Title:

The mutual relationship between the host immune system and radiotherapy: Stimulating the action of immune cells by irradiation

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Abstract:

The effects of irradiation on tumor tissue and the host immune system are interrelated. The antitumor effect of irradiation is attenuated in the immunocompromised hosts. In addition, radiation alone positively and negatively influences the host immune system. The positive effects of radiation are summarized by the ability to help induce and enhance tumor-antigen-specific immune responses. The cancer-immunity cycle is a multistep framework that illustrates how the tumor-antigen-specific immune responses are induced and how the induced antigen-specific immune cells exert their functions in tumor tissues. Irradiation affects each step of this cancer-immunity cycle, primarily in a positive manner. In contrast, radiation also has negative effects on the immune system. The first is that irradiation has the possibility to kill irradiated effector immune cells. The second is that irradiation upregulates immunosuppressive molecules in the tumor microenvironment, whereas the third is that irradiation to the tumor condenses immunosuppressor cells in the tumor microenvironment. When used in conjunction with radiotherapy, immune checkpoint inhibitors can further leverage the positive effects of radiation on the immune system and compensate for the negative effects of irradiation, which supports the rationale for the combination of radiotherapy and immune checkpoint inhibitors. In this review, we summarize the preclinical evidence for the reciprocal effects of radiation exposure and the immune system, and up-front topics of the combination therapy of immune checkpoint inhibitors and radiotherapy.

Key words: radiotherapy, immune cells, radiosensitivity, cancer-immunity cycle.

Introduction

Immune checkpoints are inhibitory pathways that are crucial for maintaining self-tolerance by regulating immune activation and by modulating the T-cell response to self-proteins [1]. In the tumor microenvironment, the immune checkpoint mechanisms driven by molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and its ligand, programmed death-ligand 1 (PD-L1), are activated to suppress the antitumor immune responses. Deactivating the checkpoint mechanisms with immune checkpoint inhibitors (ICIs), anti-CTLA4 antibody (aCTLA4), and anti-PD-1 antibody (aPD-1)/anti-PD-L1 antibody (aPD-L1), has significantly improved cancer patients' prognosis. The combination of ICIs with existing cancer treatment modalities is currently being evaluated to further improve overall survival [2].

Radiotherapy (RT) is one of the most promising cancer therapies combined with ICIs. Chemoradiotherapy followed by consolidation ICIs improves the prognosis of advanced-stage non-small cell lung cancer (NSCLC) patients without metastases [3−5]. According to an updated report, the 4-year overall survival rate for chemoradiotherapy + aPD-L1 for advanced-stage NSCLC is 49.6%, which represents a tremendous improvement compared with 36.3% for chemoradiotherapy alone [5]. Thus, this treatment protocol was established as a standard treatment of care for advanced-stage NSCLC patients. Radiation can have both positive and negative effects on host immunity. On the other

hand, ICIs can mitigate the negative impact of radiation on immunity. In addition, some immune activation mechanisms of radiation are different and non-redundant from those of ICIs, resulting in acting synergistically with ICIs.

In this review, we first provide an overview of the types of immune cells associated with tumor shrinkage following irradiation exposure and the radiosensitivity of immune cells to discuss the negative impact of radiation on host immunity. Next, we summarize the positive impact of irradiation on the immune responses focusing on multiple steps of inducing antigen-specific immune responses. In addition to the direct cell-kill of immune cells after irradiation, there are some other negative effects of radiation on the host immune responses. We discuss the potential other negative impacts of radiation on host immune responses and the rationale for combining RT and ICIs with consideration of both positive and negative impacts of radiation on immune cells. Finally, as up-front topics, we will discuss the effect of ICIs on the radiosensitivity of immune cells and the challenges of combining current ICIs with other immune-modulating agents as a new treatment strategy to discuss the potential role of RT in the era of cancer immunotherapy.

