Radiotherapy Combination Opportunities Leveraging Immunity for the Next Oncology Practice

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ABSTRACT: Approximately one-half of patients with newly diagnosed cancer and many patients with persistent or recurrent tumors receive radiotherapy (RT), with the explicit goal of eliminating tumors through direct killing. The current RT dose and schedule regimens have been empirically developed. Although early clinical studies revealed that RT could provoke important responses not only at the site of treatment but also on remote, nonirradiated tumor deposits—the so-called “abscopal effect”—the underlying mechanisms were poorly understood and were not therapeutically exploited. Recent work has elucidated the immune mechanisms underlying these effects and has paved the way for developing combinations of RT with immune therapy. In the wake of recent therapeutic breakthroughs in the field of immunotherapy, rational combinations of immunotherapy with RT could profoundly change the standard of care for many tumor types in the next decade. Thus, a deep understanding of the immunologic effects of RT is urgently needed to design the next generation of therapeutic combinations. Here, the authors review the immune mechanisms of tumor radiation and summarize the preclinical and clinical evidence on immunotherapy-RT combinations. Furthermore, a framework is provided for the practicing clinician and the clinician investigator to guide the development of novel combinations to more rapidly advance this important field.

Introduction

For the past century, radiotherapy (RT) has been at the cutting edge of cancer treatment. Thanks to its efficacy, RT is presently given as frontline therapy to approximately 60% of all patients with newly diagnosed cancer,1 usually in combinations with chemotherapy. RT also is routinely used in the palliative setting to address symptoms and enhance quality of life in patients with advanced tumors. Optimization of dosage and fractionation, along with improved technology to allow more precise delivery, has led to incremental improvements in patient outcomes. Intensity-modulated RT, for example, shapes the radiation beam to fit the area of the tumor, enabling increased and precise doses to reach the target in 3 dimensions while minimizing acute and late side effects by sparing normal tissue. The recent developments of image-guided RT and stereotactic radiosurgery (SRS) better define tumor geometry by incorporating medical imaging before or even during treatment. This has enabled the targeted delivery of higher doses of RT per fraction in so-called hypofractionated treatments with minimal damage to healthy tissues and organs.1

Historically, the clinical efficacy of ionizing radiation has been attributed to its ability to induce DNA damage, which can result in direct tumor cell death.2 However, the existence of radiation-induced, cancer cell-extrinsic mechanisms of tumor control has been increasingly recognized, whereby RT not only contributes to local control of the target lesions but also may result in the control of metastases distant to the treatment site. In 1953, Mole coined the term “abscopal” to describe the systemic effects of radiation on “out-of-field” tumor deposits,3 which have been
Radiation-Immunotherapy Combinations

Radiated tumor cells, under certain circumstances, can undergo a so-called immunogenic death: i.e., cell death that effectively exposes tumor antigen and triggers an antitumor immune response. One of the hallmarks of immunogenic cell death is the increased maturation of APCs taking up antigen released by dying cells. The translocation of an endoplasmic reticulum (ER) protein complex formed by calreticulin and disulfide isomerase ERP57 to the plasma membrane in stressed and dying cancer cells facilitates uptake by phagocytic cells and in part mediates their activation. Calreticulin provides an “eat-me” signal, which promotes the uptake of dying tumor cells by dendritic cells (DCs) and macrophages. This signal is countered by cluster differentiation 47 (CD47 [integrin-associated protein]), which acts as a “do-not-eat-me” signal and protects tumor cells from macrophage attack and phagocytosis. Loss of CD47 expression and the coordinated upregulation of cell-surface calreticulin promotes APC uptake. Inhibition of CD47 also increases the radiosensitivity of mouse tumors while conferring radioprotective effects to normal tissues. Calreticulin also activates the production of proinflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) in APCs. Complement anaphylatoxins, released after complement activation by RT-induced immunoglobulin M (IgM) binding to necrotic tumor cells, may directly contribute to DC recruitment and maturation and, ultimately, to T-cell immunity. Radiation-induced cyclic guanosine monophosphate adenosine monophosphate synthase (cGAS)-dependent and “stimulator of interferon (IFN) genes” (STING)-dependent cytosolic, nucleic acid-sensing pathways trigger type I IFN signaling on DCs, which regulates the adaptive immune response induced by RT.

Radiation-damaged tumor cells also activate APCs through the release of damage-associated molecular pattern molecules (DAMPs), which signal through pattern-recognition receptors, including Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene I-like helicases, nucleotide-binding domain receptors, and leucine-rich repeats. Such signals induce maturation of DCs, which acquire the ability to present antigen effectively and mobilize adaptive immunity. Radiation-triggered DAMPs include adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1) protein released by dying and stressed cells. Release can be very rapid (minutes) and is also triggered by low doses of ionizing radiation (eg, 0.5 Gy). The mechanism includes active exocytosis and the secretion of cytoplasmic ATP via gap-junction hemichannels. Extracellular ATP acts as a “find-me” signal for monocytes and DCs and is sensed by the purinergic receptor P2X7, which functions as a key regulatory element of the inflammasome, leading to the secretion of proinflammatory cytokines like IL-1β and IL-18. Irradiated tissues show strong IL-1β upregulation. The stimulation of APCs in vitro with ATP leads to increased expression of the surface costimulatory ligands CD80 and CD86; and, in vivo, it elicits a cascade of events, including signal transducer and activator of transcription 1 (STAT1) phosphorylation, IFNγ production, T-cell expansion, and a reduction of regulatory T (Treg) cells. Autophagy-related stress is required for the optimal release of ATP from dying cancer cells killed by cytotoxic agents, and genetic approaches have confirmed an important role of autophagy in the response of human and murine tumor cells to RT. Although, in immunodeficient hosts, autophagy loss increases the sensitivity of tumor cells to the cytotoxic effects of RT in vitro and in vivo, autophagy deficiency in immunocompetent mice leads to the poor response of tumors to RT. Pharmacological inhibition of extracellular nucleotidases,
| AUTHOR      | NO. | HISTOLOGY                      | PRIMARY TUMOR | TREATMENT OF PRIMARY SITE | TYPE OF IMMUNE THERAPY | RT DOSE, Gy/FRACTIONS | AREA OF ABScopAL EFFECT | TIME INTERVAL BETWEEN ABScopAL EFFECT AND RT, MO | DURATION OF RESPONSE | PATIENTS OUTCOME |
|------------|-----|--------------------------------|----------------|---------------------------|------------------------|-----------------------|-------------------------|-----------------------------------------------|----------------------|------------------|
| Wersall 2006 | 4   | Clear cell carcinoma          | Kidney        | Surgery                   | IL-2 + IFN-γ before RT in 1 patient | 15 × 2, 8 × 4         | Lung metastases         | 3-5                                           | 2-4 y                | Alive without disease |
| Kingsley 1975 | 1   | Melanoma                      | Skin          | Surgery                   | —                      | 14.4 × 12             | Para-aortic lymph nodes | 3                                            | 17 mo                | Death without disease |
| Ohba 1998 | 1   | Hepatocellular carcinoma      | Liver         | Surgery and chemoembolization | —                      | 36 Gy                 | Hepatic metastases      | 10                                           | 29 mo                | Alive with minimal disease |
| Brody 2010 | 15  | Refractory low-grade B-cell lymphoma stage III-IV | Lymph nodes | Multiple lines of chemotherapy | TLR9 agonist injected intra-tumoral in the irradiated site | 4 × 2 | Three metastatic sites outside the irradiated area | 1-3                          | 2-4 mo             | CR, 1 patient; PR, 3 patients; SD, 2 patients |
| Grimaldi 2014 | 21  | Melanoma                      | Skin          | Surgery                   | CTLA4 blockade before RT | 30 × 10 or 20-24 single fraction for brain; 20 × 5 or 30 × 10 or 50 × 25 for extracranial sites | 62% brain metastases; 38% extracranial metastases | NR | Median OS for patients with abscopal responses, 22.4 mo vs 8.3 mo without | PR, 43%; SD, 10% |
| Postow 2012 | 1   | Melanoma                      | Skin          | Surgery                   | CTLA4 blockade before, concomitant and after RT | 28.5 × 3            | Hilar lymphadenopathy and splenic lesions | 4                                           | NR                   | SD 10 months post-RT |
| Golden 2013 | 1   | NSCLC                          | Lung          | Chemotherapy              | CTLA4 concomitant and after RT | 30 × 5               | Multiple metastatic sites | 3                                           | 12 mo                | Alive without evidence of disease |
| Hiniker 2012 | 1   | Melanoma                      | Skin          | Surgery                   | CTLA4 concomitant and after RT | 54 × 3               | Multiple metastatic sites | 6                                           | 12 mo                | Alive without disease |
| Seung 2012 | 12  | Melanoma and clear cell carcinoma | Skin, kidney | Surgery                   | IL-2 concomitant and after RT | 20 × 1               | Multiple metastatic sites | NR                                          | 2-24 mo              | CR, 50%; PR, 16.7%; SD, 8.4% |

