Postablation Modulation after Single High-Dose Radiation Therapy Improves Tumor Control via Enhanced Immunomodulation
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ABSTRACT

Purpose: Radiotherapy (RT) is frequently used for local control of solid tumors using equal dose per fraction. Recently, single high-dose radiation has been used for ablation of solid tumors. In this report, we provide a novel immunological basis for radiation dose fractionation consisting of a single high-dose radiotherapy, followed by postablation modulation (PAM) with four daily low-dose fractions (22 Gy + 0.5 Gy × 4) to reprogram the tumor microenvironment by diminishing immune suppression, enabling infiltration of effector cells and increasing efficacy of tumor control.

Experimental Design: Palpable 3LL and 4T1 tumors in C57Bl/6 and Balb/c mice were irradiated with the Small-Animal Radiation Research Platform irradiator, and tumor growth and survival were monitored. Immunomodulation of tumor and immune cells in vitro and in vivo characterization of tumor-infiltrating immune effector cells were performed by FACS. For systemic application of PAM-RT, whole-lung irradiation was administered in 4T1-bearing Balb/c mice.

Results: We report significant tumor growth delays and increased survival in 3LL tumor–bearing mice with PAM. Primary tumor PAM-RT increased infiltration of immune effector cells and decreased Treg in irradiated tumors and secondary lymphoid organs. In a model of murine metastatic breast cancer (4T1), we demonstrated that systemic PAM-RT to the whole lung, 12 days after primary tumor ablative radiotherapy, increased survival with suppression of pulmonary metastases.

Conclusions: We provide a novel immunologic basis for radiation dose fractionation consisting of a single high dose of radiotherapy followed by daily low-dose PAM-RT fractionation to improve the immunogenic potential of ablative radiotherapy.

Introduction

Currently over 50% of solid malignancies are treated with radiotherapy. Historically, radiotherapy fractionation was developed to reduce toxicity on the basis of DNA repair in normal tissues with regimens divided into daily fractions of equal doses of radiation over a prolonged period of time (5–9 weeks). The advent of image-guided radiotherapy techniques led to the introduction of stereotactic body radiotherapy (SBRT), where short course (1–5 fractions) of high ablative doses of radiotherapy can be safely delivered to a small, well-defined target with high accuracy and steep dose gradients. These capabilities for hypofractionation, delivery of fewer than 7 fractions, open up the radiation oncology field to questions on dose variation and scheduling.

Radiation’s efficacy is primarily through direct cell death occurring after dose-dependent damage to DNA and DNA repair mechanisms, resulting in senescence, mitotic catastrophe, apoptosis, and necrosis of treated tumor cells (1, 2). This genotoxic stress can sensitize cells to immune killing and lead to an in situ vaccination through immunogenic cell death (ICD), one of the first steps in antitumor immunity (3–5). Preclinical radio-curability of transplantable tumors depends upon an intact immune system (6) and local control of irradiated tumors depends upon the induction of this adaptive immunity, especially of CD8⁺ CTLs (7, 8). Ablative, or lethal, radiotherapy, has immunomodulatory properties that, dependent upon dosage, fractionation, and tumor disposition, can have differing immunomodulatory effects. Clinical trials overwhelming show that a single fraction of high-dose radiation, immune-ablative radiation, is effective for tumor control, with a 90% response rate, regardless of histologic type (9–11). This is, in part, possibly due to the ability to overcome the radio-resistance of cancer stem cells (12–14). However, large doses of radiation are seen as an injury and trigger an immunosuppressive wound healing response that includes upregulation of immune-inhibitory molecules as well as the influx of myeloid-derived suppressor cells (MDSC) and immunosuppressive T regulatory cells (Treg) in the tumor, contributing to a tissue regeneration and protumorigenic phenotype (15, 16). After single-fraction ablative radiotherapy, tumor control and induction of CTLs increase with the size of radiotherapy dose but there is also a dose-dependent increase in infiltration of Tregs in tumors (17) and normal tissue (18). Although it was postulated that Tregs are relatively radioresistant (17), there are several reports showing reduction in Treg pools after low-dose irradiation (19–21).

