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REVIEW

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Radiotherapy as a means to increase the efficacy of T-cell therapy in solid tumors

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ABSTRACT

Chimeric antigen receptor (CAR)-T cells have demonstrated significant improvements in the treatment of refractory B-cell malignancies that previously showed limited survival. In contrast, early-phase clinical studies targeting solid tumors have been disappointing. This may be due to both a lack of specific and homogeneously expressed targets at the surface of tumor cells, as well as intrinsic properties of the solid tumor microenvironment that limit homing and activation of adoptive T cells. Faced with these antagonistic conditions, radiotherapy (RT) has the potential to change the overall tumor landscape, from depleting tumor cells to reshaping the tumor microenvironment. In this article, we describe the current landscape and discuss how RT may play a pivotal role for enhancing the efficacy of adoptive T-cell therapies in solid tumors. Indeed, by improving homing, expansion and activation of infused T cells while reducing tumor volume and heterogeneity, the use of RT could help the implementation of engineered T cells in the treatment of solid tumors.

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1. Introduction

Despite great advances in B-cell malignancies including acute lymphoblastic leukemia (B-ALL),[1,2] chronic lymphocytic leukemia,[3,4] mantle cell lymphoma,[5] large B-cell lymphoma (LBCL),[6–8] and multiple myeloma,[9] the majority of clinical investigations involving CAR-T cells in solid tumors provided poor outcomes.[10] To date, CAR-T cell therapies have been experimented in approximately 250 clinical trials as a treatment for a large variety of solid tumors, from primitive brain tumors to pelvic malignancies.[11] Except for the slight effects observed in gastric and pancreatic cancers treated with anti-claudin 18.2 CAR-T cells[12] and encouraging results with local administration of anti-mesothelin CAR-T cells in malignant pleural disease,[13] results obtained in these trials were disappointing. Of note, even when targeting relevant antigens, such as PSMA in prostate cancer, the efficacy of CAR-T cells remains low and their engraftment is uncertain. This underscores the fact that, in solid tumors treated with CAR-T cells, targeting the right antigen is necessary but not sufficient. Moreover, the use of CAR-T cells in solid tumors often resulted in on-target/off-cancer toxicities.[14] Indeed, compared to hematological malignancies, the specificities of the tumor microenvironment (TME) in solid tumors are of critical importance in thwarting the action of adoptive cells. Herein, we aim to describe how TME limits both homing and activation of infused T cells, preventing them from reaching tumor cells and exerting their antitumor effects. Finally, we will provide a speculative point of view on how RT could be involved in combating these deleterious conditions.

2. Optimizing the efficacy of T-cell therapies in solid tumors using radiotherapy: rationale

As demonstrated notably in esophageal squamous cell carcinoma,[15,16] primitive brain tumors[17] including glioblastoma,[18] head and neck cancers[19] and lung tumors,[20] a key issue for the development of solid tumors and their escape to immune surveillance is the polarization of the immune microenvironment into an immunosuppressive and tolerant phenotype.[21] This can be done through various pathways, of which the most known are the secretion of immunosuppressive chemokines such as interleukin (IL)-10 or transforming growth-factor (TGF)-β,[22] or the upregulation of several druggable inhibitory immune checkpoints which will be described later in this review. This results in a progressive change from "hot" tumors (T-cell inflamed) to "cold" tumors, either showing no immune cell infiltration (called "immune deserts") or with effector cells located at the periphery of tumor and not infiltrating tumor core (called "immune excluded"). In addition to limiting the infiltration of CAR-T cells into their core, cold tumors also promote the recruitment of regulatory T cells (Tregs), leading to the exhaustion of patient's effector cells. Tregs also dampen the efficacy of infused T cells, the reason why they have been used to prevent graft versus host disease after hematopoietic stem cell transplantation.[23] Radiotherapy has interesting properties prone to combat the antagonistic immune landscape of advanced solid tumors and by this way improve the efficacy of CAR-T cells in this setting.

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2.1. RT-induced tumor debulking may increase antitumor efficacy while decreasing toxicity of CAR-T cells

As shown in B-ALL and LBCL,[24,25] a lower initial tumor burden is a major predictor of response to CAR-T cell treatment. Indeed, a strong correlation was observed between durable response and the peak CAR-T cell levels in blood normalized to pretreatment tumor burden in LBCL. These observations highlight a minimum threshold of specific T cells relative to the number of tumor cells for the treatment to be efficient, which refers to the classical effector to tumor cell ratio in immunology.[26] Furthermore, decreasing tumor burden and tumor heterogeneity before CAR-T cell infusion may reduce the risk of inducing tumor resistance due to the selection of refractory clones characterized by loss or decrease of the antigen targeted by CAR-T cells.[27,28] Moreover, a high marrow tumor burden has been identified as an independent predictor of cytokine release syndrome (CRS) in 133 patients enrolled in a prospective phase I/II trial receiving anti-CD19 CAR-T cells.[29] Therefore, by debulking tumors, RT aims to increase the efficiency of CAR-T cells by depleting some resistant clones while limiting the rate of CRS by decreasing tumor burden.

2.2. Promoting the homing of CAR-T cells into solid tumors

Solid tumors include a large number of stromal cells of various types. These cells, by forming a natural barrier, restrict the possibility for immune cells to penetrate TME.[30] Radiotherapy (RT) has shown its ability to permeabilize the peritumoral stroma on various levels:

Remodeling vascularization

The dysfunctional vascularization often encountered in solid tumors hampers the homing of effector T cells within the tumor microenvironment, leading to tumor immune escape.[31] Therefore, normalizing vascularization and reducing hypoxia are required for the efficacy of immunotherapies, including adoptive T cells.[32] Mondini et al. first demonstrated by using a murine orthotopic model of head and neck squamous cell carcinoma (HNSCC) expressing HPV16 that a combination of single-dose of 7.5 Gy and anti-HPV vaccine increased both pericyte coverage and the expression of ICAM-1 on tumor vessels. This led to enhanced intra-tumor vascular permeability, an increased number of intra-tumor $CD8^+$ T cells, and decreased tumor-growth rate and improved survival that were not observed when using vaccine or RT alone.[33] Moreover, human CAR-T cells show both reduced expansion and cytokine secretion when cultured in hypoxic conditions.[34] From this point of view, hypofractionated ablative RT has shown its ability to normalize tumor vasculature in murine lung tumors,[35] as well as in non-small cell lung cancer patients' derived xenografts.[36] Moreover, it induced both an intense infiltration by $CD8^+$ T cells and a loss of myeloid-derived suppressor cells (MDSC) in two murine colon carcinoma models.[37] A similar pattern of vasculature normalization was assessed using functional imaging in murine pancreatic carcinoma models receiving 35 to 45 Gy in 4 to 5 fractions.[38,39] Therefore, using RT may provide an option for better homing of adoptive T-cell therapies. RT also promotes the growth of new blood vessels through an increase in endothelial nitric oxide synthase (NOS) abundance, contributing to revascularization.[40] Finally, irradiated endothelium is prone to show a more adhesive phenotype, thus facilitating the transmigration of immune cells.[41,42] Therefore, since radiotherapy has showed its effects on both revascularization and reoxygenation of solid tumors, with benefits observable with a large array of doses ranging from low doses of 1–2 Gy to dozens of grays,[43–45] further experimentations should consider it as a prime partner in CAR-T cell-based approaches, with the aim to improve the homing of infused cells.

