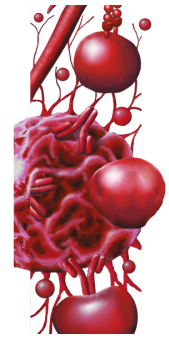
Review

Immunotherapy



Radiation therapy and immunotherapy: what is the optimal timing or sequencing?

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Radiotherapy is a component of the standard of care for many patients with locally advanced non-metastatic tumors and increasingly those with oligometastatic tumors. Despite encouraging advances in local control and progression-free and overall survival outcomes, continued manifestation of tumor progression or recurrence leaves room for improvement in therapeutic efficacy. Novel combinations of radiation with immunotherapy have shown promise in improving outcomes and reducing recurrences by overcoming tumor immune tolerance and evasion mechanisms via boosting the immune system's ability to recognize and eradicate tumor cells. In this review, we discuss preclinical and early clinical evidence that radiotherapy and immunotherapy can improve treatment outcomes for locally advanced and metastatic tumors, elucidate underlying molecular mechanisms and address strategies to optimize timing and sequencing of combination therapy for maximal synergy.

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Radiotherapy (RT) exerts its effects on tumors indirectly by generating free radicals that result in DNA damage or directly by DNA double-strand breakage. In addition to DNA damage, radiation causes mitochondrial redox imbalances, lipid peroxidation of cell membranes and a variety of signaling cascades that lead to cells either succumbing to radiation-induced cell death or temporarily arresting cell division to repair the damage. Traditionally, tumor cells are understood to evade the cytotoxic effects of radiation by one or more of four classical processes, namely repair, repopulation, reoxygenation and reassortment, the so called four 'R's of radiobiology [1]. Escape from immune-mediated tumor rejection has been proposed as a fifth process [2]. In addition to direct and indirect damage of DNA, RT has both immunostimulatory and immunosuppressive roles depending on the radiation dose, number of fractions, type of tumor and site of irradiation. On the one hand, RT's immunostimulatory effects take place in many ways, including increased expression of major histocompatibility complex antigens on the tumor cells; release of tumor-associated antigens (TAAs), leading to an *in situ* vaccine effect; and enhanced expression of immunostimulatory signals like calreticulin, ATP, heat shock proteins and high mobility group box proteins [3–5]. On the other hand, RT can suppress the immune system through depletion of hematopoietic progenitor cells; upregulation of PDL1); and proportional increases in Treg populations [6].

Tumors have the potential to evade the immune system through a complex series of processes collectively referred to as 'immunoediting'. Downregulation of major histocompatibility complex antigens, loss of TAAs, anergy of T cells, methylation of TAA-processing complexes, disordered vascularity and increased infiltration of immunosuppressive cells (Tregs, myeloid-derived suppressor cells, cancer-associated fibroblasts and M2 macrophages) are

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all potential ways of evading immune detection and eradication [4]. Despite advances in surgery, chemotherapy and radiation for cancer treatment, the development of long-term resistance and tumor recurrence has remained a challenge, and the underlying mechanisms remain elusive. One common denominator, however, is thought to be the immune system.

The discovery of immune checkpoints that modulate immune function has led to exploration of new combinations of multimodality therapies for cancer. Immunotherapeutic agents act via mechanisms that depend on expression of immune checkpoints, like CTLA4, T-cell immunoglobulin and TIM3, PD1 and its ligand PDL1; on T-cell costimulatory molecules like OX40 (also known as CD134), 4-1BB (CD137) and the glucocorticoid-induced TNF receptor-related protein GITR; and on circulating serum cytokines such as TGF-β and IL-2, -7, -10 and -15. Radiation's unique effects on the immune microenvironment can be used to enhance the efficacy of co-administered immunotherapeutic agents. Together, RT and immunotherapy mediate their antitumor effects in a dynamic interplay between effector and regulatory cells [7,8]. RT can modulate the expression of tumor antigens and immune checkpoints and influence the circulating cytokine profile. RT-induced changes in the tumor milieu facilitate the action of immunotherapeutic agents. In turn, immunotherapy facilitates the action of RT by targeting and modulating various T-cell populations. This synergistic action varies, however, depending on when the RT and immunotherapies are given and on the presence of certain immune molecules in the tumor microenvironment. Hence, a better understanding of how radiation and immunotherapy influence the tumor immune profile will provide insights that will help to optimize strategies for timing and sequencing the two forms of therapy. Here we review preclinical and clinical studies of the effects of RT given in combination with immunotherapy agents, in the context of optimizing the timing and sequencing of the two complementary treatment modalities to enhance their effectiveness while minimizing treatment-related toxicity.

RT & cancer vaccines

Cancer vaccines have long been sought as a means of generating antitumor immune responses. However, effective vaccines have been difficult to develop because they are responsible for overcoming only part of the complex set of mechanisms driving tumor-mediated immunosuppression. Radiation, like therapeutic cancer vaccines, has been shown to release TAAs such as carcinoembryonic antigen (CEA) and mucin-1 [9] and to generate tumor-specific T cells [10]. However, repeated exposure to moderate doses of radiation may have detrimental effects on the immune system, resulting in the clearance of effector cell types required within the tumor microenvironment for potent antitumor activity [10]. In that context, administering therapeutic cancer vaccines before or concomitant with radiation doses may ultimately be futile because of the lack of an intact adaptive immune system at the tumor site. For this reason, it may prove most useful to administer vaccines after RT, thereby using the vaccines as a 'booster' for the immune cells generated by RT. Also, RT induces the production of the chemokine CXCL16 by tumors such as 4T1 breast cancer cells, which in turn attracts and recruits $CD8^+$ effector T cells expressing the CXCR6 receptor [11]. How long this effect lasts, however, is unclear, because the experiments demonstrating those effects extended only to 72 h after the irradiation. Nevertheless, these findings provide a rationale for administering immunotherapeutic boosters and vaccines after RT to further expand adaptive immune responses.

Dendritic cell vaccines

Experimental data

One strategy for administering vaccines with radiation involves targeting and using dendritic cells (DCs), which are highly efficient at presenting antigens to immune cells. In preclinical experiments, Kim *et al.* demonstrated in a mouse model of a fibrosarcoma that intratumoral injections of DCs, given after irradiation to 15 Gy, led to immune-mediated cytotoxic activity against tumors even at distant metastatic locations [12].

