Clinical Cancer Research

Fractionated Radiation Therapy Stimulates Antitumor Immunity Mediated by Both Resident and Infiltrating Polyclonal T-cell Populations when Combined with PD-1 Blockade

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Abstract

Purpose: Radiotherapy is a highly effective anticancer treatment forming part of the standard of care for the majority of patients, but local and distal disease recurrence remains a major cause of mortality. Radiotherapy is known to enhance tumor immunogenicity; however, the contribution and mechanisms of radiotherapy-induced immune responses are unknown.

Experimental Design: The impact of low-dose fractionated radiotherapy (5 × 2 Gy) alone and in combination with αPD-1 mAb on the tumor microenvironment was evaluated by flow cytometry and next-generation sequencing of the T-cell receptor (TCR) repertoire. A dual-tumor model was used, with fractionated radiotherapy delivered to a single tumor site to enable evaluation of the local and systemic response to treatment and ability to induce abscopal responses outside the radiation field.

Results: We show that fractionated radiotherapy leads to T-cell infiltration at the irradiated site; however, the TCR landscape remains dominated by polyclonal expansion of preexisting T-cell clones. Adaptive resistance via the PD-1/PD-L1 pathway restricts the generation of systemic anticancer immunity following radiotherapy, which can be overcome through combination with αPD-1 mAb leading to improved local and distal tumor control. Moreover, we show that effective clearance of tumor following combination therapy is dependent on both T cells resident in the tumor at the time of radiotherapy and infiltrating T cells.

Conclusions: These data provide evidence that radiotherapy can enhance T-cell trafficking to locally treated tumor sites and augment preexisting anticancer T-cell responses with the capacity to mediate regression of out-of-field tumor lesions when delivered in combination with αPD-1 mAb therapy. *Clin Cancer Res; 23(18); 5514–26. ©2017 AACR.*

Introduction

Radiation therapy is delivered to approximately 50% of all cancer patients, improving local disease control and reducing recurrence (1–3). Radiotherapy can modulate the tumor microenvironment in several ways to enhance immunogenicity, including modulation of class I MHC and novel peptide antigen expression on tumor cells (4), generation of type I IFN (5–7), activation of the complement pathway (8), induction of immunogenic cell death (9), and direct effects on immune effector cells (10). Irradiated tumor cells may act as an "*in situ* vaccine" through the provision of increased tumor-associated antigens, damage-associated molecular patterns, and modulation of the tumor microenvironment promoting dendritic cell (DC) recruitment and T-cell priming (4, 7, 11–14). However systemic antitumor immune responses outside of the irradiated tumor field termed the "abscopal effect" are rare in routine clinical practice. Furthermore, tumor recurrence frequently occurs following radiotherapy and remains the leading cause of patient mortality. Therefore, in the clinic, radiotherapy alone appears to be generally insufficient to elicit durable, therapeutic antitumor immunity (15).

The PD-1/PD-L1 axis is known to mediate peripheral tolerance and attenuation of acute inflammatory responses through modulation of T-cell receptor (TCR) signal transduction, metabolic reprogramming, anergy, and apoptosis (16–18). Furthermore, we and others have previously shown across a range of tumor models that signaling through the PD-1/PD-L1 pathway can limit the ability of radiotherapy to generate systemic immune responses (19–21). In most of these preclinical studies, enhanced tumor

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Translational Relevance

Radiotherapy is well documented to be immunogenic; however, systemic antitumor immune responses outside of the irradiated tumor field, termed the "abscopal effect," are rare in patients. The lack of abscopal effect is poorly understood, particularly in the context of low-dose daily fractionated radiotherapy, the most common regimen used in clinical practice. We demonstrate that five daily fractions of 2 Gy induce a polyclonal T-cell response at the irradiation site that is dominated by the expansion of preexisting T-cell clones. However, systemic anticancer immunity appears to be limited locally by adaptive immunologic resistance, which can be overcome by blockade of the PD-1/PD-L1 pathway, leading to long-term tumor control. Furthermore, although T cells resident in the tumor prior to radiotherapy are important, newly infiltrated polyclonal T-cell populations are required for maximal systemic tumor control. These results provide important new insights for clinical translation to improve outcomes using radiotherapy through combination with αPD-1/PD-L1 mAb.

immune responses have been generated by high doses of hypofractionated radiotherapy in combination with a range of immunomodulatory agents (19, 21–25). In contrast, the potential effects on local and systemic T-cell responses of low-dose daily fractionation as routinely delivered using a series of daily fractionated doses (1.8–2 Gy) are less well studied. Here, we have investigated how daily fractionation modulates the TCR repertoire diversity within the irradiated tumor and how this may be modified with radiotherapy in combination with αPD-1 mAb to result in a systemic immune response. By sequencing the CDR3 regions of TCRβ in both irradiated and out-of-field tumors, and in peripheral blood, we reveal that radiotherapy leads to local expansion of preexisting T-cell clones within the tumor, which dominate the TCR repertoire. In contrast, there was little evidence of T-cell clonal expansion as a consequence of radiotherapy-induced DNA damage with all of the dominant T-cell clones present in both the irradiated and out-of-field tumors following treatment with radiotherapy and αPD-1 mAb. In addition, we show that the immunologic effects of fractionated radiotherapy appear to be limited to the irradiated tumor site through adaptive upregulation of PD-L1. Importantly, blockade of the PD-1/PD-L1 signaling axis circumvents this local immunosuppression facilitating the generation of systemic anticancer immunity capable of mediating distal tumor regression. These observations provide new mechanistic insights into the impact of daily fractionated radiotherapy on the generation of adaptive antitumor immunity.

Materials and Methods

Mice and cell lines

BALB/c and C57Bl/6 mice were obtained from Harlan. Animal experiments were approved by a local ethical committee and performed under a United Kingdom Home Office license. CT26 murine colon carcinoma cells (ATCC) and 4434 cells isolated from $BRAF^{V600E}$ $p16^{-/-}$ mice (Richard Marais, Cancer Research UK Manchester Institute, Manchester, United Kingdom) were maintained in DMEM, supplemented with 10% FCS, 1% L-glutamine (Invitrogen). Upon receipt, cells were cultured for up to four passages, screened to confirm the absence of mycoplasma contamination by PCR, and aliquots frozen in liquid nitrogen to create a batch of authenticated stock lines. Cell lines were defrosted and cultured to limited passage for 1 to 2 weeks prior to implantation with regular rescreening for mycoplasma contamination. Mice were housed under specific pathogen-free conditions in Tecniplast 1284 IVC cages holding a maximum of 7 animals with aspenchips-2 bedding, sizzlenest nesting material, and a cardboard tunnel. Mice were housed on a 12/12 light/dark cycle and were given filtered water and fed *ad libitum* on Teklad Global 19% protein extruded rodent diet.

Tumor therapy

Mice were inoculated subcutaneously with 5×10^5 CT26 cells or 5×10^6 4434 cells at one or more distinct sites. Irradiations were performed 7 to 10 days after the primary tumor was inoculated (when primary tumors were at least 100 mm^3) using a Pantak HF-320 320 kV X-ray unit (Gulmay Medical). The machine was operated at 300 kV, 9.2 mA, with filtration fitted in the x-ray beam to give a radiation quality of 2.3 mm Cu half-value layer. Mice were positioned at a distance of 350 mm from the X-ray focus, where the dose rate was 0.80 Gy/minute and treated using tangential beam delivery. Administration of αPD-1 (clone RMP1-14), αPD-L1 (clone 10F.9G2), or isotype control mAb (Biolegend) commenced on day 1 of the fractionated radiotherapy cycle via intraperitoneal injection 3 times a week at a dose of 10 mg/kg in a dose volume of 100 μL/10 g in PBS. Tumor volume (up to 1,200 mm^3) was the primary endpoint for efficacy studies. Peripheral blood was sampled during therapy to confirm cellular depletion. Administration of FTY-720 (Fingolimod; Enzo Life Sciences) commenced either the day prior to tumor inoculation or the day prior to the start of radiotherapy and was delivered by oral gavage at a dose of 25 μg/mouse in a dosing volume of 200 μL. Subsequent daily administration was continued for 30 days (after the start of radiotherapy) at a dose of 5 μg/mouse in a dosing volume of 100 μL as described previously (26).

