

# Ablative Tumor Radiation Can Change the Tumor Immune Cell Microenvironment to Induce Durable Complete Remissions

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## Abstract

**Purpose:** The goals of the study were to elucidate the immune mechanisms that contribute to desirable complete remissions of murine colon tumors treated with single radiation dose of 30 Gy. This dose is at the upper end of the ablative range used clinically to treat advanced or metastatic colorectal, liver, and non-small cell lung tumors.

**Experimental Design:** Changes in the tumor immune microenvironment of single tumor nodules exposed to radiation were studied using 21-day (>1 cm in diameter) CT26 and MC38 colon tumors. These are well-characterized weakly immunogenic tumors.

**Results:** We found that the high-dose radiation transformed the immunosuppressive tumor microenvironment resulting in an intense CD8<sup>+</sup> T-cell tumor infiltrate, and a loss of myeloid-derived suppressor cells (MDSC). The change was dependent on

antigen cross-presenting CD8<sup>+</sup> dendritic cells, secretion of IFN $\gamma$ , and CD4<sup>+</sup>T cells expressing CD40L. Antitumor CD8<sup>+</sup> T cells entered tumors shortly after radiotherapy, reversed MDSC infiltration, and mediated durable remissions in an IFN $\gamma$ -dependent manner. Interestingly, extended fractionated radiation regimen did not result in robust CD8<sup>+</sup> T-cell infiltration.

**Conclusions:** For immunologically sensitive tumors, these results indicate that remissions induced by a short course of high-dose radiotherapy depend on the development of antitumor immunity that is reflected by the nature and kinetics of changes induced in the tumor cell microenvironment. These results suggest that systematic examination of the tumor immune microenvironment may help in optimizing the radiation regimen used to treat tumors by adding a robust immune response. *Clin Cancer Res*; 21(16); 3727–39. ©2015 AACR.

## Introduction

Because of recent advances in image guidance and radiation treatment delivery techniques, single ablative doses as high as 30 Gy can be safely delivered to many tumor sites by a procedure known as stereotactic radiosurgery (SRS), stereotactic body radiotherapy (SBRT), or stereotactic ablative body irradiation (SABR; refs. 1–5). High total doses of radiation achieved by a single treatment (extreme oligofractionation),

or by 2 to 5 high-dose treatments (oligofractionation or hypofractionation) have been used as an alternative to conventional daily low-dose fractionated treatments (<3 Gy) over several weeks. Limited clinical results show improved efficacy compared with fractionated radiotherapy in managing advanced or metastatic colorectal, liver, and non-small cell lung tumors. The outcome can be comparable with that of surgery for resectable tumors, and SRS can be applied to unresectable tumors (2, 3). Also, new radiation regimens are proposed that can deliver radiation in short pulses at ultrahigh dose rates while minimizing normal tissue injury (FLASH; ref. 4).

The goal of this study was to systematically examine the role of tumor immunity in a mouse model in which high-dose single-fraction tumor radiation induces complete durable remissions. We used the CT26 and MC38 colon tumors, because they are well characterized (6–8). Although these tumors express retroviral encoded antigens, they are weakly immunogenic, and vaccination with irradiated tumor cells fails to induce immune responses that protect against tumor growth after subsequent tumor challenge (9).

Large CT26 tumors as well as other advanced solid tumors can evade antitumor immunity partly by promoting the development of an immunosuppressive/tolerogenic microenvironment that includes regulatory cells such as myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), and regulatory CD4<sup>+</sup> T cells (Tregs; refs. 10–15). In addition, the

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

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**doi:** 10.1158/1078-0432.CCR-14-2824

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### Translational Relevance

The results of the study can provide information to optimize the efficacy of radiotherapy used alone or in combination with immunotherapy. These results suggest that for clinical trials of immune stimulation by radiotherapy alone or in combination with immunotherapy, the high-dose regimen should be rapid rather than extended for at least some tumors.

conventional T cells in the tumor infiltrate are dysfunctional due to the expression of negative costimulatory receptors, such as PD-1 and Tim-3, which can interact with ligands, such as PD-L1 and galectin-9, on tumor or stromal cells (13). A high percentage of suppressive myeloid cells and/or expression of negative costimulatory receptors and their ligands predict an unfavorable outcome for patients with a variety of cancers, including colorectal cancers, and a high percentage of infiltrating conventional CD8<sup>+</sup> T cells predicts a favorable outcome of cancers (16–19).

Radiotherapy can be curative not only by killing tumor cells and their associated stromal and vascular cells, but also by inducing T-cell immunity (12, 20–27). The antitumor T-cell immunity can induce remissions at distant sites from the radiated tissues ("abscopal" effect) alone or in combination with immunotherapy (27–31). Radiation-induced injury causes release of tumor antigens, activation of dendritic cells (DC), and stimulation of CD8<sup>+</sup> T-cell immunity by the production of innate immune stimuli, including the TLR-4 agonist, high-mobility group protein 1 (HMGB), as well as type I interferons, adenosine triphosphate (ATP), and calreticulin (32–38).

We found that the immunosuppressive microenvironment in the tumors was altered by a single 30-Gy dose of radiation that rapidly increased the infiltration of CD8<sup>+</sup> tumor-killing T cells. Infiltration of the latter was dependent on the CD8<sup>+</sup> subset of antigen cross-priming DCs, help via CD40L on CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T-cell production of IFN $\gamma$ . The CD8<sup>+</sup> T cells eliminated MDSCs in the stroma, and induced remissions.

## Materials and Methods

### Animals

Wild-type male BALB/c (H-2<sup>d</sup>) and C57BL/6 (H-2<sup>b</sup>) mice, BALB/c RAG2<sup>-/-</sup>, BALB/c Batf3<sup>-/-</sup> mice were purchased from The Jackson Laboratory. The Stanford University Committee on Animal Welfare (APLAC) approved all mouse protocols used in this study.

### Cell lines

The CT26 cell line was purchased from the ATCC. CT26–LUC/GFP cell line was transduced as described previously (39–41).

The MC38 cell line was provided by D. Bartlett (University of Pittsburgh, Pittsburgh, PA). All cell lines were authenticated according to the ATCC cell line authentication test recommendations that included a morphology check by microscope, growth curve analysis, and standard Mouse Pathogen PCR Panel 1 to rule out *Mycoplasma* infection (performed June 24, 2009).

### Irradiation

Irradiation was performed with a Phillips X-ray unit operated at 200 kV with the dose rate of 1.21 Gy/min (2.0-mm alumi-

num with added filtration of 0.5-mm copper, the distance from X-ray source to the target of 31 cm, and a half value layer of 1.3-mm copper) The unanesthetized mice were placed in individual lead boxes with a cutout that allowed the tumor to be irradiated tangentially with full shielding of the rest of the body. To ensure the maximum uniformity of the dose delivered, the animals were turned by 180° halfway through each irradiation (giving the equivalent of parallel opposed fields). This ensures that the dose inhomogeneity within the tumor from edge to center is less than 2%. The depth dose is defined by the half value layer, which is 7.5 cm. The dose to the skin was 100% of the tumor dose and we did not see significant skin reactions other than late scarring and contraction (42).

### Analysis of tumor-infiltrating cells

In order to analyze percentages among mononuclear cells, Collagenase D (Roche, 11088882001) was used to digest tumors for 25 minutes, and a single-cell suspension was layered over 3-mL Lympholyte M (CL5030, Cedarlane), a well-defined layer of mononuclear cells was collected. Cells were stained with fluorochrome conjugated monoclonal antibodies (mAb) and analyzed by flow cytometry, and correlated with histopathology as described previously (9, 43). In order to determine absolute number of immune cells per mg of tumor, tumor weight was recorded, tumors were digested as described above, and the absolute number of cells in the suspension was counted.

### DC preparation and T-cell depletion

DCs were isolated from the spleen using the Dendritic Cell Isolation Kit according to the manufacturer's instructions (130-091-169; refs. 42, 43). CD4<sup>+</sup> and CD8<sup>+</sup> T cells were depleted *in vivo* by injection of mAbs as described previously (44, 45).

### MDSC suppression assay

MDSCs from 21-day CT26 tumors were isolated using a modification of the MDSC isolation kit (Miltenyi Biotech), in which tumor cells after collagenase digestion were stained with biotinylated anti-Gr-1 mAb, and incubated with streptavidin microbeads. After column purification, cells were >90% Gr-1 hi CD11b<sup>+</sup> as judged by flow cytometry (see Supplementary Fig. S1F). MDSCs were added to cultures of purified splenic T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Cell Trace, Molecular probes). Proliferation was stimulated with anti-CD3/CD28 beads, and cells were analyzed by FACS (9, 46).

### Statistical analysis

The Kaplan–Meier survival curves were generated using Prism software (SAS Institute Inc.), and statistical differences were analyzed using the log-rank (Mantel–Cox) test. Survival was defined as the time point after tumor inoculation when the mice were euthanized according to veterinary guidelines because they were moribund and unable to reach food and/or water, or when the tumor reached a diameter of more than 2 cm or when the enlarging tumor ulcerated. In some cases, the mice were found dead in their cages. Statistical significance in differences between mean percentages of cells in spleens and tumors was analyzed using the two-tailed Student *t* test of means.

### Tumor cell labeling with luciferase

The GFP-firefly luciferase fusion (GLF) gene was subcloned from pJW.GFP-yLuc (kindly provided by Dr. C.G. Fathman, Stanford University) into pHR2 to generate pHR2-GLF. 293T cells were plated in 175 cm<sup>2</sup> flasks, and the next day, near-confluent cells were cotransfected with 45- $\mu$ g lentiviral vector together with packaging and VSV-G-expressing vectors (3:2:1 ratio) in the presence of 25  $\mu$ mol/L chloroquine (Sigma; refs. 39, 40, 41). CT26 cells were seeded in a 6-well plate at  $0.25 \times 10^6$  cells per well and incubated overnight in a 37°C incubator. Titrated virus was then used to transduce the cell lines in the presence of protamine sulfate (10  $\mu$ g/mL) to enhance transduction efficiency. Stable lentiviral transductants were then sorted four times for GFP fluorescence (100% purity) using a FACS DIVA cell sorter. Sorted cells were expanded and screened for bioluminescence using a Xenogen IVIS spectrum (Caliper Life Sciences), as well as GFP. Cell lines were maintained in RPMI-1640 complete medium supplemented with 10% fetal calf serum, L-glutamine, 2 mercaptoethanol, streptomycin, and penicillin.

### Histopathology

Animals were euthanized when moribund as per Stanford Animal Welfare protocol guidelines, or at 100 days after transplantation if they survived without morbidity. Histopathologic specimens were obtained from lungs and livers of hosts. Tissues were fixed in 10% formalin, stained with hematoxylin and eosin, and images were obtained using an Eclipse E1000M microscope (Nikon).

