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Novel Radiation Therapy Paradigms and Immunomodulation: Heresies and Hope

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Radiation therapy benefits the majority of patients across the spectrum of cancer types. However, both local and distant tumor recurrences limit its clinical success. While departing from the established tenet of fractionation in clinical radiotherapy, ablative-intensity hypofractionated radiotherapy, especially stereotactic radiosurgery and stereotactic ablative radiotherapy, has emerged as an alternative paradigm achieving unprecedented rates of local tumor control. Direct tumor cell killing has been assumed to be the primary therapeutic mode of action of such ablative radiation. But with increasing recognition that tumor responses also depend on the immunostimulatory or immunosuppressive status of the tumor microenvironment, the immunologic effect of ablative radiotherapy is emerging as a key contributor to antitumor response. More recently, novel radiation modalities, such as spatially fractionated radiotherapy and ultrahigh dose rate FLASH irradiation, that venture even further from conventional paradigms have shown promise of increasing the therapeutic index of radiation therapy with the potential of immunomodulation. Here, we review the immunomodulatory impact of novel radiation therapy paradigms, heretofore considered radiobiological heresies, a deeper understanding of which is imperative to realizing fully their potential for more curative cancer therapy.
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Introduction

Radiation therapy (RT) has long been a pillar of curative cancer therapy. Over a century of clinical experience has established that fractionation of RT in daily doses protracted over weeks is a primary approach to achieving therapeutic index. For most solid tumors, to reach potentially curative doses while avoiding intolerable collateral damage to normal tissues, typical conventionally fractionated radiation therapy (CFRT) regimens involve small doses of 1.8-2 Gy fractions per day, 5 days per week, over 6-8 weeks with total doses of 60-80 Gy. The emergence of stereotactic radiosurgery for intracranial tumors[1] and later stereotactic ablative radiotherapy (SABR), also known as stereotactic body radiation therapy, for other body sites[2,3] defied the conventional wisdom of the time with the use of extremely hypofractionated regimens, such as large single-fraction treatments of up to 25-34 Gy or up to 60 Gy in 3-8 fractions over 1-2 weeks. This departure from established fractionation dogma is possible only in the setting of limited volume targets and highly conformal dose delivery, producing therapeutic index through physical separation of ablative doses from normal tissues and achieving unprecedented local tumor control outcomes in this setting.[4] Severe or fatal toxicity when excessively intensive SABR dosing is applied too close to critical normal structures or to too large a volume[5] highlights the continued need for fundamentally new strategies to improve the therapeutic index.

More recently, and in a much more nascent state of development, novel RT approaches that challenge tenets of classical radiobiology even further include ultra-rapid FLASH RT and spatially fractionated radiation therapy (SFRT). That increased therapeutic index can be achieved by the same dose of radiation given in a fraction of a second rather than

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the conventional several minutes, or by dose distributions in which substantial portions of a tumor are not directly targeted, seemingly defies explanation by conventional models.

Preclinically FLASH has been shown to achieve substantial normal organ sparing and equal or improved tumor killing in vivo, when compared to conventional dose rate irradiation, with evidence of changes in the immunologic microenvironment in both tumors and normal tissues.[6] FLASH delivers subsecond doses at rates of >40 Gy/s compared to conventional dose rates of 0.01-0.2 Gy/s. This ultrarapid dose has been achieved using dedicated experimental electron linear accelerators as well as specially configured clinical linear accelerators that generate 4.5-20 MeV electrons suitable for preclinical mouse and in vitro experiments, at a high beam current producing average dose rates as high as >200 Gy/s.[7-10] Additional beams adapted for preclinical experiments include synchrotron x-rays[11] and protons.[12,13]

In mice, substantially decreased normal organ injury has been observed with FLASH compared to the same doses of conventional dose rate irradiation in lung (inflammation and fibrosis),[6] brain (cognition and neuroinflammation),[11,14-16] skin (necrosis),[17] and gastrointestinal tract (intestinal crypt ablation and GI syndrome).[18] These studies collectively suggest that FLASH may provide an effective additional strategy to escalate radiation doses to optimize antitumor control while reducing the complications of RT. Next generation technologies are now under development to deliver ultrarapid and highly conformal RT to clinically relevant targets, typically large volume and deep-seated, simultaneously overcoming the detrimental impact of physiologic motion on RT precision and leveraging the potential biological advantage of FLASH and giving it translational relevance.[19]