For tumor rechallenge experiments, long-term surviving (LTS) mice were implanted with tumor cells at a site distal to the original tumor a minimum of 80 days after previous tumor implantation. Experimental groups contained at least 5 mice per group and are representative of at least two independent experiments.

Measurement of cytokine production by $CD8^+$ T cells isolated from LTS mice

Splenocytes were isolated from either LTS or control mice and cocultured for 5 days with either irradiated tumor cells (50 Gy) or 1 μmol/mL of the H2-Ld restricted peptides SPSYVYHQF (AH1) or TPHPARIGL (β-galactosidase) (Anaspec). Cells were restimulated at a 1:1 ratio with 50 Gy irradiated CT26 cells for 16 hours in the presence of 1 μl/mL Brefeldin A (BD Pharmingen) and 100 IU/mL human recombinant IL2 as described previously (27). Experimental groups contained at least 4 mice and are representative of two independent experiments.

Tumor and immune cell phenotyping by flow cytometry

To obtain single-cell suspensions, tumors were processed using a gentle MACS dissociator and a murine tumor dissociation kit (Miltenyi Biotec). For analysis, nonspecific binding was blocked as described above and expression of CD4, CD8 (BD Biosciences), CD45, CD11b, Gr1, PD-1, and PD-L1 examined by multiparameter flow cytometry (all eBioscience unless otherwise stated). For

analysis, live cells were gated (by vital dye exclusion, Invitrogen) and populations phenotyped (as described above). An example of the gating strategy employed for selection of either $CD45^-$, $CD45^+$, or $CD45^+CD11b^+Gr1^+$ cells is provided in Supplementary Fig. S1.

Immunosequencing of the TCRβ-expressing repertoire in peripheral blood and tumors

Immunosequencing of the CDR3 regions of murine TCRβ chains was performed on samples isolated 7 days after the last dose of radiotherapy (along with time-matched control cohorts and those treated with αPD-1 mAb) using the ImmunoSEQTM Assay (Adaptive Biotechnologies). This assay uses 54 forward V gene primers and 13 reverse J gene primers, which are employed in a bias-controlled multiplexed PCR reaction to amplify the variable region of TCRB chains. Synthetic control templates were also spiked into each sample, thereby enabling quantitation of input TCRB templates from the read counts (28). Sequences were collapsed and filtered to identify and quantitate the abundance of each unique TCRβ CDR3 region for further analysis (28, 29).

Statistical analyses of TCRβ sequencing results

The clonality metric is defined as 1 − Peilou's evenness and is calculated as:

$$\frac{1 - \sum_{i}^{N} p_i \ln(p_i)}{\ln(N)}$$

Clonality values range from 0 to 1 and describe the shape of the frequency distribution: clonality values approaching 0 indicate a very even distribution of frequencies, whereas values approaching 1 indicate an increasingly asymmetric distribution in which a few clones are present at high frequencies. To estimate the fraction of T cells in the tissue samples, we considered 6.5 pg of DNA per diploid cell, which is equal to approximately 154 productive TCR loci per ng of DNA, and normalized the total T-cell estimates in each sample to the amount of input DNA multiplied with the value of 154 productive TCR loci per ng of input DNA. Pair-wise comparisons within therapy cohorts (i.e., tumor 1 vs. tumor 2) were performed using a paired *t* test. For comparisons across multiple therapy groups, we used the nonparametric Kruskal–Wallis test. We identified clones with significantly different abundance in two samples using a Fisher exact test with Benjamini–Hochberg corrected *P* values such that FDRs were held at 5% (30).

Results

Fractionated radiotherapy in combination with αPD-1 mAb generates systemic anticancer immunity

Low doses of fractionated radiotherapy (delivered as 5 daily fractions of 2 Gy) resulted in transient tumor control followed by regrowth in the majority of mice bearing either CT26 or 4434 tumors. In both models, local tumor control following treatment with fractionated radiotherapy can be improved when combined with mAbs targeting the PD-1/PD-L1 pathway (Fig. 1A and B). Moreover, LTS mice that undergo a complete response following combination therapy are able to completely reject tumors following rechallenge (Fig. 1C; $P < 0.01$ log-rank; Mantel–Cox test). Furthermore, in responding mice, we detected an increased frequency of antigen-specific memory $CD8^+$ T cells capable of IFNγ expression following coculture with either irradiated CT26 cells or a CT26 tumor-associated peptide antigen (AH1: SPSYVYHQF) but not with a control peptide (β-galactosidase: TPHPARIGL; Fig. 1D). To address the impact of radiotherapy dose and fractionation on therapeutic response, radiotherapy was also administered as a hypofractionated regimen (12 Gy in 3 fractions) or as a single dose (7 Gy) in combination blockade of the PD-1/PD-L1 axis (Fig. 1E). Importantly, our data reveal similar combinatorial activity for these different radiotherapy dosing regimens.

Dual tumor-bearing mice were used to determine whether fractionated radiotherapy delivered to a single tumor could lead to "abscopal" responses in out-of-field tumors. We found that low doses of fractionated radiotherapy resulted in transient local tumor control followed by regrowth in the majority of mice treated. However, complete tumor regression was observed in 18.8% mice (3/16) at the irradiated site. Complete regression of the out-of-field tumor following radiotherapy was a rare event (observed in 12.5%, 2/16 mice), occurring only in mice in which the response of the irradiated tumor was such that the mice survived to the study endpoint [day 60; Fig. 2A (for experimental schema) and B; Supplementary Fig. S2A]. To determine whether local treatment with an ablative dose of radiotherapy would be more effective at generating out-of-field responses, we treated mice locally with 10 Gy radiotherapy [which led to complete responses in 4/7 tumors (57%)]. Our data demonstrate that treatment with ablative doses of radiotherapy alone was insufficient to generate systemic anticancer immune responses capable of mediating complete regression of out-of-field tumors, with all mice exhibiting progressive tumor growth at the out-of-field tumor site (Supplementary Fig. S2B).

In contrast, radiotherapy delivered concurrently with αPD-1 mAb led to the regression of both the irradiated and out-of-field tumors, with >70% of mice undergoing complete responses (Fig. 2B). These data demonstrate that the combination of fractionated radiotherapy and αPD-1 mAb generates systemic antitumor responses and tumor control in both irradiated and out-of-field tumors.

Combination therapy leads to convergence of tumoral and systemic TCR repertoires

To understand how these treatments impacted the clonal populations of tumor resident T cells, we sequenced the TCR repertoire 7 days after the last dose of radiotherapy. Analysis of the TCR-β sequences confirmed that fractionated radiotherapy increases the frequency of T cells in the irradiated but not out-of-field tumor (Fig. 2C). Although TCR quantitation does not distinguish between tumor-resident proliferating T cells or infiltrating T cells, increased overlap between the TCR repertoire of the irradiated (but not the out-of-field) tumor and that of peripheral blood suggests that T-cell infiltration occurs after local fractionated radiotherapy (Fig. 2D). When delivered as a monotherapy, αPD-1 mAb did not enhance T-cell content in either tumor relative to the non-treated (NT) mice. In contrast, concurrent radiotherapy and αPD-1 mAb therapy resulted in increased T-cell infiltration/expansion in both the irradiated and out-of-field tumors relative to NT mice or αPD-1 mAb–treated mice (Fig. 2C and D).

