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Critical Review

# Generating antitumor immunity by targeted radiation therapy: Role of dose and fractionation

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**Abstract** Accumulating evidence supports the role of radiation therapy in the induction of antitumor immunity. With recent advancements in stereotactic radiation therapy, there is increasing appreciation that, when combined with immune checkpoint blockade, the type of radiation dose and fractionation regimen selected may both influence local tumor control and also affect the generation of immune responses that are important for systemic control. Although a broad range of radiation dose and fractionation schema have been tested in both the preclinical and clinical settings, recent preclinical evidence suggests the existence of a dose per fraction threshold beyond which radiation becomes less effective in generating tumor immune responses. Such a threshold seems to be tumor dependent, probably reflecting different genetic mutations of cancer. In this review we discuss the key preclinical and clinical evidence relating to radiation dose and fractionation considerations. Future clinical trials should focus on identifying optimal radiation dose and fractionation schedules, which may depend on the clinical context.


## Introduction

Radiation therapy (RT) is an integral component of modern oncology care, spanning a broad range of indications from palliative to definitive intent therapy. Historically, radiation has been viewed almost exclusively as a local modality. From early radiobiological studies, the major mechanism of action of radiation has been found to be mediated by DNA damage, leading to the death of irradiated cells mostly at the time of cell division. However, a growing body of evidence in both the preclinical and clinical settings have yielded important insights on other radiation effects that can be sensed by both the innate and adaptive immune system. In some cases, radiation-induced antitumor effects contribute to cross priming and succeed at eliciting an immune response against the tumor.

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From classical radiobiology, it has been well established that RT exerts different effects when given at different dose-fractionation schemes, an observation that is summarized in the principles termed the 4 Rs: repair, reassortment, reoxygenation, and repopulation. These principles have provided the rationale that underlies most dose-fractionation strategies used in radiation oncology today. In addition, there is now recognition that cells of different origins respond differently to radiation even if all other variables are the same, pointing to an intrinsic property of the cell described as the fifth R: radiosensitivity. All of these properties are neatly encapsulated in the $\alpha/\beta$ ratio, which describes the curvature of the cell survival curve and directly provides an assessment of how sensitive a tumor (or target tissue) is to radiation fractionation. Tumors with a low $\alpha/\beta$ ratio are considered relatively resistant to low doses of radiation per fraction, thus implying that hypofractionated radiation (ie, fewer fractions at larger doses per fraction) would be more effective at achieving cell killing and tumor control. Conversely, normal tissues generally exhibit a high $\alpha/\beta$ ratio for acute side effects and thus are sensitive to relatively low doses of radiation per fraction, rendering a standard (conventionally fractionated) or hyperfractionated strategy more appropriate for normal tissue sparing.

Studies to optimize radiation dose and fractionation have explored a variety of altered fractionation regimens with the goal of improving the therapeutic ratio. In settings when the $\alpha/\beta$ ratio is estimated to be low (eg, prostate or breast carcinomas), hypofractionated radiation has been investigated. The advent of advanced image guidance as an integral part of radiation treatment delivery has facilitated the adoption of hypofractionated, high-dose radiation regimens in the form of stereotactic body RT (SBRT). Whether cell killing and tumor control using SBRT can be adequately described by the classical linear quadratic model, particularly when $<3$ fractions are used, has been a matter of debate. Regardless of the exact radiobiological principles at hand, multiple lines of evidence now demonstrate that excellent local control rates are achieved with SBRT across a broad range of clinical settings, leading to its widespread adoption.

SBRT has also been tested in selected patients with oligometastatic disease, in whom it has occasionally resulted in durable control with long progression-free intervals.[1] Another interesting application of SBRT in the metastatic setting of cancer is its combination with modern immunotherapy. Out-of-field effects of RT are a rare phenomenon, originally defined by Mole et al[2] and known as the abscopal effect, and illustrate the generation of a clinically significant response at a distant metastatic site. A thorough review of reports of abscopal effects from radiation identified a total of 35 cases over 45 years.[3] The immunologic nature underlying the abscopal effect has been reported in the preclinical setting.[4–8] A plausible explanation of the rarity of abscopal response despite the demonstrated proimmunogenic effects of focal RT is the strong immunosuppressive microenvironment that characterizes established cancers.[9] The availability of immune checkpoint blocking agents has enabled their testing in combination with RT, helping to potentiate tumor-directed immune responses in a clinically significant manner.