Contrary to the assumption that tumor sterilization requires comprehensive irradiation of the entire tumor to high doses, SFRT is the use of intentionally heterogeneous dose delivery comprising high dose peaks, typically much higher doses per fraction than CFRT, separated by low-dose valleys within the same tumor target volume.[20,21] It was originally developed in the early 1900s as a skin sparing approach, but more recently has demonstrated promising results for achieving tumor responses in bulky tumors too large to treat safely with CFRT, either alone or in combination with lower dose CFRT. SFRT has been delivered as 2-D arrays of pencil beams (GRID) or as 3-D lattices of high-dose vertices (lattice radiation therapy, [LRT]) using either photons or protons. GRID therapy is the most commonly used SFRT in which blocks or multi-leaf collimators (MLCs) are used to deliver nonuniform radiation to the target volume. GRIDs that have hybrid collimation (1 block and 1 MLC) are faster than MLC based GRIDs.[20] Some patients treated with GRID therapy showed induction of TNFα that strongly correlated with complete clinical response,[22] An improvement of GRID therapy uses helical tomotherapy (TOMOGRID) and has been shown to achieve superior sparing of normal tissues than commercially available GRID blocks.[23]

The immunologic effects of RT are increasingly recognized as critical to its success or failure and may in fact underly the promise of novel RT paradigms including SABR, FLASH, and SFRT. Immunologic studies in preclinical models indicate that high dose hypofractionated RT can be far more immunogenic and efficacious than conventionally fractionated RT.[24,25]

The Radiation Research branch of the National Cancer Institute organized a workshop in 2018 to encourage approaches that integrate new radiation technologies and biology into therapeutic strategies.[26] The workshop highlighted the role of immune responses in enhancing the therapeutic response of RT. There is a large body of preclinical data[27-29] and promising results from clinical trials that suggest synergy between ablative RT and immunotherapy[30,31] Here, we discuss the immune-stimulatory effects of ablative radiation that depend on doses and fractionation that seem contradictory to the classical dogma of conventional fractionation. We are optimistic that designing new treatment regimens that integrate technological advances of delivery of ablative RT and immunobiology of radiation will significantly improve clinical outcomes.

Radiation Induced Cell Death—Immunogenic or Immunosuppressive

Local tumor control by high dose RT has classically been considered to be mediated through direct cytotoxic effects on the cancer cells. Radiation induces double strand DNA breaks,[32] single strand DNA breaks,[33] and chromosomal aberrations.[34] These effects lead to cell death through inhibition of mitotic cell cycle or apoptosis, necrosis, autophagy or senescence.[35] The role of immunologic effects has been appreciated more recently. Preclinical studies show that double stranded DNA damage activates innate immune signaling pathways. The activation of cytoplasmic double stranded DNA sensor Absent in Melanoma 2 (AIM2) results in activation of caspase 1 that leads to release of proinflammatory cytokines and pyroptotic cell death.[36] Radiation produces cytosolic double strand DNA that is sensed by cyclic GMP-AMP synthase and activates stimulator of interferon genes (STING). STING recruits tank-binding kinase 1 to promote transcription of type interferon-1 (IFN-1) genes. Preclinical in vivo studies show that type-I IFN signaling in DCs promotes cross priming of tumor associated antigens to $CD8^+$ T cells which mediate antitumor response.[37,38] In contrast, activation of STING pathway in myeloid derived suppressor cells (MDSCs) that are recruited to tumors through CCR2 chemokine following local ablative radiation (20 Gy), are detrimental to antitumor responses.[39] However, $CD8^+$ T cells can reduce the infiltration of MDSCs into tumors.[25] Another preclinical study showed radiation dose greater than 24 Gy in 3 fractions (8 Gy/fraction) led to accumulation of cytosolic Three-Prime Repair exonuclease 1 that degrades cytoplasmic double stranded DNA abrogating the immunostimulatory effect of STING[40] in tumors cells. Further studies are needed to determine the contribution of STING and Three-Prime Repair exonuclease 1 in optimizing antitumor response by different hypofractionated SABR radiation regimens.

