Harnessing Radiation Effects on the Immune System: Balancing Acts

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ABSTRACT: The potential of radiotherapy (RT) to induce immune recognition of cancer cells is a growing topic of research. It has been suggested that partial volume irradiation used in GRID therapy/Lattice RT, a type of RT in which radiation does not cover the entire tumor intentionally but rather is spatially fractionated, sometimes induces an immune response. The therapeutic benefits of RT may not be limited solely to the targeted volume, but also present systemic anti-tumor effects, called the abscopal effect. The next challenge of RT now is to balance and control this immune response in patients. Here, we review what is known about the impact of RT on the innate and adaptive immune response.

KEY WORDS: radiation, immunotherapy, inflammation, tumor microenvironment

I. INTRODUCTION

Clinical radiotherapy (RT) is a cornerstone in oncologic management and aims to deliver an ablative or sterilizing dose of radiation to the tumor while minimizing damage to adjacent normal tissue. RT was thought to locally control tumor cells by inducing unrepairable DNA damage either by a direct action or indirectly when mediated through reactive oxygen species.[1,2] However, it is becoming more apparent that the therapeutic benefits of RT may perhaps extend beyond the targeted volume, encompassing additional systemic antitumor effects, such as an abscopal anti-tumor effect that is mediated by the immune system. This rare effect has been observed over several decades[3,4] and seems to be a consequence of immune-mediated clearance of tumors.[5] The biggest challenge for the future of RT is to modulate the immune responses involved in the tumor control including distant metastatic lesions while minimizing inflammatory processes involving normal tissues, to improve cancer patient care. However, an important idea to consider is that RT-induced activation of the immune system leads to compensatory mechanisms of suppression within the tumor and tumor microenvironment (TME), thus negating the effects of RT and perhaps providing the rationale for combining both RT and immunotherapy.

There is a very delicate balance between the activation of the immune system and the immunosuppression induced by RT in the tumor and the communicating TME. The effects of different RT doses and tumor volume exposure on the immune system and the TME remain unclear. A better understanding of these interactions will contribute to the optimization of RT treatment, which may prevent the recurrence of cancer and the demise of patients.

II. RT-INDUCED INFLAMMATION LEADING TO ANTI-TUMOR

A. Induction of Adhesion Molecules, Cytokines, and Chemokines

As part of the RT-induced inflammation process, there is an activation of the dendritic cells (DCs) which migrate to the adjacent lymph nodes, where they activate the T lymphocytes.[6,7] In addition, RT increased expression on the tumor cells of costimulatory molecules (CD80), adhesion molecules like intercellular adhesion molecule 1 (ICAM-1), or stress ligand (NKG2DL).[8–10] RT also increased expression of MHC-I, tumor-associated antigens (TAA), and the Fas/Fas ligand pathway rendering tumor cells more sensitive to cytotoxic T lymphocyte attack.[11–14] Moreover, high-mobility group protein

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B1 (HMGB1) released from dying cells stimulated the TLR4/MyD88/TRIIF pathway, which also activated T cells.[15]

RT activates pro-inflammatory factors including interferons and chemokines that attract activated T cells into tumors,[11] induced the C-X-C motif ligand CXCL9 and CXCL16 chemokines that recruit both effector $CD8^+$ T cells and helper $CD4^+$ T cells.[15] RT was shown to induce the production of type I interferon (IFN) by activating a stimulator of interferon genes (STING) pathway in tumor-infiltrating DCs, as well as the increased secretion of CXCL10, a C-X-C chemokine receptor ($CXCR3^+$) that recruits IFN-γ secreting $CD8^+$ T cells.[16,17] STING protein is activated by cyclic GMP-AMP (cGAMP) produced by cGAMP synthase (cGAS), which detects cytosolic dsDNA fragments in irradiated cancer cells.[16,18–20] The binding of cGAMP by STING activated several transcription factors like NF-κB, IRF3, IRF7 STAT3, and STAT6, which stimulated the immune system to respond against pathogens and cancer cells.[21,22] Type I IFN stimulates DCs to present tumor-associated antigens to T lymphocytes, thereby activating the specific T-cell response both within the irradiated site and in the lymph nodes. Therefore, RT stimulation of the immune system starts with the activation of the innate immune response followed by activation of the adaptive immune response. Activated T and natural killer cells secrete type II IFN, i.e., IFN-γ, which triggers the expression of MHC-I on the surface of tumor cells.[19,23] RT also activates an inflammatory response, in part, by induction of cell adhesion molecules (CAMs).[24]

