

# From DNA Damage to Nucleic Acid Sensing: A Strategy to Enhance Radiation Therapy

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## Abstract

Local irradiation (IR) is widely used in the treatment of primary and metastatic tumors. However, the impact of IR on the immune response is currently being defined. Local and distant relapse after radiotherapy often occurs. The current rationale for the use of IR is based on direct cytotoxicity to cancer cells; however, recent studies have shown that reduction of tumor burden following ablative (large-dose) IR largely depends on type I IFN signaling and CD8<sup>+</sup> T-cell response. Here, we review recent findings indicating that antitumor effects of radiation are contributed by both innate and adap-

tive immune responses. We focus on immune mechanisms, including cytosolic DNA sensing pathways that bridge the traditional view of IR-mediated DNA damage to DNA-sensing immune pathways. Also, we discuss how the efficacy of radiotherapy might be enhanced by targeting nucleic acid-sensing pathways. These findings highlight the mechanisms governing tumor escape from the immune response and the therapeutic potential of synergistic strategies to improve the efficacy of radiotherapy via immunotherapeutic intervention. *Clin Cancer Res*; 22(1); 20–25. ©2015 AACR.

## Introduction

Radiotherapy is used to treat approximately 60% of all cancer patients and currently is used mostly for localized disease (e.g., head and neck, prostate cancer, and lung cancer) alone or in combination with surgery and/or chemotherapy (1, 2). Stereotactic body radiotherapy (SBRT) is a technique that takes advantage of the technologic advances in image guidance and radiation dose delivery (3). SBRT directs ablative doses (10–20 Gy) to tumors with acceptable toxicity that was not previously achievable (3). The tumor response to radiation includes DNA damage, modulation of signal transduction, and alteration of the inflammatory tumor microenvironment (1). High-dose radiation can also induce apoptosis of tumor endothelial cells (4). Improvements in radiotherapy have traditionally focused on advances in the technology of radiotherapy delivery, and combining radiotherapy with chemotherapy, radiosensitizers, gene therapy, and most recently, immunotherapy (2). Although high-dose radiation might kill or suppress lymphocytes, including suppressive cells and pathways, or induce immunosuppression by activating tissue-repairing pathways, this review mainly focuses on the potential enhancing effects of radiotherapy on the immune system.

It has been increasingly observed that the use of local radiotherapy may stimulate antitumor immune responses by increase of both apoptosis and necrosis of tumor cells and the subsequent

increase in antigen presentation and expression of immunomodulatory genes (5). Most studies have focused on the immunomodulating effects directly induced on tumor cells. Radiation can modulate the peptide repertoire and enhance MHC class I expression on tumor cells, which boosts the efficacy of adoptive cytotoxic T lymphocyte (CTL) immunotherapy (6). Other reports have illustrated that local radiation of tumors alters the phenotype of tumor cells, rendering them more susceptible to vaccine-mediated T-cell killing (7, 8). Local radiation may also work by altering the tumor microenvironment to promote greater infiltration of immune effector cells (9–11). Although many studies do show the potential immunomodulatory effects of localized ionizing radiation (IR) on the primary tumor, the mechanisms for radiation-mediated immunomodulation and whether these effects can be translated for the treatment of metastases remain unclear. It is acknowledged that radiation can trigger host immunity against tumors; however, the extent of tumor reduction by this process is poorly defined.

## Adaptive Immunity Is Essential for Antitumor Effects of Radiotherapy

Our laboratory and other groups have observed that rapid reduction of tumor burden after ablative radiation clearly depends largely on T-cell response (12, 13). Radiation-induced equilibrium or dormancy, a common feature in both clinical and preclinical cases, is also a balance between tumor cell proliferation and T-cell-mediated killing (14). Ablative radiation increases T-cell priming in draining lymphoid tissues and reduction of the primary tumor or distant metastasis in a CD8<sup>+</sup> T-cell-dependent fashion (Fig. 1). However, it is unclear whether an increase in T-cell activation in the draining lymph nodes (DLN), an increase in T-cell trafficking to tumor tissues, or both, is responsible for tumor regression. We have demonstrated that ablative radiation-initiated immune response and tumor reduction are sometimes abrogated by conventional fractionated radiation or certain adjuvant chemotherapies, but are greatly amplified by local

