TGFβ inhibition prior to hypofractionated radiation enhances efficacy in preclinical models

Kristina H Young, Pippa Newell, Benjamin Cottam, et al.

TGFβ inhibition prior to hypofractionated radiation enhances efficacy in preclinical models
Kristina H. Young¹, Pippa Newell²,³, Benjamin Cottam², David Friedman², Talicia Savage², Jason R. Baird², Emmanuel Akporiaye², Michael J. Gough², Marka Crittenden²,³

¹Department of Radiation Medicine, Oregon Health & Science University, Portland, OR
²Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR
³The Oregon Clinic, Portland, OR

Running Title: TGFβ inhibition before radiation improves efficacy

Keywords: TGFβ, radiation, immune infiltrate, colorectal cancer, immunity

Financial Support:
This research was supported by the RSNA Research & Education Research Resident Grant, the American Cancer Society Research Scholar Grant RSG-12-168-01-LIB, and a grant from the MJ Murdoch Charitable trust.

Corresponding Author:
Marka Crittenden, MD, PhD
Address: Earle A Chiles Research Institute, Providence Cancer Center, 4805 NE Glisan St, Portland OR 97213.
Phone: 503-215-6705
Fax: 503-215-6841
Email: marka.crittenden@providence.org

Conflict of Interest: The authors disclose no conflicts of interest.
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 disease stage. For patients with lower immune infiltrates, overall survival is limited. Since the colorectal cancer patients studied have received conventional cancer therapies, these data may indicate that the pre-treatment tumor environment increases the efficacy of treatments such as chemotherapy, surgery and radiation. 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 radiation therapy. 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 radiation therapy. This effect was not due to changes in radiosensitivity, epithelial to 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 pre-treatment improves the efficacy of radiation therapy.
**Introduction**

Individualized therapy aims to identify which patient might benefit from specific targeted therapy. While 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 there is an international effort underway 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 radiation therapy, 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 radiation therapy.

Radiation is a common treatment primarily related to its efficacy as a focal cytotoxic agent. An under-recognized benefit to radiation 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 radiation therapy and improved therapeutic efficacy if radiation is followed by systemic immunomodulatory therapy (12–14). Anecdotal reports have demonstrated that immune therapy followed by radiation can lead to tumor regression (15). Investigators are testing the hypothesis that radiation can act in synergy with immunotherapy leading to immune activation with resultant systemic antitumor immune activity. This has been described when combining radiation with systemic IL-2 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 prior to treatment (16), indicating the pretreatment immune response may affect therapeutic response. Additionally, in a preclinical model of metastatic colorectal cancer, changing the pre-treatment immune environment by tumor-associated antigen vaccination followed by high-dose radiation lead to antigen cascade and metastatic tumor clearance (17).

Transforming growth factor beta (TGFβ) is a multipotent cytokine that has opposing effects on carcinogenesis in a cell context and timing-specific manner (18). Prior to 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, decreases antigen presentation and effector function, while increasing macrophage trafficking to the site of inflammation (23,24). TGFβ inhibitors have been combined with radiation in preclinical cancer models and have increased radiosensitivity, and reduced radiation-related mucositis, pneumonitis, and fibrosis (25–30). Early phase trials have demonstrated the safety of TGFβ inhibitors, which are being tested either alone or in combination with traditional cytotoxic therapy.

We hypothesize that an improved immune environment at the time of treatment will increase the efficacy of radiation therapy. 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 radiation therapy. We show that the efficacy is dependent on CD8 T cells. These data demonstrate that manipulation of the pre-treatment 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 radiation therapy.
Materials and Methods

Cell lines and animals
The CT26 murine colorectal and the 4T1 mammary carcinoma cell lines (31) were obtained from ATCC (Manassas, VA). The Panc02 murine pancreatic adenocarcinoma cell line (32) was kindly provided by Dr. Woo (Mount Sinai School of Medicine, NY). All three cell lines were tested and validated to be mycoplasma-free; no other authentication assay was performed. C57BL/6 mice and BALB/c were obtained from Jackson Laboratories (Bar Harbor, ME). Animal protocols 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, IFNγ-APC, IFNγ-FITC, CD62L-AF700, CD69-PE-Cy7, CD45-e650, CD11c-e450, CD40-FITC, B220-PE-Cy7, CD80-APC, and CD86-PE were purchased from eBioscience (San Diego, CA). CD4-v500 and Ly6G-FITC were purchased from BD Biosciences (San Jose, CA). CD8-PE-TxRD was purchased from Invitrogen (Carlsbad, CA). CD44-APC-Cy7 was purchased from BioLegend (San Diego, CA).

