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High versus low dose irradiation for tumor immune reprogramming

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Local administration of ionizing radiation to tumors can promote anticancer immune responses that lead to the abscopal regression of distant metastases, especially in patients receiving systemic immune-checkpoint inhibitors. Growing preclinical evidence indicates that high-dose irradiation administered locally to destroy malignant lesions, can promote the release of danger-associated molecular patterns that lead to the recruitment of immune cells, thus inducing a systemic response against tumor antigens that protects against local disease relapse and also mediates distant antineoplastic effects. An accumulating body of preclinical evidence supports also the implementation of low-dose irradiation to induce tumor immune reprogramming. Here, we provide the rationale for a clinical research agenda to refine future clinical practice based on innovative combinations of radiation-immunotherapy.

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Introduction

Radiation therapy (RT) is one of the four central pillars of cancer treatment, along with surgery, chemotherapy, and immunotherapy [1•,2•]. Approximately 60% of all cancer patients will receive at least one radiation treatment course during their lifetime [3]. In the last 20 years, the field of radiation oncology has undergone dramatic technological innovations, leading to the delivery of RT based on tumor imaging (image guided radiation therapy). Intensity modulated radiation therapy (IMRT) uses several X-ray beams of varying intensity directed towards the tumor, angled from different directions around the patient improving the precision of the delivered dose to the target volume(s), while sparing adjacent normal structures [4–6]. This has allowed the delivery of high precision radiation doses (*so called* hypofractionated stereotactic radiosurgery or stereotactic radiation therapy-SRT) [7,8,9••,10••,11••].

In contrast to standard fractionated RT, where radiation is usually given in more than 25 fractions of 2 Gy in order to avoid collateral damage to the normal tissue, SRT allows the delivery of more than 5 Gy per fraction, and the cancer can be eliminated in no more than 6–7 sessions of treatment.

Ionizing radiation kills cancer cells through direct or indirect induction of DNA damage, with DNA double-strand breaks (DSB) [12] and single strand breaks [13] being the most important lesions. However, new research points to the presence of a systemic immunological response induced by RT known as the *abscopal effect*. The word 'abscopal' was first used by Mole in 1953 [14••] to describe the systemic effects of local irradiation (i.e. disappearance of distant metastases outside the irradiated field). Over the years, anecdotal cases of abscopal effects were published, and most of the reported cases were on melanoma [15–17], renal cell carcinoma [18], lymphoma [19], and lung cancer [20], tumors that are traditionally considered as infiltrated by T cells. In animal models, Demaria *et al.* observed that the abscopal effect was tumor-specific and only occurred in wild-type mice that were treated with a combination of radiation (6 Gy single fraction) and Flt3-L, a growth factor that stimulates the production of dendritic cells (DCs) [21•]. However, no growth delays of secondary non-irradiated tumors were found in immunodeficient athymic mice or in wild-type mice treated with a single dose of radiation alone, further suggesting that the abscopal effect was mediated by immune mechanisms [21•].

Over the past decade, immunotherapy (IMT) treatments inhibiting immune checkpoint receptors such as program death 1/ligand 1 (PD-1/PDL1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) have shown remarkable clinical responses against a variety of tumors and

have emerged as a game changer in oncology [1•,2•]. However, the percentage of patients obtaining clinical benefit when used as single modality is relatively low, at only 15–20% [22••,23•]. Certainly, a prerequisite for response to immune checkpoint inhibitors (ICI) is the presence of tumor infiltrating lymphocytes (TILs) in the tumor microenvironment (TME) [24•,25•,26•,27•]. In the context of TIL infiltrated (so-called 'hot') tumors, immune checkpoint inhibitors, such as PD-1/PDL1, reinvigorate the activity of pre-existing antitumor T cells [27•,28••,29••]. Tumors that lack immune infiltration, so-called 'cold' tumors, are refractory to ICI [28••,30••]. Therefore, there is an urgent need to establish novel combination regimens that increase T-cell infiltration in tumors to make them responsive to IMT. Case reports describing the abscopal effect in patients unresponsive to ICI who also received RT for palliative purposes have renewed the interest of radiation as an immune-stimulatory agent [31••,32,33]. RT could become a valuable, non-invasive modality to be used in combination with ICI to amplify anti-tumor immune response in order to increase the incidence of abscopal effects [34••,35•]. The key question in the field is which radiation fractionation schemes dosing, and drug combinations are required to achieve an abscopal effect. This article examines the growing evidence behind the abscopal effects, and discusses the use of high-dose versus low-dose irradiation as immune enhancing agents in combination with IMT.

