

PD-1 Restrains Radiotherapy-Induced Abscopal Effect

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Abstract

We investigated the influence of PD-1 expression on the systemic antitumor response (abscopal effect) induced by stereotactic ablative radiotherapy (SABR) in preclinical melanoma and renal cell carcinoma models. We compared the SABR-induced antitumor response in PD-1-expressing wild-type (WT) and PD-1-deficient knockout (KO) mice and found that PD-1 expression compromises the survival of tumor-bearing mice treated with SABR. None of the PD-1 WT mice survived beyond 25 days, whereas 20% of the PD-1 KO mice survived beyond 40 days. Similarly, PD-1-blocking antibody in WT mice was able to recapitulate SABR-induced antitumor responses observed in PD-1 KO mice and led to increased survival. The combination of SABR plus PD-1 blockade induced near complete regression of the irradiated primary tumor (synergistic effect), as opposed to SABR alone or SABR plus control anti-

body. The combination of SABR plus PD-1 blockade therapy elicited a 66% reduction in size of nonirradiated, secondary tumors outside the SABR radiation field (abscopal effect). The observed abscopal effect was tumor specific and was not dependent on tumor histology or host genetic background. The CD11a^{high} CD8⁺ T-cell phenotype identifies a tumor-reactive population, which was associated in frequency and function with a SABR-induced antitumor immune response in PD-1 KO mice. We conclude that SABR induces an abscopal tumor-specific immune response in both the irradiated and nonirradiated tumors, which is potentiated by PD-1 blockade. The combination of SABR and PD-1 blockade has the potential to translate into a potent immunotherapy strategy in the management of patients with metastatic cancer. *Cancer Immunol Res*; 3(6); 610–9. ©2015 AACR.

Introduction

Ionizing radiotherapy is a widely used cancer treatment. Among the effects of radiotherapy is its potential for inducing necrotic tumor-cell death, a prerequisite for eliciting an antitumor immune response. Stereotactic ablative radiotherapy (SABR) optimizes local control by delivering tumor-ablative doses while sparing the surrounding normal tissues. SABR is increasingly viewed as a noninvasive alternative to surgery (1–4) with comparable local control rates. Although a detailed understanding of the effects of SABR on the immune system is incomplete, reports of partial or complete eradication of tumors outside the radiotherapy field, defined as the abscopal effect, suggest that SABR is capable of priming and expanding tumor-reactive T cells

within the irradiated tumor and among the draining lymph tissues. Presumably, these activated, tumor-specific T cells then migrate to and eliminate nonirradiated (henceforth referred to as "secondary") tumors. Preclinical models have established that the abscopal effect is T-cell dependent (5).

The infrequent incidence of the abscopal effect in patients following SABR may be due to the suboptimal radiation doses or the inhibition of immune responses (induction, expansion, or effector phases) by conventional immunoregulatory mechanisms. Immune checkpoint blockade at the level of immune priming (CTLA-4 blockade) or effector function (B7-H1/PD-1) is being investigated in many clinical trials and is yielding promising results. This study addresses the question of whether a distinct radiotherapy regimen—i.e., SABR—synergizes with an immune checkpoint blockade and elicits a potential systemic curative response.

The combination of anti-CTLA-4 and radiotherapy is capable of inducing abscopal effects in melanoma patients (6, 7). A B7-H1 or PD-1 blockade in preclinical radiotherapy models increased the rate of tumor regression, thereby recapitulating the results characteristic of PD-1 blockade in conjunction with other therapies in advanced cancer patients. However, the impact of PD-1 blockade on SABR-mediated abscopal effects is largely unexplored.

In this study, we demonstrate that SABR is a potent inducer of local and systemic (i.e., abscopal effect) antitumor immunity and that PD-1 is a major impediment to antitumor immune responses induced by SABR. Accordingly, PD-1 inhibition [in knockout (KO) and blocking antibody models] significantly enhances SABR-induced antitumor immunity and results in the suppression of both irradiated and nonirradiated (primary and

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secondary, respectively) tumor growth. We have established that PD-1 expression is upregulated in a tumor-reactive CD11a^{high} CD8⁺ T-cell population and contributes to restraining the T-cell effector activity (8). Here, we confirm the correlation between the tumor-reactive CD11a^{high} CD8⁺ T cells and the SABR-induced antitumor response. The combination of SABR and PD-1 blockade may be a promising therapeutic approach for managing patients with metastatic cancer.

Materials and Methods

Mice

Six- to eight-week-old BALB/c and C57BL/6 mice were obtained from Taconic Animal Laboratory (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions in the animal facility at Mayo Clinic Comparative Medicine Department. PD-1 KO C57BL/6 mice were provided by L. Chen (Yale University, New Haven, CT) with the permission of Dr. T. Honjo (Kyoto University). All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Mayo Clinic.

Cell line and reagents

RENCA and 4T1 are BALB/c mouse-derived immunogenic renal cell carcinoma and poorly immunogenic mammary carcinoma cell lines, respectively. RENCA (CRL-2947) and 4T1 (CRL-2947) cells were obtained from the American Type Culture Collection (ATCC). Both cell lines were passaged for less than 6 months for these studies. ATCC authenticates cell lines by short tandem repeat analysis. B16-OVA is a C57BL/6 mouse-derived poorly immunogenic melanoma cell line obtained from Dr. Richard G. Vile (Mayo Clinic). B16-OVA cells had undergone quality control analysis by reverse PCR in Dr. Vile's laboratory before receipt and were passaged for less than 6 months for the described experiments. All cell lines were proven free of *Mycoplasma* contamination (*Mycoplasma* Detection kit; Roche Diagnostics). Tumor cells were cultured in DMEM (Invitrogen Corporation) or RPMI 1640 medium (Cellgro) with 10% to 20% FBS (Life Technologies), 1 U/mL penicillin, 1 µg/mL streptomycin, and 20 mmol/L HEPES buffer (all from Mediatech). Anti-B7-H1 (PD-L1; Clone MIH5), LAG-3 (Clone C9B7W), and Tim-3 (Clone B8.2C12) mAbs were used to detect tumor or immune cells for their expression of B7-H1, LAG-3, and Tim-3. Anti-PD-1 hamster mAb G4 was purified as previously described (9). Anti-CTLA-4 hamster mAb (Clone 9H10), anti-CD11a (Clone M17/4) mAb, and appropriate isotype control Ig were purchased from Bio X Cell.