Radiation induced tumor cell necrosis releases adenosine triphosphate as well as high mobility group B1 protein, both of which have been shown to activate DCs in tumors and enhance antigen presentation to T cells.[34] Expression of TLR4 by DCs and high mobility group B1 are necessary for activation of DCs and presentation of tumor antigens released by dying tumor cells after RT.[41] Upregulation of damage associated molecular patterns (DAMPs) in dying tumor cells such as calreticulin and heat shock proteins (HSP) stimulates phagocytosis in tumor associated DCs and macrophages.[42,43] In addition, NK cells can also be activated by DAMPs.[44] The cell death pathways can vary with the dose, fractionation, tumor type, and tumor stage. High radiation dose promotes tumor cell necrosis and favors immunogenic cell death through expression of DAMPs while low dose radiation of less than 5 Gy promote nonimmunogenic apoptotic cell death.[45,46] Uptake of apoptotic bodies by DCs has been shown to prevent DC maturation and induce tolerance.[47-49] In addition, tumor radiation induced apoptosis has been shown to activate Caspase-3 which facilitates repopulation of tumor cells through prostaglandin E_2 production.[50] We have recently shown that 30 Gy in 10 fractions (3 Gy/fraction) radiation of B cell lymphoma caused significantly enhanced tumor cell necrosis and expression of calreticulin and HSP70 and HSP90 and better tumor control when delivered over 4 days (accelerated schedule) compared with over 10 days (conventional schedule).[51] The inability of the tumor cells to repair endoplasmic reticulum stress within the shorter duration (4 hours) between radiation doses compared with conventional duration (24 hours) may account for higher expression of DAMPs in tumors treated with the accelerated schedule. Thus, ablative radiotherapy regimens that activate immunogenic cell death pathways and promote antigen presentation to $CD8^+$ T cells are likely to induce durable complete remissions.

Immunogenic or Immunosuppressive Tumor Microenvironment Following Ablative Radiation Therapy

Cancer cells survive the host immune system attack by creating immunosuppressive tumor microenvironments including immune cells such as regulatory T cells, MDSCs and tumor associated macrophages (TAMs) that impair infiltration of $CD8^+$T cells that mediate antitumor responses. These immune cells have varying degrees of radiation sensitivity.[52-54] T cells, and B cells are radiation sensitive while regulatory T cells, macrophages, DCs, and NK cells are radiation resistant.

Studies show that ablative or hypofractionated regimens induce superior $CD8^+$ T cell antitumor responses compared to conventional fractionation regimens.[24,25,55] A single high radiation dose of 20 Gy upregulates Fas expression on MC38 colon cancer tumors expressing carcinoembryonic antigen. This makes the tumor cells sensitive to killing by $CD8^+$ T cells through a Fas and Fas-ligand mediated mechanism.[56] Compared to fractionated radiotherapy (15 Gy in 3 fractions, 5 Gy/fraction), a single dose of 15 Gy radiation-enhanced infiltration of antigen presenting cells and $CD8^+$ T cells into tumors, and showed superior $CD8^+$ T cell antitumor cytolytic activity.[57] Addition of fractionated radiation dose (10 × 3 Gy (30 Gy in 10 fractions, 3 Gy/fraction)) after a single ablative dose of 30 Gy abrogated the robust antitumor immunity observed with a single 30 Gy alone in a CT26 colon cancer tumor model.[25] This observation suggests that addition of fractionated radiation may result in death of infiltrating T cells that kill tumor cells.

We have recently shown that fractionated radiation (10 × 3 Gy) of A20 lymphoma tumors over 10 days results in reduced $CD8^+$ T cell infiltration as compared to (10 × 3Gy) over 4 days. The prolonged daily irradiation over 10 days depleted T cells that infiltrated the tumor a few days after the start of local tumor irradiation.[51] In a preclinical model of TSA breast cancer, anti-CTLA4 antibody therapy along with fractionated radiation of 24 Gy in 3 fractions (8 Gy/fraction) was more effective in eliciting an abscopal effect on secondary tumors than either a single dose of 20 Gy or 30 Gy in 5 fractions (6 Gy/fraction).[55] This suggests that high dose hypofractionated RT regimens may be more immunogenic than a single dose per fraction. In Figure 1, we summarize the effect of prolonged CFRT and hypofractionated accelerated high dose regimens on antitumor response.

