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# The Optimal Partnership of Radiation and Immunotherapy: from Preclinical Studies to Clinical Translation

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**The main role of the immune system is to restore tissue homeostasis when altered by pathogenic processes, including neoplastic transformation. Immune-mediated tumor rejection has been recognized as an extrinsic tumor suppressor mechanism that tumors need to overcome to progress. By the time a tumor becomes clinically apparent it has successfully escaped immune control by establishing an immunosuppressive microenvironment. Ionizing radiation applied locally to a tumor alters these tumor-host interactions. Accumulating evidence indicates that standard therapeutic doses of radiation have the potential to recover tumor immunogenicity and convert the tumor into an *in situ* personalized vaccine. Radiotherapy induces an immunogenic tumor cell death promoting cross-presentation of tumor-derived antigens by dendritic cells to T cells. In addition, radiotherapy stimulates chemokine-mediated recruitment of effector T cells to the tumor, and cellular recognition and killing by T cells that is facilitated by upregulation of major histocompatibility antigens, NKG2D ligands, adhesion molecules and death receptors. Despite these effects, radiotherapy alone is only rarely capable of generating enough proinflammatory signals to sufficiently overcome suppression, as it can also activate immunosuppressive factors. However, our group and others have shown that when combined with targeted immunotherapy agents radiotherapy significantly contributes to a therapeutically effective anti-tumor immune response. To illustrate this partnership between radiation and immunotherapy we will discuss as an example our experience in preclinical models and the molecular mechanisms identified. Additionally, the clinical translation of these combinations will be discussed.** 

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## INTRODUCTION

The primary role of the immune system is to protect against infectious agents, a function that has been successfully exploited by the development of many vaccines that prevent diseases. However, the immune system is also responsible for the maintenance of tissue homeostasis with important implications for many chronic diseases including cancer. In cancer, the immune system plays a dual role as an enabler to cancer development and progression and as an extrinsic tumor suppressor mechanism. While the purpose of inflammatory responses is to restore homeostasis, incomplete resolution of inflammation leads to chronic tissue stress, a maladaptive response that can promote genomic instability and cancer progression (*1, 2*). Conversely, the genomic instability associated with neoplastic transformation leads to the generation of neoantigens recognized by T cells (*3*), and to the expression of stress-induced ligands on cancer cells, for example members of the family of NKG2D ligands, which are recognized by natural killer (NK), γδ T cells and effector CD8 T cells (*4, 5*). Unscheduled cell death and local alterations in the stroma associated with tissue invasion generate degraded extracellular matrix components (e.g., heparin sulfate, hyaluronan), and other damage-associated molecular pattern (DAMP) molecules that act as danger signals and activate antigen-presenting cells by binding to Toll-like receptors (TLRs) (*6*). Overall, incipient tumors invariably attract the attention of the immune system, which is often successful at completely removing them. This process is known as the elimination phase of the tumor immuno-editing theory (*7*). Since complete elimination is not always successful, surviving cancer cells that have acquired the ability to evade immune recognition or suppress the anti-tumor response can emerge under the pressure of the immune system. The result is that by the time a tumor is clinically detectable it has usually become resistant to immune-mediated rejection (*8*). In fact, escape from immune-mediated control is now considered a hallmark of cancer (*9*). Importantly, many tumors co-exist with a concomitant anti-tumor T-cell response that has been shown to be associated with a better prognosis (*10–12*), providing evidence of tumor cell plasticity and of immune

escape. The recognition of this active process implies the possibility to intervene therapeutically and restore the ability of the immune system to hinder tumor progression or even cause its regression, even in the setting of overt metastatic disease. This is demonstrated by the clinical success of checkpoint inhibitors that, at least in a subset of patients, block negative regulatory pathways of T cells and recover an effective immune rejection (*13*).

However, an anti-tumor immune response that is powerful enough to control a tumor when it re-emerges by blocking immunosuppressive mechanisms or providing cytokines or other immune stimulatory factors is possible only in a minority of patients (*14*). In addition, while tumor types such as melanoma and renal cell carcinoma have been shown to respond to different immunotherapeutic interventions, most other solid tumors are refractory. Novel agents targeting the programmed death-1 (PD-1)/PD ligand-1 (PD-L1) pathway have enhanced the rates of durable tumor response to 38% used alone and >50% when used in combination with anti-CTLA-4 in metastatic melanoma, and shown activity in other cancer types (*15–19*), but overall the majority of patients with advanced cancer do not respond to immunotherapy alone.

Multiple obstacles hinder the priming and activation of anti-tumor T cells, their recruitment to the tumor site as well as their function, resulting in a formidable challenge to effective tumor rejection (*20, 21*). Ionizing radiation therapy has been known for a long time to cause inflammation in a dose-dependent manner, a side effect that oncologists have tried to minimize by manipulating fractionation and avoiding as much as possible the inclusion of normal tissue in the field of radiation. The appreciation of the potential benefits of the radiation-induced proinflammatory response has only recently emerged (*22, 23*). Work by several groups has identified molecular changes in the tumor microenvironment that contribute to conversion of the tumor into an *in situ* vaccine [reviewed in ref. (*24*)]. Radiation has been demonstrated to promote both, the priming and effector phases of the anti-tumor immune response. Priming results from the induction of an immunogenic tumor cell death by radiation (*25, 26*). In addition, radiation contributes to the effector phase by inducing chemokines and cytokines to recruit effector T cells to the tumor, and through the upregulation of major histocompatibility complex class I (MHC-I), adhesion molecules, death receptors and NKG2D ligands that enable recognition and elimination of cancer cells that have been damaged, but have survived the cytocidal effects of radiotherapy (*27–31*). The contribution of radiation-induced anti-tumor T cells to the response of the irradiated tumor, initially proposed by Stone and colleagues (*32*) is increasingly recognized (*33, 34*). Nevertheless, in most cases these responses are insufficient to result in an immune response capable to achieve systemic tumor control. Interestingly, the latter has been reported occasionally in patients undergoing radiotherapy to one site and responding at tumor sites outside of the radiation field, a phenomenon known as the abscopal (*ab-scopus*, away from the target) effect (*35*). Data in experimental models and patients suggest that the abscopal effect occurs when the anti-tumor immune response is sufficiently activated (*36, 37*). When we first made this observation in a preclinical model (*36*), it seemed reasonable to hypothesize that combining radiation with immunotherapy would provide the optimal therapeutic partnership to achieve immune-mediated systemic tumor control (*23*). Here we review our experience with the different combinations of radiation and immunotherapy tested so far.

