Published in final edited form as:

Cancer Immunol Res. 2017 November ; 5(11): 992–1004. doi:10.1158/2326-6066.CIR-17-0040.

Stereotactic Radiotherapy Increases Functionally Suppressive Regulatory T Cells in the Tumor Microenvironment

Yuki Muroyama¹, Thomas R. Nirschl¹, Christina M. Kochel¹, Zoila Lopez-Bujanda¹, Debebe Theodros¹, Wendy Mao¹, Maria A. Carrera-Haro¹, Ali Ghasemzadeh¹, Ariel E. Marciscano², Esteban Velarde², Ada J. Tam¹, Christopher J. Thoburn¹, Muniza Uddin³, Alan K. Meeker³, Robert A. Anders³, Drew M. Pardoll¹, and Charles G. Drake¹

¹Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland ²Department of Radiation Oncology and Molecular Radiation Sciences, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland ³Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

Radiotherapy (RT) enhances innate and adaptive antitumor immunity; however, the effects of radiation on suppressive immune cells, such as regulatory T cells (Treg), in the tumor microenvironment (TME) are not fully elucidated. Although previous reports suggest an increased Treg infiltration after radiation, whether these Tregs are functionally suppressive remains undetermined. To test the hypothesis that RT enhances the suppressive function of Treg in the TME, we selectively irradiated implanted tumors using the small animal radiation research platform (SARRP), which models stereotactic radiotherapy in human patients. We then analyzed tumor-infiltrating lymphocytes (TIL) with flow-cytometry and functional assays. Our data showed

Corresponding Author: Charles G. Drake, Herbert Irving Comprehensive Cancer Center at Columbia University, 177 Fort Washington Avenue, Suite 6GN-435, New York, NY 10032. Phone: 646-317-5290; Fax: 212-305-3035; cgd2139@cumc.columbia.edu. Current address for C.G. Drake: Division of Hematology and Oncology, Columbia University Medical Center, New York, New York.

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

Disclosure of Potential Conflicts of Interest

C.G. Drake reports receiving a commercial research grant from the Bristol-Myers Squibb International Immuno-Oncology Network (II-ON), has ownership interest (including patents) in Compugen, Inc., Potenza Therapeutics, Tizona Therapeutics, and UroGen Pharma, and is a consultant/advisory board member for Compugen, Inc., Roche/Genentech, Merck, Bayer, and Kleo Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Y. Muroyama, C.M. Kochel, A. Ghasemzadeh, A.E. Marciscano, E. Velarde, R.A. Anders, C.G. Drake

Development of methodology: Y. Muroyama, C.M. Kochel, A. Ghasemzadeh, A.E. Marciscano, C.G. Drake

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Muroyama, T.R. Nirschl, Z.

Lopez-Bujanda, D. Theodros, W. Mao, M.A. Carrera-Haro, E. Velarde, A.J. Tam, C.J. Thoburn, M. Uddin, A.K. Meeker, R.A. Anders
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Muroyama, R.A. Anders, C.G. Drake

Writing, review, and/or revision of the manuscript: Y. Muroyama, T.R. Nirschl, C.M. Kochel, Z. Lopez-Bujanda, D. Theodros, W. Mao, A.E. Marciscano, E. Velarde, D.M. Pardoll, C.G. Drake

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Muroyama, A.E. Marciscano, A.J. Tam

Study supervision: C.G. Drake

that RT significantly increased tumor-infiltrating Tregs (TIL-Treg), which had higher expression of CTLA-4, 4-1BB, and Helios compared with Tregs in nonirradiated tumors. This observation held true across several tumor models (B16/F10, RENCA, and MC38). We found that TIL-Tregs from irradiated tumors had equal or improved suppressive capacity compared with nonirradiated tumors. Our data also indicated that after RT, Tregs proliferated more robustly than other T-cell subsets in the TME. In addition, after RT, expansion of Tregs occurred when T-cell migration was inhibited using Fingolimod, suggesting that the increased Treg frequency was likely due to preferential proliferation of intratumoral Treg after radiation. Our data also suggested that Treg expansion after irradiation was independent of TGF β and IL33 signaling. These data demonstrate that RT increased phenotypically and functionally suppressive Tregs in the TME. Our results suggest that RT might be combined effectively with Treg-targeting agents to maximize antitumor efficacy.

Introduction

Approximately 50% of cancer patients receive radiotherapy (RT) as part of their treatment regimen (1). Although the primary antitumor effects of RT involve the induction of single- and double-stranded DNA breaks that lead to apoptosis (2), studies by several groups (3, 4) showed that RT also has immunological effects, including the occasional induction of a systemic antitumor response, the so-called abscopal effect (5). RT modulates several elements of the immune response: (i) it promotes the secretion of proinflammatory cytokines such as IL1 and IL6 (6); (ii) it enhances expression or release of damage-associated molecular patterns (DAMP), including calreticulin, high mobility group box 1 (HMGB1), and ATP from affected tumor cells (4, 6, 7); (iii) it increases MHC-I expression on tumor cells (6, 8); and (iv) it activates the stimulator of interferon genes (STING) pathway leading to type-I interferon secretion (9). Together, these factors may lead to the maturation of dendritic cells, resulting in improved cross-presentation of tumor antigens and subsequent antigen-specific CD8⁺ T-cell responses (4, 10).

Despite these multiple proinflammatory effects, the abscopal effect is clinically rare, suggesting that RT may also exert immunosuppressive effects, or amplify preexisting suppressive components of the tumor microenvironment (TME) (11, 12). The protumor microenvironment incorporates numerous mechanisms by which tumors evade host antitumor immune responses. For instance, RT has been correlated with increased levels of TGF β (13), a pluripotent cytokine that suppresses immune responses (14). Another evasion method involves regulatory T cells (Treg), which suppress immune responses and are of clinical relevance (15). Treg depletion enhances CD8⁺ T-cell activity and limits tumor growth in animal models (16); treatments targeting Tregs are in various stages of clinical development (17). However, the effects of RT on suppressive immune cells, especially Tregs, have not been fully elucidated. Several reports suggest an increase of Tregs after radiation, both in animals and humans (10, 18–21), but whether radiotherapy may render tumor-infiltrating Tregs (TIL-Treg) phenotypically or functionally more suppressive has yet to be determined.

