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Targeting DNA damage response to enhance antitumor innate immunity in radiotherapy

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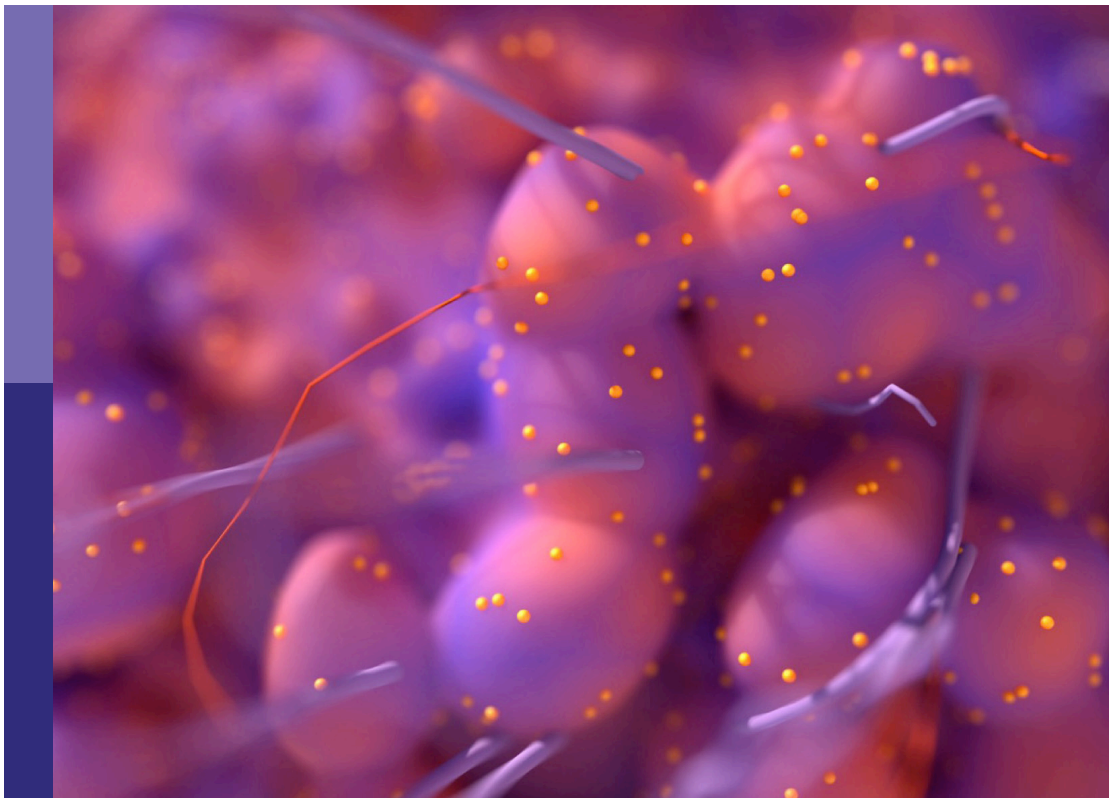

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Targeting DNA damage response to enhance antitumor innate immunity in radiotherapy

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Editorial: Targeting DNA damage response to enhance antitumor innate immunity in radiotherapy

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Editorial on the Research Topic
Targeting DNA damage response to enhance antitumor innate immunity in radiotherapy

Radiotherapy is a mainstay of cancer treatment that is used to treat approximately half of all cancers (1) with cure rates second only to surgery. The efficacy of radiotherapy has been largely attributed to the direct killing of tumor cells. Yet, recent research efforts highlighted considerable indirect effects of radiation on the tumor microenvironment (TME), especially the immune compartment, with clinical implications. This active field of research has revealed a complex relationship between radiation and the local/systemic immune system, yielding both immunostimulatory and immunosuppressive effects. Mechanistically, radiation creates a pro-immunogenic environment through the direct release of damage associated molecular patterns (DAMPs) during immunogenic cell death (2). Cells that survive after radiation modulate the immune system by: *1)* intracellular sensing of DAMPs by innate immunity sensors such as cGAS/STING and RIG-I-like receptors followed by production of type 1 interferons, and *2)* tumor-associated antigen cross-presentation (3–5). However, these initial immunostimulatory effects are often counterbalanced by immunosuppression. For instance, intracellularly, autophagy and mitophagy contribute to the clearance of immunostimulatory DAMPs (6). In the TME, longer-term immunosuppressive effects are driven by tumor-associated macrophages and myeloid-derived suppressor cells (7, 8). In addition, immune cell repopulation can occur post radiation as the irradiated tissue is driven towards a wound-healing microenvironment (9). Thus, a complex balance of several factors determines whether radiation induces a suppressed or stimulated immune environment. Current efforts are focused on understanding how the interaction between radiation and immunity plays out in the TME, with the goal of designing interventions to promote an immunostimulatory environment.

Shifting the balance toward the immune stimulatory effects of radiation, requires an in-depth knowledge of the biological effects of radiation on the tumor innate immune response and on the different immune cellular compartments. Furthermore, the contribution of tumor specific characteristics, like tumor type and stage, needs also to be considered. In this special edition, Beach et al. review the differential effects of radiation on macrophage populations in the TME. Tumor associated macrophages can be polarized by radiation into anti-inflammatory/pro-tumorigenic macrophages or pro-inflammatory/anti-tumorigenic macrophages depending on the context (Beach et al.). This exemplifies the dual potential of a single immune cell population within the TME to either promote or eradicate tumor cells, depending on factors including radiation dose, the immune profile of the TME, and the tumor type. Further insight regarding the interplay between the tumor and immune response to radiation is described by Gehre et al. Specifically, the authors demonstrate that radioresistant triple negative breast cancer cells upregulate multiple immune checkpoint molecules on their surface compared to radiosensitive cells upon radiation (Gehre et al.). Whether or not radiation leads to immune stimulation is dependent on a combination of factors including tumor intrinsic properties and the broader immune landscape.

Beyond the direct interactions of radiation with tumor cells and intratumoral immune cells, radiation may also have beneficial effects on peripheral immune cells leading to an adaptive immune response. Craig et al. comprehensively review the abscopal effect, a phenomenon whereby radiotherapy efficacy is extended beyond the tumor in the radiation field to tumor(s) outside of the radiation field by engaging a systemic/adaptive immune response. The presence of an abscopal effect has important implications in the context of metastatic and recurrent disease. Although abscopal responses remain rare in clinical settings, there is growing interest in investigating strategies to enhance the presence and consistency of abscopal responses. For instance, a recent study suggested blocking CD47/SIRPα axis increases radiation-induced phagocytosis and immune priming, leading to enhanced systematic tumor control (10, 11).

Therapeutic strategies that enhance anti-tumoral immune responses to radiotherapy such as those targeting the DNA damage and replication stress responses as well as immune checkpoints are currently an intense area of investigation with potential to further improve patient outcomes to radiotherapy. Daley et al. and Jungles et al. provide comprehensive reviews on the biological rationale and current clinical investigation of combining radiation with other treatment modalities in Ewing sarcoma and breast cancer, respectively. For example, several clinical trials are underway to evaluate the combination of PARP inhibitors, radiotherapy, and immunotherapy in breast cancer patients with or without BRCA deleterious mutations.

Inhibitors of the DNA damage response (DDR) are effective radiation sensitizers targeting multiple protective pathways, such as cell cycle checkpoints and DNA repair, that have recently emerged as promising strategies for sensitizing to immunotherapy (12, 13). Combining DDR inhibitors with radiation is an active area of both pre-clinical and clinical research reviewed by Carlsen and El-Deiry and Chan Wah Hak et al. The ability of DDR inhibitors to enhance radiation-induced immune effects including increased type 1 interferon production and immune cell infiltration is highlighted (Chan Wah Hak et al.). Interestingly, inhibition of different DDR targets enhances radiation efficacy with varying magnitudes by synergizing with different pathways of innate immune signaling (14–17). In this Research Topic, Mariampilla et al. describe how ATR inhibition following radiation enhances interferon signaling mediated by cGAS signaling in human lung cancer and osteosarcoma cells. Additional radiosensitizers, including those which target the replication stress response, are being investigated clinically and are reviewed in Zhang et al. Based on the capacity for DDR inhibitors to enhance the immune effects caused by radiation, it is conceivable that these combinations may further sensitize tumor cells to immunotherapy.

Future investigation into the foundational mechanisms behind radiation-induced immune modulation, as well as the synergies with existing treatment modalities, might provide a rationale for leveraging combinatorial strategies in clinical settings aimed at enhancing radiation-induced immune stimulation and sensitization of tumors to immunotherapy. While these concepts are thoroughly covered in the Research Topic, additional work should focus on determining the differential properties of each treatment, alone or in combination, to reveal which settings provide the best clinical outcomes while minimizing toxicity that could arise in the presence of excess systemic inflammation. This will provide clinicians with needed information to accurately match patients with the most effective treatment to ultimately improve the prognosis of the >18 million of new cancer patients diagnosed each year.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

Author RE was employed by the company AstraZeneca.

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SYSTEMATIC REVIEW
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Replication Stress: A Review of Novel Targets to Enhance Radiosensitivity-From Bench to Clinic

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DNA replication is a process fundamental in all living organisms in which deregulation, known as replication stress, often leads to genomic instability, a hallmark of cancer. Most malignant tumors sustain persistent proliferation and tolerate replication stress *via* increasing reliance to the replication stress response. So whilst replication stress induces genomic instability and tumorigenesis, the replication stress response exhibits a unique cancer-specific vulnerability that can be targeted to induce catastrophic cell proliferation. Radiation therapy, most used in cancer treatment, induces a plethora of DNA lesions that affect DNA integrity and, in-turn, DNA replication. Owing to radiation dose limitations for specific organs and tumor tissue resistance, the therapeutic window is narrow. Thus, a means to eliminate or reduce tumor radioresistance is urgently needed. Current research trends have highlighted the potential of combining replication stress regulators with radiation therapy to capitalize on the high replication stress of tumors. Here, we review the current body of evidence regarding the role of replication stress in tumor progression and discuss potential means of enhancing tumor radiosensitivity by targeting the replication stress response. We offer new insights into the possibility of combining radiation therapy with replication stress drugs for clinical use.

Keywords: replication stress, DNA damage repair, radiation therapy, radioresistance, radiosensitizer

BACKGROUND

Although radiation therapy (RT) is used to treat ~50% of malignant tumors (1), it accounts for only 5% of the total cost of cancer patient care, making it the most cost-effective cancer treatment (2). RT is also an effective treatment for patients exhibiting a poor performance status who cannot tolerate surgery (3). Although new technologies, such as CyberKnife®, Tomotherapy®, and proton and heavy ion radiotherapy have been developed, radioresistance remains a crucial factor limiting our ability to cure cancer (4). Primary radioresistance can be caused by genomic or epigenetic changes

in tumor cells, and radiation-induced genomic changes lead to secondary radioresistance, which is the most common cause of treatment failure and disease recurrence (5). Owing to limitations associated with normal tissue tolerance, increasing radiosensitivity in only cancer cells remains challenging.

Replication stress (RS) is the slowing or stalling of replication fork progression and is a major cause of genomic instability in cancer cells, which induces the accumulation of mutated and damaged DNA (6). In normal tissues, RS is a factor in the natural aging process (7). Cellular response to RS activates checkpoints to arrest cell cycle and repair DNA damage. Importantly, RS is selectively higher in cancer cells than in normal cells, and makes cancer cells more dependent on RS response pathways to survive (8, 9). Oncogene activation drives continuous proliferation, which is the basis for the generation of RS known as oncogene-induced RS. It is an important source of genome instability and might therefore be the basis of intratumor heterogeneity (10). Moreover, RS-induced DNA damage in tumors activates specific DNA damage repair pathways due to different genomic background cancer types. It also causes cells to enter mitosis with under-replicated regions that can cause genomic instability, thus potentially enhancing malignant behaviors (11). If the cellular response to RS is ineffective, then cells enter mitosis with an excess of damaged DNA, resulting in genomic instability or cell death due to mitotic catastrophe (12). These differences between normal and tumor cells suggest that targeting RS may contribute to the specific elimination of tumors (13).

Abbreviations: RT, radiation therapy; RS, replication stress; DDR, DNA damage response; ATR, ataxia telangiectasia and rad3-related; CHK1, checkpoint kinase 1; ssDNA, single-stranded DNA; RPA, replication protein A; DSBs, double-strand breaks; UPR, unfolded protein response; HR, homologous recombination; T-LAK, T-lymphoid-activated killer; TOPK, T-LAK cell-derived protein kinase; Mcl-1, myeloid cell leukemia sequence 1; PARPs, poly (ADP-ribose) polymerases; IR, ionizing radiation; ATM, ataxia telangiectasia mutated; MRE11, meiotic recombination 11; MDM2, mouse double minute 2; POLQ, DNA polymerase theta; BRCA, breast cancer related protein; mTOR, mammalian target of the rapamycin; SUMO, small ubiquitin-like modifier; HIF, hypoxia inducible factor; RSF-1, spacing factor-1; NHEJ, non-homologous end joining; CDC, cell division cycle; SAC, spindle assembly checkpoint; APC/C, anaphase-promoting complex or cyclosome; PI3K, phosphoinositide 3-kinase; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; TAME, tosyl-L-arginine methyl ester; TOP3A, topoisomerase IIIα; RMI, RecQ-mediated genome instability; BTR, BLM-Topoisomerase IIIα-RMI1-RMI2; BLM, bloom syndrome helicase; MAC, MOS4-associated complex; MRN, MRE11/RAD50/NBS1; hTERT, human telomerase reverse transcriptase; RFWD3, RING finger and WD repeat domain 3; CHK1, checkpoint kinase 1; AKT, protein kinase B; mTOR, mammalian target of rapamycin; GBM, glioblastoma; USP9X, ubiquitin-specific protease 9X; KDM4C, lysine-specific demethylase 4C; TGF-β2, transforming growth factor-β2; UBE2O, ubiquitin-conjugating enzyme E2O; Mxi1, MAX interactor 1; SENP, SUMO-specific protease; 53BP1, p53-binding protein 1; Rnf4, Ring finger protein 4; MDC1, mediators of DNA damage checkpoint protein 1; ER, endoplasmic reticulum; SETX, senataxin; PKR, protein kinase R; PERK, PKR-like ER kinase; ATF4, activating transcription factor 4; PRRs, pattern recognition receptors; DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; IFNs, interferons; cGAMP, cyclic GMP-AMP; STING, stimulator of the interferon gene; cGAS, cGAMP synthase; IRF3, interferon regulatory factor 3; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cell; EVs, extracellular vesicles; TME, tumor microenvironment; PD-L1, programmed death ligand 1; PD-1, programmed cell death protein 1.

RS has been highlighted as a hallmark of malignant tumor radiosensitivity (6, 14). Impaired responses to RS sensitize tumors to radiation (15), highlighting the importance of RS-aimed therapy for radiation treatment. Here, we summarize the current body of evidence concerning RS in cancer radiosensitivity, including known inhibitors and other potential targets. Treatments targeting RS-related pathways are suggested as an ideal radiosensitizer for cancer treatment.

RS

Accurate DNA information is crucial for ensuring genomic stability. Conserving DNA integrity during DNA replication requires coordination between multiple cis- and trans-acting factors, such as regulating fork movement, nucleotide supply, transcription machinery, cellular checkpoints, and DNA repair pathways (16, 17). Here, we briefly summarize how RS occurs in malignant cells and the differences between cancer and normal cells, and then reason why RS is an ideal target for cancer treatment.

Sources

Several major exogenous and endogenous factors that cause RS are listed here. Endogenous factors include alternative structures of DNA, centromeres, telomeres, DNA binding non-histones, replication, and transcription conflicts. All replication stressors affect the replication fork timing, causing the replication fork to slow down or even stall. Exogenous factors including DNA damage caused by radiation or cytotoxic substances, nucleotide loss, and abnormal replication, which activate DNA damage response (DDR) (**Figure 1**) (18).

RS Responses

Cells have several strategies for dealing with RS called "RS responses", including re-priming, fork reversal and restart, translation synthesis, template switching, and break-induced replication (16). RS response dysregulation is a typical characteristic of tumors, which may be caused by the loss of tumor suppressor factor or abnormal oncogene expression. Chronic RS increases the chance of breakage or gap formation in fragile sites, resulting in genomic instability, promoting further activation of oncogenes, and inducing malignant tumors in the early stage (8). Although mild or moderate levels of RS may induce tumorigenesis and promote tumor progression by accumulation genomic instability, in the event of severe and persistent RS, cells will finally develop mitotic disaster, senescence, or apoptosis (19). In the absence of active ataxia telangiectasia and rad3-related (ATR) and checkpoint kinase 1 (CHK1), replication forks cannot be stalled and thus continue to trigger dormant replication origins, leading to deoxynucleotide triphosphate pool depletion as well as slowing and stalling replication fork progression (12). When single-stranded DNA (ssDNA) is no longer protected by replication protein A (RPA), the replication fork collapses, resulting in double-strand breaks (DSBs). When these cells enter mitosis, unduplicated chromosomes trigger cell death through mitotic disasters

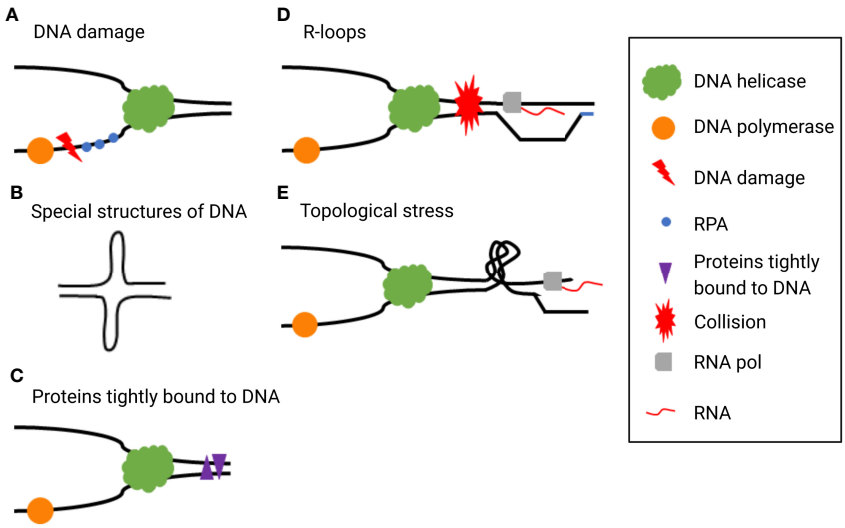

FIGURE 1 | Typical exogenous and endogenous sources cause replication stress (RS), such as **(A)** DNA damage, **(B)** special DNA structures, **(C)** proteins tightly bound to DNA, **(D)** R-loops, and **(E)** topological stress.

(20, 21). Moreover, mutations produced during cancer development enhance RS and cause tumor cells to be hyper-dependent on RS response (18), which may be a potential target for cancer therapy (**Figure 2**).

RS AND RADIORESISTANCE IN CANCER

It is well-established that tumor radiation sensitivity greatly varies among individuals. As a result, some drugs have been

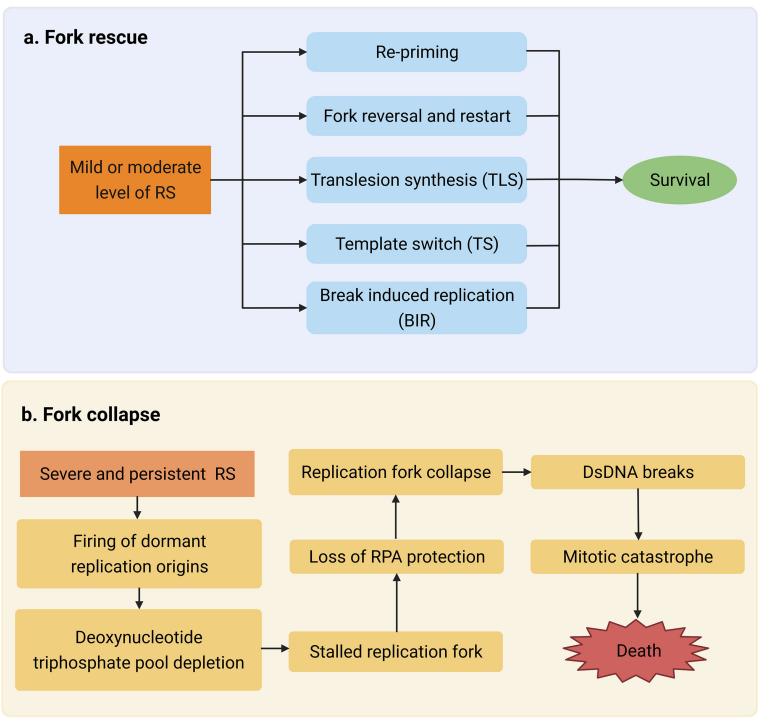

FIGURE 2 | **(A)** Mild or moderate level of replication stress (RS) activates multiple mechanisms such as re-priming to repair DNA damage. **(B)** Severe and persistent RS leads to double-stranded DNA (dsDNA) break accumulation and eventually causes mitotic catastrophe which triggers cell death.

reported to target multiple sensitivity or resistance factors (18, 22–24). Tumor radiosensitivity is mainly related to the intrinsic sensitivity of tumor cells and the cancer microenvironment (25). Here, we summarize the well-known mechanisms of radiation resistance and analyze the relationship between RS and the resistance factors (**Figure 3**).

Hypoxia

Hypoxia is a common feature of malignant tumors resulting from rapid cell proliferation coupled with abnormal vasculature formation (26) and plays a pivotal role in tumor progression and treatment resistance (27). Hypoxia inducible factor (HIF), especially HIF-1, is the key regulator response to hypoxia. Clinical data have shown that eliminating the hypoxic state of tumors is an effective radiosensitizer (28, 29). Preclinical research has shown that NVX-108 increases tumor oxygen levels by 400%, significantly enhancing radio sensitivity (30). Phase I/II clinical trials have indicated the safety of NVX-108, and studies evaluating its efficacy are ongoing (29). Hypoxia also alters cell cycle response to ensure survival and minimal errors throughout cell division (31). Recent research claimed that hypoxia-induced RS was linked to the unfolded protein response (UPR) (32). There are few proteins that link hypoxic DDR and UPR, which suggests that they could be novel therapeutic targets to improve radiotherapy response (33, 34).

Cell Apoptosis

Apoptosis is a key part of the intrinsic tumor suppression mechanism, which is triggered when proliferation becomes aberrant (35, 36). Targeting tumor cell apoptosis also contributes to radiosensitization. A high proportion of cells die through apoptosis, which is a positive indicator of radiosensitivity (37), and enhancing apoptosis effectively enhances tumor radiosensitivity. Knocking down remodeling and spacing factor-1 (RSF-1) enhanced the radiosensitivity of cervical cancer cells by redistributing the cell cycle, inducing cell apoptosis, and eventually inhibiting cell proliferation (38). Astaxanthin enhances irradiation-induced apoptosis in esophageal squamous cell carcinoma cells (39). Deficient RS response also leads to cell apoptosis, which suggests a role as a synergistic factor to RT (40).

Cell Cycle Distribution

The cell cycle distribution of cancer cells affects radio sensitization, especially for some cancer types that depend more on other DDR pathways rather than homologous repair (HR) (41). In different cell cycles, the differences in chromosome structure lead to unequal radiosensitivity. Clinicians believe G2/M is the most sensitive phase since the radiation induces more complex damage that induce longer cell cycle arrest and therefore need proficient HR for repair (42). Meanwhile, the damage that occurs during G2/M can more easily cause premature entry into mitosis, which can lead to a higher possibility of passing incorrect genomic information to the next generation, or even cause mitotic catastrophe directly (43). Eurycomalactone, an active quassinoid isolated from *Eurycoma longifolia*, has been shown to sensitize non-small cell lung cancer cells to X-rays through a G2/M block (44). Further studies have focused on the G2/M arrest after receiving radiation. When DDR is activated, it temporarily stops the cell cycle to provide more time for repair, or if the damage is too severe, induces apoptosis. Eliminating the radiation-induced G2/M arrest or forcing damage cells to enter into mitosis both sensitizes cancer cells to radiation treatment (45, 46). This cell-cycle-dependent radiosensitization mechanism provides potential directions for further research into radiosensitizers.

DNA Damage and Repair

Cells respond to DNA damage by activating the DDR pathway. Abnormal activation of DDR in tumor cells leads to the generation

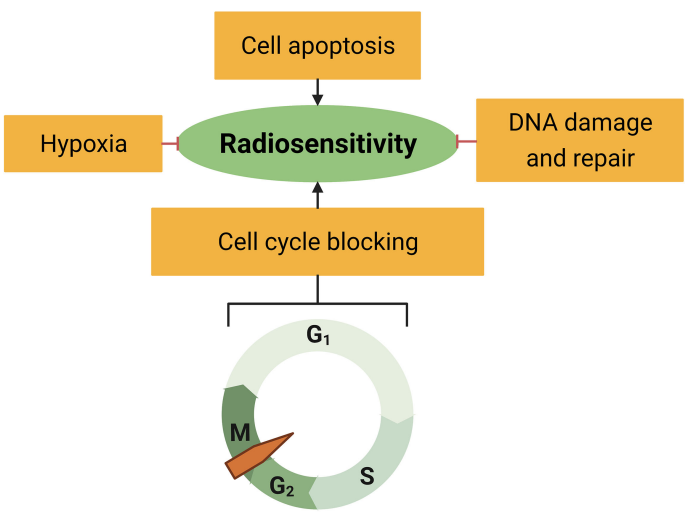

FIGURE 3 | Radiosensitivity is associated with hypoxia, cell apoptosis, cell cycle distribution, and DNA damage response.

of radiotherapy resistance (46). High RS also leads to DNA damage and activate the DDR pathway. The five major DNA repair pathways are base excision repair, nucleotide excision repair, mismatch repair, HR, and non-homologous end joining (NHEJ). Any impaired pathway can be compensated for by the overactivation of other pathways (47). These compensatory mechanisms in tumor cells lead to different responses to treatment with DNA damage agents, as well as RT (1, 48). Both RS and radiation activate a similar DDR pathway, providing the possibility of a synergistic effect of targeting RS with RT (49, 50).

TARGETING RS AS RADIATION SENSITIZER

Cancer cells relying more on RS response than normal cells to survive provides a potential target of anti-tumor treatment sensitization (51). In this section, we summarized and discussed the specific application of reagents targeting the RS response or RS-induced DDR that have already been demonstrated to be effective or have the potential to enhance tumor radiosensitization (**Table 1**, **Figure 4**).

TABLE 1 | Targeting replication stress as radiation sensitizer.

Targeted Marker	Mechanism	Drug	Phase	Details (Including NCT Number)	Status
Inducing exorbitant RS					
CDC6	Decreased CDC6 expression in tumor cells effectively inhibits tumor cell growth and promotes apoptosis by preventing G1/S and S/G2 transition.	–	–	–	–
TOPK	TOPK sensitizes cancer cells to radiotherapy, owing to the preservation of irradiation-induced damage and reduced tolerance to RS.	–	–	–	–
CDC20	Reduced CDC20 expression disrupts the APC-CDC20 interaction and shows great effect on suppressing tumor proliferating and metastasis.	TAME	–	–	–
		pro-TAME	–	–	–
		Apcin	–	–	–
Mcl-1	Mcl-1 blocks radiation-induced apoptosis and inhibits clonogenic cell death.	BAY1143572 (Atuveciclib)	Phase I	Phase I Dose Escalation of BAY1143572 in Subjects With Acute Leukemia (NCT02345382)	Completed
			Phase I	Open Label Phase I Dose Escalation Study With BAY1143572 in Patients With Advanced Cancer (NCT01938638)	Completed
		UMI77	–	–	–
Targeting RS response					
PARP	Inhibition of PARP forces PARP to trap onto DNA thus preventing replication restart, causing RS-induced DNA damage.	Rucaparib (AG014699)	Phase I	A Study of Rucaparib Administered With Radiation in Patients With Triple Negative Breast Cancer With an Incomplete Response Following Chemotherapy (NCT03542175)	Recruiting
		Niraparib (MK-4827, Zejula)	Phase I/II	A Safety Study Adding Niraparib and Dostarlimab to Radiation Therapy for Rectal Cancers (NCT04926324)	Not yet recruiting
			Phase II	The Efficacy and Safety of Radiotherapy Plus Niraparib and Toripalimab in Patients With Recurrent Small Cell Lung Cancer (NCT05162196)	Not yet recruiting
			Phase I/II	Study of Niraparib With Radiotherapy for Treatment of Metastatic Invasive Carcinoma of the Cervix (NCT03644342)	Recruiting
			Phase II	Radiation, Immunotherapy and PARP Inhibitor in Triple Negative Breast Cancer (NCT04837209)	Recruiting
			Phase II	Niraparib With Standard Combination Radiation Therapy and Androgen Deprivation Therapy in Treating Patients With High Risk Prostate Cancer (NCT04037254)	Recruiting
			Phase II	Androgen Ablation Therapy With or Without Niraparib After Radiation Therapy for the Treatment of High-Risk Localized or Locally Advanced Prostate Cancer (NCT04947254)	Recruiting
			Phase II	Niraparib Combined With Radiotherapy in rGBM (NCT04715620)	Recruiting
			Phase II	Niraparib + Dostarlimab + RT in Pancreatic Cancer (NCT04409002)	Active, not recruiting
			Phase I/II	A Multi-Center Trial of Androgen Suppression With Abiraterone Acetate, Leuprolide, PARP Inhibition and	Recruiting

(Continued)

TABLE 1 | Continued

Targeted Marker	Mechanism	Drug	Phase	Details (Including NCT Number)	Status
		Talazoparib (BMN673, Talzenna)	Phase I	Stereotactic Body Radiotherapy in Prostate Cancer (NCT04194554)	Recruiting
				Talazoparib and Radiation Therapy in Treating Patients With Locally Recurrent Gynecologic Cancers (NCT03968406)	
			Phase II	A Study to Evaluate TAlazoparib, Radiotherapy and Atezolizumab in gBRCA 1/2 Negative Patients With PD-L1 + Metastatic Triple Negative Breast Cancer (NCT04690855)	Recruiting
			Phase I	Talazoparib and Thoracic RT for ES-SCLC (NCT04170946)	Recruiting
		Olaparib (AZD2281, KU0059436)	Phase I	Olaparib & Radiation Therapy for Patients Triple Negative Breast Cancer (TNBC) (NCT03109080)	Active, not recruiting
			Phase I/II	Phase I/IIa Study of Concomitant Radiotherapy With Olaparib and Temozolomide in Unresectable High Grade Gliomas Patients (NCT03212742)	Recruiting
			Phase II	Focal Radiation With Pulsed Systemic Therapy of Abiraterone, Androgen Deprivation Therapy (ADT), Lynparza Towards Castration Sensitive Oligometastatic Prostate Cancer (FAALCON) (NCT04748042)	Recruiting
			Phase II	Radiation Therapy With or Without Olaparib in Treating Patients With Inflammatory Breast Cancer (NCT03598257)	Recruiting
			Phase I	Study of Olaparib With Radiation Therapy and Cetuximab in Advanced Head and Neck Cancer With Heavy Smoking History (NCT01758731)	Completed
			Phase I	Olaparib and Radiotherapy in Inoperable Breast Cancer (NCT02227082)	Completed
			Phase I	Olaparib and Radiotherapy in Head and Neck Cancer (NCT02229656)	Active, not recruiting
			Phase II	A Study of Radiation Therapy With Pembrolizumab and Olaparib in Women Who Have Triple-Negative Breast Cancer (NCT04683679)	Recruiting
			Phase I	A Study of Olaparib and Low Dose Radiotherapy for Small Cell Lung Cancer (NCT03532880)	Recruiting
			Phase I	Radiotherapy & Olaparib in COmbination for Carcinoma of the Oesophagus (NCT01460888)	Unknown
			Phase I	A Study of Olaparib With Concomitant Radiotherapy in Locally Advanced/Unresectable Soft-tissue Sarcoma (NCT02787642)	Recruiting
			Phase I/II	Olaparib and Durvalumab With Carboplatin, Etoposide, and/or Radiation Therapy for the Treatment of Extensive-Stage Small Cell Lung Cancer, PRIO Trial (NCT04728230)	Recruiting
			Phase I	Radiotherapy and Durvalumab/Durvalumab Combo (Tremelimumab/Olaparid) for Small Cell Lung Cancer (NCT03923270)	Recruiting
			Phase I	Olaparib Dose Escalating Trial + Concurrent RT With or Without Cisplatin in Locally Advanced NSCLC (NCT01562210)	Completed
			Phase I	A Study to Investigate Biomarker Effects of Pre-Surgical Treatment With DNA Damage Repair (DDR) Agents in Patients With Head and Neck Squamous Cell Carcinoma (HNSCC) (NCT03022409)	Completed
			Phase I	A Platform Study of Novel Agents in Combination With Radiotherapy in NSCLC (NCT04550104)	Recruiting
			Phase I/II	Lu-177-DOTATATE (Lutathera) in Combination With Olaparib in Inoperable Gastroenteropancreatico Neuroendocrine Tumors (GEP-NET) (NCT04086485)	Not yet recruiting
			Phase I	Phase I Study of Olaparib With Cisplatin Based Chemoradiotherapy in Squamous Cell Carcinoma of the Head and Neck (NCT01491139)	Withdrawn
			Phase II/III	Refining Adjuvant Treatment IN Endometrial Cancer Based On Molecular Features (NCT05255653)	Not yet recruiting

(Continued)

TABLE 1 | Continued

Targeted Marker	Mechanism	Drug	Phase	Details (Including NCT Number)	Status
		Veliparib (ABT-888, NSC 737664)	Phase I	A Phase I Study of ABT-888 in Combination With Conventional Whole Brain Radiation Therapy (WBRT) in Cancer Patients With Brain Metastases (NCT00649207)	Completed
			Phase I	A Clinical Study Conducted in Multiple Centers Evaluating Escalating Doses of Veliparib in Combination With Capecitabine and Radiation in Patients With Locally Advanced Rectal Cancer (NCT01589419)	Completed
			Phase I	Veliparib in Combination With Gemcitabine and Intensity Modulated Radiation Therapy in Patients With Pancreatic Cancer (NCT01908478)	Completed
			Phase I/II	Veliparib, Radiation Therapy, and Temozolomide in Treating Younger Patients With Newly Diagnosed Diffuse Pontine Gliomas (NCT01514201)	Completed
			Phase II	Comparison of Veliparib and Whole Brain Radiation Therapy (WBRT) Versus Placebo and WBRT in Adults With Brain Metastases From Non-Small Cell Lung Cancer	Completed
			Phase I	Veliparib and Radiation Therapy in Treating Patients With Advanced Solid Malignancies With Peritoneal Carcinomatosis, Epithelial Ovarian, Fallopian, or Primary Peritoneal Cancer (NCT01264432)	Completed
			Phase I	Veliparib With Radiation Therapy in Patients With Inflammatory or Loco-regionally Recurrent Breast Cancer (NCT01477489)	Completed
			Phase I	Pre-Operative Radiation and Veliparib for Breast Cancer (NCT01618357)	Recruiting
			Phase II	Veliparib, Radiation Therapy, and Temozolomide in Treating Patients With Newly Diagnosed Malignant Glioma Without H3 K27M or BRAFV600 Mutations (NCT03581292)	Active, not recruiting
			Phase I	ABT-888, Radiation Therapy, and Temozolomide in Treating Patients With Newly Diagnosed Glioblastoma Multiforme (NCT00770471)	Completed
			Phase I/II	Veliparib With or Without Radiation Therapy, Carboplatin, and Paclitaxel in Patients With Stage III Non-small Cell Lung Cancer That Cannot Be Removed by Surgery (NCT01386385)	Active, not recruiting
			Phase I/II	A Study Evaluating the Efficacy and Tolerability of Veliparib in Combination With Paclitaxel/Carboplatin-Based Chemoradiotherapy Followed by Veliparib and Paclitaxel/ Carboplatin Consolidation in Adults With Stage III Non-Small Cell Lung Cancer (NSCLC) (NCT02412371)	Terminated
RPA	Overexpression of RPA significantly increases the radiation resistance in multiple cancer types.	–	–	–	–
TopBP1	TopBP1 is known to form phase-separated nuclear condensates that amplify ATR activity to CHK1 and slow down replication forks.	–	–	–	–
ATR-CHK1	Inhibition of ATR-related signaling pathways increases cell apoptosis and effectively improves tumor radiosensitivity.	AZD6738 (Ceralasertib)	Phase I	Phase I Study to Assess Safety of AZD6738 Alone and in Combination With Radiotherapy in Patients With Solid Tumours (NCT02223923)	Unknown
			Phase I	A Study to Investigate Biomarker Effects of Pre-Surgical Treatment With DNA Damage Repair (DDR) Agents in Patients With Head and Neck Squamous Cell Carcinoma (HNSCC) (NCT03022409)	Completed
		VE-821	–	–	–
		SAR-020106	–	–	–
		BAY1895344 (Elimusertib)	Phase I	First-in-human Study of ATR Inhibitor BAY1895344 in Patients With Advanced Solid Tumors and Lymphomas (NCT03188965)	Active, not recruiting
			Phase I	Testing the Addition of an Anti-cancer Drug, BAY1895344, With Radiation Therapy to the Usual Pembrolizumab	Recruiting

(Continued)

TABLE 1 | Continued

Targeted Marker	Mechanism	Drug	Phase	Details (Including NCT Number)	Status
				Treatment for Recurrent Head and Neck Cancer (NCT04576091)	
RAD51	Inhibition of RAD51 induces RS to promote apoptosis.	Berberine	–	–	–
		Valproate	Phase II	Valproic Acid, Radiation, and Bevacizumab in Children With High Grade Gliomas or Diffuse Intrinsic Pontine Glioma (NCT00879437)	Completed
			Phase I/II	Preoperative Valproic Acid and Radiation Therapy for Rectal Cancer (NCT01898104)	Recruiting
			Phase II	Valproic Acid With Temozolomide and Radiation Therapy to Treat Brain Tumors (NCT00302159)	Completed
			Phase I	Phase I Study of Temozolomide, Valproic Acid and Radiation Therapy in Patients With Brain Metastases (NCT00437957)	Terminated
			Phase I/II	Valproic Acid With Chemoradiotherapy for Non-Small-Cell Lung Cancer (NCT01203735)	Unknown
BLM	The high expression of BLM is a poor prognostic biomarker for multiple cancers. Though there's no data published about the links between BLM inhibitor and radiation sensitivity till now, it's a promising target worth further research.	ML216 (CID-49852229)	–	–	–
WEE1	Inhibition of WEE1 impairs RS response activated by ATR, and thus increasing tumor cell radiosensitivity.	AZD1775 (Adavosertib, MK-1775)	Phase I	Adavosertib, Radiation Therapy, and Temozolomide in Treating Patients With Newly Diagnosed or Recurrent Glioblastoma (NCT01849146)	Active, not recruiting
			Phase I	Testing the Addition of an Anti-cancer Drug, Adavosertib, to Radiation Therapy for Patients With Incurable Esophageal and Gastroesophageal Junction Cancers (NCT04460937)	Suspended
			Phase I	Adavosertib and Local Radiation Therapy in Treating Children With Newly Diagnosed Diffuse Intrinsic Pontine Gliomas (NCT01922076)	Active, not recruiting
			Phase I	Testing AZD1775 inC Combination With Radiotherapy and Chemotherapy in Cervical, Upper Vaginal and Uterine Cancers (NCT03345784)	Active, not recruiting
			Phase I	Dose-escalating AZD1775 + Concurrent Radiation + Cisplatin for Intermediate/High Risk HNSCC (NCT02585973)	Completed
			Phase I/II	Dose Escalation Trial of AZD1775 and Gemcitabine (+Radiation) for Unresectable Adenocarcinoma of the Pancreas (NCT02037230)	Completed
			Phase I	WEE1 Inhibitor With Cisplatin and Radiotherapy: A Trial in Head and Neck Cancer (NCT03028766)	Completed
Targeting RS induced DDR					
p53	Activation of p53 activates cell cycle block and apoptosis.	–	–	–	–
MRE11	Low MRE11 expression reduces phosphorylated DNA-PKcs expression, further increases tumor radiosensitivity.	Mirin	–	–	–
		Selenium	Phase II	Capecitabine, Oxaliplatin, Selenomethionine, and Radiation Therapy in Treating Patients Undergoing Surgery For Newly Diagnosed Stage II or III Rectal Adenocarcinoma (NCT00625183)	Terminated
			Phase II	Carboplatin, Paclitaxel, Selenomethionine, and Radiation Therapy in Treating Patients With Stage III Non-Small Cell Lung Cancer That Cannot Be Removed by Surgery (NCT00526890)	Terminated
			Phase II	Selenomethionine in Reducing Mucositis in Patients With Locally Advanced Head and Neck Cancer Who Are Receiving Cisplatin and Radiation Therapy (NCT01682031)	Terminated
			Phase II	Selenomethionine and Finasteride Before Surgery or Radiation Therapy in Treating Patients With Stage I or Stage II Prostate Cancer (NCT00736645)	Completed
			Phase II		Withdrawn

(Continued)

TABLE 1 | Continued

Targeted Marker	Mechanism	Drug	Phase	Details (Including NCT Number)	Status
				Selenomethionine in Treating Patients Undergoing Surgery or Internal Radiation Therapy for Stage I or Stage II Prostate Cancer (NCT00736164)	
		OBP-301 (Telomelysin)	Phase I	A Study of OBP-301 With Radiation Therapy in Patients With Esophageal Cancer (NCT03213054)	Unknown
ATM-CHK2	Deficiency of ATM shows radiation sensitizer effect in multiple cancer types. The effect of ATM on radiation sensitivity is more depend on cell cycle regulation rather than DDR pathway.	AZD0156	–	–	–
		AZD1390	Phase I	A Study to Assess the Safety and Tolerability of AZD1390 Given With Radiation Therapy in Patients With Brain Cancer (NCT03423628)	Recruiting
			Early Phase 1	AZD1390 in Recurrent Grade IV Glioma Patients (NCT05182905)	Recruiting
			Phase I	A Platform Study of Novel Agents in Combination With Radiotherapy in NSCLC (NCT04550104)	Recruiting
			Phase I	Sarcomas and DDR-Inhibition; a Combined Modality Study (NCT05116254)	Not yet recruiting
MDM2	Inhibition of MDM2 phosphorylation leads to cell apoptosis and cell cycle arrest, thus repressing tumor cell proliferation.	MI-219	–	–	–
		APG-115 (Alrizomadlin)	–	–	–
POLQ	Reduced POLQ expression inhibits DSB repair and tumor cell survival.	Novobiocin	–	–	–
BRCA	Mutations in BRCA is synthetic lethal with PARP inhibition.	–	–	–	–
PI3K/AKT/mTOR	Inhibition of PI3K/AKT/mTOR signaling pathway leads to cell cycle arrest in the G2/M phase and reduces tumor cell radio-resistance.	Dactolisib (BEZ235, NVP-BEZ235)	–	–	–
		Apitolisib (GDC-0980, RG7422, GNE 390)	–	–	–
		Torin2	–	–	–
Others					
Ubiquitin and SUMO	SUMO/ubiquitin equilibrium at active DNA replication forks controls CDK1 activation.	–	–	–	–
UPR	Activated UPR reduces the oxidative phosphorylation thus impairing cell cycle arrest and DNA repair factors after radiation also enhance radiation induced cell death.	ONC201 (TIC10)	Phase II	Combination Therapy for the Treatment of Diffuse Midline Gliomas (NCT05009992)	Recruiting
			Phase I	ONC201 and Radiation Therapy Before Surgery for the Treatment of Recurrent Glioblastoma (NCT04854044)	Withdrawn

Data retrieved from: https://clinicaltrials.gov/ct2/home Retrieval data 04/19/2022.
RS, replication stress; DDR, DNA damage response; CDC6, cell division cycle 6 homologue; TOPK, t-lymphoid-activated killer (T-LAK) cell-derived protein kinase; CDC20, cell division cycle protein 20 homologue; TAME, tosyl-L-arginine methyl ester; Mcl-1, myeloid cell leukemia sequence 1; PARP, poly (ADP-ribose) polymerases; RPA, replication protein A; TopBP1, topoisomerase II-binding protein 1; ATR, ataxia telangiectasia and rad3-related; CHK, checkpoint kinase; MRE11, meiotic recombination 11; ATM, ataxia telangiectasia mutated; MDM2, mouse double minute 2; POLQ, DNA polymerase theta; BRCA, breast cancer related protein; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; SUMO, small ubiquitin-like modifier; UPR, unfolded protein response.

Inducing Exorbitant RS

In this section, we summarized and discussed the known factors that contribute to the normal DNA replication process. Losing control of them triggers RS thus synthetically sensitizing radiation.

CDC6

Cell division cycle 6 homologue (CDC6) is an important regulator of DNA replication in eukaryotic cells (52, 53) involved in replication complex assembly during G1 phase. Replication fork stall accumulation caused by RS triggers G2/M checkpoint activation. CDC6 promotes the response of the G2/M checkpoint (54) and is positively correlated with tumor progression. Decreased CDC6 expression in tumor cells effectively inhibits tumor cell growth and promotes apoptosis by preventing G1/S and S/G2 transition (55). CDC6 overexpression has been observed in radiation-resistant cells, contributing to an increase in radiation resistance in cancer cells (56). CDC6 downregulation enhanced cisplatin-resistant bladder cancer cell sensitivity in a clinical trial, which is also related to DSB damage (57). Therefore, CDC6 inhibition in tumor cells might be an effective target for enhancing tumor radiosensitivity. Although CDC6 has druggable sites for a chemical molecular, it is an essential protein in most cell lines that makes it difficult for clinical transformation (58). Thus, further study on the

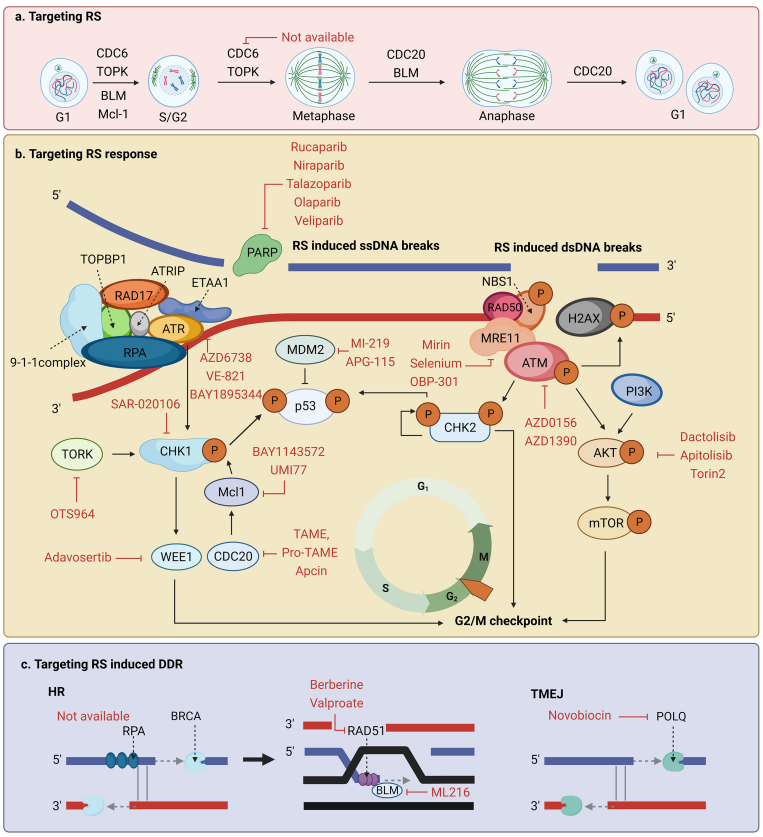

FIGURE 4 | Potential targets and corresponding inhibitors of **(A)** the replication stress (RS), **(B)** the RS response, or **(C)** RS-induced DNA damage response (DDR) that have been previously reported.

regulatory mechanism of CDC6 in radiation resistance will help to develop clinical practical drugs in the future.

TOPK

T-lymphoid-activated killer (T-LAK) cell-derived protein kinase (TOPK) is a mitogen-activated protein kinase kinase-like kinase that plays an important role in cell cycle regulation. TOPK overexpression is a pathophysiological feature in different tumors (59).

TOPK knockdown does not change the radiation response of normal tissues but significantly enhances cancer cell radiosensitivity, and TOPK disruption may lead to tumor-specific radiosensitivity (60). Thus, TOPK, as a cancer-specific biomarker and biochemical target, may enhance the efficacy of cancer treatment while causing minimal damage to normal tissues (59). TOPK was found to enhance tumor radiosensitivity by enhancing intratumor RS (61). Further experiments demonstrated that TOPK helps to restart the stopped replication fork. However, when TOPK was depleted, increased levels of stalled replication forks were observed, with or without external DNA damage (61). Therefore, TOPK suppression increases internal replication damage. Owing to the preservation of irradiation-induced damage and reduced tolerance to RS, TOPK sensitizes cancer cells to radiotherapy.

TOPK interacts with CHK1 and cell division cycle 25 homologue C (CDC25C) complex (key participants in the replication of the damage induced) (61). It facilitates mitotic progression at the G2/M checkpoint *via* cyclin-dependent kinase 1 (CDK1), and also occurs in response to replication stressors (such as irradiation) by influencing the action of key intermediates such as CHK1 (61). Therefore, the synergistic effect of TOPK inhibition and radiotherapy is likely to produce DSBs after replication. However, unlike CHK1, the toxicity of TOPK inhibitors is limited in normal tissues due to low expression. Therefore, TOPK appears to be a promising target for further research.

CDC20

Cell division cycle 20 homologue (CDC20) has important functions in chromosome segregation and mitotic exit. It is the target of the spindle assembly checkpoint (SAC) and the key cofactor of the anaphase-promoting complex or cyclosome (APC/C) E3 ubiquitin ligase, thus regulating APC/C ubiquitin activity on specific substrates for their subsequent degradation by the proteasome

(62). CDC20 is overexpressed in tumor cells and acts as a poor prognostic factor in multiple cancers (63, 64). It further increased after radiation and has been reported to increase radiation resistance *via* regulating B-cell lymphoma-2 (Bcl-2)/Bcl-2-associated X protein (Bax), forkhead box proteins O1 (FoxO1), or myeloid cell leukemia sequence 1 (Mcl-1)/p-CHK1 in different cancer types (65–67). Suppression of CDC20 expression reverses the radioresistance (65–67). There are multiple available inhibitors of CDC20, including tosyl-L-arginine methyl ester (TAME), Pro-TAME, and apcin. Their main effects involve disrupting the APC-CDC20 interaction (68, 69). Some of them showed great efficacy in suppress tumor proliferating and metastasis (70, 71). However, there has been no evidence on the effects of the CDC20 inhibitors on radiosensitivity. Therefore, CDC20 could be a potential target as a radiosensitizer, but more evidence in future studies is needed.

Mcl-1

As the first anti-apoptotic protein in the Bcl-2 family, Mcl-1 is regulated by the cell cycle and reach peak expression levels in the S/G2 phase. It acts as a functional switch in selecting between HR and NHEJ pathways after DNA damage (72). It blocks radiation-induced apoptosis and inhibits clonogenic cell death (73). Targeting Mcl-1 by a small molecule enhances RS sensitivity to cancer therapy (72). BAY1143572 downregulated Mcl-1 by inhibiting binding of HIF-1α to the Mcl-1 promoter (74). UMI77 is a selective inhibitor of Mcl-1 that dissociates Mcl-1 from the pro-apoptotic protein Bak and produced significant radiosensitization in pancreas cancers (75).

Targeting RS Response

Here, we summarize important RS response factors that are essential for cells to survive. Inhibition of these factors leads to uncontrolled replication collapse and even mitotic catastrophe, which makes them ideal targets for radiosensitization.

PARP

Poly (ADP-ribose) polymerases (PARPs) are involved in DDR and recruit DNA repair proteins to damaged sites by catalyzing ADP-ribosylation, leading to the formation of poly (ADP-ribose) polymers (76). PARP1, the most abundant PARP, plays a similar role to PARP2 in the DDR process and is an important regulator of fork reversal (77). Inhibition of PARP directly increases the speed of fork elongation and does not cause fork stalling, which contrasts with the accepted model in which inhibitors of PARP induce fork stalling and collapse. Aberrant acceleration of fork progression by 40% above the normal velocity leads to DNA damage (78).

However, the effects of PARP inhibitor do not directly decrease the expression of PARP. Rather, the inhibitor forces PARP to become stuck on DNA, thus preventing replication restart and causing RS-induced DNA damage (79). It was also linked to decreased replication fork length with greater ssDNA gaps, which in turn cause more genomic instability at G2/M (80). With all the evidence of PARP inhibitors in RS-induced DNA damage, researchers have reported on various preclinical models of combination therapy with PARP inhibitors and ionizing radiation (IR) (81). Olaparib, a PARP inhibitor that has been widely used in cancer treatment, has been reported to have strong tumor-specific radiosensitization effects (82, 83).

RPA

The RPA complex is one of the first responders to coordinate DNA replication (18, 84). It consists of three subunits, RPA1 (RPA70), RPA2 (RPA32), and RPA3 (RPA14), which are essential to protect ssDNA at replication forks and recruits DNA polymerases α, δ, and ϵ for the initiation and elongation steps of DNA replication (84). It has been reported that RPA1 phosphorylation upon RS decreases the ubiquitination of chromatin-loaded RPA1, leading to an accumulation of RPA1 on stalled replication forks. This helps the DNA-binding domains of RPA2 to bind with RPA1-coated ssDNA, thus contributing to increased RPA2 binding stability (85). Loss of RPA accelerates fork breakage, whereas overexpression of RPA is sufficient to delay a "replication catastrophe" (86). It also plays an important role in DDR in relation to the HR pathway (87). Furthermore, overexpression of RPA significantly increases the radiation resistance in multiple cancer types (88–90). However, there has been no reported inhibitors of RPA because it is an essential protein to all cells. Furthermore, it is a downstream factor of ATR, and thus the regulation of ATR may produce similar effects (86). RING finger and WD repeat domain 3 (RFWD3)-mediated ubiquitination of RPA helps to remove RPA from the damage site, which is a crucial step for HR (91), and thus provides a possible target for increasing radiation sensitivity *via* ubiquitination regulation.

TopBP1

DNA topoisomerase II-binding protein 1 (TopBP1) serves as a scaffold to assemble protein complexes in a phosphorylation-dependent manner *via* its multiple breast cancer C-terminal (BRCT) repeats. It is repurposed to scaffold different processes dependent on cell cycle-regulated changes in phosphorylation of target proteins (92). It is known to form phase-separated nuclear condensates that amplifies ATR activity to CHK1 and slow down replication forks (93). TopBP1 also stabilized bloom syndrome helicase (BLM) to maintain genome stability (94). It is often overexpressed in cancer and can bypass control by CDK2 to interact with treslin, leading to enhanced DNA replication (95).

However, it has been reported that at low levels, TopBP1 activates ATR/CHK1, but once TopBP1 protein accumulates above an optimal level, it paradoxically leads to lower activation of ATR/CHK1. This is due to the perturbation of ATR-TopBP1 interaction and ATR chromatin loading by excessive TopBP1. Depletion of TopBP1 in some specific cancer cells enhanced ATR/CHK1 activation and S-phase checkpoint response after RS (96). Thus, simply inhibiting TopBP1 may lead to unexpected results, which makes it not an ideal target for radiation sensitization.

ATR-CHK1

ATR of the phosphoinositide 3-kinase (PI3K) family is a central regulator of RS. After ssDNA fragments are coated with PRA, ATR and ATR-interacting protein are recruited and activated. It further phosphorylates various proteins, including CHK1 kinase, which inhibits mitotic entry and dormant origin activation. Mitotic entry is inhibited by CDC25 phosphatase phosphorylation, which prevents subsequent mitotic CDK activation (97). Cancer genome sequencing showed a very low ATR or CHK1 mutation or

deletion frequency. Instead, these genes are often amplified in cancer cells, probably because they need to process high levels of RS to survive. ATR and CHK1 inhibition can increase RS, leading to mitotic catastrophes that trigger cell death (98). Furthermore, inhibition of ATR-related signaling pathways can increase cell apoptosis and effectively improve tumor radiosensitivity (99). ATR inhibitors, such as AZD6738 and VE-821 as well as the CHK1 inhibitor SAR-020106 were effective radiosensitizers in preclinical studies (100–102). An ongoing phase I clinical trial (NCT03188965) is assessing the safety profile of ATR inhibitors (BAY1895344) (103).

RAD51

RAD51 is a master regulator of DNA replication and plays important roles in DSB repair, RS, and mitosis (104). RAD51 is a core factor in overcoming RS by slowing or stalling replication forks, which threatens replication integrity (105). It facilitates fork inversion, protects reverse forks, repairs and restarts broken replication forks, and post-replication gap filling (104, 106).

RAD51 inhibition may lead to increased tumor radiosensitivity, and it has been reported as a potential target of berberine in osteosarcoma radiosensitization (107). Valproate was found to increase tumor tissue cell radiosensitivity by increasing levels of RFWD3 and inhibiting RAD51 (108). The inhibition of nucleophosmin1 (NPM1) by YTR107, a small molecule that binds with NPM1, inhibits pentamer formation and represses RAD51 formation after IR. The synergistic effect of YTR107 and the PARP1/2 inhibitor ABT-888 increased RS and radiation-induced cell mortality (109).

BLM

BLM is a 3'-5' ATP-dependent RecQ DNA helicase that is one of the most essential genome stabilizers involved in the regulation of DNA replication, recombination, and both homologous and non-homologous pathways of DSB repair (110). It interacts with topoisomerase IIIα (TOP3A), RecQ-mediated genome instability (RMI) 1, and RMI2 to form the BLM-Topoisomerase IIIα-RMI1-RMI2 (BTR) complex, which dissolves double Holliday junctions to produce non-crossover HR products. It also promotes DNA-end resection, restart of stalled replication forks, and processing of ultra-fine DNA bridges in mitosis (111). BLM helicase-deficient cells exhibit multiple defects in DNA replication, including accumulation of abnormal DNA replication intermediates, slower replication fork velocity, and excessive firing of dormant origins, thus exhibit increased levels of chromatid breakage and HR (112). It interacts directly with both RAD51 and RPA, and the function in DNA replication is regulated by sumoylation (113).

The high expression of BLM is a poor prognostic biomarker for multiple cancers (114, 115). Biallelic pathogenic variants in BLM cause bloom syndrome with severe pre- and postnatal growth deficiency, immune abnormalities, sensitivity to sunlight, insulin resistance, and a high risk for many cancers that occur at an early age (116). The symptoms of bloom syndrome including sensitivity to ultraviolet damage, which is similar to radiation, provide the possibility of transforming this genomic defect into a treatment sensitizer. ML216 is a small molecule inhibitor of BLM, and inhibits cell proliferation of BLM-proficient cells and increases the frequency of sister chromatid exchanges (117). Though there has been no data published on the links between a BLM inhibitor and radiation sensitivity till now, it is a promising target worth further research.

WEE1

When ssDNA accumulation at stalled replication forks activates ATR, it phosphorylates CHK1, which in turn activates WEE1 kinase and inhibits CDC25 phosphatase. While WEE1 inhibits CDKs, the key drivers of cell cycle progression, by phosphorylating the conserved threonine 14 (Thr14) and tyrosine 15 (Tyr15) residues, CDC25 activates CDKs by dephosphorylating the same residues (118). Elevated WEE1 expression reduces RS and activates G2/M checkpoints, conferring cell resistance to CHK1 inhibitors (98). Recently, it has been reported that the ATR-WEE1 module inhibits the MOS4-associated complex (MAC) to regulate RS responses (118).

The evidence suggests that WEE1 inhibition impairs the RS response activated by ATR, and thus increases tumor cell radiosensitivity (119). WEE1 kinase inhibitors sensitize tumor cells to proton and X-ray irradiation by inducing RS, independent of TP53 mutation status, such as AZD1775 (120–122). Clinical trials have shown that the WEE1 inhibitor adavosertib could potentiate the efficacy of RT; however, its clinical application is limited by its unfavorable safety profile (123).

Targeting RS-Induced DDR

The RS response shares many biological pathways with DDR. They are widely intertwined and thus hard to completely distinguish (124). Here, we grouped the proteins that are typically related to the DDR pathway but are not necessarily involved in the RS response. Targeting these proteins usually impairs the DDR processing to enhance radiosensitivity, which makes them the most promising targets.

p53

The p53 signaling pathway plays a key role in determining radiosensitivity in normal tissues but is often inactivated during cancer. Loss of p53 in tumor cells allows them to escape cell cycle arrest and apoptosis checkpoints and promotes the growth of early-stage cancer cells by skipping the cell cycle checkpoint caused by RS (125). During DNA replication, IR-induced DNA damage stalls replication forks, and single-strand breaks (SSBs) can be transformed into DSBs, thereby activating the ataxia telangiectasia mutated (ATM)/ATR pathway. ATM and ATR phosphorylate p53 to increase its stability and activate target genes. RS induced by chemotherapy drugs such as trifluridine leads to cell senescence or apoptosis of tumor cells according to the state of p53 (126). Acetylation of p53 may modulate cancer cell radiosensitivity, which provides a promising strategy for radiosensitization (127).

MRE11

Meiotic recombination 11 (MRE11), the core of the MRE11/RAD50/NBS1 (MRN) complex, is involved in DNA break end

detection, phosphorylation-dependent signal amplification, and DSB repair (128). The complex is critical for ATM activation of DSBs and downstream activation of G2/M and p53-dependent G1/S cell cycle checkpoints (129, 130). MRE11 also has endonuclease and exonuclease activities residing in the phosphodiesterase domain. These nuclease activities are crucial for the pathway choice of HR and NHEJ (131). Cancer cells rely on DNA repair for survival during cancer therapies, and thus MRE11 might be a promising synergistic therapeutic target.