T-cell immune responses contribute to the antitumor effect of irradiation

In the past, the reason for tumor shrinkage after irradiation was considered to be mediated by only the

direct cell-killing effect of irradiation through deoxyribonucleic acid (DNA) damage and the subsequent DNA damage responses. However, it has become clear that the host immune responses also contribute to tumor shrinkage after irradiation [6]. Even when RT is given to the tumor derived from the same tumor cell line under the same irradiation conditions, RT is less effective when tumors are implanted in immunocompromised nude mice than when they are implanted in immunocompetent mice [6]. Nude mice used for *in vivo* experiments lack a thymus and have immature T-cell functions. This result suggests that T-cell immunity is involved in maintaining tumor volume reduction after irradiation.

Among T-cells, the cluster of differentiation eight positive (CD8+) T-cells have been shown to have a powerful influence on the antitumor effect after irradiation [6–9]. In an *in vivo* mouse tumor model, simultaneous elimination of CD8+T-cells with irradiation significantly attenuates the antitumor effect of RT, and the irradiated tumor rapidly regrows a few days later even after a curative dose of irradiation. CD4+T-cell depletion is less effective for modifying the antitumor effect of radiation, showing a weak or no significant difference compared with RT alone [7, 10]. On the other hand, the selective removal of the forkhead box P3 positive (Foxp3+)CD25+CD4+regulatory T-cells (Tregs), which act as CD8+T-cell suppressors, enhances the effect of irradiation [11–13]. Immune cells other than T-cells have also been shown to influence the antitumor effects of RT. The removal of dendritic cells (DCs) from mice

using genetic manipulation abrogated the effects of RT [7, 9], while the removal of myeloid-derived suppressor cells (MDSCs) enhances the effects of RT [14, 15]. Considering DCs are involved in the activation process of antigen-specific CD8+T-cells, and MDSCs suppress CD8+T-cell function [16], the effect of removing those immune cells on RT depends upon subsequent effects on CD8+T-cells mainly. Taken together, these data suggest that the immune responses mediated by CD8+T-cells contribute to the antitumor effect of RT in addition to the direct cell-killing effect of irradiation.

Activated tumor-infiltrating lymphocytes are radioresistant compared with naïve lymphocytes

The radiosensitivity of immune cells depends on whether they are in an activated or naïve state as well as the type of immune cell. Classically, lymphocytes are believed to be equally highly radiosensitive without considering their condition and situation [17]. Actually, irradiation of peripheral blood, bone marrow, and lymphoid tissues is immunosuppressive as a number of the immune cells in these tissues die, even at low doses of irradiation [18, 19]. However, phytohemagglutinin-treated activated lymphocytes have been reported to be radioresistant [20, 21]. Another activator, anti-CD3/CD28 antibody, also makes T-cells radioresistant by downregulating the expression of ataxia-telangiectasia mutated (ATM) kinase, a major regulator of the cellular response to DNA double-strand breaks, which results in decreased ATM phosphorylation following irradiation [22].

Among the T-cell subsets, memory T-cells survive eight times more than naïve T-cells after irradiation and exhibit resistance to irradiation-induced apoptosis [23]. In this study, after 18 h of whole-body irradiation (6 Gy), the number of naïve T-cells in the spleen decreased from 4×10^6 to 1×10^5 cells (1/40), whereas memory T-cells decreased from 1.5×10^6 to 3×10^5 (1/5) in the lymphocytic choriomeningitis virus (LCMV)-immune mouse model [23]. In addition to memory T-cells, Arina *et al.* demonstrated that tumor-infiltrating T-cells are radioresistant [24]. They examined the effects of a transforming growth factor beta (TGF-β) blockade on the mortality of the tumor-infiltrating T-cells following irradiation. TGF-β blockade transformed the radioresistant T-cells into a radiosensitive phenotype, indicating that this signal plays a role in the radioresistance of the tumor-infiltrating T-cells.

