Stereotactic Radiation Therapy Augments Antigen-Specific PD-1–Mediated Antitumor Immune Responses via Cross-Presentation of Tumor Antigen

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Abstract

The immune-modulating effects of radiotherapy (XRT) have gained considerable interest recently, and there have been multiple reports of synergy between XRT and immunotherapy. However, additional preclinical studies are needed to demonstrate the antigen-specific nature of radiation-induced immune responses and elucidate potential mechanisms of synergy with immunotherapy. Here, we demonstrate the ability of stereotactic XRT to induce endogenous antigen-specific immune responses when it is combined with anti–PD-1 checkpoint blockade immunotherapy. Using the small animal radiation research platform (SARRP), image-guided stereotactic XRT delivered to B16-OVA melanoma or 4T1-HA breast carcinoma tumors resulted in the development of antigen-specific T cell– and B cell–mediated immune responses. These immune-stimulating effects of XRT were significantly increased when XRT was combined with either anti–PD-1 therapy or regulatory T cell (Treg) depletion, resulting in improved local tumor control. Phenotypic analyses of antigen-specific CD8 T cells revealed that XRT increased the percentage of antigen-experienced T cells and effector memory T cells. Mechanistically, we found that XRT upregulates tumor-associated antigen–MHC complexes, enhances antigen cross-presentation in the draining lymph node, and increases T-cell infiltration into tumors. These findings demonstrate the ability of XRT to prime an endogenous antigen-specific immune response and provide an additional mechanistic rationale for combining radiation with PD-1 blockade in the clinic.

Introduction

Ionizing radiation is a locally directed therapy that induces lethal chromosomal aberrations and activates the DNA damage response pathways, including ATM and p53, resulting in cell-cycle arrest and apoptosis or mitotic catastrophe (1, 2). However, radiotherapy (XRT) also activates other signal transduction pathways and transcription factors, including protein kinase C (PKC) and mitogen-activated protein kinases (MAPK; refs. 3, 4), as well as nuclear factor-κB (NF-κB; ref. 5), which is a master regulator of immune responses. Activation of these signaling pathways and transcription factors can result in significant changes to the phenotype of cancer cells before cell death. Supporting this is a growing body of literature demonstrating how XRT can change the immunophenotype of cancer cells and alter how the immune system interacts with cancer cells (6–12). For example, in a study of 23 human carcinoma cell lines treated in vitro with radiation, 91% of the cell lines upregulated one or more of the surface molecules, including Fas, intercellular adhesion molecule-1 (ICAM-1), mucin-1, carcinoembryonic antigen (CEA), and/or major histocompatibility (MHC) complexes, enhances antigen cross-presentation in the draining lymph node, and increases T-cell infiltration into tumors. These findings demonstrate the ability of XRT to prime an endogenous antigen-specific immune response and provide an additional mechanistic rationale for combining radiation with PD-1 blockade in the clinic. More recent data show that CD8 T cells play a key role in the antitumor effect of standard XRT applied to B16 melanoma tumors. Specifically, depleting CD8 T cells reduced the antitumor effect of XRT (13–17). Early data showed that the radiation dose required to control a fibrosarcoma tumor in 50% of mice (TD50) was significantly increased in immunocompromised mice as compared with control mice (13). Conversely, when the immune system was activated with bacterial pathogens, the radiation dose required to control the tumor was significantly reduced (13). More recent data show that CD8 T cells play a key role in the antitumor effect of standard XRT applied to B16 melanoma tumors. Specifically, depleting CD8 T cells reduced the antitumor effect of XRT and decreased survival of mice with melanoma tumors (14, 15). These findings run counter to the conventional paradigm that XRT induces tumor cell kill primarily through DNA damage alone and instead suggest that the immune system may play an underappreciated role in the therapeutic effects of XRT.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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Immunotherapy has recently gained mainstream recognition as a viable anticancer therapy (18, 19). Much of the excitement about immunotherapy revolves around checkpoint blockade using antibodies blocking the negative regulatory molecules cytotoxic T-lymphocyte antigen-4 (CTLA-4) and/or programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1; refs. 20, 21). These blocking antibodies have shown activity in multiple different tumor types, and when combined have shown synergistic effects in metastatic melanoma (22–24). Given that immunotherapy is now a likely fourth pillar in the armamentarium against cancer, additional efforts are required to understand how immunotherapy can be best incorporated with surgery, chemotherapy, and XRT (25). Along these lines, XRT may be uniquely suited to synergize with immunotherapy because it can be delivered precisely to the tumor and may enhance expression of targets for the immune system (8, 26–28). Moreover, several clinical case reports provide evidence of synergy between combined XRT and immune checkpoint blockade (29, 30).