Dysfunction of it in neoplastic breast tumors results in the accumulation of R-loops, replication-associated DSB, abundance of genomic deletions, and uncontrolled proliferation (132). Evidence suggests that its expression in cancer cells is critical for radioresistance (133). Low MRE11 expression in colorectal cancer cells reduced phosphorylated DNA-PKcs expression and further increases tumor radiosensitivity (134). There are different small and large molecular inhibitors targeting MRE11. Mirin is the first inhibitor found to specifically target MRE11 exonuclease activity with radiosensitizing properties (135). Lung cancer cells treated with Selenium, which is an essential trace element, showed decreased expression of MRE11 and significantly reduced colony formation relative to IR (136). OBP-301, with the insertion of the human telomerase reverse transcriptase (hTERT) promotor, also showed reduced MRE11 expression and thus enhanced radiosensitivity of lung cancer cells (137). Therefore, MRE11 inhibitors are clinically significant for enhancing radiosensitivity, and several clinical trials investigating their potential are ongoing (131).

ATM-CHK2

ATM kinase is a member of the PI3K-like protein kinase (PIKK) family with extensive roles in DDR signaling (138). Upon recruitment by the MRN complex to DSBs, ATM autophosphorylates at different serine sites resulting in the activation of CHK2, p53, and H2AX, which are involved in DNA repair processes and cell cycle arrest (139). The most important transducer of ATM signaling is CHK2, a kinase that signals to DNA repair, cell cycle arrest, and apoptosis. ATM phosphorylates CHK2 on threonine 68 (Thr68), thereby causing CHK2 dimerization and autophosphorylation of the kinase domain and is required for full activation (140).

ATM orchestrates the cellular DDR to cytotoxic DNA DSBs induced by radiation (141, 142). Overexpression of ATM indicates radiation resistance in breast cancer cells (143), whereas deficiency of ATM showed radiation sensitizer effects in multiple cancer types (144–147). Interestingly, more studies have focused on the radiation sensitizer effects that are dependent on the cell cycle and proliferation status (148, 149). After inhibition of proliferation, ATM status did not alter cell death or micronucleus formation after radiation, which suggests that ATM in endothelial cells was immaterial if a cell cycle block was present at the time of irradiation. It is consistent with other data showing that the effect of ATM on radiation sensitivity is more dependent on cell cycle regulation rather than the DDR pathway (148,149). Considering that ATM is a large protein with extensive regions of unknown function, the inhibition of its kinase activity may produce better synergistic effect on treatment. AZD0156, as a potent and selective bioavailable inhibitor of ATM, showed strong radiosensitizer effects *in vitro* and in a lung xenograft model (150). Specially, the ATM inhibitor AZD1390 is optimized for penetration of the blood-brain barrier with radiosensitizing effects on glioma and lung cancer cell lines, even in a brain metastasis model (141, 151). All of the evidence suggests that treatments targeting ATM may be promising in clinical trials.

MDM2

Mouse double minute 2 (MDM2) protein is a major negative regulator of p53 (152). When activated, p53 suppresses tumors in response to cell damage by mediating cell proliferation, cell cycle arrest, DNA repair, metabolism, angiogenesis, senescence, and apoptosis (153). In normal cells, the self-regulating feedback loop between MDM2 and p53 controls p53 expression (154, 155). The rescue of p53 function in cancer cells by inhibiting the interaction between p53 and MDM2 restored cycle arrest and apoptosis (156). Furthermore, inhibition of MDM2 phosphorylation leads to cell apoptosis and cell cycle arrest, thus repressing tumor cell proliferation in esophageal cancer cells (157). Additionally, MDM2 inhibitors, such as MI-219, increase tumor cell radiosensitivity in a p53-dependent manner. MI-219 combined with radiation resulted in increased p53-dependent DNA damage (158). A novel small-molecule inhibitor, APG-115, was found to enhance gastric adenocarcinoma cell radiosensitivity by blocking the interaction between MDM2 and p53 (159). Therefore, blocking the MDM2/p53 pathway has broad application prospects for treating tumors and enhancing tumor radiosensitivity, especially for tumors with low TP53 mutation levels, such as those of myeloid leukemia.

POLQ

DNA polymerase theta (POLQ) is a DNA polymerase that protects against error-prone transduction DNA synthesis and error-prone DSB (160). It is involved in a major DNA repair pathway that was initially named as alternative end-joining or microhomology-mediated end joining, and was later termed polymerase theta-mediated end joining because POLQ is indispensable in this process (161). POLQ overexpression reduces replication fork speed and impairs cell cycle progression (162). Furthermore, breast cancer related protein (BRCA) 2 and POLQ co-inhibition significantly improves tumor cell sensitivity to cisplatin (163). Reduced POLQ expression inhibits DSB repair and tumor cell survival. Several hepatocellular carcinoma cell lines (Huh7, HepG2, MHCC-92L, SK-HEP-1, and BEL-7404) with low POLQ expression after knockdown were found to be significantly sensitive to chemotherapeutic drugs (160). Depletion of POLQ in POLQ-dependent cancers (i.e., malignancies deficient in HR) leads to synthetic lethality. Furthermore, POLQ depletion was shown to synergize with PARP inhibition (164, 165), and the antibiotic novobiocin was recently reported as a selective POLQ inhibitor (166). Thus, combining novobiocin with radiotherapy should be a new research direction for targeting radioresistance.

BRCA

BRCAs (including BRCA1 and BRCA2) are thought to be the predominant proteins involved in HR in the DDR pathway. In addition, as a master regulator of HR, BRCA1 and BRCA2 also mediate fork protection (167, 168). BRCA mutations increase the susceptibility to various cancer types, including breast, ovarian, prostate, and pancreatic cancers (167). It is also well known that mutations in BRCA result in synthetic lethality with PARP inhibition. The underlying mechanism includes HR deficiency and increasing replication gaps. PARP inhibition results in replication fork collapse, chromosomal instability, cell cycle arrest in G2, and subsequent apoptosis in BRCA-deficient cells (169). Therefore, targeting PARP has become a reliable therapeutic strategy for eliminating BRCA1/2-mutated malignancies at diverse sites including the breast, primary peritoneum, fallopian tubes, ovaries, and pancreas (also see section 4.1.2) (170).

It has been reported that BRCA-deficient tumors are more sensitive to chemotherapeutic agents that induce RS (171). Furthermore, mutations in BRCA1/2 enhance radiosensitivity, indicating the possibility of BRCA as a biomarker of radiation sensitivity (172, 173). Since BRCA1/2 are both large proteins and have complex multiple functions, the development of inhibitors directly targeting BRCA1/2 is difficult to achieve. Therefore, PARPi has been suggested to patients with BRCA1/2 mutations for the synergistic lethal effects. The function of PARPi in radiosensitization are summarized in 4.2.1. Further research may focus on inhibitors that specifically affect the function of BRCA.

PI3K/AKT/mTOR

The PI3K/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway activates the downstream mediator mTOR to translate specific mRNA transcripts (174, 175). They synergistically work with CHK1 to repress DSB-induced RAD51 foci, thus impairing the HR process and enhancing RS in tumor cells. In addition, PI3K/mTORi slows the fork speed by increasing cell division cycle 45 homologue (CDC45) to promote a new origin of replication, thus enhancing CHK1-induced RS (176). The PI3K/AKT/mTOR signaling pathway is hyperactivated or altered in many cancer types (177). Inhibition of the pathway reduces tumor cell radioresistance (178, 179). For example, dactolisib, a dual PI3K/mTOR inhibitor, causes cell cycle arrest in the G2/M phase and improves the radiosensitivity of DU145 cell lines. Dactolisib also inhibits radiation-induced DSB repair in glioblastoma (GBM) cell lines by inhibiting DNA-PKcs and ATM and improves the radiosensitivity of radioresistant prostate cancer cell lines (180).

Torin 2 is a special class of PI3K pathway drugs, which not only inhibits the cell cycle at G1/S but also interferes with S phase progression, causing ssDNA accumulation, DNA damage, and increased checkpoint signaling in triple-negative BRCA cells (181). Furthermore, the dual PI3K/mTOR inhibitor apitolisib (GDC-0980) was demonstrated to inhibit growth and induce apoptosis in human GBM cells (182).

Others

Despite all the classic proteins we discussed above, several novel concepts have been suggested in recent research. A large number of accessory factors involved in the assembly of replisomes have been reported, which includes multi-protein complexes that monitor replication fork progression, generate checkpoint and damage signals, and coordinate DNA synthesis with chromatin assembly (183). We list below several newly identified processes that may be related to RS and radiation sensitivity that may provide ideas for translating basic research into clinical trials.

Ubiquitin and SUMO

Post-translational modification of the DNA replication machinery by ubiquitin and small ubiquitin-like modifier (SUMO) plays key roles in cell division, DNA replication/repair, signal transduction, and cellular metabolism (184). Recent research revealed that ubiquitin/SUMO pathways are essential regulators of DNA replication during initiation, the S phase or elongation, and DNA replication termination (185). SUMO/ubiquitin equilibrium at active DNA replication forks controls CDK1 activation. An increase in ubiquitination of the replisome results in premature disassembly of the replication machinery and generation of CDK1-dependent DNA damage in the S phase (186).

Our group has identified ubiquitination factors that affect radiation sensitivity. We showed that ubiquitin-specific protease 9X (USP9X) mediates lysine-specific demethylase 4C (KDM4C) deubiquitination, which activates transforming growth factor-β2 (TGF-β2)/Smad/ATM signaling to promote radioresistance in lung cancer (142). Furthermore, ubiquitin-conjugating enzyme E2O (UBE2O) facilitates tumorigenesis and radioresistance by promoting MAX interactor 1 (Mxi1) ubiquitination and degradation (187). The SUMO-specific protease (SENP) pathway is also involved in tumor radiation sensitization (188). SUMO E3 ligase PIAS4, which is an essential signal for p53-binding protein 1 (53BP1) loading to the damage site, promote radiation resistance by increasing DDR (189). Ring finger protein 4 (Rnf4), an E3 ubiquitin ligase that targets SUMO-modified proteins, target SUMOylated mediators of DNA damage checkpoint protein 1 (MDC1) and SUMOylated BRCA1 loading at sites of DNA damage. Rnf4-deficient cells and mice exhibit increased sensitivity to IR by suppressing DDR (190). These findings identify ubiquitylation/SUMO as possible radiosensitization targets, but further research is needed.

UPR

UPR is the master regulator of endoplasmic reticulum (ER) stress. A deficiency in UPR results in apoptosis (191). Recent research revealed the link between hypoxia-induced RS and UPR (192). The induction of RNA/DNA helicase senataxin (SETX) in hypoxia is reliant on the protein kinase R (PKR)-like ER kinase (PERK)/activating transcription factor 4 (ATF4) arm of the UPR (32). Hypoxia is present in the majority of human tumors and is associated with poor prognosis due to the protection it affords to radiotherapy and chemotherapy (27). As we described earlier (section 3.1), anti-hypoxia treatments provide additional radiation benefits through cell apoptosis, which establishes a link between UPR and radiation sensitivity.

UPR is widely involved in the establishment and progression of cancers, including BRCA, prostate cancer, and GBM multiforme (193). Elevated mitochondrial UPR markers (mtHSP70 and HSP60) are associated with poor prognosis in patients with lung

adenocarcinoma, which is activated by Maf1 through ATF5. Suppressing IR-induced mitochondrial UPR activation by rapamycin resulted in increased sensitivity to IR-mediated cytotoxicity (194). ONC201, an UPR activator, reduced oxidative phosphorylation and thus impairs cell cycle arrest, and the inhibition of DNA repair factors after radiation also enhanced radiation-induced cell death (34). As a new concept of radiosensitization, the clinical significance of UPR still requires further studies.

RS-INDUCED INNATE IMMUNE RESPONSE IN RADIATION SENSITIVITY

Nowadays, the immune microenvironment is the hotspot in cancer research. It involves all processes of tumorigenesis, cancer progression, and treatment resistance. Innate immunity refers to nonspecific defense mechanisms that act immediately after antigen appearance. The activation of innate immune responses relies on pattern recognition receptors (PRRs). These PRRs detect endogenous damage-associated molecular patterns (DAMPs) or exogenous conserved pathogen-associated molecular patterns (PAMPs) to initiate a signaling cascade resulting in the production of interferons (IFNs) and inflammatory mediators (195, 196).

RS-Induced Innate Immune Activation

As research progressed, some evidence revealed the relationship between RS and innate immune response, which plays a key role in cancer treatment resistance (197). In this study, we have summarized and discussed the potential relationship between targeting RS and innate immune activation.

Innate Immunity Activation by RS in Immune Cells

Excessive RS or RS deficiency leads to the accumulation of replication blockage-derived DNA in the cytoplasm or the formation of micronuclei, resulting in activating the cyclic GMP-AMP (cGAMP) and the cGAMP receptor stimulator of the interferon gene (STING) pathway. cGAMP synthase (cGAS) is a DNA sensor that recognizes and binds with DNA fragments in the cytoplasm, enabling cGAMP synthesis. cGAMP subsequently activates STING. The activation of STING further increases interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) levels (198). IRF3 and NF-κB act as transcription factors to trigger the transcription of IFN-I and cytokines (199). Apart from cGAS, γ-interferon-inducible protein-16, a cytosolic DNA sensor, can detect both self and non-self dsDNA to promote IRF3 and NF-κB-dependent interferon production *via* STING (195, 200, 201). IFN-1 plays a crucial role in both basal and therapeutic-induced immune responses to cancer. It is a potent immune cell activator, resulting in the activation and maturation of antigen presenting cells (198). The promotion of dendritic cell migration to the tumor site and their maturation depends on IFN-1 signaling (202, 203). Innate immune cells respond to IFN-1 by increasing antigen presentation and the production of immune response mediators, such as cytokines and chemokines. These events help in antigen presentation and chemokine production in innate cells as well as induce antibody production and enhance T-cell responses (198).

Innate Immunity Activation by RS in Tumor Cells

Innate immunity activation by tumor cells is a complex phenomenon. As we mentioned above, cancer cells usually experience higher RS, leading to more cytoplasmic DNA and micronuclei formation. They activate innate immunity by secreting IFN-1 *via* the cGAS-STING pathway, exocrine exosomes, or extracellular vesicles (EVs), which can be captured by immune cells for inducing a further immune response.

Cancer cells exposed to RS-inducing agents or deficient in RS response show the increased production of IFN-1 and proinflammatory cytokines that can foster an innate immune response (204, 205). One study found that the inhibition of the ATM/CHK2 DNA damage checkpoint axis led to excessive RS and cytosolic DNA accumulation, which subsequently activated the DNA sensor STING-mediated innate immune response in ARID1A-deficient tumors (206). Cytosolic DNA can also be released in exosomes or EVs (207, 208). Exosomes/EVs containing DNA works as DAMPs to innate immune cells. Study found that EVs and exosome dsDNA promoted inflammation *via* activating the STING pathway in macrophages (209).

The activation of STING in dendritic cells is essential for radiation-induced antitumor immunity (210). In contrast, cGAS-STING activation in tumor cells impairs HR in DDR, which promotes tumorigenesis (211). Moreover, cGAS can act as a decelerator of DNA replication forks, suppressing replication-associated DNA damage (212). The complex network mechanism made it hard to simply target or enhance cGAS-STING to reverse cancer treatment resistance. In contrast, high RS or RS-response deficiency always leads to simultaneous cell damage and immune activation. Hence, it would be a better choice for cancer treatment sensitization.

Targeting RS Response Enhances Radiation Sensitivity by Innate Immunity

The immune response caused by RT remains controversial. The inflammatory responses caused by RT are different depending on the RT pattern (213). Immune cells are highly radiosensitive compared with tumor cells (214). Conventional RT-induced myeloid-derived suppressor cell filtering leads to the suppressive tumor microenvironment (TME) rather than the active TME (215). Though the hypothesis that the damage signal released from tumor cells alone can activate a systemic antitumor immune response called the abscopal effect has been observed in a small-sample study (203), confirming the hypothesis without combining the signal with checkpoint inhibitors is difficult. The basic research revealed that RT may increase programmed death ligand 1 (PD-L1) levels in tumor and immune cells, contributing to immunosuppression and in part explaining the clinical success of the combination of RT with programmed cell death protein 1 (PD-1)/PD-L1 immunotherapy (216). As basic research data are available, more clinical trials regarding the combination of anti-PD-1/PD-L1 antibody with radiation are going on (217–220).

Polymorphonuclear neutrophils recruited in the TME post-RT can facilitate tumor progression by forming neutrophil extracellular traps (221). Taken together, the tumor immune microenvironment is thought to be suppressed rather than activated after radiation, which plays a key role in radioresistance. Promoting immune response activation of TME is the key to enhance radiosensitivity (**Figure 5**).

Enhancing RS and targeting RS response are good choices to manage tumors. As mentioned above, excessive RS or RS response deficiency results in more DNA damage, which is the synergy effect of RS and RT from the direct tumor side. As they lead to dsDNA accumulation in the cytoplasm and cell apoptosis, which activate innate immunity, they may enhance radiation sensitivity from the indirect immune side (215, 222).

RAD51-depleted cells accumulate more cytosolic DNA after radiation, activating the STING pathway to increase innate immune response (223). ATR inhibition and radiation drive immune cell infiltration *via* tumor cell-intrinsic cytokine release to boost immunogenic response to radiotherapy and modulate the radiation-induced inflammatory TME (224). PARP inhibitor and radiation work synergistically to kill lung cancer cells by activating antitumor immunity in the form of increased CD8+ T lymphocytes and the activated STING/TANK-binding kinase 1/IRF3 pathway (225). WEE1 inhibitor increases tumor-specific cytotoxicity and shows a positive effect on immune response after radiation by dendritic cell activation, which can be combined with immune therapy (226, 227).

These studies indicate that the RS-induced activation of innate immune response may be crucial to enhance the radiosensitivity of tumor cells. However, more evidence is needed to draw a general conclusion. Moreover, further studies are needed on the interaction between the effect of RS-induced innate immune response on tumor-cell radiosensitivity and radiation-induced antitumor immunity to achieve the optimal radiotherapy efficacy.

CONCLUSION

We have summarized the mechanisms of how RS response affects tumor radiosensitivity from the direct tumor side and indirect innate immune side and have further discussed potential targets and drugs to increase radiosensitization. We have reviewed several strategies including directly increasing RS, targeting RS response or RS-induced DDR, and other novel pathways. Although these strategies are predominantly based on preclinical evidence, they provide promising new ideas for

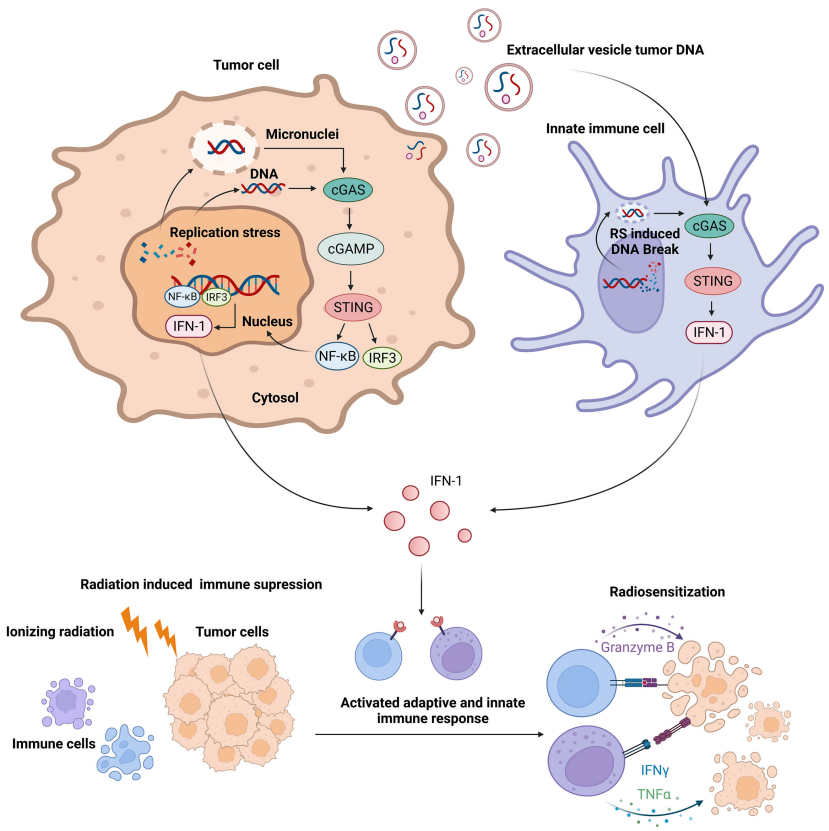

FIGURE 5 | Replication stress-induced activation of innate immune response enhances radiosensitivity *via* cyclic GMP-AMP synthase–STING signaling.

enhancing radiosensitivity. As the relationship between RS and tumor radiosensitivity will be explored in the future, we expect these new strategies to bring substantial benefits to patients suffering from radioresistant malignancies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

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Enhancing anti-tumour innate immunity by targeting the DNA damage response and pattern recognition receptors in combination with radiotherapy

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Radiotherapy is one of the most effective and frequently used treatments for a wide range of cancers. In addition to its direct anti-cancer cytotoxic effects, ionising radiation can augment the anti-tumour immune response by triggering pro-inflammatory signals, DNA damage-induced immunogenic cell death and innate immune activation. Anti-tumour innate immunity can result from recruitment and stimulation of dendritic cells (DCs) which leads to tumour-specific adaptive T-cell priming and immunostimulatory cell infiltration. Conversely, radiotherapy can also induce immunosuppressive and anti-inflammatory mediators that can confer radioresistance. Targeting the DNA damage response (DDR) concomitantly with radiotherapy is an attractive strategy for overcoming radioresistance, both by enhancing the radiosensitivity of tumour relative to normal tissues, and tipping the scales in favour of an immunostimulatory tumour microenvironment. This two-pronged approach exploits genomic instability to circumvent immune evasion, targeting both hallmarks of cancer. In this review, we describe targetable DDR proteins (PARP (poly[ADP-ribose] polymerase); ATM/ATR (ataxia–telangiectasia mutated and Rad3-related), DNA-PKcs (DNA-dependent protein kinase, catalytic subunit) and Wee1 (Wee1-like protein kinase) and their potential intersections with druggable immunomodulatory signalling pathways, including nucleic acid-sensing mechanisms (Toll-like receptors (TLR); cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) and retinoic acid-inducible gene-I (RIG-I)-like receptors), and how these might be exploited to enhance radiation therapy. We summarise current preclinical advances, recent and ongoing clinical trials and the challenges of therapeutic combinations with existing treatments such as immune checkpoint inhibitors.

KEYWORDS

DNA damage, innate immunity, radiotherapy, immunotherapy, combination therapy, cancer therapy

1 Introduction

Radiotherapy continues to be one of the most effective treatments for a wide range of cancers since its discovery over a century ago. Approximately half of cancer patients receive radiotherapy at some point in their cancer treatment (1), whether in the curative or palliative settings.

Radiotherapy exploits ionising radiation to cause cell death or senescence *via* DNA damage. Broadly, necrotic or apoptotic cell death occurs depending on cell type, radiotherapy dose and fractionation schedule (2). Cancer cells that evade apoptosis and continue to divide with accumulated DNA damage can die *via* mitotic catastrophe. Also, excess autophagy can force the cell into apoptotic or necrotic cell death (3, 4). Classically, the response of tumours to conventional fractionated radiotherapy is governed by the principles of the 4 "R"s of radiobiology: *repair* of sublethal DNA damage after exposure to ionising radiation, *redistribution* of cells in the cell cycle whereby cells in the G2/M-phase are most radiosensitive and are preferentially killed in comparison to the more radioresistant late S-phase, *repopulation* of tumour cells and r*eoxygenation* of previously hypoxic tumour areas (5). A 5th "R" of intrinsic *radiosensitivity* has also postulated by Steel, after observing the varying survival curves of different tumour cell lines following irradiation, which is thought to be independent of their DNA repair capacity (6). Combining agents that can target DNA damage repair pathways, as one of the 4 "R"s, with radiotherapy holds considerable potential to enhance therapeutic outcomes.

In addition to direct cell killing, radiotherapy can induce immunogenic cell death (ICD) and modulate the immune tumour microenvironment to lead to anti-tumour innate immune activation (7). Due to these immunostimulatory effects, there is increased interest in radiotherapy as a promising combinatorial agent with other immuno-oncology agents such as DNA-damage response (DDR)-targeting agents (8). This two-pronged approach exploits two hallmarks of cancer, namely genomic instability and evasion of immune surveillance (9, 10). The DDR sensing and signalling pathway are the collective mechanisms evolved by cells to combat the threat of DNA damage, namely the detection of DNA lesions, signalling of their presence and promotion of DNA repair (11). Promising DDR druggable targets include those within DNA repair pathways and cell cycle checkpoints, as well as damage-associated molecular pattern (DAMP)-sensing receptors which can amplify the DDR-induced immune response when combined with radiotherapy.

2 Radiotherapy and the anti-tumour immune response

Radiotherapy has both immunostimulatory and immunosuppressive effects. The difference in the ability of radiotherapy to initiate pro-immunostimulatory effects and turn immunogenically "cold" (low T-cell infiltrated) tumours "hot" (high T-cell infiltrated) may account for the enhanced response to radiotherapy of some pre-clinical models and clinical cancer histotypes.

2.1 Immunostimulatory effects mediated by radiotherapy

2.1.1 Immunogenic cell death

As a defence against microbial infection, the innate immune system has evolved pattern-recognition receptors (PRRs) that detect microbial pathogenic molecules known as pathogen-associated molecular patterns (PAMPs). However, these pathways do not exclusively sense foreign molecules. Immune activation can also occur in the absence of microbial infection, instead being triggered by inflammatory signals released from stressed or dying cells collectively known as damage-associated molecular patterns (DAMPs) (12). Radiotherapy-induced cellular stress and ICD can stimulate an immune response through the generation of DAMPs (13) detected by their cognate pattern recognition receptors (PRRs) (14). ICD has been defined as the chronic exposure of DAMPs in the tumour environment (TME), which can induce an innate and adaptive anti-tumour immune response in the host (15).

A characteristic DAMP induced by ICD is the secretion of adenosine triphosphate (ATP) from dying cancer cells into the extracellular space. Extracellular ATP functions as a "find-me" chemoattractant signal for the recruitment and activation of dendritic cells (DCs) (15–17). High-mobility group box-1 (HMGB1), secreted from the nucleus during ICD, binds to Toll-like receptor (TLR-4) and is critical for activating DCs and facilitating antigen processing and presentation to T cells (18). Translocation of calreticulin to the cell surface on dying cells provides an "eat-me" signal to antigen-presenting cells (APCs) and results in their phagocytosing target cells (19). In the context of cancer, ICD leads to release of tumour-associated antigens (TAA) and subsequent priming of a cancer-specific immune response. Another characteristic of ICD is the expression of heat shock proteins (HSP) HSP70 and HSP90 on dying cell membranes that drives cross-presentation of tumour-derived antigens on major histocompatibility complex class I (MHC-I) (15).

2.1.2 Secretion of pro-inflammatory mediators

Radiotherapy-induced DNA damage can function as a viral mimic through the accumulation of cytosolic DNA or RNA in irradiated cells (20). Cytosolic DNA and RNA activate cyclic GMP-AMP synthase (cGAS)/stimulator of interferon (IFN) genes (STING) and retinoic acid-inducible gene I (RIG-I)/mitochondrial antiviral-signalling protein (MAVS) pathways, respectively (21). These pathways activate complex

downstream signalling *via* interferon regulatory factor 3 (IRF3)/TANK-binding kinase 1 (TBK1) and nuclear factor kappa B (NF-κB) that results in production of Type I IFN and other inflammatory cytokines (e.g. interleukin (IL)-1, tumour necrosis Factor (TNF)-α) (20).

Radiotherapy is a form of ionising radiation that hydrolyses water and forms reactive molecules, such as reactive oxygen species (ROS) and nitric oxide species (NOS), which can directly alter DNA, cellular components, and molecules in the extracellular matrix (ECM) (22). ROS and NOS can be derived both from these direct ionisation events or activated immune cells, and work with other DAMPs to accelerate lymphocyte and DC recruitment. These activated immune cells generate pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, IL-12) (14, 23, 24), chemokines and growth factors leading to a sustained inflammatory response (22, 25).

2.1.3 Immune cell recruitment and tumour-specific T-cell activation

Recent data suggest that radiation can enhance cancer cell antigenicity through upregulation of genes involved in DNA damage repair and cellular stress responses (20). Immune cell recruitment is subsequently increased *via* expression of adhesion molecules (e.g. intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin) (26) and chemokines (e.g. chemokine (C-X-C motif) ligand 16 (CXCL16)) (27). Within the appropriate inflammatory environment, DCs take up antigens in peripheral tissues and mature and migrate to draining lymph nodes, where they induce activation of naïve T-cells and differentiation into effector T-cells (28). Radiotherapy-induced ICD, as discussed above, increases tumour-associated antigen presentation that can lead to specific tumour-associated antigen T-cell priming, expansion of tumour reactive CD8+ T cells and infiltration into the tumour microenvironment (TME) (29). In summary, inflammatory DAMP signalling generates a favourable environment for activated DCs to process and cross-present tumour-derived antigens from irradiated cells as a "tumour vaccine", to naïve T cells. These T cells subsequently can be primed and sustain a systemic tumour-specific immune response. The T-cell receptor (TCR) repertoire is also known to be shaped following radiotherapy, including when used in conjunction with immune checkpoint inhibitors (ICI) (30–32).

2.2 Immunosuppressive mechanisms triggered by radiotherapy

2.2.1 Immunosuppressive cells within the tumour microenvironment

Whilst pro-inflammatory signalling can lead to a positive anti-tumour effect, cancer cells adapt to survive with mechanisms such as hypoxia resistance and unrestricted proliferation that can result in a state of chronic inflammation and evasion of immune surveillance (33–35). Evasion of immune recognition or immune escape (36) is now a recognised hallmark of cancer (9) and this inclination towards pro-tumour growth is mediated by changes in cytokine signalling (TNF-α, IL-1β, IL-6, IL-10 and TGF-β) (37, 38) and recruitment of TME-immunosuppressive immune cells such as tumour-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) (39) and regulatory T cells (Tregs) (40, 41).

PD-L1 (programmed death-ligand 1) expression is found to be elevated on tumour cells following irradiation due to interferon gamma (IFN-γ) release from tumour-infiltrating lymphocytes (TILs) (42) and TILs have increased expression of PD-1 (programmed death-1) following ex-vivo irradiation (43). A recent publication found that irradiation of colorectal cancer cells triggered an ATR-mediated DNA repair signalling pathway to upregulate CD47 and PD-L1, through engagement of signal-regulator protein α (SIRPα) and PD-1, respectively, to limit tumour-associated cross-presentation and suppression of innate immune activation (44).

Recruited MDSCs and TAMs can suppress T-cell function through antagonistic cytokine signals (45). Supporting data includes that from a phase I/II clinical trial testing the combination of radiotherapy and a primed DC vaccine in which non-responders had significantly higher baseline tumour levels of MDSCs (46).

Tregs are relatively more radioresistant than other lymphocyte subsets and radiotherapy may increase the infiltration by phenotypically and functionally suppressive Tregs within the TME (40, 41, 47). In several pre-clinical mouse models (B16/F10, RENCA and MC38), Tregs in irradiated tumours expressed higher levels of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), 4-1BB (CD137, tumour necrosis factor receptor superfamily 9) and Helios compared with Tregs in non-irradiated tumours (47).

Cancer-associated fibroblasts (CAFs) can be the predominant component of the stroma in the TME and facilitate stroma-mediated radioprotection through multiple mechanisms. Following radiotherapy, CAFs can survive through formation of integrin-mediated attachments (48) and radioprotective integrin β-1 signalling (49). CAFs can promote an oxygen-rich, immunosuppressive and pro-inflammatory TME (50–52) resulting in increased tumour growth, invasion and metastasis (53).

Conversion of ATP to adenosine by CD39 and/or CD73 is a mechanism by which tumour cells can escape immune-surveillance by limiting the functionality of multiple potentially protective immune infiltrates, while enhancing the activity of immunosuppressive cell-types (54). CD39 and/or CD73 (over)expression has been found on the surface of tumour cells (55), CAFs (56) MDSCs (57), TAMs (58), Tregs and exhausted conventional CD4+ and CD8+ T cells (59–61).

2.2.2 Tumour repopulation

One of the 4 "R"s of radiobiology is repopulation (5), and tumour repopulation during radiotherapy and chemotherapy is an important cause of treatment failure (62). Some tumours exhibit accelerated tumour repopulation following irradiation by paracrine caspase 3-dependent prostaglandin E_2 (PGE2)-mediated signalling (63). Tumour repopulation may also be driven by a small number of cancer stem cells (CSC) which promote tumour growth following an insult, such as radiotherapy (64). Rapid proliferation of cancer cells is generally accepted as a prerequisite for most conventional chemotherapies and radiotherapy to be effective, and any senescent and/or quiescent tumour cells, such as CSCs, may be treatment-resistant (64). The CSC response to therapy may underpin why macroscopic tumour response to (chemo)radiation is not a robust predictor for clinical outcome, since small numbers of these relatively resistant and less immunogenic CSCs may survive to repopulate the tumour (64). However, *in vitro* pre-clinical data from human breast cancer cell lines (MCF-7 and T47D) have shown that radiotherapy can recruit CSC cells from a quiescent state into the cell cycle (65) and a CSC-druggable target in combination with radiotherapy would be useful.

As we have seen, radiotherapy can trigger key events leading to potent anti-tumour immune responses *via* production of immunostimulatory cytokines, DC recruitment, and T-cell recruitment and activation. However, these are negatively balanced by the potential for concurrent triggering of immunosuppressive cells within the TME and accelerated tumour cell repopulation. Targeting the DNA-damage response pathway (DDR) is an attractive approach to tip the scales towards maintaining positive immune anti-tumour states, which can be characterised as 'pro-immunogenic' and 'pro-inflammatory'.

3 Targeting the DNA-damage response pathway

Radiotherapy causes cell damage, stress and death through induction of DNA lesions in the form of crosslinking, single-strand breaks (SSBs) and, most significantly, double-strand breaks (DSBs) (66). These processes induce a plethora of intracellular signalling pathways involved in detecting and repairing DNA damage. Targeting both DNA damage repair and DDR's downstream cytosolic nucleic acid sensing pathways with small molecules in combination with radiotherapy can lead to increased immune activation and anti-tumour efficacy of these treatments (Figure 1).

3.1 DNA damage repair pathways

Radiotherapy induces double-strand breaks (DSBs) in cancer cell DNA, which results in genomic instability, cell cycle arrest, apoptosis or death *via* mitotic catastrophe (66). In response to radiotherapy, cancer cells can respond to exploit individualised DNA damage repair mechanisms for survival (67). Three primary DNA repair pathways have evolved to process DSB repair and maintain genomic integrity: homologous recombination, non-homologous end-joining (NHEJ) and alternative end-joining (68). Upregulation of these pathways is a mechanism by which cancer cells may acquire radioresistance and, accordingly, radiosensitisation strategies which inhibit radiation-induced DNA damage repair are expected to provide increased cancer control (66). When DNA repair is inhibited in cancer cells, this leads to accumulation of DNA damage, cellular stress and cell death which subsequently increases the likelihood of these cells triggering innate immune pathways and being recognised by anti-tumour immune surveillance.

3.1.1 ATM and ATR inhibitors

ATM and ATR are both key mediators of the DSB signalling response that induce cell cycle arrest to facilitate DNA repair (69). In addition, conditions that activate ATM and ATR as part of DDR may also participate in regulating the innate immune system and alert it to potentially 'dangerous' tumour cells (70).

In response to DSB, the MRE11-RAD50-Nibrin (NBS1) (MRN) complex assembles at DSB sites to act as a DNA damage sensor that activates and recruits ATM to DSB sites (71). Briefly, when a cell triggers the DDR, ATM initiates a massive signalling cascade with the phosphorylation of hundreds of substrates, including p53 and checkpoint kinase 2 (Chk2). Activated p53 transactivates the expression of $p21^{Cip1/kip1}$, which inhibits Cyclin Dependent Kinase (CDK) 2 and CDK4/6 to induce G1/S arrest (66). Chk2 in turn phosphorylates and inactivates Cell Division Cycle 25 (CDC25C), maintaining the inhibitory phosphorylation of CDK1 by Wee1-like protein kinase (Wee1) and Myelin Transcription Factor 1 (Myt1) to induce G2/M cell cycle arrest or apoptosis (66, 72). Inhibition of the ATM/Chk2 axis can lead to replication stress and accumulation of cytosolic DNA that subsequently activates the cGAS-STING-mediated innate immune response (73).

ATM was recognised as the defective gene in the inheritable human disorder, ataxia-telangiectasia (A-T) (74), and these patients have characteristic features including genomic instability and profound radiosensitivity (75). Deficiency of ATM-mediated signalling reactions causes sensitisation of cells to radiation (76), which has sparked interest in ATM as a therapeutic target for cancer treatment (69). Inhibition of ATM and ATR have the potential to improve radiotherapy outcomes as they are both key mediators of the DDR (69). Indeed, ATM inhibitors such as caffeine (77), wortmannin (78), CP-466722 (79), KU-55933 (80), KU-60019 (81) and KU-59403 (82) increase cell

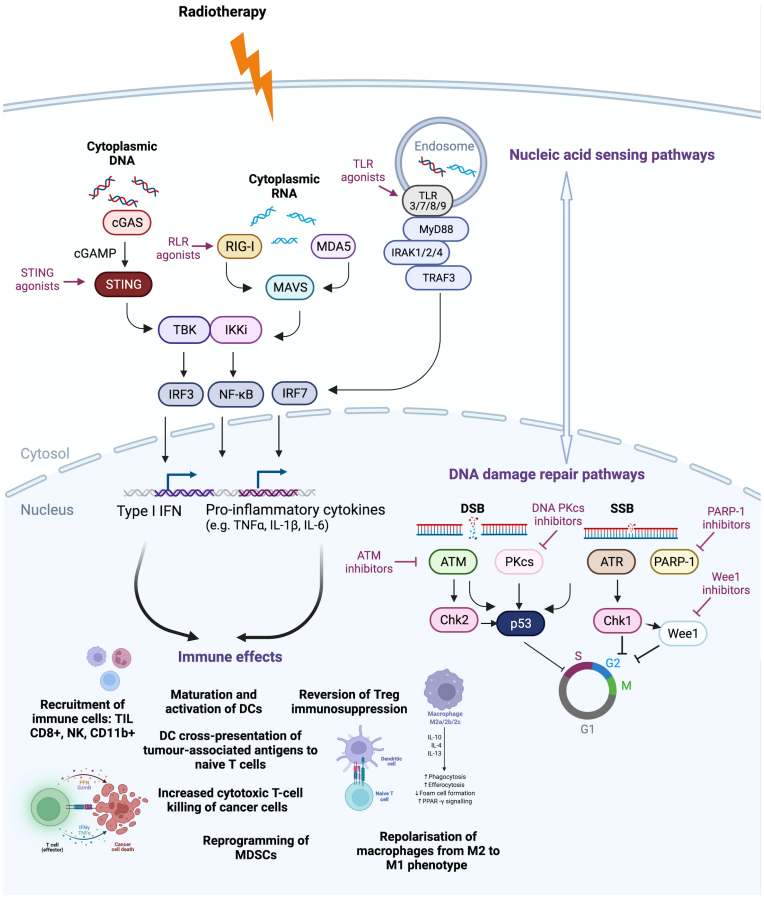

FIGURE 1
Druggable targets of the DNA damage response (DDR) pathway currently tested in clinical trials. Radiotherapy induces DNA damage and cell death. Nucleic acid sensing pathways detect cytoplasmic DNA and RNA to stimulate downstream pathways. Cytoplasmic DNA activates the Cyclic GMP–AMP synthase (cGAS) to produce cyclic GMP–AMP (cGAMP) that activates the stimulator of interferon genes (STING) pathway, leading to type I interferon (IFN) production. Radiotherapy-induced type I interferon (IFN) can induce RNA sensor activation through RNA polymerase III conversion of DNA to double-stranded RNA (dsRNA), radiotherapy-induced small non-coding RNA (sncRNA) or STAT1-induced dsRNA synthesis from endogenous retroviral elements (ERVs). These activate (RIG-I)-like receptors (RLRs), melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene-I (RIG-I), which also drives pro-inflammatory signalling through type I IFN and pro-inflammatory cytokine production. Toll-like receptors (TLRs) can recognise damage-associated molecular patterns (DAMPs) of single-stranded RNA (ssRNA), dsRNA or unmethylated CpG DNA in intracellular compartments such as endosomes, to lead to activation of nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPKs) and interferon regulatory factors (IRFs). DNA damage repair mechanisms of single- (SSB) and double-strand breaks (DSB) are often upregulated by cancer cells to avoid cell cycle arrest or death. Inhibitors of DNA damage repair components, such as ataxia telangiectasia- mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR), DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), poly(ADP- ribose) polymerase 1 (PARP-1) and Wee1 (Wee1-like protein kinase) function to propel the cell through the cell cycle, despite the presence of unrepaired damage, leading to accumulation of cytosolic DNA. This leads to cross-talk with the nucleic acid sensing pathway *via* activation of the cGAS-STING pathway and dsRNA stress pathway *via* promotion of ERV expression. These two pathways, through positive and negative cross-talk, shape the radiotherapy-induced DDR response that feeds into anti-tumour immune effects, including recruitment of tumour-infiltrating CD8+ T-cells, natural killer (NK) cells and CD11b+ innate immune cells, such as macrophages and neutrophils. Maturation and activation of dendritic cells (DCs) is increased, including DC cross-presentation of tumour-associated antigens to naive T-cells, which can become activated leading to T-cell-mediated cytotoxic-killing of cancer cells. Furthermore, the immunosuppressive effects of myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs) can be reversed and macrophages can be repolarised from M2 to an M1 pro-inflammatory phenotype. Chk, checkpoint kinase; IKKi, inducible IκB kinase; IL, interleukin; IRAK, Interleukin 1 Receptor-Associated Kinase; MAVS, mitochondrial anti-viral-signalling protein; MyD88, Myeloid differentiation primary response 88; TBK, TANK-binding kinase 1; TNFα, tumour necrosis factor alpha; TRAF3, TNF Receptor-Associated Factor 3. Created with BioRender.com.

radiosensitivity (83, 84), particularly in p53 low/deficient and phosphatidylinositol 3-kinase (PI3K) highly-expressing cells (77, 85). In a preclinical study *in vivo* with KU60019 and radiotherapy, combination treatment enhanced TBK1 activity, type I IFN production, antigen presentation and increased CD8+ TILs; moreover, complete responders had established immunological memory (86) (Table 1). The ATM inhibitor (AZD1390) and radiotherapy is being investigated in a phase I

TABLE 1 Preclinical RT and DDR combination studies.

Target (drug), route	Additional therapy	Radiotherapy (RT)	Murine tumour model	Immunological effects	References
DNA repair inhibitors					
ATR inhibitor (AZD6738, ceralasertib), PO	-	2 Gy x 2	CT26 (colorectal cancer)	Combination treatment increased TIL CD8+ T cell infiltration, decreased TIL Treg cells, and promoted immunological memory. AZD6738 blocked radiation-induced PD-L1 upregulation to reduce number of TIL Tregs.	(87)
ATR inhibitor (AZD6738, ceralasertib), PO	-	2 Gy x 4	TC-1 (HPV-transformed lung epithelial cells)	Combination treatment showed enhanced type I and type II IFN signature, increased PD- L1 expression, increased numbers of DCs, T cells and NK cells.	(88)
ATR inhibitor (AZD6738, ceralasertib), PO	Anti-PD-L1	18 Gy in 3 fractions on days 1, 3, and 5	Hepa 1–6 cells (a C57/L murine liver cancer cell line) and H22 cells	AZD6738 further increased RT-stimulated CD8+ T cell infiltration and activation and reverted the immunosuppressive effect of radiation on the number of Tregs in mice xenografts. Triple combination with anti-PD-L1 boosted the infiltration, cell proliferation, enhanced IFN-γ production ability of TIL CD8+ T cells, decreased trend in number of TIL Tregs and exhausted T cells in mice xenografts. Triple therapy led to more long-lasting immunity with tumour rechallenge rejection.	(89)
ATR inhibitor (AZD6738, ceralasertib), PO	Anti-TIGIT, Anti-PD-1	20 Gy in four 5 Gy fractions per day (MOC2); 24 Gy in three 8 Gy fractions per day over 5 days (SCC7)	MOC2 and SCC7 HPV-negative murine oral squamous cell carcinoma cell lines	ATRi enhanced radiotherapy-induced inflammation in the TME with NK cells playing a central role in maximizing treatment efficacy. Anti-tumour activity of NK cells can be further boosted with ICI targeting TIGIT and PD-1.	(90)
ATM inhibitor (KU60019), PO	Anti-PD- L1	8 Gy single fraction	mT4 and KPC2 pancreatic cancer cell lines	Combination treatment further enhanced TBK1 activity, type 1 IFN production, and antigen presentation. ATM inhibition also increased PD-L1 expression, increased intratumoural $CD8^+$ T cells and established immune memory.	(86)
DNA-PK inhibitor (M3814, peposertib), PO	Anti-PD-L1	5 Gy or 8 Gy single fraction	mT4 pancreatic cancer cell line	Radiation with DNA-PK inhibition increased cytosolic dsDNA and tumoural type 1 IFN signalling in a cGAS- and STING-independent, but an RNA POL III, RIG-I, and MAVS-dependent manner. Triple combination with anti-PD-L1 potentiated anti-tumour immunity with a significant increase in the number of CD4+ , CD8+ , and Granzyme B+ cells compared to radiation alone or radiation with M3814.	(91)
Wee1 inhibitor					
MK1775/AZD177, adavosertib, PO	Anti-PD-1	8 Gy single fraction	MOC-1 murine oral squamous cell carcinoma	Triple combination treatment efficacy is CD8-dependent. Radiation alone reduced neutrophilic myeloid-derived suppressor cells and increased Treg tumour accumulation, unchanged with the addition of AZD1775. T-cells from tumour-draining lymph nodes (TDLNs) from mice treated with the triple therapy demonstrated the greatest activation and IFNγ production upon exposure to MOC1 tumour antigen. Mice cured following triple agent treatment did not engraft tumours following rechallenge.	(92)
STING agonists					
Modified CDN derivative molecules, IT injection	-	10 Gy single fraction	Panc02 murine pancreatic adenocarcinoma cell line; SCC7 head and neck cancer model, MMTV-PyMT mammary carcinoma; 3LL lung	Combination treatment showed early T-cell-independent and TNFα-dependent haemorrhagic necrosis, followed by later CD8+ T-cell-dependent control of residual disease.	(93)

(Continued)

TABLE 1 Continued

Target (drug), route	Additional therapy	Radiotherapy (RT)	Murine tumour model	Immunological effects	References
			adenocarcinoma model		
Toll-like receptor agonists					
Imiquimod, topical	Cyclophosphamide	8 Gy x 3 consecutive days	TSA mouse breast carcinoma	Increased tumour infiltration by CD11c+, CD4+ and CD8+ cells. Tumour control abolished by CD8+ depletion. Combination treatment led to abscopal effect, long-term tumour-free mice rejected rechallenge showing immunological memory.	(94)
Imiquimod, topical	-	Whole-body RT 2 Gy single fraction	B16-F10 and B16-F1 melanoma	Combination treatment led to enhanced cell death via autophagy. Autophagy accelerated via ROS-mediated MAPK and NF-κB signalling pathways. Combination increased number of CD8+ T cells and decreased numbers of Treg and MDSCs in the tumour lesions. Combination enhanced systemic anti-cancer immunity by increasing the abundance of T cell populations expressing IFN-γ and TNF-α.	(95)
TLR7 agonist (R848), IV	-	10 Gy single fraction	B-cell lymphoma line A20, the T-cell lymphoma line EL4, and its ovalbumin-expressing derivative EG7	Combination treatment led to the longstanding clearance of tumour in T- and B-cell lymphoma-bearing mice. Combination therapy led to the expansion of tumour antigen-specific CD8+ T. Mice that achieved long-term clearance of tumour were protected from subsequent tumour rechallenge.	(96)
TLR7 agonist (DSR-6434), IV	-	KHT and CT26 tumours received a single dose of 25 or 15 Gy, or 5 daily fractions of 2 Gy, respectively.	CT26 colorectal or KHT fibrosarcoma tumours	Combination led to induction of type 1 interferon and activation of T and B lymphocytes, NK and NKT cells. Combination treatment primed an anti-tumour CD8+ T cell response. Long-term surviving mice had significantly greater frequency of tumour antigen-specific CD8+ T cells.	(97)
TLR7-selective agonist (DSR-29133), IV	-	2 Gy x 5	Syngeneic models of renal cancer (Renca), metastatic osteosarcoma (LM8) and colorectal cancer (CT26)	Administration of DSR-29133 led to the induction of IFNα/γ, IP-10, TNFα, IL-1Ra and IL-12p70. Combined therapy resulted in curative responses in a high proportion of mice bearing established CT26 tumours which was dependent on the activity of CD8+ T-cells, but independent of CD4+ T-cells and NK/NKT cells. Long-term surviving mice treated with combination were protected from subsequent tumour rechallenge.	(98)
TLR7/8 agonist (3M-011 (854A)), IP injection	-	2 Gy x 5	CT26 (murine colorectal carcinoma cell line) or Panc-02 (murine pancreatic carcinoma cell line)	*In vivo* depletion identified NK and CD8 T cells as the cell populations mediating the cytotoxic effects of treatment, while *in vivo* depletion of CD11c+ dendritic cells (DC) in CD11c-diphtheria toxin receptor (DTR) transgenic mice revealed DC as the pivotal immune hub in this setting.	(99)
TLR9 agonist (CpG oligodeoxynucleotide 1826), SC peritumoural or IT injection	-	Single dose (unspecified) or fractionated RT delivered in 1-9 Gy fractions twice daily, separated by 6-7 hours for 5 consecutive days for total dose of 10-90 Gy	Murine immunogenic fibrosarcoma tumour	Mice cured of their tumours by combined CpG oligodeoxynucleotide 1826 plus radiotherapy were highly resistant to SC tumour take or development of tumour nodules in the lung from IV injected tumour cells when rechallenged with fibrosarcoma cells 100 to 120 days after the treatment, suggesting the development of a memory response. CpG oligodeoxynucleotide 1826 also increased the radioresponse of the non-immunogenic fibrosarcoma tumour by a factor of 1.41 and 1.73 when CpG oligodeoxynucleotide 1826 was given SC and IT, respectively.	(100)
TLR9 agonist (CpG oligodeoxynucleotide 1826), peritumoural injection	-	20 Gy single fraction	Immunogenic sarcoma (FSa)	The CpG ODN-induced enhancement of tumour radioresponse was diminished in tumour-bearing mice immunocompromised by sublethal whole-body radiation. Tumours treated with combination showed increased necrosis, heavy infiltration by host inflammatory cells (lymphocytes and granulocytes), and reduced tumour cell density.	(101)

(Continued)

TABLE 1 Continued

Target (drug), route	Additional therapy	Radiotherapy (RT)	Murine tumour model	Immunological effects	References
TLR9 agonist (CpG oligodeoxynucleotides), peritumoural injection		30 Gy in 10 fractions of 3 Gy over 12 days, or a single dose (2, 6 or 10 Gy)	Rat glioma cell lines 9L and RG2	Combination treatment efficacy was lost in nude mice compared to immunocompetent mice, underlining the role of immune cells in anti-tumour effects. Tumour infiltration by immune cells and expression within tumours of the CpG receptor, TLR9, were not modified by irradiation.	(102)
TLR9 agonist CpG oligodeoxynucleotides, SC injection	-	20 Gy single fraction	Lewis lung carcinoma (3LL) cells	TLR9 agonist alone expanded and activated B cells and plasmacytoid dendritic cells in wild-type mice and natural killer DCs (NKDCs) in B cell-deficient (B−/−) tumour-bearing mice. Combined treatment led to a strong tumour-specific humoral immune response with deposition of mouse IgG auto-antibodies in tumour tissue in wild-type mice whereas the number of tumour-infiltrating NKDCs increased in $B^{-/-}$ mice.	(103)
(RIG-I)-like receptor agonist (RLR)					
dsRNA mimic polyIC by polyethylenimine (PolyIC(PEI)), IT cytoplasmic delivery	Low-dose cyclophosphamide, TLR agonist (polyIC), decitabine	Diffusing alpha-emitting radiation therapy (DaRT) Intratumoural Ra-224-coated seeds	4T1 triple-negative breast tumours Squamous cell carcinoma (SCC) tumour model SQ2	Splenocytes from PolyIC(PEI) and DaRT-treated mice, adoptively transferred to naive mice in combination with 4T1 tumour cells, delayed tumour development compared to naïve splenocytes. Delay in tumour development on re-challenge was demonstrated.	(104)

IV, intravenous; SC, subcutaneous; IP, intraperitoneal; IT, intratumoural; PO, oral.

clinical trial in brain cancer (NCT03423628). A dual ATM and DNA-PKc inhibitor (XRD-0394) and radiotherapy phase I trial is also recruiting (NCT05002140) (Table 2).

ATR is activated by single-stranded DNA (ssDNA) structures that may arise at resected DNA DSBs or stalled replication forks. ATR is recruited *via* interaction of ATR-interacting protein (ATRIP) with ssDNA-bound replication protein A (RPA) (105). RPA-ssDNA complexes stimulate loading of the RAD9–HUS1–RAD1 (9–1–1) heterotrimer, that recruits DNA topoisomerase II binding protein 1 (TopBP1) which activates ATR (106). Once ATR is activated, downstream targets, including checkpoint kinase 1 (Chk1), promote DNA repair (107, 108), restart of stalled replication forks (109) and intra-S and G2/M cell cycle arrest (110, 111). In response to DNA damage, activation of the intra-S-phase cell cycle checkpoint slows progression of DNA replication to allow time for resolution (110, 111). In addition, the ATR-dependent G2/M cell cycle checkpoint is activated through degradation of cell division cycle 25A (Cdc25A) (111), and phosphorylation of Cdc25C phosphatase inhibits its ability to activate nuclear cell division cycle 2 (Cdc2) and, hence, mitosis entry (112). Most cancer cells are defective in DNA damage-induced checkpoints through e.g. p53 pathway mutations, which leads to dependence on the intra-S-phase and G2/M checkpoints for cell survival (69). Therefore, ATR inhibition will lead to accumulation of DNA damage, premature entry into mitosis, mitotic catastrophe and cell death (69).

ATR inhibitors include schisandrin B (113), NU6027 (114), NVP-BEZ235 (115), VE-821 (116), VE-822 (117), AZ20 (118) and ceralasertib (AZD6738) (119, 120). NVP-BEZ235 has been reported to induce marked radiosensitivity in Ras-overexpressing cancers (121), and NU6027 has been shown to increase sensitivity to DNA-damaging agents in breast and ovarian cell lines (114). VE-822 results in selective sensitisation of pancreatic tumours to radiation *in vivo* by increasing persistent DNA damage, decreasing cell cycle checkpoint maintenance and reducing homologous recombination repair (117). *In vitro*, ATR inhibition downregulates radiotherapy-induced programmed death-ligand 1/2 (PD-L1/2) expression to sensitise cancer cells to T-cell killing, in addition to potentiating DNA damage (122). Promising preclinical *in vivo* studies (Table 1) of the ATR inhibitor ceralasertib (AZD6738) in combination with radiotherapy have shown an enhanced type I/II interferon response and increased immune cell infiltrate (88), increased RT-stimulated CD8+ T cell infiltration (87, 89), NK-mediated anti-tumour immunity (90), as well as reversal of the Treg immunosuppressive effect (87, 89). In addition, further addition of ICI (i.e. anti-PD-1, anti-PD-L1, anti-TIGIT (T-cell immunoglobulin and ITIM domain)) to the ceralasertib (AZD6738) and radiotherapy combination further improved response and long-lasting immunity in a CD8+ (87, 89) and NK-dependent manner (90).

There are, to date, three early phase clinical studies investigating ATR inhibition and radiotherapy. PATRIOT, a phase I study of ceralasertib (AZD6738) in combination with palliative radiotherapy, has completed recruitment and is awaiting report (NCT02223923). BAY1895344 in combination with radiotherapy and pembrolizumab in recurrent head and neck squamous cell carcinoma (HNSCC) (NCT04576091) and M6620 with radiotherapy and chemotherapy in solid cancers (NCT03641547) are ongoing studies (Table 2).

TABLE 2 Selected clinical trials investigating radiotherapy in combination with DDR inhibitor and/or other agents.