We next examined the impact of these treatments on the clonality of TCR repertoires at the different tumor sites. We found that monotherapy with αPD-1 mAb did not alter T-cell clonality in either the local or distal tumor when compared with NT controls. In contrast, radiotherapy-treated mice had increased TCR repertoire clonality in the irradiated but not out-of-field tumor when compared with either NT or αPD-1 mAb–treated

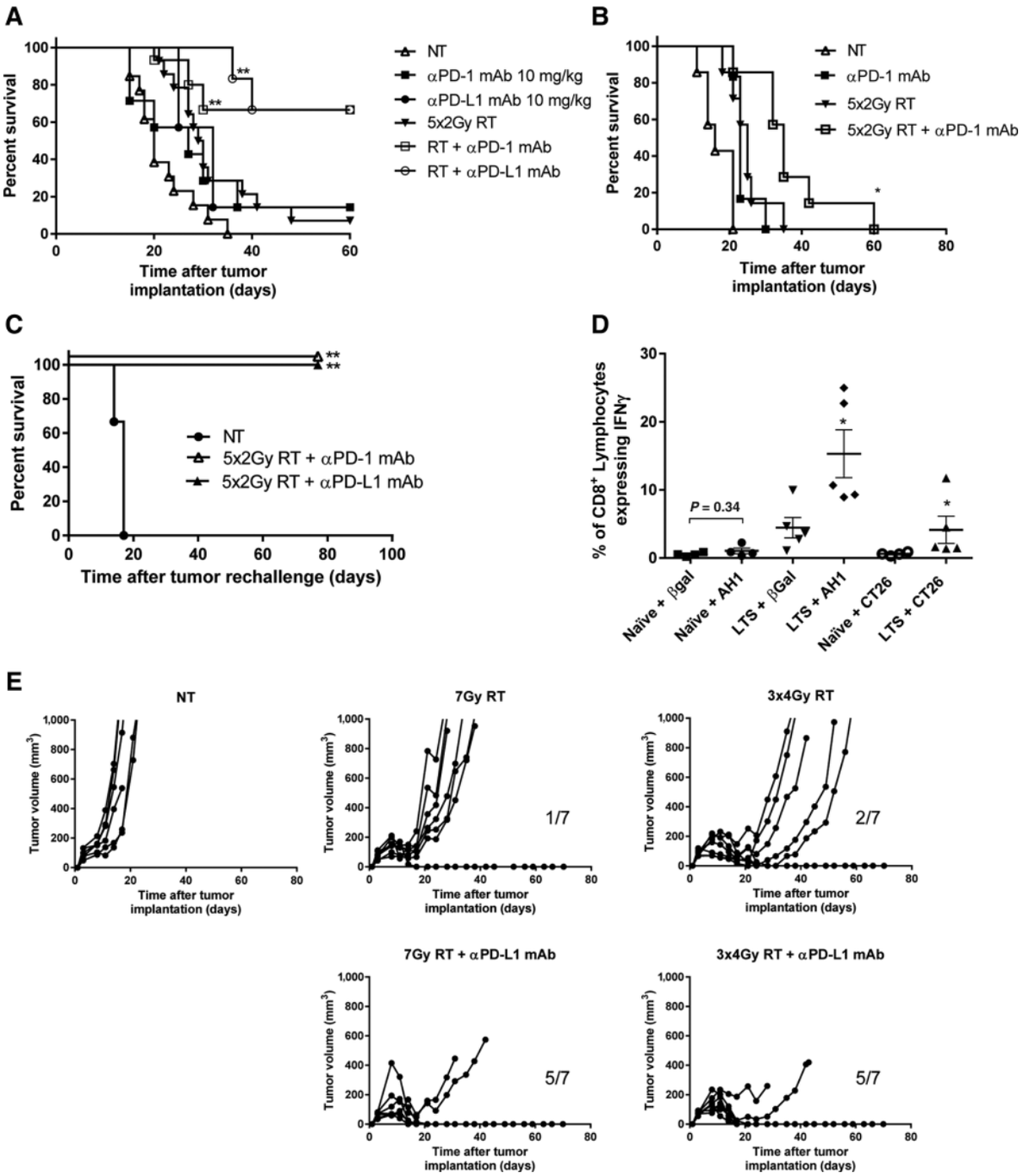

Figure 1.

Combination of fractionated radiotherapy (RT) and blockade of the PD-1/PD-L1 axis leads to improved survival and generation of tumor-specific memory immune responses. **A** and **B,** CT26 (**A**) or 4434 (**B**) tumor-bearing mice received fractionated radiotherapy delivered in five daily fractions of 2 Gy either in combination with either αPD-1/αPD-L1 mAb dosed at 10 mg/kg 3 times a week. Treatments started 7 days after tumor inoculation. Experimental groups contained at least 7 mice, except NT and radiotherapy groups, which contained 14 mice. Data are representative of two independent experiments. **C,** Survival curve of LTS mice originally treated with radiotherapy and αPD-1 mAb/αPD-L1 mAb following rechallenge with 5×10^5 CT26 cells. Experimental groups contained at least 4 mice. **D,** Frequency of IFNγ$^+$ CD8$^+$ T cells isolated from either tumor-naïve, or LTS mice originally treated with radiotherapy and αPD-L1 mAb following coculture with either H2-Ld restricted peptides (AH1 (SPSYVYHQF); a defined CT26 tumor-associated antigen or β-galactosidase (TPHPARIGL); control peptide of prokaryotic origin) or 50 Gy irradiated CT26 cells for 5 days, followed by priming with 50 Gy irradiated CT26 cells. Experimental groups contained at least 4 mice, and the data shown are representative of at least two independent experiments. **E,** CT26 tumor-bearing mice received either 7 Gy or three daily fractions of 4 Gy alone or in combination with an αPD-L1 mAb dosed at 10 mg/kg 3 times a week. The proportion of mice that experienced complete tumor resolution is indicated in each panel. **A–C,** **, $P < 0.01$; *, $P < 0.05$, log-rank (Mantel–Cox) test. **D,** Mean ± SEM. *, $P < 0.05$, Mann–Whitney test.

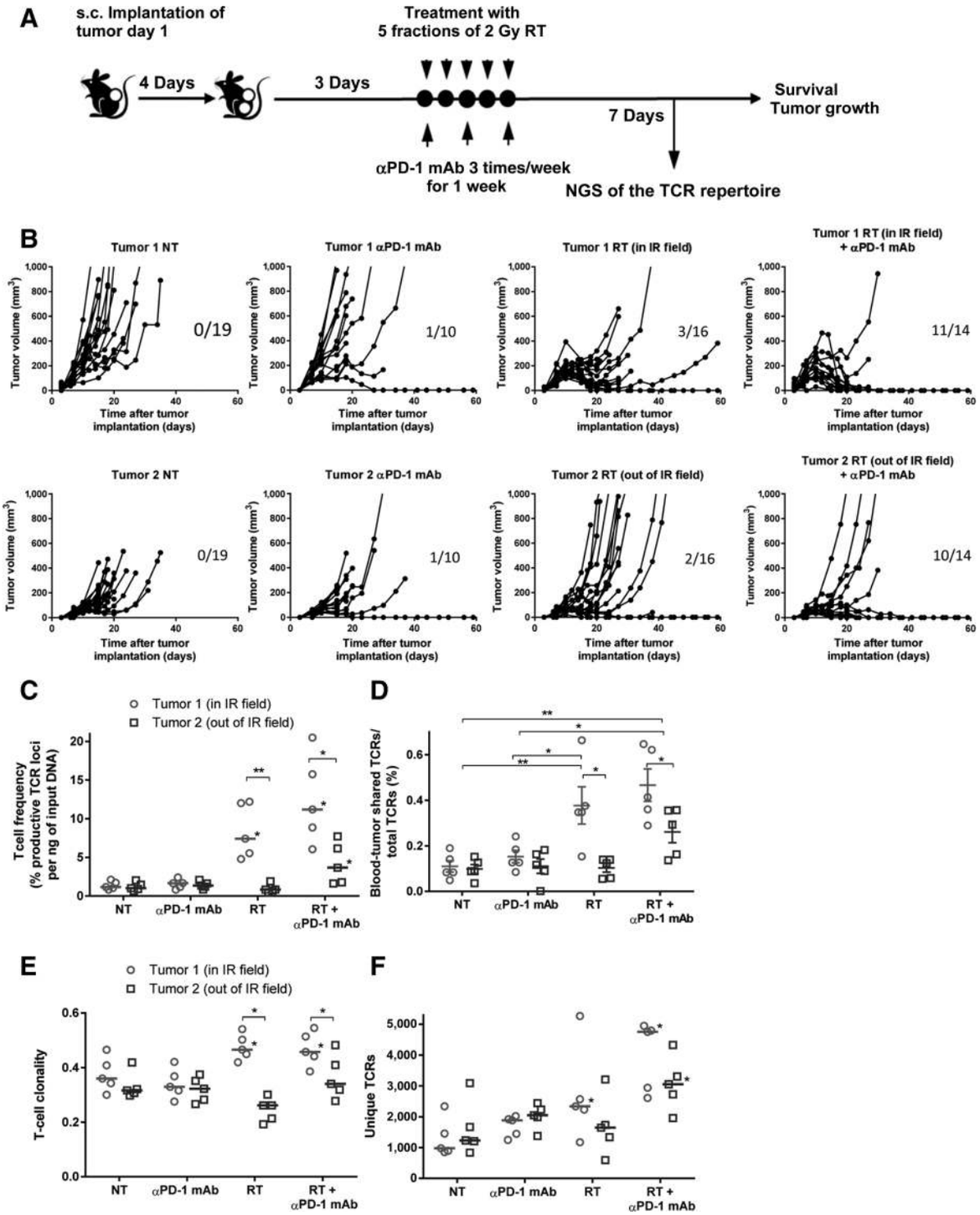

Figure 2.

