**TGFβ Blockade Enhances Radiotherapy Abscopal Efficacy Effects in Combination with Anti-PD1 and Anti-CD137 Immunostimulatory Monoclonal Antibodies**

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**Abstract**

Radiotherapy can be synergistically combined with immunotherapy in mouse models, extending its efficacious effects outside of the irradiated field (abscopal effects). We previously reported that a regimen encompassing local radiotherapy in combination with anti-CD137 plus anti-PD-1 mAbs achieves potent abscopal effects against syngeneic transplanted murine tumors up to a certain tumor size. Knowing that TGFβ expression or activation increases in irradiated tissues, we tested whether TGFβ blockade may further enhance abscopal effects in conjunction with the anti–PD-1 plus anti-CD137 mAb combination. Indeed, TGFβ blockade with 1D11, a TGFβ-neutralizing mAb, markedly enhanced abscopal effects and overall treatment efficacy against subcutaneous tumors of either 4T1 breast cancer cells or large MC38 colorectal tumors. Increases in CD8 T cells infiltrating the nonirradiated lesion were documented upon combined treatment, which intensely expressed Granzyme-B as an indicator of cytotoxic effector capability. Interestingly, tumor tissue but not healthy tissue irradiation results in the presence of higher concentrations of TGFβ in the nonirradiated contralateral tumor that showed smad2/3 phosphorylation increases in infiltrating CD8 T cells. In conclusion, radiotherapy-induced TGFβ hampers abscopal efficacy even upon combination with a potent immunotherapy regimen. Therefore, TGFβ blockade in combination with radioimmunotherapy results in greater efficacy.

**Introduction**

The role of radiotherapy as a means to augment response to immunotherapy has been explored in preclinical models (1, 2) and is currently being studied in multiple clinical trials (3–7). The most interesting experiments are those in which radiation elicits efficacy outside the irradiation fields, the so-called abscopal effects of radiotherapy (1, 8). Although rare with radiation alone, distant immune response from radiotherapy is potentiated in mouse models with systemic treatments in conjunction with mAbs antagonizing PD-1 (9), TGFβ (10), or CTLA-4 (11). Such an effect can also be observed with mAbs acting as CD40 (12) or CD137 (13) agonists. Moreover, local treatment with proinflammatory adjuvant agents such as CpG oligonucleotides (14), imiquimod (15), or poly I:C (3) is also known to enhance systemic immunity in mouse models (1, 2). In humans treated with radiotherapy and pembrolizumab, expression of IFNγ-associated genes from post-SBRT tumor biopsy specimens significantly correlated with nonirradiated tumor response underscoring the role of the immune system in abscopal effects of radiotherapy (16).

In a recent report, we showed that radiation abscopal effects can be readily demonstrated in mice bearing two subcutaneous tumors in opposite flanks in which one tumor lesion is irradiated using external beam (17) or brachytherapy (18) in combination with systemic immunostimulatory mAbs directed to CD137 and PD-1. This combination of immunotherapy agents simultaneously agonize on CD137 (4-1BB) and block PD-1/PD-L1 coinhibitory receptors (19). Abscopal effects of radiotherapy in this setting were contingent on cytolytic T lymphocytes (CTL), the integrity of the Type I IFN system, and the activity of BAFF3-dependent dendritic cells (17). These dendritic cells are also known as cDC1 and are specialized in antigen cross-presentation and cross-priming to CD8 T lymphocytes (20). Presumably, radiotherapy-induced immunogenic cell death (21) leads to the release of...
tumor antigens that can be cross-presented by this specialized dendritic cell subset.

Nonetheless, there were limits to the effectiveness of this radiopharmacologic combination because efficacy was lost when at the time of treatment the bilateral subcutaneous tumors had grown beyond a certain size. This occurred in the case of MC38 and B16-OVA bilateral tumor-bearing mice on day +15 after tumor cell inoculation, when mean diameters of tumors averaged 8–10 mm. Furthermore, although survival was prolonged in tumors derived from the 4T1 breast cancer cell line, which could be considered as examples of poorly immunogenic tumors, treatments were not finally curative in any instance.