Effects of RT on chemotaxis

In some aspects, RT has the ability to polarize the tumor microenvironment into an inflammatory phenotype prone to exert an efficient antitumor action and to show a synergistic action with immunotherapy.[46] This polarization is associated with type I and II interferon responses[47,48] with cGAS-STING pathway activation and with the secretion of multiple chemokines within tumor microenvironment,[49] such as CXCL9,[50] CXCL10,[51] CXCL11[52] or CXCL16,[53] leading to chemoattraction of either endogenous or adoptive lymphocytes to the tumor site. Moreover, immunogenic cell death induced by RT acts as a trigger for harnessing the host's immune system to attack remaining tumor cells.[54] All these features contribute to an inflammatory immune environment, which could foster the homing of CAR-T cells. However, RT also promotes immunosuppressive mechanisms, by notably increasing the infiltration of regulatory T cells (Tregs),[55] myeloid-derived suppressor cells (MDSCs)[56] and developing tolerant anti-inflammatory macrophages.[57,58] Faced with this observation, whether RT will promote or limit the attraction and homing of infused T cells will depend on a balance between subsequent activating and inhibiting signals. In this regard, the scheme of RT more prone to potentiate immune antitumor signaling is still debatable, with scarce preclinical elements indicating a deleterious profile mediated by the expression of exonuclease TREX1 following high doses per fraction beyond 12 Gy.[59] Therefore, the translation of these findings requires further clarification among preclinical models used, as well as considerations about radiation timing and dose.

Cancer-associated fibroblasts (CAF)

Lastly, tumor stroma and CAFs are known to play a crucial role in immune escape.[60] In a mouse model of intrarectal orthotopic tumor, Nicolas et al. demonstrated RT's ability to modulate the phenotype of inflammatory CAF (iCAF), shown as predictors of poor response to neoadjuvant therapy in rectal cancer patients,[61] depending on IL-1. In another intrarectal orthotopic model, RT-activated CAFs promoted survival of tumor cells through the activation of IGF1 receptor (IGF1R), and RT followed by the neutralization of IGF1R reduced the number of mice with organ metastases.[62] These studies demonstrate the relevance of associations using RT and agents targeting tumor stroma.

2.3. To enhancing activation of adoptive T-cells in the solid tumor core

TCR engineered T cells (TCR-T cells), in contrast to CAR-T cells, bind to their corresponding antigen after the antigen has been processed by major histocompatibility complex class I (MHC I). This process is represented in Figure 1. This makes TCR-T cells able to recognize mostly intracellular antigens, whereas CAR-T cells only bind cell surface antigens. RT is known for its ability to enhance the presence of MHC I at the surface of tumor cells. This was first-assessed in the early 2000's by Garnett et al. in preclinical work in which eight human carcinoma cell lines (five colon, three lung) showed increased expression of MHC I 72 hours after non-lethal irradiation of 10 to 20 Gy, with subsequently enhanced susceptibility of tumor cells to T-cell mediated attack.[63] Two years later, Reits et al. confirmed these findings in human cutaneous melanoma cells for doses beyond 7 Gy, as well as in MC38 mouse colon carcinoma cells. In addition, in MC38 subcutaneous tumors, the combination of 10 Gy irradiation and adoptive T-cells led to marked inhibition of tumor growth,[64] thus creating a solid view for the future of such an association in clinical trials. Finally, since RT is able to enhance the expression of MHC class I at the surface of tumor cells, it may also limit immune escape which is related to the loss of MHC class I expression in multiple cancers.[65–67] This could enhance the objective response rate of TCR-T cell therapies and limit further tumor immune escape in the setting of combinatorial approaches.

In regard to CAR-T cells, particular attention should be given to those targeting cell surface antigens for which high expression levels are associated with poor response to RT, i.e. the ErbB family including epidermal growth factor receptor EGFR (ErbB1)[68,69] and ErbB2 (HER2).[70,71] Thus, targeting these pathways using CAR-T cells could result in limited survival of radio-resistant clones prone to drive tumor progression or relapse. Although, even if more than 40 clinical trials experimenting HER2 (NCT03182816; NCT05341492; NCT02873390; NCT03198052; NCT0368167) or EGFR (NCT03696030; NCT04650451; NCT04995003; NCT0092044) targeted CAR-T cells in solid tumors are ongoing, only two trials experiment associations between CAR-T cells targeting EGFR and radiotherapy in glioblastoma. These trials are included in Table 1. In the other trials, conditioning regimens, when described in protocols, use chemotherapy alone and do not include radiotherapy as a possible option.

Another issue to consider for the activation of adoptive T cells in solid tumors is the up-regulation of immune suppressive checkpoints within the tumor microenvironment likely to thwart the action of both endogenous and adoptive T cells and drive tumor immune escape. This challenge is of critical importance since high doses of RT are likely to enhance the expression of several of these checkpoints, including programmed-cell death ligand 1 (PD-L1),[72–74] cytotoxic T-lymphocyte associated protein 4 (CTLA-4),[75,76] lymphocyte-activation gene 3 (LAG-3)[77] and T cell immunoreceptor with Ig and ITIM domains (TIGIT).[78]

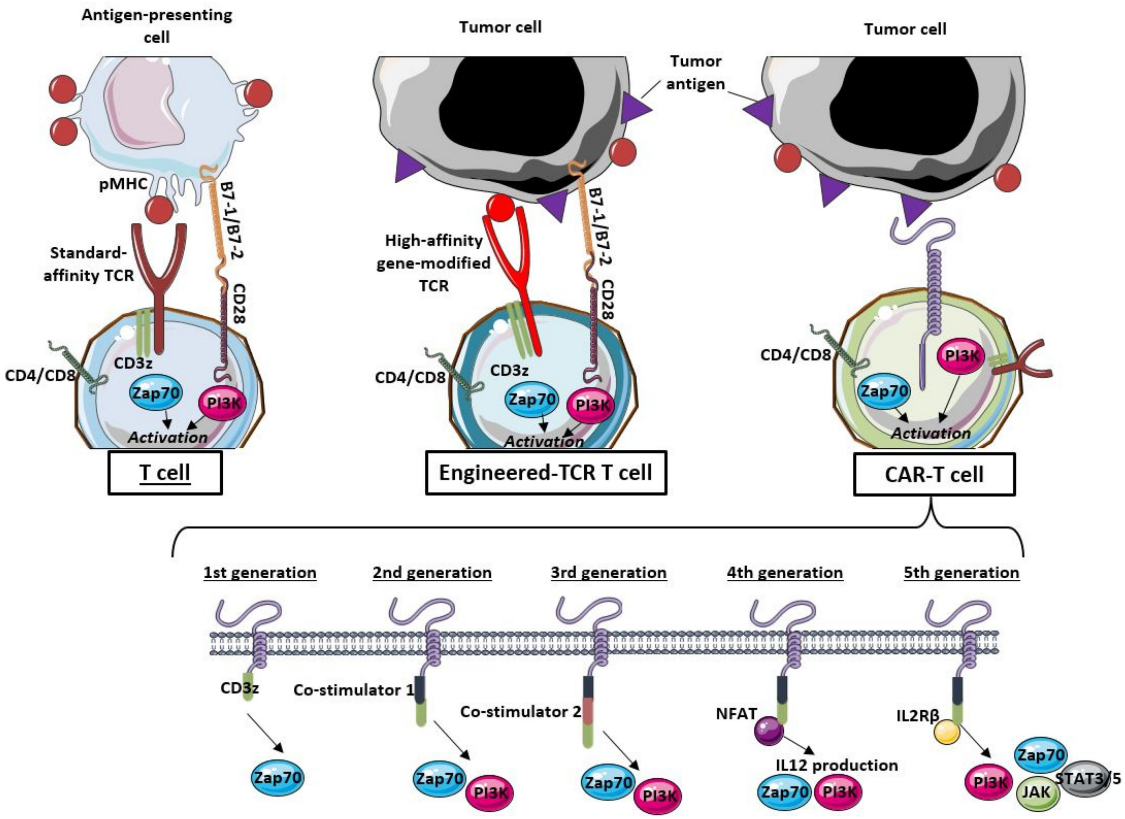

Figure 1. Patterns of activation of conventional T cells, gene-modified TCR-T cells and CAR-T cells in the tumor immune microenvironment. (pMHC: peptide presented by major histocompatibility complex; TCR: T-cell receptor; PI3K: phosphoinositide-3 kinase; JAK: janus kinase; STAT: signal transducer and activator of transcription)

Table 1. Brief summary of current clinical trials involving adoptive T-cell therapies and radiotherapy in solid tumors.