### *Mouse Models of Cancer*

To test whether local radiotherapy could induce an abscopal effect when combined with immunotherapy, we employed two main experimental settings that were designed to mimic both early and late metastatic disease (Fig. 1). The 4T1 mammary carcinoma is a poorly immunogenic and highly metastatic tumor. Circulating tumor cells are found within a week from implantation of 4T1 cells subcutaneously, and within a few weeks mice die of lung metastases outgrowth (*38*). The subcutaneous tumor was treated with local radiotherapy once it became palpable, 12–14 days post-implantation. At this time surgical resection of the tumor does not lead to a significant reduction in lung metastases (*39*) and, therefore, inhibition of lung metastases indicates an abscopal effect on visceral metastases rather than reduced dissemination from the irradiated tumor.

Another experimental setting that mimics more advanced metastatic disease with multiple detectable tumor nodules was employed for mouse carcinomas without (67NR and MCA38) or with low (TSA) ability to spontaneously metastasize when cells are injected subcutaneously (*38, 40, 41*). The cancer cells were injected at two separate sites in contralateral flanks, and radiotherapy was delivered to one nodule, mimicking the palliative use of radiation in metastatic disease.

We also tested the role of radiotherapy in the GL261 mouse model of high-grade glioma implanted intracranially. While this tumor type does not spread outside of the brain, it often recurs locally due to the highly infiltrative nature, a behavior that is reproduced in the mouse by GL261 cells (*42*). In this model we tested if immunotherapy could improve the response to whole brain radiotherapy (WBRT) and increase survival, a critical end point for this rapidly fatal tumor model.

### *Combination of Local Radiotherapy with a Dendritic Cell (DC) Growth Factor*

Dendritic cells are professional antigen-presenting cells (APCs) with the unique ability to cross-present antigens from dying cells and activate T-ell responses (*43*).

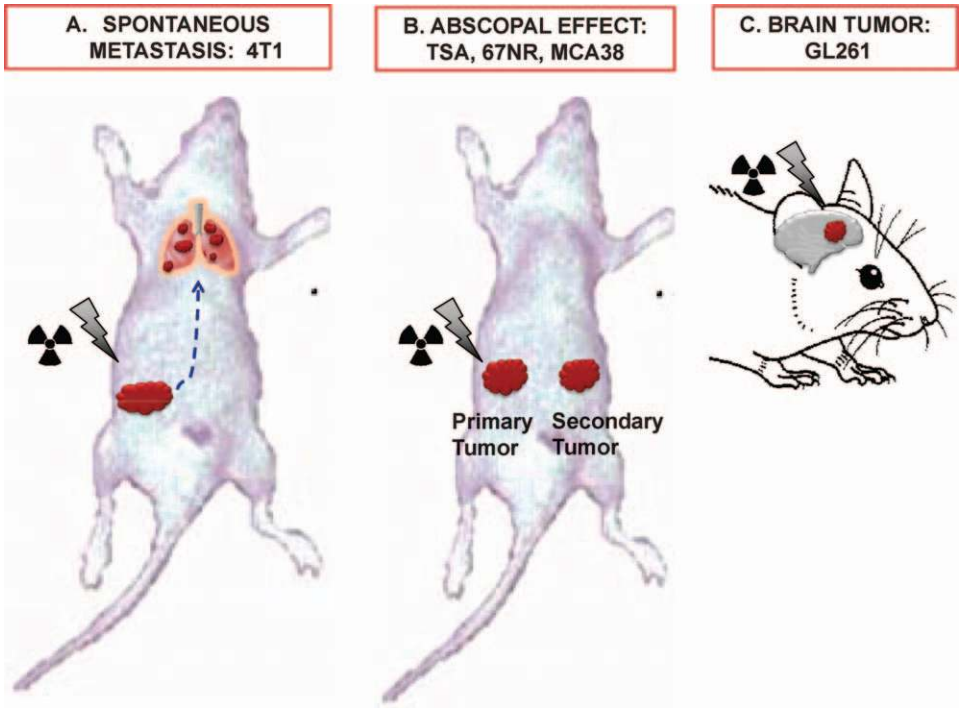


**FIG. 1.** Mouse models used to test combinations of radiotherapy and immunotherapy. Synergistic interaction between radiotherapy and immunotherapy were studied *in vivo* using 5 transplantable murine tumor models of breast (4T1, TSA, 67NR), colon (MCA38) and brain (GL261) malignancies. Panel A: 4T1 cells spontaneously metastasize from the ''primary'' subcutaneous tumor by the vascular route to the lungs. Outgrowth of lung metastases is responsible for death of the animals. Radiotherapy given to the primary tumor once it becomes palpable does not inhibit lung metastases. Panel B: TSA and 67NR are BALB/c-derived tumors. MCA38 is derived from C57BL/6 mice. Irradiation of one subcutaneous tumor module, by itself, does not affect the growth of another identical tumor outside of the radiation field. Panel C: GL261 cells are derived from C57BL/6 mice and grow with infiltrative borders when implanted stereotactically in the brain of syngeneic mice.

Therefore, the suboptimal function of DC in tumor-bearing hosts may be a critical barrier to induction of therapeutically effective anti-tumor T cells by radiotherapy. To overcome this barrier we treated mice bearing the mammary carcinoma 67NR with local radiation and Flt3-ligand (Flt3-L), a growth factor that improves DC numbers and function (*44*) (Fig. 2). Radiation by itself was unable to induce an abscopal effect, despite the fact that 67NR is a relatively more immunogenic tumor compared to 4T1 and TSA. Flt3-L did not have any significant effect by itself on tumor growth, but led to an abscopal effect when combined with radiotherapy (*36*). Expansion of tumor-specific $CD8^+$ T cells able to kill 67NR cells was detected only in mice receiving the combination of radiation and Flt3-L, and T cells were required for the abscopal effect. Overall, data support the concept that radiation generates an *in situ* vaccine by inducing an immunogenic tumor cell death but DC are required to uptake and present the released antigens. In the absence of optimally fit DC the immune response does not develop. This concept has received further support by the results of several studies showing that DC growth factors or injection of DC into irradiated tumors leads to development of anti-tumor T-cell responses (*45–47*).

