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FOCUSSED RESEARCH REVIEW

Immune-modulating properties of ionizing radiation: rationale for the treatment of cancer by combination radiotherapy and immune checkpoint inhibitors

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Abstract Radiotherapy (RT) utilizes the DNA-damaging properties of ionizing radiation to control tumor growth and ultimately kill tumor cells. By modifying the tumor cell phenotype and the tumor microenvironment, it may also modulate the immune system. However, out-of-field reactions of RT mostly assume further immune activation. Here, the sequence of the applications of RT and immunotherapy is crucial, just as the dose and fractionation may be. Lower single doses may impact on tumor vascularization and immune cell infiltration in particular, while higher doses may impact on intratumoral induction and production of type I interferons. The induction of immunogenic cancer cell death seems in turn to be a common mechanism for most RT schemes. Dendritic cells (DCs) are activated by the released danger signals and by taking up tumor peptides derived from irradiated cells. DCs subsequently activate T cells, a process that has to be tightly controlled to ensure tolerance. Inhibitory pathways known as immune checkpoints exist for this purpose and are exploited by tumors to inhibit immune responses. Cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) on T cells are two major checkpoints. The biological concepts behind the findings that RT in combination with anti-CTLA-4 and/or anti-PD-L1 blockade stimulates CD8+ T cell-mediated anti-tumor immunity are reviewed in detail. On this basis, we suggest clinically significant combinations and sequences of RT and immune checkpoint inhibition. We conclude that RT and immune therapies complement one another.

Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
CpG	Cytosine–guanine-rich motifs
CRT	Calreticulin
CT	Chemotherapy
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FDA	US Food and Drug Administration
Flt3-L	Fms-related tyrosine kinase 3 ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMGB1	High-mobility group box 1
Hsp70	Heat shock protein 70
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase

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MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
NSCLC	Non-small cell lung cancer
PD-1	Programmed cell death protein 1
ROS	Reactive oxygen species
RT	Radiotherapy
RT5	Rat insulin promoter (RIP)1-Tag5 tumor mouse model
TNF	Tumor necrosis factor
X-ray	Ionizing radiation
zVAD-fmk	Z-Val-Ala-DL-Asp-FMK

Introduction

Neoplastic diseases are very complex and tumors are far more than just an accumulation of tumor cells. Non-cancerous cells such as fibroblasts, cells that comprise the blood vessels and immune cells are highly interconnected with the tumor cells and form the cancer microenvironment. The tumors are characterized by sustained proliferation, avoidance of growth regulation, cell death resistance, genomic instability and mutations, modified energy supply, angiogenesis, invasion and metastases, and immortalization, as well as inflammation and active immunosuppression [1]. Tumor cells and immune cells are in a dynamic process and the immune system therefore not only protects against cancer development but also shapes and impacts on the phenotype of emerging tumors [2]. The immunoediting concept of Schreiber and colleagues that comprises the elimination, equilibrium and escape phase [3] opened the minds of oncologists of different specializations to the prospect that modulation of the immune system can strongly contribute to cancer therapy success.

Immunogenicity of chemotherapy and radiotherapy

But how do classical treatment modalities such as chemotherapy and radiotherapy that are commonly known to induce immunosuppression fit into this concept?

For chemotherapy, it has become clear that distinct chemotherapeutic agents such as oxaliplatin and anthracyclines induce immunogenic cell death which is characterized by the exposure of endoplasmic reticulum-derived proteins such as calreticulin (CRT) and by the release of immune-activating danger signals such as high-mobility group box 1 (HMGB1) protein, heat shock protein 70 (Hsp70) and adenosine triphosphate (ATP) [4, 5]. Such immunogenic drugs are currently being tested in clinical trials [6].