Clinicaltrials.gov identifier	Study phase	Conditions	Interventions	Locations	Status
NCT03412526	Phase II	Ovarian Cancer	Total body irradiation 2GyIL-2 Tumor-infiltrating lymphocytes (TIL)	Ramat Gan, Israel	Recruiting
NCT03396575	Phase I	Pediatric glioma	Radiotherapy Temozolomide Ex-vivo expanded TIL	Gainesville, USA	Recruiting
NCT02584829	Phase I	Merkel cell carcinoma	Radiotherapy Avelumab	Seattle, USA	Active, not recruiting
	Phase II		Autologous T-CD8+ cells Interferon beta	Washington, USA	
NCT03347097	Early Phase I	Glioblastoma	Chemoradiotherapy TIL expressing PD1	Shanghai, China	Active, not recruiting
NCT01758458	Phase I Phase II	Merkel cell carcinoma	Radiotherapy Recombinant human IL-2 Intralesional injection of interferon beta	Seattle, USA Washington, USA	Terminated
NCT00512889	Phase I	Melanoma	Radiotherapy GM-SF Autologous activated lymphocytes	Boston, USA	Completed
NCT03344250	Phase I	Glioblastoma	Anti-EGFR TCR-T cells Chemoradiotherapy	Charlottesville, USA	Active, not recruiting
NCT05022849	Phase I	Castration-resistant prostate cancer	KLK2 CAR-T cells Radiotherapy (option) Chemotherapy (option)	Multiple centers, USA	Recruiting
NCT03132922	Phase I	Multiple solid tumors	Low-dose radiotherapy 1.4 Gy Gene-modified TCR-T cells	Multiple centers, USA	Active, not recruiting
NCT02664363	Phase I	Gliblastoma	Chemoradiotherapy EGFRvIII CAR-T cells	Durham, USA	Terminated

However, these findings were the substrate for breakthrough trials experimenting combinatorial approaches associating RT and immune-checkpoint blockades, leading to practice changes and improvements in patients' survival.[79,80] Therefore, this negative feature does not impair the potential benefits of combining RT with adoptive T-cell therapies in solid tumors. Indeed, several options may be used to counteract these effects. These options may include the association of immune-checkpoint blockades to adoptive T cells and RT, or the use of newly engineered CAR-T cells either showing dominant negative TGF β,[81] or switch receptors PD1/CD28,[82] or even producing immune checkpoint blockades (ICB) targeting PD1.[83] Moreover, since RT may up-regulate these inhibitory checkpoints in a dose- and fractionation-dependent manner,[78] research must be conducted on the best RT schedule to both maximize activating and minimize inhibiting features.

2.4. Radiation-induced lymphopenia as support for proper expansion of engineered T cells

Radiation-induced lymphopenia (RIL) is a common adverse event in radiotherapy treatments, often reported as jeopardizing the efficacy of either concurrent or subsequent immunotherapy administration.[84–86] This frequent condition is largely due to the high radiosensitivity of lymphocytes, assessed in-vitro with near 90% of lethality in a population of human lymphocytes irradiated with a single dose of 2 Gy.[87] This observation was the basis for further studies highlighting the impact of RT on the reduction of circulating lymphocytes following conventional fractionation RT for the treatment of high-grade gliomas.[88] Of note, even before the era of intensity-modulated RT and radioimmunotherapy combinations, the effects of RT on reducing lymphocyte counts were well established.[89–91] Therefore, even when practiced in non-myeloablative conditions, *e.g.* as a local ablative treatment, RT can induce lymphopenia in the patient and therefore may favor the expansion of infused T cells in the setting of CAR-T cell transplantation. In this setting, the sequence between ablative RT and CAR-T cell infusion is crucial, since RT should be delivered before the infusion of T cells.

Interestingly, the ability of RT to deplete patient's lymphocytes led to consider total-body irradiation (TBI) as a valuable conditioning option before hematopoietic cell transplantation (HCT) in combination with chemotherapy.[92] In this setting, two trials have demonstrated the equivalence of chemoradiotherapy (CRT) and chemotherapy (CT)-based regimens in chronic myeloid leukemia. Moreover, in acute myeloid leukemia,[93,94] one trial has showed both clinical and biological superiority of the CRT-based approach over CT alone.[95] Although a strategy of lymphocyte depletion may be controversial in case of a metastatic patient, it remains crucial before CAR-T cell infusion to allow their proper expansion, whether it uses CT, RT or both. From this point of view, adding a large-volume and low-dose irradiation to CT during the conditioning phase would combine an efficient lymphocyte depletion to the biological benefits of low-dose RT in solid tumors relying upon mobilizing both innate and adaptive immunity. These benefits are detailed later in this article and notably include the secretion of IFN γ,[96] the recruitment of Th1 $CD4^+$ and activated dendritic cells,[97] the polarization of macrophages, the decrease of TGF β secretion and the infiltration of NK cells.[98] All these features are prone to enhance the action of subsequently infused T cells.

However, the duration and depth of lymphopenia after conditioning are crucial issues. Indeed, patients have to recover an appropriate lymphocyte count fast enough not to compromise long-term adaptive response against solid tumors. Moreover, lymphocyte depletion must not lead to the progression of patients before T-cell infusion. From this perspective, experiments remain to be done about the best CT and RT doses and timing to achieve positive and safe outcomes before HCT. A recent case series of patients with relapsed or refractory large B-cell lymphoma has demonstrated the safety and efficacy of a CRT-based conditioning option.[99] In this retrospective review, 12 patients received bridging RT on target lesions before the infusion of Axicabtagene Ciloleucel. RT was delivered on a large volume, ranging from one hip to the whole abdomen. The dose of RT was flexible, ranging from 6 Gy in 3

fractions to 36.5 Gy in 14 fractions, according to irradiated volume. Four patients out of 12 did not receive any concurrent bridging systemic therapy. No significant toxicity was observed during bridging RT. At 30 days, the objective response rate was 81.8%, with complete response in 27% of evaluable patients. These outcomes were observed despite a slight decrease in lymphocyte counts in 10 patients.

Therefore, RT in a large volume with a controlled dose may represent an interesting conditioning option with CT instead of myeloablative TBI questionable in a patient with metastatic solid tumor. A comparison of the various available regimens of CRT in the setting of CAR-T cells according to their respective immunomodulatory effects is needed.

2.5. Promoting a durable response despite radiation-induced lymphopenia: an impossible challenge?

The currently scarce use of CRT-based conditioning regimens before CAR-T cell infusion may be explained by the apprehension of inducing excessive lymphopenia and therefore compromising the generation of a durable antitumor adaptive immunity driven by endogenous memory T cells. Indeed, because the presence of infused T cells is time-limited, especially in the case of allogeneic T cells eliminated by the host immune system, the generation of an adaptive immunity is necessary to achieve a long event-free survival. By using an oncolytic vaccine and a preclinical model of methylcholanthrene-induced fibrosarcoma, Walsh et al. demonstrated the contribution of both transferred and endogenous T cells in providing long-term survival after adoptive T-cell therapy.[100] Thus, transferred T-cells destroyed tumor masses, while endogenous T-cells prevented immune escape by limiting the emergence of new tumor-associated antigens (TAA). Another study by Alizadeh et al. illustrated in a syngeneic model of murine glioblastoma the critical role played by adoptive T cells in the induction of endogenous T-cell memory response through activation of the IFNγ pathway and activation of intratumoral macrophages.[101]

Moreover, beyond the issue of radiation-induced lymphopenia, high doses of focal RT have shown their ability to release great amounts of tumor-specific antigens that further act like danger-associated molecular patterns (DAMPs) for the activation of endogenous T-cells mediated by antigen-presenting cells (APC).[102,103] This process leads to the generation of endogenous memory T-cells acting as an antitumor in-situ vaccine represented in Figure 2.