Even if T-cells survive radiation exposure, it is of no use if the irradiated T-cells lose their function as effector immune cells in the tumor microenvironment. There is one report that examined the motility and function of the tumor-infiltrating T-cells following irradiation [24]. The authors used longitudinal *in vivo* imaging and discovered that irradiated pre-existing intratumoral T-cells maintained, or even rather increased, their motility as well as IFNγ production [24]. These results were surprising, but irradiated intratumoral T-cells have the possibility to maintain their motility and cytotoxic function after irradiation [24].

Effects of irradiation on each step of the cancer-immunity cycle

"The cancer-immunity cycle" is a multistep framework used to describe how the tumor-antigen-specific immune cells are activated as well as recognize and kill tumor cells (Fig.1) [25]. In the first step (step 1), the cancer-immunity cycle is initiated by regulated cell death known as immunogenic cell death (ICD). ICD is a form of stress-driven cell death that elicits sufficiently immune responses through the extracellular release of tumor-associated antigens and damage-associated molecular patterns (DAMPs), including extracellularly secreted adenosine-5'-triphosphate (ATP) and high-mobility group box 1 (HMGB1) as well as surface-exposed calreticulin by translocation, from the dying tumor cells in the tumor microenvironment [26]. Radiation is a bona fide ICD inducer [27] as is a consequence of the production of reactive oxygen species and endoplasmic reticulum stress in the irradiated tumor (Fig.1) [28, 29]. The next step (step 2) is the tumor-antigen presentation by DCs. In addition to the release of DAMPs and tumor antigens, irradiation induces DC maturation, as measured by the increased expression of costimulatory molecules, CD80/CD86 (ligands to CD28 or CTLA4 on T-cells) on DCs following irradiation [7]. The release of DAMPs and tumor antigens, and the maturation of DCs cooperatively enable the DCs to present tumor antigens to responsive T-cells as the next step of the cancer-immunity cycle.

147 The third step of the cancer-immunity cycle is the T-cell priming and activation phase. After step 1
148 and step 2, DCs transport from the tumor tissue to draining lymph nodes and serve as antigen-
149 presenting cells to effector cells in the lymph nodes, by cross-presenting captured antigens to effector
150 cells and priming them [9, 30]. The efficient initiation of the priming step requires type I interferon
151 (IFN) [31, 32], and starts from the activation of cyclic GMP-AMP synthase (cGAS) and the stimulator
152 of IFN genes (STING) pathway in both tumor cells and DCs [32−34]. Micronuclei are extra-nuclear
153 bodies that form following DNA damage whenever a chromosome or its fragment is not incorporated
154 into the daughter nuclei during cell division. Micronuclei and chromatin bridges, which are bridges
155 formed between the separating groups of anaphase chromosomes accompanied by acentric
156 chromosome fragments, are vital cGAS activators [35, 36]. Radiation-induced DNA damage forms
157 micronuclei and chromatin bridges in the irradiated tumor cells, and activates cGAS and subsequent
158 STING pathway in a dose-dependent manner, which results in the production of type I IFN (Fig.1)
159 [34, 37]. After priming, the activated CD8+T-cells divide and proliferate. Thus, the degree of priming
160 is estimated *in vivo* by monitoring the division of the CD8+T cells in the tumor-draining lymph nodes.
161 By measuring the enhanced division of CD8+T-cells after irradiation in the draining lymph nodes,
162 irradiation of the tumor is demonstrated to induce robust priming compared with unirradiated tumors
163 (Fig.1) [6].
164

Activated antigen-specific CD8+T-cells in the draining lymph nodes return to the tumor microenvironment and infiltrate the tumor tissue. This represents the next steps of the cancer-immunity cycle and is known as the T-cell trafficking (step 4) and infiltration (step 5) phase. Radiation-induced chemokines and integrin ligand receptors cooperatively enhance T-cell trafficking at the irradiated tumor site [38]. Chemokine receptors, C-X-C chemokine receptor 3 (CXCR3) and CXCR6, are receptors for C-X-C chemokine ligand 9 (CXCL9)/CXCL10 and CXCL16, respectively. CXCR3 and CXCR6 are expressed at low levels on naïve T cells, but are upregulated upon activation [39, 40]. Radiation induces CXCL9/CXCL10 and CXCL16 expression in the tumor microenvironment in an IFNγ-dependent manner, which helps activated T-cells to traffic to the irradiated tumor (step 4) [41, 42]. Regarding T-cell infiltration to the tumor (step 5), irradiation induces the expression of integrin ligand receptors, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion protein-1 (VCAM-1), on the endothelium in the tumor microenvironment in a radiation-dose-dependent manner from 2 Gy up to 20 Gy (Fig.1) [43, 44]. The induced integrin ligand receptors of the T-cells assist in migration to the irradiated tumor and prevent recirculation to draining lymph nodes, which results in the enhanced localization of antigen-specific CD8+T-cells and condensation of them in the tumor tissue [45, 46].