CR indicates complete response; CTLA4, cytotoxic T-lymphocyte–associated protein 4; Gy, gray; IFN-γ, interferon gamma; IL-2, interleukin-2; OS, overall survival; NR, not reported; NSCLC, nonsmall cell lung carcinoma; PR, partial response; RT, radiotherapy; SD, stable disease; TLR9, toll-like receptor 9.
which increase the pericellular concentration of ATP, restored the radiosensitivity of autophagy-deficient cancers by immune-mediated mechanisms. This is in line with evidence that RT as well as chemotherapy treatments that induce autophagy are more effective in immunocompetent mice.19,20,41,42

HMGB1, another key hallmark of immunogenic cell death, is a histone–chromatin binding protein released from stressed or dying cells, especially necrotic ones after exposure to RT.43 HMGB1 exerts potent immunomodulatory effects by binding to TLR4 and TLR9 and activating the myeloid differentiation primary response protein 88 (MyD88) pathway, leading to the activation of nuclear factor-κB (NF-κB) and a downstream inflammatory cytokine response. Both TLR4 and TLR9 play crucial roles in driving inflammatory responses in response to RT. Notably, the level of HMGB1 after chemoradiation therapy (chemo-RT) was significantly elevated in patients with esophageal cancer who had antigen-specific T-cell responses.44 Furthermore, a TLR4 polymorphism (Asp299Gly) that affects binding to HMGB1 predicted early relapse after RT and anthracycline-based chemotherapy in patients with breast cancer.45,46

Interestingly, the abscopal effect of radiation does not occur in tumor protein 53 (p53)-deficient animals.46 This may be related to mechanisms of cell death or cell stress that are activated by radiation in the presence of functional p53. Indeed, p53 deficiency attenuates danger signals47 that are normally induced by high-dose irradiation and potentiate immune activation.48 In addition, p53 is involved in the regulation of specific natural killer (NK) group 2, member D (NKG2D) ligands, which are upregulated upon cell stress and induce the potent activation of NK cells in addition to providing strong costimulation to cytolytic CD8+ T cells.49,50

RT has the potential to trigger antigen-specific, adaptive immunity, a phenomenon referred to as “in situ” vaccination (Fig. 1). In mice, this phenomenon was responsible for the therapeutic efficacy of a single 10-Gy RT dose.51 Antitumor immune responses after RT also have been reported in patients with cancer.52,53 Circulating, tumor-specific, CD8+ T cells have been detected de novo in patients with colorectal cancer after the completion of chemo-RT with 45 Gy54 as well as in patients with prostate cancer after prostate and pelvic RT with curative intention.55 Furthermore, in patients with prostate cancer, the development of treatment-associated autoantibody responses was documented after neoadjuvant androgen-deprivation therapy and external-beam RT or brachytherapy, but not after radical prostatectomy, and was absent in untreated controls.56

**RT Reprograms the Tumor Microenvironment**

In addition to mobilizing antitumor immunity, RT renders the tumor microenvironment conducive to effector T-cell recruitment and function. Ionizing radiation has the ability to induce chemokines involved in the recruitment of effector T cells, effectively converting tumors into “inflamed” tissues, which are susceptible to T-cell attack.57 In addition, RT may enhance T-cell trafficking in tumors through local vascular endothelial inflammation. For example, RT enhanced expression of the cell surface intercellular adhesion molecule-1 (ICAM-1) in endothelial cells, facilitating leukocyte endothelial transmigration.58,59 Importantly, a single 10-Gy dose of irradiation significantly enhanced the efficacy of adoptive T-cell transfer in vivo, with significant tumor regression.60

Macrophages are widely distributed, innate immune cells that play key roles in the response to pathogens, tissue injury, and homeostasis.61 Macrophages can be polarized to different functional phenotypes. Classically activated (M1) macrophages express proinflammatory cytokines, such as IL-12, IL-23, and TNFα, as well as nitric oxide (NO) and high levels of major histocompatibility complex (MHC) class I (MHC-I) and MHC-II. In contrast, alternatively activated (M2) macrophages express anti-inflammatory cytokines IL-10 and transforming growth factor β (TGFβ) as well as arginase-1, which contribute to depleting extracellular L-arginine and drives T-cell suppression.62 Tumor-associated macrophages (TAMs) are mostly of the M2 phenotype, contributing to the immunosuppressive tumor microenvironment as well as to angiogenesis, growth, and metastasis.63 Importantly, Klug et al have recently shown that low doses of RT (≤2 Gy) reprogrammed TAMs to an M1 phenotype, which, in turn, led to normalization of the tumor vasculature with a reduction of CD31+ vessels and upregulation of vascular cell adhesion protein-1 (VCAM-1) in tumor endothelium. Consequently, low-dose RT enhanced the efficacy of adoptive immunotherapy because of enhanced tumor homing of T cells.64