Target (drug) & route	Additional therapy	Radiotherapy	Phase	Patient population	n	Response	Toxicity	NCT ID
DNA repair inhibitors								
ATM kinase inhibitor (AZD1390)	N/A	35 Gy over 2 weeks; 30 Gy over two weeks; 60 Gy over 6 weeks	I	Brain cancer	120	Recruiting	Recruiting	NCT03423628
ATR inhibitor (AZD6738)	None	20 or 30 Gy	I	Solid tumours	46	Active, not recruiting	Active, not recruiting	NCT02223923
ATR kinase inhibitor (BAY1895344)	Pembrolizumab	SBRT 3 fractions with 2-3 days between fractions	I	Recurrent head and neck squamous cell carcinoma	37	Recruiting	Recruiting	NCT04576091
ATR inhibitor (M6620)	Cisplatin; capecitabine	Not specified	I	Oesophageal cancer and other solid cancers	65	Recruiting	Recruiting	NCT03641547
DNA- PK inhibitor (M3814)	Avelumab	Hypofractionated in 5 fractions	I/II	Advanced hepatobiliary malignancies	92	Not yet recruiting	Not yet recruiting	NCT04068194
DNA- PK inhibitor (M3814)	Cisplatin	3 Gy x 10; 2 Gy x 33-35	I	Locally advanced tumours	52	Preliminary efficacy: in-field response (n=16): one patient had pCR, 4 PR, 7 SD, and 3 have not yet been evaluated. One patient was not evaluable.	Dose-escalation results reported (n=16 patients enrolled). The most frequent AEs were fatigue in 12/16 and nausea 8/16. No patients discontinued due to DLTs. Four DLTs were reported: grade 3 mucositis lasting > 7 days in 3/16 and odynophagia in 1/16, all recovered without sequelae. One fatal suspected unexpected serious AE considered as radiation pneumonitis occurred.	NCT02516813
DNA- PK inhibitor (M3814)	Capecitabine	45–50 Gy in 25–28 fractions over 5 weeks	Ib/II	Rectal cancer	165	Recruiting	Recruiting	NCT03770689
DNA- PK inhibitor (M3814)	Avelumab	30 Gy in 10 fractions over 2 weeks	I	Various advanced solid tumours	24	Recruiting	Recruiting	NCT03724890
DNA- PK inhibitor (M3814)	Temozolomide	60 Gy in 30 fractions over 6 weeks	I	MGMT promoter unmethylated glioblastoma or gliosarcoma	29	Recruiting	Recruiting	NCT04555577
DNA- PK inhibitor (M3814)	N/A	Not specified	I	Advanced head and neck cancer	42	Recruiting	Recruiting	NCT04533750
DNA-PK inhibitor (XRD-0394)	N/A	20 Gy in 5 fractions over 1 week	I	Various advanced solid tumours	38	Recruiting	Recruiting	NCT05002140
Dual ATM and DNA-PK	N/A	20 Gy in 5 fractions over 1 week	I	Metastatic, locally advanced,	38	Recruiting	Recruiting	NCT05002140

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TABLE 2 Continued

Target (drug) & route	Additional therapy	Radiotherapy	Phase	Patient population	n	Response	Toxicity	NCT ID
inhibitor (XRD-0394)				or recurrent cancer				
PARP inhibitor (olaparib)	Durvalumab; Tremelimumab	30 Gy in 10 fractions over 2 weeks	I/II	Extensive stage small cell lung cancer	54	Recruiting	Recruiting	NCT03923270
PARP inhibitor (olaparib)	N/A	Not specified	I	Triple-negative breast cancer	24	Awaiting report	2/24 (8.7%) patients experienced acute grade 3 dermatitis related to RT. Olaparib-related toxicity grade 3-4 haematological toxicity was lymphopenia in 11/24 (45.8%) patients.	NCT03109080
PARP inhibitor (olaparib)	N/A	Unspecified standard radiotherapy treatment 5 days per week for 6 weeks	II	Inflammatory breast cancer	300	Recruiting	Recruiting	NCT03598257
PARP inhibitor (olaparib)	Durvalumab; carboplatin; etoposide	Not specified consolidative thoracic radiotherapy	I/II	Extensive-stage small cell lung cancer	63	Recruiting	Recruiting	NCT04728230
PARP inhibitor (olaparib)	N/A	High-dose 70 Gy in 35 fractions; elective neck 54.25 Gy in 35 fractions	I	Head and neck cancer	12	Active, not recruiting	Active, not recruiting	NCT02229656
PARP inhibitor (olaparib)	Temozolomide	2 Gy per fraction given once daily five days per week over 6 weeks, for a total dose of 60 Gy	I/IIa	High-grade gliomas	79	Recruiting	Recruiting	NCT03212742
PARP inhibitor (niraparib)	N/A	Not specified	I	Triple-negative breast cancer	20	Recruiting	Recruiting	NCT03945721
PARP inhibitor (niraparib)	Dostarlimab	Not specified	II	Triple-negative breast cancer	32	Recruiting	Recruiting	NCT04837209
PARP inhibitor (veliparib)	Temozolomide	30 daily fractions of radiation therapy 5 days per week for 6-7 weeks	II	Newly diagnosed malignant glioma without H3 K27M or BRAFV600 mutations	115	Active, not recruiting	Active, not recruiting	NCT03581292
PARP inhibitor (Veliparib)	N/A	50 Gy to the chest wall and regional lymph nodes plus a 10-Gy boost	I	Inflammatory or loco-regionally recurrent breast cancer	30	15 disease control failures during the 3 years of follow-up. 13 died (all after recurrence)	5 dose-limiting AEs occurred: 4 moist desquamation, 1 neutropenia. Crude Grade 3 toxicity was 10% at year 1, 16.7% at year 2, and 46.7% at year 3. At year 3, 6 of 15 surviving patients had severe fibrosis in the treatment field.	NCT01477489

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TABLE 2 Continued

Target (drug) & route	Additional therapy	Radiotherapy	Phase	Patient population	n	Response	Toxicity	NCT ID
PARP inhibitor (Veliparib)	Capecitabine	50·4 Gy in 1.8 Gy fractions daily, 5 consecutive days per week for 5·5 weeks	!b	Locally advanced rectal cancer	32	Tumour downstaging at surgery was noted in 22 (71%) of 31 patients; nine (29%) of 31 patients achieved a pathological complete response.	Common AEs included nausea in 17 patients (53%), diarrhoea in 16 (50%), and fatigue in 16 (50%). Grade 3 diarrhoea in three (9%) of 32 patients; no Grade 4 events.	NCT01589419
Wee 1 inhibitor								
Adavosertib (AZD1775)	Cisplatin	IMRT 5 days a week, once daily, Monday to Friday, for 6 weeks	I	Head and neck cancer	9	Completed	Completed	NCT03028766
Adavosertib (AZD1775)	Cisplatin	45 Gy or greater	I	Cervical, upper vaginal and uterine Cancers	33	Active, not recruiting	Active, not recruiting	NCT03345784
Adavosertib (AZD1775)	Cisplatin	70 Gy at 2Gy per fraction, 35 fractions, Monday to Friday over 7 weeks	I	Intermediate/ high risk squamous cell carcinoma of head and neck	12	Completed	Completed	NCT02585973
Adavosertib (AZD1775)	Gemcitabine	52.5Gy in 25 fractions (2.1Gy/ fraction), using intensity-modulated radiation therapy (IMRT) after chemotherapy	I/II	Unresectable adenocarcinoma of the pancreas	34	Median overall survival for all patients was 21.7 months (90% CI, 16.7 to 24.8 months) which was substantially higher than prior results combining gemcitabine with radiation therapy.	8/34 patients (24%) experienced a dose-limiting toxicity, most commonly anorexia, nausea, or fatigue.	NCT02037230
Toll-like receptor agonists								
TLR9 agonist (SD-101) intratumoural	N/A	4 Gy in 2 fractions over 2 days	I/II	Untreated indolent lymphoma	29	26/29 (89.7%) patients had tumour reduction at treated site. 24 (82.8%) patients had tumour reduction at non-treated sites.	Grade 1-2 drug-related AEs reported by all patients. Most common treatment-related side effect was a flu-like systemic reaction. 8/29 patients (27.6%) had grade 3 drug-related AEs. No drug-related grade 4 or serious AEs.	NCT02266147
TLR9 agonist (SD-101) intratumoural	Anti-OX40 (BMS-986178)	Low-dose not specified over 2 fractions	I	Low-grade B cell non-Hodgkin lymphoma	15	Recruiting	Recruiting	NCT03410901
TLR9 agonist (SD-101) intratumoural	Epacadostat	24 Gy in 8 fractions, 20 Gy in 5 fractions, 4 Gy in 2 fractions	I/II	Advanced solid tumours	20	Early outcome reported for 7 patients refractory to prior therapy with anti-PD-L1 checkpoint inhibition. In these patients, disease control rate (DCR) and abscopal DCR was 86% (6/7) and 100% (7/7), response rate was 43% (3/7), and abscopal response rate was 29% (2/7) including 2 patients with long-term durable complete responses.	Awaiting report	NCT03322384
TLR9 agonist (SD-101) intratumoural	Pembrolizumab; leuprolide acetate; abiraterone	35 Gy in 7 fractions	II	Oligometastatic prostate cancer	42	Recruiting	Recruiting	NCT03007732

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TABLE 2 Continued

Target (drug) & route	Additional therapy	Radiotherapy	Phase	Patient population	n	Response	Toxicity	NCT ID
	Acetate; prednisone							
TLR9 agonist (SD-101) intratumoural	Ibrutinib	Not specified	Ib/II	Lymphoma	30	Early outcome reported for 13 patients treated with a median follow-up of 7.7 months. 6 of 12 evaluable patients had achieved a partial response (50% ORR) and 3 had achieved >50% reduction in distal tumour burden. Eight of 12 patients (66.7%) had experienced at least a 30% reduction in distal tumour burden.	AEs were consistent with known effects of ibrutinib and of CpG with no unexpected AEs to suggest synergistic toxicity. There were no grade 4 or 5 events. AEs led to ibrutinib dose reduction or discontinuation in 3 patients.	NCT02927964
TLR9 agonist (SD-101) intratumoural	Nivolumab	6-10 Gy per fraction to the injected lesion given on days 1, 3, 5, 8, and 10	I	Metastatic pancreatic adenocarcinoma	6	Active, not recruiting	Active, not recruiting	NCT04050085
CMP-001 intratumoural	Nivolumab; ipilimumab	Radiosurgery	I	Colorectal cancer metastatic to liver	19	Recruiting	Recruiting	NCT03507699
SD-101 intratumoural	Ipilimumab	Low-dose radiation therapy to 1 site of disease	I/II	Recurrent low-grade B-cell lymphoma	9	Completed	Completed	NCT02254772
Imiquimod (topical)	Cyclophosphamide	30 Gy in 5 fractions	I/II	Metastatic breast cancer	31	Completed	Completed	NCT01421017
Poly(ICLC) intratumoural	rhuFlt3L/CDX-301	2 Gy x 2	I/II	Lymphoma	11	Partial or complete response of treated tumour in 8/11 (72.7%). Six (54.5%) had stable disease/minor regressions at non-treated sites and three (27.3%) showed significant distant disease regression.	All AEs Grade 1 apart from 1 patient with G2 fever	NCT01976585
CpG-enriched TLR9 agonist (PF-3512676) intratumoural		4 Gy in 2 fractions over 2 days	I/II	Mycosis fungoides	15	One (6.7%) patient with complete clinical response, distant site clinical response seen in 5 patients (33.3%).	Mild injection site reaction and mild flu- like symptoms	NCT00185965

AEs, Adverse effects; DLTs, Dose-limiting toxicities; NCT, National Clinical Trial; N/A, Not Applicable.

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A downstream target of ATR, Chk1, has also been investigated as a potential therapeutic target, due to its ability to activate intra-S and G2/M cell cycle checkpoints and modulate the replication stress response (123), particularly as a sensitiser to radiotherapy (124). Chk1 inhibitors, to date, include UCN-01 (125), LY2606368 (126), PF-00477736 (127), MK8776 (128) and CCT244747 (129), AZD7762 (130) and LY2603618 (131). Although there have been promising results in refractory acute myeloid leukaemia and advanced cancer with MK-8776 (132, 133) and LY2606368 (134), unfortunately severe adverse effects such as drug-related cardiac toxicity have also been reported during the clinical development of these drugs, e.g. AZD7762 (135). Thus far, no clinical trials are investigating the combination of Chk1 inhibition and radiotherapy.

3.1.2 DNA-PKcs (DNA-dependent protein kinase, catalytic subunit) inhibitors

DNA-PK is pivotal for the initiation of DNA repair following DSBs, which ultimately results in recruitment of proteins involved in DNA damage repair progressing and ligating the broken DNA ends most recognised *via* the NHEJ pathway (136). Various cancer cell lines with reduced levels of DNA-PKcs show increased radiosensitivity compared to unirradiated controls (137–139) due to defective DNA DSB repair, inhibition of phosphorylated protein kinase B (Akt) on Ser473 and reduction of radiotherapy-induced transcription factor hypoxia-inducible factor-1 α levels (HIF-1 α) (138).

Given that DNA-PKcs is critical in radiotherapy-induced DDR, DNA-PKcs inhibition is an emerging therapeutic target for potentiating radiotherapy responses (140, 141), and many agents have already been tested in clinical trials. Non-selective DNA-PKcs inhibitors include wortmannin, which also inhibits ATM (142), and LY294002, which has a similar structure (143, 144). More selective DNA-PKcs inhibitors include NU7026 (145), NU7441 (146), IC86621, IC87102, IC87361 (147), vanillin (148), OK-1035 (149), SU11752 (150), BVAN08 (151), IC486241 (152) and NK314 (153). More recently, novel inhibitors have been discovered including M3814 (154), AZD7648 (155) and VX-984 (156). Doxycycline was first approved by US Food and Drug Administration (FDA) in 1967 as a broad-spectrum antibiotic and has recently been recognised to function also as an DNA-PK inhibitor (157). Mechanisms by which DNA-PKcs helps to sensitise to radiotherapy include prolongation of radiotherapy-induced G2/M phase arrest (158) and reduced repair of radiotherapy-induced DSB (147, 150, 159) leading to the induction of autophagic cell death and mitotic catastrophe (66).

In terms of DNA-PKcs inhibition leading to stimulation of the innate immune system, a recent study showed that combining radiation with M3814-induced DNA-PK inhibition increased cytosolic dsDNA and tumour type I interferon signalling in a cGAS-STING-independent, but RNA Polymerase III-, RIG-I- and MAVS-dependent manner, in pancreatic cancer models (91). Furthermore, radiotherapy and M3814 increased PD-L1 expression and sensitised to anti-PD-L1 treatment in poorly immunogenic pancreatic cancers (91). DNA-PKcs itself also functions as a DNA sensor that activates innate immunity. It has been reported to function as a PRR by binding to cytoplasmic DNA and can trigger a type I IFN response in a STING/IRF-3/TBK1-dependent manner (160) as well as a STING-independent manner *via* phosphorylation of heat shock protein HSPA8/heat shock cognate HSC70 (161). It is still unclear whether pharmacological inhibition of DNA-PKcs kinase activity may dampen anti-tumour immunity in contrast to inhibition of other DDR kinases described such as ATM or ATR.

Clinical studies of DNA repair inhibitors, M3814 (NCT04533750) and XRD-0394 (NCT05002140), in combination with radiotherapy are recruiting. In addition, triple combination of M3814 with radiotherapy and chemotherapy (NCT02516813, NCT03770689, NCT04555577) or anti-PD-L1 (NCT04068194, NCT03724890) are also awaiting report (Table 2).

3.1.3 PARP inhibitors

PARP-1 has been the most extensively studied of the PARP superfamily and is a key regulator of DNA damage repair (162, 163). In response to DNA damage, such as that induced by radiotherapy, an initial response is poly(ADP-ribosyl)ation (PARylation) of proteins including nuclear DDR proteins, such as DNA-PKcs, to provide a local signal of DNA damage (163–165). Inhibitors of PARP generally function by inhibiting PARylation or suppressing PARP-1 release by 'trapping'. PARP-1 inhibition has been reported to sensitise cancer cells to various forms of ionising radiation including conventional gamma irradiation (166, 167), proton-beam irradiation (167) and radionuclide therapy (168, 169) (Table 2). Although SSBs are primary repaired by PARP-1, its inhibition may not be lethal due to other available repair pathways, such as homologous recombination. However, deficiency in BRCA1/2 functionality, which are key components in the HR pathway of DSB repair, leads to synthetic lethality and selective sensitivity to PARP inhibition (170).

Beyond DNA repair, PARP-1 also plays an immunomodulatory role by regulating gene transcription of several immune cell types, modulating the stimulatory ability of DCs, and by directly affecting the differentiation and function of T and B cells (171, 172). PARP-1 knockout mice show reduced T helper type 2 (Th2) differentiation responses (172). PARP-1 is also involved in the differentiation of Foxp3+ regulatory T cells (Treg) and promotion of Treg cell apoptosis during inflammatory responses (172). PARP inhibitors generate cytoplasmic chromatin fragments with micronuclei characteristics which activate cGAS-STING, downstream type I interferon signalling and chemokine ligand 5 (CCL5) secretion in excision repair cross-complementation group 1 (ERCC1)-

defective non-small cell lung cancer (NSCLC) cells (173). The capacity of PARP1 inhibitors to upregulate innate immune and inflammasome-like signalling events, such as cGAS-STING signalling, closely depends on their PARP1-trapping abilities (174, 175). In the context of viral infection, activated DNA-PK has been reported to phosphorylate PARP1 leading to its cytoplasmic translocation (176). Cytoplasmic PARP1 can then interact with and directly PARylate cGAS to inhibit its DNA-binding ability (176). This has implications to how PARP inhibition, in the context of cancer-induced genome instability, can positively modulate the host anti-tumour immune response.

Early PARP-1 inhibitors were non-specific and non-selective, such as nicotinamide (177), AG14361 (178) and 4-amino-1,8-naphthalimide (179). Newer PARP-1 inhibitors, such as olaparib and niraparib, are now used in routine clinical practice following approval by the FDA and European Union (180, 181). They are licensed for use in patients with advanced BRCA-mutated ovarian cancer, metastatic-castration-resistant prostate cancer with BRCA1/2 or ATM mutation (182), suspected germline HR repair gene mutated mCRPC who have progressed on enzalutamide or abiraterone (183) and, most recently, recurrent epithelial ovarian, fallopian tube or primary peritoneal cancer which has responded to first-line platinum chemotherapy (184, 185).

Combining PARP-1 inhibition and radiotherapy has been supported by preclinical studies. Particularly in BRCA1-mutant cancers, PARP inhibition showed radiation hypersensitivity in lymphoblastoid cells (186). In various models, PARP-1 inhibitors KJ-28d (187), ABT-888 (188) and the PARP-1/2 inhibitor MK-4827 (189) increased cancer cell radiation sensitivity.

Many clinical trials are underway investigating the combination of PARP inhibitors and radiotherapy, with addition of chemotherapy and/or immunotherapy agents (Table 1). The mechanisms underlying radiosensitisation by PARP inhibitors are still not completely clear and, indeed, recent studies have revealed a wider immunological role for PARP-1 that could potentially be exploited through new therapeutic approaches (190). For example, one study showed through multiomics profiling that macrophage-mediated immune suppression is a liability of PARP inhibition (191). Following this evidence, the rationale for combining CSF-1R blocking antibodies with PARP inhibitors led to reprogramming of the TME and significantly enhanced innate and adaptive anti-tumour immunity, which was CD8+-mediated in BRCA-deficient tumours *in vivo* (191).

3.1.4 Wee1-like protein kinase (Wee1) inhibitors

Wee1 is a cell cycle checkpoint negative regulator at the G2/M transition. The process by which Wee1 activation leads to phosphorylation and inactivation of the cyclin B1/CDK1 complex blocking entry into mitosis is well described (192).

Emerging studies have highlighted the role of Wee1 directly and indirectly in immune signalling (193). For example, ineffective CDK-1-dependent nuclear laminin degradation abrogates apoptosis induction, leading to immune resistance in tumour cells (194). Accordingly, Wee1 inhibition reconstitutes CDK1 activity to reverse resistance of these cancer cells to immune attack (194). In various cancer models, Wee1 inhibition promotes accumulation of cytosolic dsDNA, leading to activation of the cGAS-STING pathway (Figure 1), increased type I interferon target gene expression when delivered alone (195), as well as in combination with ATR inhibitors (196) or immune checkpoint blockade (197). A STING-independent pathway by which Wee1 inhibition induces the interferon response has also been reported. In cGAS-STING-defective tumour models, Wee1 inhibition can upregulate immune signalling through the dsRNA anti-viral defence pathway by promoting expression of endogenous retroviral element (ERV) (198). ERVs trigger dsRNA stress and the interferon response, resulting in the recruitment of anti-tumour T-cells, and increased expression of PD-L1 with sensitisation to anti-PD-L1 blockade in multiple cancer models (198).

Wee1 inhibitors, some of which are concomitant CDK1 inhibitors, are promising as a combination partner with radiotherapy (199). This combination has shown synergistic effects in various cancer models (200–202). Wee1 inhibitors such as 681641 (203), PD0166285 (204) and adavosertib (MK1775/AZD1775) (92, 202, 205) have been reported to increase the radiosensitivity of cancer cells. Cancer cells very frequently harbour G1 checkpoint deficiencies and Wee1 inhibitor-mediated prevention of DNA repair following radiotherapy may lead to premature entry into mitosis and, ultimately, cell death *via* mitotic catastrophe (206). Other mechanisms include blocking radiotherapy-induced DNA damage repair (204) by impairing DNA repair protein RAD51 homolog 1 (RAD51) focus formation (202) and suppression of Sirt1 (silent mating type information regulation 2 homolog 1). Sirt1 interacts with and deacetylates HR-repair machinery proteins including Nibrin (NBS1) and RAD51, thus, Wee1-induced Sirt1 suppression impairs HR-repair activity (207).

Several clinical trials are exploring the combination of Wee1 inhibition by adavosertib (MK1775/AZD1775) with radiotherapy and chemotherapy (NCT03028766, NCT03345784, NCT02585973, NCT02037230) (Table 2). The emerging immune-mediating effects of Wee1 inhibition provide a strong rationale for its combination with immune checkpoint inhibitors (198).

3.2 Cytosolic nucleic acid sensing pathways

The ability to detect cytosolic nucleic acids by PRRs, arising from pathogens or disruption of cellular functions from genotoxic stress such as DNA damage, is part of the protective cellular response against infection or injury. These mechanisms

are an evolutionary product of anti-microbial responses and can trigger an inflammatory signalling cascade and subsequent activation of the innate immune system. Targeting these nucleic acid sensing mechanisms has the potential to further amplify the DDR-induced anti-tumour innate immunity in conjunction with radiotherapy.

3.2.1 Direct DNA sensing

3.2.1.1 STING agonists

Stimulator of interferon genes (STING) is an endoplasmic reticulum adaptor that senses self and foreign cytoplasmic DNA, *via* cyclic GMP–AMP synthase (cGAS), and is crucial for effective innate immune signalling (208). Cytosolic DNA induces synthesis of the cyclic dinucleotide (CDN) cyclic GMP–AMP (cGAMP) from ATP and GTP by a cyclase enzyme called cGAS. cGAMP directly binds to STING to cause its dimerization and activation (209, 210), leading to activation of both NF-κB and IRF3 transcription pathways to induce expression of type I interferon, recruitment of immune cells, promotion of DC maturation and antigen-specific immune priming (211).

The cGAS-STING pathway is essential for anti-tumour T cell responses (212). One proposed mechanism is that CD8α$^+$ DCs engulf apoptotic or necrotic tumour cells, and tumour cell-derived DNA triggers STING signalling in DCs (212–214). The subsequent type I IFN production by these DCs facilitates antigen cross-presentation and T-cell priming independent of the TLR or RIG-I/MAVS pathways (212). Recent studies have also suggested that STING signalling in the TME can suppress the immunosuppressive activity of MDSCs (215, 216). STING signalling is critical for radiation-induced anti-tumour responses (214) and, thus, it is an attractive potential treatment combination with radiotherapy. Preclinical data have shown that consideration needs to be given to radiotherapy dose per fraction as doses above 12-18 Gy induce the DNA exonuclease Trex1, which degrades the cytosolic DNA required to stimulate an effective STING-dependent type I IFN response (217).

The first generation STING agonist, 5,6-Dimethylxanthenone-4-acetic Acid (DMXAA), was originally developed as a vascular-disrupting agent (218, 219) and its anti-tumour effect is based on vascular necrosis leading to tumour starvation and haemorrhagic necrosis (218, 220). DMXAA has previously been shown to synergise with radiotherapy in mouse models in a hypoxia-preferential manner (221). However, the TME was found to remain immunologically sterile and tumours eventually progressed with time without durable protective anti-tumour immunity (222, 223). High local STING concentrations can lead to rapid T-cell apoptosis (224) whereas low-dose administration can lead to 'vascular normalisation' and favourably transform the TME to allow use of effective combinatorial anti-tumour immunotherapy (225–227).

There are two categories of STING agonists in clinical development: synthetic cyclic dinucleotides (CDNs) or non-CDN small molecules (228). These drugs are generally administered intratumourally due to their poor stability and bioavailability. This caveat limits their use to accessible tumours and recent efforts have been focused on development of STING agonists for systemic delivery (intravenously (228), orally (229, 230) and even as an inhalable nanoparticulate (231)). In addition, novel STING antibody-drug conjugates show promising preclinical results (232). There have only been a handful of preclinical studies investigating novel STING agonists with radiotherapy *in vivo* (Table 1). In mouse models, STING agonists synergise with radiotherapy to control local and distant disease and mediate rejection of tumour rechallenge (93, 231) *via* early T-cell-independent and TNF-α-dependent haemorrhagic necrosis, followed by a later stage of CD8 T-cell-dependent control (93). A number of clinical trials have looked into combining STING agonists with ICI or conventional chemotherapy (233); however, at the time of this review no radiotherapy and STING agonist combination clinical trials are in progress.

3.2.2 Crosstalk with RNA sensors

3.2.2.1 Toll-like receptor agonists

Toll-like receptors (TLRs) are a form of PRR expressed on sentinel immune cells which activate innate defence systems by detecting PAMPs. Genotoxic stress and DNA damage are increasingly recognised to signal through TLRs and cause the upregulation of TLR expression (234) *via* p53 (235). TLR signalling leads to maturation of APCs such as DCs, which are key mediators of T-cell activation and subsequent adaptive immunity. There is growing preclinical evidence that TLR agonists in combination with radiotherapy may lead to enhanced anti-tumour immunity, particularly through the mechanism of enhanced DC-mediated T-cell priming following radiotherapy (236). This occurs at various stages of this pathway; for example, TLR activation enhances type I IFN-signalling in many immune cells, modulates chemokine expression to enhance DC migration to lymphoid tissues (237–239) and upregulates CD80 and CD86 co-stimulatory molecules on DCs, which bind to CD28 on naïve T-cells for antigen/MHC-complex mediated TCR stimulation (240). TLRs can also stimulate DC-mediated release of IL-6 to dampen Treg suppressive signalling (241).

Given these observations, TLR agonists are seen as an attractive combination partner with radiotherapy. There have been numerous preclinical studies (Table 1) and early phase clinical trials (Table 2) of different TLR agonists, particularly of TLR3, TLR7/8 and TLR9, in combination with radiotherapy.

TLR3 senses dsRNA as a PAMP and polyinosinic-polycytidylic acid or poly (I:C) is a synthetic mimic of dsRNA which can stimulate TLR3-signalling pathways and lead to type I-IFN-dependent (242, 243) DC antigen cross-priming *in vivo*

(244, 245). Poly(I:C) also has several immunostimulatory effects, including maturation and activation of DCs (246–248), T-cell stimulation (249, 250), enhanced cytotoxicity of Natural Killer (NK) cells (251–253), reprogramming of MDSCs (254) and repolarisation of macrophage populations from an M2 (classically activated macrophages) to M1 (alternatively activated macrophages) phenotype (255) (Figure 1). Pre-clinical studies exploring TLR3 agonists with radiotherapy in a radioresistant mouse model of lung cancer showed that poly(I:C) enhanced radiotherapy anti-tumour effects (256). The results from initial clinical trials have been disappointing, likely due to the short half-life of poly(I:C) (257). To address this, a degradation-resistant derivative polyinosinic-polycytidylic acid, and poly-L-lysine or poly(ICLC) was developed that has shown efficacy in clinical trials, although toxicity remains an issue (257). Preclinical studies in a murine lymphoma model have investigated the Fms-like tyrosine kinase 3 (Flt3)-ligand with radiotherapy and poly(ICLC) (258). Flt3-ligand is a cytokine which increases migration of DCs into the tumour and radiotherapy then stimulates maturation of DCs *via* ICD and HMGB-1 signalling for antigen uptake and processing (259). This combination with the addition of poly(ICLC) further maximises DC maturation and activation (246–248). There is a clinical study investigating intratumoral delivery of poly (ICLC) in combination with an in-situ vaccine rhuFlt3L/CDX-301 and radiotherapy which was well-tolerated and showed promising results (258) (NCT01976585) (Table 2). Two phase 2 studies in glioblastoma patients are also investigating the efficacy of poly(ICLC) in combination with radiotherapy (260, 261).

TLR7 and TLR8 detect guanosine or uridine-rich single-stranded RNA and their activation can directly induce MDSCs to lose their immunosuppressive function and acquire an APC-like phenotype that can induce tumour-specific T-cell responses (262), convert MDSCs to M1-like macrophages (263), activate NK cells (264–267) and revert Treg immunosuppressive effects (268). The imidazoquinolines are synthetic agonists for TLR7/8 of which topical imiquimod is the most extensively studied as well as being currently licensed for the treatment of superficial basal cell carcinoma (269). A preclinical study in breast cancer has investigated topical imiquimod in combination with radiotherapy and low-dose cyclophosphamide (94), and found that this triple combination had synergistic anti-cancer effects at both irradiated and unirradiated (abscopal) sites. Long-term surviving mice were able to reject tumour rechallenge, likely due to the establishment of anti-tumour immunological memory (94) (Table 1). A phase 2 clinical trial in metastatic breast cancer testing the efficacy of this triple therapy has finished recruiting (NCT01421017) (Table 2). Synergistic effects of subcutaneous TLR7 agonist and radiotherapy have also been observed in a preclinical model of melanoma (95) (Table 1). The efficacy of systemic delivery of the TLR7 agonists R848 (96), DSR-6434 (97), DSR-29133 (98) and 3M-011 (99), in combination with radiotherapy, has been explored in the treatment of several preclinical models of solid cancers. Dual therapy works synergistically to enhance tumour control, generate tumour-antigen-specific T-cells, suppress tumour growth (96–99) after rechallenge in long-term surviving mice (97) (98) and reduce the formation of distant metastases (99). Systemically-administered TLR7/8 agonists are not currently being investigated in a clinical setting; notably a phase I clinical trial investigating systemic TLR7 agonist ANA975 in chronic hepatitis C virus (270) had to be withdrawn due to excessive toxicity in extended preclinical studies (271), highlighting the need for caution when delivering systemic TLR7/8 agonists, especially in combination with radiotherapy (236).

Finally, TLR9 is expressed on APCs and B-cells and senses unmethylated CpG oligonucleotides present in bacterial and viral DNA (272–274). Again, TLR9 agonism can lead to activation and maturation of DCs, cytokine release from T helper type 1 (Th1) cells, differentiation of MDSC towards an M1 phenotype (275–279) and inhibition of Treg immunosuppressive effects (280). Several preclinical studies (281–284) have shown that TLR9 agonists can lead to anti-tumour effects in an NK- and CD8 T-cell-dependent manner (285). Preclinical studies showed enhanced tumour control in combination with radiotherapy in a model of murine fibrosarcoma and lung cancer (100–103), and induction of immunological memory by mice rejecting tumour rechallenge (102). The synergistic effects of radiotherapy and TLR9 agonists are dependent on a competent host immune system (102). Early clinical studies, although in small patient numbers, have tested TRL9 agonists in combination with radiotherapy. CpG-enriched oligodeoxynucleotide delivered intratumorally in combination with radiotherapy, 4 Gy in two fractions, led to overall objective response rates of 27% in the non-treated lesions of patients with relapsed low-grade B cell lymphoma (286).

3.2.2.2 (RIG-I)-like receptor (RLR) agonists

RIG-I and melanoma differentiation-associated gene 5 (MDA5) are collectively (RIG-I)-like receptors (RLR) which detect cytosolic RNA and are a key PRR in anti-viral responses (287). RIG-I preferentially binds to short (>10 bp) dsRNAs whereas MDA5 detects long accessible dsRNAs (>2 kbp) (288, 289), and downstream signalling of either activates IRF3 and NF-κB pathways to induce type I IFN and other inflammatory cytokines. In the context of DNA damage, RIG-I interacts with X-ray repair cross complementing 4 (XRCC4) to impede formation of the XRCC4/LIG4 (DNA ligase 4)/XLF (XRCC4-like factor) at DSBs. High expression of RIG-I compromises DNA repair and sensitises cancer cells to irradiation treatment. In contrast, depletion of RIG-I renders cells resistant to irradiation *in vitro* and *in vivo* (290).

In the anti-tumour response, there is increasing evidence that RLR activation in various cancer models by RNA ligands can induce cancer cell apoptosis in a type I IFN-dependent (291), or -independent manner (292, 293). RIG-I signalling can

induce ICD of ovarian and pancreatic cancer cells *in vivo* by systemic activation of DCs, NK cells and CD8+ T cells (294, 295). In a pancreatic cancer model, tumour-derived type I IFN activates DCs and CD8α^+ DCs engulf apoptotic tumour material and cross-present tumour-associated antigen to naïve CD8+ T cells (296). RIG-I may also inhibit tumour growth indirectly through regulation of tumour hypoxia (297) and the gut microbiota (298). The efficacy of anti-cancer treatments such as radiotherapy and many chemotherapy agents has also been shown to depend on the RLR pathway through endogenous non-coding RNAs, and depletion of RIG-I in human tumours confers treatment resistance (299).

Harnessing the RLR-pathway through RLR agonists is an attractive therapeutic target and several RLR mimetics or agonists have been developed which have shown promise in preclinical studies. For example, a unique RIG-I agonist in the form of RNA stem-loop of 14 bp (SLR14), when delivered intratumorally, significantly inhibited B16 tumour growth locally and systemically in bilateral and tumour metastasis models, with cured mice developing immunological memory (300). SLR14 was mainly taken up by CD11b+ myeloid cells in the TME leading to subsequent increase in the number of CD8+ T lymphocytes, NK cells, and CD11b+ cells in SLR14-treated tumours (300). MK4621 (or RGT100), a synthetic RNA oligonucleotide RIG-I activator is currently in phase 1 clinical trials for the treatment of advanced/metastatic solid tumours (NCT03739138).

Combining RLR agonists and radiotherapy is an attractive strategy to activate multiple DDR pathways *via* cytosolic RNA sensing and radiotherapy-induced cytosolic DNA/DNA damage detection. *In vitro*, an RLR agonist Poly(I:C)-HMW (High Molecular Weight)/LyoVec^{TM} [Poly(I:C)-HMW] sensitised *in vitro* human lung cancer cells to Fas ligand (FasL)-induced apoptosis by radiotherapy (301). *In vivo* intratumoral cytoplasmic delivery of the dsRNA mimic poly(I:C) by polyethylenimine (PEI), prior to diffusing alpha-emitting radiation therapy (DaRT), resulted in synergistic tumour and metastatic disease control. Furthermore, immunological memory was demonstrated, whereby splenocytes from treated mice adoptively transferred to naïve tumour-bearing mice, resulted in delayed tumour development and protection from rechallenge (104). Combining RLR-agonists and radiotherapy has not yet been translated into clinical practice and to the best of our knowledge there are no clinical trials investigating this combination.

4 Discussion

We have discussed in detail the various druggable targets related to the DDR pathway, in particular agonists of the nucleic acid sensing pathways and inhibitors of DNA damage repair mechanisms. Next, this review will explore the clinical challenges and implications of combining radiotherapy with DDR-targeted agents.

4.1 The role of conventional chemotherapy

Conventional chemotherapy has historically been used in the backbone of radical chemoradiation (CRT) in many locally advanced tumours such as rectal, cervical and head and neck cancers. Chemotherapy agents traditionally used as radiosensitisers include platin salts (e.g. Cisplatin, Carboplatin) or fluoropyrimidines (e.g. 5-fluorouracil or its prodrug Capecitabine), which trigger cell death by instigating DNA damage (302). Chemotherapy-induced cell death can lead to DNA leakage into the cytosol and trigger intrinsic STING pathway stimulation and activation of the immune system (303). Some may argue that investigating novel DDR-pathway specific agents is redundant given that chemotherapy may exert its anti-cancer effects partly by stimulating the innate immune system (303). However, it is recognised that chemotherapy (304), radiotherapy (305) or concomitant CRT (306) in various cancers can result in lymphocyte depletion which can potentially negate a sustained effective anti-tumour response. Lymphocyte depletion post-treatment is a poor prognostic factor in patients who have undergone radiotherapy for Stage III lung cancer (305) or CRT for newly diagnosed glioblastoma (306). Furthermore, defects in DDR signalling may contribute to chemoresistance in some cancer types (303) and, as such, development of specific DDR-targeting agents remains an important avenue for research.

4.2 Maintaining anti-tumour immunity using ICIs

The anti-tumour innate immunity initiated by radiotherapy and DDR inhibitors is likely to be complementary to the effect of immune checkpoint inhibitors (ICIs), which can sustain and maintain the adaptive arm of the anti-tumour immune response. For example, preclinical studies in lymphoma have shown that treatment with Flt3L, radiation and poly(ICLC) led to PD-L1 upregulation in both tumour cells and intratumoural DCs, and that the further addition of anti-PD-1 antibody led to improved local and systemic tumour control (258). There is an increasing number of early phase clinical studies investigating the addition of ICI with radiotherapy and DDR-targeted agents, such as TLR agonists (NCT03007732, NCT04050085, NCT03507699, NCT02254772) and DNA-PK inhibitors (NCT04068194, NCT03724890, NCT04576091, NCT03923270).

Clinical response to ICIs is typically predicted by tumour mutational burden and neoantigen load (307, 308). Preclinical data suggests that radiotherapy and DDR inhibitors may

replicate the phenotype of high mutational and neoantigen burden and rationally direct therapeutic combinations with ICIs. However, the caveat is that radiotherapy-induced subclonal neoantigens may translate into poorer responses to ICI in some tumour types (307). The combination of radiotherapy and anti-CTLA-4 increases the diversity of TIL TCR repertoire, leading to increased tumour control *in vivo*; however, these tumours remain dominated by a small number of high-frequency T-cell clones (30, 32). It is still unknown whether it is more important to have an immune response against pre-existing tumour antigens or new radiotherapy-generated tumour antigens. As we await the results of the ongoing triple combination treatments (RT + DDR agents + ICI) in early phase clinical trials, further work is needed to investigate such combinations in the context of creation of subclonal neoantigens.

4.3 Tumour-specific radiosensitisation and the safety profile of combination therapy

A key principle of radiation oncology is that the dose delivered to the tumour is limited by the surrounding normal tissue organs-at-risk (OARs). Hence, strategies in designing clinical trials arguably should have some basis for a selective effect of any combination drug on the tumour (309). Preclinical studies in mouse models, for example, show that M3814, a DNA-PK inhibitor given with radiotherapy, shows marked improvement in tumour control (310). However, when translated into clinical practice, a clinical trial of M3814 with radiation (NCT02516813) reported enhanced normal tissue reactions including dysphagia, prolonged stomatitis and radiation dermatitis (311). Pre-clinical models are also severely limited in predicting long-term treatment toxicity in humans.

A further therapeutic challenge of using DDR pathway agents with radiotherapy is that there may be high variability in drug pharmacokinetics leading to varying degrees of radiosensitisation between tumour versus normal tissues, which makes it difficult to predict the therapeutic index for each individual patient (309). Therefore, unless there is a clear mechanism for tumour-specific radiosensitisation, clinical trials combining DNA repair inhibitors and radiotherapy may be severely compromised by unacceptable toxicity. Potential solutions may be an intratumoural route of drug delivery, as taken by certain trials of TLR9 agonists and STING agonists (Table 2), or conditional drug activation, such as with a hypoxia-activated DNA-PK inhibitor (312, 313). Increased knowledge of biomarkers and access to routine tumour profiling may guide the best selection of which DDR agent to use in a particular cancer subtype, for example PARP-inhibitors in BRCA-mutant or ATM/ATR inhibitors in p53-mutant tumours. Advances in radiotherapy delivery techniques using stereotactic techniques to irradiate tumour volumes highly selectively is a further way to reduce off-target combination effects of DDR-targeting agents. For example, a Phase I trial in recurrent head and neck squamous cell carcinoma investigating combining an ATR kinase inhibitor BAY1895344 with pembrolizumab and stereotactic body radiotherapy (SBRT) (NCT04576091) represents one such promising approach.

4.4 Radiotherapy planning, modality and scheduling with DDR delivery

In some occasions, radiotherapy can result in the regression of disease outside of the irradiated field in the so-called abscopal effect, which is thought to be immune-mediated (314). Inducing such systemic anti-tumour immune responses is likely highly dependent on radiotherapy dose and fractionation and these factors, therefore, need to be an important consideration in combination treatments with DDR agents and/or ICI (315).

Irradiation of regional lymph nodes in cancer treatment is common practice either with high doses in macroscopic disease or prophylactic lower doses, if lymph nodes are deemed to be at risk of harbouring micrometastatic disease. This approach has recently become more controversial given that we know these lymphoid organs have an important role in DC-mediated T-cell priming, activation and subsequent tumour infiltration following radiotherapy (31). Routine irradiation of regional lymph nodes may potentially deplete important immune cells and have a detrimental effect on the anti-tumour immune response (316).

The biological effects of radiotherapy, such as DNA damage complexity, depend on radiation quality and degree of linear energy transfer (LET). High LET radiation (e.g. protons, carbon ions, α-particle-emitting radionuclides) can differentially affect cell fate (317). For example, protons mainly induce apoptosis not necrosis which may reduce the leakage of nucleic acids into the cytoplasm to serve as danger signals, hence impacting on the innate immune response (317). The effects of radiotherapy were previously thought to be mainly due to nuclear DNA damage and their repair mechanisms. However, the outcome of irradiation depends also on the activation and regulation of other organelles that determine cellular metabolism, survival and immunological responses such as the mitochondria (318). Recent studies have shown that mitochondrial DNA DSBs activate a type I IFN response and mitochondrial RNA release into the cytoplasm triggers a RIG-I-MAVS-dependent immune response (319, 320). Low-dose versus high-dose radiation, as well as radiation quality, can also have different effects on mitochondria-mediated innate and adaptive immune responses (318). Interestingly, high LET particle radiotherapy which are more efficient in ROS production is reportedly more

likely to lead to mitochondria-mediated apoptosis and anti-tumour immune responses (318, 321).

The most appropriate scheduling of DDR agents with respect to radiotherapy also needs to be investigated further. For example, a study investigating a novel TLR7/8 agonist in combination with radiotherapy showed that the optimal combination efficacy required the drug to be administered concurrently at the start rather than end of radiotherapy (98). However, another investigation of a TLR9 agonist showed maximum synergy was observed when mice received the agent three days after radiotherapy in the adjuvant setting (102). Clinical trials investigating TLR3 agonists used in the concurrent or adjuvant setting with respect to radiotherapy both showed activity (258, 260, 261, 322). More preclinical studies investigating the biological basis of optimal scheduling are required, although it may be that optimal scheduling may ultimately be both treatment- and tumour-specific.

5 Conclusion

Our increasing knowledge of the mechanisms of how radiotherapy-induced DDR interacts intimately with the host immune response is critical to the discovery of novel therapeutic targets and effective strategies against cancer. DDR-targeted agents are an exciting avenue for overcoming radioresistance and improving patient outcomes through enhancement of anti-tumour immunity. Understanding the molecular mechanisms and immunological effects of these DDR agents, through rigorous preclinical testing and translational analyses, is key to guiding rational clinical trial design in terms of drug route of delivery, schedules and choice of additional combination treatments, such as chemotherapy or immunotherapy.

Author contributions

CC conceptualised this review and drafted the manuscript. AR, EP, MP, AM and KH contributed to the writing and critical revision of this article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The effects of radiation therapy on the macrophage response in cancer

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The efficacy of radiotherapy, a mainstay of cancer treatment, is strongly influenced by both cellular and non-cellular features of the tumor microenvironment (TME). Tumor-associated macrophages (TAMs) are a heterogeneous population within the TME and their prevalence significantly correlates with patient prognosis in a range of cancers. Macrophages display intrinsic radio-resistance and radiotherapy can influence TAM recruitment and phenotype. However, whether radiotherapy alone can effectively "reprogram" TAMs to display anti-tumor phenotypes appears conflicting. Here, we discuss the effect of radiation on macrophage recruitment and plasticity in cancer, while emphasizing the role of specific TME components which may compromise the tumor response to radiation and influence macrophage function. In particular, this review will focus on soluble factors (cytokines, chemokines and components of the complement system) as well as physical changes to the TME. Since the macrophage response has the potential to influence radiotherapy outcomes this population may represent a drug target for improving treatment. An enhanced understanding of components of the TME impacting radiation-induced TAM recruitment and function may help consider the scope for future therapeutic avenues to target this plastic and pervasive population.

KEYWORDS

radiotherapy, tumor microenvironment, hypoxia, extracellular matrix, macrophage polarization, macrophage recruitment, tumor associated macrophages (TAM), complement system

Introduction

Within neoplastic lesions, immune and mesenchymal cells interact with malignant tumor cells and influence many facets of tumor progression (1–3). Tumor-associated macrophages (TAMs) often make up a large proportion of the immune cell population within the TME. Macrophages are a highly plastic immune cell population, and their

phenotypes are shaped by the microenvironments in which they reside (4, 5). In the context of cancer, macrophages are exploited by the tumor cells to adopt phenotypes which counterintuitively, help facilitate disease progression through providing a suitable microenvironment for the progression of multiple carcinomas (6). It is possible to consider the role of TAMs in tumor progression as occurring in phases (Figure 1) which include initial recruitment of TAM progenitors, subsequent polarization to an immunosuppressive phenotype and prevention of anti-tumor immune responses. TAMs can also facilitate angiogenesis to meet the metabolic demands of the cancer while assisting the passage of tumor cells into circulation and setting up the site for secondary tumor growth (7–9). Interestingly, the TAM population is phenotypically diverse to the extent that both pro- and anti-tumoral phenotypes of these cells can reside in the same tumor (10, 11). The prevalence of the TAM population correlates with poor patient prognosis in all cancers (except colorectal) (12–14) highlighting this population as a potential therapeutic target in cancer.

Radiotherapy is still a mainstay of cancer treatment for approximately 50% of all cancer patients. It is increasingly recognized that radiotherapy is a strong immune modulator, with the capacity to induce both pro- and anti-inflammatory processes (15, 16). As such, radiation can elicit macrophage recruitment into the tumor (17–20). TAM polarization away from tissue-protection and towards anti-tumoral/immunostimulatory functions could be a potential approach to boost the anti-cancer effects of radiotherapy and capitalize on the immune-stimulating effects of this treatment (16, 19). Here, the effect of radiation on TAM recruitment and polarization will be described. We will particularly focus on changes to soluble and physical components of the tumor microenvironment (TME) which may limit the positive effects of radiation on macrophage plasticity and highlight key examples that could be therapeutically targeted to improve radiation response.

Phase 1: Recruitment of TAMs

Recruitment overview

TAMs within the tumor are either present as tissue-resident macrophages or are formed after circulating monocytes are recruited and subsequently polarized into mature TAMs (21, 22). Resident macrophages are present during embryonic development and tend to exist in specific tissues such as Kupffer cells in the liver, and alveolar macrophages in the pulmonary alveolus of the lungs (23). These macrophages can provide a pro-tumorigenic niche and assist with initial tumor growth from a very early stage (24).

Soluble factors impacting TAM recruitment following radiotherapy

Soluble factors that mediate mobilization are critically associated with recruiting monocytes/macrophages to the TME (Figure 1). A well-documented signaling molecule involved in this process is chemokine (C-C motif) ligand 2 (CCL2, also known as monocyte chemoattractant protein 1; MCP1) (25–27).

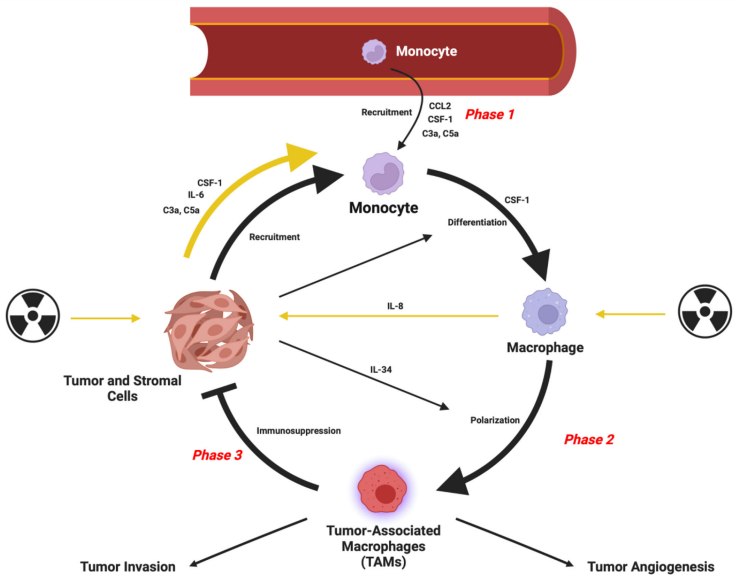

FIGURE 1
Schematic representation of the role of TAMs in tumor progression. Radiation can contribute to recruitment and polarization as indicated by the yellow arrows. Figure created in Biorender. Agreement number: RO24DGQPW4.

Radiotherapy is known to induce the expression of CCL2 within the TME (28, 29). Increased CCL2 expression can also be regulated by components of the humoral arm of innate immunity such as the complement system (30–32) and the long pentraxin PTX3 (33). Both of these innate immunity components appear to work in concert since PTX3 deficiency results in complement-dependent TAM recruitment in 3-Methylcholanthrene carcinogenesis models (33). Signaling of complement anaphylatoxins C3a and C5a through their respective receptors, C3aR and C5aR1, has been further demonstrated to result in TAM recruitment and polarization towards an immunosuppressive phenotype (30, 31). This includes reduced CD206 expression and upregulation of CD11c, major histocompatibility complex class II, CD80 and CD86 in TAMs from C3 and C3aR1$^{-/-}$ mice (32). Interestingly, expression of C3a, C5a and their receptors C3aR and C5aR1 is induced in melanoma murine tumors following irradiation (20 Gy) (34). Furthermore, complement inhibition at the level of C3 (with a CR2-Crry fusion protein) in combination with radiation has been demonstrated to enhance the numbers of macrophages with an M1-like phenotype (F4/80^{+}, CD11c^{+}, CD206^{-}) in lymphoma tumor models (35).

In addition to chemokines and complement soluble factors, cytokines are also involved in the recruitment of monocytes/macrophages to the TME. Colony-stimulating factor 1 (CSF-1, also known as macrophage colony stimulating factor; M-CSF), which typically is associated with a differentiation/survival signal for monocyte/macrophages, also has chemotactic properties for the recruitment of these cells to a site of inflammation (36, 37). In several tumor types and murine models, radiation has been demonstrated to induce CSF-1 production which can facilitate macrophage recruitment (17, 18). Following irradiation of tumors the DNA damage-induced kinase ABL1 (c-Abl) is recruited into the nuclei of tumor cells to enhance CSF1 transcription (38). CSF-1 production is also induced in response to IL-8, which can be secreted by the macrophages themselves, contributing to a positive feedback axis further perpetuating macrophage recruitment. However, this axis is not necessarily macrophage-specific as cancer cells can also produce IL-8 themselves post-irradiation (39). IL-34 is a cytokine that shares its receptor with CSF-1, binding CSF1-R, and as such they have similar biological properties. Like CSF-1, IL-34 expression is induced after irradiation (40). This induction has also been demonstrated to promote monocyte recruitment to the TME and subsequent polarization to an immunosuppressive phenotype (41).

Furthermore, tumor cells produce IL-6 in response to radiation-induced damage which promotes monocytes/macrophage recruitment to the TME (42–44). In a double-edged role for IL-6, once monocyte recruitment occurs, the cytokine also blocks dendritic cell differentiation and promotes monocytes to differentiate towards a TAM-like cell with an immunosuppressive phenotype (6, 45).

Physical changes in the TME affecting TAM recruitment following radiotherapy

Hypoxia (low oxygen tension) is a common physical feature of the TME that arises due to insufficient oxygen supply to support rapidly growing tumors. Hypoxia is particularly relevant to radiotherapy since cells irradiated under reduced oxygen levels are more resistant to the lethal effects of radiation (46). Hypoxia-inducible factors (HIFs) are key to the transcriptional response to hypoxia. HIF heterodimers consist of an oxygen-sensitive subunit (HIF-1α, HIF-2α or HIF-3α), and a constitutively expressed HIF-β subunit. Under ambient oxygen concentrations, HIF-α subunits are continually degraded by ubiquitination and proteasomal degradation. However, under low oxygen tensions, HIF-α subunits are stabilized and trafficked to the nucleus where they modulate gene expression through binding hypoxia-responsive elements of specific genes associated with the hypoxic response (47–49). Both HIF-1α and HIF-2α can accumulate in macrophages exposed to hypoxic conditions *in vitro* (50, 51). *In vivo*, HIF-1α has been found to be essential for maintenance of appropriate cellular ATP pools necessary for myeloid cell motility and function (52). Furthermore, following tumor irradiation, nitric oxide (NO) generation in TAMs results in s-nitrosylation of HIF-1α at its oxygen-dependent degradation domain which prevents its destruction. Pharmacological inhibition of NO production is associated with reduced tumor growth following irradiation (53). Furthermore, studies using mice specifically lacking HIF-2α in myeloid cells have demonstrated reduced TAM infiltration in hepatocellular and colitis-associated colon carcinoma models through regulation of cytokine receptor CSF-1R and chemokine receptor CXCR4. Interestingly, this observed reduction in TAM infiltration was associated with reduced tumor cell proliferation (54). HIF-dependent induction of CCL2 also further supports monocyte/macrophage recruitment (55). A recent study has demonstrated that vascular endothelial growth factor-A (VEGF-A), another HIF-regulated gene, also plays a key role in both the recruitment of macrophages and the polarization toward an immunosuppressive phenotype as shown by the increase of the marker CD163 (56).

Extracellular matrix

The extracellular matrix (ECM), which constitutes the protein scaffold around the tumor and stromal cells, has a role in providing a platform for innate immune cell infiltration, with many of its components and post-degradation fragments sharing the ability to recruit monocytes. Much focus has been directed to proteolytic fragments of the ECM which have been demonstrated to represent endogenous ligands for binding and activating toll-like receptors (TLRs). The release of glycosaminoglycan hyaluronan (HA) after irradiation of the

tumor has been documented (57). HA can also play a role in facilitating macrophage infiltration into the tumor stroma through an interaction with the HA receptor CD44 expressed by macrophages (58). Monocytes/TAMs recruited by the CD44: HA axis have an immunosuppressive phenotype. This is facilitated by the upregulation of IL-10 expression while concurrently downregulating NF-κB signaling (59).

In addition to HA, latent TGF-β (an inactive form of the cytokine) is also released by the ECM post-irradiation. Once activated, TGF-β has a potent influence on TAM recruitment. This can occur directly through enhanced integrin expression and type IV collagenase secretion (60) and indirectly through the upregulation of CXCR4 on monocytes, with perivascular fibroblast expression of CXCL12 attracting the monocytes to the tumor bed (61).

Additionally, damaging the ECM leads to macrophage recruitment due to the attraction of immunosuppressive TAMs through the scavenger receptor CD206 (mannose receptor). This allows the phagocytosis and degradation of collagen fragments to form a strong chemoattractant for macrophages (62, 63). This leads to a feedback loop where initial radiation-induced damage to the ECM leads to recruitment of TAMs that themselves facilitate a continuous wound-healing state within the tumor site, further increasing monocyte/TAM recruitment. In a similar fashion, elastin fragments generated by the activity of macrophage-derived MMPs (9 and -12) have been demonstrated to act as chemotactic factors for monocytes, creating a positive feedback loop which increases the prevalence of TAMs in the TME (64).

Phase 2: Macrophage polarization

Polarization overview

Previously, monocyte polarization into mature macrophages was thought to be binary, with TAMs either acting as inflammatory or immunosuppressive agents within the stroma (65). However, it is becoming increasingly clear that, once polarized, the TAMs phenotypically fall on a spectrum (4). Data, mostly gathered from *in vitro* studies, has indicated that polarization on this spectrum may depend on the presence of specific factors such as IL-4, IL-10, IL-13, IFNγ, and lipopolysaccharide (LPS) (66, 67) (Figure 2). Once these factors bind to their respective receptor, monocytes undergo polarization and maturation into more specialised TAM phenotypes through downstream signal transduction pathways altering transcription within these cells (68). Recently, it has been identified that TAM polarization can be refined to a three-way polarization program in a spontaneous murine model of breast cancer (11). This three-way program is broadly split into an alternatively-activated-like, angiogenic/immunosuppressive, and inflammatory phenotypic specialization of these cells (11).

Pathways involved in radiation-induced polarization

Following irradiation, macrophage polarization towards either pro- or anti-inflammatory sides of the spectrum may be dependent on irradiation dose and which transcription factors are formed to drive downstream gene expression (69, 70). NF-κB is a key modulator of macrophage polarization and NF-κB p65-p50 heterodimers can initiate transcription of pro-inflammatory genes such as TNFα, IL1β, IL6, IL12, IFNγ and CXCL10 (70). Increased p65/RelA expression following 2 Gy irradiation of the RAW264.7 macrophage cell line or $CD11b^{+}$ peritoneal macrophages, is associated with increased levels of inducible nitric oxide synthase (iNOS, which is an M1-associated marker) (71). Low dose (2 Gy) whole body irradiation has also been demonstrated to induce iNOS, and concurrently reduce M2-associated markers such as Ym-1 and Fizz-1 in peritoneal macrophages. iNOS expressing TAMs in turn appear important for effector T-cell recruitment into the tumor

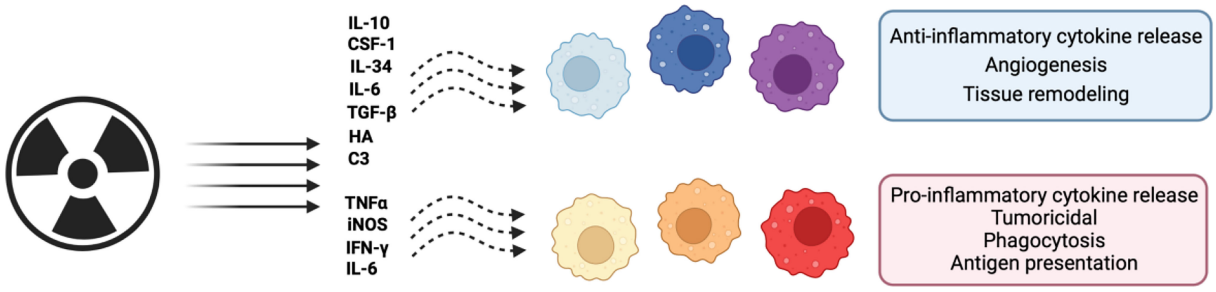

FIGURE 2
Schematic representation of the effects of radiation on macrophage polarization. Macrophages can adopt both pro- and anti-tumoral phenotypes across a spectrum of possible polarization states. Shown are the effects of radiation on these phenotypes and effector molecules. Figure created in Biorender. Agreement number: LH24DGQ49Q.

through vascular normalization (69). Irradiation of human monocyte-derived macrophages with 2, 6 or 10 Gy, results in increased RelB expression which is accompanied by reduced expression of anti-inflammatory genes (such as CD163, and IL-10) (72). Conversely, loss of NF-κB p50 expression has been associated with a pro-inflammatory macrophage phenotype including enhanced TNFα and reduced IL10 expression in bone marrow-derived macrophages incubated with both LPS and irradiated 4T1 cancer cells (10 Gy) (17) (Figure 2).

Enhanced radiation-induced NF-κB signaling can occur following activation of the apical DNA damage kinase, ATM. ATM-dependent NF-κB activation occurs following ubiquitination of NEMO (NF-κB essential modulator) which releases the cytoplasmic p50-p65 heterodimer allowing its translocation to the nucleus to act as a transcriptional activator (73).

ATM activation can also occur downstream of reactive oxygen species (ROS) production. NADPH oxidase 2 (NOX2)-dependent ROS production was reported to be important in ATM-dependent polarization of macrophages towards a pro-inflammatory phenotype through regulation of IRF5 at the mRNA and post-translational level. Therapeutically targeting other DNA damage response components, such as poly (ADP-ribose) polymerase (PARP) also appeared to activate macrophages towards a pro-inflammatory phenotype following increased ATM and IRF5 activation (74). Importantly enhanced expression of $iNOS^+CD68^+$ and $NOX2^+CD68^+$ TAMs was observed in resected specimens of rectal cancer patients with good responses to neoadjuvant radiotherapy (74). A recent study also suggested that targeting the angiogenic factor, fibroblast growth factor 2 (FGF2), in combination with radiotherapy can increase the $iNOS^+/CD206^+$ TAM ratio and improve tumor responses following fractionated radiotherapy (75). These data suggest that FGF2 could be considered as a therapeutic target to be exploited in combination with radiotherapy.

Examples of potential barriers to effective polarization by radiation

As previously mentioned, radiotherapy induces the expression of CCL2 within the TME (28, 29). CCL2 acts to shift the recruited monocytes towards a more immunosuppressive phenotypic type directly by downregulating polarization-related gene expression and indirectly *via* T helper 2 cells (Th2) releasing anti-inflammatory cytokines such as IL-4, IL-6 and IL-10 (76). In a preclinical pancreatic ductal adenocarcinoma model, the inhibition of CCL2 in isolation had little impact on tumor growth unless used in combination with radiotherapy (77). It was found that irradiation of the tumor caused a significant increase in CCL2 production and radiation-dependent recruitment of monocytes/macrophages (77). Inhibiting this CCL2/CCR2 recruitment axis led to a decrease in tumor growth and vascularity (77). Additionally, the inhibition of CCL2 led to a decrease in TAM presence and a decrease in metastasis (78). This decrease in metastasis was caused by CCL2 inhibition reducing the production of CCL3 by immunosuppressive TAMs thereby reducing the ability of these macrophages to assist with tumor intravasation (78).

There has also been a lot of interest in therapeutically targeting CSF-1 signaling to modulate macrophage polarization following irradiation in a variety of cancers. In glioblastoma tumor models, CSF-1R inhibition delays recurrence following irradiation by reducing radiation-induced monocyte recruitment and differentiation to immunosuppressive TAMs (40). Interestingly, TAM survival in the context of CSF-1R inhibition appears to be facilitated by granulocyte-macrophage CSF (GM-CSF) and IFNγ (79). Altered TAM polarization and a reduction in macrophage migration was also seen in a preclinical prostate cancer model (38). Furthermore, in preclinical colorectal and pancreatic models, macrophage depletion using CSF-1 blocking antibodies, enhances the effectiveness of combined radiotherapy and immune checkpoint inhibitor (anti-PD-L1) treatment suggesting that macrophages act to hinder productive anti-tumor immune functions of radiotherapy (19).

Complement activation and signaling of complement anaphylatoxins through their respective receptors can also impact macrophage polarization. This is relevant in the context of radiotherapy since irradiation has been found to increase the local tumor expression of several complement factors in murine models (following 5 and 20 Gy irradiation) and in patient samples (treated with 1.5-2 Gy) (34). Of note, in the TME, the presence of stromal $CD34^{high}$ fibroblasts expressing high levels of central complement component C3 (which when cleaved will result in C3a production) may also support the recruitment of macrophages with immunosuppressive phenotypes and results in attenuation of T-cell mediated responses (80). Interestingly, C3aR activation in TAMs can occur following intracellular production of C3a by tumor cells; and activation of PI3Kγ signaling downstream of C3aR activation contributes to suppression of anti-tumor responses (81). The effects of irradiation on intracellular C3a or C5a levels across tumor cells, however, is still unclear. Previously published work suggested that the presence of C5a and C3a might be essential for effective tumor radiation responses (34). However, the well-documented impact of C3a and C5a on macrophage recruitment and polarization towards immunosuppression may indicate that targeting the C3a-C3aR or C5a-C5aR signaling axes might prove to be beneficial in certain contexts. In combination with anti-PD-1 blocking antibodies, blocking C5a/C5aR1 signaling has indeed proven effective at improving primary and metastatic disease in lung tumor models (82). Similarly, in the B16-F10 melanoma model, blocking the PD-1/PDL-1 axis alongside C3a-C3aR or C5a-C5aR resulted in improved tumor control (83). The effects of radiotherapy in combination with immune checkpoint and C5a/C5aR1 inhibition, however, has yet to be determined.

The use of TGF-β inhibition in combination with PD-1/PD-L1 inhibition has also found success in a multitude of clinical trials, with phase two trials commencing in non-small cell lung (NCT03631706), triple negative breast (NCT03579472), colorectal (NCT03724851), and pancreatic (NCT02734160) cancers. A summary of additional recent clinical trials combining radiotherapy and macrophage targeting is shown in Table 1. Interestingly, combining TGF-β and PD-1 inhibition with radiotherapy in a preclinical colorectal cancer model demonstrated improved survival plus reduced tumor growth (84). Additionally, this study demonstrated a reduction in TAM recruitment to both primary tumors as well as non-irradiated bilateral lesions (84).

Conclusion

Effectively modulating the immunostimulatory effects of radiation has the enticing potential to improve local and distant tumor control (85). Given the relatively high numbers of macrophages in the TME (relative to other cell types) and the enhanced macrophage recruitment observed following irradiation, it is likely that combination therapies will have to consider how to polarize this immune population to the pro-inflammatory, tumoricidal side of the spectrum (86). Indeed, investigation into targeting TAMs is currently at the forefront of cancer immunotherapies and, a greater understanding of mechanisms of recruitment and pro-tumor activity of these macrophages may provide new therapeutic opportunities to improve the efficacy of existing treatments (39). Targeting the soluble factor-receptor axes interactions that may pose a barrier to the most effective polarization could be considered. For example, CSF1-CSF1R, C5a-C5aR1, FGF2 or TGFβ/TGFβR blockade in combination with immune checkpoint inhibitors such as PD1/PDL-1 could be promising strategies (19, 84). Further research into the effect of different radiation doses and fractionation regimes on macrophage recruitment and plasticity will help optimize the timing and nature of the most effective combination therapies. A consideration of the effect of an altered macrophage response to normal tissue toxicity following radiotherapy will also be important since maximal therapeutic benefit relies on effective tumor control with minimal normal tissue toxicity.