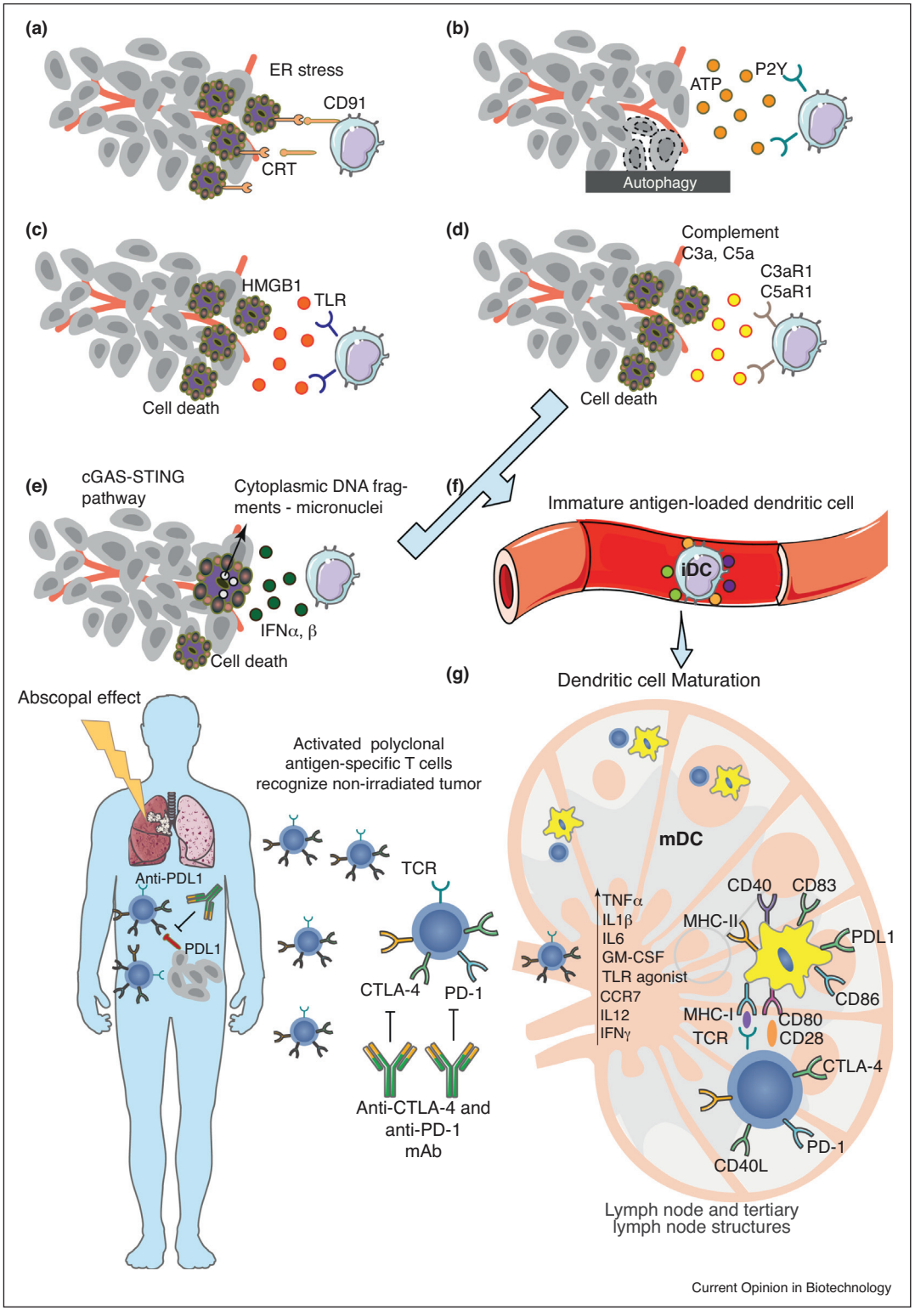
In situ vaccination induced by radiation therapy

Many forms of DNA damage arise when ionizing radiation hits tumor cells, but most of this damage can be remedied by DNA repair systems [36,37]. However, due to their relatively high difficulty of repair (compared with other damage processes), DSBs are thought to be a major factor leading to cell death [38]. DSBs can be visualized by means of immunofluorescent staining with γ-H2AX antibody, called the γ-H2AX foci formation assay [39,40]. Beyreuther *et al.* demonstrated that the number of γ-H2AX foci reaches a peak within two hours after irradiation and that radiation doses below 2 Gy delivered with 25 kV can induce approximately 8 γ-H2AX foci [41]. Cell stress caused by radiation leads to the release of tumor associated antigens (TAA) in the context of necrotic and apoptotic tumor cell death. These DNA fragments are also 'sensed' by the cyclic GMP-AMP synthase (cGAS), a pattern recognition receptor that triggers interferon I (IFN-I) production through the downstream adaptor stimulator of interferon genes (STING) [42••]. Induction of IFN from cGAS/STING signaling is required to achieve optimal DC recruitment and cross-priming of effector T cells, essential to convert the tumor to an *in situ* vaccine [43]. Danger signals cause an inflammatory response, also called immunogenic cell death, which leads to innate immune activation [34••]. Hallmarks of immunogenic cell death upon irradiation include the translocation of calreticulin from the endoplasmic reticulum to the cell surface, which acts as an 'eat-me' signal inducing maturation of DCs, with subsequent release of cytokines such as interleukin-6 and tumor necrosis factor alpha (TNF-α) [44••]. In addition, radiation-damaged tumor cells activate APCs also through the release of damage-associated molecular patterns (DAMPs), which include high-mobility group box 1 (HMGB1), a chromatin nuclear protein that is released mainly after necrotic cell death and serves as a toll like receptor 4 (TLR4) ligand on APCs [45••], and the release of adenosine triphosphate (ATP), which acts as a 'find-me' signal for monocytes and DCs [46••], leading to the secretion of pro-inflammatory cytokines such as interleukin-1β and interleukin-18 [47]. Similarly, complement anaphylatoxins, released following complement activation by RT-induced immunoglobulin M (IgM) binding to necrotic tumor cells, may directly contribute to DC recruitment and maturation, and ultimately to T cell immunity [48••] (Figure 1).

Radiation reprograms the tumor microenvironment

The *in situ* vaccination effect of RT contributes to the uptake, processing and presentation of TAA (also called cross-presentation) by DCs [42••,49]. Discoveries over recent years suggest that a specific subset of DCs excels at cross-presentation. This DC subtype works under the control of basic leucine zipper ATF-like transcription factor 3 (Batf3) [50••], and expresses XCR1, CLEC9A/DNGR-1, CD8α and/or CD103 in mice, while in humans it can be best identified by XCR1, CLEC9A/DNGR-1 and BDCA3 (CD141) staining [51]. This subset efficiently cross-presents extracellular antigens, particularly cell-associated antigens, to $CD8^+$ T cells [50••]. The combination of RT with immunostimulatory anti-PD-1 and anti-CD137 mAbs was conducive to abscopal effects on distant non-irradiated tumor lesions in transplanted MC38 (colorectal cancer), B16OVA (melanoma), and 4T1 (breast cancer) models [49]. However, the effect was completely abolished in $Batf3^{-/-}$ mice, due to the impaired cross-priming of cytotoxic T lymphocytes against tumor antigens, highlighting the importance of CD8α DCs in mediating anticancer immune responses [52]. The cross-priming process requires also peptide-major histocompatibility complex (MHC) recognition by cognate T cell receptors (TCR). RT upregulates MHC class I molecules on tumor cells, enabling enhanced presentation of TAAs [53]. The upregulation begins 18 hours after irradiation and lasts 10 days [53]. Radiation doses from 1 to 25 Gy can induce an increase in MHC class I antigen presentation by two different mechanisms: *i)* increased degradation of proteins in the proteasome that gives rise to a new peptide pool, *ii)* activation of the mTOR pathway which results in increased translation of proteins and an increased generation of peptides from these new proteins [53]. These new proteins are called tumor neoantigens (nonsynonymous mutations acquired during tumorigenesis) and are able to trigger new tumor-