Treg cells are important in maintaining peripheral tolerance, limiting inflammation, and preventing autoimmunity by self-reacting T-effector cells, and they play a key role in tumor immune tolerance.65 RT increases Treg cells,66–69 limiting the positive immunomodulatory effects of hypofractionated RT. Release of adenosine by tumor cells70,71 as well as upregulation of tumor TGFβ72,73 both of which are implicated in homeostatic tissue repair, may contribute to Treg cell accumulation after RT. Indeed, the balance between T-helper 1 (Th1) and Treg cell responses is part of the homeostatic healing response to RT, and T-bet+/− (Th1-deficient) mice develop an increased acute fibrotic response to 10-Gy irradiation, characterized by higher TGFβ and collagen deposition and significantly lower levels of IFNγ compared with wild-type mice.74 This homeostatic response
attenuates inflammation and promotes normal tissue recovery, but it also may attenuate the potential antitumor immunomodulatory activity of RT. The effects of RT on Treg cells have not been well characterized and may be dose-dependent. Some experiments found that Treg cells had an attenuated suppressive phenotype after RT. Although forkhead box 3 (Foxp3)+ Treg cells are relatively resistant to RT-induced death, RT can suppress the proliferation of Treg cells, specifically at a dose of 0.94 Gy.
RT can also render tumor cells more susceptible to T-cell attack. In mouse models, hypofractionated RT administered at a high dose (ie, >7 Gy on no more than 5 consecutive days), increased the local production of type I interferons as well as IFNγ, which enhances MHC-I and antigen presentation in tumor cells. In vitro and in vivo studies with human melanoma cells have confirmed that hypofractionated RT (10–25 Gy in a single fraction)
enabling effects on the stromal cells of the tumor micro-
immune-mediated tumor rejection (Fig. 2). The establishment of a permissive microenvironment for the tumor cells themselves, which, together, promote upregulation of the death receptor Fas in tumor cells, rendering them sensitive to killing by activated T cells, increases cell surface MHC-I expression in a dose-dependent and time-dependent manner, enhancing antigen presentation and rendering tumor cells more susceptible to T-cell attack.\textsuperscript{60} Such an effect was observed up to 11 days after irradiation, leading to the elimination of a large proportion of tumor cells by tumor-specific cytotoxic T lymphocytes (CTLs). Furthermore, RT induces expression of Fas ligand.\textsuperscript{58} Thus, RT may have enabling effects on the stromal cells of the tumor microenvironment along with direct proinflammatory effects on the tumor cells themselves, which, together, promote the establishment of a permissive microenvironment for immune-mediated tumor rejection (Fig. 2).

**Clinical Development of Rational Radioimmunotherapy Combinations**

The immunomodulatory properties of RT offer important opportunities toward the development of rational combinations with immunotherapy, aiming to maximize local tumor control and eliminate the potential for systemic metastases. However, a considerable amount of work is still required to fully understand how to develop effective combinations of immunotherapeutics and radiation. Here, we propose a framework with 3 main clinical scenarios in which combinations can be tested to enhance current clinical practice:

1. **Immune therapy added to standard-of-care hypofractionated RT in the treatment of oligometastatic disease.** The clinical goal here is mainly to reduce distant failures by capitalizing on the in situ vaccination effect of RT coupled to the local and systemic effects of immune therapy.

2. **Immune therapy added to standard-of-care chemoradiotherapy.** The clinical goal of adding immune therapy here is to enhance the efficacy of chemoradiotherapy locally and reduce distant failures by building local and systemic synergies between RT and immune modulation, ultimately prolonging progression-free survival and, hopefully, cure rates.

3. **Radiation added to immune therapy.** The clinical goal here is to maximize the efficacy of immune therapy against specific tumor deposits, using RT as a biological response modifier. Each of these conditions requires careful understanding of the clinical goals, the biological effects of each of the therapeutic components, and the desired interactions that should be elicited by the new combination. In particular, whereas the first condition aims at maximizing therapeutic interactions outside of the field of radiation, the second condition also aims at maximizing the local synergies between RT and immune therapy as well as maximizing the systemic effects of immunotherapy. Finally, the third condition aims at achieving maximal effects of the combination within the field of radiation. Understanding these clear biological and therapeutic goals is key to designing and testing rational combinations (Fig. 3).

### 1. Immune Therapy Added to Hypofractionated RT to Treat Oligometastatic Tumors

In the setting of relapsed, metastatic tumors, RT has historically been used to provide symptom palliation. However, in patients with oligometastatic disease—an intermediate state of cancer spread with few metastases detectable by current imaging methods—\textsuperscript{85} stereotactic body RT (SBRT) is increasingly being used to control disease, altering its natural course to extend progression-free and overall survival.\textsuperscript{20} Because of safety issues related to the dose or volume of RT, the current standard of care for patients with oligometastatic disease is to deliver SBRT simultaneously up to 3 metastatic sites. Such treatment has shown promising local control rates (range, 67%-97%) and a good toxicity profile (<5% of serious adverse events).\textsuperscript{83,84} However, in most of these patients, recurrence is usually at distant sites outside of the irradiated area, with a median time to progression of 4 to 6 months, indicative of occult, metastatic deposits at the time of treatment. Several experiments suggest that hypofractionated SBRT (10-24 Gy in a single fraction) leads to the release of large amounts of immunogenic tumor antigens for several days,\textsuperscript{60,85} as well as endogenous DAMP ligands, which can stimulate TLRs on APCs,\textsuperscript{45} thus providing the basis for developing rational immunotherapy combinations. Below, we propose rational combinations based on the existing literature.

#### Activation of APCs

Because of potent immune suppression in the tumor microenvironment and the draining lymph nodes,\textsuperscript{86} the immunomodulatory effects of RT in most patients are not sufficient for triggering effective antitumor immune responses, which explains why abscopal tumor regression is seldom observed in clinical practice with SBRT alone. Based on mouse experiments, additional pharmacological APC activation can be rather beneficial in this setting to boost the in situ vaccination effect of RT. Drugs currently under clinical development include agonists to the stimulator receptor CD40 and to TLRs. CD40 is a member of the TNF receptor superfamily and is expressed on B cells, DCs, monocytes, hematopoietic precursors, endothelial cells, smooth muscle cells, epithelial cells, platelets, as well as many human tumor cells.\textsuperscript{87} CD40 activates antigen-processing and presentation pathways in DCs and enhances their migration to lymph nodes, and CD40 agonists have shown activity in different cancer types in preclinical models and early phase clinical trials.\textsuperscript{88-90} In a B-cell lymphoma mouse
model, combined anti-CD40 plus 5 Gy total body irradiation (TBI) resulted in increased survival with long-term T-cell–mediated protection in more than 80% of animals. In addition, cancer vaccines could provide opportunities for further boosting the radiation-mediated in situ vaccination effect. Different RT doses and fractionations can be combined with agonistic antibodies directed against costimulatory molecules on T cells (eg, tumor necrosis factor receptor superfamily member 4 [OX-40], cluster of differentiation 137 [CD137], CD27) and/or blocking antibodies against coinhibitory molecules (eg, cytotoxic T-lymphocyte-associated protein 4 [CTLA-4], programmed cell death 1 [PD-1]), to increase T-cell function. The immunomodulatory effects of RT at different doses and schedules on the tumor microenvironment could be harnessed to enhance the therapeutic efficacy of immunotherapy approaches that aim to activate T cells, such as antibodies against coinhibitory T-cell receptors (eg, PD-1, PD-1 ligand [PD-L1], T-cell immunoglobulin and mucin-domain containing 3 [TIM-3], lymphocyte activation gene 3 protein [LAG-3], etc) or transforming growth factor β (TGF-β)–blocking drugs. Finally, low-dose RT could increase the homing capacity of activated T cells and could be useful particularly in the context of adoptive T-cell therapy or cancer vaccines. BTLA/HVEM, indicates B-lymphocyte and T-lymphocyte attenuator/herpes virus entry mediator; Gy, grays; IDO, indoleamine 2,3-dioxygenase.