TABLE 1 Table summarizing latest clinical trials combining radiotherapy and approaches which may impact macrophage recruitment or function.

Target	Drug	Combination	Cancer Type	Phase	Year	Reference
ATM	AZD1390	RT	Glioblastoma	I	2018	NCT03423628
CD47/SIRPα	RRx-001	RT + Temozolomide	Gliomas	I	2016	NCT02871843
CD40	CDX-1140	RT + Poly-ICLC + FLT3-L	Breast	I	2020	NCT04616248
CSF-1R	Cabiralizumab	RT + Nivolumab	Pan-	I	2018	NCT03431948
	Sunitinib	RT	Head and Neck, Pelvic, Nervous System, Thoracic	I	2007	NCT00437372
	Sunitinib	RT	Metastatic	I/II	2007	NCT00463060
	Sunitinib	RT	Soft Tissue Sarcoma	I/II	2008	NCT00753727
	Sunitinib	RT	Glioblastoma	II	2010	NCT01100177
	Sunitinib	RT + Temozolomide	Glioblastoma Multiforme	II	2016	NCT02928575
	Sunitinib	RT + Surgery + Irinotecan + Cisplatin	Esophageal	II	2006	NCT00400114
	Sunitinib	RT + Leuprolide + Goserelin + Casodex	Prostate	I	2008	NCT00631527
	Nilotinib	RT	Chordoma	I	2011	NCT01407198
	PLX3397	RT + Temozolomide	Glioblastoma	I/II	2013	NCT01790503
	PLX3397	RT + Anti-hormone Therapy	Prostate	I	2015	NCT02472275
CCR2/CCR5	BMS-813160	RT + Nivolumab + GVAX	Pancreatic Ductal Adenocarcinoma	I/II	2018	NCT03767582
PI3Kγ	BYL719	RT + Cetuximab	Head and Neck Squamous Cell	I	2014	NCT02282371
	BYL719	RT + Cisplatin	Head and Neck Squamous Cell Carcinoma	I	2015	NCT02537223
	BKM120	RT + Temozolomide	Glioblastoma	I	2011	NCT01473901
	BKM120	RT + Cisplatin	Multiple	I	2014	NCT02113878
TLR3	Poly-ICLC	RT + Temozolomide	Glioblastoma Multiforme	II	2005	NCT00262730
TLR7/9	Imiquimod	RT + Cyclophosphamide	Breast	I/II	2011	NCT01421017
TLR9	SD-101	RT	B-Cell Lymphoma	I/II	2014	NCT02266147
	SD-101	RT + Ibrutinib	Follicular Lymphoma	I/II	2016	NCT02927964
	SD-101	RT + Nivolumab	Pancreatic	I	2019	NCT04050085

Search conducted on ClinicalTrials.gov using search criteria "Cancer", "Radiation" and "Macrophage". CSF-1R, Colony Stimulating Factor Receptor 1; CCR2, C-C Chemokine Receptor; PI3Kγ, Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma; TLR, Toll-Like Receptor; RT, Radiotherapy.

Author contributions

Conceptualization: CB and MO. Writing original draft: CB, DMac, DMaj, and MO. Writing review and editing: CB, DMac, DMaj, JA, and MO. Resources: JA and MO. Supervision: JA and MO. Funding acquisition: JA and MO. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Anti-cancer immune responses to DNA damage response inhibitors: Molecular mechanisms and progress toward clinical translation

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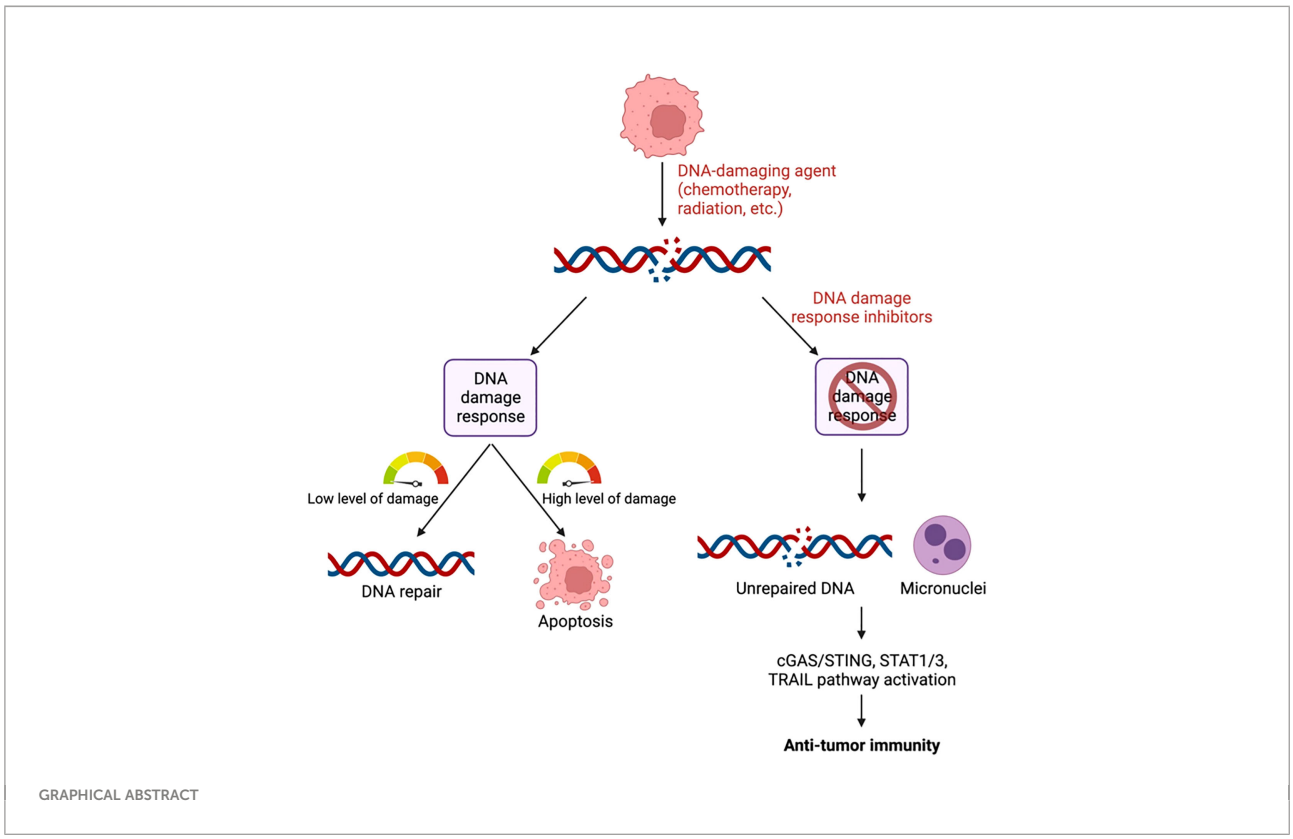
DNA damage response inhibitors are widely used anti-cancer agents that have potent activity against tumor cells with deficiencies in various DNA damage response proteins such as BRCA1/2. Inhibition of other proteins in this pathway including PARP, DNA-PK, WEE1, CHK1/2, ATR, or ATM can sensitize cancer cells to radiotherapy and chemotherapy, and such combinations are currently being tested in clinical trials for treatment of many malignancies including breast, ovarian, rectal, and lung cancer. Unrepaired DNA damage induced by DNA damage response inhibitors alone or in combination with radio- or chemotherapy has a direct cytotoxic effect on cancer cells and can also engage anti-cancer innate and adaptive immune responses. DNA damage-induced immune stimulation occurs by a variety of mechanisms including by the cGAS/STING pathway, STAT1 and downstream TRAIL pathway activation, and direct immune cell activation. Whether or not the relative contribution of these mechanisms varies after treatment with different DNA damage response inhibitors or across cancers with different genetic aberrations in DNA damage response enzymes is not well-characterized, limiting the design of optimal combinations with radio- and chemotherapy. Here, we review how the inhibition of key DNA damage response enzymes including PARP, DNA-PK, WEE1, CHK1/2, ATR, and ATM induces innate and adaptive immune responses alone or in combination with radiotherapy, chemotherapy, and/or immunotherapy. We also discuss current progress in the clinical translation of immunostimulatory DNA-damaging treatment regimens and necessary future directions to optimize the immune-sensitizing potential of DNA damage response inhibitors.

KEYWORDS

DNA damage response (DDR), immunotherapy, cGAS/STING, DNA-PK, WEE1, CHK1/2, ATR, ATM



Introduction

The DNA damage response (DDR) involves several pathways including base excision repair (BER) and nucleotide excision repair (NER) to repair single-stranded DNA breaks as well as homologous recombination (HR) and non-homologous end joining (NHEJ) to repair double-stranded DNA breaks. Activation of these pathways results in cell cycle arrest, DNA repair, senescence, and/or apoptosis depending on the extent of DNA damage (Figure 1) (15). Inhibition of DDR proteins including poly-ADP ribose polymerase (PARP), DNA-dependent protein kinase (DNA-PK), WEE1, checkpoint kinase 1/2 (CHK1/2), ataxia telangiectasia and Rad3 related (ATR), or ataxia telangiectasia mutated (ATM) serine/threonine kinase results in cell cycle progression and accumulation of unrepaired DNA (16). This accumulation eventually leads to cell death and/or DNA leakage into the cytosol in the form of micronuclei (17). DDR inhibitor (DDRi) therapy is used to treat cancer patients with tumors that harbor alterations in DDR proteins such as BRCA1/2. In these tumors, inhibition of additional DDR proteins renders the cell incapable of any type of DNA repair, resulting in cell death (18). This mechanism is known as synthetic lethality, a situation in which inhibition or mutation of two proteins separately is viable, but mutation or inactivation of both is lethal to the cell (19). Even in the absence of DDRi agents, cancer cells with defects in DNA repair pathways tend be more sensitive to anti-cancer therapies (20) including chemotherapy as compared to cells without genetic alterations in these pathways (21, 22). In addition, cells with DNA damage repair defects tend to be sensitive to immunotherapy as a result of enhanced neoantigen generation, upregulation of programmed death ligand 1 (PD-L1), and induction of the cyclic GMP–AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway (23–26).

DDRi therapy may be used as a single agent or in combination with DNA-damaging agents such as chemotherapy and radiation

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; BER, base excision repair; cGAS, cyclic GMP-AMP synthase; CHK1/2, checkpoint kinase 1/2; DCR, disease control rate; DDR, DNA damage response; DNA-PK, DNA-dependent protein kinase; DR5, death receptor 5; HR, homologous recombination; ICI, immune checkpoint inhibition; IFN, interferon; ISG, interferon stimulated gene; MDSC, myeloid-derived suppressor cell; MSI, microsatellite instable; NER, nucleotide excision repair; NHEJ, non-homologous end joining; NK, natural killer; ORR, overall response rate; PARP, poly-ADP ribose polymerase; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; RT, radiation therapy; SASP, senescence-associated secretory phenotype; STAT1, signal transducer and activator of transcription 1; STING, stimulator of interferon genes; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

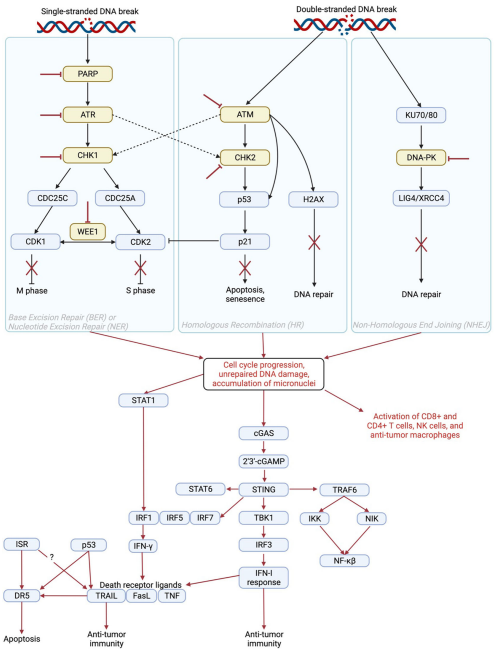


FIGURE 1
Mechanism of immune activation by inhibition of DNA damage repair proteins. PARP, ATR, CHK1/2, WEE1, ATM, and DNA-PK play roles in DNA repair pathways including base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end joining (NHEJ) to induce cell cycle arrest, apoptosis, senescence, and/or DNA repair. Inhibition of DNA damage repair proteins (red inhibitor lines) results in cell cycle progression, unrepaired DNA damage, and accumulation of cytosolic micronuclei that contain fragments of DNA (1). This results in activation of the STAT1 (2, 3), cGAS/STING (4), and TRAIL pathways as well as direct activation of immune cells (5, 6) to induce an anti-tumor immune response. The STAT1 pathway induces IFN-γ, which can increase levels of death receptor ligands including TRAIL (7), FasL (8), and TNF (9). The cGAS/STING pathway induces an IFN-I response, which also contributes to increased levels of death receptor ligands (10–13) as well as contributes directly to anti-tumor immunity (14). Created in BioRender.com.

therapy (RT) (1) (Table 1). Certain PARP inhibitors (PARPi) are FDA-approved to treat breast, prostate, and gynecologic cancers including ovarian cancer (21–23), and there are numerous clinical trials underway to extend their use to other malignancies (Table 1). The WEE1 inhibitor ZN-c3 has been granted fast track designation by the FDA for treatment of patients with uterine serous carcinoma (27, 28) and is included in nine other clinical trials testing its efficacy in various other types of cancer. Additional clinical trials are ongoing to investigate other DDRi therapies including inhibitors of DNA-PK, and CHK1/2, ATR, and ATM.

In addition to inducing cancer cell death by synthetic lethality, it is now well-recognized that DDRi therapy induces innate and adaptive immune responses (29, 30). DDRi-induced immune stimulation primarily occurs *via* the cGAS/STING pathway (29), but also occurs through signal transducer and activator of transcription 1 (STAT1) pathway activation (2) and direct activation of immune cells including T cells, NK cells, and anti-tumor macrophages (5, 6). As a result of extensive preclinical evidence supporting DDRi-induced immune responses, several clinical trials have been initiated to test the combination of DDRi with immunotherapy, primarily immune checkpoint inhibition (ICI) (Table 1). In this Review, we will discuss the mechanisms of different DDR proteins, their interactions with the immune system, and clinical translation of DDRi + immunotherapy. We also discuss necessary future directions for optimal clinical translation including clarification of variation across different DDRi therapies and across cancer types, as well as the need for a stronger focus on combining DDRi + immunotherapy strategies with DNA-damaging agents such as chemotherapy and RT.

cGAS/STING pathway

The cGAS/STING pathway is heavily implicated in the immunomodulatory effects of DNA damaging drugs and DDRi therapies. The first step of the pathway involves cGAS interaction with double-stranded DNA in the cytosol (31). These segments of DNA are often referred to as micronuclei (32). Then, cGAMP acts as a second messenger to activate STING, which activates TBK1 to recruit and activate IRF3. IRF3 then translocates to the nucleus to induce transcription of immune-stimulated genes (ISG) and type 1 interferons (IFNs). STING also activates IKK and NIK to mediate the induction of canonical and non-canonical NF-κB-driven inflammatory genes (31).

cGAS/STING-mediated IFN signaling enhances the infiltration of anti-tumor T cells and NK cells into the tumor. Though further study is needed to confirm this mechanism, it is also thought that cytosolic DNA from tumor cells can be transferred to the cytosol of immune cells to induce cGAS/STING signaling and enhance antigen presentation and cross-priming in DCs and T cells, respectively (31). Lastly, c-GAS-STING also promotes the senescence-associated secretory phenotype (SASP), which is characterized by cancer cell secretion of pro-inflammatory cytokines, chemokines, proteases, and growth factors that induce senescence and tumor control (31). However, it is important to mention that SASP can also induce an immunosuppressive TME, promoting cancer progression (33).

It is important to note that the effects of cytosolic DNA on cancer progression are likely dependent on cancer stage. In early stages, cytosolic DNA likely leads to immune surveillance through mechanisms such as the cGAS/STING pathway. In late stages, cancer cells are more likely to have lost functional checkpoints of cell cycle and immune regulation, and therefore cytosolic DNA can induce chronic inflammatory signaling that

TABLE 1 Ongoing, completed, and recruiting clinical trials testing the combination of DNA damage inhibitors with immunotherapy in various cancer types.

DDR target	Interventions	Cancer type	Phase	Trial #
PARP	Niraparib + Dostarlimab + RT	TNBC	II	NCT04837209
	Pembrolizumab + Olaparib	Cervical cancer, cervical carcinoma	II	NCT04483544
	Olaparib +/- Pembrolizumab	Metastatic pancreatic adenocarcinoma, stage IV pancreatic cancer AJCC v8	II	NCT04548752
	Olaparib + Durvalumab +/- Carboplatin, Etoposide, and/or RT	Extensive stage lung small cell carcinoma, stage IV, IVA, IVB lung cancer AJCC v8	I/II	NCT04728230
	Olaparib +/- Tremelimumab	Recurrent ovarian, fallopian tube or peritoneal cancer	II	NCT04034927
	Pembrolizumab + Olaparib	Breast cancer	II	NCT03025035
	Durvalumab + Olaparib + RT	Locally advanced, unresectable pancreatic adenocarcinoma, stage II & III pancreatic cancer AJCC v8	I	NCT05411094
	Durvalumab + Olaparib	Metastatic TNBC	I	NCT03544125
	Atezolizumab +/- Niraparib & Temozolomide	Advanced solid tumors	I/II	NCT03830918
	Olaparib +/- Atezolizumab	BRCA mutant non-HER2-positive breast cancer	II	NCT02849496
	Olaparib+ Pembrolizumab + Paclitaxel	Recurrent/advanced gastric and gastro-esophageal junction cancer with HRR mutation and MSS	I/II	NCT04592211
	Durvalumab + Olaparib + Copanlisib HCl	Advanced solid tumors with selected mutations	I	NCT03842228
	Durvalumab + Olaparib	Prostate cancer with high neoantigen load	II	NCT04336943
	Niraparib + Dostarlimab	BRCA-mutated unresectable or metastatic breast, pancreas, ovary, fallopian tube, or primary peritoneal cancer	I	NCT04673448
	Cabazitaxel + Carboplatin + Cetrelimab + Niraparib	Metastatic prostate cancer	II	NCT04592237
	Atezolizumab + Talazoparib	SLFN11 + small cell lung cancer	II	NCT04334941
	Cediranib Maleate + Durvalumab + Olaparib	Ovarian, primary peritoneal, or fallopian tube cancer after Pt therapy	II	NCT04739800
	Niraparib + Dostarlimab	HPV-negative head and neck squamous cell carcinoma	II	NCT04681469
	Olaparib + Tremelimumab	BRCA-deficient ovarian cancer	I/II	NCT02571725
	Dostarlimab + Niraparib	BRCA1/2 and PALB2-mutated metastatic pancreatic cancer	II	NCT04493060
	Rucaparib + Nivolumab	Solid tumors	II	NCT03824704
	NK cells + Talazoparib	Acute myeloid leukemia	I/II	NCT05319249
	Olaparib + Pembrolizumab	Advanced melanoma with homologous recombination mutation	II	NCT04633902
	Paclitaxel + Olaparib + Pembrolizumab	Advanced gastric adenocarcinoma	II	NCT04209686
	Busulfan + Gemcitabine + Melphalan + Olaparib + Rituximab + Vorinostat	Relapsed or refractory lymphomas undergoing stem cell transplant		NCT03259503
DNA-PK	M3814 + Avelumab +/- RT	Solid tumors	I	NCT03724890
	Avelumab + RT +/- Peposertib	Advanced/metastatic solid tumors and hepatobiliary malignancies	I/II	NCT04068194
	Radium 223 dichloride alone, + Peposertib, or + Peposertib and Avelumab	Advanced prostate cancer not responsive to hormonal therapy	I/II	NCT04071236
WEE1	Adavosertib + Durvalumab	Advanced solid tumors	I	NCT02617277
	ZN-c3 + Pembrolizumab (27)	Solid tumors		NCT04158336
ATR	Elimusertib + Pembrolizumab + RT	Recurrent head and neck cancer	I	NCT04576091
	M1774 + immune checkpoint inhibitor	Metastatic or locally advanced unresectable solid tumors	I	NCT05396833
	Elimusertib + Pembrolizumab	Advanced solid tumors	I	NCT04095273
ATM	None			
CHK1/2	None			

Search performed on 7/10/2022 using keywords "immunotherapy" and "PARP inhibitor, DNA-PK inhibitor, WEE1 inhibitor, ATR inhibitor, ATM inhibitor, or CHK1/2 inhibitor".

may be associated with survival and metastasis. Thus, the tumor microenvironment should be carefully monitored during the therapeutic induction of cytosolic DNA accumulation and cGAS/STING pathway activation using DDRi therapy (31).

STING agonists are being investigated to treat many types of cancer either as a single agent or combined with ICI or chemotherapy (34). STING-based therapeutics have yet to be combined with DDRi therapy, though there is rationale for combination to enhance DDRi-induced immune activation (34, 35). STING agonists can activate the cGAS/STING pathway in the absence of cytosolic DNA and therefore circumvent the need for DNA damage to induce the type 1

IFN response (35). Some limitations of targeting cGAS/STING in cancer exist, including evidence of cGAS/STING silencing or loss-of-function mutations in certain tumors (35, 36) and cGAS/STING-driven IL-6-dependent survival of chromosomally instable cancers (37). In these cases, administration of cGAS/STING agonists may have limited to no efficacy or may be pro-tumorigenic. Careful consideration of STING agonist combination therapies and evaluation of patients who may not benefit or be harmed by these therapies is needed prior to clinical translation of cGAS/STING + DDRi combination therapies.

TRAIL pathway

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is primarily expressed on the surface of immune cells including NK cells, T cells, NK tumor (NKT) cells, DCs, and macrophages (38). It can also be expressed in soluble form after proteolytic cleavage from the cell surface (39). Both membrane-bound and soluble TRAIL bind to death receptor 5 (DR5) and death receptor 4 (DR4) on cancer cells to induce apoptosis (39). TRAIL is induced by the IFN-γ/STAT1 and cGAS/STING pathways (7, 40–42) that are activated after DDRi therapy. The TRAIL pathway is anti-tumorigenic, as evidenced by the increased susceptibility of TRAIL receptor-deficient mice to chronic inflammation and tumorigenesis (43, 44).

In addition to its role in apoptosis, TRAIL also plays an important role in the anti-cancer immune response. For example, some immune cells kill cancer cells in a TRAIL-dependent manner (38, 45) and targeted delivery of TRAIL to cell surface antigens on T cells may enhance their cytotoxic activity (46). TRAIL-TRAIL receptor interaction on MDSCs can limit their lifespan, supporting an anti-tumor immune microenvironment (47, 48). Due to its ability to induce both apoptosis and anti-tumor immune responses, activation of the TRAIL pathway is a promising clinical strategy (49). Various therapeutic approaches have been considered including TRAIL receptor agonists, DR4/5 agonistic monoclonal antibodies, and different formulations such as PEGylated TRAIL (49). Other exciting new directions are being pursued preclinically such as engineering tumor-homing, TRAIL-expressing mesenchymal stem cells (50, 51). TRAIL-based therapies have been studied extensively in the clinic and some have shown early signs of efficacy in non-Hodgkin's lymphoma (52) and non-small cell lung cancer (53, 54). Limitations include short half-life, limited induction of receptor clustering, binding to decoy receptors such as DcR1, DcR2, and osteoprotegerin (55, 56), and development of resistance (49). There are some ongoing clinical trials with TRAIL-based therapies such as the TRAIL-receptor agonists ABBV-621 in combination with bortezomib and dexamethasone (NCT04570631) and INBRX-109 alone (NCT04950075) or in combination with DNA damaging agents (NCT03715933) (49). No trials are currently investigating the combination of TRAIL-based therapy with DDRi agents.

Combination therapies may overcome some of these limitations, and various preclinical investigations support the combination of TRAIL-based therapies with DDRi. For example, one study found that PARPi enhanced the efficacy of a DR5 antibody in a pancreatic cancer mouse xenograft model (57). Others found that DNA-PKi potentiates p53-dependent apoptosis after treatment with a DNA damaging agent in AML cells, and that the TRAIL pathway plays a major role in this apoptotic response (58). Others have described upregulation of TRAIL-mediated apoptosis after ATMi treatment of melanoma cells (59). These findings provide rationale for combining TRAIL agonists and DDRi therapy in the clinic to enhance induction of apoptosis. Whether or not these types of combination treatments will enhance the anti-tumor immune response remains to be investigated.

DDRi-induced upregulation of PD-L1

PD-L1 is a ligand that binds programmed cell death protein 1 (PD-1) on activated T cells. Binding of PD-L1 to PD-1 inhibits T cell activity (60) and elevated expression of PD-L1 is a major biomarker of favorable response to immune checkpoint inhibitors (61). Experimental evidence suggests that PARPi agents can upregulate PD-L1 expression by blocking glycogen synthase kinase-3 beta (GSK3β), a regulator of glycogen metabolism, cell cycle, inflammation, and proliferation (62). GSK3β also plays a role in the repair of both single- and double-stranded DNA breaks. PARPi-induced inhibition of GSK3β causes inhibition of DNA damage repair and upregulation of PD-L1 (63–65).

ATR inhibitors, on the other hand, seem to upregulate PD-L1 mRNA but downregulate PD-L1 protein expression (66–68). Further, studies have shown that DNA damage-induced upregulation of PD-L1 by cisplatin or ionizing radiation was suppressed by co-administration with ATRi agents (67). DNA-PK inhibitors seem to upregulate PD-L1 (69) along with WEE1 inhibitors (2) and Chk1/2 inhibitors (70, 71), likely by preventing the repair of double-stranded DNA breaks, which activates STAT1/3 signaling through ATM/ATR/Chk1 kinases, resulting in an upregulation of PD-L1 levels (60).

PARP inhibitors

Poly-ADP ribose polymerase (PARP) is an enzyme that plays a critical role in the DNA repair pathways NER and BER, which repair DNA damage that is caused by therapeutic agents such as alkylating agents and chemotherapy (72). The PARP family contains 17 different proteins, but most studied are

PARP1 and PARP2. PARP1 binds to DNA regardless of phosphorylation state and PARP2 preferentially binds phosphorylated DNA breaks, but otherwise these proteins largely function similarly (73). PARP inhibitors (PARPi) lead to death by synthetic lethality in cancer cells with deficiencies in the homologous recombination (HR) DNA repair pathway (74). PARPi is FDA-approved to treat breast cancer, prostate cancer, and gynecologic cancers including ovarian cancer (75–77).

PARP plays an important role in the normal functioning of the immune system. PARP2 contributes to the development of mature CD4+ and CD8+ T cells and *in vivo* data suggests that dual inhibition of PARP1 and PARP2 leads to a measurable decrease in T cell populations. PARP1 and PARP2 also contribute to normal T cell functioning as demonstrated by experiments in which PARP1/2 inhibition resulted in decreased IL-2 and IFN-γ-secreting T cells. PARP1 is also responsible for marking Foxp3-expressing T regulatory cells (T regs) for degradation. Additionally, PARP1 regulates NFAT, a family of transcription factors that that regulates CD4+ T cell differentiation, but it is unclear if inhibition of PARP1 biases CD4+ cells toward a Th1 or Th2 phenotype (73). Lastly, PARP1 may cooperate with IFI16 to induce noncanonical STING activation in response to chemotherapy-induced DNA damage (31).

Interestingly, in BRCA1-deficient ovarian cancer models, PARP inhibition with olaparib increased CD4+ and CD8+ T cells in the tumor and in circulation, reduced their expression of inhibitory receptors PD-1, Tim-3, and Lag-3, and increased their levels of TNF-alpha and IFN-γ secretion. In dendritic cells, PARPi upregulates costimulatory molecules CD80/CD86 and MHC class II which enhances antigen presentation and interactions with T cells. PARPi may increase expression of cell death receptor ligands and NKG2D ligands, which increases cancer cell sensitivity to NK cell-mediated killing. In macrophages, the impact of PARPi is dependent on factors in the tumor microenvironment including certain cytokines. The DNA damage caused by PARPi leads to cytosolic DNA, activating the cGAS/STING pathway and the type I IFN response (73). PARPi can also increase the amount of DNA in the cytosol, leading to the accumulation of neoantigens (78).

Due to the immune-stimulating properties of PARPi therapy, there is clinical interest in combining PARPi with immunotherapy. Clinical trials testing such combinations are ongoing for ovarian, ovarian, lung, urothelial, prostate, and gastrointestinal cancers (78). The results of these trials have been most promising in ovarian and breast cancer. In ovarian cancer, overall response rates (ORR) ranged from 45-63% and disease control rate (DCR) was 73-81% depending on the patient population. In breast cancer, ORR was 53% and DCR was 47-83% depending on patient population. PARPi alone is effective in patients with prostate cancer and has been combined with IT in several clinical trials. Results of the completed trials have been promising, with 9/17 patients with metastatic castration-resistant prostate cancer (mCRPC) treated with durvalumab and olaparib experiencing a PSA decline of >50% and 4/17 patients experiencing a radiographic response. A combination of pembrolizumab and olaparib in a cohort of patients with wild-type HR proteins had slightly less exciting results, with 7% partial response and 29% DCR. Studies in gastric cancer combining durvalumab and olaparib have reported a 10% ORR and 12-week DCR of 26% (78).

DNA-PK inhibitors

DNA-PK is a serine/threonine protein kinase that plays a critical role in the DNA repair pathways classical NHEJ and HR. DNA-PK inhibitors (DNA-PKi) interfere with its kinase function and sensitize cells to DNA-damaging agents. DNA-PKi can be used as a single agent in some cancers with ATM deficiency by inducing synthetic lethality (79). No DNA-PKi therapies are FDA approved, however there are several ongoing clinical trials involving compounds such as XRD-0394, CC-115, VX-984 (M9831), AZD7648, and M3814 (nedisertib, peposertib, MSC-2490484A) to treat various type of cancer, typically advanced solid tumors (80, 81).

DNA-PK phosphorylates cGAS and suppresses its enzymatic activity. DNA-PK inhibition or deficiency correlates with decreased levels of phosphorylated cGAS and promotes antiviral immune responses (82). Additionally, as DNA-PK is critical to maintaining genomic stability, the loss or inhibition of this kinase may lead to high mutation load secondary to the development of genomic instability. Mutation of the gene encoding DNA-PK protein *PRKDC* is associated with high mutation load or microsatellite instable (MSI)-high status in The Cancer Genome Atlas pan-cancer cohort. Further, *PRKDC* knockout and DNA-PKi enhanced the efficacy of ICI (83, 84). The DNA-PKi AZD7648 sensitizes mice with colorectal tumors or melanoma to radiotherapy and induces a tumor control that is dependent on type I IFNs. There are phase I/II clinical trials involving AZD7648 in combination with chemotherapy (NCT03907969) and radiotherapy (NCT04550104) currently ongoing (85). Due to the dependence of AZD7648 on type I IFN responses, it would be interesting to combine this drug with immunomodulatory drugs that enhance the type I IFN response. Additionally, the DNA-PKi peposertib enhanced RT-induced TGFβ/PD-L1-targeted immunotherapy in mice, further supporting the combination of DNA-PKi, RT, and immunotherapy (69).

Three clinical trials are evaluating the combination of the DNA-PKi M3814 combined with the anti-PD-L1 ICI avelumab (86). M3814 has demonstrated monotherapy activity in several tumor cell lines, and M3814 + radiotherapy (RT) combined with avelumab significantly delayed tumor growth as compared to either agent alone + RT in MC38 syngrafts, indicating the benefit of combining DNA-PKi and immunotherapy (87). One trial is investigating M3814 and avelumab +/- radiotherapy for

treatment of patients with advanced solid tumors (NCT03724890) (87). Another is investigating avelumab and RT +/- M3814 in advanced solid tumors and hepatobiliary malignancies (NCT04068194) (88). Lastly, one trial is evaluating RT vs. RT + M3814 vs. RT + M3814 + avelumab in patients with advanced prostate cancer that is unresponsive to hormonal therapy (NCT04071236) (89).

WEE1 inhibitors

The WEE1 kinase family consists of three serine/threonine kinases: WEE1, PKMYT1, and WEE1B (WEE2). WEE1 and PKMYT1 play a crucial role in cell cycle regulation and DNA damage repair, while WEE2 regulates cell cycle progression and largely regulates meiosis. WEE1 and PKMYT1 can act like oncogenes and are a major focus in anti-cancer drug development (90). One WEE1 inhibitor, ZN-c3, has been granted fast track designation by the FDA for treatment of patients with uterine serous carcinoma. Another WEE1 inhibitor adavosertib (AZD1775, MK-1775) is highly developed and has been included in over fifty clinical trials to treat various types of cancer since 2008 (28).

WEE1 overexpression abrogates immune cell killing, for example by protecting cancer cells from granzyme B/TNFα induced cell death. One study found that cancer cells develop resistance to granzyme B/TNFα-mediated cytotoxic T cell killing by activating the G2/M cell cycle checkpoint. Further, they found that administration of WEE1i adavosertib reversed this effect, enhanced T cell killing, and synergized with an anti-PD-1 monoclonal antibody in murine models of oral cavity carcinoma, melanoma and colon adenocarcinoma with various TP53 mutations (91). WEE1 inhibition activates the STING and STAT1 pathways in SCLC and enhances the antitumor immune response to PD-L1 inhibition (2). Like the STING pathway, the STAT1 pathway is a major contributor to the anti-tumor immune response. Along with STAT2, STAT1 induces IFN-regulated genes, enhances antigen presentation, and contributes to an inflammatory, anti-cancer response. It is important to differentiate STAT1 and STAT2 from other STAT family members such as STAT3 and STAT5, which contribute to cancer cell survival, proliferation, and angiogenesis (3). It has also been shown that WEE1 induces anti-tumor immunity by activating endogenous retroviral elements and the dsRNA pathway (92). WEE1i also sensitizes head and neck cancers to natural killer (NK) cell therapies (93).

One ongoing clinical trial is evaluating adavosertib with the anti-PD-L1 ICI durvalumab for treatment of patients with advanced solid tumors (NCT02617277). DCR for the total cohort was 36%, suggesting antitumor activity (94). Notably, adavosertib + immunotherapy has a better safety profile compared to adavosertib + chemotherapy, warranting continued investigation (95). Another actively recruiting trial will test the safety, tolerability, efficacy, pharmacokinetics and pharmacodynamics of ZN-c3 alone and in combination with other drugs including the anti-PD-1 ICI pembrolizumab (NCT04158336) (27). As p53 mutations and overexpression of SKP2 and CUL1 may be biomarkers of a favorable response to WEE1i, additional clinical trials in these patient populations in combination with immunotherapy are needed (27, 91).

CHK1/2 and ATR inhibitors

ATR and its major downstream effector checkpoint kinase 1 CHK1 play a role in the DNA damage response. In response to single-stranded DNA breaks, ATR activates CHK1 to trigger intra-S and G2/M phase checkpoints. In response to double-stranded DNA breaks, the MRE11/NBS1/RAD5 complex activates ATM and CHK2 to trigger the G1/S-phase checkpoint (96). Because ATR has a broader range of biological functions than CHK1, it is thought that ATRi may have greater toxicity in normal cells. Therefore, the clinical development of CHK1i is more advanced than ATRi (96). There are over twenty CHK1/2 and ten ATR inhibitors in various stages of clinical trials for many different cancer types mostly in combination with chemotherapy but also with RT and histone deacetylase inhibitors (HDACi) (96). No CHK1/2 or ATR inhibitors are FDA-approved yet (97).

One study found that in the leukemia cell line THP-1, CHK1i increased TBK1 but did not increase IRF3 phosphorylation, induce IRF3 or NF-κB reporter activation, nor induce a type 1 IFN response (98). The same group found that in solid tumor cell lines, addition of CHK1i to chemotherapy treatment such as gemcitabine or camptothecin increased the accumulation of cytosolic DNA but decreased the level of chemotherapy-mediated IRF1 and STAT1 phosphorylation. Interestingly, similar results as far as lack of type 1 IFN response were found using ATRi and WEE1i, indicating that context such as cancer type may affect the ability of DDRi to induce the cGAS/STING pathway (99). Another study found that in murine melanoma models, CHK1i induces an immunogenic signaling and increased levels and activity of CD8+ T cells (100). Similarly, others observed that treatment of patients with head and neck squamous cell carcinoma with CHK1i led to an upregulation of transcripts associated with T-cell activation and inflammatory cytokines and chemokines but also T regs (101). Interestingly, others have shown that the combination of CHK1i and ionizing RT increases micronuclei formation and induces an abscopal tumor regression response in a murine melanoma model (102).

Despite the advanced preclinical development of CHK1i alone or in combination with chemotherapy or RT, there are currently no ongoing or completed clinical trials testing the combination of CHK1/2i with immunotherapy. There are three trials that are evaluating ATRi with immunotherapy. One study

(NCT04576091) is investigating sensitization to pembrolizumab with the ATRi elimusertib in combination with RT for treatment of patients with recurrent head and neck cancer. As of February 2022, no patients were enrolled in this study. Another trial is currently recruiting patients with advanced solid tumors to evaluate elimusertib + pembrolizumab without RT (NCT04095273). Lastly, one trial will evaluate the combination of ATRi M1774 with immune checkpoint inhibition for treatment of patients with metastatic or locally advanced unresectable solid tumors (NCT05396833). The results of these trials are highly anticipated.

ATM inhibitors

ATM is activated by double stranded breaks in DNA, and cells that are deficient in ATM experience abnormal DNA repair. Activated ATM phosphorylates p53 at serine 15 to activate it and phosphorylates MDM2 to prevent its inhibitory binding to p53. ATM also phosphorylates and activates CHK2, which phosphorylates p53 at another activating site (serine 20). p53 induces p21 to inhibit CDK2/cyclin E to induce arrest at the G1 phase of the cell cycle. Activated ATM also phosphorylates NBS1, which is necessary for RT-induced S phase cell cycle arrest, but the complete mechanism remains to be clarified (103).

In *Drosophila* models, ATR deficiency causes an innate immune response (104). In murine and human cancer cell lines, ATM deficiency induces ISG expression and tumor infiltration of immune cells in a cGAS/STING-dependent manner. Further investigation revealed this effect was mediated specifically by leakage of mitochondrial DNA rather than nuclear DNA into the cytoplasm. The same group found that ATM expression levels negatively correlate with type 1 IFN gene expression in human tumor tissues and that patients with tumors harboring ATM mutations have a favorable response to ICI (105). Similar findings as far as ATM mutations serving as a biomarker of favorable response to ICI have been made in bladder cancer (106). Other studies in pancreatic cancer have shown that ATMi induces type 1 IFNs in a cGAS/STING-independent manner, but this response was dependent on TBK1 and SRC (107). Despite this preclinical evidence of ATMi-induced immune stimulation, there are no clinical trials testing the combination of ATMi and immunotherapy.

Inhibition of oxidative damage repair

Chemotherapy and radiation therapy are well-known inducers of oxidative stress, a condition in the cell characterized by excess reactive oxygen species and the resulting processes that detoxify the cell and repair oxidative damage (108). Oxidative stress plays a major role in inducing cellular damage after treatment with DNA-damaging agents (109). Oxidative stress increases levels of intracellular Ca^{2+}, induces Fenton reaction DNA lesions, and triggers DNA repair mechanisms (110). BER plays a major role in the cellular response to oxidative DNA damage. During BER, damaged bases are excised, generating apurinic/apyrimidinic (AP) sites. At these sites, apurinic/apyrimidinic endonuclease 2 (APE2, APN2, or APEX2) creates a single-strand break which is then fixed by other DNA repair enzymes. Thus, APE2 plays a critical role in the repair of oxidative damage, and in fact knockdown of APE2 led to increased micronuclei formation in the PANC1 pancreatic cancer cell line (111). Oxidative stress plays many roles in the immune microenvironment of the tumor (112). For example, APE2 is involved in B cell development and immunoglobulin class switch recombination and APE2-knockout mice develop defects in immune responses. BER and ATR pathways, both of which are heavily involved in regulating PD-L1 expression, rely on APE2. APE2 involvement in the response to immunotherapy is likely but has not been investigated. There are no APE2 inhibitors in clinical trials for the treatment of cancer. Future studies should investigate the impact of APE2 and other oxidative damage repair enzymes on the immune response and response to immunotherapy (113).

Conclusion and open questions

The clinical applicability of DDRi has been clearly demonstrated for cancers with deficiencies in the DDR pathway. The combination of DDRi with DNA-damaging agents has improved the efficacy of these agents in certain contexts. It is now well-recognized that DDRi compounds stimulate the immune system against cancer and that this effect may be enhanced by combinations with DNA-damaging agents. The cGAS/STING pathway is a major regulator of DDRi-induced immune stimulation, though the STAT1 pathway, TRAIL pathway, and direct activation of anti-cancer immune cells also play important roles. The effects of DDRi on the immune system provide rationale for their combination with immunotherapy such as ICI, as is being tested in various clinical trials.

TRAIL is induced by the IFN-γ/STAT1 and cGAS/STING pathways (7, 40–42) that are activated after DDRi therapy. TRAIL-based therapies have therapeutic potential because of their ability to induce both apoptosis and lasting anti-cancer immunity. There are no FDA-approved TRAIL-based treatments (49), however numerous clinical trials are continuing to investigate new approaches and combination treatments (49, 55, 56). Many preclinical investigations support the combination of TRAIL-based therapies with DDRi (57–59), providing rationale for clinical translation. The addition of ICI to this treatment regimen should also be considered given the heavy involvement of both DDRi and the TRAIL pathway in anti-cancer immune activation.

Less than half (12/33) of the clinical trials that are testing combinations of DDRi and immunotherapy involve combination with a DNA damaging agent such as chemotherapy or

radiotherapy (Table 1). Most of the trials that do not include a DNA damaging agent are for treatment of malignancies in which alterations in DDR proteins are common, such as breast cancer and ovarian cancer. While DDRi therapy can induce accumulation of cytosolic DNA and stimulate the immune system in the presence of these alterations, co-administration of DNA damaging agents should be considered to expand the use of this combination therapy to patients without such alterations.

Cancer stem cells are cancer cells with stem-like phenotypes that are slow-cycling and have highly efficient DNA repair, which grants them resistance to chemotherapy and radiotherapy (114). Cancer stem cells present a major challenge as far as overcoming drug resistance and cancer recurrence. Cancer stem cells may also be able to evade the immune system (115), thus immunotherapy alone may not be active against this subset of the tumor. Inhibiting the highly efficient DNA repair processes in cancer stem cells, especially in combination with DNA-damaging agents, may be a promising approach to eliminate this cell population (116). Bulk tumor reduction and elimination of cancer stem cells with the combination of DDRi and DNA damaging agents sequenced with immunotherapy for lasting tumor regression may be a viable treatment option for patients with tumors characterized by cancer stemness. Optimization and validation of treatment dose, timing, and sequencing is necessary *in vivo*.

Another area in need of further investigation is the differential effects of various DDRi agents on the immune system. Though inhibition of each DDR protein has similar effects in most studies, there seem to be context-specific differences especially for CHK1i. Similar context-dependency may be found with complementary study of other DDR proteins. Further investigation is critical to the application of DDRi + immunotherapy to wider patient populations.

Author contributions

Conceptualization, WE-D and LC. Investigation, WE-D and LC. Writing—original draft preparation, LC. Writing—review and editing, WE-D and LC. Supervision, WE-D. Project administration, WE-D. Funding acquisition, WE-D. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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Systemic benefit of radiation therapy *via* abscopal effect

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Evidence of a systemic response related to localized radiation therapy (RT) in cancer management is rare. However, enhancing the immune response *via* immunotherapy followed by localized RT has shown evidence of tumor shrinkage to non-irradiated metastatic disease thereby inducing an "abscopal effect." Combined induction of the cGAS-STING pathway and activation of IFN-gamma signaling cascade related to RT within an activated immune environment promotes neoantigen presentation and expansion of cytotoxic effector cells enabling enhancement of systemic immune response. A proposed mechanism, case examples, and clinical trial evidence of "abscopal effect" benefit are reviewed. Results support strategic therapeutic testing to enhance "abscopal effect."

KEYWORDS

abscopal effect, irradiation, cancer management, immune response, autologous tumor immunotherapy, radiation therapy

Abbreviations: AFP, alpha-fetoprotein; ATM, ATM serine/threonine kinase; CCL2, C-C Motif Chemokine Ligand 2; CCL22, C-C Motif Chemokine Ligand 22; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate synthase; cGAS, cGAMP synthase; CIRT, carbon-ion radiation therapy; CR, complete response; EBRT, external beam radiation therapy; Flt3-L, Fms Related Receptor Tyrosine Kinase 3 Ligand; Gy, grey; HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitor; IFI16, interferon gamma inducible protein 16; IMRT, intensity modulated radiotherapy; MDM2, MDM2 proto-oncogene; MHC, major histocompatibility complex; OS, overall survival; PARP-1, poly ADP-ribose polymerase 1; PFS, progression-free survival; PIVKA-II, protein induced by vitamin K absence or antagonists II; RFS, recurrence-free survival; SBRT, stereotactic body radiation therapy; STING, stimulator of interferon genes; TGFß, transforming growth factor beta; TNF, tumor necrosis factor; TRAF6, TNF receptor associated factor 6; TP53, tumor protein P53; WBRT, whole-brain radiotherapy; XRT, radiation therapy.

Introduction

The abscopal effect is a phenomenon seen when irradiation at a distinct anatomic site induces a systemic antitumor response throughout the body. It was first described using cell lines and was known as the "bystander effect." Researchers found that in addition to direct cellular damage *via* reactive oxygen and nitrogen species induced by radiation therapy (RT), irradiated cells could also induce changes in distant non-irradiated cells through cell signaling molecules (1). Initial studies demonstrated that cell culture media taken from irradiated cultures could be transferred to non-irradiated cultures and induce DNA damage (1). Similarly, tumor cells can elicit cellular and DNA changes within normal cells when media used to grow tumor cells is transferred to normal cultures. This conditioned media demonstrates increased levels of many cytokines including transforming growth factor beta (TGFß) and C-C Motif Chemokine Ligand 2 (CCL2) (2, 3). In addition, *in vivo* experiments using both C57BL/6 wild-type and CCL2-knockout mice subjected to ionizing radiation identified six differentially expressed genes implicated in the abscopal effect in tissue outside the field of radiation. These include TGFß (4) and CCL2 (5), as well as tumor protein P53 (TP53) (6), tumor necrosis factor (TNF) (7), C-C Motif Chemokine Ligand 22 (CCL22) (8), and the proto-oncogene, MDM2 (9).

CCL2 is particularly important, as it is involved in the propagation of the immune effects associated with abscopal activity. Specifically, CCL2 is a member of the monocyte chemoattractant protein family and not only serves an important role in the recruitment of monocytes, but also has been shown to recruit T cells, B cells, NK cells, macrophages, and dendritic cells (10–14). It is induced by multiple pro-inflammatory molecules (15–18) and by reactive oxygen and nitrogen species generated by RT supporting the idea that it contributes significantly to the immune response associated with the abscopal effect (19).

While the precise mechanism for the abscopal effect is complex and continues to be elucidated, current evidence supports that it is primarily a T cell-mediated process. Ultimately, irreparable DNA damage in tumor cells induced by RT increases tumor immunogenicity by providing dendritic cells with tumor-specific antigens to present to, and activate, $CD8^+$ T cells *via* major histocompatibility complex (MHC) class 1. Clinical case examples, which we summarize, have stimulated ongoing preclinical and clinical trials focusing on strategies to stimulate dendritic cell proliferation and T cell activation to more consistently induce an abscopal effect concurrent with RT.

In an effort to understand abscopal activity, we can look at the molecular mechanism behind RT-induced DNA damage and the immune response. Specifically, RT of tumor cells induces double-stranded DNA breaks and unique nucleotide adducts that leak into the cytosol and bind to the DNA sensor cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) protein resulting in an increase in intracellular cGAMP (20, 21). Increasing levels of cGAMP then bind to the stimulator of interferon genes (STING) protein leading to the production of type 1 interferons (IFN-1). (IFN-1 binds to IFNAR1/2 receptors that result in a signaling cascade that activates immune-stimulating genes that promote activation of dendritic cell populations (22).

However, in addition to the anti-tumor and immunogenic effects of the cGAS-STING pathway, this pathway has also been shown to induce pro-tumorigenic factors, such as IL-6 through activation of the NFkB pathway and PD-L1 through activation of the JAK-STAT pathway (23, 24). This is important in the context of induction of abscopal effect because IL-6 has been implicated in resistance to RT by suppressing oxidative stress, and efforts to pharmacologically block the production of IL-6, in addition to PD-L1, may help increase the abscopal effect (25). In addition to the canonical cGAS-STING pathway, an alternative STING pathway is also capable of sensing DNA damage independent of the cytosolic DNA receptor cGAS. Instead, this non-canonical STING pathway utilizes a protein complex consisting of a DNA binding protein called Interferon Gamma Inducible Protein 16 (IFI16), DNA damage response factors (ATM serine/threonine kinase [ATM] and Poly ADP-Ribose Polymerase 1 [PARP-1]), tumor suppressor protein p53 (TP53), and a E3 ubiquitin ligase called TNF Receptor Associated Factor 6 (TRAF6). This protein complex ultimately leads to the activation of the NFkB pathway resulting in expression of IFN-ß and thus its downstream targets (26). Each of these unique pathways demonstrates the complexity of RT-induced DNA damage and the diverse molecular mechanisms that play a role in both anti-cancer and pro-cancer response.

Importantly, as the dose of radiation increases, larger amounts of damaged DNA products precipitate in the cytosol activating TREX1, a cytosolic DNA exonuclease that functions to degrade and eliminate cytosolic DNA, precluding the activation of the cGAS-STING signaling cascade that is thought to trigger the abscopal effect. In an effort to fine-tune induction of abscopal effect, Vanpouille-Box et al. sought to determine an optimal radiation dose that maximized production of IFN-1 while minimizing TREX1 expression using a mammary carcinoma mouse model treated with RT and an antibody against anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). CTLA-4 is a cell-surface protein expressed by regulatory T cells to inhibit T cell functions by increasing the activation energy necessary for T cell activation (27). This is especially important in the context of cancer due to the weakly immunogenic self- and tumor-antigens. Ipilimumab is an anti-CTLA-4 antibody and was the first immune checkpoint inhibitor approved for treating cancer (28, 29). Their results showed that

an irradiation scheme of 8 grey (Gy) x 3 coupled with an anti-CTLA-4 antibody did not result in TREX1 gene expression, while a single large dose (20 and 30 Gy) coupled with an anti-CTLA-4 antibody did (30). Activation of IFN-1 was preserved in both examples.

In addition to optimizing expression of both immune-stimulating and immune-suppressing genes, several studies have demonstrated the importance of an intact T cell response and a sufficient dendritic cell population to induce an abscopal response. For example, Stone et al. published one of the first preclinical experiments seeking to understand the abscopal effect using a syngeneic fibrosarcoma mouse model. They showed that the radiation dose necessary to reduce tumor size by 50% was significantly smaller in T cell-competent mice compared to T cell-depleted mice. In addition, they noticed that the likelihood of metastasis was lower in T cell-competent mice compared to their T cell-depleted counterparts (31). More recently, Demaria et al. utilized a murine model with both wild-type and *nu/nu* T cell-deficient BALB/C mice to compare (a) the effect of irradiation alone or irradiation supplemented with the dendritic cell stimulator, Fms Related Receptor Tyrosine Kinase 3 Ligand (Flt3-L), and (b) the effect of tumor immunogenicity using two cell lines (32). The two groups of mice were injected at two distinct anatomic sites with either the highly immunogenic 67NR BALB/C mouse-derived mammary carcinoma cell line or the low immunogenicity A20 BALB/C mouse-derived B-cell leukemia/lymphoma cell line creating a pseudo-primary site that would receive direct RT (2 Gy, single dose) and a secondary site that would not. Their results showed that not only were T cells necessary to induce a response at the secondary, non-irradiated site, the addition of Flt3-L significantly increased the tumor response at the non-irradiated site in the wild-type mice. In addition, the low immunogenicity A20 B cell leukemia/lymphoma cell line did not show a significant increase in response at the secondary site in both the irradiation alone and irradiation + Flt3-L groups demonstrating the importance of an immunogenic tumor in activating a T cell response.

The combination of RT with immune checkpoint inhibition (ICI)—pharmacologic agents has also resulted in a more potent tumor response than either treatment alone in preclinical studies examining head and neck cancer, metastatic melanoma, metastatic pancreatic cancer, and lung cancer (33–35). This has resulted in investigators examining ideal radiation dosing and fractionation schemes when coupled with ICI to induce abscopal responses. For example, Dewan et al. utilized a mouse model of bilateral mammary adenocarcinoma to identify an ideal radiation dose. In their study, they treated mice with either 3 fractions (8 Gy each) coupled with an anti-CTLA-4 monoclonal antibody (mAb), or a single dose (30 Gy) coupled with the anti-CTLA-4 mAb. Their results showed an abscopal response in the group treated with 8 Gy x 3 + anti-CTLA-4 mAb but did not see the same response in the group treated with a single dose of RT (30 Gy) coupled with the anti-CTLA-4 mAb (36).

Clinical case reports of abscopal effect following irradiation without systemic treatment

Early evidence of abscopal activity has initially been portrayed in published case reports which are described below. Each demonstrate systemic clinical response following local site RT as consistent with abscopal activity.

A 57-year-old male was diagnosed with multiple lung nodules, vertebra metastases, and brain metastases (37). The results of pathological examination suggested adenocarcinoma of the lung. RT of 39 Gy in 13 fractions was administered to the ninth thoracic vertebra for destructive extension. However, all the lesions including the brain metastases spontaneously shrunk, thereby supporting abscopal activity as no systemic therapy had been administered. Two months after RT, complete regression to the lung and other non-irradiated thoracic vertebra was achieved. Whole-brain radiotherapy for a total dose of 36 Gy in 12 fractions was performed. Unfortunately,15 months after initial RT, the brain metastasis recurred.

A 61-year-old male with renal cell carcinoma and metastatic lesions to the brain, bone, spine, lung, and lymph nodes underwent stereotactic body radiation therapy (SBRT) to the brain metastases and external beam radiation therapy (EBRT) to the metastatic lesions in his bone and spine (38). 1 month later, lesions that were not subjected to radiotherapy showed regression as evidenced by CT scan. In addition, follow-up CT scans taken 2 months later and 3 months later demonstrated continued response of these untreated lung lesions suggesting a possible abscopal response. Unfortunately, this patient went on to develop new brain metastases requiring additional stereotactic radiosurgery.

A 66-year-old female with clear cell renal cell carcinoma was treated with a nephrectomy (39). Ten years later, the patient had a metastatic lesion of the renal cell cancer in the neck, and was treatment with pazopanib, but then terminated due to intolerability. CT scans of the thorax and the neck showed progression in the neck, portacaval lymph node, hypochondrium subcutaneous node, and new and progressive lung metastases. Palliative RT was given to the neck, but the patient did not resume systemic therapy. Eleven months after (radiation therapy) XRT, the patient had complete regression of the lung metastases, the subcutaneous abdominal node remained, but growth of the portacaval lymph node persisted. 17 months after XRT, the patient's stable disease remained, demonstrating abscopal activity.

A 93-year-old female with melanoma on the fifth metatarsal (Breslow depth: 2.8 millimeters) underwent therapeutic

amputation. Despite amputation, the patient demonstrated disease progression 13 months later. He presented with a 4-centimeter painful inguinal lymph node mass along with 5 cutaneous nodules located on the anterolateral leg below the knee. These cutaneous nodules were hard to palpation and macroscopically consistent with metastasis. The patient underwent palliative RT to the inguinal lymph node for pain management. Interestingly, the patient also demonstrated near complete resolution of the non-irradiated cutaneous lesions one month after RT. The patient was lost to follow-up 14 months later due to relocation to a new city, but demonstrated stable disease throughout that period (40).

A 75-year-old male with a history of stage IV colorectal cancer with liver metastasis (November 2007) status post anterior resection with partial hepatectomy and rectal cancer (January 2010) status post anterior resection presented with abdominal pain in November 2010. Abdominal imaging showed two masses: a 35-millimeter mass located on the left side of the abdomen and a 15-millimeter mass invading the right common iliac artery. The left-sided mass was irradiated using carbon-ion radiation therapy (CIRT) with a regimen of 73.6 Gy x 16 fractions over a 28-day period in January 2011. The mass invading the right common iliac artery was not irradiated due to its proximity to the small intestine. Interestingly, a PET-CT scan performed 1 month after therapy showed significant reduction in tumor size in both the irradiated and non-irradiated tumors as evidenced by decreased fludeoxyglucose accumulation. Unfortunately, the patient died 46 months after CIRT due to myelodysplastic syndrome with no evidence of progression of the two tumors as evidenced by annual PET-CT scans. Taken together, it is likely that this patient had a durable abscopal response to CIRT (41).

An 85-year-old male with a history of recurrent colon cancer in the ascending colon presented with back pain in February 2009 after a 10-month stable period post hemicolectomy (April 2008). CT imaging revealed a 45-millimeter para-aortic tumor along with two 10-millimeter tumors in the mediastinum and right clavicle. The patient was not eligible for chemotherapy due to comorbidities, so the decision was made to perform CIRT (Gy x 12 fractions over 21 days) on the para-aortic mass as part of an ongoing clinical trial. Following completion of therapy, there was a significant reduction in size of the irradiated para-aortic tumor as well as the non-irradiated mediastinal and subclavian tumors as evidenced by both CT and PET-CT imaging. The patient received no additional therapy, which suggests the patient had an abscopal response to CIRT. The patient's condition has remained stable for 92 months at the time of publication with no change in tumor size (41).

A 63-year-old male presented with a 10.5 cm x 9 cm x 11 cm hepatocellular carcinoma (HCC) with 3 daughter nodules <1 centimeter each. He underwent an extended right lobectomy and was stable for 18 months until metastatic nodules were found in the right lower lobe of the lung and left mediastinal lymph node as evidenced by CT scan. This was confirmed to be HCC metastatic disease due to an alpha-fetoprotein (AFP) of 4,869 ng/mL and a protein induced by vitamin K absence or antagonists II (PIVKA-II) >20,000 mAU/mL. Trans-catheter arterial embolization of the mediastinal tumor was attempted but ultimately aborted due to risk of spinal artery embolism. The decision was made to perform palliative external beam radiation therapy (2.25 Gy x 27 fractions) on the mediastinal node. Following RT, both the mediastinal lymph node and the right lower lobe lung tumor demonstrated significant response as evidenced by CT scan along with a decrease in AFP from 4,869 ng/mL to 23 ng/mL and a decrease in PIVKA-II from >20,000 mAU/mL to 13 mAU/mL. His disease remained stable for 4 years until a 3.5 cm lymph node was found near the left gastric artery. He was treated with stereotactic body radiotherapy and showed no additional disease after 6 months (42).

A 76-year-old female was diagnosed with pulmonary adenocarcinoma (cT1bN0M0) in November 2015, and subsequently underwent a right upper lobectomy with confirmation of pathological pT1bN2M0, stage IIIA disease. The patient did not receive adjuvant chemotherapy. In February 2018, multiple new mediastinal and right hilar lymph node metastases were identified. A total dose of 60.0 Gy of RT was given over 6 weeks to selected lesions. The target area included multiple mediastinal, and several (but not all) right hilar lymph nodes. Twelve weeks after completion of RT, a chest CT scan showed complete disappearance of the treated and untreated pulmonary metastases. Another follow up CT scan was completed (6 months after completion of RT) showing no reappearance of multiple metastatic pulmonary nodules both non irradiated and irradiated pulmonary nodules supportive of abscopal effect (43).

An 81-year-old female was diagnosed with a pT2a, pN0 (0/5), cM0, UICC stage IB squamous cell carcinoma of the left upper lung lobe (44). She underwent a lobectomy with lymphadenectomy, and subsequently had no relapse for 5 years during follow up. Thirteen years later, recurrence was confirmed *via* biopsy, chest CT showed a mass in the left lung, negative for brain metastasis on MRI, and PET showing left sided pleural carcinomatosis, left sided periclavicular lymph node metastases, and bone metastases in the 12^{th} thoracic and 4^{th} lumbar vertebra. The patient declined systemic treatment. She thus underwent palliative radiotherapy to the symptomatic pulmonary tumor. Four weeks after RT completion, restaging was performed showing a partial remission of the tumor, the nodal metastases and the previously untreated vertebral lesions. During follow-up, further decrease in tumor size and complete metabolic remission of the bone, pleural and lymph node metastases was seen. 25 months after radiotherapy, the patient still had evidence of stable disease, but remained free of disease symptoms.

In June 2018, a 69-year-old male was diagnosed with squamous cell carcinoma of the right lower lobe with

involvement of mediastinal nodes (45). The patient was initially treated with vinorelbine and cisplatin however, after four cycles, his symptoms worsened, and chest CT scan confirmed progressive disease. Hence, the chemotherapy regimen was shifted to paclitaxel, but the primary lung lesion was still not controlled, and he showed disease progression in the chest, and as well as a bone scan that showed a new lesion in the right tibia, indicating the occurrence of bone metastases. After initial response, this patient showed progression on the PD-1 inhibitor, camrelizumab, and the tyrosine kinase inhibitor that selectively inhibits VEGFR2, apatinib, and went on to receive palliative CT-guided microwave ablation to the primary lung tumor. One month later, chest CT scan showed the right lower lobe mass and mediastinal lymph nodes were also reduced, indicating an abscopal effect following local ablation.