Tumor-cell challenge and treatment

C57BL/6 and BALB/c mice were injected s.c. with 1×10^5 B16-OVA or 5×10^5 RENCA cells in the right hindlimb (primary tumor; irradiated) on day 0 and in the left flank (secondary tumor; nonirradiated) on days 3 to 4. The third party tumor cells (EL4 or 4T1) were injected s.c. in the right flank (secondary tumor; nonirradiated) on days 3 to 4. Perpendicular tumor diameters were measured using a digital caliper, and tumor sizes were calculated as length \times width (mm²). On day 8, when primary tumors were palpable, animals were randomly assigned to treatment groups. Radiotherapy (15 Gy) in a single fraction was administered locally to the primary tumors in the right hindlimb.

Briefly, all mice were positioned in a modified 50-mL conical plastic tube, which allowed the area of the tumor to be irradiated while keeping the rest of the body outside the radiotherapy field. Radiotherapy was delivered using 12 MeV electrons to a field including the tumor with a TrueBeam Linear Accelerator (Varian Medical Systems) fitted with a 20×20 cm² cone. EBT3 Gafchromic film (Ashland ISP Advanced Materials) dosimetry confirmed homogeneity of the prescribed dose within $\pm 5\%$. Superflab bolus (1.0 cm tissue equivalent material) was placed over the tumor, and the tumor received a dose of 15 Gy in one fraction at a rate of 600 cGy/min. PD-1-blocking mAb G4, CTLA-4-blocking mAb (Clone 9H10), or isotype control Ig was administered as an i.p. injection at a dose of 200 µg/mouse (10 mg/kg) alone or as a combination of PD-1- and CTLA-4-blocking mAbs. Tumor growth was evaluated every 2 to 3 days until days 35 to 40, when all mice were euthanized. In compliance with animal care guidelines, mice were euthanized when either primary or secondary tumors reached 300 mm² in size.

Flow cytometry analysis of IFN γ -producing CD8 T cells

For the *in vitro* tumor-cell challenge, 5 to 10×10^5 tumor-infiltrating lymphocytes (TIL), which were isolated from tumor tissues or lymphoid organs, were cultured with ovalbumin (OVA) peptide (1 µl/mL) for 4 to 5 hours in the presence of 1 µl/mL of Brefeldin A (Sigma), washed, and incubated with rat anti-mouse CD16/CD32 mAb (2.4G2) to block nonspecific binding, and then stained with CD8-PE-Cy5 and IFN γ -FITC or control antibodies according to the manufacturer's instructions (BD Pharmingen). Tumor antigen-specific CD8 T cells were identified by staining with OVA tetramer (Beckman Coulter) and TRP-2 pentamer (ProImmune). Cells were analyzed using FACScan flow cytometer and FlowJo version X.10 (Tree Star) software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software 5.0 (GraphPad Software, Inc.). A two-sided, unpaired or paired Student *t* test was used to assess statistical differences in experimental groups. A *P* value of <0.05 was considered statistically significant.

Results

In the absence of PD-1 expression, the SABR-induced abscopal effect is enhanced

To examine to what extent PD-1 may influence the abscopal effect induced by SABR, B16-OVA melanoma cells were injected into the right hindlimb (primary; irradiated) and left flank (secondary; nonirradiated) of wild-type (WT) and PD-1-deficient (PD-1 KO) C57BL/6 mice. Eight days following tumor-cell injection, tumors in the right hindlimb (primary tumors) were administered a single dose of 15 Gy. The secondary tumors (left flank) were kept out of the radiation field. The results in Fig. 1A show that SABR resulted in a 5-fold reduction ($P < 0.05$, $n = 5$) in primary tumor size 24 days after SABR in the PD-1 KO mice, as compared with that in the WT mice. Importantly, nonirradiated secondary tumors in PD-1 KO mice exhibited a significant reduction in growth (i.e., an abscopal effect; Fig. 1B, $P < 0.05$), as compared with that of the secondary tumors in WT mice. As shown in Fig. 1C, none of the WT mice survived beyond 25 days, whereas 60% of PD-1 KO mice were alive at day 25 and 20% survived beyond 40 days. From these observations, we conclude that PD-1 expression

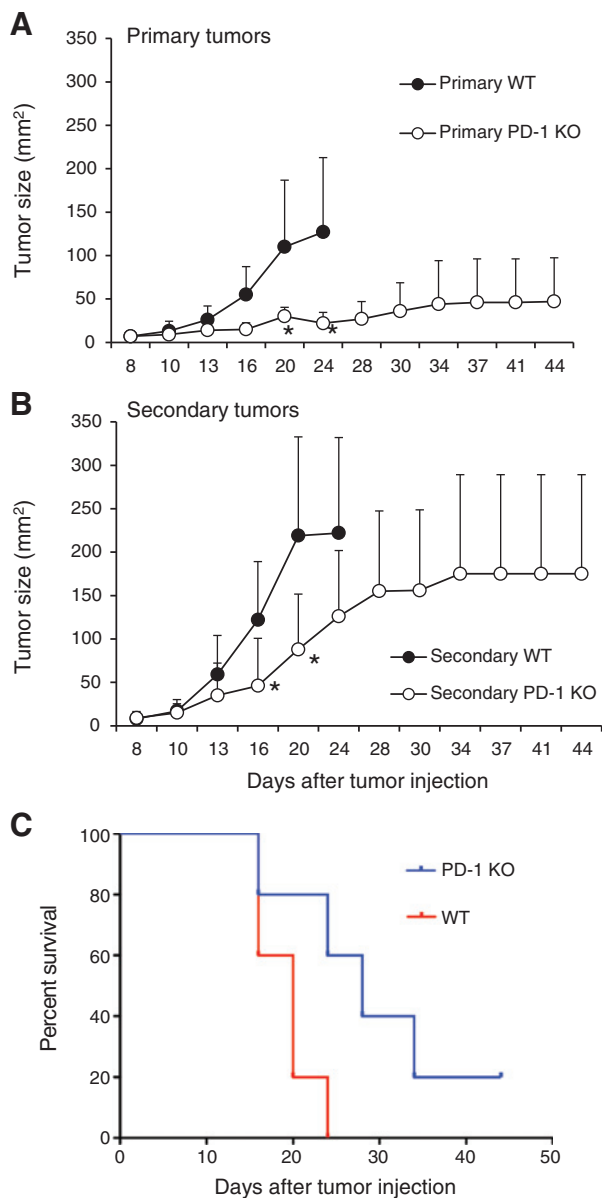


Figure 1. Enhanced antitumor abscopal effect in PD-1-deficient mice induced by radiotherapy. Both primary and secondary B16-OVA tumors (2×10^5) were injected into WT or PD-1-deficient (KO) mice (5 mice per group). Tumor growth was measured following radiotherapy. Data show mean size \pm SD of 5 mice per group of primary (A) and secondary tumors (B). *, $P < 0.05$ compared with WT mice. C, the survival of WT mice was shorter than that of PD-1 KO mice ($P < 0.05$, $n = 5$) following radiotherapy.

suppresses the SABR-induced antitumor response and that the abscopal effect is enhanced in the absence of PD-1, thereby improving survival.