The final steps of the cancer-immunity cycle are tumor cell recognition (step 6) and killing (step 7) by

183 immune cells. The recognition of tumor cells by the activated antigen-specific CD8+T-cells requires
184 major histocompatibility complex I (MHC-I) expression on the tumor cells. The CD8+T-cells respond
185 better to tumor cells expressing abundant peptide-MHC-I complexes than those with low or no MHC-
186 I expression [47, 48]. Importantly, radiation induces MHC-I expression on the tumor cell surface in a
187 dose-dependent manner and enhances tumor immunogenicity (Fig.1) [49–51]. Moreover, radiation
188 enhances not only MHC-I expression but also both the peptide repertoire in the tumor cells and the
189 expression level of tumor-associated antigens [49, 52, 53]. These make irradiated tumor cells more
190 likely to be recognized by immune cells, and help tumor-antigen-specific CD8+ T-cells to kill tumor
191 cells (Fig.1) [52, 53].
192
193 **Synergistic effects of combining ICIs and RT**
194 Among the multistep of the cancer-immunity cycle, immune checkpoint molecules suppress especially
195 step 3 (priming) and step 7 (killing of targets) [25]. T-cell mediated antigen recognition requires the
196 interaction of T-cell receptor (TCR) with MHC molecules and costimulatory signals by CD28 on the
197 T-cells through CD80/CD86 molecules expressed on antigen-presenting DCs (Fig. 1). CTLA4 is a
198 CD28 homolog with the higher binding affinity of CD80/CD86 and competitively suppresses the
199 binding of CD80/CD86 to CD28 [54, 55]. CTLA4 expression on both DCs and Tregs deprives the
200 effector T-cells' chance to get a sufficient costimulatory signal via CD28, leading to the attenuation of

the antigen-specific activation processes (step 3). In contrast to CTLA4, mainly expressed on immune cells, PD-1 ligands, PD-L1 and PD-L2, are expressed on various normal tissues [56, 57], and these tissues protect themselves against the potentially autoreactive effector T-cells by suppressing their activities with PD-1 ligands [58]. By expressing the PD-L1, cancer cells escape the effector cell-killing step of the cancer-immunity cycle by inducing the exhaustion of effector immune cells and attenuating the effector functions [59]. ICIs, both aCTLA4 and aPD-1/aPD-L1, reverse the inhibitory responses on the cancer-immunity cycle and enhance tumor-antigen-specific immune responses. Irradiation activates multistep of the cancer-immunity cycle, while ICIs deactivate immunosuppressive responses in the cancer-immunity cycle. This is one of the rationales for the combination of RT and ICIs.

In addition, RT enhances the antitumor reactivity of T-cells in a non-redundant way of ICIs' mechanisms of action, which also strengthens the rationale for combining ICIs with RT. The effective antigen-specific T-cell responses are induced only if the intratumoral TCR repertoire is intrinsically tumor-reactive and appropriate T-cell clones are ready to respond to the tumor [60], which is consistent with the clinical finding that the increased diversity of TCR of tumor-infiltrating T-cells is predictive for the efficacy of ICIs in metastatic melanoma patients [61]. Victor *et al.* demonstrated that irradiation to the tumor diversifies the TCR repertoire of tumor-infiltrating lymphocytes compared with unirradiated tumors [62]. This RT-induced diversification of the TCR repertoire helps to broaden the

window of the T-cell response [63]. In summary, radiation increases the diversity of the T-cell clones, while ICIs, if combined with RT, expand the RT-induced variety of T-cell clones, working synergistically to enhance the more robust immune responses [62, 64].