One of the central issues in optimizing radiation and immunotherapy combinations is how to identify the best radiation dose-fractionation regimen. In contrast to clinical experience suggesting that a higher biologically effective dose (BED) may improve clinical outcomes (local control) across multiple tumor types,[10] when RT was combined with immune checkpoint blockade to induce a systemic effect, a threshold dose to the generation of effective antitumor immunity was identified in the preclinical setting.[11]

In this review article we discuss the preclinical and clinical evidence regarding dose-fractionation considerations for RT and the influence of these variables on the generation of effective antitumor immunity.

## Radiation-induced immune responses

Several lines of investigation have provided a greater understanding that not only does radiation directly influence tumor immunity, it also exerts its effects via a series of distinct mechanisms. These mechanisms include triggering immunogenic cell death,[12] generating neoantigens and enhancing antigen processing and cross presentation,[13] decreasing immunosuppression in the tumor microenvironment,[14,15] overcoming T-cell exclusion from the tumor microenvironment,[16] and increasing tumor cell recognition by the immune system.[13]

RT has been found to directly increase the immunogenicity of tumor cells by increasing the translocation of calreticulin to the tumor cell surface, the extracellular release of high mobility group protein 1, and the extracellular release of adenosine triphosphate, leading to immunogenic cell death.[12] Additionally, the use of radiation has been found to generate neoantigens and could enhance antigen processing.[13] Third, radiation has been found to reduce the degree of immunosuppression in the tumor microenvironment, in part because of the production of cytokines such as type I interferon.[17,18] In addition, radiation can decrease immunosuppressive components in the tumor microenvironment,[15] including T regulatory cells, myeloid-derived suppressor cells, and tolerogenic dendritic cells. It is also known that radiation can reprogram tumor-associated macrophages to convert an immunosuppressive tumor microenvironment to one that is more favorable for tumor immunity.[14] Fourth, radiation treatment may help to overcome T-cell exclusion from the tumor microenvironment by normalizing the

vasculature and increasing production of immune-attractant chemokines such as CXCL16.[16,19] Lastly, radiation treatment leads to greater recognition of the tumor by the immune system. Radiation has been found to lead to downregulation of CD47 on tumor cells[20] and upregulation of MHC class I[18,21] with enhanced degradation of existing proteins, enhanced peptide production, and increased antigen presentation.[13]

## Preclinical evidence

### In vitro data

Radiation treatment can increase the immunogenicity of tumor cells by increasing their surface expression of MHC class I complexes, and this effect has been found to be dose dependent.[13] Across a single-fraction dose range of 1 to 25 Gy, Reits et al[13] found that higher radiation dose leads to increased degradation of proteins, leading to an increased intracellular peptide pool. In addition, higher radiation dose leads to increased activation of the mammalian target of rapamycin pathway, triggering increased peptide production and antigen presentation. Lastly, higher radiation dose also leads to the generation of novel proteins as a consequence of DNA damage, thus allowing the formation of neoantigens and presentation of neoepitopes on MHC class I molecules. As a proof of concept, these investigators found in their MC38 murine colorectal adenocarcinoma model that neither adoptive transfer of gp70-specific cytotoxic T lymphocytes nor radiation (10 Gy) alone was sufficient to cure mice bearing MC38 tumors, but radiation followed by adoptive transfer of cytotoxic T lymphocytes led to significant regression of all tumors, including cure in 63% of treated mice. Although a dose titration was not performed in their in vivo tumor experiment, it nevertheless was consistent with the role of radiation in increasing tumor immunogenicity.