B. RT-Induced Tumor Infiltration of Immune Cells

The cascade of RT-induced inflammation includes leukocyte infiltration into the tumor by: (1) a change in vascular structure, (2) increased expression of adhesion molecules, and (3) chemokine secretion.[25] This cascade has recently been demonstrated in work comparing whole tumor irradiation vs partial volume RT (PVRT) from our group and others[26–28] (Fig. 1). Similar to RT, PVRT induces inflammatory cytokine interleukin (IL)-1β, tumor necrosis factor

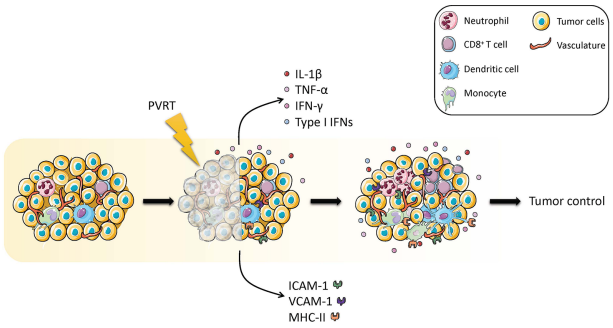

FIG. 1: Anti-tumor immune response following PVRT. PVRT stimulates the production of cytokines such as IL-1*β*, TNF-*α*, IFN-*γ*, and type I IFNs. In addition, PVRT stimulates the expression of adhesion molecules (ICAM-1 and VCAM-1) by the endothelial cells. These pro-inflammatory factors stimulate the tumor infiltration by immune cells such as neutrophils, monocytes, dendritic cells, and $CD8^+$ T cells, resulting in tumor control, similar to 100% tumor volume exposure to RT. In addition, PVRT elicited abscopal effect in pre-clinical models, indicating the urgency to apply it to the clinical setting.

(TNF)-α, and type I and II IFNs secretion which affects the upregulation of vascular cell adhesion molecule 1 (VCAM-1) on tumor endothelium.[8,29,30] Increased expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and VCAM, in tumor vessels, enables tumor infiltration by T lymphocytes.[11,31] ICAM-1 also mediates the migration of neutrophils into the tumor.[29] RT induces the rapid and transient infiltration of neutrophils that eliminate tumor cells by releasing reactive oxygen species (ROS).[16]

The essential role of T cells in the tumor response to RT has been demonstrated in several animal models suggesting that T cells likely polarize RT-induced inflammation and immune stimulation to inhibit tumor regrowth[32–35] and a similar response was observed in our work using PVRT.[27] Enhanced T cell infiltration into the tumor microenvironment following RT has been reported in several models.[33,35–38] The mechanisms that regulate RT-induced T cell recruitment are mechanistically like those that regulate myeloid cell infiltration and involve modulation of adhesion molecule and chemokine expression. In addition to the effects of TNF and IL-1 on endothelial

adhesion molecule expression, IFN-γ production by infiltrating T cells drives enhanced expression of VCAM-1 as a positive feedback loop to further enhance T cell infiltration.

The PVRT paradigm was further corroborated by Arina et al.[39] in which they demonstrate that activated and memory T cells appear to be more radioresistant than naive T cells within the tumor.[40–42] They have shown that antigen-experienced tumor-infiltrating T cells are more radioresistant as compared with activated/memory cells in the spleen from mice exposed to a similar RT dose. These results suggest that the influence of the tumor (and certain non-lymphoid tissue) microenvironments could be key to fully understand this phenomenon. Intra-tumoral T cells might have different gene expression patterns and degrees of radio-resistance depending on tumor type and location, thus acquiring phenotypic characteristics of the tumor microenvironment, as part of their ability to adapt to the microenvironment.[43]

In addition, Arina's group showed that irradiated preexisting intra-tumoral T cells (Trm, T resident memory) had a severely diminished proliferative capacity, but they retained their motility and showed increased production of IFN-γ when compared with unirradiated T cells. Subsequently, a myeloid cell infiltrate that was both less suppressive and more enriched in DCs expressing higher MHC-II expression could explain the improved effector function. IFN-γ production also mediates T cell-induced tumor ischemia[44] which might be a key contributor for tumor control by preexisting intra-tumoral T cells.