Histology: F4/80 was purchased from AdB Serotec (Raleigh NC); E-cadherin from BD Bioscience; CD3, CD31 from Spring Bio (Pleasanton, CA); and Cy3-conjugated Smooth Muscle Actin from Sigma (St. Louis MO). Hypoxypyrobe and detection antibody was purchased from Hypoxypyrobe (Burlington, MA); and FITC-conjugated 40,000MW Dextran was purchased from Invitrogen.

Western Blot: pSMAD2, SMAD2, GAPDH, and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Arginase I was purchased from BD Biosciences, and iNOS from Cayman Chemical Corporation (Ann Arbor, MI).

SM16: SM16 was obtained under a material transfer agreement from Biogen Idec (Cambridge, MA) and was incorporated into standard Purina rodent chow (#5001) by Research Diets (New Brunswick, NJ) 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 ~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 Radiation Therapy Models**

1x10^4 CT26 or 2x10^5 Panc02 cells were injected sc in the right hind limb of immunocompetent BALB/c or C57BL/6 mice, respectively. SM16 or control chow was fed from d7-13. Radiation was delivered using the clinical linear accelerator (6MV photons, Elekta Synergy linear accelerator, Atlanta, GA) with a halfbeam block to protect vital organs and 1.0cm bolus. For CT26 tumors, 20Gyx1 was delivered on d14; for Panc02 tumors 20Gyx3 was delivered on d14-16, as described previously (33). Mice cured of CT26 tumors were rechallenged with 5x10^4 4T1 and 1x10^4 CT26 cells in opposite flanks to assess tumor-specific immunity. For concomitant immunity experiments, mice were challenged with 1x10^4 CT26 cells in the right hind limb on d0, then 1x10^4 CT26 in the left hind limb on d3. Mice were treated with control or SM16 chow beginning on d7. 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 5mm incision in anesthetized C57BL/6 mice. 8x10^4 Panc02 cells were injected in 8µL PBS with 2µL Lipiodol Ultra-Fluide (Guerbet, Bloomington, IN, 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, Suwanee, GA). 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 5x5mm collimator was utilized to deliver 8Gyx3, mice were treated every other day beginning on d14.

**Immune analysis**

For analysis of tumor-infiltrating immune cells, the tumor was digested in 10mL of PBS with 1mg/mL collagenase (Invitrogen), 100µg/mL hyaluronidase (Sigma), and 20mg/mL DNase (Sigma) for up to 1hr at room temperature. Single-cell suspensions were filtered through 100µm 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 LSRII.
Cell numbers in the peripheral blood were measured using a whole blood bead assay (34). Whole blood was harvested into EDTA-containing tubes, and 5µL 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 LSRII flow cytometer. We determined the absolute number of cells in the sample based on comparing cellular events to 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 cyropreserved by equilibration in 30% sucrose then flash frozen in OCT. For paraffin sections, tissue was dehydrated through graded alcohol to xylene, incubated in molten paraffin, and then buried in paraffin. 5µm sections were cut and mounted for analysis. Tissue sections were boiled in EDTA buffer as appropriate for antigen retrieval. Primary antibody binding was visualized with AlexaFluor 488-, AlexaFluor 568-, or AlexaFluor 647-conjugated secondary antibodies (Molecular Probes, Eugene, OR) 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 (Perkin Elmer); a Leica SCN400 whole slide scanner or a SCN400F fluorescence whole slide scanner. Images displayed in the manuscript 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 4 tumors per cohort were utilized for analysis.