## Results

### The microenvironment of CT26 colon tumors is highly immunosuppressive

We established CT26 tumors to model advanced disease, and  $2.5 \times 10^4$  tumor cells were injected subcutaneously (s.c.) into the hind quarter of BALB/c mice, and allowed to grow for 21 days when the tumor diameter was about 1 to 1.5 cm. Tumors were excised and the mononuclear cells were purified before immunofluorescent staining for T-cell markers as well as the CD11b and Gr-1 markers of MDSCs and TAMs (47). Figure 1A compares representative profiles from the 21-day tumors, from spleen cells obtained at the same time, from the tumor-bearing mice, and spleen cells from untreated normal mice. The latter cells were used to show the balance of immune cells in normal lymphoid tissues, and normal receptor expression. Whereas the percentage of CD4<sup>+</sup> T cells in the tumor-bearing and normal spleen was about twice as high as CD8<sup>+</sup> T cells, in the tumor CD8<sup>+</sup> T cells were at least twice as high as CD4<sup>+</sup> T cells (Fig. 1A and B). Among the gated CD8<sup>+</sup> cells in tumors, about 74% expressed the PD-1<sup>+</sup>Tim-3<sup>+</sup> phenotype that has been described for "exhausted" cells in mice with other tumors or with chronic viral infections (13, 48). In contrast, among CD8<sup>+</sup> T cells in the normal and tumor-bearing spleens, about 5% expressed the PD-1<sup>+</sup>Tim-3<sup>+</sup> phenotype. Among the CD4<sup>+</sup> cells in tumors, about 33% were CD25<sup>+</sup>, and among the latter, about 60% were FoxP3<sup>+</sup> Treg cells (data not shown). In addition, the majority of these CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells expressed the negative costimulatory receptor, PD-1. Foxp3<sup>+</sup> Treg cells were about twice as high among CD4<sup>+</sup> T cells in tumors as compared with the spleens (Fig. 1A and B). Interestingly, the mononuclear cells in tumors contained about 26%

CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells (MDSC phenotype), and 19% that were CD11b<sup>+</sup>Gr-1<sup>lo</sup> (TAM phenotype). Tumor-bearing and normal spleens contained less than 5% of each cell type (Fig. 1A and B). MDSC and TAM phenotype cells in tumors expressed high levels of PD-L1 (Supplementary Fig. S2).

In order to confirm that the CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells in the tumors were capable of immune suppression, these cells were purified from the 21-day tumors and assayed for the ability to suppress the proliferation of T cells isolated from the normal spleen and stimulated *in vitro* with anti-CD3 and anti-CD28 mAb-coated beads. Figure 1C shows representative staining patterns of CFSE-labeled T cells that were stimulated in the presence or absence of an equal number of CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells. Whereas about 68% of T cells expressed low levels of CFSE staining in the absence of the CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells, only 1.9% expressed low levels after the addition of the latter cells. Alteration of the ratios of the suppressive cells to the T cells showed that significant suppression was observed with 1:1 and 1:5 ratios, but not with ratios of 1:10 or above (Fig. 1D). Suppression was no longer significant when the CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells were separated from T cells in Transwell (Fig. 1C) or when a combination of inhibitors of arginase-1 and inducible nitric oxide synthase (iNOS) were added to the standard wells at 1.5- $\mu$ mol/L concentrations (Fig. 1C and E). Thus, the suppressor cells required both cell contact and the production of NO and arginase-1 for optimum suppression. On the basis of the above experiments, we used the term "MDSCs" to describe CD11b<sup>+</sup>Gr-1<sup>hi</sup> phenotype cells, in subsequent experiments reported herein, and did not repeat the suppressor assays except when noted.

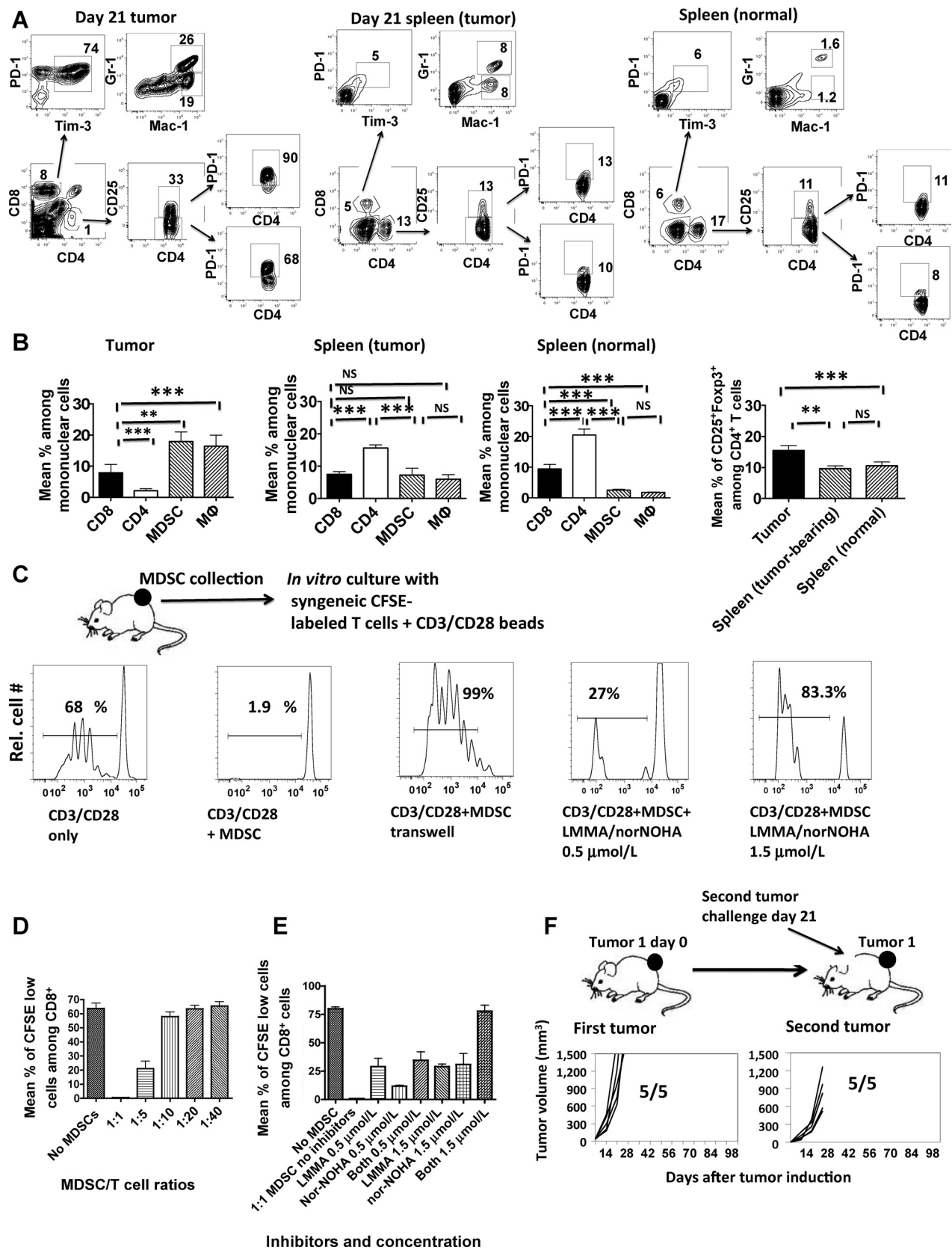
We found no difference in the growth patterns of the primary tumor as compared with the growth of the same number of tumor cells injected s.c. into the opposite flank on day 21 (Fig. 1F). In both cases, there was a marked increase in volume between days 14 and 28, and all injected sites grew large tumor nodules. Thus, growth of the first tumor does not induce "concomitant" immunity to prevent distant tumor growth at day 21.

### Single high-dose radiation of CT26 tumors induces durable complete remissions mediated by T-cell immunity that can be adoptively transferred

In further studies, tumors were given a single dose of 15-Gy local tumor irradiation (LTI) at day 21 using lead jigs developed for targeting only the 1.0- to 1.5-cm diameter tumor nodule (42). A complete remission was observed in tumors of 1 of 14 mice and 13 of 14 did not survive beyond 100 days despite slowing of tumor growth (Fig. 2A). Untreated tumor-bearing mice did not survive beyond 40 days. When the dose was increased to 20 Gy, then 3 of 5 mice developed complete tumor remissions. When the dose was increased to 30 Gy, 13 of 15 mice achieved complete remissions, and the latter mice survived for at least 100 days (Fig. 2A). Further observations showed no recurrence of tumors up to 180 days (data not shown).

The cured wild-type mice observed for 100 to 150 days were challenged with an s.c. injection of  $5.0 \times 10^5$  CT26 tumor cells, and 9 of 12 tumors resolved after a brief increase in volume (Fig. 2B). Three out of 12 tumors grew progressively, and mice with the latter tumors died within 100 days (Fig. 2B). In a previous study (9), we showed that a single s.c. vaccination with  $1 \times 10^6$  CT26 tumor cells that were irradiated *in vitro* with 50 Gy and mixed with the adjuvant, CpG, was able to protect

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about 50% of BALB/c mice from subsequent challenge with  $2.5 \times 10^4$  tumor cells. However, when the vaccinated mice were challenged with  $5.0 \times 10^5$  tumor cells, most tumors grew progressively, and about 90% of challenged hosts did not survive (Fig. 2B).

In order to determine whether T cells from mice with complete remissions of tumors for at least 100 days after LTI treatment can adoptively transfer the ability to effectively treat CT26 tumors, we used the scheme outlined in the diagram in Fig. 2C. T cells were purified from the spleens of the cured mice using anti-Thy1.2 columns, and combined with T cell-depleted bone marrow cells from the donors. The marrow and T cells were injected i.v. into irradiated adoptive recipients that had been given an s.c. injection of CT26 tumor cells, and then a single dose of 8 Gy TBI 7 days later. The tumor-bearing recipients all developed complete remissions and survived for at least 100 days (Fig. 2C). When the experiment was repeated using T cells from the spleen of untreated normal mice combined with T cell-depleted marrow cells, the adoptive transfer did not induce remissions in tumor growth, and all recipients died by day 40 (Fig. 2C). The survival of the latter recipients was similar to that of recipients given tumors without subsequent radiation and transplantation.

When mice with 21-day tumors were given 30 Gy LTI at day 21 along with a contralateral tumor challenge on the same day, all second tumors grew progressively (Fig. 2D). In contrast, if challenge was delayed until 30 days after LTI, then only one of five second tumors grew progressively. This indicated that there was no "abscopal" effect on second tumor growing simultaneously because systemic tumor immunity did not develop immediately after LTI, but become manifest after a few weeks.