C. RT-Mediated Trafficking of Tumor-Specific Lymphocytes

Several investigators have suggested that RT can expose tumor-associated antigens[45] and even alter phenotypes,[46] as well as upregulate expression of certain molecules such as MHC class I[35,47,48] and II[49] and TNF ligands.[50,51] These changes promote apoptosis as well as antigen presentation to dendritic cells and lymphocytes. RT has also been shown to upregulate the production of CXCL16, which causes migration and activation of $CD8^+$ T cells and Th1 Lymphocytes expressing CXCR6 receptors.[52,53] In addition, RT can promote the release of signaling molecules directly from damaged cells, such as HMGB1 and ATP.[54,55] ATP has been shown to stimulate the production of cytokines from dendritic cells, whereas HMGB1 interacts with Toll-like receptor 4 (TLR4) on macrophages and dendritic cells to promote their activation and migration into the TME.[56,57] Kroemer's group also demonstrated translocation of calreticulin (CALR) from the mitochondria to the plasma membrane after RT, facilitating processing by dendritic cells.[58,59]

D. RT-Induced Systemic Abscopal Effects

The RT-induced abscopal effect refers to regression or disappearance of lesions outside of the irradiated field (first described in the 1950s by Mole). Several investigators have demonstrated that targeted radiation treatments can produce tumor shrinkage at sites distant from the target. However, the effect is usually seen in combination with immunotherapy, and the exact mechanism has yet to be elucidated. Demaria and colleagues were the first to propose that the abscopal effect is an immune-driven phenomenon, indicating that local RT produces systemic effects.[60] Subsequently, with Formenti they reported that localized RT in combination with ICB (in this case: CTLA4 blockade), inhibited the development of lung metastases and that hypofractionated RT + CTLA4 blockade resulted in an abscopal effect in a mouse tumor model. One investigation that utilized RT with anti-CTLA-4 antibody demonstrated that this effect was dependent on fractionated RT as well as CD4 and CD8 infiltration of the secondary sites, and showed that the effect was greater with higher doses per fraction.[61]

Similarly, RT + anti-PD-1 antibody treatment has produced abscopal effects in mouse models of melanoma, renal cell carcinoma, adenocarcinoma, and other carcinoma models. Park and colleagues[62] demonstrated that a combination of SABR with anti-PD-1 treatment resulted in almost complete regression of the primary (melanoma and RCC) tumors treated and a 66% reduction in distant tumors via abscopal responses.

More recent clinical studies reported by Luke et al. suggested that radiation may augment anti-PD-1 immunotherapy as they observed marked control of tumors. In an exploratory subset analysis of partially irradiated tumors [due to large size lesions, making stereotactic body RT (SBRT) unsafe], they observed similar tumor control compared with total tumor volume irradiation, suggesting a compensatory immune effect of nontarget RT in combination with anti-PD-1 immunotherapy leading to equivalent tumor control. The most intriguing finding was that the low dose of RT applied to the nontargeted portion of the tumor may have been sufficient to elicit an immune response,[63] i.e., that SBRT might have immune-activating effects by itself.

E. RT-Induced Activation of Innate and Adaptive Immune Responses

Although for almost a century, RT has been considered as a direct approach targeting the exposed cells, there is an enormous amount of evidence showing that irradiated cells are the source for both bystander response and the abscopal effect. The bystander response to radiation is due to the communication between the irradiated and the non-irradiated adjacent cells.[64] The death of the non-irradiated cells can be provoked by the transmission of cytotoxic and genotoxic compounds from the irradiated cells.[65–68] The long-range effects are linked with the activation of the immune system. RT-induces immunogenic tumor cell death which involves the production and the release of several pro-inflammatory factors by the irradiated cells, such as TAA,[69] stress-induced molecules such as NKG2DL,[70] damage-associated molecular patterns (DAMPs) such as HMBG1, the expression of CALR on dying tumor cells, which facilitates phagocytosis and activation of dendritic cells,[71] as well as pro-inflammatory cytokines such as type I interferons (IFNs).[72]