To test the hypothesis that stereotactic radiotherapy increases numbers of phenotypically and functionally suppressive TIL-Treg, we used the small animal radiation research platform (SARRP), which uses CT imaging to guide therapeutic radiation of tumors (22) and models stereotactic radiotherapy in human patients. We used this model to investigate the phenotype and functional ability of TIL-Treg. We also evaluated several possible mechanisms for the observed increase in numbers of Tregs after RT, including signaling by TGF β and IL33, and the role of trafficking of Tregs into the tumor versus intratumoral proliferation.

Materials and Methods

Experimental animals

C57BL/6J and BALB/cJ mice were purchased from The Jackson Laboratory. Female mice 4 to 8 weeks in age were used. To provide a congenic marker for cells used in suppression assays, 4- to 8-week-old, sex-matched CD45.1 (B6-Ly5.1/Cr) mice were purchased from Charles River Laboratories. In some studies, Tregs were isolated using sex-matched mice that express GFP under the control of the Foxp3 promoter (B6.129(Cg)-*Foxp3^{tm3(DTR/GFP)Ayr/J}*), referred hereafter as Foxp3 reporter mice (23). A breeding pair was purchased from The Jackson Laboratories and experimental animals were bred in-house. Animals were bred and housed in specific pathogen-free facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine (Baltimore, MD).

Cell lines

B16/F10, MC38, and RENCA cell lines were purchased from ATCC: B16/F10 and RENCA were cultured in complete RPMI (RPMI (Mediatech, Inc.)) with 10% fetal bovine serum (Gemini Bio Products), 1% antibiotic/anti-mycotic solution (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), and 1% MEM non-essential aminoacids (Thermo Fisher Scientific). MC38 cells were cultured in complete DMEM (DMEM (Mediatech, Inc.)) with 10% fetal bovine serum (Gemini Bio Products), 1% antibiotic/anti-mycotic solution (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), and 1% MEM non-essential aminoacids (Thermo Fisher Scientific) in 37°C, 5% CO₂ incubator.

Flow cytometry

Single-cell suspensions of tumor-infiltrating lymphocytes (TIL), draining lymph nodes (DLN), and spleens were prepared as previously described (10). Briefly, suspensions were prepared by mechanical dissociation, followed by density gradient centrifugation on an 80%/40% Percoll (GE Healthcare) gradient. Cells were Fc-blocked with purified rat anti-mouse CD16/CD32 (Clone: 2.4 G2, Becton Dickinson: BD) for 30 minutes in 4°C. Dead cells were discriminated using the LIVE/DEAD (LD) Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific) and samples were stained with the following antibodies: CD3 (Clone 17A2, BioLegend), CD4 (Clone RM4-5, Thermo Fisher Scientific), CD8a (Clone 53-6.7, BioLegend), Foxp3 (Clone FJK-16s, eBioscience), CCR4 (Clone 2G12, BioLegend), 4-1BB (Clone 17B5, BioLegend), Ki-67 (Clone SolA15, BioLegend), CTLA-4 (Clone UC10-4B9, BioLegend), and Helios (Clone 22F6, BioLegend). Stained samples were

analyzed on an LSR II flow cytometer (BD). Flow data were quantified using FlowJo software.

Radiotherapy (RT)

Palpable tumors were irradiated with 10 Gy using the Small Animal Radiation Research Platform (SARRP; Xstrahl) as previously described (22). Briefly, mice with palpable subcutaneous tumors (B16/F10, RENCA, and MC38) were anesthetized with isoflurane and treated with SARRP radiotherapy (RT). Seven days after radiation, tumors, DLNs, and spleens were harvested and analyzed by flow cytometry.

Tumor growth and TIL preparation

On day 0, 5×10^5 B16/F10, 1.5×10^6 MC38, or 3×10^5 RENCA cells were implanted subcutaneously (s.c.) to the flank of either wild-type C57BL/6J mice (in B16/F10 or MC38 experiments) or wild-type BALB/c mice (in RENCA experiments). On either day 7 (B16/F10), day 16 (RENCA), or day 10 (MC38), mice received 10 Gy of stereotactic radiation (RT) via the SARRP. Seven days after RT, mice were sacrificed. Tumors, DLNs, and spleens were harvested and used for flow-cytometry analysis. Tumor diameters were measured every 2 to 3 days with an electronic caliper and are reported as volume using the formula $(W^2 \times L)/2$, where W represents the shorter diameter and L stands for the longer tumor diameter.

Immunohistochemistry (IHC)

Immunostaining for Foxp3 was performed on formalin-fixed, paraffin-embedded sections. Briefly, following dewaxing and rehydration, slides were immersed in 1% tween-20, and heat-induced antigen retrieval was performed in a steamer using Target Retrieval Solution (Dako) for 45 minutes. Slides were rinsed in PBST and endogenous peroxidase and phosphatase was blocked with Dual Endogenous Enzyme Block (Dako), and sections were then incubated with primary antibody; Foxp3 Rabbit monoclonal antibody (Clone D6O8R, Cell Signaling Technology) for 45 minutes at room temperature. The primary antibodies were detected via a 30-minute incubation with HRP-labeled secondary antibody (Leica Microsystems) followed by detection with 3,3'-diaminobenzidine (Sigma-Aldrich), counterstaining with Harris hematoxylin, rehydration and mounting.