In order to determine the extent of tumor killing by 30 Gy LTI in the absence of T or B cells, we used luciferase-labeled CT26 cells to establish s.c. tumors in RAG2<sup>-/-</sup> BALB/c mice. Supplementary Fig. S3 shows that 30 Gy slowed labeled tumor growth, but all tumors relapsed, and no tumor-bearing mice survived beyond day 70.

#### High single dose radiation increases CD8<sup>+</sup> T cells and reduces MDSCs in the stroma of tumors with induced remissions

The tumor-infiltrating mononuclear cells in CT26 tumors given a single dose of 30 Gy on day 21 were compared with mononuclear cells in unirradiated tumors 14 days later as shown in the representative flow cytometry patterns in Fig. 3A. Whereas the unirradiated tumor mononuclear cells contained about 26%

MDSCs and 20% TAMs on day 35, the irradiated tumors contained about 6% and 1%, respectively. In contrast, the unirradiated tumor contained about 19% CD8<sup>+</sup> T cells, and the irradiated tumor contained about 70%. Thus, the ratio of MDSCs to CD8<sup>+</sup> T cells changed from about 1:1 in the unirradiated tumor to about 1:10 in the irradiated tumor. Although, the ratios were markedly changed, the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that expressed high levels of Tim-3 and/or PD-1 did not. The change in the composition of tumor-infiltrating cells in untreated and irradiated mice is clearly seen in the immunofluorescent staining of tumor tissue sections for CD11b<sup>+</sup> myeloid cells (red) and CD3<sup>+</sup> T cells (green) on day 35 (Fig. 3B). Whereas there is a dense infiltration of myeloid cells with rare T cells in the untreated tumor, there is a dense infiltrate of T cells with rare myeloid cells in the treated tumor.

Figure 3C shows the kinetics of changes in the mean percentages of MDSCs, TAMs, CD11c<sup>+</sup> cells (APCs), CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells during the 14-day interval after 30-Gy tumor radiation. Interestingly, there was a transient significant increase in the percentage of MDSCs that peaked at about 50% 3 days after LTI (day 24), and then decreased to below 5% at day 35. MDSCs that infiltrated the tumor 3 days after LTI retained their suppressive function *in vitro* (Supplementary Fig. S1A). The decrease in the MDSC percentage after day 24 was associated with the significant increase in the percentage of CD8<sup>+</sup> T cells that began at day 27 and continued until the peak value of about 70% at day 35. A similar pattern was observed after 15-Gy LTI, but the changes were not as robust as with 30 Gy (Supplementary Fig. S1B). The marked reduction of MDSCs and increase in CD8<sup>+</sup> T cells in tumor infiltrates during the 14 days after 30-Gy LTI was also observed when the mean absolute number of the latter cells per mg of tumor were analyzed (Fig. 3D). It is of interest that the mean absolute number of live cells per mg of tumor peaked at day 6 after LTI, and that the mean tumor weight did not significantly decrease until 14 days after LTI (Supplementary Fig. S1C and S1D).

#### Increased tumor infiltration by CD8<sup>+</sup> T cells and reduced infiltration by MDSCs after high-dose LTI are dependent on cross-presenting CD8<sup>+</sup> DCs and IFN $\gamma$

Depletion of either CD8<sup>+</sup> or CD4<sup>+</sup> T cells by repeated injections of anti-CD8 or anti-CD4 mAb during the 14-day interval after high-dose LTI significantly reduced survival ( $P < 0.001$ ) as compared with nondepleted mice given LTI, and all tumor-bearing hosts failed to survive beyond day 73 (Fig. 4A). The marked

#### Figure 1.

Immunosuppressive tumor microenvironment in CT 26 tumors established for 21 days. A, mononuclear cells from day 21 s.c. CT26 tumors, spleens from day 21 tumor-bearing mice, and spleens from normal mice were analyzed for expression of CD25, PD-1, and Tim-3 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and for MDSC phenotype cells (CD11b<sup>+</sup> Gr1<sup>hi</sup>) and TAM phenotype cells (CD11b<sup>+</sup>Gr1<sup>lo</sup>). Percentages of each subset in boxes on representative two-color analysis panels are shown, and arrows identify gated subsets. Staining for CD11b, Gr-1, CD4, and CD8 used a live mononuclear cell gate. B, CD8<sup>+</sup>, CD4<sup>+</sup>, CD11b Gr-1<sup>hi</sup> (MDSCs;  $n = 6$ ), and CD11b Gr-1<sup>lo</sup> (M $\Phi$ ;  $n = 6$ ) cells are shown as a mean percentage  $\pm$  SE among mononuclear cells in tumor and spleens at day 21 after tumor implantation, and mean percentage of CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> cells shown among total CD4<sup>+</sup> T cells ( $n = 6$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; NS,  $P > 0.05$ . C, representative staining of cultures in which tumor-derived MDSCs were incubated with syngeneic splenic T cells loaded with CFSE and with CD3/CD28 beads *in vitro* for 5 days. MDSC:T-cell ratio was 1:1. HTS Transwell-96-well plates with 0.4- $\mu$ m membranes were used for Transwell studies. iNOS (L-NMMA) and L-arginase (nor-NOHA) inhibitors were used at two different concentrations (0.5 and 1.5  $\mu$ mol/L). Percentage of gated CD8<sup>+</sup> T cells that diluted CFSE is shown. D, CFSE dilution by CD8<sup>+</sup> T cells is shown as a mean percentage  $\pm$  SE of triplicate wells. CFSE-labeled T cells were cultured with tumor-derived MDSCs in the presence of CD3/CD28 beads. MDSC:T-cell ratios were 1:1, 1:5, 1:10, 1:20, and 1:40 ( $n = 7$ ). E, CFSE dilution by CD8<sup>+</sup> T cells is shown as a mean percentage  $\pm$  SE of triplicate wells. CFSE-labeled T cells were cultured with tumor-derived MDSCs in the presence of CD3/CD28 beads at the 1:1 ratio, and L-NMMA and nor-NOHA inhibitors were added in concentrations 0.5 or 1.5  $\mu$ mol/L ( $n = 7$ ). F, primary CT26 tumors were established at day 0. Tumor-bearing animals were challenged with  $5 \times 10^6$  CT26 cells on the opposite flank at day 21. Growth curves of the second tumor and fraction of mice with progressive second tumor growth are shown ( $n = 5$ ).

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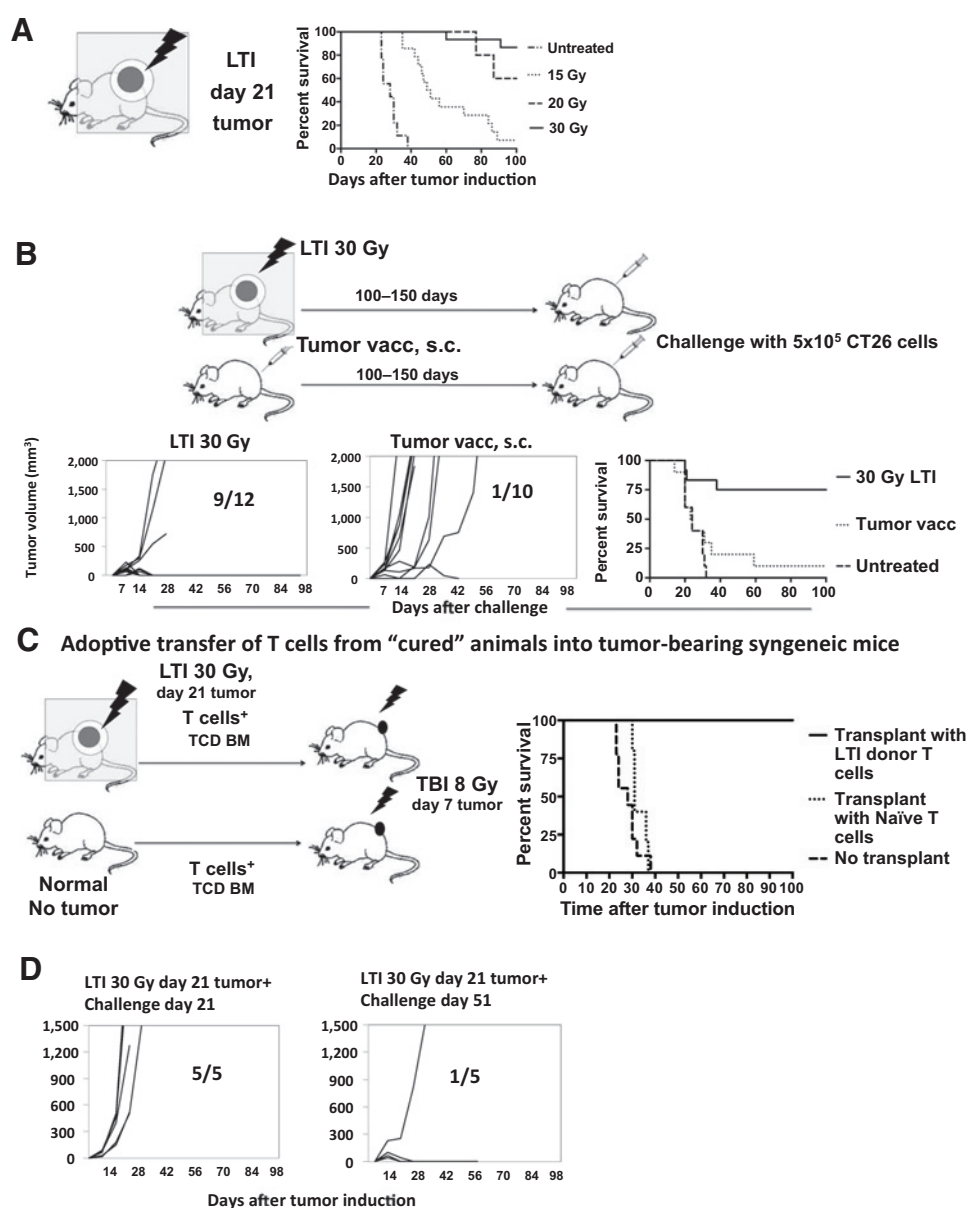


Figure 2.

