Research Article

TGFβ Inhibition Prior to Hypofractionated Radiation Enhances Efficacy in Preclinical Models

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Abstract

The immune infiltrate in colorectal cancer has been correlated with outcome, such that individuals with higher infiltrations of T cells have increased survival independent of the disease stage. For patients with lower immune infiltrates, overall survival is limited. Because the patients with colorectal cancer studied have received conventional cancer therapies, these data may indicate that the pretreatment tumor environment increases the efficacy of treatments such as chemotherapy, surgery, and radiotherapy. This study was designed to test the hypothesis that an improved immune environment in the tumor at the time of treatment will increase the efficacy of radiotherapy. We demonstrate that inhibition of TGFβ using the orally available small-molecule inhibitor SM16 improved the immune environment of tumors in mice and significantly improved the efficacy of subsequent radiotherapy. This effect was not due to changes in radiosensitivity, epithelial–mesenchymal transition, or changes in vascular function in the tumor; rather, this effect was dependent on adaptive immunity and resulted in long-term protective immunity in cured mice. These data demonstrate that immunotherapy is an option to improve the immune status of patients with poor tumor infiltrates and that pretreatment improves the efficacy of radiotherapy. Cancer Immunol Res; 2(10); 1011–22. ©2014 AACR.

Introduction

Individualized therapy aims to identify which patient might benefit from specific targeted therapy. Although many targeted therapies focus on aberrations in growth signaling pathways, recently strides have been made in targeting immune alterations (1–3). Immune evasion is a critical step in carcinogenesis and occurs through an array of mechanisms (4–6). Studies report that decreased T-cell infiltrate and increased macrophage infiltrate correlate with decreased survival (7, 8). These data are particularly dramatic in colorectal cancer, for which an international effort is under way to evaluate immune infiltrates in tumors as a prognostic tool (7). For patients identified with poor “immune scores,” the question remains as to whether the tumor immune status can be improved. Infiltrate and survival correlations were made in patients treated with some combination of surgical resection, chemotherapy, and radiotherapy, suggesting that the immune environment of tumors influences outcome via conventional therapies. Thus, we hypothesize that an improved immune environment in the tumor at the time of treatment will increase the efficacy of radiotherapy.

Radiotherapy is a common treatment primarily related to its efficacy as a focal cytotoxic agent. An underrecognized benefit to radiotherapy is its ability to expose tumor antigen and create a focal inflammatory response (9–11). Preclinical studies have demonstrated a dependence on adaptive immunity for the full efficacy of radiotherapy and improved therapeutic efficacy if radiotherapy is followed by systemic immunomodulatory therapy (12–14). Anecdotal reports have demonstrated that immune therapy followed by radiotherapy can lead to tumor regression (15). Investigators are testing the hypothesis that radiotherapy can act in synergy with immunotherapy, leading to immune activation with resultant systemic antitumor immune activity. This has been described when combining radiotherapy with systemic IL2 therapy in patients with metastatic melanoma and renal cell carcinoma (16). In this trial, patients who responded to therapy had higher levels of proliferating effector memory T cells before treatment (16), indicating that the pretreatment immune response may affect therapeutic response. In addition, in a preclinical model of metastatic colorectal cancer, changing the pretreatment immune environment by tumor-associated antigen vaccination followed by high-dose radiotherapy leads to antigen cascade and metastatic tumor clearance (17).

Transforming growth factor β (TGFβ) is a multipotent cytokine that has opposing effects on carcinogenesis in a cell-context and timing-specific manner (18). Before invasion, TGFβ acts mainly as a tumor suppressor; at later invasive stages, TGFβ acts as a tumor promoter via effects on proliferation, differentiation, angiogenesis, and inflammation (19–21). TGFβ...
is largely immunosuppressive, with effects on virtually all immune-cell lineages. TGFβ decreases CD8 T-cell proliferation and effector function via regulation of IFNγ, granzyme, and perforin expression, while promoting regulatory T cell (Treg) activation and proliferation (22). In addition, TGFβ promotes alternative macrophage differentiation and decreases antigen presentation and effector function, while increasing macrophage trafficking to the site of inflammation (23, 24). TGFβ inhibitors have been combined with radiotherapy in preclinical cancer models and have increased radiosensitivity and reduced inhibition is a tool to improve the in vivo efficacy of radiotherapy.