In vitro suppression assays (microsuppression assay)

Small-cell number suppression assays were performed as previously described (24). To reliably sort Tregs from spleen and tumors, Foxp3 reporter mice (B6.129(Cg)-*Foxp3^{tm3(DTR/GFP)/Ayr/J}*) were used (23). These mice were used for isolation of GFP-positive Treg, not for Treg-depletion. B16/F10 cells (5×10^5) were implanted s.c. into Foxp3 reporter mice. Mice were irradiated, and tumors and spleens were harvested as described above. TIL-Tregs and spleen Tregs from Foxp3 reporter mice were sorted as CD4⁺ GFP⁺ (CD4⁺ Foxp3⁺) cells; congenically marked responder cells (Tresponder) from the spleen and inguinal lymph nodes of CD45.1 (B6-Ly5.1/Cr) mice were sorted as CD45.1⁺ CD4⁺ CD25⁻. Antigen presenting cells (APC) were sorted as CD4⁻ Foxp3⁻ cells from spleen of Foxp3 reporter mice. Cells were sorted on a Fluorescence-Activated Cell Sorter (FACS) Aria II (BD). Sorted responder cells were stained with CellTrace Violet (CTV; Thermo Fisher

Scientific) and plated in a 96-well round-bottom plate at a density of 2×10^4 responder cells per well. GFP⁺ Tregs were added at 2-fold dilutions starting from 1×10^4 cells, in the presence of mitomycin C-fixed (Sigma-Aldrich), 4×10^4 APCs, and 1 µg/mL anti-CD3 (Clone: 145-2C11, BioLegend) and incubated for 80 to 90 hours. Proliferation of responder cells (gated as L/D⁻CD4⁺CD45.1⁺Foxp3⁻) was quantified by flow cytometry based on the dilution of Cell Trace Violet (CTV). Percent suppression (% Suppression) was calculated by the following formula: % Suppression = $[1 - (\% \text{ divided cells of the condition} / \text{the average of \% divided cells of Trespender-only conditions})] \times 100$.

TGFβ inhibition

Galunisertib (LY2157299; Selleck Chemicals) or vehicle (10 g glucose, 30 g cremophor, 30 g polyethylene glycol, 10 mL ethanol, and 30 mL distilled water) were delivered at a dose of 300 mg/kg/day in a volume of 100 µL via oral gavage every 12 hours, starting 1 day prior to RT and continued until the day of harvest.

IL33 signaling blockade

Mouse anti-ST2/IL-33 R monoclonal antibody (Clone 245707, R&D) was diluted in PBS and administrated intraperitoneally at a concentration of 200 µg in a volume of 100 µL per mouse every 3 days (25), starting 1 day before RT, for a total of three doses.

Fingolimod experiments

Fingolimod (FTY720, Enzo Life Sciences, Farmingdale, NY) was prepared and administered as previously described (26). Briefly, mice received a dose of 25 µg FTY720 or vehicle (PBS containing DMSO) via oral gavage in a volume of 100 µL per mouse every 3 days, starting 1 day prior to RT, for a total of three doses. Sequestration of peripheral lymphocytes was monitored using the Hemavet whole blood cell counter (Drew Scientific).

Luminex assays and ELISA

Tumors collected at different postradiation time points were minced, lysed in CellLytic MT (Sigma) containing halt protease and phosphatase inhibitor (Thermo Fisher Scientific) in a 1:100 ratio, and incubated on ice for 30 minutes with intermittent vortexing. Tumor lysates were assayed for raw protein concentration with Coomassie assay (Bio-Rad Laboratories). A panel of cytokines and chemokines (IL2, Exodus-2/CCL21/6Ckine, MCP-5/CCL12, Fractalkine/CXCL1, TARC/CCL17, MIP-3β, MDC2/CCL22, MIP-3α/CCL20, Eotaxin/CCL11, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, MCP-1/CCL2, MIG/CXCL9, RANTES/CCL5, and IP-10/CXCL10) was analyzed using a Millipore Mouse Cytokine/Chemokine Panel (Millipore). In addition, TGFβ and IL33 were analyzed using a Milliplex map Kit (Millipore), and a Mouse/Rat IL33 Quantikine ELISA Kit (R&D), respectively.

NanoString

RNA extraction was performed using the TRIzol reagent (Thermo Fisher Scientific) as per manufacturer's instructions. For NanoString analysis, the nCounter mouse PanCancer Immune Profiling panel was used using the nCounter Analysis System (both NanoString Technologies). Analysis was conducted using nSolver software (NanoString Technologies).

Statistical analyses

Group means were compared with Student *t* tests. Tumor growth and lymphocyte counts in the Fingolimod experiment were analyzed using two-way ANOVA with multiple comparisons. All statistical tests were two-sided, and *P* values equal or below 0.05 were considered statistically significant. All analyses were conducted using GraphPad Prism 5 (GraphPad Software Inc.).

Results

Stereotactic radiation increases Tregs in multiple tumor types

We first sought to investigate whether the previously observed RT-mediated increase in intratumoral Tregs (10, 18–21) was a general phenomenon, i.e., whether it occurred across multiple tumor types. For these studies, we used the SARRP (ref. 22; Fig. 1A). At the dose studied, RT suppressed B16/F10 (melanoma) tumor growth, but did not completely eradicate the tumors (Fig. 1B). Similar trends were observed in the RENCA (kidney cancer) and MC38 (colorectal cancer) models (Supplementary Fig. S1A and S1B). These effects were confirmed by a comprehensive profiling of the post-RT TME, which revealed additional changes in the immunobiology of the TME involving CD4⁺ T cells, CD8⁺ T cells, and myeloid cells (Supplementary Fig. S2). In all three tumor types studied, RT significantly increased both the proportion (Fig. 1D and E) and absolute numbers of Tregs (Fig. 1C and data not shown). This effect was local; no significant increases in Tregs were observed in either the DLN or spleen in any of the three models examined (Supplementary Fig. S3). This increase in Tregs was accompanied by an increase in Foxp3 levels as assayed by MFI (Fig. 1F) and was verified by IHC (Fig. 1G). Proportions and numbers of Tregs remained higher two weeks after the administration of RT (Supplementary Fig. S4). Taken together, these data established a model to study Tregs following RT.