Clinical data, perspectives & ongoing trials

The first cancer vaccine approved by the US FDA was sipuleucel-T (Provenge®), a DC-based vaccine, in April 2010 for the treatment of prostate cancer. Treatment involves obtaining DCs from patients by leukapheresis, loading them with the prostate cancer cell antigen prostatic acid phosphatase and GM-CSF, and injecting the activated product back into the patients. Because of concerns that continuing RT after the adoptive transfer might lead to loss of the antigen-presentation capabilities of the DCs, one of five currently ongoing trials, the randomized Phase II clinical trial NCT01807065, involves giving patients external beam RT in weeks 1–2 followed by intravenous sipuleucel-T on days 22, 36 and 50. Another Phase II trial (NCT01818986) involves giving sipuleucel-T concurrently with

stereotactic ablative RT to metastatic sites in an effort to enrich the antigenic pool and immune response. A pilot study (NCT01833208) is evaluating whether giving a single high-dose RT fraction to at least one bone lesion at 2 days after the first administration of sipuleucel-T affects the vaccine's immunogenicity. A third Phase II trial (NCT02232230) involves giving RT to at least one metastatic site of advanced castrate-refractory metastatic prostate cancer, followed by sipuleucel-T 28 days later, and testing the effects on immune cell profiles at various time points after treatment. The fourth trial, a Phase II multicenter study of men with limited bone-metastatic castrate-resistant prostate cancer (NCT02463799), is investigating the effects of intravenous sipuleucel-T on weeks 6, 8 and 10, given with or without intravenous radium-223 on weeks 0, 4, 8, 12, 16 and 20. End points include antitumor effects and measures of immune activation through T-cell proliferation. Results from these studies, now pending, will help to evaluate outcomes based on the sequencing of RT relative to the vaccine administration.

Viral vaccines

Experimental data

Viral vaccines represent a unique strategy in which a virus is used not only to attack the tumor but also to produce immunostimulatory compounds and immune-cell recruitment. In one preclinical investigation, Chakraborty *et al.* studied the effects of an 8-Gy dose of RT combined with the plaque-forming units of a vaccine containing recombinant vaccinia and fowlpox vectors (rV-CEA/TRICOM and rF-GM-CSF) to treat an animal model of colon cancer expressing CEA antigen (MC38-CEA$^+$) [13]. Tumors were implanted on day 0, followed by vaccine administration on day 8, RT on day 14 and three more rounds of vaccination on days 15, 22 and 29. Neither vaccine nor RT alone had significant therapeutic benefits, but the combination had significant antitumor activity, leading to cure rates in excess of 50%. In 2008, that same group tested the efficacy of this recombinant vaccine and RT regimen with an yttrium-90-radiolabeled murine antibody against CEA and again found superior survival after the combined therapy relative to vaccine or antibody alone. As was the case for the previous study, vaccine treatment was begun on day 8 and radiolabeled antibody was given on days 15, 22 and 29 [14]. These findings underscore the diversity in potential applications of RT and therapeutic cancer vaccines.

Clinical data, perspectives & ongoing trials

Three clinical trials have been conducted to date in which RT was combined with viral vaccines. One Phase II study from the US National Cancer Institute was designed to determine if a poxviral vaccine encoding prostate-specific antigen (PSA) would induce a PSA-specific T-cell response when combined with RT for men with localized prostate cancer. The combination, given with the costimulatory molecule B7.1 as well as local GM-CSF and low-dose systemic IL-2, was well tolerated and generated a PSA-specific immune response [15]. Of the 19 patients enrolled in the combination therapy group, 17 completed the vaccination regimen and 13 showed evidence of PSA-specific T cells versus none in the RT-only group ($p < 0.0005$). A Phase I study by the same group showed that 17 of 18 patients receiving combination RT, IL-2 and rV-PSA/rV-B7.1 tolerated the regimen well, and some patients exhibited high numbers of PSA-specific T cells [16]. Long-term follow-up of a different group of 26 patients given a poxvirus vaccine with external-beam RT, however, showed no differences in PSA control, overall survival or late toxicity relative to external-beam RT alone [17].

Protein & peptide vaccines

Experimental data

In other preclinical studies of RT in combination with cancer vaccines, Mondini *et al.* used the TC-1 murine model to study HPV-related head and neck cancer. They gave tumor-bearing mice intraperitoneal Shiga toxin-based E7 vaccine once before and once after a dose of 7.5-Gy RT [18]. The survival rate for the mice given the combination therapy was 70%; however, the study did not include control groups of vaccine only or RT only. Moreover, substituting the single-fraction 7.5-Gy dose with four consecutive daily fractions of 2.6 Gy given with the vaccine reduced the survival rate to 40%, a finding that could reflect the negative effect of repeated rounds of RT on the immune system. Another study with a different HPV cervical cancer model involved giving subcutaneous E7 protein + CpG-oligodeoxynucleotide subunit vaccine three-times, 1 week apart, after a single 20-Gy dose of RT [19]. That regimen led to clearance of established tumors and generation of long-term immune memory. Those investigators also reported that giving RT first, followed by vaccine, reduced the RD_{50} value (radiation dose yielding 50% complete response rate) by 16 Gy relative to RT alone.

Another preclinical study reported by Harris *et al.* provided direct evidence that giving active immunotherapy within a specific temporal window (3–5 weeks after RT) led to enhanced antitumor efficacy compared with giving the drug before or during radiation [20]. That study involved use of a double-transgenic murine model that spontaneously develops prostate cancer; the cancer cells were made to express the influenza hemagglutinin antigen (HA) as a TAA. The RT was given in a single fraction of 15 Gy to the pelvic site, followed 5 weeks later by the adoptive transfer of $CD4^+$ T cells specific for HA and followed 2 weeks after that by vaccination with recombinant Vaccinia-HA. The RT + vaccine group showed significant increases in percentages of HA-specific $CD4^+$ T cells in prostate-draining lymph nodes and in axillary lymph nodes as compared with the vaccine-only arm. Another experiment in which RT was given as three 10-Gy fractions on days 0, 5 and 10, with adoptive transfer of $CD4^+$ T cells on days 6, 16, 33 or 94, showed that $CD4^+$ transfer on day 6 or day 16 abrogated the subsequent therapeutic effect of Vaccinia-HA given 2 weeks after the adoptive transfer [21]. The peak $CD4^+$ T-cell expansion was detected on day 33 (i.e., at about 5 weeks), but again the effect was diminished when the HA-specific $CD4^+$ T cells were transferred on day 94. These findings are useful for establishing a time frame and demonstrate that using vaccine as a 'booster' may help to reactivate immune cells generated by RT.

Clinical data, perspectives & ongoing trials

Vitespen® (marketed as Oncophage®) is a vaccine based on isolated heat shock protein peptide complex 96, and serves as a tumor antigen for kidney carcinoma and melanoma [22,23]. A clinical Phase I trial for pediatric patients with glioma (NCT02722512) involves administering 4 weekly doses of the vaccine beginning 28 days after completion of RT. A similar Phase II trial using the same vaccine (NCT00905060, now completed) gave the protein intradermally after standard treatment with radiation and temozolomide.

Additional studies in which vaccines are combined with RT are reviewed elsewhere by Garnett-Benson *et al.* [24]. Collectively, these studies highlight the potential for achieving better outcomes when RT is given first, followed by the cancer vaccine. This pattern also seems to hold true for combination treatments that involve RT and adoptive immunotherapy agents, as discussed in the following section.

RT & adoptive immunotherapy

Although currently available adoptive immunotherapy strategies such as T-cell and natural killer (NK)-cell-based therapies have been studied as monotherapy or in combination with chemotherapy for leukemia, to date few have evaluated the effects of these strategies in combination with RT, especially for solid tumors. Current evidence from preclinical and clinical studies is summarized below.

