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Whole-body irradiation increases the magnitude and persistence of adoptively transferred T cells associated with tumor regression in a mouse model of prostate cancer

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Abstract

Adoptive immunotherapy has demonstrated efficacy in a subset of clinical and preclinical studies, but the T cells used for therapy often are rendered rapidly non-functional in tumor-bearing hosts. Recent evidence indicates that prostate cancer can be susceptible to immunotherapy, but most studies using autochthonous tumor models demonstrate only short-lived T-cell responses in the tolerogenic prostate microenvironment. Here, we assessed the efficacy of sublethal whole-body irradiation (WBI) to enhance the magnitude and duration of adoptively transferred CD8⁺ T cells in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. We demonstrate that WBI promoted high-level accumulation of granzyme B (GzB)-expressing donor T cells both in lymphoid organs and in the prostate of TRAMP mice. Donor T cells remained responsive to vaccination in irradiated recipients, but a single round of WBI-enhanced adoptive immunotherapy failed to impact significantly the existing disease. Addition of a second round of immunotherapy promoted regression of established disease in half of the treated mice, with no progressions observed. Regression was associated with long-term persistence of effector/memory phenotype CD8⁺ donor cells. Administration of the second round of adoptive immunotherapy led to reacquisition of GzB expression by persistent T cells from the first transfer. These results indicate that WBI conditioning amplifies tumor-specific T cells in the TRAMP prostate and lymphoid tissue, and suggest that the initial treatment alters the tolerogenic microenvironment to increase antitumor activity by a second wave of donor cells.

Keywords

TRAMP mice; SV40 T antigen; immunotherapy; whole body irradiation; CD8⁺ T cell

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Introduction

Prostate cancer is the most commonly diagnosed form of cancer, and the second leading cause of cancer-related deaths among American men (1). Although early-stage prostate cancer tends to respond well to radiation or surgical excision, there are few options for men with advanced or recurrent prostate cancer. FDA approval of Sipuleucel-T, the first therapeutic cancer vaccine (2), has rekindled the pursuit of immunotherapy for the control of prostate cancer. Adoptive T-cell transfer strategies have demonstrated efficacy in delaying onset of adenocarcinoma (3–7), or increasing survival through disease regression (8) in autochthonous murine prostate tumor models. Together, these results suggest that prostate cancer is a good candidate disease for further development of immunotherapeutic treatment strategies, but a better understanding of this complex tumor microenvironment is needed to promote durable antitumor immunity.

The transgenic adenocarcinoma of the mouse prostate (TRAMP) model recapitulates the disease progression observed in humans, providing a realistic but challenging model in which to develop prostate cancer therapies. TRAMP mice express the oncogenic SV40 large T antigen (T Ag) under the control of the rat probasin promoter in the dorsolateral and ventral lobes of the prostate (9). Tumor progression in TRAMP mice on the C57BL/6 background is characterized by the appearance of prostatic intraepithelial neoplasia (PIN) at week 8, with well-differentiated adenocarcinoma becoming predominant in the dorsal prostate as early as 16 weeks (9, 10). Concurrent with the onset of T Ag expression at 6 weeks of age (11), TRAMP mice develop tolerance to T Ag within the CD8⁺ T-cell compartment (5, 6, 11, 12) with the residual population of endogenous T Ag-specific CD8⁺ T cells exhibiting little impact on tumor progression (13–15). Tumors maintain T Ag expression throughout disease progression (5), indicating the importance of oncogene expression in tumor-cell survival, and providing a tumor-associated immune target.

Previous studies in TRAMP mice have demonstrated that transferred tumor-specific CD8⁺ T cells are rapidly tolerized or converted to a suppressor phenotype (3, 7, 16, 17). However, a recent study using adoptive transfer of genetically modified T cells into irradiated TRAMP mice resulted in extended survival (8), suggesting that WBI conditioning could maximize the antitumor effect. WBI has been shown to enhance antitumor immunity of CD8⁺ T cells in mouse models (18–21) and in human cancer patients (22). Our group previously demonstrated that WBI-enhanced adoptive T cell-mediated immunotherapy targeting T Ag produced durable regressions of established T Ag-induced choroid plexus tumors, and persistence of donor T cells at the tumor site (23). We hypothesized that conditioning TRAMP mice with WBI would augment T-cell activity and persistence within the negative regulatory microenvironment of the TRAMP prostate. Thus, we investigated the impact of a sublethal dose of WBI on the accumulation and maintenance of adoptively transferred CD8⁺ T cells in TRAMP mice, and the associated impact on the disease.

Materials and Methods

Mice

TRAMP mice on the C57BL/6J background were purchased from The Jackson Laboratories (C57BL/6-Tg(TRAMP)8247Ng/J, stock number 003135), bred, and maintained under barrier SPF conditions in the animal vivarium at the Penn State Hershey Medical Center. Mice were screened by PCR for the presence of the T Ag transgene as described previously, and transgene-positive males were used in experiments at 18 weeks of age (9, 24). gBT-I.1 transgenic mice on the C57BL/6 background were generously provided by Dr. Francis Carbone (25). TCR-IV mice on the C57BL/6 background have been described (23). In some cases TCR-IV hemizygote males were bred with homozygous C57BL/6-Tg(UBC-GFP)30Scha/J (stock number 004353) or B6.PL-Thy1a/CyJ (stock number 000406) females purchased from The Jackson Laboratories to yield marked TCR-IV cells. All animal studies were approved by the Penn State Hershey Institutional Animal Care and Use Committee.