TGFβ is a pleiotropic cytokine whose activity is known to be potently immunosuppressive (22, 23). TGFβ downregulates effector CD8 and CD4 T-cell activation (24) and promotes differentiation and function of immunosuppressive regulatory T cells (Treg; ref. 25). Furthermore, it reportedly fosters immunosuppressive myeloid-derived suppressor cells thereby enhancing their deleterious functions on cancer immunity (26).

Recent evidence indicates that TGFβ plays a key role in restraining T cells from infiltrating the malignancy by inducing the formation of fibrotic networks that impede tissue penetration (27). Importantly, this mechanism seems to be prominent in tumors refractory to current immunotherapies such as mesenchymal-type colon cancer (23), ductal pancreatic carcinoma, and a subgroup of urothelial carcinomas (27). In the context of radiotherapy, TGFβ is known to play a key role opposing tumor control by promoting survival (28) as a mediator in profibrotic pathogenic reactions that give rise to chronic and often severe side effects (29).

Pharmacologic blockade of TGFβ has been a long-term goal of the pharmaceutical industry. Many strategies have been followed (30), but neutralizing mAbs such as fresolizumab and small-molecule inhibitors of SMAD2/3 phosphorylation are the most advanced options under current clinical development (31, 32). Although the safety of systemic blockade of TGFβ is a concern due to the housekeeping functions of these cytokines in mesenchymal tissues and wound healing, the agents have an excellent toxicity profile in short-term (<1 year) treatment regimens. However, the efficacy of these agents as cancer monotherapy is as yet very modest (4, 30). As mentioned above, TGFβ blockade in conjunction with local radiotherapy enhances efficacy in mouse models (33). This has warranted a phase I/II clinical trial combining radiotherapy and fresolizumab, which has provided hints of certain clinical benefit in a few cases and shows translational evidence for the relief of TGFβ-suppressive effects on antitumor immune responses in human patients with cancer in the context of radiotherapy (4).

Given the induction of TGFβ by radiotherapy and the demonstration that inhibition of TGFβ promotes tumor control mediated by immune cells (33), we decided to test whether TGFβ inhibition could enhance the efficacy achieved by local radiotherapy in conjunction with anti–PD-1 plus anti–CD137 mAbs conforming a triple-pronged approach. Because TGFβ is secreted in a latent form and needs to be released from the latent protein to exert its biological functions on its cell surface receptors, TGFβ can be safely blocked in mice with the 1D11 mAb (34), which selectively recognizes and neutralizes the active state of the three isoforms of this cytokine (10). Radiotherapy elicits both latent TGFβ activation (35) and induces the transcription of TGFβ1 (36).

In our hands, TGFβ systemic inhibition in conjunction with the radioimmunotherapy regimen encompassing anti–CD137 and anti–PD-1 mAbs further enhances abscopal effects of radiotherapy. T-cell infiltrates in the contralateral nonirradiated tumor, which mimics occult or known distant metastases, appear to be more abundant and functionally fit.

Materials and Methods

Mice and cell lines

Female C57BL/6 and BALB/c mice were obtained from Harlan Laboratories. All animal procedures were conducted under institutional guidelines that comply with national laws and approved policies (study number 117/14). These experiments conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes (Real Decreto 1201/2005).

MC38 is a colon adenocarcinoma cell line of C57BL/6 origin whose identity was confirmed by Radyll (Case 6592-2012) and was originally provided to us by Dr. Karl E. Hellström (University of Washington, Seattle, WA). The 4T1 breast carcinoma cells of BALB/c origin were originally provided by Dr. Claude Leleuc (Institute Pasteur, Paris, France) and were verified in the master cell bank at the Institute Pasteur (Paris, France). Cell lines were cultured in RPMI1640 supplemented with 10% FBS, 2 mmol/L l-glutamine, 0.05 mmol/L 2-mercaptoethanol, HEPES, penicillin, and streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. All these cell lines were checked monthly to be free from contamination by Mycoplasma using the Mycoplasma detection kit (MycAAlert Mycoplasma Detection Kit from Lonza).