Therefore, facing these data, solutions must be found to limit lymphopenia during conditioning phase in the setting of combinatorial approaches associating adoptive T cells with RT. This is a highly challenging condition, since lymphopenia must be present to ensure an appropriate expansion of adoptive cells, but must not be too high, as to hamper further antitumor adaptive immunity. The following options may be explored to reach this purpose.

Choosing an appropriate conditioning regimen

The question of the best conditioning regimen before HCT remains controversial. Moreover, RT is inconsistently used with CT in this setting. Nissani et al. made a comparison between different non-myeloablative conditioning regimens

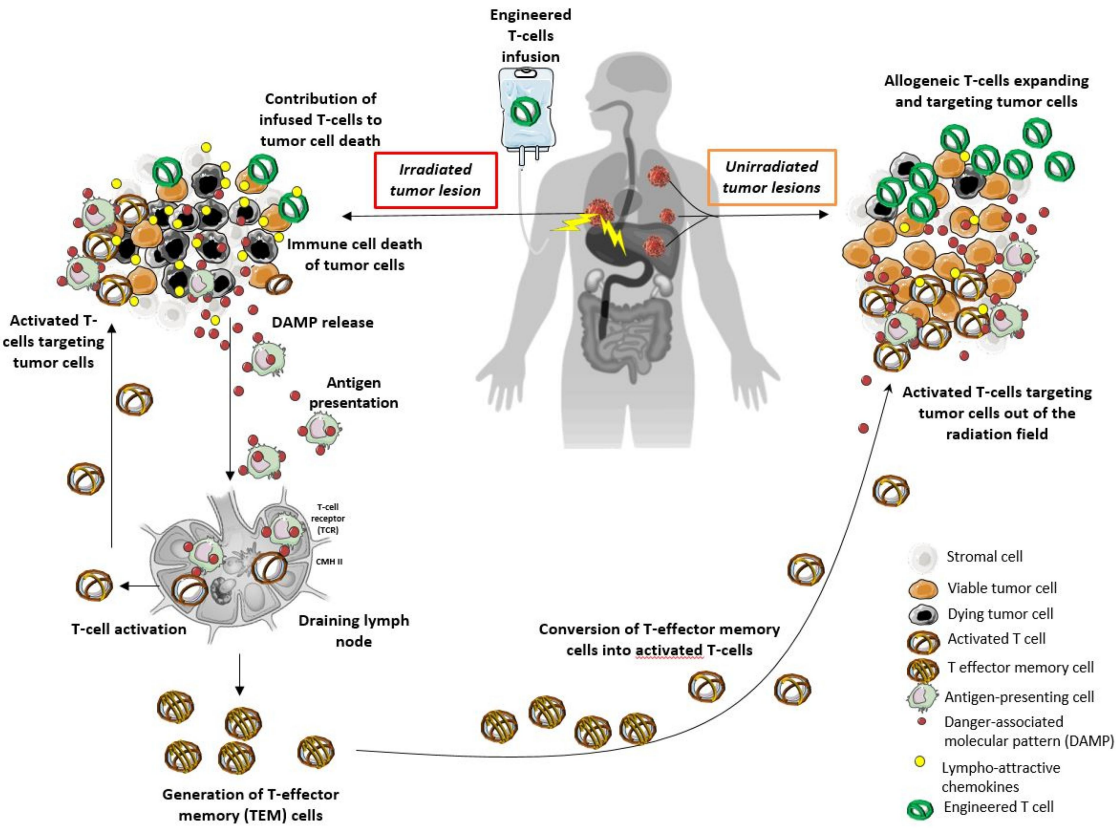

Figure 2. Radiotherapy as an in-situ vaccine in combination with adoptive T-cell therapies.

in patients with metastatic melanoma treated using an infusion of tumor-infiltrating lymphocytes (TILs).[104] They demonstrated that a combination of low-dose TBI of 2 Gy and 75 mg/m2 fludarabine was less efficient in depleting lymphocytes compared to chemotherapy regimens with cytarabine and fludarabine. The authors negatively concluded about the association between TBI and fludarabine, arguing its use resulted in insufficient bone marrow suppression. However, this treatment provided at least 2 days of severe lymphopenia in a large proportion of patients by using a low dose of RT and therefore showed a good safety profile. Based on the assumption that excessive lymphopenia would compromise long-term immune response, these results should reconsider the option of CRT-based conditioning regimens, with the possibility for clinicians to make TBI dose vary according to the desired depth of lymphopenia. Alternatives to TBI may include high-dose focal irradiation of target lesions, with surrounding areas irradiated with scattered low-doses. A brief overview of clinical trials associating CAR-T or TCR-T cells and RT is exposed in Table 1. Of note, only two trials are currently reported as experimenting a combination of chemotherapy and low-dose irradiation followed by TCR-T or CAR-T cell infusion in solid tumors.

Challenging the conditions of irradiation

Beyond the question of conditioning regimen, radiation oncologists must pay careful attention to dosimetry, notably within areas surrounding high-dose fields. Indeed, these volumes are often significant and receive scattered low-doses likely to impact lymphocyte survival. From this point of view, a comprehensive understanding of the expected occurrence, duration and depth of lymphopenia caused by his treatment is a difficult but crucial challenge to the radiation oncologist. It may require usual analysis of dose-volume histograms[105–107] as well as a composite set of criteria[108] or use recent machine learning solutions.[109]

Moreover, in the setting of metastatic patients, internal radiotherapy delivered using α particles may be a promising option, as it deposits high energy over a short path length. In hematological malignancies, this approach is experimented in acute myeloid leukemia (AML) with the anti-CD33 antibody lintuzumab conjugated with actinium-225. The results of a recent phase I trial showed a maximum tolerated dose of 111 kBq/kg.[110] Up to that dose, no event of myelosuppression longer than 35 days was reported. The prevalence of treatment-related lymphopenia was difficult to evaluate due to coexisting baseline suppression of hematopoiesis by active AML present in 11 patients out of 18.

Menager et al. tested the combination of α-radiotherapy with adoptive T cell transfer in a murine model of myeloma expressing the tumor antigen CD138 and ovalbumin (OVA).[111] By using an anti-CD138 antibody coupled to bismuth-213 and followed by adoptive transfer of OVA-specific $CD8^+$ T cells, they observed a significant tumor growth control and an improved survival in the animals treated with multimodal treatment. Interestingly, at the end of the experiment, infused T-cells were still present in tumor and lymph nodes, assessing the persistence of infused lymphocytes despite the concurrent presence of α emitters.

In the setting of solid tumors, α-radiotherapy is a source of major advances in castration-resistant prostate cancer. In a phase III trial, the use of lutetium-177-prostate specific membrane antigen (PSMA) led to improvements in progression-free survival (PFS) and overall survival (OS) compared to standard of care.[112] Moreover, according to a recent study of biodistribution and safety, lutetium-177-PSMA lost 90% of its initial activity within the first 70 hours following administration.[113] This resulted in only 2 patients out of 51 presenting leukocytopenia of grade 3 or more, which is congruent with the 2.5% rate of leukopenia grade 3 or more presented in the phase III trial previously mentioned. Therefore, in prostate cancer, α-radiotherapy should not impair the efficacy of a subsequent adoptive T-cell transfer due to cytotoxicity against infused T cells. A similar approach of combined treatments using α-radiotherapy may be experimented in a wide variety of solid tumors, as the field of available radiopharmaceuticals is rapidly growing.[114]

Finally, an appropriate solution to improving homing and activation of infused T cells and allow their proper expansion by depleting endogenous lymphocytes without compromising further immunity would be to combine both high-dose and low-dose irradiation. In this way, treating different volumes with different levels of dose could help to treat a high number of lesions in a patient. Indeed, in patients with advanced and refractory diseases, it may be difficult to treat a significant number of lesions with conventional or high-dose RT because of unacceptable toxicity. In such a setting, the complementary use of low-dose RT could make these patients benefit from the radiobiological properties of both high-dose and low-dose RT in combination with CAR-T cells. From such a perspective, an overview of the features of RT within the tumor microenvironment according to fractionation is presented in Figure 3. The issues of combining low-dose RT, high-dose RT and adoptive T-cell therapies are described later in this article.