PD-1 blockade recapitulates SABR-induced abscopal effect observed in PD-1 KO mice

We observed that SABR induced an abscopal effect in the absence of PD-1 and conferred increased survival by suppressing the growth of secondary nonirradiated tumors, as shown in Fig. 1.

Here, we addressed whether PD-1 blockade in WT mice, when combined with SABR, would recapitulate the abscopal effect induced by SABR in the KO model. B16-OVA tumor cells were injected into the right hindlimb (primary tumor) and left flank (secondary tumor) of WT mice, and SABR was administered 8 days after injection. On days 7, 9, 11, 14, and 16 following tumor-cell inoculation, either PD-1-blocking or isotype-matched control antibody (200 μ g) was administered.

Without PD-1 blockade, SABR treatment resulted in the retardation of tumor growth only at the site of irradiation (primary tumor). PD-1 blockade alone modestly suppressed primary and secondary tumor growth. However, the combination of SABR and PD-1 blockade resulted in a near complete response of the primary tumor when compared with SABR plus control Ig or PD-1 blockade alone (Fig. 2A). Therefore, a synergistic effect between SABR and PD-1 blockade was noted in the primary tumor.

To address whether PD-1 blockade would enhance the SABR-induced abscopal effect, secondary tumor growth (outside of the radiation field) was assessed. Whereas neither PD-1 blockade alone or SABR plus control Ig treatment had any effect on the secondary tumor outgrowth, the combination of SABR and PD-1 blockade was effective in eliciting a 66% reduction in the secondary tumor size (100 mm vs. 300 mm; Fig. 2B, $P < 0.05$) 20 days after treatment. PD-1 blockade alone or SABR plus control Ig had minimal effect on survival, as compared with mice treated solely with control Ig (day 22 survival vs. day 19; Fig. 2C). In contrast, the incorporation of PD-1 blockade in conjunction with SABR elicited a significant increase ($P < 0.05$) in survival (20% of mice were alive at day 33; Fig. 2C). Together, results shown in Figs. 1 and 2 demonstrate that SABR is capable of inducing antitumor immunity, which is restrained by PD-1. Furthermore, PD-1 inhibition by KO or blockade potentiates the SABR-induced abscopal effect and leads to increased survival. Because CTLA-4 also has been reported to promote abscopal effects (10), we next tested whether the combined PD-1 and CTLA-4 blockade would lead to synergistic effects. SABR with PD-1 or CTLA-4 blockade alone produced minimal reduction of primary and secondary tumor growth in our melanoma animal model with larger tumor burden. However, SABR and the combined PD-1 and CTLA-4 blockade significantly reduced the growth of secondary tumors (Supplementary Fig. S1). These results suggest that the combined checkpoint inhibition (PD-1 and CTLA-4 blockade) has synergistic effects in enhancing SABR-induced abscopal effects and warrants clinical investigation in patients with a large metastatic tumor burden.

Combination SABR and PD-1 blockade therapy is tumor antigen-specific and is not dependent on tumor histology or host genetic background

To demonstrate that the applicability of SABR and PD-1 combination therapy is not limited to a specific tumor histology or host genetic factors and that the combination therapy is tumor antigen-specific, mouse renal carcinoma (RENCA) as primary and secondary tumors and breast cancer (4T1) as the third (experimental) tumor histology were injected into Balb/c mice that had a different genetic background from C57BL/6 mice (Fig. 3A). Primary RENCA tumor (grown on the right hindlimb) was administered as a single 15-Gy dose. Tumor growth was measured at (i) the primary irradiated RENCA tumor (right

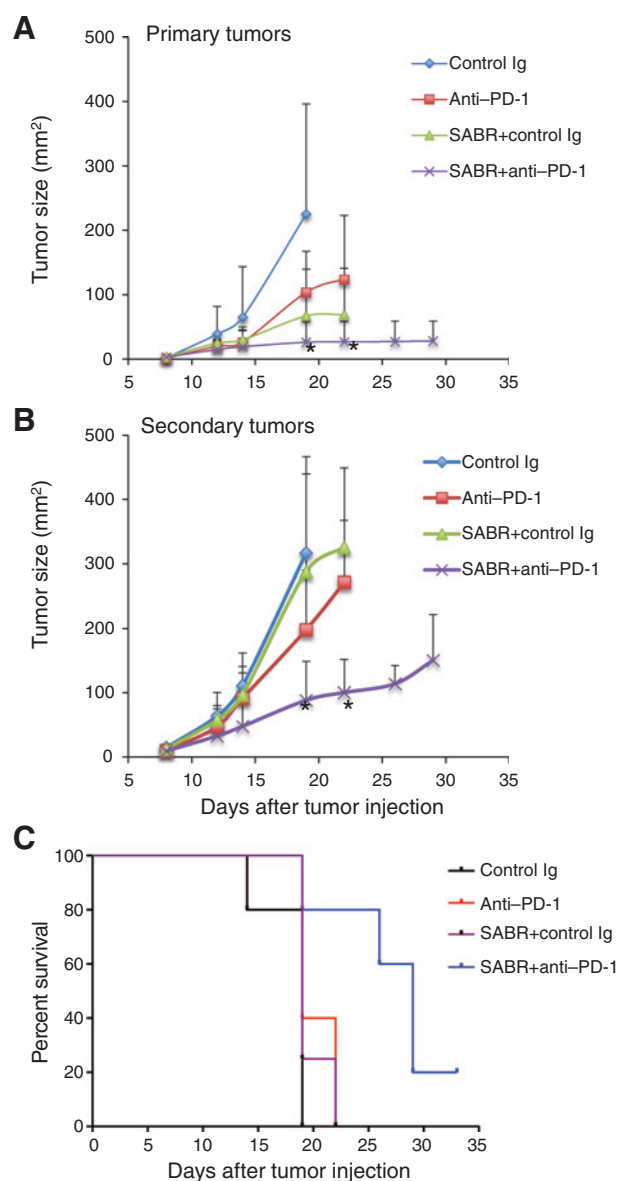


Figure 2. PD-1 blockade enhanced antitumor abscopal effects induced by radiotherapy. The primary tumors were treated with local radiotherapy at 15 Gy on day 8 after tumor injection. Anti-PD-1 mAb (Clone G4) or control Ig (200 μ g/mouse) were injected i.p. on day 7 and every other day thereafter for a total of 5 doses. Tumor growth was measured in primary (A) and secondary (B) tumors and was shown as mean size \pm SD of 5 mice per group. *, $P < 0.05$ compared with mice treated with anti-PD-1 mAb alone ($n = 5$). C, the survival of mice treated with anti-PD-1 mAb and radiotherapy was longer than that of mice treated with anti-PD-1 mAb alone ($P < 0.05$, $n = 5$) or without treatment. Results of one of three independent experiments are shown.

