Whole-Body Irradiation Increases the Magnitude and Persistence of Adoptively Transferred T Cells Associated with Tumor Regression in a Mouse Model of Prostate Cancer

Lindsay K. Ward-Kavanagh, Junjia Zhu, Timothy K. Cooper, and Todd D. Schell

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 nonfunctional 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, Gzmb)–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 affect significantly the existing disease. Addition of a second round of immunotherapy promoted regression of established disease in half of the treated mice, with no progression 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. Cancer Immunol Res; 2(8); 777–88. ©2014 AACR.

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 immunotherapeutic strategies to delay or reverse disease (3). 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 regression 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 whole-body irradiation (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 patients with cancer (22). Our group previously demonstrated that WBI-enhanced adoptive T cell-mediated immunotherapy targeting TAg produced durable regressions of established TAg-induced choroid plexus tumors, and persistence of donor T cells at the tumor site (23). We hypothesized that conditioned 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/6 background were purchased from The Jackson Laboratory [C57BL/6-Tg(TRAMP)8247Ng/J, stock number 003135], bred, and maintained under barrier pathogen-free (SPF) conditions in the animal vivarium at the Penn State Hershey Medical Center (Hershey, PA). Mice were screened by PCR for the presence of the TAg transgene as described previously, and transgene-positive males were used in experiments at 18 weeks of age (9, 24). gBTF-L1 transgenic mice on the C57BL/6 background were generously provided by Dr. Francis Carbone (University of Melbourne, Australia; ref. 25). TCR-IV mice on the C57BL/6 background have been described previously (23). In some cases, TCR-IV hemizygote males were bred with homozygous C57BL/6-Tg(UBC-GFP)30Scha/J (stock number 003135) or B6.PL-Thy1a/CyJ (stock number 000406) females purchased from The Jackson Laboratory to yield marked C57-TRACP/Luci-Luciferase mice. 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 TAg were derived by transformation of primary mouse kidney cells with wild-type SV40 (26), and were originally provided by Dr. Satvir S. Tevethia (Penn State University College of Medicine, Hershey, PA). Cell lines were authenticated by Western blotting and flow cytometry for the expression of full-length SV40 TAg and mouse H-2Kb and H-2Db MHC class I molecules, respectively, and were grown as previously described (26). Prostates were digested using a 1 mmol/L collagenase (Life Technologies) and 50 U/mL DNase I (Roche) mixture prepared in RPMI-1640 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 donor cells were labeled with commercially available antibodies to cell-surface and intracellular proteins, as well as phycoerythrin (PE)-labeled H-2Kb/IV (TetIV) or H-2Kb/gB tetramers that recognize TAg site IV- and HSV gB 498-505–specific T cells, respectively (23). Cells stained only for surface markers were fixed with 2% paraformaldehyde, whereas the CytoCytokine Kit (BD Pharmingen) was used to permeabilize and intracellularly stain cells for granzyme B (GzB). Samples were run on a FACSCanto II or BD LSRII instrument (BD Biosciences), and data were analyzed using the FlowJo software (TreeStar, Inc.). The following antibody cocktails were used: CD8-V450 and CD45.2-V500 (BD Pharmingen), TetIV-PE, CD90.1-APC, CD62L-APC-e780, CD44-FITC, and CD11c-APC. Data were analyzed using the FlowJo software (TreeStar, Inc.). The following antibody cocktails were used: CD8-V450 and CD45.2-V500 (BD Pharmingen), TetIV-PE, CD90.1-APC, CD62L-APC-e780, CD44-FITC, CD11c-APC, CD127-PE-Cy5.5, or GzB-PerCP Cy5.5 (Invitrogen). All antibodies were purchased from eBioscience unless otherwise noted.

Adoptive immunotherapy

Eighteen-week-old male TRAMP mice were exposed to 475 cGy γ-ray WBI using a 60Co-source GammaCell 220 irradiator (Nordion International) 1 day before adoptive transfer. CD8+ donor cells from the spleens and lymph nodes (LN) of naive TCR-IV or gBTF-L1 mice were enriched by positive magnetic sorting using an autoMACS cell sorter (Miltenyi Biotec). Purity was determined by flow cytometry, and ranged between 85% and 95%. Irradiated and age-matched unirradiated male TRAMP mice received 1×10^6 CD8-enriched cells in sterile PBS by intravenous tail vein injection. For some experiments, CD90.1+ donor cells were labeled with 5 μmol/L carboxyfluorescin succinimidyl ester (CFSE) in 0.1% BSA in PBS before transfer.