In contrast to these positive effects of RT, there are some negative effects on the immune system. Combining RT with ICIs compensates for the negative impact of radiation on the antitumor immune responses. First, irradiation induces the expression of PD-L1 on tumor cells [65]. Sato *et al.* demonstrated that tumor cells upregulate PD-L1 in response to DNA double-strand break in an ATM/ataxia telangiectasia and Rad3-related protein (ATR)/checkpoint kinase 1 (Chk1) pathway-dependent manner [66]. Other studies indicate that increased tumor PD-L1 expression and subsequent T-cell exhaustion cause resistance to RT + aCTLA combination therapy using a genetic PD-L1-deficient tumor [62]. The combination of aPD-1/aPD-L1 with RT reverses the effect of irradiation-induced PD-L1. The second negative effect of radiation on the antitumor immune responses involves Tregs. Several reports have shown that Tregs are more radioresistant than other lymphocytes, resulting in their selection after irradiation [67, 68]. Irradiated Tregs in the tumor are able to exert suppressive effects equally and are also able to proliferate in the tumor tissue even after irradiation [68]. CTLA-4 is expressed by Tregs constitutively. aCTLA4 drives the loss of Treg stability in the tumor microenvironment [69]. Targeting Tregs with aCTLA4 or other drugs is a strategy to reverse the Treg-

237 related negative impact on the immune responses following irradiation [70–72].

238

239

240 **Up-front topics and future perspectives of the combination therapy of ICIs and RT**

241 The sequence of combination therapy of RT plus ICIs (e.g., RT 1st & ICIs later or ICIs 1st & RT later)
242 was one of the controversial issues that need to be resolved to maximize the combination effect [73].
243 As for an up-front topic, a recent report provides clues to a solution to the optimal order of the
244 combination in relation to the radiosensitivity of immune cells [13]. To determine whether aPD1 prior
245 to RT affects radiation-induced DNA damage of T-cells, the amount of fluorescent-stained
246 phosphorylation of histone 2A family member X (γH2AX), a marker for DNA damage, of CD8+T-
247 cells was evaluated by flow cytometry. T-cells treated with aPD1 showed a significantly higher level
248 of γH2AX median fluorescence intensity than those with isotype control. In addition, treatment with
249 aPD1 before irradiation induced more apoptosis of intratumor T-cells than isotype control. These
250 results suggest that RT first protocol may be better in combination with RT and aPD1. To elucidate the
251 underlying mechanism, the authors used an unsupervised hierarchical clustering algorithm, and found
252 that aPD-1 administration before irradiation expanded the intratumor CD8+T-cell population, which
253 resulted in a more naïve/non-activated phenotype in the tumor tissue [13]. This is consistent with the
254 previous reports demonstrating that the administration of aPD1 not only reinvigorates exhausted

immune cells, but also induces the proliferation of CD8+T-cells [64, 74]. Naïve/non-activating CD8+T-cells are more radiosensitive compared with the activating ones, as mentioned above. The administration of aPD1 prior to RT results in an increased percentage of radiosensitive, unactivated intratumor CD8+T-cell populations, and the following RT may eradicate the radiosensitive populations expanded in the tumor tissue by aPD1. The potential survival superiority of "RT 1st & ICIs later protocol" was demonstrated in a retrospective analysis of patients with resected melanoma brain metastases [75]. Although further translational researches and prospective clinical trials are still needed, these findings may imply that aPD1/aPD-L1 administration has the potential to be better used following irradiation.