2.6. CAR-NK cells better than CAR-T cells in combination with RT? The latest arrival in the field of engineered-cell therapies

Natural killer (NK) cells constitute an important component of innate immunity, accounting for 5–15% of peripheral blood leucocytes in human. Conversely to T cells, NK cells can generate tumor cell death through a large variety of mechanisms that do not require the presence of antigen-specific TCR. Their activation is mostly based on a balance between germline-encoded activating and inhibiting signals, with thus a natural trend towards killing tumor cells known for their ability to down-regulate the expression of HLA.[115] In a study by Weiss et al., a CAR-T cell based on the activating NK signal NKG2D has demonstrated prolonged survival in mice bearing glioblastoma, and even cured a fraction of them. Moreover, the combination of NKG2D-based CAR-T cells with RT resulted in a synergistic activity in two different mouse glioma models.[116]

After the large success of engineered T-cell therapies in the treatment of refractory hematological malignancies, researchers have shifted their attention towards the creation of modified NK cells targeting antigens of interest, including chimeric antigen receptor NK cells, or CAR NK-cells. The early-phase

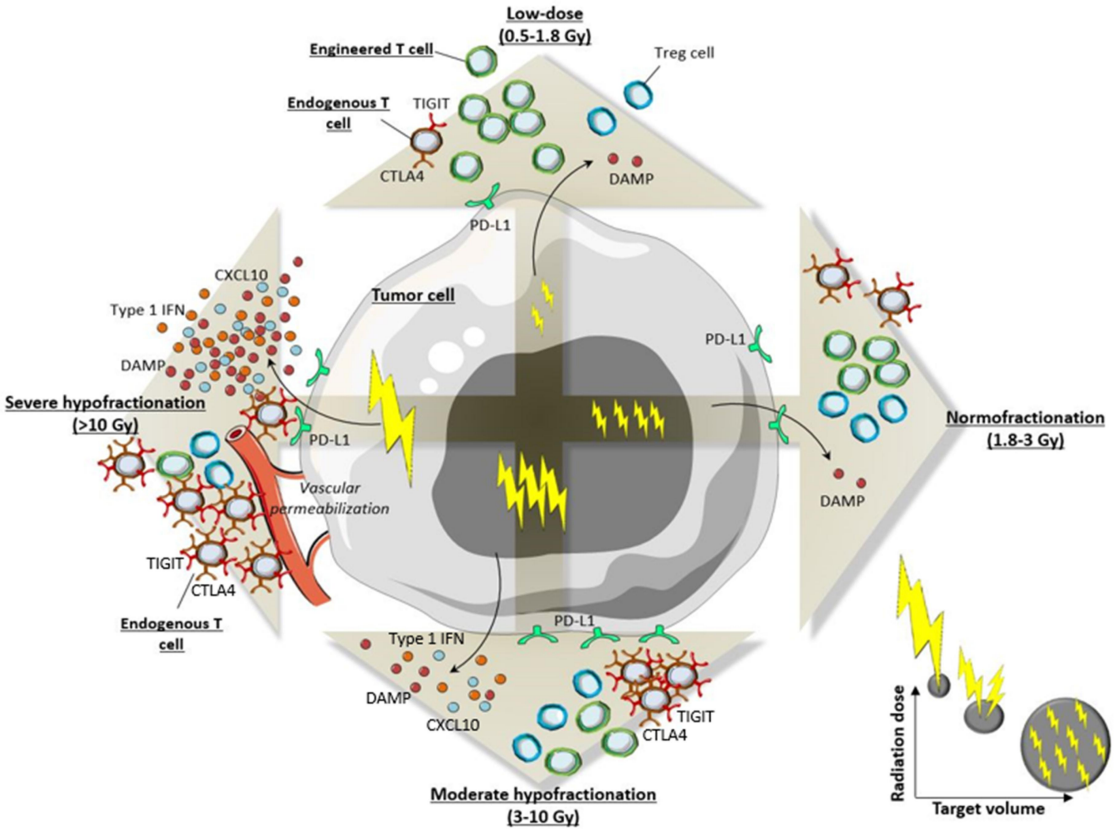

Figure 3. Comparative biological effects of RT within tumor microenvironment according to fractionation. (DAMP: danger-associated molecular pattern; CTLA4: cytotoxic T-lymphocyte-associated protein 4; PD-L1: programmed-death ligand 1; TIGIT: T cell immunoreceptor with Ig and ITIM domains; IFN: interferon; Gy: Gray)

experimentation of these new agents in CD-19 positive tumors showed low rates of cytokine release syndrome and neurotoxicity with encouraging signs of efficacy.[117] This resulted in widening the scope of CAR-NK-based treatments by using new NK-based constructs targeting HER2 in breast cancer,[118] EGFR in glioblastoma[119] or mesothelin in ovarian cancer.[120] However, the activity of CAR-NK cells can be thwarted by the up-regulation of inhibitory ligands at the surface of tumor cells, e.g. HLA-G in Ewing sarcoma.[121,122]

From this perspective, high doses of RT beyond 10 Gy are prone to up-regulate activating ligands at the surface of tumor cells, notably those recognized by NKG2D, as it has been demonstrated *in-vitro*.[123–125] Furthermore, in an *in-vivo* model of canine sarcoma, a single-fraction of 2 Gy followed by injection of a plasmid encoding recombinant human interleukin 15 (rhIL-15) enhanced both intra-tumor homing and cytotoxicity of subsequently infused NK cells.[126] Finally, RT in combination with blocking of PD-L1/PD-1 pathway increased antitumor cytotoxicity of NK cells in human nasopharyngeal carcinoma.[127]

Considering these encouraging data, RT, either used alone or in association with chemotherapy or immune-checkpoint blockades, should be considered in further early-phase clinical trials in association with CAR-NK cells. Such a combination may challenge the line between their inhibiting and activating signals and thus polarizing them into an inflammatory and antitumor phenotype. For this purpose, targeting the inhibitory checkpoint NKG2A[128] or the heterogeneous KIR family[129] at the surface of host NK cells may also constitute an interesting option in association with RT.

3. Optimizing the efficacy of T-cell therapies in solid tumors using radiotherapy: how?

The outstanding technical improvements in RT practices over the last decade have greatly broadened the indications and modalities of RT in the treatment of solid tumors, with more than 60% of cancer patients requiring RT.[130] Therefore, at the era of widespread use of immunotherapy, an important concern remains, regarding the scheme of RT more prone to enhance the action of immunotherapy in a combinatorial setting.[131] While this issue is the field for a large body of research regarding the sequences of immune checkpoint blockades and RT, a similar reflection should be engaged as part of the optimization in further combinatorial strategies associating T-cell therapies and RT.[132,133] Different settings regarding the volume to irradiate and the dose to deliver can be compared one with another with the aim to improve the efficacy of adoptive T-cell therapies using RT.