Figure 1



In situ vaccination induced by radiation therapy. Cellular stress and death of cancer cells during the course of radiation therapy lead to the release of danger-associated molecular patterns that foster maturation of dendritic cells with subsequent cross-priming of T cells. **(a)** Exposure of calreticulin (CRT) on the plasma membrane of apopotic cells acts as a phagocytosis-promoting signal (eat me signal) attracting mature dendritic cells that express the counterpart receptor CD91. **(b)** The secretion of ATP is facilitated by radiation-induced autophagy, extracellular ATP acts as a chemoattractant to immature dendritic cells that express P2Y2 counterpart receptor. **(c)** The release of high mobility group B1 (HMGB1) acts on

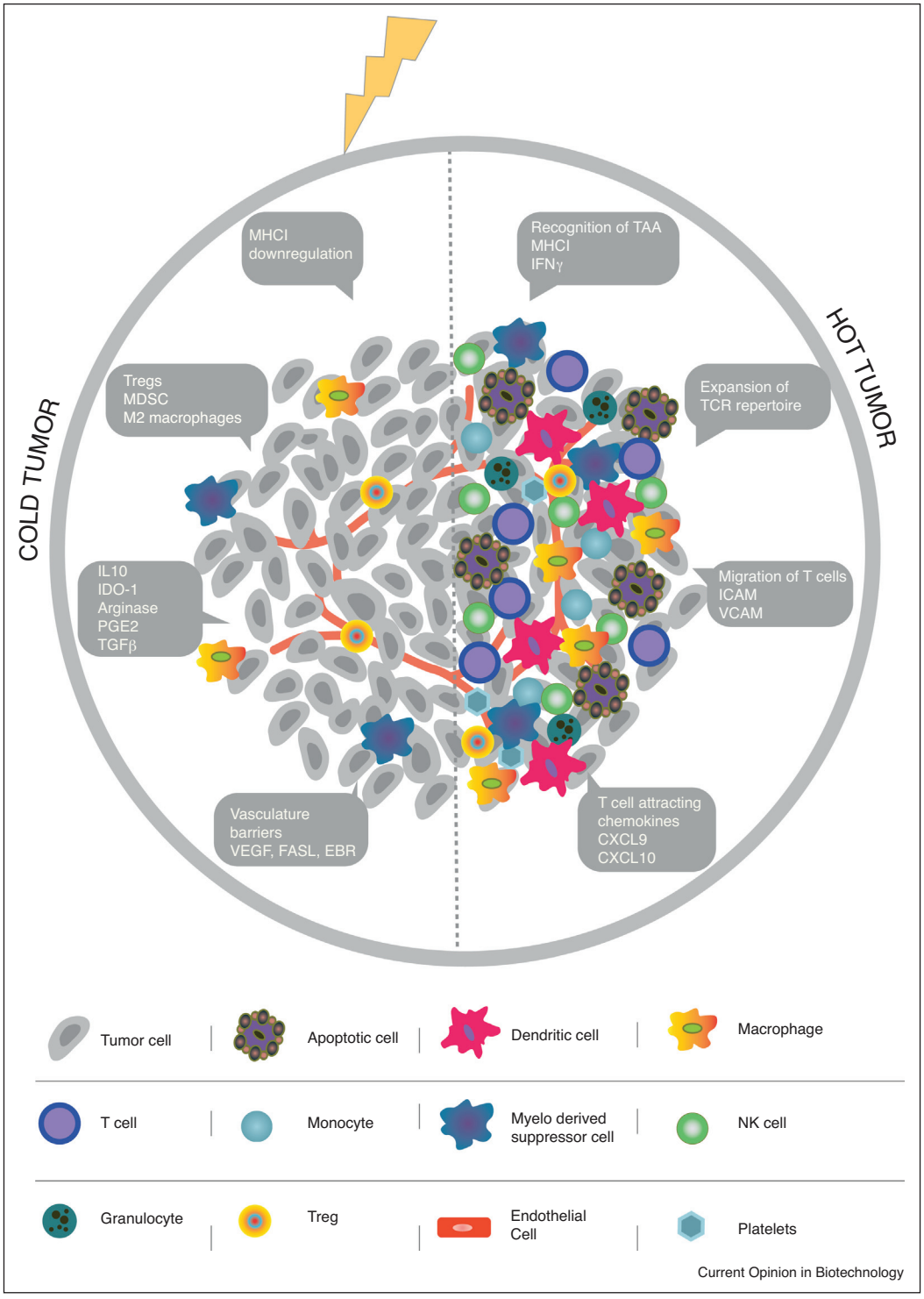
specific TCR clones. Formenti *et al.* [54••] reported TCR clones reacting to radiation-induced tumor neoantigens in a patient with complete response to CTLA4 blockade and SRT. In addition, local high-dose RT can trigger production of type I IFN that initiates a cascade of events able to activate innate and adaptive immunity against the tumor [55]. Induction of Natural Killer Group 2D (NKG2D) receptor ligands upon irradiation acts as an activating receptor for the adaptive immune system [56–58]. The expression of Fas, TNF-α [59], and TRAIL [60] death receptors on tumor cells can also be induced by RT. The ligands for these receptors are expressed on activated cytotoxic T lymphocytes. Thus, RT can induce T cell mediated apoptosis of tumor cells. Many co-stimulatory ligands are members of the TNF superfamily (TNFSF), including 4-1BBL, OX40L, CD70, CD40L [61]. For instance, CD40 activates antigen processing and presentation pathways in DCs and enhances their migration to lymph nodes, and CD40 agonists have shown activity in different types of cancer, both in preclinical models and early phase clinical trials [62–64]. In the B cell lymphoma mouse model, anti-CD40 and 5 Gy total body irradiation (TBI) combined resulted in increased survival with long-term T cell-mediated protection in more than 80% of the animals [65]. In addition, concomitant activation of CD40 and CD137 (a costimulatory receptor expressed on activated T cells) enhanced the antitumor effects of local hypofractionated RT (single fraction of 12 Gy) and promoted the rejection of established subcutaneous syngeneic 4T1.2 breast tumors in a $CD8^+$ T and NK cell dependent manner, inducing immunologic memory capable of controlling a secondary tumor challenge [66].

