Chapter 7
Combining Radiotherapy and Immunotherapy: Emerging Preclinical Observations of Lymphocyte Costimulatory and Inhibitory Receptor Modulation

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Abstract A greater understanding of immune system biology has translated into more effective cancer immunotherapeutics. This has prompted exploration of the combination of these agents with other cancer treatments such as radiotherapy, which has also been shown to promote antitumor immunity independently. This review will present data from reports of immune modulators and radiotherapy and will discuss common themes and observations. Costimulatory molecules including CD40 and CD134/OX40; glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), CD137/4-1BB; and inhibitory molecules CD152/cytotoxic T lymphocyte-associated protein 4 (CTLA4), lymphocyte activation gene 3 (LAG3), programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1), and T cell immunoglobulin and mucin domain 3 (TIM-3) will be discussed. Observations regarding radiotherapy sequencing, dose, and fractionation will also be addressed. We conclude that a strategy combining immune modulation and radiotherapy is rational and holds promise for future successful translation in clinical trials.

Keywords Radiotherapy • Radiation • Immunotherapy • Immune checkpoint • CTLA4 • PD-1 • PD-L1 • Abscopal effect • Checkpoint blockade

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Introduction

A greater understanding of immune system biology has translated into more effective cancer immunotherapeutics. This has prompted exploration of the combination of these agents with other cancer treatments such as radiotherapy, which has also been shown to promote antitumor immunity independently. This review will present data from reports of immune modulators and radiotherapy and will discuss common themes and observations. Costimulatory molecules including CD40 and CD134/OX40; glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), CD137/4-1BB; and inhibitory molecules CD152/cytotoxic T lymphocyte-associated protein 4 (CTLA4), lymphocyte activation gene 3 (LAG3), programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1), and T cell immunoglobulin and mucin domain 3 (TIM-3) will be discussed. Observations regarding radiotherapy sequencing, dose, and fractionation will also be addressed.

Cancer Immunity and Radiation Response

In order to generate a robust and sustained immune response to a pathogen or cancer, several key elements are required. These include the presence of an immunogenic antigen at sufficient quantities to be picked up, processed, and presented by antigen-presenting cells (APCs) such as dendritic cells (DCs) for T cell recognition. Antigen presentation by APCs in the context of MHC molecules and subsequent recognition by the TCR complex on a T cells are a critical first step for mounting an immune response. In order for the APC-T cell interaction to result in activation of the T cell and subsequent immune response, a second costimulatory signal is required either directly from the APC or from the surrounding microenvironment to promote T cell maturation. The immune response can also be modulated by the presence of inhibitory molecules on the surface of the dendritic cell, T cell, or target cancer cell. In addition, the microenvironment can dramatically affect the degree and type of immune response via circulating cytokines and chemokines as well as direct cell-cell interactions. Suppressor cells such as regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) can promote an anti-inflammatory milieu and thus curtail the antitumor immune response. All these factors contribute to the activation, efficacy, and duration of an antigen-specific immune response, and cancers have thus developed mechanisms to modulate these pathways in order to subvert anticancer immunity.

The interplay of radiotherapy and the local and systemic immune response has been demonstrated in numerous preclinical studies. The efficacy of RT is severely reduced in the absence of an immune response in nude mice, which are deficient in B and T cells, and is significantly dependent on local CD8 T cell infiltration [1]. The absence of an innate immune response also results in reduced efficacy of RT [2]. Radiation can promote tumor antigen availability and presentation via immunogenic

cell death via cell apoptosis and modification of the microenvironment with upregulation of damage-associated molecular patterns (DAMPs) including calreticulin, secreted ATP, and HMGB1 [3–5]. Tumor irradiation also results in upregulation of major histocompatibility complex class I (MHC-1) expression [6] and chemokine and cytokine secretion promoting an inflammatory infiltrate within the tumor as well as draining lymph node [7, 8]. Of note, tumor irradiation has been shown to induce some immunosuppressive properties such as increased proportion of Treg cells and promotion of inhibitory factors such as TGF-beta and PD-L1 expression which can be overcome with some of the immunotherapeutics discussed [9].

Cancer cells have thus been shown to evade recognition and elimination by the immune system via a variety of mechanisms including antigen variation or editing, downregulation of MHC, immunosuppressive cytokines, recruitment of regulatory cells, and overexpression of inhibitory ligands. Irradiation of the tumor can result in reversal or neutralization of many of these mechanisms supporting its potential synergy in attempts to promote tumor immunity via systemic immunotherapy.

Modulation of Lymphocyte Costimulatory or Inhibitory Receptors and Experimental Methods

A variety of cell surface receptors are present on lymphocytes and are critical to function of the immune system [10]. These are generally grouped into costimulatory or inhibitory receptors, with corresponding ligands (see Table 7.1). Recently, therapeutic strategies have evolved to antagonize inhibitory molecules or agonize costimulatory molecules with monoclonal antibodies independently or in combination. Some of these lymphocyte receptor modulators are used in clinical practice, while others are still undergoing preclinical development. Importantly, the therapeutic target of these agents is the lymphocyte signaling process, not the cancer cell itself. In addition, modulating some of these targets can also lead to activation of the innate immune system.

Several investigations combining radiotherapy and lymphocyte receptor modulators in preclinical models have been reported. Many of these studies use similar immunologic experimental methods. For readers unfamiliar with these methods, they are explained briefly here. The reader is also referred to several recent reviews on the immunologic effects of radiation therapy for further understanding of the effect of radiation on the immune system, in the absence of lymphocyte receptor modulators [11, 12].