The IFN family represents a widely expressed group of cytokines. The term "interferon" derives from the ability of these cytokines to interfere with viral replication.[73] To detect pathogens, the innate immune system developed several systems of extranuclear DNA recognition. For example, TLR9 specifically recognizes the unmethylated cytidine-phosphate-guanosine (CpG) motifs in bacterial DNA,[74] whereas ZBP-1 mediates the host defense by sensing viral nucleic acids.[75–80] To date, one of the main innate mechanisms is the cGAS/STING pathway. It starts with the DNA-sensing by the enzyme cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP–AMP) synthase (cGAS).[81] cGAS is activated upon binding to cytosolic double-strand DNA. Once activated, cGAS converts adenosine 5′-triphosphate (ATP) and guanosine 5′-triphosphate (GTP) into cyclic GMP–AMP (cGAMP). Then, cGAMP acts as a secondary messenger that binds to and activates the stimulator of interferon genes (STING). The activation of STING induces a conformational change[82,83] allowing its translocation from the endoplasmic reticulum to the Golgi.[84,85] Subsequently, STING recruits and activates tank-binding kinase 1 (TBK1), which phosphorylates STING and the transcription factor IRF3.[86–88] This triggers a signaling cascade, which leads to the production of a series of immune and inflammatory mediators such as type-I IFNs and other cytokines.[89–91]

Similar to viral infection, RT induces DNA accumulation in the cytosol which activates cGAS,[92] that subsequently binds and activates STING, essential for the production of type I interferons (IFNs) following radiation.[93] IFNs are essential for the function of $CD8^+$ T cells after RT, stimulating innate and adaptive immune responses in tumors.[37,81,94,95] However, it has been suggested that in addition to the canonical cGAS/STING pathway, STING can also be activated in response to DNA damage, independent of cGAS activation, via other cytosolic DNA receptors. It seems that DNA damage receptor MRE11,[96] DEAD-box helicase 41 (DDX41), and members of the Aim2-like receptor (ALR) family,[97,98] can also sense cytosolic DNA and induce type I IFN production via the STING pathway.

Recently, another STING activation pathway has been described that is independent of cGAS. In this non-canonical pathway, earlier recognition of the DNA damages is mediated by the DNA-binding

protein IFI16, together with the DNA damage response factors, ATM and PARP-1, resulting in the formation of an alternative STING signaling complex. In this complex, TRAF6 catalyzes the formation of K63-linked ubiquitin chains on STING. Once ubiquitinylated, STING will activate the transcription factor NF-κB, rather than IRF3, inducing the expression of multiples genes such as type I IFNs, cytokine IL-6, and chemokine CCL20.[99] Another cGAS-independent STING activation triggering the recruitment of the transcription factor IRF7 has been described in response to DNA vaccines.[100] These data show the complexity of the mechanisms involved in the immune system activation following pathogens infection, but also in response to RT. Although the canonical cGAS/STING pathway has been shown to be activated after RT,[101–103] it might not be always the case.

Spatially-fractionated GRID RT (SFGRT) such as GRID/LRT, which are the clinical equivalent of the PVRT previously discussed, have been linked to a bystander and an abscopal effect,[104] through the activation of the host immune system.[28,105] Markovsky et al.[27] showed that a single dose of RT (10–20 Gy) delivered to half of the tumor volume (PVRT) not only reproducibly activated an anti-tumor immune response comparable to the response of a 100%-irradiated tumor volume but can also elicit a significant abscopal effect in a bilateral 67NR breast orthotopic mouse model. Tumors exposed to PVRT showed a significant infiltration of cytotoxic $CD8^+$ T cells 24 hours after RT, concomitant with an increased expression of adhesion molecules responsible for lymphocytes recruitment, with ICAM[106,107] expression being the most substantial. This response to PVRT was abrogated by treatment with either anti-CD8 or anti-ICAM antibodies, indicating that the involvement of the immune system in this tumor response was crucial. Indeed, numerous pre-clinical studies show that $CD8^+$ T cells are essential for the anti-tumor effects of RT and depleting these cells will completely abolish the effects of RT.[34,108,109] RT can mediate antitumor immunity through the maturation of dendritic cells (DCs) and activation of T cells by enhancing DNA sensing–mediated type I/II interferon (IFN) production,[27,101,110] but can also stimulate the antitumoral adaptive immunity by inhibiting pro-tumoral factors such as tumor-induced $CD45^-$ $Ter119^+CD71^+$ erythroid progenitor cells.[111] These cells called "Ter cells," promote tumor progression by secreting artemin (ARTN) and represents the majority of splenocytes in animals with advanced solid tumors.[112,113]