The exposure of a tumor to radiation therapy can either directly or indirectly attract and/or activate cytotoxic T cells, thus, explaining its ability to turn a cold and non-inflamed tumor into a hot tumor that responds to immunotherapy [34••]. For instance, chemokines CXCL9, CXCL10, and CXCL16, which promote the recruitment of effector CD8 and T-helper 1 CD4 T cells, have been induced by radiation in different tumors [67,68]. RT can also help CD8 T cell migration into the tumor bed through the upregulation of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) on the endothelial tumor vasculature [69,70], which facilitates leukocyte endothelial transmigration [71–73] (Figure 2).

However, some of the RT effects on the TME have a negative impact on immunity. Ahn *et al.*, for example, showed that RT increases the recruitment of myeloid derived suppressor cells (MDSCs) which promote blood vessel formation and tumor regrowth [74]. Tumor associated macrophages (TAMs), which are typically M2, have also been implicated in the promotion of angiogenesis, tumor growth and metastasis following RT [75,76]. M2 macrophages express the anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGFβ), as well as the enzyme arginase-1 (which depletes extracellular L-arginine), which cause T cell suppression [77]. Through the upregulation of TGFα RT also leads to the recruitment of suppressive $Foxp3^+$ T regulatory cells (Tregs) [78,79]. In addition, TGFβ can promote extracellular matrix production and angiogenesis [80], enabling tumor cell proliferation, adhesion and metastasis [81]. The cascade of immune inhibitory events in the TME following radiation exposure are part of the homeostatic repair process activated to promote normal tissue recovery, which unfortunately attenuates RT's immunomodulatory and tumor cell killing ability [82,83]. Counteractive steps, however, can be taken. For example, in pre-clinical glioblastoma models, inhibition of MDSCs by blocking CSF1 increased radiation sensitivity [74,84,85]. Similarly, radiation promoted MDSC infiltration in a pre-clinical model of colon cancer and the association of RT with a CCR2 inhibitor enhanced radiation response [86]. In addition, the deleterious effect of TGFβ could be reversed in the murine breast cancer model by combining RT (5×6 Gy) with TGFβ blockade and PDL1 blockade [87]. As a final example, low-dose irradiation (0.5−2 Gy) has been shown to effectively transform M2 macrophages to M1 phenotypes in the murine pancreatic cancer model and to synergize with adoptively transferred T lymphocytes for tumor control [88••]. In summary, although radiation can potently promote the recruitment and

(Figure 1 Legend Continued) toll like receptor 4 (TLR4) to stimulate optimal antigen cross-presentation to T cells. **(d)** Radiation induced tumor cell death activates anaphylatoxins C3a and C5a. **(e)** Cytosolic DNA fragments induced by DNA damage activate the cGAS/STING pathway. Activation of the cGAS/STING pathway induces IFN type I (IFNα and IFNβ) further increasing DC activation and maturation. **(f)** Antigens from damaged tumor cells are taken up by immature dendritic cells (iDCs), which travel to the lymph node to present tumor antigen-peptides to T cells. **(g)** Inflammatory mediators induce terminal differentiation of immature DC into fully matured immunogenic DC (mDCs). This process is associated with a dramatic change in their morphology with spiculate extensions that improve the interaction with T cells. The maturation process also drastically enhances their migratory capacity and through the upregulation of homing receptors like CCR7, migration to lymph nodes is accelerated. The upregulation of MHC and costimulatory molecules on their surface (CD40, CD80, CD83, CD86) provide strong costimulatory and T cell activating capacity. Modulation of T cell activation is mediated by an interplay of different costimulatory/co-inhibitory molecules expressed on DC that have either immunogenic or tolerogenic function. Under these molecules, CD80/86/CD28, and CD40/CD40L play a prominent role. At the same time, T cells also express inhibitory molecules such as CTLA-4 or PD-1, that down regulate T cell activation. Immunotherapeutic agents blocking tolerogenic signals (i.e. CTLA-4 and PD-1 or PDL1) or increasing co-stimulatory signals (agonistic CD40 mAb) in combination with SRT can boost abscopal responses by targeting different aspects of the immune-mediated abscopal response process. For example, anti-CD40 monoclonal antibody could be employed to increase activation of the DC, while antibodies against cytotoxic T lymphocyte-associated antigen (CTLA4), programmed cell death protein 1 (PD-1) or PD-1 ligand 1 (PDL1) can act as immune checkpoint inhibitors, increasing the T cell activity directed against tumor cells at irradiated as well as non-irradiated tumor sites.

Figure 2



Radiation induced changes in the tumor immune landscape.
Tumors not infiltrated by T cells are often referred to as cold tumors. Barriers to T-cell infiltration and activity include: i) downregulation of MHC-I. ii) Immune suppressive immune infiltrate MDSCs, Tregs, and M2 macrophages. iii) Suppressive factors like IL-10, PGE_2, TGFβ, arginase, and IDO-1. iv) Vasculature barriers like FASL upregulation on endothelial cells, endothelin B receptor and VEGF will abrogate T cell entry into the TME. RT promotes an inflammatory response in cold tumors converting them into hot. 'Hot' tumors are characterized by expression of inflammatory mediators, IFNs, and appropriate chemokines that attract T cells. Upregulation of MHC-I in tumor cells by RT allows recognition by incoming T cells with subsequent release of effector cytokines, expansion of the TCR repertoire and killing of tumor targets. Radiation reprograms tumor macrophages to iNOS-expressing M1 cells, which increase the expression of adhesion molecules ICAM1 and VCAM1 in the tumor endothelium,

activation of DCs and cytotoxic T cells through a variety of mechanisms, this may be counteracted by the migration of suppressive immune cells. This presents a tremendous opportunity for combining RT with immunomodulatory agents for improved tumor control.