We hypothesize that an improved immune environment at the time of treatment will increase the efficacy of radiotherapy. The data presented below demonstrate that TGFβ inhibition improves the immune environment of colorectal tumors in mice by increasing CD8 T-cell infiltrate and decreasing Treg infiltrate, resulting in increased efficacy of radiotherapy. We show that the efficacy is dependent on CD8 T cells. These data demonstrate that manipulation of the pretreatment tumor immune environment is a potential therapy for patients with poor immune scores, and TGFβ inhibition is a tool to improve the in vivo efficacy of radiotherapy.

Materials and Methods

Cell lines and animals

The CT26 murine colorectal and the 4T1 mammary carcinoma cell lines (31) were obtained from the ATCC. The Panc02 murine pancreatic adenocarcinoma cell line (32) was kindly provided by Dr. Woo (Mount Sinai School of Medicine, New York, NY). All three cell lines were tested and validated to be Mycoplasma free; no other authentication assay was performed. C57BL/6 and BALB/c were obtained from The Jackson Laboratory. Animal protocols were approved by the Earle A. Chiles Research Institute IACUC (Animal Welfare Assurance No. A3913-01).

Antibodies and reagents

Flow cytometry. Fluorescently conjugated antibodies CD11b-AF700, Gr1-PE-Cy7, IA(MHC class II)-e780, Ly6C-PerCP-Cy5.5, CD4-e450, FoxP3-e450, CD25-APC, CD4-PerCP-Cy5.5, CD8-FITC, CD45-e650, CD11c-e450, CD62L-AF700, CD49d-FITC, CD11b-AF700, Gr1-PE-Cy7, IA(MHC class II)-e780, Ly6C-e780, CD80-APC, and CD86-PE were purchased from eBioscience. PE-Cy7, CD45-e650, CD11c-e450, CD40-FITC, B220-PE-Cy7, CD80-APC, and CD86-PE were purchased from eBioscience. Hypoxyprobe and detection antibody was purchased from Hypoxyprobe, and FITC-conjugated 40,000 MW dextran was purchased from Invitrogen. CD44-APC-Cy7 was purchased from BioLegend. CD8-PE-TxRD was purchased from Invitrogen. CD4-v500 and Ly6G-FITC were purchased from BD Biosciences. CD40-PerCP-Cy7, B220-PE-Cy7, CD80-APC, and CD86-PE were purchased from eBioscience. CD4-e450, FoxP3-e450, CD25-APC, CD4-PerCP-Cy7, F4/80 was purchased from AbD Serotec, E-cadherin from BD Biosciences, CD3 and CD31 from Spring Bioscience, and Cy3-conjugated smooth muscle actin (SMA) from Sigma. Hypoxyprobe and detection antibody was purchased from Hypoxyprobe, and FITC-conjugated 40,000 MW dextran was purchased from Invitrogen.

Western blot analysis. pSMAD2, SMAD2, GAPDH, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology. Arginase I was purchased from BD Biosciences, and iNOS from Cayman Chemical Corporation.

SM16. SM16 was obtained under a material transfer agreement from Biogen Idec and was incorporated into standard Purina rodent chow (#5001) by Research Diets at a concentration of 0.3 g SM16 per kg chow (0.03%). A calorie and nutrient-matched diet without SM16 (Purina) was used as the control diet.

Clonogenic assay

Cells were irradiated in a single fraction using a cesium irradiator at the indicated doses and plated in triplicate. When colonies reached approximately 50 cells in size (5–7 days), they were fixed with methanol and manually counted with average colony counts plotted relative to unirradiated control.

In vivo radiotherapy models

CT26 (1 × 10^6) or Panc02 cells (2 × 10^6) were injected s.c. in the right hind limb of immunocompetent BALB/c or C57BL/6 mice, respectively. SM16 or control chow was fed from day 7 to day 13. Radiation was delivered using the clinical linear accelerator (6 MV photons; Elekta Synergy linear accelerator), with a halfbeam block to protect vital organs and 1.0-cm bolus. For CT26 tumors, 20 Gy × 1 was delivered on day 14; for Panc02 tumors, 20 Gy × 3 was delivered from day 14 to day 16, as described previously (33). Mice cured of CT26 tumors were rechallenged with 5 × 10^3 4T1 and 1 × 10^5 CT26 cells in opposite flanks to assess tumor-specific immunity. For concomitant immunity experiments, mice were challenged with 1 × 10^6 CT26 cells in the right hind limb on day 0, then 1 × 10^4 CT26 in the left hind limb on day 3. Mice were treated with control or SM16 chow beginning on day 7. Radiation was delivered to only the right limb tumor, as described above.