Using several tumor models Dovedi et al[58] reported that CFRT induced upregulation of PD-L1 on cancer cells and MDSCs by increasing IFNγ produced by $CD8^+$ T cells. This led to attenuation of antitumor responses. Tumor irradiation can induce the chemokine CCL2 that can attract monocytes to the tumor microenvironment and promote their differentiation into TAMs. Radiation increases the expression of HIF-1α which promotes the transcription of genes such as monocyte colony stimulating factor 1, vascular endothelial growth factor alpha (VEGF-α), and CXCL12. Monocyte colony stimulating factor 1 polarizes TAMs to a more immunosuppressive M2 type that secrete transforming growth factor β (TGFβ) that converts $CD4^+$ T cells to T_{reg} cells.[59] Additionally, VEGF-α facilitates proliferation for T_{reg} cells. A single radiation dose of 15 Gy to glioblastoma tumors induces CXCL12 which promotes influx of bone marrow derived $CXCR4^+CD11b^+$ monocytes to tumors.[60] These myeloid cells promote formation of new blood vessels and tumor recurrences. There was a 10-fold increase in $CD11b^+$ cells in glioblastoma tumor biopsies from patients after RT.[60] Thus, radiation may have both proinflammatory or antiinflammatory effects on the tumor microenvironment.

High ablative doses of RT can reduce the intratumoral levels of MDSCs while CFRT failed to induce this effect.[25] Patients with cervical cancer receiving CFRT showed increases in MDSCs in their peripheral blood.[61] In contrast, hypofractionated radiotherapy can decrease hypoxia and VEGF in tumors, and reduce PDL-1 expression and VEGF receptors on MDSCs.[62] In addition, high dose ablative RT has been shown to inhibit VEGF/VEGF receptor signaling which is essential for trafficking of MDSCs to tumors.[63] An