Current approaches leveraging the *in situ* vaccination effect of SRT

Optimism regarding the potential synergy between RT and IMT has contributed to a significant increase in the number of clinical trials testing IMT–SRT combinations. Most of the patients treated in these non-randomized trials were naïve of ICI, making it difficult to evaluate the contribution of abscopal effects as the observed responses could be due to the systemic effect of ICI [17,33,89–93] (Table 1). Despite these caveats, those trials have shown encouraging results. For example, the recently published PEMBRO-RT study [94] in which 92 patients with advanced non-small cell lung cancer (NSCLC) EGFR and ALK wild type who have received at least 1 line of treatment were enrolled and randomized regardless of their PDL1 status to pembrolizumab (200 mg/kg every 3 weeks) either alone (control arm) or after SRT (3 doses of 8 Gy, experimental arm) to a single tumor site until progression, unacceptable toxic effects, or death. The ORR at 12 weeks was 18% in the control arm versus 36% in the experimental arm ($p = 0.07$). Median progression-free survival was 1.9 months (95%CI, 1.7–6.9 months) versus 6.6 months (95%CI, 4.0–14.6 months, HR, 0.71; 95%CI, 0.42–1.18; $p = 0.19$), and median overall survival was 7.6 months (95%CI, 6.0–13.9 months) versus 15.9 months (95%CI, 7.1 months to not reached, HR: 0.66; 95%CI, 0.37–1.18; $p = 0.16$) [94]. These results although not statistically significant showed a trend in favor of SRT and pembrolizumab and merit further investigation.

The question remains as to whether such trials could underpin others, helping to improve abscopal effects as a primary objective. Here, we propose some ideas which could help tailor the design of clinical trials:

First, we believe that many of the previously mentioned trials were designed to use radiation as an *in situ* vaccine in tumors. The idea behind this is that hypofractionated RT will stimulate intratumoral T cells and induce systemic immune responses, even though only one site (or, in some patients, a few sites) is irradiated. This however assumes that all other non-irradiated tumor lesions are already infiltrated by T cells. Tumor heterogeneity and divergent clonal evolution are major factors contributing to the resistance of immune mediating killing by SRT and immunotherapy. As one of the purposes of SRT is to generate tumor neoantigens, the irradiation of several tumor metastases may be more appropriate to induce a broader range of neoantigen specificities. Several papers in the literature support this. For instance, Joshi *et al.* [95] demonstrated that somatic mutations together with immunoediting drive extensive heterogeneity within NSCLC. They showed that some TCRs were found ubiquitously throughout the tumor and others only in particular locations corresponding to regional mutations. The number of ubiquitous and regional TCRs correlates with the number of ubiquitous and regional nonsynonymous mutations, respectively. This means that the immune system, through T cells, is actually mirroring the genetic diversity of the tumor. Thus, radiation to most tumor deposits may be used as a non-invasive approach to stimulate tumor-reactive TCRs. This is also supported by data from TIL cultures established from excisional tumor biopsies where independent TIL cultures derived from different tumor biopsies from the same patient demonstrated qualitatively better patterns of HLA-A2 restricted tumor antigen recognition than TIL cultures from a single tumor biopsy [96].

Similarly, the study by Arina *et al.* [97] supports the notion that most, if not all, tumor deposits would require hypofractionated irradiation in order to induce more abscopal responses in future clinical trials. The investigators have shown that in murine tumor models representative of 'hot' tumors, many of the pre-existing T cells survived large doses (20 Gy) of localized radiation without the need for newly infiltrative T cells. In that pre-clinical study, the effect of radiation was shown to be exclusively local, as intratumoral T cells exhibited increased motility and IFNγ production upon SRT. Future phase I/II clinical trials could assess the safety and clinical efficacy of irradiation in multiple tumor deposits with concomitant IMT.

Second, which radiation doses should be used for potential combinations of radio-immunotherapy? Regimens like 3×8 Gy were proposed by Vanpouille-Box *et al.* [98] who demonstrated that double strand DNA fragments accumulated in the cytoplasm with hypofractionated doses below 10 Gy. Above that RT dose threshold, induction of 3' repair exonuclease 1 (Trex1), an enzyme that degrades cytoplasmic DNA, mediates rapid degradation of cytosolic DNA, precluding the activation of the cGAS/STING pathway and abrogating the abscopal

(Figure 2 Legend Continued) enabling T cell homing.
Abbreviations: TGFβ, transforming growth factor beta; IDO1, indoleamine 2,3-dioxygenase; PGE2, prostaglandin E2; MDSC, myeloid-derived suppressor cells; Tregs, regulatory T cells; MHC, major histocompatibility complex; TAA, stumor-associated antigens; IFNγ, interferon gamma; VEGF, vasculature endothelial growth factor; EBR, endothelin B receptor; ICAM1, inter-cellular adhesion molecule 1; FASL, FAS ligand and VCAM1, vascular cell adhesion molecule 1; RT, radiation therapy.

Table 1

Clinical trials of stereotatic body radiation therapy (SRT) and immune checkpoint inhibitors (ICI) in different disease types

Author	Disease	N	RT (dose in Gy x number of fractions)	ICI	Schedule	Abscopal
Twyman-Saint Victor *et al.* [91]	Melanoma	22	6 Gy x 2–3 8 Gy x 2–3 (one metastatic site)	Ipilimumab 3 mg/Kg/3 w x 4	Ipilimumab 3–5 days after SRT	18% No CR
Hiniker *et al.* [32]	Melanoma	22	8 Gy x 3 4 Gy x 10 (1–2 metastatic sites)	Ipilimumab 3 mg/Kg/3 w x 4	SRT within 5 days of ipilimumab	CR 14% PR: 14% SD: 23%
Tang *et al.* [90]	NSCLC, CRC, RCC, others	35	12 Gy x 4 6 Gy x 10 (1 metastatic site)	Ipilimumab 3 mg/Kg/3 w x 4	SRT 1 day after ipilimumab or 1 week after 2nd ipilimumab	PR 10% SD 13% No CR
Luke *et al.* [129]	Ovarian, endometrial, CRC, others	73	30–50 Gy x 3–5 (2–4 metastatic sites)	Pembrolizumab 200 mg/3 w until progression, death or toxicity	Pembrolizumab 7 days after SRT	1 CR 8 PR 21 SD
Maity *et al.* [130]	Cohort1: NSCLC, melanoma (progression on anti-PD-1) and Cohort 2: pancreas, breast, H&N, colon, kidney (no prior anti-PD-1)	12 12	8 Gy x 3 to the first 6 patients in each cohort and 17 Gy x 1 to the following patients (1 metastatic site)	Pembrolizumab 200 mg/3 w x 6	SRT 6–10 days after pembrolizumab	Cohort 1: PR: 2 PD: 9 NE: 1 Cohort 2: CR: 1 SD: 1 PD: 9
Sundhal *et al.* [131]	Urothelial carcinoma	9 9	8 Gy x 3 (1 metastatic lesion)	Pembrolizumab 200 mg/3 w	Cohort A sequential Cohort B concomitant	CR, PR, SD: 0% CR: 11% PR: 33% SD: 0% PD: 56%

Abbreviations: Gy: Gray, mg: milligram, Kg: kilogram, CR: complete response, PR: partial response, SD: stable disease, SRT: stereotactic body radiation therapy, NE: not evaluable.

effect of radiation and synergy with CTLA-4 blockade [98].