For orthotopic pancreatic tumors, the spleen was displaced to reveal the pancreas via a 5-mm incision in anesthetized C57BL/6 mice. Panc02 cells (8 × 10^5) were injected in 8 μL of PBS with 2 μL Lipidol Ultra-Fluide (Guerbet; donated by Dr. Kolbeck, Oregon Health and Sciences University, Portland, OR) into the pancreas. The peritoneum, abdominal wall, and skin were sutured closed in two layers. Radiation was delivered using the Small Animal Radiation Research Platform (SARRP; XStrahl; Gulmay Medical). Using a cone-beam CT scan, the tumor was visualized with lipiodol enhancement. Isocenter was placed within the tumor adjacent to the lipiodol stain. A 180° arc using a 5 × 5 mm collimator was used to deliver 8 Gy × 3, mice were treated every other day, beginning on day 14.

Immune analysis

For analysis of tumor-infiltrating immune cells, the tumor was digested in 10 mL of PBS with 1 mg/mL collagenase (Invitrogen), 100 μg/mL hyaluronidase (Sigma), and 20 mg/mL DNase (Sigma) for up to 1 hour at room temperature. Single-cell suspensions were filtered through 100-μm nylon mesh and stained with antibodies specific for surface antigens, then washed, and fixed using a Treg staining kit (eBioscience), and intracellularly stained for FoxP3 as previously described (34). Immune organs were crushed, filtered, and stained as
described above. The proportion of each infiltrating cell type was analyzed on a BD LSR II.

Cell numbers in the peripheral blood were measured using a whole-blood bead assay (34). Whole blood was harvested into EDTA-containing tubes, and 5 μL was stained directly with fluorescent antibody cocktails. AccuCheck fluorescent beads (Invitrogen) were added to each sample, then red blood cells were lysed with Cal-Lyse whole-blood lysing solution (Invitrogen), and samples analyzed on a BD LSR II flow cytometer. We determined the absolute number of cells in the sample based on comparing cellular events with bead events (cells/μL).

For immunohistology, tumors were fixed in formalin or Z7 zinc–based fixative (35) overnight. Tissue was processed for frozen or paraffin tissue sections. For frozen sections, tissue was cryopreserved by equilibration in 30% sucrose and then flash frozen in optimal cutting temperature embedding media. For paraffin sections, tissue was dehydrated through graded alcohol to xylene, incubated in molten paraffin, and then buried in paraffin. Sections (5 μm) were cut and mounted for analysis. Tissue sections were boiled in EDTA buffer as appropriate for antigen retrieval. Primary antibody binding was visualized with Alexa Fluor488–, Alexa Fluor568–, or Alexa ControlSM16 Control + RT

![Image](https://example.com/figure1.png)

**Figure 1.** SM16 alters the composition of tumor immune infiltrate and improves survival in combination with radiotherapy (RT). BALB/c mice bearing day 7 CT26 tumors were treated with control or SM16 chow for 7 days. A, flow cytometry of tumor-infiltrating cells on day 14. Each symbol represents the tumor from one animal, n = 5 mice per group (mpg). B, i, average diameter of CT26 tumors treated with control chow or SM16 chow as described above (gray). On day 14, mice received 20 Gy of RT (dashed). ii, individual tumor diameters at day 21, 7 days following RT, and cessation of SM16 treatment. C, survival of mice treated as in B; n = 6 mpg. Median survival: control, 25 days; SM16, 28 days; RT, 49.5 days; RT + SM16, 74 days. Experiments shown are representative of two or more replicates. NS, not statistically significant; *, P < 0.05; **, P < 0.01; ****, P < 0.001. t test, infiltrates; ANOVA, multiple comparisons; log-rank, survival.
Fluorescein-conjugated secondary antibodies (Molecular Probes) and mounted with DAPI (Invitrogen) to stain nuclear material. Images were acquired using a Nikon TE2000S epifluorescence microscope, Nikon DsFi1 digital camera, and Nikon NIS-Elements imaging software; a Zeiss Axioimager, Zeiss Icc5 digital camera, and Zen imaging software; a Nuance multispectral imaging camera and imaging software (PerkinElmer); a Leica SCN400 whole-slide scanner or a SCN400F fluorescence whole-slide scanner. Images displayed in this article are representative of the entire tumor and their respective experimental cohort. Multispectral images were separated into their single marker components using Nuance software. NIH image was used to quantify the number of pixels present in each image. A minimum of three images per section and a minimum of four tumors per cohort were used for analysis.