Staining and flow cytometric analysis of lymphocyte populations

Spleens and prostates were removed and processed individually, whereas 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 1 mmol/L collagenase (Life Technologies) and 50 U/mL DNase I (Roche) mixture prepared in RPMI-1640 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.

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Intracellular cytokine staining

Cells (2 × 10^6) from the spleen- or prostate-derived cell suspensions were stimulated with 1 μmol/L site-IV C411L-substituted peptide (VYDFFKL) or the unrelated H-2Kb/gB glycoprotein B (gB) peptide (SIEFFARL) plus 1 μmol/L 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 IFNγ and TNFα as 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 of sterile PBS followed by 10 mL of 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 (H&E), provided in an anonymous fashion to a board-certified veterinary pathologist for tumor-stage scoring according to the Herman-Booey scoring system (27). Images were obtained using an Olympus BX51 microscope with a ×4, ×40, or ×100 (oil-immersion) objective fitted with an Olympus DP71 digital camera and CellSens Standard 1.6 imaging software (Olympus).

**Statistical analysis**

The 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 as it was not significant. The Dunnett test was used for 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 the SAS software version 9.3 (SAS Institute) or Prism software (GraphPad Software, Inc.). The significance level was set at 0.05 for all analyses.

**Results**

**The initial donor T-cell response in the tumor-draining LN 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 TAg site IV–specific TCR-IV transgenic mice were transferred into TRAMP mice 1 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 tumor-draining LN (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 and 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 2 days after transfer, but substantial dilution of CFSE by day 5 (Fig. 1D and E) when the majority of cells in both treatment groups exhibited a differentiated CD4+)CD62Llo phenotype (Fig. 1F and G). In contrast, distant LNs contained few donor cells that had undergone multiple rounds of division (unpublished data), 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), whereas immunization with TAg–expressing cells produced strong proliferation and effector differentiation (Fig. 1H, right). 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 after 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 after transfer (Fig. 2C and D). Nearly half of TCR-IV cells in the spleen at day 5 retained CD62L expression (Fig. 2E and F). This CD62Llo population was predominantly CFSE+ (Fig. 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 CFSElo 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, because CD8+ T cells specific for the herpes simplex virus gB underwent only limited proliferation in the spleens of irradiated TRAMP mice by day 7 (Supplementary Fig. S1A). 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 3 weeks, similar to the kinetics observed in the spleen (Fig. 3A). Prostate-infiltrating TCR-IV cells were restricted to CFSElo, differentiated cells independent of irradiation (Fig. 3C and 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. S1C and S1D). 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\(\gamma\) or TNF\(\alpha\) in response to tumor antigen (Fig. 4A and B). Furthermore, WBI failed to enhance the production of either cytokine at any time tested despite enhanced T-cell accumulation (Fig. 4A and B and unpublished data).

Despite limited IFN\(\gamma\) and TNF\(\alpha\) 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 nontransgenic littermates did not express GzB (Fig. 4D and 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 3 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 data), 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 IFNγ-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 the disease score at early times after treatment

We evaluated the impact of WBI-enhanced TCR-IV transfer on the disease score in TRAMP mice. Therapy was initiated at 18 weeks of age, and the disease score was measured over time. Histologic scoring of the prostate lobes at 7 days after transfer demonstrated that mice in all groups remained in the high-grade PIN stage (Fig. 5F, day 7). At 21 days after transfer, mice treated with WBI or WBI-enhanced adoptive immunotherapy had stable high-grade PIN, whereas 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 F, 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
A second round of adoptive transfer with WBI promotes high-level T-cell accumulation, T-cell persistence, and disease regression