Although low-dose radiotherapy (e.g., 0.5–2 Gy) is incapable of controlling tumor burden, it has been shown to reprogram the immunosuppressive, protumorigenic tumor-associated macrophages (TAM) toward a more inflammatory antitumoral phenotype (22, 23). This regimen alters the cytokine milieu, and increases the infiltration of immune effector cells in tumors (24, 25). Because radiotherapy is associated with both immune activating (26) and immune-suppressing roles (27), it is critical to study the optimal dose and fractionation of radiotherapy for designing clinical trials.

Much research is being done with variations in dose and fractionation, but the exact role of treatment parameters, such as duration

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Translational Relevance
Radiation therapy is frequently used for local control of solid tumors. We have developed a novel paradigm by shifting the radiobiological focus from DNA damage to immunomodulatory potential using a large single-dose followed by four fractions of low-dose radiation for optimal immunomodulation. This concept can be applied to both primary tumor and metastatic lesions to improve the local and systemic therapeutic outcomes of patients after ablative radiation treatment (stereotactic body radiotherapy and stereotactic ablative radiotherapy).

Materials and Methods

Cell lines and mice
The murine cell line Lewis Lung Carcinoma, 3LL, was purchased from the ATCC and grown in supplemented DMEM (10% FBS, 5% sodium pyruvate, 2.5% NEAA, 1% antibacterial/antimycotic). The murine breast carcinoma cell line, 4T1, was purchased from ATCC and grown in supplemented DMEM (10% FBS, 1% antibacterial/antimyotic). Cell lines were used between 4 and 8 passages and Mycoplasma was tested every 4 months with MycoAlert (Lonza LT07-705). Six- to 8-week-old C57BL/6 mice and 10- to 12-week-old BalB/C mice were ordered from NCI and athymic nude mice were ordered from Charles River Laboratories. The Institutional Animal Care and Use Committee approved all studies performed.

In vivo tumor studies
C57BL/6 mice were challenged with $1.5 \times 10^5$ 3LL cells subcutaneously and BalB/C mice were challenged orthotopically with $2 \times 10^5$ 4T1 cells in the mammary fat pad. Treatments were started once the tumors reached approximately 5 mm in diameter. Tumor size was measured twice a week. Tumor volume was calculated using an ellipsoid formula: $V = (\pi/6 \times \text{length} \times \text{width} \times \text{height})$. Survival was determined by death of mouse or sacrifice for ethical considerations.

CT-guided radiotherapy of tumor-bearing mice
Radiation is delivered using Xstrahl’s Limited Small Animal Radiation Research Platform (SARRP). Image-guided radiotherapy is performed using the SARRPs on-board cone beam CT (CBCT).

Following CBCT acquisition, the treatment plan was constructed using Muriplan.

Tumor and immune cell analysis
Tumor cells were cultured, plated, and treated with the specified radiation schemes. Cells were harvested 6 and 24 hours after the last treatment on day 5. Cells were harvested and stained for flow cytometry. Bone marrow-derived macrophages were polarized to M1 (100 ng/mL LPS + 50 ng/mL IFNγ), M2 (10 ng/mL IL4), and treated with radiation 24 hours later. T-cell populations were sorted from spleens of naïve C57BL/6 mice with CD3, CD8, CD4, and CD25 antibodies and allowed to rest overnight before radiation treatment. Immune cells were harvested 6 hours after final radiation dose.

Tumor processing
Tumors or whole lungs were harvested on ice, weighed, and washed. After manual dissection with razor, the tumor or whole lung was transferred to 1 mL Digestion buffer [10% FBS, Collagenase I and IV at 100 U/mL (Sigma) and 1× DNase I (Thermo Fisher Scientific)] in a 15 mL conical tube with a magnetic stir bar. Tubes are incubated at 37°C for 15 minutes while rotating and transferred to a stir plate for manual digestion for 15 minutes. Single-cell suspensions are filtered. Cells are resuspended for flow cytometry.

Flow cytometry analysis
Cells were stained at 4°C for 30 minutes with surface stain antibodies. LIVE/Dead fixable dye was used to give the total death of the cells irrespective of the mode of the death. Antibodies used included CD45, CD3, CD4, FOXP3, CD8, GrB, CD11b, MHCII, IL10, TNFα and CD206. After washing, the cells were fixed with 4% PFA or permeabilized for intracellular staining BD Pharmingen Transcription Factor buffer set as per instructions. Intracellular stains are FOXP3, TNFα, IL10, and Granzyme B, followed by fixation with 4% PFA. Cells were collected on the LSRII flow cytometer (BD Biosciences) and analyzed via Flow Jo software (Tree Star).