Radiotherapy (RT) leads to local T-cell infiltration/expansion and broadening of the TCR repertoire, but systemic responses are only observed when combined with αPD-1 mAb. **A,** Schema for studies. Fractionated-dose radiotherapy (as 10 Gy in five daily fractions of 2 Gy) was delivered to tumor 1 with tumor 2 shielded from the ionizing beam in combination with αPD-1 mAb dosed at 10 mg/kg 3 times a week for 1 week. **B,** Individual tumor growth curves. The proportion of mice that experienced complete tumor resolution is indicated in each panel. Experimental groups contained at least 4 mice, and the data shown are representative of at least three independent experiments (for αPD-1 mAb-only arms, these data comprise a minimum of 5 mice and two independent experiments). **C–F,** Cohorts of 5 mice per group were euthanized 7 days after the last dose of radiotherapy and TCR metrics determined. **C,** T-cell infiltration/expansion. **D,** The number of TCR molecules detected in post-therapy blood samples and the respective tumor sample divided by the total number of TCR molecules in the two samples. **E,** TCR clonality. **F,** Number of unique TCRs identified in both tumors stratified by therapy. Asterisks with bars indicate pair-wise comparisons between the tumors within a therapy group. **, $P < 0.01$; *, $P < 0.05$; paired *t* test. Stand-alone asterisks denote significance when compared with NT control. **, $P < 0.01$, or *, $P < 0.05$ level. NGS, next generation sequencing.

mice (Fig. 2E). Moreover, radiotherapy increased the number of unique TCRs (thereby increased the diversity of the TCR repertoire) in the irradiated but not out-of-field tumor when compared with NT controls (Fig. 2F). The largest increase in TCR diversity in both the irradiated and out-of-field tumors occurred in mice treated with radiotherapy/αPD-1 mAb therapy, demonstrating that this combination generated an immunologic response that extended beyond the radiotherapy-treatment field. However, the precise contribution of these individual T-cell clones to tumor control remains unknown.

Analysis of clone sharing and dynamics between the irradiated and out-of-field tumors (tumor #1 and #2, respectively) revealed that most TCR clones and all high abundance clones (>10 copies detected) were present in both tumors in the NT mice (Fig. 3A, first panel). Moreover, the frequencies of individual clones in both tumor repertoires showed a strong concordance (slope = 0.85, R^2 = 0.94). These observations indicate that similar TCR repertoires, derived from common progenitor clones, were established in both tumors prior to therapy. Similar results were observed in the mice treated with αPD-1 mAb as a monotherapy (slope = 0.90, R^2 = 0.95; Fig. 3A, second panel), which is consistent with the lack of T-cell infiltration/expansion observed in either tumor (Fig. 2C). In contrast, fractionated radiotherapy led to preferential clonal expansion in the irradiated tumor and infiltration of many unique clones [clones shown below the dotted line; slope = 0.12, R^2 = 0.84; Fig. 3A, third panel; median = 3,015, interquartile range (IQR): 2,759–3,124], which corresponded to 80% of unique clones but only 13.3% of T cells (IQR = 5.7%–16.3%). Interestingly, a large portion of the clones identified as having a significantly greater frequency in tumor 1 than in tumor 2 (median: 90%, IQR: 92.6%–89.1%) was also detected at low abundance in the out-of-field tumor. Treatment with radiotherapy and αPD-1 mAb restored some of the concordance of T-cell clones observed in the tumors of NT mice and led to greater convergence in expanded T-cell clones present in both the irradiated and out-of-field tumors (slope = 0.5, R^2 = 0.66; Fig. 3A, last panel and Fig. 2D). Moreover, the majority of high abundance clones in the irradiated tumor were also observed in the out-of-field tumor (median = 99.53%). These data demonstrate that there is a high degree of concordance in the TCR clonotypes infiltrating into both the irradiated and out-of-field tumors.

Although radiotherapy led to an increase in the TCR repertoire overlap between the tumors, the number of clones shared between the irradiated and out-of-field tumors only increased in response to combination therapy (Fig. 3B and C). Correspondingly, few T-cell clones had higher abundance in the out-of-field tumor than in the irradiated tumor except in mice that received combined therapy (Fig. 3D). These results are consistent with T-cell trafficking either from the irradiated tumor and/or the periphery to the out-of-field tumor. Analysis of the top 25 clones identified in the irradiated tumor and tracking the frequency of these clones in the out-of-field tumor and in peripheral blood reveal that expansion of these principal clones in the second tumor only happens following combination therapy with radiotherapy and αPD-1 mAb (Fig. 3E; Supplementary Fig. S3). Importantly, these data demonstrate that fractionated radiotherapy stimulates a polyclonal T-cell response that is restricted to the irradiated tumor site and ultimately fails to control tumor growth. When radiotherapy is delivered in combination with PD-1 blockade, this polyclonal response extends outside of the irradiated field with propagation to the out-of-field tumor and to generation of a systemic therapeutic antitumor response. Furthermore, dose scheduling was critical for antitumor activity, with concomitant but not sequential administration of αPD-1 mAb required to mediate tumor regressions in the nonirradiated tumor (Supplementary Fig. S4).

The effects of fractionated radiotherapy rarely extend beyond the treatment site

To provide further context to the TCR sequencing data, we also characterized the changes in the tumor microenvironment by flow cytometry (experimental schema outlined in Fig. 4A). Profiling of tumor-infiltrating effector T cells revealed that fractionated radiotherapy leads to an acute reduction in the number of $CD8^+$ and $CD4^+$ T cells (at day 1 and day 1 and 3, respectively) in the irradiated but not out-of-field tumors when compared with time-matched NT controls (Fig. 4B, left and middle). By day 7, there is a small expansion in $CD8^+$ (1.47-fold compared with time-matched controls) but not $CD4^+$ T cells in the tumor that received radiotherapy. In contrast, radiotherapy led to a significant increase in the number of $CD11b^+$ $Gr1^+$ cells infiltrating into the in-field, but not the out-of-field tumors when compared with time-matched NT controls (Fig. 4B, right). This change was also transient, and by day 7 postradiotherapy, no difference in $CD11b^+$ $Gr1^+$ cell numbers was observed between the in- and out-of-field tumors. Given that tumor cell expression of PD-L1 may represent a biomarker of a local effector T-cell response, we evaluated the impact of local radiotherapy on tumor cell PD-L1 expression both in and out of the radiotherapy field. We have previously shown that low-dose fractionated radiotherapy can lead to $CD8^+$ T-cell–dependent expression of PD-L1 at an irradiated tumor site (27). Here, we demonstrate that expression of PD-L1 is elevated on $CD45^-$ tumor cells only in the irradiated, but not out-of-field lesion (at all time points tested; Fig. 4C; Supplementary Fig. S5A) and on $CD11b^+Gr1^+$ cells (transiently at day 3; Fig. 4D; Supplementary Fig. S5B). We have previously demonstrated that this increase in PD-L1 following local fractionated radiotherapy is mediated by $CD8^+$ T-cell issued IFNγ (20). In this context, tumor cell expression of PD-L1 may be a biomarker of local effector T-cell responses, suggesting that these responses are restricted to the site of treatment.