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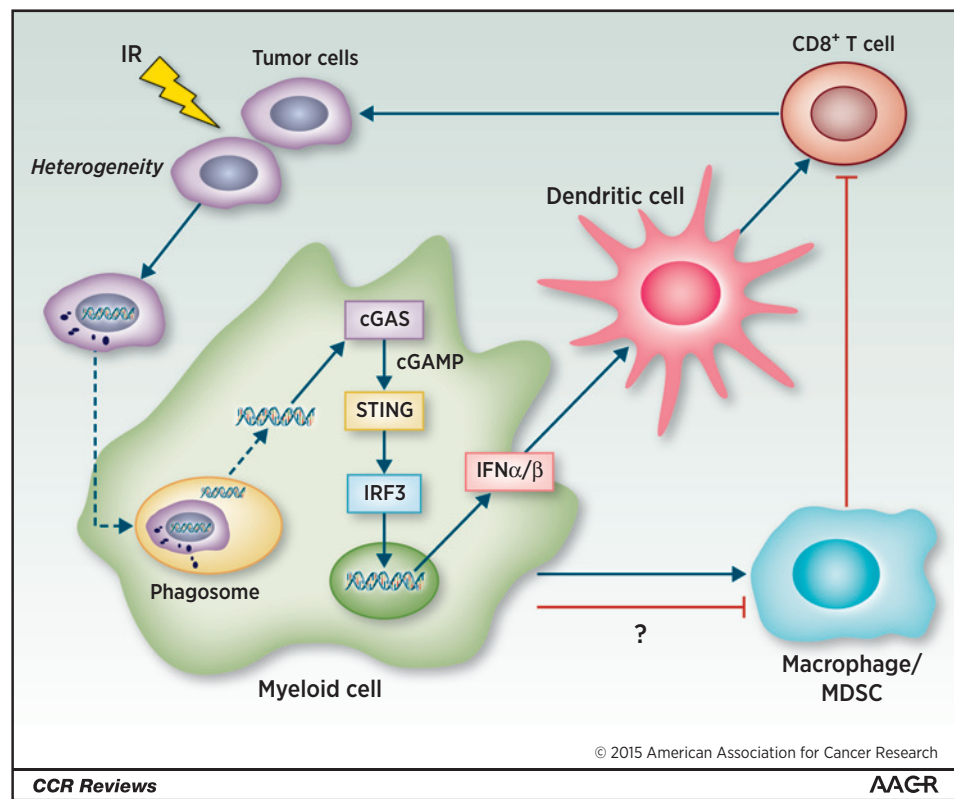
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**Figure 1.**

Cytosolic DNA sensing orchestrates tumor immunity during radiotherapy. Radiation results in the upregulation of "find-me" and "eat-me" signals from tumor cells. During phagocytosis in myeloid cells, the DNA fragments hidden in irradiated tumor cells are released from phagosomes to cytoplasm, acting as a danger signal. The cyclase cGAS (GMP-AMP synthase) binds this DNA, becomes catalytically active, and generates cGAMP as a second messenger. cGAMP binds to STING, which in turn activates IRF3 to induce type I IFN production. Type I IFN signaling in DCs promotes the cross-priming of CD8<sup>+</sup> T cells, leading to tumor control. However, the influx of macrophage and MDSCs after radiation attenuates CD8<sup>+</sup> T-cell responses to help tumor escape.



immunotherapy (12). These data support the rationale for synergy between current radiotherapy/chemotherapy strategies and immunotherapy, emphasizing the need for proper radiotherapy that not only reduces tumor burden but also enhances immune activation. Therefore, subsequent immunotherapy can sustain or amplify IR-initiated immune responses.