As the target for experimental manipulation is the immune system, most studies are performed in vivo, rather than in vitro. For this reason, the models must use immunocompetent syngeneic species-specific (often murine) tumor grafts, rather than xenografts from human tumors in immunocompromised hosts. Investigators have studied tumor grafts placed subcutaneously or intradermally (on the flank or hind limb) and orthotopically (in the organ of tissue origin, such as the breast, brain,

Table 7.1 Costimulatory and inhibitor lymphocyte receptors and ligands. Representative costimulatory and inhibitory lymphocyte receptors and ligands studied in combination with radiotherapy are presented, with example agonistic and antagonistic therapies listed

	Cell surface receptor	Cell surface receptor ligand	Therapeutic agonist/ antagonist examples
Costimulatory	CD40	CD40L	CP-870,893 (Pfizer), dacetuzumab (Seattle Genetics)
	CD134/OX40	CD252/OX40L	MEDI0562, MEDI6469, MEDI6383 (AstraZeneca)
	GITR	GITRL	TRX518 (GITR Incorporated)
	CD137/4-1BB	CD137L	PF-05082566 (Pfizer), lipocalin (Pieris Pharmaceuticals), urelumab (Bristol-Myers Squibb)
Inhibitory	CD152/CTLA4	CD80, CD86	Ipilimumab (Bristol-Myers Squibb), tremelimumab (AstraZeneca)
	LAG3	MHC II	BMS-986016 (Bristol-Myers Squibb), IMP321 (Immuntep)
	PD-1	PD-L1, PD-L2	Nivolumab (Bristol-Myers Squibb), pembrolizumab (Merck), pidilizumab (Cure Tech), AMP-224, AMP-514 (Amplimmune)
	TIM-3	Galectin-9, HMGB1, PS, CEACAM-1	Anti-TIM-3 (Tesaro)

GITR glucocorticoid-induced TNFR family-related gene, *CTLA4* cytotoxic T lymphocyte-associated protein 4, *LAG3* lymphocyte activation gene 3, *PD-1* programmed death 1, *PD-L1* programmed death ligand 1, *PD-L2* programmed death ligand 2, *TIM-3* T cell immunoglobulin and mucin domain 3, *HMGB1* high-mobility group box 1, *PS* phosphatidylserine, *CECAM-1* carcinoembryonic antigen-related cell adhesion molecule 1

or skin). Tumor size, tumor growth delay, tumor response, metastasis, and overall survival are often the simplest measures of treatment effect. To demonstrate immune-mediated response to cancer distant from the radiotherapy target, some models incorporate two tumors, where one is irradiated and the other is not irradiated. This allows for demonstration of an abscopal effect (effect of radiation away from the target of radiotherapy) [13].

Immunologic response to tumor, radiotherapy, and lymphocyte receptor modulation can also be characterized at the treated tumor or in the peripheral lymphoid organs. Often immune cell populations (lymphocytes, myeloid cells, macrophages) are characterized based on cell surface markers (of differentiation, activation, exhaustion, etc.) in different anatomic compartments (infiltrating the tumor, draining the lymph node basin, spleen, etc.). The dependency of the treatment effect on specific immune cell populations can be interrogated by performing experiments in animal models deficient for immune function (through genetic knockout) or through

depletion of immune cell populations by neutralizing monoclonal antibodies against cell surface markers (CD4, CD8, etc.) and ligands (PD-L1, TIM-3, etc.). Determining whether immune cells recognize tumor-specific antigens can be carried out using ex vivo assays to determine if lymphocytes can kill tumor cells or if they elaborate cytokines such as interferon gamma in response to tumor antigens. Finally, immunologic memory can be tested after complete tumor regression by rechallenging the host with the tumor graft and assessing for the presence or absence of tumor growth. Similarly, immune cells from hosts with complete tumor regression can be adoptively transferred to naïve, tumor-bearing animals to assess for antitumor properties of the transferred immune cells.

Combinations of Costimulatory Receptor Modulation and Radiotherapy

CD137/4-1BB

CD137 or 4-1BB is a member of the tumor necrosis factor receptor (TNFR) superfamily and is expressed on T cells and other immune subsets following activation. Ligation of the receptor via its ligand or agonist antibodies results in enhanced T cell proliferation and production of cytokines. CD137 activation has also been shown to provide a strong survival signal for CD8 T cells via upregulation of anti-apoptotic pathways [14]. In 2006, investigators first reported on the combination of 4-1BB agonism (with a monoclonal antibody, BMS-469492) and radiotherapy (5–15 Gy/1 fraction or 40 Gy/10 fractions) in a preclinical breast (EMT6) and lung (M109) cancer model. The authors found that 4-1BB agonism could effectively delay the growth of tumors in the breast cancer model, but not the lung cancer model. In the breast cancer model, when treated with the combination of single-dose or fractionated radiotherapy followed by 4-1BB agonism, investigators observed a delay in tumor growth significantly longer than either therapy when given alone. In the lung cancer model, only the highest single dose of radiotherapy (15 Gy), but not fractionated treatment, yielded a significant delay in tumor growth compared to either therapy given alone. The lung cancer cell line was found to have high basal expression of 4-1BBL which could not be increased by irradiation, while the breast cancer cell line had low basal expression of 4-1BBL, which could be increased by irradiation [15]. This suggests that the expression of 4-1BB ligand may be a good biomarker for combining RT with 4-1BB agonism.

In a preclinical orthotopic model of glioma using the GL261 cell line, investigators observed that the 4-1BB agonist antibody (BMS-469492) in combination with whole-head radiotherapy (8 Gy/2 fractions) yielded significantly longer survival rates than either treatment alone. Of the long-term survivors treated with radiotherapy alone ($n=2$) or in combination with BMS-469492 ($n=6$), 50 % and 83 % demonstrated no evidence of tumor regrowth after tumor rechallenge, respectively.