T-cell therapies

Experimental data

Filatenkov *et al.* tested the effectiveness of combining cyclophosphamide and RT with or without autologous T-cell infusions in the 4T1 murine model of metastatic breast cancer [25]. The authors concluded that an RT regimen of 60 Gy (three fractions of 20 Gy each) before autologous T-cell infusions resulted in significant improvement of survival and remission rates relative to mice receiving only cyclophosphamide and RT. In another approach, Ménager *et al.* combined RT with an α-particle emitter, bismuth-213, with adoptive T-cell transfer (α-radioimmunotherapy [RIT]) [26]. Using an immunocompetent murine multiple myeloma model that expresses CD138 and ovalbumin, these investigators administered an anti-CD138 antibody coupled to bismuth-213 followed by adoptive T-cell transfer of $CD8^+$ T cells specific to ovalbumin. The combination of RIT and adoptive T-cell transfer modestly extended median survival times (31 vs 27 days for T-cell transfer alone and 28 days for RIT alone). Data published in abstract form by DeSelm *et al.* showed potentiation of the antitumor effect when RT (in doses as low as 2 Gy) was given in the neoadjuvant setting 2 days before infusion of chimeric antigen receptor (CAR) T cells [27]. Moreover, another abstract published by Cortez *et al.* showed that RT given with CAR T cells targeted to EGFR in A431 epidermoid murine models caused increased targeted T-cell infiltration relative to either therapy alone [28]; the timing in those experiments involved giving the RT 9 days before the CAR T cells.

Clinical data, perspective & ongoing trials

In clinical studies, Dudley *et al.* reported that treating patients with metastatic melanoma with chemotherapy (cyclophosphamide and fludarabine) plus total body irradiation to 2 or 12 Gy followed by infusion of tumor-infiltrating lymphocytes led to higher objective response rates (52 and 72%) than treatment that did not include

total body irradiation (49%) [29]. More recently, Rosenberg *et al.* found similar objective response rates with that treatment regimen in 93 patients, noting complete response rates of 12% for those given chemotherapy alone, 20% for chemotherapy plus 2-Gy total body irradiation, and 40% for those given chemotherapy plus 12 Gy [30]. In a case study of a patient with poorly differentiated adenocarcinoma (stage IIIC) that recurred after surgery who was given T-cell and DC therapy before and concurrently with RT [31], Sato *et al.* observed signs of tumor shrinkage and improved performance status after the concurrent treatment. These results emphasize the diversity in tested combinations of T-cell therapies and RT, and suggest that the effects of these regimens depend on the type, dose and timing of RT relative to T-cell administration.

NK-cell therapies

Experimental data

Like regimens involving T-cell therapy, very few studies have evaluated the effects of administering NK cells with RT. In one preclinical study, Heo *et al.* hypothesized that concurrent RT and NK-cell treatment of the non-small cell cancer cell line NCI-H23 would result in increased NK-cell cytotoxicity, perhaps via RT-induced increases in the expression of NKG2DLs-activating ligands [32]. That study highlights a potential mechanism underlying synergy between NK cells and RT. Finkel *et al.* performed several experiments with B16 melanoma cells to evaluate the immunogenic potential of irradiated cells and the mechanisms through which this might occur [33]. They found that NK-cell depletion just before RT and hyperthermia led to retardation of tumor growth, which suggests that immunization with B16 cells followed by RT and hyperthermia could induce a memory response, and that NK cells might hinder proper immune activation during the immunization. These results suggest that tumor cell irradiation could participate in promoting NK-cell activation. Although this study involved testing NK cells in conjunction with hyperthermia, these findings could set the stage for strategies in which RT is used for tumor immune activation before NK-cell therapy.

RT & cytokines

Some of the studies already discussed included IL-2 in the treatment regimens, highlighting the trend toward inclusion of cytokines to stimulate the innate and adaptive immune cells and treat certain tumors. Such cytokines include but are not limited to TGF-β, TNF-α, GM-CSF, IL-2, IL-7 and IL-15. However, use of these cytokines is still limited because of their toxic side effects, especially in combination therapy. Studies involving these agents are described below.

TGF-β

Experimental data

TGF-β represents a classic double-edged sword – it has tumor-suppressive properties during the early phase of carcinogenesis but acts to favor malignant progression in later stages. TGF-β suppresses immune-cell infiltration into tumors by modulating lymphocyte activation and macrophage function. Among the numerous approaches to inhibiting TGF-β signaling tested to date are monoclonal antibodies, antisense oligonucleotides, small molecule inhibitors, peptide aptamers and stabilized soluble proteins [34]. The time at which the TGF-β-modifying drugs are administered is an important factor. In rat breast cancer models, TGF-β levels were found to increase after cyclophosphamide and RT [35], an undesirable effect potentially leading to tumor resistance. Conversely, inhibition of TGF-β with neutralizing antibodies restored the drug sensitivity of the tumor, suggesting that giving TGF-β inhibitors with chemoradiation could enhance therapeutic efficacy. A small molecule inhibitor of TGF-β, LY364947, given with a TGF-β-neutralizing antibody was found to radiosensitize the breast cancer cell line MDA-MB231 by augmenting dsDNA damage [36]. Further support for the observation that inhibiting TGF-β before RT delays tumor growth comes from Vanpouille-Box *et al.*, who reported that this combination had significant effects on both irradiated and nonirradiated metastatic 4T1 tumors [37]. In that study, tumor cells were injected subcutaneously in the right flank (day 1) and left flank (day 2) of mice; on days 13–17, RT to 6 Gy was delivered only to the right flank tumor followed by intraperitoneal injection of anti-TGF-β every other day from days 12 to 28. In another study, giving the anti-TGF-β antibody after 8 Gy of lung RT was shown to reduce RT-induced lung fibrosis, which was thought to be mediated through active TGF-β secretion by macrophages in thoracic tumors [38].

Clinical data, perspectives & ongoing trials

Clinical trials are evaluating the strategy of initial priming with TGF-β inhibitors followed by concurrent RT plus TGF-β inhibitors in two different patient populations. One such trial includes patients with non-small-cell lung cancer (NCT02581787), and the other, the ExIST trial (NCT02688712), is exploring this approach in patients with rectal cancer. Another recently completed trial (NCT01220271) also involved giving RT with concurrent TGF-β inhibitors and temozolomide to patients with newly diagnosed gliomas.

FMS-like tyrosine kinase 3 ligand

Experimental data

Binding between the FMS-like tyrosine kinase 3 ligand (Flt3L) and Flt3 (CD135) has been shown to stimulate hematopoietic cell differentiation [39]. In one preclinical study of Flt3L, a growth factor that induces and enhances DC function, Chakravarty *et al.* showed that 60 Gy of RT followed by 10 days of Flt3L, starting 1 day after RT, had great potential for limiting primary and metastatic tumors in a murine model of Lewis lung carcinoma [40]. Another group showed that treatment with Flt3L for 10 consecutive days after low-dose RT (2 or 6 Gy) promoted a systemic abscopal response in the 67NR murine model of mammary carcinoma [41]. An abscopal response refers to a systemic effect in response to local irradiation, for example, a reduction in size or extent of metastatic disease outside the scope of the radiation field. Additional confirmation that the success of this combination treatment depends on a functioning immune system is that the antitumor effect was lost in nude mice, as they lack T cells.