Radiotherapy uses high energy radiation to locally treat cancer. It primarily aims to induce DNA damage and ER stress via reactive oxygen species (ROS). These targeted effects result in cell cycle arrest of the tumor cells that try to repair the induced damage and concomitantly in reduction in the clonogenic potential. The latter is termed "clonogenic survival" by radiobiologists. It describes the capability of a single cell to form colonies again, but does not state anything about cell death. Cells whose cell cycle is arrested, as also occurs in senescence, should be regarded as alive, and the expression "replicative cell death" which alludes to the loss of the clonogenic capacity should be avoided [7]. Besides the targeted effects of ionizing radiation (X-ray) used in RT, non-targeted, systemic effects also exist [8]. In response to radiation, an increased expression of MHC-I and MHC-II molecules, CD80, adhesion molecules, stress ligands, Hsp70 and death receptors on tumor cell surfaces can be observed [9]. Furthermore, immune-activating chemokines, cytokines, exosomes and danger signals are released [10]. To summarize, this may result in activation of dendritic cells (DCs) that then initiate innate and adaptive immune responses [11] (Fig. 1).

Systemic immune responses induced by radiation with further immune stimulation

However, the clinical routine shows that patients who receive RT very rarely develop regression of metastases outside of the irradiation field. This suggests that out-of-field reactions of RT need further immune modulation. This has been proven by preclinical models, such as the syngeneic mammary carcinoma 67NR mouse model. Regression of the non-irradiated ectopic tumor, outside of the irradiation field of the other tumor on the opposite flank, only occurred when additional immune activation was initiated. In this case, the number of DCs was enhanced with the growth factor Fms-related tyrosine kinase 3 ligand (Flt3-L). The resulting systemic immune reaction was tumor specific and dependent on T cells [12]. We found that local antitumor immune reactions that are induced by multimodal treatment of the tumor with RT, CT, hyperthermia and additional immune stimulation with the pan-caspase inhibitor zVAD-fmk are also mediated by T cells and dependent on the danger signals HMGB1 and nucleotides like ATP [13]. We are currently analyzing how zVAD-fmk impacts on systemic immune responses against tumor masses outside the irradiation field, since it fosters the induction of necroptosis by blocking apoptosis. Necroptosis is a programmed form of necrosis [14], considered to be highly immunogenic and therefore ideal for triggering systemic immune responses.

Nevertheless, radiation by itself can also be a beneficial trigger to recruit and activate immune cells. In the rat
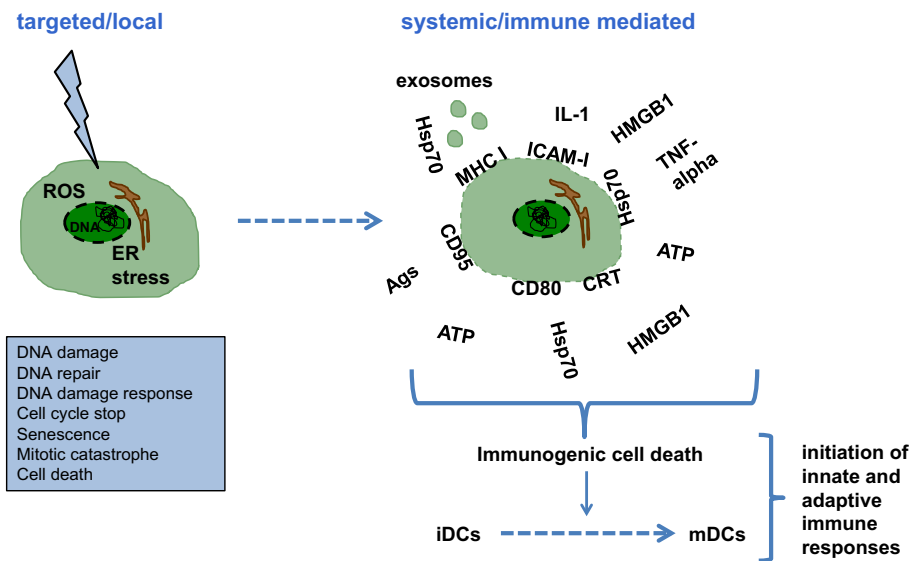
Fig. 1 Contribution of local and systemic effects of radiotherapy to tumor cell killing. Radiotherapy-induced increased generation of reactive oxygen species (ROS) induces DNA damage that is immediately sensed and, if possible, repaired (DNA damage response). Cells are arrested in the cell cycle and no longer proliferate. Senescent cells may result or the cells are driven toward death during the course of mitosis (mitotic catastrophe). Unrepaired DNA damage finally results in cell death. Additionally, systemic responses can be initiated arising from ROS and endoplasmic reticulum (ER) stress by modification of the tumor cell surface after exposure to ionizing radiation and by release of danger signals, inflammatory cytokines, exosomes and (modified) tumor antigens. Dendritic cells (DCs) mature are activated and initiate innate and adaptive immune responses. *Ags* antigens, *CRT* calreticulin, *iDC* immature DC, *mDC* mature DC