The phenotype of Tregs after RT is consistent with activation

We next tested whether TIL-Tregs treated with RT developed a phenotype different from that observed in untreated tumors. For this, we focused on four molecules: (i) CCR4, which has been associated with Treg trafficking (27); (ii) the checkpoint molecule cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; ref. 28); (iii) the transcription factor Helios (IKZF2), which is upregulated with activation (29–31); and (iv) 4-1BB (CD137), which also increases with T-cell activation (32). As shown in Fig. 2, in the B16/F10 model, CCR4 was not significantly altered by RT. By contrast, CTLA-4, Helios, and 4-1BB expression were all significantly upregulated after RT, potentially consistent with a more activated Treg phenotype. Similar trends were noted in the RENCA and MC38 models (Supplementary Figs. S5 and S6). Supporting a local immunological role for RT, neither expression nor MFI of any of these four markers was significantly altered in either the DLN or the spleen of RT-treated mice (Supplementary Figs. S7 and S8). We also quantified expression of these markers in the non-Treg CD4⁺ T cells (Tconv) and found that in both the B16 and RENCA models, 4-1BB and CTLA-4 were induced after RT on Tconv as well; this was not observed in the MC38 model (Supplementary Figs. S9–S11). In none of the three models was 4-1BB significantly induced on CD8⁺ T cells after RT (Supplementary Fig. S12).

TIL-Tregs retain suppressive function after RT

Based on their increased expression of CTLA-4 (28) and Helios (29–31), we queried whether TIL-Tregs demonstrated increased suppressive function after RT. To investigate the suppressive capabilities of post-RT Treg, we conducted microsuppression assays (24). Briefly, GFP-labeled TIL-Tregs were sorted from Foxp3 reporter mice (B6.129(Cg)-*Foxp3^{tm3(DTR/GFP)Ayr/J}*), which express GFP under the Foxp3 promoter (23). As shown in Fig. 3, we found that TIL-Tregs from irradiated mice (RT TIL-Treg) were more suppressive than those from non-irradiated tumors (Control TIL-Treg) or unmanipulated spleens at low Treg:Tresponder ratios, but that at a higher ratio the three populations were functionally indistinguishable (Fig. 3A and B). Collectively, these data show that the post-RT Tregs from TILs are indeed functional and demonstrate enhanced suppression in standard *in vitro* suppression assays.

RT-induced expansion of TIL-Tregs is not TGFβ dependent

Given the well-described influence of TGFβ on Treg induction (33), we next investigated whether the post-RT Treg expansion observed above was dependent on TGFβ signaling. To test this hypothesis, we treated B16/F10 tumor-bearing mice with galunisertib (LY2157299), a small-molecule TGFβR1 kinase inhibitor that nearly completely abrogates TGFβ signaling (34–36). To optimize potential treatment effects, we began treatment with daily oral gavage (every 12 hours) 1 day prior to RT (Fig. 4A). As expected, treatment with galunisertib did not significantly alter tumor outgrowth, independent of the presence or absence of RT (Fig. 4B). Blockade of TGFβ did not significantly abrogate the RT-induced TIL-Tregs increase as compared with the vehicle-treated control groups (Fig. 4C). Neither the proportion of Foxp3⁺ cells (Fig. 4D) nor the absolute numbers (Fig. 4E) decreased, suggesting the post-RT TIL-Tregs increase was not critically dependent on TGFβ in this model. In addition, the overall composition of the T-cell infiltrate in these aggressive B16/F10 tumors was not significantly affected by galunisertib; there was no significant difference in total CD3⁺, CD4⁺ or CD8⁺ proportions (Supplementary Fig. S13). We also tested whether RT was associated with increased levels of TGFβ1 or β2. As shown in Supplementary Fig. S14, levels of both TGFβ molecules increased as tumors progressed, but significant differences were not observed between irradiated and nonirradiated tumors.

RT-induced expansion of TIL-Tregs is not IL33 dependent

Because data suggest the possibility of an increase in IL33 levels after RT (37), we next tested whether this cytokine might be responsible for the expansion of TIL-Tregs after RT. To investigate this hypothesis, we quantified IL33 levels in B16/F10 tumors after RT. As shown in Fig. 5A, IL33 levels increased after RT, consistent with previous data. Therefore, we treated mice with a neutralizing ST2 (IL-33R) antibody (25), starting 1 day prior to RT (Fig. 5B). Anti-ST2 treatment did not significantly affect tumor outgrowth (Fig. 5C). In addition, the blockade of ST2 did not significantly abrogate the post-RT TIL-Treg increase (Fig. 5D–F). Taken together, these data suggest that, in this model, the post-RT Treg increase is most likely not dependent on either TGFβ or IL33 signaling.

Radiation enhances the proliferation of TIL-Tregs

We next tested whether the observed increase in TIL-Tregs post RT was due to Treg migration into tumors as opposed to being driven by intratumoral proliferation of preexisting TIL-Treg. To perform these studies, we treated B16/F10-bearing mice with Fingolimod (FTY720), a blocker of the sphingosine 1-phosphate receptor 1 (SP1); this drug blocks T-cell egress from lymphoid organs (26, 38). As in the studies above, treatment was initiated 1 day prior to radiation (Fig. 6A). As expected, administration of Fingolimod resulted in an approximately 75% to 80% decrease in peripheral blood lymphocytes counts from baseline (Supplementary Fig. S15), and did not significantly affect tumor growth (Fig. 6B). Fingolimod slightly mitigated the TIL-Treg increase, but did not abrogate it completely (Fig. 6C and D). We next investigated the proliferation of intratumoral Tregs via Ki-67 staining. RT increased Treg proliferation within tumors both in the presence or absence of Fingolimod (Fig. 6E–G). Although post-RT proliferation occurred to some extent in all T-cell subsets, after RT the proliferation of Tregs was approximately 2-fold greater than that of T-cell subsets examined (Fig. 6G).

Discussion

Radiation therapy is a mainstay of cancer treatment and is used across a spectrum of tumor types. RT induces single- and double-stranded DNA breaks, which lead to apoptotic cell death. Tumor cells are preferentially affected (2, 39). However, evidence suggests that RT has immunological effects as well, including the occasional induction of a systemic antitumor response, the so-called abscopal effect (3, 4).