**FITC-dextran penetrance and Hypoxypyrobe**

To measure hypoxia, mice were injected 1 hour before euthanasia with 1.5-mg Hypoxyprobe in PBS intraperitoneally (Hypoxyprobe, Inc.) and analyzed by immunohistology. To measure perfusion, 10 minutes before euthanasia, mice were injected i.v. with 100 μL of FITC-dextran (40 kDa; Sigma). On

![Figure 2. SM16 has a minimal effect on the physical properties of the tumor. A, in vitro clonogenic assay using vehicle control or SM16 with the survival fraction of clones recorded for each dose of radiation delivered. Means of triplicates are displayed. B, immunofluorescence for E-cadherin (red) versus nuclear DAPI counterstain (blue) in i, small bowel positive control; ii, CT26 tumors with no primary antibody control; iii, day 14 CT26 tumors in mice given 7 days of control chow; or iv, day 14 CT26 tumors in mice given 7 days of SM16 chow; n = 5 mice per group (mpg). C, immunofluorescence histology for SMA in CT26 tumors treated as in B with control or SM16 chow. D, immunofluorescence for CD31 in CT26 tumors treated as in B with control or SM16 chow; n = 5 mpg. E, molecular penetrance, measured by FITC-dextran fluorescence per milligram of tissue in day 14 CT26 tumors in mice given 7 days of control or SM16 chow, with controls of liver and brain tissue; n = 5 mpg. F, immunofluorescence histology for hypoxia in mice treated as in B, but given i.v. Hypoxyprobe 1 hour before harvest, with the formation of Hypoxyprobe adducts detected with specific antibodies. Scale bar, 100 μm; n = 5 mpg.](image-url)
euthanasia, cardiac perfusion was used to flush the vasculature with PBS before tissue harvest. Tissue was digested as described above and the fluorescence present in the supernatant detected using a Modulous microplate reader (Turner Biosystems/Promega).

**Western blot analysis**

Tumors were established as described above and treated with either control or SM16 chow for 1 week beginning on day 7. Tumors were harvested on day 14 and homogenized into RIPA buffer (Sigma) containing protease inhibitors. Bone marrow–derived macrophages were obtained as described previously (33). Equal amounts of protein were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked, and incubated overnight at 4°C with primary antibodies. Secondary antibodies were applied for 1 hour at room temperature. Chemiluminescence was detected using the FluorChemE scanner (Protein Simple) or film and analyzed using ImageJ.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software. Kaplan–Meier survival curves were compared using a log-rank test. The difference in cell percentages, dextran penetrance, tumor weight, tumor area, and tumor infiltrates between specific groups was compared using the Student t test, ANOVA, or Mann–Whitney.