Lymphoid organ harvest for immune analysis
Spleens and draining lymph nodes were harvested on ice and processed to single-cell suspensions. In spleens, red blood cells were lysed with ACK lysis buffer (Lonza). Cells are counted before resuspension in complete RPMI (10% heat-inactivated FBS, 1% antibacterial/antimycotic). For intracellular cytokine analysis, Golgistop and monensin were added for 3 hours at 37°C. Single-cell suspensions are further processed for flow cytometry analysis as done with tumor.

ELISOPT assay
Spleens processed for immune analysis were plated in coated ELISOPT plates at $1 \times 10^6$ cells per well and incubated overnight before further processing. Reagents for IFNγ ELISOPTs were ordered from BD Biosciences and manufacturer’s protocol was followed. Reagents for granzyme B ELISOPTs were ordered from R&D Systems and manufacturer’s protocol was followed.

Histologic staining of tumor
Tumors and lungs were harvested and transferred into 4% PFA or 10% formalin and stored at 4°C. Samples were then transferred to 70% and embedded in paraffin. Blocks were sectioned and dual stained in the histology core with CD8 and FOXP3.
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Statistical analysis

Statistical analysis was performed using PRISM 7 (GraphPad Software) software. Analyses were performed using Student's t test or ANOVA analysis with multiple group comparison. Data are representative mean ± SD. Survival curves were analyzed by log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests. P values are represented as *P < 0.05; **P < 0.005; ***P < 0.0005; ****P < 0.0001 for statistical significance.

For RT-PCR, PET imaging, and India ink methods, see Supplementary Data.

Results

Local PAM-RT delays tumor growth and increases survival in mice

To investigate the efficacy of PAM-RT for local tumor control, C57BL/6 mice with palpable, subcutaneous, 3LL tumors were divided into five treatment cohorts for pilot studies—untreated, 24 Gy at day 1 or day 5 of treatment, 1 Gy × 4 followed by 20 Gy or 20 Gy followed by 1 Gy × 4 (Fig. 1A). Basically, we compared four fractions of 1 Gy that were either delivered before or after a single fraction of 20 Gy to the primary tumor with a 24 Gy single fraction of radiotherapy. When compared with single-dose radiotherapy, pretreatment with low-dose radiotherapy showed minimal tumor control, but there was a significant growth delay and improvement in survival after PAM-RT of 1 Gy × 4 fractions (Fig. 1B and C). Because 3LL tumors grow rapidly, priming radiotherapy efficacy could have been affected by the tumor size difference for ablative radiotherapy on the fifth day of treatment. Further studies of tumor growth led to the adoption of PAM-RT with 0.5 Gy × 4 fractions as the optimal modulation doses for all further experiments. As before, we compared a single-dose ablative fractionation of 24 Gy with 22 Gy followed by four fractions of 0.5 Gy PAM-RT (Fig. 1A). When compared with 24 Gy, PAM-RT delayed tumor growth (Fig. 1D) and significantly improved survival in these animals (Fig. 1E). Finally, the effects of PAM-RT were lost in immunocompromised mice (Fig. 1F and G). These data demonstrate the ability of PAM to delay local tumor progression and significantly increase survival in 3LL tumor–bearing mice, although all mice eventually succumbed to local tumor growth. Furthermore, it clearly demonstrated that the PAM-RT effects are contingent on a competent immune system.

Immunomodulatory effects of local PAM-RT in vitro

We examined the immunomodulatory effects of PAM-RT on 3LL cells in vitro (Fig. 2A). Cells were harvested at 6 and 24 hours after fifth treatment day. With no difference seen at 6 hours, PAM-RT caused significantly more cell death at 24 hours than ablation alone (Fig. 2B); however, as seen in in vivo experiments using nude mice, this change alone was not enough to attenuate tumor progression. When compared with untreated cells and low-dose radiation–treated cells, ablative radiation, with or without PAM-RT, increased cell surface immunomodulatory (CD80), stress (CRT, Hsp70, Fas, and MHC I), and immunosuppressive (CD47 and PD-L1) markers at 6-hour postradiotherapy. Interestingly, at 24 hours, PAM-RT–treated cells had greater cell surface expression of 4-1BBL, CRT, Fas, Hsp70, and PD-L1, as compared with ablation alone (Fig. 2C).