hindlimb); (ii) the secondary nonirradiated RENCA tumor (left flank); and (iii) the nonirradiated experimental 4T1 tumor (right flank). Because of the institutional mouse tumor burden guidelines, survival endpoints were not a component of this experiment. In both the primary irradiated and secondary nonirradiated RENCA tumor sites, SABR in combination with PD-1 blockade elicited either complete or nearly complete remission of RENCA

tumors (Fig. 3B and C). However, the growth of 4T1 tumors was not affected by SABR, PD-1 blockade, or the combined therapy (Fig. 3D). These data support the hypothesis that PD-1 restrains tumor-specific antitumor immunity in mice treated with SABR and PD-1 blockade. We conclude that the SABR-induced antitumor abscopal effect is potentiated by PD-1 blockade and that it is tumor-specific and independent of tumor histology or host genetic background.

Tumor-reactive CD11a^{high} CD8⁺ T cells from primary and secondary tumor sites express PD-1

Our observations are consistent with the ability of SABR to induce an *in situ* antitumor response at the irradiated site, which then traffics to secondary tumor sites outside the radiation field. The observation of PD-1 blockade augmenting SABR-induced antitumor immunity is consistent with PD-1 functioning as an immune checkpoint inhibitory molecule. Because CD11a expression is required in the rejection of tumors (11), we previously established that CD11a^{high} CD8⁺ T cells from primary and secondary tumors was examined (Fig. 4). B16-OVA cells were injected into the right hindlimb and the left flank of C57BL/6 mice. A single SABR fraction of 15 Gy was administered to the right hindlimb on day 8 after injection. Seven days after SABR, CD11a^{high} CD8⁺ T cells were identified from irradiated (right hindlimb), nonirradiated (left flank), and control tumor-bearing mice that were not irradiated. Levels of PD-1 (represented by percentages of positive) expressed by CD11a^{high} CD8⁺ T cells are higher in the primary tumor site as compared with those of the secondary tumor site ($P < 0.05$ on day 15). In contrast with mice that received SABR, CD11a^{high} CD8⁺ T cells in the tumor tissues of nonirradiated mice expressed only modest levels of PD-1 (Fig. 4A, $P < 0.01$ on day 15). To confirm whether these PD-1⁺ CD8 T cells are indeed tumor antigen-reactive effector T cells, we measured their intracellular IFN γ production following a brief restimulation with surrogate tumor-antigen peptide (OVA peptide) *ex vivo*. Figure 4B shows that 33% to 61% of PD-1⁺ CD8 T cells produced IFN γ at the primary and secondary tumor sites, suggesting that PD-1⁺ CD11a^{high} CD8⁺ T cells are effector T cells within the tumors. The expression of PD-1 ligand (PD-L1; B7-H1) increased in tumor cells after SABR in both primary and secondary tumors, but it did not change in that of the nontumor host (leukocyte) cells (Fig. 4C). In addition to PD-1, we measured the expression of other immune checkpoint molecules expressed by CD8 T cells. The results presented in Fig. 4D show that tumor-reactive CD11a^{high} CD8⁺ T cells from both primary and secondary tumors express high levels of LAG-3, but not Tim-3, and SABR did not alter the LAG-3 expression by CD8⁺ T cells in either of the tumor sites. In contrast, CD11a^{low} CD8⁺ T cells did not express either LAG-3 or Tim-3 before or after SABR at both primary and secondary tumor sites. These results suggest that radiotherapy does not induce the expression of LAG-3-regulatory molecules within tumors.

A direct correlation between the number of tumor-reactive CD11a^{high} CD8⁺ T cells within both primary and secondary tumors and the extent of tumor growth delay was also noted in PD-1 KO mice (Fig. 5A). The tumor-antigen specificity of

