Inflammatory microenvironment remodeling by tumor cells after radiotherapy

Martin McLaughlin[1], Emmanuel C. Patin[1], Malin Pedersen[2], Anna Wilkins[3], Magnus T. Dillon[1,3], Alan A. Melcher[2,3], Kevin J. Harrington[1,3]*

[1]Targeted Therapy Team, The Institute of Cancer Research, London, UK

[2]Translational Immunotherapy Team, The Institute of Cancer Research, London, UK

[3]The Royal Marsden Hospital, London, UK

*Correspondence to M.M. e-mail: martin.mclaughlin@icr.ac.uk

Abstract | The development of immune checkpoint inhibitors (ICIs) is revolutionizing the way we think about cancer treatment. Even so, for most types of cancer, only a minority of patients currently benefit from ICI. therapies. Intrinsic and acquired resistance to ICIs has focused research towards new combination therapy approaches that seek to increase response rates, the depth of remission and the durability of benefit. In this review, we describe how radiotherapy, through its immunomodulating effects, represents a promising combination partner with ICIs. We describe how recent research on DNA damage response (DDR) inhibitors in combination with radiotherapy may be used to augment this approach. Radiotherapy can kill cancer cells while simultaneously triggering the release of pro-inflammatory mediators and increasing tumor-infiltrating immune cells – phenomena often described colloquially as turning immunologically 'cold' tumors 'hot'. Here, we focus on new developments illustrating the key role of tumor cell-autonomous signaling after radiotherapy. Radiotherapy-induced tumor cell micronuclei activate cytosolic nucleic acid sensor pathways, such as cyclic GMP-AMP synthase (cGAS)– stimulator of interferon genes (STING), and propagation of the resulting inflammatory signals remodels the immune contexture of the tumor microenvironment. In parallel, radiation can impact immunosurveillance by modulating neoantigen expression. Finally, we highlight how tumor cell-autonomous mechanisms might be exploited by combining DDR inhibitors, ICIs and radiotherapy.

[H1] Introduction

After decades of effort, attempts to enlist the aid of the immune system in cancer treatment have begun to bear fruit with the emergence of immune checkpoint inhibitors **[G]** (ICIs), such as antibodies against programmed cell death protein 1 (PD-1), PD1 ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4). Although some patients show dramatic and potentially durable responses, the majority derive no benefit. The reasons underpinning these diverse outcomes from ICIs, the opportunities presented by alternative immuno-oncology agents and the role of combination approaches to improve tumor responses are all currently areas of intense research activity.

Approximately half of all cancer patients will receive radiotherapy as part of their treatment [1]. First used to treat cancer over a century ago, radiotherapy has become a mainstay of first-line treatment in a number of solid tumors. We now know that radiotherapy can have profound immunostimulatory effects and, as such, it is increasingly viewed as a promising combination partner with ICIs and other immuno-oncology agents [2,3].

In addition to its ability to mediate DNA damage-induced cancer cell death [4], radiotherapy can modulate both the immunogenicity and adjuvanticity of tumors by triggering release of pro-inflammatory (and anti-inflammatory) mediators, increasing tumor-infiltrating immunostimulatory (and immunoinhibitory) cells and enhancing the expression of neoantigens [2,5-7]. Collectively, in their positive, immunostimulatory manifestations, these phenomena are often summarised as turning immunologically 'cold' tumors 'hot'. This binary classification has become a powerful concept for patient classification [8]. Two further immune classifications of tumors, immunosuppressed and T-cell excluded, have been described [9]. Immunosuppression has been linked to chronic interferon signaling [10], while exclusion has been linked to pathways such as β-catenin and transforming growth factor-β (TGFβ)[9,11]. Tumors with a lower mutational or neoantigen burden respond poorly to ICIs [11,12]. Even so-called "hot" tumors with high levels of tumor mutations (leading to more tumor neoantigens **[G]**) and immune cell infiltrates may show poor responses to ICIs due to subclonal neoantigens **[G]** [12]. Radiotherapy, by driving immune cell infiltration and enhancing immunogenicity, has the potential to increase the immunoresponsiveness of tumours.

Radiation has a direct impact on the tumor stroma, including on cancer-associated fibroblasts (CAFs), blood vessels and immune cells [13]. Recent studies on these direct effects are discussed in Box 1. However, rapid recent advances have revealed that tumor cell-intrinsic events driven by DNA damage are central to the immunomodulatory actions of radiotherapy [5,6]. Such tumor cell-autonomous effects and how they may guide future therapeutic combinations are the focus of this review.

We summarize how radiotherapy-induced tumor genome fragmentation initiates an inflammatory response through cytosolic nucleic acid sensors. This is discussed in the context of new research in the area of DNA damage response (DDR) inhibitors and with a tumor-cell centric focus. Signal propagation to the tumor microenvironment occurs through tumor cell production of cytokines as well as indirect immunostimulatory signaling by the cyclic dinucleotide cyclic GMP-AMP **[G]** (cGAMP). DNA damage and altered gene transcription due to radiotherapy can modulate tumor neoantigen expression. These events result in activation of innate and/or adaptive anti-tumor immune priming. We highlight how these tumor cell-autonomous characteristics may be harnessed to rationally direct new combinations of DDR inhibitors and ICIs with radiotherapy.

[H1] Cytoplasmic Nucleic Acid Sensing

[H2] Radiation Induces Cytoplasmic DNA Sensing Through cGAS–STING

Cytoplasmic nucleic acid sensors were initially described as intracellular pattern recognition receptors **[G]** which initiate innate immune responses to viral and other pathogenic infections [14]. Our understanding of the mechanistic basis of radiotherapy as an anti-cancer treatment has been transformed by the recent discovery that DNA damage in cycling tumor cells (discussed later) can activate these intracellular sensors [5,6]. The cytoplasmic DNA-sensing cyclic GMP-AMP synthase (cGAS)– stimulator of interferon genes **(**STING) pathway appears to be phenotypically dominant [15,16] in this process and recent findings, described in this section, have begun to highlight the complexity of both induction and regulation of this pathway. These findings have substantial implications for how genomic instability, both basal and in response to radiation-induced DNA damage, can impact inflammatory responses. The regulatory mechanisms and feedback loops described in the following section are illustrated in Figure 1

Initial studies discovered that cytoplasmic B-form DNA binding to cGAS triggered production of the second messenger and immunotransmitter cGAMP [17]. Upstream of cGAS, the cytoplasmic deoxyribonuclease TREX1 (induced by radiotherapy at doses between approximately 12 and 18 Gy) degrades cytoplasmic double-stranded DNA (dsDNA) and therefore prevents the production of cGAMP by cGAS [18]. Binding of cGAMP to STING induces type-I interferon **[G]** (IFN) production [19,20]. This was followed by findings that STING and interferon-α/β receptor 1 (IFNAR1) in immune cells play critical roles in therapeutic responses to radiotherapy [15]. Finally, exogenous cGAMP or synthetic STING agonists can enhance the efficacy of radiation in mouse models [15,21,22]. STING activation can also result in nuclear factor-κB (NF-κB) activation [23-25], but much of the literature has focused on type-I IFN signaling.

The cellular compartment in which STING activation mediates its biological effects remains unclear at present, but non-tumor cell STING is consistently reported as a crucial factor in immune activation [15,21,26].In some *in vivo* models, tumor cell-intrinsic STING activation is necessary [5,27,28]. In others, preclinical data suggest that tumor cell-derived DNA in exosomes **[G]** and/or cGAS-derived cGAMP can activate immune cell cGAS–STING signaling *in trans* and this contributes to radiotherapeutic responses [18,22,27,29,30].

[H2] Inter- and Extra-Cellular Signaling via cGAMP and Exosomes

Conflicting data on the exact roles of cGAS and STING in tumor versus non-tumor cells is linked to the concept of cGAMP as an immunotransmitter. Cytoplasmic tumor-derived cGAMP can diffuse to adjacent cells via gap junctions **[G]** [15,16]. Recent studies have uncovered novel regulatory and transmembrane transport mechanisms for cGAMP [31,32] which, in its extracellular form, preliminary data indicates is largely tumor-derived [22]. Mammalian cGAMP is degraded by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), an ectoenzyme **[G]** present both in membrane-bound and cleaved soluble forms [33]. Preliminary data have shown that loss of ENPP1 enhanced the efficacy of both radiotherapy alone and in combination with anti-CTLA4 [22]. In parallel with the discovery of the importance of extracellular cGAMP, SLC19A1 has recently been identified as the first known importer of cGAMP [31,32], and the presence of an as yet unidentified cGAMP export mechanism is implied in another preliminary study [22].

In addition to cGAMP, exosomes represent another mechanism of immunostimulatory inter-cellular signaling. Radiation alters the composition of tumor-derived exosomes [30,34,35]. For example, tumor-derived exosomes produced following radiation can shuttle immunostimulatory tumor DNA to dendritic cells (DCs). The resulting IFN response in DCs was dependent on STING in DCs and abolished by expression of dsDNA-degrading TREX1 in tumor cells [30].

[H2] Co-Factors and Post-Translational Modifications Regulate cGAS and STING Activation

One might expect that a key role in anti-tumor immunity would drive selection for loss of cGAS–STING function in tumors. From The Cancer Genome Atlas (TCGA) analysis, inactivation of cGAS or STING is rare, although 4-6% of tumors exhibit deep deletion in the IFN gene cluster [25]. Although allelic variation (STING

has five haplotypes), epigenetic silencing or loss-of-function mutations [26,36-38] may have consequences for radiotherapy-induced immune effects, complex pathway regulation may be more critical.