We next examined the effect of PAM-RT on immune cells in vitro. We harvested bone marrow–derived macrophages and splenic T cells from wild-type C57BL/6 mice. Macrophages were polarized with cytokines to M1 or M2 differentiation or left untreated. Splenic T cells were sorted into the three main T-cell populations: CD8⁺, CD4⁺CD25⁻, and CD4⁺CD25⁺ (Tregs). All populations were treated with our low-dose radiotherapy (0.5 Gy × 4), one day after polarization or sorting. Ablative doses were not included in treatment due to the radiosensitivity of immune cells. Characterization of T-cell populations 6 hours after final dose showed a significant decrease in Treg viability, that was not seen in the CD8 or CD4⁺CD25⁺ T-cell populations (Fig. 2D). Within the sorted Treg population, cells treated low-dose radiotherapy showed a trend toward decreased CD25 surface expression and significantly decreased FOXP3 and CD25 dual expression (Fig. 2E). M2-polarized macrophages treated with low-dose radiotherapy showed a significant decrease in CD206 expression, a M2 marker (Fig. 2F). M2-polarized macrophages also tended to express less IL10 after radiotherapy, indicating a repolarization after treatment (Fig. 2G). These data indicate that PAM-RT increases the cytotoxicity of ablative radiotherapy without inhibiting the immunomodulatory effects. Furthermore, our low-dose radiotherapy reduces Treg and reprograms M2 macrophages toward a less immunosuppressive phenotype.

Local PAM-RT promotes TME remodeling by reducing Treg and M2 macrophages

To investigate the immunologic consequences of PAM-RT on the TME, irradiated 3LL tumors were harvested on days 6 and 10 after start of radiotherapy (Fig. 3A). There was a significant increase in leukocyte infiltration in tumors treated with PAM-RT, as compared with ablative radiotherapy alone. Upon phenotyping of the infiltrating leukocytes, there was a significant decrease in intratumoral Tregs (Fig. 3B) and significantly increased CD8/Treg ratio at day 6 in PAM-RT–treated mice, as compared with ablative radiotherapy alone. RT-PCR of whole-tumor RNA showed a significant decrease in FOXP3 mRNA expression in PAM-RT–treated tumors at day 6 (Fig. 3C). This remodeling was accompanied by a trend toward an increase in granzyme B–secreting intratumoral CD8⁺ T cells at days 6 and 10 (Fig. 3D), indicating an increase toward effector CTL responses. Characterization of the intratumoral myeloid cell population revealed a significant decrease in IL10-secreting macrophages and a slight decrease in CD206 expression at day 6 as well as a trend toward a decrease in IL10 secretion and a significant decrease in CD206 expression at day 10 after start of treatment (Fig. 3E). Taken together, PAM-RT promotes TME remodeling by reducing immunosuppressive Treg and M2 macrophages that could help potentiate CTL activity.

Local PAM-RT increases systemic T-cell responses and decreases Tregs

We next examined the immune cells in the secondary lymphoid organs. Tumor-draining lymph nodes and spleens were harvested, 6 and 10 days after start of radiotherapy, as mentioned in previous studies (Fig. 3A). At days 6 and 10, there was a significant increase in leukocytes in spleen and draining lymph nodes in PAM-RT–treated mice (Supplementary Fig. S1). In the tumor-draining lymph node, there were significantly more CD8⁺ T cells with no increase in Tregs in PAM-RT–treated mice at both days 6 and 10 (Fig. 4A). Splenic analysis revealed minimal changes in CD8 T cells, but significant decreases in Tregs at day 6 that was reversed by day 10 with significantly, more CD8⁺ T cells and minimal changes in Tregs in PAM-RT–treated mice, compared with a single ablative dose (Fig. 4B). Investigations in the functional status of splenic T cells at days 6 and 10 with ELISPOTs revealed a trend toward increased granzyyme B–secreting effector cells and a significant increase in IFNγ-secreting effector cells at day 6 in PAM-RT–treated mice. Day 10 analysis revealed a significant increase in granzyyme B–secreting effector cells and a trend toward increased IFNγ-secreting effector cells (Fig. 4C). Although...
treatment in these mice was localized to the primary tumor, systemic modulation occurred similar to trends occurring in the tumor with decreased immunosuppression and increased T-cell responses.