In the natural history of cancer development, the picture of how the immune system functions inside the tumor microenvironment remains unclear (15). Tumors are able to grow progressively in immune-competent hosts, despite tumor antigenicity and the presence of tumor-reactive lymphocytes in the tumor-bearing hosts (16). Accumulating evidence indicates that many human cancers are indeed antigenic and can be recognized by T cells (17–19). Furthermore, infiltration of T cells, especially CD8<sup>+</sup> cells, into immunogenic human tumors is often associated with better prognoses (20). However, the immune system fails to reject these tumors despite adequate immune recognition and competent T-cell function in peripheral tissues. The tumor microenvironment is populated by various types of inhibitory immune cells, including regulatory T cells (Treg), alternatively activated macrophages, and myeloid-derived suppressor cells (MDSC), which shut down T-cell activation and assist tumor outgrowth (21, 22). This failure could be attributed to the suppressive tumor environment that impedes the proper recruitment and function of the immune system (23–26). An alternative hypothesis is that excessive immune-suppression factors dominate the tumor microenvironment, which favors tumor recurrence following radiation. Biswas and colleagues (27) showed that radiation induces the production of TGF $\beta$  to promote increased circulating tumor cells and lung metastases in the MMTV/PyVmT transgenic model of metastatic breast cancer, which has the mouse mammary tumor virus

(MMTV) long terminal repeat upstream of a cDNA sequence encoding the polyoma virus middle T antigen (PyVT). Neutralization of TGF $\beta$  signaling restored the T-cell response to endogenous tumor antigens following radiation (28). Kioi and colleagues (29) showed that radiation upregulates HIF-1 to induce the influx of bone marrow–derived cells that mediate the repair of damaged vasculature, allowing surviving tumor cells to regrow. Taken together, these findings suggest that immune-suppressive factors that hinder the extent of T-cell activation result in a permissive environment that allows tumor resistance to radiation.

### Type I IFN Signaling Induces Increased Function of Adaptive Immunity after Radiation

Innate immunity and adaptive immunity are two integral components for host recognition of tumor cells (16). However, a question remains as to which immunologic pathway associates radiation with the activation of innate immunity to produce an effective adaptive response. Type I IFNs (IFN $\alpha/\beta$ ) are a family of cytokines known to have potent antiviral effects (30). The role of IFNs in immune response to viral infection has been studied extensively, but many questions remain unaddressed with regard to tumor immunity. All vertebrates have a gene-encoding IFN $\beta$ , and a few genes encoding IFN $\alpha$ . Both IFN $\alpha$  and IFN $\beta$  use a common IFN $\alpha/\beta$  receptor that is ubiquitously expressed (31). Type I IFNs are well known as critical mediators capable of bridging innate responses to adaptive immunity (30, 31). IFN $\alpha/\beta$  can directly activate T cells as well and promote their expansion, activation, survival, and memory formation (30, 32–34). Gresser and colleagues (35) showed that type I IFN may

contribute to antitumor responses using an IFN $\alpha/\beta$ -neutralizing antiserum. Interestingly, it has been shown that endogenous type I IFN production plays a critical role in tumor immunoeediting in the MCA-induced tumor formation model (36). More recently, two studies showed that IFN $\alpha/\beta$  promotes maturation of bone marrow-derived cells and splenic dendritic cells (DC) and stimulates the process of cross-priming of CD8<sup>+</sup> T cells against tumor invasion (37, 38). Therefore, type I IFNs are indispensable components of host defense during tumor initiation and progression.

It has been shown that the efficacy of radiotherapy depends on the host immune response, particularly the adaptive immune response. Little is known about whether and how radiation alters the tumor microenvironment to support or hinder concurrent immunotherapy in the early treatment phase. By inducing apoptosis/necrosis of tumor tissues and subsequent release of DNA fragments and other danger signals, local high-dose radiation can promote inflammation (39). Therefore, we investigated the potential role of type I IFNs in bridging the initial inflammation induced by radiation and demonstrated a productive tumor-reducing adaptive immune response (40). Through real-time PCR and ELISA analyses, we showed that radiation indeed increases production of IFN $\beta$  inside tumors. More importantly, the physiologic implications of this increase are significant: The therapeutic effect of radiation is diminished in an IFN $\alpha/\beta$  receptor knock-out host (40). We further showed that type I IFN is essential for bridging innate and adaptive immunity for tumor regression (Fig. 1; ref. 40).