**Results**

To test our hypothesis that an improved immune environment in the tumor at the time of treatment will increase the overall survival of mice treated as in B. Median survival: control, 27 days; SM16, 32 days; radiotherapy (RT), 58 days; RT + SM16, 70 days; n = 6 mgp. D, FITC-dextran fluorescence per milligram of tissue in day 14 Panc02 tumors in mice given 7 days of control chow or SM16 chow, with controls of liver and brain tissue; n = 5 mgp. E, immunofluorescence in mice treated as in D, but given i.v. hydroxyprobe 1 hour before harvest, with the formation of Hypoxyprobe adducts detected with specific antibodies; n = 5 mgp. Experiments shown are representative of two or more replicates. NS, not statistically significant; *, P < 0.05; **, P < 0.01; ***, P < 0.005. t test, infiltrates; log-rank, survival.
from immunocompetent BALB/c mice. Seven days later, we injected CT26 colorectal carcinoma cells into the right hind limb of immunocompetent BALB/c mice. Effective TGFβ inhibition in the tumor was demonstrated by reduced SMAD signaling in tumor lysates (Supplementary Fig. S1). Mice were euthanized on day 14 and tumors were analyzed for immune-cell infiltrate and activity, we aimed to determine whether radiotherapy at this time point would be more efficacious. Seven days after tumor cell injection, mice were fed with either control or SM16 chow for 1 week. TGFβ inhibition was discontinued to exclude its potential effect on immune function in the posttreatment environment. Similar to many small-molecule inhibitors, SM16 has a short half-life in vivo, with SMAD signaling in the tumor returning within 24 hours after treatment cessation (39). We delivered 20 Gy of radiation in a single fraction to the right hind limb on day 14. Mice were followed for tumor growth, and were euthanized when tumor diameter reached 12 mm. The tumors in mice fed with control or SM16 chow alone had similar growth kinetics and survival (Fig. 1B and C). At very early time points, SM16 chow seemed to provide a small growth delay compared with control chow–fed animals, but once treatment was discontinued, this difference rapidly corrected itself and provided no survival advantage. While mice fed with SM16 chow had tumors that were on average 1 mm smaller at the time of radiotherapy, within each experimental group there was tumor size variance that did not demonstrate variable radiation responses, such that 4-mm tumors in control chow–fed mice were not cured by radiotherapy, whereas 6-mm tumors in SM16 chow–fed mice were cured, suggesting that tumor size at the time of radiotherapy could not account for the differences observed (Supplementary Fig. S2A). Mice treated with control chow and radiotherapy showed a period of tumor control followed by tumor outgrowth leading to euthanasia in all mice (Fig. 1B and C). Mice pretreated with SM16 chow followed by radiotherapy demonstrated prolonged tumor control, with a proportion of mice having tumor resolution without recurrence (Fig. 1B and C). Modification of the tumor environment by treatment with SM16 followed by radiotherapy provided a statistically significant survival benefit compared with all other groups. Previous studies have reported that TGFβ inhibition increases radiosensitivity (40). To evaluate whether SM16 treatment increased the efficacy of radiotherapy by increasing the radiosensitivity of CT26 cells, we performed an in vitro clonogenic assay. SM16 increased radiosensitivity for single doses up to 6 Gy, but for larger single doses, radiosensitivity was equivalent to that of the control chow (Fig. 2A). Given that we delivered 20 Gy per fraction, altered radiosensitivity is unlikely to explain our observed effect. Because TGFβ drives epithelial–mesenchymal transition (EMT), it is possible that TGFβ inhibition may restore cells in vivo to a more differentiated state and affect radiosensitivity. To address this, we evaluated the treated tumors for expression of the epithelial marker, E-cadherin, and mesenchymal marker, SMA. Control structures demonstrated clear membranous E-cadherin, whereas cancer cells poorly expressed E-cadherin and its distribution did not change in the presence or absence of TGFβ inhibition (Fig. 2B). Similarly, SMA was expressed on stromal structures but not cancer cells, and was not affected by TGFβ inhibition (Fig. 2C). Histologic analysis showed no evidence of morphologic changes associated with EMT in vivo. These findings suggest that these tumors are highly undifferentiated and do not regain epithelial differentiation following TGFβ inhibition.

TGFβ inhibitors have been used successfully in wound injury and tumor models to reverse TGFβ-induced fibrosis, vessel density, tumor vascularity, and angiogenesis (41, 42). Remodeling of the tumor vasculature could increase oxygen delivery to the tumor by reversing tumor-directed neoangiogenic vasculature. Because oxygenation is a critical feature of radiation-mediated cell killing, improved vascular function could explain the increased efficacy of radiotherapy following TGFβ inhibition. SM16 treatment did not alter the vascularity of CT26 tumors at the time of radiotherapy, as evaluated by quantification of staining for the endothelial marker CD31 (Fig. 2D and

Figure 4. Orthotopic pancreatic adenocarcinomas demonstrate treatment effect of radiotherapy (RT) and SM16. A, cone-beam CT scan of C57BL/6 mice bearing orthotopic Panc02 tumors with lipiodol for CT localization (white arrow). 8-Gy RT delivered using a 180° arc treatment and a 5 × 5 mm collimator, with dose cloud. B, mice treated with control or SM16 chow for 1 week beginning day 7. 8-Gy RT delivered on day 14 and every other day for three treatments. Tumor size determined by weight of (i) pancreas and (ii) cross-sectional area via histology. n = 5 mice per group (mpg). C, quantitation of CD3 cell infiltrate from immunofluorescence images; n = 5 mpg, a minimum of three fields per tumor measured for infiltrate quantification. D, H&E staining of tumors given, (i) control chow (NT), (ii) SM16 chow, (iii) control chow plus 8 Gy × 3 fractions, or (iv) SM16 chow plus 8 Gy × 3 fractions; n = 5 mpg. E, immunofluorescence for E-cadherin (red), F4/80 (green), CD3 (magenta), and DAPI nuclear staining (blue) in tumors treated as per C; n = 5 mpg. Scale bar, 50 μm. NS, not statistically significant; **, P < 0.005; ***, P < 0.0001. ANOVA, tumor area; Mann–Whitney, histology.
Supplementary Fig. S2). To evaluate vascular function, mice were treated i.v. with FITC-dextran and tumors were harvested to evaluate drug penetration into the tumor. CT26 tumors showed relatively high penetration of FITC-dextran into the tumor, with levels comparable with those in the liver, which has a readily penetrable fenestrated vasculature, and is significantly above that of the relatively impermeable brain (Fig. 2E). SM16-treated tumors did not exhibit any change in penetration (Fig. 2E). To evaluate tumor oxygenation, tumor-bearing mice were treated with Hypoxyprobe, which forms detectable adducts in the presence of hypoxia. Immunohistology for Hypoxyprobe adducts demonstrated regions of hypoxia in CT26 tumors, but the distribution of hypoxia was not changed following TGFβ inhibition (Fig. 2F). These data demonstrate that the improved efficacy of radiotherapy following TGFβ inhibition cannot be explained by vascular modification.