Cell Lines and Reagents

B6/WT-19 cells expressing full-length wild-type SV40 T Ag were derived by transformation of primary mouse kidney cells with wild type SV40 (26) and were originally provided by Satvir S. Tevethia. Cells were authenticated by western blot and flow cytometry for the expression of full length SV40 T Ag and mouse H-2K^b and H-2D^b MHC class I molecules, respectively, and were grown as previously described (23). Processing and short-term culture of single-cell lymphocyte suspensions were performed using RPMI 1640 media supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 10mM HEPES, 50µM 2-ME, and 2% FBS.

Adoptive Immunotherapy

18-week-old male TRAMP mice were exposed to 475 cGy gamma-ray WBI using a ⁶⁰Co-source GammaCell 220 irradiator (Nordion International) one day prior to adoptive transfer. CD8⁺ donor cells from the spleens and lymph nodes (LNs) of naïve TCR-IV or gBT-I.1 mice were enriched by positive magnetic sorting using an AutoMACS cell sorter (Miltenyi Biotech). Purity was determined by flow cytometry, and ranged between 85–95%. Irradiated and age-matched unirradiated male TRAMP mice received 1×10⁶ CD8-enriched cells in sterile PBS by intravenous tail vein injection. For some experiments, CD90.1⁺ donor cells were labeled with 5 µM carboxyfluorescein succinimidyl ester (CFSE) in 0.1% BSA in PBS prior to transfer.

Staining and flow cytometric analysis of lymphocyte populations

Spleens and prostates were removed and processed individually, while medial iliac LNs, considered the tumor-draining LN (TLN), were pooled by treatment group. Single-cell suspensions of splenocytes and LN cells were generated by mechanical disruption and depletion of red blood cells as described previously (23). Prostates were digested using a 1mg/ml collagenase (Life Technologies) and 50U/ml DNase I (Roche) mixture prepared in RPMI complete media supplemented with 1% FBS for 1 hour at 37° C with rocking. The

cell suspension was passed through a wire mesh to remove particulates, and the single-cell suspension was treated according to the protocol used for splenocytes.

Aliquots of 2×10^6 cells were stained with commercially available antibodies to cell-surface and intracellular proteins, as well as PE-labeled H-2K^b/IV (TetIV) or H-2K^b/gB tetramers that recognize T Ag site IV- and HSV gB 498–505-specific T cells, respectively (23). Cells stained only for surface markers were fixed with 2% paraformaldehyde, while the Cytofix/Cytoperm kit (BD Pharmingen) was used to permeabilize and intracellularly stain cells for GzB. Samples were run on a FACSCanto II or BD LSRII instrument (BD Biosciences), and data were analyzed using FlowJo software (Treestar, Inc). The following antibody cocktails were used: CD8-V450, CD45.2-V500 (BD Pharmingen), TetIV-PE, CD90.1-APC, CD62L-APCe780, CD44-FITC, CD4-PECy7, CD127-PerCP Cy5.5 or GzB-PerCP Cy5.5 (Invitrogen). All antibodies were purchased from eBioscience unless otherwise noted.

Intracellular cytokine staining

2×10^6 cells from the spleen- or prostate-derived cell suspensions were stimulated with 1 μ M site-IV C411L-substituted peptide (VVYDFLKL) or the unrelated H-2K^b/HSV glycoprotein B (gB) peptide (SSIEFARL) plus 1 μ g/ml brefeldin A for 4 hours at 37° C. Cells were stained for surface expression of CD8 and CD90.1, and permeabilized with the Cytofix/Cytoperm kit for intracellular staining with antibodies to IFN γ and TNF- α per the manufacturer's instructions. The percentage of cytokine-producing CD8⁺ T cells capable of responding to the site IV determinant was calculated by subtracting the frequency of gB-reactive T cells from the frequency of C411L-reactive T cells.

Histology

TRAMP mice were euthanized by CO₂ asphyxiation, and perfused with 10 mL sterile PBS followed by 10 mL 10% neutral buffered formalin (NBF). The entire urogenital tract was removed for immersion fixation in NBF overnight. Tissues were then transferred into 70% ethanol for at least 24 hours before embedding in paraffin and sectioning. Prostate tissue sections were stained with hematoxylin and eosin, provided in an anonymous fashion to a board-certified veterinary pathologist for tumor-stage scoring according to the Berman-Booty scoring system (27). Images were obtained using an Olympus BX51 microscope with a 4 $\times$, 40 $\times$ or 100 $\times$ (oil-immersion) objective fitted with an Olympus DP71 digital camera and cellSens Standard 1.6 imaging software (Olympus).