A number of preclinical studies have combined XRT and immunotherapy with intriguing results, including effects outside of the radiation field—termed the abscopal effect. Initial pioneering work by Demaria and colleagues (31) combined XRT with Flt3-L and documented an abscopal effect in contralateral shielded tumors that was immune mediated (31, 32). A subsequent study combined XRT with anti–CTLA-4 antibody in TSA breast carcinoma and MC38 colorectal carcinoma and reported abscopal effects that correlated with the frequency of IFNγ−CD8 T cells (33). Our group previously used the small animal radiation research platform (SARRP; ref. 34) to combine XRT with a cell-based vaccine in an autochthonous model of prostate cancer, and showed an additive treatment effect (35). In addition, we were the first to use the SARRP to deliver stereotoxic XRT combined with anti–PD-1 antibody in a glioma model and reported long-term survival of mice receiving combination therapy (36). Recently, a report combining XRT with anti–PD-L1, an antibody against the ligand of PD-1, demonstrated enhanced efficacy through a cytotoxic T cell–dependent mechanism with a synergistic reduction in tumor-infiltrating myeloid-derived suppressor cells (MDSC; ref. 37). Furthermore, a recent study combining radiation with blockade of PD-L1 demonstrated improved local control, survival, and protection against tumor rechallenge in colorectal and breast cancer mouse models (38). Although these preclinical studies have shown additive or synergistic effects on tumor control, the question of whether XRT-induced priming augments an endogenous antigen-specific immune response, specifically in combination with antibody-mediated blockade of the PD-1 receptor, requires further investigation.

Here, we report that combining XRT with anti–PD-1 antibody results in the development of endogenous antigen-specific antitumor immune responses in models of melanoma and breast cancer in conjunction with enhanced tumor control. Importantly, we identify that the immune responses induced by combined XRT and checkpoint blockade are not limited to T cells and include development of specific B cell–mediated antitumor antibodies. Mechanistically, we found that XRT is capable of increasing immune-cell tumor infiltration and direct presentation of tumor antigens, although in vivo the increase in antigen recognition mediated by XRT likely requires cross-presentation.

Materials and Methods

Mouse strains and cell lines

C57BL/6, BALB/cJ, and MHC class I knockout female 6- to 8-week-old mice were purchased from The Jackson Laboratory. OT1-Rag knockout mice were bred in-house. Animal experiments were performed in specific pathogen-free facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine (Baltimore, MD). MC38-OVA cells were a kind gift from Dr. Mark Smyth (QIMR Berghofer Medical Research Institute, Melbourne, Australia) on April 2013. B16-OVA melanoma cells were obtained from the laboratory of Dr. Hyam Levitsky (Johns Hopkins University) and cultured in RPMI complete media plus G418 selection. 4T1-HA breast carcinoma cells were obtained from Dr. Katharine Whartenby (Johns Hopkins University). B3ZT-cell hybridoma was a kind gift from Dr. Nilabh Shastri (University of California, Berkeley, CA) on September 2013. All tumor cell lines were tested before in vivo use and found to be free of Mycoplasma. In addition, model antigen expression for ovalbumin (OVA) and hemagglutinin (HA) was confirmed by flow cytometry and Western blotting.

Flow cytometry, intracellular cytokine staining, and cytokine analysis

Single-cell suspensions were prepared from spleens, inguinal lymph nodes, and tumors. Cells were stained with fluorescent-labeled antibodies (BioLegend, BD-Bioscience Pharmingen, or eBiosciences) and analyzed by either FACSCalibur or LSR II flow cytometer (BD). The following clones were used: CD4 (RM4–5), CD8 (5H1), CD11c (HL3), Cdlb (M170), CD25 (PG6.1.5), SIINFEKL/H-2Kb (25-D1.16), CD44 (IM7), CD62L (MEL-14), IFNγ (XMG1.2), TNFα (MP6-XT22), FAS (2495), CD40 (3/23), and Foxp3 (FJK-16s). For pentamer staining, H-2Kd (SIINFEKL) or H-2Kd (HYSTVASSL) pentamers were used followed by Pro5 R-PE Fluorotag (ProImmune). For intracellular cytokine staining, cells were activated with OVA peptide-pulsed splenocytes or with PMA (100 ng/mL) plus ionomycin (500 ng/mL) for 4 hours in the presence of GolgIPlug (32), processed with a Cytofix/Cytoperm kit (32), and stained as indicated. Gates and quadrants were set based on isotype control staining.