All had pathologic complete response in the brain. When examining the tumor-infiltrating lymphocytes, significantly higher numbers of CD8 and CD4 lymphocytes were noted in the group treated with radiotherapy alone compared to the untreated control group, and even higher numbers were observed in those treated with 4-1BB agonism and radiotherapy. Finally, the production of interferon gamma, indicative of T cell effector function, in a tumor-specific manner by splenocytes was greatest in the group treated with 4-1BB agonism and radiotherapy [16].

CD134/OX40

CD134 or OX40 is another member of the TNFR superfamily expressed on activated CD4 and CD8 T cells as well as neutrophils, DCs, and Treg cells. The natural ligand (OX40L) is found on APCs as well as activated T cells. Engagement of OX40 promotes T cell activation, maturation, survival, and cytokine production [17]. Investigators have also observed that single-dose radiotherapy (20 Gy) followed by OX40 agonism (with a monoclonal antibody clone OX86) increased the rate of cure in a preclinical lung (Lewis lung carcinoma) model, compared to either treatment alone. This effect was found to be dependent on CD8 lymphocytes, but not CD4 lymphocytes or natural killer cells. The combination of OX40 agonism and radiotherapy significantly increased the proportion of CD8 lymphocytes in the draining lymph node compared to either treatment alone. The CD8 lymphocytes had the ability to kill the lung cancer cell line in an antigen-specific manner. Finally, the combination of OX40 agonism and radiotherapy was found to yield immunologic memory and tumor rejection after rechallenge, compared to animals not previously treated [18].

Other investigators found that in a preclinical model of lung cancer (Lewis lung carcinoma, LLC), radiotherapy (60 Gy/3 fractions) followed by OX40 agonism (starting one day after the first fraction of radiotherapy) yielded significantly longer survival compared to either treatment alone. Tumor rechallenge after combination radiotherapy and OX40 agonism demonstrated immunologic memory [19].

GITR

Glucocorticoid-induced TNFR family-related (GITR) gene is expressed on CD4 and CD8 T cells and is upregulated after activation. Similar to other TNFR family members, ligation with its natural ligand (GITRL) expressed on activated APCs and endothelial cells (EC) results in enhanced T cell proliferation, survival, and effector function [20]. Investigators explored the effect of radiotherapy (30 Gy/1 fraction) with or without GITR agonism using a monoclonal antibody (DTA-1) in a lung carcinoma (Lewis lung carcinoma, LLC) model. The authors observed that irradiation of LLC significantly delayed tumor growth and increased survival, compared

to no irradiation. Depletion of CD8 lymphocytes significantly decreased the tumor growth delay and survival suggesting that the effect is CD8 dependent. When GITR agonism and radiotherapy were combined, there was a nonsignificant tumor growth delay greater than either treatment alone, but no association with longer survival [21].

CD40

CD40 is another member of the TNFR superfamily constitutively expressed on APCs, and its ligation results in promotion of functional maturation with enhanced antigen presentation and cytokine production resulting in increased activation of T cells [22]. In 2003, investigators reported on studies of two syngeneic models of B cell lymphoma (A31 and BCL1) treated with total body irradiation (TBI, 2–8 Gy/1 fraction) for systemic lymphoma and/or costimulation by CD40 agonist monoclonal antibody 4 h after irradiation. The investigators observed a significant increase in survival with the combination of TBI and CD40 agonism, compared to either treatment alone. However, the effect was dependent on the dose of TBI used; 5 Gy yielded the highest proportion of survivors, with higher or lower doses of radiation proving inferior. The authors found that a wide range of doses of the CD40 agonist were effective at promoting survival, but that other B cell-depleting antibodies (against CD19, MHC II, CD22) did not yield the same effect as the CD40 agonist suggesting that the CD40 antibody is not acting by simply depleting B cells. In vitro analyses of apoptosis and clonogenic survival suggest that CD40 agonism did not increase the cellular radiosensitivity of the lymphoma cell lines. Interestingly, in an experiment where variable numbers of lymphoma cells were inoculated, the authors observed that a minimum amount of lymphoma cells must be treated to yield long-term immunity, again suggesting that CD40 is not a general sensitizer to radiation. By tracking the number of lymphoma cells present after combination treatment, investigators found that TBI alone yielded a dose-dependent decrease in the number of lymphoma cells, which regrew in the absence of CD40 agonism. The combination of TBI and CD40 agonism led to a two-phase (early and late) pattern of lymphoma regression. Importantly, a significant increase in CD8 cells was noted in animals treated with 5 Gy of TBI and CD40 agonism compared to those given 5 Gy of TBI alone. However, this was not observed with higher (8 Gy) or lower (2 Gy) doses of radiation, or in animals not bearing lymphoma, or with the use of other monoclonal antibodies. The authors observed that CD8 cells in the group receiving CD40 agonism and TBI had a significantly greater lymphoma-specific cytotoxic activity. In addition, CD8, but not CD4, lymphocyte depletion abrogated the therapeutic effect of TBI and CD40 agonism. In long-term survivors of the CD40 agonism and TBI treatment, rechallenge with lymphoma cells demonstrated immunologic memory in 80 % of the treated animals. Finally, adoptive transfer of T cells from survivors of the CD40 agonism and TBI combination to untreated lymphoma-bearing animals significantly increased the duration of survival in the recipient mice [23].