Third, which is the right combination of IMT to enable abscopal responses when combined with SRT?

We believe that orthogonal combinatorial strategies are required to induce abscopal responses. An important immune cell type directly involved in tumor cell destruction following RT and IMT are cytotoxic T lymphocytes, but their effectiveness is dependent upon support received from other immune cells, such as antigen presentation and co-stimulation by activated DCs. Moreover, effector T cells can quickly be rendered anergic or exhausted by a plethora of suppressive mechanisms that can be upregulated in the TME. Thus, as we aim to improve patient outcome in the era of immuno-oncology, it is evident that all aspects of the cancer immunity cycle, including, (i), the release of cancer antigen, (ii) cancer antigen presentation by activated APCs, (iii), priming and activation of T cells, (iv) trafficking and infiltration of T cells into the tumor, and, (v) recognition and killing of cancer cells, as well as specific barriers that are present in a given tumor, must be targeted in order to obtain the desired abscopal effect [99].

While SRT itself can promote tumor antigen upregulation and presentation by tumor cells, as well as recruit DCs and T cells, the phenotype of the APCs may be such that they do not provide sufficient co-stimulation. In such situations, anti-CD40 antibody, or TLR agonists, can be employed to differentiate and maturate the DCs [66,100,101].

ICI of the PD-1/PDL1 axis, and CLTA-4 could provide further synergy to the combination by releasing the brakes on T cells and enhancing their priming [99,102]. It is conceivable that a combination of RT, anti-CD40 Ab, or TLR agonist, plus ICI, will be sufficient to overcome barriers to an abscopal effect in a patient. Agonistic antibodies to costimulatory T cell receptors offer a complementary strategy to activate antitumor T cells, and could be combined with RT. Tumor-reactive $CD8^+$ tumor infiltrating lymphocytes (TILs) that co-expressed PD-1 and CD137 were detected in a breast cancer mouse model 12 hours after exposure to RT [66], explaining the benefit of combination therapy with anti-PD-1, anti-CD137 mAbs and RT either at 12 Gy in a single fraction or 4–5 Gy in four fractions [103]. Additional evidence from lung, breast and glioma mouse models indicates that checkpoint blockade and costimulatory antibody therapy such as CD137 can be successfully combined with RT, and particularly with SRT, leading to increased survival and tumor control [104–107]. OX-40 interaction with its ligand (OX-40L) also provides a costimulatory signal for T-cell proliferation.

Agonistic anti-OX-40 antibody combined with RT resulted in a significant survival advantage in tumor-bearing mice, which was mediated by $CD8^+$ cells [108]. Finally, interleukin 2 (IL-2) is a potent cytokine used in clinical practice to activate T cells. The combination of IL-2 with 15 Gy single dose RT was able to induce T cell-mediated complete responses in mice bearing MC38 colon adenocarcinoma compared with 12% responses with either treatment alone [109]. In the same way, patients with melanoma and renal cell carcinoma treated with SRT given in one, two or three doses of 20 Gy in combination with IL-2 showed higher than expected abscopal responses [110]. These strategies will require careful implementation in the context of phase I/II clinical trials with an in depth translational investigation of paired tumor biopsies.

In order to prevent significant systemic toxicity and off-target effects of these combinations, intra-tumoral immunotherapy may favor safer administration of such regimens. Dewan *et al.* [111] topically administered TLR7 agonist imiquimod in combination with 3 fractions of 8 Gy localized radiation to the TSA breast cancer mouse model. Local imiquimod and radiation improved tumor response compared with either treatment alone. Importantly, the addition of topical imiquimod also resulted in a growth inhibition of secondary tumors outside the radiation field. Low-dose cyclophosphamide given before starting treatment with imiquimod and RT further improved tumor inhibition and reduced tumor recurrence [111]. More recently, in a rodent model of advanced limb sarcomas, oncolytic vaccinia virus (GLV-1h68) therapy was administered by isolated limb perfusion in combination with anti-PD-1 therapy before surgical resection and RT (2 fractions of 3.25 Gy). Tumor bearing animals achieved long-lasting local disease control and the regimen was able to prevent metastatic disease [112]. Enhanced therapy was associated with marked modulation of the tumor microenvironment, an increase in the number and penetration of intratumoural $CD8^+$ T cells and the expansion and activation of dendritic cells [112].