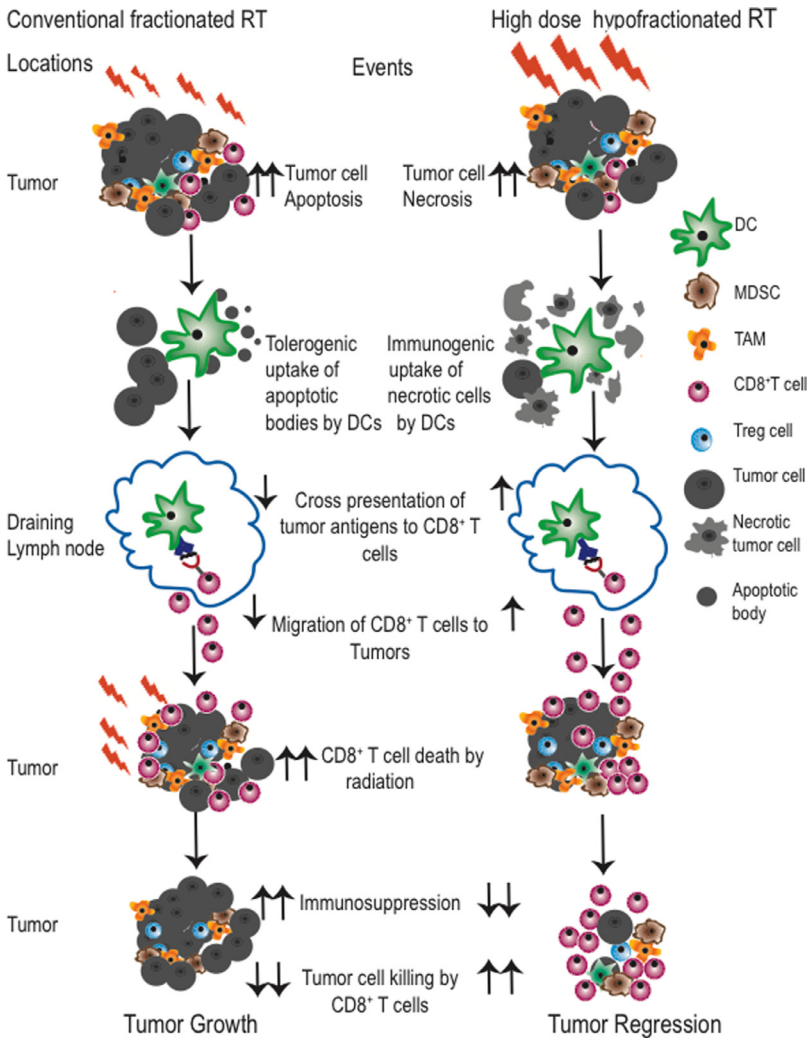

Figure 1 Schematic diagram of immunomodulatory effects of conventional fractionated radiation and high dose hypofractionated radiation therapy on antitumor response. In conventional fractionated RT, small daily radiation doses cause tumor cells to either die by apoptosis or repair of radiation induced DNA damage and recover. High dose hypofractionated RT cause cancer cell death by necrosis. Uptake of apoptotic bodies by dendritic cells (DCs) can be tolerogenic while uptake of necrotic cells by dendritic cells is immunogenic. These DCs migrate to the draining lymph node and present tumor antigens to $CD8^+$ T cells. These crossprimed $CD8^+$ T cells migrate to the tumor and kill cancer cells that lead to tumor regression. As conventional RT is administered over prolonged period, radiation kills tumor infiltrating $CD8^+$ T cells while sparing immunosuppressive cells such as MDSCs, Treg cells, and TAMS. In contrast, in hypofractionated RT the radiation schedule is completed before $CD8^+$ T cell infiltrate the tumor.

important goal of clinical ablative therapy is to optimize radiation doses and fractionation strategies that achieve elimination of immunosuppressive cells such as M2 phenotype TAMs and $CD11b^+$ monocytes and MDSCs from tumors and circulation to achieve durable anti-tumor responses while stimulating the infiltration of $CD8^+$ T cells.

Ultrahigh dose rate FLASH radiotherapy (≥40 Gy/s) as a single fraction 15 Gy was compared with conventional dose rate (≤0.03 Gy/s) in an orthotopic lung cancer model.[6] The conventional dose rate caused lung fibrosis along with activation of the immunosuppressive cytokine TGF-β. There was no fibrosis observed below 20 Gy of FLASH radiotherapy. Consequently, it was possible to escalate the dose in FLASH sufficiently to achieve 70% survival in mice with TC-1 Luc+ orthotopic lung tumors whereas it was not possible with conventional dose rate. An in vitro study of normal human lung fibroblasts also found reduced expression of TGFβ after FLASH compared to conventional dose rate irradiation with 4.5 MeV protons.[13] Recently, a dose escalation clinical trial in cats found that a single dose of 25-41 Gy FLASH RT achieved complete remission without dose limiting toxicity in 6 domestic cats treated for spontaneous squamous cell cancer of the nasal planum.[17] However, the effect of FLASH RT on the immune cells in the tumor microenvironment has not been reported. It is speculated that the reduced exposure time of FLASH may spare more circulating lymphocytes than conventional dose rate RT.[64]

The immunomodulatory role of high dose LRT was evaluated in a murine cancer model.[65] A dose of 20 Gy LRT was delivered locally to 20% volume of subcutaneous model of

LLC1 lung tumor while the second tumor was not irradiated. This 20% volume irradiation demonstrated reduction in volume in both irradiated and unirradiated tumors. There were increases in $CD3^+$ T cell infiltration in both tumors of LRT group compared with untreated tumor in the open field group. The reduced levels $CD3^+$ T cells in unirradiated tumors suggest that open field radiation kills these circulating $CD3^+$ T cells before they can infiltrate unirradiated tumors. Significant increases in serum Th1 IFNγ and reduction in Th2 cytokines IL-4 and IL-10 compared with untreated group were observed after 3 days of tumor radiation. Using a 67NR breast cancer model, Markovsky et al[66] observed similar growth delays when 100% or 50% of the tumor volume was irradiated with a single dose of 10 Gy or 20 Gy using X-RAD 225C with a 2×2 collimator. There were Infiltrations of $CD8^+$T cells in both irradiated and nonirradiated parts of the tumor at 24 hours after 10 Gy irradiation. The nonirradiated part of the tumor showed a significant increase in endothelial adhesion molecule ICAM that is critical for T cell filtration. Similar results were observed in LLC1 lung tumors that were irradiated with 15Gy.

Clinical Studies

In early stage non−small-cell lung cancer (NSCLC), a study found increases in activated $CD4^+$ and $CD8^+$ T cells with decrease in $CD4^+$ $FOXP3^+$ T_{reg} cells in the circulation of patients who received SABR of 48 Gy in 6-8 fractions (6-8 Gy/fractions).[67] Increases in the number of $CD8^+$ T cells, $CD4^+$ T cells and decreases in $CD4^+CD25^+FOXP3^+$ T_{reg} were observed after stereotactic body radiation therapy of the lungs.[68] A small study investigated radiation induced lymphopenia in patients with stage I-II NSCLC who received small-field hypofractionated SABR. The degree of drop in absolute lymphocyte counts after SABR compared with pre-SABR levels were associated with overall survival, disease free survival, distant progression-free survival and local progression free survival.[69] Lymphopenia was also observed in NSCLC patients who received SABR.[70]