TLRs are a family of pattern-recognition receptors expressed broadly on hematopoietic cells, including APCs, monocytes, and B cells, which recognize DAMPs, activate innate immune responses, and facilitate the development of adaptive immune responses. In humans, the TLR family consists of 10 members (TLR1–TLR10). Different DC subsets express different TLRs in humans and in mice. TLR9 recognizes unmethylated 5′–C–phosphatethy- G-3′ (CpG) motifs and functions through the MyD88 pathway, leading to NF-κB activation, cytokine secretion, and an inflammatory response. TLR9 plays crucial roles in the innate immune system and is a target for cancer immunotherapy.
in driving inflammatory responses in mouse myeloid cells in reaction to tissue stress or RT injury. Interestingly, it was shown that endogenous TLR9 ligands released by tumors after RT promoted tumor regrowth of syngeneic B16 melanoma, CT26 colon tumors, or MB49 bladder tumors after a single dose of RT at 13 Gy. This effect depended on TLR/MyD-88/NF-kB-mediated upregulation of IL-6 in myeloid cells, which, in turn, induced STAT3 phosphorylation and promoted a unique expression of genes linked to neovascularization. Indeed, thrl2−/−-bearing mice had a delay in tumor regrowth relative to TLR9-proficient mice.96 However, pharmacologic TLR9 activation by intratumoral delivery of exogenous CpG markedly enhanced tumor responses to single-fraction RT at 20 Gy in a murine fibrosarcoma model97,98 and in the Lewis lung adenocarcinoma model.99 Furthermore, the administration of imiquimod (1-isobutyl-1H-imidazo[4,5-c][quinolin-4-amin]), a TLR-7/TLR-8 agonist, with low-dose cyclophosphamide (to deplete Treg cells) and 8 Gy irradiation in 3 fractions (on days 12-14) suppressed tumor growth in a breast cancer mouse model with skin metastasis.100 Similarly, intravenous administration of a TLR-7/TLR-8 agonist with single-dose RT at 10 Gy led to T-cell–mediated tumor clearance and generation of tumor-specific immune memory in a mouse T-cell and B-cell lymphoma model.101 These studies provide a strong rationale to combine TLR agonist therapy with SBRT, although expression of TLRs differs markedly between humans and mice, and the translation of mouse data to human data will require careful validation with respect to the choice of a TLR agonist. Encouragingly, abscopal responses were reported in a clinical study of patients with low-grade B-cell lymphoma who received RT at 2 Gy in 2 fractions to a single tumor site plus local injection of TLR-9 agonist.8

**Release of T-cell–inhibitory signals**

In addition to direct APC activation, additional immunotherapy interventions that activate T cells could be very useful in combination with SBRT, and preliminary clinical evidence supports their added value. CTL antigen 4 (CTLA-4) is a key negative regulatory receptor that is recruited to the plasma membrane of activated T cells, where it binds to the B7 family costimulatory ligands CD80 and CD86, which are expressed by DCs and other APCs, thereby attenuating T-cell activation.102 Transient CTLA-4 blockade enhances memory CD8+ T-cell responses during vaccination and augments memory formation and maintenance102; therefore, CTLA-4 blockade during RT could increase the in situ vaccination effect of RT.103,104 This combination has indeed proven to be synergistic and therapeutically effective in numerous mouse models, in which RT or CTLA-4 blockade alone was ineffective to control tumors, but the combination has produced improved local control of irradiated tumors as well as control of distant metastases.103-109 CD8+, but not CD4+, T cells were required for the observed therapeutic effect in the mouse,103 and the development of tumorspecific, CD8+ T-cell responses required functional DCs in tumors and draining lymph nodes.109 The synergy between RT and CTLA-4 blockade appears to be dose-dependent and fractionation-dependent in mouse experiments. For example, hypofractionated RT (in 3 fractions of 8 Gy) with anti-CTLA-4 antibody were effective in inducing an antitumor immune response that was able to inhibit the tumor locally and systemically, whereas 5 fractions of 6 Gy were inferior, and a single fraction of 20 Gy was ineffective when combined with anti-CTLA-4.104

The combination of RT with ipilimumab, a monoclonal antibody that blocks human CTLA-4 and has been approved by the US Food and Drug Administration for the treatment of metastatic melanoma,110 has produced encouraging, concordant results in tests between mice and humans. In addition to enhancing T-cell priming, ipilimumab may also deplete intratumoral Treg cells111 through antibody-dependent, cell-mediated cytotoxicity,112,113 a further synergistic interaction with RT. A retrospective study reported abscopal effects in 11 of 21 patients (52%) with metastatic melanoma who, after progression on ipilimumab, received hypofractionated RT to different metastatic lesions. Nine of those patients experienced partial objective responses (43%), and 2 had stable disease (10%), and their overall survival was increased compared with patients who did not experience an abscopal effect. Interestingly, abscopal responses were only observed in patients who had complete local regression of irradiated tumor(s). Complete abscopal tumor regression also has been reported anecdotally as a result of this combination. Postow et al reported that immunological changes associated with an abscopal effect led to a complete response in a patient with melanoma who received local RT (9.5 Gy in 3 fractions) and ipilimumab, despite previous progression observed while on ipilimumab alone.10 In another case report, a patient with metastatic melanoma received SBRT (17 Gy in 3 fractions) to 2 of 7 liver metastases after 2 cycles of ipilimumab. The patient then received an additional 2 cycles of ipilimumab and experienced complete regression of all irradiated and nonirradiated tumors.12 Golden et al reported abscopal effects in multiple nonirradiated liver and bone lesions in a patient with metastatic chemotherapy-refractory nonsmall cell lung cancer who received SBRT (6 Gy in 5 fractions) to a single liver lesion in combination with ipilimumab starting the day after the first radiation dose followed by 3 more doses of the drug.11

It is important to note that positive interactions between SBRT and ipilimumab have been reported in patients who
received SBRT while receiving concomitant ipilimumab. This concomitant treatment possibly enables robust T-cell priming, an effect that presumably is lost with the sequential administration of ipilimumab after RT. Indeed, in a phase 1 clinical trial of 22 patients with multiple melanoma metastases, a single index lesion was irradiated with hypofractionated radiation delivered over 2 or 3 fractions, followed by 4 cycles of ipilimumab. Although 18% of those patients had partial responses in the nonirradiated lesions, the majority of patients did not respond. The median progression-free and overall survival was 3.8 and 10.7 months, respectively. Furthermore, the sequential combination of ipilimumab and SBRT fell short of expectations in a randomized, double-blind, phase 3 trial in which 799 patients with metastatic, castration-resistant prostate cancer who had at least one bone metastasis progressed after docetaxel chemotherapy. Patients were randomly assigned to receive bone-directed RT (8 Gy in a single fraction; to at least one and up to five bone fields) followed by either ipilimumab 10 mg/kg or placebo every 3 weeks for up to 4 doses. Nonprogressing patients could continue to receive ipilimumab at 10 mg/kg or placebo as maintenance therapy every 3 months until disease progression, unacceptable toxic effect, or death. The study nearly missed its primary end-point of survival benefit, with a hazard ratio (HR) of 0.85 (95% confidence interval [CI], 0.72-1.00; \(P = .053\)), although there was a significant improvement in progression-free survival (HR, 0.70; 95% CI, 0.61-0.82; \(P < .0001\)), and a subgroup analysis showed improved overall survival in patients who had less tumor burden (HR, 0.62; 95% CI, 0.45-0.86; \(P = .038\)). Additional studies must address the potentially critical issue of schedule when combining SBRT and ipilimumab.