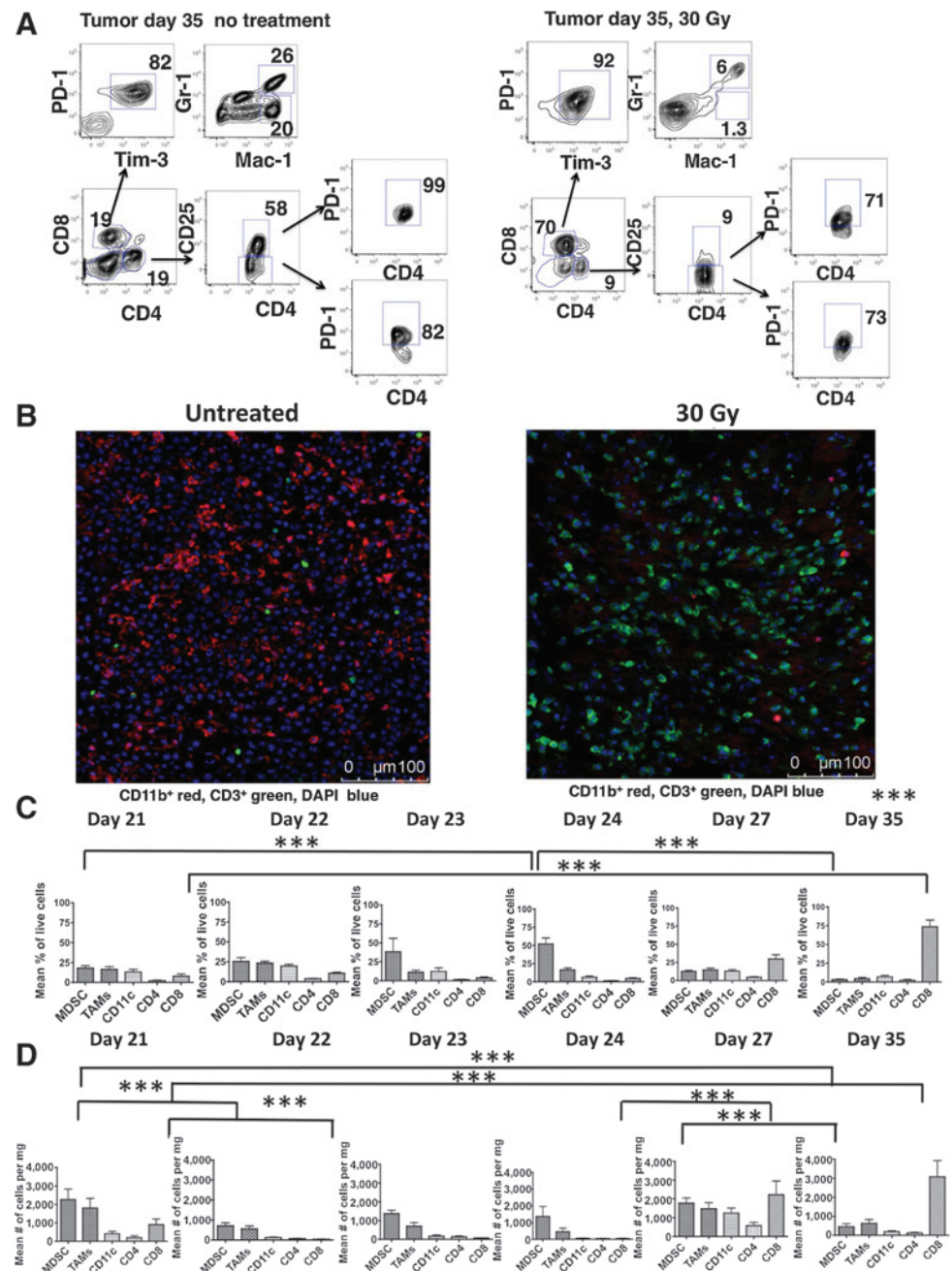
Treatment of advanced CT26 tumors by single high-dose radiation leads to complete remission, and development of systemic long-term immunity that can be adoptively transferred by T cells. A, experimental scheme. CT26 colon tumors were established for 21 days s.c. and mice received a single dose of LTI. Survival after single doses of irradiation 15 ( $n = 8$ ), 20 ( $n = 5$ ), and 30 Gy ( $n = 15$ ), or without radiation ( $n = 9$ ) is shown. There were significant differences in survival in groups with untreated tumors versus tumors treated with 15 Gy ( $P < 0.01$ ) or in groups treated with 30 Gy versus 15 Gy ( $P < 0.05$ ) by the Mantel-Cox test. B, experimental scheme. Mice with complete remissions of 21-day tumors after 30 Gy of LTI ( $n = 12$ ) were selected for this study. As controls, a group of normal mice was vaccinated s.c. with  $1 \times 10^6$  irradiated tumor cells (50 Gy *in vitro*) and 30  $\mu$ g CpG ( $n = 10$ ). Vaccinated ( $n = 10$ ) or irradiated ( $n = 12$ ) mice were challenged with  $5 \times 10^6$  of CT26 cells s.c. 100 to 150 days after treatment. Tumor growth curves, fraction of protected mice and survival are shown. There were significant differences in survival of vaccinated or untreated versus irradiated mice ( $P < 0.05$ ). C, experimental scheme. T cells ( $6 \times 10^6$ ) and T cell-depleted (TCD) bone marrow cells ( $1 \times 10^7$ ) were harvested from mice that were in remissions after 30 Gy for at least 100 days, and transferred into syngeneic tumor-bearing mice (7-day tumors) conditioned with 8 Gy of total body irradiation (TBI;  $n = 5$ ). T cells and TCD bone marrow transfer from untreated mice served as controls ( $n = 5$ ). Survival for 100 days is shown. There was a significant difference in survival between groups without the transplant procedure ( $n = 9$ ) versus with transplants from LTI donors ( $P < 0.001$ ;  $n = 5$ ), but not with transplants from naïve mice ( $P > 0.1$ ;  $n = 5$ ). D, primary CT26 tumors were established at day 0. Thirty-Gy LTI to primary tumor was given at day 21, and mice were challenged with  $5 \times 10^6$  of CT26 cells on the opposite flank at days 21 ( $n = 5$ ) or 51 ( $n = 5$ ) after primary tumor implantation. Growth curves are for second tumors on the contralateral flank. There was a significant difference in the fraction without tumor growth in groups with LTI challenged at day 21 versus 51 ( $P < 0.05$  by  $\chi^2$  test).

reduction of MDSCs observed 14 days after LTI (Fig. 3C) was dependent on T cells, because CD8<sup>+</sup> or CD4<sup>+</sup> depletion resulted in a substantial increase in the mean percentage of MDSCs at

day 35 as compared with irradiated nondepleted hosts ( $P < 0.01$ ; Fig. 4C). The increase in the percentage of MDSCs in T cell-depleted mice was not associated with a significant increase

**Figure 3.**

Change of balance of tumor-infiltrating cells after 30-Gy LTI. A, mice with 21-day tumors received a single dose of LTI (30 Gy). Tumor-infiltrating mononuclear cells were analyzed 14 days after LTI completion. Control tumor-bearing mice received no LTI and cells were analyzed at day 35. Representative stainings of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, MDSCs, and TAMs are shown as well as the expression of PD-1 and Tim-3. B, immunohistochemistry of tumors at day 35 that were untreated or received 30-Gy LTI at day 21. Tumor tissue sections were stained with anti-CD3 and anti-CD11b antibodies using a two-stage procedure. CD11b<sup>+</sup>-red, CD3<sup>+</sup> is green and DAPI staining is blue. C, kinetics of tumor-infiltrating cells after LTI was analyzed at days 1, 2, 3, 6, and 14 after LTI that are days 22, 23, 24, 27, and 35 after tumor induction, respectively. Cell subsets (CD11b Gr-1<sup>hi</sup>, CD11b Gr-1<sup>lo</sup>, CD11c, CD4<sup>+</sup>, and CD8<sup>+</sup>; *n* = 8) are shown as a mean percentage  $\pm$  SE among live mononuclear cells in tumor. D, kinetics of tumor-infiltrating cells after LTI was analyzed at days 1, 2, 3, 6, and 14 after LTI that are days 22, 23, 24, 27, and 35 after tumor induction, respectively. Cell subsets (CD11b Gr-1<sup>hi</sup>, CD11b Gr-1<sup>lo</sup>, CD11c, CD4<sup>+</sup>, and CD8<sup>+</sup>) are shown as a mean absolute number  $\pm$  SE per mg of tumor (*n* = 8).

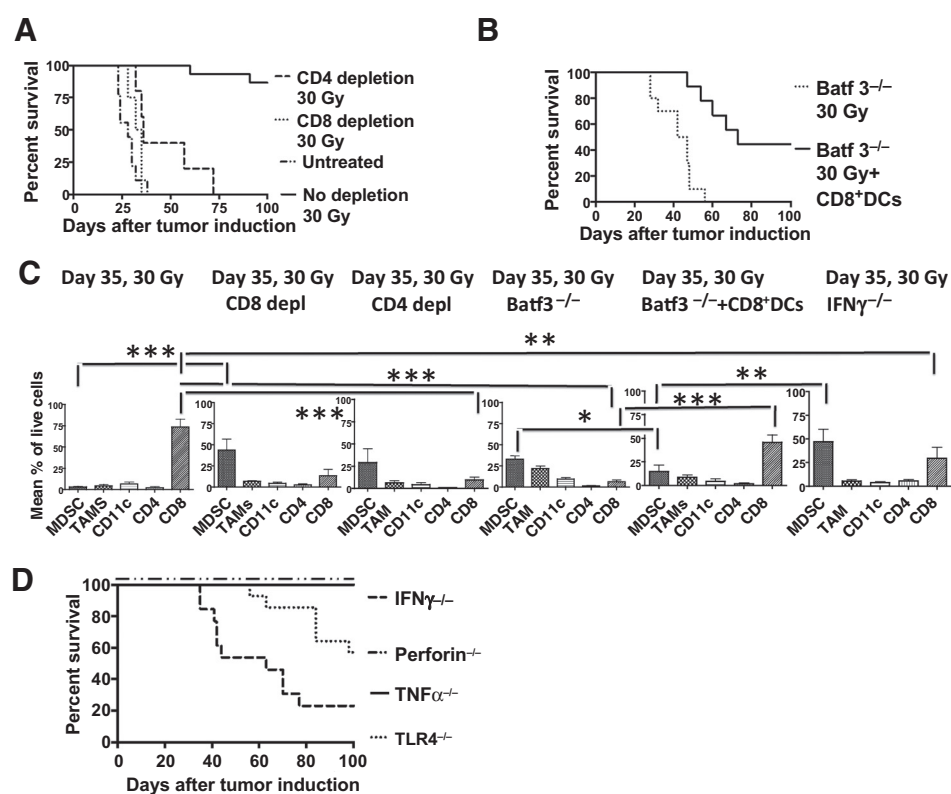


( $P > 0.05$ ) in the percentage of TAMs (Fig. 4C). Interestingly, the significant increase in the percentage of tumor CD8<sup>+</sup> T cells 14 days after LTI was not observed with CD4<sup>+</sup> T-cell depletion (Fig. 4C), and indicates that CD4<sup>+</sup> T-cell help was required.