**Systemic PAM-RT delays metastatic progression and increases survival**

We next investigated whether PAM-RT of metastases-prone organs can prevent progression after a course of hypofractionated ablative radiotherapy to the primary tumor in a poorly immunogenic, highly metastatic 4T1 breast cancer in Balb/c mice. We first administered a course local PAM-RT (22 Gy + 0.5 Gy × 4) to orthotopic 4T1 breast cancer in Balb/c mice. Although primary tumor PAM-RT trended to delay local tumor progression, there was, however, no increase in survival with all treated mice succumbing to metastatic disease (Supplementary Fig. S2). Considering local treatment with PAM-RT remodeled the TME, we translated...
the use of PAM-RT to treat metastatic organs after primary tumor ablation. For adequate local tumor control, BalB/C mice with palpable 4T1 tumors were treated with three doses of 20 Gy delivered over 3 consecutive days to the primary tumor. As expected, primary tumor ablation alone could not rescue the animals from pulmonary metastases. We reasoned that despite induction of an antitumoral immune response after primary tumor radiotherapy, radiotherapy-induced CTLs might be excluded from...

![Figure 2. In vitro immunogenic changes to tumor and immune cells after PAM-RT. A-C, In vitro treatment of 3LL tumor cells. A, Treatment schema for PAM on tumor cells. B, Cell death 6 and 24 hours after treatment measured by LIVE/Dead fixable dye. C, FACS of immunomodulatory, stress, and immunosuppressive cell surface markers on 3LL cells at 6 and 24 hours after final treatment. D-G, In vitro treatment of immune cell subsets with 0.5 Gy x 4. D, Viability of sorted T-cell subsets after treatment. E, Expression of CD25 and FOXP3 after treatment on sorted CD4⁺CD25⁺FOXP3⁺ (Tregs). F, Expression of CD206, an M2 macrophage marker, on cytokine-polarized bone marrow-derived macrophages. G, Cytokine secretions of cytokine polarized bone marrow-derived macrophages after treatment (*, P < 0.05; **, P < 0.005; *** P < 0.0005; ****, P < 0.0001 unpaired t test; all panels).]
Figure 3.
PAM-RT remodels TME by increasing leukocyte infiltration, while decreasing immunosuppressive Treg and M2 macrophages. A, Treatment and harvest schematic for local PAM treatment in 3LL tumor-bearing mice. In vivo infiltration for local PAM treatment at days 6 and 10 after start of treatment of leukocytes and Tregs by flow cytometry (B). C, Whole-tumor lysate RNA expression of FOXP3 at day 6. D, Intratumoral infiltration of granzyme B–secreting effector cells at days 6 and 10 by flow cytometry. E, Phenotyping for polarization of tumor-infiltrating macrophages at days 6 and 10 after start of treatment by flow cytometry (*, P < 0.05; **, P < 0.005; *** P < 0.0005 ANOVA multiple comparison; B and D, unpaired t test, C and E).
We, therefore, treated the metastasis-prone organ, whole lung with daily doses of 0.5 Gy over 4 days, 12 days after completion of the primary tumor radiotherapy (Fig. 5A). Whole-lung treatment was delayed to allow for induction of an immune response after ablative radiotherapy of the primary tumor. Survival was significantly increased in these animals after whole-lung PAM-RT, as compared with primary tumor radiotherapy alone (Fig. 5B). Investigation of metastatic burden of treated mice revealed fewer metastatic lesions in PAM-RT–treated lungs by gross examination after India ink injection (Fig. 5C) and in histologic specimens (Fig. 5D); however, there was no significant difference. PET scans of 4T1 mice showed similar results to the lung harvest, with lungs receiving PAM-RT having less metastatic burden (Supplementary Fig. S3). These data indicate that PAM-RT doses of 0.5 Gy/C2 can be administered either, directly to the primary tumor for local control or when treating systemic disease, delayed PAM-RT can be administered to the metastasis-prone organ to slow tumor progression and increase survival.