Statistical Analysis

Two-sample student T-test or one-way ANOVA were used to compare the quantitative outcome variables between treatment groups at individual time points. A linear mixed-effect model was used to analyze the effect of treatment and time on tumor scores. Grades measured in different lobes of the same mouse were used as repeated measure inputs in the statistical model. The interaction effect between time and treatment was removed from the model since it was not significant. Dunnett's test was used for the multiple comparisons between a specified treatment group (serving as control) and other groups. The family-wise Type-I error rate was controlled. All statistical analyses were performed using SAS software

version 9.3 (SAS Institute, Cary, NC) or PRISM software (GraphPad Software, Inc., San Diego, CA). The significance level was set at 0.05 for all analyses.

RESULTS

The initial donor T-cell response in the tumor-draining lymph node (TLN) is not altered by WBI

We initiated therapy of TRAMP mice at 18 weeks of age when the epithelium transitions from high-grade PIN to overt carcinoma (9, 10). One million CD8⁺ donor cells enriched from T Ag site IV-specific TCR-IV transgenic mice were transferred into TRAMP mice one day after recipient mice received a lymphodepleting dose of 475 cGy WBI. Transfer of naïve T cells was used to allow visualization of early T-cell triggering in the lymphoid organs. WBI had no significant effect on total accumulation of TCR-IV cells in the TLN (Fig. 1A). However, TCR-IV cells composed a larger proportion of cells in irradiated mice due to the depletion of endogenous populations (Fig. 1B–C). We observed a reproducible increase in the accumulation of endogenous CD8⁺ T cells within the TLN of unirradiated mice immediately following transfer (Fig. 1C), suggestive of a brief period of inflammation.

CFSE-labeled TCR-IV cells showed minimal proliferation two days after transfer, but substantial dilution of CFSE by day 5 (Fig. 1D–E) when the majority of cells in both treatment groups exhibited a differentiated CD44^{hi}CD62L^{lo} phenotype (Fig. 1F–G). In contrast, distant LNs contained few donor cells that had undergone multiple rounds of division (unpublished observation), consistent with previous reports (13, 17). Proliferation was dependent upon antigen recognition, as TCR-IV cells transferred into irradiated or unirradiated C57BL/6 hosts remained undivided (Fig. 1H; left panels), while immunization with T Ag-expressing cells produced strong proliferation and effector differentiation (Fig. 1H, right panels). These results demonstrate that the kinetics of T-cell priming in the TLN of TRAMP mice are not altered by WBI despite host lymphodepletion.

WBI promotes increased donor T-cell accumulation in the spleen of TRAMP mice

In the spleen, we observed an initial drop in the total number of TCR-IV cells between days 2 and 3 post-transfer (Fig. 2A). TCR-IV cells then expanded to high levels in irradiated mice between days 5 and 7, and remained at increased levels between 7 and 21 days, before contracting to baseline levels by day 28 (Fig. 2A). Endogenous CD8⁺ T cells remained at reduced levels over this time period (Fig. 2B). In contrast, contraction of TCR-IV cells in unirradiated recipients occurred much earlier, and with faster kinetics (Fig. 2A).

To evaluate whether this discrepancy could be explained by differences in T-cell proliferation, CFSE dilution was monitored among TCR-IV cells that accumulated in the spleen. Unlike results in the TLN (Fig. 1C), accumulation of TCR-IV cells that had undergone multiple rounds of division was accelerated in irradiated mice at both days 5 and 7 post-transfer (Figs. 2C and 2D). Nearly half of TCR-IV cells in the spleen at day 5 retained CD62L expression (Fig. 2E and 2F). This CD62L^{hi} population was predominantly CFSE⁺ (Figure 2E), consistent with previous findings that T-cell differentiation correlates with the extent of cell proliferation (28). By day 7 the majority of TCR-IV cells in both

groups downregulated CD62L (Fig. 2E), despite the lack of accumulation of CFSE^{lo} cells in unirradiated mice (Fig. 2D). No CD127⁺ cells were detected among TCR-IV cells that persisted at day 21 (Fig. 2G), suggesting the absence of classical memory T-cell development in TRAMP mice. Extensive proliferation of donor T cells was antigen-dependent, since CD8⁺ T cells specific for the herpes simplex virus glycoprotein B (gB) underwent only limited proliferation in the spleens of irradiated TRAMP mice by day 7 (Supplementary Fig. 1A). Thus, WBI promotes the systemic accumulation of recently divided TCR-IV cells, while such cells are confined mainly in the TLN of unirradiated TRAMP mice. Our results are consistent with previous studies demonstrating abortive proliferation of tumor-specific CD8⁺ T cells in unirradiated TRAMP mice (7), and suggest that WBI conditioning enhances T-cell expansion or protects a population of antigen-experienced T cells from deletion.

WBI enhances accumulation of TCR-IV cells in the prostate

Accumulation of TCR-IV cells in the prostate was significantly enhanced in irradiated mice as early as day 5 (Fig. 3A and inset), but dramatically increased by day 7 coincident with peak accumulation of TCR-IV cells in the spleen (Fig. 2A). TCR-IV cells in the prostate of irradiated mice contracted over the next three weeks, similar to the kinetics observed in the spleen (Fig. 3A). Prostate-infiltrating TCR-IV cells were restricted to CFSE^{lo}, differentiated cells independent of irradiation (Fig. 3C–D).