Subsequently it was reported that increasing radiation dose increases the induction of immunogenic cell death.[22] In TSA murine mammary carcinoma cells radiated with single-fraction doses ranging from 2 to 20 Gy, there was a dose-dependent increase in the release of adenosine triphosphate into the extracellular matrix, translocation of calreticulin to the cell surface, and release of high mobility group protein 1 from dying tumor cells, all of which are key components of immunogenic cell death.[12]

### Animal models

The preclinical evidence supporting a radiation-induced systemic immune response falls mostly to the evolving story of the abscopal phenomenon in immunocompetent mouse models.[4,5] In general there has been significant heterogeneity in how preclinical studies are conducted, and relatively few include a rigorous comparison of outcomes after different dose-fractionation regimens.[23] A wide range of murine tumors have been tested, including mammary carcinomas (TUBO, FM3A, TSA, 4T1, 67NR), colon carcinomas (MCA38, HCT116, Colon 26), sarcomas (MethA, T241, MCA205), lung carcinoma (LLC), squamous cell carcinoma (VII), cervical carcinoma (C3), and melanoma (D5). Most of these tumor models are implanted heterotopically in a flank location, allowing for partial body irradiation and monitoring of the tumor response at a nonirradiated site. Another distant (nonirradiated) tumor readout used in a few studies is the incidence of distant metastases, often in the lung or liver.

In an early experiment an abscopal response was identified against 67NR murine mammary carcinomas when radiation was given with Flt3 ligand.[4] In this study there was no significant difference in the identified abscopal response at a single-fraction dose of 2 Gy or 6 Gy, suggesting no significant difference in the generation of systemic immune responses against this tumor across this single-fraction dose range in the presence of Flt3 ligand. When looking at single-fractionation strategies in the preclinical setting, prior studies have reported abscopal effects with single-fraction doses up to 60 Gy.[24] However, no dose titration was specified in most of these studies, and thus it remains unclear whether there is a significant dose response to justify the extremely high radiation doses.

Subsequently, different groups of investigators have approached preclinical studies from different angles. There have been conflicting preclinical results regarding whether a high-dose, single-fraction approach is superior in generating an abscopal response compared with a moderate- or low-dose, multiple-fraction approach. In one study, multiple dose-fractionation regimens (20 Gy × 1, 8 Gy × 3, and 6 Gy × 5) were compared for abscopal effects against TSA mammary carcinomas.[7] Although all 3 radiation approaches limited primary tumor growth to a similar extent, the fractionated regimens were able to generate a greater abscopal response in the nonirradiated tumor. A parallel experiment found that the multiple-fraction approach of 8 Gy × 3 (in combination with anti-CTLA-4 mAb) was able to generate a greater abscopal response against MCA38 colorectal adenocarcinomas than the single-fraction approach of 20 Gy × 1. However, other investigators have found that a single-fraction approach was superior for the generation of systemic antitumor responses. In one study there was a greater degree of immune activation in tumor-draining lymph nodes when mice with B16-F0 tumors were treated with 15 Gy × 1 rather than 5 Gy × 3, even though both reportedly had a marked effect on tumor growth.[25] In another study it was reported that an ablative radiation regimen of 20 Gy × 1 was superior to 5 Gy × 4 (given over 2 weeks) in terms of generating an antitumor immune response in a B16-SIY melanoma model.[8] One of

the problems with this comparison is the radiobiological inferiority of 5 Gy × 3 to 4; equivalence to a single dose of 15 to 20 Gy is usually achieved with 8 to 9 Gy × 3 or 6 Gy × 5 given within 1 week. In addition, these studies were conducted in the setting of strongly immunogenic tumor rejection antigens (OVA and SIY, respectively), which may not recapitulate what would typically occur with weakly immunogenic self-antigens in the clinical setting. Most importantly, neither of these studies used an immune-modulating agent (eg, CTLA-4 mAb) alongside RT.

Another preclinical report compared 2 different fractionated regimens, observing that the abscopal antitumor response was significantly more potent when mice bearing LLC tumors were treated with 10 Gy × 5 rather than 2 Gy × 12.[26] Again, these radiation dose-fractionation schemes differ significantly not only in terms of their total dose but also because of the BED. Thus it is not clear if the enhanced abscopal effect from the 10 Gy × 5 regimen simply reflected greater BED, greater total dose, greater dose per fraction, or some combination of these factors.