Systemic PAM-RT remolds the metastatic niche with decreased Tregs in lungs

Characterization of immune cells 19 days after primary tumor ablation in PAM-RT–treated lungs showed similar results to local PAM-RT treatment, with a decrease in immunosuppressive phenotype of these cells. There was a significant decrease in Tregs (Fig. 6A) leading to a significantly increased CD8/Treg ratio in PAM-RT–treated whole lungs (Supplementary Fig. S4). Phenotyping of T cells in the lungs revealed significant increases in granzyme B secretion in both CD8+ and CD4+ T cells (Fig. 6B). IHC of micrometastases in the few metastatic lesions of PAM-RT–treated lungs showed massive infiltration of CD8+ T cells, whereas FoxP3+ cells decreased, compared with lesions in mice receiving primary tumor ablation alone (Fig. 6C). Next, we examined the systemic immunomodulation in these animals 19 days after primary tumor ablation. In untreated animals, there were splenic metastases with enlargement of spleen and a reduction of CD45+ leucocytes (Supplementary Fig. S5). Upon primary tumor radiotherapy and primary tumor radiotherapy + lung
Targeted systemic PAM increases survival and reduces metastases. 

**A.** Treatment schematic for systemic PAM with whole-lung irradiation after primary tumor ablation. Twenty-six to 27 mice per group. (gray arrow indicates 2 months after inoculation with P value).

**B.** Overall survival for systemic PAM treatments compared with primary tumor ablation alone. Twenty-six to 27 mice per group. (gray arrow indicates 2 months after inoculation with P value).

**C.** India ink–injected lungs 28 days after primary tumor ablation with and without whole-lung irradiation (red arrows indicate macrometastases) and graph of enumerated visible macrometastases.

**D.** Histologic section of lung 19 days after treatment completion (red asterisks indicate metastatic lesions) and graph of enumerated lesions (*, P < 0.05 log-rank (Mantel–cox) test; n = 26–27 mice (B); ns by unpaired t test (C and D)).

**Figure 5.**

Immunologic Consequences of Radiation Fractionation
PAM-RT, splenic size decreased with subsequent increase in CD45\(^{+}\) leucocytes (Supplementary Fig. S5A) and CD3\(^{+}\) T-cell number (Supplementary Fig. S5B), with radiotherapy + lung PAM-RT returning to baseline levels as seen in wild-type animals without tumors. A peripheral myeloid expansion is associated with G-CSF–secreting 4T1 tumors and in line with previous reports, there were massive increases in...
in splenic macrophages among CD45\(^+\) leucocytes in mice with 4T1 tumors, with progressive reduction of these cells in mice treated with ablation alone versus ablation + PAM-RT \((P = 0.033\) by ANOVA multiple comparison; Fig. 6D). Interestingly, in animals with untreated tumor, splenic macrophages were immature with a reduced MHC Class II expression. Upon treatment with primary tumor ablation \(\pm\) lung PAM-RT, Class II MHC expression increased in macrophages significantly, indicating activation of macrophages with PAM-RT treatment (Fig. 6E). These experiments indicate that PAM-RT treatment can promote remodeling of the TME in the primary tumor, as well as, in metastatic site by reducing Tregs, activating macrophages to an inflammatory phenotype, and promoting infiltration of CD8\(^+\) CTLs in metastatic tumors. Figure 7 provides a summary of the effect of PAM-RT treatment on local and systemic immunomodulation.

Discussion

Radiotherapy is one of the primary cytotoxic modalities used in the treatment of malignancies, and advances in technology have made treatment planning more precise and the options for delivering ablative doses of radiotherapy over a short period of time have greatly expanded. Much research is being done on the immunomodulatory potential of differing fractionations and possible combination with immune therapies. Our goal is to optimize radiation dose and fractionation for optimal immunomodulation so that the immune-activating properties of radiotherapy can be retained while reducing the immune-suppressing features.