These cases highlight systemic abscopal effect related to localized RT. The effect involved a broad range of cancer patients that include a robust age range up to 93 years old.

Case reports of abscopal effect with irradiation and enhancing immune modulation

Evidence of abscopal activity related to systemic immune induction of RT may be enhanced with combination immune modulatory therapy. The following case reports support evidence of abscopal activity with combined RT in a setting of failed systemic response prior to ongoing immunotherapy followed by immune response with same immune therapy (abscopal effect) after local RT.

A 54-year-old male patient presented with a stageT4N0M1b disease. He had a pulmonary large cell neuroendocrine carcinoma of the right upper lobe, associated with bilateral adrenal metastases and a PD-L1 tumor proportion score of 20% (46). After four cycles of chemotherapy (pemetrexed, cisplatin) and the VEGF inhibitor, bevacizumab, CT scans revealed disease progression in the right upper lobe as well as in both adrenal glands. Second-line therapy with nivolumab, a PD-1 inhibitor, was started, but increasingly symptomatic spinal cord compression, due to tumor invasion occurred. Hemilaminectomy of the third thoracic vertebra combined with resection of the epidural tumor mass was thus performed. Postoperative radiotherapy (30 Gy) was applied targeting the involved thoracic vertebrae. Nivolumab continued, CT scans 4 months after the first radiotherapy showed partial regression of the lung tumor and adrenal metastases. The patient showed disease progression 10 months after radiotherapy but is still alive, supportive of abscopal effect, 25 months after the initial diagnosis.

A 64-year-old male patient presented with T2N3M1c disease which included an adenocarcinoma of the left upper lobe, mediastinal contralateral lymph nodes and distant metastases (brain and ocular). The patient's PD-L1 results are currently blinded due to the requirements of a clinical trial (Impower130 trial: ClinicalTrials.gov identifier NCT02367781). The radiological images after 5 months of treatment (four cycles of nab-paclitaxel/carboplatin with atezolizumab, a PD-L1 inhibitor, followed by four cycles of atezolizumab alone) showed an excellent response of the ocular metastasis, but progression of the brain metastasis. The thoracic tumor manifestations showed partial remission after four cycles of combined chemotherapy and immunotherapy with no further shrinkage after the four additional cycles of atezolizumab monotherapy. Whole-brain radiotherapy (WBRT) was performed and atezolizumab was continued. Radiological follow-up 4 months after WBRT showed a partial response in the brain (complete response [CR] of ocular disease and remission of brain disease) as well as complete remission of lung and mediastinal tumor masses, supportive of potential abscopal effect. The patient is still alive with radiologically nearly complete remission 28 months after the initial diagnosis of metastatic lung cancer (46).

A 70-year-old male patient presented with a T3N2M1a disease involving central adenocarcinoma of the middle lung lobe, associated with positive mediastinal lymph nodes and a malignant ipsilateral pleural effusion. There were no EGFR or ALK mutations and the PD-L1 tumor proportion score was 70%. First-line therapy with pembrolizumab, a PD-1 inhibitor, was started, leading to a partial response. After a year of treatment, pulmonary and pleural disease progression occurred, and a clinically symptomatic brain metastasis associated with perimetastatic cerebral edema appeared. Pembrolizumab was continued and WBRT added (30 Gy in ten fractions). Radiography of the thorax after radiotherapy showed partial regression of the lung tumor and pleural effusion, supportive of abscopal activity. The patient is still alive 19 months after the initial diagnosis (46).

A 65-year-old female presented with mucosal melanoma in June 2015 with no evidence of metastatic disease as evidenced by CT scan of the neck and MRI with gadolinium. The decision was made to perform a right partial maxillectomy to remove the lesion followed by targeted intensity modulated radiotherapy (IMRT). Unfortunately, the patient relapsed 9 months later, and evidence of disease progression was found in the neck and lungs. The patient was then enrolled in a trial comparing epacadostat + pembrolizumab or placebo + pembrolizumab, which initially

showed tumor response, but ultimately resulted in disease progression. The treatment was stopped, and the patient started a palliative course of IMRT to the neck due to increased symptoms. Interestingly, both the neck lesion and the pulmonary lesions responded to IMRT based on CT scans before and after IMRT suggesting an abscopal response (47).

A 67-year-old female presented with metastatic pancreatic uncinate carcinoma to the right liver lobe in August 2015 with a CA 19-9 of 1,814 U/mL. The patient was initially treated with single-agent gemcitabine, but this was discontinued due to poor response and worsening abdominal pain. The patient was then switched to albumin-bound paclitaxel, which demonstrated partial response based on Response Evaluation Criteria in Solid Tumors (RECIST 1.0), but ultimately demonstrated disease progression with additional metastasis to the right pleura and worsening side effects. The patient was then switched to Apatinib, but this was quickly discontinued due to severe gastrointestinal distress. The decision was made to initiate palliative radiotherapy (45 Gy x 15 over 3 weeks) coupled with GM-CSF to the primary pancreatic tumor due to abdominal pain and jaundice requiring percutaneous transhepatic-cholangial drainage. 1 month later, the patient demonstrated significant response to the primary tumor as evidenced by CT scan, but also demonstrated significant abscopal response to the metastatic sites in the liver and pleura that were outside the cone of radiation (48).

A 33-year-old female presented with a mole on her upper back concerning for melanoma in April 2004. Biopsy of the lesion revealed melanoma with a Breslow thickness of 1.53 millimeters. The decision was made to perform a wide local excision of the malignant lesion along with sentinel lymph node biopsy. The patient remained disease free until 2008 when a PET-CT revealed a 2-centimeter pulmonary nodule suggestive of metastatic disease that was confirmed *via* CT percutaneous biopsy. She was treated with cisplatin, vinblastine, and temozolomide (CVT) chemotherapy due to lack of a targetable mutation (e.g., BRAF) followed by surgical resection. The patient demonstrated stable disease until surveillance CT scan showed a metastatic paraspinal mass along with hilar lymphadenopathy in August 2009. The decision was made to initiate 4 doses of ipilimumab, a CTLA-4 inhibitor, (10 mg/kg) every 3 weeks which resulted in an initial slight enlargement of the paraspinal mass but effectively stabilized her disease for 14 months. Unfortunately, the patient demonstrated continued enlargement of the paraspinal mass with additional evidence of metastatic splenic lesions. The patient was experiencing significant back pain due to mass effect from the paraspinal mass, so the decision was made to initiate palliative RT to the paraspinal mass (950 Gy x 3 fractions over 7 days). Ten months after therapy, there was evidence of abscopal effect as evidenced by CT-scan demonstrating significant reduction in size of both the treated paraspinal mass and the splenic lesions that were outside the cone of radiation (49).

A 71-year-old male was diagnosed with stage IV lung adenocarcinoma, and began treatment with atezolizumab (50). After 19 months of atezolizumab, there was a complete response to the primary lung tumor. A brain metastasis then developed two years later, which was treated with gamma knife radiotherapy. However, after radiation, the patient's lung disease recurred. Two months later, the lesions in the lung had shrunk, indicating that the

TABLE 1 Abscopal case reports following irradiation without systemic treatment.

Patient	Disease	Sites of involvement	Treatment	Response	Reference
57-year-old male	Unknown primary	Lung nodules, vertebra, and brain metastases	Radiation (XRT) to 9th vertebra	All lesions	(37)
61-year-old male	Renal Cell carcinoma	Bone, spine, brain, lung lymphadenopathy mets	XRT to brain, spine, and bone	Regression of untreated lung metastases and lymphadenopathy	(38)
66-year-old female	Renal Cell carcinoma	Neck, lung and portacaval node	XRT to the neck	Regression of irradiated neck mass and non-irradiated lung metastases	(39)
93-year-old female	Melanoma (toe)	Thigh and inguinal node	Surgery, RT to inguinal region	Regression of thigh lesions	(40)
75-year-old male	Colorectal cancer	Liver mets	Resection and RT	Reduction in both the treated and untreated liver masses	(41)
85-year-old male	Colorectal Cancer	Nodes (near abdominal aorta, mediastinal, and subclavian)	Resection and XRT to aortic lymph node	Untreated subclavian node shrank, and mediastinal node remained stable.	(41)
63-year-old male	Hepatocellular Carcinoma	Lung and mediastinal nodes	XRT to mediastinal lymph node	Mediastinal lymph node and untreated lung mass	(42)
76-year-old female	NSCLC	Mediastinal and hilar lymph nodes, lung disease	XRT only, to mediastinal and hilar lymph nodes.	Complete response to multiple untreated lung lesions	(43)
81-year-old female	NSCLC	Lung, pleura, periclavicular node, and vertebra	XRT only	Complete remission of untreated vertebral lesions and periclavicular node	(44)

TABLE 2 Abscopal case reports of irradiation and systemic treatment following systemic treatment failure.

Patient	Disease	Sites of involvement	Treatment	Response	Reference
69-year-old male	NSCLC	Lung, mediastinal and other nodes	Vinorelbine/cisplatin, paclitaxel, camrelizumab/apatinib, CT-guided microwave ablation of lung lesion, following systemic treatment failure	After progression on systemic treatment and then XTR, right lower lobe mass and mediastinal lymph nodes reduced	(45)
54-year-old male	Neuroendocrine	Lung, adrenal glands, para-spinal cord	After progression with systemic therapy, Pemetrexed/cisplatin/bevacizumab, nivolumab, XRT to para spinal vertebrae	Lung tumor and adrenal metastases underwent regression	(46)
64-year-old male	NSCLC	Mediastinal contralateral lymph nodes and distant metastases (brain and ocular)	Nab-paclitaxel/carboplatin/atezolizumab, atezolizumab alone, after progression of all lesions, WBRT (whole brain radiation therapy)	PR to brain, CR to lung and mediastinal masses was seen after WBRT	(46)
70-year-old male	NSCLC	Mediastinal lymph nodes and malignant pleural effusion, PD-L1 70%	Failed Pembrolizumab, then WBRT added	Partial regression of the lung tumor and pleural effusion after WBRT	(46)
65-year-old female	Melanoma (mucosal)	Neck and pulmonary mets	Failed Pembrolizumab, then given XRT to neck	Tumor regression of the pulmonary metastases after XRT to the neck	(47)
67-year-old female	Pancreas Cancer	Liver and right pleura	Failed Gemcitabine, paclitaxel, apatinib, then given palliative XRT (to pancreas)/GM-CSF	XRT to pancreatic tumor, non-irradiated systemic metastases significantly decreased	(48)
33-year-old female	Melanoma (cutaneous)	Pulmonary nodule, paraspinal mass, hilar node, splenic lesion	Failed Cisplatin/vinblastine/temozolomide (CVT), ipilimumab, XRT to paraspinal mass	Regression to non-irradiated hilar lymphadenopathy and splenic lesions	(49)
71-year-old male	NSCLC	Brain mets, mediastinal lymph nodes lung disease	Failed nedaplatin/paclitaxel, and Atezolizumab, then given Atezolizumab/brain XRT	Primary lung lesion and hydrothorax decrease after systemic treatment failure and brain XRT	(50)

prior changes in the lung may have been pseudo-progression as abscopal effect was later demonstrated.

Combination irradiation with immunotherapy may be associated with more frequent abscopal effect as suggested by preclinical testing and preliminary clinical results (31–36, 51). In summary (see Tables 1, 2), these 17 case reports provide evidence of abscopal effect and support combination with immunotherapy is well tolerated and may enhance abscopal activity related to RT.

Current studies evaluating abscopal effect

The clinical trial landscape for abscopal effect and development of clinical trials is increasing. There are several prospective clinical trials investigating the abscopal effect (Table 3). However, consistent results regarding occurrence of abscopal effect and level of benefit are highly variable. Moreover,

TABLE 3 Select ongoing clinical trials involving radiotherapy and checkpoint inhibitors to achieve abscopal effect.

Cancer type	Irradiation scheme/combination	Clinical trial number
NSCLC	30-50 Grays in 5 fractions Bevacizumab toripalimab	NCT04238169
NSCLC	20 Gray nivolumab	NCT03480334
NSCLC	20 x 2 Gray (daily for 4 weeks) 5 x 5 Gray (daily over 1 week) 3 x 8 Gray (every other day over 1 week) Durvalumab	NCT04245514
Metastatic Cancer	1 dose of SBRT Durvalumab and trememlimumab	NCT03212469
Hepatocellular	4 fractions over 8-15 days Pemrolizumab	NCT03316872

trials exploring expansion of abscopal effect looking at various irradiation doses, schedules and immune modulating combination therapy still only provide relatively low occurrence rate of abscopal activity. In general, though, when abscopal effect is observed compared to those receiving the same regimen without abscopal effect clinical benefits with respect to response, progression-free survival (PFS) and overall survival (OS) is observed (52–54). In one early retrospective study involving melanoma patients treated with ipilimumab followed by RT, 52% showed evidence of abscopal activity and those who did had significantly improved OS (54). Another retrospective study involving melanoma showed similar results (55). Moreover, in a third small trial of 10 prostate cancer patients improved durable disease control was observed with combined ipilimumab and irradiation (56). Although in a larger later Phase 3 trial of advanced prostate cancer undergoing irradiation and ipilimumab vs. irradiation alone OS was not different (57).

Similarly, several combination PD-1/PD-L1 checkpoint inhibitor treatments with RT have also demonstrated evidence of abscopal activity. KEYNOTE-001 trial demonstrated improved PFS and OS in NSCLC patients who received prior RT and pembrolizumab compared to pembrolizumab alone although actual abscopal events were not well defined (58). Another retrospective study looking at PD-1 inhibitors involving melanoma patients, some receiving RT, showed significant improvement in response rate but no improvement in PFS and OS with combination checkpoint inhibitors/RT. However, only one patient of 59 demonstrated abscopal activity (59). Not all clinical results have reproduced the same result. For example, a Phase I clinical trial examining the ideal radiation dose in patients with metastatic NSCLC or melanoma on pembrolizumab showed abscopal responses in patients treated with either 24 Gy x 3 fractionation scheme or a single 17 Gy fraction (NCT02303990) (60). This suggests that the abscopal response to irradiation is multi-factorial and radiation fractionation regimens may not be universal.

Interestingly, GM-CSF combination RT involving a general group of 41 solid tumor patients showed a high fraction (over 25%) of patients with abscopal activity (breast cancer, NSCLC, thymic cancer) when combined with localized irradiation (61). In addition, Formenti et al. performed a proof-of-principle trial where they supplemented RT with subcutaneous GM-CSF, a cytokine that promotes dendritic cell differentiation and expansion, in patients with metastatic tumors including breast cancer, bladder cancer, and eccrine cancer (51, 62). Their results showed that 30% of patients who received RT supplemented with GM-CSF over the course of 2 weeks had an abscopal response as evidenced by PET/CT. Also, breast cancer patients receiving high dose vs. low dose TGFβ blockade (fresolimumab) along with RT had significantly prolonged OS (63).

Consideration GM-CSF expression/ TGFβ knockdown to induce abscopal effect

There continues to be strong evidence that radiation is able to activate the immune system although the mechanism for this has not been fully elucidated (62). RT has also been shown to promote the development of an immunosuppressive tumor microenvironment, specifically by upregulation of PD-L1 (64, 65). Therefore, it has been hypothesized that radiation coupled with immunotherapy would elicit an abscopal effect. However, results have been limited with studies evaluating the dose of radiation and sequencing of combination immunotherapy (66). The abscopal effect has largely been observed in highly immunogenic tumors including melanoma, renal cell, and hepatocellular carcinoma. The tumor microenvironment in these "hot" tumor types are characterized by T cell infiltration and expression of proinflammatory cytokines (67).

In addition to combination with checkpoint inhibitor therapy, autologous tumor cellular immunotherapy may also be considered as a clinical testing direction. Vigil is a triple function immune therapy constructed from patient tumor cells. Vigil mechanism involves the introduction of bifunctional short-hairpin RNA to knockdown furin in the autologous tumor cells. Furin knockdown results in decreased cleavage of TGFβ into TGFβ1 and TGFβ2 (68). TGFβ is an immune suppressive cytokine associated with poor prognosis and therapeutic resistance in many solid tumors (69–71). Vigil plasmid also encodes for human GM-CSF which is also an immune stimulatory cytokine that increases tumor antigen presentation by dendritic cells (72). Moreover, Vigil provides personalized, clonal cancer specific neoantigens to enable the immune system to recognize tumor cells and mount an effective, targeted T-cell mediated response. Vigil has demonstrated improved clinical outcomes which correlated with IFNγ-ELISPOT positivity (73, 74). IFNγ is known to activate a multitude of immune cells, including effector T cells. Vigil has shown clinical benefit in advanced solid tumor patients with overall survival correlation with TIS^{HIGH} vs. TIS^{LOW} (one year OS 75% vs. 25%, p=0.03795) and elevated MHC-II expression (p=0.038). In recurrent ovarian cancer patients, the OS rate was observed to be 58% compared to historical rate of <20% with standard of care. In a Phase IIb double-blind, randomized, placebo-controlled trial in frontline ovarian cancer maintenance, Vigil patients demonstrated a trend towards benefit (11.5 months vs. 8.4 months for placebo, p=0.078). The result for the secondary endpoint of recurrence-free survival (RFS) for the BRCA-wt subpopulation however, was statistically significant, demonstrating benefit in RFS from procurement (time of initial debulking surgery) and

randomization (time of initial Vigil administration; 18.3 months vs. 14.8 months, HR=0.478, p=0.02; 11.5 months vs. 8.0 months, HR=0.514, p=0.02 respectively) and OS from procurement and randomization (not reached vs. 48.3 months, HR=0.490, p=0.047; not reached vs. 41.4 months, HR=0.493, p=0.049 respectively). Based on a *post hoc* exploratory analysis in the BRCA-wt, HRP subpopulation, RFS and OS were increased in a statistically significant fashion relative to the control arm, demonstrating a benefit with Vigil in RFS from procurement and randomization (18 months vs. 12 months, HR=0.363, p=0.005; 10.6 months vs. 5.7 months, HR=0.386, p=0.007 respectively) and OS from procurement and randomization (not reached vs. 37.3 months, HR=0.340, p=0.018; not reached vs. 26.9 months, HR=0.342, p=0.019 respectively). Long term follow up analysis also revealed that 83% of Vigil treated patients were still alive three years after their initial debulking surgery versus 40% who received placebo (p=0.0006). Clinical testing of Vigil with RT to induce and augment the abscopal effect is under consideration.

Conclusion

Clearly sufficient preclinical and clinical evidence exists which support benefit to patients who incur abscopal effect while undergoing RT. There does not appear to be any concerning toxic effect related to abscopal activity. Benefit associated with response, PFS, duration of PFS and OS has been observed. However, results are inconsistent and hard to predict. Biomarkers indicative of abscopal development are not known. Combination of RT with immune modulatory therapy appear to suggest enhancement in abscopal activity but results are variable. Further research towards enhancement in abscopal activity is warranted. Consideration in modulation of GM-CSF expression and TGFβ knockdown is justified.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Author contributions

DC, SA, LS, and AW contributed to writing and editing the manuscript. JN was responsible for manuscript conception, supervision and writing and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

Authors SA, LS, AW, and JN were employed by Gradalis, Inc.

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Updates in combined approaches of radiotherapy and immune checkpoint inhibitors for the treatment of breast cancer

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Breast cancer is the most prevalent non-skin cancer diagnosed in females and developing novel therapeutic strategies to improve patient outcomes is crucial. The immune system plays an integral role in the body's response to breast cancer and modulating this immune response through immunotherapy is a promising therapeutic option. Although immune checkpoint inhibitors were recently approved for the treatment of breast cancer patients, not all patients respond to immune checkpoint inhibitors as a monotherapy, highlighting the need to better understand the biology underlying patient response. Additionally, as radiotherapy is a critical component of breast cancer treatment, understanding the interplay of radiation and immune checkpoint inhibitors will be vital as recent studies suggest that combined therapies may induce synergistic effects in preclinical models of breast cancer. This review will discuss the mechanisms supporting combined approaches with radiotherapy and immune checkpoint inhibitors for the treatment of breast cancer. Moreover, this review will analyze the current clinical trials examining combined approaches of radiotherapy, immunotherapy, chemotherapy, and targeted therapy. Finally, this review will evaluate data regarding treatment tolerance and potential biomarkers for these emerging therapies aimed at improving breast cancer outcomes.

KEYWORDS

immune checkpoint inhibitors (ICI), radiotherapy, breast cancer, tumor immunology, radiation biology, immunotherapy

Introduction

Breast cancer (BC) is the most common non-cutaneous malignancy diagnosed in females, accounting for nearly one-third of all new cancer diagnoses (1). During 2022, in the United States, approximately 287,850 females will be diagnosed with breast cancer, while over 43,000 females will ultimately succumb to their disease (1). Breast cancer incidence has increased in female patients, coinciding with an increase in obesity and decline in fertility rates (1, 2). Early detection and improved loco-regional and systemic therapies have led to improved outcomes among breast cancer patients in recent years (3). However, breast cancer is a heterogenous disease with diverse molecular subtypes, clinical classifications, and genetic variations (3, 4). Using the most common definition, breast cancer is divided into four molecular subtypes—luminal A, luminal B, $HER2^+$, and triple negative breast cancer (TNBC)—based upon the presence or absence of important hormone receptors, including the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (4). This heterogeneity at the tumor level results in different responses to therapy (3–5). Importantly, TNBC is the most aggressive breast cancer subset that disproportionately impacts patients of color and younger patients (4, 6–8). Significantly, more effective therapies for TNBC are desperately needed.

Locally advanced breast cancer is treated via a trimodal approach that includes surgery, chemotherapy, and radiotherapy. Recent advances in precision medicine have been developed to target the molecular differences that exist in breast cancer (3). Endocrine therapies, including the selective estrogen receptor modulator (SERM) tamoxifen, selective estrogen degrader (SERD) fulvestrant, or the aromatase inhibitors anastrozole and exemestane, target the estrogen receptor found in ER^+ breast cancer (9). Other precision medicine advancements used in the management of metastatic breast cancer include small molecule inhibitors of key modulators of breast cancer growth and survival. For example, inhibiting the cyclin dependent kinases 4 and 6 (CDK4/6) mechanistically prevents the progression of cancerous cells through the cell cycle, while inhibiting poly (adenosine diphosphate-ribose) polymerase (PARP) impairs DNA repair (10, 11). While these targeted therapies improve survival, therapeutic resistance is common, and the discovery of additional treatment options are warranted.

An emerging therapeutic option for treating breast cancer is immunotherapy, which enables a patient's immune system to recognize and eliminate cancerous cells. Cancer cells evade the immune system by expressing immune checkpoints: inhibitory molecules that hinder the immune system's ability to eliminate cancer. Immune checkpoint inhibitors (ICIs) block these immune checkpoints or "brakes" on the immune system, resulting in an increase in antitumor immunity and the eradication of cancerous cells. Currently, clinically utilized ICIs target the programmed death receptor 1 (PD-1)-programmed death ligand 1 (PD-L1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) axes (12). ICIs have been most clinically successful in the management of melanoma (13), non-small cell lung cancer (NSCLC) (14), and bladder cancer (15). Overall, more than 40% of all cancer patients are eligible to receive ICIs (16, 17). Importantly, recent studies suggest that ICIs are effective for the treatment of breast cancer patients, although it was originally believed that these patients would respond poorly to immunotherapies due to this disease being a relatively nonimmunogenic cancer (18). Of all breast cancer subtypes, immunotherapy is particularly promising for the treatment of TNBC that cannot be treated via hormone therapies due to not expressing commonly targeted hormone receptors—including the ER, PR, and HER2. Immunotherapy may also be promising for treating this subset of breast cancer, since treatment resistance to standard therapies—like chemotherapy and radiotherapy—remains a significant clinical issue for TNBC patients (19, 20).

Combining radiotherapy with immunotherapy for the treatment of aggressive breast cancers may improve treatment efficacy. Early preclinical studies demonstrate that radiotherapy promotes antigen presentation in tumor cells by causing DNA damage, altering transcription, and potentially leading to presentation of immunogenic peptides (21, 22). By promoting the presentation of immunogenic peptides, the recognition of cancer cells by T cells can be enhanced to reactivate the body against the tumor, thus, overcoming the immunosuppressive effects of immune checkpoints. Clinical studies assessing the effectiveness of multimodal approaches incorporating radiotherapy and immunotherapy in breast cancer are ongoing. While combining immunotherapy and radiotherapy to treat aggressive breast cancers is clinically promising, additional research is necessary to determine the mechanisms underlying this therapeutic approach. This review will cover the cellular and molecular regulators of antitumor immunity as well as review the preclinical and clinical advances supporting immunotherapy as a treatment option for breast cancer patients. Throughout this review, we place a special emphasis on emerging therapeutic approaches and clinical trials combining immunotherapy with radiotherapy to treat breast cancer.

Immune microenvironment in breast cancer

The immune system is a powerful mediator in protecting the body against foreign pathogens, and importantly plays a crucial role in safeguarding the body from self-cells that become

cancerous. Paradoxically, the immune system can play both supportive and inhibitory roles in breast cancer progression and is an important pharmacological target to improve patient outcomes (23). Tumors are classified based upon the presence and location of immune cells in the tumor microenvironment (TME), where noninflamed ("cold") tumors have a low infiltration of lymphocytes and inflamed ("hot") tumors have a high infiltration of lymphocytes (24). Noninflamed tumors can also have an absence of infiltrating lymphocytes or have lymphocytes only on the peripheral edges of the tumor ("excluded") (25). Additionally, antitumor immunity is dependent on the immune tone of the TME, with both immunosuppressive and immunostimulatory milieu being common. This is relevant in breast cancer carcinogenesis, where both the innate and adaptive immune system contribute to cancer development and immune evasion (26).

Tumor-associated macrophages (TAMs) are innate immune cells found within the TME that have pro-tumorigenic and anti-tumorigenic effector mechanisms in the context of cancer (27). Macrophages are divided into M1-like macrophages that exert antitumor effects and M2-like macrophages that exert pro-tumorigenic effects; however, these phenotypes are plastic and can be pharmacologically reprogrammed (27). In breast cancer, it has been known for the past two decades that macrophages can promote malignant transformation (28), while monocyte-derived macrophages additionally contribute to breast cancer metastasis (29). FOLR2$^+$ macrophages are a specific subset of TAMs enriched predominantly in healthy mammary glands that positively correlate with CD8$^+$ T cells (30). Contrastingly, TREM2$^+$ macrophages are a subset of TAMs expressed primarily in cancerous breast tissue that contribute to tumor development (30). Additionally, in both TNBC and hormone receptor-positive (HR$^+$) breast cancer, CD163$^+$ TAMs are derived from circulating monocytes and contribute to immunosuppression (31). Neutrophils, another innate cell lineage, can also exert multifaceted pro-tumorigenic and anti-tumorigenic effects under different contexts (32). Within TNBC, there are dichotomous neutrophil-enriched subtypes (NES) and macrophage-enriched subtypes (MES). Specifically, the NES subtype displays an abundance of immunosuppressive neutrophils and is resistant to ICIs, whereas the MES subtype demonstrates mixed responses to ICIs (33). Furthermore, myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells of the innate immune system that suppress CD8$^+$ T cells and other immune cells in the TME, promoting tumor progression (34). Elevated levels of circulating MDSCs were present more often in breast cancer patients than in healthy volunteers and were even higher in patients with metastatic disease (35). MDSC crosstalk signaling promotes breast cancer progression in part through the STAT3 and NOTCH signaling pathways (36). In all, these cells of the innate immune system exert multifaceted effects in the TME and execute significant roles in cancer progression and immune surveillance.

Tumor-infiltrating lymphocytes (TILs) collectively refer to the populations of lymphocytes in the tumor. This population of white blood cells includes T lymphocytes (T cells), B lymphocytes (B cells), and natural killer (NK) cells (37, 38). T cells compose approximately 75% of TILs and consist of different subsets including cytotoxic CD8$^+$ T cells, CD4$^+$ T cells, and regulatory T cells (Tregs) that all contribute to the adaptive immune response (38, 39). The presence of TILs is associated with improved disease outcomes in breast cancer patients (40, 41). CD8$^+$ T cells are directly cytotoxic to tumor cells, while CD4$^+$ T cells can promote antitumor immunity through the secretion of inflammatory cytokines (42). Meanwhile, some immune cell populations may induce immunosuppressive effects in the TME. For example, CD4$^+$ Tregs restrain the activation and function of CD8$^+$ T cells (43). While it is well-established that CD8$^+$ TILs are a favorable prognostic indicator and positively correlate with relapse-free survival in breast cancer (44), the T cell subtypes present in breast cancer are not fully understood (45). CD8$^+$ tissue-resident memory (T_{RM}) cells are one subset of CD8$^+$ TILs contributing to immunity that express cytotoxic molecules and immune checkpoint proteins (46). Interestingly, CD8$^+$ TRM cells are associated with improved relapse-free survival (RFS) in TNBC cancer patients (45). In early-stage TNBC patients, the presence of T_{RM}s is associated with improved patient outcomes—including increased survival and decreased rates of recurrence (46). Increased intra-tumoral expression of CD39$^+$PD-1$^+$CD8$^+$ T cells, another subset of CD8$^+$ TILs, correlates with longer disease-free survival in breast cancer patients (47). In breast cancer, FOXP3$^+$ Tregs are a distinct population of T cells associated with more aggressive forms of breast cancer, including a higher risk of relapse and decrease in survival (48). Additionally, intratumoral Tregs from breast cancer tumors have increased expression of the chemokine receptor CCR8, suggesting a unique phenotype and function of these cells in human breast cancer patients (49). B lymphocytes are a humoral cell population of the adaptive immune system that can contribute to both antitumor immune responses and potentiate cancer development (50). B lymphocytes are less prevalent in invasive breast cancers in comparison to early ductal carcinoma *in situ* (50). Importantly, the presence of immune infiltrates in the breast tumor may correlate to patient response to therapy. In the SweBCG91RT trial, immune infiltrates, in the form of CD8$^+$ T cells and FOXP3$^+$ T cells, were examined in early-stage breast cancer patients that received breast-conserving surgery (BCS) and postoperative radiotherapy. In this trial, early-stage breast cancer patients with antitumoral immune infiltrates had a decreased risk of recurrence, while the addition of radiotherapy to these patients was found to have limited benefits (51). In summary, a variety of lymphocytes are present in breast tissue and many of these lymphocytes play dual roles in carcinogenesis and immune recognition.

Of the breast cancer subtypes, TNBC is associated with the highest lymphocyte infiltration, followed by $HER2^+$ breast cancer, and finally by HR^+, $HER2^-$ breast cancer (41). Importantly, lymphocyte infiltration in breast cancer patients varies significantly from 1.1% to 44%, which is independent from tumor size (52). In a study that examined $CD8^+$ T cell infiltration among 12,439 breast cancer patients, the presence of intratumoral $CD8^+$ T cells was associated with a significant reduction in risk of death in both ER^- and ER^+, $HER2^+$ breast cancer. Specifically, intratumoral $CD8^+$ T cell expression was associated with a 28% reduction in mortality for TNBC and $HER2^+$ tumors and 27% reduction in mortality for ER^+, $HER2^+$ tumors (53). Furthermore, there have also been differences found in the tumor immune microenvironment of African American breast cancer patients compared to non-African American patients, which may be contributed to socioeconomic and ancestry factors. African American TNBC patients display an increase in gene expression of immune pathways and an increase in immune infiltration—providing rationale for the application of immunotherapies for these patient populations (54). Inflammatory breast cancer (IBC) is a rare type of breast cancer which clinically presents with distinct rapid and substantial inflammation of the breast (55). IBC has a unique tumor microenvironment composition compared to other breast cancers (56). Emerging evidence suggests that the tumor microenvironments of IBC tumors is associated with an increase in $CD8^+$ T cell infiltration (57, 58) and tumor-associated macrophages (59, 60); however, the effects of the immune system and underlying molecular pathways of IBC carcinogenesis are not fully defined (61). In summary, more research is necessary to understand the implications of immunotherapy for other breast cancer subsets, including HR^+ breast cancers and IBC.

Regulators of immune responses in breast cancer

The immunogenicity of tumors is influenced by multiple factors, including the mutational load of the tumor. Cancerous cells accumulate variable levels of somatic mutations, which may result in the production of neoantigens and tumor-specific antigens (TSAs) (62–64). These antigens are recognized by the immune system to distinguish cancer cells from healthy, noncancerous cells (62). The ability of cytotoxic $CD8^+$ T cells to recognize neoantigens produced by tumor cells was reported in the early 1990s and provided an important insight into the antitumor effects of T cells in cancer (65, 66). Cancer immunotherapies are often developed to target these neoantigens because they are tumor-specific and, thus, an attractive target for minimizing on-target, off-tumor effects (63, 67). Compared to other malignancies, breast cancer has less than the median number of somatic mutations (64). Only 5% of all breast cancers are hypermutated and carry a significant load of somatic mutations. Additionally, in breast cancer, the APOBEC signature, a signature that represents dysregulated AID/APOBEC cytidine deaminases, is the primary mutational process leading to these hypermutations (68). As tumor mutational load correlates with response to immunotherapy, from the perspective of antigen presentation, breast cancer is deemed relatively non-immunogenic.

Disruption and dysregulation of the cancer immunity cycle promotes carcinogenesis. Data from The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) breast cancer cohorts suggest that malfunction of the cancer immunity cycle contributes to disease progression and serves as a prognostic biomarker (69). Avoiding immune clearance is an important hallmark of cancer that enables cancer cells to expand independently from the inhibitory effects of the immune system (65, 70). In the cancer immunity cycle, antigens produced by cancer cells are sampled by antigen-presenting cells (APCs) such as macrophages, dendritic cells, and B cells (65). APCs then present the antigens via major histocompatibility complexes I or II (MHCI/II) (65). Naïve T cells can recognize these antigens when their T cell receptor (TCR) binds to the MHC on the APC, and this interaction is stabilized by the co-receptors CD4 or CD8. This TCR recognition of the peptide-MHC complex is insufficient to fully activate T cells. An additional co-stimulation signal is required, which occurs when costimulatory molecules, such as CD28, on the T cell recognize signals, such as CD80/86, on the APC. Following these two signals, the APC will release cytokines, such as IL-2, to further direct the activation and differentiation of T cells. Once activated, T cells egress from the lymph nodes, traffic through the blood, and enter the TME (65). Trafficked T cells may then utilize their tumor antigen-specific TCRs to bind to neoantigens presented on MHC-I by the cancer cell, allowing for granzyme and perforin driven cytotoxicity. The overall effect of this pathway is dependent on which population of T cells is recruited to the tumor microenvironment.

In breast cancer, there are several mechanisms utilized by cancer cells to avoid recognition by the cancer immunity cycle (71). One way tumor cells can avoid immune recognition is via loss of MHC class I antigen presentation, which prevents the tumor cells from being recognized by $CD8^+$ T cells (72). In breast cancer cells, this may occur in part through the protein MAL2 that promotes endocytosis of tumor antigens (73). Moreover, breast cancer cells can deplete the costimulatory receptor needed for T cell activation when CTLA-4 on tumor cells and CD80 on APCs promote trans-endocytosis of CD80 (74). Furthermore, by expressing immune checkpoints, cancer cells can target and inhibit the effector functions of T cells, including suppression of antitumor cytokine secretion and T cell proliferation (71). Collectively, these studies illustrate the many ways that breast cancer can avoid recognition by the cancer immunity cycle.

An immune pathway especially critical for modulating immune responses to cancer is the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS/STING) pathway, as represented in Figure 1 (75). The stimulator of interferon genes (STING) is an endoplasmic reticulum (EnR)-bound, transmembrane protein that stimulates the transcription of numerous immune pathways following the recognition of cyclic dinucleotides (CDNs) and cytosolic DNA (cDNA) (75–77). CDNs and cDNA can be produced from viruses, bacteria, and diseased states including cancer (76). These cytoplasmic molecules of genetic information are consequently recognized by cyclic GMP-AMP synthase (cGAS), which produces cyclic GMP-AMP (cGAMP) (76, 77). Chromosomal instability (CIN)—another hallmark of cancer—occurs following chromosomal segregation errors during mitosis and can also activate the cGAS/STING pathway in cancer cells (70, 78). Moreover, in addition to promoting an antitumor immune response through the cGAS/STING pathway, CIN can also promote the activation of other immune cells, including natural killer cells to promote antitumor immunity (79). Micronuclei formation can additionally promote the cGAS/STING pathway to activate an immune response (80). Production of cGAMP by such means activates STING via binding with two STING molecules in the EnR, which leads to STING interacting with TANK-binding kinase 1 (TBK1) (76, 77). TBK1 can then phosphorylate type 1 interferon (T1IFN) transcription factors including interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB) that promote gene transcription after translocation to the nucleus (76, 77). The cGAS/STING pathway and activation of T1IFNs also plays critical roles in cancer (81). For example, T1IFN production is often associated with T cell infiltration that promotes immune responses against tumors (76, 82, 83). In breast cancer, perinuclear expression of STING was recently found to be associated with improved prognosis in ER^+ breast cancers (84). Consequently, the development of STING agonists has been explored as a therapy for the treatment of breast cancer to induce an antitumor response and improve the efficacy of additional immunotherapeutic approaches (85, 86). In short, the cGAS/STING pathway plays a

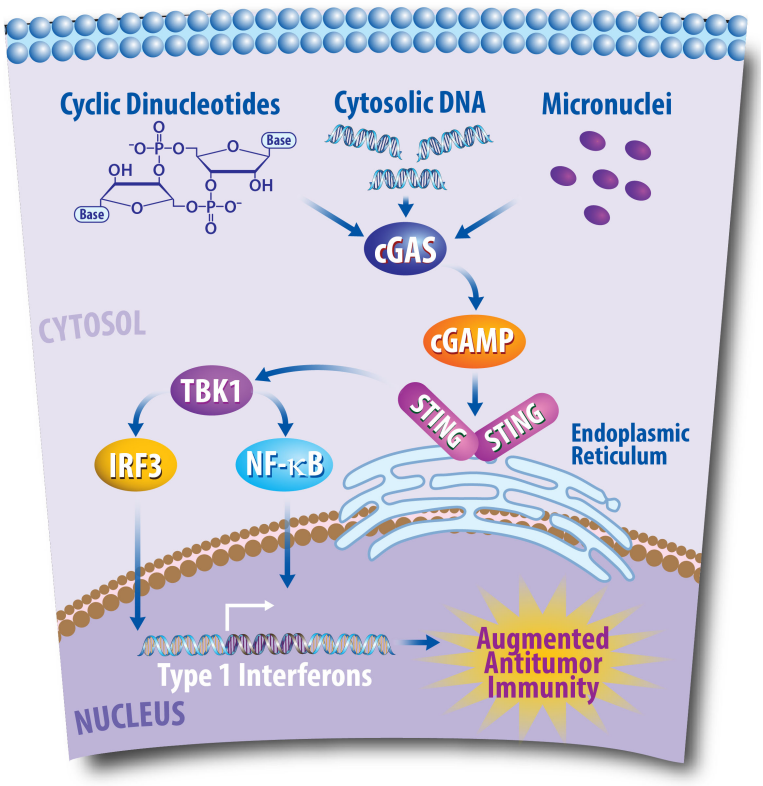

FIGURE 1
The Cyclic GMP-AMP Synthase-Stimulator of Interferon Genes (cGAS/STING) Pathway Plays a Critical Role in Antitumor Immunity. Following DNA damaging events, DNA fragments enter the cytoplasm of cancer cells. This cytosolic DNA is then recognized by the cytoplasmic sensor cGAS, which can then produce cyclic GMP-AMP (cGAMP). Consequently, cGAMP promotes the recruitment of STING molecules in the endoplasmic reticulum, which leads to TANK-binding kinase 1 (TBK1) phosphorylating interferon regulatory factor 3 (IRF3), and nuclear factor-κB (NF- κB). IRF3 and NF- κB then translocate to the nucleus to promote transcription of type I interferons, which can lead to an antitumor response via the promotion of T cell infiltration into the tumor microenvironment.

critical role in cancer and is a potential pharmacological target for treating cancer patients.

The role of immunotherapy in breast cancer

Immunotherapeutic approaches aimed at improving cancer control rates in breast cancer patients include cancer vaccines, adoptive cell transfer, and ICIs (87, 88). Cancer vaccines target distinct antigens upregulated in the tumors of cancer patients and provide immunological memory (89). Mechanistically, cancer vaccines seek to trigger an immune response via machinery that promotes the presentation of tumor antigens to the immune system and via adjuvants that cause a proinflammatory response to activate the immune system (89). Current research is focused on developing vaccines that can prevent the progression of aggressive breast cancers—such as triple negative disease (NCT04674306)—and combining breast cancer vaccines with other treatment approaches (NCT00082641, NCT03789097). For instance, mRNA vaccines have recently been successful in the context of COVID-19 and are currently being explored for use in breast cancer (90). Significant work has been done to study the efficacy of breast cancer vaccines both preclinically and clinically; however, most studies have failed to produce significant responses in patients, which may be attributed to the heterogeneity of breast cancer (89, 91).

ICIs have revolutionized cancer therapeutics, leading to Dr. James P. Allison and Dr. Tasuku Honjo being awarded the Nobel Prize in Physiology or Medicine in 2018 (92). One class of ICIs target programmed death-ligand 1 (PD-L1 or B7-H1), which serves to inhibit the immune system by binding to PD-1 on T cells and dampening their cytotoxic abilities (93). PD-L1 is expressed on a myriad of immune cells, including antigen presenting cells, T cells, and B cells, and interacts with its receptor, PD-1, expressed on T cells (94, 95). Mechanistically, PD-L1 and PD-1 interactions suppress tumor immunity by causing T cell apoptosis, anergy, exhaustion, and IL-10 expression (94). Expression of PD-L1 and PD-1 in the tumor microenvironment is a common cancer immune evasion strategy (94). Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 or CD152) is another immune checkpoint receptor expressed on T cells that has a high affinity for CD80 and CD86, which are necessary for T cell co-stimulation (96, 97). CTLA-4 outcompetes the co-stimulatory molecule CD28 to induce immune suppression (97, 98). In breast cancer, TCGA analyses suggest that TNBC patients express higher levels of PD-L1 as compared to patients with other breast cancer subtypes with approximately 20% of TNBC samples expressing significant levels of PD-L1 (99). While PD(L)-1 inhibition is clinically efficacious in many cancer types, PD-L1 expression poorly predicts clinical benefit, emphasizing the demand for clinical trials evaluating efficacy as well as the need for better biomarkers of treatment response (100).

Importantly, clinical trials have tested the efficacy of ICIs in TNBC. The Phase Ib KEYNOTE-012 clinical trial (NCT0184883) tested whether pembrolizumab (anti-PD-1) was tolerable in patients with PD-L1$^+$ advanced TNBC. This study found that pembrolizumab had an acceptable safety profile, with an overall response rate of 18.5% (101). In the Phase II KEYNOTE-086 trial (NCT02447003), 254 female patients with metastatic TNBC received pembrolizumab in either the second line setting or the first line setting. In the second line setting, patients unselected for PD-L1 expression had an objective response rate (ORR) of 5.3%, while in the first line setting, PD-L1$^+$ patients had an ORR of 21.4%. Tolerability was reaffirmed in both cohorts (102, 103). This trial led to the randomized, open-label Phase III KEYNOTE-119 (NCT02555657) trial that examined the efficacy of pembrolizumab versus single agent chemotherapy in patients with PD-L$^+$ metastatic TNBC. In this trial, PD-L1$^+$ status was characterized by patient PD-L1 combined positive scores (CPS), defined as the ratio of PD-L1$^+$ tumor cells, lymphocytes, and macrophages out of total tumor cells multiplied by 100. Pembrolizumab improved the median overall survival (OS) from 11.6 months to 12.7 months as compared to chemotherapy in patients with a CPS of 10 or higher (104). KEYNOTE-119 motivated the Phase III, double-blind, randomized trials KEYNOTE-355 (NCT02819518) and KEYNOTE-522 (NCT03036488) (105, 106). In KEYNOTE-355, 847 patients with metastatic TNBC or previously untreated, locally recurrent inoperable breast cancer were randomized 2:1 to pembrolizumab and chemotherapy (specifically, paclitaxel, nab-paclitaxel, or gemcitabine plus carboplatin) or placebo and chemotherapy. The co-primary endpoints of this trial were overall survival and progression-free survival, and patients were stratified by PD-L1 expression. Pembrolizumab and chemotherapy improved the median progression-free survival from 5.6 months to 9.7 months for patients with high PD-L1$^+$ scores, providing the clinical rationale for using this combined therapy as a first-line treatment for metastatic TNBC (105). Furthermore, recent data supports that in patients with advanced TNBC with a CPS of 10 or more, the median overall survival increased from 16.1 months in the placebo-chemotherapy group to 23.0 months in the pembrolizumab-chemotherapy group. Similarly, in patients with a CPS of 1 or more, the median overall survival increased from 16 months in the placebo-chemotherapy group to 17.6 months in the pembrolizumab-chemotherapy group (107). In KEYNOTE-522, 1,174 patients with either previously untreated stage II breast cancer or stage III TNBC were randomly assigned 2:1 to receive neoadjuvant and adjuvant pembrolizumab with chemotherapy (either carboplatin or paclitaxel) or placebo with chemotherapy. All patients also received standard of care neoadjuvant doxorubicin–cyclophosphamide or epirubicin–

cyclophosphamide. KEYNOTE-522 had two primary endpoints of pathological complete response (pCR, defined as the absence of invasive disease) and event-free survival. Pembrolizumab and chemotherapy significantly increased the pCR compared to chemotherapy alone (51.2% to 64.8%), and these data were foundational to the FDA-approval for pembrolizumab use in combination with chemotherapy for this patient population (106). Thus, these trials have established pembrolizumab as an important treatment for both metastatic and non-metastatic TNBC. Additionally, preliminary data suggests atezolizumab, a humanized anti-PD-L1 IgG1 antibody, is active in PD-L1$^+$ locally advanced or metastatic TNBC; however, accelerated approval was later rescinded based on subsequent demonstration of limited clinical efficacy (108–110).

Clinical trials have also assessed the efficacy of ICIs in the management of HR$^+$ breast cancers. In the Phase 1b KEYNOTE-028 study, patients with ER$^+$, HER2$^-$ breast cancer with PD-L1$^+$ tumors received pembrolizumab and achieved an ORR of 12% (NCT02054806) (111). Furthermore, in the Phase 1b JAVELIN study, which tested the safety of avelumab, 43% of patients had HR$^+$, HER2$^-$ breast cancer and the ORR was 3% (NCT01772004) (112). The combination of pembrolizumab with chemotherapy (113) and cyclin-dependent kinase inhibitors (114) in this patient population has also not led to improvements in clinical outcomes. These trials highlight that ICIs have limited clinical activity in HR$^+$ breast cancer. The poor efficacy of ICIs for the treatment of HR$^+$ breast cancer may be, in part, due to the limited immune cell infiltrate in these tumors (115). The effects of ICIs are also currently being examined for the treatment of inflammatory breast cancer (116). A Phase II study (NCT02411656) is currently assessing the effects of pembrolizumab in metastatic or recurrent inflammatory breast cancer patients. Moreover, a Phase II study is currently examining the effect of pembrolizumab in combination with hormone therapy during or after radiotherapy for patients with HR$^+$ inflammatory breast cancer who did not respond to neoadjuvant chemotherapy alone (NCT02971748). Clinical trials are currently recruiting patients to assess the effect of ICIs in combination with chemotherapy (NCT03515798, NCT05093387) for the treatment of inflammatory breast cancer. Furthermore, a recent case study suggests clinical promise in combining pembrolizumab and chemotherapy for treating inflammatory breast cancer (117), while additional studies are underway to identify novel biomarkers for anti-PD-1 therapy in this disease, including peripheral T cell exhaustion and clonality markers (118). Moreover, beyond the scope of immunotherapy, current clinical trials are also examining combined therapies of radiotherapy and PARP inhibition for the treatment of inflammatory breast cancer (NCT03598257).

Adoptive cell transfer (ACT) therapy functions by transferring immune cells into cancer patients. Chimeric antigen receptor (CAR)-T cells enable improved T cell recognition of cancers via bypass of the common cancer immune evasion strategies of MHC downregulation and co-stimulation blockade (119). CAR-T cells are composed of single-chain variable fragments (scFv) fused to a costimulatory molecule which is fused to the intracellular CD3ζ signaling domain. The scFv recognizes antigen expressed on the surface of tumor cells. The CD3ζ immunotyrosine activation motif (ITAM) generates T cell activation signal 1 and the intracellular costimulatory domain generates signal 2. This allows CAR-T cells to become fully activated following recognition of peptide without the need for MHC presentation or additional co-stimulation. CAR-T cells, are engineered for each individual patient by first collecting T cells from the peripheral blood of cancer patients, transducing them *ex vivo* to express the appropriate CAR, expanding, and validating these CAR-T cells, and then reintroducing these cells into patients (120). CAR-T cell therapies are a powerful tool for treating cancer patients in that these modified cells can also persist in patients for extended periods, providing significant support to the immune systems of patients undergoing CAR-T cell therapy (119). Currently, there are six CAR-T cell therapies approved for clinical use in hematologic malignancies (121). However, there are no CARs currently approved for use in breast cancer. In developing CAR-T cell therapies, it is important that the antigens being targeted are enriched in the tumor and not the healthy tissues of patients to prevent "on-target off-tumor" adverse events (119, 120). Additionally, CARs are limited in that they can only be directed towards surface-expressed antigens. CAR-T cells have shown limited promise in solid tumors due to a variety of challenges, including poor T cell infiltration into tumors and immunosuppressive tumor microenvironments, although there is significant work underway to overcome these obstacles. For the treatment of breast cancer, preclinical studies are ongoing to examine the effects of CAR-T cell therapy on various tumor specific antigens including mucin 1 (MUC1), HER2, Lewis Y, mesothelin, and folate receptor alpha (FR-α) (119). Clinical trials are underway to assess the effects of CAR-T cell therapy for treating breast cancer, including CAR-T cells recognizing epithelial cell adhesion molecule (EpCAM) (NCT02915445), cleaved MUC1 (NCT04020575, NCT02792114), and ROR1 (NCT05274451). In addition to CAR-T cell therapy, tumor-infiltrating lymphocytes (TILs) are being examined as a type of adoptive cell transfer for the treatment of breast cancer. TIL therapy involves isolating tumor-infiltrating lymphocytes from patients, expanding them *ex vivo* with large amounts of IL-2 and other cytokines, then re-infusing them into the patient (122). Importantly, TIL therapy does not significantly modify the lymphocytes, and, unlike CAR-T therapy, TIL therapy assumes patient lymphocytes are able to recognize tumor neoantigens that exist in small quantities. Whole exome sequencing of breast cancer tissues revealed TNBC expresses more neoantigens than non-TNBC, suggesting TNBC patients may be good candidates for TIL

therapy (123). An ongoing clinical trial (NCT01174121) seeks to use TIL therapy in metastatic breast cancer, and preliminary data has shown tumor regression in a subset of patients (124). Collectively, these studies suggest the importance of ACT therapies as a potential therapeutic approach for breast cancer.

Despite promise of these therapies as single-agent therapies, additional studies are underway to find ways to increase patient responses to ACT by combining with radiotherapy or other forms of immunotherapy. For example, studies are currently examining combining radiotherapy with CAR-T cell therapy as a means to improve patient response to adoptive T cell transfer and overcome resistance in solid tumors (125). The effect of CAR-T cell therapy and internal radiotherapy are beginning to be evaluated for the treatment of liver metastases in breast cancer patients in a Phase 1b trial (NCT02416466), and results demonstrated some efficacy of the combination therapy with minimal toxicities (126). Moreover, a study recently examined the impact of combining infusion of *ex vivo* expanded NK cells into a human TNBC xenograft model with radiotherapy and found that the combination therapy significantly decreased primary tumor growth while minimizing toxicity (127). Combining CAR-T cell therapy with anti-PD-1 led to reduced tumor weight and improved CAR-T cell infiltration into the TME in a murine breast cancer model, demonstrating this combination therapy strategy may also be promising for treating breast cancer patients (128). While adoptive cell transfer strategies have shown some promise in the treatment of breast cancer in preclinical models, there has yet to be significant clinical efficacy in these solid malignancies.

In addition to immunotherapy, monoclonal antibodies (mABs) directed either towards tumor-specific antigens or mediators of oncogenic signaling have been used in breast cancer for more than twenty years. Monoclonal antibodies that target growth signaling can prevent cancer cell proliferation and ultimately lead to apoptosis. Additionally, these monoclonal antibodies can mediate antibody-dependent cellular cytotoxicity (ADCC), engaging the immune system to recognize cancer cells coated with antibodies bound to the surface of the cell (129). Trastuzumab is a clinically approved anti-HER2 mAb which improves the overall survival of patients with $HER2^+$ breast cancers (130). Pertuzumab targets a distinct epitope of HER2 and is another mAB used in the management of $HER2^+$ breast cancer. Consequently, mABs are a promising immunotherapy strategy for the treatment of breast cancer patients; however, these therapies are not efficacious for the treatment of triple negative disease that does not express the HER2 receptor. Interestingly, even in HER2-low expression tumors, the DESTINY-Breast04 trial recently demonstrated improved survival in women with metastatic HER2-low expressing tumors using the HER2 targeted therapy trastuzumab deruxtecan (131). Whether HER2-targeted therapies combined with ICIs will be even more effective remains an area of active clinical interest.

The impacts of immunotherapy and radiotherapy in breast cancer

Unfortunately, only 10% of patients with TNBC respond to immune checkpoint inhibitor monotherapy (85). Thus, there is an unmet need to develop more effective therapeutic strategies to improve patient responses to ICIs. One strategy to improve therapeutic efficacy of ICIs may be to combine immunotherapy with other effective breast cancer treatment modalities such as radiotherapy. For this review, we will primarily focus on combined approaches with immunotherapy—in the form of ICIs—and radiotherapy. However, other reviews have examined the effects of combining radiotherapy with cancer vaccines (132, 133), anti-HER2 therapies (134), or CAR-T cell therapy (135).

Radiotherapy is a mainstay breast cancer therapy first used to treat breast cancer patients in as early as the 1800s (136, 137). Clinical radiotherapy involves the delivery of fractionated doses of ionizing radiation to the affected cancerous breast tissue while sparing the surrounding benign tissues. This results in targeted disruption of tumor cells through induction of DNA damage, alterations in the cell cycle, and ultimately cancer cell death (138–140). Multiple randomized clinical trials have effectively established that radiotherapy reduces local recurrence in both invasive and noninvasive breast cancers, in addition to reducing the risk of breast cancer death (141–143). Specifically, after breast-conserving therapy, radiotherapy reduced the 10-year risk of a local or distant recurrence from 35.0% to 19.3% and reduced the 15-year breast cancer death risk from 25.2% to 21.4% (141). Despite such benefits, radiotherapy can have pleotropic effects on the immune system. For instance, large field and total body irradiation, which is clinically indicated in the management of hematologic malignancies (144), is used to induce profound lymphopenia. Meanwhile, localized radiotherapy may promote antitumor immune responses. An early study in the 1950s first described a phenomenon known as the "abscopal effect" that showed a correlation between the immune system and localized radiotherapy (145). The abscopal effect (in Latin, *ab*: away from, *scopus*: target) postulates that radiotherapy delivered to one part of the body can reduce tumor size systemically, in regions outside of where radiation is delivered (145–147). Literature suggests that this phenomenon occurs in part through the immune system (148–150), and immunotherapy is believed to promote abscopal effects (151). However, studies show that the abscopal effect is rare (146, 152) and unlikely to be broadly applicable clinically. An additional hallmark study of the late 1970s further expanded upon the connections between radiotherapy and the immune system to show that the efficacy of RT is dependent upon the immune system (153). Significantly, radiotherapy and immunotherapy provide synergistic tumor control when combined in preclinical

models (154, 155). In fact, radiotherapy can sensitize even poorly immunogenic cancers including pancreatic cancer (156), head and neck squamous cell carcinoma (157), and breast cancer (158) to ICIs—which emphasizes the promise of combined radiotherapy and immunotherapy treatment modalities.

Notably, the effects of combination radiotherapy with ICIs in breast cancer models have been explored. A crucial study by Demaria et al. in 2005 illustrated the effects of combined radiotherapy and immune checkpoint inhibition in murine models of breast cancer (159). Specifically, combined local radiation with anti-CTLA-4 immune checkpoint inhibition in a poorly immunogenic murine breast cancer model resulted in prolonged survival and decreased lung metastases (159). Furthermore, later studies suggest that fractionated radiotherapy—as opposed to single-dose radiotherapy—induces systemic antitumor effects in combination with anti-CTLA-4 treatment in murine breast cancer models (160). These studies mutually suggest that radiotherapy combined with anti-CTLA-4 therapy promotes antitumor immunity in preclinical breast cancer models—providing rationale for combined use in the clinic (159, 160). Studies suggest that these effects of combined therapy depend on the immune cells present. In fact, in murine breast cancer models, the effects of radiotherapy and anti-CTLA-4 immunotherapy are dependent upon the presence of invariant natural killer T cells (161). Radiotherapy has also been found to induce CXCL16 release by breast cancer cells to attract effector T cells in murine models (162). Moreover, it has been proposed that the synergistic effects of radiotherapy and immune checkpoint inhibitors depend upon MTOR signaling (163) and tumor heterogeneity (164) in murine breast syngeneic models. While these studies display the synergistic effects of combined radiotherapy and ICIs for the treatment of breast cancer, more research is warranted to further understand the implications of these combined therapies.

Radiotherapy has been found to improve innate antitumor responses, deplete immunosuppressive cell types, and augment adaptive immune responses in combination with PD-1 blockade (165). Functionally, it is believed that radiotherapy activates the innate immune system via a process known as cross priming (166). As radiotherapy induces tumor cell death, these cells release neoantigens (167) that may be phagocytosed by nearby APCs. APCs can then activate the adaptive immune system, specifically $CD8^+$ effector T cells, to kill cancer cells (166, 168). Consequently, the efficacy of radiotherapy specifically depends upon the presence of these cytotoxic cells (169). Interestingly, combining radiotherapy with immunotherapy has also been shown to jointly promote tumoral lipid oxidation-dependent ferroptosis via SLC7A11 (170). Radiotherapy can further induce the DNA damage response often associated with the synergistic effects of radiotherapy and immunotherapy. Targeting ataxia telangiectasia mutated (ATM)—a kinase that plays a role in the DNA damage response to double stranded DNA breaks induced by radiotherapy—sensitizes pancreatic cancer to ICIs, providing a mechanistic link for this observed synergy (171). Additionally, inhibition of DNA-dependent protein kinase (DNA-PK) has been shown to synergize with radiotherapy and modulate the immune system in pancreatic cancer models by increasing cytosolic double-stranded DNA and type 1 interferon signaling. Moreover, combined anti-PD-L1 with radiotherapy and DNA-PK inhibition further potentiates antitumoral immunity in preclinical pancreatic cancer models (172). These studies emphasize the complexity underlying the synergistic effects of combined radiotherapy and immunotherapy and can importantly be extended into the breast cancer space to determine the underlying mechanisms of such approaches.