B3Z assay

B3Z cells were cultured in complete media and split 1:20 every 2 days. Semi-confluent B16-OVA cells were trypsinized and washed in complete media and irradiated using a fixed Cs source Gamma Irradiator with 0, 10, or 20 Gy. B16-OVA cells were washed and then immediately seeded into 24-well plates at 1 × 10^5 cells per well and cultured in complete media + G418 for 36 hours. Wells were washed twice in serum-free media and 5 × 10^5 B3Z cells and/or whole splenocytes were then added to the wells and cocultured for 16 hours. For control experiments, OVA peptide-pulsed splenocytes alone were added to wells 4 hours before B3Z cells. Supernatant was aspirated and IL2 concentrations were measured by IL2 ELISA (eBioscience) according to the manufacturer’s specifications.

Adaptive transfer experiments and CFSE labeling

T cells from spleens and lymph nodes of OT1-Rag knockout mice were washed twice in PBS and labeled in 5 nmol/L CFSE

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Tumor growth experiments and TIL preparation
B16-OVA melanoma and 4T1-HA breast carcinoma models were performed as previously described with some modifications (33, 34). Briefly, on day 0, mice were injected with 1.25 to 5.0 × 10^6 B16 cells intradermally (i.d.) in the back or 2.0 to 5.0 × 10^6 4T1-HA cells subcutaneously (s.c.) in the right flank. Tumor diameter was measured every 2 to 3 days with an electronic caliper and reported as volume using the formula (m_1 × m_2)^2/2. Mice were treated with 200 μg anti–PD-1 antibody via i.p. injection every 3 days for a total of three injections per mouse. To isolate tumor-infiltrating lymphocytes (TIL), solid tumors were excised after 12 to 14 days, single-cell suspensions prepared by mechanical dissociation, followed by density gradient centrifugation on an 80%/40% Percoll (GE Healthcare) gradient.

SARRP irradiation
For in vivo experiments, mice with established palpable tumors were treated using the SARRP previously described by Wong and colleagues (34). The anesthetized mice underwent CT imaging on the SARRP for image-guided localization of the tumor and placement of isocenter before irradiation. The dose rate was 1.9 Gy/min.

Statistical analysis
Statistical analysis was performed using Prism 5 (GraphPad). Unpaired two-tailed t tests were conducted and results were considered statistically significant at P ≤ 0.05 (**), 0.01 (**), and 0.001 (***).

Results
XRT enhances presentation of tumor-associated antigens
Tumor cell lines expressing model antigens, such as OVA or HA, are useful models with which to evaluate the effects of XRT on the development of antigen-specific immune responses. To better understand how XRT affects antigen presentation, we used B16 melanoma and MC38 colorectal carcinoma cell lines that were engineered to express OVA. The gross morphologic effects of XRT on these cell lines were as expected; light microscopy of cell cultures 48 hours after XRT showed increased cell size, an increased nuclear-to-cytoplasmic ratio, and prominent nucleoli (Fig. 1A, top). These effects were mirrored in the increased forward scatter and side scatter of the viable cell population as assayed by flow cytometry (Fig. 1A, bottom). To test whether XRT could result in increased direct (i.e., tumor-cell intrinsic) antigen presentation, we used a commercially available antibody that binds specifically to the immunodominant OVA peptide SIINFEKL complexed within the MHC class I molecule Kb (39–41). Using this reagent, we observed a significant increase in antigen presentation on irradiated cells (Fig. 1B). Although B16-OVA has a lower baseline expression of MHC class I as compared with MC38-OVA, both cell lines upregulated MHC and OVA antigen presentation upon irradiation in a dose-dependent manner (Fig. 1C). As a positive control for the proimmunogenic effects of XRT, we confirmed that XRT upregulated expression of FAS, as previously described (Fig. 1D; ref. 7). As a negative control, we evaluated CD40 expression and found no significant upregulation (Fig. 1D), suggesting that XRT does not lead to a general, nonspecific expression of cell-surface antigens on these tumor cell lines.