Combinations of Multiple Costimulatory Receptor Modulators and Radiotherapy

In 2012, investigators explored the combination of targeting multiple costimulation modulators in combination with radiotherapy in preclinical models. Using two triple-negative (estrogen/progesterone/Her-2/neu receptor negative) breast cancer cell (4T1.2 and AT-3) models, the authors explored the effect of 4-1BB and CD40 agonism alone, in combination, or immediately after radiotherapy (12 Gy/1 fraction). It was observed that 4-1BB agonism alone, or in combination with CD40 agonism, significantly delayed tumor growth compared to control. Notably, CD40 agonism alone did not significantly delay tumor growth. Likewise, when given radiotherapy, 4-1BB agonism alone, or in combination with CD40 agonism, significantly delayed tumor growth. This effect was not observed with CD40 agonism after radiotherapy. In the 4T1.2 cell line, tumor cure was observed with radiotherapy or in combination with CD40 and 4-1BB agonism; tumor cure occurred most often in the group receiving the combination of CD40 and 4-1BB agonism and radiotherapy. The antitumor effect was noted to be dependent on CD4, CD8, and natural killer cells. Moreover, rechallenge of the host with a tumor demonstrated immunologic memory. The authors hypothesized that the differences in response to the combination of immunotherapy and radiotherapy in the two cell lines were associated with 4T1.2 tumors supporting a necrotic core and undergoing an immunogenic, non-apoptotic death after radiotherapy, while AT-3 cells expressed PD-L1, possibly conferring resistance to the combination of costimulation and radiotherapy. The authors conducted further experiments to explore ways to overcome resistance (described further below) [24].

Combinations of Inhibitory Receptor Modulation and Radiotherapy

CTLA4

APCs present antigen in the context of MHC to a specific T cell receptor on the surface of T cells. However, for resulting T cell activation, costimulation is required by a variety of other cell surface receptors including CD28 on the T cell interacting with CD80/B7.1 and CD86 B7.2 on APCs. CTLA4 is a member of the CD28 family of receptors and is upregulated on activated T cells. CTLA4 has a higher affinity for CD80/CD86 than the costimulatory receptor CD28 and can therefore competitively bind ligand more avidly than CD28. Through this mechanism, it acts as a negative feedback loop for T cell activation after TCR stimulation. CTLA4 is also expressed constitutively at high levels on Treg cells and is important for their suppressive functions. The administration of anti-CTLA4 antibodies results in blockade of inhibitory signals as well as direct depletion of Treg cells resulting in immune activation [25].

In 2005, investigators reported on the effects of radiotherapy (12 Gy/1 fraction or 24 Gy/2 fractions 48 h apart) alone or followed by CTLA4 blockade with the antibody 9H10 in a breast cancer (4T1) model. The growth of implanted 4T1 tumors was significantly delayed only in animals treated with radiotherapy, with or without 9H10, compared to untreated controls. Treatment with 9H10 alone did not delay tumor growth. Moreover, radiotherapy or CTLA4 blockade did not significantly increase survival compared to the group that did not receive treatment. However, the combination of CTLA4 blockade and RT did significantly increase survival compared to the untreated control group. Compared to untreated controls, a significantly lower number of lung metastases were observed only with the combination of CTLA4 blockade and radiotherapy, but not either treatment alone. This effect was abrogated with CD8 lymphocyte depletion, but not CD4 lymphocyte depletion. The authors further demonstrated that a higher total dose of radiation (24 Gy/2 fractions) yielded a 57 % rate of complete regression of the primary tumor, which was not observed for the lower dose of radiation (12 Gy/1 fraction). Despite improvement in primary tumor control with a higher dose of radiation, similar to prior experiments, the combination of CTLA4 blockade with 9H10 and radiotherapy yielded significantly longer survival than either treatment alone or no treatment at all. In the group with long-term survival, tumor rechallenge demonstrated protective immunity with 4T1-specific cytolytic activity in the spleen [26].

A subsequent study from the same group investigated the effect of single-dose (20 Gy/1) or fractionated radiotherapy (30 Gy/5 fractions or 24 Gy/3 fractions) with or without concurrent or subsequent CTLA4 blockade with a monoclonal antibody (9H10) in breast cancer (TSA) or colon cancer (MCA38) models. Using a two-tumor model where tumors were implanted on each flank of the mice but only one tumor was irradiated (as illustrated in Fig. 7.1), the authors observed that 9H10

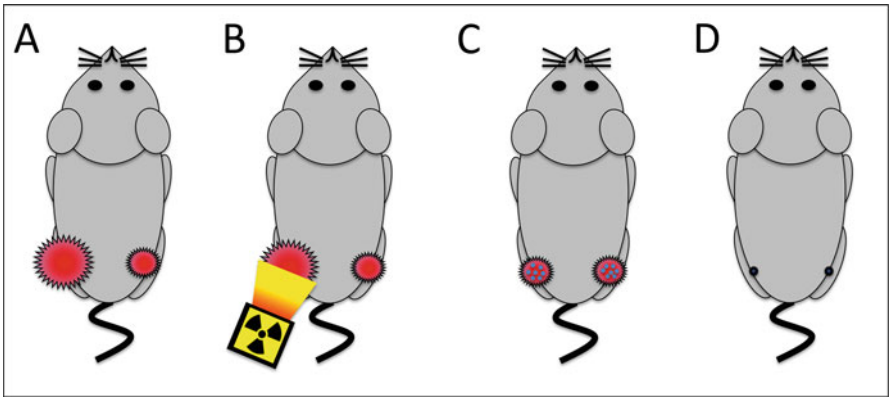

Fig. 7.1 Two-tumor model for the assessment of the abscopal effect. In this model, bilateral tumor grafts are placed, typically with one tumor being smaller than the other (**a**). Radiotherapy is administered to the larger of the two tumors (**b**), and the immune response in the tumors can be assessed after treatment (**c**). The unirradiated tumor is observed for abscopal response, or response away from the target of radiotherapy (**d**). This effect is thought to be immune mediated