III. FIRST BALANCING ACT: RT-INDUCED IMMUNE ACTIVATION-SPARING NORMAL TISSUE TOXICITY

RT is the most commonly used therapeutic modality for most cancers in combination with/without chemotherapy and surgery.[28] RT is based on the principle that radiation will produce lethal dsDNA breaks in exposed cells. Ionization and excitation of molecules contained in cells, lead also to the production of radical species, such as ROS and nitric oxide synthase, that will damage cell constituents, notably nuclear DNA but other subcellular targets too, such as cell membrane,[114,115] mitochondria[116,117] and lysosomes.[118] Since the beginning of RT in the clinical setting, protocols and devices have undergone multiple revisions leading to better accuracy of radiation delivery and less normal tissue toxicity. We have seen the emergence of new and advanced technologies such as intensity-modulated RT (IMRT), SBRT, three-dimensional conformal RT (3D-CRT), image-guided RT (IGRT), particles radiation such as proton, photon, electron therapies,[119] and more recently, FLASH RT[120,121] which allows the deliverance of RT at ultra-high dose rates (> 40 Gy/s) that are much higher than conventional RT dose rates (~ 5 Gy/min) usually used in the clinical setting.[122] These new and improved technologies allow for the accurate delivery of high doses of radiation and excellent coverage of the tumor avoiding healthy surrounding tissues. Furthermore, these new developments allow for different total dosing to certain areas, which is known in the field as "dose painting" while taking into account the different hypoxic areas of the tumor which are more resistant to the prescribed RT and therefore higher total RT doses are delivered.

Conventional RT modalities may not be the most suitable treatment in case of disseminated or diffuse tumors, potentially located very close to radiosensitive organs. In this configuration,

instead of targeting the entire volume of the tumor, methods such as GRID/Lattice RT (LRT) delivers high-dose radiation to small volumes within a tumor target.[123–125] In GRID/LRT or SFGRT, grids were usually composed of a shield with an array of openings of circular or square shapes ranging in size from 0.5 to 1.5 cm. This technique was developed in the 1930s and the rationale behind it was that such a treatment permitted higher dose delivery with acceptable skin toxicity.[126–128] Although fewer patients have received GRID treatment compare to conventional RT, significant and dramatic tumor regressions have been observed and reported.[129–132] Nevertheless, GRID therapy is limited to treating complex anatomical tumor targets. Therefore, Wu et al. developed a new technical concept using modern RT instrumentation to advance traditional two-dimensional (2D) GRID treatment to modern 3D high-dose LRT.[133] Its basic principle is to create multiple localized high-dose small spheres called vertices with a certain degree of separation to form low-dose regions while keeping the dose level lower in the periphery of the tumor to avoid related toxicity. LRT has been utilized clinically in patients and has resulted in improved local control without added toxicity.[123,125,134,135] Although its use is limited to small tumors, a recent phase I trial suggests that it is also safe and efficient in patients presenting larger tumors.[124] According to initial pre-clinical and clinical data, it is becoming apparent that LRT activates the immune system.[27,28,39,63]

Basically, by using RT delivered more precisely to the tumor, avoiding normal tissues toxicity, using state of the art procedures such as SFRT, GRID/LRT, or FLASH, and "dialing down" the cytokine/chemokines generated to avoid a "cytokine storm," we might be able to stimulate the immune response and limit the normal tissue toxicity. This is particularly important now since RT is used in combination with immune checkpoint blockade (ICB) and shows improved results as compared to the ICB therapy alone. Conversely, this combination therapy while more successful and longer-lasting may increase the potential for the development of "cytokine storm." Future studies are necessary to determine the use of these new combinations, as fas as RT dose, fractionation, tumor volume exposure, and in the optimal combination with ICB to achieve this goal. In addition, the response will also depend on the tumor type, stage of the disease, patient immune background, and previous treatments received.