Here, we found an increased frequency of Tregs in the TME of several tumor types (B16/F10, RENCA, and MC38) following treatment with RT (Fig. 1). The consistency across these disparate tumor types (using two separate mouse strains) is in agreement with prior results (10, 18–21). We investigated Treg-specific expression of several molecules after RT: chemokine receptor CCR4 (27), the checkpoint molecules CTLA-4 (28) and 4-1BB (32), and the transcription factor (Helios). After RT, we found increased expression of CTLA-4, 4-1BB, and Helios, consistent with an activated/suppressive phenotype. Based on the increased expression of CTLA-4 (28) and Helios (29–31) observed in RT-treated TIL-Tregs, we next sought to investigate whether these cells demonstrated greater suppressive capability than their non-treated counterparts. Tregs from RT-treated tumors not only retained their ability to suppress effector-cell proliferation, but were more suppressive at low Treg:Tresponder ratios. Our development of a microsuppression assay using sorted TIL-Tregs to acquire data on mice treated with stereotactic RT may help clarify the suppressive mechanisms that counterbalance RT-induced immune activation (4, 40–42).

We interrogated several potential molecular mechanisms underlying the RT-mediated increase in the frequency of TIL-Treg. A primary consideration was TGF β for three reasons. First, TGF β induces the expression of Foxp3, the master transcription factor for Treg (43), in CD4⁺Foxp3⁺ effector CD4⁺ T cells (33, 44). Second, RT correlates with increased TGF β expression (13). Lastly, several prior studies suggest synergistic antitumor effects when RT is combined with TGF β blockade (45–47). Thus, we blocked TGF β signaling using the small-molecule inhibitor galunisertib (LY2157299), which prevents phosphorylation of

SMAD2 by inhibiting TGF β receptor I kinase (34–36). We were not able to influence the TIL-Treg increase induced by RT with TGF β blockade. Our results are not consistent with studies by Wu and colleagues (48), in which TGF β blockade mitigated the TIL-Treg increase after irradiation in a murine prostate cancer model. The reasons for the discrepancy between these results are not obvious, but may include differences in the models used, or in agents used; the prior studies used intraperitoneal delivery of a neutralizing antibody against TGF β 1 or transfection with TGF β 1 shRNA, whereas we orally administered a small-molecule inhibitor that is currently under investigation in a number of clinical trials (35).

In addition, we investigated IL33, a cytokine that has been described as important in Treg expansion in several models (49–51). As in (37), we found that RT increased the levels of IL33 in the TME. We thus blocked IL33–IL33R (ST2) signaling using antibodies against ST2 to test whether IL33 might play a role in the increment of TIL-Tregs after RT. IL33 blockade did not abrogate the TIL-Treg increase after irradiation (Fig. 5). What molecules or signaling pathways drive Treg expansion in our models remains a matter for ongoing investigation. We also found that the post-RT TME undergoes a number of dynamic changes in the expression of multiple immunological transcripts involving diverse immune cell subsets. This not only highlights the complexity and dynamics of the TME but also suggests additional avenues for intervention (Supplementary Fig. S2). Some of the effects of RT appeared to transcend cell subtype; for example, we noted increased proliferation of CD4⁺, CD8⁺, and Tregs after RT. In contrast, some cell surface proteins were differentially affected, with 4-1BB expression upregulated after RT on Tregs but not on CD8⁺ T cells. These data highlight the complex effects of RT on the immune components of TME.

We next sought to determine whether the observed increase was due to Treg proliferation *in situ*, or due to an increased trafficking of peripheral Tregs to the TME. For these studies, we used Fingo-limod, a small-molecule S1P signaling inhibitor that blocks T-cell emigration from lymph nodes. We hypothesized that if the RT TIL-Treg increase was due to an influx of new immune cells into irradiated tumors, then the increase would be blocked or attenuated by Fingolimod treatment. In multiple iterations, the post-RT Treg increase appears to be blunted but not blocked in the presence of this agent (Fig. 6). From a technical standpoint, Fingolimod-treated mice displayed less infiltration when considering the density of T cells (ubiquitously across all immune cell types) in the TME, these low numbers may be a source of variation in population frequencies. Therefore, it is possible that trafficking of Tregs may play some role in the increased Treg frequency in the TME, as suggested by an increased level of the CCR4 ligands: CCL17 and CCL22 in RT-treated tumors (Supplementary Fig. S16). Finally, we turned our attention to the possibility of Treg expansion/proliferation driving the post-RT increase of TIL-Treg. We found an increase in TIL-Treg proliferation after RT, suggesting that proliferation explains most of the post-RT Treg increase. Nearly all T-cell subtypes in the tumor showed increased proliferation after RT, but the effect was most pronounced in the Treg population (Fig. 6), leading to a potential suppressive counterbalance to the stimulatory effects of RT (4, 40, 41, 52).

The strengths of these studies include a well-defined, localized, and consistent RT treatment regimen using the SARRP platform (22), which we used to model single-dose stereotactic radiation. In addition, we controlled for tumor size at the time of treatment. Potential

limitations include the use of implanted tumor models, which may not accurately recapitulate the TME in human tumors, and that we did not test other radiation doses or fractionation regimens. Thus, it is possible that our results may not translate to other treatment regimens. Nevertheless, the results obtained here are consistent with prior work (10, 18–21) showing that RT may induce proliferation of Treg, an outcome that may need to be considered in clinical regimens that combine RT with immunotherapy. In addition, we documented that the post-RT intratumoral Tregs have suppressive function, providing a rationale for regimens combining Treg-targeted strategies with RT. Indeed, such combination regimens may be required to optimize the immune effects of RT in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Dario Vignali, Dr. Greg Delgoffe, Dr. Tullia Bruno, Abigail E. Overacre-Delgoffe, and members of Dr. Dario Vignali's lab at University of Pittsburgh for their technical input on *in vitro* microsuppression assay; the Johns Hopkins University Sidney Kimmel Comprehensive Cancer Center Flow Core (Julie Nauroth and Ada J. Tam), Immune Monitoring Core (Christopher J. Thoburn) for Luminex; Dr. Robert A. Anders, Farah Succaria, and Qingfeng Zhu for their help in evaluating immunohistochemistry slides; and members of Dr. Charles Drake's lab, Dr. Drew Pardoll's lab, Dr. Jonathan Powell's lab, Dr. Fan Pan's lab, Dr. Elizabeth Jaffee's lab, and Dr. Leisha Emens' lab in the Bloomberg/Kimmel Institute for Cancer Immunotherapy at Johns Hopkins University School of Medicine.