### *Combination of Local Radiotherapy with a TLR7 Agonist*

Toll-like receptors are a family of receptors expressed by innate immune cells that sense the presence of infectious agents and cellular damage by binding to a variety of pathogen-associated molecular pattern (PAMPs) and DAMPs molecules (*48*). Triggering of TLRs leads to activation of the type I interferon (IFN) and NFkB pathways resulting in production of IFN and proinflammatory cytokines, which enhance DC maturation and antigen presentation ability. Therefore, a variety of synthetic TLR agonists are under investigation as promising immunotherapy agents (*49*). Radiation induces the release from dying tumor cells of high-mobility group protein B1 (HMGB-1) which acts as a DAMP and binds to TLR4 (*25*). However, the ability of radiation to induce sufficient proinflammatory signals to optimally stimulate DC maturation is limited (*22*), suggesting that it could be complemented by administration of a TLR agonist. In support of this hypothesis, intratumoral delivery of the TLR9 agonist CpG has been shown to increase tumor response to radiation (*50*).

We chose to test the TLR7 agonist imiquimod (IMQ), which can be applied topically, in a mouse model of cutaneous breast cancer metastasis. The choice was

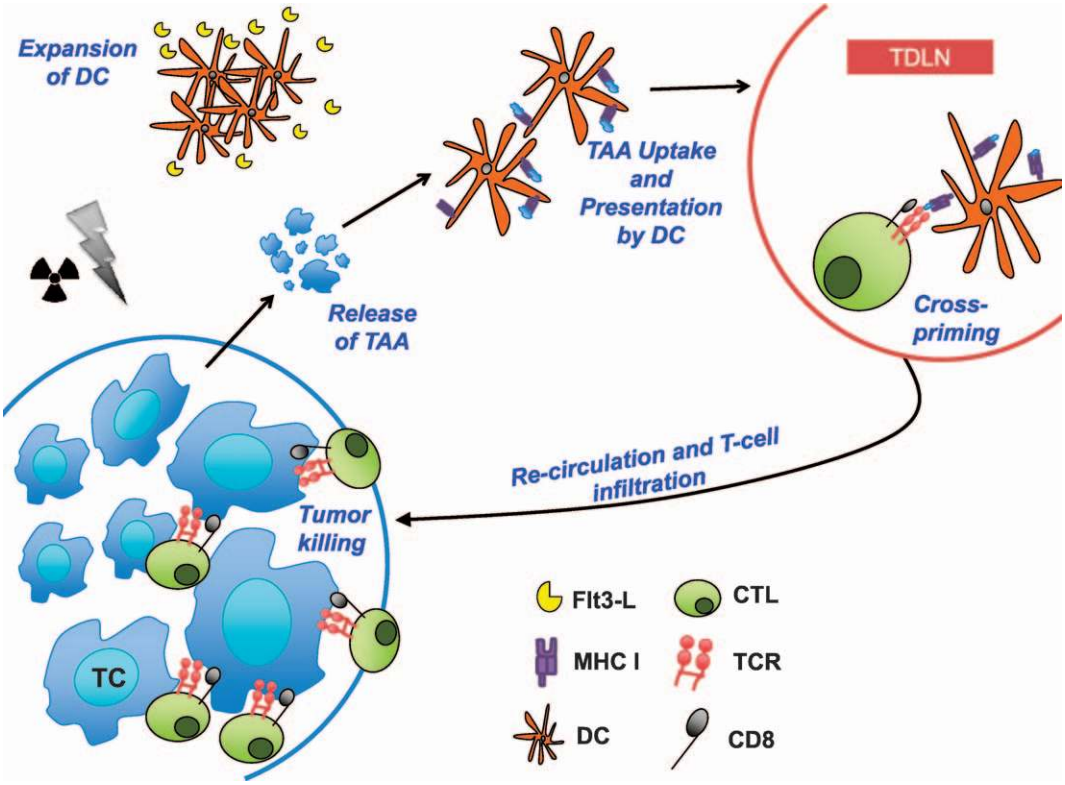


**FIG. 2.** Combination of radiotherapy and Flt3L. Ionizing radiation promotes cross-priming of anti-tumor T cells by inducing release of tumor-associated antigens (TAA) from tumor cells (TC). Dendritic cells (DC), which are expanded by administered FLt3L, uptake and process the TAA and present them as complexes with major histocompatibility (MHC) molecules. TAA-loaded DC travel to the tumor-draining lymph nodes (TDLN) where they activate naïve $CD8^+$ T-cells to become cytotoxic T-lymphocytes (CTL). Tumor-specific CTLs are recruited to the tumor where they kill tumor cells.

motivated by the fact that we had evidence of some activity of IMQ in breast cancer patients (*51*), and that it is FDA approved for topical treatment of some early skin cancers and known to have limited toxicity. IMQ was applied on the skin above TSA mammary carcinoma growing subcutaneously in mice 3 times/week. As single agent, IMQ caused increased tumor infiltration by DC, $CD8^+$ and $CD4^+$ T cells and slower tumor growth, an effect that was dependent on $CD8^+$ T cells (*52*). However, tumors kept growing despite treatment with IMQ. In contrast, when tumors were treated with local radiation given in 3 fractions of 8 Gy together with topical IMQ the majority of tumors showed complete regression. Like IMQ, radiation alone slowed tumor growth but did not induce complete regression. Importantly, in mice bearing two tumors, application of IMQ to the irradiated tumor induced an abscopal effect, which was enhanced by application of IMQ also on the tumor outside of the radiation field (*52*). Priming of tumor-specific T cells was confirmed in the lymph nodes draining the tumors treated with radiation and IMQ. In addition, IMQ-treated tumors showed increased expression of intercellular adhesion molecule-1 (ICAM-1) and MHC class I, suggesting that IMQ can sensitize tumor cells to rejection by $CD8^+$ T cells which are optimally activated and primed by the combination of radiation and IMQ. Thus, radiation and IMQ synergize in inducing tumor regression by multiple mechanisms, some of which are distinct but others may be overlapping (Fig. 3). Interestingly, the anti-tumor immune response elicited by radiation and imiquimod was not long lasting in all mice. In some mice tumors recurred after a variable tumor-free interval. Recurrence was reduced by administration of a single low-dose cyclophosphamide, which decreased IL-10 and Treg cells, suggesting a need to overcome immunosuppressive mechanisms to achieve long-term tumor control (*52*).