The cGAS–STING pathway is subject to both negative and positive crosstalk with other nucleic acid sensors. Alongside cGAS, NLRC3 (NOD-like receptor family CARD domain containing 3), IFI16 (IFNγ-inducible protein 16) and DDX41 (DEAD-box helicase 41) all bind dsDNA with positive consequences for STING-mediated type-I IFN signaling. NLRC3 binds to and blocks STING activity through sequestration, and binding of dsDNA to NLRC3 facilitates release of STING [39]. IFI16 can bind DNA via two HIN domains **[G]** and subsequently interacts via a pyrin domain with STING [40,41]. While one study found no role for IFI16 in IFNβ expression [42], overall the literature suggests IFI16–STING interaction enhances STING-dependent IFNβ production in response to cGAMP [40,41,43,44]. DDX41 can bind dsDNA [45,46]. Bruton's tyrosine kinase (BTK)-mediated phosphorylation of DDX41 promotes dsDNA binding to DDX41 [47], with the E3 ubiquitin ligase TRIM21 negatively regulating DDX41 by ubiquitination and degradation [48]. Binding of DNA to DDX41 leads to DDX41 binding to STING and enhanced downstream type-I IFN production [45-48].

Both cGAS and STING are subject to extensive post-translational modifications regulating activity. TRIM56 monoubiquitinates cGAS, increasing dimerization, DNA binding and cGAMP production [49]. G3BP1 also promotes dsDNA binding to cGAS [50]. Polyglutamylation by TTLL6 (removed by CCP6) blocks DNA binding to cGAS, while TTLL4 monoglutamylation (removed by CCP5) blocks cGAMP synthase activity [51]. Sumoylation and desumoylation of cGAS and STING by TRIM38 or sentrin-specific protease 2 (SENP2), respectively, regulate degradation due to phosphorylation of STING by TANK-binding kinase 1 (TBK1) [52]. Regulation through TBK1 also occurs through HER2-dependent recruitment of AKT1, which phosphorylates a site on TBK1 that decreases the association between STING and TBK1 in response to cGAMP [53]. A comprehensive overview of post-translational modifications in the cGAS–STING pathway has recently been published [54].

The caveat with research in this area still being at an early stage is that detailed mechanistic studies into cGAS–STING signaling are based on model cell lines known to be fully pathway-competent. The cancer cell-specific status of many of these regulatory mechanisms and how they might influence tumor cell-intrinsic effects of radiotherapy is not currently known.

[H2] Negative-Regulation of cGAS–STING by Caspases

Although the nucleic acid sensor Toll-like receptor 9 (TLR9) is upregulated post-radiotherapy [7], evidence does not support a role for TLR9 or downstream signaling through MYD88 or TRIF in the type-I IFN response to endogenous DNA [15,20,40,55]. Cytoplasmic DNA can activate inflammasome **[G]** signaling pathways with positive and negative crosstalk between cGAS-STING and the inflammasome shown in studies. cGAMP or IFNβ upregulate the inflammasome linked genes AIM2 (absent in melanoma 2), NLRP3 (NACHT, LRR and PYD domains-containing protein 3), caspase-1, IL-1β [56] and ZBP1 (Z-DNA-binding protein 1) [7,57,58]. AIM2 is a cytosolic dsDNA sensor that forms the AIM2–ASC–caspase-1 inflammasome, resulting in cleavage of the pro- forms and secretion of interleukin 1β (IL-1β) and IL-18, and subsequent pyroptosis **[G]** [53]. Exogenous IL-1β-mediated activation of the IL-1 receptor (IL-1R) can induce mitochondrial DNA release and activation of cGAS [59]. Similar to cGAS, AIM2 is activated by cytoplasmic DNA release with activation reduced by TREX1 [60]. However, AIM2 has been shown to be dispensable for type-I IFN production [42]. ZBP1 was originally identified to bind dsDNA leading to type-I IFN production [61], but this is not supported by data from knock-out mice [62]. ZBP1 has been reported to bind Z-form, rather than B-form, dsDNA but the exact ZBP1 ligand in the context of radiotherapy remains unclear. The emerging consensus is that ZBP1 is upstream of receptor-interacting serine/threonine kinase 3 (RIPK3)–caspase-8 and the NLRP3–ASC–caspase-1 inflammasome, subject to negative regulation by RIPK1 [57,58].

Negative regulation of cGAS –STING appears to converge on caspase activation. Caspase-1 has been shown to directly cleave and inactivate cGAS [63]. Caspase-1 also cleaves and activates gasdermin D. This forms a K^+ efflux pore at the cell membrane that negatively regulates cGAS activity [64]. This K^+ efflux can also activate NLRP3, though it is not known if ZBP1 is required [57,58]. In addition to inflammasome linked caspase-1 activity, apoptotic caspase activation can also negatively regulate the cGAS-STING pathway. Caspase-3

cleaves cGAS, interferon regulatory factor 3 (IRF3) and mitochondrial antiviral-signaling protein (MAVS) with cGAS cleavage enhanced if it is bound to DNA [65]. Loss of caspase-3 (*Casp3* knockout) boosts type-I IFN production due to radiotherapy and the efficacy of radiotherapy alone or in combination with anti-CTLA4 in mice[66]. Together, these studies have shown substantial crosstalk between cGAS-STING and caspase activity that regulates the inflammatory response to cytosolic dsDNA.

[H2] Crosstalk with RNA Sensors

Many nucleic acid sensors are upregulated in tumor cells in mice post-radiotherapy, including the RNA sensors and pattern recognition receptors retinoic acid inducible gene-I (RIG-I, also known as DDX58) and melanoma differentiation-associated protein 5 (MDA5, also known as IFIH1) [7]. A degree of crosstalk has been identified between DNA-sensing pathways and cytoplasmic RNA-sensing pathways. Binding of double-stranded RNA (dsRNA) to cytoplasmic RIG-I or MDA5 leads to type-I IFN signaling through the adaptor protein MAVS. MAVS and RIG-I have been shown to be necessary to achieve maximal type-I IFN production induced by radiotherapy [67,68]. Radiotherapy can induce endogenous small non-coding RNAs (sncRNAs) [68], dsRNA from endogenous retroviral elements (ERVs) downstream of signal transducer and activator of transcription 1 (STAT1) activation [69], and RNA with a 5'-triphosphate moiety, which is synthesized by DNA-dependent RNA polymerase III from AT rich dsDNA [70,71]. These can all activate RNA-sensing MDA5 and RIG-I pathways. An interesting recent finding is pro-survival effects and negative regulation of type-I IFN signaling by radiation-dependent induction of the helicase LGP2 (also known as DHX58)[67,68]. This is mediated through LGP2 interaction with TNF receptor-associated factor 2 (TRAF2), TRAF3, TRAF4 and TRAF6 downstream of MAVS. LGP2 negatively regulates both type-I IFN and NF-κB signaling downstream of RNA sensors and cGAMP-mediated STING activation [72].

[H1] Radiotherapy and the DDR

[H2] Surveillance of Micronuclei by cGAS

At the time that cytoplasmic DNA-induced type-I IFN production through cGAS–STING pathway activation was described, the precise mechanism linking this process to radiotherapy was not fully understood. A number of pivotal studies revealing surveillance of micronuclei **[G]** by cGAS [5,6,73] have transformed our understanding of how radiotherapy and DNA repair defects in cancer cells intersect with the immune system. The mechanisms discussed in this section are illustrated in Figure 2.

Initial studies into micronuclei were carried out in the context of chromothripsis **[G]** , where whole chromosome-containing micronuclei undergo defective asynchronous DNA replication and exhibit defective nuclear import [74]. This is due to bundled spindle microtubules inhibiting the assembly of non-core nuclear envelope proteins including nuclear pore complexes (NPCs) [75]. Micronuclei irreversibly lose compartmentalization of nuclear material from the cytoplasm in interphase, linked to depletion of lamin B1 [76,77]. The ESCRT-III (Endosomal Sorting Complex Required for Transport-III) membrane-remodeling complex subunits CHMP7 and CHMP4B are enriched in NPC-negative micronuclei, likely as a result of attempted but defective nuclear envelope repair [77]. Micronuclear membrane breakdown (rupturing) is followed by DNA damage and invasion of endoplasmic reticulum (ER) membranes into micronuclear chromatin [76,77].

Induction of micronuclei in cancer cells by radiotherapy is a well-established phenomenon [78], as is DNA damage- or radiation-induced type-I IFN production [79,80]. The discovery that cGAS localizes to ruptured micronuclei [5,6,77] linked these two areas of radiation research. Interferon-stimulated gene (ISG) transcripts were present only in micronucleated cells, in a cGAS- and STING-dependent manner [5,6,77]. Regulatory feedback loops linked to micronuclei are already being identified. Direct inhibition of DNA repair by cGAS may promote micronucleus formation [81,82], while STING activation induces WIPI2- and ATG5-dependent autophagic clearance of micronuclei and cytosolic DNA [6,83,84]. Autophagy and NF-κB activation downstream of STING have been postulated to be evolutionarily conserved functions pre-dating interferon signaling [84,85]. The critical observations outlined above have bridged the knowledge gap as to how radiotherapy, through cytoplasmic micronucleus-derived DNA, propagates an inflammatory type-I IFN response from irradiated tumor cells.