## Elucidating the cellular mechanisms underlying the abscopal response

Preclinical insights culminated recently in a direct comparison of subablative hypofractionated radiation versus ablative single-fraction SBRT. This study identified a threshold dose of >10 to 12 Gy, beyond which there was an increase in immunosuppression and loss of the abscopal effect.[11] In a variety of tumor models, including the murine TSA and MCA38 models, the use of a single large radiation dose of 20 to 30 Gy, in combination with anti–CTLA-4 blockade, led to reduced tumor immunogenicity and loss of the abscopal effect compared with a moderate subablative dose of 8 Gy × 3.[11] It was noted that at very high radiation doses (>10-12 Gy) per fraction, tumor cells release greater quantities of double-stranded DNA into the cytosol, triggering upregulation of the DNA exonuclease Trex1. Greater Trex1 expression then leads to increased clearance of double-stranded DNA from the cytosol. Decreased cytosolic DNA levels lead to decreased binding to cyclic guanosine monophosphate–adenosine monophosphate (GMP-AMP) synthase and decreased production of cyclic GMP-AMP. As a consequence, there is decreased binding of cyclic GMP-AMP to the protein stimulator of interferon genes, leading to decreased interferon regulatory factor 3 phosphorylation and nuclear translocation, thus decreasing the transcription of inflammatory genes such as type I interferon.

## Radiation effects on the tumor microenvironment

In addition to the influence of radiation dose-fractionation on tumor cells, there is also preclinical evidence that the radiation dose used may generate different effects in the tumor microenvironment. It is known that low radiation doses of 2 Gy can promote inducible nitric oxide synthase expression by tumor-associated macrophages, suggesting that a proimmunogenic environment can be induced by radiation treatment at a low dose.[14] In contrast, higher radiation doses have been found to result in tumor infiltration by protumorigenic macrophages,[27] suggesting that there may be a window of radiation dosing that is most effective in supporting tumor immunity. There is also evidence that single radiation doses of 5 to 10 Gy cause relatively mild vascular changes, but doses >10 Gy cause significant vascular damage and reduce vascular flow as a result of endothelial cell death, leading to reduced effector T-cell recruitment to the tumor.[28]

## Lessons learned from preclinical evidence

The preclinical evidence to date suggests that abscopal effects are generally more prominent at larger fraction sizes, thus supporting hypofractionation, although there is a demonstrated dose threshold beyond which the frequency of such effects declines, and conflicting evidence exists on whether single or multiple fractions are more effective. Lessons learned from preclinical evidence have indicated that the optimal dose-fractionation regimen must take into account not only the best strategy to elicit an immunogenic cell death[12] but also the optimal strategy to establish a proimmunogenic tumor microenvironment.

# Clinical evidence

## Clinical investigations of abscopal response

In the clinical setting there has been a constellation of radiation dose-fractionation schema either in conjunction with immunotherapy or with radiation delivered as a single modality.[29–36] A recent review found a total of 46 abscopal cases reported in 31 articles with a median dose of 31 Gy (range, 0.45-60.75 Gy) and a median dose per fraction of 3 Gy (range, 0.15-26 Gy).[3] Although the primary mechanism remained unclear in the setting of early case reports, the immune system as primary mediator of abscopal response has subsequently been proven in clinical investigations. To date, no clinical studies have directly compared different dose-fraction regimens in their ability to elucidate optimal abscopal responses. Thus, from a clinical perspective, there has been a divergence between ablative versus subablative radiation dose fractionation approaches, and the resultant immune responses are likely to be varied.

Early in the clinical investigations of immune-mediated tumor regression, it became clear that the likelihood of achieving abscopal response would be greatest

with the combination of an immune adjuvant—a conclusion based in the hypothesis that a bolstered immune response mounts tumor responses both locally and distantly.

In a recently published post hoc analysis of patients with advanced non-small cell lung carcinoma (NSCLC) treated on KEYNOTE-001, Shaverdian et al[37] found that patients who received any RT before pembrolizumab had a significant improvement in median overall survival (OS) compared with those who never received RT (10.7 months vs 5.3 months; hazard ratio [HR], 0.58; $P = .026$).[37] Additionally, patients who received prior RT had a significantly longer median progression-free survival (PFS) compared with those who never received RT (4.4 months vs 2.1 months; HR, 0.56; $P = .019$). However, this analysis could not account for whether different dose-fractionation options led to different immune or clinical effects in this patient population. Although interpretation of the findings is limited by the post hoc nature of this analysis and the lack of immune correlative studies, it does raise the interesting possibility that one mechanism to explain the benefit of RT before pembrolizumab is the generation of effective antitumor immunity by RT; pembrolizumab then potentiates this response in a clinically significant manner, thus leading to improvements in PFS and OS endpoints in this patient cohort.