### *Combination of Local Radiotherapy with Checkpoint Receptor Blockade*

Multiple pathways and mechanisms tightly regulate the activation of $CD4^+$ and $CD8^+$ T cells, resulting in productive immune responses that can be rapidly turned off once the offending agent has been cleared. This exquisitely orchestrated regulation is mediated by an array of costimulatory and coinhibitory or checkpoint receptors expressed by T cells (*53*). CD28 is a key costimulatory receptor that delivers a second signal required for T-cell activation in addition to T-cell receptor (TCR) engagement. CD28 binds to B7-1 and B7-2 molecules expressed on APC and induces interleukin (IL)-2 production culminating in robust T-cell proliferation.

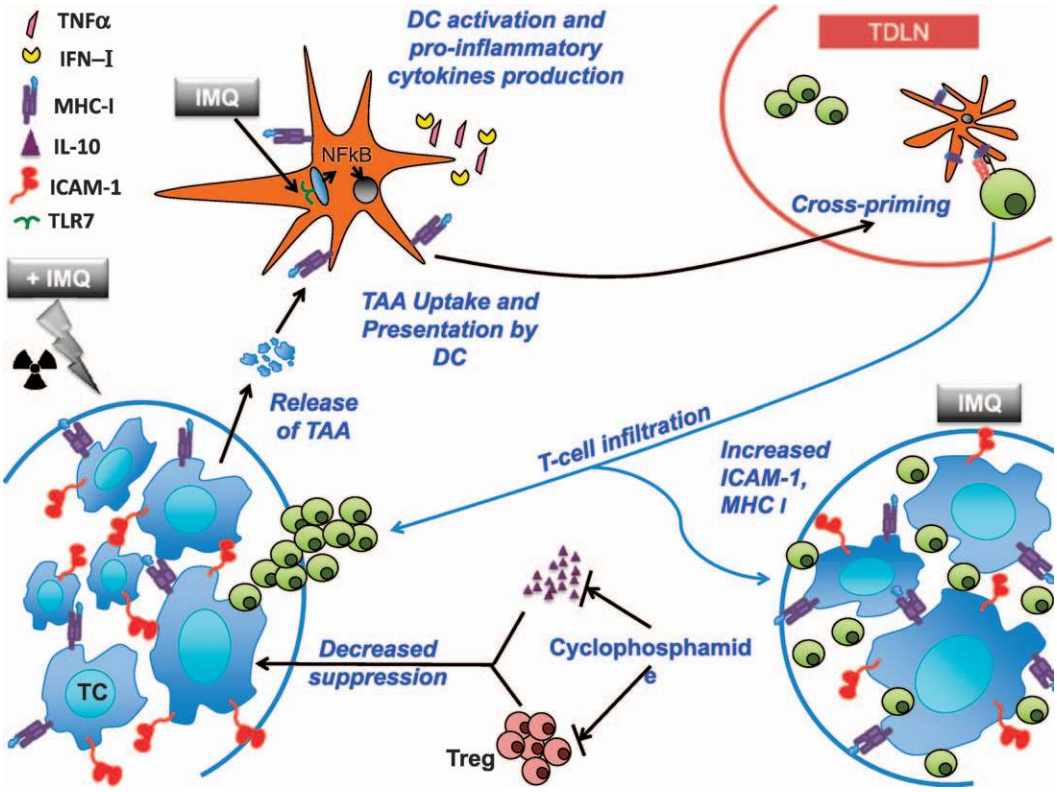


**FIG. 3.** Combination of radiotherapy and TLR7 agonist. Imiquimod stimulates production of type I IFN and proinflammatory cytokines by a toll-like receptor (TLR)-7 expressed mainly in DCs. This results in enhanced maturation and activation of DC and improved cross-priming of anti-tumor T cells to TAA released by radiation. Primed CTLs migrate to irradiated and nonirradiated tumors. Here imiquimod-induced upregulation of ICAM-1 and MHC-1 molecule on tumor cells (TC), increases their susceptibility to killing by CTL. Administration of cyclophosphamide reduces IL-10 levels and Treg numbers and results in a more sustained anti-tumor T cell response.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is the prototypical checkpoint receptor, limiting T-cell activation and proliferation to prevent autoimmunity (*54*). Induced shortly after TCR signaling is triggered through cognate interaction with peptide-MHC, CTLA-4 is rapidly recruited to the immune synapse where it binds to B7-1 and B7-2 with greater affinity than CD28, thus outcompeting CD28 when co-stimulatory molecules are present in limiting amounts (*55*). In addition, CTLA-4 constitutively expressed on regulatory T cells (Treg) exerts its inhibitory function by removing B7-1 and B7-2 from the surface of APC (*56*). Chronic antigen exposure in the context of cancer leads to T cell exhaustion and increased expression of CTLA-4 on effector T cells. Together with reduced costimulation of APC and increased Treg presence, this promotes tolerance of anti-tumor T cells (*54*). The importance of this checkpoint receptor in cancer has been clearly demonstrated by the ability of monoclonal antibodies (mAb) against CTLA-4 to induce effective anti-tumor immunity (*57*). However, the response is limited in the clinic to a subset of patients and in pre-clinical tumor models is seen only in relatively immunogenic tumors (*54, 57*).

We hypothesized that radiotherapy could convert tumors unresponsive to anti-CTLA-4 into responsive ones by its ability to convert the irradiated tumor into an immunogenic hub. This was first tested in the poorly immunogenic 4T1 carcinoma model (Fig. 1). While radiation given to established tumors delayed significantly the growth of the subcutaneous irradiated tumor, it did not reduce lung metastases and median survival of treated mice was comparable to control cohorts (*58*). As expected, anti-CTLA-4 mAb did not show any anti-tumor activity by itself, but synergized with radiation improving control of the irradiated tumor and inhibiting lung metastases. This response was mediated by induction of anti-tumor $CD8^+$ T cells and led to a significant extension of mice survival (*58*). The therapeutic synergy of the combination of local radiotherapy and anti-CTLA-4 was confirmed in two additional tumor models, TSA and MCA38, syngeneic to mice of different genetic background (Fig. 1). Interestingly, we found that the radiation regimen employed was a critical determinant of the ability of radiation to synergize with anti-CTLA-4 mAb and induce anti-tumor T cells able to mediate an abscopal effect (*59*). A fractionated regimen of 8 Gy $\times$ 3 given on consecutive days was the most effective, while a