[H2] Interferonopathies and DNA Repair-Defective Cancer

Defects in cGAS–STING signaling have been identified in disorders such as STING-associated vasculopathy with onset in infancy **[G]** (SAVI) [36] and Aicardi-Goutières syndrome **[G]** (AGS) [86]. AGS and SAVI are interferonopathies, a group of autoinflammatory disorders mechanistically linked by chronic interferon production. This is driven by cytosolic DNA in the case of AGS, through defective TREX1**,** or constitutive STING activation in SAVI. Following the discovery of cGAS surveillance of damaged DNA in micronuclei, a number of studies have identified upstream DNA repair defects to be responsible for similar autoimmune disorders. Mutations in ATM (ataxia telangiectasia-mutated), Artemis and BLM (Bloom syndrome protein) result in upregulation of ISGs associated with cytosolic DNA [87-89]. These DDR-defect driven inflammatory disorders are mechanistically identical to STING-driven interferonopathies.

An inextricable link between DDR signaling directly upstream of type-I IFN signaling has consequences for our understanding of cancer biology and cancer treatment. While ataxia telangiectasia patients are more susceptible to multiple cancers [88], homologous recombination defects are best known for their role in breast cancer [90]. In keeping with the interferonopathies described, *BRCA1*- and *BRCA2*-mutant cell lines are characterized by cGAS bound to cytoplasmic DNA in micronuclei and type-I IFN signaling [91,92]. DDR-deficient breast cancer patient samples with BRCA or Fanconi anemia **[G]** pathway-mutations contain higher T-cell infiltration [91]. Cancers containing defective DNA repair pathways have spurred the development of numerous DDR inhibitors driven by the concept of synthetic lethality [90]. It is now becoming clear that this therapeutic approach, alone or in combination with radiotherapy, may have profound immunostimulatory consequences through generation of micronuclei and downstream type-I IFN signaling.

[H2] DDR Inhibitors and Radiosensitization

Cancer-associated DDR defects in G1 cell cycle checkpoint control and homologous recombination repair **[G]** (HRR) have been seen as exploitable traits for drug development. This approach has led to the development of poly(ADP-ribose) polymerase (PARP) inhibitors for cancers with BRCA1 or BRCA2 defects and checkpoint kinase 1 (CHK1), WEE1 and ataxia telangiectasia and Rad3-related protein (ATR) inhibitors that prevent S and G2/M cell cycle arrest after DNA damage. Other radiosensitization approaches such as inhibition of non-homologous end joining by DNA-PK, or depletion of CHK1, ATR and RAD51 through inhibition of the chaperone HSP90 have also been developed. Since radiation doses are constrained by normal tissue toxicity, the use of tumor-selective radiosensitizers may allow improved tumor control without increased normal tissue toxicity. A common theme of tumor-centric radiosensitization studies of ATR, CHK1, DNA-dependent protein kinase (DNA-PK), and HSP90 inhibition has been increased micronucleus generation in combination with radiotherapy [4,93-95]. The confluence of DDR defects, micronucleus generation and inflammatory pathway activation, has led to a number of recent studies showing that DDR inhibitors can enhance the inflammatory response to radiotherapy. These studies are listed in Table 1.

ATR and WEE1 kinases are critical components in cell cycle arrest post-radiotherapy. Cancer cells treated with radiation and the ATR inhibitor AZD6738 showed abrogation of G2 arrest coinciding with the generation of micronuclei that bore the hallmarks of nuclear envelope rupture [4]. Studies in mice indicated that ATR inhibition potentiated the radiation-induced type-I IFN response, significantly enhancing immune cell infiltration [7]. *In vitro*, WEE1 inhibition in combination with radiotherapy increased granzyme B-dependent killing of tumor cells by cytotoxic T-lymphocytes (CTLs) [96]. The addition of anti-PD-L1 to radiotherapy plus WEE1 inhibition significantly increased survival in a MOC1 syngeneic mouse model. Maximal T-cell tumor antigen-specific responses were observed with this triple combination [96]. Tumor cell killing by NK cells was also enhanced by WEE1 inhibition alone, linked to reversal of granzyme B-induced G2/M arrest [97].

While ATR is the apical DNA replication stress-response kinase, ATM is the apical kinase responsible for global cellular responses to DNA double-stranded breaks (DSBs). ATM loss corresponds to an increased IFN response signature in pancreatic TCGA data [98]. ATM loss or inhibition boosted type-I IFN production in response to radiotherapy [98]. One study found STING-dependence while the other showed this to be cGAS-STING-independent, but SRC-dependent [87,98]. Efficacy of ATM knockdown or ATM knockdown plus radiotherapy was increased in combination with anti-PD-L1, with $CD8^+$ T cell infiltration significantly improved by the triple combination [98].

PARP inhibition is synthetically lethal with HRR defects [90]. This synthetic lethality may go hand-in-hand with type-I IFN signaling. PARP inhibition stimulates an interferon response in tumors lacking BRCA1 or BRCA2, with no effect in wild-type cells [99]. The DNA excision repair protein ERCC1 has also been linked to sensitivity to PARP inhibitors. Low ERCC1 expression in non-small cell lung cancer (NSCLC) correlated with higher numbers of tumor-infiltrating lymphocytes, while PARP inhibition induced higher cGAS-positive chromatin fragments and type-I IFN signaling in ERCC1-deficient cell lines versus wild-type [100]. In wild-type HCT116 colon cancer cells, radiosensitization by PARP inhibition markedly increased ISG expression [101]. In keeping with these data, depletion of the HRR protein RAD51 and radiotherapy leads to cytoplasmic DNA, STING activation and production of *IL6* and *TNF* transcripts [102].

These recent studies highlight the role of DDR inhibitors alone or in combination with radiotherapy to enhance tumor inflammation. A number of early findings highlight the potential this has in combination with ICIs, as discussed below.

[H1] Radiation-Induced Antigen Presentation

[H2] Radiation-Induced Tumor-Associated Neoantigen Presentation

As outlined, activation of nucleic acid sensors triggers production of type-I IFNs and inflammatory cytokines. This is frequently referred to as "viral mimicry" with the potential to stimulate anti-tumor $CD8^+$ T-cell responses [2,18]. The addition of ICIs may extend this effect to non-irradiated distal lesions, referred to as the abscopal effect [18,103-105]. However, elimination of cells by cytotoxic $CD8^+$ T-cells requires recognition of antigens presented on major histocompatibility complex I **[G]** (MHC-I) on the target cell's surface. In this regard, radiotherapy has been shown both to increase and modulate antigen presentation on cancer cells.

A number of studies have investigated changes in the tumor cell surface presentation of both existing and novel radiation-upregulated neoantigens **[G]**. Radiation increases MHC-I expression on the surface of tumor cells [7,106-109]. The addition of DDR inhibitors can further increase radiation-induced tumor cell MHC-I surface expression [7]. Radiation expands intracellular peptide pools, altering the MHC-I-associated peptide profile [107], while enhancing levels of existing peptide presentation as shown by MHC-I–SIINFEKL **[G]** *in vivo* [109]. Radiation upregulated MHC-I on CEA-expressing human tumor cell lines *in vitro*. This was associated with enhanced cell killing by CEA-specific $CD8^+$ T-cells [106]. In the syngeneic 4T1 mouse breast carcinoma model, $CD8^+$ T-cells reactive with the known AH1 antigen increased in response to radiation plus anti-CTLA4 blockade [110]. Clinical evidence of this occurrence has also been reported. In colorectal cancer patients, radiotherapy increased the percentage of survivin-reactive T-cells in circulation [111].

The acute genotoxic stress induced by radiation triggers a transcriptional program necessary for the resolution of DNA damage. This also extends to genes downstream of nucleic acid sensors, with radiation dose and fractionation shown to influence the extent of these changes [79]. The finding that radiation can trigger anti-tumor immunity through the expression of poorly or unexpressed tumor neoantigens has caused substantial excitement. CTLA4 blockade in combination with radiotherapy induced systemic anti-tumor T cells in chemo-refractory metastatic NSCLC. In a patient with complete response, neoantigen prediction identified a mutation in *KPNA2*, with *KPNA2* gene expression upregulated by radiotherapy. Peptides corresponding to mutant, but not wild-type, *KPNA2* led to IFNγ production from the patient's $CD8^+$ T cells [2]. It has been postulated that this IFNγ may trigger antigen spread. This may occur when initial rounds of T-cell mediated destruction lead to recognition and responses against secondary non-radiation-induced tumor-associated antigens. Excellent reviews on the topics of antigen spread and radiation-induced exposure of immunogenic mutations have recently been published [112,113].

[H2] Radiation-created Neoantigens and T-Cell Receptor Repertoire

It is now accepted that mutational burden and tumor neoantigen load typically predict clinical response to ICIs [8,12,114]. Cancers can develop high mutational burdens through exposure to mutagens (UV, smoking), DNA modification and replication errors (via APOBEC3B expression, or mutation of POLE or POLD1) or either inherited or acquired DNA repair defects [115]. Somatic alterations and epigenetic silencing of genes in DDR pathways is prevalent across many cancer types [116]. Tumors in this group of patients may possess both high mutation and neoantigen burden as well as cytosolic DNA-driven inflammatory signaling [91].