In this work, we designed a novel radiation scheme, a single-fraction ablative radiation followed by 4 daily low-dose fractions of PAM-RT. Although high-dose single-fraction radiotherapy is effective in achieving \(>90\%\) local control (9, 10) and releasing damage-associated molecular pattern signals, inducing ICD (4) with an increase in antigen presentation and cell surface HLA (5), there are several immunosuppressive features in ablative dose regimens of radiotherapy that might preclude induction of systemic immunity (28, 29). Ablative fractionation regimens reduce tumor perfusion (30, 31), increase TGF\(\beta\) secretion (28), increase intratumoral Tregs (16), maintains immunosuppressive features of carcinoma-associated fibroblasts (32), promotes deposition of collagen-I (33), and reprograms macrophages to anti-inflammatory M2 phenotype (34). Although hypofractionated subablative radiation (e.g., 8 Gy \(\times\) 3) may produce abscopal effects in combination with anti-CTLA4 immunotherapy (35), such radiotherapy regimens by itself may not achieve local tumor control and induce the recruitment of bone marrow–derived myeloid cells (30, 31), and reprogram TAMs to a M2 phenotype (34, 36). In contrast, low-dose radiation, although not tumoricidal, is capable of increasing blood perfusion (37), reprogramming TAM into an inflammatory M1 phenotype (22), and selectively deplete Tregs (19–21). We, therefore, chose to combine ablative radiotherapy regimens with postablative, TME-modulating, low-dose radiotherapy, thereby, mitigating the immunosuppressive consequences incurred after ablative radiotherapy.

In our studies with 3LL lung adenocarcinoma, local PAM-RT increased immune cell infiltration, decreased intratumoral Tregs, reprogrammed immunosuppressive CD206\(^+\) macrophages (Fig. 3), thereby increasing local control and survival (Fig. 1). This was accompanied by increased T cells responses in secondary lymphoid organs (Fig. 4). Thus, we propose a novel fractionation scheme for
radiosurgery and ablative radiotherapy, where a single high-dose radiotherapy with four fractions of low-dose primary tumor PAM-RT should be examined for increased efficacy in the clinic (Fig. 7). We also introduce the concept of systemic PAM-RT that could target large metastatic organs to reprogram the metastatic niche to prevent progression of micrometastatic lesions. Systemic PAM-RT reduces Tregs, increases intratumoral effector T-cell infiltration in pulmonary metastases (Fig. 6), thereby reducing metastatic progression in lungs and spleens and improving survival (Fig. 5) of a poorly immunogenic, highly metastatic 4T1 breast cancer without the aid of any systemic immunotherapy.

Currently, hypofractionated radiotherapy regimens are being favored over single-fraction ablative radiotherapy to minimize the immunosuppressive consequences of conventional fractionation and/or single-fraction high-dose radiation (35, 38). In this work, we show that combining variations in fraction dose within a treatment scheme can have beneficial effects on not only tumor growth and survival, but also immunomodulation of the systemic immune response after treatment. Thus, low-dose radiotherapy can be used as PAM for two applications: (i) local control with PAM administered directly after immune-ablative radiation to the primary tumor, and (ii) systemic control with PAM administered to potential metastatic prone organs, such as, whole lungs for targeted systemic control of tumors that can be locally controlled by ablative radiotherapy. Interestingly, low-dose radiation appears to augment the systemic immune response induced by hypofractionated radiotherapy and improve survival in mice with 4T1 breast cancer (39). Similarly, clinical data suggest that low-dose radiation may increase the systemic response rates seen after high-dose radiotherapy and immunotherapy (40). By optimizing the immunomodulatory properties of radiation fractionation regimens, we are building a foundation on which various immunotherapeutic combinations can be designed. This novel fractionation scheme is easily adaptable to the clinic given the use of low-dose radiation in previous clinical trials (41) and could become the new standard in clinical treatment with radiosurgery and ablative radiotherapy. This radiation scheme also has the potential as a chemosensitizer (42). Further studies investigating the impact of immunomodulation after other variations on fractionation need to be done, but this work looks to further unlock radiation’s immunogenic potential.

**Disclosure of Potential Conflicts of Interest**

C. Guha is an employee/paid consultant for Varian Medical Systems. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

**Conception and design:** T. Savage, C. Guha

**Development of methodology:** T. Savage, S. Pandey, C. Guha

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** T. Savage, S. Pandey

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** T. Savage, S. Pandey, C. Guha

**Writing, review, and/or revision of the manuscript:** T. Savage, S. Pandey, C. Guha

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

**Study supervision:** C. Guha

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