The influx of TCR-IV cells into the prostate of irradiated TRAMP mice was paralleled by a smaller magnitude increase in endogenous CD8⁺ T cells (Fig. 3B), suggesting that TCR-IV cell infiltration promotes inflammation within the prostate. T-cell accumulation was antigen-dependent, however, as gB-specific T cells failed to accumulate or drive recruitment of endogenous CD8⁺ T cells in the prostate of irradiated TRAMP mice (Supplementary Fig. 1C–D). These results demonstrate that WBI promoted a significant but transient spike in antigen-specific T-cell accumulation in the prostate that was accompanied by an increase in endogenous CD8⁺ T-cell accumulation.

Irradiation facilitates the survival of tumor antigen-responsive GzB⁺ CD8⁺ T cells in TRAMP mice

Previous studies in TRAMP mice have demonstrated a block in T-cell effector function despite the initial T-cell priming, and accumulation in the prostate (3, 6, 7, 13, 17). Likewise, we found that few CD8⁺ T cells recovered from either the spleen or prostate of TRAMP mice produced IFN γ or TNF α in response to tumor antigen (Fig. 4A–B). Furthermore, WBI failed to enhance the production of either cytokine at any time tested despite enhanced T-cell accumulation (Fig. 4A–B and unpublished observations).

Despite limited IFN γ and TNF α production, high proportions of TCR-IV cells expressed GzB; particularly prostate-infiltrating TCR-IV cells (Fig 4C). Although WBI failed to significantly enhance the total number of GzB⁺ TCR-IV cells in the spleen (Fig. 4D), irradiation enhanced the accumulation of GzB⁺ TCR-IV cells in the prostate by greater than 100-fold at day 7 (Fig. 4E). Accumulation of GzB⁺ TCR-IV cells was antigen-dependent, as TCR-IV cells recovered from non-transgenic littermates did not express GzB (Fig. 4D–E).

Although a population of TCR-IV cells persisted in the prostates of irradiated mice at day 21 (Fig. 3A), these cells no longer expressed GzB (Fig. 4E). This observation suggests that GzB⁺ cells were either eliminated, became quiescent, or converted to an anergic/suppressor phenotype in the tumor microenvironment (7, 16).

To assess the functionality of the persisting cells, TRAMP mice were immunized with B6/WT-19 cells three weeks after adoptive transfer (Fig. 4F). TCR-IV cells in unirradiated TRAMP mice failed to expand in either the spleen or prostate following immunization (Fig. 4G), and exhibited no effector functions (unpublished observations), consistent with the development of T-cell anergy. Although we cannot rule out that TCR-IV cells are redistributed to unrelated tissues in these mice, such cells were not recruited by immunization. Conversely, immunization significantly increased the number of TCR-IV cells in the spleen of irradiated TRAMP mice (Fig. 4G). Immunization also significantly increased the proportion of donor cells expressing GzB in the spleen and prostate of irradiated mice (Fig. 4H), but promoted only a minimal increase in IFN γ -producing cells in the prostate (Fig. 4I). These data indicate that irradiation facilitates the persistence of a subset of TCR-IV cells that retain responsiveness to antigenic challenge, and can acquire effector functions late in the antitumor response.

Adoptive transfer with WBI fails to reduce disease score at early times post-treatment

We evaluated the impact of WBI-enhanced TCR-IV transfer on disease score in TRAMP mice. Therapy was initiated at 18 weeks of age, and disease score was measured over time. Histological scoring of the prostate lobes at 7 days post-transfer demonstrated that mice in all groups remained in the high-grade PIN stage (Fig. 5F; day 7). At 21 days post-transfer, mice treated with WBI or WBI-enhanced adoptive immunotherapy had stable high-grade PIN, while a subset of mice in the other treatment groups had progressed to well-differentiated invasive adenocarcinoma (score 13) or poorly-differentiated neuroendocrine tumors (score 19 or 21) (Fig. 5A–D and 5F; 21 days). We noted a significant increase in inflammation within the prostate at day 21 for mice that received adoptive T-cell transfer either alone or in combination with WBI, but not in WBI-only treated mice (Fig. 5E). However, disease scores did not significantly differ among the groups at either 7 or 21 days post-treatment, and tumor regressions were not observed within this 21-day window.