We compared the survival of tumor-bearing mice and CD8<sup>+</sup> T-cell infiltration of tumors in wild-type BALB/c mice given 30 Gy to tumor-bearing *Batf3*<sup>-/-</sup> BALB/c mice given 30 Gy. The latter mice have an isolated deficiency of CD8<sup>+</sup> antigen cross-priming DCs (49–51). The latter cells play a critical role in the development of antiviral and antitumor immunity by stimulating CD8<sup>+</sup> T cells with tumor or viral antigens (23, 49–52). As shown in Fig. 4B, none of the *Batf3*<sup>-/-</sup> tumor-bearing mice

given 30 Gy survived more than 60 days, and survival was not significantly different from unirradiated mice ( $P > 0.05$ ). These mice failed to show the marked increase in the percentage of CD8<sup>+</sup> T cells among tumor mononuclear cells at day 35, and the mean percentage of CD8<sup>+</sup> T cells was below 10% ( $P < 0.001$  as compared with wild-type mice; Fig. 4C). Thus, the tumor infiltration of CD8<sup>+</sup> T cells and remissions after 30 Gy were dependent on the presence of CD8<sup>+</sup> cross-priming DCs. The percentage of the cells was significantly increased in the tumors 14 days after LTI (Supplementary Fig. S1E). These data are consistent with the findings that efficacy of radiotherapy depends on cross-presenting DCs (23).

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**Figure 4.**

Curative effect of radiation requires CD4 and CD8 T cells, as well as cross-presenting DCs, and is mediated by IFN $\gamma$ . A, survival of tumor-bearing animals after 30-Gy LTI depends on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Wild-type animals with 21-day CT26 tumors received 30-Gy LTI ( $n = 14$ ) and anti-CD8 ( $n = 8$ ) or CD4 ( $n = 5$ )-depleting antibodies. B, curative effect of LTI depends on CD8<sup>+</sup> DCs. Survival after single dose of 30 Gy LTI at day 21 in Batf3<sup>-/-</sup> mice with ( $n = 9$ ) and without ( $n = 10$ ) add back of purified CD8<sup>+</sup> CD11c<sup>+</sup> spleen cells is shown. The latter cells ( $5 \times 10^6$ ) were injected intratumorally within 1 day after irradiation. C, cell subsets (CD11b<sup>+</sup>Gr<sup>-hi</sup>, CD11b<sup>+</sup>Gr<sup>-lo</sup>, CD11c, CD4<sup>+</sup>, and CD8<sup>+</sup>) are shown as a mean percentage  $\pm$  SE among mononuclear cells in tumor at day 14 after LTI in wild-type mice with or without CD4<sup>+</sup> ( $n = 5$ ) or CD8<sup>+</sup> T cells ( $n = 5$ ) depletion, in Batf3<sup>-/-</sup> mice with ( $n = 5$ ) and without ( $n = 5$ ) addback of CD8<sup>+</sup> CD11c<sup>+</sup> cells, and IFN $\gamma$ <sup>-/-</sup> mice ( $n = 5$ ). D, IFN $\gamma$  is necessary for curative effect of LTI. IFN $\gamma$ <sup>-/-</sup> ( $n = 13$ ), perforin<sup>-/-</sup> ( $n = 5$ ) and TNF $\alpha$ <sup>-/-</sup> ( $n = 5$ ), and TLR4<sup>-/-</sup> ( $n = 14$ ) tumor-bearing mice were used. Survival after single dose of LTI at day 21 is shown.

In further experiments, CD8<sup>+</sup>CD11c<sup>+</sup> DCs were purified from normal spleens, and added back to the Batf3<sup>-/-</sup> tumor-bearing mice given LTI. Figure 4B also shows that the addback of the DCs significantly increased the survival of the Batf3<sup>-/-</sup> mice ( $P < 0.01$ ). The increased survival was reflected in a significant increase in the percentage of CD8<sup>+</sup> T cells among mononuclear cells ( $P < 0.001$ ) in tumors after addback (Fig. 4C). Although, the TLR-4 receptor on APCs has been reported to play an important role in the induction of tumor immunity after radiation or chemotherapy (38), the survival of TLR4<sup>-/-</sup> tumor-bearing mice was about 60% at 100 days after 30 Gy (Fig. 4D). The survival of the latter mice did not differ significantly from that of wild-type mice ( $P > 0.05$ ), and suggests that expression of TLR4 is not required to achieve durable remissions.

Because CD8<sup>+</sup> T cells that infiltrate tumors can reduce tumor cell growth and increase host survival by the production of effector molecules, such as IFN $\gamma$ , TNF $\alpha$ , and perforin (52–54), we determined the impact of the 30-Gy treatment on the survival of IFN $\gamma$ <sup>-/-</sup>, TNF $\alpha$ <sup>-/-</sup>, and perforin<sup>-/-</sup> mice deficient in each of these molecules. Figure 4D shows that all irradiated TNF $\alpha$ <sup>-/-</sup> and perforin<sup>-/-</sup> tumor-bearing mice survived at least 100 days with durable remissions. However, the survival of IFN $\gamma$ <sup>-/-</sup> mice was significantly reduced ( $P < 0.01$ ) as compared with the latter mice, and only 20% survived beyond 80 days (Fig. 4D). Consistent with our earlier findings in mice with tumors that were not irradiated, the poor survival of the irradiated IFN $\gamma$ <sup>-/-</sup> mice was associated with a significantly increased percentage of MDSCs in tumors at day 35 as compared with wild-type mice (Fig. 4C;  $P < 0.01$ ), and a significantly reduced percentage of CD8<sup>+</sup> T cells ( $P < 0.05$ ).

#### Daily fractionated radiation does not result in robust CD8<sup>+</sup> T-cell infiltration

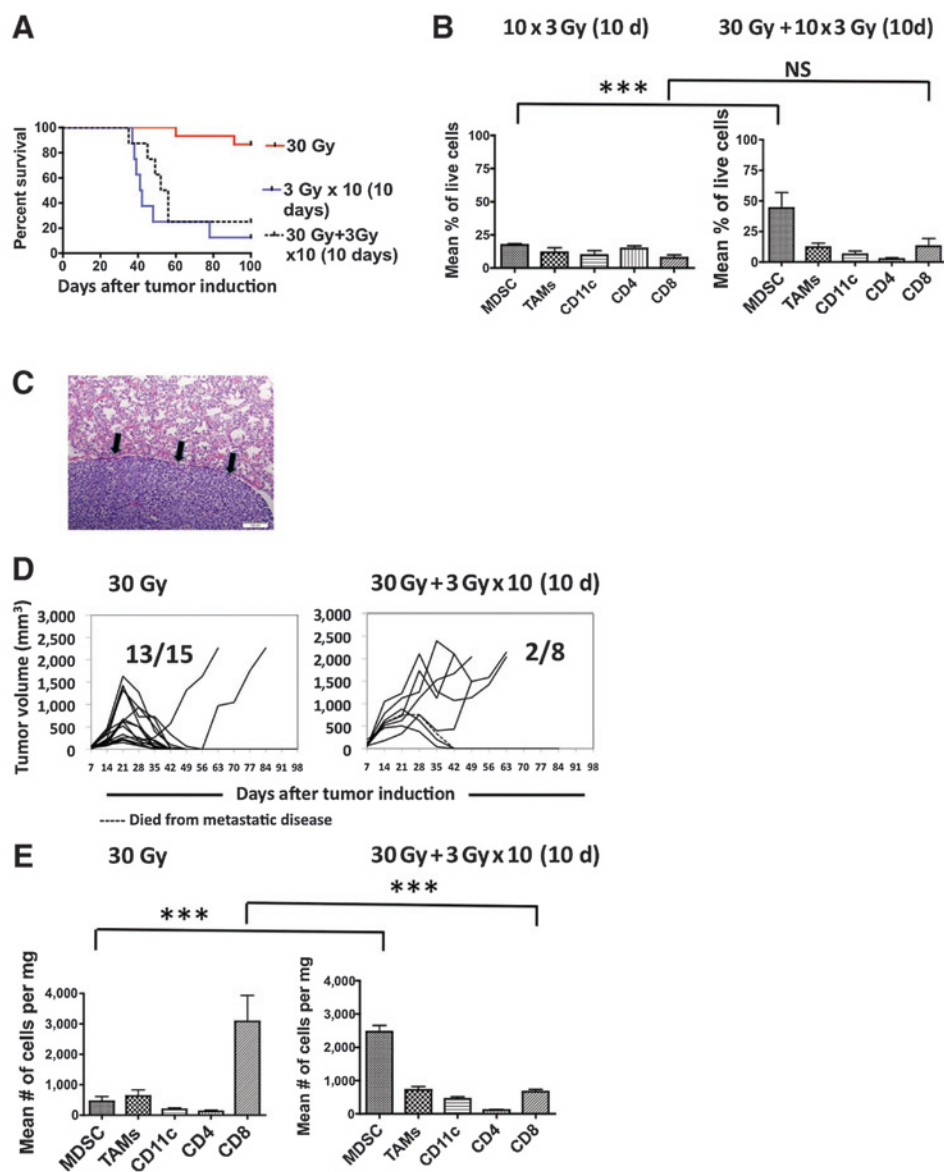
The single dose of 30 Gy administered to tumors is a model for the clinical use of SRS and SBRT. Although tumor control after the single 30-Gy dose administered to 21-day CT26 was about 90%, 10 daily radiation doses of 3 Gy each radiation alone resulted in the control of only about 10% by day 100 (Fig. 5A). Addition of the 10 daily doses of 3 Gy each to the single dose of 30 Gy significantly reduced survival ( $P < 0.01$ ) such that only 30% of mice had tumor control by day 100 (Fig. 5A). The poor survival of the mice given 10 doses of 3 Gy each or the combination of 30 Gy plus 10 doses of 3 Gy was associated with a marked reduction in the mean percentage of CD8<sup>+</sup> T cells in the tumor infiltrate at day 35 from about 70% with the single dose alone (Fig. 3C) to about 4% to 8% with fractionated radiation alone or in combination with the single high-dose ( $P < 0.01$ ; Fig. 5B).