We evaluated whether SM16 provided a similar therapeutic benefit in a less favorable model. Pancreatic adenocarcinomas are highly angiogenic and fibrotic tumors, and TGFβ overexpression in pancreatic cancer correlates with decreased survival (43-44). The Panc02 murine pancreatic adenocarcinoma cell line is highly resistant to chemotherapy and radiotherapy when injected into syngeneic mice (32). While in vitro Panc02 cells are similarly radiosensitive as CT26 cells, in vivo CT26 tumors are markedly more radiosensitive (Supplementary Fig. S1). To determine the effect of TGFβ inhibition on the immune environment of Panc02 tumors, C57BL/6 mice were inoculated with 2 x 10^6 Panc02 cells in the right hind limb and 7 days later were given control chow or SM16 chow for 1 week. On day 14, mice were euthanized and tumors were analyzed for tumor-infiltrating leukocytes (TIL) via flow cytometry. The TIL alterations in Panc02 tumors following SM16 treatment were different than those in CT26 tumors (Fig. 3A and 1A). Control Panc02 tumors exhibited a high myeloid infiltrate, comprising approximately 50% of live cells within the digested tumor suspension (Fig. 3A). SM16 treatment significantly decreased the myeloid infiltrate, including the Gr1 hi population, consistent with decreasing both macrophages and MDSCs (Fig. 3A). There was a trend to increased CD8 infiltrate (although not statistically significant; Fig. 3A). Nevertheless, a decrease in the proportion of immune-suppressive myeloid cells mediated by TGFβ inhibition could be a positive change in the tumor immune environment (45), as myeloid depletion has been shown to increase the response to radiotherapy (46). To determine whether this change in the tumor immune environment improved the efficacy of radiotherapy, mice were injected with Panc02 cells, then treated with control chow or SM16 chow for 1 week, and on day 14, mice underwent 20 Gy of radiotherapy daily for 3 consecutive days, totaling 60 Gy (33). Chow was discontinued before radiotherapy to minimize the effects on the postradiation immune environment. Panc02 tumor-bearing mice treated with SM16 chow alone showed slightly improved growth kinetics and survival compared with mice receiving control chow (median survival, 27 days vs. 32 days; P < 0.05), and the combination of SM16 chow and radiotherapy improved the efficacy of radiotherapy (median survival, 56 days vs. 70 days; P < 0.05; Fig. 3B and C), but it did not generate long-term tumor-free mice.

To determine whether TGFβ inhibition changed the vascular function of Panc02 tumors, the in vivo permeability of Panc02 tumors was measured via FITC-dextran, as described above. FITC-dextran penetration was between those for the liver and the brain, consistent with Panc02 tumors being less permeable than CT26 tumors at baseline (Fig. 3D). Permeability of Panc02 tumors was not altered by SM16 treatment (Fig. 3D). Similarly, we saw no difference between groups in the hypoxic area (Fig. 3E).