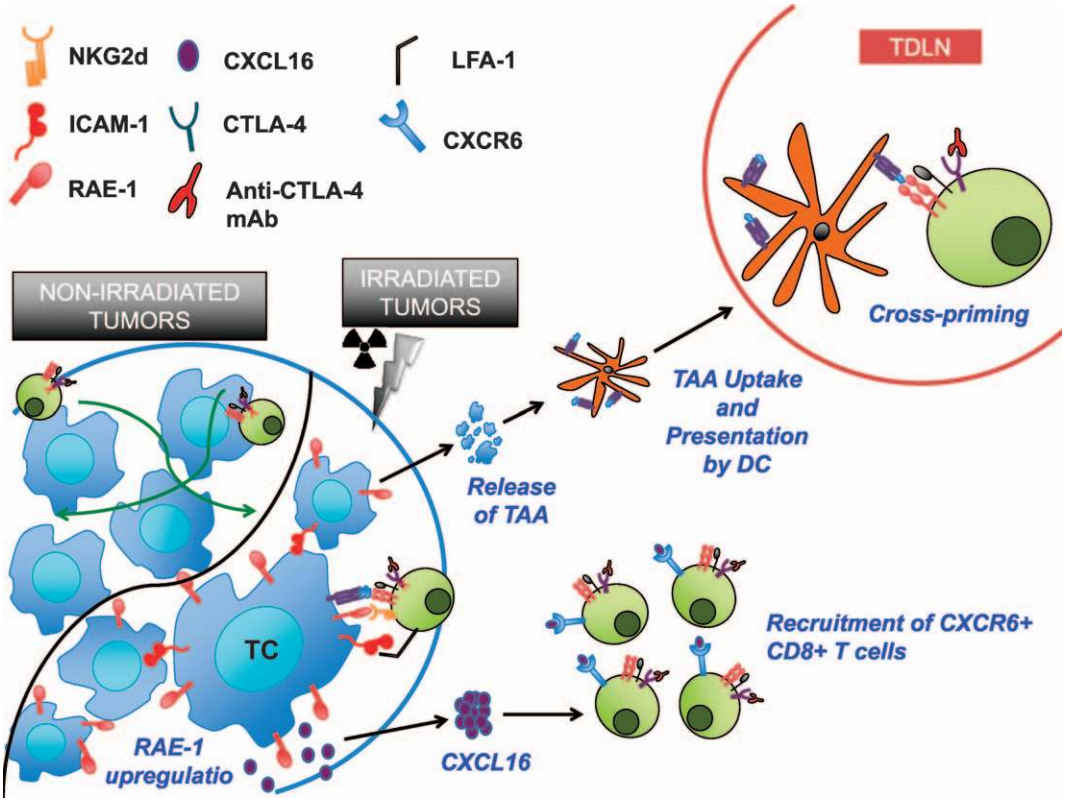


**FIG. 4.** Combination of radiotherapy and anti-CTLA-4 antibody. Multiple mechanisms underlie the cooperative effects of ionizing radiation and CTLA-4 checkpoint blockade. The uptake and presentation by DC of TAA released from dying cells promotes cross-priming of tumor-specific T cells, which is mediated by engagement of T-cell receptor (TCR) by MHC/antigen complexes and lymphocyte function-associated antigen 1 (LFA-1), and is enhanced by blocking CTLA-4. Primed $CD8^+$ effector T cells are recruited to the tumor by radiation-induced CXCL16. Inside the tumor, upregulation of RAE-1 promotes immune synapse formation between the cancer cells and $NKG2D^+$ CTLs leading to cancer cell killing and tumor regression.

single large dose (20 Gy) was unable to induce an abscopal effect in combination with anti-CTLA-4. The molecular bases for this difference between radiation regimens are currently being investigated.

Mechanisms responsible for the synergy of radiation with anti-CTLA-4 mAb were further investigated in the 4T1 model (Fig. 4). We found that regressing tumors were infiltrated by $CD8^+$ T cells expressing the activation marker CD69 and chemokine receptor CXCR6 (*27*). CXCR6 was responsible for recruitment of tumor-infiltrating lymphocytes (TILs) to the irradiated 4T1 tumors, since reduced numbers of $CD8^+$ TILs were seen in $CXCR6^{-/-}$ mice. Consistently, the ligand for CXCR6, the chemokine CXCL16, was significantly upregulated in 4T1 tumor cells by radiation, both in vitro and in vivo. Chemotaxis assays confirmed that CXCL16 released by irradiated 4T1 cells attracted activated $CD8^+$ T cells towards the tumor cells. Importantly, $CXCR6^{-/-}$ mice that have T cells unable to sense CXCL16 showed impaired tumor control after treatment with radiation and anti-CTLA-4 mAb (*27*). Collectively, these studies implicate the key role of CXCR6/CXCL16 interactions in driving radiation-induced recruitment of effector anti-tumor T cells in the 4T1 model. We found that CXCL16 was induced by radiation in several human breast cancer cells, as well as in other mouse cells, including prostate and colorectal carcinoma, suggesting that enhanced recruitment of activated T cells may be a common effect of radiotherapy (*27, 60*).

Additional analysis of the dynamic behavior of $CD8^+$ TILs by two photon laser scanning microscopy (TPLSM) revealed a molecular interaction that is critical for tumor rejection in mice treated with radiation and anti-CTLA-4 (*30*). Stable interactions between effector $CD8^+$ T cells and target tumor cells are required for the formation of an immune synapse and tumor cell killing (*61*). Radiation-induced expression of the NKG2D ligand retinoic acid early inducible-1 (RAE-1) on tumor cells was required to promote the formation of such immune synapse. TILs moved faster without stopping in contact with target tumor cells in mice treated with anti-CTLA-4 or radiation as monotherapy, while the opposite was seen when the two modalities were combined. Blocking the interaction of NKG2D receptor expressed on effector $CD8^+$ T cells with RAE-1 induced by radiation on tumor cells abrogated the therapeutic response to anti-CTLA-4 treatment in 4T1 tumor-bearing mice (*30*). These data suggest that NKG2D