In addition, the mean percentage of MDSCs in tumors after the combination of single and daily doses was increased to about 45% (Fig. 5B) as compared with about 5% with the single dose alone (Fig. 3C;  $P < 0.01$ ), and about 20% with 10 doses of 3 Gy each (Fig. 5B). Interestingly, autopsy of 6 of the 8 mice with the combination that were moribund showed the development of metastatic tumor nodules in the lungs in all 6 (Fig. 5C, arrows), whereas none of the autopsies of 8 unirradiated tumor-bearing mice showed lung tumors. The difference in survival of mice in the 30 Gy versus 30 Gy + 10  $\times$  3 Gy groups is reflected in the tumor growth curves shown in Fig. 5D. The marked differences in the MDSC and CD8<sup>+</sup> T cells infiltration between these groups after treatment is shown by comparison of the mean absolute number of cells



**Figure 5.**

Addition of conventional daily fractionated to single high-dose radiation results in decreased survival of tumor-bearing mice and metastatic spread of the tumor. A, Balb/c mice with 21 d CT 26 tumors received a single dose 30 Gy LTI ( $n = 15$ ), fractionated LTI 3 Gy daily for 10 days ( $n = 8$ ), and combined regimen single dose 30 Gy LTI on day 21 and fractionated 3 Gy  $\times$  10 started on day 24 ( $n = 8$ ). Survival of tumor-bearing mice is shown. There were significant differences in survival in groups with LTI 30 Gy versus fractionated or combined regimens ( $P < 0.01$ ) by the Mantel-Cox test. B, cell subsets ( $CD8^+$ ,  $CD4^+$ ,  $CD11b Gr-1^{hi}$ , and  $CD11b Gr-1^{lo}$ ) are shown as a mean percentage  $\pm$  SE among live mononuclear cells in tumor at day 35 after tumor implantation (day 14 after LTI started;  $n = 5$ ). C, H&E staining of the lungs (day 14 after LTI) of a tumor-bearing animal that received combined LTI regimen (30 Gy + 3G  $\times$  10). D, tumor growth curves and fraction of mice that survived at least 100 days are shown after single dose of 30 Gy or combined regimen of 30 Gy + 3 Gy  $\times$  10. E, cell subsets ( $CD8^+$ ,  $CD4^+$ ,  $CD11b Gr-1^{hi}$ , and  $CD11b Gr-1^{lo}$ ) are shown (after a single dose of 30 Gy ( $n = 5$ ) or combined regimen of 30 Gy + 3 Gy  $\times$  10;  $n = 5$ ) as a mean number  $\pm$  SE per mg of tumor at day 35 after tumor implantation (day 14 after LTI started).



per mg of tumor (Fig. 5E). Although SBRT regimen is not combined with daily fractionated radiation in clinical regimens, this experiment demonstrates that  $CD8^+$  T-cell infiltration and antitumor immunity can be reduced by extended radiation.

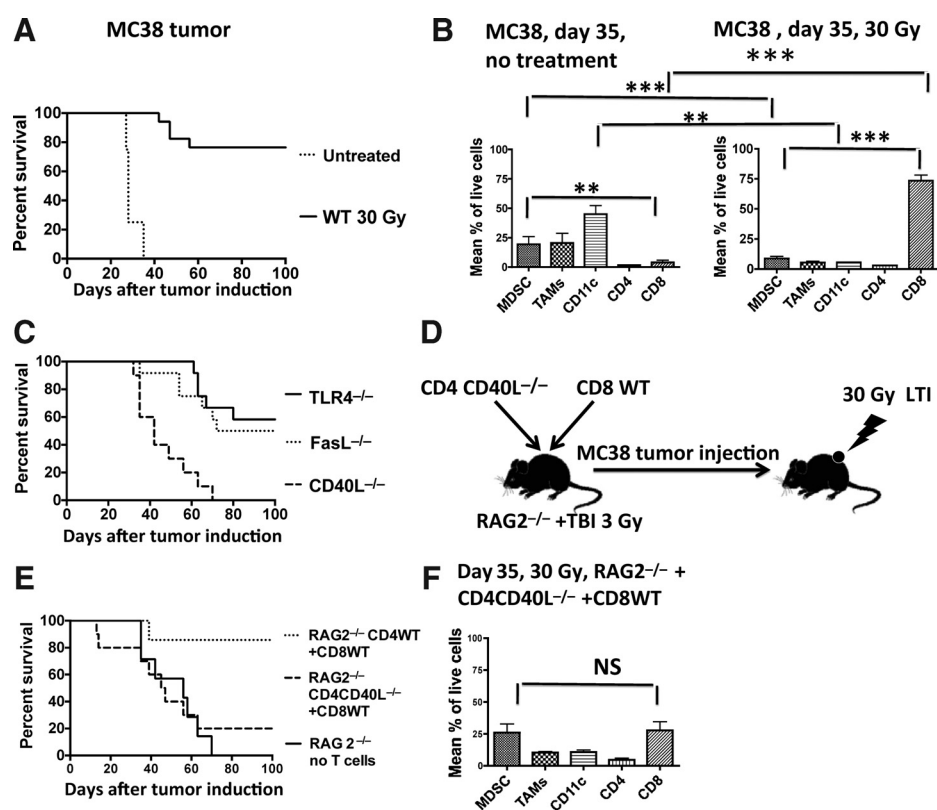
#### MC38 colon tumors respond to accelerated LTI in a manner similar to CT26 tumors

All of the experiments described above examined CT26 tumors growing in BALB/c mice. In further experiments, we extended our studies to another colon tumor, MC38, that is derived from C57BL/6 mice. The MC38 tumor cells were injected s.c. in the hind quarter of the latter mice, and nodules grew progressively as described above for the CT26 tumor. None of the untreated wild-type mice survived more than 35 days, but about 80% of those treated with a single dose of 30 Gy on day 21 survived at least 100 days (Fig. 6A).

The tumor-infiltrating cells in the MC38 tumors showed a pattern similar to that of the CT26 tumors at day 35 in

unirradiated wild-type mice, because the mean percentage of MDSCs (about 20%) was greater than that of the  $CD8^+$  T cells (about 5%;  $P < 0.01$ ; Fig. 6B). There was a significant increase ( $P < 0.001$ ) in the mean percentage of  $CD8^+$  T cells to about 65% at day 35 in the irradiated mice. In contrast to the CT26 studies, the percentage of MDSCs in irradiated mice showed no significant decrease as compared with unirradiated controls ( $P > 0.05$ ). Interestingly, the  $CD11c^+$  cells were the predominant mononuclear subset in the unirradiated MC38 tumors, whereas the latter cells were a minor subset in the CT26 tumors. There was a significant reduction in the mean percentage of the  $CD11c^+$  cells after radiation (Fig. 6B). When  $TLR4^{-/-}$  or  $FasL^{-/-}$  C57BL/6 mice were used, instead of wild-type mice, about 60% of irradiated tumor-bearing hosts survived for at least 100 days, and this was not significantly different from the survival of wild-type mice ( $P > 0.05$ ; Fig. 6C). In contrast,  $CD40L^{-/-}$  irradiated tumor-bearing hosts all died by day 70 (Fig. 6C).

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**Figure 6.**

Studies with the MC38 colon tumor. Curative effect of single high-dose radiation requires CD40L expression on CD4<sup>+</sup> T cells. A, MC38 colon tumors were established for 21 days s.c. in C57/B6 mice or C57/B6 RAG2<sup>-/-</sup> mice, and the mice received a single dose of LTI 30 Gy. Survival after single doses of irradiation 30 Gy ( $n = 17$ ), or without radiation is shown ( $n = 8$ ). There were significant differences in survival in WT mice treated with 30 Gy versus RAG2<sup>-/-</sup>-treated group ( $P < 0.05$ ), or WT untreated group ( $P < 0.01$ ) by the Mantel-Cox test. B, cell subsets (CD8<sup>+</sup>, CD4<sup>+</sup>, CD11b<sup>+</sup> Gr-1<sup>hi</sup>, and CD11b<sup>+</sup> Gr-1<sup>lo</sup>) are shown as a mean percentage  $\pm$  SE among mononuclear cells in tumor at day 35 after tumor implantation in untreated WT ( $n = 5$ ) versus mice treated with LTI 30 Gy ( $n = 5$ ) at day 21. C, MC38 tumors were established for 21 day. Survival of TLR4<sup>-/-</sup> ( $n = 7$ ), FasL<sup>-/-</sup> ( $n = 12$ ), and CD40L<sup>-/-</sup> ( $n = 10$ ) tumor-bearing mice that received 30-Gy LTI at day mice is shown. Survival of unirradiated wild-type and gene inactivated mice given MC38 tumors was not significantly different (data not shown). D, scheme:  $6 \times 10^6$  of CD4<sup>+</sup> T cells from CD40L<sup>-/-</sup> or WT mice and  $2 \times 10^6$  of CD8<sup>+</sup> from wild-type mice were transferred into RAG2<sup>-/-</sup> mouse within 6 hours after they received 3-Gy total body irradiation. Six weeks after TBI, MC38 tumors were established s.c. MC38 tumors received 30-Gy LTI at day 21. E, survival of MC38 tumor-bearing RAG2<sup>-/-</sup> mice or mice reconstituted with CD40L<sup>-/-</sup> CD4<sup>+</sup> ( $n = 10$ ) or CD4 WT T cells ( $n = 7$ ) and CD8<sup>+</sup> WT T cells. All mice received 30-Gy LTI at day 21. F, cell subsets (CD8<sup>+</sup>, CD4<sup>+</sup>, CD11b<sup>+</sup> Gr-1<sup>hi</sup>, CD11c<sup>+</sup>, and CD11b<sup>+</sup> Gr-1<sup>lo</sup>) are shown as a mean percentage  $\pm$  SE live among mononuclear cells in tumors in RAG2<sup>-/-</sup> mice reconstituted with CD40L<sup>-/-</sup> CD4<sup>+</sup> T cells and CD8<sup>+</sup> WT T cells 2 weeks after 30-Gy LTI ( $n = 5$ ).

When tumor-bearing hosts were immunodeficient C57BL/6 RAG-2<sup>-/-</sup> mice, the efficacy of the radiation treatment was markedly reduced ( $P < 0.01$ ), and none of the tumor-bearing mice survived more than 70 days (Fig. 6E). Injection of RAG2<sup>-/-</sup> mice with CD4<sup>+</sup> and CD8<sup>+</sup> T cells from wild-type mice 6 weeks before tumor inoculation restored survival of irradiated hosts to more than 80% by 100 days (Fig. 6D and E).

Injection of CD4<sup>+</sup> T cells from CD40L<sup>-/-</sup> donor mice in combination with CD8<sup>+</sup> T cells from wild-type mice was less effective in prolonging survival ( $P < 0.01$ ), and only 20% of irradiated hosts survived for 100 days (Fig. 6D and E). Analysis of day 35 tumor infiltrates in the latter hosts showed equal mean percentage (about 25%) of MDSCs and CD8<sup>+</sup> T cells, instead of the marked imbalance favoring CD8<sup>+</sup> T cells in wild-type hosts (Fig. 6F). The results suggest that CD4<sup>+</sup> T-cell help for MC38 tumor immunity is dependent on CD40L expression on CD4<sup>+</sup> T cells.