The inhibitory immune receptor programmed cell death 1 (PD-1)\(^{115}\) is upregulated in T cells upon antigen-dependent activation and drives T-cell suppression when engaged by its cognate ligands, PD-1 ligand (PD-L1) and PD-L2, which are expressed by tumor cells and/or tumor-infiltrating myeloid cells in many tumors.\(^{115,116}\) Neutralizing antibodies to either PD-1 or PD-L1 have been reported to generate potent antitumor activity in clinical trials,\(^{117-119}\) and expression of PD-L1 in tumors is a positive predictor of response to PD-1 or PD-L1 blockade.\(^{119,120}\) Pembrolizumab and nivolumab—2 checkpoint inhibitors that target PD-1—received US Food and Drug Administration approval in 2014 for patients with metastatic melanoma who did not respond to prior treatment\(^ {121,122}\) and was subsequently approved for nonsmall cell lung cancer (both drugs) as well as renal cell carcinoma and classical Hodgkin lymphoma (nivolumab). Hypofractionated RT can lead to the upregulation of PD-L1 in experimental tumor models, which was shown to be mediated by IFN-\(\gamma\).\(^ {123}\) Tumor-infiltrating, CD8\(^{+}\) CTLs also exhibited increased expression of PD-1 after RT in vivo, and the depletion of CD8\(^{+}\) T cells (but not CD4\(^{+}\) T or NK cells) abrogated the upregulation of PD-L1 induced by RT in tumor cells.\(^ {124}\) Taken together, these findings suggest that upregulation of the PD-L1/PD-1 pathway may be an important adaptive resistance mechanism to RT; therefore, PD-1/PD-L1 blockade could be an important addition to radioimmunotherapy combinations. In addition, the combination of SRS and PD-L1 blockade generated equally tumor-specific immune responses in the mouse.\(^ {125}\) Anti–PD-L1 antibody and 12-Gy local irradiation in a mouse breast carcinoma model significantly decreased tumor growth compared with either treatment alone and generated long-term immunological memory, protecting animals against tumor rechallenge.\(^ {123}\) In addition, the combination of anti–PD-L1 with RT at 12 Gy in a single fraction mediated the regression of distant tumors.\(^ {123}\) Accordingly, in a phase 1 study combining hypofractionated RT followed by 4 cycles of ipilimumab in metastatic melanoma, patients who had low PD-L1 expression experienced significantly higher overall and progression-free survival compared with those who had high PD-L1 expression.\(^ {105}\) This observation was reproduced in a mouse melanoma model, in which the addition of anti–CTLA-4 and anti–PD-L1 antibodies to radiation (20 Gy) improved irradiated tumor responses and caused growth suppression in nonirradiated, distant tumors. Subsequent tumor progression was associated with PD-L1 upregulation in melanoma cells and lymphocyte exhaustion. Optimal therapy required double CTLA-4 and PD-L1 blockade in combination with RT, in which CTLA-4 blockade predominantly inhibited Treg cells and increased the CD8\(^{+}\) T-cell to Treg-cell ratio, radiation enhanced the diversity of the T-cell receptor (TCR) repertoire of intratumoral T cells, and PD-L1 blockade reversed T-cell exhaustion.\(^ {105}\)

Agonistic antibodies to costimulatory TCRs offer a complementary strategy to activating antitumor T cells and could be combined with RT. Tumor-reactive, CD8\(^{+}\) tumor-infiltrating lymphocytes (TILs) that coexpressed PD-1 and CD137 were detected in a breast cancer mouse model 12 hours after exposure to RT,\(^ {92}\) explaining the benefit of combination therapy with anti–PD-1 and anti–CD137 monoclonal antibodies and RT either at 12 Gy in a single fraction or at 4 or 5 Gy in 5 fractions.\(^ {126}\) Additional evidence in lung, breast, and glioma mouse models indicates that checkpoint blockade and costimulatory antibody therapy, such as CD137, can be successfully combined with RT, and particularly with SRS, leading to increased survival and tumor control.\(^ {93,106,127,128}\) CD134 (TNF receptor superfamily member 4 [OX-40]) interaction with its ligand...
proliferation. Agonistic anti–OX-40 antibody combined with RT resulted in a significant survival advantage in tumor-bearing mice that were mediated by CD8+ cells.\textsuperscript{129} Finally, IL-2 is a potent cytokine used in clinical practice to activate T cells. The combination of IL-2 with 15-Gy single-dose RT was able to induce T-cell–mediated complete responses in mice bearing MC38 colon adenocarcinoma versus a 12% complete response rate with either treatment alone.\textsuperscript{130} In the same way, patients with melanoma and renal cell carcinoma who received SBRT in 1, 2, or 3 20-Gy doses combined with IL-2 showed higher than expected abscopal responses.\textsuperscript{13}

Indoleamine 2,3 dioxygenase 1 (IDO1) is an intracellular tryptophan-catabolizing enzyme that is produced by several cell types, including tumor cells and APCs (both conventional myeloid and plasmacytoid) and mediates immune suppression through local reduction of tryptophan levels and production of tryptophan metabolites, such as kynurenin, which suppress T-cell proliferation and cause T-cell apoptosis.\textsuperscript{131} 1-Methyltryptophan, a putative IDO1 inhibitor, has been shown to enhance the effects of single-fraction, localized RT (5 Gy) in combination with temozolomide in mice bearing orthotopic glioblastoma.\textsuperscript{132} IDO1 can be upregulated in the lymph node microenvironment by TLR stimulation,\textsuperscript{133} attenuating the priming effects of APC activation. Thus, the therapeutic effect of TLR agonists when combined with SBRT might be significantly increased by the simultaneous delivery of IDO inhibitors. Similar to PD-L1, IDO1 is upregulated by IFNγ\textsuperscript{134} and is also responsible for mediating adaptive resistance of tumors to CTLA-4 or PD-1/PD-L1 blockade.\textsuperscript{135} Taken together, these data suggest that IDO1 blockade would be a suitable partner for combinations with RT and checkpoint blockade.

Another important immunosuppressive factor to be neutralized in the context of RT is TGFβ, a pleiotropic cytokine important in the regulation of tissue homeostasis and cell proliferation that attenuates inflammation and immune responses. TGFβ suppresses T-effector cells\textsuperscript{136,137} and promotes the accumulation of Treg cells.\textsuperscript{73,138,139} It also drives DCs toward a tolerogenic polarization, and its blockade greatly enhances DC polarization toward an immunogenic phenotype.\textsuperscript{140} It is activated in the tumor microenvironment of irradiated tumors because of specific redox-sensitive, conformational changes in the TGFβ complex induced by RT.\textsuperscript{141} In addition, TGFβ is upregulated and actively released in the tumor microenvironment in response to acute RT injury.\textsuperscript{56,74} Studies on radiation-induced enteropathy demonstrated that, 2 weeks after RT, regenerating intestinal crypts, inflammatory cells, smooth muscle cells, and mesothelium exhibited increased levels of different TGFβ isoforms.\textsuperscript{142} TGFβ reduces the radiosensitivity of tumors and contributes to tumor invasion, metastasis, and poor clinical outcomes.\textsuperscript{143–146} In mice bearing flank breast tumors, a neutralizing antibody to TGFβ combined with 5 daily doses of 6 Gy (starting on day 13) resulted in DC activation and a robust CD8+ T-cell response, which contributed to suppressing the growth of both irradiated and distant tumors. Despite an initial response, however, tumors progressed, which was associated with the upregulation of PDL-1 and PDL-2 both in tumor cells and in CD45+/CD11b+ myeloid cells. The addition of PDL-1 blockade to the RT/TGFβ blockade combination further improved tumor responses and mouse survival.\textsuperscript{147}

**Developing rational combinations in the clinic**

Taken together, the preclinical and clinical evidence described above strongly suggests that hypofractionated SBRT should be combined in clinical trials with drugs that activate APCs (to maximize in situ vaccination) and with drugs that enhance T-cell priming and function in the tumor microenvironment. The addition of APC stimulation in this context appears to be particularly attractive, because mature APCs may exert beneficial pleiotropic effects in the tumor microenvironment that provide opportunities for multiple synergistic interactions with RT and drugs that stimulate T cells. For example, activating tumor APCs will enhance tumor antigen presentation and help prime or boost antitumor T cells in lymph nodes or in tumor-embedded tertiary lymphoid structures. In addition, properly activated APCs provide potent costimulatory signals to TILs in the tumor microenvironment and release appropriate chemokines, which guide effector T cells to home in the tumor microenvironment. However, important work needs to be done to understand how best to combine RT and these drugs in patients.