3.1. Option 1: a small volume irradiated with high doses of RT, improving the homing and efficacy of CAR-T cells

The implementation of stereotactic radiosurgery (SRS) has improved local control in most solid tumor types.[134] By

delivering high doses of radiation per fraction on a small volume, i.e. macroscopic tumor with limited margins, SRS is able to increase the biologically equivalent dose (BED) delivered, leading to higher local control rate.[135–137]

Congruently with its direct antitumor effects, SRS is also able to reshape the tumor microenvironment (TME) in a way that could synergize with adoptive T-cell therapies to various levels. First, high doses of RT are able to induce immunogenic cell death (ICD), a critical feature for dendritic cell (DC) activation and effector T-cell priming.[138,139] This condition can be enhanced by the concomitant use of radiosensitizers, as demonstrated with hafnium oxide nanoparticles NBTXR3. In a preclinical model of murine colorectal tumor, NBTXR3 injected intratumorally and then activated by 12 Gy in 3 fractions increased $CD4^+$, $CD8^+$ and $CD68^+$ cell infiltrates compared to radiotherapy alone. Moreover, significant modifications in TCR repertoire diversity were found in radiotherapy-activated NBTXR3 group, not only in tumors treated with combination but also in distant untreated tumors.[140] Other alternatives of radiosensitizers prone to increase ICD in combination with high-dose RT include gold nanoparticles[141] or repurposing anti-alcoholism drug disulfiram.[142]

Furthermore, the ability of high-dose RT to activate immune microenvironment in solid tumors depends on the dose and fractionation.[143] As an example, Vanpouille-Box et al. demonstrated 24 Gy delivered in 3 fractions of 8 Gy increased $CD8^+$ and Th1 cells within tumor microenvironment in TSA murine tumors through the secretion of activating cytokines and cGAS-STING pathway. However, in three different murine cell lines, when the single-fraction dose exceeded 12 Gy, the activation of DNA exonuclease TREX1 impaired the activation of cGAS-STING pathway by decreasing the amount of double-stranded DNA within the cytoplasm of tumor cells.[59] The overexpression of several immune checkpoints may also participate to this relative immunosuppression induced by high doses, as demonstrated with PD-L1, Galectin-9 and herpes virus entry mediator (HVEM) in B16-F10 cells.[144] This supports the association of immune checkpoint blockades to high-dose RT described later in this section.

Similarly, high doses of RT above 5 Gy increase the expression of cell-adhesion molecules such as intracellular adhesion molecule (ICAM-1) and vascular-cell adhesion molecule (VCAM-1),[145] enhancing the adhesion of lymphocytes to endothelial cells and then their extravasation to TME. Moreover, the high level of lethality observed in tumor cells following SRS may lead to the depletion of clones likely to develop mechanisms of resistance to adoptive T-cells and therefore limit further immune escape.

On the other hand, high doses per fraction can also promote the recruitment within TME of regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs), and anti-inflammatory tumor-associated macrophages, known for their pro-tumor/immunosuppressive properties.[131] Such findings force the development of strategies prone to maintain a positive antitumor immune balance within TME and hence ensure the action of both endogenous and infused T cells. Facing this challenge, recent improvements in functional imaging and radiomics are prone to estimate in a non-invasive manner the amount of effector cells[146–148] as well as the presence of hypoxic areas[149] within tumors. This could help to select lesions more likely to benefit from RT while increasing the dose in the immunological "no man's land" constituted by hypoxic areas.[150]

Several other options can also be experimented, including the simultaneous use of ICB targeting PD-1, CTLA-4, PD-L1 or TIGIT that are up-regulated by RT in a dose- and fractionation-dependent manner as mentioned earlier in this section. Newly engineered CAR-T cells harboring switch receptors or producing ICB may also be a solution and are currently challenged in early-phase experiments. Other options may include administrations of cyclophosphamide, known for efficiently depleting Tregs, as demonstrated in animals as in humans with good tolerance.[151–153] Therefore, the association of such treatments to associations between CAR-T cells and RT should deserve more consideration from further experiments, since it is likely to enhance the efficacy of CAR-T cells by combating immunosuppressive pathways within tumor microenvironment.

3.2. Option 2: a large volume irradiated with low doses of RT, facilitating the expansion of CAR-T cells and making the most of the surprising properties of low-dose RT

3.2.1 The definition of low-dose RT

From a technical point of view, there is no clear consensus on the definition of a low dose of RT. In preclinical settings, a recent review analyzed 37 studies challenging combinations between RT and immunotherapy in various animal models from 1996 to 2019.[154] Of the 36 studies delivering local irradiation, 24 delivered a total dose of at least 10 Gy and 17 delivered at least 15 Gy. Moreover, RT was delivered in a single fraction in 15 studies, and in 1 to 4 fractions in 20 studies, with such hypofractionation increasing biologically effective dose (BED). As a comparison, most of preclinical studies experimenting low-dose RT and presented later in this section use total irradiation doses ranged from 0.1 to 2 Gy. Therefore, even if no clear definition exists about low-dose RT, such a level of dose may legitimately be evaluated as low. Furthermore, some authors differentiate a low dose of RT defined as up to 0.1 Gy from a moderate dose ranging from 0.1 to 2 Gy.[96] Obviously, even a dose of 2 Gy is far different from the usual preclinical radiation schemes described earlier and therefore may be considered as a low dose.

In a clinical setting, in contrast to preclinical experiments, there are clear recommendations on the prescription of dose according to the histopathology and staging of tumors. This dose usually varies between 50 and 80 Gy in 25 to 40 fractions for tumors treated with curative intent and may be over 100 Gy BED in case of hypofractionated stereotactic radiosurgery (SRS). Therefore, in this setting, as experimented in publications mentioned later in this review, 1 to 4 Gy delivered in a few fractions may legitimately be regarded as low-dose RT.

3.2.2 The interesting properties of low doses and applications

Historically, incidental RT has been presented as increasing the risk of cancer. This observation is largely due to the dose–response curves made on the basis of Hiroshima and Nagasaki bomb survivors.[155] However, these conclusions are unclear for

doses inferior to 1 Gy and for low-dose rate irradiation.[156] In addition, the incidence of cancer in patients after radiation exposure varies according to their age when exposed, with a relative decrease in older patients. Moreover, in this study, the dose received by survivors was retrospectively estimated according to their location and shielding at the time of bombing, and radiation consisted in gamma rays and neutrons which differ from X-rays in their radiobiological properties. For these reasons, low doses received by these cohorts should not be systematically extrapolated to medical workers or patients exposed to a low dose from a known source of x-rays. From that perspective, an epidemiologic study demonstrated male radiologists registered after 1955 in England have a negative standard mortality ratio (SMR) for death from cancer compared to all male medical practitioners (SMR 0.68).[157] This trend was similar in 145,915 radiologic technologists registered between 1926 and 1982 in the United States.[158,159] Finally, in patients, a recent retrospective work showed that, after RT for breast cancer, radiation-induced sarcomas mostly occur, not within areas irradiated with low doses but within those irradiated with doses superior to 30 Gy.[160]

Low-dose RT has been an expanding field of research over recent years. In contrast to high-dose conventional RT, it is able to treat large targets without compromising surrounding healthy tissues. Beyond depleting endogenous lymphocytes to allow a proper expansion of subsequently infused T cells, low-dose RT has also interesting radiobiological properties to exploit in solid tumors. When low doses of RT are delivered to non-transformed cells surrounding tumor cells, they are prone to induce apoptosis in cancer cells through reactive-oxygen species (ROS) signaling.[161,162] The unexpected properties of low doses translate into multiple animal studies showing tumor reduction and changes in host immune response after low-dose irradiation[163–172]

According to clinical practice, several applications of low-dose RT have emerged in recent years. By using a pre-clinical model of lung adenocarcinoma, Barsoumian et al. showed the synergistic effect of the association between a single low-dose of 2 Gy and a combination of both anti-PD1 and anti-CTLA4[98]. This result was explained by the ability of low-dose RT to reshape tumoral stroma into a proactive, antitumor phenotype. Moreover, in a pre-clinical model of advanced ovarian carcinoma, Herrera et al. used an association of low-dose RT with three different immunotherapies and cyclophosphamide to obtain a significant tumor growth delay through the activation of various pathways.[97] This resulted in early-phase experimentations of low-dose RT and immunotherapy in patients harboring advanced and metastatic ovarian carcinomas (NCT03728179), with expected results to come.