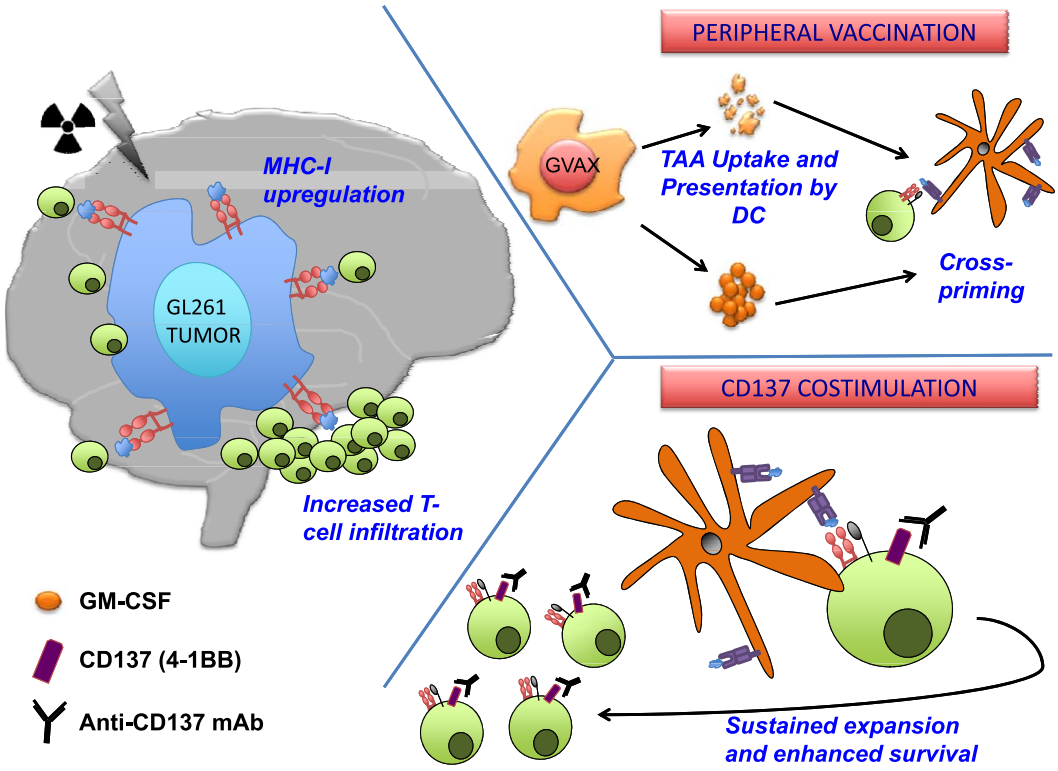


**FIG. 5**. Combination of whole brain radiotherapy (WBRT) and immunotherapy. Ionizing radiation promotes immune recognition of GL261 glioma cells by upregulating tumor expression of MHC class I molecules and promoting influx of effector T-cells in the tumor microenvironment. Robust anti-tumor T cells sufficient to induce tumor regression are generated by either vaccination with autologous tumor cells modified to produce GM-CSF (GVAX), or by promoting expansion and survival of anti-tumor T cells primed by endogenously released antigens after WBRT by agonistic mAb to the co-stimulatory CD137/4-1BB receptor.

ligand expression may be a determinant of tumor response to anti-CTLA-4 immunotherapy, and provides a novel molecular mechanism for the synergy between radiotherapy and CTLA-4 treatment (*62*).

### *Combination of Local Radiotherapy with Vaccination*

Granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes the maturation and antigen presenting ability of DCs, which play a central role in T-cell priming. Vaccination with autologous tumor cells modified to secrete GM-CSF was shown to be effective at inducing a robust and sustained anti-tumor immune response in preclinical models and some clinical trials (*63, 64*). In 2006, we tested whether peripheral vaccination with autologous tumor cells transduced with GM-CSF could enhance the effectiveness of WBRT in the GL261 glioma model (*65*). Response of established intracranial GL261 glioma was significantly improved when WBRT was combined with peripheral vaccination, resulting in increased overall survival. Mice with intracranial tumors typically succumbed within 33 days from initial implantation, and survival was modestly increased by monotherapy with either WBRT alone (median survival of 55 days) or vaccine alone (median survival of 45 days). On the other hand, 80% of animals given WBRT + vaccine survived more than 75 days, and most survivors rejected a secondary tumorigenic GL261 inoculum.

*In vitro*, irradiation (4 or 6 Gy) of GL261 cells enhanced expression of MHC class I molecules, increasing their susceptibility to killing by $CD8^+$ T cells (*65, 66*). *In vivo* WBRT given in 2 fractions of 4 Gy induced strong surface expression of MHC class I on invading glioma cells. WBRT also enhanced tumor infiltration by $CD4^+$ and $CD8^+$ T cells, suggesting that radiation was effectively enhancing tumor rejection by T cells activated by the vaccine (Fig. 5).

### *Combination of Local Radiotherapy with Co-stimulation by CD137/4-1BB*

CD137 (4-1BB, TNFRSF9) is a member of the tumor necrosis factor receptor (TNFR) superfamily which is expressed following activation by T cells, natural killer (NK) cells, neutrophils, monocytes and DCs (*67*). CD137 ligation enhances T cell proliferation, functional maturation and production of cytokines. CD137 provides a strong survival signal especially for $CD8^+$ T cells, primarily by upregulation of anti-apoptotic Bcl-2 molecules. Importantly, anti-CD137 mAb have shown anti-tumor activity in several preclinical models (*68*).