insulin promoter (RIP)1-Tag5 (RT5) mouse model of spontaneous tumors, the capability of tumor antigen-reactive T cells to infiltrate malignant tissue is lost during tumor progression. This results in tumor outgrowth. A single irradiation with 2 Gy restored and even increased the capability of tumor-specific CD4+ and CD8+ T cells to migrate into the tumor [15]. Radiation triggers the normalization of the vasculature by reprogramming M2 macrophages into M1 like macrophages that display inducible nitric oxide synthase (iNOS) activity. Combination of radiation and NO-dependent normalization of the tumor vasculature in combination with adoptive T cell transfer therefore results in efficient tumor cell killing and control. Systemic application of pro-inflammatory reagents such as cytosine–guanine-rich (CpG) motifs render tumors permissive for immune infiltration [16] and RT could be an inducer of such immune stimulators [17].

Importance of the timing of combination of RT with immune stimulation

To summarize, combination of RT with additional immunotherapy is a promising approach for inducing specific antitumor immune responses. One must always keep in mind that the sequence of the applications is crucial. As outlined above, first, the immune cell recruitment into the tumor by RT has to be enabled, and then additional immunotherapy

should be performed. Witek et al. [18] demonstrated that radiation serves as an adjuvant and preparatory step for tumor vaccination. Tumor-specific T cell responses were amplified in mice with tumors irradiated before or after vaccination with an adenoviral-based vaccine against the colorectal cancer antigen guanylyl cyclase C. Here, fractionated radiation-induced similar effects compared to single high-dose irradiation with 8 Gy.

Impact of the radiation dose and fractionation on immune stimulation

The activation of human monocyte-derived DCs was also similar when coming into contact with norm- or hypofractionated irradiated human colorectal cancer cells [19]. Nevertheless, more data are urgently needed in order to draw conclusions on whether continued fractionated application of 2 Gy that is used in classical tumor therapy is as immunogenic as fewer applications with higher single doses or a very high single dose (radiosurgery). Irradiation of intracranial GL261-luc glioblastoma mouse tumors with 10 Gy with a dose rate of 1.9 Gy/min resulted in tumor growth retardation that was significantly improved through combination with immunotherapy using anti-programmed cell death protein 1 (PD-1) monoclonal antibody (see also below). The local RT with 10 Gy enhanced the pro-inflammatory profile of GL261 gliomas and paved the way for an increased

influx of CD8+ effector T cells when combined with anti-PD-1 immunotherapy [20]. However, in a TSA mouse breast carcinoma model, fractionated irradiation with single doses of 8 or 6 Gy, in particular, in combination with an antibody against the immune checkpoint protein cytotoxic T lymphocyte antigen 4 (CTLA-4) resulted in significant enhanced tumor growth retardation in the tumor outside of the irradiation field [21]. Nevertheless, single doses of 6 or 8 Gy are still high doses. Of note is that a single high dose of 20 Gy was as effective as 3×8 Gy or 5×6 Gy in retarding growth of the irradiated tumor. This indicates that local and systemic responses always have to be considered when RT is part of multimodal tumor therapies. Furthermore, it is most likely that promotion of immune responses can be induced by many single doses and fractionations of X-ray. The mechanistic basis, as the CD8+ T cell-mediated tumor cell killing, might be common, but further mechanisms might differ in part. Lower single doses impact on tumor vascularization and consecutive infiltration of immune cells in particular [22], while higher doses impact on intratumoral induction and production of type I interferon (IFN) with consecutive triggering of innate and adaptive immune mechanisms [23]. The induction of immunogenic cell death by the many doses of radiation and fractionation schemes that are applied in tumor therapy seems in turn to be a common mechanism [24].