We developed an orthotopic pancreatic tumor model to test the effect of therapy on the posttreatment tumor immune environment. One week after tumor implantation, mice were fed with either control chow or SM16 chow. Subsequently, the mice underwent image-guided stereotactic radiotherapy using the SARRP, with 8 Gy per fraction doses delivered on days 14, 19, and 21 (Fig. 4A). One week after the final radiation treatment, which resulted in markedly smaller tumors, only the SM16-treated tumors exhibited a statistically significant reduction in pancreatic weight and tumor area following radiotherapy (Fig. 4B). This finding was similar to results with subcutaneous Panc02 tumor in which there was no difference in leg diameter 1 week after radiotherapy between the radiotherapy-alone group and the SM16 plus radiotherapy group, but the extension in survival in the subcutaneous experiment results from a delayed outgrowth of recurrent tumors (Fig. 3B and C). Interestingly, changes in immune infiltrate remained, despite the discontinuation of TGFβ inhibition 2 weeks before tumor harvest (Fig. 4D and E). Similar to the subcutaneous tumors, quantitation of immunofluorescent images demonstrated that mice that received SM16 alone had a nonsignificant decrease in macrophages, as stained by F4/80 (Fig. 4E, quantitation not shown); and only mice that received both SM16 and radiotherapy had an increased CD3 infiltrate compared with untreated mice (Fig. 4C and E).

To compare differences in CD4 and CD8 memory, effector, and activated phenotypes within the tumor-draining lymphoid organs, we performed a FACs-based multiparameter analysis of TIL in Panc02 tumors. TDLNs were harvested on day 15. A, CD4 and B, CD8 T cells analyzed via flow cytometry for (i) naïve, (ii) memory, (iii) effector, and (iv) activated subpopulations. Each symbol represents one animal; n = 5 mice per group (mpg). C, concomitant CT26 tumors established in bilateral hind limbs and treated with control or SM16 chow for 1 week followed by 20 Gy of RT to the right hind limb tumor on day 14, i, average tumor diameter; and ii, the untreated left hind limb tumors on day 20. D, CT26 tumor-bearing mice treated with control or SM16 chow as described above. Anti-CD8 antibody was injected i.p. on days 10, 17, and 24. Flow cytometry of peripheral blood for (i) CD8 T cells and (ii) CD4 T cells (i) to confirm selective depletion; n = 4 mpg; E, i, average tumor size for mice treated as in C, with control or SM16 chow as described above (gray). Day 14 tumor RT (dotted) and CD8-depletion (dashed). At day 22, mean tumor area of the treated tumors is significantly smaller than that of the untreated (control P < 0.001; SM16 P < 0.01) but not when CD8 T cells are depleted. ii, survival of mice treated as in i with control chow treated (left graph) and SM16 treated (right graph). Median survival: control, 31 days; control RT, 55 days; control + RT = anti-CD8, 38 days; SM16, 36 days; SM16 + RT, undefined; SM16 + RT + anti-CD8, 38 days; n = 6 mpg. NS, not statistically significant; * P < 0.05; ** P < 0.01; *** P < 0.005; t test, infiltrates; ANOVA, tumor means; log-rank, survival.
nodes (TDLN) between mice receiving radiotherapy alone or SM16 pretreatment followed by radiotherapy. TDLNs were harvested 1 day after radiotherapy in mice pretreated with 1 week of control chow or SM16 chow. We found that mice pretreated with SM16 chow had an increase in memory effector, and activated CD4 T cells within the TDLNs (Fig. 5A). SM16 pretreatment did not significantly alter CD8 T cells in the TDLNs in the first 24 hours after radiotherapy (Fig. 5B), despite increases in activated CD8 T cells in the tumor (Fig. 1A). Thus, the major change within the TDLNs within 24 hours of radiotherapy is within the CD4 T-cell compartment.

To address the effect of combination treatment and the role of immunity, we established a model of concomitant tumor control in which CT26 tumors were established on opposing limbs, and treated with SM16 or control chow, and only one tumor site was irradiated. Improved tumor control was demonstrated in the opposing unirradiated tumor only when both SM16 and radiotherapy treatments were applied to the primary tumor (Fig. 5C). These data demonstrate that enhanced adaptive immune response is necessary for the improved efficacy of TGFβ pretreatment before radiotherapy. To test whether the effect of TGFβ inhibition before radiotherapy was mediated by adaptive immune control of residual disease, we evaluated the role of CD8 T cells in tumor control. CD8 T cells were depleted weekly beginning on day 10 for three total doses, and their loss was confirmed by a quantitative FACS analysis of peripheral blood (Fig. 5D). CD8 depletion limited the efficacy of radiotherapy in the control group [median survival; radiotherapy, 55 days; radiotherapy + αCD8, 38 days; P < 0.01], consistent with previous reports demonstrating its necessity for the efficacy of hypofractionated radiation (Fig. 5E; refs. 12, 14). CD8 depletion also abrogated the synergistic effect of SM16 and radiotherapy (median survival: radiotherapy + SM16, not reached; radiotherapy + SM16 + αCD8, 38 days; P < 0.01), such that there was no survival benefit of adding SM16 to radiotherapy to mice in which CD8 T cells were depleted (Fig. 5E).