**FITC-Dextran Penetrance and Hypoxyprobe**

To measure hypoxia, mice were injected 1 hour before euthanasia with 1.5mg hypoxyprobe in PBS ip (Hypoxyprobe, Inc) and analyzed by immunohistology. To measure perfusion, ten minutes before euthanasia mice were injected iv with 100µL of FITC-Dextran (40kDa, Sigma, St. Louis, MO). On 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 Biosoynets/Promega, Madison, WI).

**Western Blot**
Tumors were established as described above and treated with either control or SM16 chow for one week beginning on d7. Tumors were harvested on d14 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 1h at room temperature. Chemiluminescence was detected using the FluorChemE scanner (Protein Simple, San Jose, CA) or film and analyzed using Image J.

Statistics
Statistics 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 Student’s 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 efficacy of radiation therapy, we first tested whether a TGFβ inhibitor could change the tumor immune environment. SM16 is readily bioavailable and has been successfully formulated in the diet for oral delivery in animals. Studies have demonstrated that chronic administration of dietary SM16 suppresses tumor growth and metastases, re-polarizes tumor-infiltrating myeloid cells with antitumor activity without apparent toxicity (36–38), and synergizes with T cell-targeted immunotherapies (36). We injected CT26 colorectal carcinoma cells into the right hind limb of immunocompetent BALB/c mice. Seven days later, we began treatment with either control or SM16 chow for one week. Effective TGFβ inhibition in the tumor was demonstrated by reduced SMAD signaling in tumor lysates (Figure S1). Mice were euthanized on d14 and tumors were analyzed for immune-cell infiltrate. Mice fed with SM16 chow had a statistically significant increase in T-cell infiltrate as detected by CD3+ cells, increased early activated CD8 T cells as detected by CD8+CD25+ cells, and decreased Treg as detected by CD4+CD25+FoxP3+ cells (Figure 1a). These changes are consistent with the known ability of TGFβ to suppress CD8 T-cell effector function and to drive Treg differentiation in the tumor. The effects of TGFβ inhibition could not be explained by modulation of γδ or NKT cells as the CD3+CD4−CD8− population represented less than 20% of the T-cell population and was not altered by SM16 treatment (data not shown). No difference was seen in the myeloid populations (Figure 1a). Other immune-cell populations were evaluated including CD3, CD4, CD8, CD25, FoxP3, Granzyme B, Ki67, live/dead, CD11b, MHCII, and Gr1, but none was altered in a statistically significant manner (data not shown). Given the differences in immune-cell infiltrate and activity, we aimed to determine whether radiation therapy at this time point would be more efficacious. 7 days after tumor cell injection, mice were fed with either control or SM16 chow for one week. TGFβ inhibition was discontinued to exclude its potential effect on immune function in the post-treatment 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 following treatment cessation (39). We delivered 20Gy of radiation in a single fraction to the right hind limb on d14. Mice were followed for tumor growth, and were euthanized when tumor diameter reached 12mm. The tumors in mice fed with control or SM16 chow alone had similar growth kinetics and survival (Figure 1b and 1c). At very early time points, SM16 chow appeared to provide a small growth delay compared to control chow-fed animals, but once treatment was discontinued, this difference rapidly corrected and provided no survival advantage. While mice fed with SM16 chow had tumors that were on an average 1mm smaller at the time of radiation, within each experimental group there was tumor size variance which did not demonstrate variable radiation responses, such that 4mm tumors in control chow-fed mice were not cured by radiation while 6mm tumors in SM16 chow-fed
mice were cured, suggesting that tumor size at the time of radiation could not account for the differences observed (Supplemental Figure 2a). Mice treated with control chow and radiation showed a period of tumor control followed by tumor outgrowth leading to euthanasia in all mice (Figure 1b and 1c). Mice pretreated with SM16 chow followed by radiation demonstrated prolonged tumor control with a proportion of mice having tumor resolution without recurrence (Figure 1b and 1c). Modification of the tumor environment by treatment with SM16 followed by radiation provided a statistically significant survival benefit compared to all other groups.