Clinical data, perspectives & ongoing trials

A clinical study is now underway combining Flt3L with stereotactic ablative body RT aimed at isolated lung lesions in patients with advanced non-small-cell lung cancer (NCT02839265).

TNF-α

Experimental data

The term TNF refers to a superfamily of numerous protein homologs that act primarily on two receptors: TNFR1, expressed on all cells and TNFR2, expressed only on immune cells and endothelial cells. TNF-α is predominantly produced by activated macrophages, T cells and NK cells. It acts to enhance the production of IL-1, IL-6, IL-8 and IFN-γ in NK cells; reactive oxygen species in macrophages; and nitric oxide synthase in endothelial cells, thereby augmenting the antitumor immune response [42,43]. Preclinical studies of human tumor cell lines have shown that TNF-α can intensify the effect of RT. One study showed that treating mice with human colon cancer xenograft tumors with four cycles of TNF-α, the first given 1 day before the first of 4 weekly RT for 4 weekly doses, led to better tumor control than RT alone [44]. In another study, human xenograft models of squamous cell carcinoma and malignant glioma showed increased RT-induced damage to vasculature in tumors that had been treated with TNF-α [45,46]. TNF-α gene therapy given before RT was also found in a preclinical study to enhance RT-induced cytotoxicity in a human prostate cancer xenograft model [47]. Similarly, TNF-α gene therapy combined with RT led to better local control in human esophageal cancer xenograft models than did either therapy alone [48].

Clinical data, perspectives & ongoing trials

In clinical studies, an early-1990s Phase I dose-escalation trial to explore the toxicity of TNF-α given once daily during RT indicated good response rates [49] but at the cost of severe side effects such as angina, cardiac arrhythmias, leukopenia, allergies and respiratory distress. These findings prompted the search for novel TNF-α delivery methods, one of which was TNFerade, a nonreplicating adenoviral vector containing human TNF-α DNA with a radiation-inducible promoter. Citrin *et al.* tested the feasibility of using TNFerade in combination with capecitabine and RT in a pilot study of patients with locally advanced rectal cancer [50]. The first dose of TNFerade was given with RT and followed with 5 weekly injections thereafter. This treatment led to a complete pathologic response in two of nine patients tested. Herman *et al.* reported findings from a Phase III clinical trial testing the combination of TNFerade with chemoradiation for patients with locally advanced pancreatic cancer [51]. In that trial, giving TNFerade intratumorally before the first fraction of RT along with standard treatment did not improve survival, a finding the authors attributed to ineffective delivery of TNFerade, inadequate activation of the radiation-inducible promotor and the presence of metastatic disease in most patients. These results suggest that TNF-α therapy should be given, perhaps intratumorally, before RT for maximum benefit in terms of local tumor control.

IL-2

Experimental data

IL-2 was the first immunotherapy agent to be approved for use in humans and is known to increase populations of $CD8^+$ T cells, $CD4^+$ helper T cells and Treg cells. The major limiting toxicity that has been reported is capillary leak syndrome. Attempts to overcome this toxicity have included conjugating IL-2 to L19, a small immunoprotein, which allows selective localization of IL-2 to the tumor by targeting the extradomain-B of fibronectin, a marker of tumor neoangiogenesis that is expressed only in the vasculature of solid tumors [52,53]. Rekers *et al.* used an F9 tumor model in which tumor-bearing 129/FvHsd mice received a single 10-Gy dose of RT followed by three rounds of L19–IL-2 cytokine treatment, or RT alone, or L19–IL-2 alone. The optimal schedule in terms of tumor growth delay was found to be giving RT 24 h before starting L9–IL-2 therapy. On the other hand, when these two therapies were combined with systemic IL-2 administration, the most effective schedule was presensitization with IL-2 followed by RT. That kind of sensitization could not be achieved by L19–IL-2, because the latter stimulates the immune system only in the immediate tumor area and not systemically, an important consideration given that NK cells (which require activation by IL-2) are lacking in the tumor stroma. Overall, the combination of RT and IL-2 led to better responses than did IL-2 alone. Zegers *et al.* [53] used three mouse models to study the antitumor effects of RT followed by L19–IL-2 treatment in light of extradomain-B expression: C51 colon carcinoma, with high levels; Lewis lung carcinoma, with intermediate levels; and 4T1 mammary carcinoma cells, with low levels. The highest response rates – up to 75% – were found in the C51 model; because depletion of $CD8^+$ cytotoxic T cells negated the antitumor response, this effect was thought to be mediated through these cells.

Clinical data, perspectives & ongoing trials

From the clinical perspective, numerous trials are being conducted that test IL-2 in combination with RT for a variety of tumor types, but few results are available on the best sequencing of these two therapies. In one Phase I trial, Seung *et al.* studied the combination of IL-2 with stereotactic ablative RT in patients with metastatic melanoma and renal cell carcinoma. The treatment regimen included three rounds of radiation at 20 Gy per fraction, followed 3 days later by various numbers of cycles of IL-2 (600,000 IU/kg given intravenously every 8 h for up to 14 doses per cycle, with a 2-week rest period between cycles). At least a partial response was observed in >60% of patients [54]. Immune-cell profiling of samples from the patients who responded showed a higher frequency of proliferating $CD4^+$ T cells with an activated memory phenotype. In summary, combinations of IL-2 and RT show promise, but the timing and sequence of the two therapies need further optimization, and specific consideration of agents and modes of administration is needed as well.

GM-CSF

Experimental data

GM-CSF is secreted by macrophages, NK cells and T cells, and stimulates presentation of tumor antigens by DCs (via Flt-3 ligand), which activates cytotoxic T-cell responses that mediate antitumor immunity.

Clinical data, perspectives & ongoing trials

Golden *et al.* published their observations of GM-CSF and RT leading to abscopal effects in a trial of patients with advanced solid tumors [55]. In that trial, GM-CSF was given subcutaneously daily for 14 days, beginning 1 week after the start of palliative RT (35 Gy given in ten fractions) to one of three distinct measurable sites of metastatic disease in combination with single-agent chemotherapy or hormonal therapy. Overall, >20% of patients demonstrated an abscopal response, defined as a 30% decrease in the longest diameter of an index off-target lesion. In another study, treatment with GM-CSF given 2 weeks after initiation of an accelerated 3-week course of RT was shown to reduce RT-induced mucositis in patients with laryngeal cancer without adversely affecting tumor control outcomes, presumably via improved mucosal wound healing as a result of proliferation of immune stem cells, surviving mucosal cells or both [56]. Another mechanism of GM-CSF administration is via GVAX, a vaccine shown to enhance immune responses in patients with non-small-cell lung cancer [57]. Currently, eight active clinical trials are studying the combination of GM-CSF and RT as concurrent or adjuvant therapy (NCT02946138, NCT00091052, NCT02663440, NCT03113851, NCT02623595, NCT01595321, NCT02648282 and NCT00727441).