In addition, one has to keep in mind that low and intermediate doses of ionizing radiation (≤ 1.0 Gy) have opposed immunological effects. Here, inflammation is attenuated and the viability of many cells is not strongly influenced [25]. This might, however, also be beneficial for anti-tumor immunity, since chronic inflammation promotes tumor outgrowth [26]. A deeper understanding of the interconnection of inflammation, cancer and DNA damage responses should lead to further improvements in radio- and immunotherapy used to treat cancer in the future.

Immune checkpoint inhibition in combination with RT

As outlined above, DCs play a central role in starting innate and adaptive immune responses by being activated by damage-associated molecular patterns (DAMPs) and by taking up tumor peptides derived from irradiated cells. Subsequently, DCs have to deliver diverse signals to T cells to activate them and ensure their survival, and to initiate their differentiation. As a safety system of the immune system, T cell activation has to be highly controlled to ensure tolerance and to avoid severe autoimmune reactions. For this purpose, in addition to multiple co-stimulatory signals, inhibitory pathways exist that are known as immune checkpoints.

It has become clear that tumors exploit immune checkpoint pathways as a mechanism of inhibiting immune responses, particularly against tumor-specific cytotoxic T cells [27]. Activated T cells are inhibited via the binding of CD80 or CD86 on DCs to CTLA-4 on T cells. CTLA-4 (CD152) was discovered in the late 1980s by French researchers who discovered that it avoids overreactions of the immune system [28]. It regulates early T cell activation. The antibody ipilimumab against CTLA-4 was approved by the US Food and Drug Administration (FDA) in 2011, and together with the checkpoint inhibitors anti-PD-1/anti-PD-L1 (approved in 2014), a breakthrough in melanoma therapy was made [29]. PD-1 was discovered in the early 1990s by a Japanese biologist who found it as a molecule expressed on dying T cells [28]. It was therefore named programmed cell death protein 1 (CD279). It limits activity of T cells in peripheral tissues at the time of inflammatory response and therefore later than CTLA-4. Hence, PD-1 is the major immune resistance mechanism in the tumor microenvironment. Figure 2 depicts the immunobiological concept of why RT in combination with anti-PD-L1 blockade stimulates CD8+ T cell-mediated anti-tumor immunity. Currently, in 2015, the largest group of the over 250 trials registered at clinicaltrials.gov with immunotherapies is of checkpoint inhibitors [30]. However, one has to acknowledge that although the responses in some patients are impressive, they are often of limited duration and not present in the majority of patients. This implies that combination of checkpoint inhibitors and classical therapies such as RT have to be reinforced.