Radiation increases T-cell recognition of tumor cells in vitro
To study whether increased MHC–OVA expression secondary to XRT leads to specific T-cell recognition in vivo, we used the B3Z hybridoma (42, 43). These immortalized T cells have a TCR specific for the OVA peptide SIINFEKL, and secrete IL2 in response to antigen recognition in a dose-dependent manner (42). We first verified B3Z function by coculturing these cells with peptide-pulsed, syngeneic splenocytes as antigen-presenting cells (APC), and confirmed their dose-dependent IL2 secretion as assayed by ELISA (Fig. 2A). Surprisingly, XRT of B16-OVA cells in the absence of APC resulted in increased IL2 secretion by cocultured B3Z T cells, showing that XRT enhanced direct antigen presentation from tumor cells (Fig. 2B). In addition to this direct presentation, it is also possible that XRT-induced cell death could result in the release of antigen, which could then be taken up by APCs and cross-presented. To investigate this possibility, we irradiated B16-OVA cells, harvested the cell-free supernatant from those cultures 24 hours after radiation, and pulsed APCs with the cell-free conditioned medium. As shown in Fig. 2C, the addition of conditioned media from irradiated B16-OVA to APCs did not result in detectable IL2 secretion (i.e., activation) from B3Z. Along with the data from Fig. 1, these data suggest that increased direct antigen presentation could be one of the effects of XRT on tumor cells.

Radiation increases T-cell recognition of tumor antigen in vivo by cross-presentation
To further explore the effects of XRT on tumor antigen presentation, we used an in vivo model, in which congenically marked, CFSE-labeled OVA-specific CD8 T cells (OT-1) were adoptively transferred into animals bearing established B16-OVA tumors in their flanks. Using the SARRP (34), animals received 12 Gy of image-guided stereotactic XRT to their flank tumors 1 day before adoptive transfer of CFSE-labeled OT-1, and their draining lymph nodes (DLN) were harvested on day 4. As shown in Fig. 2D, XRT resulted in significantly increased antigen-specific proliferation as measured by CFSE dilution. We tested whether this recognition was proinflammatory by performing intracellular staining for effector cytokines, and found increased expression of IFNγ and TNFα (Fig. 2D and E, and data not shown). Similar levels of specific T-cell activation were noted with a fractionated XRT scheme of 7 Gy × 3 (Supplementary Fig. S1). We next tested whether CD11c+ dendritic cells (DC) in the tumor DLNs were presenting antigen by staining them with the SIINFEKL/Kb-specific antibody used in Fig. 1. For these studies, we used mice that express OVA ubiquitously (CAG-OVA) as a positive control. As shown in Fig. 2F, CD11c+ DCs in the tumor DLNs indeed expressed the specific MHC–OVA complex, suggesting that in addition to direct presentation, in vivo XRT might also augment tumor antigen recognition via cross-presentation. To evaluate the relative importance of direct versus cross-presentation in XRT-
mediated upregulation of tumor antigen recognition, we repeated these studies using MHC class I knockout mice, in which only direct presentation would be possible. In these animals, we observed a reduction in the proliferation of adoptively transferred OT-1 T cells (Fig. 2G). Furthermore, the absence of MHC class I on host cells also abrogated the ability of radiation to enhance activation (i.e., cytokine secretion) of tumor antigen–specific T cells (Fig. 2G). Taken together, these results demonstrate that locally directed XRT can increase the activation and proliferation of an antigen-specific antitumor T-cell population in the DLN and that this effect likely involves cross-presentation via MHC class I–expressing professional APCs. It should be noted that although we did not directly measure the radiation dose to the DLNs in these experiments, in previous studies, we used IHC for γH2AX to demonstrate that the SARP is capable of precisely irradiating a target structure while sparing nearby normal tissues (35).