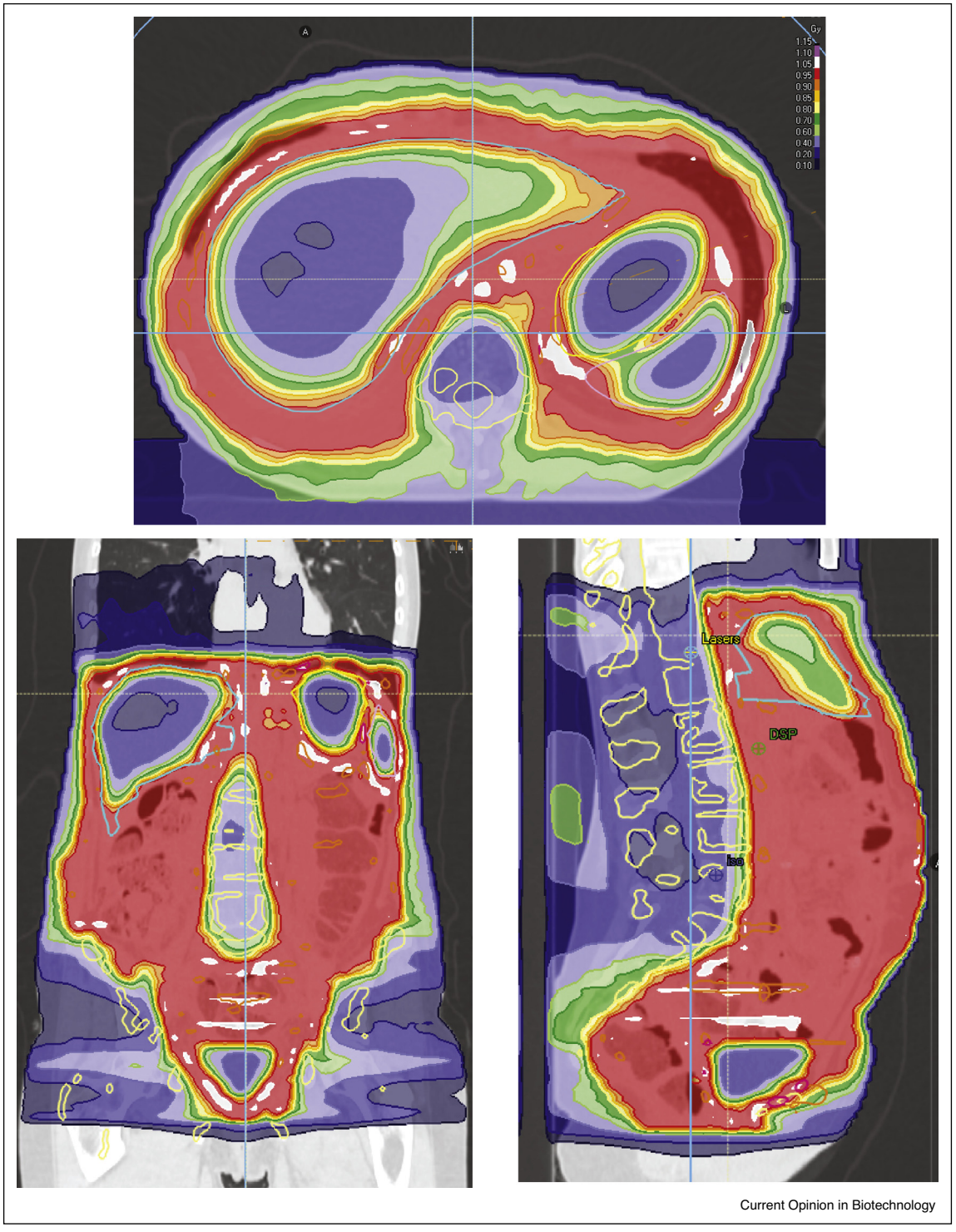
Rodriguez-Ruiz *et al.* [113] conducted a phase I clinical trial in 15 advanced cancer patients that were treated with four weekly cycles of intradermal daily doses of monocyte-derived dendritic cells pre-loaded with autologous tumor lysate and matured for 24 hour with poly-ICLC (Hiltonol), TNF-α and IFN-α. On days 8 and 10 of each cycle, patients received intratumoral injections of the dsRNA-analogue Hiltonol. Cyclophosphamide 600 mg/m^2 was administered 1 week before. Six patients were treated with SRT on selected tumor lesions (24 Gy in three fractions administered every other day from day 7 to 11). The combination treatment was safe and well tolerated. The only complete response was observed in a heavily pretreated castration-resistant prostate cancer patient who received SRT and immunotherapy [113].

Low dose irradiation to reprogram the tumor microenvironment

If the purpose of delivering RT is immune modulation to enable IMT, low-dose irradiation (e.g. radiation doses below 2 Gy) could be delivered to all tumor deposits with little or no toxicity which could facilitate the combination with several IMT drugs. In this case, radiation is not used for the purpose of direct tumor cell killing but rather as an immune reprogramming agent. Low dose metronomic ionizing irradiation increases the immunogenicity of cold tumors by activating inflammatory mechanisms that enable innate and adaptive immune activation [34••]. In preclinical trials, BALB/c mice irradiated with single doses of 0.1 or 0.2 Gy to the entire body and injected intravenously 2 hours later with syngeneic L1 sarcoma cells had significantly less lung metastases relative to sham-irradiated control mice [114]. This anti-cancer effect of low dose irradiation has been abrogated by the nature killer (NK)-suppressive anti-asialo GM1 antibody, suggesting that NK cells play a key role in the anti-cancer effect of LDR [114]. Likewise, in rats implanted locally with a hepatocellular carcinoma cell line, lung metastasis were suppressed by 0.2 Gy total-body irradiation, although the same dose of local irradiation had no effect in controlling distant metastases [115]. In this study, low-dose TBI increased IFNγ and TNFα gene expression signatures as well as $CD8^+$ cells in the tumor microenviroment [115].

Low-dose irradiation is able to induce DNA damage [116], and activation of cGAS-STING pathway [117••] by double-stranded DNA which is critical to induce DC activation and maturation [42••,118,119]. Translational research in humans shows that low-dose irradiation can induce positive regulation of TLR signaling pathway; for example, human monocytes irradiated with 0.05 and 0.1 Gy have shown significant upregulation of TLR signaling molecules (HMGB1, TLR4, TLR9, MyD88 and IRAK1) [120]. Shigematsu *et al.* reported that 0.05 Gy-pre-irradiated DCs exhibited the highest proliferation capacity of T cells when co-cultured together, and augmented the production of IL-2, IL-12, and IFNγ in the supernatant of the co-culture system [121]. In a mouse model of pancreatic cancer, it has been described that a single fraction of localized low dose irradiation (i.e. 0.5−2 Gy) could reprogram the TME, inducing reprogramming of TAMs towards an iNOS + M1 phenotype, which in turn produced the appropriate chemokines to recruit effector T cells. In addition, M1 macrophages drove normalization of the tumor vasculature, increasing CD31 and VCAM-1 expression, and allowing T cell infiltration in tumors. Together, these effects enabled T cell mediated tumor rejection in the context of adoptive T cell therapy [88••]. Moreover, the results were corroborated in patients with pancreatic adenocarcinomas treated in the neo-adjuvant setting, where a single dose of 2 Gy was sufficient to increase T cells in the TME [88••]. Spary *et al.* demonstrated that 0.6–2.4 Gy radiation

Figure 3



Low dose whole abdominal irradiation in a patient with ovarian cancer.
Intensity modulation radiation therapy (IMRT) plan for whole abdominal radiotherapy. The planning target volume (whole abdominal cavity) is red depicting a delivered dose of 1 Gy. The liver, kidneys and active bone marrow are protected and received less than 0.2 Gy (blue isodose line).

enhanced T cell function by increasing T cell proliferation, T cell receptor signaling, and $CD8^+$ cell polyfunctionality [122].