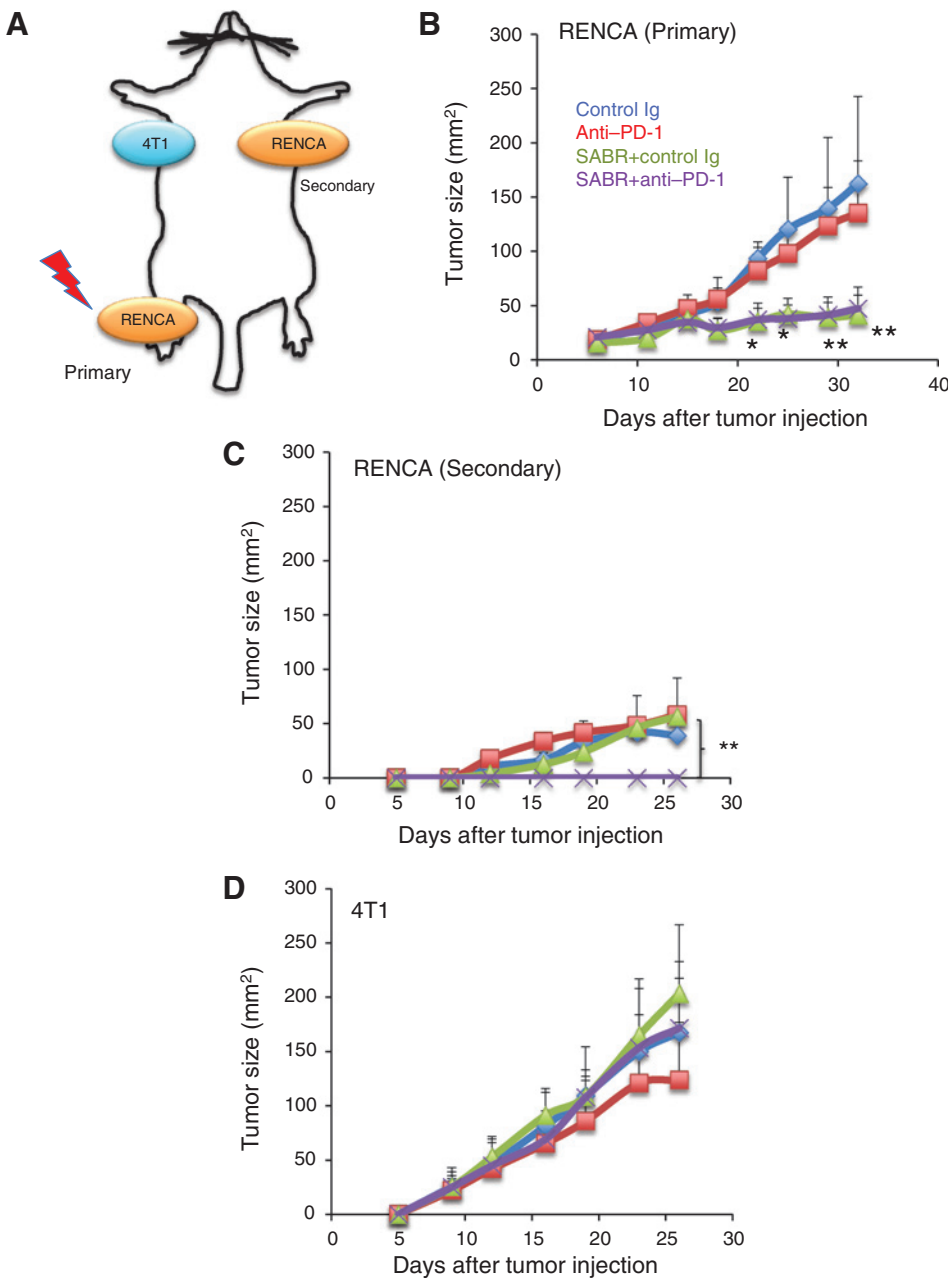


Figure 3. PD-1 blockade enhanced tumor-specific antitumor abscopal effects induced by radiotherapy. A, tumor models treated with radiotherapy and/or anti-PD-1 mAb. The primary RENCA tumor cells (5×10^5) were injected into the right hindlimb of Balb/c mice. The secondary RENCA tumor cells (2.5×10^5) and a different tumor cell line 4T1 (5×10^4) were injected into the left or right flanks, respectively, 4 days after primary tumor-cell injection in the same mice. The primary tumors were treated with 15 Gy on day 8 after tumor-cell injection. Anti-PD-1 mAb (Clone G4) or control Ig (200 μ g/mouse) were injected i.p. on day 7 and every other day thereafter for a total of 5 doses. Tumor growth (RENCA tumors) was measured in the primary (B) and secondary sites (C) and was shown as mean size \pm SD of 5 mice per group. *, $P < 0.05$; **, $P < 0.01$ compared with mice treated with anti-PD-1 mAb alone. D, the growth of 4T1 tumors did not respond to radiotherapy targeting RENCA tumors and anti-PD-1 treatment. Results from one of two independent experiments are shown.

CD11a^{high} CD8⁺ T cells within both tumor sites was identified by antigen peptide tetramer or pentamer staining. As shown in Fig. 5B, CD11a^{high} but not CD11a^{low} CD8⁺ T cells isolated from both primary (irradiated) and secondary (nonirradiated) tumors demonstrated tumor-antigen (OVA) specificity by binding to OVA tetramer, and to a lesser degree to TRP-2 pentamer, suggesting that the CD11a^{high} CD8⁺ T-cell population indicates the presence of tumor-specific CD8 T cells. As shown in Fig. 5C, CD11a^{high} CD8⁺ T cells isolated from tumor sites produced IFN γ upon OVA peptide stimulation but not with control peptide stimulation. These new data collectively suggest that CD11a^{high} CD8⁺ T cells inside tumors are tumor-specific functional effector cells and that their function is enhanced by SABR in PD-1 KO mice.

We next determined to what degree CD8⁺ or CD4⁺ T cells contribute to the SABR-enhanced abscopal effects in the absence of PD-1 by depleting CD8⁺ or CD4⁺ T cells in WT and PD-1 KO mice treated with SABR. As shown in Fig. 6, the depletion of CD8⁺ T cells, but not the depletion of CD4⁺ T cells, abolished the synergistic and abscopal effects in the primary and secondary tumors in PD-1 KO mice. Although the high expression of CD11a by CD8 T cells was used as a surrogate marker to identify tumor-reactive T cells, the blockade of CD11a did not affect the SABR-induced abscopal effects in PD-1 KO mice (Fig. 6), suggesting that the disruption of CD11a alone may not affect CD8 T-cell-mediated antitumor effects. In conclusion, our data suggest that PD-1 is a T-cell regulator that restrains the immune-mediated abscopal effect induced by radiotherapy.