## Discussion

The goals of this study were to elucidate the cellular and molecular basis by which high-dose single-fraction tumor radiation changes this microenvironment in the murine CT26 and MC38 colon tumors. The results of the study can provide information to optimize the efficacy of radiotherapy used alone or in combination with immunotherapy. SRS with single doses of at least 30 Gy has been suggested to be more effective than daily fractionated radiation in early clinical trials (2, 3). In addition, SBRT used in combination with immunotherapy involving the negative costimulatory agonist, ipilimumab, resulted in complete remissions in some patients with melanoma (31). It was not clear whether daily fractionated radiation that is usually administered over weeks or months can synergize with immunotherapy in clinical trials. Preclinical studies have demonstrated synergy between the immunostimulation of a hypofractionated radiation regimen given over

a short duration (5 days) and immunotherapy to treat 4T1 tumors (28). However, extended periods of tumor immunotherapy may kill tumor-infiltrating immune cells, and the preclinical model used herein was designed to study this potentially negative effect of radiotherapy.

The single high-dose radiation protocol was able to induce T-cell immune-mediated durable remissions in the CT26 tumor. The oligofractionation radiation regimen dramatically altered the immunosuppressive microenvironment in the tumors, and within 14 days the percentage and absolute number per mg of tumor of MDSCs was markedly reduced in association with a dramatic increase in the percentage of CD8<sup>+</sup> T cells. This was confirmed by immunofluorescent staining of tumor tissue sections. The CD8<sup>+</sup> T-cell infiltration began about 6 days after single high-dose radiation. A similar pattern of infiltration was observed with a single dose of 15 Gy, but the changes were not as robust (Supplementary Fig. S2B). The result suggests that effective immunity may be achieved with two to three daily fractions of 15 Gy, an SBRT dose in common clinical use.

Mice that developed durable remissions after radiation treatment were resistant to a second challenge with CT26 tumors due to development of systemic immunity that became potent about 1 month after treatment. Second tumors injected at the time of treatment continued to grow while systemic tumor immunity was developing, and the evasive microenvironment of second tumors likely prevented antitumor T-cell infiltration. Second tumor evasion may be overcome by enhancing the rapidity and potency of tumor immunity by combining SBRT with immunotherapy. On the other hand, the lack of late relapse of primary tumors after radiotherapy induced complete remissions of the CT26 tumors may be due to the slowly developing tumor immunity. The splenic T cells obtained after development of systemic immunity were able to transfer anti-CT26 tumor immunity to adoptive hosts, but T cells from untreated mice could not.

Tumor-bearing RAG-2<sup>-/-</sup> mice lacking T and B cells and wild-type mice depleted of CD4<sup>+</sup> or CD8<sup>+</sup> T cells by mAb treatment did not develop remissions after radiation. The percentage of MDSCs in the stroma of the tumors in the latter mice remained high 14 days after radiation in association with the progressive tumor growth. Addback of T cells to RAG2<sup>-/-</sup> mice resulted in the reduction of the tumor MDSCs. Despite the tumor infiltration of CD8<sup>+</sup> T cells, but not CD4<sup>+</sup> T cells, after radiation, durable remissions were dependent on CD4<sup>+</sup> T cells and their expression of CD40L.

Both the development of remissions and the reduction of MDSCs were dependent on IFN $\gamma$ , because durable remissions and loss of tumor MDSCs were significantly reduced in IFN $\gamma$ <sup>-/-</sup> as compared with wild-type hosts. Previous studies have shown that local tumor radiation of B16 OVA melanoma cells with a single dose of 15 Gy increases intratumoral inflammatory responses by IFN $\gamma$ -dependent upregulation of VCAM-1 on the vasculature, increased expression of chemokines, MIG and IP10, and upregulation of MHC class I on tumor cells (55). In this study, we found that the source of IFN $\gamma$  was tumor-infiltrating CD8<sup>+</sup> T cells based on adoptive transfer studies using RAG2<sup>-/-</sup> mice that were treated with cyclophosphamide in addition to local tumor radiation (data not shown). The results suggest that CD8<sup>+</sup> T-cell production of IFN $\gamma$  controls the survival and infiltration of MDSCs in the tumor, and reverses the immunosuppressive environment. Furthermore, antitumor immune CD8<sup>+</sup> T cells can kill MDSCs via their pro-

duction of TNF $\alpha$ , IFN $\gamma$ , or expression of FasL, and thereby reduce MDSC tumor infiltration (52–54).

The release of HMGB from dying tumor cells can stimulate immunity to tumors by activating DCs via the TLR-4 receptor (38). However, TLR-4 was dispensable in the model of radiation-induced remissions described here, because tumor remissions were obtained in the majority of TLR-4<sup>-/-</sup> hosts. Tumor remissions were also dependent on the CD8<sup>+</sup> subset of DCs that have been reported to stimulate CD8<sup>+</sup> T-cell immunity to both tumor and viral antigens via cross-priming (49–51). It is not clear which CT26 tumor antigens are targeted by the radiation-induced CD8<sup>+</sup> T-cell immunity, because we did not observe a significant increase in CD8<sup>+</sup> T cells staining with the tetramer that identified the dominant retroviral antigen, AH-1, (data not shown).

When tumors that had been previously treated with single high-dose radiation were given conventional fractionated daily dose radiation for 10 days, the therapeutic effect of the former treatment was abrogated by the additional irradiation. The additional fractionated radiation resulted in a marked decrease in the percentage and absolute number (per mg of tumor) of CD8<sup>+</sup> T-cell tumor infiltrates and in an associated increase in MDSCs. A likely explanation of the latter finding is that the extended radiation killed the tumor-infiltrating CD8<sup>+</sup> T cells (56). In addition, a recent study has shown that fractionated radiation can upregulate PD-L1 on tumor cells such that tumors have increased resistance to immune eradication (57). This resistance can be overcome by the concomitant treatment with anti-PD-1 or anti-PD-L1 antibodies (57) when small (7 to 10 day) CT26 tumors were studied. However, it is not clear whether the combined treatment is effective with the large CT26 tumors (21 day) used in this study.

The ability of high-dose oligofractionated radiation to stimulate a robust antitumor immune response may not occur with all tumors. The success of conventional fractionated radiation over several weeks in the induction of complete remissions in prostate, and head and neck tumors suggests that T-cell infiltration may provide less important contribution to control of the latter tumors after radiation. In summary, the model depicted in Supplementary Fig. S4 shows that the induction of durable tumor remissions by high-dose single-fraction radiotherapy involves the activation of both innate and adaptive immune cells that result in desirable changes in the tumor microenvironment.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A. Filatenkov, J. Baker, N. Zhang, H. Kohrt, S. Dejbakhsh-Jones, S. Strober

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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S. Strober

**Study supervision:** E.C. Engleman, S. Strober

**Other (helped designing experiments):** S. Dutt

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## Acknowledgments

The authors thank Dr. G. Fisher for advice, G. Letsinger for help with article preparation, and Dr. J.M. Brown for helpful discussions.

## Grant Support

The work was supported by grant RO1 CA-163441 from the NIH, from the Nishimura Fund for Cancer Research and the Hsiao Fund.

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Received October 31, 2014; revised March 6, 2015; accepted March 15, 2015; published OnlineFirst April 13, 2015.

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# Clinical Cancer Research

## Ablative Tumor Radiation Can Change the Tumor Immune Cell Microenvironment to Induce Durable Complete Remissions

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*Clin Cancer Res* 2015;21:3727-3739. Published OnlineFirst April 13, 2015.

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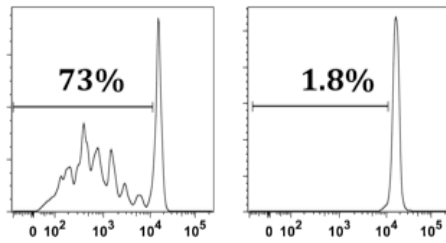
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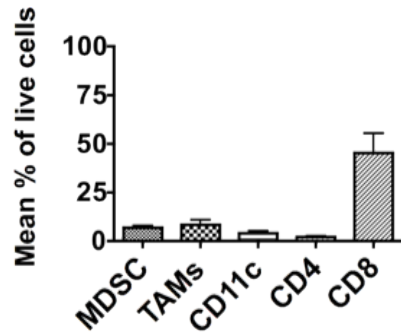
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**A 3 day MDSC suppression study**

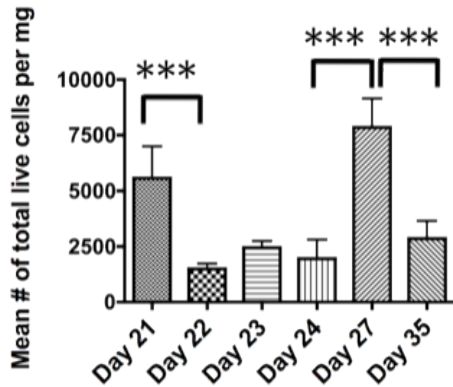
T cells no MDSCs 3 day MDSC +T cells



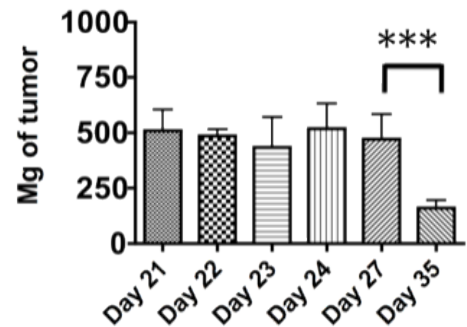
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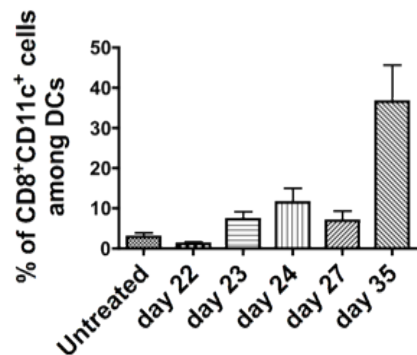
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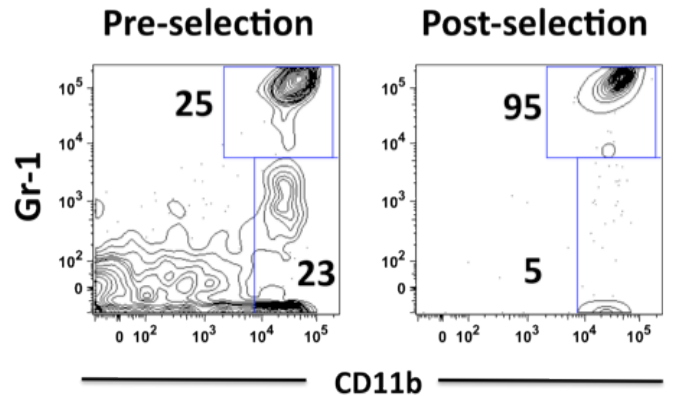
**D 30 Gy**