Tumor radiotherapy procedures

All mice were lightly anesthetized by intraperitoneal injection of Ketamine (Imalgene Merital Laboratorios S.L.) at dose of 100 mg/kg, while positioned on a dedicated methylacrylate platform over the linac couch. CT scans were performed with a Siemens Somatom Emotion scanner, using the narrowest thickness allowed (0.6 mm) and sequential (nonhelical) capture, to increase spatial accuracy on axis direction for dose planning. Field delimiting light was used to correctly orient the beam. Radiotherapy was delivered to a field including the tumor with 0.5 mm margins using a Versa HD Linear Accelerator (Elekta AB) fitted with a 10–15 mm radiosurgery conical collimator, which is designed to deliver very sharp and limited radiation–dosed fields. A Superflab bolus (5 mm thick tissue equivalent material) was placed over the tumor as a skin surrogate, and a source to axis distance (SAD) of 100 cm was set. The use of collimator-based radiosurgery allows a more homogenous and more concentrated radiation dose to be delivered to the tumor and a very low dose outside the radiation field. Ninety eight percent of treatment volume was covered by the prescription dose (8 Gy), and the average distance between the isodoses of 8 Gy and 2 Gy, inside the mouse, was approximately 2 mm. Radiation was delivered at about 10 Gy/min with 6 MV FFF (fattening filter free) X-rays. In our Versa HD linear accelerator, the quality index of the energy is TPR_{20,10} = 0.59. Mice received 3 fractions of 8 Gy on alternate days.

In vivo tumor experiments

C57BL/6 female mice were injected subcutaneously with 5 x 10⁵ MC38 cells, in the right flank (primary tumor) and with 3 x 10⁵ MC38 cells in the left shoulder area (secondary...
tumor, inoculated with fewer tumor cells to stay within ethical size limits during treatment). A similar scheme was used to subcutaneously and bilaterally engraft 3 × 10³ 4T1 cells in female BALB/c mice. Perpendicular tumor diameters were measured with a Vernier precision caliper every 2–3 days, and tumor volumes were calculated. On day +14 for MC38 cells or on day +11 for 4T1, when both tumors were palpable (always >3 mm in average diameter), animals were randomly assigned to 6 groups receiving or not radiotherapy (8 Gy × 3 fractions), to only one of the two subcutaneous tumors (right), in combination or not with intraperitoneal immunostimulatory mAbs (anti-PD1 plus anti-CD137 and/or anti-TGFβ). A combination of both anti-PD1 and anti-CD137 (kindly provided by Dr. Alan Korman of Bristol Myers Squibb), or anti-Rat IgG control antibodies (Sigma-Aldrich) was administered intraperitoneally with repeated corresponding doses of 200 μg/mouse (5 mg/kg) or 100 μg/mouse (2.5 mg/kg). Pan-isofrom TGFβ-neutralizing 1D11 mAb (purchased from Bio X Cell) was given on indicated days at 200 μg per dose. Mice were sacrificed when tumor size reached 4 cm³ or showed distress according to protocol.

Flow cytometry and ELISA assays

Tumor tissue was processed to obtain single-cell suspensions for flow cytometry analyses. To estimate absolute numbers in cell suspension, perfect count microspheres were used as an internal standard as per the manufacturer’s instructions (Cytognos). For gating and costaining, the following mAbs were used: CD45.2 (clone 104 from BioLegend), CD4 (clone RM4-5 from BioLegend), CD8 (clone 53-6.7 from BioLegend), CD4 (clone FJK-16S from eBioscience), CD25 (clone 104 from BioLegend), CD8 (clone 53-6.7 from BioLegend), CD4 (clone RM4-5 from Biosciences), and CyExpert software. viSNE analysis was performed using FlowJo (TreeStar Inc.) and CytExpert software. viSNE analysis was performed using CytOBANK software.

Levels of TGFβ1 in mouse tumor tissue homogenates were measured by a commercial ELISA (Human TGFβ Elisa Set, BD OptEIA, BD Biosciences), following the manufacturer’s instructions. All samples were measured in at least duplicates. The detection cut-off concentration for the assay was 4 pg/mL and the coefficient of variation was <15%.

Levels of IFNγ in mouse plasma samples were measured by a commercial ELISA (mouse IFNγ Elisa Set, BD OptEIA, BD Biosciences), following the manufacturer’s instructions. All samples were measured in duplicate. The detection cut-off levels of the IFNγ assay were 4.7 pg/mL and the coefficient of variation was <15%.

