively transferred to 4–5 months old TRAMP mice. **A**: Overall orthotopic tumor-free survival of the TRAMP hosts was monitored for up to 68 weeks before the survivor mice were euthanized for further studies ($n = 9–18$ mice/group; $P = 0.002$). **B**: Two-colored flow cytometry was performed for the different groups of donor CTLs with FITC-labeled anti-CD8 and PE-labeled anti-CD25 antibodies. Numbers indicate mean fluorescent intensity of CD25 staining on gated $CD8^+$ cells. **C**: Histopathology studies revealed normal prostate epithelium (**left panel**) in four survivor TRAMP mice that received vac-mTag immunized C57BL/6 CTL whereas others demonstrated prostate cancer/high grade dysplasia (**middle** and **right panels**). Numbers indicate proportion of mice from each therapy group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mice (Fig. 5B, lower panel) or from V69-immunized C57BL/6 mice (data not shown). These results indicate that the expression of IL-2 receptor is defective in the $CD8^+$ CTLs from TRAMP mice following immunization with vac-mTag. More interestingly, C57BL/6-derived non-transgenic Tag-specific CTLs not only delayed the initial palpable time of prostate tumors in significant numbers of the recipient TRAMP mice (data not shown), but also completely prevented tumor incidence in 4 out of 13 recipient TRAMP mice tested (Fig. 5C). Histologic analysis showed that the four long-term survivor TRAMP mice exhibit entirely normal prostate histology at 68 weeks of age whereas the rest of the TRAMP hosts demonstrate prostate cancer or high-grade prostate dysplasia upon tumor-related death (Fig. 5C).

Finally, we determined the extent to which Tag-specific immunotherapy affected the targeted oncogene antigen in the prostate. Prostate Tag expression in the long-term survivor TRAMP mice was evaluated by

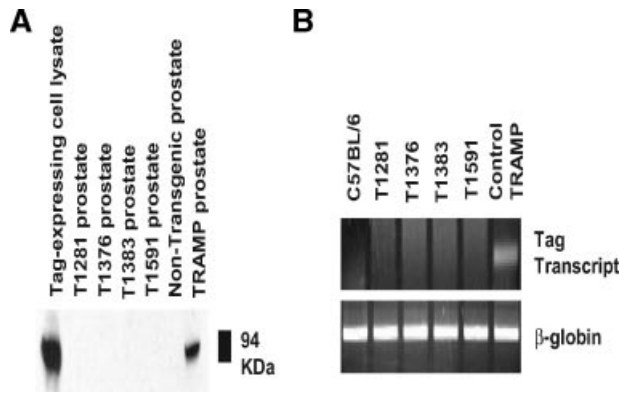


Fig. 6. Absence of Tag expression in prostates of the long-term survivor TRAMP mice that received vac-mTag-induced non-transgenic CTLs. **A**: Western blot was performed using prostates from the four long-term survivor TRAMP mice to identify the expression of the 94 kDa Tag. An untreated TRAMP prostate and a Tag-expressing tumor cell line were used as positive controls while a non-transgenic prostate was used as negative control. **B**: Tag RT-PCR was performed using the same set of prostates as above to determine the expression of Tag transcript. Genotyping was performed with tail DNA to confirm the Tag-positive identities of the tumor-free TRAMP mice. β-globin served as internal control for both RT-PCR and genotyping PCR.

Tag Western blot and RT-PCR (Fig 6A,B). Interestingly, neither Tag protein nor Tag transcripts were detectable in any of the prostates from the long-term survivor TRAMP mice (Fig. 6A,B, upper panel). This indicates that malignant epithelial cells expressing the Tag oncogene in the prostate were successfully targeted and eliminated by the immune system following Tag-specific immunotherapy. The genotype of the four survivor TRAMP mice was reconfirmed by PCR using tail DNA (data not shown).

DISCUSSION

In the present study, we used a unique transgenic mouse model of prostate cancer (TRAMP) to determine the contribution of peripheral (prostatic) expression of antigen and tumorigenesis toward potentiation and maintenance of prostatic TAA tolerance, and evaluate an alternative method of TAA delivery for immunization using a TAA-encoding vaccinia virus to circumvent this tolerance.