alone had no effect on tumor growth compared to untreated controls. Radiotherapy alone caused tumor growth delay of the irradiated tumor of a similar magnitude across the three-dose schedules, but no growth delay in the unirradiated distant tumor. The combination of CTLA4 blockade and fractionated radiotherapy (but not single-dose radiotherapy) was associated with regression of both irradiated and unirradiated tumors demonstrating an abscopal effect. The effect was greatest in the 24 Gy/3 fraction regimen. The investigators further explored the effect of delaying the start of immunotherapy after initiating radiotherapy. They found that the longest delay between immunotherapy and radiotherapy was associated with the most rapid rate of tumor regression. Examination of the unirradiated tumors in the group receiving 9H10 and radiotherapy (24 Gy/3 fractions) demonstrated a significantly greater number of tumor-infiltrating CD4 and CD8 lymphocytes, compared to either treatment alone. Finally, the ex vivo tumor-specific production of interferon gamma by splenocytes was greatest in animals exhibiting rejection of the unirradiated tumor [27].

Another group of investigators examined the effects of combining radiotherapy (2–30 Gy/1 fraction) with or without CTLA4 blockade using a monoclonal antibody (9H10) in a lung carcinoma (Lewis lung carcinoma, LLC) model. The authors assessed secretion of HMGB1, a protein released after immunogenic cell death, after LLC cell irradiation in vitro. They noted no difference in HMGB1 levels between cells irradiated with 2 Gy and those not irradiated. However, cells irradiated with 6 or 30 Gy released threefold more HMGB1 than those not irradiated. The authors then carried out in vivo experiments, observing that radiotherapy (30 Gy/1 fraction) significantly delayed tumor growth and increased overall survival, compared to no radiotherapy. CD8 lymphocyte depletion significantly decreased the tumor growth delay and survival. CTLA4 blockade and radiotherapy significantly increased tumor growth delay and overall survival, compared to radiation or CTLA4 blockade alone [21].

Subsequently, other investigators studied radiotherapy (5–15 Gy/1–3 fractions) in combination with CTLA4 blockade with a monoclonal antibody in a mesothelioma model (AB12) in immunocompetent or immunodeficient (nonobese diabetic/severe combined immunodeficient, NOD/SCID) hosts. Using a two-tumor model, the authors found that irradiating one tumor leads to significant delay in growth of the irradiated tumor, as well as the unirradiated tumor. However, immunodeficient (NOD/SCID) hosts did not demonstrate delay in growth of the unirradiated tumor suggesting that the adaptive immune system is important in controlling the growth of the unirradiated tumors. The combination of CTLA4 blockade and radiotherapy delayed irradiated and unirradiated tumor growth significantly longer than either treatment alone. These same observations were made whether the irradiated tumor and unirradiated tumor were implanted synchronously or metachronously. Assessment of immune infiltrates in the tumor, draining lymph nodes, and spleen 10 days after treatment revealed significantly higher levels of activated (ICOS+) and proliferating (Ki67+) CD4 and CD8 lymphocytes, as well as dendritic cells in the draining lymph nodes (but not the spleen) of hosts with irradiated tumors, compared to control tumors. Significantly more CD8 T cells were noted in irradiated tumors in

the group treated with CTLA4 blockade and radiotherapy, compared to those treated with either treatment alone. In the group treated with CTLA4 blockade and radiotherapy, a significant increase in the number of activated (but not total) CD4 and CD8 T cells in the spleen was observed compared to the group treated with radiotherapy alone. Finally, compared with irradiated or unirradiated tumors treated with radiotherapy alone, those treated with CTLA4 blockade and radiotherapy demonstrated an increase in the expression of pro-inflammatory markers including interferon gamma, perforin, IP-10, TNF alpha, granzyme B, ICOS, IL-4, IL-12, IL-12p70, IL-5, IL-6, IL-17A, and MCP-1 [28].

In a colon carcinoma (CT26) model, investigators studied the combination of radiotherapy (10 Gy/1 fraction) with intratumoral injection of immature dendritic cells (iDCs) with or without CTLA4 blockade (9H10). Using a two-tumor model, they observed a significantly longer delay in tumor growth, overall survival, and greater tumor-specific cytolytic T cell activity with the combination of radiotherapy and iDC injection with CTLA4 blockade, compared to either treatment alone [29].

PD-1/PD-L1

Programmed cell death protein-1 (PD-1) is a coinhibitory member of the CD28 superfamily expressed on activated T cells in a more delayed fashion than CTLA4 and thought to be involved in more chronic inflammation to induce T cell exhaustion or anergy. PD-1 binds to B7-family ligands PD-L1 and PD-L2 on APCs on other nonimmune cells which induce inhibitory signals within the T cells [30]. Tumor cells have been shown to dramatically upregulate PD-L1 to dampen the antitumor immune response [31]. Using two breast carcinoma (4T1 and AT3) models, investigators studied the effect of radiotherapy (12 Gy/1 fraction) followed by PD-1 blockade with a monoclonal antibody (RMP1-14). The investigators found that PD-L1 was not expressed on AT3 cells in vitro, but was present ex vivo whether taken from a subcutaneous or orthotopically grown tumor. Radiotherapy did not affect the expression of PD-L1 on explanted tumor cells. However, 12 h after radiotherapy, there was an enrichment of tumor-infiltrating CD8 cells that expressed high levels of PD-1 (PD-1^{High}) through the reduction of CD8 cells expressing low levels of PD-1 (PD-1^{Low}), with a resultant increase in the ratio of PD-1^{High} to PD-1^{Low} CD8 cells in treated AT3 tumors. In addition, 36 h after radiotherapy, tumor-infiltrating CD8 cells were noted to be actively proliferating and productive of interferon gamma, indicating preservation of functionality. Additional experiments confirmed these CD8 cells were tumor antigen specific. Finally, the combination of PD-1 blockade and radiotherapy in vivo did not delay tumor growth more than radiotherapy or PD-1 alone in the subcutaneous AT3 model. However, in the subcutaneous orthotopic AT3 model, the combination of radiotherapy and PD-1 blockade delayed tumor growth significantly longer than either treatment alone, with a long-term cure rate of 17 % [24].