IHC

Tumors were excised from mice at necropsy, formalin-fixed and embedded in paraffin. IHC was performed using the following mAbs against CD3 (SP7 clone) and FoxP3 (FJK-16S clone). Sections stained for CD3 and FOXP3 were digitally scanned (sScan XT, Ventana). The whole-section images were visualized using VENTANA Virtuosso software. A trained pathologist selected representative areas (50,000 μ² and more than 1,000 cells) and software automatically estimates percentage of positivity, which is calculated by dividing the number of positive cells by the total nucleated cells in the area.

Statistical analysis

Statistical differences between survival curves were analyzed with the Mantel–Cox, log-rank test, nonlinear-regression and differences between other groups were analyzed with the Mann–Whitney U test using GraphPad Prism (GraphPad Software Inc.). The data shown in the analyses are the results of three independent experiments.

Results

TGFβ blockade enhances radiotherapy abscopal effects in synergy with anti–PD-1 and anti-CD137 mAbs

In a previous article (17), we reported that mice bilaterally bearing MC38-derived tumors showed abscopal effects as a result of a combined radiation and immunotherapy regimen encompassing three 8 Gy doses of unilateral radiotherapy (24 Gy/8 Gy/dose; ref. 37) and three every other day doses of anti–PD-1 and anti-CD137 mAbs (17). However, if treatment onset was delayed beyond day +11 after tumor cell inoculation, abscopal effects were largely lost.

To explore the potential of TGFβ blockade in these settings, experiments were undertaken delaying treatments until as late as 14 days following bilateral tumor cell inoculation, when tumors reached an average diameter of approximately 300 mm³ (Fig. 1A). As shown in Fig. 1B, abscopal efficacy of local radiotherapy plus systemic anti-PD1 plus anti-CD137 mAb triple combination (henceforth RT + Combo), was very weak at this level of tumor progression. However, if the 1D11 antibody anti-TGFβ mAb was given prior to irradiation on day 14, as well as on days 16, 18, 20, and every two weeks thereafter (Fig. 1A), curative antitumor activity was seen against the nonirradiated lesion (Fig. 1B). Of note, TGFβ blockade as a single agent plus radiotherapy without immunotherapy does not exert any beneficial effect in this model. Figure 1C shows the survival benefit attained in 37% (3/8) mice by RT + Combo while addition of anti-TGFβ mAb resulted in 87% survival (7/8). Figure 1D shows higher serum concentrations of IFNγ on day +22 in the groups in which TGFβ blockade was added to the radioimmunotherapy regimen, which is consistent with stronger antitumor cellular immunity.

In our prior studies, abscopal effects affect beyond day +7 were very seldom observed in mammary carcinomas derived from 4T1 cells (17). To confirm that TGFβ blockade synergizes with RT + Combo, female mice bearing bilateral 4T1 tumors started such combined treatment on day +11 (Fig. 2A). In this difficult-to-treat setting, meaningful abscopal effects of the radioimmunotherapy combination were only seen when TGFβ was blocked (Fig. 2B). However, in this model, curative effects were not achieved, as was previously observed when 4T1 were treated with RT, 1D11, and anti-PDL1 (33).

In this case, the combination of our radioimmunotherapy regimen plus TGFβ blockade did not result in higher concentrations of circulating IFNγ on day +18, levels that were already quite high in the RT + Combo group (Fig. 2C).

1D11 mAb treatment partially blocks TGFβ as elicited by radiotherapy

To ascertain whether 1D11 mAb treatment was actually downregulating TGFβ, we performed ELISA assays on tissue...
Homogenates from the irradiated and nonirradiated tumors in a setting as shown in Fig. 2A. Homogenates from tumors excised on day +18 showed reduction of TGFβ concentrations in the contralateral nonirradiated tumor but not significantly in the irradiated lesion.

Biological activity of TGFβ on T cells is best assessed by the level of intracellular Smad 2/3 phosphorylation. FACS analyses of TILs from the nonirradiated and irradiated 4T1 lesions showed a clear decrease of phosphorylated Smad2/3 as a result of 1D11 mAb treatment (Fig. 3B). The reduction was more prominent in the irradiated tumor site. Residual p-Smad2/3 may indicate that further inhibition of the TGFβ pathway with TGFβR inhibitors is possible to perhaps attain even greater efficacy.