Combining cancer vaccines and T-cell stimulating conventional therapy might be synergistic and produce greater efficacy than either treatment alone. Wu and colleagues (41) have demonstrated that type I IFN signaling orchestrates the synergy of radiation and antigenic peptide vaccine. The deficiency of type I IFN results in the attenuation of the priming and expansion of antigen-specific CTLs after the combination of radiation and local vaccine delivery (41). Similar to immune selection in spontaneous tumors, these studies identify type I IFNs as critical mediators of radiation-induced tumor immunity. This finding indicates that agonists of type I IFN induction warrant further investigation; such agonists could be aimed at disruption of tumor resistance to radiation.

### Cytosolic Nucleic Acid Sensing Mediates the Induction of Type I IFNs after Radiation

Several pathways have been documented to trigger production of type I IFN (42, 43), in which nucleic acids have been considered as potent stimuli for type I IFN production (44). RNA-sensing pathways have been shown to mediate tumor sensitivity to radio- and chemotherapy. The first key players in the pathway(s) to be identified were TLRs, a well-characterized class of pattern recognition receptors (PRR) that are responsible for detecting nucleic acids in intracellular endosomes and lysosomes (45). Conventional chemotherapy also has been shown to induce the production of IFNs by tumor cells in an autocrine fashion (46). This process has been demonstrated to depend on TLR3 recognition, suggesting that endosomal RNA sensing might be the stimulus (46). In contrast, other groups have shown that DNA-damaging agents stimulate TLR3 expression in a p53-dependent manner to induce cell death, and some of them generate double-stranded RNAs that can activate TLR3-dependent secretion of cytokines or

cell death (47–49). To reconcile these results, the interpretation is that the downstream signaling of TLR3 depends on the type of stimulus and the environment. On the other hand, cytosolic RNA sensing is mediated by the adaptor protein mitochondrial antiviral signaling (MAVS) for type I IFN production (43). RIG-I (retinoic acid inducible gene-1) and its homolog MDA5 (melanoma differentiation associated gene 5) are identified as the sensors of cytosolic viral RNA (43). RIG-I detects viral RNA containing 5'ppp, panhandle-like secondary structures, and short dsRNA, whereas MDA5 recognizes long dsRNA in the viral genome (43). RIG-I and MDA5 induce prion-like polymerization of MAVS, which in turn activates TBK1 and IRF3, leading to the induction of type I IFNs (43). Widau and colleagues (50) demonstrated that LGP2 (Laboratory of Genetics and Physiology 2), a suppressor of RIG-I, mediates human tumor cell radioresistance by suppressing type I IFN production. Multiple doses of radiation produced a signature of IFN-inducible genes, a subset of which was later demonstrated to be predictive of chemotherapy and radiotherapy resistance in patients with breast cancer (51, 52). Experimentally, an IFN-related DNA damage resistance signature (IRDS) has been determined by analysis of the activation of RIG-I-MAVS signaling through RNA transfer from stromal cells to tumor cells (53). Because all the evidence supporting RIG-I/MAVS function was derived from a xenograft model, the extent to which RIG-I influences host innate and adaptive immune responses will need to be further determined. It is likely that RNA-sensing pathways in tumor cells, not in immune cells, play an important role in the genotoxic stress induced by radiotherapy and chemotherapy. Particularly, the possibility that radiation and chemotherapeutic agents directly stimulate the expression of TLR3 and RIG-I, respectively, should be considered.

The cytosolic DNA-sensing pathway is mediated by adaptor protein STING (stimulator of IFN genes), which in turn binds to TBK1 (TANK-binding kinase 1), activating the transcription factors IRF3 (IFN regulatory factor 3) for type I IFN induction. We have found that radiation creates stress for tumor cells, causing them to release danger signals that are recognized by patrolling DCs (54). Tumor-derived DNA is released to the cytosol of DCs by an unknown mechanism, and in turn activates the cGAS (cyclic GMP-AMP synthase)–STING–IRF3–IFN $\beta$  axis (54, 55). Radiotherapy can enhance the activation significantly in a more quantitative manner compared with the natural immunity of the tumor. This recently redefined mechanism has bridged the tumor DNA damage response and host cell cytosolic DNA-sensing pathways in the context of radiotherapy (Fig. 1). Despite remaining uncertainties, the evidence at minimum indicates that cytosolic DNA sensing in immune cells potentially plays an important role during the antitumor immune response, and supports the overall hypothesis that the nucleic acid-sensing pathways are responsible for the induction of type I IFN and are essential for an effective adaptive immune response after radiation.