Using an orthotopic glioblastoma model (GL261), other investigators tested stereotactic radiosurgery (10 Gy/1 fraction) with or without immediate PD-1 blockade with a monoclonal antibody (G4). In vitro, the investigators found that GL261 expressed PD-L1, a potential biomarker of the efficacy of PD-1 blockade. In addition, radiotherapy increased the surface expression of MHC I, ICAM1, and CXCL16 in vitro. In vivo, the investigators found that the combination of PD-1 blockade and radiotherapy yielded significantly longer overall survival than either treatment alone, or no treatment. Depletion of CD8 (more than CD4) was associated with abrogation of the survival benefit. In long-term survivors, tumor rechallenge demonstrated long-term immunity. On studying the brain immune infiltrates of mice treated with radiation or PD-1 blockade, investigators found that the combination significantly increased the number of CD8 cells, while radiation (with or without PD-1 blockade) seemed to decrease regulatory T cells. The net result was a significant increase in the ratio of CD8 to regulatory T cells in the group treated with PD-1 blockade and radiotherapy [32].

Other investigators subsequently reported on single-dose or fractionated radiotherapy (at various doses) and PD-1 blockade with an antibody in models of colorectal cancer (MC38-OVA), breast cancer (4T1-HA), and melanoma (B16-OVA). In vitro, they observed that radiotherapy (10–20 Gy/1 fraction) resulted in a dose-dependent increase in antigen presentation. In vivo, B16-OVA tumor growth delay was significantly longer with PD-1 blockade and radiotherapy, compared to either treatment alone. The investigators observe a significantly greater proportion of antigen-specific immune infiltrates in the spleen and draining lymph nodes after treatment with PD-1 blockade and radiotherapy, compared to either treatment alone. Adoptively transferred splenocytes from hosts treated with PD-1 blockade and radiotherapy significantly delayed tumor growth longer than splenocytes from untreated hosts, or hosts treated with PD-1 blockade alone. Greater numbers of tumor-infiltrating lymphocytes were noted after PD-1 blockade and radiotherapy, compared to radiotherapy alone. Greater numbers of CD4 and CD8 cells were noted to infiltrate irradiated tumors, compared to those not treated with radiotherapy. There was an increase in regulatory T cells in irradiated tumors (but not draining lymph nodes or spleens) not treated with PD-1 blockade. The combination of PD-1 blockade and radiotherapy yielded a significantly greater increase the CD8 to regulatory T cell ratio, compared to either treatment alone. The combination of PD-1 blockade and radiotherapy increased the frequency of effector memory T cells in the tumors to a greater extent than either treatment alone. Similar findings were observed in the 4T1-HA model [33].

Other investigators explored the effect of stereotactic ablative radiotherapy (15 Gy/1 fraction) and PD-1 blockade (G4) in breast cancer (4T1), renal cancer (RENCA), or melanoma (B16) in PD-1 wild-type or knockout mouse models. Using a two-tumor model, the authors observed that both irradiated and unirradiated tumors grew significantly slower in the PD-1 knockout model. Survival was also significantly longer in the PD-1 knockout model, compared to the wild-type model. In the wild-type model, the combination of PD-1 blockade and radiotherapy was associated with a significantly longer delay in irradiated and unirradiated tumor

growth, compared to either treatment alone. The combination treatment was also associated with significantly longer survival than either treatment alone. The investigators went on to demonstrate that antitumor immune effect is antigen specific for the irradiated tumor. In a three tumor model, with two of the unirradiated tumors being of different origin (one 4T1, one RENCA), only the irradiated RENCA tumor and the unirradiated RENCA tumor responded to the combination of PD-1 blockade and radiotherapy; the 4T1 did not respond to treatment. In the two-tumor model, significantly more PD-1 expressing tumor-infiltrating reactive CD8 cells ($CD11a^{High}$) were present in irradiated or unirradiated tumors, compared to untreated controls. This population of cells was found to be tumor antigen specific and responsive. Moreover, PD-L1 expression on leukocytes (but not tumor cells) in the irradiated and unirradiated tumors significantly increased after irradiation. The expression of LAG3 and TIM-3 on tumor-infiltrating CD8 cells was not affected by irradiation. Finally, CD4, CD8, and CD11a depletion in vivo demonstrated dependence of unirradiated tumor regression on CD8 cells [34].

Investigators studied the combination of radiotherapy (12–20 Gy/1 fraction) and PD-L1 blockade (10F.9G2) in breast (TUBO) and colorectal cancer (MC38) models. The authors found that radiotherapy (12 Gy/1 fraction) increased PD-L1 expression on tumor cells and dendritic cells, but not on myeloid-derived suppressor cells or macrophages. PD-1 expression was slightly downregulated on CD8 (but not CD4) cells after radiotherapy. In vivo, the combination of PD-L1 blockade and radiotherapy delayed irradiated MC38 and TUBO tumor growth significantly longer than either treatment alone. In a two-tumor model, this combination also delayed the growth of an unirradiated TUBO tumor longer than either treatment alone. In the group with complete tumor regression after the combination of PD-L1 blockade and radiotherapy, tumor rechallenge experiments demonstrated long-lasting immunity. Depletion of CD8 cells was noted to abrogate the therapeutic effect of PD-L1 blockade and radiotherapy. The combination of PD-L1 blockade and radiotherapy was associated with tumor-specific T cell functionality that was greater than either treatment alone. On the investigation of the immune cells infiltrating the tumor and present in the spleen, the authors observed a significantly greater reduction in myeloid-derived suppressor cells (MDSCs) 10 days (but not 3 days) after PD-L1 blockade and radiotherapy, and this reduction was greater than those observed from either treatment alone. They noted that depletion of MDSCs could significantly delay tumor growth in animals treated with radiotherapy alone. Finally, the authors observed CD8 cells were in part responsible for the reduction in MDSCs, through the cytokine tumor necrosis factor alpha (TNFa). TNFa blockade abrogated the suppression of tumor growth in the combination of PD-L1 and radiotherapy [35].