IV. SECOND BALANCING ACT: RT-INDUCED IMMUNOSUPPRESSION WITHIN THE TUMOR AND/OR TME

We have already mentioned above in the First Act the immunostimulatory RT effects on the tumor cells and the TME, and while it may activate the immune cells within both compartments, it may concomitantly generate tumor resistance in the TME, via different mechanisms. RT has been shown to recruit regulatory T cells,[136,137] myeloid-derived suppressive cells (MDSCs) immune populations including myeloid cells (mostly immunosuppressive). In addition, RT activates immunosuppressive cytokines such as transforming growth factor-beta (TGF-β)[138] and induces hypoxia by modifications of the tumor-associated stroma and endothelium. Moreover, the efficacy of RT may be impaired by (a) intrinsic tumor cell radio-resistance; (b) tumor heterogeneity; (c) a diversity in radiation responses; and (d) a resistance-promoting microenvironment.[139–143] Beyond biological features of RT dose, fractioning and timing regulate the net immune stimulating/inhibitory effect.[61,144] Consequently, whereas RT has the potential to become a game-changer in the field of immuno-oncology due to its capability to modulate the immune responses, for a positive outcome it can also concomitantly result in immunosuppressive effects. Mechanistic insight from preclinical models underscores the intricacy of host and tumor responses that are dependent on tumor intrinsic factors (the radiosensitivity of the tumor, the composition of the TME), and parameters pertaining to RT itself (i.e., radiation dose, dose rate, fractionation, and source of RT). Indeed, the optimal RT dose and regimen to initiate anti-tumor immunity is currently debated and the immunogenic background of the patient should also be part of the formula.

Although the involvement of the immune system following RT plays an important anti-tumoral effect, if not properly controlled, it can lead to

various adverse effects and cause damage to various organs for years after exposure.[145] Inflammation (acute and chronic) may be the most important mediator of normal tissue responses to RT, involving damage of the vasculature, infiltration of leukocytes, and the secretion of numerous immune system mediators.[146,147] This response to RT should be inhibited by anti-inflammatory mechanisms so as to "dial down" the pathological effects on the normal tissues. If not, the chronic inflammation induced by inflammatory cytokines and free radicals generated by RT may disrupt the normal function of a number of crucial organs.[148,149] Several studies suggest that RT-induced inflammation diseases such as pneumonitis, nephrotoxicity including fibrosis, could be treated with antioxidants such as selenium, beta-carotene, and silibinin, known to be able to ameliorate the levels of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, IFN-γ, and TGF-β.[150–154]

Similarly, RT-induced cardiovascular diseases (CVD), which are an emerging problem in a growing population of cancer survivors receiving thoracic radiation, have been linked to inflammation.[155,156] ROS production is known to impair endothelial and vascular smooth muscle cell functions, contributing to the development of CVD.[157] Inhibition of the receptor of pro-inflammatory cytokine IL-1 seems to prevent the RT-induced arterial inflammation[158] and the development of the RT-related CVD that might be mediated by endothelial inflammation.[159] The chronic activation of the endothelial cells, leading to upregulation of adhesions molecules such as ICAM-1 seems to play an important role in the RT-induced side effects.[160–163] Indeed, it was shown that ICAM-1 knockout abrogates pulmonary inflammation[164] and its serum levels could be used as an early detection marker for radiation pneumonitis.[165] Several studies suggest that STING could play a pivotal role in this inflammation process.[166–169]

V. CONCLUSIONS

Our discussion highlights the potential of RT to stimulate an anti-tumoral immune response not only locally, but also to trigger an abscopal effect as well (enhanced perhaps by SFGRT/PVRT), and additional work is needed to take advantage of these procedures in the future. The immune system activation seems able to be induced by numerous pathways and further systematic studies will determine which pathway are involved in each case and which need a fine tuning to protect normal tissues. In adition, RT induces suppression of the immune response, that also needs to be identified specifically in each tumor and patient and minimized to optimize the RT effects on the tumor response.

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