Previous studies have reported that TGFβ inhibition increases radiosensitivity (40). To evaluate whether SM16 treatment increased the efficacy of radiation therapy by increasing the radiosensitivity of CT26 cells, we performed an in vitro clonogenic assay. SM16 increased radiosensitivity for single doses up to 6Gy, but for larger single doses radiosensitivity was equivalent to that of the control chow (Figure 2a). Given that we delivered 20Gy per fraction, altered radiosensitivity is unlikely to explain our observed effect. Since 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, smooth muscle actin (SMA). Control structures demonstrated clear membranous E-cadherin, while cancer cells poorly expressed E-cadherin and its distribution did not change in the presence or absence of TGFβ inhibition (Figure 2b). Similarly, SMA was expressed on stromal structures but not cancer cells, and was not affected by TGFβ inhibition (Figure 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. Since oxygenation is a critical feature of radiation-mediated cell killing, improved vascular function could explain the increased efficacy of radiation following TGFβ inhibition. SM16 treatment did not alter the vascularity of CT26 tumors at the time of radiation, as evaluated by quantification of staining for the endothelial marker CD31 (Figure 2d and Figure 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 demonstrated relatively high penetration of FITC-dextran into the tumor, with levels comparable to those in the liver, which has a readily penetrable fenestrated vasculature, and is
significantly above that of the relatively impenetrable brain (Figure 2e). SM16-treated tumors did not exhibit any change in penetration (Figure 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 (Figure 2f). These data demonstrate that the improved efficacy of radiation therapy following TGFβ inhibition cannot be explained by vascular modification.

We evaluated if 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 Figure 1). To determine the effect of TGFβ inhibition on the immune environment of Panc02 tumors, C57BL/6 mice were inoculated with 2x10⁵ Panc02 cells in the right hind limb and seven days later were given control chow or SM16 chow for one week. On day fourteen, 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 (Figure 3a and 1a). Control Panc02 tumors exhibited a high myeloid infiltrate, comprising approximately 50% of live cells within the digested tumor suspension (Figure 3a). SM16 treatment significantly decreased the myeloid infiltrate, including the Gr1⁷ population consistent with decreasing both macrophages and MDSCs (Figure 3a). There was a trend to increased CD8 infiltrate (although not statistically significant, Figure 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 improve the response to radiation therapy (46). To determine whether this change in the tumor immune environment improved the efficacy of radiation therapy, mice were injected with Panc02 cells, then treated with control chow or SM16 chow for one week, and on d14 mice underwent 20Gy of radiation treatment daily for three consecutive days totaling 60Gy (33). Chow was discontinued prior to delivery of radiation to minimize the effects on the post-radiation immune environment. Panc02 tumor-bearing mice treated with SM16 chow alone demonstrated slightly improved growth kinetics and survival compared to mice receiving control chow (Median survival 27 days versus 32 days, p<0.05) and the combination of SM16 chow and radiation improved the efficacy of
radiation therapy (Median survival 56 days versus 70 days, p<0.05) (Figure 3b and 3c), but it did not generate long-term tumor-free mice.

To determine whether TGFβ inhibition changed the vascular function of Panc02 tumors in mice, 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 (Figure 3d). Permeability of Panc02 tumors was not altered by SM16 treatment (Figure 3d). Similarly, we saw no difference between groups in hypoxic area (Figure 3e).

We developed an orthotopic pancreatic tumor model to test the effect of therapy on the post-treatment 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 radiation using the SARRP, with 8Gy per fraction doses delivered on d14, d19, and d21 (Figure 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 radiation (Figure 4b). This finding was similar to results with subcutaneous Panc02 tumor in which there was no difference in leg diameter one-week post radiation between the radiation alone group and the SM16 plus radiation group, but the extension in survival in the subcutaneous experiment results from a delayed outgrowth of recurrent tumors (Figures 3b, 3c). Interestingly, changes in immune infiltrate remained despite discontinuation of TGFβ inhibition two weeks prior to tumor harvest (Figure 4d, 4e). Similar to the subcutaneous tumors, quantitation of immunofluorescent images demonstrated that mice that received SM16 alone had a non-significant decrease in macrophages, as stained by F4/80 (Figure 4e, quantitation not shown); and only mice that received both SM16 and radiation had an increased CD3 infiltrate compared to untreated mice (Figure 4c, 4e).