While the precise mechanisms underlying the synergistic effects of radiotherapy and immunotherapy are not well established, studies have suggested that the cGAS/STING pathway may contribute to these combined effects as summarized in Figure 2. As discussed above, the cGAS/STING pathway plays a critical role in the antitumoral immune response by inducing interferon signaling following the recognition of cytosolic DNA (76). It is also well established that radiotherapy induces the cGAS/STING pathway to activate interferon signaling (173, 174). Importantly, interferon signaling can promote antitumor T cell responses (76, 81). It was also recently discovered that STING regulates radiotherapy sensitivity *in vivo* in part through the production of reactive oxygen species (ROS) (175). In human breast cancer cell lines and murine breast cancer models, inhibition of ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1), a hydrolase of cGAMP, was recently found to increase extracellular cGAMP levels and synergize with radiotherapy to prevent tumor growth. The radiotherapy-induced increased production of extracellular cGAMP was subsequently sensed by STING and promoted the infiltration of dendritic cells and cytotoxic T cells into the tumor. Furthermore, depletion of extracellular cGAMP abrogated this immune cell infiltration in breast cancer models, suggesting that these radiation-induced immune effects are dependent upon the presence of extracellular cGAMP and the cGAS/STING pathway (176). Mechanistically, in human breast cancer cell lines, it has also been shown that the cGAS/STING pathway is required for interferon activation induced by combined radiotherapy and anti-CTLA-4 immune checkpoint inhibition (177). In addition to studying the effects of combined radiotherapy with anti-CTLA-4 treatments, preclinical studies suggest that radiotherapy and anti-PD-1/L1 therapy synergistically potentiate antitumor immunity in murine breast cancer models (178–180). Specifically, this antitumor immunity occurs in the form of reduced accumulation of myeloid-derived suppressor cells in the tumor (178), promotion of $CD8^+$ T cell expansion (179), expansion of antigen-specific T cell responses (180), and reduction in tumor growth in non-irradiated tumor sites (181). Importantly, additional work is required to understand the contribution of other innate

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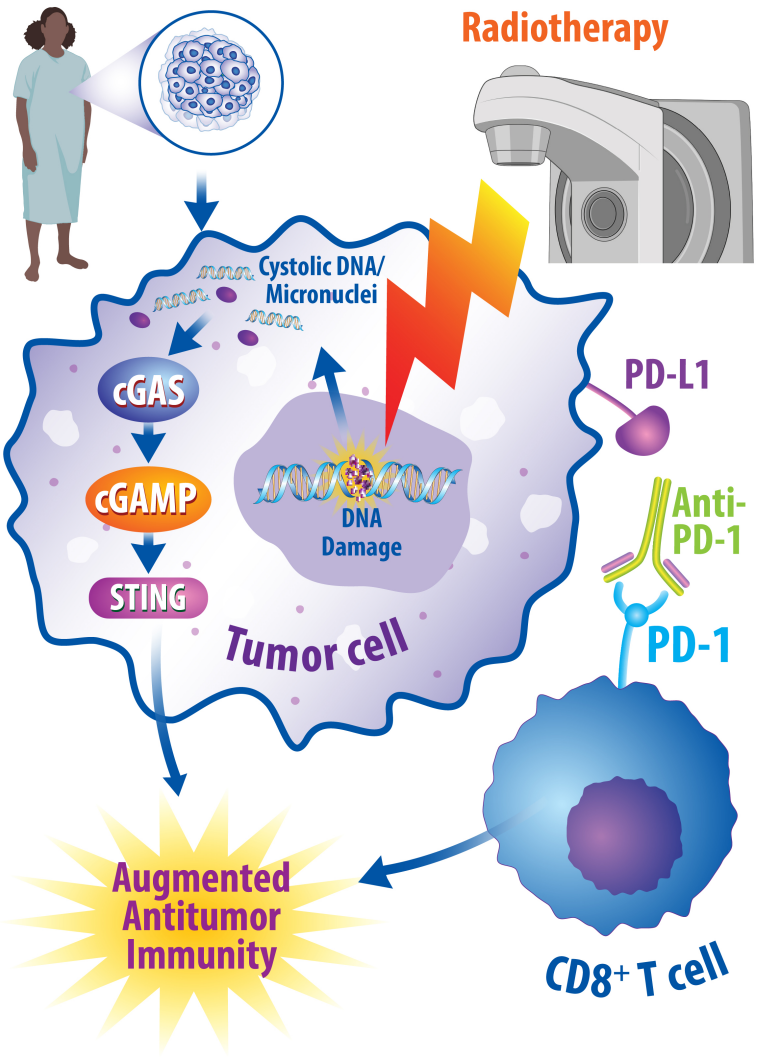

FIGURE 2
Radiotherapy and Immunotherapy Synergistically Promote Antitumor Immune Responses. One potential combined therapeutic approach is to combine radiotherapy with immune checkpoint inhibition. Radiotherapy promotes DNA damage within cancerous cells, which can consequently be recognized by cGAS and lead to activation of the cGAS/STING pathway to promote antitumor immunity through interferon signaling. Likewise, immune checkpoint inhibitors, such as anti-PD-1 monoclonal antibodies, can modulate an augmented antitumor immune response by turning off immune checkpoints. Under normal conditions, these checkpoints result in a decrease in the cytotoxic abilities of T cells; however, when turned off, this enhances the cytotoxic effects of T cells and results in enhanced antitumoral effects. Numerous preclinical and clinical studies suggest synergy exists in combining radiotherapy and immune checkpoint inhibitors in breast cancer patients and studies are currently underway to determine the best ways oncologists can implement these interactions.

immune sensors and immune signaling pathways governing the synergistic interactions between radiotherapy and immunotherapy in breast cancer.

STING-dependent cytosolic sensing of DNA has been found to contribute to innate immunostimulatory responses following radiotherapy (173). However, there are also other pathways that link DNA damage to innate immune signaling. Nucleic acids can also be sensed by retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), Nod-like receptors (NLRs), and Toll-like receptors (TLRs) (182). Furthermore, the recognition of cytosolic DNA following viral infection has been found to activate a type I interferon response independently from toll-like receptors—further adding to the complexity of such pathways (183). When RIG-1 engages single and double stranded RNA, RIG-I complexes with mitochondrial antiviral-signaling protein (MAVS) and activates the TBK1 complex which ultimately promotes interferon signaling (184). In breast cancer, RIG-I agonists have been found to induce inflammatory transcription factors, type I interferons, and lymphocyte-recruiting chemokines (185).

The DHA-dependent protein kinase (DNA-PK) which, is required for nonhomologous end joining (NHEJ), also serves as another STING-independent innate immune sensor. DNA-PK can be activated by viral DNA leading to IRF3 and IRF7 dependent innate immune sensing (186). Interestingly, inhibition of DNA-PK has also been shown to augment radiation-induced interferon signaling in an RNA Polymerase III, Rig-I, and MAVS dependent fashion (172). TLRs have also been found to contribute to innate immune signaling in breast cancer (187). Specifically, Toll-like Receptor 9 (TLR9) can detect DNA released by tumor cells following chemotherapy leading to enhanced antigen presentation and improved antitumor immune responses (188). Consequently, TLR9 agonists have been examined as potential cancer therapeutics delivered in combination with other therapies (189). Combined TLR9 agonism and radiotherapy promotes systemic antitumor immunity in models of metastatic lung cancer and colon cancer (190). In a preclinical breast cancer mouse model resistant to PD-1, TLR9 agonists increased infiltration of $CD8^+$ T cells into tumors and promoted IFN signaling (191). Collectively, these studies articulate the breadth of the pathways linking DNA damage and innate immune signaling.

While preclinical studies have illustrated the importance of combining radiotherapy with immunotherapy, clinical trials are also underway to assess these combined approaches. The single-arm Phase II clinical trial (NCT02730130) assessed the combination of pembrolizumab and radiotherapy in patients with metastatic TNBC and observed a 17.6% overall response rate, with minor adverse events as a result of combined therapy (192). In this study, radiotherapy was delivered at 30 Gy at five daily fractions to both PD-$L1^+$ and PD-$L1^-$ patients. Of the 9 patients observed through this trial, 3 patients with baseline PD-$L1^+$ expression received a complete, durable response, which was similar to responses in studies where all patients had PD-$L1^+$ metastatic TNBC (192). Phase II trials have also evaluated the combination of pembrolizumab and radiotherapy in patients with HR^+, $HER2^-$ heavily pretreated metastatic breast cancer (NCT03051672). This trial observed that pembrolizumab delivered prior to palliative radiotherapy (20 Gy in 5 fractions) did not result in any objective responses (193). These studies suggest that combined radiotherapy and immunotherapy may be more efficacious for patients with triple negative disease as opposed to HR^+ breast cancers; however, additional research is necessary to fully determine the mechanisms of resistance in luminal breast cancer to immunotherapy.

Clinical trials are underway to study the effects of radiotherapy and ICIs in patients with breast cancer. These trials are summarized in Table 1. In addition to examining the effects of combined ICIs with radiotherapy in metastatic TNBC as discussed above (NCT02730130), such clinical trials are also examining combined therapies in metastatic HR^+ breast cancer (NCT04756505). Importantly, many clinical trials are aimed at determining the survival outcome of combined therapies, as well as understanding the immune-enhancing effects of radiotherapy and immunotherapy in breast cancer patients. For example, preoperative delivery of radiation boost is being examined in combination with ICIs to enhance ICI efficacy in operable breast cancer (NCT04454528) and in TNBC and $HR^+/HER2^-$ breast tumors (NCT03366844) (194). Another study is assessing the effects of ICIs on the tumor microenvironment of TNBC patients prior to intraoperative radiotherapy (IORT) (NCT02977468). Trials are also examining the effects of novel therapeutic immune agents, including an antagonistic OX40 monoclonal antibody (NCT01862900) and the STING agonist TAK-676 (NCT04879849) combined with radiotherapy for the treatment of breast cancer patients. While many studies are examining the effects of the ICI pembrolizumab, studies are also examining the effects of the ICI nivolumab in combination with radiotherapy for the treatment of metastatic breast cancer brain metastases (NCT03807765) and patients with TNBC (NCT03818685). Together, these studies will help understand the effects of combined radiotherapy and ICIs in breast cancer patients and provide clinical rationale for combining these therapeutics with other available therapies such as chemotherapy.

The clinical and preclinical promise of combining immunotherapy, radiotherapy, and chemotherapy in breast cancer

Importantly, one potential multimodal therapeutic approach is combining immunotherapy, radiotherapy, and chemotherapy. This approach is summarized in Figure 3. The combination of chemotherapy, radiotherapy, and surgery is the standard of care for breast cancer treatment, while numerous studies support the therapeutic potential of combining radiotherapy with chemotherapy for treating breast cancer patients. The evidence supporting the integration of radiotherapy with chemotherapy has been more extensively reviewed elsewhere (140, 195, 196). Importantly, many chemotherapies function by inducing DNA damage, consequently resulting in synergistic effects when combined with radiotherapy in the preclinical and clinical setting (140, 197). Cytotoxic chemotherapeutic agents—such as platinums, taxanes, and antimetabolites—have been found to promote synergistic, radiosensitizing effects in breast cancer (198). Platinum chemotherapies—such as cisplatin and carboplatin—are alkylating agents delivered to breast cancer patients that bind to and crosslink DNA to inhibit proper replication, leading to the formation of double stranded breaks in the DNA (199, 200). Consequently, when platinum therapies are combined with radiotherapy, studies support that this promotes radiosensitization in various subsets of breast cancer, including metastatic IBC (201) and early-stage TNBC (202). Taxanes—such as paclitaxel and docetaxel—inhibit microtubule

TABLE 1 Trials examining the effects of combined radiotherapy and immune checkpoint inhibitors.

ClinicalTrials.gov identifier	Study title	Conditions	Therapeutic agent(s)	Radiotherapy	Phase and patients	Status (at time of publication)
NCT02730130	A Multicenter Single Arm Phase II Study to Assess the Efficacy of Pembrolizumab Plus Radiotherapy in Metastatic Triple Negative Breast Cancer Patients	- Breast cancer - Metastatic triple negative breast cancer	- Pembrolizumab (200 mg intravenous) (anti-PD-1)	- 30 Gy radiotherapy delivered in 5, 6 Gray (Gy) × 5 fractions	- Phase II - 17 participants - Clinical trial	- Active, not recruiting
NCT03051672	A Phase II Study Of Pembrolizumab In Combination With Palliative Radiotherapy For Metastatic Hormone Receptor Positive Breast Cancer	- Metastatic breast cancer	- Pembrolizumab (200 mg intravenous)	- Palliative radiotherapy, 20 Gy in × 5 fractions	- Phase II - 8 participants - Clinical trial	- Terminated
NCT04756505	REINA: A Phase I Study of Radiation Enhanced IL 12-Necrosis Attraction in Hormone Receptor Positive, HER2 Negative Metastatic Breast Cancer Patients	- Stage IV breast cancer - Hormone receptor positive breast adenocarcinoma - Metastatic/ metastatic HER2- breast carcinoma - Stage IV breast cancer	- Bintrafusp Alfa (intravenous) - Immunocytokine NHS-IL12 (subcutaneous)	- Radiotherapy	- Phase I - 20 participants - Clinical trial	- Recruiting
NCT04454528	Preoperative Use of Radiation Boost to Enhance Effectiveness of Immune Checkpoint Blockade Therapy in Operable Breast Cancer	- Operable breast cancer	- Pembrolizumab (200 mg intravenous)	- Hypofractionated radiotherapy boost of 7 Gy x 1 fraction	- Phase 1b/2 - 27 participants - Clinical trial	- Recruiting
NCT03366844	Preoperative Combination of Pembrolizumab and Radiation Therapy in Patients With Operable Breast Cancer	- Breast cancer	- Pembrolizumab	- Radiotherapy boost, 8 Gy x 3 fractions	- Phase I and II - 60 participants - Clinical trial	- Active, not recruiting
NCT02977468	Effects of MK-3475 (Pembrolizumab) on the Breast Tumor Microenvironment in Triple Negative Breast Cancer With and Without Intra-operative RT: a Window of Opportunity Study	- Triple negative breast cancer	- Pembrolizumab (MK-3475) (intravenous)	- Intraoperative radiotherapy (IORT) on day of surgery	- Phase I - 15 participants - Clinical trial	- Recruiting
NCT01862900	Phase I/II Study of Stereotactic Body Radiation Therapy to Metastatic Lesions in the Liver or Lung in Combination With Monoclonal Antibody to OX40 (MEDI6469) in Patients With Progressive Metastatic Breast Cancer After Systemic Therapy.	- Metastatic breast cancer - Lung metastases -Liver metastases	- Biological: MEDI6469 (anti-OX40) (0.4 mg/kg intravenous)	- Stereotactic body radiotherapy (SBRT) - Three arms: 15 Gy, 20 Gy, or 25 Gy SBRT	- Phase I/II - 14 participants - Clinical trial	- Completed
NCT04879849	An Open-label, Phase I, Dose-escalation Study to Evaluate the Safety and	- Triple negative breast neoplasms - Non-small-cell lung	- Pembrolizumab (200 mg intravenous)	- Image-guided radiotherapy	- Phase I - 65	- Recruiting

(Continued)

TABLE 1 Continued

ClinicalTrials.gov identifier	Study title	Conditions	Therapeutic agent(s)	Radiotherapy	Phase and patients	Status (at time of publication)
	Preliminary Antitumor Activity of TAK-676 With Pembrolizumab Following Radiation Therapy in the Treatment of Non-small-cell Lung Cancer, Triple-negative Breast Cancer, or Squamous-cell Carcinoma of the Head and Neck That Has Progressed on Checkpoint Inhibitors	carcinoma - Squamous cell carcinoma of head and neck	- TAK-676 (0.2 mg and above intravenous)		participants - Clinical trial	
NCT03807765	Phase Ib Study of Stereotactic Radiation and Nivolumab in the Management of Metastatic Breast Cancer Brain Metastases	- Metastatic breast cancer brain metastases	- Nivolumab (anti-PD-1) (480 mg intravenous)	- Stereotactic radiosurgery delivered to brain metastases	- Phase I - 14 participants - Clinical trial	- Active, not recruiting
NCT03818685	A Multicenter, Randomised, Open-label Phase II Study to Evaluate the Clinical Benefit of a Post-operative Treatment Associating Radiotherapy + Nivolumab + Ipilimumab Versus Radiotherapy + Capecitabine for Triple Negative Breast Cancer Patients With Residual Disease After Neoadjuvant Chemotherapy	- Breast cancer - Triple negative breast neoplasms	- Nivolumab (360 mg intravenous) - Ipilimumab (anti-CTLA4) (1mg/kg intravenous) - Capecitabine (1000mg/m2)	- Radiotherapy delivered per standard practice	- Phase I - 114 participants - Clinical trial	- Active, not recruiting

function, inducing cell cycle arrest at the G2/M Phase, consequently leading to cancer cell death (203). Combining taxane chemotherapy with radiotherapy has been examined in several settings. Combined paclitaxel and radiotherapy led to a 34% complete response in patients with early-stage breast cancer (204). When tested in patients with locoregional recurrence, radiotherapy combined with taxanes or with taxanes combined with cisplatin found increased recurrence-free survival regardless of whether cisplatin was added (205). In the context of locally advanced breast cancer, paclitaxel treatment with concurrent radiotherapy improved disease-free survival and overall survival (206). Antimetabolite chemotherapeutic agents —such as fluoropyridines or gemcitabine—are well-established radiosensitizers that function by mimicking natural metabolites found in the body to become incorporated into DNA or RNA, leading to DNA damage (207, 208). These antimetabolite therapeutics have also been examined in combination with radiotherapy. When treating breast cancer chest wall recurrences with combined gemcitabine and radiotherapy, 100% locoregional control was achieved, although normal tissue toxicity limits this combination clinically (209). Chemotherapy resistant breast cancer treated with capecitabine and radiotherapy was retrospectively analyzed to find that there were no increased toxicities associated with the combination therapy (210). Patients with advanced, non-TNBC treated with capecitabine and radiotherapy led to 73% partial or complete response (211). Collectively, these studies provide the rationale for combining chemotherapy with radiotherapy for the treatment of breast cancer patients.

Chemotherapy, like radiotherapy, has pleotropic effects on the immune system. It is well established that chemotherapy is immunosuppressive, rendering patients undergoing treatment

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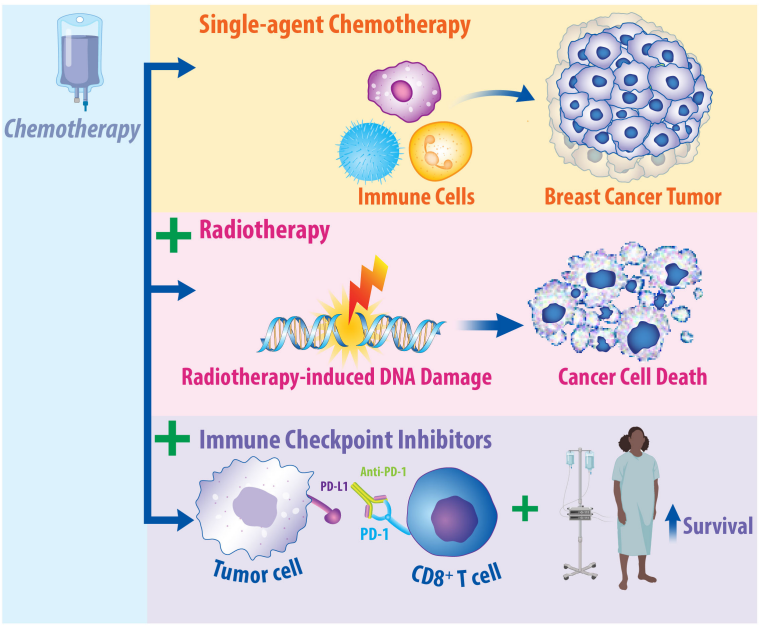

FIGURE 3
Chemotherapy Has Immunomodulatory Effects on the Tumor Microenvironment and May Promote Synergy in Combination with Radiotherapy and Immune Checkpoint Inhibitors. Chemotherapy is a standard of care therapy for the treatment of breast cancer and has significant implications on the immune response. Studies suggest that single-agent chemotherapy can recruit immune cells to the microenvironment of breast cancer tumors. Additionally, in breast cancer patients, response to chemotherapy is dependent upon the presence of tumor-infiltrating lymphocytes. When chemotherapy is combined with radiotherapy, this can induce radiosensitization in preclinical and clinical models, resulting in enhanced cancer cell death. Clinical promise may exist in combining immune checkpoint inhibitors, radiotherapy, and chemotherapy for the treatment of breast cancer. When chemotherapy is combined with immunotherapy, this enhances its efficacy and increases patient survival. Clinical trials are currently underway to ascertain the effects of combined approaches in breast cancer patients.

more susceptible to infection (212). However, chemotherapy—particularly in the neoadjuvant setting—has also been found to result in pro-inflammatory, antitumor effects. Neoadjuvant chemotherapy induces immune responses in breast cancer patients, including increasing concentrations of TILs and $CD8^+$ T cells (213, 214). Furthermore, the immune response induced by neoadjuvant chemotherapy predicts survival of breast cancer patients and may prime tumors for treatment with immunotherapy (213, 214). The presence of TILs is predictive of response to chemotherapy in breast cancer, further supporting the complex interaction between the immune system and chemotherapy (215). DNA damage immune response signatures have also been confirmed as prognostic biomarkers in TNBC patients treated with adjuvant doxorubicin and cyclophosphamide (216). Additionally, activation of immune responses mediated by the cGAS/STING pathway have been found to predict patient response to neoadjuvant chemotherapy (217). Collectively, these studies support the complex interactions that exist between chemotherapy and the immune system in breast cancer patients. Moreover, these studies also emphasize the importance of further understanding these complex interactions in both preclinical and clinical breast cancer models.

Many clinical trials are currently evaluating the combination of chemotherapy and immunotherapy in breast cancer patients (218). While the focus of this review is trimodal combinations, primarily with radiotherapy, immunotherapy, and additional agents, others have extensively reviewed the effects of combined chemotherapy and immunotherapy (218–220). The I-SPY2 trial (Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging And Molecular Analysis 2) is one such important trial examining ICIs in combination with chemotherapy. This randomized, adaptive clinical trial aims to assess the effects of novel agents combined with standard therapies for stage II or stage III breast cancer patients (NCT01042379) with high-risk MammaPrint scores, a gene signature used to predict breast cancer patient clinical outcomes (221, 222). The primary endpoint for I-SPY 2 is pCR. One arm of I-SPY 2 examined the therapeutic effects of combining pembrolizumab with neoadjuvant chemotherapy in approximately 250 patients. Pembrolizumab more than

doubled the pCR rate in the HR^+, HER2-negative subset (13% to 30%) as well as the TNBC subset (22% to 60%) (223). Jointly, these studies support the clinical promise of combining chemotherapy and immunotherapy.

Clinical trials are currently underway to assess the effectiveness of combining chemotherapy with immunotherapy and/or radiotherapy as summarized in Table 2. Trials are currently evaluating the effects of preoperative pembrolizumab combined with neoadjuvant chemotherapy (paclitaxel, carboplatin, cyclophosphamide, doxorubicin, and/or capecitabine) for TNBC or HR^+, $HER2^-$ breast cancer (NCT04443348), in addition to radiotherapy combined with chemotherapy (nab-paclitaxel and paclitaxel) and pembrolizumab in PD-$L1^+$ TNBC (NCT05233696). Moreover, a Phase III trial is examining the effects of adjuvant pembrolizumab in combination with radiotherapy on disease-free survival in TNBC patients (NCT02954874). The priming effects of radiotherapy on breast cancer patients prior to neoadjuvant chemotherapy are also being examined to further understand the role of the immune response following radiotherapy (NCT03978663). The TONIC trial is a Phase II, randomized, open-label trial examining whether chemotherapy or radiotherapy prior to immune checkpoint inhibition with nivolumab induces an inflamed tumor microenvironment in metastatic TNBC patients (NCT02499367). In this study, chemotherapy resulted in the most significant patient responses, where cisplatin treated patients had an ORR of 23% and doxorubicin treated patients had an ORR of 35% in addition to an increase in immune cell infiltration. Interestingly, patients pretreated with radiotherapy did not see an increase in immune cell infiltration in the form of $CD8^+$ T cells and TILs. However, results from this study suggest that delivering chemotherapy prior to PD-1/PD-L1 inhibition can prime tumors for response to immune checkpoint inhibition (224). These studies highlight the clinical promise of combining chemotherapy, ICIs, and radiotherapy for treating breast cancer patients, and the important research underway to understand the clinical effects of these combined approaches.

The clinical and preclinical promise of combining immunotherapy, radiotherapy, and PARP inhibitors

Another approach for improving the efficacy of immunotherapy exists in combining immunotherapy and radiotherapy with DNA damage inhibitors, as summarized in Figure 4. Poly(ADP-ribose) polymerase (PARP) proteins help mediate effective DNA damage responses, and PARP inhibitors hold promise for the treatment of breast cancer by inhibiting this repair process (225). Mechanistically, PARP proteins are recruited to sites of damaged DNA and complete a posttranslational modification termed PARylation (225, 226). PARylation recruits DNA repair proteins to induce repair of single-strand breaks (SSBs) (140, 225, 226). PARP inhibitors prevent the accumulation of DNA damage repair proteins, resulting in increased DNA double-strand breaks (DSBs) (225, 226). Approximately 5% of breast cancer patients carry a deleterious mutation in the Breast Cancer (*BRCA1/2*) genes, which are required for proper DNA damage repair and correlate with increased risk of developing breast cancer (225, 227, 228). In patients with BRCA deleterious mutations, PARP inhibitors cause "synthetic lethality," wherein loss of multiple DNA repair pathways results in synergistic tumor cell death (229). The PARP inhibitors olaparib and talazoparib are currently FDA-approved for the treatment of HER2-negative, BRCA-mutated breast cancer (225). Combining PARP inhibitors with radiotherapy can promote breast cancer cell death. Mechanistically, radiotherapy induces DNA damage, while PARP inhibitors prevent DNA damage repair (140). PARP1 inhibition was found to radiosensitize breast cancer models to ionizing radiotherapy preclinically (230, 231). Thus, there is a strong preclinical rationale to combine radiotherapy and PARP inhibitors for the treatment of breast cancer clinically.

Clinical trials have begun to evaluate the combination of PARP inhibitors with radiotherapy and/or immunotherapy, which are summarized in Table 3. The PARP inhibitor veliparib has been combined with radiotherapy for breast cancer patients with inflammatory disease or locoregionally recurrent disease (NCT01477489) and is currently being examined in breast cancer patients in combination with preoperative radiotherapy (NCT01618357). The PARP inhibitor rucaparib is also currently being investigated in combination with radiotherapy for TNBC patients who do not respond to chemotherapy (NCT03542175). Furthermore, studies are also combining olaparib and radiotherapy (NCT03109080, NCT03598257). For example, the RADIOPARP Phase I trial examined the effects of olaparib combined with 50 Gy radiotherapy for patients with inflammatory, metastatic, or locoregionally advanced TNBC (NCT03109080) (232). While trimodality therapy can cause an increase in acute self-limited adverse events, overall, the combination is well tolerated (233). However, more research is needed to continue monitoring potential toxicities caused by this treatment modality in patients over time (232, 234).

In addition to contributing to radiation-induced DNA damage, studies also suggest that PARP inhibition regulates antitumor immunity (226). Many studies suggest a connection between *BRCA* mutations, PARP inhibition, and the immune system in breast cancer. In *BRCA*-deficient TNBC models, PARP inhibition with olaparib induces a $CD8^+$ T cell response *in vivo* through the activation of the cGAS/STING pathway (235). PARP inhibition also modulates immunosuppressive

TABLE 2 Trials currently assessing combined immune checkpoint inhibition, chemotherapy, and/or radiotherapy.

ClinicalTrials.gov identifier	Study title	Conditions	Therapeutic agent(s)	Radiotherapy	Phase and patients	Status (at time of publication)
NCT04443348	P-RAD: A Randomized Study of Preoperative Chemotherapy, Pembrolizumab and No, Low or High Dose RADiation in Node-Positive, HER2-Negative Breast Cancer	- Triple negative breast cancer - Hormone receptor positive breast cancer - Biopsy-proven, positive lymph nodes	- Pembrolizumab - Paclitaxel - Carboplatin - Cyclophosphamide - Doxorubicin - Capecitabine	- Radiotherapy boost	- Phase II - 120 Participants - Clinical trial	- Recruiting
NCT05233696	Phase II Study of Radiotherapy in Combination With Chemotherapy and Immunotherapy in Patients With PD-L1-Positive Metastatic Triple-Negative Breast Cancer	- Triple negative breast cancer - Locally advanced breast cancer - Unresectable breast carcinoma - Metastatic breast cancer	- Nab-paclitaxel (100 mg/m2 intravenous) - Paclitaxel (80 mg/m2 intravenous) - Pembrolizumab (200 mg)	- One to four metastatic sites will be treated at the discretion of the radiation oncologist	- Phase II - 29 participants - Clinical trial	- Recruiting
NCT03978663	Evaluating the Use of Stereotactic Radiation Therapy Prior to Neoadjuvant Chemotherapy for High-risk Breast Carcinoma (a SIGNAL Series Clinical Trial): Three Fraction Radiation to Induce Immuno-Oncologic Response (TRIO Trial)	- High risk cancer - Locally advanced breast cancer	- Neoadjuvant anthracycline and taxane based chemotherapy	- Neoadjuvant radiotherapy - 8 Gy x 3 fractions, with a fall off dose of 4 Gy x 3 fractions	- N/A - 40 participants - Clinical trial	- Recruiting
NCT02499367	Adaptive Phase II Randomized Non-comparative Trial of Nivolumab After Induction Treatment in Triple-negative Breast Cancer (TNBC) Patients: TONIC-trial	- Breast cancer	- Nivolumab (3 mg/kg) - Low dose doxorubicin (15 mg) - Cyclophosphamide (50 mg oral) - Cisplatin (40 mg/m2)	- Radiotherapy; 20 Gy to metastatic lesions	- Phase II - 84 participants - Clinical trial	- Active, not recruiting
NCT02954874	A Randomized, Phase III Trial to Evaluate the Efficacy and Safety of Pembrolizumab (MK-3475) as Adjuvant Therapy for Triple Receptor-Negative Breast Cancer With >/= 1 CM Residual Invasive Cancer or Positive Lymph Nodes (ypN1mi, ypN1-3) After Neoadjuvant Chemotherapy	- Invasive breast carcinoma - Stage 0-III breast cancer - Triple negative breast carcinoma	- Pembrolizumab (intravenous)	- Radiotherapy within 12 weeks post treatment or 12 weeks of last breast cancer operation	- Phase III - 1155 participants - Clinical trial	- Active, not recruiting
NCT02971748	A Phase II Study of Anti-PD-1 (Pembrolizumab) in Combination With Hormonal Therapy During or After Radiation in Patients With Hormone Receptor (HR)-Positive Localized Inflammatory Breast Cancer (IBC) Who Did Not Achieve a Pathological Complete Response (pCR) to Neoadjuvant Chemotherapy	- Stage III breast cancer - Breast inflammatory carcinoma	- Pembrolizumab (intravenous)	- Radiotherapy	- Phase II - 37 participants - Clinical trial	- Active, not recruiting
NCT03515798	A Prospective Multicenter Open-label, Randomized Phase II Study of Pembrolizumab in Combination With	- HER2-negative, inflammatory breast cancer	- Epirubicine-cyclophosphamide (EC) paclitaxel chemotherapy	- None	- Phase II - 81 participants - Clinical trial	- Recruiting

(Continued)

TABLE 2 Continued

ClinicalTrials.gov identifier	Study title	Conditions	Therapeutic agent(s)	Radiotherapy	Phase and patients	Status (at time of publication)
	Neoadjuvant EC-Paclitaxel Regimen in HER2-negative Inflammatory Breast Cancer.		- Pembrolizumab (MK3475) (intravenous)			
NCT05093387	A Pilot Study of SGT-53 With Carboplatin and Pembrolizumab in Metastatic Triple Negative Inflammatory Breast Cancer	- Metastatic, triple negative inflammatory breast cancer	- Carboplatin (intravenous) - Pembrolizumab (intravenous) - SGT-53 (Transferrin Receptor-Targeted Liposomal p53 cDNA) (intravenous)	- None	- Phase I - 9 participants - Clinical trial	- Not yet recruiting

macrophages in the TME of *BRCA1*-associated TNBC models and treating these models with CSF-1R antibodies combined with PARP inhibitors overcomes PARP inhibitor acquired resistance (236). Moreover, knock down of BRCA2 in human breast cancer cells activates the cGAS/STING pathway (237). Surprisingly, PARP inhibition in some BRCA proficient ovarian and colorectal cancer models can also activate immune responses through the STING pathway (238). Moreover, combining PARP inhibitors with anti-PD-L1 improves tumor control in preclinical breast cancer models (239). These

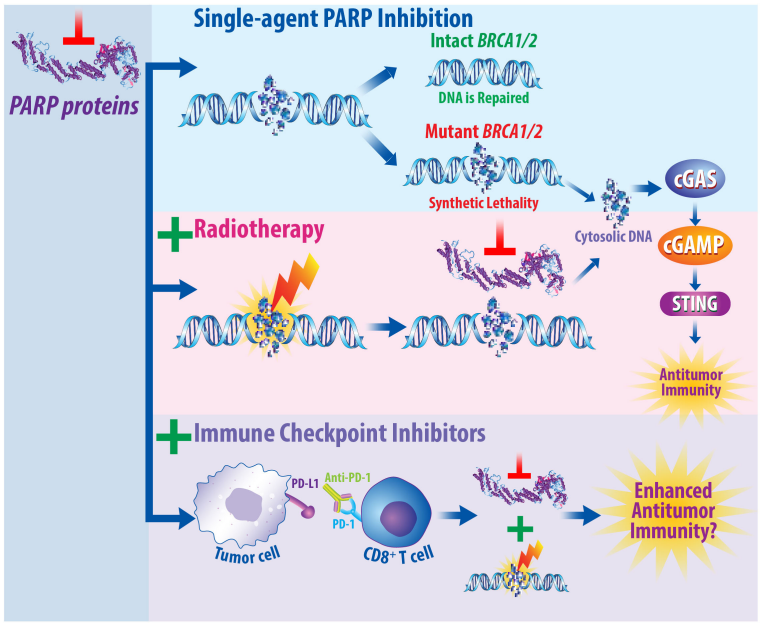

FIGURE 4
PARP Inhibitors Prevent DNA Damage Repair and May Synergize with Both Radiotherapy and Immune Checkpoint Inhibition. Mechanistically, PARP proteins are recruited to regions of DNA damage to assist in the repair of single-strand breaks. When PARP proteins are inhibited, this prevents proper DNA repair and promotes the accumulation of double-strand breaks. In patients that express the *BRCA1/2* genes, this damage can be repaired; however, in patients with a deleterious *BRCA1/2* mutation, this results in synthetic lethality due to the absence of multiple DNA repair pathways. It is well established that radiotherapy induces DNA damage. When radiotherapy is combined with PARP inhibitors, this prevents DNA damage repair in BRCA mutant cancers. Furthermore, the DNA damage induced by radiotherapy that is then not repaired following PARP inhibition can result in the production of cytosolic DNA molecules. As single agents, immune checkpoint inhibitors illicit immune responses by turning off immune checkpoints, resulting in pro-inflammatory, antitumor effects. Studies are currently underway to determine whether combined PARP inhibition, radiotherapy, and immune checkpoint inhibition will promote enhanced antitumor immunity and be efficacious for the treatment of breast cancer patients.

TABLE 3 Clinical trials assessing the effects of PARP inhibitors combined with radiotherapy and/or immune checkpoint inhibitors.

ClinicalTrials.gov identifier	Study title	Conditions	Therapeutic agent(s)	Radiotherapy	Phase and patients	Status (at time of publication)
NCT01477489	A Phase I Study of Veliparib Administered Concurrently With Chest Wall and Nodal Radiation Therapy in Patients With Inflammatory or Loco-regionally Recurrent Breast Cancer	- Breast cancer	- Veliparib (50 mg – 200 mg)	- Standard radiotherapy (limited to 60 Gy)	- Phase I - 33 participants - Clinical trial	- Completed
NCT01618357	Pre-Operative PARPi and Irradiation (POPI) in Women With an Incomplete Response to Neo-Adjuvant Chemotherapy for Breast Cancer	- Breast cancer	- Lumpectomy/ Mastectomy - Veliparib	- Radiotherapy; 2.35 Gy per fraction for 16 fractions for a total of 37.5 Gy	- Phase I - 41 participants - Clinical trial	- Suspended
NCT03542175	A Phase I Study of Rucaparib Administered Concurrently With Postoperative Radiotherapy in Patients With Triple Negative Breast Cancer With an Incomplete Pathologic Response Following Neoadjuvant Chemotherapy	- Breast cancer	- Rucaparib (300 mg, 400 mg, 500 mg, or 600 mg)	- Radiotherapy; 50 Gy in 2 Gy per fraction, plus 10 Gy boost to lumpectomy cavity	- Phase I - 30 participants - Clinical trial	- Recruiting
NCT03109080	A Phase I of Olaparib With Radiation Therapy in Patients With Inflammatory, Loco-regionally Advanced or Metastatic TNBC (Triple Negative Breast Cancer) or Patient With Operated TNBC With Residual Disease	- Malignant and triple-negative breast neoplasms	- Olaparib	- Radiotherapy	- Phase I - 24 participants - Clinical trial	- Active, not recruiting
NCT03598257	A Phase II Randomized Trial of Olaparib (NSC-747856) Administered Concurrently With Radiotherapy Versus Radiotherapy Alone for Inflammatory Breast Cancer	- Breast inflammatory carcinoma	- Olaparib (oral)	- Radiotherapy	- Phase II - 300 participants - Clinical trial	- Recruiting
NCT02657889	Phase 1/2 Clinical Study of Niraparib in Combination With Pembrolizumab (MK-3475) in Patients With Advanced or Metastatic Triple-Negative Breast Cancer and in Patients With Recurrent Ovarian Cancer	- Triple negative breast cancer - Breast cancer - Metastatic breast cancer - Advanced breast cancer - Stage IV breast cancer - Neoplasms - Ovarian cancer - Fallopian tube cancer - Peritoneal cancer	- Niraparib (up to 300 mg/day oral) - Pembrolizumab (200 mg intravenous)	- None	- Phase I/II - 122 participants - Clinical trial	- Completed
NCT03544125	A Pilot Study of Olaparib and Durvalumab in Patients With Metastatic Triple Negative Breast Cancer	- Stage IV breast cancer - Estrogen receptor negative - HER2 negative - Progesterone receptor negative - Stage IV breast cancer	- Durvalumab (intravenous) - Olaparib (oral)	- None	- Phase I - 3 participants - Clinical trial	- Completed

(Continued)

TABLE 3 Continued

ClinicalTrials.gov identifier	Study title	Conditions	Therapeutic agent(s)	Radiotherapy	Phase and patients	Status (at time of publication)
		- Triple-negative breast carcinoma				
NCT03025035	Open Label, Phase II Pilot Study of Immune Checkpoint Inhibition With Pembrolizumab in Combination With PARP Inhibition With Olaparib in Advanced BRCA-mutated or HDR-defect Breast Cancers	- Breast cancer	- Pembrolizumab (intravenous) - Olaparib (oral)	- None	- Phase II - 20 participants - Clinical trial	- Recruiting
NCT02849496	A Phase II Open-Label, Randomized Study of PARP Inhibition (Olaparib) Either Alone or in Combination With Anti-PD-L1 Therapy (Atezolizumab; MPDL3280A) in Homologous DNA Repair (HDR) Deficient, Locally Advanced or Metastatic Non-HER2-Positive Breast Cancer	- Locally advanced - unresectable breast carcinoma - Metastatic breast carcinoma - Stage III breast cancer - Stage IV breast cancer	- Atezolizumab (intravenous) - Olaparib (oral)	- None	- Phase II - 81 participants - Clinical trial	- Suspended
NCT04683679	A Phase II Study of Pembrolizumab and Ablative Radiotherapy With or Without Olaparib in Metastatic Triple-Negative Breast Cancers : Initial Test Cohorts of a Platform Trial to Sequentially Investigate Combinations of DNA-Damage Response Inhibitors and Immunotherapy for the Augmentation of Immune Responses	- Triple negative breast cancer	- Pembrolizumab (200 mg intravenous) - Olaparib (600 mg oral)	- 8-9 Gy x 3 fractions or 30 Gy in 6 Gy per fraction for larger tumors	- Phase II - 56 participants - Clinical trial	- Recruiting
NCT04837209	A Phase II Study of NirAparib, Dostarlimab and Radiotherapy in Metastatic, PD-L1 Negative or Immunotherapy-Refractory Triple-Negative Breast Cancer (NADiR)	- Breast cancer - Triple negative breast cancer	- Niraparib (oral) - Dostarlimab (anti-PD-1) (intravenous)	- Radiotherapy	- Phase II - 32 participants - Clinical trial	- Recruiting

preclinical data suggest that PARP inhibition may promote antitumor immunity.

Furthermore, studies have examined the mechanisms underlying the interactions between resistance to PARP inhibitors and ICIs. PARP inhibitors have been found to upregulate PD-L1 expression, resulting in immunosuppression (240). Glycosylation of PD-L1 is required for its interaction with PD-1 and subsequent suppression of T cell activity (240, 241). However, inhibition of PD-L1 glycosylation via 2-deoxyglucose (2-DG) promotes T-cell mediated cytotoxicity and potent antitumor activity in combination with PARP inhibitors (240). Human and murine TNBC cell lines resistant to PARP inhibitors display an increase in epithelial-mesenchymal transition and upregulation of PD-L1 (242). These effects are abrogated by the application of metformin to block pAkt S473—potentially providing a synergistic approach to increase PARP inhibition and immunotherapy efficacy (242). In short, various studies suggest that PD-L1 upregulation may regulate PARP inhibitor resistance.

Clinical trials are beginning to report the efficacy of PARP inhibition combined with ICIs in breast cancer patients. In the

TOPACIO/KEYNOTE-162 trial, the PARP inhibitor niraparib was combined with pembrolizumab for the treatment of advanced or metastatic TNBC (NCT02657889). Preliminary results from this study suggest that combining PARP inhibition with ICIs may be effective in metastatic TNBC regardless of BRCA status (243). Additionally, ongoing studies are examining the combination of olaparib and durvalumab for patients with metastatic TNBC (NCT03544125) (244), as well as examining the combination of pembrolizumab and olaparib in patients with DNA damage response pathway mutations (NCT03025035). Furthermore, a Phase II, open-label, randomized trial was recently underway to assess the effects of olaparib alone and in combination with atezolizumab in HDR deficient locally advanced or metastatic non-HER2$^+$ breast cancer, although it was recently suspended (NCT02849496) (245). To conclude, these clinical data suggest that PARP inhibition may enhance patient responses to immunotherapy; however, additional research is merited.

Based upon the promise of combining both PARP inhibition with radiotherapy and PARP inhibition with immunotherapy, trials are also examining trimodal approaches with radiotherapy, ICIs, and PARP inhibition. A Phase II trial is currently recruiting patients to ascertain the efficacy and safety of talazoparib combined with radiotherapy and atezolizumab (anti-PD-L1) for PD-L1$^+$ metastatic TNBC patients (NCT04690855). Additionally, a randomized, Phase II study is recruiting breast cancer patients to understand the effects of radiotherapy in combination with pembrolizumab and olaparib to treat patients with triple negative disease (NCT04683679). Moreover, a Phase II trial is currently assessing the effects of combined niraparib, dostarlimab (anti-PD-1), and radiotherapy in metastatic, PD-L1$^-$, or immunotherapy-refractory TNBC (NCT04837209). Importantly, more time is necessary to define the tolerability and efficacy of these trimodal approaches in breast cancer patients.

Safety, tolerability, and cost-effectiveness of combined therapy approaches

Importantly, while combining targeted therapies with radiotherapy and immune checkpoint inhibitors is a promising approach for the treatment of breast cancer patients, more studies are warranted to further examine the safety and tolerance of such combinations. All pharmaceutical agents are associated with potential adverse events and combining therapeutic agents and modalities can heighten the risk of toxicity. Combining therapeutics also has the potential of reducing toxicity if combined therapies are synergistic and require lower doses of these agents in combination compared to when delivered as monotherapies. Clinical and preclinical studies are currently underway to screen for potential adverse effects and unwanted toxicities of combined approaches for the treatment of breast cancer.

Collectively, in breast cancer patients, single agent targeted therapies can result in various toxicities, including cardiovascular (246, 247), endocrine, dermatologic, and pulmonary toxicities (248). While advancements in the delivery of radiotherapy as a monotherapy have allowed for the precise delivery of radiation rays directly to cancerous lesions, radiotherapy can also damage nearby, non-malignant cells, resulting in acute and late-onset toxicities (249). ICIs are associated with idiosyncratic inflammatory adverse events which can occur in potentially any organ system, emphasizing the importance of closely monitoring patients receiving such therapies (250). Anti-CTLA-4 immunotherapies are associated with a higher incidence of immune-related adverse events (irAEs) compared to inhibitors of the PD-1 axis, which may coincide with their different mechanisms of action (249). Anti-PD-1 therapies (i.e., pembrolizumab) may be associated with fewer adverse events than anti-PD-L1 therapies (i.e., atezolizumab) in breast cancer patients (250, 251). Collectively, as more patients receive ICIs as part of their treatment regimens, more screening is warranted to understand why these adverse events take place and how these events can be prevented in patients undergoing treatment.

Combination therapies involving the application of both radiotherapy and ICIs may result in complex effects on the immune system which may promote enhanced therapy efficacy and also therapy toxicity. To date, the combination of radiotherapy and ICIs has been found to be safe and well-tolerated in patients undergoing treatment (249, 252). Combined anti-PD-1 and anti-CTLA-4 ICIs with palliative radiotherapy was found to be associated with few adverse events in patients with non-small cell lung cancer, melanoma, renal cell cancer, and breast cancer (192, 253). Toxicity can also occur in studies combining chemotherapy with ICIs. For instance, in the KEYNOTE-522 trials, while combination of chemotherapy and pembrolizumab improved pathological complete response in patients with early TNBC, this therapy resulted in 78% of patients having grade 3 or higher adverse events, compared to only 73% of patients in the placebo-chemotherapy group (106). Targeted therapy can also cause adverse events. Single-agent PARP inhibition has been found to be less toxic compared to single-agent chemotherapy; however, when PARP inhibitors are used in combination with radiotherapy, toxicity must be closely monitored (248). In a study that combined PARP inhibition (veliparib) with radiotherapy in patients with inflammatory or locoregionally recurrent breast cancer, 1 year post treatment resulted in grade 3 toxicity of 10%. However, 3 years following combined therapy, 46.7% of patients experienced grade 3 toxicity, with 6 out of a total 15 patients having severe fibrosis in the field of treatment (233). Collectively, more studies are needed to screen for such toxicities and determine the proper doses of targeted therapies, ICIs, and radiotherapy that can be efficacious, while inducing minor adverse events and low toxicities in patients with aggressive forms of breast cancer,

While the safety profiles of combined approaches are important to consider when determining the optimal treatment plan, another important aspect to consider is the cost-effectiveness of such therapeutics. Financial toxicity is a growing concern in breast cancer care (254). While ICIs are an emerging and promising therapeutic option for cancer patients, they are costly services for patients, which is a critical factor when patients are deciding what course of therapy to pursue. In a study assessing the cost effectiveness of immunotherapy in non-small cell lung cancer, the median yearly cost of ICIs was $148,431. Importantly, while the costs of ICIs may vary based upon drug rand and mechanism of action, overall, prolonged usage of such therapies beyond two years was not found to be financially feasible for patients (255). Consequently, numerous studies are focused on accessing the cost-effectiveness (CE) of immunotherapies, which is often measured as the incremental cost-effectiveness ratio (ICER), a ratio that represents the cost required for one additional year of life (256). In breast cancer, results from studies assessing the cost-effectiveness of immunotherapies are often mixed and are drug-dependent—supporting the need to further analyze the benefit of prescribing ICIs to cancer patients—especially in combination with other targeted therapies. In solid tumors, ICIs provide significant clinical benefits to patients and certain types of ICIs have been found to be cost-effective in different types of cancer compared to chemotherapy treatment alone (256). In PD-1$^+$, metastatic TNBC, the combination of pembrolizumab with chemotherapy was found to be cost-effective (257). Combined chemotherapy and pembrolizumab was also cost-effective in high risk, early-stage TNBC (258). Combining ICIs with radiotherapy is also cost-effective in non-small cell lung cancer; however, this has not been examined as thoroughly in the context of breast cancer and more studies are warranted (259). More work is also necessary to determine how cost-effective trimodal approaches are for breast cancer patients—such as for combined ICIs, radiotherapy, and targeted therapy. Furthermore, this also starts conversations regarding the overall cost of therapeutics and accessibility to affordable healthcare—which may vary based upon where patients are receiving their cancer care and influence their decisions to receive such therapies.

Future directions

Future clinical trials are focused on assessing whether combination approaches increase immunotherapy efficacy in patients with breast cancer as demonstrated in Table 4 (260). CDK4/6 inhibitors are mainstay treatments for women with metastatic HR+, HER2$^-$ breast cancer and induce radiosensitization in preclinical models of ER$^+$ breast cancer and TNBC (261, 262). Furthermore, the CDK4/6 inhibitor abemaciclib enhances the efficacy of anti-PD-L1 ICIs by augmenting antigen presentation and T cell activation in human breast cancer cells (263). These data motivate the assessment of combining CDK4/6 inhibitors with radiotherapy and ICIs in future studies. Currently, the effects of combined stereotactic body radiation (SBRT), ICIs, and hormone therapies are being examined in ER$^+$ breast cancer (NCT04563507). In addition to analyzing the effects of already developed pharmacological agents with radiotherapy and ICIs, future studies should investigate the combined effects of novel cancer therapeutic agents. For instance, combining a phosphoinositide 3-kinase δ (PI3Kαδ) inhibitor with radiotherapy and anti-PD-1 was found to increase CD8$^+$ T cell accumulation and delay tumor growth in a murine syngeneic TNBC model (264). STING agonists are also currently being examined in preclinical breast cancer models in combination with ubiquitinated protein nanovaccines (265), anti-CD47 monoclonal antibodies (266), and CAR-T cell therapy (267). These studies suggest that combining STING agonists, ICIs, and radiotherapy may have clinical potential.

Additional studies are crucial to determine the most effective radiotherapy dose and fractionation in patients. The optimal dose fractionation to induce effective antitumor immune responses has not yet been determined, with preclinical literature supporting both ablative single fractions (268) as well as moderate hypofractionation (160, 166). For example, ablative stereotactic body radiotherapy delivered at 15 Gy delivered in 3 fractions or 30 Gy radiotherapy delivered in 1 fraction combined with immunotherapy decreased primary tumor size in a 4T1 murine breast cancer model, while ablative radiotherapy delivered at 1 fraction of 30 Gy transforms the tumor suppressive microenvironment of colon tumors into a pro-inflammatory, CD8$^+$ T cell enriched environment (268, 269). Hypofractionated radiotherapy delivered at 9.18 Gy in 3 fractions or 6.43 Gy in 5 fractions also induces systemic antitumor effects and promotes synergy in combination with anti-PD-1 in syngeneic breast cancer models (270). Conversely, radiotherapy delivered at doses above 12-18 Gy induces Trex1 in other breast cancer models, which can hinder the pro-immune effects of radiotherapy by degrading cellular DNA upstream of the cGAS/STING pathway (177). Prospective clinical evaluations are needed to define the optimal radiotherapy regimens in patients.

In addition to better understanding the mechanisms involved in radiotherapy, it is also critical to further understand the underlying mechanisms involved in immunotherapy efficacy and patient response to immunotherapy. Importantly, many factors play a role in the efficacy of ICIs, such as age (85), sex (NCT04435964), gut microbiome (NCT03383107, NCT05037825), and oncogenic signaling/mutations (NCT01351103) (271). Immunotherapy efficacy may also depend on sites of metastatic involvement. In

TABLE 4 Additional studies assessing combinatorial therapies for the treatment of breast cancer.

ClinicalTrials.gov identifier	Study title	Conditions	Therapeutic agent(s)	Radiotherapy	Phase and patients	Status (at time of publication)
NCT04563507	CIMER: Combined Immunotherapies in Metastatic ER+ Breast Cancer	- Breast cancer	- Letrozole (2.5 Mg tablet) - Palbociclib (125 mg)	- SBRT at 50 Gy × 5 fractions	- Phase II - 102 participants - Clinical trial	- Recruiting
NCT04435964	Gender Difference in sidE eFfects of ImmuNotherapy: a Possible Clue to Optimize cancEr tReatment	- Breast Cancer - Melanoma - Lung cancer - Head and neck cancer - Urogenital neoplasms	- Immune checkpoint inhibitors as a monotherapy or in combination with radiotherapy and/or chemotherapy	- Varies	- 400 participants - Observational trial	- Recruiting
NCT03383107	Effect of Radiotherapy Variables on Circulating Effectors of Immune Response and Local Microbiome	- Breast cancer - Prostate cancer	- Radiotherapy	(For breast cancer) - Standard fractionation breast and nodal radiotherapy to 50 Gy in × 25 fractions - Partial breast RT to 30 Gy in × 25 fractions and × 5 fractions	- 66 participants - Observational trial	- Completed
NCT05037825	The Gut Microbiome and Immune Checkpoint Inhibitor Therapy in Solid Tumors	- Triple-negative breast cancer - Non-small-cell lung carcinoma - Malignant melanoma - Renal cell carcinoma	- Anti-PD-1, anti-PD-L1, or anti-CTLA-4 in combination with other checkpoint inhibitors or agents including radiotherapy, surgery, and/or chemotherapy	- Varies	- 800 participants - Observational trial	- Recruiting
NCT01351103	A Phase I, Open-label, Dose Escalation Study of Oral LGK974 in Patients With Malignancies Dependent on Wnt Ligands	- Triple negative breast cancer - Pancreatic cancer - BRAF mutant colorectal cancer - Melanoma - Head and neck squamous cell cancer - Cervical squamous cell cancer - Esophageal squamous cell cancer - Lung squamous cell cancer	- Drug: LGK974 (PORCN inhibitor) - Biological: PDR001 (anti-PD-1)	- None	- Phase I - 185 participants - Clinical trial	- Recruiting

both patients and preclinical models, liver metastases are associated with diminished immunotherapy efficacy (272). Moreover, it is essential to continue investigating the effects of the cGAS/STING pathway and its implications in both the radiotherapy response and immune response in human cancers. Numerous studies are currently investigating the preclinical implications of the cGAS/STING pathway in cancer and how other mediators of this pathway can be modulated to promote pro-immune, antitumor effects. In all, the mechanisms underlying combined therapies are complex and more research is justified to further understand these interactions.

Moreover, it is also critical to define treatment tolerance since adverse events may occur following combined treatments. Finally, another crucial future direction is developing predictive and prognostic biomarkers indicative of response to combination therapies. While studies suggest TILs, tumor mutation burden (TMB), and immune gene signatures may be potential biomarkers for response to ICIs in breast cancer, biomarkers indicative of combined therapy efficacy have not yet been identified (273, 274). In short, more research is necessary to discover biomarkers to help identify which patient populations will respond best to these novel therapeutic approaches.

Discussion

Breast cancer is the leading non-cutaneous cancer diagnosed among females and is a heterogeneous disease that can result in poor clinical outcomes, especially in patients with triple negative disease. Immunotherapy is an emerging therapeutic option for aggressive forms of breast cancer and combining immunotherapy with radiotherapy may hold clinical benefit. Preclinical studies are underway to understand the potential benefit of combining radiotherapy with immune checkpoint inhibitors and to examine the molecular mechanisms that contribute to potential synergy between these therapies. Additional studies are needed to develop therapeutic approaches targeting canonical and noncanonical regulators of innate immunity in conjunction with radiotherapy and immunotherapy. Clinical trials are currently examining the prognostic benefits of combined ICIs and radiotherapy with other available cancer therapeutics in breast cancer patients. Collectively, these studies support the importance of improving combined therapy efficacy with the ultimate goal of improving outcomes in breast cancer.

Author contributions

KMJ conceptualized this review and drafted the original manuscript. EH, AP, KRJ, CB, LP, MG, and CS contributed to the writing and critical revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Caspase activation counteracts interferon signaling after G2 checkpoint abrogation by ATR inhibition in irradiated human cancer cells

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Recent studies suggest that inhibition of the ATR kinase can potentiate radiation-induced antitumor immune responses, but the extent and mechanisms of such responses in human cancers remain scarcely understood. We aimed to assess whether the ATR inhibitors VE822 and AZD6738, by abrogating the G2 checkpoint, increase cGAS-mediated type I IFN response after irradiation in human lung cancer and osteosarcoma cell lines. Supporting that the checkpoint may prevent IFN induction, radiation-induced IFN signaling declined when the G2 checkpoint arrest was prolonged at high radiation doses. G2 checkpoint abrogation after co-treatment with radiation and ATR inhibitors was accompanied by increased radiation-induced IFN signaling in four out of five cell lines tested. Consistent with the hypothesis that the cytosolic DNA sensor cGAS may detect DNA from ruptured micronuclei after G2 checkpoint abrogation, cGAS co-localized with micronuclei, and depletion of cGAS or STING abolished the IFN responses. Contrastingly, one lung cancer cell line showed no increase in IFN signaling despite irradiation and G2 checkpoint abrogation. This cell line showed a higher level of the exonuclease TREX1 than the other cell lines, but TREX1 depletion did not enhance IFN signaling. Rather, addition of a pan-caspase inhibitor restored the IFN response in this cell line and also increased the responses in the other cell lines. These results show that treatment-induced caspase activation can suppress the IFN response after co-treatment with radiation and ATR inhibitors. Caspase activation thus warrants further consideration as a possible predictive marker for lack of IFN signaling.

KEYWORDS

cell cycle checkpoints, type I interferon (IFN) signaling, radiation therapy (radiotherapy), micronuclei (MN), ATR, caspase, cGAS, TREX1

Introduction

Local radiotherapy can increase tumor immunogenicity, yielding systemic, abscopal effects on distal metastases in rare cases (1, 2). However, the influence of radiotherapy on the immune system is complex, and radiotherapy may also stimulate immunosuppressive mechanisms (3). Immune checkpoint inhibitors combined with radiotherapy has shown promise in enhancing the antitumor immune effects (4–6). Nevertheless, therapeutic responses remain limited, urging the need for more knowledge and new, efficacious strategies.

The serine/threonine protein kinase ATR is a central regulator of the G2 cell cycle checkpoint and DNA repair following irradiation (7, 8). When ATR inhibitors (ATRi) are combined with irradiation, cells will enter mitosis with unrepaired DNA lesions, which ultimately causes micronucleus formation and cell death (9). ATRi are therefore promising radiosensitizers under clinical evaluation (10, 11). Interestingly, recent studies suggest that ATRi, besides their effects on cell cycle checkpoints and DNA repair, may also increase radiation-induced antitumor immune responses. Increased immune effects, such as activation of $CD8^+$ T cells and immunological memory, have been observed in murine cancer models after treatment with the ATR inhibitor AZD6738 and ionizing radiation (IR) (12–14). Mechanistically, ATRi may stimulate tumor immunogenicity through downregulation of programmed cell death 1 ligand 1 (PD-L1) in irradiated cancer cells (3, 14, 15). In addition, ATR inhibition can potentiate radiation-induced type I IFN responses, likely through generation of cytosolic DNA resulting from increased micronucleus formation after abrogation of cell cycle checkpoints (16, 17). In this scenario, the DNA sensor cGAS recognizes *de facto* cytosolic DNA in ruptured micronuclei, and triggers induction of type I IFN through the cGAS–STING–IRF3–TBK1 signaling cascade (18–20). Noteworthy, the cGAS–STING–IFN pathway is negatively regulated by three-prime repair exonuclease 1 (TREX1), which degrades the DNA substrates of cGAS (21, 22). In addition, this pathway can be negatively regulated by caspase-mediated protein cleavage (23).

The potentiation of IFN responses after IR and ATRi were mostly shown in murine cancer or human normal cells, and it remains elusive whether similar effects commonly occur in human cancer cells. Furthermore, in some cell lines, IFN responses were rather stimulated through immune recognition of cytosolic RNA (16, 17). Opposing results regarding whether the IFN response was dependent on the cytosolic RNA sensor RIG-I or the DNA sensor cGAS have even been reported for the same cells (MCF10A) (16, 17), underlining the mechanistic uncertainty of the response.

Here, we investigated the hypothesis that combined treatment of human cancer cells with IR and ATRi stimulates cGAS-mediated type I IFN responses, due to G2 checkpoint abrogation and consequently enhanced generation of micronuclei. We found that the combined treatment caused increased cGAS-mediated type I IFN secretion in all tested cell lines except for one, which contained very high basal levels of the exonuclease TREX1. However, downregulation of TREX1 in this cell line did not restore IFN signaling. Rather, the IFN response was restored upon co-treatment with a pan-caspase inhibitor. The caspase inhibitor also further increased the IFN responses in the other cell lines.

Results

Radiation-induced type I interferon signaling declines at high radiation doses, coinciding with a prolonged G2 checkpoint arrest

To explore how ATR inhibitors affect radiation-induced IFN signaling, we first assessed the effects of irradiation alone. We treated the human osteosarcoma cell line U2OS with different radiation doses (2-20 Gy), and measured IFN signaling three to six days post treatment by immunoblotting of phosphorylated STAT1 (pSTAT1). STAT1 is phosphorylated upon autocrine and paracrine type I IFN signaling, rendering pSTAT1 indicative of IFN secretion (18, 24). At six days post treatment, a marked increase in pSTAT1 was observed after lower radiation doses (2 and 5 Gy), whereas higher doses (>10 Gy) gave only minor increases in pSTAT1 level (Figures 1A, B). Similar radiation dose responses have been reported in a previous study, where the lack of IFN secretion after higher doses (> 10 Gy) was attributed to radiation-induced increases in *TREX1* expression (25). Contrastingly, we did not find any increase in TREX1 levels in U2OS cells after irradiation with 10-20 Gy (Figure 1A). Our results thus suggest other mechanisms to be responsible for suppression of IFN responses after high-dose irradiation in this cell line. Induction of type I IFN responses has been linked to formation of micronuclei resulting from mitosis with unrepaired DNA after irradiation (18, 19). As arrest at the G2 checkpoint delays mitotic entry, we compared cell cycle progression after low- and high-dose irradiation. The cells arrested notably longer in the radiation-induced G2 checkpoint after higher doses than after lower doses, as expected (Figure 1C). The lack of IFN signaling after exposure to high doses of radiation thus coincides with prolonged G2 checkpoint arrest, suggesting that the arrest counteracts IFN signaling.