While preclinical data suggest that radiotherapy and DDR inhibitors may therapeutically replicate this phenotype, there are some concerns that this may not translate to clinical benefit. Lung cancer patients in the upper quartile of clonal neoantigen **[G]** burden had higher levels of *CD8A* and *CD8B* transcripts, the T-cell migration chemokines CXCL9 (chemokine (C-X-C motif) ligand 9) and CXCL10, increased PD-L1 and granzymes compared to the lower quartile [12]. This is similar to changes induced by radiotherapy or DDR inhibitors in preclinical models[7]. The lung and DDRi studies both described a similar inflamed tumor microenvironment enriched with activated effector T cells and immune checkpoint molecules [7,12]. However, in melanoma patients who received dacarbazine chemotherapy before anti-CTLA4 therapy, there were concerns that chemotherapy-induced DNA damage may have generated subclonal neoantigens that resulted in poorer responses. This was in keeping with findings that the baseline prevalence of subclonal neoantigens in NSCLC and melanoma predicted poor response to anti-CTLA4, or anti-PD-1 [12].

With the caveat that preclinical models study homogenous cell populations, dilution of response due to excessive T cell receptor (TCR) diversity has not been reported [105,110,117]. Radiation increases the number of unique TCRs and T-cell clonality, with additional anti-PD-1 therapy necessary to extend this increased diversity to tumor sites out of the radiation field. The majority of TCR clones were high abundance, with the level of concordance observed between tumors in- and out-of-field suggestive of an expansion of pre-existing clones [105]. In studies of the TCR repertoire in response to radiotherapy and anti-CTLA-4 blockade in mice, radiation increased the diversity of the TCR repertoire of intratumoral T cells and increased tumor control in combination with anti-CTLA-4 [110,117]. Nevertheless, tumors treated with these radiation and anti-CTLA-4 combinations were still dominated by a small number of high frequency T cell clones [110].

The clinical importance of immune responses against new radiation-created tumor antigens versus increased activity against pre-existing tumor antigens is not yet clear. High mutational burden may increase the probability that radiotherapy triggers transcription of a tumor- associated antigen as has been shown in lung cancer [2]. Preclinical data suggests increased antigen presentation of basally detectable MHC-I peptides (such as SIINFEKL, AH1, CEA) is beneficial to radiotherapy and ICI combinations [106,109,110]. Likewise, upregulation of pre-existing but poorly or non-expressed tumor neoantigens (such as KPNA2) has been shown clinically to correlate with response to radiotherapy and anti-CTLA4 combinations [2]. Radiation-created highly subclonal neoantigens, potentially exacerbated by the addition of DDR inhibitors, would be a distraction to the goal of expansion of T-cells against clonal tumor neoantigens. Detailed studies of radiation, DDR inhibitors and ICI clinical combinations are needed to address these concerns about subclonal neoantigen creation.

[H1] TME Remodeling by Irradiated Tumor Cells

This section is restricted to tumor-driven changes in the tumor microenvironment (TME) post-radiation, with a focus on areas where substantial recent advances in knowledge have occurred. Key non-tumor effects are summarized in Box 1, with extensive reviews available covering the broader implications of radiation on the tumor microenvironment [13,118]. The interaction between the immunostimulatory and immunosuppressive effects of radiotherapy outlined below are illustrated in Figure 3.

[H2] Impact of Radiotherapy on Dendritic Cells

In addition to the effects of radiation and DDR inhibition on tumor MHC-I presentation, cross presentation of tumor antigens post-radiotherapy has been demonstrated preclinically. MHC-I–SIINFEKL on DCs was shown in lymph nodes, with radiotherapy increasing MHC-I–SIINFEKL-specific effector memory $CD8^+$ T-cells at this site [109]. Human papillomavirus (HPV)-driven cancer models have also demonstrated that radiotherapy contributes to HPV E7-based vaccination strategies [119,120], with radiation dose-dependently increasing DC maturation and peptide-specific T-cell responses [120].

Immunogenic cell death corresponds to the release of damage-associated molecular patterns **[G]** (DAMPs), such as calreticulin, HMGB1 (High mobility group box 1) and ATP, all of which are increased by radiotherapy [121]. Calreticulin acts as a pro-phagocytosis eat-me signal in opposition to CD47 [122]. Release of HMGB1 from tumor cells, via TLR4 activation, promotes antigen presentation by DCs by blocking lysosomal degradation of phagosomes [123]. DC function has been shown to be vital to the immune response to radiation. $CD11c^+CD8\alpha^+$ BATF3-lineage DCs have been shown to be key to the therapeutic efficacy of radiation and anti-CTLA-4 responses [18], while deletion of *IFNAR1* on $CD11c^+$ DCs in mice reverses efficacy [15]. ATP released into the tumor microenvironment binds $P2X_7$ purinergic receptors on DCs, resulting in IL-1β release via NLRP3. This has been shown to be required for the priming of IFNγ-producing tumor antigen-specific $CD8^+$ T-cells in mice [124].

[H2] T-Cell Infiltration

Parallel to effects on DC function, radiotherapy promotes cytokine secretion necessary for T-cell infiltration. Radiation induces secretion of tumor cell-intrinsic CXCL16, which binds to C-X-C chemokine receptor type 6 (CXCR6) on T helper 1 (Th1) cells and activated CD8 T cells [125]. The T-cell chemoattractants CXCL9 and CXCL10 bind to CXCR3 on T-cells. CHK1 inhibitors or PARP inhibitors induce STING-dependent *CXCL10* transcription from small cell lung cancer (SCLC) cell lines *in vitro* [27]. Use of the pan-immune cell marker CD45 to sort irradiated tumors in mice into $CD45^-$ and $CD45^+$ samples indicate both populations significantly upregulate *CXCL10* transcription following combined radiation and ATR inhibitor treatment [7]. In a separate study, $CD103^+CD8\alpha^+$ BATF3-lineage DCs have been shown to be important for CXCL9 and CXCL10 production [126]. ICAM1 and the NKG2D ligand RAE-1γ (*Raet1g*) are upregulated on tumor cells *in vivo* after irradiation. Upon T cell infiltration, MHC-I, ICAM1, RAE-1γ and NKG2D play a role in T-cell arrest, tumor cell engagement, and the therapeutic efficacy of radiation and anti-CTLA4 in combination in mice [127].

Unfortunately, positive aspects of radiotherapy in relation to antigen presentation, DC function and $CD8^+$ T cell infiltration are counter-balanced by suppressive signaling. In cancer, this can lead to a chronic inflamed, but suppressed, immune response or subsidence of therapy-induced inflammation. Regulatory $CD4^+FOXP3^+$ T-cells (Treg cells) also increase in mouse tumors following radiotherapy and are further elevated by ATR inhibition [7,128,129]. Treg cells contribute to immunosuppression through CTLA4 signaling, production of TGFβ and IL-10, as well as ATP conversion to adenosine by CD39 and CD73 [13,129-131]. An extensive body of literature indicates that targeting Treg cells in combination with radiotherapy can be beneficial [2,18,110,117,132].

[H2] Suppressive Myeloid Populations

Classical inflammatory monocytes are recruited to tissues during inflammation where they can differentiate into macrophages or DCs that can exhibit pro- or anti-inflammatory characteristics, depending on the cytokine milieu. Such immunosuppressive populations have earned the alias myeloid-derived suppressor

cells (MDSCs). C-C motif chemokine 2 (CCL2)– C-C chemokine receptor type 2 (CCR2) signaling has been shown to increase such populations with immunosuppressive consequences post-radiotherapy in mouse models [129,133-135]. Radiotherapy-induced CCL2 can be tumor cell-derived [7,129,134], with *Ccr2* knockout or CCL2 blockade able to counteract such populations and increase tumor control by radiotherapy [129,133-135].

CCR5, the receptor for CCL5, has also been associated with immune infiltration. Radiation increases the production of CCL5 and CCL2, driving the infiltration of $CCR2^+$ $CCR5^+$ inflammatory macrophages intratumorally and in the circulation in mice [7,133]. A CCR2 and CCR5 antagonist reversed this radiation-induced increase and enhanced radiation efficacy [133]. *Ccl2* and *Ccl5* were two of the most significantly increased tumor-specific transcripts in mice treated with the combination of radiotherapy and an ATR inhibitor [7]. A parallel increase in tumor infiltration of macrophages and MDSCs was observed, indicating that both immunosuppressive and immunostimulatory signals are increased by DDR inhibitor combinations with radiotherapy.

Radiation alone, or in combination with an ATR inhibitor, increased PD-L1 expression on MDSCs in mice [7,105,128]. In one study, ATR inhibition increased radiation-induced PD-L1 on tumor cells [7], while another study indicated that ATR inhibition reduced PD-L1 [136]. Numerous anti-PD-1 or anti-PD-L1 plus radiotherapy combination trials are ongoing. In this context, recent studies in mice found that PD-L1 on tumor cells was dispensable for ICI efficacy [137,138]. As was the case for CTLA4, targeting PD-1 or PD-L1 combined with radiotherapy was beneficial in multiple preclinical studies [98,105,109,128,139,140].

[H2] Tumor Cell versus Immune Cell Responses to Type-I IFNs

Radiation-induced or exogenous IFNβ is critical for tumor control and is dependent on non-tumor cells, in particular IFNAR on $CD11c^+$ DCs [15,18,55,141]. However, pre-existing expression of ISGs are predictive of resistance to radiation and/or chemotherapy in a number of human cancers [142-145]. Recent evidence suggests that this is driven by autocrine or paracrine type-I IFN signaling on tumor cells.