Collectively, our data suggest that at least partial interference with the TGFβ signaling in the distant nonirradiated tumor may underlie the observed abscopal efficacy.

Figure 1.
TGFβ blockade enhances to a curative level the abscopal effects of a radioimmunotherapy regimen that combines local irradiation with systemic anti-CD137 and anti-PD1 mAbs. A, Scheme of treatment of MC38-derived bilateral distant subcutaneous tumors that were allowed to engraft for 14 days and were then treated with 200 μg of 1D11 TGFβ-neutralizing mAb or control intraperitoneally as indicated. On day +15, +17 and +19 one of the tumor lesions received external beam radiotherapy (8 Gy/dose). On days +16, +18, and +20 mice received 200 μg of anti-PD1 and anti-CD137 mAbs. Corresponding control antibodies (Rat IgG) were injected into the indicated groups. B, Bilateral tumor size follow-up (mean volume ± SEM) of the mice in the indicated groups of treatment as assessed in the irradiated tumor site (left) or the concomitant nonirradiated tumor lesion (right). C, Kaplan–Meier curves representing survival data statistically compared by log-rank tests. D, Shows circulating IFNγ concentrations in serum samples drawn on day +22 from the indicated groups. ***: P < 0.0001; **: P < 0.001; *: P < 0.05.

Two experiments performed showed comparable results.
Anti-TGFβ to Enhance Radioimmunotherapy Abscopal Effects

Figure 2. Abscopal effects of radioimmunotherapy are enhanced by TGFβ blockade against the 4T1 breast carcinoma model. A, Experimental design of treatments started on day +10, a condition in which radioimmunotherapy with anti-CD137 and anti-PD-1 mAbs attains limited abscopal effects. Treatments and days of treatment are indicated by colored arrows. B, Tumor size follow-up (volume ± SEM) in the irradiated (left) and nonirradiated tumor lesions. C, Shows circulating IFNγ concentrations on day +18 of the experiment in individual mice from the indicated experimental groups. A representative experiment of two performed is shown.

TGFβ blockade enhances T-cell infiltrates in distant nonirradiated tumors as elicited by the radioimmunotherapy combination

Given the antitumor effects on the distant nonirradiated tumor, we set up experiments to excise contralateral 4T1-derived tumors on day +18 as shown in Fig. 2A. Excised tumors were analyzed by IHC and numbers of CD3+ T cells and FOXP3+ Tregs were counted to estimate their density and location. Total CD3+ T cells were further enhanced upon the addition of TGFβ blockade over the effect of the radioimmunotherapy combination (RT + Combo), whereas Foxp3+ cells tended to be reduced or at least remain stable, as shown in Fig. 4A and B.

To further study in more detail such tendencies on the lymphoid infiltrates in independent experiments, we obtained single-cell suspensions of the nonirradiated 4T1 tumor on day +18 as indicated in Supplementary Fig. S2A. Indeed, distant tumors under radiotherapy plus the triple mAb regimen (RT + Combo + 1D11) had started to stabilize and reduce weight at that time point (Supplementary Fig. S2B and S2C). In 4T1-derived malignancies, tissue density of CD8 and CD4 T cells was increased by TGFβ blockade throughout the radioimmunotherapy combination regimen (Fig. 5A). However, FOXP3+CD25+ Tregs also increased to some extent in these nonirradiated lesions, even though the CD8/Treg ratios remained favorably increased (Fig. 5B). In the case of MC38-derived tumors, similar CD8 increases were observed, which was not the case with CD4 T cells (Supplementary Fig. S3A).

Cytolytic granules were studied in the 4T1 tumor–infiltrating lymphocytes (TIL) by FACS assessment of intracellular Granzyme-B. Interestingly, both CD8 and more prominently CD4 T cells increased their Granzyme-B content under concomitant TGFβ inhibition with 1D11 (Fig. 5C). In MC38 TILs, Granzyme-B content was increased in CD8 but not in CD4 T cells, suggesting differences in the immune response between tumor models (Supplementary Fig. S3B).