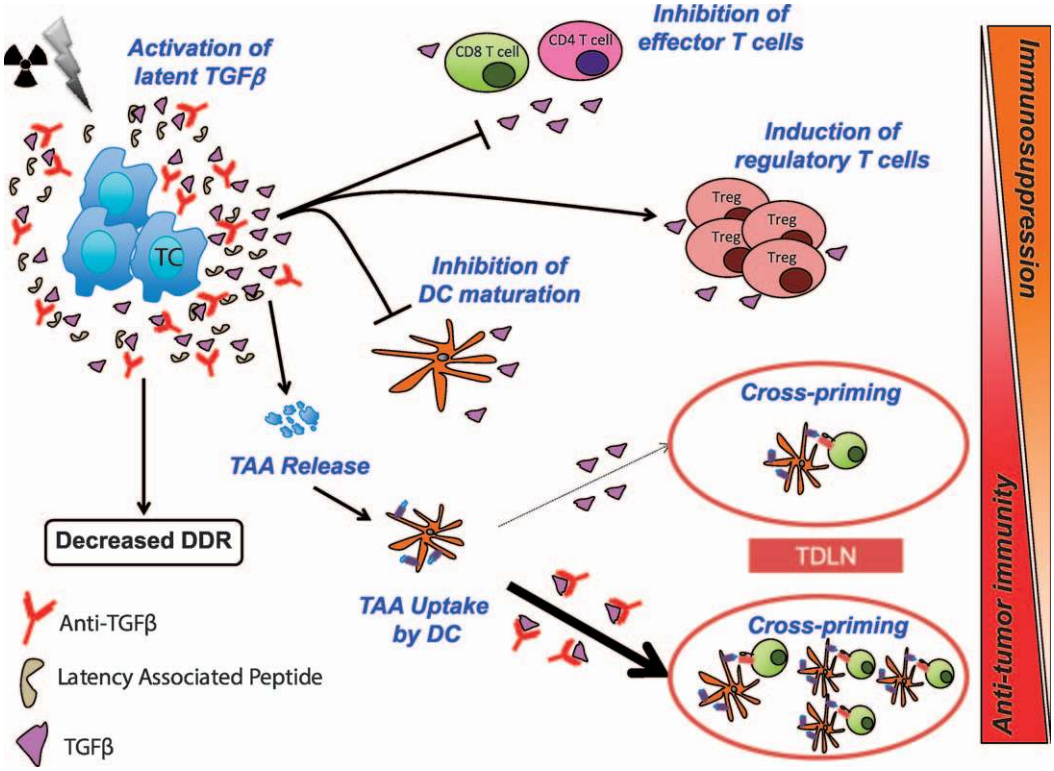


**FIG. 6.** Combination of radiotherapy and TGFβ blockade. Ionizing radiation kills tumor cells releasing TAA, but also activates the immunosuppressive cytokine TGFβ by promoting its dissociation from the latency-associated peptide (LAP). TGFβ inhibits the antigen-presenting function of DC and the differentiation of T cells into effectors, while promoting their differentiation into regulatory T cells. TGFβ neutralization by anti-TGFβ mAb enhances antigen-presentation by DC, promoting cross-priming and acquisition of effector function by anti-tumor T cells, leading to a shift from immunosuppression to anti-tumor immunity. Neutralization of TGFβ also increases radiosensitivity of tumor cells by inhibiting the DNA damage response (DDR).

To determine if radiation induced an immune responses to an intracranial tumor that could be enhanced by CD137 co-stimulation, 15 days after intracranial GL261 implantation mice were given WBRT in two fractions of 4 Gy and anti-CD137 mAb starting on the day after the last irradiation (*69*). The combination of WBRT and anti-CD137 mAb improved significantly survival with a median of 114 days compared to 31 days in the untreated control group, 37 days with WBRT alone and 42 days with anti-CD137 alone, thereby indicating a synergistic effect elicited by the combination treatment. The majority of animals treated with WBRT and anti-CD137 became long-term survivors and showed anti-tumor memory responses able to reject a secondary challenge of viable GL261 cells. A massive increase in TILs was seen in mice treated with WBRT and anti-CD137, which was more pronounced for $CD8^+$ T cells (36-fold over the background in untreated mice), while WBRT alone and anti-CD137 alone caused only a 4- to 6-fold increase in TILs (*69*). Tumor-specific production of IFNγ by spleen T cells was markedly increased only in mice treated with WBRT and anti-CD137. Collectively, the data is consistent with the interpretation that radiation induces priming of anti-tumor T-cells that require additional costimulatory signals to acquire effector functions and to persist (Fig. 5). In addition, WBRT facilitates tumor rejection in the brain by improving T cell recruitment and infiltration. Therefore, complementary effects of radiation and CD137 costimulation seem to underlie the synergy between these two treatments.

### *Combination of Local Radiotherapy with TGFβ Neutralization*

As discussed above, tumors able to progress have a highly immunosuppressive microenvironment that allows them to escape immune-mediated control. One key mediator of immunosuppression is the cytokine transforming growth factor (TGF)β which is produced by cancer cells and by some immune cells with regulatory function such as Treg and myeloid-derived suppressor cells (*70*). Importantly, radiation activates latent TGFβ (*71, 72*). In addition to suppression of T cell and DC function, TGFβ enhances multiple processes that support tumor progression and resistance to radiation, including angiogenesis, epithelial to mesenchymal transition and DNA damage response (*73*). Therefore, blocking TGFβ in the context of radiotherapy may yield multiple benefits (Fig. 6). In support of this notion, we have shown that antibody-mediated neutralization of TGFβ increased radiation sensitivity of 4T1 cells by

impairing DNA damage repair, and significantly increased tumor growth delay in response to single and fractionated radiation *in vivo* (*74*). Importantly, our recent data indicate that TGFβ is a key regulator of radiation-induced anti-tumor immune responses (Vanpouille-Box *et al*., manuscript in preparation). Overall, these data provide a strong rationale for testing the combination of radiotherapy and strategies to block TGFβ in cancer patients.

*Clinical Translation*

Radiation and chemotherapy are currently used to palliate patients with metastatic or recurrent local-regional disease. While long lasting remissions are rare, most patients derive some measurable benefit from either treatment. With the advent of novel immunotherapies the possibility of sustained anti-tumor immune responses is emerging (*75*). However, to date it is unknown whether anti-cancer immunity developed from radiation in conjunction with immunotherapy can lead to tumor regression and long lasting systemic effects. Thus, based on our preclinical data, we designed several clinical studies to detect the abscopal effects of radiation and immunotherapy and assess for sustained immunological responses.

Because the clinical responses to immunotherapy do not exactly mirror the responses to chemotherapy, several criteria to standardize assessment of immunologic responses to immunotherapies were proposed (*76*). In each of the clinical trials we are conducting, the sites of disease for each patient are assessed with clinical/radiological evaluation, including PET/CT, at baseline and after treatment. Whenever possible, additional serial blood draws and/or biopsies are obtained for in depth immunological assessment.

*GM-CSF*

The findings from our experiments in the preclinical models highlighted above suggest that adding a treatment that increases DC numbers and function to radiation can induce effective anti-tumor immunity. We hypothesized that the induction of tumor cell death by concomitant chemotherapy and radiation to a specific metastatic site may enhance tumor immunogenicity by promoting cross-priming and eliciting anti-tumor T-cell responses in patients. Similarly to Flt3L, GM-CSF has the potential to enrich the DC compartment and could improve anti-tumor immunity elicited by concurrent chemotherapy and radiotherapy. A clinical trial in patients with metastatic solid tumors was designed to test this hypothesis (*77*).