ISGs linked to resistance to ICIs are predominantly expressed in cancer cells [146]. An earlier study showed this resistance could develop if tumors in mice were allowed to establish longer before the start of radiation and anti-CTLA4 treatment. Knock-out of *Ifnar* and *Ifngr* on tumor cells showed that type-I IFN signaling through these receptors was responsible for the upregulation of an array of T-cell inhibitory ligands, including PD-L1, Galectin 9, MHC-II and HVEM [10].

Across multiple cancer types, in patients who had T-cell infiltration but markers of T[147]. In melanoma patients treated with anti-CTLA4, higher serpin family B member 9 (*SERPINB9*) corresponded to poorer prognosis [147]. *Serpinb9* in mouse models has been shown to be upregulated by IFNα and IFNγ , and mediates immune escape by inactivating granzyme B [147,148]. Dual *Ifnar* and *Ifngr* knockout restored responsiveness to radiotherapy plus anti-CTLA4 treatment [10,146]. *Ifnar1* knock-out or Janus kinase (JAK) inhibition in mouse models enhanced tumor response to radiation due to increased $CD8^+$ T-cell-mediated cell killing linked to reduction in SERPINB9. Overexpression of SERPINB9 reversed the effect of *Ifnar1* knockout [10,149]. In this context, radiotherapy, DDR inhibitors, and ICI combinations may only benefit tumors with low ISG signatures, without the addition of inhibitors targeting tumor IFNAR1 signaling. It remains to be determined if a transient radiation-induced type-I IFN signal suffers the same consequences as a chronic IFN-driven basal ISG dysfunctional phenotype.

[H1] Clinical Implications

[H2] Radiotherapy and Immune Checkpoint Inhibitor Trials

A huge number of radiation plus ICI combination studies are currently recruiting patients. A comprehensive list of combination trials with reported results (radiotherapy plus either anti-CTLA4, anti-PD-1 or anti-PD-L1) is shown in Supplementary Table 1. After numerous phase I studies showed the combination of radiation plus an ICI to be safe, further studies are now investigating the benefit from concurrent immunotherapy and chemoradiotherapy in various patient groups.

The PACIFIC trial showed significantly longer overall survival when durvalumab (anti-PD-L1) was given after standard chemoradiotherapy in patients with unresected stage III NSCLC [3]. Ipilimumab (anti-CTLA4) in combination with palliative radiotherapy in chemorefractory metastatic NSCLC resulted in objective responses in 18% of patients, with changes in serum IFN-β and early changes of T cell clonality predictive of response [2]. The two studies described benefit either from large patient numbers [3], or extensive analysis of immune biomarkers in a focused patient population [2].

Many trials listed in Supplementary Table 1 are early phase with toxicity endpoints and hence conclusions regarding efficacy are limited by small, heterogeneous patient groups. Multiple agents have been investigated including ipilimumab, anti-PD-1 (pembrolizumab or nivolumab) and anti-PD-L1 (durvalumab or atezolizumab). Currently tumor types responsive to ICIs are lead candidates for combination studies with radiotherapy. This can make the contribution of radiation to response unclear. This is further compounded by the variety of dose-fractionation schedules being tested and the number of lesions that are irradiated. Most commonly, moderately hypofractionated radiotherapy **[G]** such as three 8 Gy fractions or five 6 Gy fractions have been selected based on preclinical data suggesting that these offer the highest degree of favorable immunomodulation [18,105]. Few clinical trials are directly comparing different radiation dose-fractionations in combination with ICIs. The use of concomitant chemotherapy, where even few preclinical studies have been done, further adds to this complexity (Table 1). Well-designed later phase combination clinical trials may help to unpick some of these issues.

Clinical trials involving some of the targets outlined in earlier sections (for example, CD47, CD73, CCR2 or CCR5) in combination with radiotherapy, as well as other novel immunotherapies, are listed in Table 2. The goal of many of these early phase I and II studies is to establish tolerable dose levels. However, many of the studies share some of the limitations outlined above (patient heterogeneity or concomitant chemotherapy) which may complicate the interpretation of results. Studies on CCR2 and CCR5 antagonists with the JAK1 and JAK2 inhibitor ruxolitinib are supported by strong preclinical mechanistic datasets [10,129,133-135]. Replication of preclinical analyses in these clinical trials would be hugely beneficial in directing both future trials and preclinical research in this area.

[H2] Future Trials Investigating DDR inhibitors and Immune Readouts

Based on the preclinical data presented earlier, we believe that additional immunomodulation, such as that induced by DDR inhibitors with radiation, may further improve response rates. While radiation and ICI combination trials are now a highly active area, immune readouts from clinical trials of radiation combined with DDR inhibitors are rare. This is changing after seminal publications on cGAS surveillance of micronuclei in response to DNA damage [5,6]. The ATR inhibitor AZD6738 increases radiation-induced $CD8^+$ T-cell infiltration and enhance effector functions in preclinical studies [7,136]. An ongoing phase I trial of AZD6738 in combination with palliative radiotherapy [150] may be one of the first to evaluate immune modulation by a DDR inhibitor combined with radiotherapy [150].

While DNA-PK inhibition has not been studied preclinically in combination with radiotherapy and ICIs, clinical trials in combination with the anti-PD-L1 agent avelumab are already recruiting (Table 2). A trial of the PARP inhibitor olaparib and durvalumab is also recruiting in patients with SCLC. The addition of DDR inhibitors may, however, further complicate interpretation of clinical findings. As DDR inhibitor plus radiotherapy trials incorporating immune readouts, or DDR inhibitor plus radiotherapy and an ICI trials begin to appear, it would be prudent to be aware of the limitations observed so far for radiotherapy plus ICI trials. These early stage clinical trials will do much to direct future trial design and preclinical research. The

progression of preclinical and clinical analyses for radiotherapy and anti-CTLA4 [2,18,110,151] is a benchmark in this regard.

[H1] Conclusions

Intrinsic tumor cell signaling events following radiotherapy have a profound effect in remodeling the inflammatory tumor microenvironment. Huge progress has been made in understanding the underlying biology whereby sensing of damaged host DNA is transformed into intensified immunosurveillance of tumor cells. Understanding the variability within this response will be critical to unlocking the full potential of the immune system to improve outcomes for patients treated with radiotherapy. Challenging questions remain around the best way to potentiate the immunostimulatory effects of radiotherapy without eliciting the negative effects of immunosuppression. Enhancing the immunogenic effects of radiotherapy through DDR inhibitors while negating immunosuppressive aspects through ICIs represents a particularly promising therapeutic approach.

Acknowledgements
The authors acknowledge support from the Rosetrees Trust, the Oracle Cancer Trust, the Anthony Long Charitable Trust, the Wellcome Trust, the Stoneygate Trust and the Royal Marsden/Institute of Cancer Research NIHR Biomedical Research Centre.

Author contributions
The authors contributed equally to all aspects of the article.

Competing interests
A.A.M. reports: research grant funding from AstraZeneca/Medimmune, Bristol Myers Squibb and Oncolytics Biotech; advisory board membership, honoraria and/or speakers' bureaus from Amgen, AstraZeneca, Bristol-Myers-Squibb, Merck-Serono, Turnstone Biologics. K.J.H. reports: research grant funding from AstraZeneca/Medimmune, Boehringer-Ingelheim, Merck Sharp Dohme and Replimune; advisory board membership, honoraria and/or speakers' bureaus from Amgen, AstraZeneca, Bristol-Myers-Squibb, Boehringer-Ingelheim, Merck-Serono, Merck Sharp Dohme, Oncolys, Pfizer and Replimune. The other authors declare no competing interests.

Peer review information
Nature Reviews Cancer thanks S. Demaria, R. Greenberg and R. Weichselbaum for their contribution to the peer review of this work.

Table 1 | **Preclinical radiotherapy and DDR inhibitor or cisplatin combination studies with immune readouts**

RT and DDR inhibitor Combination	Immunotherapy/Immunostimulant	Murine tumor model	Immunological effects	Reference
RT and ATR inhibitor (AZD6738)	None	CT26 (colorectal)	Increased CD8 infiltration, decreased Tregs, promotion of immunological memory, decreased PD-L1 due to AZD6738	[136]
RT and ATR inhibitor (AZD6738)	None	TC-1 (HPV positive)	Enhanced type-I and type-II IFN signature, increased PD-L1, increased numbers of DCs, T-cells and NK cells due to AZD6738	[7]
RT and Cisplatin (+ CTX and NOS inhibitor)	None	mEER (HPV positive)	Increased proportions of inflammatory monocytes and M1-macrophages, increased CD8 T-cell activity, increased CD8:Treg ratio	[152]
RT and Cisplatin	Anti-PD-1 and CD137 agonist	AT-3 (breast cancer)	Small increase of CD8:CD4 T-cell ratio and small decrease of $CD43^+$ CD8 T cell percentage	[153]
RT and ATM silencing	Anti-PD-L1	mT4 and KPC2 (pancreatic)	Increased numbers of CD8 T cells	[98]
RT and WEE1 inhibitor (AZD1775)	Anti-PD-1	MOC-1 (HNSCC)	Increased lymphocyte activation and IFN-γ production	[96]
RT and Cisplatin	Anti-PD-1	MC38 and C51 (colorectal)	Increased CD8 T-cells in primary, secondary tumors (biflank model), increased chemokine expression	[154]
RT and Cisplatin	CXCR4 inhibitor (plerixaflor)	Cervial cancer PDX models	Decreased CXCL12–CXCR4 signaling and myeloid cell infiltration	[155]

Cisplatin, as a radiosensitizer, was included alongside DDRi studies. CTX, cyclophosphamide; ND, none described; NK cell, natural killer cell; NOS, nitric oxide synthase; PDX, patient-derived xenograft; RT, radiotherapy.