Grant Support

These studies were supported by NIH R01CA127153 and P30CA006973, the Patrick C. Walsh Prostate Cancer Research Fund, the One-in-Six Foundation, the Prostate Cancer Foundation, and the Melanoma Research Alliance. Y. Muroyama was the recipient of the scholarship from Japan Student Services Organization (JASSO).

References

- Harrington KJ, Billingham LJ, Brunner TB, Burnet NG, Chan CS, Hoskin P, et al. Guidelines for preclinical and early phase clinical assessment of novel radiosensitisers. *Br J Cancer*. 2011; 105:628–39. [PubMed: 21772330]
- Liau SL, Connell PP, Weichselbaum RR. New paradigms and future challenges in radiation oncology: an update of biological targets and technology. *Sci Transl Med*. 2013; 5:173sr2. [PubMed: 23427246]
- Demaria S, Golden EB, Formenti SC. Role of local radiation therapy in cancer immunotherapy. *JAMA Oncol*. 2015; 1:1325–32. [PubMed: 26270858]
- Sharabi AB, Lim M, DeWeese TL, Drake CG. Radiation and checkpoint blockade immunotherapy: radiosensitisation and potential mechanisms of synergy. *Lancet Oncol*. 2015; 16:e498–509. [PubMed: 26433823]
- Postow MA, Callahan MK, Barker CA, Yamada Y, Yuan J, Kitano S, et al. Immunologic correlates of the abscopal effect in a patient with melanoma. *N Engl J Med*. 2012; 366:925–31. [PubMed: 22397654]
- Drake C. Radiation-Induced Immune Modulation. In: DeWeese, TL., Laiho, M., editors. *Molecular determinants of radiation response, current cancer research*. New York: Springer-Verlag; 2011. p. 251–63.
- Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med*. 2007; 13:1050–9. [PubMed: 17704786]

8. Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK, et al. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J Exp Med*. 2006; 203:1259–71. [PubMed: 16636135]
9. Deng L, Liang H, Xu M, Yang X, Burnette B, Arina A, et al. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity*. 2014; 41:843–52. [PubMed: 25517616]
10. Sharabi AB, Nirschl CJ, Kochel CM, Nirschl TR, Francica BJ, Velarde E, et al. Stereotactic radiation therapy augments antigen-specific PD-1-mediated antitumor immune responses via cross-presentation of tumor antigen. *Cancer Immunol Res*. 2015; 3:345–55. [PubMed: 25527358]
11. van der Burg SH, Arens R, Ossendorp F, van Hall T, Melief CJ. Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nat Rev Cancer*. 2016; 16:219–33. [PubMed: 26965076]
12. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011; 331:1565–70. [PubMed: 21436444]
13. Martin M, Lefaix JL, Delanian S. TGF- β 1 and radiation fibrosis: a master switch and a specific therapeutic target? *Int J Radiat Oncol Biol Phys*. 2000; 47:277–90. [PubMed: 10802350]
14. Thomas DA, Massagué J. TGF- β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell*. 2005; 8:369–80. [PubMed: 16286245]
15. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Curr Opin Immunol*. 2014; 27:1–7. [PubMed: 24413387]
16. Bos PD, Plitas G, Rudra D, Lee SY, Rudensky AY. Transient regulatory T cell ablation deters oncogene-driven breast cancer and enhances radiotherapy. *J Exp Med*. 2013; 210:2435–66. [PubMed: 24127486]
17. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010; 363:711–23. [PubMed: 20525992]
18. Kachikwu EL, Iwamoto KS, Liao YP, DeMarco JJ, Agazaryan N, Economou JS, et al. Radiation enhances regulatory T cell representation. *Int J Radiat Oncol Biol Phys*. 2011; 81:1128–35. [PubMed: 21093169]
19. Price JG, Idoyaga J, Salmon H, Hogstad B, Bigarella CL, Ghaffari S, et al. CDKN1A regulates Langerhans cell survival and promotes Treg cell generation upon exposure to ionizing irradiation. *Nat Immunol*. 2015; 16:1060–8. [PubMed: 26343536]
20. Schuler PJ, Harasymczuk M, Schilling B, Saze Z, Strauss L, Lang S, et al. Effects of adjuvant chemoradiotherapy on the frequency and function of regulatory T cells in patients with head and neck cancer. *Clin Cancer Res*. 2013; 19:6585–96. [PubMed: 24097865]
21. Wirsdorfer F, Cappuccini F, Niazman M, de Leve S, Westendorf AM, Ludemann L, et al. Thorax irradiation triggers a local and systemic accumulation of immunosuppressive CD4⁺ FoxP3⁺ regulatory T cells. *Radiat Oncol*. 2014; 9:98. [PubMed: 24766907]
22. Wong J, Armour E, Kazantzides P, Iordachita I, Tryggestad E, Deng H, et al. High-resolution, small animal radiation research platform with X-ray tomographic guidance capabilities. *Int J Radiat Oncol Biol Phys*. 2008; 71:1591–9. [PubMed: 18640502]
23. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*. 2007; 8:191–7. [PubMed: 17136045]
24. Turnis ME, Sawant DV, Szymczak-Workman AL, Andrews LP, Delgoffe GM, Yano H, et al. Interleukin-35 limits anti-tumor immunity. *Immunity*. 2016; 44:316–29. [PubMed: 26872697]
25. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol*. 2011; 12:1045–54. [PubMed: 21946417]
26. Spranger S, Koblish HK, Horton B, Scherle PA, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. *J Immunother Cancer*. 2014; 2:3. [PubMed: 24829760]
27. Chang DK, Peterson E, Sun J, Goudie C, Drapkin RI, Liu JF, et al. Anti-CCR4 monoclonal antibody enhances antitumor immunity by modulating tumor-infiltrating Tregs in an ovarian