Failure in rejection of Tag-expressing tumor engraftments by tumor-bearing old TRAMP as well as tumor-free juvenile TRAMP mice (Table I) suggests that minimal levels of Tag expression undetectable by Western blot in juvenile TRAMP prostates might cause immune ignorance of Tag-expressing tumors by the TRAMP hosts.

In TRAMP mice, levels of tolerogen (Tag) expression increase in concert with cancer progression (Fig. 1). The dynamic effects of concurrent cancer progression and increased tolerogen expression on oncogene-specific tolerance have not been extensively studied. Although the absence of Tag transcripts in TRAMP mice thymi in our hands as shown by RT-PCR (Fig. 1) is consistent with a previous study that demonstrated exclusive expression of Tag in the prostate [14], it contradicts another previous work that reported the presence of Tag RNA transcripts in TRAMP mice thymi [23]. This contradiction can be reconciled in our study for a variety of reasons: First, we could not detect any Tag protein even in the 2-months-old TRAMP prostates which exhibit evident Tag mRNA transcripts. Presence of minute amounts of Tag mRNA transcripts in the thymus might therefore not be able to result in deletion of Tag-specific CTL through negative selection. Second, in the transgenic TCR setting, the proportion of CTL expressing a single Tag-specific TCR is much higher than that in the absence of transgene-driven TCR, and the impact of thymic deletion in the transgenic TCR-bearing mice may be accentuated as compared to tolerance processes in tumor-bearing mice without transgenic TCR, as the latter are capable of producing a broader repertoire of CD8+ T cells. Third, complete absence of peripheral Tag specific CTLs was shown in young tumor-free mice (i.e., 25 days), where T cells who escaped negative selection in the thymus did not have the chance to encounter their cognate antigen and proliferate to detectable amounts.

Unlike previous studies that evaluated CD4 T lymphocyte- and B lymphocyte-driven peripheral tolerance in autoimmunity [24–28], our study was primarily focused on induction of cytotoxic T lymphocytes that are TAA-specific and may harbor a potential therapeutic value in anti-tumor immunotherapy. First, immunization with vac-mTag promptly primed CTL activity in young tumor-free TRAMP mice (Fig. 3). Consistent with previous studies [29–34], this result implies that tumor reactive CTLs were not deleted in the hosts that were peripherally tolerant to the extra-thymic antigen. Although these CTLs readily kill Tag-expressing tumor targets in vitro in a MHC class I-restricted fashion, they are phenotypically and functionally different from those derived from non-transgenic mice. Unlike B6 CTLs, Tag-specific CTLs from TRAMP mice failed to expand in vitro following repetitive stimulation with syngeneic tumor cells and IL-2 (unpublished data). Ohlen et al. [35] reported a different finding in which they successfully rescued and expanded the transgenic CTL by in vitro restimulation with syngeneic tumor and IL-2, although they failed to detect any CTL expansion following repetitive in vivo stimulation in the tolerant mice. Our data provide evidence that tumor antigen-specific CTL precursors do exist in the periphery of tolerant

tumor-bearing hosts. When properly activated, these CTLs can recognize the tolerogen and demonstrate effective cytolytic activity against tumor targets.

Additionally, our findings indicate that the level of tolerogen plays a pivotal role in determining the depth of peripheral tolerance against the extra-thymic tumor antigen [36–38]. Vac-mTag immunization failed to induce Tag-specific CTL activity in the profoundly tolerant older TRAMP mice, such lack of responsiveness was tumor antigen specific and not driven by the generalized tumor growth as evidenced by the strong CTL response to β-gal (Fig. 3).

Breaking tolerance to TAAs in hosts with large tumor burden proved to be challenging. Tumor cells and the intratumoral milieu have been shown to interfere with in vivo function of tumor reactive lymphocytes [3] by directly acting on T lymphocytes [39–41] or by compromising antigen processing and presentation by antigen-presenting cells (APCs) [42,43]. Additionally, a variety of mechanisms have been shown to impede immune responses to tumors (Reviewed in Ref. [44]), such as the release of the immunosuppressive substances IL-4, IL-6, and IL-10 [45–48], TGF-β [49,50], L-arginine and nitric oxide [51,52], prostaglandin E_2 [53], MUC1 glycoprotein [54], and PSA [55,56]. Furthermore, it has been reported that tumor cells express Fas-L and induce CTL apoptosis through Fas–FasL interaction (reviewed in Refs. [57] and [58–60]), although this claim remains a subject of controversy [61].