An important issue that will require careful evaluation is the schedule of the different interventions in radioimmunotherapy combinations. For example, should drugs be given concomitantly or sequentially, and in which order? During pathogen recognition, antigen uptake and activation of APCs by DAMPs occur simultaneously, resulting in potent immune activation. To maximize pathogen mimicry during RT and trigger an efficient immune response, perhaps APC activation by CD40 or TLR agonists should occur at the time of antigen release. The kinetics of antigen release after SBRT have not been studied carefully, especially in humans, but in vitro and in vivo experiments suggest that RT-induced tumor cell death starts within 8 hours after the delivery of a large RT dose, peaks at 24 hours, and persists for at least 7 days.\textsuperscript{22,27} Ionizing radiation increases the MHC class I-associated peptide pool starting 18 hours after irradiation, an effect that persists for up to 10 days thereafter.\textsuperscript{62} TLR agonists as well as small-molecule CD40...
agonists have a very short half-life, with biological effects that vanish within 48 to 72 hours. Therefore, these drugs should be timed very carefully to coincide with the peak of tumor antigen release. CD40 antibodies or other macromolecules have longer half-lives and achieve more sustained activation of APCs, permitting more flexibility in the timing of radioimmunotherapy.

Given the important role in T-cell priming, the administration of CTLA-4–blocking antibodies also should be initiated before or at the time of delivering RT. Similarly, TGFβ–neutralizing therapies would be initiated best at the same time to prevent the reactive upregulation of TGFβ in the irradiated tumors and its deleterious effects on antigen presentation. Once a T-cell response is primed, support of effector T cells through the delivery of PD-1/ PD-L1–blocking, TGFβ–blocking, and IDO–1–blocking agents as well as IL-2 appears to be ideal for maximizing effects in irradiated tumors and distant metastases. This area will require careful optimization in appropriate mouse models and in clinical studies with translational endpoints to prepare suitable combinations that result in maximizing the in situ vaccination and antitumor effects of SBRT for the treatment of relapsed oligometastatic tumors.

The dose and fractionation of SBRT will require careful optimization in the context of radioimmunotherapy combinations, because the immunomodulatory effects of RT may be sensitive to these parameters. For example, the in situ vaccination effect of RT seems to be dose-dependent and schedule-dependent. In the mouse, a single dose of 20 Gy or 3 fractions of 15 Gy increased T-cell priming and led to CD8+–dependent reduction/eradication of primary melanoma and distant metastases, whereas 5 Gy in 4 fractions over a 2-week period led to inferior tumor growth inhibition. Overall, tumor control as well as the frequency of tumor-reactive T cells increased with the size of the RT dose, with maximum effects observed between 7.5 and 15 Gy. However, fractionated treatment with medium-sized radiation doses of 7.5 Gy in 2 fractions produced the best tumor control, the best tumor-specific T-cell responses, and the lowest Treg-cell numbers. In combination with TLR agonists, 8 Gy in 3 fractions or 10 to 13 Gy in a single fraction seemed to bolster the desirable immune responses in animal models. Similarly, in combination with anti–CTLA-4, anti–PD-1, or anti–PD-L1 antibodies, 8 Gy in 3 fractions increased immune-mediated tumor-cell killing; but alternate schemes, such as 17 to 20 Gy in 1 to 3 fractions, 12 Gy in a single fraction, 9.5 Gy in 3 fractions, and 6 Gy in 3 to 5 fractions, also have been successfully tested in the mouse. When used in combination with IL-2, higher doses have been necessary in humans to achieve systemic effects (15-20 Gy). Preclinical and clinical experience to date is insufficient to make specific recommendations on dose/fractionation schemes, and systematic evaluation must be undertaken in the context of comparative clinical studies (Supporting Table 1 summarizes current and ongoing clinical trials combining hypofractionated RT with immunotherapy; see online supporting information).

It is also important to note that the selected dose and fractionation scheme largely depends on the size and anatomic location of the tumor and the dose tolerance to radiation of the specific organs at risk. In most SBRT trials, the inclusion criteria have been limited to patients with up to 5 metastatic locations (oligometastatic disease), but the maximum number of metastases treated simultaneously has been 3, probably because of safety issues related to the large volumes to be exposed to SBRT. Notwithstanding this, the immunological effect of SBRT could potentially benefit patients who have more than 5 metastatic sites, broadening the use of SBRT to all visible irradiated lesions. Under the same framework, if the desired effect is antigen release, then it is conceivable that partial tumor volume irradiation would be sufficient to induce in situ vaccination with fewer side effects. This is an unexplored area in radiation oncology that would require careful testing in clinical trials.

2. Immune Therapy as Adjuvant to Standard-of-Care Chemo-RT
Approximately one-half of cancer patients receive upfront treatment with some combination of chemo-RT. Such conventional chemo-RT regimens have been developed empirically based on tolerability and best clinical responses, but not based on immunological principles. In these regimens, RT is used at a conventional dose and fractionation schemes ranging between 1.8 and 2 Gy per fraction, for a total dose up to 50 to 60 Gy, adapting known radiobiology principles to maximize the total RT dose delivered to the tumor while minimizing side effects to the surrounding normal tissues. However, such dose/fraction schemes are associated with a strong immune-suppressive response in tumors, including expanding Treg cells and myeloid-derived suppressor cells, as well as significant upregulation of TGFβ. In addition, chemotherapy is given at the maximal tolerated dose (MTD). Traditionally, chemotherapy has been viewed as deleterious in the context of immune therapy, because it was believed to exert broad immunosuppressive effects, including suppressing T cells, in addition to its well known myelosuppression. Indeed, select chemotherapy agents, eg, methotrexate and taxanes, have been used to treat recalcitrant forms of autoimmune pathologies driven by T cells, such as severe rheumatoid arthritis. The observation that chemotherapy-naïve patients benefitted more from PD-L1 blockade relative to previously treated patients also suggests that
chemotherapy at the MTD may exert long-term immunosuppressive effects. Nevertheless, numerous studies have documented that the presence and quantity of TILs pretherapy are strongly correlated with increased response and survival after standard chemo-RT treatments.\textsuperscript{52,53,154–156} This epidemiologic evidence also suggests the possibility of positive long-term interactions between antitumor immune response and conventional chemo-RT schemes, which, if well understood, could provide the basis for developing rationally optimized combinations with available immunotherapy approaches. If successful, effective combinations could both enhance the local control of the primary tumor in the radiation field and provide systemic protective immunity to minimize distant failures and increase cure rates.