**Priming of endogenous antigen-specific T cells and B cells by XRT**

To test whether XRT could increase specific recognition of tumors by endogenous (as opposed to adoptively transferred) T cells, we performed antigen-specific pentamer staining in an in vivo treatment model. We first established such a model by treating implanted B16-OVA tumors with either XRT, anti–PD-1, or the combination. For these studies, we chose to block the immune checkpoint PD-1, because of clinical activity, as well as its generally tolerable side effect profile. As previously demonstrated by our group (36) and others (33, 37), the combination of directed XRT and immune checkpoint blockade resulted in increased inhibition of tumor outgrowth (Fig. 3A). Using SIINFEKL–MHC pentamer staining, we next tested whether the combined treatment increased antigen-recognition and drove the expansion of tumor antigen–specific T cells. As shown in Fig. 3B, XRT alone resulted in a moderate increase in OVA-specific T cells in the DLNs and spleens. However, when single-fraction XRT was combined with PD-1 blockade, we observed significant increases in OVA-specific T cells (Fig. 3B and C). Of interest, the increased prevalence of pentamer-positive T cells in the spleens supports the notion that local irradiation can result in the development of a systemic immune response outside of the radiation field. To assay the functionality of the endogenous immune cells induced by either single or combined treatment, we assayed the ability of these cells to prevent tumor outgrowth after adoptive transfer into naïve mice. To perform those studies, we adoptively transferred splenocytes from untreated or treated mice into wild-type (WT) naïve mice and then challenged those animals with primary B16-OVA tumor cells and followed tumor outgrowth. As shown in Fig. 3D, endogenous immune cells primed by XRT + anti–PD-1 were able to significantly delay tumor cell outgrowth in naïve hosts, supporting their functionality. Although previous groups have described the ability of XRT to induce or expand antibodies (44), the relative specificity of those antibodies for tumor cells is less clear. To address that issue, we assayed the ability of XRT + PD-1 blockade to induce OVA-specific antibodies in mice bearing OVA-expressing tumors. As shown in Fig. 3E, although the ability of XRT + PD-1 blockade to induce IgG antibodies to OVA was quite variable, we observed an increased frequency and magnitude of IgG1 OVA-specific antibodies in the sera from animals in the combined treatment group. Taken together, these data suggest that combined XRT + checkpoint blockade can induce tumor antigen–specific responses among endogenous immune cells.

**Combining XRT with PD-1 blockade increases endogenous T-cell infiltration of established B16 tumors**

To assay the effects of increased antigen recognition mediated by XRT + PD-1 blockade on the endogenous T-cell population, we tested whether the combined treatment increased tumor infiltration with either bulk or specific (OVA pentamer–positive) T cells.
As shown in Fig. 4A, untreated B16-OVA tumors or tumors treated with anti–PD-1 alone had scant immune-cell infiltrates and a notable absence of CD4 or CD8 lymphocytes. Remarkably, XRT alone drove a significant intratumoral infiltrate, composed of both endogenous CD4 and CD8 T cells (Fig. 4A, top). Adding anti–PD-1 to XRT further enhanced the percentage of CD8 T cells within B16-OVA tumors, with a significant proportion of these cells being specific for OVA as determined by pentamer staining (Fig. 4A, bottom). To further quantify these changes, we calculated the absolute numbers of CD4 and CD8 T cells per 50,000 gated events. As shown in Fig. 4B and C, XRT alone significantly increased CD4 and CD8 T-cell infiltration, but this was not further increased by the addition of PD-1 blockade. As the immunologic effects of effector cells within the tumor parenchyma likely
represent a balance between regulatory T cell (Treg) and effector T-cell function, we also quantified Treg infiltration, as well as the ratio of CD8:Treg for each of the treatment groups. These data (Fig. 4D and E) show that XRT alone significantly increases the percentage of CD4 TILs that are FoxP3⁺, but that this effect is significantly abrogated by the addition of PD-1 blockade to XRT. Indeed, the combination of XRT + PD-1 blockade results in an increase in the ratio of antigen-specific (OVA pentamer–positive): Treg ratio; this parameter in the tumor microenvironment appears to correlate with the treatment effects shown in Fig. 4A. To further explore the role of Tregs in this model, we performed depletion studies using the anti-CD25 antibody PC-61. As shown in Supplementary Fig. S2, PC-61–mediated Treg depletion added to XRT in tumor control, confirming the notion that Tregs likely attenuate the capacity of XRT to augment antitumor immune responses.