The effect of the double combination of RT and ICIs is still insufficient in clinical situations, and triple combination therapy of RT, ICIs, and the addition of immunomodulatory and radiosensitizing drugs is a promising future strategy. One such candidate agent is an indoleamine 2, 3-dioxygenases (IDO) inhibitor, which compensates for the negative impact of RT on host immune function and improves the efficacy of the double combination. IDO is a tryptophan catabolic enzyme that catalyzes the essential amino acid tryptophan into its immunosuppressive metabolite, kynurenine. Irradiation induces not only PD-L1 on the tumor, but also IDO overexpression in the tumor cells through type I and II IFN signaling [76]. The depletion of tryptophan and the increase in kynurenine in the

273 microenvironment impair the proliferation of effector immune cells, induce their apoptosis, and
274 stimulate the differentiation process into Tregs [77]. Additionally, IDO inhibitors improve the
275 radiosensitivity of tumor cells, and synergistic effects are observed when used in combination with RT
276 [78]. Free heme and heme-derived iron show pro-oxidant activity in the irradiated sites. IDO contains
277 heme [79], and IDO inhibitors release IDO-bound heme, which increases the concentration of free
278 heme in the microenvironment and contributes to increased radiosensitivity [80]. With the
279 aforementioned mechanisms of action, we hypothesized that the combination of IDO inhibitors and
280 RT as well as aPD1 would have a synergistic effect by mitigating the negative impact of radiation. We
281 showed that the combination of RT + IDO inhibitor (1-methyl-D-tryptophan) + aPD1 enhances the
282 effect of the double combination of RT and aPD1 in preclinical models [81]. The combination of RT
283 and IDO inhibitors (+ chemotherapy) is being tested in the clinical studies based on the compatibility
284 of each (NCT04049669, NCT02052648). The treatments in these clinical trials are not combined with
285 ICIs, leaving room for further combination with them if the clinical trials are successful. Although the
286 result of a randomized phase III study (ECHO-301) of the combination of ICIs and an IDO inhibitor
287 (without RT) was negative [82], the triple treatment strategy with ICIs + IDO inhibitors + RT would
288 still be promising considering that RT has immune-activating effects that do not overlap with
289 immunotherapy and IDO inhibitors can mitigate the radioresistance of tumor cells.
290

Conclusion

We summarized the preclinical data related to the interaction of irradiation and the immune responses mainly from the viewpoint of the radiosensitivity of immune cells, the rationale for combining ICIs with RT considering the positive and negative impacts of radiation on the immune system, and future perspectives of the combination strategy of RT + ICIs. The results of preclinical studies may help us to effectively combine ICIs with RT to further improve treatment response in clinical practice.

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Compliance with ethical standards

Conflict of interest

The authors declare no potential conflicts of interest.

Figure legend

Fig.1 The effects of radiation on multistep of cancer-immunity cycle

The cancer-immunity cycle is a multistep framework to induce antigen-specific host immune response,

starting from (1) initiation by immunogenic cell death of tumor cells phase to the subsequent phases: (2) cancer antigen presentation by DC, (3) T-cell priming and activation by antigen-presenting cells, (4) T-cell trafficking from draining lymph nodes, (5) infiltration of T-cells to the tumor tissue, (6) T-cell recognition and (7) killing of tumors. Radiotherapy (RT) enhances each step of the cancer-immunity cycle and antigen-specific immune responses.