A second round of adoptive transfer with WBI promotes high-level T-cell accumulation, T-cell persistence and disease regression

Since TCR-IV cell numbers in the prostate contract dramatically by 21 days post-treatment (Fig. 3A), we administered a second round of WBI-enhanced adoptive immunotherapy to prolong the period of T-cell activity (Fig. 6A). T cells from the first (CD90.1⁺) and second (GFP⁺) transfers were evaluated seven days after the second transfer. Analysis of GFP⁺ TCR-IV cells revealed a significantly higher accumulation in both the spleen and prostate of mice that received the initial treatment at 18 weeks relative to mice that only received treatment at 21 weeks (Fig. 6B–C). This result suggests that the first treatment alters the immune environment to allow a more robust T-cell response with the second treatment. Conversely, administration of the second treatment did not significantly alter the number of CD90.1⁺ cells detected in the spleen or prostate, indicating that T cells from the first transfer

did not expand in response to the second treatment. Analysis of effector functions demonstrated that neither adoptive transfer produced a significant population of IFN γ -producing TCR-IV cells (Fig. 6D), and the proportion of GFP⁺ T cells in the prostate that acquired GzB expression by 7 days post-transfer was independent of treatment at 18 weeks (Fig. 6E; right panels). However, we observed a dramatic increase in the frequency of CD90.1⁺GzB⁺ TCR-IV cells in the prostate following the second treatment (Fig. 6E; left panels; $p < 0.005$). Together these results demonstrate that a second round of immunotherapy promotes high-level accumulation of donor T cells in the lymphoid organs and the prostate, and facilitates reactivation of persisting tumor-specific CD8⁺ T cells.

We also performed histological analysis of the prostate lobes at 70 days post-treatment for mice that received two rounds of WBI and adoptive transfer or the individual components. Data are reported as histological score (Fig. 5F; day 70) and disease outcome (Fig. 5G), including mice that succumbed to tumor progression prior to 28 weeks of age. Histological analysis revealed that half of the mice that received two rounds of WBI with TCR-IV cell transfer had Berman-Booty scores reduced to low- or moderate-grade PIN (Fig. 5F; day 70), indicated by reduced herniated glands, and papillary and cribriform structures (Fig. 6F). Given that all mice exhibited high-grade PIN at the time of the second treatment (Fig. 5F; day 21), the decrease in lesion scoring is consistent with disease regression (Fig. 5G), and was demonstrated to be widespread throughout the tissue (Supplementary Fig. 2). The remaining mice in the 2 $\times$ WBI+TCR-IV transfer group showed stable high-grade PIN, with none exhibiting disease progression (Fig. 5F–G). The 2 $\times$ WBI+TCR-IV group differed significantly from all other groups except for mice that received two rounds of TCR-IV cells without WBI, which was marginally insignificant (Fig. 5F; $p = 0.0522$). However, neither the 2 $\times$ TCR-IV group ($p = 0.8666$) nor any other treatment group, excluding the 2 $\times$ WBI+TCR-IV group, differed significantly from untreated mice (Fig. 5F). Instead, a proportion of the TRAMP mice in all other groups exhibited progression to higher-grade prostate cancer (Fig. 5F; day 70 and 5G), including well-differentiated focally invasive adenocarcinoma (Fig. 6G and 6I; grade 13), and poorly-differentiated neuroendocrine carcinomas (Fig. 5F; grade 21). Some mice in Untreated, 2 $\times$ WBI and 2 $\times$ TCR-IV treatment groups succumbed to tumor-associated death prior to Day 70 (Fig. 5G), preventing their inclusion in the histological scoring (Fig. 5F, asterisks). In the case of untreated mice, deaths prior to the end of the experiment explain the apparent drop in Berman-Booty score between days 21 and 70. Taken together, these data indicate that two rounds of WBI with TCR-IV transfer, but not a single round, can promote disease regression in a subset of TRAMP mice, and block disease progression for an extended period of time.

We determined the level of TCR-IV cell persistence in the lymphoid organs of the same mice at 28 weeks, and found that TCR-IV cells were present in mice that received two rounds of WBI with adoptive transfer, but not in those that received only two rounds of TCR-IV cells without WBI (Fig. 6J). Given that T cells from a single treatment dropped to undetectable levels by four weeks post-transfer (Fig. 2A), these results indicate that two treatments increased the persistence to at least seven weeks. Recovered TCR-IV cells were predominantly CD44^{hi}CD62L^{lo}, and a subset of these cells expressed CD127, consistent with memory T-cell development within the lymphoid organs (Fig. 6J). We noted that total splenocyte numbers appeared to be slightly reduced seven weeks after the second round of

therapy, but were not significantly below normal at this time post-treatment (Supplementary Fig. 3B). Taken together, our results demonstrate that autochthonous prostate lesions are susceptible to T cell-based therapy, but disease regression requires multiple rounds of adoptive T-cell transfer with low-dose WBI conditioning. These data also suggest that disease regression is associated with a change in the immune microenvironment that allows the persistence of tumor-specific CD8⁺ T cells.

DISCUSSION

Our results show that irradiation-enhanced adoptive T-cell transfer can result in durable T-cell responses in TRAMP mice. WBI increased the accumulation and persistence of GzB⁺ effector-phenotype TCR-IV cells in recipient TRAMP mice, particularly in the neoplastic prostate, and protected a subset of donor cells from deletion or anergy. Furthermore, two successive rounds of WBI-enhanced adoptive immunotherapy led to reactivation of persisting T cells, high-level accumulation of donor cells, and long-term survival of memory phenotype T cells. These immunological findings were associated with regression of established autochthonous lesions or the maintenance of stable disease through 28 weeks of age. We suggest that the combination of WBI and T-cell transfer moderates the immune suppressive environment in the prostate, promoting long-term immunity.