- cancer xenograft humanized mouse model. *Oncoimmunology*. 2016; 5:e1090075. [PubMed: 27141347]
28. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 2008; 322:271–5. [PubMed: 18845758]
 29. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. *PLoS One*. 2011; 6:e24226. [PubMed: 21918685]
 30. Zabransky DJ, Nirschl CJ, Durham NM, Park BV, Ceccato CM, Bruno TC, et al. Phenotypic and functional properties of Helios+ regulatory T cells. *PLoS One*. 2012; 7:e34547. [PubMed: 22479644]
 31. Fuhrman CA, Yeh WI, Seay HR, Saikumar Lakshmi P, Chopra G, Zhang L, et al. Divergent phenotypes of human regulatory T cells expressing the receptors TIGIT and CD226. *J Immunol*. 2015; 195:145–55. [PubMed: 25994968]
 32. So T, Lee SW, Croft M. Immune regulation and control of regulatory T cells by OX40 and 4-1BB. *Cytokine Growth Factor Rev*. 2008; 19:253–62. [PubMed: 18508403]
 33. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limón P. The polarization of immune cells in the tumour environment by TGF β . *Nat Rev Immunol*. 2010; 10:554–67. [PubMed: 20616810]
 34. Bueno L, de Alwis DP, Pitou C, Yingling J, Lahn M, Glatt S, et al. Semi-mechanistic modelling of the tumour growth inhibitory effects of LY2157299, a new type I receptor TGF- β kinase antagonist, in mice. *Eur J Cancer*. 2008; 44:142–50. [PubMed: 18039567]
 35. Herbertz S, Sawyer JS, Stauber AJ, Gueorguieva I, Driscoll KE, Estrem ST, et al. Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway. *Drug Des Devel Ther*. 2015; 9:4479–99.
 36. Serova M, Tijeras-Raballand A, Dos Santos C, Albuquerque M, Paradis V, Neuzillet C, et al. Effects of TGF-beta signalling inhibition with galunisertib (LY2157299) in hepatocellular carcinoma models and in ex vivo whole tumor tissue samples from patients. *Oncotarget*. 2015; 6:21614–27. [PubMed: 26057634]
 37. Lee EJ, Kim JW, Yoo H, Kwak W, Choi WH, Cho S, et al. Single high-dose irradiation aggravates eosinophil-mediated fibrosis through IL-33 secreted from impaired vessels in the skin compared to fractionated irradiation. *Biochem Biophys Res Commun*. 2015; 464:20–6. [PubMed: 26047701]
 38. Halin C, Scimone ML, Bonasio R, Gauguier JM, Mempel TR, Quackenbush E, et al. The S1P-analog FTY720 differentially modulates T-cell homing via HEV: T-cell-expressed S1P1 amplifies integrin activation in peripheral lymph nodes but not in Peyer patches. *Blood*. 2005; 106:1314–22. [PubMed: 15870184]
 39. Deloch L, Derer A, Hartmann J, Frey B, Fietkau R, Gaipl US. Modern Radiotherapy concepts and the impact of radiation on immune activation. *Front Oncol*. 2016; 6:141. [PubMed: 27379203]
 40. Demaria S, Formenti SC. Radiation as an immunological adjuvant: current evidence on dose and fractionation. *Front Oncol*. 2012; 2:153. [PubMed: 23112958]
 41. Burnette B, Weichselbaum RR. The immunology of ablative radiation. *Semin Radiat Oncol*. 2015; 25:40–5. [PubMed: 25481265]
 42. Gough MJ, Crittenden MR. Combination approaches to immunotherapy: the radiotherapy example. *Immunotherapy*. 2009; 1:1025–37. [PubMed: 20635917]
 43. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003; 299:1057–61. [PubMed: 12522256]
 44. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 2003; 198:1875–86. [PubMed: 14676299]
 45. Vanpouille-Box C, Diamond JM, Pilonis KA, Zavadi J, Babb JS, Formenti SC, et al. TGFbeta is a master regulator of radiation therapy-induced antitumor immunity. *Cancer Res*. 2015; 75:2232–42. [PubMed: 25858148]
 46. Hardee ME, Marciscano AE, Medina-Ramirez CM, Zagzag D, Narayana A, Lonning SM, et al. Resistance of glioblastoma-initiating cells to radiation mediated by the tumor microenvironment can be abolished by inhibiting transforming growth factor- β . *Cancer Res*. 2012; 72:4119–29. [PubMed: 22693253]

47. Young KH, Newell P, Cottam B, Friedman D, Savage T, Baird JR, et al. TGFbeta inhibition prior to hypofractionated radiation enhances efficacy in preclinical models. *Cancer Immunol Res.* 2014; 2:1011–22. [PubMed: 25047233]
48. Wu CT, Hsieh CC, Yen TC, Chen WC, Chen MF. TGF- β 1 mediates the radiation response of prostate cancer. *J Mol Med.* 2015; 93:73–82. [PubMed: 25228112]
49. Turnquist HR, Zhao Z, Rosborough BR, Liu Q, Castellaneta A, Isse K, et al. IL-33 expands suppressive CD11b+ Gr-1int and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. *J Immunol.* 2011; 187:4598–610. [PubMed: 21949025]
50. Matta BM, Lott JM, Mathews LR, Liu Q, Rosborough BR, Blazar BR, et al. IL-33 is an unconventional Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. *J Immunol.* 2014; 193:4010–20. [PubMed: 25217167]
51. Matta BM, Reichenbach DK, Zhang X, Mathews L, Koehn BH, Dwyer GK, et al. Peri-alloHCT IL-33 administration expands recipient T-regulatory cells that protect mice against acute GVHD. *Blood.* 2016; 128:427–39. [PubMed: 27222477]
52. Burnette B, Weichselbaum RR. Radiation as an immune modulator. *Semin Radiat Oncol.* 2013; 23:273–80. [PubMed: 24012341]

Figure 1.