Combined treatment with fractionated radiotherapy and αPD-1 mAb facilitates $CD8^+$ T-cell expansion in the irradiated and out-of-field tumors

As we previously observed with radiotherapy alone, combination therapy with radiotherapy and αPD-1 mAb (experimental schema outlined in Fig. 5A) also led to a significant reduction in the number of $CD8^+$ T-cells (but not $CD4^+$ T cells) infiltrating the tumor (when compared with out-of-field lesions; Fig. 5B). However, this reduction in $CD8^+$ T cells was acute, and by day 7, both the irradiated and out-of-field tumors had significantly greater numbers of $CD8^+$ T cells (2.15- and 1.96-fold, respectively) when compared with the time-matched NT controls. Moreover, the numbers of $CD11b^+$ $Gr1^+$ cells in the irradiated tumor were increased on day 1 after combined radiotherapy/αPD-1 mAb therapy in the irradiated tumor when compared with the out-of-field tumor (Fig. 5B). However, in contrast to our observations after radiotherapy alone, this increase was transient and followed by a significant reduction in $CD11b^+$ $Gr1^+$ cell numbers in the irradiated tumor, when compared with the out-of-field tumor at days 3 and 7. In contrast to treatment with radiotherapy alone,

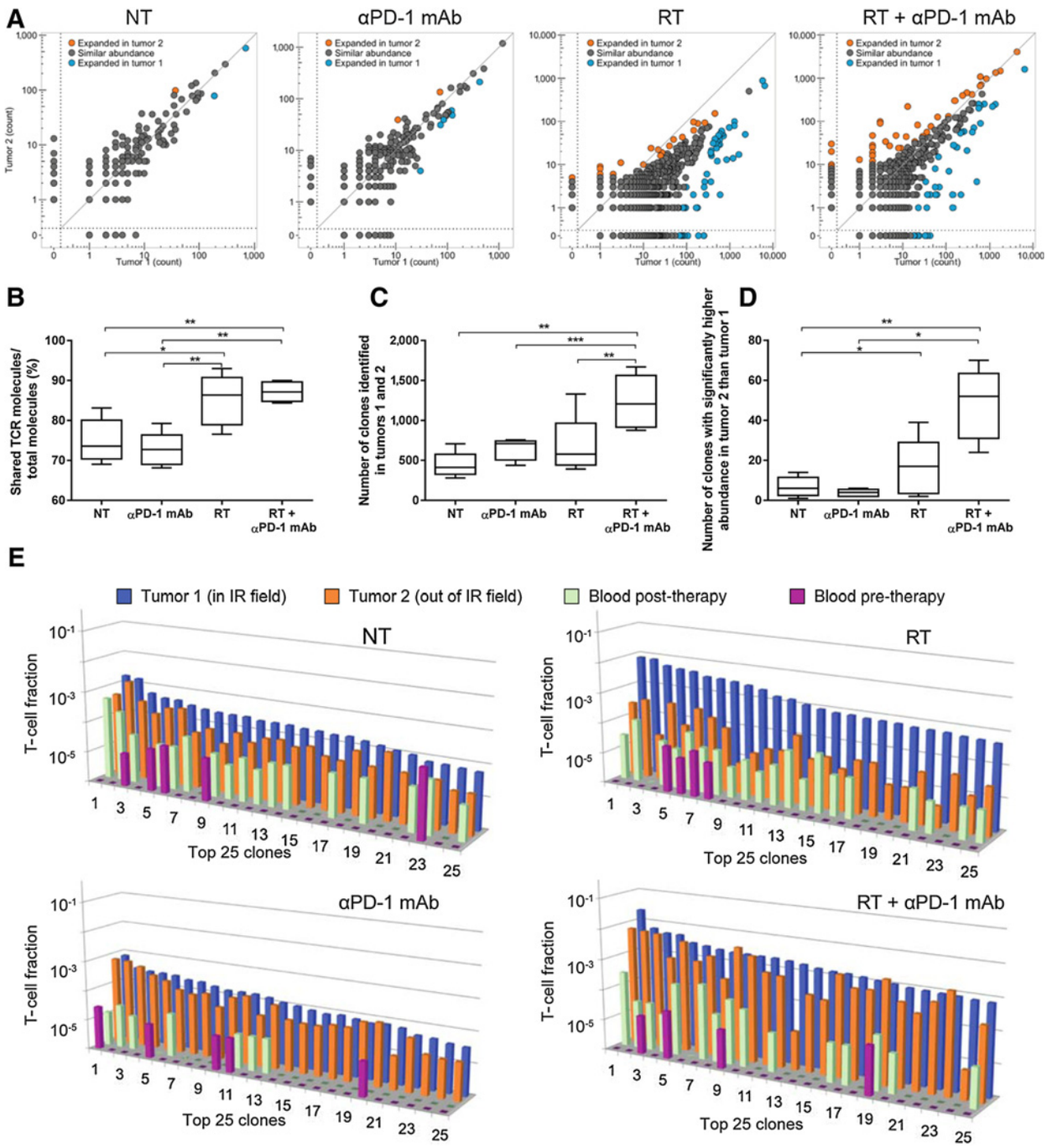

Figure 3.

Combination therapy leads to convergence of the TCR repertoires in both irradiated and out-of-field tumors. **A,** Scatterplots of clone abundance in tumor 2 versus tumor 1 following treatment. Points to the left of or below the dotted line were TCR clones detected in only tumor 2 or tumor 1, respectively. Quantitation of overlap metrics between the TCR repertoires of tumor 2 and tumor 1 are: the number of shared TCR molecules identified in sequencing divided by the total number of TCR molecules in the two samples (**B**); number of unique TCR clones that were found in both tumors (**C**); and the number of clones with significantly greater frequency in tumor 2 than in tumor 1 (**D**). **E,** The top 25 clones in tumor 1 were tracked in pre-therapy blood, post-therapy blood, and in post-therapy tumor 2 samples; the plots quantify the T-cell fraction (T cells per nucleated cell) for each clone in each sample. Each graph is representative of an individual animal. Asterisks with bars indicate pair-wise comparisons between the tumors within a therapy group: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; paired t test. Experimental groups contained 5 mice.