A dominant impact of WBI conditioning in the TRAMP model was increased T-cell accumulation in both the spleen and the prostate. Since TCR-IV T cells initially proliferated in the TLN of both irradiated and unirradiated TRAMP mice, increased accumulation may be explained by better T-cell survival or prolonged T-cell proliferation in irradiated mice. This interpretation is supported by the accumulation of CFSE^{lo} T cells in the spleen of irradiated, but not unirradiated, mice beginning at day 5 post-transfer. Multiple mechanisms have been proposed to explain the immune-enhancing effects of irradiation, including upregulation of costimulatory function by dendritic cells (DCs) (29). Sublethal WBI has also been shown to induce microbial translocation, critical for adoptive immunotherapy in a melanoma model, which promotes the systemic circulation of bacterial lipopolysaccharide and maturation of DCs (30). Such a mechanism may be particularly important for achieving productive, rather than abortive, activation of naïve donor T cells. Administration of higher doses of radiation (10–15 Gy) can induce immunogenic tumor-cell death, which may facilitate increased acquisition of tumor antigen by DCs to promote more efficient cross-presentation to T cells (31, 32). However, we have no direct evidence that the lower dose of WBI used in the current study can induce immunogenic cell death in the TRAMP prostate.

A second impact of irradiation was increased persistence of responsive T cells. A subset of tumor-specific T cells remained responsive to immunization in irradiated mice three weeks post-transfer. This finding contrasts with studies showing lack of response to vaccination in unirradiated TRAMP mice following T-cell contraction (17). Increased persistence could be a product of the initially higher T-cell accumulation in irradiated TRAMP mice. Indeed, TCR-IV T-cell numbers in unconditioned mice were highest immediately following transfer, and did not increase following initiation of proliferation. Development of persisting T cells could alternatively be explained by unique differentiation of TCR-IV T cells in irradiated versus unirradiated TRAMP mice. Lymphopenia induced by WBI can enhance access to

survival cytokines, resulting in increased functional differentiation of T cells in tumor-bearing mice (33), and the promotion of a memory-like phenotype (34–36). Thus, lymphopenia may be essential to develop long-term immunity in this model.

Despite strong GzB expression by donor TCR-IV cells recovered from irradiated TRAMP mice, these cells produced limited IFN γ and TNF α , consistent with results in human and mouse prostate cancer (37, 38). In fact, the majority of studies in TRAMP mice show that tumor antigen-specific CD8 $^{+}$ T cells are rapidly tolerized or converted to a suppressive phenotype (3, 7, 13, 16, 17, 39). Immunosuppression was delayed by either DC-based vaccination or CD4 $^{+}$ T cell co-transfer, but was rapidly re-exerted once these supportive regimens were discontinued (3, 7, 40). TGF- β plays a major suppressive role in the TRAMP model (4, 8, 14, 17, 39). However, the effects of TGF- β signaling may not explain our observations of split anergy (GzB $^{+}$ without cytokine production) since TGF- β signaling has been shown to activate the Smad complex and Atf1 to reduce the expression of both IFN- γ and GzB (41). Since only a minor fraction of TCR-IV cells ever developed into IFN γ^{+} effectors in irradiated TRAMP mice, even as early as 3 days post-transfer (unpublished observations), this mechanism of T-cell anergy may be distinct from the sequential loss of cytokine and cytotoxic molecule expression described for T-cell exhaustion (42). Rather, our results suggest a block in T-cell differentiation following initial antigen encounter. Alternatively, the impact of TGF- β on the T cells may be temporarily reduced by WBI, allowing partial differentiation to GzB-expressing cells. Further studies are required to identify the upstream signals that regulate this phenotype.

Naïve donor T cells were used to allow visualization of early events in the lymphoid organs and to allow direct comparison of our results with those obtained in previous studies with other T Ag-induced cancers (23). TCR-transduced T cells, which require *in vitro* activation, were recently shown to induce localized elimination of T Ag-expressing cells in the prostate of irradiated TRAMP mice (8), suggesting that irradiation also can enhance the effectiveness of *in vitro* expanded and redirected T cells in this system. From a translational perspective, the current requirement for large numbers of donor T cells precludes the use of antigen-specific naïve donor T cells for clinical use (43). However, naïve T cells are readily abundant in the peripheral blood and, along with the more recently defined T-memory stem cells, have been shown to out-perform central and effector memory T cells in experimental models of adoptive T-cell therapy (44, 45) and as the starting population for genetic engineering of donor T cells (46). This is likely due to their enhanced ability to undergo differentiation into effector T cells needed for tumor elimination while retaining their renewable potential (43). Current efforts in the field are focused on expanding T cells that retain a renewable phenotype, including genetic reprogramming of cells to retain characteristics of naïve T cells and T-memory stem cells to provide a source of donor cells (45, 47).