Single immune-modulatory agents have been shown to enhance the anti-tumor effects of RT, even in poorly immunogenic tumors. In the metastatic mouse mammary carcinoma 4T1 model, for example, only combination of RT with a single dose of 12 Gy with anti-CTLA-4 mAb 9H10 extends the survival of mice in a CD8+ T cell-dependent manner [31]. Nevertheless, tumor-induced immunosuppression often restricts the success of radio-immunotherapies. Therefore, combination of antibodies (Abs) designed to stimulate immunity (e.g., anti-CD137 or anti-CD40) or relieve immunosuppression (anti-PD-1) with RT might overcome this suppression. Mice bearing established orthotopic AT-3 mammary tumors were all cured when anti-CD137 and anti-PD-1 monoclonal Abs were combined with single (12 Gy) or fractionated (4×4 Gy or 4×5 Gy) RT [32]. Furthermore, combination of two checkpoint inhibitors that target immune checkpoints both at early (anti-CTLA-4) and later (anti-PD-1) time points of T cell activation in concerted action with RT could be another solution. Addition of PD-L1 blockade reinvigorates exhausted T cells and improves the response to radiation plus anti-CTLA4 Ab treatment. While anti-CTLA-4 treatment predominantly decreased regulatory T cells,
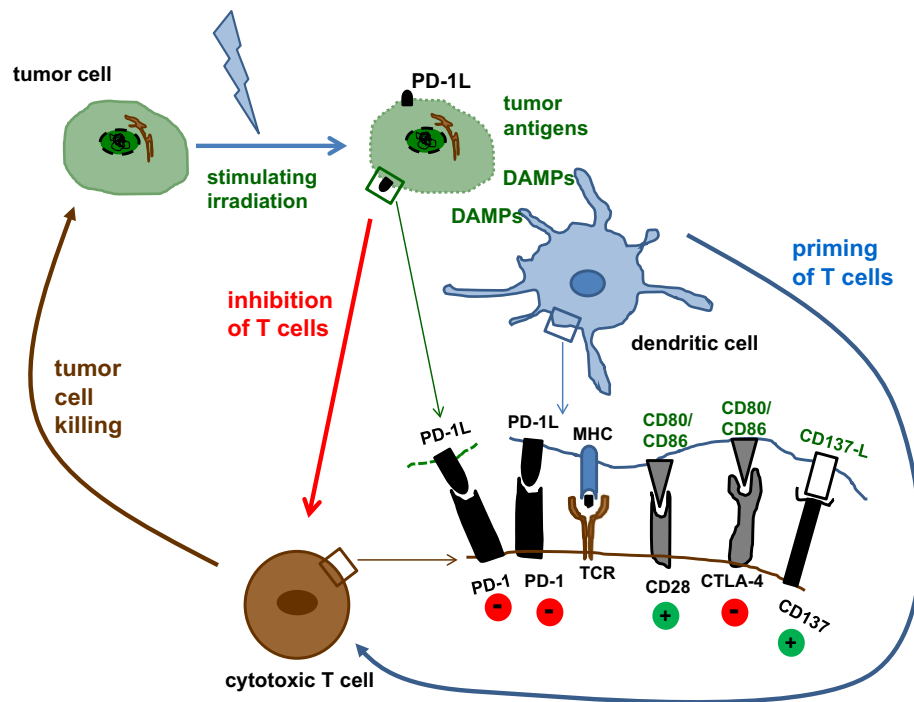
Fig. 2 Immune-modulating irradiation as a basis for combination of radiotherapy with checkpoint inhibitors. Immune-stimulating irradiation results in the release of damage-associated molecular patterns (DAMPs) and (modified) tumor antigens. Dendritic cells (DCs) are recruited and activated and take up the antigens (Ags). DCs then migrate to lymph nodes and cross-present the tumor Ags to T cells. The priming of T cells is highly controlled to avoid autoimmune reactions and to maintain self-tolerance. There are various ligand–receptor interactions between antigen-presenting cells such as DCs and T cells that regulate the T cell response to Ag. The up-regulation of the co-stimulatory molecules CD80 and CD86 on DCs in response to radiation-induced DAMPs delivers co-stimulatory signals to T cells. However, mostly pairs of co-stimulatory–inhibitory receptors bind the same ligand(s) (e.g., CD28 and cytotoxic T lymphocyte antigen 4 (CTLA-4) both bind to CD80/CD86). Of note is that the inhibitory receptor is commonly up-regulated later on, namely after T cell activation. A longer-lasting T cell activation can therefore be achieved when antibodies designed to relieve immunosuppression such as anti-CTLA-4 are administered in addition to RT. Besides these immune-activating effects of RT, potentially deleterious effects exist. Tumor cells can up-regulate programmed cell death protein 1 ligand (PD-L1) in response to radiation. PD-L1 interaction with PD-1 on T cells then shuts down the cytotoxic T cell response within the tumor. This is another reason for combining RT with Abs that inhibit immune checkpoint proteins or the interaction with their respective receptors. Since anti-CTLA-4 and anti-PD-L1 use non-redundant immune mechanisms to enhance anti-tumor immunity and act at different time points during establishment of anti-tumor immune reactions, dual immune checkpoint inhibition in combination with RT might be the therapy of choice for the future