Myeloid populations in leukocyte infiltrates of 4T1-derived nonirradiated lesions were also analyzed. As shown in Supplementary Fig. S4, radioimmunotherapy with RT + Combo reduced the content of myeloid-derived suppressor cells, macrophages, and dendritic cells. Adding TGFβ blockade with 1D11 did not result in further changes (Supplementary Fig. S4). Accordingly, the additional beneficial effect of TGFβ-blockade to RT + Combo cannot be attributed to numeric myeloid cell changes.

Next, we examined whether TGFβ effects and concentrations were increased in the contralateral nonirradiated tumor site following tumor irradiation. For this purpose, we set up experiments as schematically depicted in Fig. 6A. Animals received the three fractionated radiotherapy regimen on tumor lesions or on healthy nontransformed hind limb tissue. We observed that when tumors were irradiated, the phosphorylation of SMAD2/3 in CD8+ tumor–infiltrating T cells and the content of TGFβ were augmented in the contralateral nonirradiated tumor lesion (Fig. 6B and C). Remarkably, if healthy nontransformed tissue was irradiated, such increases were not substantiated (Fig. 6B and C).

Collectively, these data indicate that adding at least partial blockade of TGFβ to the previously reported regimen of local radiotherapy plus anti–PD-1 plus anti–CD137 (RT + Combo), results in more prominent T-cell infiltrates in the nonirradiated lesion. These T-cell infiltrates expressing more prominent cytotoxic features are likely to explain the more efficacious abscopal effects observed when TGFβ blockade was added to the regimen.

Discussion

To improve our previously reported effects combining radiotherapy with anti-PD1 plus anti-CD137 mAb combinations, we...
explored concomitant TGFβ blockade with the 1D11 mAb to enhance effects using syngeneic bilateral tumor models in which only one of the tumor lesions receives radiotherapy. Efficacy against the distant nonirradiated tumor was significantly enhanced by adding TGFβ inhibition. The rationale to enhance radioimmunotherapy antagonizing TGFβ is well documented in the literature (4, 7) and is to be clinically tested in radioimmunotherapy strategies. Indeed, radiotherapy is described to elicit TGFβ activation in the irradiated tumor tissue (10). Such an effect is considered undesirable because it opposes tumor control (10, 38), is profibrogenic (39), and also exerts potent immunosuppressive effects (40). 1D11 is a mAb that neutralizes the active state of all TGFβ isoforms.

Cancer immunotherapy achieves its best results in synergistic combinations that have resulted in over a thousand clinical trials testing immuno-oncology combinations as can be found at Figure 3.

**Figure 3.**
1D11 anti-TGFβ mAb actually targets the TGFβ pathway to attain abscopal effects. A, ELISA measurements of TGFβ1 concentration in tumor tissue homogenates from experiments as those shown in Fig. 2A and Supplementary Fig. S2A. B, Level of SMAD2/3 phosphorylation in 4T1 tumor–infiltrating CD8 and CD4 T cells assessed by intracellular immunostaining and flow cytometry in the indicated groups treated as in Fig. 2A and Supplementary Fig. S2A. Results represent mean ± SEM and represent one experiment of two similarly performed.
ClinicalTrials.gov (41). Triplet and quadruplet immunotherapy combinations are not unthinkable according to results in animal models (42). In fact, higher order of magnitude combinations are reaching the clinical trial arena. External beam radiotherapy, if given in adequate hypofractionated dosing schedules is clearly proimmunogenic and can be exploited in immunotherapy schemes (43). Unfortunately, its proimmunogenic effects as a single-treatment modality are suboptimal and very seldom control nonirradiated lesions.

The proimmune effects of radiotherapy are ascribed to its ability to induce immunogenic cell death (44) and activation of the type I IFN pathway (37, 45). Consistent with these findings, the role of BATF-3–dependent dendritic cells mediating tumor antigen cross-priming is invoked by solid experimental evidence (17, 46). Because radiotherapy-elicted immune effects are insufficient, combinations with other immunotherapy agents are advisable. An ample number of preclinical reports provide evidence for the synergistic effects, both local and abscopal, of radiotherapy with immunostimulatory mAbs. Published previous experience using TGFβ blockade with mAbs in monotherapy showed improved local tumor control of radiotherapy without abscopal effects (33). We have observed that TGFβ blockade achieves abscopal effects beyond the previously reported spectacular effects reached by unilateral radiotherapy plus anti–PD-1