The effect of radiation dose to the immune system on local tumor control and overall survival was evaluated in patients stage III NSCLC enrolled in a randomized phase III clinical trial (RTOG0617) with CFRT and cetuximab. Higher radiation dose to the immune system correlated with poorer survival.[71] Recently, a secondary dosimetric analyses of patients in the RTOG0617 trial receiving CFRT with chemotherapy for stage III NSCLC showed similar results. Patients who received high estimated doses of radiation to immune cells had shorter survival than those who received low-estimated doses. This suggests that radiation to circulating immune cells causes lymphopenia, and may be an important predictor of tumor control and survival.[72] The clinical outcome of radiation induced lymphopenia has also been reported in breast cancer patients.[73]

Tumor biopsies from patients with cervical cancer after 10 Gy, 20 Gy, and 30 Gy local irradiation showed reduction in $CD4^+$ and $CD8^+$ T cells in tumor tissue as compared to the levels before irradiation. Interestingly, there were no differences in the numbers of $FOXP3^+T_{reg}$ cells before and after irradiation, indicating that $FOXP3^+$ T_{reg} cells were more resistant to ionizing radiation than T cells.[74] This study suggests that radiation induced killing of $CD8^+$ T cells may compromise local-antitumor response.

Recently a clinical study evaluated the systemic immune responses following SABR to lung, liver, bone, and brain. The study found increases in $CD4^+$ memory T cells in peripheral blood with increased expression of ICOS and CD25 activation markers after SABR to parenchymal sites but not in brain or bone. There were no changes in the memory $CD8^+$ T cell compartment.[75] $ICOS^+CD4^+$ T cells have been associated with improved clinical outcomes in anti-CTLA4 and anti-OX40 immunotherapies.[76,77]

The first in human FLASH RT treatment has now been reported, in which patient with multiresistant $CD30^+$ T-cell cutaneous lymphoma received a single fraction of 15 Gy delivered in 90 milliseconds using 5.6 MeV electrons to a symptomatic forearm tumor. This produced rapid, complete tumor control without clinically significant toxicity at 5 months of follow-up, in contrast to protracted wound healing after prior courses of CFRT.[78]

A pilot study used GRID therapy as a palliative treatment for sarcomas, recurrent gastrointestinal cancers, liver metastases, melanoma, prostate cancer, renal cell carcinoma, and squamous cell carcinoma.[79] A single radiation dose ranging from 10-15 Gy was given using a grid consisting of 50% open and 50% closed areas. Palliation of symptoms and objective response was observed in 20 out of 22 patients without acute effects. In a follow-up study,[80] 2 groups of head and neck cancer patients were treated with a conventional external beam median radiation dose of 70 Gy and GRID therapy (15 Gy) (Group 1) or 59 Gy and GRID therapy (15 Gy) to the neck disease followed by planned surgery (Group 2). Local regional control was 93% and disease specific survival was 50% in Group 1 at a median follow up of 10 months, while in Group 2 pathologic complete response and disease specific survival were both 85% and local control was 92% at a median follow up of 38 months. A recent review summarizes clinical trials in SFRT.[20] In a recent study, 10 patients with voluminous NSCLC were treated with initial LRT fraction of 18 Gy in the vertices and 3 Gy in the periphery followed by conventional radiation of 25-29 daily fractions of 1.8 Gy-2 Gy. There was no LRT related toxicity with 42 % mean decrease in tumor volume.[81] The immune parameters were not evaluated in this study.

Future Perspectives

There is profound clinical interest in optimizing RT for maximum antitumor immune responses. Novel ablative RT paradigms include SABR, FLASH, and SFRT. Perhaps heresies of classical radiobiology, may produce better immunologic synergy than conventional RT fractionation, dose rate, and targeting. Although preclinical studies have provided insights into the immunostimulatory role of ablative RT, there is a relative lack of clinical studies that correlate radiation fractionation and dose-dependent induction of immune

responses with patient outcomes. Hence, there is a compelling need for translational studies to evaluate the antitumor responses through immune monitoring of large cohorts of patients treated with SABR, FLASH, and SFRT as they mature or begin to enter clinical use, to determine the immune profile and biomarkers that are associated with long term remissions or disease recurrences. The knowledge obtained from such studies would assist in designing future clinical trials that will ultimately help realize the hope of optimal synergy between RT and antitumor immunity.

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