To compare differences in CD4 and CD8 memory, effector and activated phenotypes within the tumor-draining lymph nodes (TDLN) between mice receiving radiation alone or SM16 pretreatment followed by radiation, TDLNs were harvested one day after radiation in mice pretreated with one 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 (Figure 5a). SM16 pretreatment did not significantly alter CD8 T cells in the TDLNs in the first 24 hours after radiation (Figure 5b) despite increases in activated CD8 T cells in the tumor (Figure 1a). Thus, the major change within the TDLNs within 24 hours of radiation 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 radiation treatments were applied to the primary tumor (Figure 5c). These data demonstrate that enhanced adaptive immune response is necessary for the improved efficacy of TGFβ pretreatment before radiation therapy. To test whether the effect of TGFβ inhibition prior to radiation 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 d10 for three total doses, and their loss was confirmed by quantitative FACS analysis of peripheral blood (Figure 5d). CD8-depletion limited the efficacy of radiation in the control group (median survival RT 55 days, RT+αCD8 38 days (p<0.01)) consistent with previous reports demonstrating their necessity for the efficacy of hypofractionated radiation (Figure 5e) (12,14). CD8-depletion also abrogated the synergistic effect of SM16 and radiation (median survival RT+SM16 not reached, RT+SM16+αCD8 38 days (p<0.01)), such that there was no survival benefit of adding SM16 to radiation therapy to mice in which CD8 T cells were depleted (Figure 5e).

To determine whether immune-mediated clearance of residual disease following radiation had a tumor-specific protective effect, mice cured of local CT26 tumors by radiation therapy 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 by SM16 and radiation 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 prior to radiation therapy both enhances local tumor control and establishes protective immunity.
Discussion

Colorectal cancer patients 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 radiation therapy. 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 radiation therapy, 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 radiation therapy.

While we saw a statistically significant survival advantage in the combination of SM16 with radiation over radiation 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 to Panc02 tumors, T-cell depletion has little effect on the radiation response in the Panc02 model (data not shown) and the Panc02 tumors are dominated by highly immunosuppressive macrophages that infiltrate the tumors following radiation treatment (33). We have shown previously that redirecting the differentiation of these macrophages is key to control of Panc02 tumors by radiation therapy (33). Thus, pre-treatment 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 radiation (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 prior to development of invasive cancer (21). Prior to using TGFβ inhibitors clinically considerations must be given to the duration of inhibition particularly in patients that have a “field cancerization effect” who may have other 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 radiation may depend on tumor type and immune infiltrate. While it seems our hypothesis is correct, that an improved immune environment in the tumor at the time of treatment will increase the efficacy of radiation therapy, it may be optimal to extend TGFβ inhibition to prevent inflammatory resolution in the post-treatment tumor (45) and to decrease the negative effects of radiation-induced fibrosis (26).
References


**Figure 1. SM16 alters the composition of tumor immune infiltrate and improves survival in combination with radiation.** BALB/c mice bearing d7 CT26 tumors were treated with control or SM16 chow for 7 days. a) Flow cytometry of tumor-infiltrating cells on d14. 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 (grey). On d14, mice received 20Gy of radiation (dashed). ii) individual tumor diameters at d21, 7 days following radiation therapy and cessation of SM16 treatment. c) Survival of mice treated as in b). n=6 mpg. Median survival: Control 25d; SM16 28d; RT 49.5d; RT+SM16 74d. Experiments shown are representative of two or more replicates. NS=not significant; *p<0.05; **p<0.01; ***p<0.005; ****p<0.001. Statistics:t-test – infiltrates, ANOVA – multiple comparisons, Logrank – survival.

**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) d14 CT26 tumors in mice given 7 days of control chow; or iv) d14 CT26 tumors in mice given 7 days of SM16 chow. n=5 mpg. c) Immunofluorescence histology for smooth muscle actin (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 d14 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. hypoxypothesis one hour prior to harvest, with the formation of hypoxypothesis adducts detected with specific antibodies. Scale bar 100µm. n=5 mpg.