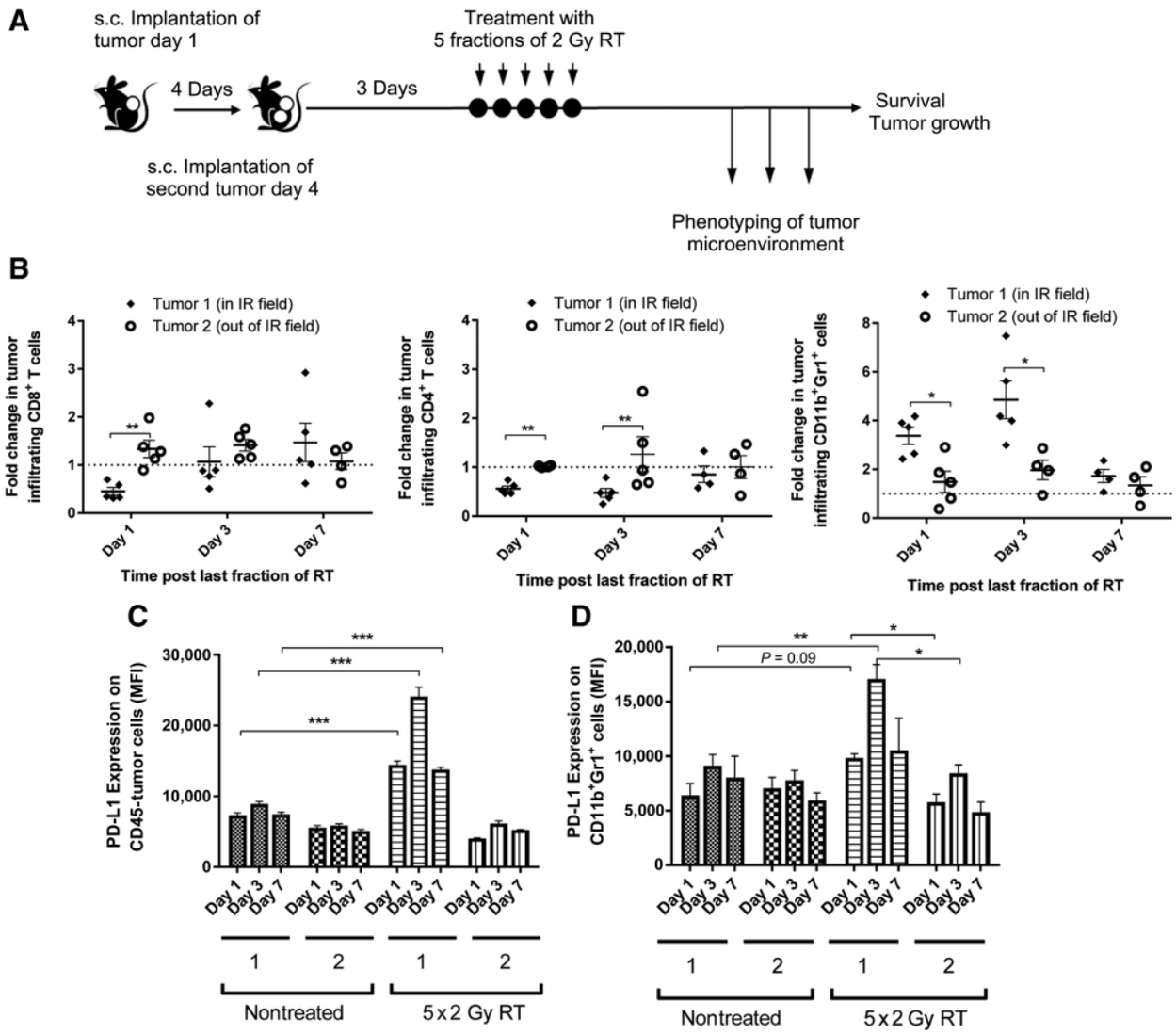

Figure 4.

Fractionated radiotherapy (RT) leads to transient depletion of T cells and expansion of $CD11b^+$ $Gr1^+$ cells at the site of treatment. **A,** Schema for studies. Fractionated-dose radiotherapy (as 10 Gy in five daily fractions of 2 Gy) was delivered to tumor 1 with tumor 2 shielded from the ionizing beam. **B,** Fold change in $CD8^+$ (left), $CD4^+$ (middle), and $CD11b^+Gr1^+$ (right) cells compared with nontreated time-matched controls in the irradiated (1) and out-of-field (2) tumors. **C** and **D,** Expression of PD-L1 on CT26 cells (**C**, gated as $CD45^-$ cells) and on $CD45^+CD11b^+Gr1^+$ (**D**) 24 hours, 72 hours, and 7 days posttreatment with five fractions of 2 Gy radiotherapy. Fold changes were calculated by determining the frequency of cells as a population of $CD45^+$ and then expressing these relative to their frequency in time/anatomic implant site-matched NT control mice. Dashed line, baseline for each tumor implanted in mice receiving NT. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; Mann-Whitney test. Experimental groups contained 4 to 5 mice, and the data shown are representative of at least two independent experiments.

combined therapy with radiotherapy and αPD-1 mAb leads to the upregulation of PD-L1 expression in both the irradiated and out-of-field tumors at all time points tested (Fig. 5C; Supplementary Fig. S5A). A similar pattern of PD-L1 expression was also observed on tumor-infiltrating $CD11b^+$ $Gr1^+$ cells (Fig. 5D; Supplementary Fig. S5B). Together, these data demonstrate that fractionated radiotherapy, when delivered in combination with αPD-1 mAb, leads to dynamic changes in tumor-infiltrating $CD8^+$ effector T-cell populations capable of mediating regression of both irradiated and out-of-field tumors.

Both tumor-resident and infiltrating T cells contribute to therapeutic activity following combination therapy

To determine the relative contribution of tumor-resident versus infiltrating T cells on the therapeutic response following combined radiotherapy/αPD-1 mAb therapy, we used FTY-720 (a sphingosine 1-phosphate receptor agonist), which prohibits T-cell emigration from lymphoid tissues (31). FTY-720 has been shown to have direct antitumor effects (32, 33). We initially confirmed that treatment with low doses of FTY-720 had no effect on tumor growth while retaining the capacity to reduce both

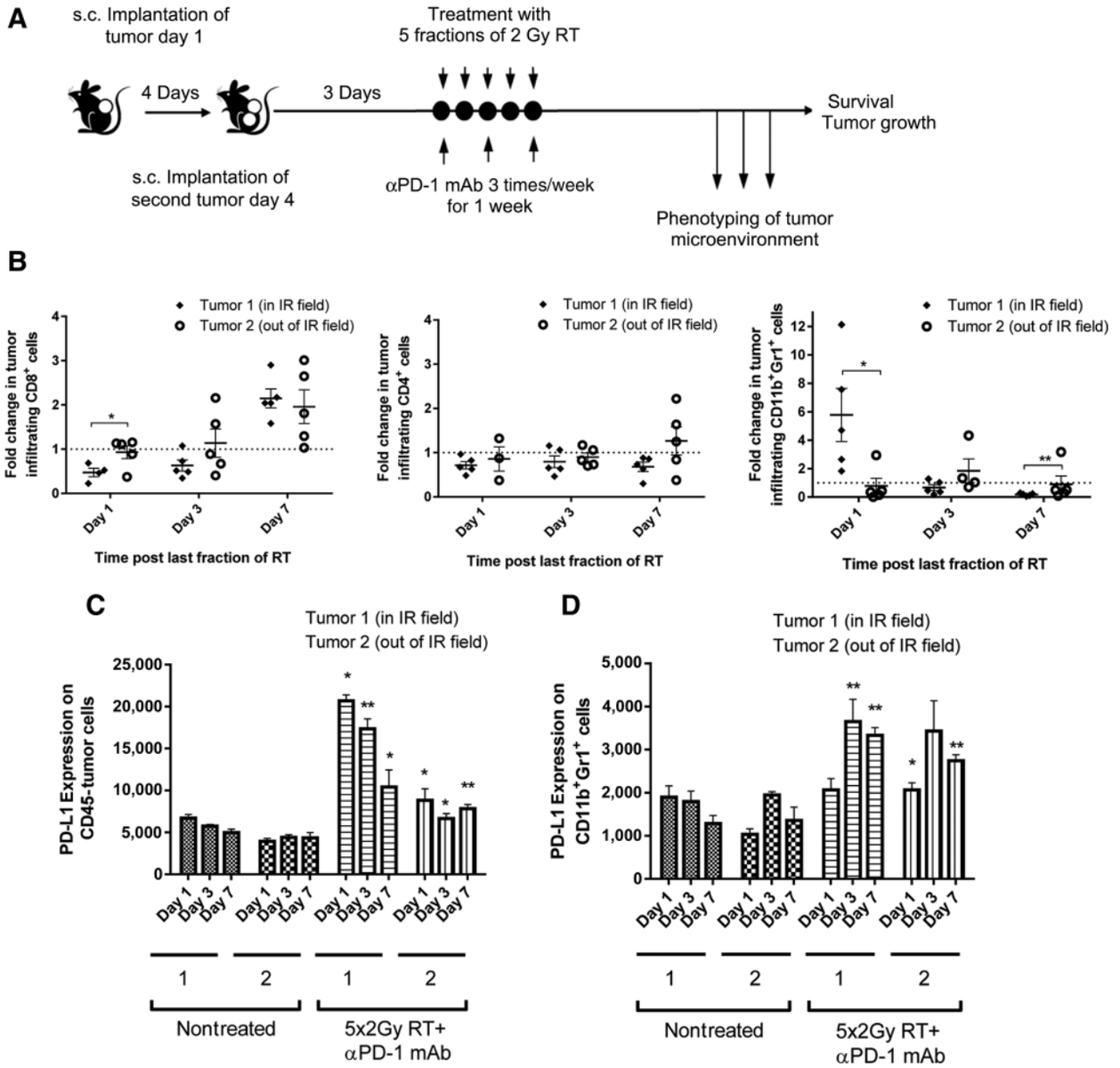

Figure 5.