Combining XRT with PD-1 blockade increases endogenous T-cell infiltration of established 4T1 tumors

To investigate whether the ability of combined XRT + PD-1 blockade to enhance an endogenous antigen-specific immune response was confined to the poorly immunogenic B16 cell line, we used an engineered version of the 4T1 breast carcinoma line that expresses HA as a model antigen. Similar to B16-OVA, established 4T1-HA tumors treated with anti–PD-1 or XRT alone showed a significant growth delay (Fig. 5A). However, when XRT was combined with anti–PD-1, we observed significant regression of tumors and tumor control (Fig. 5A). We analyzed the development of antigen-specific CD8 T-cell responses using HA peptide–MHC pentamers and found that either anti–PD-1 or radiation alone resulted in increased HA-specific CD8 T cells (Fig. 5B and C). However, when XRT was combined with anti–PD-1, there was a statistically significant increase in HA-specific CD8 T cells compared with that treated with anti–PD-1 alone. Interestingly, and as was the case for B16-OVA, we found that XRT increased the proportion of intratumoral CD4 T cells with a Treg phenotype, and this increase was abrogated by the combined treatment (Fig. 5D). This XRT-mediated increase in Treg percentages appeared to be a localized phenomenon; we did not observe any increase in Tregs in the DLNs (Fig. 5E) or in the spleens (data not shown) in either the 4T1-HA or B16-OVA models.

Figure 3. Stereotactic XRT combined with anti–PD-1 immunotherapy significantly improves tumor control and enhances development of antigen-specific T-cell– and B-cell–mediated antitumor immune responses. A, tumor volumes measured at day 18 in WT (non–tumor-bearing) or B16-OVA tumor-bearing mice treated as indicated; representative of three independent experiments. B, percentages of CD8⁺ SIINFEKL Pentamer⁺ T cells isolated on day 10 after irradiation from WT (non–tumor-bearing) or B16-OVA tumor-bearing mice treated as indicated; representative of three independent experiments. C, scatter plot quantifying significant increase in SIINFEKL Pentamer⁺ T cells (*, P < 0.05; **, P < 0.005) in mice treated with XRT + anti–PD-1 Abs. D, tumor volumes measured at day 18 in WT mice that received 12 × 10⁶ iv. adoptively transferred splenocytes from mice treated as indicated (harvested on day 14) followed 1 day later by tumor challenge with 2 × 10⁵ B16-OVA cells. E, OVA Ab ELISA measuring concentration of anti-OVA IgG1 present in sera of mice (n = 8–11 mice/group) treated as indicated harvested on day 14 after XRT.

XRT + anti–PD-1 increases tumor-antigen specific central memory cells in the DLN

Our ability to identify tumor antigen–specific endogenous CD8 T cells in two different models afforded us the opportunity to quantify the effects of either single or combined treatment on the phenotype of induced/expanded CD8 T cells. For these studies, we defined naive cells as CD62L⁺ and CD44⁻ (Fig. 6A). In a similar manner, the effector memory phenotype was defined as CD44⁺CD62L⁻, and central memory cells as CD44⁺CD62L⁺ (45). As in previous experiments, we identified and gated on tumor antigen–specific CD8 T cells in the DLNs using pentamer staining (see Supplementary Fig. S3 for gating scheme). In the poorly immunogenic B16-OVA model, we found that XRT
decreased the relative percentage of naïve tumor-specific CD8 T cells and increased the proportion of tumor-specific CD8 T cells with an effector memory phenotype (Fig. 6A and B), but that PD-1 blockade did not significantly add to this skewing. We also observed significant increases in the absolute numbers of tumor-specific effector CD8 T cells induced by XRT alone or XRT combined with anti–PD-1 immunotherapy (Fig. 6C). The phenotype of tumor antigen–specific CD8 T cells was also altered by XRT in the 4T1-HA model (Fig. 6D–F), but here XRT alone was less effective in increasing the absolute number of effector memory CD8 T cells compared with that of combination therapy (Fig. 6F), possibly reflecting differences in the tumor microenvironment and immunogenicity of 4T1 compared with B16 cells. Taken together, these two datasets support the concept that XRT combined with PD-1 blockade increases tumor-specific CD8 T cells with a memory phenotype.