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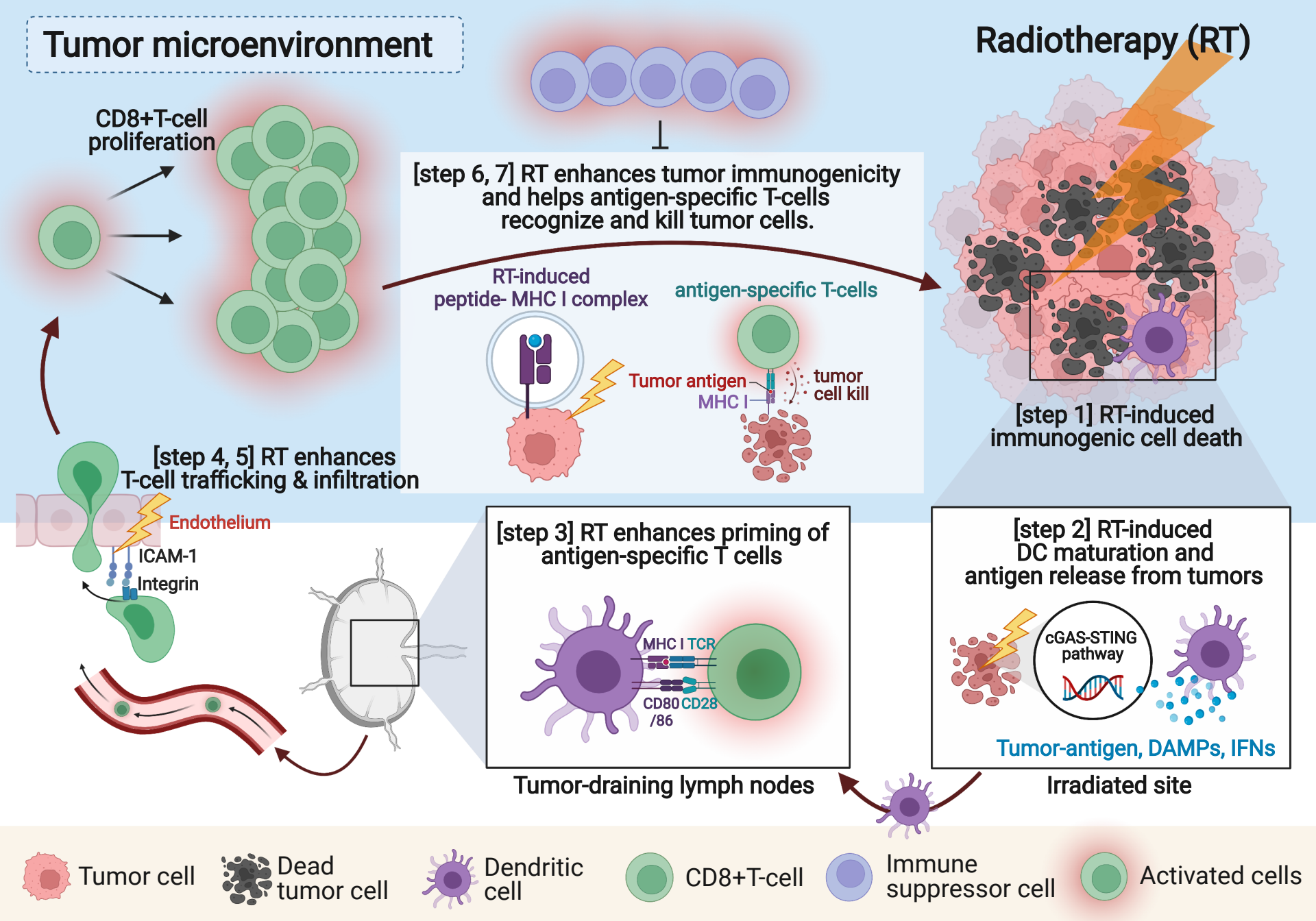
Tumor microenvironment
Radiotherapy (RT)
CD8+T-cell proliferation
[step 6, 7] RT enhances tumor immunogenicity and helps antigen-specific T-cells recognize and kill tumor cells.
RT-induced peptide- MHC I complex
antigen-specific T-cells
Tumor antigen
MHC I
tumor cell kill
[step 1] RT-induced immunogenic cell death
[step 4, 5] RT enhances T-cell trafficking & infiltration
Endothelium
ICAM-1
Integrin
[step 3] RT enhances priming of antigen-specific T cells
MHC I TCR
CD80 CD28 /86
[step 2] RT-induced DC maturation and antigen release from tumors
cGAS-STING pathway
Tumor-antigen, DAMPs, IFNs
Tumor-draining lymph nodes
Irradiated site
Tumor cell
Dead tumor cell
Dendritic cell
CD8+T-cell
Immune suppressor cell
Activated cells