ATR inhibition-induced G2 checkpoint abrogation accelerates micronucleus formation after irradiation

We next investigated whether ATR inhibition can abrogate the G2 checkpoint after irradiation with low and high doses. We

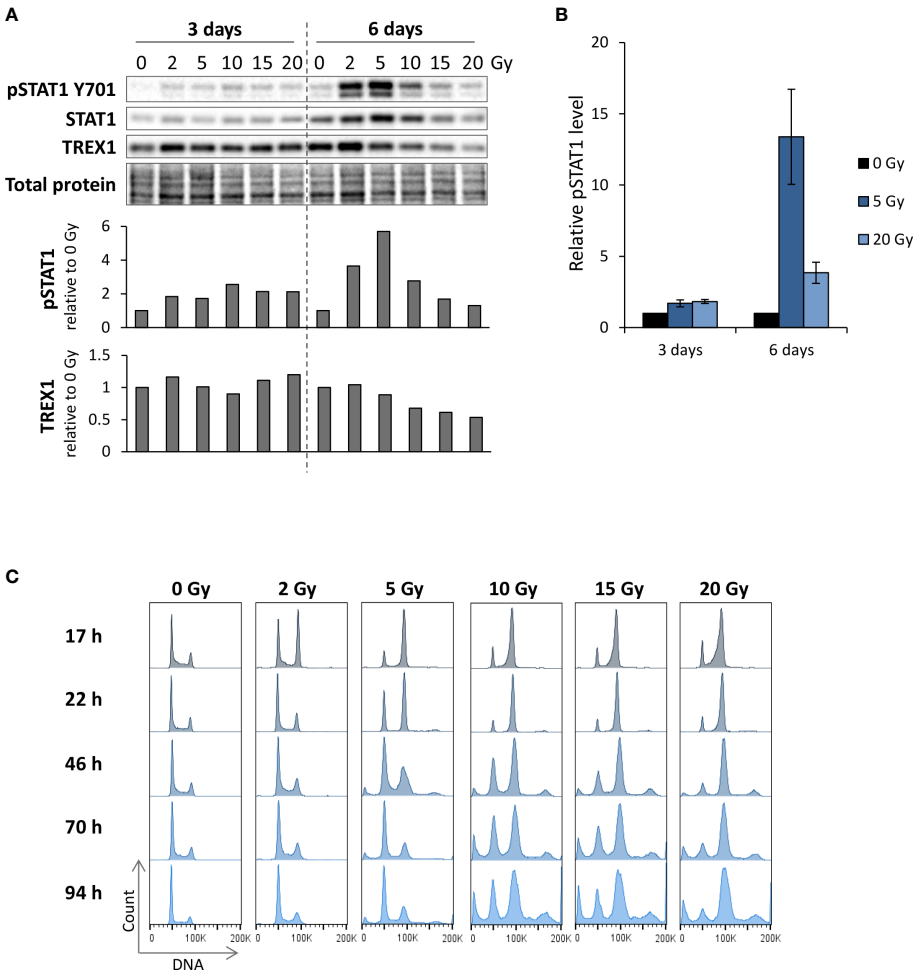

FIGURE 1
Reduction of radiation-induced IFN signaling at high IR doses coincides with prolonged G2 checkpoint arrest. **(A)** Immunoblots of U2OS cells harvested at three and six days after IR. Bar charts show pSTAT1 and TREX1 levels relative to total protein and normalized to mock. **(B)** Quantification of pSTAT1 levels relative to the corresponding mock sample for multiple independent experiments with 5 and 20 Gy as in **(A)**. (3 days: n = 5 for 5 Gy and n = 4 for 20 Gy; 6 days: n = 3) **(C)** DNA histograms from parallel samples in the same experiment as in **(A)**. The '100K' annotation marks the G2/M phase peak. Results in **(A, C)** are representative for three independent experiments performed at different time points within 0-6 days post treatment.

employed the ATR inhibitor VE822 (berzosertib) at a high concentration (250 nM), which caused ~80% reduction in cell viability (Supplementary Figure S1A, left). Treatment with 250 nM VE822 efficiently abrogated the checkpoint after 2 and 5 Gy irradiation, but less so after irradiation with 10 or 20 Gy (Supplementary Figure S1B). Hence, ATR inhibition is less effective in abrogating G2 checkpoint arrest after higher radiation doses, in agreement with previous studies showing that the G2 checkpoint is regulated by multiple factors (26–30). In our subsequent studies with radiation and ATRi, we therefore irradiated with 5 Gy. U2OS cells showed a pronounced G2 checkpoint arrest at 17 hours after 5 Gy, with the cell cycle profile slowly beginning to redistribute at 22-41 hours post treatment (Figure 2A). Cells co-treated with 5 Gy and 250 nM VE822 showed no sign of checkpoint arrest, with no accumulation of cells in G2 phase at 17-22 hours post treatment (Figure 2A). Furthermore, the G2 checkpoint was almost completely abrogated at 0-6 hours post treatment, as detected by presence of mitotic cells (data not shown). The checkpoint was correspondingly abrogated by a high concentration (1250 nM) of the ATR inhibitor AZD6738 (ceralasertib) (Figures 2B, C). This concentration of AZD6738 caused ~50% reduction in cell viability (Supplementary Figure S1A, right). We also tested lower, less toxic concentrations of both VE822 and AZD6738 (50 nM and 250 nM, respectively), yielding 5-10% reduction in viability (Supplementary Figure S1A). The lower concentrations gave a partial abrogation of the G2 checkpoint (Figures 2B, C). The effect of ATR inhibition

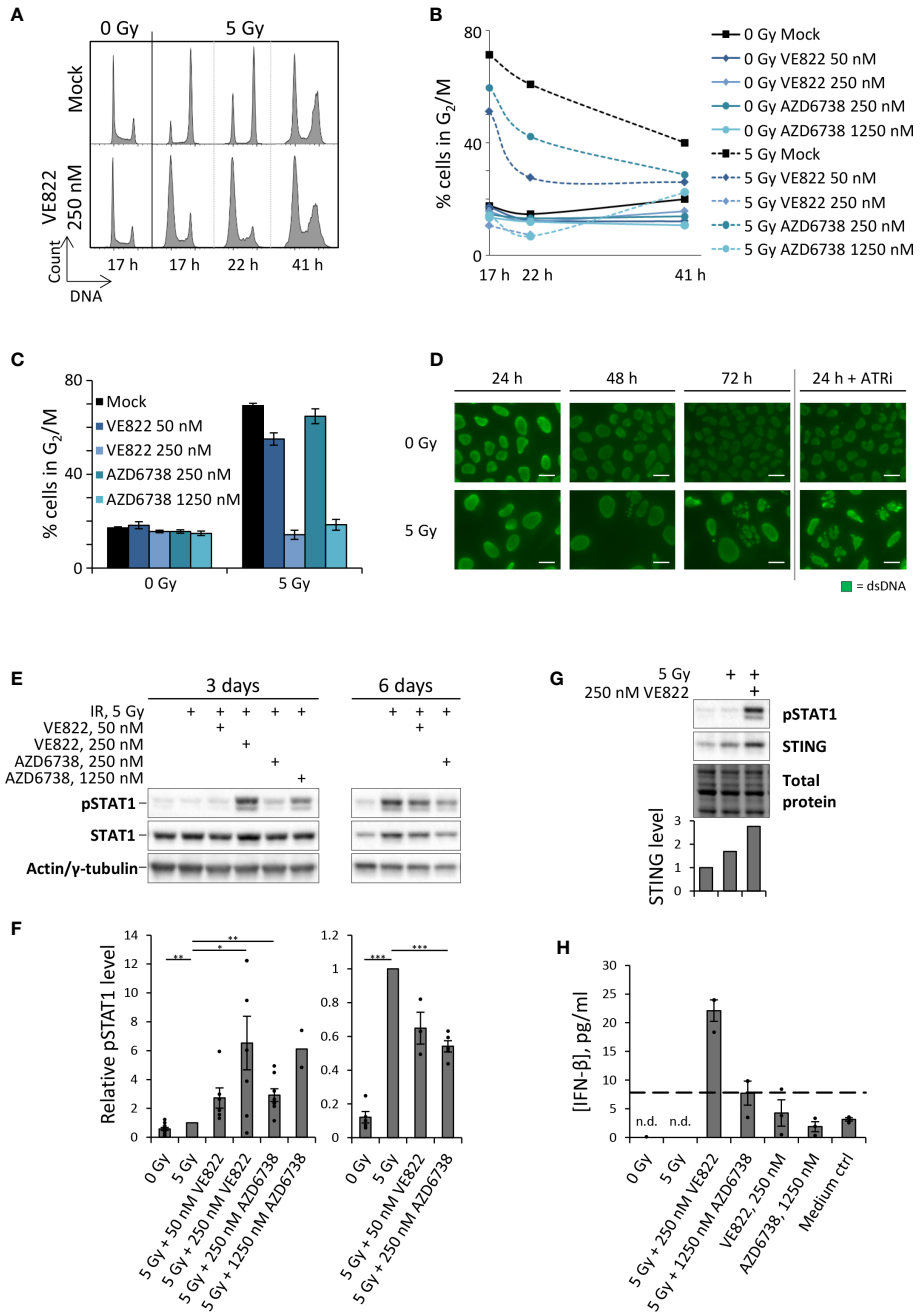

FIGURE 2
ATRi abrogates radiation-induced G2 checkpoint arrest, resulting in expedited generation of micronuclei and induction of type I IFN response in U2OS cells. **(A)** DNA histograms after treatment with IR and VE822. **(B)** Quantification of proportion of cells in G2/M phase from the experiment in **(A)**. **(C)** Bar-plotted quantification of G2/M proportions from three independent experiments performed as in **(A)**, at 17 hours after treatments. **(D)** Micrographs showing anti-dsDNA immunofluorescence staining. ATRi: 250 nM VE822. Scale bars: 20 μm. **(E)** Immunoblot of phosphorylated STAT1 (pSTAT1) and total STAT1 (STAT1) three and six days after IR with or without VE822 and AZD6738. Pan-actin and γ-tubulin were used as loading controls at three and six days, respectively. **(F)** Quantification of pSTAT1 levels relative to loading controls for experiments as in **(E)**. Values are normalized to 5 Gy. **(G)** Immunoblot of pSTAT1 and STING in U2OS cells at three days after the indicated treatments. Bar chart shows STING level relative to total protein and normalized to mock. **(H)** ELISA of IFN-β in 20X up-concentrated growth media from U2OS cells harvested three days after treatment. Dashed line indicates the lowest interferon-β concentration tested in the standard curve in Supplementary Figure S1E (7.81 pg/ml). n.d. = not detectable.

was also assessed by immunofluorescence microscopy. Cells treated with 5 Gy IR and 250 nM VE822 generated micronuclei already within 24 hours post treatment (consistent with finalized mitosis), whereas micronuclei were observed at 72 hour post treatment for irradiated mock cells (Figure 2D). These results indicate that ATR inhibitors at high concentrations efficiently abrogate G2 checkpoint arrest and thereby accelerate the generation of micronuclei.

Combined treatment with IR and ATRi expedites radiation-induced interferon response in U2OS cells

Type I IFN responses upon treatment of U2OS cells with IR and ATRi were measured by pSTAT1 levels and IFN-β ELISA. Irradiation (5 Gy) alone gave nearly no increase in pSTAT1 levels at three days post treatment (Figures 2E, F). Co-treatment with IR and high concentrations of ATRi (250 nM VE822; 1250 nM AZD6738) markedly increased this response, whereas a smaller increase was obtained with the lower concentrations (50 nM VE822; 250 nM AZD6738) (Figures 2E, F). The biggest effect was obtained with the high concentration of VE822 (250 nM), which also caused the highest reduction of cell viability (Supplementary Figure S1A). At six days post treatment, IR alone induced the highest pSTAT1 levels, but this induction nevertheless appeared lower than after the aforementioned high-concentration co-treatments at three days (Figures 2E, F; Supplementary Figure S1C). ATRi thus causes an earlier and more pronounced wave of IFN response, which declines with time. The latter might be related to reduced kinase activities in dying or dead cells. Indeed, the higher concentrations of ATRi rendered measurements unattainable at six days due to too much cell death (data not shown). We also observed increased levels of total STAT1 after the treatments (Figure 2E; Supplementary Figure S1D), consistent with previous work in other cell lines showing that radiation-induced increase in pSTAT1 is accompanied by increased levels of total STAT1 (18). Of note, a previous study has reported that the cGAS–STING–IFN pathway is defective in U2OS cells due to very low or undetectable expression levels of *STING1* (31). However, we consistently observed an increase in STING level after treatment with IR and ATRi (Figure 2G), supporting that this pathway may likely be active in U2OS cells after the treatment.

To verify that pSTAT1 levels represent an activated type I interferon signaling cascade, we measured levels of IFN-β in growth medium supernatants by ELISA three days post treatment. Whereas the unirradiated mock samples and the samples treated with IR or ATRi alone failed to give detectable levels of IFN-β, the combined treatment with IR + 250 nM VE822 – which produced the highest increase in pSTAT1 levels – gave clearly elevated IFN-β concentrations in the medium (Figure 2H; Supplementary Figure S1E). The ELISA measurements thus confirm that the increased pSTAT1 levels correlated with IFN-β secretion. Altogether, these results indicate that whereas IR alone induces an IFN response at around six days, the combined treatment with IR and ATRi can induce an expedited response at three days post treatment.

The effect of co-treatment with IR and ATRi on interferon signaling varies between human lung cancer cell lines

As done for U2OS, we next assayed pSTAT1 levels in the non-small cell lung cancer (NSCLC) cell lines SW900, H1975, A549 and H460. Irradiation alone caused a small increase in pSTAT1 for SW900 and H1975 at three days post treatment, and in A549 at six days post treatment (Figures 3A–C). We detected further increased pSTAT1 levels upon co-treatment with IR and ATRi for SW900, H1975 and A549 (Figures 3A–C), albeit to a lesser extent than for U2OS. The highest levels of pSTAT1 were observed after treatment with IR + 250 nM VE822 for SW900 and A549 (Figures 3A, C), in concordance with the results for U2OS (Figures 2F, H). For H1975, the differences between IR and IR + ATRi were not statistically significant, but nevertheless, the pSTAT1 level was increased both after IR alone and in combination with ATRi when compared to the non-irradiated cells (Figure 3B). At six days post treatment, all the treatments of H1975 resulted in pSTAT1 levels around or below the mock sample background level (Supplementary Figure S2C). SW900, on the other hand, showed a marked radiation-induced increase in pSTAT1 level at six days, but still lower than after IR + ATRi at three days (Supplementary Figures S2A, B), resembling the results for U2OS.

Notably, no increase in pSTAT1 levels was observed for H460 after treatment with either IR alone or in combination with ATRi, neither at three nor six days post treatment (Figure 3D; Supplementary Figure S2E). This was confirmed by ELISA measurements of IFN-β in H460 (Figure 3E). H460 thus deviates from the other tested cell lines, all of which showed an increase in pSTAT1 levels after treatment with IR and/or IR + ATRi. To address whether H460 also deviated in terms of G2 checkpoint abrogation, we performed cell cycle analyses after treatment with 5 Gy IR + 250 nM VE822. However, all four lung cancer cell lines showed a clear G2 arrest at 17 hours after irradiation, which was abrogated upon ATR inhibition (Figures 3F, G; Supplementary Figures S3A, B). Of note is that A549 had less accumulation of cells in G2 phase after irradiation, likely due to a more pronounced G1 checkpoint (Supplementary Figure S3A). Thus, A549 may cycle more slowly than the other cell lines after the treatment, which could possibly explain the delayed IFN response in this cell line relative to the others. Together, these results show that ATRi can increase the IFN response after irradiation in three out of the five cell lines tested, and weakly in further one cell line, while the G2 checkpoint was abrogated in all five cell lines.

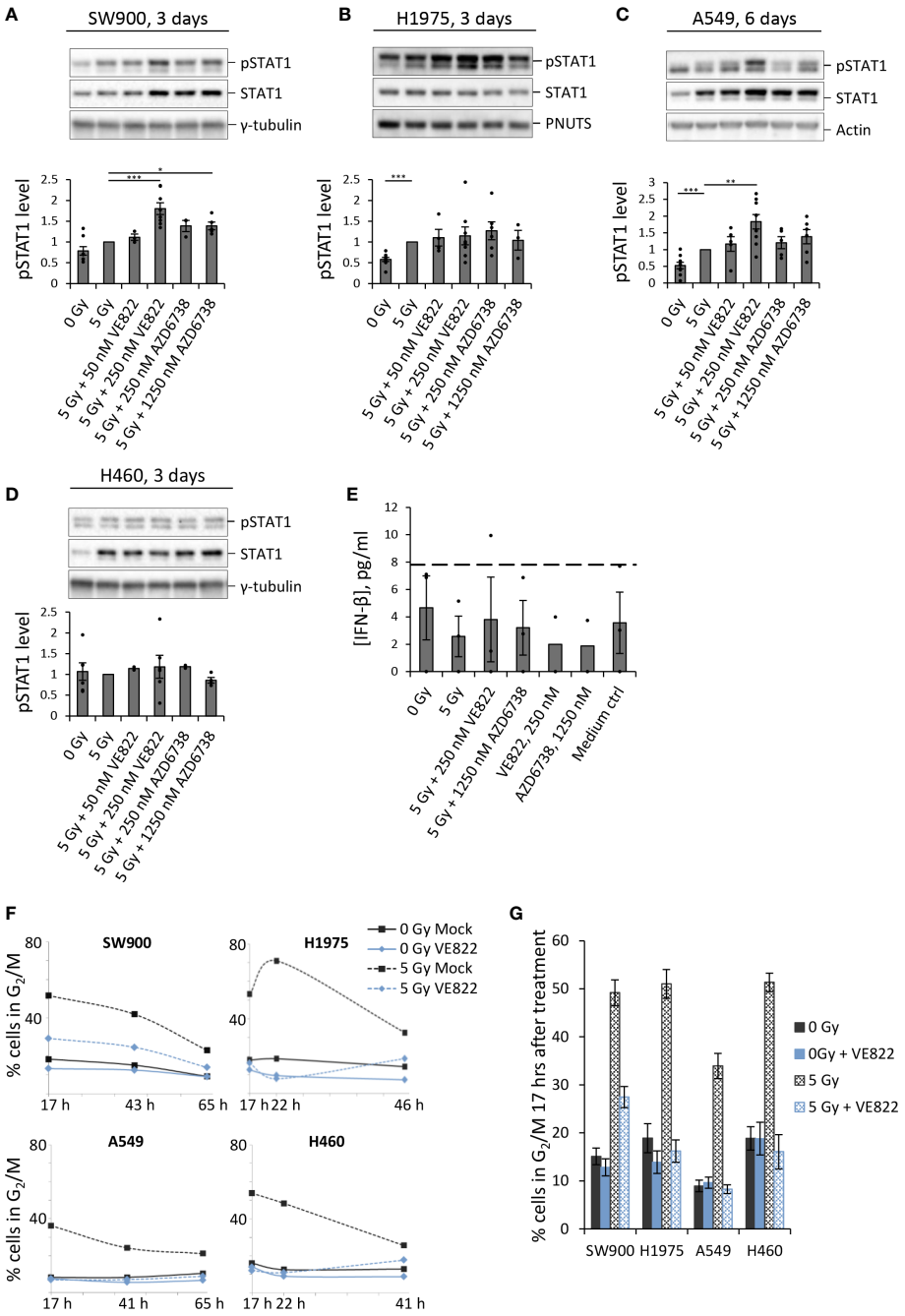

FIGURE 3
ATRi abrogates radiation-induced G2 checkpoint arrest in four human lung cancer cell lines, and gives increased IFN response in three of these. **(A–D)** Immunoblots for indicated NSCLC cell lines after treatment with IR and ATRi. Bar charts show pSTAT1 levels relative to loading controls and normalized to 5 Gy. (Results for A549 at three days after treatment and at six days for the other cell lines are shown in Supplementary Figure S2). γ-tubulin, PNUTS and pan-actin were used as loading controls. **(E)** ELISA of IFN-β in H460 cells treated and analyzed as in Figure 2H. (The zero values are from an experiment where all IFN readings were equal to or lower than the lowest value of the standard curve). **(F)** Proportion of cells in G2/M phase after treatment with IR and 250 nM VE822. The corresponding DNA histograms are shown in Supplementary Figure S3. **(G)** Bar-plotted quantification of G2/M proportions from three independent experiments performed as in **(F)**, at 17 hours after treatments.

Increased pSTAT1 levels after combined treatment with IR and ATRi are dependent on cGAS

To investigate whether the treatment-induced increases in pSTAT1 levels were dependent on the cytosolic DNA sensor cGAS, we performed siRNA transfection to deplete cGAS in U2OS, A549 and SW900. For all three cell lines, the increase in pSTAT1 level was abolished or heavily diminished upon cGAS depletion (Figure 4A). This result substantiates the hypothesis of IFN secretion in response to detection of cytosolic DNA by cGAS after treatment with IR and ATRi. To further elucidate cGAS' role in the response, we performed immunofluorescence microscopy of U2OS at three days after treatment with IR with and without 250 nM VE822. If cGAS initiates the type I IFN response after detection of *de facto* cytosolic DNA in micronuclei, cGAS should localize to the micronuclear lumen. Indeed, cGAS formed distinct foci localized to micronuclei in U2OS cells after the combined treatment (Figure 4B). Transfection with siRNA targeting *CGAS* abolished this effect despite presence of micronuclei (Figure 4C). Furthermore, siRNA-mediated depletion of STING also abolished the IFN response after IR and ATRi, highly consistent with activation of the cGAS–STING–IFN pathway in U2OS cells (Supplementary Figure S4A). In contrast, transfection with three different non-targeting control siRNAs did not eliminate the IFN response (Supplementary Figure S4A). Taken together, these results show that the IFN response is dependent on cGAS–STING, and that there is a link between micronuclear cGAS localization and induction of the type I IFN response.

Caspase inhibition restores the IFN response in H460 cells and increases the responses in the other cell lines.

As H460 deviated from the other cell lines by the lack of IFN response after treatment, and as TREX1 can degrade the DNA substrate of cGAS, we assessed the protein level of TREX1 in all the cell lines (Figure 5A). The level of TREX1 was considerably higher in H460 than in the other cell lines (Figures 5A, B), which could imply that TREX1 is responsible for the lack of IFN response in H460. To address this, we depleted TREX1 by siRNA transfection. However, depletion of TREX1 in H460 caused massive cell death and growth arrest, and did not produce any IFN response upon treatment with IR and ATRi (data not shown). We therefore titrated the siRNA concentration to obtain a partial depletion of TREX1 in H460, reaching approximately similar level of TREX1 as in the other cell lines (Figure 5C). In this experiment we also included a pan-caspase inhibitor (Q-VD-OPh) to address whether apoptotic cell death might camouflage the effect of TREX1 depletion. Remarkably, the caspase inhibition, but not the TREX1 depletion, resulted in a high pSTAT1 level after treatment with IR and ATRi in H460 (Figure 5C). The magnitude of this response after the triple-treatment was comparable to the IFN response in U2OS cells after IR + ATRi (Figure 5D). The caspase inhibitor also increased the pSTAT1 levels after IR + ATRi in U2OS, SW900 and A549 cells, but no increase was seen in H1975 cells (Figure 6A). Of note is that these differences were not statistically significant for U2OS and A549, but all the experiments anyway showed a similar trend (Figure 6A). To further validate these findings, we measured IFN-β by ELISA in H460, U2OS, H1975 and SW900 cells after treatment with ATRi and/or IR in the presence and absence of the caspase inhibitor. The ELISA results confirmed that caspase inhibition restores the IFN response in H460 and increases the responses in U2OS and SW900 cells (Figures 6B, C). Intriguingly, caspase inhibition also increased IFN-β secretion in H1975 cells (Figures 6B, C), despite the lack of increase in pSTAT1 level (Figure 6A). The amount of secreted IFN-β was even higher for H1975 than for the other cell lines. The pSTAT1 response occurring downstream of IFN-β secretion must thus somehow be downregulated in H1975 cells. Treatment-induced cleavage of caspase-3 and PARP1 were detected in H460, U2OS and H1975 cells (Supplementary Figure S4B), which also were the three cell lines showing biggest increases in IFN response upon caspase inhibition. Altogether, these results strongly suggest that treatment-induced caspase activation is responsible for the lack of IFN response in H460 cells after IR + ATRi. Furthermore, caspase activation also counteracts the IFN response in the other cell lines.

Discussion

Combined treatment with ATRi and radiotherapy is a promising strategy under evaluation in clinical trials (10, 11, 32). While the rationale until recently has been ATR's function in DNA damage repair and cell cycle checkpoints, a new role for ATR is also emerging in the suppression of antitumor immune responses [reviewed in (33–35)]. However, the mechanisms of how ATRi regulate immune effects, and to what extent these are important in human cancers, have been unclear. We show that the ATR inhibitors VE822 and AZD6738 can potentiate radiation-induced, cGAS-dependent type I interferon signaling in several cell lines from human osteosarcoma and NSCLC. On the other hand, IFN signaling was not observed in one of the NSCLC cell lines, H460, despite abrogation of the G2 checkpoint and presence of micronuclei. Remarkably, upon addition of a pan-caspase inhibitor, the IFN response was restored in this cell line after irradiation and ATR inhibition. Moreover, the caspase inhibitor also increased the IFN responses in the other cell lines. Our results are consistent with a model where the ATR inhibitors' abrogating effect on the G2 checkpoint leads to an

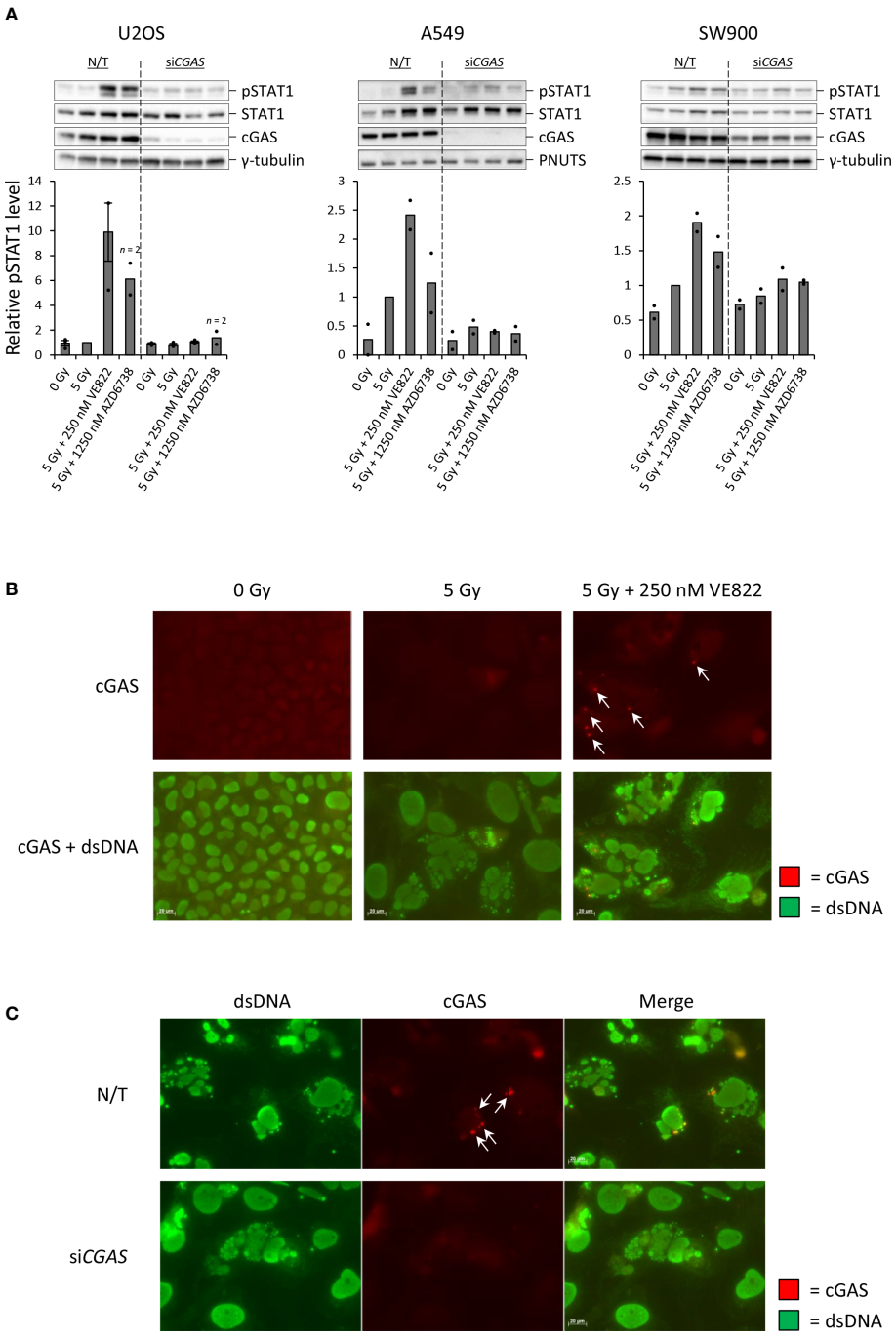

FIGURE 4
Increased IFN signaling after IR and ATRi is dependent on the cytosolic DNA sensor cGAS. **(A)** Immunoblots and quantifications of pSTAT1 levels in non-transfected (N/T) and si*CGAS*-transfected cells at three (U2OS, SW900) or six (A549) days after treatment. Bar charts show pSTAT1 levels relative to loading controls (γ-tubulin or PNUTS) and normalized to 5 Gy. **(B)** Micrographs of U2OS cells stained with antibodies against cGAS (red) and dsDNA (green) at three days after treatment. **(C)** Micrographs of U2OS cells transfected with si*CGAS* as in **(A)** and harvested at three days after treatment with 5 Gy and 250 nM VE822. Arrows in Figure B-C indicate cGAS foci localized to micronuclei. Scale bars: 20 μm.

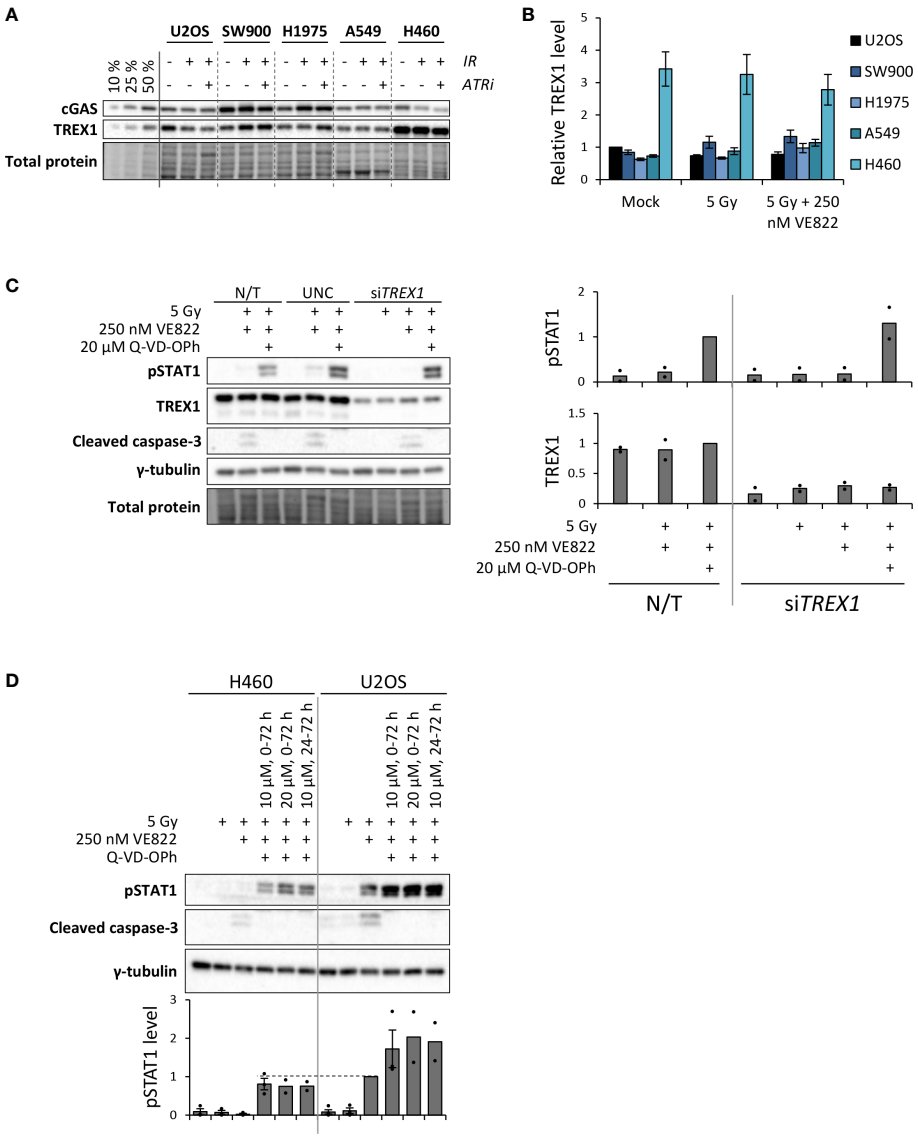

FIGURE 5
Caspase inhibition restores IFN signaling in the H460 cell line, which initially lacked the IFN response after IR + ATRi. **(A)** Immunoblots showing cGAS and TREX1 levels at three days after treatment. Three leftmost lanes: 10, 25 and 50% loading of the co-treated SW900 sample. ATRi: 250 nM VE822. **(B)** Quantification of immunoblots from three independent experiments as shown in **(A)** for TREX1, relative to total protein levels and normalized to U2OS mock. **(C)** Left: Immunoblots of H460 cells at three days after treatment with IR (5 Gy), ATRi (250 nM VE822) and a pan-caspase inhibitor (20 µM Q-VD-OPh). Cells were transfected with control siRNA (UNC; universal negative control) or siRNA targeting TREX1 at six hours prior to the treatment. Right: Quantification of immunoblots for pSTAT1 and TREX1 from two independent experiments, relative to total protein and normalized to the triple-treated non-transfected (N/T) cells (third lane). **(D)** Immunoblots of H460 cells and U2OS cells at three days after the indicated treatments. The caspase inhibitor was present at 10 µM or 20 µM for 0-72 h or 24-72 h after irradiation, as indicated. The ATR inhibitor (VE822) was present for 0-72 h. Bottom bar chart shows quantification of pSTAT1 levels from three (two for the two latter triple-treatments for both cell lines) independent experiments, relative to γ-tubulin and normalized to the co-treated U2OS sample (5 Gy + 250 nM VE822). Dashed line is included to compare pSTAT1 levels for the triple treated H460 cells with the co-treated U2OS cells.

IFN response *via* detection of micronuclear DNA by the cytosolic DNA sensor cGAS. The ATR inhibitors thereby accelerate and increase the radiation-induced IFN response. However, treatment-induced caspase activation can suppress this response (Figure 7).

Our finding, that caspase inhibition increases interferon signaling, is consistent with previous studies showing caspase-dependent suppression of the cGAS–STING–IFN pathway during DNA virus infection [reviewed in (23)]. A previous study has reported that caspase inhibition also can increase

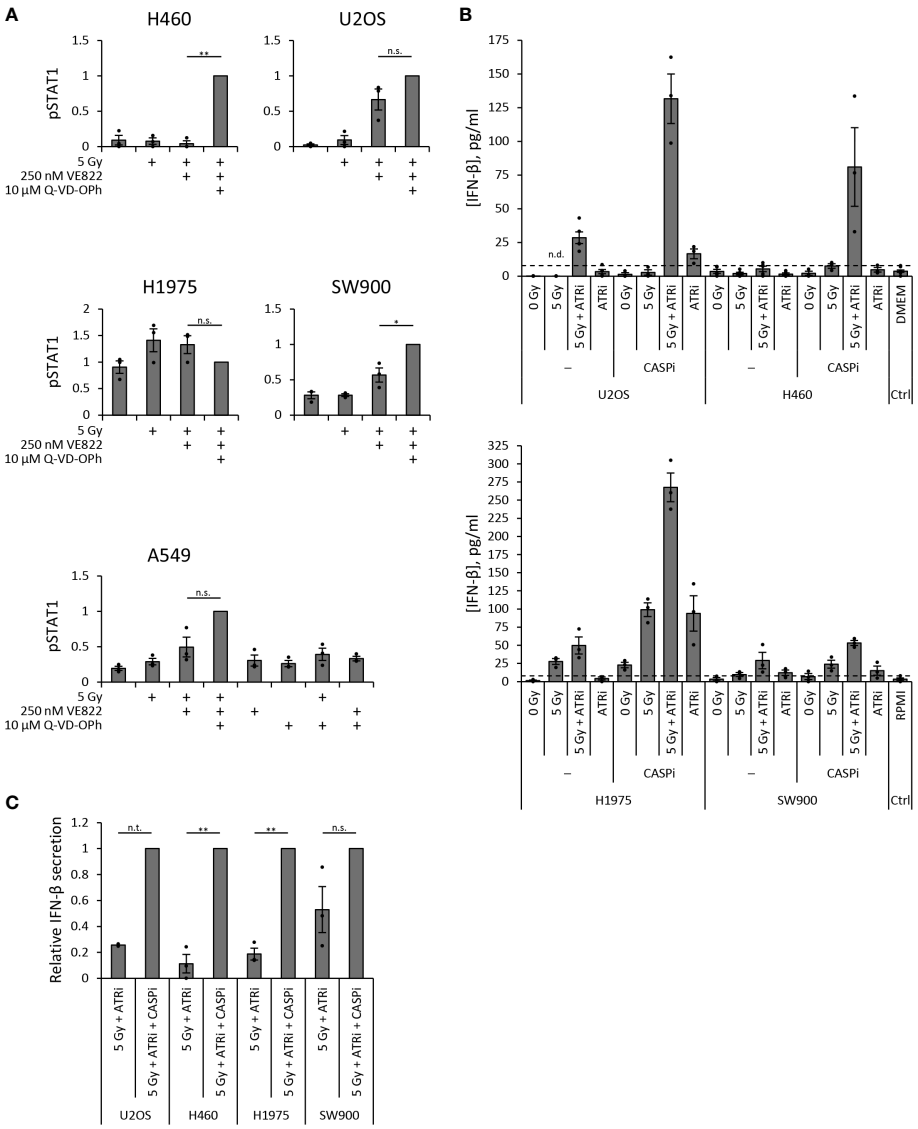

FIGURE 6
Caspase inhibition increases secreted IFN-β. **(A)** Quantification of pSTAT1 levels from three independent immunoblot experiments in each cell line at three days (H460, U2OS, H1975, SW900) or six days (A549) post treatment. Values are relative to γ-tubulin or total protein and normalized to the triple-treated sample. One sample *t* test was conducted for differences between co-treated (5 Gy + ATRi) and triple-treated (5 Gy + ATRi + CASPi) samples. **(B)** ELISA measurements of secreted IFN-β in 20X upconcentrated growth medium supernatants from samples three days after IR and ATRi. ATRi: 250 nM VE822, CASPi: 10 µM Q-VD-OPh (24–72 h). Top: U2OS and H460 with DMEM medium control; bottom: H1975 and SW900 with RPMI medium control. Results from Figures 2H and 3E are included in the plots for U2OS and H460 without CASPi. **(C)** The IFN-β values in **(B)** for co-treated (5 Gy + ATRi) normalized to the values for triple-treated (5 Gy + ATRi + CASPi) samples. (n.t.: not tested (U2OS, *n* = 2), n.s.: not significant).

radiation-induced IFN secretion (36). However, to our knowledge, it has not previously been shown that caspase inhibition increases the IFN response after combined treatment with IR and ATRi. We propose that treatment-induced caspase activation counteracts the IFN response mediated by cGAS-detection of DNA from ruptured micronuclei. This finding may partly explain why different cancer cell lines show large variations in the extent of IFN response after irradiation and ATR inhibition [this study and (17)]. In some cell lines, the treatment induces strong caspase activation which suppresses the response. The underlying molecular mechanism of how pan-caspase inhibition increases the IFN response after IR and ATRi remains to be elucidated. Caspases may potentially cleave cGAS or other factors in the cGAS–STING signaling cascade (23, 37). Furthermore, the previous study with radiation-induced IFN suggested that

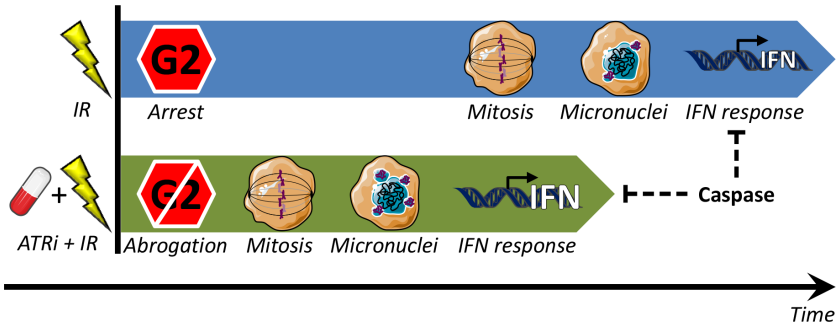

FIGURE 7
Model for regulation of type I IFN response by G2 checkpoint arrest. Treatment with IR alone (top) can induce a delayed IFN response, occurring after completion of the IR-induced G2 checkpoint arrest. When the G2 arrest is abrogated by ATRi (ATRi + IR; bottom), the IFN response comes earlier, and it is also stronger (because more micronuclei are formed when there is less time for DNA repair prior to mitosis). In both cases IFN is induced due to immune recognition of DNA from ruptured micronuclei, *via* the cGAS–STING–IFN pathway. This pathway can be suppressed by treatment-induced caspase activation.

caspase inhibition prevents breakdown of irradiated cells with cytosolic DNA (36). Notably, the IFN response is regulated by multiple factors. In addition to the micronuclei, mitochondrial DNA or endogenous retroviruses can also cause IFN induction after irradiation (38, 39). An important task for the future is therefore to better understand the relative contribution from each of these pathways.

The lack of IFN response in H460, after both irradiation alone and co-treatment with IR and ATRi, coincided with a higher baseline level of TREX1 in this cell line than in the responding ones. We therefore hypothesized that TREX1 might be a regulating factor for the cGAS–IFN signaling pathway in H460. However, neither partial depletion nor full depletion of TREX1 by siRNA transfection did increase IFN signaling in H460. Furthermore, we failed to see an increase in TREX1 levels after treatment with high radiation doses (10-20 Gy). The observed reduction of IFN response after high doses did therefore not correlate with an induction of *TREX1* expression, in contrast to the results of a previous study (25). However, while we assessed TREX1 protein levels, the previous study examined *TREX1* mRNA levels and also applied other cell lines than us, which might explain differences between the results.

In our study, we employed two different concentrations of the ATR inhibitors. While the highest, most toxic concentrations of the inhibitors (250 nM VE822; 1250 nM AZD6738) abrogated the G2 checkpoint and induced IFN signaling, the lower concentrations (50 nM VE822; 250 nM AZD6738), which were less toxic, only moderately abrogated the checkpoint and showed minor increases in IFN signaling. Of note is that the higher inhibitor concentrations are toxic even without irradiation, and the concentrations typically used for radiosensitization of cancer cell lines are closer to the lower concentrations in our study. Radiosensitizing effects have for instance been reported with 25-50 nM VE822 in U2OS and A549 cells (40) and with 100-300 nM AZD6738 in A549 and H460 cells (41), as measured by clonogenic survival. Interestingly, in order to cause pronounced increases in IFN signaling, the cells required higher concentrations of the inhibitors than what is needed for a mere radiosensitizing effect. The effects of ATRi in IFN signaling nevertheless required co-treatment with radiation, as treatment with the inhibitors in the absence of irradiation caused no or only small increases in IFN response (Figures 2H; 3E; 6A, B).

The reduction in IFN response after high IR doses (10-20 Gy) correlated with a prolonged G2 checkpoint arrest. This correlation is in line with previous reports showing reduced IFN signaling and a longer G2 checkpoint arrest after irradiation of DNA repair-deficient cells, as compared to repair-proficient cells (16, 18). In repair-deficient cells, the higher level of unrepaired DNA damage with low radiation doses will cause a longer G2 checkpoint arrest, analogous to the prolonged checkpoint arrest seen in repair-proficient cells with high radiation doses. Our results thus strongly support the notion that radiation-induced cell cycle arrest functions to suppress the type I IFN response (16). Previously, a phenomenon of checkpoint adaptation and G2 checkpoint imperfectness, allowing cells to escape checkpoint arrest even with remaining DNA breaks, has been described (42, 43). Interestingly, the link between micronuclei and induction of IFN signaling suggests an important functional role of checkpoint adaptation in stimulating antitumor immune responses.

In conclusion, the combined treatment of irradiation and ATR inhibition can potentiate radiation-induced type I IFN responses, and thus be a candidate immunostimulatory radiotherapeutic strategy. The clinically relevant immune effect of such co-treatment will likely depend on the type of cancer, the heterogeneity of the tumors and possibly also treatment-induced caspase activation. Adding caspase inhibitors could potentially also be a future strategy to increase antitumor immune effects,

although it is far from clear how they will affect both normal tissue and other antitumor responses. Further *in vivo* investigation will unveil the fuller potential of these combined treatments, which may also be further combined with immune checkpoint inhibition.

Materials and methods

Cell culture, irradiation and inhibitor treatment

Human H460 and A549 NSCLC and U2OS osteosarcoma cells were grown in DMEM with GlutaMAX-I, and SW900 and H1975 NSCLC cells in RPMI 1640 medium with GlutaMAX-I (both media from Gibco by Life Technologies), at 37°C with humidified 5% CO_2 atmosphere. The media were supplemented with 10% fetal bovine serum (Biowest) and 1% penicillin–streptomycin solution (50 IU/ml) (Gibco). Cells were tested for *Mycoplasma* infection, and their identity was confirmed by short tandem repeat analysis. ATR inhibitors VE822 (berzosertib/VX970, Selleckchem) and AZD6738 (ceralasertib, Selleckchem) were added 10–30 minutes before irradiation (160 kV X-rays, 1 Gy/min, Faxitron CP-160).

Cell cycle analysis

Cells were fixated with 70% ethanol, stained with Hoechst 33258 (Sigma-Aldrich) and analyzed with a LSR II flow cytometer (BD Biosciences) coupled to the BD FACSDiva v8 software. DNA histograms were analyzed in FlowJo v10. Cell cycle analysis was conducted by the Watson algorithm.

Immunoblotting

Cells were lysed in whole-cell lysis buffer (20 mM NaCl, 2 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100) with protease and phosphatase inhibitor cocktails (cOmplete mini (EDTA-free) and PhosSTOP EASYpack, Roche) and benzonase (100 IU/ml; Merck/Sigma-Aldrich). Protein concentration was measured by Micro BCA Protein Assay kit (ThermoFisher Scientific), and adjusted. Lane Marker Reducing Sample Buffer (Pierce) was added and the samples were boiled for 10 minutes at 95°C. SDS-polyacrylamide 4-15% gradient gels (Bio-Rad) were used for electrophoresis and nitrocellulose membranes (Bio-Rad) for blotting. The resulting membrane was blocked in 5% non-fat skimmed-milk powder in PBS with 0.1% Tween (PBST) at room temperature for a minimum of 30 minutes. Membranes were stained with primary antibodies at 4°C overnight and secondary antibodies at room temperature for 30-45 minutes (antibodies were diluted in the aforementioned blocking solution), before addition of enhanced chemiluminescence solution (ThermoFisher Scientific). Washing of membranes after transfer and antibody incubations was done in room-tempered PBST. Images were processed and quantifications were performed in Image Lab 4.1 (Bio-Rad). Range of detection was verified by excluding saturated signals and by including a dilution series of one of the samples (see Figure 5A). The resulting standard curve allowed for accurate quantification. Antibodies are listed in Supplementary Table 1.

Immunofluorescence microscopy

Cells were cultured on glass coverslips and fixated with 10% formalin solution (Sigma-Aldrich) for 10 minutes at room temperature. Cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS, and stained with primary antibodies for 1 hour followed by secondary antibodies for 30 minutes. For blocking, the antibodies were diluted in room-tempered DMEM with 10% FBS upon staining of coverslips. The coverslips were washed three times in PBS after fixation, permeabilization and antibody incubations. Coverslips were mounted with mowiol solution (Sigma-Aldrich). Antibodies are listed in Supplementary Table 2.

siRNA transfection for gene knockdown

For cGAS depletion, cells were transfected with 20 nM si*CGAS* (M-015607-01-0005, SMARTpool, Dharmacon). For STING depletion, cells were transfected with 10 nM si*STING1* (si*TMEM173*, ID 128591, Ambion). For partial TREX1 depletion, cells were transfected with 5 nM si*TREX1* (ID s535182, Ambion). All transfections were performed with Lipofectamine RNAiMax (Invitrogen), at six hours before treatment. For siRNA sequences, consult Supplementary Table 3.

Enzyme-linked immunosorbent assay (ELISA) of interferon-β

Growth medium supernatants were centrifuged to exclude floating cells. Resulting supernatants were 20X up-concentrated by centrifuge filtering through 10 kDa cut-off columns (Amicon Ultracel-10, Merck). ELISA (Human IFN-beta DuoSET ELISA, R&D Systems) was conducted according to supplier's protocol. Optical density was measured at 450 nm with pathlength correction at 540 nm in a microplate spectrophotometer (PowerWave XS2, BioTek) coupled to the Gen5 software v2.09.1. IFN-β standards were included in all experiments, and a best-fitting 2^{nd} degree polynomial function was used for calculation of measured IFN-β in the samples.

Statistics

Error bars represent standard error of the mean (SEM; $n \geq 3$). Dots in bar charts indicate individual experiments. p values (one-sample Student's t test for pairs involving normalization value (*i.e.* 5 Gy for most plots); two-tailed, paired-samples Student's t test for the remaining pairs) were calculated with IBM SPSS Statistics v28, with significance level set to 0.05. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Data availability statement

The raw data supporting the conclusions of this article will be made available upon request to the corresponding author.

Author contributions

Conceptualization: RGS, SH, AEM. Experiments: AEM, SH, GER. Supporting experiments: IØ. Data analysis: AEM, SH, IØ, RGS. Figures: AEM, SH. Supervision: RGS, AC. Critical review of work: all authors. Writing—original draft preparation: AEM, RGS. Writing—editing: all authors. Funding acquisition: RGS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.981332/full#supplementary-material

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Harnessing immunomodulation during DNA damage in Ewing sarcoma

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Ewing sarcoma is a fusion-oncoprotein-driven primary bone tumor most commonly diagnosed in adolescents. Given the continued poor outcomes for patients with metastatic and relapsed Ewing sarcoma, testing innovative therapeutic approaches is essential. Ewing sarcoma has been categorized as a 'BRCAness' tumor with emerging data characterizing a spectrum of DNA damage repair defects within individual Ewing tumors, including the presence of EWSR1::FLI1 itself, recurrent somatic mutations, and rare germline-based defects. It is critical to understand the cumulative impact of various DNA damage repair defects on an individual Ewing tumor's response to therapy. Further, in addition to DNA-damage-directed therapies, subsets of Ewing tumors may be more susceptible to DNA-damage/immunotherapy combinations given the significant cross-talk between DNA damage and inflammatory pathways in the tumor microenvironment. Here we review potential approaches utilizing DNA-damaging agents as modulators of the Ewing tumor immune microenvironment, with a focus on radiation and opportunities during disease metastasis and relapse.

KEYWORDS

Ewing sarcoma, radiation, immunobiology, DNA damage, immunomodulation, relapse

Introduction

Ewing sarcoma is the second most common bone tumor diagnosed in adolescents and young adults. Ewing sarcoma is driven by a fusion oncoprotein derived from the translocation of *EWSR1* on chromosome 22 with an ETS family member, most commonly *FLI1* on chromosome 11 (1). Patients with upfront metastatic or relapsed Ewing sarcoma continue to have very poor outcomes (2), and new therapeutic approaches continue to be in high demand. The exquisite sensitivity of Ewing tumors to DNA damage has been recognized for decades and DNA damaging agents such as chemotherapy and radiation continue to be the mainstays of Ewing sarcoma therapy, even for aggressive disease (3).

DNA damage can elicit significant alterations in tumor biology, including modulation of the tumor immune microenvironment (TIME). Historically, the impact of DNA damage on the Ewing TIME has been understudied given a paucity of tumor biopsies at the time of relapse and the lack of syngeneic or transgenic (immunocompetent) mouse models of Ewing sarcoma (4). DNA-damaging agents can promote immunogenicity through multiple mechanisms including increasing the neoantigen repertoire, increasing antigen presentation, and shifting the cytokine profile to promote an inflamed tumor microenvironment (5, 6). Understanding TIME alterations elicited by DNA damage specifically in Ewing sarcoma is a high priority, as TIME modulation during DNA damage may offer a new avenue for therapy for patients with aggressive disease. Therapeutically, it can be challenging to increase chemotherapy doses or add additional marrow-suppressive agents into existing chemotherapy backbones for the treatment of Ewing sarcoma, also highlighting why multi-modality approaches, such as TIME modulation, are in need.

Immunotherapy includes medications and cell-based therapies that broadly act by enhancing the anti-tumor immune response through various mechanisms (7) and have been utilized successfully in many adult carcinomas and soft tissue sarcomas (8, 9) (10). Clinical trials investigating single-agent immunotherapy, such as PD1 inhibition, have not demonstrated a significant clinical benefit in advanced Ewing sarcoma (11, 12). Given the importance of immunotherapy type and timing in disease response (13) such results are neither surprising nor discouraging when currently so little is known about the Ewing TIME. Primary Ewing sarcoma is known to have low overall immune infiltration compared to other tumors types. However, some studies have demonstrated a correlation between increased infiltration of CD8+ T cells and improved outcomes (14, 15). Our recent work demonstrated the Ewing TIME can evolve and demonstrate enhanced immune cell infiltration upon disease metastasis and relapse, possibly due to a combination of prior chemotherapy exposure and changes in tumor microenvironments (bone versus lung) (16). This work again highlights the need to better understand Ewing tumor immunobiology, especially in the setting of relapse.

In this mini-review we will discuss the layers of DNA damage repair defects in Ewing sarcoma, how DNA damaging agents can influence the TIME, and ways in which immunomodulation during DNA damage could provide new therapeutic opportunities for Ewing sarcoma in the future.

DNA damage and Ewing sarcoma

EWSR1::FLI1 and DNA damage sensitivity

Ewing tumors demonstrate high sensitivity to DNA damage. DNA damaging agents, including doxorubicin and cyclophosphamide, have formed the chemotherapy backbone for the treatment of Ewing sarcoma since the first use of adjuvant therapy in the 1970s (17). Ewing sarcoma is also sensitive to radiation therapy (18). Decades later, a screen of hundreds of cancer cell lines seeking to identify biomarkers for targeted cancer agents discovered EWSR1::FLI1 was significantly associated with sensitivity to the PARP [Poly (ADP-ribose) polymerase] inhibitor (PARPi) olaparib (19). PARP1 is an enzyme involved in DNA damage repair and a drug target in BRCA-mutant cancers deficient in homologous recombination repair (20). PARP1 drives transcription and accelerates base excision repair (21, 22), and inhibition of PARP1 leads to cell death in cancers deficient in homologous repair by causing defects in the replication fork needed to repair DNA damage. Further studies elucidated that EWSR1::FLI1 interacts directly with PARP (20). Gorthi et al. demonstrated that expression of the EWSR1::FLI1 fusion oncoprotein correlated with increased chemosensitivity (23). Mechanistically, they found that EWSR1::FLI1 promotes R-loop accumulation, and ultimately deranges DNA damage repair machinery by impairing normal BRCA1 functionality. A study in 2002 by Spahn et al. also demonstrated that the N-terminal portion of EWSR1::FLI1 can interact with the C-terminal portion of BRCA1-Associated Ring Domain 1 (BARD1), thus providing another potential link between EWSR1::FLI1 and BRCA1 biology (24). Such studies provided rationale for phase II clinical trial of olaparib as single-agent therapy in patients with refractory Ewing sarcoma (25) and subsequent studies have demonstrated that sensitivity to PARP inhibition in Ewing sarcoma is increased in the setting of other DNA damaging agents (irinotecan, temozolomide) (26). Despite this, the overall clinical response of Ewing tumors to PARPi has been underwhelming. Lastly, elegant work has demonstrated the importance of the level of EWSR1::FLI1 fusion oncoprotein expression on Ewing cell behavior. EWSR1::FLI1 expression can vary between cells within a tumor. It is plausible that Ewing cells with low versus high EWS::FLI1 expression may demonstrate altered sensitivity to DNA damage (27–29), thus allowing for tumor cell subpopulation targeting.

Somatic and germline variants in Ewing sarcoma

In addition to DNA-damage-repair defects imparted by EWSR1::FLI1 in all Ewing tumors, there is the potential for Ewing tumors to harbor additional defects in DNA damage repair through the presence of somatic and germline variants or post-transcriptional modifications resulting in loss of protein expression. When comparing Ewing tumors to adult carcinomas, and even other pediatric primary bone tumors such as osteosarcoma, Ewing sarcoma demonstrates a very low tumor mutational burden (30–32). A handful of recurrent somatic variants, such as *STAG2*, *CDKN2A*, and *TP53* have

been reported in Ewing sarcoma (31, 32). Ewing tumors harboring one or more of these somatic mutations may demonstrate altered responses to DNA damage, as each of the corresponding proteins have been shown to participate in DNA damage repair through different mechanisms. For example, *in vitro* studies of STAG2-deficient glioblastomas demonstrated increased sensitivity to PARP inhibition (33). In Ewing sarcoma, loss of STAG2 expression can be secondary to *STAG2* somatic mutations or loss of protein expression in the absence of a mutation (34).

A third layer of DNA-damage-repair deficiency to consider in Ewing sarcoma derives from germline pathogenic variants. Multiple sequencing studies of pediatric cancers have noted a small fraction of germline pathogenic variants in patients with Ewing sarcoma (35, 36). In a germline variant analysis of sequencing data from 175 patients with Ewing sarcoma, likely pathogenic variants were identified in 13.1% of patients (37). In the variants found, involving 22 different genes, a strong enrichment for DNA repair pathways and DNA double-strand break repair was noted on pathway analysis. Our work and others continue to add to the growing number of germline variants in DNA damage repair genes noted in patients with Ewing sarcoma (38, 39). Our group's prior work demonstrated that loss of additional DNA damage repair machinery, such as BARD1 expression, can indeed confer Ewing cells more susceptible to DNA damage as compared wuth the sensitivity imparted by the presence of EWSR1::FLI1 alone (40). Figure 1 depicts a brief summary of the spectrum of DNA damage repair deficiencies in Ewing sarcoma.

DNA damaging agents used in Ewing sarcoma therapy

Given the spectrum of DNA damage repair defects in Ewing sarcoma, DNA damaging agents will continue to be a mainstay of therapy. Following the original treatment schema with doxorubicin and cyclophosphamide discussed above, in the 1980s it was noted that ifosfamide and etoposide, which also exert their anti-neoplastic effect through induction of DNA damage, were effective in treating patients with relapsed Ewing sarcoma (41). This led to the development of the National Cancer Institute protocol INT-0091 (CCG-7881 and POG-8850) in which ifosfamide and etoposide were added to the standard therapy backbone. Improved overall and event free survival was seen in patients with newly diagnosed, localized Ewing sarcoma using this five-drug approach (42). Alternating cycles of VDC (vincristine, doxorubicin, cyclophosphamide) and IE (ifosfamide and etoposide) thus remain the standard of care for patients with upfront localized or metastatic Ewing sarcoma. AEWS0031 later demonstrated that shortening the time between cycles (interval compression) provides additional benefit (43).

In addition to chemotherapy, radiation is also an important component of Ewing sarcoma treatment. Radiation is a curative-intent treatment modality option for local control, either as definitive treatment or as adjuvant treatment following surgical resection. The commonly prescribed radiation dose for definitive treatment of primary tumors is 55-60 Gy in 1.8-2 Gy fractions (44). The most recent Children's Oncology Group protocols for Ewing sarcoma recommend 45 Gy be delivered to the original tumor volume with an additional 10.8 Gy boost delivered to the post-induction chemotherapy volume (3). Gross residual disease post-surgical resection is treated with 55.8 Gy, and microscopic disease treated with 50.4 Gy. There has recently been data suggesting that dose escalation up to a total dose of 70.2 Gy may be of benefit in improved local control (45), although this strategy has not been widely adopted to date. More recent studies show that hypofractionation (5-10 Gy doses over 5-10 fractions) may be as or more effective at treating sarcomas, including Ewing sarcoma (46).

Radiation therapy is also a key component in the treatment of metastatic and relapsed Ewing sarcoma. For patients presenting with pulmonary metastases at diagnosis, there have been multiple studies demonstrating the survival benefit of whole lung irradiation after completion of chemotherapy (47). For patients presenting with bony metastases, outcomes are worse overall; however, radiation delivery to sites of metastatic disease is beneficial (48). Patients with solitary bone metastases benefited most from radiotherapy, with doses of up to 50 Gy to sites of bony metastases being utilized. As patients with Ewing sarcoma receiving radiation are often a higher-risk patient population (incomplete resections, metastatic disease, relapse, etc.), this is a group of high interest when considering immunotherapy interventions following post-DNA damage modulation of the Ewing TIME.

Immunomodulation through DNA damage

Immunomodulation by chemotherapy

DNA damaging chemotherapeutic agents have been shown to induce immunogenicity through a variety of mechanisms (5). Given the low mutational burden of Ewing sarcoma, the mutagenic potential of DNA damaging agents is an appealing mechanism of enhancing immunogenicity by production of tumor neoantigens (49). Tumor neoantigens can induce increased anti-tumor T cell response which is again beneficial when combined with immunotherapy agents. However, increased neoantigens in the TIME are not always sufficient to induce immune response (50). DNA damaging agents additionally lead to release of damage associated molecular patterns (DAMPs) after cell death. DAMPs stimulate the

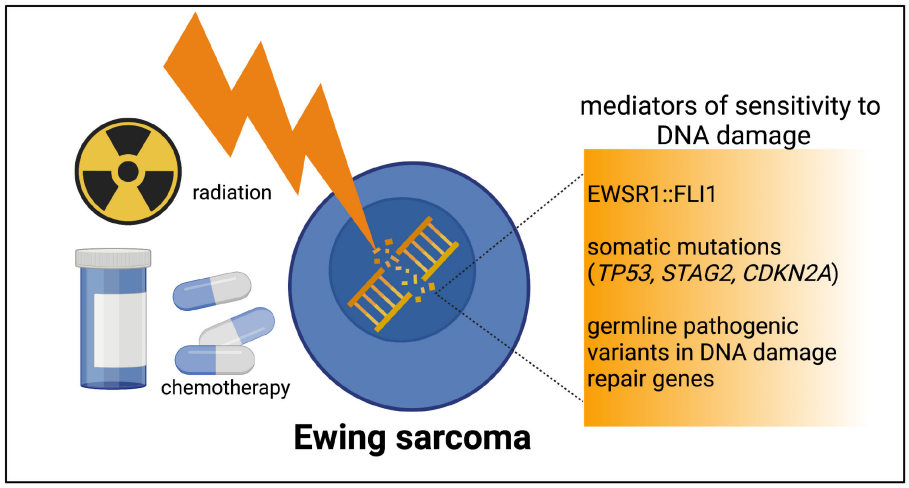

FIGURE 1
The spectrum of DNA damage repair deficiencies in Ewing tumors. Ewing tumors all have a level of DNA damage repair deficiency imparted by EWSR1::FLI1. The presence of one or more recurrent somatic mutations or rare germline pathogenic variants have the potential to contribute an additional level of DNA damage repair deficiency in a subset of Ewing tumors. Figure created by biorender.com.

recruitment of antigen-presenting cells to the site of cell death and further prime the TIME for an adaptive immune response. Doxorubicin and cyclophosphamide are utilized in the treatment of Ewing sarcoma and are known to induce immunogenic cell death (51). Cyclophosphamide additionally remains of particular interest as it has been shown to increase antigen presentation on tumor cells and expand dendritic cell populations that can promote T cell priming (52, 53).