**Discussion**

Tumor cell lines expressing model antigens are useful for studying induction of endogenous antigen-specific immune responses because of the numerous epitope-specific reagents available to detect responses to these model antigens. Here, we used these reagents to test the ability of XRT alone or in combination with immune checkpoint blockade to induce immune responses against OVA expressed by B16 melanoma and HA expressed by 4T1 breast carcinoma. Studies from several research groups have shown that XRT increases the expression of MHC class I in a dose-dependent manner in a number of different tumor types (7, 12). This is a critically important finding because many tumor types, including melanoma, may downregulate MHC expression to protect from CD8 T cell–mediated cytotoxicity. By upregulating MHC, XRT may prevent tumor cells from remaining undetected by CD8 T cells. Indeed, we found that XRT increased the expression of specific tumor model antigenic epitopes presented in MHC on the cell surface (Fig. 1). The H2kb-SIINFEKL antibody stains for the direct targets of CD8 OT1 T cells or endogenously generated CD8 T cells specific for the SIINFEKL epitope. Thus, upregulating the targets for CD8 T cells is one mechanism by which XRT directly increases the susceptibility of tumor cells to CD8 T cell–mediated cytotoxicity. One potential limitation of model antigen systems like these is that the relative
immunogenicity of exogenously introduced antigens such as OVA or HA could render them more susceptible to immunologic intervention. For B16, this is unlikely; implanted B16 and B16-OVA have similar growth curves in vivo (data not shown), but it does remain a minor concern for 4T1-HA. The interpretation of these studies should also be tempered by observation that both B16 (46) and 4T1 (47) express PD-L1 in vivo, whereas expression of PD-L1 in human tumors is variable.

In addition to upregulation of MHC class I, multiple other potential mechanisms exist by which XRT can increase tumor-cell susceptibility. Upregulation of FAS (7) could increase tumor-cell susceptibility to undergoing FAS-L-mediated apoptosis (Fig. 1D). Interestingly, FAS-L-mediated induction of apoptosis could be triggered by tumor-specific immune cells as well as bystander immune cells that are not necessarily specific for tumor antigens. Taken together, these observations would support the hypothesis generated by previous studies that the ability of XRT to control certain tumors depends on the general immune status of the host (13), and more specifically on CD8 T cells (14, 15).

The B3Z T-cell hybridoma is an important cell line for probing the expression of OVA antigen presented by tumor cells or other engineered cell lines. The B3Z T-cell hybridoma is selected to secrete IL2 in a dose-dependent manner upon TCR ligation with MHC loaded with SIINFEKL epitope, and thus is similar in some respects to an immortalized OT-1 cell line. Here, we adapted this cell line to quantify the level of OVA antigen presentation in tumor cells after irradiation. We confirmed that XRT increases direct tumor-mediated cell–cell antigen presentation as measured by IL2 ELISA (Fig. 2). This raised the question whether XRT could potentially convert tumor cells into APCs capable of priming immune responses. Using an in vitro assay coculturing irradiated B16-OVA melanoma cells with naive CFSE-labeled OT-1 T cells, we found no evidence of T-cell priming or proliferation (data not shown). Furthermore, using MHC class I knockout mice in which only the injected B16-OVA tumor cells were capable of presenting antigen to adoptively transferred OT-1 T cells, we confirmed that the ability of XRT to enhance T-cell proliferation in the DLN requires MHC class I–expressing APCs. This raises the intriguing question of whether stereotactic XRT, which does not generally include the DLNs, may be superior to larger field or conventional XRT in terms of inducing antitumor immune responses. One limitation of these results is that we did not specifically quantify the radiation dose to the DLNs, although in previous studies, we were able to target the prostate gland without irradiating either the bladder or DLNs (35). Studies in which the DLNs are specifically targeted or spared are ongoing to more precisely address this issue.

One potential downside of increased direct tumor cell–mediated antigen presentation is increased T-cell anergy or conversion of naïve T cells to Tregs. Indeed, we did observe that XRT alone increased the relative percentage of Tregs in the tumor microenvironment but not in the DLN (Figs. 4 and 5). Increased Tregs would be deleterious to antitumor immune responses, and thus we investigated strategies to block Tregs using PC61-depleting antibody. We observed increases in MHC class I–expressing APCs; however, using MHC class I knockout populations and B16-OVA tumor control when XRT was combined with Treg depletion (Supplementary Fig. S2). It remains to be determined whether this increase in Tregs is a general
phenomenon of XRT for multiple tumor types. However, strategies combining XRT with Treg-depleting antibodies, such as anti-GITR or low-dose cyclophosphamide, may be worthy of further clinical investigation.