Table 2 | **Selected trials investigating radiotherapy with novel immuno-oncology or DDR inhibitor combinations**

Target (drug)	Radiotherapy	Phase	Patient population	N	Response	Toxicity	NCT or reference
Intra-tumoral anti-CD47 (TTI-621)	Regimen not stated	I/II	Relapsed and refractory percutaneously accessible solid tumors or mycosis fungoides.*	240	Not reported	Not reported	NCT02890368
Anti-PD-1 (pembrolizumab) +/- Flt-3 ligand (CDX-301)	8 Gy x 3 fractions alternative days	II	Localized breast cancer	100	Not yet recruiting	Not yet recruiting	NCT03804944
Anti-PD-L1 (durvalumab) +/- anti-CD73 (oleclumab) combined with various chemotherapies	SBRT 8 Gy x 3 fractions pre-operatively	II	Luminal B breast cancer	147	Not yet open	Not yet open	NCT03875573
Anti-PD-1 (nivolumab) and CCR2/CCR5 dual antagonist (BMS-813160) with or without GVAX following chemotherapy and radiotherapy	6.6 Gy x 5 fractions	I/II	Locally-advanced pancreatic ductal adenocarcinoma	30	Not yet open	Not yet open	NCT03767582
JAK1 and JAK2 inhibitor (ruxolitinib) and chemotherapy (temozolomide)	60 Gy in 30 fractions over 6 weeks	I	Grade III glioma and glioblastoma	36	Recruiting	Recruiting	NCT03514069
TLR9 agonist (SD-101) plus anti-OX40 (BMS-986178)	Low dose	I	Low-grade B cell non-Hodgkin lymphoma	15	Not available	Not available	NCT03410901
Intra-tumoral injections of a CpG enriched TLR9 agonist (PF-3512676)	4 Gy in 2 fractions over 2 days	I/II	Mycosis fungoides*	15	Distant site clinical response seen in 5 patients	Mild injection site reaction and mild flu-like symptoms	[156]
Intratumoral TLR9 agonist (SD-101) at a single irradiated tumor site	4 Gy in 1 fraction	I/II	Indolent lymphomas (follicular, marginal zone, small lymphocytic, chronic lymphocytic or cutaneous B-cell)	29	24 had tumor reduction in non-treated site; 5/25 had PR and 1/25 had CR	No treatment related grade 4 or SAE occurred. 8/25 had grade 3 drug-related AE	[157]
DNA-PK inhibitor (M3814) and anti-PD-L1 (avelumab)	Hypofractionated 5 fractions	I/II	Advanced hepatobiliary malignancies	92	Not yet recruiting	Not yet recruiting	NCT04068194
DNA-PK inhibitor (M3814) and capecitabine	45-50 Gy in 25-28 fractions over 5 weeks	Ib/II	Rectal cancer	165	Recruiting	Recruiting	NCT03770689
DNA-PK inhibitor (M3814) and anti-PD-L1 (avelumab)	30 Gy in 10 fractions over 2 weeks	I	Various advanced solid tumor	24	Recruiting	Recruiting	NCT03724890
Anti-PD-L1 (durvalumab) monotherapy or combined with anti-	30 Gy in 10 fractions over 2 weeks	I/II	Extensive stage SCLC	54	Recruiting	Recruiting	NCT03923270

CTLA4 (tremelimumab) or a PARP inhibitor (olaparib)							

Trials were selected based on those showing evidence of efficacy of mechanistically related preclinical studies. *Mycosis fungoides is a type of cutaneous T-cell lymphoma.
GVAX is pancreatic cancer vaccine of irradiated patient specific cancer cells which have been modified to secrete granulocyte-macrophage colony stimulating factor. AE, adverse effect; CR, complete response; PR, partial response; SAE, serious adverse effect; SBRT, stereotactic body radiotherapy.

Figure 1 | **Crosstalk between cGAS–STING, inflammasome and ribonucleic acid sensing pathways.** Radiotherapy induces cytoplasmic DNA that is sensed by a number of intracellular sensors. cGAS–cGAMP–STING (shown in red) drives downstream signaling pathways which lead to type-I IFN production in response to cytoplasmic DNA. cGAMP acts as an immunosignaling molecule, activating STING in other cells of the tumor microenvironment. Many direct regulators (dark blue) of cGAS, cGAMP or STING function have emerged from recent research. These include TREX1, which degrades cytoplasmic DNA preventing cGAS activation; and IFI16, DDX41 and NLRC3, where DNA binding has been shown to potentiate STING activity. Extracellular cGAMP can be degraded by membrane-bound and cleaved soluble forms of ENPP1. Cytoplasmic DNA can also activate AIM2–ZBP1 inflammasome signaling (yellow). This results in pyroptosis, an inflammatory form of cell death characterized by IL-1β and IL-18 secretion, as well as gasdermin D-mediated potassium channel formation. Inflammasome activation of caspase 1, as well as apoptotic caspase 3 (also shown in yellow), regulate interferon signaling negatively through cleavage of cGAS, IRF3 and MAVS. Radiotherapy-induced type-I IFNs can also induce RNA sensor activation (pink). In addition to RNA pol III conversion of AT-rich DNA to 5'-triphosphate (5'-ppp) dsRNA, autocrine and paracrine signaling of type-I IFN through STAT1 induces dsRNA synthesis from endogenous retroviral elements (ERVs). These can activate MDA5 and RIG-I alongside small non-coding RNAs (sncRNAs) induced by radiotherapy. These three pathways, through positive and negative cross-talk, help shape the inflammatory response to radiation-induced cytoplasmic DNA. ASC, apoptosis-associated speck-like protein containing a CARD; CCP, Cytosolic carboxypeptidase; G3BP1, GAP SH3 domain-binding protein 1; IKK, IκB kinase; MLKL, Mixed lineage kinase domain-like pseudokinase; RIPK, Receptor-interacting serine/threonine-protein kinase; SENP2, Sentrin-specific protease 2; TRAF, TNF receptor-associated factor; TRIM, Tripartite motif-containing protein; TTLL, Tubulin Tyrosine Ligase-Like.

Figure 2 | **Radiation-induced micronucleus formation and the role of the DNA damage response.** Radiation-induced DNA damage, such as double- and single-stranded breaks, induce the DNA damage response (DDR). Depending on the context of damage, repair is activated and this is mediated by the three central DDR kinases, DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR), which facilitate non-homologous end-joining (NHEJ), homologous recombination repair, and stabilization of stalled replication forks. Downstream cell cycle arrest by checkpoint kinase 1 (CHK1) and WEE1, as well as parylation by poly(ADP-ribose) polymerase 1 (PARP1), also facilitate repair proficiency. Loss of function or inhibitors against a number of key DDR kinases (pink) have been show to induce type-I interferon production, as have defects or knockdown of other DNA repair proteins (light blue). Defective DNA repair results in micronuclei due to separation of chromosomal fragments from the primary nucleus at cell division. Formation of core envelope proteins (lamina-associated polypeptide 2α (LAP2α) and Emerin) is unaffected, but non-core protein incorporation (nucleoporin 133 (NUP133) and lamin-B receptor (LBR)) is blocked due to the interaction of micronuclei with spindle microtubules. This leads to nuclear import defects, loss of RNApol III activity and DNA replication, lamin B1 depletion and futile attempts at nuclear envelope repair by ESCRT-III. Rupturing of the micronuclei coincides with DNA damage, invasion of the ER membrane into micronuclear chromatin, and surveillance of cytoplasmic DNA by cGAS. By this mechanism, micronuclei link DNA damage by radiotherapy and/or DDR inhibitors, to cytoplasmic nucleic acid sensors and type-I IFN signaling. STING can also nucleate autophagosome formation at the ER–Golgi intermediate compartment (ERGIC). This is dependent on ATG5 and WIPI2 and has been shown to clear micronuclei and cytoplasmic DNA. ; CHMP, Charged multivesicular body protein.