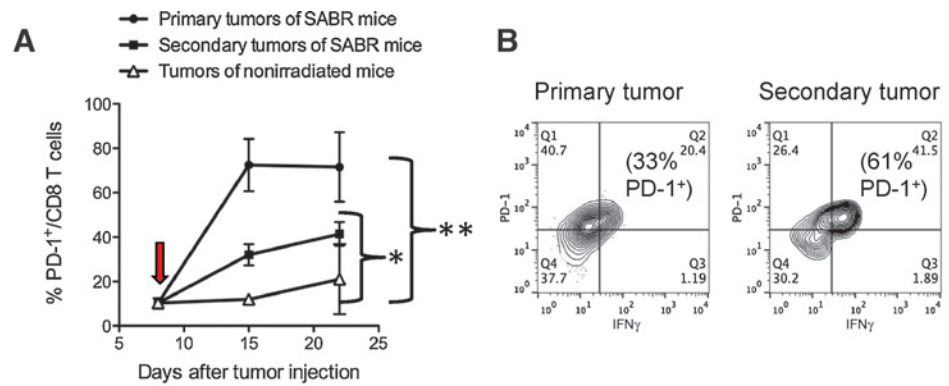
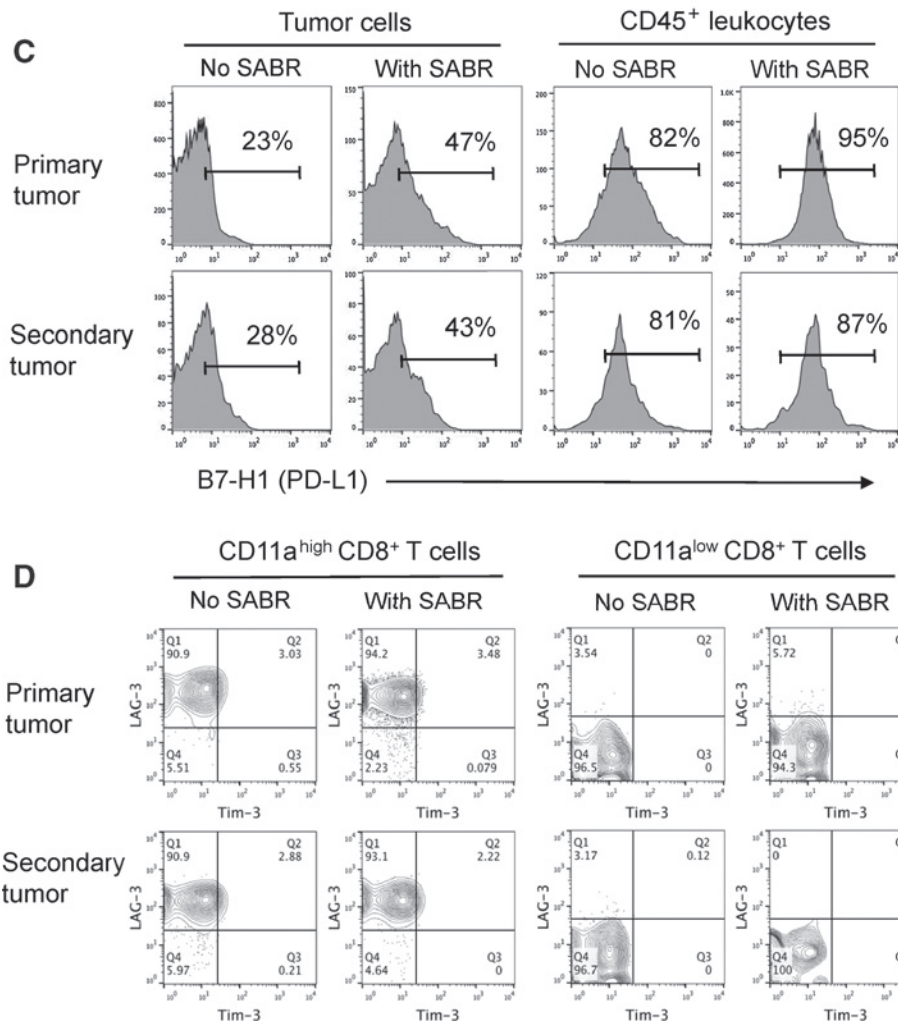


Figure 4. Immune checkpoint molecule expression in primary and secondary tumors following radiotherapy. On day 8 after injection of both primary and secondary B16-OVA tumor cells (2×10^5), primary tumors of the right hindlimb were irradiated with a single dose of 15 Gy. In the following weeks, TILs were isolated and analyzed for PD-1 expression (A) and CTL function (B) of CD11a^{high} CD8⁺ T cells. Data show percentages of PD-1⁺ in total CD11a^{high} CD8⁺ TILs of 3 to 5 mice per group. *, $P < 0.05$; **, $P < 0.01$, compared with that in mice without radiotherapy (A). Arrow indicates day of radiotherapy (day 8). The production of IFN γ was analyzed by intracellular staining for IFN γ after a brief restimulation with OVA peptide *ex vivo* for 5 hours. Numbers in parentheses show the percentages of IFN γ -producing cells per PD-1⁺ population. C, B7-H1 (PD-L1) expression by CD45⁻ (tumor cells) and CD45⁺ (leukocytes) cells within both tumor sites. D, LAG-3 and Tim-3 expression by CD11a^{high} or CD11a^{low} CD8⁺ TILs within both tumor sites.



Discussion

In this study, we demonstrate that SABR-induced antitumor immunity is tumor antigen-specific and that combination SABR and anti-PD-1 therapy is not restricted to tumor histology or host genetic factors. We show that PD-1 restrains the immune-mediated abscopal effect induced by local radiotherapy in a preclinical model. PD-1 blockade or deficiency can synergize with radiotherapy to induce tumor-specific CD8⁺ T-cell immunity and result in a

clinical response in the secondary tumors outside of the radiation field.

The demonstration of radiotherapy-induced T-cell priming within the tumor by the sensitization of the tumor stroma (13) or at tumor-draining lymph nodes via activation of antigen-presenting dendritic cells (14), as well as the accumulation of effector CD8⁺ T cells inside the tumor microenvironment (15), supports the hypothesis that radiotherapy can elicit and enhance both the priming and effector phases of antitumor T-cell response.