anti-PD-L1 treatment increased the frequency of tumor-infiltrating CD8+ T cells [33]. Kim et al. [34] recently demonstrated in C57BL/6 mice that were implanted with the mouse glioma cell line GL261 transfected with luciferase that combination of anti-TIM-3 with anti-PD-1 and stereotactic radiosurgery was synergistic and conferred a significant survival benefit.

Importance of the timing of RT with immune checkpoint inhibition

A big challenge is to identify not only the most beneficial combination of certain radiation schedules with immunotherapies but also the chronological sequence. Immune cells are recruited into the tumor after irradiation. Since RT is mostly delivered in fractions, re-irradiation of the tumor might kill the infiltrating immune cells that are already fighting the tumor or are activated for migration into the next lymph node to prime cytotoxic T cells. Our own work demonstrates that when starting 2 days after the last irradiation with 2 × 5 Gy, immune cells migrate into CT-26 colorectal tumors and rest there only for a few days (Frey et al. [35] manuscript in preparation). Additional immune activation by immunotherapy should be administered in this time frame and RT should be paused. This suggests that immunotherapies should be applied in the middle to the end of the working week to exploit the radiation pause at the week ends.

Adaptive immune resistance mechanisms further determine the design of multimodal therapies. When a weak endogenous immune response is present, no up-regulation of PD-L1 ligand on tumor cells is likely. In this case, therapy with anti-PD-L1/anti-PD-1 Abs is not effective.

Inducers of anti-tumor immunity such as RT also have some deleterious effects and can induce expression of PD-L1 ligand on tumor cells and tumor-associated antigen-presenting cells (APCs). However, here consecutive therapy with anti-PD-L1/anti-PD-1 Abs becomes beneficial and finally results in induction of anti-tumor immunity [27]. Three days after irradiation with 12 Gy of a spontaneous mammary tumor (TUBO) in BALB/c mice, an increase in the expression of PD-L1 was observed on DCs and on tumor cells. Combined treatment of the tumor with RT plus anti-PD-L1 was most effective and dependent on CD8+ T cells that induce apoptosis of myeloid-derived suppressor cells (MDSCs) through tumor necrosis factor (TNF) alpha [36]. Furthermore, interferon gamma produced by CD8+ T cells is responsible for mediating PD-L1 up-regulation on tumor cells after delivery of fractionated RT with 5×2 Gy, as demonstrated with the CT-26 colorectal tumor model. Of special note is that again the dosing schedule is critical to the outcome with RT: The survival of the mice was significantly improved only when anti-PD-L1 Ab was given concomitantly, namely at the beginning or the end of the fractionated irradiation. Delivery of anti-PD-1 Ab one week after the last irradiation did not improve the sole effects of RT on the tumor [37].

Clinical evidence of beneficial interactions of RT with immunotherapy

Although there is strong evidence from preclinical work that RT and immunotherapy fit together, clinical reports detailing the interaction of RT with immunotherapies are limited, but on the way (summarized in [38]). Immunological effects of RT alone are mostly described in patients with melanoma. Furthermore, DCs loaded with antigens from irradiated autologous proliferating tumor cells are superior to vaccination with antigens only. For the combination of IL-2 with RT, higher single doses seem to be of advantage. Abscopal, namely immune-mediated systemic, anti-tumor effects of RT are predominantly observed after combination with anti-CTLA-4 Ab and anti-PD-L1 Ab. Again, tumor regressions are only observed in a subset of patients. Metastatic melanoma patients with tumors showing high PD-L1 expression did not respond to RT plus anti-CTLA4. This suggests that radiation, anti-CTLA4 and anti-PD-L1 have distinct effects on the TCR repertoire, regulatory T cells and T cell exhaustion. Their combination promotes response and immunity through distinct mechanisms, as outlined above [33]. A further breakthrough is the study of Golden et al. [39] demonstrating that combination of radio(chemo)therapy with granulocyte-macrophage colony-stimulating factor (GM-CSF) generates systemic anti-tumor responses not only in melanoma, but also in other