Our finding that the disease score was reduced in TRAMP mice that received two rounds of WBI with adoptive transfer, but not by a single round of therapy, could be explained by several mechanisms. This result was associated with durable TCR-IV accumulation for at least 7 weeks, while T cells contracted to baseline levels within four weeks after a single treatment. This observation suggests that regression of advanced prostatic lesions requires

an extended attack by the immune system. Indeed, persistence of adoptively transferred T cells positively correlates with objective clinical responses (48). After the second treatment, but not the first, the TCR-IV population included CD127⁺ cells (Fig. 2G and 6J), suggesting that the second round of treatment uniquely produced memory-like cells. This change in TCR-IV persistence with two rounds of WBI-enhanced adoptive immunotherapy may be explained by reduced immunosuppression or increased inflammation at the tumor site produced by the first round of therapy (Figure 5E), providing an environment that allows the development of a persistent T-cell population with the second transfer. Such a change in the tumor microenvironment is also suggested from our finding that some prostate-resident TCR-IV cells from the initial transfer regained GzB expression following the second round of therapy (Fig. 6C). Reactivation of tolerant prostate-resident T cells was previously observed following intra-prostatic injection of antigen-pulsed dendritic cells (40), indicating that these T cells are not irreversibly tolerized. These encouraging results raise the possibility that endogenous prostate tumor-specific T cells might be rescued from tolerance following immune intervention.

It remains to be determined whether two complete cycles of WBI with TCR-IV cells are required to generate long-lived tumor-specific T-cell responses in TRAMP mice. For example, whether a second dose of irradiation prior to the second T-cell transfer is required to recapitulate both the long-lived T-cell response and disease regression is unknown. Clearly, the provision of multiple doses of WBI is not desirable for a translational setting due to increased potential for radiation-induced toxicities. Since WBI-induced lymphopenia is only partially recovered prior to the second treatment (Fig. 2B and Fig. S3A), WBI may not be necessary to enhance T-cell activation and accumulation during the second transfer. Lymphodepletion or innate triggering might alternatively be induced by approaches such as chemotherapy to reduce potential side effects from radiation. In addition, hematopoietic stem cells could be provided at the time of the second transfer (48). Further studies are required to identify the particular changes to the immune and prostate microenvironment induced after each round of therapy.

Previously we used WBI with TCR-IV adoptive transfer to evaluate the impact of immunotherapy in the T Ag-driven SV11 choroid plexus tumor model (23). In both the SV11 and TRAMP models WBI enhanced the persistence of donor TCR-IV cells, and increased the therapeutic effect. However, in SV11 mice a single round of WBI-enhanced adoptive immunotherapy was sufficient to produce complete regression of established tumors, and conferred long-term protection from recurrence (23). TCR-IV cells persisted at high frequencies in the brains of irradiated SV11 mice for over 2 months, while those localized to the TRAMP prostate after a single round of therapy contracted to baseline detection after 21 days. TCR-IV cells in TRAMP mice also lacked IFN γ production, while IFN γ production by TCR-IV cells in SV11 mice was essential for tumor elimination (23). This finding could explain the less efficient elimination of tumors in TRAMP mice. This comparison indicates that a similar adoptive immunotherapy approach may be applicable for tumors arising in distinct tissues that share a common antigen. However, unique contributions of the tissue microenvironments regulate the duration of the response and impact of the therapeutic approach, thus requiring a more complete understanding of the unique contribution of each environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. WBI enhances the frequency of TCR-IV cells within the tumor-draining lymph node (TLN)

18-week old male TRAMP mice received 475 cGy WBI or were left unirradiated, followed one day later by transfer of 1×10^6 CD8-enriched, CD90.1⁺ TCR-IV cells. All panels represent cells isolated from the TLN and evaluated by flow cytometry at the indicated times using TetIV staining. CD8⁺ TetIV⁺ TCR-IV cells plotted as total number (A) and percentage of total cells (B). (C) Total endogenous CD8⁺ T cells. Solid line indicates the average CD8⁺ T-cell count in the TLN of untreated TRAMP mice. (D) CFSE dilution in TCR-IV cells recovered from unirradiated (top) or irradiated (bottom) TRAMP mice. Dotted line represents C57BL/6 control recipients. (E) CFSE^{lo} TCR-IV cells were quantified on day 5 using the gate shown in panel (D). n.s.; no statistical difference. (F–G) CD44 and CD62L expression by TCR-IV cells from unirradiated (top) and irradiated (bottom) TRAMP mice 5 days post-transfer. (H) CFSE dilution in TCR-IV cells from C57BL/6 mice 5 days post-transfer into irradiated (middle), or unirradiated mice without (left) or with B6/WT-19 immunization (right). The marker on the TCR-IV+WT-19 panel indicates the CFSE^{lo} threshold. CD44 and CD62L expression (far right) by TCR-IV cells in unirradiated,

immunized C57BL/6 mice. Data are pooled from two independent experiments with 5 to 7 mice per group.