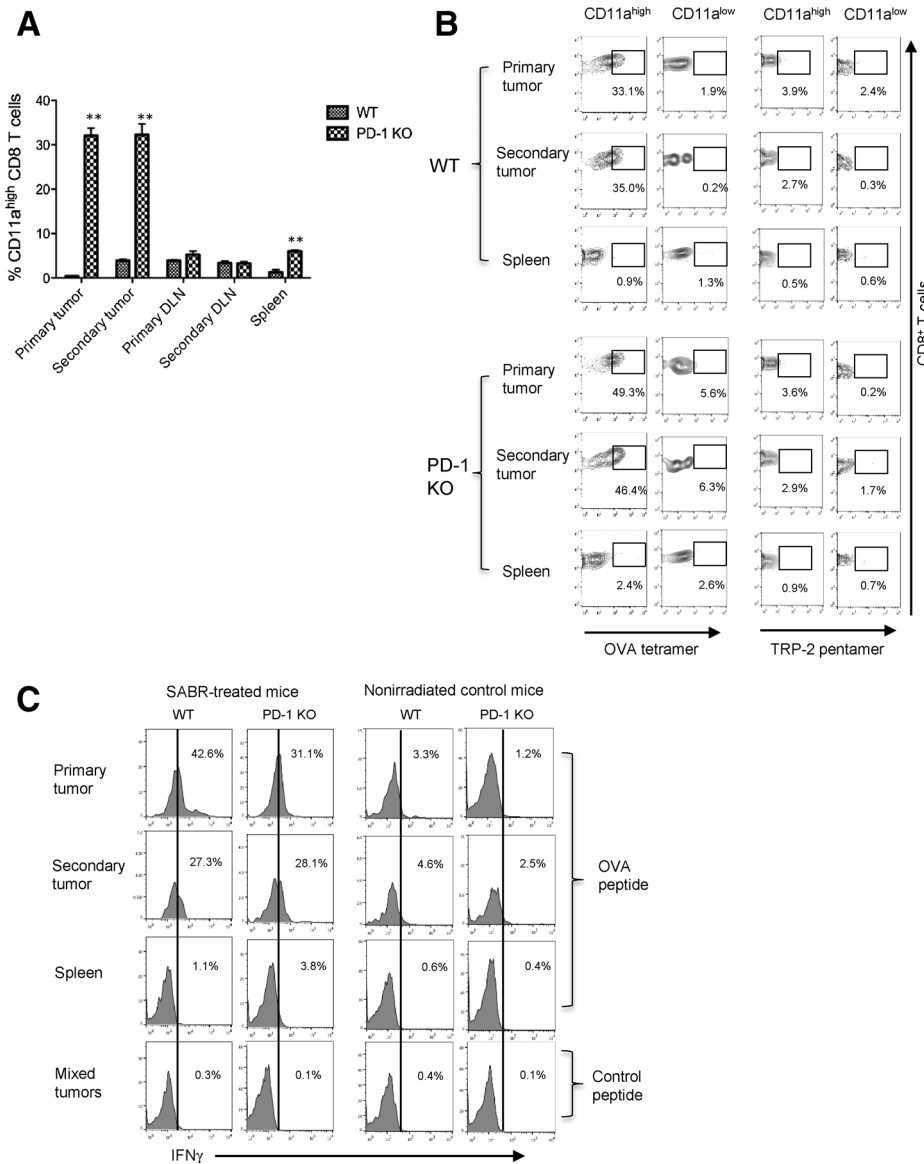


Figure 5. Increased tumor-infiltrating effector CD8⁺ T cells in PD-1-deficient mice induced by radiotherapy. One week after radiotherapy, lymphocytes containing tumor-reactive CD11a^{high} CD8⁺ T cells were identified within both primary and secondary B16-OVA tumors, as well as within draining lymph nodes and spleen tissue from WT or PD-1-deficient (KO) mice. CTL function was analyzed by their intracellular production of IFN γ . A, increased frequency of CD11a^{high} CD8⁺ T cells in tumor tissues in PD-1 KO mice. **, $P < 0.01$ compared with WT mice ($n = 3$). B, representative dot blot graph shows the binding of OVA tetramer or TRP-2 pentamer to CD11a^{high} CD8⁺ T cells, isolated from WT and PD-1 KO mice treated with or without SABR. C, representative histogram shows that CD11a^{high} CD8⁺ T cells, isolated from WT and PD-1 KO mice treated with or without SABR, produced IFN γ upon brief *ex vivo* restimulation with OVA peptide.

Consequently, if sufficient effector CD8⁺ cytotoxic lymphocytes are generated, they can migrate to and infiltrate distant metastatic tumors. Our CD8⁺ T-cell-depletion data demonstrated that CD8⁺ T cells mediated the enhanced antitumor abscopal effects in the absence of PD-1 (Fig. 6). Thus, the generation of antitumor effector CD8⁺ T cells appears to be a key mechanism underlying the abscopal effect (16, 17).

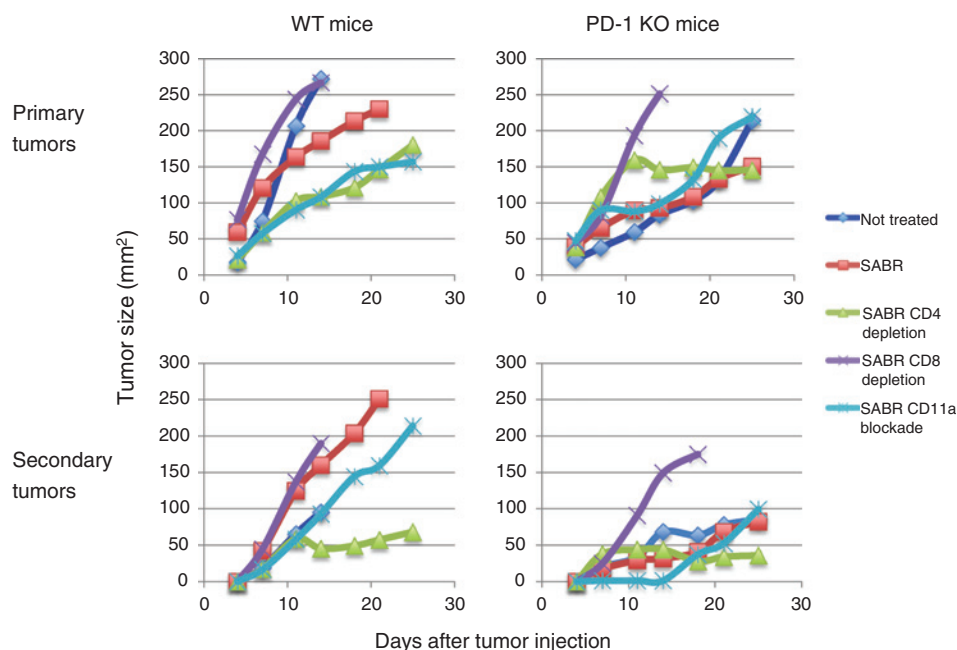
The abscopal effect is not frequently seen with radiotherapy alone, despite the observation of the radiotherapy-induced generation of tumor-specific T cells both in preclinical models and in patients (15, 18). However, local radiotherapy to the primary tumor is necessary to elicit the abscopal effect, because the anti-PD-1 blockade by itself was not effective in the suppression of secondary tumors (Figs. 2 and 3). The low objective response rate is further supported by the modest response rates of 13% to 24% in the clinical anti-PD-1 and anti-PD-L1 studies (19, 20). This observation is consistent with the hypothesis that radiotherapy induces *in situ* priming of antitumor effector T cells, which is

dependent on immunogenic tumor-cell death and the induction of danger signals (14, 21). The effector T cells generated from radiotherapy-induced tumor-cell death may not be sufficient to control a distant tumor due to the upregulation of immune checkpoint molecules, such as CTLA-4 and PD-1 (Fig. 4A), as well as PD-1 ligand (B7-H1), by tumor cells (Fig. 4C). Therefore, the addition of checkpoint blockade immunotherapy is likely to enhance the antitumor immunity mediated by the effector T cells, especially at the sites remote from irradiation. As a proof of this concept, the blockade of CTLA-4 has been shown to enhance radiotherapy-induced abscopal effects in melanoma and lung cancer patients (6, 7, 10). Our findings further demonstrate that the PD-1 blockade or deficiency promotes the immune-mediated abscopal effect induced by SABR in multiple mouse tumor models with different genetic backgrounds and tumor histologies.