An additional mechanism by which DNA damaging chemotherapeutics can increase anti-tumor immune response is through changes in the cytokine profile of the tumor environment. Cellular response to DNA damage includes activation of signaling pathways that lead to release of proinflammatory cytokines including IFN-α and cytokines triggered by activation of the NF-κB signaling pathway. Specifically, cyclophosphamide has been shown to induce IFN-γ and IL-2, pro-inflammatory cytokines that promote immunogenicity (53). Parkes et al. demonstrated that in a breast cancer model DNA-damage-repair defects lead to increased expression of the chemokines CXCL10 and CCL5 from tumor cells (16, 54).

The precise impact of chemotherapy commonly used in relapsed Ewing sarcoma [irinotecan and temozolomide (IT), topotecan and cyclophosphamide (TC), high dose ifosfamide (IFOS), and gemcitabine and docetaxel (GD) (55)] on Ewing tumor immunobiology is still largely unknown. PARP inhibitors have been shown to induce infiltration of CD8+ T cells in breast cancer, and the efficacy of PARP inhibition is due to recruitment of these cytotoxic T cells through the cGAS/STING pathway (56). In this model, depletion of CD8+ T cells decreased the efficacy of PARP inhibition. In addition to recruiting cytotoxic T cells, PARP inhibition has also been shown to increase the expression of immune checkpoint ligand PD-L1 on cancer cells (57). Our work has previously shown that PD-L1 and PD-L2 expression can be manipulated in Ewing cell subpopulations in response to inflammatory signaling (58).

In summary, the effect of DNA damaging chemotherapeutic agents used in the treatment of Ewing sarcoma can, in theory, manipulate the TIME; however, this is an understudied area. While chemotherapy has the temporary ability to alter the TIME, ultimately due to systemic effects, patients are largely overall immunosuppressed during therapy. Thus, focal delivery of DNA damage, such as radiation therapy, may be beneficial when considering immunotherapy combinations.

Immunomodulation by radiation

The interest in the immune-mediated effects of radiation date back to the 1980s when it was first noted that local radiation can lead to anti-tumor effect at distant sites of disease (59, 60). Subsequently, many studies have demonstrated that local radiation can produce systemic immune-mediated anti-tumor effects, though this is not a consistent finding in all studies (6, 61, 62). Studies examining the radiation anti-tumor effect in immunocompetent vs immunodeficient mouse models of melanoma have demonstrated that the presence of CD8+ cytotoxic T cells are necessary for this response (63). Radiation enhances the immune response to tumors through release of cytokines and chemokines in the tumor microenvironment following cell death (64). These cytokines and chemokines result in infiltration of effector immune cells (dendritic cells,

macrophages, cytotoxic T cells) as well as immunosuppressive populations (Tregs, myeloid-derived suppressor cells) (65). Similar to the effect of chemotherapy described above, radiation induces immunogenic cell death leading to release of DAMPs. This leads to increased production and recruitment of proinflammatory cytokines and chemokines, including CXCL9, CXCL10, and CXCL11 (66). The generation of this proinflammatory environment is thought to lead to recruitment of effector T cells and may enhance the priming of T cells in the TIME. Recently, the essential role of natural killer (NK) cells in controlling the radiation-induced anti-tumor has been demonstrated (67).

In addition to promoting a proinflammatory TIME, radiation can also exert immunosuppressive effects. Tregs are a well described subset of CD4+ T cells that exert immunosuppressive effects on the TIME. In some adult carcinomas, radiation has been shown to increase Tregs and the subsequent production of immunosuppressive cytokines including TGF-β and IL-10 (68) TGF-β is known to be increased following radiation and is converted from its latent to active form by reactive species generated during radiation (69). TGF-β exerts immunosuppressive effects on the TIME and it has been shown that increase in TGF-β in the TIME can lead to decreased efficacy of immunotherapy through the exclusion of CD8+ T cells (70). Several studies have demonstrated that inhibition of the immunosuppressive pathways activated by localized radiation can improve radiation-induced tumor kill and anti-tumor immunity (71, 72).

An additional immunosuppressive cell population that can be induced/increased following radiation are myeloid-deprived suppressor cells (MDSCs). MDSCs are well described to promote tumor growth and survival and are known to be recruited into the TIME of pancreatic and prostate cancer immediately following radiation (73), with a decrease in this population seen at 1-2 weeks post radiation. TGF-β is known to induce differentiation of macrophages to an M2 phenotype which is protumor and immunosuppressive. These mechanisms of immunosuppression induced by radiation represent potential targets to improve the anti-tumor immune response induced by radiation.

Radiation therapy in Ewing sarcoma: Untapped potential for multi-modality therapies

Currently, relatively little is known about the specific impact of radiation on the Ewing sarcoma TIME. New therapeutic approaches for patients with metastatic and relapsed Ewing sarcoma are long overdue. While agents that induce tumor DNA damage clearly provide some benefit for the treatment of relapsed disease, they are rarely curative. Understanding which multi-modality therapeutic approaches may circumvent Ewing tumor cell resistance to single- modality therapies is a priority. It is possible that subsets of Ewing tumors in the DNA-damage-repair deficiency spectrum (Figure 1) could demonstrate differential responses to multi-modality therapy. Radiation therapy is often utilized in patients with high-risk (metastatic and relapsed) Ewing sarcoma, and given its potential to modulate the TIME, it is a logical treatment modality to consider in combination with immunomodulation (Figure 2). There has been growing interest in oncology to combine radiation with immunomodulatory agents to improve the anti-tumor immune response (74–76). Given the concurrent immune-stimulatory and immunosuppressive effects that radiation therapy can trigger in the TIME, there has been interest in combination therapies targeting both of these sequalae (77). Broadly speaking, logical categories of immunomodulators to preclinically study in combination with radiation for the treatment of Ewing sarcoma include: 1) immune checkpoint inhibitors (ICI), 2) cytokine modulators, and 3) cell-based therapies. Here we will briefly address each of these approaches.

The combination of radiation therapy and ICI in patients with advanced solid tumors has demonstrated promising early clinical results (74, 75). It has also been reported that the presence of DNA damage repair defects, such as germline BRCA 1/2 pathogenic variants, is correlated with increased expression of immunosuppressive ligands such as PD-1/PD-L1 (78), considered one marker of response to immune checkpoint inhibition. This association provides a rationale for preclinically testing the response of Ewing tumors with additional DNA damage repair defects to the combination of radiation and immune checkpoint inhibition.

In addition to examining ICIs, modulation of cytokines in the tumor microenvironment during radiation therapy is of great interest. While not every cytokine can be addressed in this mini-review, we will highlight two. TGF-β is an immunosuppressive cytokine that is increased in tumor microenvironments following radiation and has been shown to confer resistance to radiation (72). Inhibition of TGF-β during radiation has the potential to enhance the anti-tumor immune response (79). A second cytokine, IL-6, is known to be secreted by Ewing tumors (80, 81), and can be upregulated following radiation-induced DNA damage. Further, it is thought that the presence of IL-6 in the TME confers radiation resistance (82). IL-6 inhibitors are active in clinical trials as monotherapy for cancer (83), however, combination therapy with these inhibitors during radiation offers another therapeutic avenue worthy of preclinical testing.

Lastly, there is promise for delivering cell-based therapies in the setting of radiation. Chimeric antigen receptor T-cells (CAR-T) therapies have shown great success in the treatment of hematologic malignancies but have not seen the same success in solid tumors (84). Challenges have included identification of an ideal target

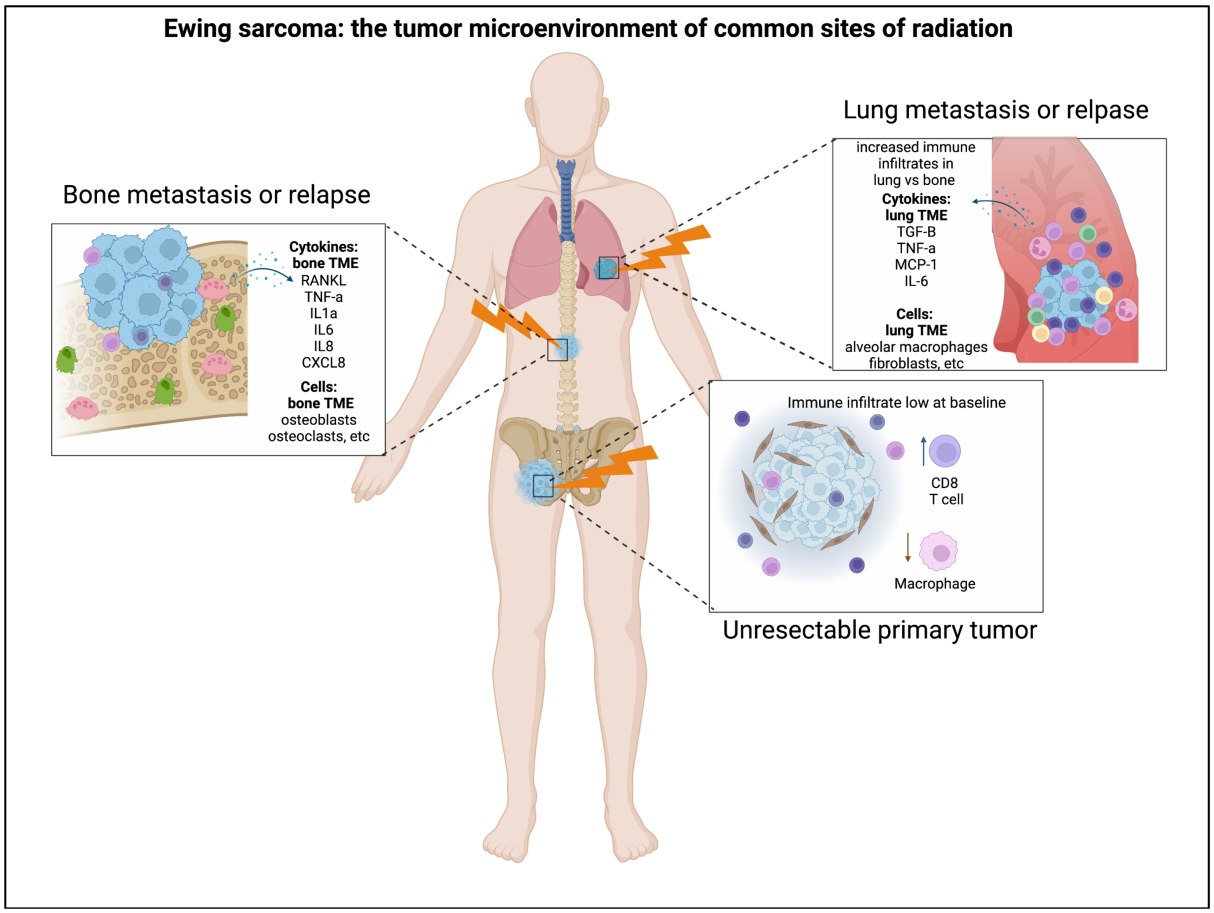

FIGURE 2
Radiation and the Ewing sarcoma tumor immune microenvironment. Radiation is often utilized for the treatment of Ewing sarcoma in the setting of unresectable primary tumors, lung metastases, relapse to the bone, etc. The Ewing tumor immune microenvironment can demonstrate differences in immune infiltration and cytokine abundance in these distinct microenvironments where radiation is utilized. Figure created by biorender.com.

antigen, cell trafficking to the tumor, and the overall immunosuppressive environment of solid tumors. Therapies targeting the DNA damage repair pathway have shown some success in solid tumors in improving response to CAR-T therapy through induction of a more pro-inflammatory TIME (85). Additionally, radiation therapy delivered prior to administration of CAR-T in a mouse model of glioblastoma demonstrated improved trafficking and efficacy of the CAR-T cells post-radiation (86). There is ongoing research in the field to identify a targetable antigen for cell based therapies for the treatment of Ewing sarcoma (87). In addition to CAR-T cell therapy, dendritic cell-based immunotherapy is a cell-based therapy that could logically be combined with DNA-damaging agents. Studies have demonstrated improved efficacy of dendritic cell vaccination when given in combination with radiation (88, 89). Lastly, as noted above, recent work has demonstrated the key role of NK cells in the radiation anti-tumor response. Understanding the role of NK cells in the TIME of Ewing tumors specifically during radiation is a priority (67, 90).

Future directions and challenges

Significant historical impediments to studying the influence of DNA damage on the immune microenvironment in Ewing sarcoma include, but are not limited to, the lack of an immunocompetent animal model of Ewing sarcoma (4) and the sparsity of samples from disease relapse and pre-/post-intervention biopsies. Recently, a genetically engineered zebrafish model of Ewing sarcoma has been developed, which may offer a new immunocompetent model (91), although studies specifically investigating immune interactions in this model have not yet been performed. A potentially valuable model for studying the TIME of Ewing sarcoma is the

development of humanized (presence of human immune cells), immunocompetent mouse models, a focus of ongoing work by our group. Developing and validating a robust preclinical model to study the impact of DNA-damaging agents used clinically for the treatment of Ewing sarcoma on the TIME is a crucial and necessary step toward determining promising immunomodulatory agents to partner with radiation or chemotherapy in an attempt to improve the outcomes for patients with advanced disease. While DNA damage, such as radiation therapy, is the focus of this mini-review, the impact of other novel agents, such as tyrosine kinase inhibitors, agents targeting EWSR1::FLI1, etc., on the Ewing sarcoma TIME are also worthy of exploration and represent a limitation of this mini-review.

Author contributions

JD, AO, and KB contributed to the mini-review concept, initial manuscript writing, and editing. JD and KB generated the figures and completed formatting. All authors contributed to the article and approved the submitted version.

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Clonogenicity-based radioresistance determines the expression of immune suppressive immune checkpoint molecules after hypofractionated irradiation of MDA-MB-231 triple-negative breast cancer cells

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Only a subset of patients with triple-negative breast cancer (TNBC) benefits from a combination of radio- (RT) and immunotherapy. Therefore, we aimed to examine the impact of radioresistance and brain metastasizing potential on the immunological phenotype of TNBC cells following hypofractionated RT by analyzing cell death, immune checkpoint molecule (ICM) expression and activation of human monocyte-derived dendritic cells (DCs). MDA-MB-231 triple-negative breast cancer tumor cells were used as model system. Apoptosis was the dominant cell death form of brain metastasizing tumor cells, while Hsp70 release was generally significantly increased following RT and went along with necrosis induction. The ICMs PD-L1, PD-L2, HVEM, ICOS-L, CD137-L and OX40-L were found on the tumor cell surfaces and were significantly upregulated by RT with 5 x 5.2 Gy. Strikingly, the expression of immune suppressive ICMs was significantly higher on radioresistant clones compared to their respective non-radioresistant ones. Although hypofractionated RT led to significant cell death induction and release of Hsp70 in all tumor cell lines, human monocyte-derived DCs were not activated after co-incubation with RT-treated tumor cells. We conclude that radioresistance is a potent driver of immune suppressive ICM expression on the

surface of TNBC MDA-MB-231 cells. This mechanism is generally known to predominantly influence the effector phase, rather than the priming phase, of anti-tumor immune responses.

KEYWORDS

radiotherapy, breast cancer, radioresistance, immune checkpoint molecules, dendritic cells, tumor cell death

1 Introduction

Triple-negative breast cancer (TNBC) is defined by the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression. It accounts for 10-20% of all breast cancer cases and is characterized by high invasiveness, early metastasis (esp. lung- and brain-metastases), and high recurrence rate. Despite similar therapeutic approaches (surgery, chemo- and radiotherapy), TNBC remains the breast cancer subtype with the worst prognosis. High heterogeneity, the lack of hormone receptors and chemoresistance (triple-negative paradox) leave little room for targeted therapy approaches (1–3). Therefore, therapeutic strategies leading to improved therapy outcomes are urgently needed.

Adjuvant radiotherapy (RT) is perceived as standard of care in patients with early-stage breast cancer undergoing breast-conserving surgery and complete mastectomy. The goal of it is to reduce the risk of locoregional recurrence and breast cancer associated mortality (4, 5). In this context moderately hypofractionated RT (HFRT) has gained importance over the last years (6). It is characterized by increased dose per fraction and simultaneously, decreased fractions in total (40 Gy in total, 15-16 fractions in 3-5 weeks). In comparison to conventional fractionation schedules (50 Gy in total, 25-28 fractions in 5-6 weeks), this approach offers lower acute toxic effects while maintaining local tumor control (7–9). Recently, the FAST-Forward trial indicated that a super-hypofractionated five-day treatment schedule of postoperative radiotherapy (26 Gy, five fractions in one week) is non-inferior to moderately hypofractionated adjuvant radiation therapy (40 Gy, 15 fractions in three weeks) in terms of local tumor control and side effects in women with early-stage breast cancer (10).

RT in general is attributed to both immune stimulatory and immune inhibitory effects. On the one hand, it can enhance anti-tumor immunity by cell death-triggered release of neoantigens, damage-associated molecular patterns (DAMPs, e.g. HMGB1, ATP, Hsp70) and proinflammatory substances (e.g. CXCL10 and CXCL16). Additionally, the activation of the cGAS/STING pathway including consequent type I interferon production and increased MHC-I expression for antigen presentation on the cell surface of cancer cells is also activated by RT (11). Besides the control of the immune checkpoint PD-L1/PD1 axis by interferons, also less well understood immune checkpoint molecules are triggered (12). Hypofractionated RT induces DNA damage and impaired DNA repair results in transfer and accumulation of DNA fragments in the cytoplasm of the tumor cells. As physiological mechanisms for detection of cytosolic DNA (e.g. resulting from invading pathogens), DNA sensing pathways as the cGAS/STING pathway are triggered that activate the innate immune response through a signaling cascade leading to upregulation of cytokine and interferon production (13). This is also a common mechanism in triple negative breast cancer that impacts on tumor cell survival and immune modulation (14, 15). On the other hand it was shown by Rückert et al., that HFRT in particular is predestined to induce immunogenic cell death (ICD) (16), which is defined as “a form of regulated cell death (RCD) that is sufficient to activate an adaptive immune response in immunocompetent syngeneic hosts” (17) leading to T cell-mediated immune responses against tumor antigens. Based on that, RT has been reported to work as *in situ* cancer vaccine making abscopal effects possible (18). On the other hand, RT can also mediate immune suppressive effects, for example by inducing an increased expression of immune suppressive immune checkpoint molecules (ICMs), the release of immune inhibitory cytokines (e.g. TGF-β) and the infiltration of T regulatory cells (Tregs) as well as myeloid derived suppressor cells (MDSC) into the tumor area (11). The T cell suppression in the effector phase of the immune response mediated *via* immune inhibitory ICM interactions, can be antagonized by immune checkpoint inhibitors (ICIs). Consequently, a tumor-antigen specific cytotoxic T cell response can be restored (19, 20). That makes combinations of radiotherapy and immune checkpoint blockade (ICB) reasonable.

Although breast cancer has been perceived historically as immunologically “cold” tumor, it becomes more and more evident that the different subtypes differ a lot regarding their respective immunogenicity. TNBC seems to be the most immunogenic subtype, because of its higher tumor infiltrating lymphocyte (TIL) counts and tumor mutational burden (TMB) (21). Supporting this, ICI therapy particularly benefits those breast cancer patients suffering from TNBC (22). Therefore, a growing number of clinical trials examining the efficacy of ICB in patients with TNBC have recently been conducted. Unfortunately, only a small minority of these patients has been shown to benefit from anti-PD-(L)1 monotherapy in terms of overall response rate (ORR) (23). However, Ho et al. reported that therapeutic approaches combining RT and ICB could be superior to ICI monotherapy (24). In this context radioresistant cancer cells remain a major

challenge in TNBC treatment because of their capacity to form local- and distant recurrence.

In the past, radioresistance of a cell has always been defined based on the ability to form new cell colonies after being irradiated (25, 26). Since presumably radiation-resistant (breast) cancer cells are responsible for recurrence or metastasis after RT, it may not only be radioresistance alone but rather the combination with immune evasion allowing breast cancer cells to survive and form clinically apparent tumors. Therefore, we hypothesized that radioresistance itself could significantly drive the immunogenic properties of breast cancer cells. To investigate this for the first time, we treated two different radioresistant (RR) and two non-RR MDA-MB-231 cell lines with hypofractionated RT (5 x 5.2 Gy) and analyzed cell death induction by AnnexinV/Propidium iodide staining, Hsp70 release and the activation of human monocyte-derived dendritic cells (DCs) after previous co-cultivation. Furthermore, the immune checkpoint molecule expression on the tumor cell surface was examined. Our key finding was that the expression of immune suppressive ICMs was significantly increased in the radioresistant cell lines after RT.

2 Materials and methods

2.1 Cell lines and cell culture

Four different human MDA-MB-231 cell lines with differences in radioresistance (according to their behaviour in the clonogenic assays) were investigated (27). Besides the wildtype (WT), a brain-metastasizing (BR) clone was used. It was created by Yoneda et al., 2001 by inoculating the MDA-MB-231 WT cells into immunodeficient mice. MDA-MB-231 cells in brain metastases were isolated, grown in culture and reinoculated. This procedure was repeated until only brain metastases occurred after injection into immunodeficient mice (28). Radioresistant (sub)clones (WT RR, BR RR) were generated by irradiation of the WT and BR clone with 4 Gy, pooling of the surviving cells, cultivating them for 10-14 days and irradiating them again. This procedure was repeated to a total dose of at least 40 Gy. Radioresistance was checked after the last irradiation with clonogenic assay (Figures 1A, B).

All four cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM, Pan-Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and 1% Penicillin-Streptomycin (PenStrep, Gibco, Carlsbad, CA, USA). Peripheral blood mononuclear cells (PBMCs) derived from healthy human donors were cultured in "DC medium" consisting of RPMI-1640 (Merck, Darmstadt, Germany) supplemented with 1% Pen/Strep, 1% L-Glutamine (Gibco, Carlsbad, CA, USA), 1% Hepes buffer (Gibco Life Technologies, Waltham, MA, USA) and 1% human serum heat inactivated (Gibco, Carlsbad, CA, USA). All cells were cultivated in a standardized and humidified environment (37°C, 5% CO_2 and 95% humidity).

2.2 Treatments and sampling

The day after seeding, the four MDA-MB-231 cell lines were irradiated for five days with 5.2 Gy of X-rays (120 kV, 22.4 mA for 0.7 min; X-Ray tube Isovolt Titan, GE Sensing & Inspection, Boston, USA), respectively. The cells were harvested with trypsin (Gibco Life

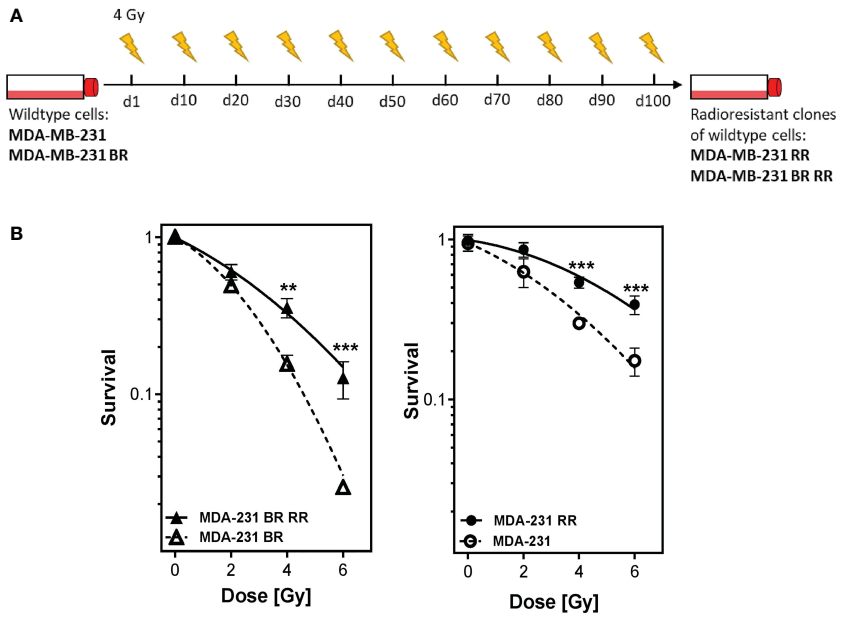

FIGURE 1
Generation of radioresistant breast cancer clones is done by repeated irradiation of MDA-MB-231 breast cancer cells. Radioresistant (sub)clones of MDA-MB-231 cells were generated by repeatedly irradiating MDA-MB-231 wildtype (MDA-MB-231) and brain metastasizing MDA-MB-231 (MDA-MB-231 BR) tumor cells **(A)**. This resulted in more radioresistant clones (MDA-MB-231 RR and MDA-MB-231 BR RR), as verified by clonogenic survival assay **(B)**. Data are from three independent experiments. **p < 0.01; ***p < 0.001 (Student's t-test).

Technologies, Carlsbad, CA, USA) on day 6, 7 and 8 for cell death analysis, on day 7 for ICM expression analysis and on day 6 to evaluate the DC activation potential of untreated and treated tumor cells after co-incubation. Hsp70 concentration in the cell culture supernatant was determined 48 hours after irradiation (day 7) *via* ELISA.

2.3 Cell death analysis and clonogenic survival assay

2×10^5 cells were stained with 100 µl of cell death staining solution (1 ml of Ringer's solution (Fresenius Kabi, Bad Homburg, Germany), 0.75 µl/ml of AnnexinV-FITC (AxV) (1 mg/ml) (GeneArt, Regensburg, Germany), and 1.0 µl/ml of Propidium iodide (Pi) (1 mg/ml) (Sigma-Aldrich, Munich, Germany)). After incubation for 30 minutes, the measurement was performed on a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with the Kaluza Analysis software (Beckman Coulter, Brea, CA, USA).

To determine the clonogenic potential of the breast cancer cells, 250 tumor cells/well were seeded in a 6-well plate 6h before irradiation. Afterwards they were irradiated with the indicated doses and cultured for two weeks, fixed and stained with 1% crystal violet in ethanol (Sigma-Aldrich, St. Louis, MO). Colonies with more than 50 cells were counted and normalized to mock-treated samples. The survival curves were calculated by adding a curve fit (dek(hx)). All calculations were performed with GraphPad Prism.

2.4 Immune checkpoint molecule expression analysis

2×10^5 cells were stained with staining solution containing FACS buffer (PBS (Sigma-Aldrich, Munich, Germany), 2% FBS and 4% 0.5 mM EDTA (Carl Roth, Karlsruhe, Germany)) and Zombie NIR alone or Zombie NIR and antibodies (Table 1). Before the measurement at the CytoFLEX S flow cytometer, the cells were incubated for 30 minutes at 4°C. To correct for treatment-related autofluorescence, the ΔMFI (mean fluorescence intensity) of every ICM was calculated by subtracting the MFI of the Zombie-only-stained sample (AF ctrl) from the MFI of the Zombie-and-antibody stained one.

TABLE 1 List of antibodies used to analyze the expression of immune checkpoint molecules on the surface of non-irradiated and irradiated MDA-MB-231 tumor cells *via* multicolor flow cytometry.

Marker	Fluorochrome	Manufacturer
PD-L1 (CD 274)	BV 605	Biolegend
PD-L2 (CD 273)	APC	Biolegend
ICOS-L (CD 275)	BV 421	BD Horizon
HVEM (CD 270)	APC	Biolegend
TNFRSF9 (CD137-L)	BV 421	Biolegend
OX40-L (CD252)	PE	Biolegend
Live/dead	Zombie NIR	Biolegend

2.5 Quantitative measurement of Hsp70 in the supernatant of untreated and treated MDA-MB-231 cells

To examine the concentration of tumor cell released Hsp70, the supernatant of the cell cultures was analyzed using a sandwich ELISA assay (Human/Mouse/Rat Total HSP70/HSPA1A ELISA, R&D Systems, Minneapolis, MN, USA). It was performed according to the manufacturer's instructions.

2.6 Isolation of human peripheral blood mononuclear cells and differentiation to human monocyte-derived dendritic cells

On day 0, human peripheral blood mononuclear cells (PBMCs) were isolated from leukoreduction system chambers (LRSC) of healthy human donors *via* density gradient centrifugation in SepMate™ PBMC Isolation Tubes (Stemcell, Vancouver, Canada) and Lymphoflot (Biotest AG, Dreieich, Germany). Consequently, they were washed twice at 4°C with PBS (Sigma-Aldrich, Munich, Germany)/0.5 mM EDTA (Carl Roth, Karlsruhe, Germany) and RMPI-1640, respectively. After that, 30×10^6 cells each were seeded on IgG-pre-coated cell culture dishes in 10 ml of DC medium and incubated for 1 h. Subsequently, non-attached cells were removed and 10 ml of fresh DC medium was added.

On day 1, the old DC medium was removed again and 10 ml of RPMI containing 800 U/ml (0.57 µl/ml) of GM-CSF (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) and 500 U/ml (5 µl/ml) of IL-4 (ImmunoTools, Friesoythe, Germany) were added to each cell culture dish. Two days later, on day 3, 4 ml of RPMI and 800 U/ml (0.57 µl/ml) of GM-CSF and 500 U/ml (5 µl/ml) of IL-4 were added. On day 5, 4 ml of RPMI with half of the previously used amount of GM-CSF (400 U/ml = 0.285 µl/ml) and IL-4 (250 U/ml = 2.5 µl/ml) were added.

2.7 Maturation induction of immature DCs *via* maturation cocktail or co-incubation with untreated and treated MDA-MB-231 cell lines

Six days after the isolation of the PBMCs, the human monocyte-derived immature DCs (iDCs) were harvested mechanically using a serological pipette. After that, 0.75×10^5 iDCs were put into each 6-well in 2 ml of DC medium. In case of co-incubation with non-irradiated or irradiated tumor cells, 1.5×10^5 tumor cells including 2 ml of their respective cell culture supernatant were added. Positive controls (without tumor cells) were established by using a maturation cocktail (MC) containing 13.16 ng/ml of IL-1β (ImmunoTools, Friesoythe, Germany), 1000 U/ml of IL-6 (ImmunoTools, Friesoythe, Germany), 10 ng/ml of TNF-α (ImmunoTools, Friesoythe, Germany) and 1 µg/ml of PGE-2 (Pfizer, Berlin, Germany).

2.8 Maturation

The expression of common activation markers on the cell surface of the DCs was analyzed 48 hours after co-incubation with untreated and treated MDA-MB-231 cells using multicolor flow cytometry. Therefore, the DCs were harvested mechanically at first. Then, the first half of all DCs in each 6-well was stained with a Zombie-only FACS buffer staining solution, the second half was stained with one containing Zombie Yellow and antibodies in addition (Table 2). After incubation for 30 minutes at 4°C, the MFI of the different samples was measured at the CytoFLEX S flow cytometer. The ΔMFI of every activation marker was calculated by subtracting the MFIs of the Zombie-only from the fully stained sample.

2.9 Statistical analyses

For statistical analyses the Student's t-test, the Mann-Whitney U test and the Kruskal-Wallis test with multiple comparisons were used as respectively indicated in the figure legends.

3 Results

3.1 Radioresistant clones of MDA-MB-231 cells can be generated by repeated irradiation

MDA-MB-231 wild type and brain metastasizing tumor cells were repeatedly irradiated with 4 Gy to generate more radioresistant clones, as being verified by standard clonogenic survival assay (Figure 1). The four different human MDA-MB-231 cell lines, MDA-MB-231 WT, MDA-MB-231 BR (brain metastasizing) and the corresponding more radioresistant clones (RR) were used for the consecutive analyses.

3.2 Radiation-induced apoptosis of MDA-MB-231 cells depends on tissue origin of the tumor cells rather than on radioresistance

We then analyzed cell death 24 h – 72 h after hypofractionated irradiation of the four MDA-MB-231 tumor cell lines (Figures 2A, B). Apoptosis, rather than necrosis (Figures 2C–E), was the predominant cell death mechanism and, differed significantly between the irradiated WT and its clones. Apoptosis increased over time, whereas the ratio between the respective cell lines remained similar. Both cell lines that had been derived from brain metastases (BR, BR RR) were very radiosensitive in terms of apoptosis induction and succumbed to it significantly more frequent in comparison to the WT. BR RR even showed the greatest apoptosis rate of all four cell lines despite its radioresistance in the clonogenic assay (Figure 1B), closely followed by BR. This was different in the WT RR clone that was significantly less sensitive to irradiation with regard to apoptosis.

Similarly, necrotic cell death (Figures 2F–H) was significantly increased in cell lines derived from brain metastases (BR, BR RR) compared to the WT cell line 24 h after the treatment. Further, only the WT RR cell line showed significantly less necrosis than the WT. However, 48 and 72 hours after irradiation necrotic cell death was significantly decreased in all clones compared to the WT.

TABLE 2 List of antibodies used to analyze the expression of various activation markers on the surface of DCs *via* multicolor flow cytometry.

Marker	Fluorochrome	Manufacturer
CD70	FITC	Biolegend
CD83	PE-Cy7	eBioscience
CD80	APC	Miltenyi Biotec (MACS)
CD86	Briliant Violet	BioLegend
Live/dead	Zombie Yellow	Biolegend

3.3 Radioresistance drives the expression of immune suppressive checkpoint molecules following irradiation

The expression of the investigated ICMs did not vary considerably between the untreated WT and its clones. There was a similar base level of ICM expression between all four different cell lines, with just one exception: the immune stimulatory ICM CD137-L was significantly lower expressed in all non-irradiated clones that were originally derived from the WT.

Irradiation with 5 × 5.2 Gy resulted in a significant upregulation of both immune suppressive (PD-L1, PD-L2 and HVEM) (Figures 3C–E) and immune stimulatory (ICOS-L, CD137-L, OX40-L) (Figures 3F–H) immune checkpoint molecules on the cell surface of the treated cells compared to the untreated ones in all examined cell lines. CD137-L as an exception thereof however, was significantly downregulated on the WT after irradiation (Figures 3A–H).

However, the radioresistant cell lines (WT RR, BR RR) were characterized by a significantly increased expression of especially the immune suppressive ICMs PD-L1, PD-L2 and HVEM in comparison to the respective non-radioresistant clone.

3.4 Danger signal Hsp70 is released after irradiation irrespective of the tumor cell clone

The release of the damage-associated molecular pattern Hsp70 was significantly increased from the irradiated compared to the respective non-irradiated MDA-MB-231 cells 48 hours after irradiation (Figure 4). However, there was no significant difference between the irradiated WT and its different clones.

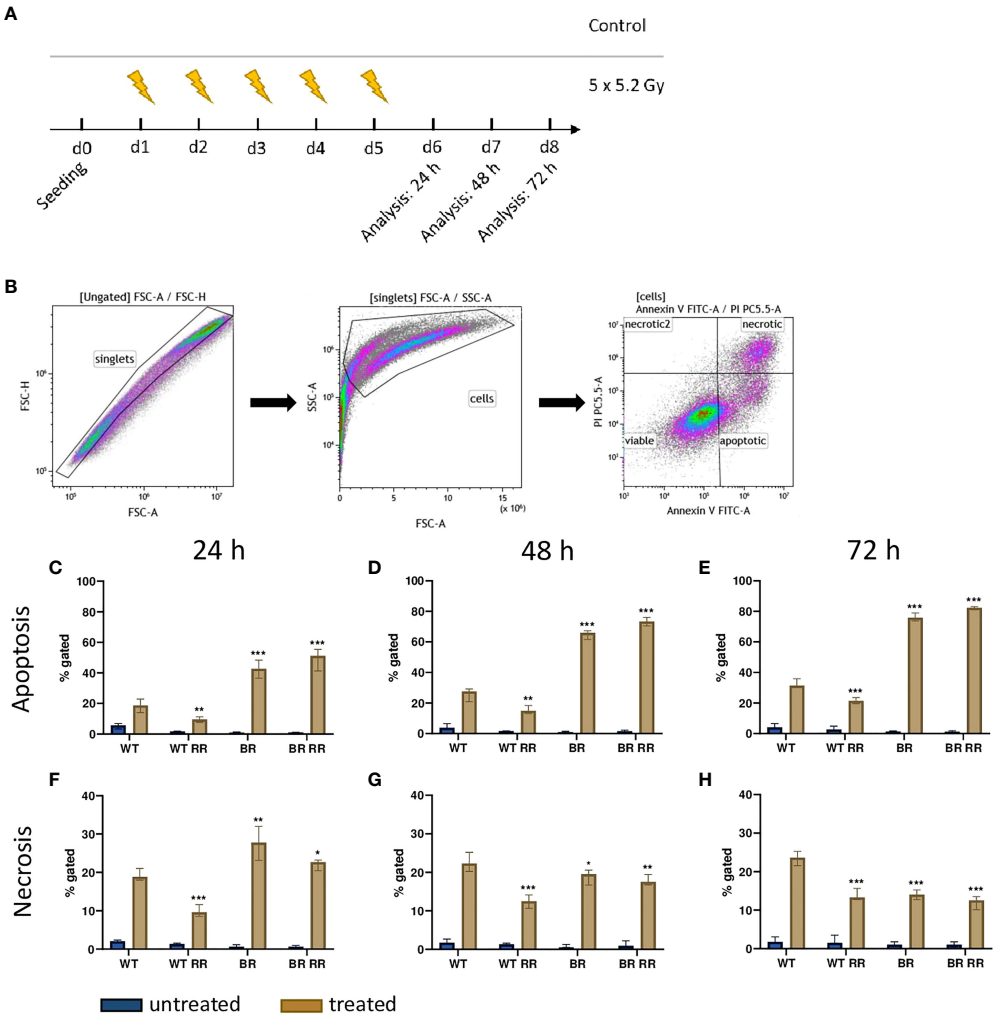

FIGURE 2
Cell death induction after irradiation of the four MDA-MB-231 cell lines is dependent on tissue origin rather than on radioresistance. **(A)** After seeding on day 0, the four MDA-MB-231 cell lines, the WT and the WT-derived brain metastasis clone (BR) as well as the radioresistant (RR) clones derived from those cells (WT RR, BR RR), were treated with 5 × 5.2 Gy. On day 6, 7 and 8, cell death forms were analyzed with Annexin V/Propidium iodide (AxVPi) staining *via* multicolor flow cytometry. The gating strategy is shown in **(B)**. After pre-gating on the singlets and consequently excluding the debris, the remaining cells were identified as viable, apoptotic, or necrotic as presented. The percentage of apoptosis **(C–E)** and necrosis **(F–H)** of the different cell lines 24 **(C–F)**, 48 **(D, G)** and 72 hours **(E–H)** after irradiation is shown as median with interquartile range. The data are from nine independent experiments. For statistical analysis, each treated clone was compared to the WT *via* Mann-Whitney U test (*p < 0.05, **p < 0.01, ***p < 0.001).

3.5 Neither non-irradiated nor irradiated MDA-MB-231 cells and their supernatants increase the expression of common activation markers on human monocyte-derived DCs

To investigate the potential of treated and untreated RR and non-RR tumor cells to prime DCs, they were co-incubated with human monocyte-derived DCs (Figures 5A–C). Incubation of the DCs with the maturation cocktail (MC) led to a significant up-regulation of the four analyzed common activation markers CD70, CD80, CD83 and CD86 (Figures 5D–G). However, DCs which were co-incubated with either non-irradiated or irradiated WT, WT RR or BR RR cells and their respective supernatants, did not increase the expression of common activation markers compared to unstimulated, immature DCs (w/o MC).

4 Discussion

According to preclinical data, immunogenic cell death induction has been attributed to hypofractionated irradiation schedules (29, 30) which are more and more clinically applied for RT of breast cancer. In contrast to apoptosis which is considered to have a rather suppressive effect on the immune system (31), necrotic cell death is more immune stimulatory because of the release of potentially immunogenic neoantigens and DAMPs such as Hsp70. Necrosis has been reported to be primarily activated by

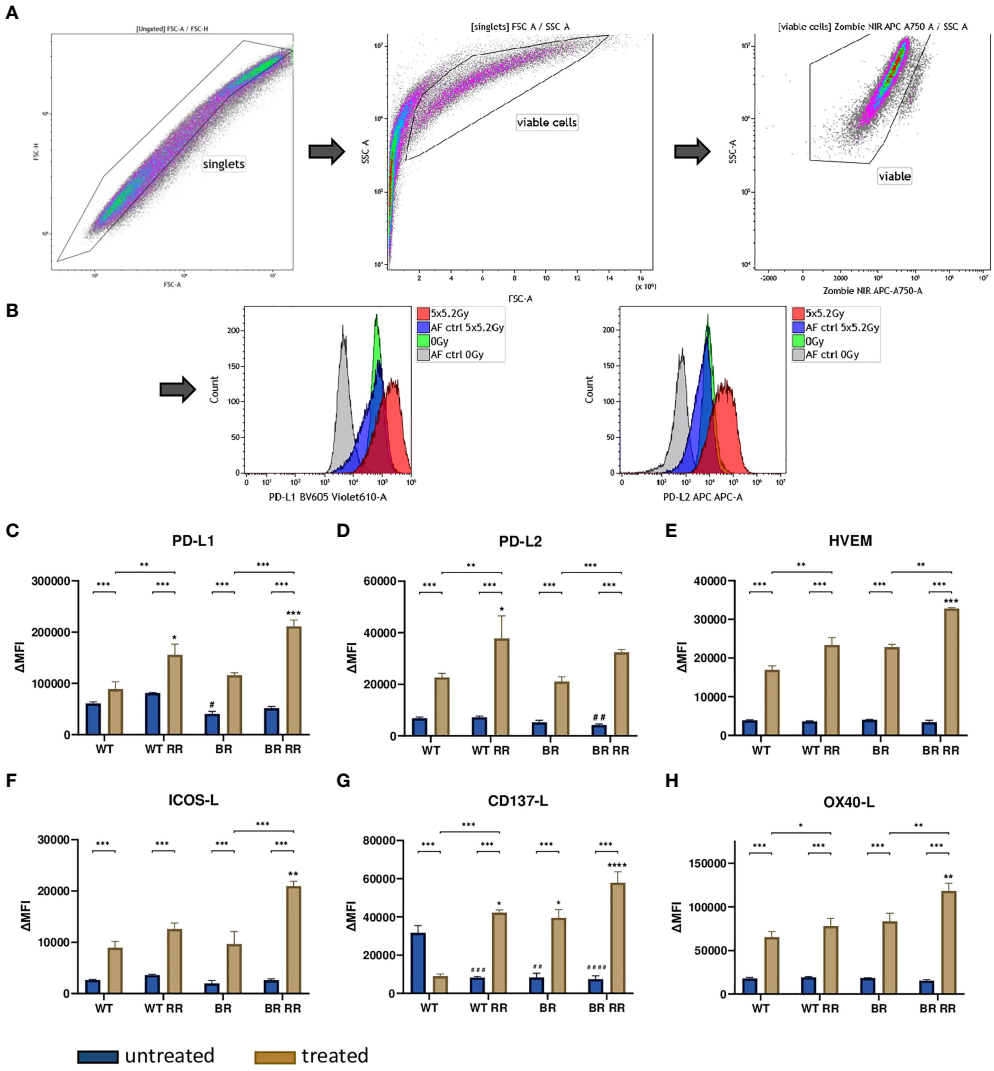

FIGURE 3
Radioresistance (RR) drives the expression of immune suppressive checkpoint molecules on the surface of the four presented MDA-MB-231 cell lines 48 hours after hypofractionated irradiation. The gating strategy is presented in **(A)** After pre-gating on the singlets, the debris was excluded. Then the viable cells were detected *via* the Zombie NIR viable/dead stain. Immune checkpoint molecule (ICM) expression is presented in the graphs as ΔMFI (mean fluorescence intensity). It was calculated by subtracting the MFI of the Zombie-only-stained samples (AF ctrl) from the respective Zombie-and-antibody-stained samples of various ICMs expressed on the cell surface of the four cell lines. Exemplarily primary data are shown for PD-L1 and PD-L2 detection. The WT and the WT-derived brain metastasis clone (BR) as well as the radioresistant (RR) clones derived from those cells (WT RR, BR RR) were treated with 5 × 5.2 Gy. **(B)** The expression of immune suppressive (PD-L1: **(C)**, PD-L2: **(D)**, HVEM: **(E)** and immune stimulatory (ICOS-L: **(F)**, CD137-L: **(G)**, OX40-L: **(H)** ICMs is presented as median with interquartile range. Data are from seven independent experiments. For statistical analysis, a Mann-Whitney U test was performed to compare untreated and treated cells within one cell line. The same test was used to compare an irradiated radioresistant cell clone with its respective non-radioresistant one. A Kruskal-Wallis test with multiple comparisons was used to examine statistical differences between the ICM expression of the different clones compared to the WT within the respective untreated (#) and treated (*) group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

higher doses of ionizing radiation (hypofractionation). It is the desired form of cell death in the context of anti-tumor immune response initiation (18, 32). Unlike other breast cancer subtypes, TNBC is characterized by a rather high TMB. That in turn, leads to the production of neoantigens (33). Consequently, its higher immunogenicity makes it a potential candidate for ICB.

To get first hints about the immunogenic phenotype of MDA-MB-231 breast cancer cells after hypofractionated irradiation and in dependence of their radioresistant properties and tissue origin (e.g. metastatic spread to the brain), cell death forms were determined of the four different clones. Hypofractionated irradiation induced a mixture of apoptosis and necrosis in the four cell lines (Figure 2). However, in comparison to the two WT cell lines, both brain-metastasized clones showed to be more sensitive to X-rays leading to strongly apoptosis-dominating cell death. It has been reported that metastatic spread to the brain has an impact on radioresistance of MDA-MB-231 cells as shown by a clonogenic assay (34) which could be due to the influence of the brain microenvironment on gene expression patterns of the tumor cells, as also indicated by the findings of Park et al. (35). Radioresistance was verified as previously described (27). The clonogenic survival (Figure 1) confirms that the RR clones have enhanced potential to still form

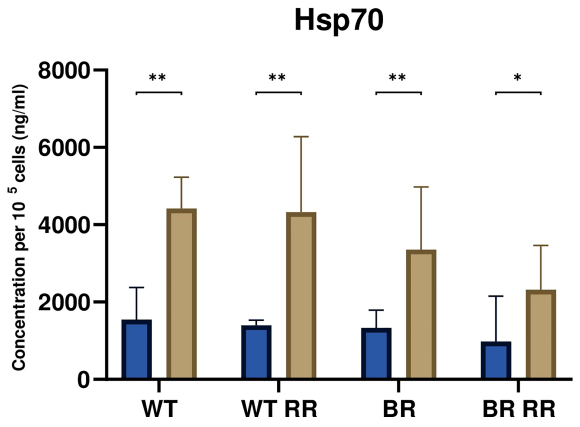

FIGURE 4
Hsp70 release was significantly increased from irradiated compared to non-irradiated MDA-MB-231 cells. The graph shows the concentration of Hsp70 per 10^5 cells (ng/ml) in the cell culture supernatant of WT and the WT-derived brain metastasis clone (BR) as well as the radioresistant (RR) clones derived from those cells (WT RR, BR RR), either untreated (blue bars) or after irradiation with 5 × 5.2 Gy (brown bars). Data is presented as median with interquartile range. Data are from six independent experiments. For statistical analysis, a Mann-Whitney U test was performed to compare untreated and treated (5 × 5.2 Gy) cells within one cell line (*$p < 0.05$, **$p < 0.01$). Furthermore, a Kruskal-Wallis test with multiple comparisons was used to compare Hsp70 concentrations between the treated WT and its clones.

colonies after radiation exposure compared the non-RR clones. Radioresistance was not generally correlated to the capability of tumor cell death induction, as the BR clones had similar amounts of apoptotic and necrotic cells after RT with 5 x 5.2Gy. The relationship between surviving fraction after radiation exposure and the percentage of apoptotic cells at the first days after the same dose of exposure is complex (36). We conclude that radioresistance of the BR cells might also be reflected by cell death forms at later time points than 72 hours and this is already indicated by slightly reduced percentages of necrotic cells of the BR RR clone.

Besides antigenicity, ICD also depends on adjuvanticity (37). Therefore, in the context of cancer cell death, this was exemplarily analyzed by quantifying the Hsp70 concentration in the supernatant of non-irradiated and irradiated tumor cells. In accordance with earlier examinations by Kötter et al., (38). Hsp70 release was significantly increased by irradiated cancer cells in comparison to the respective untreated ones (Figure 4). In sufficient quantities, Hsp70 can stimulate the uptake of tumor antigens (39) and further activate dendritic cells (40). Linder et al. suggested that extracellular Hsp70 is released predominantly by active mechanisms and not mainly during cell death (41). Compared to non-irradiated cells, our analyses showed that triple-negative breast cancer cells after radiation therapy have an increased secretion of HSP70 which is independent of the radioresistance. This suggests, as already observed for other tumor entities, that release of HSP70 is mostly connected to necrosis induction (42) of tumor cells (see Figure 2G) rather than to radiosensitivity being determined with clonogenic assays. However, radiosensitivity is linked to the immune surface phenotype of the tumor cells, most likely being triggered by activation of DNA sensing pathways in the cytosol of the cells (43).

DCs in general play a key role in T cell priming and therefore provide the basis for T cell-mediated anti-tumor immune responses. To get first hints whether the hypofractionated irradiation of the MDA-MB-231 breast cancer cells affects the priming capabilities of DCs, we examined the expression of DC-specific activation markers after co-incubation with untreated and treated tumor cells. However, although hypofractionated RT induced cell death and the release of Hsp70 in all four cell lines, we did not detect increased expression of any of the investigated activation markers (CD70, CD80, CD83 and CD86) on the surface of the DCs after co-incubation, neither with untreated nor with treated cancer cells in this *in vitro* setting. This suggests that irradiation of breast cancer cells might rather affects the effector phase of anti-tumor immune responses than the priming phase. However more detailed analyses are needed in the future such as how certain DC subsets might be affected. Pilones and colleagues recently discovered that Batf3-dependent conventional dendritic cells type 1 (cDC1) are required for priming RT-induced of tumor-specific CD8+ T cells (44).

Consequently, we analyzed whether the chosen treatment schedule would influence the expression of ICMs on the tumor cells. Most research on immune checkpoint molecules in breast cancer has focused on the PD-1/PD-L1 pathway so far. Monoclonal antibodies (mABs) which antagonize these immune suppressive ICMs and consequently help to restore a potent anti-tumor immune response, have recently led to remarkable long-lasting benefits, but unfortunately just in a minority of (metastatic) cancer patients (24). In this context, higher PD-L1 expression in the tumor has been associated with improved response rates to anti-PD-(L)1 therapies in various cancer types, including TNBC (45–47). Given the predictive value of PD-L1 expression, knowledge about the behaviour of further ICMs in response to irradiation is currently missing but may be beneficial to optimize future radioimmunotherapies (RITs). Therefore, we did not only analyse the expression of PD-L1, but also that of other key ICMs on MDA-MB-231 breast cancer clones.

Consistent with our data, the immune suppressive ICM PD-L1 has been reported to be expressed on the surface of many tumor cells (19, 20, 48, 49). In line with previous *in vitro* and *in vivo* examinations using various cancer cell lines and models, we revealed that it is not just PD-L1 which is upregulated by RT, but rather both immune inhibitory and immune stimulatory (50–53) ICM expression is significantly increased on the surface of TNBC cells following (hypofractionated) irradiation. This has already been demonstrated in other settings and tumor entities (54, 55).

However, a key new finding of our analyses is that particularly immune suppressive checkpoint molecules were significantly more upregulated on the cell surface of radioresistant MDA-MB-231 clones. This indicates for the first time that radioresistant tumour cells do not necessarily have a more stem cell-like phenotype, but might rather suppress the immune system by upregulation of immune suppressive molecules following radiation exposure. This, together with reduction of the dsDNA content in the tumor cells that attenuates the cGAS/STING pathway and consecutively

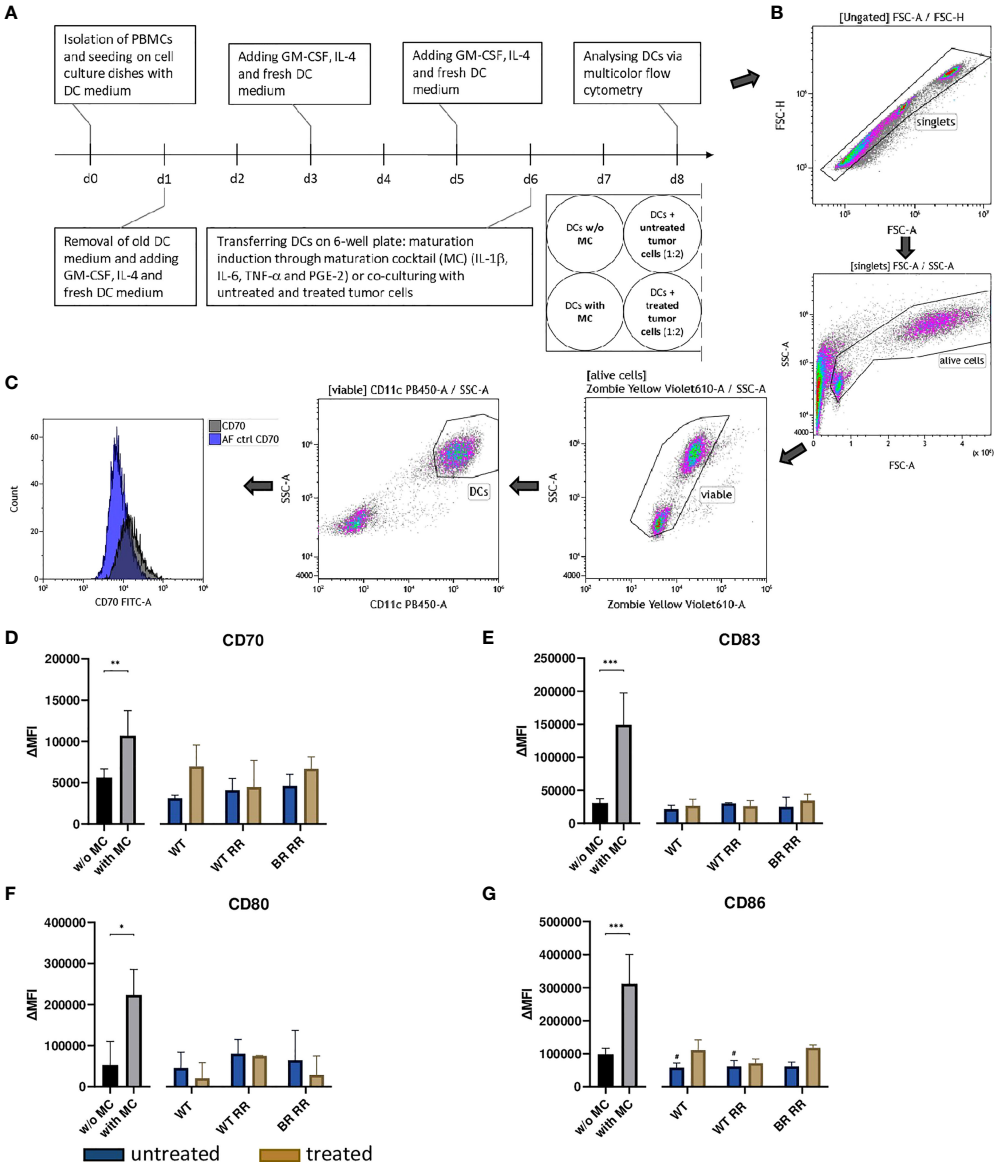

FIGURE 5
Neither untreated nor treated (5 × 5.2 Gy) MDA-MB-231 cells increased the expression of activation markers on dendritic cells (DCs) 48 hours after co-incubation. **(A)** Human monocyte-derived DCs were differentiated from peripheral blood mononuclear cells (PBMCs) for 5 days before they were co-incubated with untreated and treated wild type (WT) MDA-MB-231 cells or radioresistant (RR) clones. 48 hours later, the expression of common DC activation markers was examined using multicolor flow cytometry. The gating strategy is presented in **(B)** After pre-gating on the singlets, the viable cells were detected. Then, gating on CD11c positive cells identified DCs. CD70 **(D)**, CD83 **(E)**, CD80 **(F)**, CD86 **(G)** expression on the cell surface of DCs is presented in the graphs as ΔMFI. It was calculated by subtracting the Zombie-only-stained samples (AF ctrl) from the respective Zombie-and-antibody-stained samples, here shown exemplarily for CD70 **(C)**. The data is presented as median with interquartile range. Data are from seven independent experiments. For statistical analysis, a Mann-Whitney-U test was used to compare activation marker expression on DCs with and without (w/o) maturation cocktail (MC). Further, a Kruskal-Wallis test was performed to compare DCs w/o MC with DCs which had been co-cultured with either untreated or treated cancer cells, respectively. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $^{\#}p < 0.05$).

the INFgamma-dependent immune activation (27), contributes to immune suppression. Future analyses will have to deal with connections between RT-induced intracellular modifications and modulations on the tumor cell surface to obtain a complete picture of immune suppressive mechanisms in more radioresistant tumor cell clones. Similarly, Jang et al. found in their single cell RNA-based investigation that PD-L1 expression was increased on radioresistant – based on the radiosensitivity index (RSI) – breast cancer cells. These cells included in particular basal, HER2 and luminal B subtypes and were associated with a higher risk of recurrence (56). Based on our data, we conclude that the immunological phenotype of (breast) cancer cells is strongly shaped by radioresistance. To the best of our knowledge, the underlying mechanisms therefore have not been described in the literature so far and have to be addressed in even more detail in the future, particularly in the context of innovative radiation oncology (57). As TNBC is characterized by a higher infiltration of immune cells it has be suggested that that these patients are more responsive to

immunotherapy, but, as already stated, only a minority of these patients benefit from anti-PD-(L)1 monotherapy, which can be improved by adding RT. To achieve further improvement, targeting of additional immune checkpoint molecules should be envisaged (58). Our analyses revealed that HVEM has similar expression patterns such as PD-L1 and PD-L2 and it has been suggested that HVEM negatively correlates with overall survival in breast cancer patients (59). For exploration in clinical application double blockade of the PD-1/PD-L1/2 axis and HVEM in combination with RT should be taken into consideration.

5 Conclusion

Basically, there are two main models used to explain tumorigenesis. Both have challenged each other since their existence. On the one hand, there is the cancer stem cell concept which states that so called "cancer stem cells" (CSC) are responsible for cancer development due to their capacity to differentiate into phenotypically diverse cancer cells. Many properties have been attributed to CSCs, amongst others radioresistance. On the other hand, there is the clonal evolution/stochastic model. It assumes that normal cells can acquire distinct mutations over time and become cancer cells (60, 61). Our data indicate that – independent of the CSC concept with its radioresistant stem cells – radioresistant TNBC clones could survive radiotherapy and subsequently, evade the immune response by increased immune suppressive ICM expression. During the last decade it has become evident that the immune system plays an important role in influencing the response to RT treatment and prognosis in many solid tumor entities, including in breast cancer. The most beneficial dose of radiation for induction of anti-tumor immune responses could not be defined until today, but several preclinical, first clinical observation and *in silico* simulations support the hypothesis that hypofractionated RT is the most immunogenic one (62). Following Stereotactic Ablative Body Radiotherapy (SABR), e.g., there is evidence of systemic immune activation in patients with increased PD1 expression (63). Future studies should nevertheless additionally investigate conventional radiation therapy and moderately hypofractionated radiation therapy with regard to radioresistance and immune phenotype of breast cancer cells. In our analyses we aimed to refer to current clinical approaches focusing on more hypofractionated schedules, as already outlined above (10). Generally, the increased immune suppressive ICM expression could then in turn form the basis for recurrence and newly emerging metastases. Therefore, we speculate that the significance of ICB may increase in parallel to the number of experienced radiotherapy sessions and that targeting different ICMs at once might be necessary in breast cancer. We finally want to stress that the key focus was set on the immune phenotype of the radioresistant breast cancer cells in the here presented analyses. Future work will have to focus on even more detailed functional analyses with DC subsets and consecutive T cell activation. Furthermore, analysis of the expression of ICMs in breast cancer specimen tissue microarray with radiation sensitive- and radiation resistant-patient should provide deeper insights how radiosensitivity might be connected to immune phenotypes of breast cancer tumor cells.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The permission to use this LRSC was given by the ethics committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg (ethical approval no. 180_13 B and 48_19 B).

Author contributions

Conceptualization, UG, MR, BF, KB, and RF; methodology, SG, FM, UG, and BF; validation, MR and UG; formal analysis, SG, FM, AS, and UG; investigation, SG, FG, CR, and JA,; resources, RF, BF, and UG; data curation, CR and FG; writing—original draft preparation, SG and MR; writing—review and editing, MR, UG, FM, and KB; visualization, SG, CR and FG; supervision, MR and UG; project administration, UG and KB; funding acquisition, UG. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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