The source of DNA for cGAS-STING activation under conditions of cell stress remains undetermined. Both genomic DNA and mitochondrial DNA are able to induce the activation of the cytosolic DNA sensor, cGAS. Recently, two studies have shown that type I IFN production in apoptotic caspase activity-deficient cells is triggered by mitochondrial membrane permeabilization (MOMP) by pro-apoptotic BAK (Bcl-2-antagonist/killer-1) and BAX (Bcl-2-associated X protein; refs. 56, 57). In particular, both studies found that the trigger for IFN induction released by MOMP is mitochondrial DNA, which is subsequently sensed by the



cGAS–STING pathway (56, 57). Similarly, another study has shown that mitochondrial DNA activates cGAS–STING signaling to trigger antiviral responses in a cell-intrinsic manner, and this process responds to the instability of mitochondrial DNA in the absence of the mtDNA-binding protein TFAM (transcription factor A, mitochondrial; ref. 58). This evidence suggests that the mitochondrion could contribute to the organelle to provide DNA to initiate the cGAS–STING pathway in the context of cell stress. In contrast, it has been reported that nuclear DNA, which constitutes 99% of DNA content inside cells, is able to initiate the activation of the cGAS–STING pathway during the DNA damage response. DNA damage or loss of ATM (ataxia-telangiectasia mutated, a DNA repair apical kinase) results in the release of nuclear DNA in the cytoplasm (59). This finding mechanistically reveals why dysfunction of ATM contributes to the symptoms of patients with ataxia telangiectasia, a disease that has a variety of inflammatory manifestations and is frequently comorbid with cancer. Although these sometimes contradictory findings have thus far confounded our ability to elucidate the DNA source that initiates cGAS–STING pathways, further investigation is clearly warranted, especially in relation to radiotherapy effects.

### Strategies to Enhance Antitumor Immune Response to Improve Radiation Efficacy

Although radiation is effective in producing tumor regression, the tumors may eventually recur even after a prolonged stable or dormant phase (14). Multiple resistance mechanisms facilitate tumor relapse during the inflammatory response that occurs after radiation. Costimulatory and coinhibitory receptors play a dominant role in T-cell activation, differentiation, and function (60). It is reasonable to argue that the agonists and antagonists targeting costimulators/inhibitors are able to increase T-cell function or relieve T-cell inhibition during radiotherapy (14). We have found that the effect of radiation-induced immune-mediated tumor regression gradually diminished as tumor growth continued to progress. Because type I IFN is a potent inducer of PD-L1, we speculated that (i) radiation resistance could be due to the engagement of T-cell–negative regulatory pathways, especially IFN-induced PD-L1 upregulation inside tumor tissues; and (ii) anti-PD-L1 treatment could overcome IR resistance and completely eradicate tumor (61). Increased PD-L1 expression has recently been observed in a variety of human and mouse solid malignancies (62, 63), suggesting that PD-L1 might be a dominant mechanism of immune suppression for some tumors. Therefore, targeting the induction and action of type I IFN as well as PD-L1 blockade could be a potential strategy to pave the way for effective antitumor–adaptive immune responses following radiation.

#### Targeting adaptive immunity

During the reconstitution of the tumor microenvironment after radiation, suppressive immune responses likely override antitumor–adaptive immune response, leading to tumor relapse. Therefore, combining radiation with an immunomodulatory monoclonal antibody (mAb) targeting adaptive immunity provides promising insights to the management of cancer following radiotherapy (64). CD40 agonists have been shown to synergize with radiation to treat B-cell lymphoma in a CD8<sup>+</sup> T-cell–dependent manner (65). It is likely that immune checkpoints disrupt