**Figure 3. SM16 treatment in Panc02 tumors alters the composition of immune infiltrate and improves survival.** C57BL/6 mice bearing Panc02 tumors treated on d7 with control or SM16 chow for one week. a) Flow cytometry for tumor-infiltrating cells on d14. Each symbol represents the tumor from one animal, n=5 mpg. b) Mice bearing Panc02 tumors treated with control or SM16 chow as described above (grey). On d14-16, tumors were irradiated with 20Gy daily for three consecutive days (dashed). n=6 mpg. c) Overall survival of mice treated as in b). Median survival: Control 27d; SM16 32d; RT 56d; RT+SM16 70d. n=6 mpg. d) FITC-Dextran fluorescence per milligram of tissue in d14 Panc02 tumors in mice given 7 days of control chow or SM16 chow, with controls of liver and brain tissue. n=5 mpg. e) Immunofluorescence in mice treated as in d) but given i.v. hypoxypothesis 1hr prior to harvest, with the formation of hypoxypothesis adducts detected with specific antibodies. n=5 mpg. Experiments shown are representative of two or more replicates. Key: NS=not significant; *p<0.05; **p<0.01; ***p<0.005. Statistics:t-test – infiltrates, Logrank – survival.

**Figure 4. Orthotopic pancreatic adenocarcinomas demonstrate treatment effect of radiation and SM16.** a) Cone beam CT scan of C57BL/6 mice bearing orthotopic Panc02 tumors with lipiodol for CT localization (white arrow). 8Gy radiation delivered using a 180° arc treatment and a 5x5mm collimator, with dose cloud. b) Mice treated with control or SM16 chow for one week beginning d7. 8Gy radiation delivered on d14 and every other day for 3 treatments. Tumor size determined by i) weight of pancreas and ii) cross-sectional area via histology. n=5 mpg. c) Quantitation of CD3 cell infiltrate from immunofluorescence images. n=5 mpg, a minimum of 3 fields per tumor measured for infiltrate quantification. d) H&E staining of tumors given i) control chow, ii) SM16 chow, iii) control chow plus 8Gyx3 fractions, or iv) SM16
chow plus 8Gy x3 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 50µm. NS=not significant; *p<0.05; ****p<0.0001. Statistics: ANOVA – tumor area, Mann Whitney – histology.

**Figure 5. The increased efficacy of radiation with SM16 is dependent on CD8 T cells.** Mice bearing CT26 tumors were fed with control chow or SM16 chow for one week beginning on d7. Treated with 20Gy radiation on d14. Tumor-draining lymph nodes (TDLN) were harvested on d15. 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 mpg. c) Concomitant CT26 tumors established in bilateral hindlimbs and treated with control or SM16 chow for one week followed by 20Gy of radiation to the right hindlimb tumor on d14. i) Average tumor diameter and ii) the untreated left hindlimb tumors on d20. d) CT26 tumor-bearing mice treated with control or SM16 chow as described above. Anti-CD8 antibody was injected intraperitoneally on d10, d17, and d24. Flow cytometry of peripheral blood for i) CD8 T cells and ii) CD4 T cells 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 (grey). D14 tumor radiation (dotted) and CD8-depletion (dashed). At d22, 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 31d; Control RT 55d; Control+RT+anti-CD8 38d; SM16 36d; SM16+RT undefined; SM16+RT+anti-CD8 38d. n=6 mpg. NS=not significant; *p<0.05; **p<0.01. Statistics: t-test – infiltrates, ANOVA – tumor means, Logrank – survival.
Figure 1

a)  
   i) CD11b$^+$  
   ![Graph](image1)  
   Percent of live cells  
   Control SM16  
   Percent of CD11b$^+$  
   Control SM16  
   ii) CD11b$^+$Gr1$^{hi}$  
   ![Graph](image2)  
   Percent of live cells  
   Control SM16  
   iii) CD3$^+$  
   ![Graph](image3)  
   Percent of live cells  
   Control SM16  
   iv) CD3$^+$CD8$^+$  
   ![Graph](image4)  
   Percent of live cells  
   Control SM16  
   v) CD3$^+$CD8$^+$CD25$^+$  
   ![Graph](image5)  
   Percent of live cells  
   Control SM16  
   vi) CD3$^+$CD4$^+$  
   ![Graph](image6)  
   Percent of live cells  
   Control SM16  
   vii) CD25$^+$FoxP3$^+$  
   ![Graph](image7)  
   Percent of live cells  
   Control SM16