Because TCR-IV cell numbers in the prostate contract dramatically by 21 days after 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 7 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 and 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 IFNγ-producing TCR-IV cells (Fig. 6D), and the proportion of GFP⁺ T cells in the prostate that acquired GzB expression by 7 days after transfer was independent of treatment at 18 weeks (Fig. 6E, right). 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; 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 histologic analysis of the prostate lobes at 70 days after treatment for mice that received two rounds of WBI and adoptive transfer or the individual components. Data are reported as the histologic score (Fig. 5F, day 70) and disease outcome (Fig. 5G), including mice that succumbed to tumor progression before 28 weeks of age. Histologic 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. S2). The remaining mice in the 2× WBI+TCR-IV transfer group showed stable high-grade PIN, with none exhibiting disease progression (Fig. 5F and G). The 2× 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× TCR-IV group (P = 0.8666) nor any other treatment group, excluding the 2× 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 I, grade 13), and poorly
differentiated neuroendocrine carcinomas (Fig. 5F, grade 21). Some mice in untreated, 2× WBI and 2× TCR-IV treatment groups succumbed to tumor-associated death before day 70 (Fig. 5G), preventing their inclusion in the histologic scoring (Fig. 5F, x). In the case of untreated mice, deaths before the end of the experiment explain the apparent drop in the 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 4 weeks after transfer (Fig. 2A), these results indicate that two treatments increased the persistence to at least 7 weeks. Recovered TCR-IV cells were predominantly CD44hiCD62Llo, 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 seemed to be slightly reduced 7 weeks after the second round of therapy, but were not significantly below normal at this time after treatment (Supplementary Fig. S3B). 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

Figure 4. WBI facilitates persistence of antigen-responsive TCR-IV cells. A and B, total number of CD8+ T cells in the spleen (A) or prostate (B) of TRAMP mice that are TetIV+, or produce IFNγ or TNFα in response to ex vivo stimulation with Site IV peptide (C411L) 7 days after transfer. C, the percentage of CD90.1+ TCR-IV cells expressing GzB 7 days after 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 to I, TRAMP mice treated as in (F) were harvested 28 days after transfer and evaluated for total number of TCR-IV cells (G), and expression of GzB (H) or production of IFNγ (I). Statistical significance was evaluated by the Student t test. Data are pooled from two independent experiments with a minimum of 6 mice per group.
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.

Figure 5. Two rounds of WBI with adoptive transfer are associated with reduced disease score at late times after treatment. A–D, representative H&E sections of the most advanced lesion in the dorsal prostate of TRAMP mice at 21 days after transfer for mice shown in F. A, high-grade PIN with inflammation in WBI+TCR-IV (A) and TCR-IV (C) groups, high-grade PIN in the WBI-only group (B), and well-differentiated adenocarcinoma resembling high-grade PIN but with focal invasion and stromal reaction (inset) in the untreated group (D). E, average inflammation score for each treatment group 21 days after transfer. Statistical significance was verified by one-way ANOVA. *P < 0.05. F, Berman-Booty scoring of TRAMP mice at 19 (red), 21 (green), and 28 (blue) weeks of age. Each point indicates an individual mouse, and the most advanced lesion in the prostate; x, mice that succumbed to tumor burden before 28 weeks of age. Statistical significance was evaluated using the Dunnett 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.
cells. These immunologic 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. Because 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<sup>lo</sup> T cells in the spleen of irradiated, but not unirradiated, mice beginning at day 5 after transfer. Multiple mechanisms have been proposed to explain the immune-enhancing effects of irradiation, including upregulation of costimulatory function by dendritic cells (DC; ref. 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 this 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 3 weeks after transfer. This finding contrasts with results from 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 IFNγ and TNFα, 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 cotransfer, but was rapidly reeverted once these supportive regimens were discontinued (3, 7, 40). TGFβ plays a major suppressive role in the TRAMP model (4, 8, 14, 17, 39). However, the effects of TGFβ signaling may not explain our observations of split anergy (GzB+ without cytokine production) because TGFβ signaling has been shown to activate the Smad complex and Atf1 to reduce the expression of both IFNγ and GzB (41). Because only a minor fraction of TCR-IV cells ever developed into IFNγ+ effectors in irradiated TRAMP mice, even as early as 3 days after transfer (unpublished data), 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 TGFβ 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, whereas T cells contracted to baseline levels within 4 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 (Figs. 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 (Fig. 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 intraprostatic injection of antigen-pulsed DCs (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 before 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. Because WBI-induced lymphopenia is only partially recovered before the second treatment (Fig. 2B and Supplementary 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, whereas 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 IFNγ production, whereas IFNγ 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.K. Ward-Kavanagh, T.D. Schell


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.K. Ward-Kavanagh, T.D. Schell

Analysis and interpretation of data (e.g., statistical analysis, biosatistics, computational analysis): L.K. Ward-Kavanagh, J. Zhu, T.K. Cooper, T.D. Schell

Writing, review, and/or revision of the manuscript: L.K. Ward-Kavanagh, J. Zhu, T.K. Cooper, T.D. Schell

Study supervision: T.D. Schell

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T-cell Persistence in Irradiated TRAMP Mice

References


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Lindsay K. Ward-Kavanagh, Junjia Zhu, Timothy K. Cooper, et al.


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