Stereotactic radiotherapy increases Tregs in tumors. **A**, Experimental design. 5×10^5 B16/F10, 1.5×10^6 MC38, or 3×10^5 RENCA cells were implanted subcutaneously (s.c.) to the flank of wild-type C57BL/6J mice (in B16/F10 or MC38 experiments) or BALB/c mice (in RENCA experiment), respectively on day 0. Mice received 10 Gy of stereotactic radiotherapy (RT) via SARRP on day 7 (B16/F10), day 16 (RENCA), day 10 (MC38), respectively. Tumors, draining lymph nodes (DLN), and spleens were harvested 7 days after the radiation. **B**, Tumor growth curves of B16/F10-bearing mice as in **A**. Nonirradiated tumors (control) in black line and irradiated tumors (RT) in red line, respectively. **C**, The absolute number of TIL-Tregs per gram tumor weight in the B16/F10 model. **D–F**, Representative flow plots (**D**) and quantitative bar graph of % Foxp3⁺ cells (**E**) and MFI of

Foxp3 (**F**) in CD4⁺ cells from TILs. $N = 7-15$ per group, repeated at least 4×. **G**, Immunohistochemistry of Foxp3 in tumors (B16/F10, RENCA, MC38), 20× and 40× magnification as indicated. Foxp3⁺ cells are stained red in B16/F10 (due to the brown pigment in melanoma), and brown in RENCA and MC38 tumors. Error bars, SEM; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, determined by two-way ANOVA (**B**) or unpaired Student t test (**C**, **E** and **F**).

Figure 2.

Stereotactic radiotherapy increases the activation/suppression markers of TIL-Tregs. **A** and **B**, Representative flow plots (**A**) and quantitative scatterplot (**B**) depicting of the percentages of CCR4⁺, CTLA4⁺, 4-1BB⁺ or Helios⁺ TIL-Treg, from day 14 B16/F10 tumor-bearing mice. *N* = 8 per group, repeated 3×. Error bars, SEM; ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05, determined by an unpaired Student *t* test (**B**).

Figure 3. Radiated tumor-infiltrating Tregs (TIL-Treg) are functionally suppressive. **A**, Representative figure of the *in vitro* suppression assay. Responder T cells were gated based on the CD45.1 congenically marked and their proliferation was analyzed based on the dilution of CTV dye. Solid lines show the conditions with spleen Tregs (black line), nonirradiated control TIL-Tregs (blue line), and RT TIL-Tregs (red line); filled green histograms shows the T responder-only condition. **B**, Quantitative plots represent percent suppression at the indicated Treg:T responder ratio. A representative experiment of a total of three independent replicates is shown ($n = 2-3$ per group, repeated 3 $\times$).

Figure 4.

The effect of TGF β on the post-RT increase of Tregs in tumors. **A**, Experimental design. C57BL/6 mice were injected s.c. with 5×10^5 B16/F10 cells on day 0. Mice received 10 Gy of RT on day 7. Mice received galunisertib (300 mg/kg/day, LY2157299) or vehicle via oral gavage every 12 hours, starting a day before RT. Tumors, DLNs and spleens were harvested on day 14. **B**, Tumor growth curves of vehicle versus galunisertib treated mice (dashed lines vs. real line, respectively), with or without radiation (black line or red line, respectively, $n = 5-6$ per group, repeated $\times 2$). **C**, Representative flow plot of TIL-Tregs. **D** and **E**, Quantitative scatter plots of (**D**) the percent of tumor-infiltrating CD4⁺ cells that were

Foxp3⁺ and **(E)** the absolute number of TIL-Tregs per gram tumor weight, in galunisertib-treated versus vehicle-treated groups (pooled data from two experiments; Error bars, SEM). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, determined by two-way ANOVA (**B**) and an unpaired Student t test (**D**, **E**).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

The effect of IL33 on the post-RT increase of Tregs in tumors. **A**, The time course of the IL33 level in tumor lysates from different time points (1, 6, 24, 48, and 72 hours and 7 days post RT) measured by ELISA ($n = 5$ per group). **B**, Experimental design. C57BL/6 mice were injected subcutaneously with 5×10^5 B16/F10 cells on day 0. Mice received 10 Gy of RT on day 7. Mice received a mAb to ST2 (200 μ g/ mouse, Anti-ST2 Ab) or vehicle intraperitoneally (i.p.) every 3 days, starting 1 day before RT. Tumors, DLNs, and spleens were harvested on day 14. **C**, Tumor growth curves of vehicle control- (dashed line) versus ST2 Ab-treated mice (solid line), with or without radiation (black or red, respectively). **D**, Representative flow plot of TIL-Tregs. **E** and **F**, Quantitative scatter plots of % Fopx3⁺ cells of tumor-infiltrating CD4⁺ cells (**E**), and the absolute number of TIL-Tregs per gram tumor weight (**F**) in ST2 Ab-treated versus vehicle-treated group ($n = 5$ per group, repeated $\times 2$). Error bars, SEM; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, determined by two-way ANOVA (**A**, **C**), and an unpaired Student t test (**E**, **F**).

Figure 6.

Stereotactic radiation enhances preferential Treg proliferation in the tumor microenvironment (TME). **A**, Experimental design. C57BL/6 mice were injected subcutaneously with 5×10^5 B16/F10 cells on day 0. Mice received 10 Gy of RT on day 7. Mice received Fingolimod (25 μ g, FTY720) or PBS containing DMSO as control via oral gavage on days 6, 9, and 12. Peripheral blood was collected for monitoring lymphocyte counts. Tumors, DLNs and spleens were harvested on day 14. **B**, Tumor growth curves of vehicle versus FTY720-treated mice (dashed line vs. solid line, respectively), with or without radiation (black or red line respectively). **C**, Representative flow plot of TIL-Tregs (CD4⁺ Foxp3⁺). **D**, Absolute number Tregs per gram tumor weight in FTY720-treated versus vehicle-treated group. **E** and **F**, Representative flow plot (**E**) and quantitative scatter plot (**F**) of tumor-infiltrating Ki-67⁺ Tregs (CD4⁺ Foxp3⁺ cells). **G**, Quantitative scatter plot of Ki-67 expression in tumor-infiltrating CD3⁺, CD4⁺, CD8⁺ T cells, and Tregs ($n = 9-10$ per group, repeated $\times 2$). **D**, Pooled data from two experiments. Error bars, SEM; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, determined by two-way ANOVA (**B**), an unpaired Student t test (**D**, **F**, and **G**).