b)  
   i) Average tumor diameter  
   ![Graph](image8)  
   ii) d21 tumors  
   ![Graph](image9)  
   iii) Overall survival  
   ![Graph](image10)  
   Percent survival  
   Control SM16 Control+RT SM16+RT
a) Clonogenic assay

![Graph showing dose-response curve for clonogenic assay.]

- Control
- SM16

b) Small Bowel

![Images showing E-cadherin expression in CT26, Control, CT26 SM16.]
- CT26
- No Primary Ab
- E-cadherin
- E-cadherin

![Images showing SMA expression in CT26 Control, CT26 SM16.]
- SMA
- SMA

c) CT26 Control
- CT26 SM16

d) CT26 Control
- CT26 SM16
- CD31
- CD31

e) Vascular penetration

![Graph showing fluorescence per mg in different tissues.]

- Control
- SM16
- Liver
- Brain

f) CT26 Control
- CT26 SM16
- Hypoxia
- Hypoxia
a) i) \( \text{CD11b}^+ \) ii) \( \text{CD11b}^+\text{Gr1}^\text{HI} \)

- Percent of live cells
- Control SM16

- Percent of CD11b
- Control SM16

- **

iii) \( \text{CD8}^+ \) iv) \( \text{CD8}^+\text{CD25}^+ \) v) \( \text{CD4}^+ \) vi) \( \text{CD25}^+\text{FoxP}3^+ \)

- Percent of live cells
- Control SM16

- Percent of CD8
- Control SM16

- NS

b) Average tumor diameter

- Leg diameter (mm)
- Time (days)

- 0 20 40 60

- Control SM16

c) Overall survival

- Percent survival
- Time (days)

- 0 20 40 60 80

- Control SM16 SM16+RT

- **

- e) Panc02 Control Panc02 SM16

- Hypoxia

- Brain
Figure 4

(a) Figure images showing different experimental conditions.

(b) Graphs:

- **Pancreas weight**
  - i) NT  
  - ii) SM16  
  - iii) RT  
  - iv) RT+SM16
  
- **Tumor area**
  - i) NT  
  - ii) SM16  
  - iii) RT  
  - iv) RT+SM16
  
(c) **CD3+ infiltrate**
  - i) NT  
  - ii) SM16  
  - iii) RT  
  - iv) RT+SM16

(d) Images of histological sections:
  - i) NT  
  - ii) SM16  
  - iii) RT  
  - iv) RT+SM16

(e) Images with immunofluorescence staining:
  - i) NT  
  - ii) SM16  
  - iii) RT  
  - iv) RT+SM16
Figure 5

a) i) CD4 Naive: CD62L^CD44^{LO/INT}  
   ii) CD4 Memory: CD62L^CD44^{HI}  
   iii) CD4 Effector: CD62L^CD44^{HI}  
   iv) CD4 Activated: CD25^FoxP3^  

b) i) CD8 Naive: CD62L^CD44^{LO/INT}  
   ii) CD8 Memory: CD62L^CD44^{HI}  
   iii) CD8 Effector: CD62L^CD44^{HI}  
   iv) CD8 Activated: CD25^  

c) i) Average tumor diameter  
   ii) 2^o tumor d20  

d) i) Blood CD8^+  

e) i) Average tumor diameter  
   ii) Overall survival
Table 1: Long-term tumor-specific protection in cured mice. Mice cured of CT26 tumors by SM16 pretreatment followed by radiation therapy 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+) as compared to the number of mice challenged.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>4T1</th>
<th>CT26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor+ mice</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Mice challenged</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 1: Long-term tumor-specific protection in cured mice. Mice cured of CT26 tumors by SM16 pretreatment followed by radiation therapy 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+) as compared to the number of mice challenged.