Other investigators studied fractionated radiotherapy (10 Gy/5 fractions) and PD-1 or PD-L1 blockade with monoclonal antibodies in melanoma (4434), breast cancer (4T1), and colorectal cancer (CT26) models. The authors observed that PD-L1 expression increased on CT26 tumor cells, increased 1 day after in vivo tumor radiotherapy (10 Gy/5 fractions), reached a peak 3 days after radiotherapy, and declined significantly 7 days after radiotherapy. Subsequent experiments revealed that irradiation (2–10 Gy/1 fraction) of tumor cells in vitro had little effect

on PD-L1 expression. In vivo, depletion of CD8 cells abrogated the increase in PD-L1 expression caused by radiotherapy. Depletion of natural killer cells had no effect on PD-L1 expression, while depletion of CD4 cells increased the expression of PD-L1 on tumor cells. In the absence of radiation, interferon gamma alone and in combination with tumor necrosis factor alpha (but not tumor necrosis factor alpha alone) increased PD-L1 expression on tumor cells in vitro. In addition, depletion of interferon gamma suppressed the overexpression of PD-L1 on irradiated cells. In vivo, local tumor control and overall survival were significantly greater in the group treated with the combination of PD-L1 or PD-1 blockade and radiotherapy, compared to either treatment alone. Combined PD-1 and PD-L1 blockade and radiotherapy were not associated with an improvement in the outcome of radiotherapy and PD-1 or PD-L1 blockade. In vitro, the authors found that the PD-1 and PD-L1 blocking monoclonal antibodies did not increase tumor cell radiosensitivity. Depletion of CD8 and natural killer cells abrogated the tumor growth delay provided by PD-L1 blockade and radiotherapy. Depletion of CD4 cells significantly increased tumor growth delay after PD-L1 blockade and radiotherapy, which the authors speculated may have been due to the presence of fewer regulatory T cells. Among the group with complete tumor regression after PD-L1 blockade and radiotherapy, tumor rechallenge demonstrated long-term antigen-specific immunity. In addition, the authors found that the scheduling of PD-L1 blockade and radiotherapy was important. The authors observed significantly longer survival in the groups initiating PD-L1 blockade on the first or last day of fractionated radiotherapy, compared to 7 days after the end of fractionated radiotherapy. Consistent with this time-dependent effect, the authors observed significantly higher PD-1 expression levels on CD4 and CD8 cells infiltrating the tumor 1 day after radiotherapy (compared to untreated controls), but not 7 days after radiotherapy [36].

Combinations of Multiple Inhibitory Receptor Modulators and Radiotherapy

Using a melanoma model (B16), investigators studied the effect of stereotactic ablative radiotherapy (15 Gy/1 fraction) and CTLA4 (9H10) and PD-1 (G4) blockade. Using a two-tumor model, they observed the greatest delay in tumor growth with the combination of CTLA4 blockade, PD-1 blockade, and radiotherapy, compared to CTLA4 or PD-1 blockade and radiotherapy. This effect was noted at the irradiated and unirradiated tumor, with the latter observation being statistically significant [34].

Using models of melanoma (B16-F10), breast cancer (TSA), and pancreas cancer (PDA.4662), investigators explored the effect of CTLA4 (9H10), PD-1 (RMP1-14), and PD-L1 (10F.9G2) blockade and radiotherapy (20 Gy/1 fraction or 24 Gy/3 fractions). Using a two-tumor B16-F10 model, investigators found that the combination of CTLA4 blockade and radiotherapy was associated with the greatest delay in distant unirradiated tumor growth, compared to either treatment alone. Depletion of CD8 cells abrogated this effect. Among the 17% treated with the combination

and achieving a complete tumor response, tumor rechallenge demonstrated persistent immunity. Analysis of tumor-infiltrating lymphocytes demonstrated that resistance to therapy was associated with low numbers of infiltrating CD8 cells, and a low CD8/regulatory T cell ratio, and a higher number of "exhausted" CD8 cells. They also observed that upregulation of PD-L1 and interferon-stimulated genes in tumor cells was associated with resistance to combination treatment. The authors found that adding PD-L1 blockade to the combination of CTLA4 blockade and radiotherapy increased the response rate to 58 % and was associated with reinvigoration of the "exhausted" CD8 population infiltrating the tumor and in the periphery. They went on to characterize the effect of radiotherapy in the context of dual checkpoint blockade and found that radiotherapy was associated with diversification of the T cell receptor repertoire, while CTLA4 and PD-1 lowered the percent of Tregs and reversed T cell exhaustion in the tumor, respectively. Finally, the authors developed and tested the accuracy of a model to predict response to the combination of immunotherapy and radiotherapy which incorporated the proportion of "exhausted" CD8 cells, reinvigorated CD8 cells, and the ratio of CD8/regulatory T cells [37].