Radiotherapy can be delivered using various regimens (standard, hypofractionated, SABR, etc.) in the clinic, and the radiotherapy dose and fractionation for the optimal induction of

Figure 6.

CD8 T cells mediated an enhanced antitumor abscopal effect in PD-1-deficient mice induced by radiotherapy. Both primary and secondary B16-OVA tumors (2×10^5) were injected into WT or PD-1-deficient (KO) mice (5 mice per group). Depletion of CD8 or CD4 T cells and blockade of CD11a were performed by injection of depleting or blocking antibodies starting 2 days before radiotherapy and during the subsequent 2 weeks. Tumor growth was measured following radiotherapy. Data show the average size of primary and secondary tumors of 5 mice per group.



antitumor immunity has not been validated. Demaria and colleagues suggested that 8 Gy \times 3 hypofractionation best induces antitumor immunity in their preclinical model (17). However, this is a subcurative dose, and we chose a 15 Gy \times 1 regimen in our preclinical model because this regimen was previously used in animal models without apparent toxicity, was more readily translatable to the clinical regimen of SABR, and was superior to a 3 Gy \times 5 fractionated regimen in immune induction (H. Dong and S.S. Park; unpublished data). SABR has been demonstrated to improve local control and disease-free survival in multiple prospective clinical studies in patients with oligometastatic cancers (22–24) and may have an important role in a select group of patients with limited metastases. Our preclinical data indicate that a single 15-Gy fraction was not a curative dose but was effective in tumor growth delay at the primary irradiated tumor site. It has been demonstrated that the radiotherapy-induced tumor growth delay (subcurative radiotherapy dose) results from a dynamic balance between tumor proliferation and CD8⁺T-cell-mediated tumor-cell apoptosis (25). Our finding supports this previous report, as the combination of radiotherapy and PD-1 blockade or inhibition led to an improvement in tumor growth delay at the primary tumor site (synergism), with 50% of animals achieving a complete response when compared with radiotherapy alone.

Although radiotherapy alone led to the primary tumor growth delay, it was not able to induce tumor regression of the distant secondary tumor that was outside of the radiotherapy field. Radiotherapy can induce the expression of B7-H1 (PD-1 ligand) in the irradiated tumor tissues (25), and B7-H1 functions as a critical barrier to antitumor immunity following radiotherapy. We report herein that TILs expressed high levels of PD-1 at both irradiated primary and nonirradiated secondary tumor sites (Fig. 4A) and that the tumor growth delay was enhanced at both irradiated and nonirradiated sites when PD-1 blockade or deficiency was combined with radiotherapy. Furthermore, the restimulation of TILs with OVA antigens confirmed that these TILs were

indeed antitumor-specific effector cells that were present not only in the primary tumor site but also in the secondary tumor site (Fig. 4B), which supports the immune-mediated mechanism of the abscopal effect observed in Figs. 1–3. This observation suggests that PD-1/PD-L1 interaction may be a key checkpoint mechanism of TIL downregulation in the tumor microenvironment at both the primary and secondary tumor sites.

In our study, radiotherapy in the form of SABR combined with PD-1 KO or blockade dramatically improved the control of both primary and secondary tumors. In contrast, Dewan and colleagues (26) reported that CTLA-4 blockade required fractional irradiation to achieve secondary tumor regression. CTLA-4 and PD-1 regulate T-cell response at the priming and effector phases, respectively (27). The mechanisms underlying the way in which single-dose and fractionated radiation differ in their ability to synergize with PD-1 or CTLA-4 blockade are not clear. Nevertheless, our results show that the combination of PD-1 and CTLA-4 blockade has synergistic effects in promoting antitumor radiotherapy-induced abscopal effects in models with a larger tumor burden (Supplementary Fig. S1). Future studies are warranted to determine the optimal radiation fractionation and timing in combination with checkpoint blockade therapy.

Using a similar B16 mouse melanoma model, Lugade and colleagues (15) have shown that a single radiotherapy dose of 15 Gy resulted in the priming of tumor-specific T cells. Given the tendency toward radiotherapy resistance both *in vitro* and *in vivo*, B16 tumor models are adequate for us to address the extrinsic host factor tumor growth in control mice during radiotherapy. We have compared the accumulation of tumor antigen-specific CD8⁺T cells within B16-OVA tumors between a single dose of SABR and fractional radiotherapy (3 Gy \times 5). We found that the numbers of OT-1 CD8 T cells (with TCR specific for OVA antigen) were significantly higher in mice treated with SABR as compared with fractional radiotherapy (H. Dong and S.S. Park; unpublished data). These findings are consistent with the fact that single-dose radiotherapy promotes cross-priming of T cells within primary

tumors (14) but that the magnitude of the elicited T-cell responses is restrained by PD-1 expression following irradiation (28). It is conceivable that upregulation of PD-1 in effector CD8⁺ T cells could result in insufficient control of secondary tumors outside the radiation field. Accordingly, a blockade of PD-1 would protect the effector CD8⁺ T cells from the immunosuppressive effects of PD-1 to achieve antitumor immunity.

Antibodies targeting immune checkpoint molecules on T cells to enhance antitumor immunity have demonstrated promising effects in the control of advanced/metastatic cancers in recent clinical studies (19, 20). PD-1 blockade was shown to be active as a single treatment in melanoma, renal, and lung carcinomas; however, only a small portion of patients achieved a complete response (19). Previous preclinical studies have shown that radiotherapy synergizes with anti-PD-1 or anti-B7-H1 to produce tumor regression in several tumor models (25, 28, 29). Although the combination of radiotherapy and PD-1 blockade has not been reported in the clinic, a case report showed an abscopal effect when radiotherapy was added to an ongoing anti-CTLA-4 therapy in 1 melanoma patient (6). Results from our studies are consistent with clinical predictions of PD-1 blockade, and they support the concept that local radiotherapy can synergize with checkpoint blockade therapy to promote antitumor immunity.

In conclusion, our data could be of significant clinical interest because they suggest that the combination of anti-PD-1 blockade and local radiotherapy can lead to the systemic control of tumors that are refractory to treatment with PD-1 blockade alone. Another potential advantage is that a single SABR and short-term PD-1 blockade may focus the antitumor T-cell response in the targeted tissues and be less likely to elicit an autoimmune reaction in other organs.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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