It has been demonstrated that specific chemotherapy agents exert pleiotropic immunomodulatory effects, which may be dose-dependent and schedule-dependent. For example, cyclophosphamide at myelosuppressive doses induces rebound myelopoiesis and leads to the emergence of tumor-infiltrating DCs that secrete more IL-12 and less IL-10, which are fully capable of priming T-cell responses.\textsuperscript{157} 5-Fluorouracil, gemcitabine, and taxanes can cause a decrease in myeloid-derived suppressor cells.\textsuperscript{158–160} Nowak et al showed that relatively high doses of gemcitabine suppressed immunoglobulin G production but did not block T-lymphocyte responses and were not detrimental to specific antitumor immunity.\textsuperscript{161} Furthermore, topotecan at the MTD did not impair the frequency or response of lymphocytes in patients with ovarian cancer.\textsuperscript{162}

To maximize the immunomodulatory effects of chemotherapy or chemo-RT combinations, the schedule-sensitive effects of the ensuing immunomodulation must be recognized. For example, in the mouse, cyclophosphamide, doxorubicin, and paclitaxel at the MTD increased the efficacy of a tumor-cell–based vaccine, helping to break the tolerance to tumor-associated antigen when given before the vaccine, but dampening the efficacy of the vaccine when given after it.\textsuperscript{163} In patients with ovarian cancer, a single course of paclitaxel and carboplatin treatment decreased the number of Treg cells and increased type-1 T-helper cells and CTLs as well as NK cells in peripheral blood, with maximal effects around 2 weeks after treatment.\textsuperscript{164} Thus, transient windows of opportunity to reverse tumor immunosuppression may be offered in the context of chemotherapy, which may be particularly interesting for immunotherapy interventions. For example, in preclinical models and in a follow-on phase 1 trial in metastatic cervical cancer, carboplatin/paclitaxel chemotherapy administered approximately 10 days after vaccination, timed carefully to coincide with the nadir of immunosuppressive myeloid cells in peripheral blood, maximized T-cell responses to a human papilloma virus 16-specific peptide vaccine.\textsuperscript{165,166}

Preclinical models suggest that vaccines may combine safely with and add benefit to chemo-RT schemes, especially tumor models associated with viral antigens.\textsuperscript{167,168} While clinical experience has been disappointing to date,\textsuperscript{169,170} this may have been because of the sequential vaccine administration (which was initiated several weeks after chemo-RT had been completed, thus missing the positive interaction with chemo-RT) and the overall poor clinical efficacy of historic vaccines, such as molecularly defined, monovalent vaccines targeting a single “self” tumor antigen like mucin 1 (MUC1),\textsuperscript{169} or the use of allogeneic whole-tumor lysate.\textsuperscript{170} The discovery of private, nonsynonymous mutations (ie, a single nucleotide mutation that results in a change in the amino acid sequence of a protein) expressed at different frequencies in all tumor types—the so-called mutanome—provides renewed cancer vaccine opportunities, capitalizing on tumor immune recognition based on epitopes containing somatically mutated residues, referred to as mutant neoantigens.\textsuperscript{171} These are expected to escape central immunological tolerance, similar to virus-derived antigens expressed in virally induced cancers;\textsuperscript{172} therefore, vaccination is expected to have increased efficacy relative to historic vaccines directed at cancer-associated “self” antigens.\textsuperscript{173} Tumors with a high mutational load, such as metastatic melanoma and lung cancer, exhibit increased response rates to immune checkpoint blockade,\textsuperscript{174,175} providing further validation of the possible relevance of tumor neoantigens in cancer immunotherapy. For example, a hypothesis is that the increased clinical response rates to adoptively transferred T cells and to CTLA-4 and/or PDL-1 checkpoint blockade are mediated in part by neoantigen–specific T cells. Thus, an exciting possibility is that peptide–based vaccination with mutated peptides could induce tumor-specific T cells, which are lacking in patients who have failed to respond to checkpoint blockade, and might convert these patients into responders.\textsuperscript{176} Mutated peptides also may prove to be natural targets for tumor-specific TILs and could be used for ex vivo expansion of patient-derived T cells before adoptive T-cell therapy.\textsuperscript{177} In addition, mutated peptides could be efficiently targeted with TCR-transduced T cells. Finally, mutated peptides may be useful in immune monitoring, to evaluate specific immunity against them and to correlate with response or disease recurrence after immunotherapy.\textsuperscript{178} Thus, understanding mutanome-encoded peptides as a target for antitumor T cells is a new frontier for cancer immunotherapy, and the combination of these therapies with standard-of-care chemo-RT will need to be tested in future clinical trials.

**Building rational combinations**

Principles similar to those discussed above with respect to SBRT would apply here, with the objective of maximizing
The evidence that hypofractionated RT may be better suited for immunotherapy combinations, along with increasing evidence of safety and efficacy as well as acceptability by clinicians, suggests a potential benefit from the incorporation of hypofractionated schemas into frontline chemo-RT treatments. These would conveniently shorten the time of radiation and offer optimal schedule opportunities for combination with chemotherapy and immunomodulatory drugs. In this context, the use of cytotoxic chemotherapy drugs that induce immunogenic cell death would seem particularly opportune. Thus, the current frontline chemo-RT schemes may need to be drastically revised to maximize the benefit from mobilizing antitumor immunity.

Vaccines may be ideal partners for frontline chemo-RT combinations given their overall low toxicity. Furthermore, vaccine continuation would be an ideal maintenance strategy after frontline chemoradioimmunotherapy combinations. Ionizing radiation in standard dose-fractionation regimens induces the release of endogenous tumor antigens and has in situ vaccination effects, which can be improved with additional immunomodulation. Thus, chemo-RT could offer unique opportunities for developing integrated prime–boost schemes between the in situ vaccination effects of RT and novel, exogenous, neoepitope-based vaccines. Given the low risk of toxicity from vaccines, innovative clinical trial designs with accelerated rule-based designs, such as a titration design, may be proposed for combinations with concomitant chemo-RT as an alternative to traditional 3 + 3, dose-escalation, phase 1 trial designs for determining the MTD. Supporting Table 1 summarizes ongoing clinical trials combining immunotherapy and chemo-RT (see online supporting information).

The combinations discussed above would benefit from additional immunomodulation to activate T cells, such as checkpoint blockade; and early clinical experience of combining such drugs with conventional chemotherapy shows good tolerability and promising results. Agents activating T-cell function would be best suited in the immediate postchemo-RT period, as consolidation strategy, and for maintenance strategies, in which they also can be combined with vaccines. Careful clinical testing will require the design of phase 0 (translational) studies with biological endpoints and phase 1 studies in which the various combinations and schedules can be tested in a systematic way. Phase 2 adaptive design trials also may allow investigators to quickly identify combinations with therapeutic effect and to minimize the exposure of patient populations to less appropriate combinations.

### 3. Irradiation as Response Modifier of Immunotherapy

Recent breakthroughs with checkpoint blockade therapy as well as adoptive transfer of genetically modified T cells that express new TCRs or chimeric antigen receptors have demonstrated the clinical potential of cancer immunotherapy. However, not all patients benefit from such therapies, in part because the tumor microenvironment is not conducive to T-cell engraftment. Vascular barriers, lack of appropriate chemokines, and other stroma-immunosuppressive factors may play important roles in preventing proper T-cell homing and function. Ionizing irradiation may render these tumors immunogenic, restoring inflammatory mechanisms that allow T-cell infiltration. Recent evidence in mouse models suggests that a single fraction of low-dose irradiation (LDI) (ie, 0.5-2 Gy) can reprogram the tumor microenvironment, inducing macrophage M1 polarization. Inducible nitric oxide synthase–positive (iNOS+) M1 macrophages, in turn, produced the appropriate chemokines to recruit effector T cells and induced tumor vasculature normalization and inflammation, allowing T-cell infiltration.

The potential clinical applicability of LDI-mediated reprogramming of macrophages was supported in that study by a retrospective analysis of patients with pancreatic adenocarcinomas who previously received LDI in the neoadjuvant setting. In these tumors, LDI significantly increased the proportion of iNOS+ macrophages and CD8+ T cells and decreased the average size of the tumor blood vessels, possibly reflecting vascular normalization. These results should be reconciled with prior evidence in humans that LDI of inflammatory fields, conversely, may attenuate inflammation, as observed in patients with benign inflammatory or degenerative diseases driven by autoimmune T cells, in whom LDI (single doses ≤1 Gy) exerted anti-inflammatory properties. Clinical studies should be...
conducted to directly compare different low doses of RT with respect to their effect on the tumor microenvironment, to confirm the mouse findings, and to establish an optimal range of LDI doses capable of reprogramming human tumor macrophages and enhancing T-cell infiltration.