Low dose irradiation could reverse the immunosuppressive function of several immune cell types, such as Tregs [123], immunosuppressive TAMs [88••] and tolerogenic DCs [124], and provide a convenient therapeutic platform for immunotherapy by fostering the reprogramming of the tumor microenvironment, favoring the infiltration of T cells into the tumor. In this context, low dose irradiation could be accompanied by pharmacological interventions to further increase DC activation (CD40 agonists, TLR agonists) and ICI, such as anti-PD-1/PDL1, anti-CTLA-4, anti-LAG-3, in order to increase the tumor killing capacity of T cells. These schemes are supported by pre-clinical studies and early clinical trials. For example, the intratumoral TLR9 agonist, combined with 2×2 Gy radiation, produced important clinical responses in patients with advanced lymphoma [125••]. Analysis of paired pre-treatment and post-treatment biopsies showed a significant increase in $CD3^+$, $CD8^+$, and $CD4^+$ T cells after treatment (day 9) at the irradiated tumor site [126].

Similarly, low dose irradiation (2 Gy) was able to sensitize tumor cells to immune rejection by locally activating chimeric antigen receptors (CARs) T cells. In the mouse pancreatic adenocarcinoma model partially expressing the antigen sialyl Lewis-A (sLeA), tumor cells expressing the sLeA antigen were targeted with CAR T cells. CAR T cell that recognized sLeA + cells produced the death ligand TRAIL and were able to eliminate sLeA negative tumor cells previously exposed to systemic or local low-dose radiation [127]. These observations are provocative, and may stimulate interest in the development of clinical trials to determine the effectiveness of low dose irradiation in the context of ICI and adoptive T cell therapies.

Despite the lower doses proposed, caution should be exercised with the delivery of low radiation doses to large volumes. Therefore, similar principles as for SRT should be applied in order to avoid unnecessary radiation to healthy organs. Low dose irradiation will require careful testing in clinical trials. Several disease types where large tumor volumes require irradiation could benefit from this approach (i.e. ovarian cancer or gastro-intestinal cancers harboring peritoneal carcinomatoses, cancers exhibiting large liver or lung metastases unable to be irradiated by SRT, cancers causing pleural effusion) (Figure 3). Early phase trials may help to optimize the amplitude and magnitude of the immune response and to define the volume, doses and intensity of the LDI approach. A recent post-hoc analysis of three immuno-radiation trials using either CTLA-4 or PD-1/PDL1 monoclonal antibodies showed that patients receiving low dose irradiation as scatter from high-dose radiation fields had 58% PR or CR by RECIST criteria compared with only 18% of response in lesions not receiving scattered low dose irradiation, ($p = 0.0001$) [128]. This finding, although interesting, deserves caution since the analysis was retrospective and examined only 26 patients, of whom only 6 patients had disease progression to ICI, rendering the assessment of abscopal responses to LDI difficult to interpret. The average scattered dose given to low dose treated tumors was 7.3 Gy (1.1–19.4 Gy) in the study [128]. We believe that this data is encouraging, but the median dose of 7.3 Gy is still substantially high and has the potential to increase radiation toxicity even with highly conformal techniques. If the purpose is immune modulation, based on the above observations doses below 2 Gy should be tested in clinical practice. Our group is currently testing low dose irradiation (<2 Gy) in early phase clinical trials (NCT03728179) in combination with monoclonal antibodies targeting CTLA4 and PD-1/PDL1 as well as cyclophosphamide to deplete Tregs and aspirin to favor T cell infiltration in patients with TIL negative solid tumors. Similarly, in another trial we test low dose irradiation with adoptive transfer of autologous lymphocytes (NCT03992326).

Concluding remarks and future directions

The ability of RT to activate anti-tumor immunity is a paradigm shift in oncology and explains the synergy of RT with IMT, which is well documented in pre-clinical studies and in patients who were previously refractory to ICI and subsequently responded after receiving RT. However, the low incidence of abscopal responses when SRT is combined with IMT in clinical trials indicates that although SRT may release tumor antigens, with activation of antigen presenting cells and T cells this biological effect is suboptimal. Several barriers to innate immune activation, T cell homing, engraftment and function should be considered when combining immunotherapy with SRT [34••]. Efforts should focus at maximizing the *in situ* vaccination effects of SRT, by providing drugs that boost APC maturation (CD40 or TLR agonists), enhance T cell priming (CTLA-4 blockade) and attenuate immunosuppressive cues (TGFβ, Tregs etc.). Careful clinical testing will require the design of phase 0 (translational) studies with biological endpoints, and phase I studies where the various combinations and schedules can be tested in a systematic way. Phase II adaptive design trials may also allow investigators to quickly identify combinations with therapeutic effect, and to minimize patient populations exposed to less appropriate combinations. These trials should include mandatory translational research with paired pre-treatment and post-treatment tumor biopsies. Whenever possible, biopsies should be taken from irradiated and non-irradiated lesions, which may only be feasible in patients with accessible metastases (i.e. squamous cell carcinoma of the vulva, penis, skin or other cancer types that evolve with skin dissemination).

LDI can be applied to remodel the tumor microenvironment in combination with immunotherapy in order to *i)*

reduce tumor supportive macrophage activity, *ii)* train bone marrow derived monocytes to express co-stimulatory molecules, *iii)* enable T cell homing in patients with absence of TILs, *iv)* and increase the killing capacity of newly infiltrating T cells. LDI offers the advantage of being safe and allows the irradiation of several if not all tumor deposits. Similar to SRT, LDI should be combined with immunotherapy agents that increase priming and activation of T cells as well as reduce the immune suppressive factors of the TME. Finally, a combination of high-dose SRT to a few metastases, to trigger *in situ* vaccination, and low-dose RT to the remaining metastases, could provide an important opportunity to maximize the abscopal effects by exploiting both the potentials of RT, that is, to induce vaccination and to facilitate T cell attack.

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Conflicts of interest statement

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All other authors report no conflicts of interest.

CRediT authorship contribution statement

Maria Ochoa de Olza: Methodology, Writing - original draft. **Jean Bourhis:** Methodology. **Melita Irving:** Methodology, Project administration. **George Coukos:** Conceptualization, Writing - review & editing. **Fernanda G Herrera:** Conceptualization, Writing - review & editing, Supervision.

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