Two landmark case reports in 2012 and 2013, led by Postow et al[38] and Golden et al,[39] respectively, reported the clinical achievement of abscopal response when immunotherapy was combined with radiation. Both cases used anti-CTLA-4 therapy (ipilimumab) and subablative hypofractionated dose regimens (9.5 Gy × 3 [38] and 6 Gy × 5 [39]). Providing further evidence, 2 subsequent trials by Seung et al[40] and Golden et al[41] combined immunotherapy with differing dose-regimens to assess abscopal regression. In the ablative study by Seung et al,[40] a phase 1 trial of interleukin 2 combined with 1 to 3 fractions of 20 Gy was tested in the setting of metastatic renal cell carcinoma and melanoma. Five of eight patients with melanoma (62.5%) experienced either complete or partial response in nonirradiated lesions, whereas both patients with renal cell carcinoma experienced a partial response, a response rate superior to that achieved with interleukin 2 alone.[40] A "proof of principle" study led by Golden et al[41] delivered subablative dosing to a metastasis in patients with metastatic solid tumors (breast, NSCLC, thymic cancer) in combination with granulocyte-macrophage colony-stimulating factor and achieved similarly successful abscopal results.

In subsequent studies a variety of different radiation fractionations and dose regimens were explored in combination with immunotherapy, leading to a range of immunologic responses. These include investigations by Hiniker et al,[42] who used a mixed range of both moderately hypofractionated (2.5-3 Gy × 10-15 fractions) and subablative SBRT doses in combination with anti-CTLA-4 therapy for patients with metastatic melanoma with an overall response (complete and partial) of 27% in unirradiated lesions. A similar rate of abscopal response (27%) was found in the recently reported iMOSART (pembrolizumab immunotherapy and multi–organ site ablative SBRT in patients with advanced solid tumors) phase 1 study.[43] Tang et al[44] evaluated multiple ablative SBRT dose fractionation regimens (BED ranging from 96 to >100) for lung and liver metastases and found partial responses in unirradiated tumors in 10% of patients but no complete responses.[44]

A recently published study by Luke et al[45] evaluated the feasibility of pembrolizumab and ablative SBRT in patients with metastasis from various solid tumors. Any tumor volume was potentially eligible, but for larger tumors only a portion of the gross tumor volume (up to 65 mL) was targeted with ablative SBRT. Despite the use of an "ablative" dose of SBRT, only 1 of the 68 evaluable patients achieved an in-field CR. The ablative dosing used by Luke et al[45] resulted in 1 patient achieving a complete response in unirradiated tumor. The authors note the overall response rate (ORR) reported is comparable to that achieved in earlier preclinical models evaluating for abscopal response[46,47] and the 26.9% ORR from the Golden et al[41] "proof of principle" trial in the nonirradiated lesion per Response Evaluation Criteria in Solid Tumors. However, it should be noted that pembrolizumab monotherapy has about a 10% response rate, whereas immune adjuvants such as granulocyte-macrophage colony-stimulating factor are associated with no clinically significant responses when given as monotherapy in solid tumors. There are likely further dimensions to this finding, given the heterogeneous nature of radiation treatment planning: It is unknown whether portions of the target lesion in the subablative dose range exhibited a greater immune response compared with those falling within the ablative dose range. This study suggests a need for prospective comparisons of ablative versus subablative dose regimens to define how effectively they achieve abscopal responses. It also invites a reassessment of the dose ranges considered "ablative" in heavily pretreated patients and whether "ablation" is the appropriate descriptor in these patients.