Fractionated radiotherapy (RT) delivered concurrently with αPD-1 mAb leads to adaptive immunologic changes in both irradiated and out-of-field tumors and systemic tumor control. **A,** Schema for studies. Fractionated-dose radiotherapy (as 10 Gy in five daily fractions of 2 Gy) was delivered to tumor 1 with tumor 2 shielded from the ionizing beam in combination with αPD-1 mAb dosed at 10 mg/kg 3 times a week for 1 week. **B,** Fold change in $CD8^+$ (left), $CD4^+$ (middle), and $CD11b^+Gr1^+$ (right) cells compared with nontreated time-matched controls in the irradiated (1) and shielded (2) tumors. Fold changes were calculated by determining the frequency of cells as a population of $CD45^+$ and then expressing these relative to their frequency in time/anatomic implant site-matched NT control mice. Dashed line, baseline for each tumor implanted in mice receiving NT. **C** and **D,** Expression of PD-L1 on CT26 cells (**C**, gated as $CD45^-$ cells) and on $CD45^+CD11b^+Gr1^+$ (**D**) 24 hours, 72 hours, and 7 days posttreatment. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; Mann-Whitney test. Experimental groups contained 3 to 5 mice, and the data shown are representative of at least two independent experiments.

circulating and tumor-infiltrating T-cell populations (Supplementary Fig. S6A and S6B). We then determined the effect of blocking T-cell infiltration into the tumor either prior to implantation or prior to treatment (experimental schema outlined in Fig. 6A). Our data demonstrate that T cells resident in the tumor at the time of treatment are capable of mediating therapeutic responses following combined therapy with approximately 40% of mice that received combined radiotherapy/αPD-1 mAb therapy undergoing curative responses when FTY-720 treatment was initiated on the day prior to therapy (6 days after tumor implantation; Fig. 6B and C). However, infiltration of circulating T cells into the tumor post-therapy was required to achieve maximal responses (~80% of mice that did not receive FTY-720 achieved curative responses).

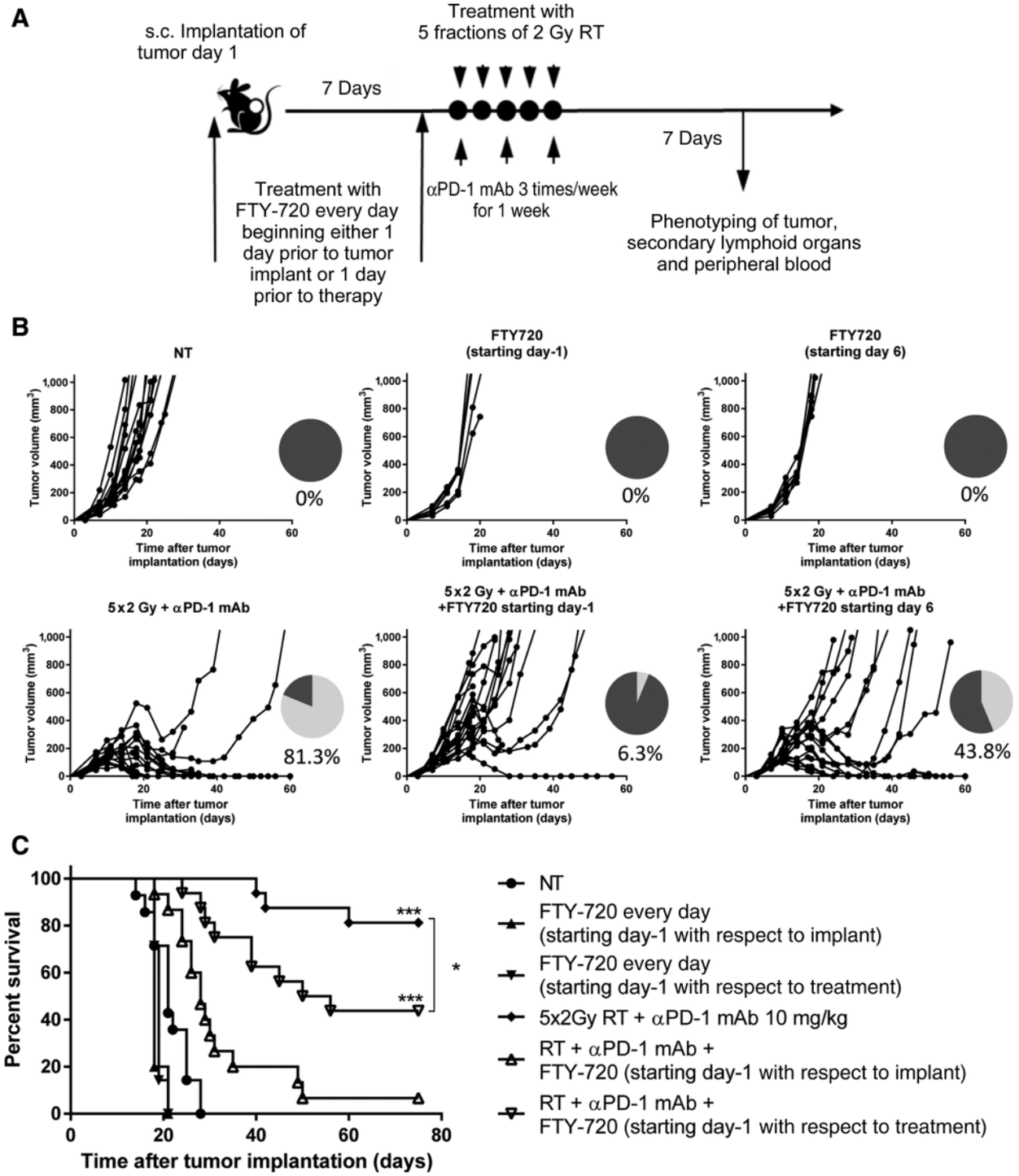

Figure 6.

Tumor-resident and infiltrating T cells contribute to the therapeutic response following fractionated radiotherapy (RT) and blockade and αPD-1 mAb therapy. **A,** Schema for studies. CT26 tumor-bearing mice received fractionated radiotherapy delivered in five daily fractions of 2 Gy in combination with αPD-1 mAb dosed at 10 mg/kg 3 times a week for 1 week. Cohorts of mice also received FTY-720 dosed at 25 μg/mouse for the first dose and 5 μg/mouse every day for up to 6 weeks. **B,** Individual tumor growth curves. Pie charts represent the proportion of mice that experienced complete tumor resolution. **C,** Kaplan–Meier curves of therapy. Experimental groups contained at least 7 mice, and the data shown are representative of at least two independent experiments. ***, $P < 0.001$; log-rank (Mantel–Cox) test; *, $P < 0.05$; Mann–Whitney test.

These data demonstrate that in a proportion of mice, tumor-resident T cells have the capacity to mediate local tumor control and clearance. However, the response mediated by tumor-resident T cells appears insufficient, and infiltrating T cells from outside of tumor are also required for successful clearance of tumor in the majority of mice following combination radiotherapy/αPD-1 mAb therapy.

Discussion

In this study, we demonstrate for the first time that low-dose daily fractionated radiotherapy leads to polyclonal T-cell infiltration and expansion at the site of treatment but that the generation of systemic antitumor immunity is suppressed through the PD-1/PD-L1 axis. Inhibition of this axis facilitated the generation of a systemic polyclonal T-cell response capable of mediating out-of-field (abscopal) effects following local radiotherapy. Our data demonstrate that both tumor-resident and infiltrating T cells contribute to tumor control following combined therapy. Furthermore, immunosequencing of the CDR3 regions of TCRβ revealed that all of the dominant T-cell clones were present in both the irradiated and out-of-field tumors.