radiation-induced adaptive immune responses to drive radiation resistance. Blockade of CTLA-4 signaling with a mAb facilitates T-cell motility to generate synergy with radiation for greater antitumor activity (66). We initially observed that the combination of anti-PD-L1 and radiation synergistically promotes CD8<sup>+</sup> T-cell function and optimizes the tumor microenvironment by reducing MDSC accumulation (61). A recent study, which confirmed our observation, also showed that the triple combination of radiation, anti-CTLA-4, and anti-PD-L1 promotes remarkable antitumor immune responses through nonredundant mechanisms (62). In this instance, radiation enhances the diversity of the TCR repertoire, anti-CTLA-4 promotes CD8<sup>+</sup> T-cell expansion by inhibiting Tregs, and anti-PD-L1 further reverses CD8<sup>+</sup> T-cell exhaustion (62). These results provide insight that the combination of radiation with a mAb targeting adaptive immunity is feasible to control tumor effectively. Considering the exciting clinical trial results for immune checkpoint inhibitors, it is expected that the combination of radiation and immune checkpoint inhibitors synergistically generates durable responses in a subset of patients and can avoid overlapping toxicities to minimize side-effects for cancer patients.

#### Targeting innate immunity

Type I IFN signaling is responsible for the antitumor effects of radiation by dictating adaptive immune responses. IFN $\alpha$  has demonstrated antitumor activity in hairy cell leukemia, melanoma, renal cell carcinoma, and other solid tumors (64). To overcome tumor resistance to radiation, it is reasonable to combine radiation with IFN $\alpha$ / $\beta$  fusion proteins in upcoming clinical trials. The combination of IFN $\alpha$ / $\beta$  could be engineered with anti-Her2 or anti-EGFR for specific target delivery (63). Agonists, which target TLRs and STING to enhance the induction of type I IFNs, have also been evaluated for synergy with radiation. Although radiation effects rely on cGAS–STING sensing and signaling, the activation of STING signaling induced by radiation is transient. In contrast, the combination of radiation and a STING agonist is capable of activating STING signaling persistently, leading to durable antitumor immune responses. Our data have shown that the STING agonist 2'3'-cGAMP synergizes with radiation to eradicate tumors effectively, and this process relies on STING function in host cells (54). However, the mechanism for the synergy is unclear because treating tumors alone using 2'3'-cGAMP did not show any antitumor effect. One can speculate that IR creates a microenvironment that allows 2'3'-cGAMP to move into the cytosol effectively, but IR-mediated DNA damage might also contribute to tumor reduction. Furthermore, other molecules that can activate type I IFN might also be valuable. In fact, in preclinical experiments, systemic delivery of TLR agonists has been documented to improve lymphoma outcomes when combined with radiation. Intravenous administration of the TLR7 agonist R848 in combination with radiation resulted in durable responses to T-cell and B-cell lymphoma (67). This combination was able to facilitate the expansion of tumor antigen-specific CD8<sup>+</sup> T cells and the generation of a tumor-specific memory immune response (67). Second, the combination of radiation and TLR9 agonists has shown potential for the treatment of cancer in phase I/II clinic trials. The combination of intratumoral injection of TLR9 agonist CpG with local radiation promotes tumor immunogenicity and induces antitumor CD8<sup>+</sup> T-cell response, leading to systemic regression of indolent B-cell lymphoma (68). This type of combination strategy has been explored in another disease, mycosis

fungoides, where the mechanism is presumably through a greater reduction of Tregs and epidermal DCs (69). Taken together, the evidence supports these IFN induction stimuli as promising adjuvants for greater radiation efficacy in the clinic.

## Conclusions

In this review, we postulate that, in addition to immune checkpoint inhibitors, enhancing cytosolic DNA sensing for type I IFN induction is an essential tool for tumor management via radiotherapy. Type I IFN signaling enhances DC activity and then promotes CD8<sup>+</sup> T-cell cross-priming, leading to tumor control. Multiple nucleic acid-sensing pathways control the induction of type I IFN, and the corresponding agonists have displayed the potential ability to improve radiotherapy. So far, the detailed mechanisms of the combinations are still not well defined. To translate these discoveries into practice, it will be necessary to

further determine the toxicity and synergy of radiation with nucleic acid-sensing agonists in the clinic, as well as to develop carriers for effective delivery of agonists to tumor sites to improve radiotherapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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