IL-7

Experimental data

IL-7 is a product of stromal cells present in lymphoid tissue that is responsible for maturation of T cells and enriching the populations of both $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells. IL-7 has been used to restore lymphocyte counts in irradiated mice, where it causes preferential expansion of $CD8^+$ T cells [58].

Clinical data, perspectives & ongoing trials

Radiation-induced lymphopenia has been linked with inferior survival in patients with high-grade glioma and other cancers [59,60]. Interest in IL-7 for treating radiation-induced lymphopenia stems from the observation that, unlike the compensatory increase in IL-7 and IL-15 noted in patients receiving total body irradiation to deplete their hematopoietic cells before bone marrow transplantation, RT for glioma causes lymphopenia but no compensatory increase in IL-7 and IL-15. One Phase I trial (NCT02659800) is exploring whether IL-7 can increase CD4 counts in patients with high-grade glioma experiencing lymphopenia after RT and temozolomide.

IL-15

Experimental data

IL-15 shares structural homology with IL-2 and results in increased $CD8^+$ cytotoxic and $CD4^+$ helper T-cell populations but has no effect on Treg cells. IL-15 has been investigated in preclinical models for its ability to synergize with RT. In two recent abstracts, Demaria *et al.* reported that concurrent RT and IL-15 plus adjuvant IL-15 produced the maximum antitumor response in mice bearing the TSA breast cancer cell line. The authors attribute this effect to increased tumor infiltration by NK cells and $CD4^+$ and $CD8^+$ cytotoxic lymphocytes [61,62].

RT & immune checkpoint inhibitors

As is true for studies of RT and cytokines, the effectiveness of combining radiation with immune checkpoint inhibitors has been shown in many models, but the question of the optimal timing of these treatment combinations remains unanswered. Interactions between myeloid cells (which are largely immunosuppressive) and antitumor cytotoxic T cells within tumors are complex. The data available to date seem to justify either simultaneous or delayed administration of checkpoint inhibitors after RT so that newly recruited T cells can destroy tumor cells, both at the primary site and systemically after being presented with novel tumor antigens. Studies of RT and immune checkpoint inhibitors are discussed briefly below.

CTLA4

Experimental data

CTLA4 is responsible for maintaining immune tolerance through inhibition of early stage T-cell development. CTLA4 inhibitors act by preventing the downregulation of helper T-cell activity while reducing Treg cell activity. Several studies have explored combinations of CTLA4 with RT in neoadjuvant, concurrent and adjuvant settings, yet the optimal combination remains elusive. Demaria *et al.* previously demonstrated that RT followed by CTLA4 blockade conferred improved survival relative to either treatment alone in a 4T1 breast cancer model [63]. Dewan *et al.* later showed that this effect holds for simultaneous delivery of immunotherapy and fractionated RT [64]. A review of preclinical studies since 2014 also reveals similar findings. For example, Son *et al.* demonstrated that CTLA4 inhibition enhanced antitumor immunity when immature DCs were intratumorally injected after an RT dose of 10 Gy in murine colon cancer models [65]. That study indicated that the RT/immature DC combination led to inhibition of distant tumors, and that the addition of anti-CTLA4 antibody significantly improved this effect by reducing Treg populations and increasing survival. Yoshimoto *et al.* also reported that RT-induced antitumor immunity has a crucial role in the therapeutic efficacy of RT, and suggested that modulating the immune microenvironment with monoclonal antibodies could further enhance efficacy [66]. In that study, they showed that lymphoma and Lewis lung carcinoma mouse models treated with a single RT dose of 30 Gy followed by anti-CTLA4 had substantially increased antitumor activity relative to control groups. Wu *et al.* also found that blocking CTLA4 on T cells after RT enhanced abscopal effects in a mouse model of mesothelioma [67]. That group showed that RT delayed growth of the primary tumor and that CTLA4 blockade significantly reduced Tregs while increasing cytotoxic T-cell populations in both primary and secondary tumors, thus leading to increased survival. Although all of these studies have reported enhanced antitumor effects from combining RT with anti-CTLA4, only one study to date has evaluated the effects of the timing of the CTLA4 blockade with RT. In that study,

Young *et al.* showed that giving CTLA4 before RT followed by OX40 had the best effects, with clearance of CT26 tumors in all mice. Although giving CTLA4 either 1 day or 5 days after RT also provided a survival advantage, tumor clearance was present in only half of the mice so treated [68]. Across these studies, most, but not all, evidence suggests that immune activation via CTLA4 blockade synergizes optimally with a single or a few large fractions of radiation rather than multiple small fractions of radiation, possibly because repetitive radiation doses deplete newly arrived activated T cells that infiltrate the tumor after CTLA4 blockade [69].

Clinical data, perspectives & ongoing trials

Among the clinical studies conducted to date, Qian *et al.* reported findings from 75 patients with melanoma and brain metastasis showing that giving immunotherapy within 4 weeks of stereotactic radiosurgery (SRS) resulted in the highest lesion response rates [70]. However, that study was designed to compare only immunotherapy given <4 weeks versus >4 weeks after the SRS procedure. In a similar study, Kiess *et al.* retrospectively evaluated 46 patients undergoing single-fraction SRS to 21 Gy and found that giving the anti-CTLA4 agent ipilimumab either before or concurrently with SRS led to the longest survival benefit with the lowest rate of local recurrence [71]. Notably, however, the patients who received ipilimumab after SRS in this study had initially responded poorly to the SRS, and the ipilimumab had been given >2 months after the SRS. Jiang *et al.* reported early findings from a retrospective comparison of patients given SRS either within 5.5 months or ≥ 5.5 months after ipilimumab for brain metastases; that comparison showed that giving SRS closer to the last dose of ipilimumab led to the best intracranial control rates and that better control also correlated with higher absolute lymphocyte counts [72]. Schoenfeld *et al.* also reported a case review of 16 patients who had received whole-brain RT + SRS with ipilimumab for brain metastatic disease [73]. In that study, receipt of SRS before ipilimumab was associated with a median survival time of 26 months versus only 6 months for those who had received SRS after ipilimumab ($p = 0.001$) and 18 months for concurrent administration; however, the authors acknowledge that delays in the receipt of RT may have confounded the results. Knisely *et al.* reported a cohort of 77 patients of melanoma with brain metastasis of whom 35% received ipilimumab before or after SRS and found no difference in median survival time ($p = 0.58$) at 19.8 months for those given the drug before SRS versus 21.3 months for those given the drug after SRS [74]. Notably, however, the brain is an immune-privileged site, with low concentrations of lymphocytes, and these studies all involved very large RT fractions ($\sim$20 Gy). These factors complicate the extrapolation of these findings to others tumors, particularly with treatment scenarios that involve more conventionally fractionated RT. Findings for patients with solid tumors have included anecdotal case reports suggesting that giving palliative RT to patients who had previously received ipilimumab could lead to good local response as well as systemic abscopal effects [75,76]. Barker *et al.* reviewed patients with unresectable stage III–IV melanoma who received nonbrain RT and found that the median survival time for those given induction ipilimumab followed by RT was 9 versus 39 months for those given ipilimumab maintenance therapy [77].