Patients who had demonstrated no change or early progression after single agent chemotherapy were eligible: they were maintained on the same systemic treatment but radiation to a site of metastatic disease and GM-CSF were added. The main endpoint for this exploratory study was to assess whether the abscopal response achieved in the preclinical model could be detected in patients. Radiation was given to a total dose of 35 Gy in 10 fractions. After completing the first week of irradiation, patients were given GM-CSF (125 μg/m$^2$ subcutaneously) administered daily for 2 weeks. Abscopal responses were assessed, thereafter, by measuring nonirradiated target lesion(s) clinically and radiologically. An abscopal response was detected in 30% of the patients (*78*).

*Imiquimod*

Based on the preclinical data indicating that the combination of local radiotherapy and imiquimod induces anti-tumor immune responses that are active both locally and systemically, we designed a single arm, open label Phase I/II clinical trial for breast cancer patients with multiple cutaneous metastasis, which is ongoing (http://clinicaltrials.gov/show/NCT01421017).

At trial entry, all skin metastases are outlined and photographed (including visible/palpable borders). Topical imiquimod is applied to all skin metastases while radiotherapy is given to one area only. The lesion to be irradiated is chosen by the radiation oncologist to limit normal tissue toxicities, especially if the patient was previously irradiated. This site is treated to a total dose of 30 Gy (with either electrons and/or photons) distributed in 5 fractions of 6 Gy delivered every other day. Responses are assessed in skin metastases treated with radiation and imiquimod and with imiquimod alone. Since many of these patients have additional metastases to internal organs, responses are also assessed radiologically in these untreated metastases. In some patients without detectable visceral metastases an area of skin is left untreated to measure the abscopal effect. Clinical and radiological assessment of untreated lesions is performed at week 9.

*Fresolimumab*

Our preclinical data with radiation and TGFβ neutralization suggest that the combination may act to radiosensitize tumor cells by reducing DNA repair mechanisms, while inducing anti-tumor immune responses. We hypothesized that similar effects may be clinically observed. Fresolimumab (GC1008) is a human mAb that neutralizes TGFβ and is being tested in early clinical trials for a few diseases, including cancer. The number of patients receiving GC1008 is small and, at this point, information regarding any possible clinical benefit remains limited.

Based on our preclinical work we designed a trial to combine Fresolimumab and radiation to one metastatic site in patients with mestastatic breast cancer, which is currently enrolling (http://clinicaltrials.gov/ct2/show/NCT01401062). Since the optimal dose of GC1008 to neutralize TGFβ in irradiated cancer patients is unknown, eligible patients are randomly assigned to two different doses of Fresolimumab, either Arm 1 (1 mg/kg of GC1008) or Arm 2 (10 mg/kg of GC1008). The antibody is administered intravenously every 3 weeks for a total of 5 infusions at the assigned dose. The chosen metastatic site receives conformal external beam

radiation 7.5 Gy per fraction, given every other day to a total of 22.5 Gy. The first lesion is irradiated at week 1 (radiation starts after 1st dose of GC1008), lesion 2 is irradiated at week 7. Patients are assessed for response by PET/CT imaging. Serial blood samples are collected to monitor changes in cytokines, lymphocytic and myeloid populations and to measure development of tumor-specific T cells.

*Ipilimumab*

In early 2011, ipilimumab (a humanized antibody to CTLA-4) was approved by the U.S. FDA to treat patients with metastatic melanoma (*79*). Since its approval, ipilimumab, when given occasionally in combination with radiation, has led to abscopal responses in some auspicious patients (*37, 80*). The most provocative abscopal response was reported in a patient that demonstrated radiographic evidence of disease progression, while on ipilimumab maintenance therapy. Growth of a paraspinal mass, which caused right-sided back pain, triggered the indication for palliative radiotherapy, administered concurrently with maintenance ipilimumab. The treatment resulted in regression of distant disease in the spleen and mediastinal lymph nodes. Interestingly, the therapeutic response temporarily correlated with an increase in antibody titers targeting NY-ESO-1 and other tumor associated antigens, an increase in $CD4^+$ T-cell and myeloid lineage activation, and a decline in the quantity of myeloid-derived suppressor cells, lending credence to the immunologic hypothesis of the abscopal effect (*37*). Encouraged by these anecdotal cases and based on our preclinical work, we designed a trial to test whether the combination of radiation and Ipilimumab can induce an anti-tumor immune response at the irradiated site capable to elicit immune-mediated abscopal effects. A phase I randomized trial tests ipilimumab immunotherapy with local radiotherapy in patients with metastatic melanoma who have at least two separate measurable sites of disease documented by CT scanning or MRI prior to entering the study (http://www.clinicaltrials.gov/ct2/show/NCT01689974). Patients are then randomized to either Arm A (ipilimumab alone) or Arm B (ipilumimab with radiation). Immune-monitoring includes T cell and B cell responses to melanoma associated tumor antigens.

## CONCLUSIONS

Accumulating data in preclinical studies and clinical observations highlight the importance of this new area of investigation, aimed at identifying the most promising combinations of radiotherapy and immunotherapy for treatment of different cancers. This new application of radiotherapy has at least two important implications. The first is that it can change the role of radiation in metastatic disease from a palliative measure to one that has the potential to extend survival and perhaps even cure some patients. The second implication is that it requires a new partnership between radiation oncologists and immunotherapists in management of patients. The latter will be greatly facilitated by incorporating training in tumor immunology in the curriculum of residents training in oncology.

Noticeably, responses to immunotherapy occur even in heavily pretreated metastatic disease, providing a real new option for patients who would normally have exhausted available therapeutic possibilities.

The growing number of clinical trials testing combinations of radiotherapy and immunotherapy represents an outstanding example of translation form preclinical models to clinical studies. The immunological consequences of tumor irradiation not only provide a therapeutic opportunity, but also highlight the critical role of the tumor microenvironment as a determinant of the response to radiation. This improved understanding of the role of the immune system in response to radiation makes a compelling case for the use of immunocompetent animals for testing response to treatment in experimental conditions.

Overall, to assure the success of the use of radiation as a partner for immunotherapy it is critical to gain more insights into the mechanisms at play. Support for basic, translational and clinical studies in this field is required to deliver the promise of this new treatment strategy.

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