Combinations of Costimulatory and Inhibitory Receptor Modulators and Radiotherapy

Using a triple-negative (estrogen/progesterone/Her-2/neu receptor negative) breast cancer cell (AT-3) model, investigators explored the combination of PD-1 antagonism with a monoclonal antibody, with 4-1BB agonism alone immediately after radiotherapy (12 Gy/1 fraction or 16–20 Gy/4 fractions). The authors found that the combination of radiotherapy, 4-1BB agonism, and PD-1 antagonism was associated with a higher rate of response than the combination of radiotherapy and 4-1BB agonism or radiotherapy and PD-1 antagonism. In an orthotopic and subcutaneous model, this combination led to a 100 % and 40 % cure rate among the group treated with the triple combination of 4-1BB agonism, PD-1 blockade, and radiotherapy, respectively. A similar effect was noted when combining fractionated radiotherapy, PD-1 blockade, and 4-1BB agonism, with approximately 80 % achieving cure [24].

Investigators using a glioblastoma (GL261-luc) model examined the effect of focal radiotherapy (10 Gy/1 fraction) followed by CTLA4 (4F10) blockade and 4-1BB (2A) agonism. The combination of radiotherapy and CTLA4 blockade or radiotherapy and 4-1BB agonism was associated with longer survival than no treatment in their model; the CTLA4 combination with radiotherapy (but not the 4-1BB combination) was associated with significantly longer survival than radiotherapy alone. They found that delivering radiotherapy 2 days before, the day of, or 2 days after the first dose of CTLA4-blocking antibody was associated with similar survival benefits, compared to no treatment, although a lower proportion of long-term survivors and shortest median duration of survival was noted in the group treated with radiotherapy, followed 2 days later by CTLA4 blockade. The authors then went on to observe that the group treated with CTLA4 blockade, 4-1BB

agonism, and radiotherapy had survival longer than any other combination treatment groups. They observed that CD4 and CD8 brain-infiltrating lymphocytes were more common in the groups treated with the combination of CTLA4 blockade and 4-1BB agonism, with or without radiotherapy, compared to non-tumor-bearing brains. The difference in infiltrating lymphocytes was not noted in draining cervical lymph nodes. Depletion of CD4 and CD8 abrogated the survival improvement with CTLA4 blockade and 4-1BB agonism, with the former being a more profound effect. Long-term survivors of the combination therapy underwent tumor rechallenge and were found to have long-term tumor-specific immunity [38].

An alternative strategy to tumor-specific irradiation is whole-body irradiation. Investigators used a combination of "lymphodepleting" whole-body irradiation (WBI) to 5 Gy in 1 dose 7 days after injection of multiple myeloma cell lines and found that this, in conjunction with a combination of checkpoint-blocking antibodies (against CTLA4, PD-1, PD-L1, TIM-3, or LAG3 or a combination thereof), improved the anti-myeloma immune response and survival. The authors could not elucidate the mechanism whereby WBI augmented immunotherapy other than to say it depleted lymphocytes with upregulated coinhibitory molecules, and this transient depletion facilitated effective immunotherapy. Notably, this strategy was only effective in hematopoietic cancer models, but not in solid tumor models [39].

Summary

Several noteworthy preclinical studies have examined the effect of combining lymphocyte costimulatory and inhibitor receptor modulators and radiotherapy. Most have demonstrated improvements in irradiated tumor control with the combination of receptor modulation and radiotherapy. In two-tumor models, this was associated with an improved control of unirradiated tumors, which translated into longer survival, cure, and immunologic memory. However, no improvement over radiotherapy alone was presented in some studies of CD40 and GITR. Moreover, some studies found that a combination of radiotherapy and more than one costimulatory or checkpoint modulator (CTLA4 and PD-1 or PD-L1, CD137/4-1BB and PD-L1, CD137/4-1BB and CTLA4) yielded the best outcomes. These effects have been observed in models of various cancers, including breast, lung, glioma, lymphoma, colon, mesothelioma, melanoma, kidney, pancreas, and multiple myeloma.

Variations in radiotherapeutic approach have been explored. The effect has been observed with single-dose and fractionated in vivo tumor radiotherapy, which most accurately recapitulates common clinical scenarios for patients with solid tumors. Importantly, some studies observed that a significant delay in checkpoint modulation after radiotherapy abrogated the therapeutic effects. In some models, a higher dose of tumor radiotherapy was associated with response, while lower doses were not. In some instances, irradiation of tumor cells demonstrated the effect, and in one study, two doses of radiotherapy appeared superior to a single dose. The target of

radiotherapy was most often a tumor, but in the context of hematopoietic disease models, whole-body irradiation increased the response to checkpoint modulation and appeared to be dose dependent.

Immunologically, induction of the receptor ligands (PD-L1, 4-1BBL) by radiotherapy was associated with response to combination therapy in some studies. One study found that the T cell receptor diversification may be another important effect that radiotherapy has on the immune system. The infiltration of immune cells after combined checkpoint modulation and radiotherapy was greater than after either treatment alone; depletion of CD8 cells (and sometimes CD4, natural killer cells, or macrophages) typically abrogated therapeutic effects. In vitro assays typically demonstrated tumor-specific functionality of the infiltrating immune cells. Resistance to immune checkpoint modulation and radiotherapy was attributed to PD-L1 expression on tumor cells in two studies, and strategies to block this immunologic barrier appeared to overcome resistance. Finally, the use of immunocompromised model organisms demonstrated a lack of an antitumor effect after radiation demonstrating a critical role of the immune system in mediating the antitumor efficacy of radiation therapy.

The findings discussed herein clearly support future investigations combining lymphocyte costimulatory and inhibitory receptor modulation and radiotherapy. Preclinical evidence has suggested approaches that hold promise for cancer patients, but additional studies will be needed to clarify the optimal therapeutic approach. The design of rationale clinical trials will be imperative to validate the potential benefit for cancer patients.

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