PD1 & PDL1

Experimental data

PD1 functions to restrict the activity of T cells in the tumor microenvironment. Increased expression of the PD1 ligand PDL1 is a resistance mechanism by which tumor cells evade the immune system. Radiation increases the expression of PDL1 in the tumor microenvironment and on $CD8^+$ cytotoxic lymphocytes [78]. Dovedi *et al.* reported that RT increased PDL1 expression in syngeneic models of colorectal cancer (CT 26), breast cancer (4T1) and melanoma (4434 p16-negative BRAFV600E) [79]. Concurrent administration of anti-PDL1 antibody with RT led to a survival benefit, whereas sequential treatment did not. In another preclinical study, Wu *et al.* gave RT on days 1 and 14, and anti-PDL1 antibody daily on days 0–14 to mice orthotopically implanted with MB49 transitional cell bladder cancer [80]. This regimen, similar to concurrent regimens, resulted in longer tumor growth delays relative to RT or anti-PDL1 alone. Another group used Panc-02 murine pancreatic cell lines to study combinations of RT with anti-PDL1 antibody and gemcitabine (given on days 0 and 3) [81]. The anti-PDL1 antibody was administered on days 0, 3, 6 and 9 with RT or on days 4, 7, 10 and 13 with RT + gemcitabine. The gemcitabine-alone treatment group received the drug on days 0 and 3. The RT was given in one of five schedules, three high dose (one 12-Gy fraction, five 3-Gy fractions or one 20-Gy fraction) and two low dose (one 6-Gy fraction or five 2-Gy fractions). The best outcomes were obtained for the mice given high-dose RT with anti-PDL1 and gemcitabine. By using a mathematical model, these authors further concluded that a delay of 7 days between RT and receipt of the anti-PDL1 antibody abolished the radiosensitization effect.

Clinical data, perspective & ongoing trials

Currently, approximately 50 Phase I/II trials are underway to evaluate combinations of RT and PD1 inhibitors [82]. PDL1 upregulation has been found to be a tumor-escape mechanism when RT + anti-CTLA4 therapy is used [83], and it remains to be seen whether combining anti-CTLA4 + RT followed by anti-PDL1 treatment or triple therapy with anti-CTLA4, anti-PDL1 and RT would circumvent this resistance mechanism. Notably, anti-PDL1 therapy is relatively well tolerated; Ahmed *et al.* studied 26 patients of melanoma with brain metastasis treated with the anti-PDL1 agent nivolumab with SRS and found no undue toxicity from this regimen [84].

RT & immune costimulatory molecules

OX40

Experimental data

OX40 is a costimulatory molecule that increases the cytolytic activity of $CD8^+$ T cells. In preclinical studies, Yokouchi *et al.* assessed the effect of an agonistic anti-OX40 monoclonal antibody, given with RT, in a mouse model of ovalbumin-transfected Lewis lung carcinoma [85]. In those experiments, anti-OX40 was given on the day of RT and also as adjuvant therapy on days 8, 11 and 14; this schedule was found to produce the longest overall survival and the most durable immunity compared with either RT or anti-OX40 given as single-modality therapy. Another group reported that adjuvant agonistic anti-OX40 given after RT or surgery in mice increased survival, presumably by increasing $CD8^+$ T cells [86]. A previously mentioned study by Young *et al.* also evaluated the timing of OX40 administration and found that giving anti-OX40 1 day after RT resulted in optimal responses [68].

Clinical data, perspective & ongoing trials

Currently two clinical trials are underway to evaluate anti-OX40, one for metastatic prostate cancer (NCT01303705) and the other for metastatic breast cancer (NCT01862900); both are giving hypofractionated RT to metastatic disease sites with anti-OX40 antibody as adjuvant therapy. Initial immunologic analyses in a Phase I/II trial of patients with metastatic prostate cancer suggest that anti-OX40, RT and cyclophosphamide had no effect on the proliferation of peripheral blood lymphocytes but may increase the percentage of circulating activated $CD8^+$ T cells [87].

Toll-like receptor agonists

Experimental data

Activation of the Toll-like receptor (TLR) can enhance the presentation of TAAs by DCs. Radiation is known to increase the release of TAAs, and TLR can potentiate the activity of RT. Several preclinical studies of TLR agonists have been published. One such study evaluated the immunogenicity of CBLB613, a TLR-2/6 agonist and a naturally occurring lipopeptide of *Mycoplasma arginini* [88]. Two other groups found that giving the TLR-7 agonist imiquimod 6 h before RT or 24 h after RT led to enhanced autophagy and $CD8^+$ T-cell-mediated cell death in a mouse model of melanoma [89], and led to synergistic effects in a mouse model of cutaneous breast cancer [90]. Dovedi *et al.* tested 5 weekly doses of another TLR-7 agonist, R848, after RT in mice bearing B-cell lymphoma (A20) and T-cell lymphoma (EL-4) [91]. The combined therapy led to greater tumor regression than did either therapy given alone, and expansion of $CD8^+$ T cells was suggested as an underlying mechanism. Another agent, a CpG oligodeoxynucleotide targeted to TLR-9, has been tested in a preclinical model of fibrosarcoma; the agent was given before and after RT total doses ranging from 10 to 90 Gy once a week for 6 weeks. A significant difference was found in the radiation dose resulting in 50% tumor cure (TCD_{50}), with 23 Gy for the combination treatment versus 83.1 Gy for RT alone [92].

Clinical data, perspective & ongoing trials

When this article was written, nine active clinical trials were evaluating TLR agonists in combination with RT. In one Phase III trial of patients with stage IIB-IVA cervical cancer [93], chemoradiation was given concurrently with the TLR agonist Z-100, followed by adjuvant Z-100 until disease progression. The planned RT dose was 50 Gy, given in 1.8- to 2-Gy fractions five-times a week. A trend toward better overall survival was noted in the group receiving Z-100. Overall, both preclinical and clinical studies have indicated that TLR agonists require a priming dose followed by concurrent dosing with RT to produce the best antitumor response.