Although the mouse preclinical models and clinical examples have shown that hypofractionated RT delivered at relatively high doses can trigger in situ vaccination and subsequent abscopal effects, there is no current evidence that LDI can trigger the same results. Rather, it has been demonstrated that LDI can reprogram the microenvironment of the radiated tumors and may be quite useful to enable T-cell homing in patients with an absence of preexisting tumor-infiltrating CD8+ T cells. LDI may be very useful as a preparatory step to induce T-cell homing in tumors lacking T cells in the context of combinations with checkpoint inhibitors. The next clinical trials should focus on different LDI schemas in combination with checkpoint inhibitors. In the future, this could be offered as a salvage treatment for patients who have become refractory to other therapeutic approaches, including checkpoint inhibitors, in which manipulating the tumor microenvironment with LDI could induce de novo tumor responses. Finally, a combination of high-dose SBRT to a few metastases, to trigger in situ vaccination, and LDI to the remaining metastases could provide an important opportunity to maximize the abscopal effects by exploiting the potential of RT both to induce vaccination and to facilitate T-cell attack.

Additional synergies could be provided by LDI based on emerging radiobiology data on the effects of RT at low doses. Studies using dynamic microscopic imaging, an optical technic that allows the performance of experiments in real time, demonstrated that x-rays could induce some tumor cell killing at doses between 0.1 and 0.5 Gy, a phenomenon called hyper-radiation sensitivity. LDI at the dose range of hyper-radiation sensitivity has been studied in combination with chemotherapy in many clinical trials with unexpectedly high rates of disease control, explained in part by the ability of LDI to sensitize tumor cells to cytotoxic chemotherapy. All clinical studies have reported excellent tolerability of LDI, with no additional toxicity over conventional chemotherapy, even in the setting of whole-abdominal LDI (0.6 Gy per fraction, twice daily, 2 days weekly, for 6 weeks). Therefore, tumor-targeted LDI could be combined with systemic chemotherapy, and combinations with immunogenic chemotherapy drugs could be developed, to provide a backbone of immunogenic chemorT for chemoinmunotherapy combinations.

A particular aspect of LDI with potentially interesting immunomodulatory functions is TBI. Various immune functions are stimulated by low-dose TBI at doses of 0.1 Gy using a dose rate of 15 milligrays per minute, including NK-cell and macrophage activation and T-cell proliferation. Maturation of murine APCs also was reported after TBI with 0.075 Gy and was accompanied by increased expression of CD28 and decreased expression of CTLA-4 on T cells at early time points after LDI. Chronic administration of LDI at total doses of 1 Gy also resulted in the activation of innate immune responses. Furthermore, several studies have reported improved tumor control after TBI in mice (dose range, 0.15–0.2 Gy).

Thus, rational combinations with immunotherapy could be developed in patients who are treated with large volumes of RT, in whom LDI schemes can be proposed.

TBI at higher doses has been used to achieve profound lymphodepletion to improve the effectiveness of adoptive T-cell transfer. Pioneering studies using the premelanosome protein 1 (Pmel-1) mouse model, with adoptive transfer of glycophitin 100-specific T cells into mice bearing B16 melanoma tumors, have convincingly demonstrated that the increased intensity of myeloablation and lymphodepletion through the use of single-dose TBI before adoptive transfer enhances the engraftment of T cells and therapeutic efficacy, with a myeloablative plateau at 9 Gy. Additional mouse experiments using mammaglobin-A2–specific CD8+ T cells or T cells transduced with a chimeric antigen receptor, which recognizes the vascular endothelial growth factor receptor 2 (VEGFR-2), have confirmed the value of TBI before adoptive T-cell transfer. The profound lymphodepletion, with the near-complete removal of T-cell cytokine sinks, resulting in elevation of endogenous homeostatic cytokines IL-7 and IL-15, and the depletion of Treg cells in part explain the beneficial effect of TBI. Elegant studies by Paulos et al further demonstrated that, in mice genetically deficient in lymphocytes (and thus lacking Treg cells and cytokine sinks), TBI preconditioning had a similar potentiating effect on adoptive T-cell transfer that was related to radiation damage of the intestinal mucosal barrier, transmigration of intestinal flora in the mesenteric lymph nodes, and potent, innate immune activation through microbial TLR4 ligands like lipopolysaccharide. The value of TBI in the context of adoptive T-cell therapy in humans was first tested in a prospective metastatic melanoma cohort, in which patients received nonmyeloablative cyclophosphamide and fludarabine chemotherapy in addition to 2-Gy or 12-Gy TBI, followed by TIL infusion, and high-dose IL-2. Patients who received TBI in association with chemotherapy also received CD34+ hematopoietic stem cell support. Patients who received 2-Gy or 12-Gy TBI had an objective response rate of 52% and 72%, respectively; whereas patients who did not receive TBI had an objective response rate of 49%. This prompted the completion of a randomized study assessing the contribution of TBI in the context of adoptive T-cell therapy. One hundred patients with
metastatic melanoma were randomly assigned to receive non-myeloablative chemotherapy with or without 1.2-Gy TBI before the transfer of TILs. The clinical response rate, which was the primary endpoint of the trial, was 24% in both groups, and overall survival was also similar (38.2 vs 36.6 months; \( P = .71 \)). Importantly, 27% of the patients who received TBI suffered from thrombotic microangiopathy, an event exclusively observed in RT-treated patients. Thus, the nonmyeloablative chemotherapy regimen seemed to provide sufficient lymphodepletion for successful adoptive transfer without the need to add TBI.209

Conclusions

The immunomodulatory effects of RT have been extensively reported in preclinical and clinical studies, while the reported abscopal effects have confirmed that immunemediated tumor responses can be triggered by RT. RT releases tumor antigen and can favorably modulate immunological pathways, leading to increased tumor antigen presentation, priming of tumor-specific cytotoxic T cells, as well as enhanced T-cell homing, engraftment and function in tumors. These effects can be substantially potentiated by combination therapies that exploit the immune effects of RT and enhance immune function. Several examples of the synergistic effect between RT, chemotherapy, and immunotherapy support the development of clinical trials to test this combination. Novel radiation technology can deliver high doses of precise radiation beams, protecting healthy tissue and avoiding side effects: this makes RT a suitable complement to immunotherapy. Important work still remains to define the optimal dose/fractionation schemes that can create maximal interactions with immune modulation and to identify the type, dose, and schedule of immunogenic chemotherapy and the type and schedule of immunomodulatory drugs that are suitable for combinations with chemo-RT. The roles of particle radiation, including protons, and of radionuclide therapy targeted to tumors through antibodies or receptor ligands will need to be evaluated for their potential immunomodulatory effects and combinations with immunomodulation following similar principles. Finally, the role of immunomodulatory radiation should be evaluated in the context of emerging adoptive T-cell therapy strategies as an important opportunity to enhance the efficacy of adoptive T-cell therapy against solid tumors. Rapid progress will require the development of innovative clinical study adaptive designs that allow high throughput testing and comparison of rational combinations, heavily focused on deep translational interrogation of patients tumor biopsies obtained pretreatment and during treatment as well as upon progression, to understand the effects of therapy along with parallel investigation in mouse models. ■

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