Regarding some other aspects of the immunosuppressive landscape in solid tumors, low-dose RT can play a key role, notably by reshaping the immune phenotype of the myeloid compartment within tumor microenvironment, with subsequent benefits on T cell migration and activity.[173–175] Moreover, a large amount of preclinical reports on various tumor models assessed the immune benefits of a low dose delivered in a large volume (whole-body irradiation), including the secretion of various pro-inflammatory cytokines as IFN.[163,166,167,176,177]

Therefore, even in the absence of preclinical data and ongoing trial associating low-dose RT and adoptive T-cell therapy, it makes sense to consider that low-dose RT may not be limited to the simple function of depleting patients' lymphocytes in order to ensure an optimal expansion of infused cells. The unique radiobiological effects provided by low doses could be helpful for the immune activation of solid tumors and thus could participate in the efficiency of infused T cells, in a different but complementary way to conventional/high-dose RT.

3.3. Final option: combining options 1 and 2, associating the properties of both low-dose and high-dose RT

Considering the data mentioned above and the different but complementary effects shown by high-dose and low-dose RT, a promising idea would be to associate both of them. This appears to be technically possible thanks to the ability of RT being spatially adjustable, which allows combining different doses delivered on different volumes in a same treatment. Such combinations between high-dose and low-dose irradiation may not require any specific process, since focal high-dose ablative RT often scatters large fields of low-dose within surrounding areas. As an example, this is illustrated in the dosimetry of a real patient treated with high-dose ablative RT, 60 Gy in 8 fractions delivered on 2 lung metastases, presented in Figure 4. In a setting of CAR-T cell therapy, this would result in the combination of positive effects underlying both high-dose and low-dose radiation. Such combinations are displayed in Figure 5, with examples of clinical outcomes already existing in literature. Menon et al. worked on a cohort of patients with various histological types receiving SRS associated with immune-checkpoint blockade.[178] Interestingly, they demonstrated that not only lesions irradiated with high-dose SRS showed good response but also lesions receiving 5 to 10 Gy (versus 50 Gy for high-dose RT) experienced a surprisingly high objective response rate, with more than half of them (58%) meeting the PR/CR criteria for RECIST. Of note, these lesions received either an intentional low-dose RT or low doses scattered from the volume treated with high-dose RT. This observation paved the way for further perspectives of associations between high-dose and low-dose RT in patients with solid metastatic tumors in association with immunotherapy, with promising results.[179–181]

Therefore, with regard to CAR-T cell therapies and similarly to observations in radioimmunotherapy combinatorial approaches, combining a low-dose of radiation delivered in a large volume with a high dose delivered in a limited volume would result in improving both the expansion, the homing and the activation of infused T cells. This hypothesis should be the starting point for the initiation of future clinical trials centered on spatially modulated bridging RT as a prime conditioning option, with the help of concurrent chemotherapy.

However, in this setting, there is no consensus regarding the optimal volume to irradiate with low or high dose. A suitable option may be to treat a limited volume of macroscopic tumor with a high-dose of RT in order to maximize homing and activation of infused T cells while preventing the patient from toxicity. For this purpose, partial irradiation could be a promising option, since it is likely to reshape the tumor microenvironment in

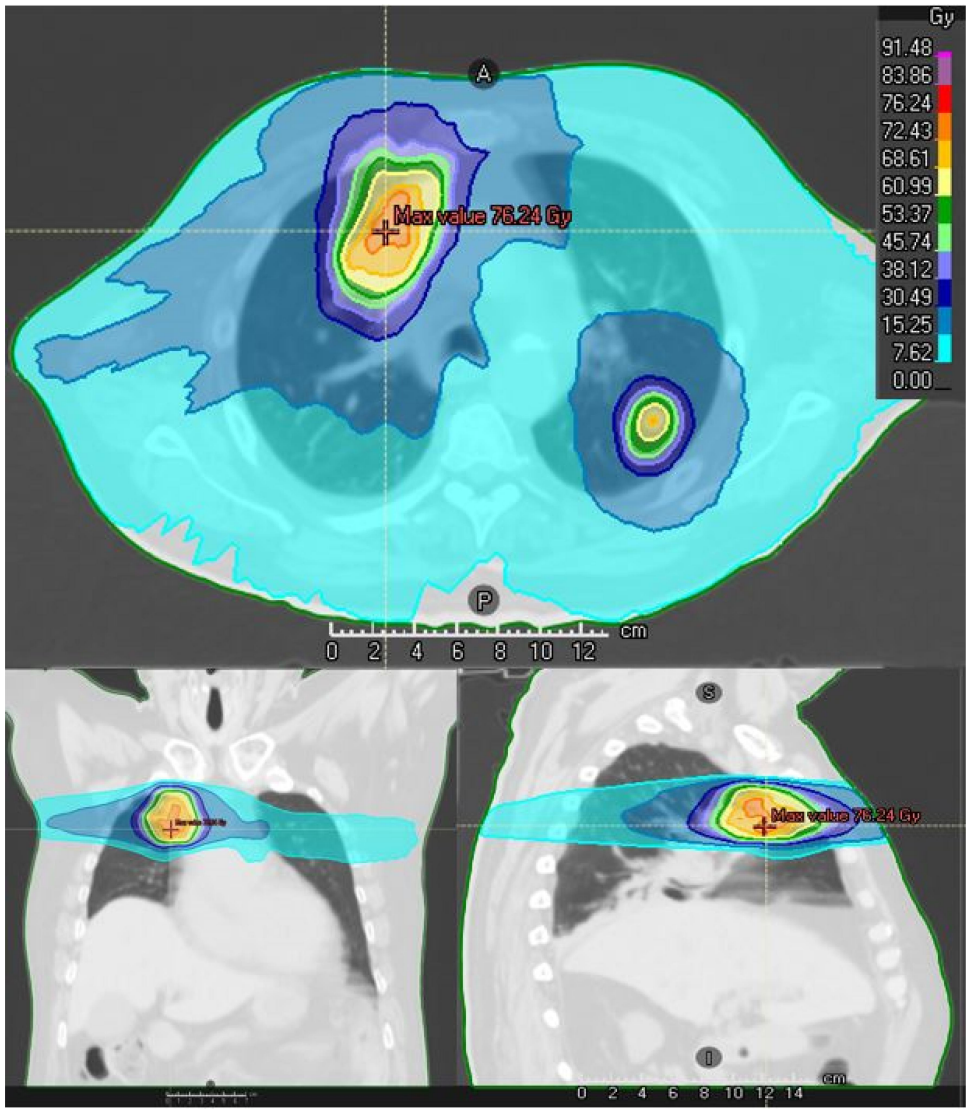

Figure 4. An example of patient treated with high-dose focal RT, 60 Gy in 8 fractions on 2 pulmonary lesions, illustrating large incidental low-dose radiation surrounding treated volumes.

a similar manner to conventional whole-tumor RT, leading to in-situ abscopal effect as demonstrated in two mice models.[182]

Finally, in the frequent setting of lung metastases, an interesting option may be the combination of low-dose total-lung irradiation (TLI) associated with focal high-dose RT on macroscopic tumors. In this setting, whole-lung irradiation may ensure the occurrence of radiation-induced lymphopenia while treating microscopic tumor involvement. In addition, according to safety profiles, it would provide a limited rate of radiation-induced lung injury for doses up to 5 Gy.[183]

4. New approaches in RT: paving the way for further preclinical and clinical experiments in the field of radioimmunotherapy combinations

As described in this review, defining the best conditions for the clinical implementation of combinatorial strategies using RT and CAR-T cells is challenging. Therefore, scientists and physicians must make the most of innovations designed in recent years.