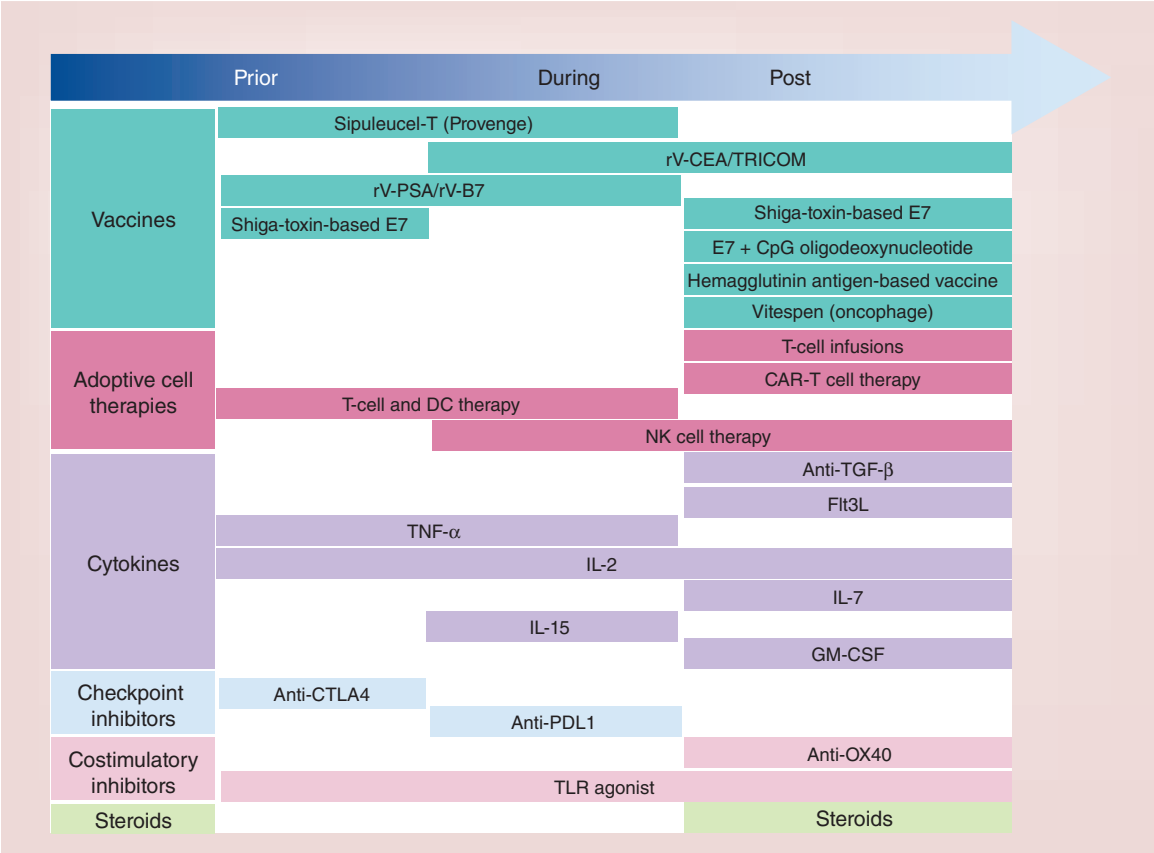

Figure 1. Combinations of immunotherapy and radiotherapy with respect to timing and sequencing.

RT & steroids

Experimental data

Steroids such as glucocorticoids are commonly used to mitigate the side effects of immunotherapy, particularly immune checkpoint inhibitors [94]. Glucocorticoids are potent immune suppressants that function partly by inhibiting proliferation at the transcriptional level and triggering apoptosis and by interfering with IL-2 signaling [95–97]. However, because the goal of immunotherapy with RT is to activate the immune system against cancer through the production of new T cells, steroids could inhibit the antitumor immune response. Thus withholding steroids from patients receiving RT and immunotherapy may prove beneficial unless those patients experience immune-related adverse effects.

Glucocorticoids have several effects on T cells. In addition to triggering apoptosis, the synthetic glucocorticoid dexamethasone can lead to increased percentages of Tregs in patients with asthma and in healthy donors [98]. Dexamethasone was shown to upregulate the glucocorticoid-induced TNF receptor GITR on $CD4^+$ T cells and its ligand GITRL on DCs; interactions between the two induce the $CD4^+$ T cells to produce indoleamine 2,3-dioxygenase (IDO), which in turn controls inflammatory reactions and can lead to tumor cell tolerance [99]. Some evidence exists that glucocorticoid-induced apoptosis can be avoided through activation of T cells, including co-stimulation via CD80/CD86 on either CD28 or CTLA4; indeed, Wagner *et al.* found that co-incubating T cells with CD80- or CD86-transfected thymocytes and dexamethasone caused a large reduction in cells undergoing apoptosis [100]. However, when a CTLA4 fusion protein was added to block CD80 and CD86, the cells did undergo apoptosis, suggesting that signaling via CD28, CTLA4 or both is probably needed for this effect. Hinrichs *et al.*, in exploring how steroids affect activated T cells used in adoptive T-cell immunotherapy, noted no difference in tumor growth after adoptive T-cell therapy in the presence or absence of dexamethasone. They also found that dexamethasone inhibited only naive antigen-specific T cells, not activated antigen-specific T cells, although IFN-γ secretion by the latter was reduced [101]. On the basis of this preclinical evidence, we recommend that steroids be avoided before RT and immunotherapy, because they may inhibit T-cell priming and activation.