Figure 2. WBI enhances TCR-IV cell accumulation in the spleen of TRAMP mice
 TRAMP mice were treated as in Fig. 1, and splenocytes were analyzed by flow cytometry. Total number of (A) TCR-IV T cells and (B) endogenous CD8⁺ T cells. Solid line in (B) indicates average CD8⁺ T-cell number in untreated TRAMP mice. * indicates statistical significance by Student's t-test. (C–D) CFSE dilution in TCR-IV cells recovered from unirradiated (top) or irradiated (bottom) TRAMP mice. Dotted line in (C) represents C57BL/6 control. (E) Representative CFSE dilution of CD44^{hi}CD62L^{hi} (solid line) and CD44^{hi}CD62L^{lo} (dashed line) TCR-IV populations in non-irradiated (top) and irradiated

(bottom) TRAMP mice 5 days post-transfer. (F) Quantification of CD44^{hi}CD62L^{lo} TCR-IV cells. (G) CD127 expression by TCR-IV cells in non-irradiated (top) and irradiated (bottom) TRAMP mice 21 days post-transfer. Quadrants were set using endogenous CD8⁺ T cells. Data are pooled from at least two independent experiments with a minimum of 6 mice per group.

Figure 3. WBI enhances accumulation of TCR-IV cells in the prostate of TRAMP mice
 TRAMP mice were treated as in Figure 1, and prostate cells were analyzed by flow cytometry. (A) Total number of CD90.1⁺ TCR-IV cells and (B) endogenous CD8⁺ T cells. The solid line indicates the average number of CD8⁺ T cells in an untreated TRAMP. Bar graph shows the data from day 5 on a reduced scale. *indicates statistical significance by Student's t-test. (C) CD44^{hi}CD62L^{lo} and (D) CFSE^{lo} cells within CD90.1⁺ TCR-IV population at 5 days post-transfer. Data are pooled from at least two independent experiments with a minimum of 6 mice per group.

Figure 4. WBI facilitates persistence of antigen-responsive TCR-IV cells

(A–B) Total number of CD8⁺ T cells in the spleen (A) or prostate (B) of TRAMP mice that are TetIV⁺, or produce IFN γ or TNF α in response to *ex vivo* stimulation with Site IV peptide (C411L) 7 days post-transfer. (C) Percentage of CD90.1⁺ TCR-IV cells expressing GzB seven days post-transfer. Total numbers of GzB⁺ TCR-IV cells in the spleen (D) and prostate (E) of irradiated or unirradiated mice. (F) Schematic of challenge experiment in TRAMP mice. (G–I) TRAMP mice treated as in (F) were harvested 28 days post-transfer and evaluated for (G) total number of TCR-IV cells, and (H) expression of GzB or (I) production of IFN- γ . Statistical significance was evaluated by Student's T test. Data are pooled from two independent experiments with a minimum of 6 mice per group.

Figure 5. Two rounds of WBI with adoptive transfer are associated with reduced disease score at late times post-treatment

(A–D) Representative H&E sections of the most advanced lesion in the dorsal prostate of TRAMP mice at 21 days post-transfer for mice shown in Fig. 5F. (A) High-grade PIN with inflammation in WBI+TCR-IV (A) and TCR-IV (C) groups, (B) high grade PIN in WBI-only group, and (D) well-differentiated adenocarcinoma resembling high-grade PIN but with focal invasion and stromal reaction (inset) in the untreated group. (E) Average inflammation score for each treatment group at 21 days post-transfer. (F) Berman-Booty scoring of TRAMP mice at 19 (red), 21 (green) and 28 (blue) weeks of age. Each point represents an individual mouse, and indicates the most advanced lesion in the prostate. * indicate mice

that succumbed to tumor burden prior to 28 weeks of age. Statistical significance was evaluated using Dunnett's test. (G) Categorization of disease outcome in TRAMP mice at 28 weeks of age using the following scale: Death = lethal tumor burden, Progression = score >9, Stable disease = score 7–9, and Regression = score <7. "n=" number of mice per group.

Figure 6. A second round of WBI with TCR-IV transfer promotes enhanced T-cell accumulation, effector function, and persistence

(A) Schematic of consecutive treatment regimen. Organs were harvested at 22 weeks of age to assess the accumulation of CD90.1⁺ or GFP⁺ TCR-IV cells in the spleen (B) and prostate (C). Some mice received treatment only at week 18 or 21 for comparison. (D) IFN γ production and (E) GzB expression by CD90.1⁺ or GFP⁺ TCR-IV T cells. (F–I) Representative H&E sections of the lesions detected in the dorsal prostate at 28 weeks of age for mice shown in Figure 5F. (F) Healthy prostate lobe indicative of disease regression but showing a small area of low-grade PIN (asterisk) in mice that received 2 $\times$ WBI+TCR-

IV. Well-differentiated adenocarcinoma with focal invasion (inset) in mice that received (G) 2× WBI or (I) no treatment. Arrows indicate invasive cell populations. (H) High-grade PIN in 2× TCR-IV only mice. (J) Analysis of splenocytes at 28 weeks showing TetIV⁺ CD8⁺ T cells (left), and CD44 and CD62L (center) or CD127 (right) on CD8⁺TetIV⁺ cells from the indicated treatment groups. Plots are representative of results obtained from two independent experiments with a minimum of 5 mice per group.