The presence of immune-cell infiltrate has been correlated with improved patient outcomes in multiple different solid tumors, including melanoma, colorectal, breast, and prostate carcinoma (48–51). In fact, the type and location of immune cells in human...
colorectal cancer was previously reported to be a better predictor of survival than traditional stage groupings [48]. Similarly, the absence of immune-cell infiltrate has been associated with worse patient outcomes, possibly as a sign of tumor immunosuppression or physical barriers to immune-cell migration and tumor infiltration. Thus, strategies that enhance immune-cell engagement and infiltration into tumors could potentially result in significant clinical benefit. Here, we demonstrated that intact, untreated B16 melanoma has a relatively scant immune-cell infiltrate consistent with the poorly immunogenic nature of this tumor line (Fig. 4A). XRT of B16 melanoma significantly increased both CD4 and CD8 T-cell infiltrates (Fig. 4). Interestingly, simply driving recruitment of T cells into a tumor is likely not sufficient to activate an antitumor immune response given the immunosuppressive tumor microenvironment. Indeed, analysis of the CD4 T-cell population showed that the majority of these cells are CD25+FoxP3+ Tregs (Figs. 4D and 5D). However, the combination of XRT and anti–PD-1 immunotherapy altered the ratio of CD4 to CD8 T cells and resulted in decreased percentages of CD4 Tregs and absolute increases in CD8 T-cell populations, likely as a result of diminished inhibitory signaling from the PD-1 pathway. These findings provide a clear rationale for combining XRT with checkpoint blockade immunotherapy and further work is certainly warranted to determine whether these findings hold true in human tumors.

Here, we chose to study radiation combined with anti–PD-1 checkpoint blockade primarily because a drug blocking the PD-1 pathway (pembrolizumab; Merck) has recently been approved by the FDA and additional agents blocking the PD-1 pathway are in phase III clinical trial development. Given that these agents are generally well tolerated with favorable adverse event profiles, strategies incorporating anti–PD-1 immunotherapy with current treatment modalities, including chemotherapy, targeted therapies, and/or XRT, are of critical importance. To this end, data suggest that XRT may be uniquely suited to combine with checkpoint blockade immunotherapy and specifically PD-1 blockade. Recently, there have been reports that radiation alone can result in increased expression of PD-1 ligands on the surface of tumor cells [37, 38], including melanoma and breast cancer model cell lines. Upregulation of PD-1 ligands might serve to dampen effector immune responses and potentially counteract the positive immunogenic effects of radiation such as increased MHC expression, antigen presentation, and immune-cell infiltration. Thus, concurrent blockade of the PD-1 pathway may synergize with radiation by specifically blocking a counterproductive effect of radiation on immune responses.

Taken together, these data have several clinical implications. First, these findings clearly support the coadministration of XRT with checkpoint blockade immunotherapy to improve local tumor control. The trend toward increased Tregs in the tumor after XRT alone suggests that strategies combining Treg depletion with XRT may have additional clinical benefit. Furthermore, the increase in antigen-specific T cells and antitumor antibodies with concurrent XRT and anti–PD-1 immunotherapy suggest that the combination may also potentially aid in systemic or distant tumor control via the abscopal effect. Finally, the requirement of host APCs and cross-presentation for XRT to enhance immune responses raises the intriguing hypothesis that stereotactic XRT could be superior to conventional field XRT by sparing irradiation of DLN regions. These data provide a preclinical rationale for evaluating these questions in prospective clinical trials.

Disclosure of Potential Conflicts of Interest
C.G. Drake reports receiving commercial research grants from Bristol-Myers Squibb and Janssen; has ownership interest (including patents) in NestImmune and Compugen; and is a consultant/advisory board member for Bristol-Myers Squibb, Compugen, Novartis, Potenza Therapeutics, Roche/Genentech, and Janssen. No potential conflicts of interest were disclosed by the other authors.

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References


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