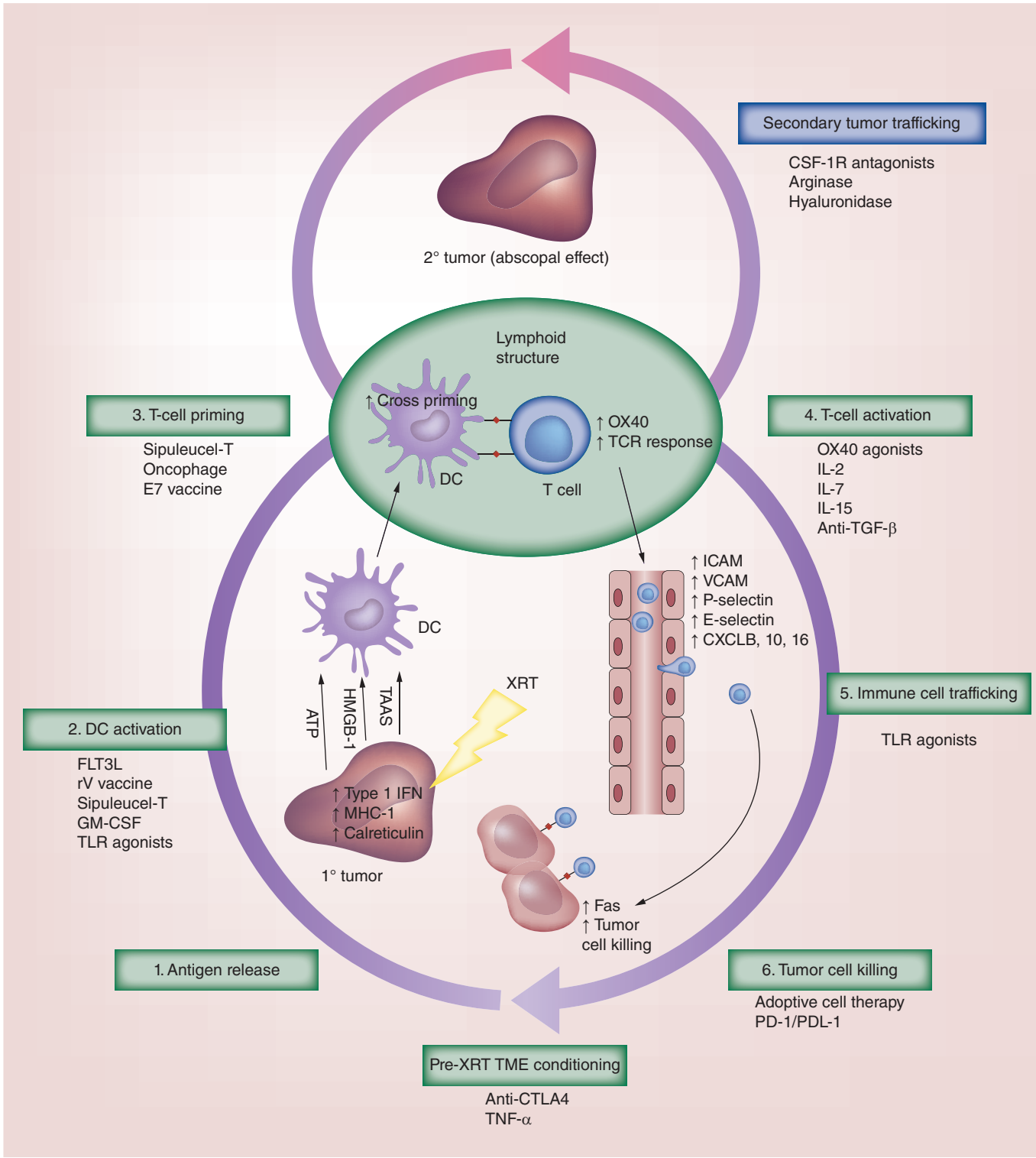

Figure 2. Radiation effects on the tumor microenvironment and potential strategies for combining radiation with immunotherapy. After radiation therapy, cancer cell antigens are released and recognized by dendritic cells, which upon activation cross present these antigens to T cells, which are then primed and activated. This cascade of events can be potentiated by using agents that enhance DC activation and T-cell priming and activation. Upon activation, circulating T cells infiltrate the tumor and engage cancer cell antigens to trigger immune-mediated cell death. Trafficking of activated T cells to both primary tumor and metastatic sites could be enhanced by stromal-disrupting agents.

Clinical data, perspective & ongoing trials

Clinically, numerous trials in melanoma in which ipilimumab was used with a prophylactic steroid regimen before SRS suggest that using steroids results in lower median survival rates [73,102,103]. However, giving steroids during treatment may not interfere with response rates, given that T cells may already be activated. Further preclinical and clinical studies are needed to address the unanswered questions as to the use of steroids with immunotherapy and RT, specifically, to address when steroids might be given without ablating the antitumor immune response and to identify the optimal interval after prior systemic steroid therapy when immunotherapy can be given without affecting antitumor response, as was done with ipilimumab for patients with brain metastases from melanoma [104].

Conclusion

Figure 1 provides a summary of all the agents discussed in this article. Figure 2 illustrates the course of events involved in immune activation induced by RT, and how those events are affected by the various immunotherapies described in this article. One or more windows are apparent, particularly after RT, during which certain events take place, such as T-cell priming and stimulation. For example, OX40 expression on $CD4^+$ T cells increases quickly after RT, which translates to a short time window in which to administer anti-OX40 agonists. Other changes, such as increased antigen presentation after RT, provide a rationale for the timing of delivering vaccines and DC-maturing factors during and especially after RT. Cytokine therapies that modulate T- and NK-cell activity should be given after RT to avoid radiation-induced T-cell apoptosis, whereas therapies that target Tregs, such as anti-CTLA4, may need to be given before RT to make the tumor microenvironment more favorable for proper antigen presentation. Furthermore, by facilitating trafficking of treatment-induced antigen-specific activated T cells deep within the primary tumor as well as to metastatic tumor sites, agents inducing stromal disruption such as arginase, hyaluronidase and CSF-1R antagonists may further enhance the benefits of the immunotherapy–RT combinations discussed here. Metastatic disease treated with RT may be an ideal scenario for exploring these questions in a systematic manner, because synergy at the treated site as well as distant sites (via induction of a systemic immune response) can be simultaneously evaluated. Furthermore, hypofractionated treatments, which tend to synergize optimally with some immunotherapy–RT combinations, are often used in this setting. Finally, the presence of multiple sites of disease in the same patient offers the opportunity to explore the relative benefits or pitfalls of different combination strategies that can initiate or re-initiate the cascade of events resulting in an effective immune response.

Future perspective

The rational application of specific immunotherapies at specific times relative to RT hinges on a nuanced understanding of each therapy's effect on tumor cells and tumor microenvironmental immune cells and how they interact. This will help in the design and effectiveness of future clinical trials in which an enhanced immune response may be effectively triggered while avoiding chronic use of immunotherapies that can result in undesired toxicity. Recognizing that use of single immunotherapeutic agent strategies may risk development of tolerance or resistance, optimal combination of multiple immune-directed strategies with RT may need to be surveyed to maximize the chances of systemic antitumor immune activation. The ultimate goal of rationally designed combination therapy is, of course, better patient outcomes with reduced side effects.

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Executive summary

Radiation therapy & cancer vaccines

- Experimental and clinical studies point to beneficial effects from delivering cancer vaccines after radiation therapy (RT).
- Sipuleucel-T, a dendritic cell-based vaccine, has been extensively tested in combination with RT. Additional clinical studies are ongoing.
- Viral-, protein- and peptide-based vaccine treatments after RT are in clinical trials.

RT & adoptive immunotherapy

- Adoptive T-cell transfer in the setting of radiation works best when the T cells are given after RT.
- Chimeric antigen receptor T cells have shown evidence of efficacy when given after RT in preliminary studies.
- Natural killer cell therapy also has synergistic effects when given after radiation in preclinical studies.

RT & cytokines

- TGF-β inhibition after RT can sensitize tumors and mitigate toxicity, and is currently being tested concurrently with RT in clinical trials.
- Flt3 ligand treatment after RT generates a systemic antitumor immune response and off-target (abscopal) effects at distant sites of disease.
- TNF-α has shown benefit when given before RT.
- GM-CSF, like vaccines, has proven beneficial when given after RT.
- Several studies are ongoing to clarify the timing and mechanism of interleukin administration with RT.
- Interest is increasing in interleukin therapy for radiation-induced lymphopenia.

RT & immune checkpoint inhibitors or immune co-stimulatory molecules

- Combination of RT and anti-CTLA4 has resulted in abscopal responses in patients with solid tumors. Most studies point to a benefit of anti-CTLA4 given after RT.
- Anti-PDL1 therapy after RT improves local tumor control in preclinical studies.
- Dual therapy with anti-CTLA4 and anti-PDL1 in addition to RT can enhance response rates.
- OX40 given after RT has resulted in extended overall survival as well as local tumor control in various *in vivo* models.
- Toll-like receptor agonists have shown benefit when given before RT and are currently being investigated in clinical trials.

RT, immunotherapy & steroids

- Prophylactic administration of glucocorticoids before RT results in lower survival.
- Further studies are needed on the future use of steroids with RT and immunotherapy given the dampening effect on antitumor immune responses.

Conclusion & future perspective

- Optimal combinations of RT and immunotherapy depend on understanding the effect of each agent on the tumor, tumor microenvironment and immune response.

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