4.1 Preclinical innovations in RT: treating mice as humans

The use of appropriate animal models remains crucial to increase the reproducibility of results and enable a good « bench to bedside » approach. Recent improvements include the use of next-generation devices able to deliver RT in similar conditions in mice than in patients. Thanks to their on-board imaging system, these devices deliver precise and controlled irradiation in mice due to visualization of the target.[184,185] In the near future, preclinical research will not be limited to TBI, subcutaneous tumors or *in-vitro* studies.

Moreover, to facilitate the transition between preclinical and clinical research, preclinical models should be as similar as possible to their clinical counterpart. In such a goal, humanized mice should be used more often. After myeloablative treatment and then transfusion with human hematopoietic precursor cells, they may represent an appropriate model to experiment new radioimmunotherapy combinations using patient-derived xenografts (PDX).[186] Of note, studies using humanized mice in the field of radioimmunotherapy combinations are currently scarce. Moreover, current preclinical associations between CAR-T cells

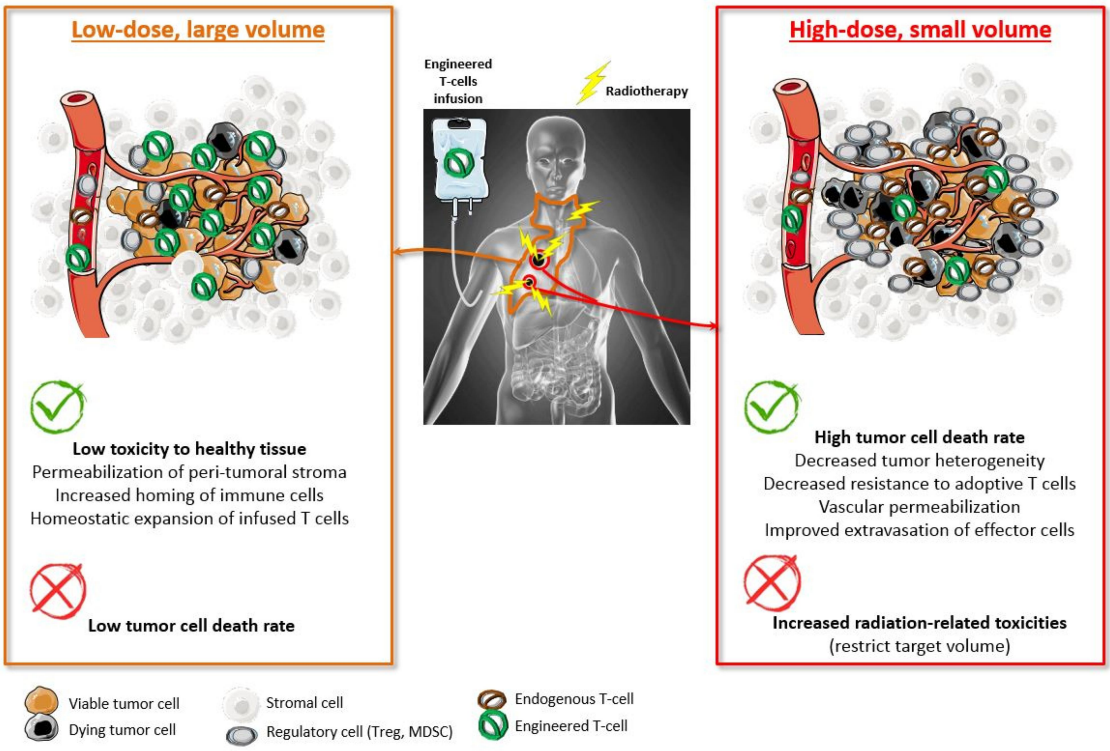

Figure 5. Complementary features between low-dose and high-dose RT in the setting of combinatorial approaches using engineered T-cell therapies.

and RT involve PDX of pancreatic[187] or prostate cancer[188] in immune deficient mice treated with fractionated low-dose. The use of a syngeneic model of murine glioblastoma in immunocompetent mice is also reported.[189]

4.2 Technological innovations in RT: FLASH ultra-high dose rate RT, proton beams, minibeams

Significant technological advances over the past decade in RT have translated into great opportunities and major changes in the landscape of cancer management, with the continuous aim to spare normal tissue as efficiently as possible. From this perspective, ultra-high dose rate RT, also called as FLASH-RT, has demonstrated its ability to enhance the differential effect of RT between tumors and normal tissues.[190] However, the biological mechanisms underlying this effect are unclear, and a high variability remains depending on the type of tissue irradiated (acute or late-responding tissue), the particle used (photon or proton), or differences in dose and dose-rate.[191]

Another breakthrough innovation is spatially-fractionated RT (SFRT) with the use of highly spatially modulated beams, such as minibeams[192] and microbeams.[193] Notably, proton minibeam RT markedly increased the tolerance of normal brain tissue in rats compared to conventional homogeneous irradiation.[194]

Therefore, using FLASH or SFRT in immunocompetent animal models and analyzing their respective impacts on lymphocyte survival may provide informative content to researchers in the field of radioimmunotherapy.

5. Conclusion

As previously mentioned, clinical trials involving engineered T cells, in particular CAR-T cells, for the treatment of solid tumors have provided disappointing results, in contrast with their increasingly wide use in refractory hematological malignancies. The heterogeneity of solid tumors as well as the variety of oncogenic drivers could result in such a failure. However, the modalities and sequence of conditioning treatments play a crucial role in the efficacy of engineered T cells. From this perspective, radiotherapy, inconsistently used in conditioning phase, and currently underused in ongoing trials experimenting CAR-T cells in solid tumors may enhance the efficacy of these latter in solid tumors. Far beyond the old example of TBI, current RT is a flexible tool, spatially adjustable, with the ability to sculpt dose-delivering and thus deliver different doses in different volumes of interest in a same treatment. Considering the differential effects of high doses versus low doses of RT and the high radiosensitivity of resident T cells depleted during the conditioning phase, radiotherapy should deserve further consideration from clinical research in this field. Notably, local RT could be widely used as part of the conditioning phase in combination with chemotherapy as it provides crucial benefits for the action of adoptive T cells:

i) Deep remodeling of the tumor microenvironment by changes in tumor vasculature and cytokine secretion, thus increasing the attraction, homing and activation of engineered T cells in solid tumors.

ii) Fighting the immune no-go zone represented by hypoxic areas within the tumor.

iii) Depleting clones likely to further escape adoptive T cell therapy.

Whether these effects will be observed only in some selected radiosensitive settings or in a wide area of cancers will depend on ongoing and future experiments involving relevant preclinical models as well as early-phase clinical trials.

For its part, CT would consolidate the depletion of host T-cells in addition to significant antitumor effects.

A major challenge for RT in the future of combinatorial approaches associating CAR-T cells will be to find the optimal dose and volumes of irradiation to both maximize the benefits mentioned above and prevent excessive radiation-induced lymphopenia to improve long-term adaptive immunity. In addition to the options mentioned earlier in this review, particular emphasis should be placed on the implementation of functional imaging and radiomics in patients. Indeed, by using these non-invasive tools, we would be able to make iterative evaluations of the tumor immune landscape either before or after infusion of engineered T cells and selectively deliver on-demand RT to cold tumors, with the objective of strengthening the action of infused cells in real time.

Therefore, in the field of engineered T cells, a fine multidisciplinary management involving hematologists, medical and radiation oncologists, radiologists, biologists and medical physicists is the path towards overcoming the resistance of solid malignancies to adoptive T-cell therapies. Thus, after recent disappointments in this field, we may revive the phoenix from its ashes.

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