Although our data did not unravel the potential mechanisms utilized by Tag-expressing tumors to escape immune surveillance, the lack of effective CTL activity following vac-mTag immunization in tumor-bearing TRAMP mice (Fig. 3), together with the age-dependent Tag expression in TRAMP mice tumors (Fig. 1), might suggest that induction of tumor antigen-specific CTL at an early age when tumor burden is relatively low might be a key factor for an effective anti-tumor immunotherapy to take place. This is in line with two recent reports that demonstrated that early immunization directed against SV40 Tag can result in overcoming peripheral T cell tolerance and lead to extensive control of pancreatic [62] and prostatic [63] tumor progression in SV40 Tag transgenic mice.

In our in vivo studies, the recombinant vaccinia-induced Tag-specific CTLs from young TRAMP mice demonstrated ability to delay the onset and growth of implanted heterotopic tumors in SCID mice (Fig. 4), consistent with their Tag-specific cytolytic activity in vitro (Fig. 3). However, a more effective protection was accomplished by Tag-specific CTLs derived from non-transgenic B6 mice, as they not only effectively controlled the outgrowth of heterotopic tumors in SCID hosts (data not shown), but also eradicated orthotopic tumors in 4 out of 13 TRAMP prostates tested (Fig. 5A). Histologic analysis (Fig. 5B), as well as Tag Western blot and RT-PCR assays (Fig. 5C,D), revealed that the subset of prostate cells that expressed Tag oncogene was eliminated by non-transgenic Tag-specific CTL therapy. This corroborates the previous observation by Granziero et al., who performed an adoptive transfer of memory T cells that succeeded in preventing tumor development in TRAMP mice without damaging the prostate tissue. Invasion of the stroma by tumor cells was completely prevented, and the few Tag positive cells that could still be observed in treated mice were apoptotic and confined above the basal membrane either in the epithelium or exfoliating in the gland lumen [21].

Absence of damaged prostate tissue in this model [21] is in agreement with our findings and with a recent work that reported that T lymphocytes specific for a prostate antigen are capable of inducing inflammatory infiltration of the prostate without causing pathological prostate injury [22].

Lack of correlation between in vitro reactivity of T lymphocytes specific for an oncogene tolerogen and their anti-tumor efficacy in vivo is well documented [64–67]. Similarly, adoptive transfer of vac-mTag-induced CTLs from juvenile TRAMP mice did not prevent the orthotopic tumor growth in TRAMP prostates in our hands (Fig. 5A), despite exhibiting a high killing potential against Tag-expressing tumor cells in vitro (Fig. 3). Phenotypic analysis of T lymphocytes revealed that expression of IL-2 receptor alpha (CD25) was hampered in the $CD8^+$ cell subset (Fig. 5E), which would subsequently interfere with the expansion of tumor-reactive T lymphocytes in vivo. Consistent with this observation, Ohlen et al. have previously reported that $CD8^+$ T lymphocytes fail to proliferate in response to a tumor-associated tolerogen despite their ability to lyse tumor cells and produce IFN-γ. This unresponsiveness was attributed to an abrogated IL-2 production by $CD8^+$ T cells and their unresponsiveness to exogenous IL-2 [65]. Others have reported that partial agonists fail to induce T cell proliferation, although they do induce cytolysis and the secretion of at least some cytokines [68,69]. Interestingly, in our hands, the same set of CTLs that were ineffective in treating orthotopic tumors were effective in treating pre-established heterotopic tumors in non-tolerant immunocompromised SCID hosts (Fig. 4). This suggests that mechanisms other than down-regulation of CD25 expression by $CD8^+$ T cells might be involved in skewing the partially activated CTLs in the Tag tolerant TRAMP hosts towards a tolerant phenotype.

Taken together, the data presented here corroborate the persistence of Tag-specific CTLs in TRAMP mice,

and point to the possibility of activating antigen-specific CTLs against endogenous Tag-induced prostate tumors. Such activation could be achieved through vaccination with safety-modified recombinant vaccinia virus carrying Tag. Nevertheless, generating efficient CTLs that can benefit anti-tumor therapy requires an early immunization to avoid peripheral tolerance that is triggered by tumor antigen encounter.

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