Figure 3 | **Tumor-cell centric immune signaling in the tumor microenvironment post-radiotherapy.** Radiotherapy-induced type-I IFN signaling is illustrated in (1) cells deficient in both cGAS and STING through exosomal transfer of tumor dsDNA to DCs, (2) indirect cGAMP signaling via cancer-associated fibroblasts (CAFs) from tumor cells competent in cGAS only, and (3) fully cGAS–STING competent tumor cells. Immunostimulatory effects are illustrated at the top left. Release of DAMPs such as ATP, HMGB1 and calreticulin (CRT) promotes phagocytosis and cross-presentation by dendritic cells (DCs). Cross-presentation

of tumor-associated antigens (shown as red ovals) leads to expansion of tumor-reactive T-cells in lymph nodes. Chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10 and CXCL16 from both tumor cells and DCs promote T-cell infiltration, with radiation-induced upregulation of MHC-I, intercellular adhesion molecule 1 (ICAM1) and RAE-1γ on tumor cells promoting T-cell engagement. Immunostimulatory effects are balanced by radiotherapy-induced immunosuppressive signaling, shown on the bottom of the figure. C-C motif chemokine 5 (CCL5) and CCL2 production from tumor cells promotes MDSC and Treg cell infiltration. This leads to the inhibition of $CD8^+$ T-cells and production of immunosuppressive transforming growth factor β (TGFβ). IFNAR1 activation on DCs has been shown to be required for effective adaptive immune responses to radiotherapy-induced type-I IFN. However, IFNAR1 and IFNGR activation on tumor cells by IFNβ and IFNγ upregulate granzyme B resistance due to serpin family B member 9 (SERPINB9) upregulation. This combined with cell surface inhibitory molecules promotes a T-cell exhaustion phenotype. BTLA, B- and T-lymphocyte attenuator; CCR, C-C chemokine receptor; CTLA4, cytotoxic T-lymphocyte-associated protein 4; CXCR, C-X-C chemokine receptor; HVEM, herpesvirus entry mediator; JAK, Janus kinase; LFA-1, lymphocyte function-associated antigen 1; STAT, signal transducer and activator of transcription; TIM3, T cell immunoglobulin mucin 3; TLR, toll-like receptor; PD-1, programmed cell death protein 1; PD-L1, PD1-ligand 1.

Box 1 | **Direct Effects of Radiotherapy on Non-Tumor Cells**

Concerns exist around radiosensitivity of immune cells and adverse normal tissue effects in response to radiotherapy. A problematic issue is the finding that STING activation is toxic to T-cells [158]. These concerns are somewhat counterbalanced by studies where achieving higher levels of cGAMP–STING activation is therapeutically beneficial [15,22,159]. Similar concerns have been raised around direct damage to T-cells from radiotherapy. New data points to tumor resident T-cells being resistant to radiotherapy [160]. However, in preclinical models $CD8^+$ T-cells in the lymph node are substantially more sensitive to radiotherapy and this has been shown to have negative therapeutic consequences [128,160]. Radiotherapy also has severe deleterious effects on vascular density and function [161]. Somewhat unexpectedly, ATR inhibition reverses reductions in vessel density due to radiotherapy in mice [7]. It is not yet clear how the balance of decreased vascular area and permeability, along with increased hypoxia, can be manipulated for therapeutic benefit in the context of inflammation post-radiotherapy [162].

The role of cancer-associated fibroblasts (CAFs) in the immune response to radiotherapy is not well understood, in part because of heterogeneity in CAF function. CAFs are predominantly thought to have immunosuppressive roles in tumors [163], and can contribute to radioresistance by the secretion of TGF-β to promote a radioresistant cancer stem cell phenotype [164]. CAFs also secrete exosomes that interact with tumor cells via RIG-I, further contributing to radioresistance [143]. More broadly, the DNA damage-induced senescent-messaging secretome from irradiated CAFs is thought to have diverse effects including the promotion of tumor cell survival via epithelial-mesenchymal transition and increased expression of β1-integrins [165,166]. Further work is needed to improve our understanding of how these diverse immune and non-immune mechanisms contribute to radioresistance.

Glossary

Immune Checkpoint Inhibitors
Therapeutic blockade of negative immune checkpoint signaling. Most notable of these are the clinically approved agents targeting CTLA4 and PD-L1–PD-1.

Tumor neoantigens
Neoantigens can arise when mutations in tumor cells alter peptide fragments presented to the immune system. The immune system can recognize these as foreign vs the non-mutated self-sequence.

cyclic GMP-AMP
Mammalian 2’3’-cyclic guanosine monophosphate–adenosine monophosphate, shortened to cGAMP, is a second messenger produced by cGAS binding to cytosolic DNA. It is frequently called an immunotransmitter due to extensive indirect signaling.

Pattern recognition receptors
Innate immune receptors that recognize viral or microbial molecules (pathogen-associated molecular patterns, PAMPs) or host cell molecules released during damage (damage-associated molecular patterns, DAMPs) activating host immune signalling

Type-I Interferon
This class of interferons includes interferon-α isoforms and interferon-β. Interferon-α/β receptor 1 (IFNAR1) and IFNAR2 form the type-I interferon receptor.

Gap Junctions
Intercellular channels composed of connexin transmembrane proteins. They permit direct cell–cell transfer of ions and small molecules.

Ectoenzyme
An enzyme that is found on the cell surface or that is secreted and functions outside a cell

Exosomes
Extracellular vesicles released from cells and shown to contain proteins, lipids, RNA and/or DNA. They are thought to act as a means of intercellular communication through transmission of bioactive macromolecules.

HIN Domain
The DNA binding domain present on IFI16 and AIM2 that facilitates recognition of cytosolic dsDNA. HIN is an acronym for hematopoietic expression, interferon-inducible nature, and nuclear localization.

Inflammasome
A multiprotein intracellular complex that activates the pro-inflammatory cytokines IL-1β and IL-18. This can be due to pathogens or sterile stimuli leading to activation of caspase-1.

Pyroptosis
A highly inflammatory form of cell death resulting from inflammasome activation of caspase-1.

Micronuclei
Small nuclear structures formed by mitotic errors or chromosome breakage. They form within a nuclear envelope isolated from the primary nucleus.

Chromothripsis
Clustered chromosomal rearrangements in one or a few chromosomes, which are thought to occur through a one-step catastrophic genomic event.

STING-associated vasculopathy with onset in infancy
(SAVI) An autoinflammatory disorder driven by activating mutations in STING.

Aicardi-Goutières syndrome
An inflammatory disorder driven by a number of mutations (*TREX1, SAMHD1, RNASEH2A-C, ADAR1 and IFIH1)* that lead to increased activation of cytoplasmic nucleic acid sensors and type-I interferon production.

Fanconi anemia
A rare genetic disorder that results in aplastic anaemia, leukaemia and cancer susceptibility, and hypersensitivity to DNA crosslinking agents. The pathway is responsible for the repair of DNA interstrand crosslinks and overlaps somewhat with homologous recombination repair.

Homologous recombination repair
An identical or nearly identical DNA sequence from a homologous chromosome is used as a template for the repair of a DNA break.

Major histocompatibility complex I
(MHC-I)
This complex is composed of an α and β chain and is expressed on all nucleated cells. It presents peptide fragments of intracellular proteins to the immune system.

Radiation Upregulated Neoantigens
Radiation can increase existing tumor neoantigens through either radiation-induced transcription or increased antigen presentation. It is also possible for radiotherapy to create neoantigens due to DNA damage-induced mutations.

SIINFEKL
A peptide sequence from chicken ovalbumin presented by MHC-I and used as a model peptide to study antigen presentation.

Antigen
In this context, an MHC-I presented peptide capable of stimulating an immune response.

Subclonal Neoantigens
Subclonal neoantigens are only present in a subset of tumor cells.

Clonal neoantigen
A clonal neoantigen is present in all tumor cells.

Damage-associated molecular patterns
Stimuli released by stressed, dying or injured cells that may trigger an inflammatory response by the activation of a number of pattern recognition receptors.

Hypofractionated radiotherapy

Radiation treatment where the total dose of radiation is divided into larger doses and given over a smaller number of fractions than standard radiation therapy.

Table of Contents Summary

This Review focuses on the role of tumor cell-autonomous signaling after radiotherapy. It describes how radiotherapy, through its immunomodulating effects, might be combined with immune checkpoint inhibitors and other immunotherapies and how DNA damage response inhibitors in combination with radiotherapy may be used to further augment this approach.

References

1. Delaney, G., Jacob, S., Featherstone, C. & Barton, M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. *Cancer* **104,** 1129–1137 (2005).
2. Formenti, S. C. *et al.* Radiotherapy induces responses of lung cancer to CTLA-4 blockade. *Nat. Med.* **24,** 1845–1851 (2018).

This study reports the induction of systemic anti-tumor T cells in metastatic NSCLC by radiation therapy and CTLA-4 blockade. This was in chemo-refractory patients where anti-CTLA-4 alone failed to demonstrate significant efficacy.

3. Antonia, S. J. *et al.* Overall Survival with Durvalumab after Chemoradiotherapy in Stage III NSCLC. *N. Engl. J. Med.* **379,** 2342–2350 (2018).
4. Dillon, M. T. *et al.* Radiosensitization by the ATR Inhibitor AZD6738 through Generation of Acentric Micronuclei. *Mol. Cancer Ther.* **16,** 25–34 (2017).
5. Harding, S. M. *et al.* Mitotic progression following DNA damage enables pattern recognition within micronuclei. *Nature* **548,** 466–470 (2017).
6. Mackenzie, K. J. *et al.* cGAS surveillance of micronuclei links genome instability to innate immunity. *Nature* **548,** 461–465 (2017).

These two studies (Harding et al., MacKenzie et al.) revealed that micronuclei produced in response to radiotherapy result in cytoplasmic dsDNA that is sensed by cGAS.

7. Dillon, M. T. *et al.* ATR Inhibition Potentiates the Radiation-induced Inflammatory Tumor Microenvironment. *Clin. Cancer Res.* **25,** 3392–3403 (2019).