Figure 4.
IHC assessment of immune infiltrates in 4T1 nonirradiated tumors. A, Representative microphotographs of IHC staining for CD3 and Foxp3 in tumors excised on day +18 from 4T1-derived subcutaneous tumors and treated as indicated in Fig. 2A. B, Quantitative estimate of the numbers of positive cells per μm² ± SEM. Results are from a single experiment with 6 mice per group.
plus anti-CD137 mAbs in combination (3, 18). In our hands, the quadruple combination approach achieves abscopal effects against large and well-established transplanted tumors.

Local radiotherapy and TGFβ blockade with fresolizumab has already been tested in a small clinical trial with modest results but with data suggesting elicitation of stronger antitumor immunity (47). Fresolizumab is a humanized version of 1D11, and this clinical trial provides evidence for safety and tolerability at the doses tested (48). It makes sense, therefore, to incorporate at least a PD-(L)1 blocking agent into this regimen because combinations of TGFβ inhibition and anti-PD-(L)1 are reportedly synergistic against mouse tumors (23, 27). Indeed, most contemporary polyimmunotherapy regimens are currently built on the PD-1/PD-L1 blockade backbone (19). An interesting attempt to simultaneously impact the TGFβ and the anti-PD1 pathway is a construction of avelumab (an anti–PD-L1 antibody) chimerized with TGFβRII to conform a soluble TGFβ trap (M7824). This bispecific immuno-biological tool has shown clearly better therapeutic activity against tumors than avelumab by itself at least in some mouse models (47). Clinical development of this compound has started and results are eagerly awaited, even if the potential disadvantage of this agent is that the doses of the TGFβ inhibitor and the PD-L1 blocker cannot be dissociated for optimization, as could be done when the two agents are administered separately. Its combination with radiotherapy regimens in search of abscopal effects and better local tumor control should be considered (47).

Our results point at increases in functional CD8 T cells in the tumor microenvironment of the nonirradiated lesions as the clear evidence that the combination increases systemic antitumor immunity, perhaps together with a relative reduction of Treg cells. This would be consistent with the literature that shows how TGFβ reduces the activation of CD8 T cells in tumor models (49) and how TGFβ augments the numbers and function of Foxp3+ Tregs (50, 51). In our case, we did not observe further reductions of myeloid-suppressive cells beyond the remarkable reductions attained by RT + Combo by itself.

Our results regarding presence and signaling effects of TGFβ in the contralateral nonirradiated site argue in favor of circulation of...
this immunosuppressive cytokine from the irradiated site to the distant lesion. Interestingly, this occurs from a tumor lesion but not from irradiated nonmalignant healthy tissue for reasons that warrant further investigation.

The magnitude of the preclinical abscopal effects seen in this study warrants inclusion of TGFβ blockade to synergize with radioimmunotherapy clinical regimens, as shown for radiotherapy and ipilimumab for melanoma (9, 52) and NSCLC (53). In the case of PD-1 inhibitors, clinical trials are ongoing in combination with radiotherapy in which effects on nonirradiated lesions are monitored (21). The addition of agonist antibodies to TNFR costimulatory members such as CD137, OX40, and CD40 makes sense since it is a potential next step forward as supported by our results. In conclusion, although TGFβ blockade might not be sufficient to unleash meaningful abscopal effects from radiotherapy, it is likely to be a valuable partner in radioimmunotherapy combinations.

Disclosure of Potential Conflicts of Interest
M.H. Barcellos-Hoff reports receiving a commercial research grant from Varian Medical Systems and Genentech; has received speakers bureau honoraria from Roche and BMS; and is a consultant/advisory board member for Roche and BMS. I. Melero reports receiving a commercial research grant from BMS, Roche, Bioncotech, and Alligator; has received speakers bureau honoraria from MSD and has ownership interest (including stocks and patents) in Tusk; and is a consultant/advisory board member for BMS, Roche, Bayer, EMD, Gemmab, Numab, Piers, Molecular partners, Immune design, and Bioncotech. No potential conflicts of interest were disclosed by the other authors.

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