**E Mean % of CD8+DC among CD11+ DCs**



**F Day 21, no treatment**

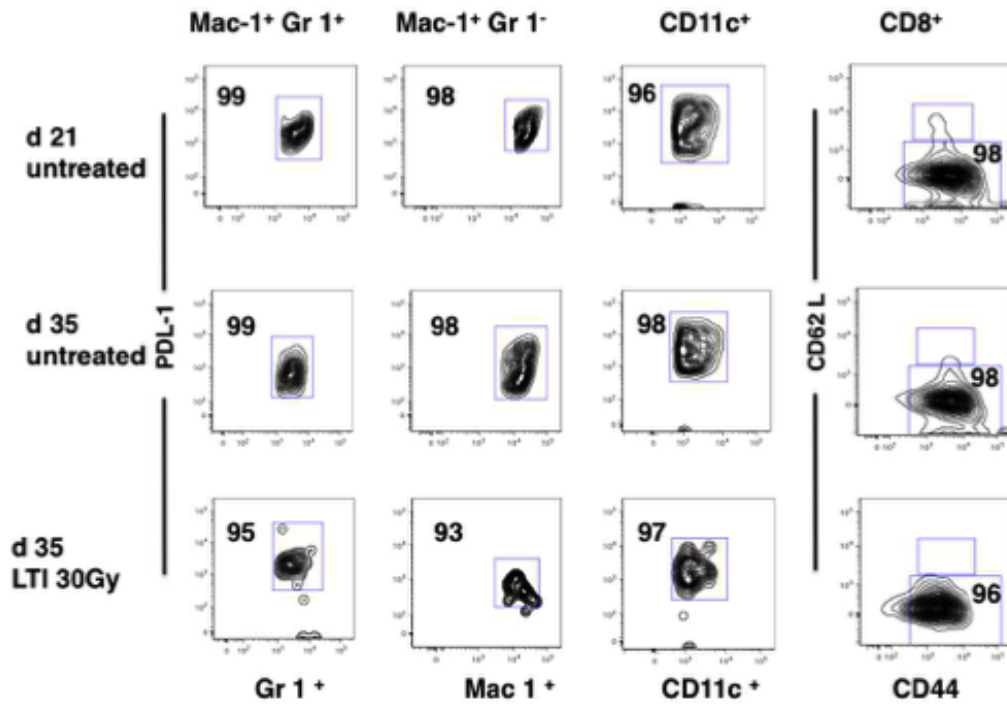


Supplementary Figure 1

Supplementary Figure 1. **In vitro suppressive function of tumor-infiltrating MDSC 3 days after LTI, and analysis of CD8<sup>+</sup>CD11<sup>+</sup> dendritic cell tumor infiltration after LTI.**

**A**, Representative staining of cultures in which CT26 tumor-derived MDSCs collected 3 days after 30 Gy LTI were incubated with syngeneic splenic T cells loaded with CFSE and incubated with CD3/CD28 beads in vitro for 5 days. MDSC:Tcell ratio was 1:1. Percentage of gated CD8<sup>+</sup> T cells that diluted CFSE is shown. **B**, Cell subsets (CD11b Gr-1<sup>hi</sup>, CD11b Gr-1<sup>lo</sup>, CD11c, CD4<sup>+</sup> and CD8<sup>+</sup>) are shown as a mean percentage among live mononuclear cells +/- SE after LTI 15 Gy (n=5). **C**, Kinetics of total live tumor-infiltrating cells after 30 Gy LTI was analyzed at days 1,2,3, 6 and 14 after LTI that are days 22, 23, 24, 27, and 35 after tumor induction, respectively (n=5 each group). Total live cells are shown as a mean number +/- SE per mg of tumor. **D**, Kinetics of tumor weight changes after 30 Gy are shown as mean weight in mgs +/- SE (n=5 each group). **E**, Percentage of CT26 tumor-infiltrating CD8<sup>+</sup> CD11c<sup>+</sup> cells is shown as a mean percentage +/- SE among total CD11c<sup>+</sup> cells at days 22, 23, 24, 27 and 35 after tumor inoculation (n=5 each group). 30Gy LTI was given at day 21. **F**, Representative flow cytometry profiles of tumor cells from day 21 untreated CT26 tumors stained with anti-Gr-1 mAbs, incubated with streptavidin microbeads, and purified with magnetic columns. Percentages of Gr-1<sup>hi</sup>CD11b<sup>+</sup>





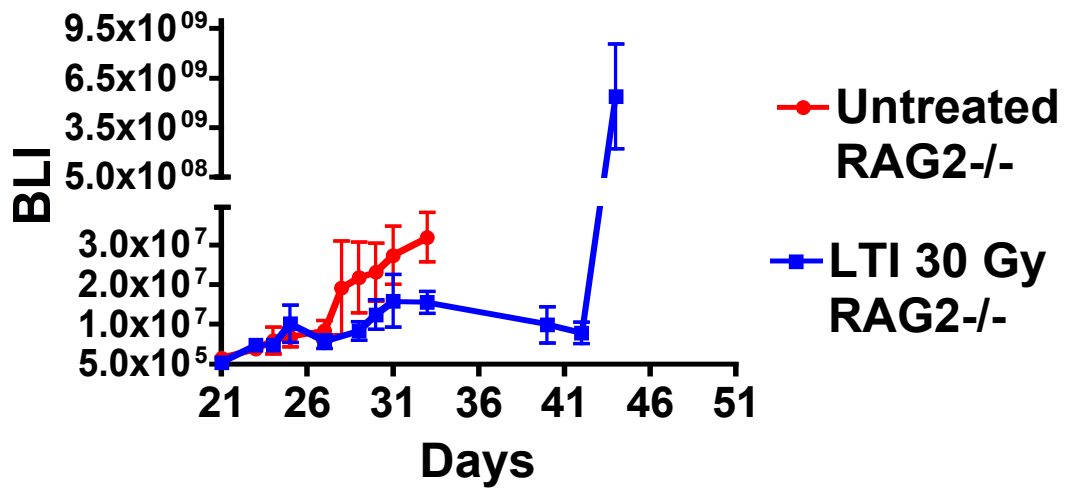
Supplementary figure 1

Supplementary Figure 2. **PDL-1 and CD62L expression of tumor-infiltrating cells.**

Tumor-infiltrating cells were analyzed at day 21 after CT26 tumor implantation, as well as at day 35 in untreated animals or animals that received LTI 30 Gy at day 21.

PDL-1 expression was analyzed on MDSCs (gated Mac-1<sup>+</sup> Gr1<sup>hi</sup>), TAMs (gated Mac-1<sup>+</sup> Gr1<sup>lo</sup>) as well as on DCs (CD11c<sup>+</sup>). CD44 and CD62L expression was analyzed on CD8<sup>+</sup> tumor

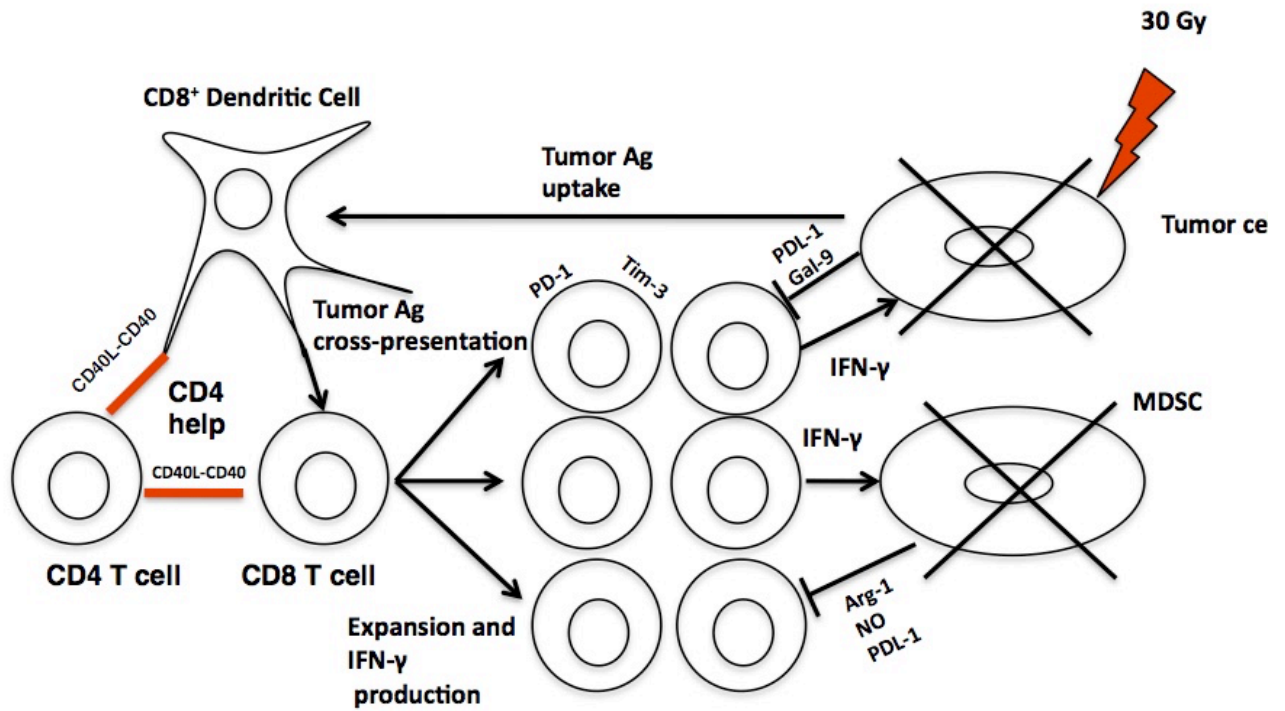
infiltrating T cells. The percentage of effector memory cells (CD62L-CD44+) are shown in the lower boxes. Representative stainings are shown.



Supplementary Figure 3

Supplementary Figure 3.

CT26 luc<sup>+</sup> tumors were established in BALB/c RAG2<sup>-/-</sup> animals. 30 Gy LTI was given on day 21 after tumor induction. Bioluminescence imaging (BLI) is shown with proton emission at serial time points.



Supplementary Figure 4

**Supplementary Figure 4. Model for T cell mediated remissions and reversal of the immunosuppressive tumor microenvironment**

High dose LTI results in tumor cell death, release of tumor antigens, and innate immune activation of CD8<sup>+</sup> dendritic cells (DCs). CD8<sup>+</sup>DCs present tumor antigen to CD8<sup>+</sup> T cells. CD4<sup>+</sup> T cells are also stimulated by tumor antigen and provide help to the CD8<sup>+</sup> T cells via CD40L. The CD8<sup>+</sup> T cells eliminate both tumor cells and the MDSCs in the stroma in an IFN- $\gamma$  dependent manner. Tumor cell expression of PDL-1 and Gal-9, inhibits CD8<sup>+</sup> T cell function by binding to the PD-1 and Tim-3 receptors, and MDSCs mediate their inhibitory effects by expression of Arg-1, NO, and PDL-1.