Most recently, investigators from the Netherlands Cancer Institute presented results from their PEMBRO-RT study (NCT02492568), a phase 2 trial of patients with advanced NSCLC after at least 1 prior line of systemic therapy who were randomly assigned to receive SBRT (8 Gy × 3) to 1 lesion followed by pembrolizumab within 7 days or to receive pembrolizumab alone.[48] At the time of their presentation, 72 patients had been enrolled and 64 were evaluable for the primary endpoint of ORR. Compared with an ORR of 19% in the control arm (pembrolizumab monotherapy), the use of SBRT to 1 lesion increased ORR to 41%; median PFS was 1.8 months in the control arm and 6.4 months in the

experimental arm ($P = .04$). Significantly, there was no increase in treatment toxicities: grade 3+ toxicities were noted in 22% of control patients and 17% of experimental patients. The findings from this study lend further support to the premise that a subablative SBRT regimen in combination with pembrolizumab can successfully elicit clinically significant abscopal effects, leading to a doubling of response rates in nonirradiated lesions. Although highly promising, there was one cautionary note from these investigators' results: In post hoc analysis, it was noted that the proportion of PD-L1–positive tumors was different between treatment groups because this variable was not used for initial patient selection or stratification. Because strongly PD-L1–positive tumors are expected to respond more robustly to immunotherapy,[49–51] this confounding variable could potentially account for at least some of the reported ORR differences.

## Lessons from a negative trial of RT and immunotherapy

It should be acknowledged that there is also evidence that RT combined with immune checkpoint blockade may not be beneficial across all clinical scenarios and endpoints. In the phase 3 CA184-043 trial of patients with metastatic castration-resistant prostate cancer who received bone-directed RT (8 Gy × 1, up to 5 lesions) and then were randomly assigned 1:1 to either ipilimumab (10 mg/kg every 3 weeks for 4 doses) or placebo, the addition of ipilimumab to RT did not result in a significant improvement in OS as the primary endpoint.[52] The OS curves were noted to cross, a violation of the proportional hazards model and implying that OS may not be an ideal clinical endpoint or a useful surrogate for the generation of potentially clinically significant tumor-directed immune responses. In support of this possibility, the addition of ipilimumab to RT did result in significantly prolonged PFS (median of 4.0 months vs 3.1 months; HR, 0.70; $P < .0001$) and led to a higher frequency of post-treatment prostate-specific antigen reductions (13.1% vs 5.2%). In addition, post hoc analysis indicated that there was an improvement in OS with RT + ipilimumab in patients with good prognostic features (alkaline phosphatase <1.5 times upper limit of normal, hemoglobin >11 g/dL, and no visceral metastases), suggesting that there are subsets of favorable patients who may still benefit in the primary endpoint of OS and that measures of tumor burden and/or performance status are important to select patient populations that may benefit from RT + immunotherapy combinations. A high tumor burden may be inherently immunosuppressive, and even if an immune response could be successfully generated in this setting, it may not be able to overcome the high tumor burden to achieve clinically meaningful endpoints (eg, survival endpoints). The radiation target may also be an important consideration: Perhaps the bone microenvironment (or bone metastases specifically) is not as conducive to immune priming. Lastly, the overall negative findings from this trial could be interpreted as reinforcing the critical importance of dose and fractionation: Here patients received only 8 Gy × 1, a palliative dose known to be inferior for palliation control.[53] No additional dose-fractionation regimens were tested in this trial. One possibility is that the dose per fraction could be sufficient, but the total dose may be inadequate for the generation of robust tumor-directed immune responses.

## Future directions

Emerging preclinical and clinical data continue to support radiation as a critical component in an immunotherapeutic regimen. Experimental and early clinical models have yet to confirm the optimal dose and fractionation scheme necessary to induce an abscopal response. However, both preclinical and clinical evidence continue to support hypofractionation, with an upper threshold to the fractional dose beyond which abscopal responses are less likely to occur. As further exploration in this area is performed, several considerations should be integrated, including the selection of the best endpoint (eg, local vs distant tumor control, or survival outcomes) as well as the ideal immunotherapy combination. Additionally, with further research the various implications of tumor type, tumor mutational burden, and host immune context (including prior and/or concurrent treatments) will become an increasingly important points of focus. The optimal radiation type (eg, photon vs charged particles) will need further exploration, as will the local and systemic implications of dose rate and frequency of delivery (daily, every other day, or once a week). Ultimately our current interpretation of what may be considered the "best" dose fractionation schemes will be limited until dedicated randomized studies comparing these concepts in combination with immunotherapy are pursued.

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