solid tumor entities, such as non-small cell lung carcinoma (NSCLC) and breast cancer. Immunotherapy works best for tumors with high somatic mutation prevalence. Melanoma, lung cancer and colorectal cancer have this prevalence [40]. Nevertheless, it clearly also works in breast cancer and glioma, which display lower prevalence. Further stress stimuli such as RT and CT might enhance the mutation rates and thereby support additional immunotherapy.

Tremelimumab and ipilimumab are two fully humanized anti-CTLA-4 antibodies that have advanced to testing in clinical trials. Again, in the beginning most studies focused on melanoma [41], but there are currently several ongoing trials on combination of RT and anti-CTLA-4 antibody therapy for other tumor entities, such as unresectable pancreatic cancer (NCT02311361), metastatic castration-resistant prostate cancer (NCT00861614), metastatic NSCLC (NCT02221739), advanced cervical cancer (NCT01711515), and metastatic cancers of the liver and lungs (NCT02239900). Single-agent trials have already been initiated with the anti-PD-1 antibodies nivolumab, pembrolizumab and pidilizumab, and current efforts focus on trials of combination treatments together with efforts to identify predictive and prognostic biomarkers [42].

Conclusions and challenges

Many preclinical and more and more clinical data prove that RT and immunotherapies fit together. Concurrent application of immune checkpoint inhibitors and RT makes sense with the current knowledge of the biological modes of action. However, more clinical studies are urgently needed in order to gain deeper knowledge of side effects, such as severe inflammation and strong autoimmune reactions. No conclusive preclinical data on the most beneficial dose and fractionation of RT are available so far. From the immunological point of view, hypofractionated RT might be ahead, since in the longer radiation pauses the immune system has time to act and react. Combined therapies work best for tumors with mutational processes such as melanoma, lung cancer, bladder cancer, esophageal cancer, colorectal cancer, cervical cancer, and head and neck cancer, but others should not remain unconsidered. Furthermore, also malignancies of hematopoietic origin have to be followed up since they are also capable of co-opting their local environment in order to escape immune attack. In selected subtypes of Hodgkin lymphoma (nodular sclerosing Hodgkin lymphoma), for example, PD-1 ligands are over-expressed due to a genetic amplification of the loci encoding them [43]. They should therefore be ideal candidates for combined treatment with RT plus immune checkpoint inhibitors. Since RCT is usually performed in clinics for most tumor entities, chemotherapeutic agents that

induce immunogenic cancer cell death, such as anthracyclines and oxaliplatin, should be considered.

Of further note is that the time frame of response differs between classical therapies and immunotherapies. The response of CT and small molecule inhibitors is within weeks, but the response of immune checkpoint inhibitors is within months. Sometimes initial increase in metastatic lesions due to infiltrating immune cells even occurs. This provides a case for re-evaluation of response criteria such as time to progression, to name just one [27]. Since multiple and diverse anti-tumor and immune escape mechanisms are triggered by distinct checkpoint inhibitors and radiation schemes, clinical trials should always be accompanied by translational research projects to define prognostic and predictive immunobiomarkers [44]. Furthermore, since damage induced by ionizing radiation strongly impacts on the tumor microenvironment, but also on the environment of healthy tissue, multiple targets for radiosensitization and radioprotection arise. Combinations of RT with immunomodulation, vascular normalization and antifibrotic therapy have to be carefully designed and evaluated in the near future [45]. Nevertheless, it has already become clear that RT complements immune checkpoint therapies and thereby acts locally and systemically to combat cancer [46, 47].

Compliance with ethical standards

Conflict of interest All authors declare that they have no competing interests.

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