To determine whether immune-mediated clearance of residual disease following radiotherapy had a tumor-specific protective effect, mice cured of local CT26 tumors by radiotherapy preceded by TGFβ inhibition were rechallenged with CT26 cells on one flank and syngeneic but immunologically distinct 4T1 mammary carcinoma cells on the opposing flank. Both tumors grew in control naïve mice; mice cured with SM16 and radiotherapy all had outgrowth of 4T1 tumors, while none developed CT26 tumors, indicating that long-term tumor-specific immunity to CT26 cells was developed with the combined therapy (Table 1). These data indicate that remodeling the tumor immune environment before radiotherapy both enhances local tumor control and establishes protective immunity.

### Discussion

Patients with colorectal cancer can present with distinct immune environments in their tumors, which can affect disease outcome (7). We tested the hypothesis that an improved tumor immune environment will increase the efficacy of radiotherapy. TGFβ is an important immunosuppressive factor in tumors, and we demonstrate that inhibition of TGFβ using the orally available TGFβ inhibitor, SM16, resulted in an improved tumor immune environment at the time of radiotherapy, leading to significantly increased tumor control by radiation. This effect could not be accounted for by changes in radiosensitivity, EMT, or vascular organization, but was dependent on the presence of CD8 T cells. This report demonstrates that therapies aimed to alter the quantity and quality of the tumor immune infiltrate can improve therapeutic efficacy, resulting in durable remission and long-term immunity. The mechanism by which this occurs may differ by tumor type related to the tumor immune environment; however, in each case, TGFβ inhibition was able to improve the local tumor environment and improve the efficacy of radiotherapy.

Although we saw a statistically significant survival advantage in the combination of SM16 with radiotherapy over radiotherapy alone in each model, we only saw long-term survivors in the CT26 tumor model. One possible explanation for this improved response is that TGFβ inhibition is more effective in enhancing an already “positive” immune environment. As noted above, CT26 tumors are markedly more radiosensitive in vivo compared with Panc02 tumors. T-cell depletion has little effect on the radiotherapy response in the Panc02 model (data not shown), and the Panc02 tumors are dominated by highly immunosuppressive macrophages that infiltrate the tumors following radiotherapy (33). We have shown previously that redirecting the differentiation of these macrophages is key to control of Panc02 tumors by radiotherapy (33). Thus, pretreatment with TGFβ inhibitors may be insufficient to cure tumors if a different immunosuppressive environment can reform later. We opted to maintain our TGFβ treatment regimen for comparability between tumor types, but a higher dose, longer duration, or rescheduling of inhibition may be required for synergy with radiotherapy (40). Further experiments will determine the ideal duration of treatment in this setting.

Given the complex role of TGFβ in tumor formation and progression, we must carefully consider translation of preclinical studies. TGFβ signaling components can act as tumor suppressors before the development of invasive cancer (21). Before using TGFβ inhibitors clinically, considerations must be given to the duration of inhibition, particularly in patients that have a “field carcinization effect” who may have other

### Table 1. Long-term tumor-specific protection in cured mice

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<th>Tumor</th>
<th>4T1</th>
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<td>Tumor cure</td>
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</table>

NOTE: Mice cured of CT26 tumors by SM16 pretreatment followed by radiotherapy were rechallenged on opposing flanks with 4T1 and CT26 cells. Mice were followed for development of palpable tumors at the injection site. Table shows the number of mice developing palpable tumors (Tumor cured) as compared with the number of mice challenged.
subclinical in situ disease that may progress to malignancy after prolonged exposure to TGFβ inhibitors. In an early-phase trial of an anti-TGFβ ligand antibody in patients with metastatic melanoma, higher dose and prolonged exposure were associated with development of eruptive keratoacanthomas and squamous cell carcinomas, which resolved upon discontinuation of anti-TGFβ therapy (47). Careful monitoring for an increased rate of secondary malignancy should be part of any translational use of TGFβ inhibitors.

In conclusion, in this study, we demonstrate that TGFβ inhibition alters the immune environment of both colorectal and pancreatic syngeneic murine tumors. The dose, timing, and duration of TGFβ inhibition in combination with radiotherapy may depend on tumor type and immune infiltrate. Although it seems that our hypothesis is correct, that an inhibition to prevent inflammatory resolution in the posttreatment tumor (45) and to decrease the negative effects of radiation-induced fibrosis (26).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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