We have previously demonstrated that fractionated radiotherapy leads to local T-cell activation and production of IFNγ, which drives adaptive resistance through the PD-1/PD-L1 axis (20). In this study, we provide additional insight into this T-cell response and demonstrate that low-dose daily fractionated radiotherapy modulates the TCR repertoire, leading to polyclonal expansion of preexisting tumor-infiltrating lymphocyte (TIL) populations within the irradiated tumor but not within the out-of-field non-irradiated tumor, coincident with the emergence of immunologic suppression. Here, we show that radiotherapy leads to increases in PD-L1 expression on both tumor cells and $CD11b^+$ $Gr1^+$ cells only within the irradiated tumor. Given that PD-L1 expression in the tumor microenvironment appears to be a biomarker for local $CD8^+$ T-cell activation, these data suggest that the immune response is restricted to the site of radiotherapy. Complete regression of both the irradiated and out-of-field tumors following treatment with fractionated radiotherapy alone was a rare event across independent experiments, and it remains unclear what local and systemic events underpin these responses. In contrast, concurrent treatment with radiotherapy and αPD-1 mAb overcomes this local immunosuppression, facilitating systemic antitumor immunity capable of mediating the regression of distal, nonirradiated lesions, which also exhibit increased expression of PD-L1 on tumor and $CD11b^+$ $Gr1^+$ cells. Sequential therapy where PD-1 blockade began 7 days after completion of the fractionated radiotherapy cycle was ineffective, suggesting that although fractionated radiotherapy can lead to changes in the TCR repertoire in the irradiated tumor, exhaustion and subsequent atrophy of tumor-reactive TILs may occur rapidly after radiotherapy unless signaling through the PD-1/PD-L1 axis is blocked. Although regression of irradiated tumor has previously been shown to be $CD8^+$ T-cell dependent (27), further mechanistic studies would be required to determine the relative contribution of the distinct T-cell clones at the irradiated and out-of-field tumor sites and confirm causality to response.

The frequency of T cells in the tumor is well documented to predict outcome (34); however, the relative contribution of tumor-resident versus infiltrating T cells on therapeutic response following combination radiotherapy and αPD-1 mAb therapy is unclear. The analysis of the TCR repertoires in the blood and tumor demonstrate that fractionated radiotherapy leads to T-cell infiltration in the irradiated tumor but not the distal nonirradiated tumor site. However, although both tumor-resident and infiltrating T cells were required for complete responses in approximately 80% of mice following combined therapy, a proportion of mice elicited complete tumor regressions mediated by resident T cells alone. This is despite the radiosensitivity of lymphocytes and subsequent observed reduction in TIL number within 24 hours after a fractionated radiotherapy cycle. Furthermore, by day 7 after combination therapy, both the irradiated and out-of-field tumors had significantly greater numbers of $CD8^+$ T cells after daily fractionated radiotherapy. Although it cannot be ruled out that T-cell numbers increased due to shrinkage of the tumor, these data suggest that those T cells that are activated following radiotherapy may be more radioresistant, potentially through modulation of antiapoptotic proteins, such as BCL-2, BCL-xl, and Bim (35). However, maximal therapeutic responses required both resident and infiltrating T cells. Preclinical studies demonstrate that naïve TILs can undergo activation and differentiation within the tumor microenvironment in the presence of sufficient antigen and costimulation provided by local APC (36, 37). Given the immunogenic nature of radiotherapy, further studies are required to delineate the relative contribution of *in situ* priming by tumor-resident APC versus classical priming in secondary lymphoid organs.

The generation of neoantigens secondary to DNA damage following ionizing radiation is hypothesized to contribute to tumor control through broadening of the TCR repertoire (4). Our data suggest that following fractionated radiotherapy, only a small fraction of T cells (<0.5%) is unique to the irradiated tumor, and therefore, we speculate may be specific for a neoantigen generated by radiotherapy. In addition, the high level of concordance in the high-abundance clones present in both the irradiated and out of field tumor suggests that the antigens may be shared across the two tumors. These data suggest that following low-dose fractionated therapy, the T-cell response may be dominated by clones responding to preexisting tumor antigens, and we speculate that the response against potential neoantigens generated as a consequence of radiotherapy-induced DNA damage may be minimal. Interestingly, the addition of αPD-1 mAb to radiotherapy does not significantly alter the clonality or diversity of the T-cell repertoire above that of radiotherapy alone. Presumably, this is because inhibition of signaling through PD-1 may principally operate to reinvigorate T cells. However, next-generation sequencing of TCRs does not enable identification of specific peptide antigens or define the lineage of the TCR-expressing cell, and further mechanistic studies would need to be undertaken to confirm the specificity of TCR clonotypes restricted to $CD4^+$ and $CD8^+$ T cells. Moreover, targeting coactivating immune checkpoints, such as CTLA-4, has been shown to broaden the peripheral TCR repertoire (38, 39). Therefore, combining radiotherapy and αCTLA-4 mAb may cooperate to further enhance repertoire diversity and potentially improve therapeutic outcome (22, 25).

Recent advances in the delivery of radiotherapy, such as stereotactic ablative radiotherapy, permit high single-fraction doses to be administered to patients with minimal collateral damage to normal tissue. Preclinical evidence across a range of syngeneic models demonstrates that radiotherapy dose fractionation can influence systemic immune response and subsequent tumor control (23, 27, 40, 41). A number of recent publications have

demonstrated the enhancement of antitumor immune responses when mAbs targeting coinhibitory/activation receptors, such as PD-1/PD-L1, CTLA-4, and CD137, are combined with high ablative single [e.g., 10/12 Gy (42), 12 Gy (19, 24), or 20 Gy (25)] or hypofractionated radiotherapy-regimens [e.g., 3 × 8 Gy (21, 23) or 5 × 6 Gy (22)]. Moreover, increased TCR diversity in irradiated tumors was also described in a preclinical model following treatment with a single dose of 20 Gy radiotherapy (25). Despite our data in the CT26 model demonstrating that varying radiotherapy dose fractionation has little effect on therapeutic response when combined with blockade of the PD-1/PD-L1 pathway, the extent to which these different radiotherapy dose and fractionation approaches may differently modulate neoantigen generation, TCR diversity, and therapeutic response in the clinical setting remains unclear and requires further investigation. The impact of radiotherapy delivered as low-dose fractionated regimens, higher dose hypofractionated, and single-dose ablative radiotherapy is not only likely to affect the amount, kinetics, and type of tumor cell death, but is also likely to be affected by the intrinsic radiosensitivity and microenvironment of the tumor. Higher radiotherapy doses are more likely to have very different effects on the tumor microenvironment and antitumor immune response compared with the lower 2 Gy per day fractionated radiotherapy, which was used in this study and routinely given to a majority of cancer patients. The effect of radiotherapy dose and fractionation has been shown in a recent study demonstrating that a single 30 Gy dose of radiotherapy stimulated curative $CD8^+$ T-cell–dependent responses but that this effect was abrogated when followed by 10 days of fractionated therapy (3 Gy/day; ref. 41). These data suggest that that repeated doses of radiotherapy to the tumor may be detrimental to the antitumor immune response, and this may have profound implications for the current practice of irradiating tumor draining lymph nodes, which needs further study.

A number of clinical trials are currently ongoing to further delineate optimum radiotherapy dose and fractionation schedules to improve immunologic response (43). Although radiotherapy alone has been shown to induce abscopal effects in a limited number of patients (44), reports of abscopal effects following concurrent radiotherapy and immunotherapy are currently limited to case reports, and results from clinical trials investigating radiotherapy plus immune checkpoint blockade are eagerly awaited. In conclusion, the combination of radiotherapy and immunotherapy holds great promise to improve cancer outcomes. Unlocking this potential requires further investigation, with well-designed clinical trials investigating the effect of radiotherapy dose fractionation in different tumors to guide the next phases of clinical development.

Disclosure of Potential Conflicts of Interest

S.J. Dovedi reports receiving commercial research grants from Medimmune. R. Stewart holds ownership interest (including patents) in AstraZeneca. H. Robins is an employee of and reports receiving commercial research grants from Adaptive Biotechnologies. T. Illidge reports receiving commercial research grants from Medimmune. No potential conflicts of interest were disclosed by the other authors.

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