This study profiles type-I/II IFN and tumour-infiltrating lymphoid and myeloid immune populations due to the combination of ATR inhibition with radiotherapy. It breaks down PD-L1 and cytokine expression in tumour vs immune cells.

8. Chakravarthy, A. *et al.* Pan-cancer deconvolution of tumour composition using DNA methylation. *Nat. Comm.* **9,** 3220 (2018).
9. Galon, J. & Bruni, D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat. Rev. Drug Discov.* **18,** 197–218 (2019).
10. Benci, J. L. *et al.* Tumor Interferon Signaling Regulates a Multigenic Resistance Program to Immune Checkpoint Blockade. *Cell* **167,** 1540–1554.e12 (2016).
11. Mariathasan, S. *et al.* TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature* **554,** 544–548 (2018).
12. McGranahan, N. *et al.* Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science* **351,** 1463–1469 (2016).

This study reports that mutations due to cytotoxic chemotherapy may reduce the efficacy of immune checkpoint blockade due to the creation of subclonal neoantigens.

13. Barker, H. E., Paget, J. T. E., Khan, A. A. & Harrington, K. J. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nat. Rev. Cancer* **15,** 409–425 (2015).
14. Cao, X. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. *Nat. Rev. Immunol.* **16,** 35–50 (2016).
15. Deng, L. *et al.* STING-Dependent Cytosolic DNA Sensing Promotes Radiation-Induced Type I Interferon-Dependent Antitumor Immunity in Immunogenic Tumors. *Immunity* **41,** 843–852 (2014).

This paper showed that STING is required for the type-I IFN-dependent anti-tumour effects of radiotherapy. It also showed that exogenous cGAMP enhanced radiation induced anti-tumour immunity.

16. Vance, R. E. Cytosolic DNA Sensing: The Field Narrows. *Immunity* **45,** 227–228 (2016).
17. Sun, L., Wu, J., Du, F., Chen, X. & Chen, Z. J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **339,** 786–791 (2013).
18. Vanpouille-Box, C. *et al.* DNA exonuclease Trex1 regulates radiotherapy-induced tumour immunogenicity. *Nat. Commun.* **8,** 15618 (2017).

This paper describes how high radiation doses above 12-18 Gy can induce TREX1, degrading cytoplasmic DNA and attenuating the immunogenicity of radiotherapy.

19. Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455,** 674–678 (2008).
20. Burdette, D. L. *et al.* STING is a direct innate immune sensor of cyclic di-GMP. *Nature* **478,** 515–518 (2011).

21. Francica, B. J. *et al.* TNFα and Radioresistant Stromal Cells Are Essential for Therapeutic Efficacy of Cyclic Dinucleotide STING Agonists in Nonimmunogenic Tumors. *Cancer Immunol. Res.* **6,** 422–433 (2018).
22. Carozza, J. A. *et al.* 2'3'-cGAMP is an immunotransmitter produced by cancer cells and regulated by ENPP1. *bioRxiv* (2019). doi:10.1101/539312
23. Abe, T. & Barber, G. N. Cytosolic-DNA-mediated, STING-dependent proinflammatory gene induction necessitates canonical NF-κB activation through TBK1. *J. Virol.* **88,** 5328–5341 (2014).
24. Hou, Y. *et al.* Non-canonical NF-κB Antagonizes STING Sensor-Mediated DNA Sensing in Radiotherapy. *Immunity* **49,** 490–503.e4 (2018).
25. Bakhoum, S. F. & Cantley, L. C. The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment. *Cell* **174,** 1347–1360 (2018).
26. Corrales, L. *et al.* Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell Reports* **11,** 1018–1030 (2015).
27. Sen, T. *et al.* Targeting DNA damage response promotes anti-tumor immunity through STING-mediated T-cell activation in small cell lung cancer. *Cancer Discov.* **9,** 646-661 (2019).

This study showed that PARP or CHK1 inhibition increases PD-L1 expression potentiating the effect of PD-L1 blockade. This was shown to be dependent on activation of cGAS-STING in tumour cells.

28. Wang, Z. *et al.* cGAS/STING axis mediates a topoisomerase II inhibitor-induced tumor immunogenicity. *J. Clin. Invest.* **130,** 4850-4862(2019).
29. Marcus, A. *et al.* Tumor-Derived cGAMP Triggers a STING-Mediated Interferon Response in Non-tumor Cells to Activate the NK Cell Response. *Immunity* **49,** 754–763.e4 (2018).
30. Diamond, J. M. *et al.* Exosomes Shuttle TREX1-Sensitive IFN-Stimulatory dsDNA from Irradiated Cancer Cells to DCs. *Cancer Immunol. Res.* **6,** 910–920 (2018).
31. Luteijn, R. D. *et al.* SLC19A1 transports immunoreactive cyclic dinucleotides. *Nature* **573,** 434–438 (2019).
32. Ritchie, C., Cordova, A. F., Hess, G. T., Bassik, M. C. & Li, L. SLC19A1 Is an Importer of the Immunotransmitter cGAMP. *Mol. Cell* **75,** 372-381.e5 (2019).
33. Li, L. *et al.* Hydrolysis of 2'3'-cGAMP by ENPP1 and design of nonhydrolyzable analogs. *Nat. Chem. Biol.* **10,** 1043–1048 (2014).
34. Mrowczynski, O. D. *et al.* Exosomes impact survival to radiation exposure in cell line models of nervous system cancer. *Oncotarget* **9,** 36083–36101 (2018).
35. Yuan, D. *et al.* Extracellular miR-1246 promotes lung cancer cell proliferation and enhances radioresistance by directly targeting DR5. *Oncotarget* **7,** 32707–32722 (2016).
36. Patel, S. & Jin, L. TMEM173 variants and potential importance to human biology and disease. *Genes Immun.* **20,** 82–89 (2019).
37. Zhang, X. *et al.* Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. *Mol. Cell* **51,** 226–235 (2013).
38. Konno, H. *et al.* Suppression of STING signaling through epigenetic silencing and missense mutation impedes DNA damage mediated cytokine production. *Oncogene* **37,** 2037–2051 (2018).
39. Li, X. *et al.* Viral DNA Binding to NLRC3, an Inhibitory Nucleic Acid Sensor, Unleashes STING, a Cyclic Dinucleotide Receptor that Activates Type I Interferon. *Immunity* **50,** 591–599.e6 (2019).
40. Unterholzner, L. *et al.* IFI16 is an innate immune sensor for intracellular DNA. *Nat. Immunol.* **11,** 997–1004 (2010).
41. Almine, J. F. *et al.* IFI16 and cGAS cooperate in the activation of STING during DNA sensing in human keratinocytes. *Nat. Commun.* **8,** 14392 (2017).
42. Gray, E. E. *et al.* The AIM2-like Receptors Are Dispensable for the Interferon Response to Intracellular DNA. *Immunity* **45,** 255–266 (2016).
43. Jønsson, K. L. *et al.* IFI16 is required for DNA sensing in human macrophages by promoting production and function of cGAMP. *Nat. Commun.* **8,** 14391 (2017).
44. Dunphy, G. *et al.* Non-canonical Activation of the DNA Sensing Adaptor STING by ATM and IFI16 Mediates NF-κB Signaling after Nuclear DNA Damage. *Mol. Cell* **71,** 745–760.e5 (2018).
45. Zhang, Z. *et al.* The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat. Immunol.* **12,** 959–965 (2011).
46. Omura, H. *et al.* Structural and Functional Analysis of DDX41: a bispecific immune receptor for DNA and cyclic dinucleotide. *Scientific Reports* **6,** 34756 (2016).

47. Lee, K.-G. *et al.* Bruton's tyrosine kinase phosphorylates DDX41 and activates its binding of dsDNA and STING to initiate type 1 interferon response. *Cell Reports* **10,** 1055–1065 (2015).
48. Zhang, Z. *et al.* The E3 ubiquitin ligase TRIM21 negatively regulates the innate immune response to intracellular double-stranded DNA. *Nat. Immunol.* **14,** 172–178 (2013).
49. Seo, G. J. *et al.* TRIM56-mediated monoubiquitination of cGAS for cytosolic DNA sensing. *Nat. Commun.* **9,** 613 (2018).
50. Liu, Z.-S. *et al.* G3BP1 promotes DNA binding and activation of cGAS. *Nat. Immunol.* **20,** 18–28 (2019).
51. Xia, P. *et al.* Glutamylation of the DNA sensor cGAS regulates its binding and synthase activity in antiviral immunity. *Nat. Immunol.* **17,** 369–378 (2016).
52. Hu, M.-M. *et al.* Sumoylation Promotes the Stability of the DNA Sensor cGAS and the Adaptor STING to Regulate the Kinetics of Response to DNA Virus. *Immunity* **45,** 555–569 (2016).
53. Wu, S. *et al.* HER2 recruits AKT1 to disrupt STING signalling and suppress antiviral defence and antitumour immunity. *Nat. Cell Biol.* **21,** 1027–1040 (2019).
54. Motwani, M., Pesiridis, S. & Fitzgerald, K. A. DNA sensing by the cGAS-STING pathway in health and disease. *Nat. Rev. Genet.* **72,** 447 (2019).
55. Woo, S.-R. *et al.* STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* **41,** 830–842 (2014).
56. Swanson, K. V. *et al.* A noncanonical function of cGAMP in inflammasome priming and activation. *J. Exp. Med.* **214,** 3611–3626 (2017).
57. Kuriakose, T. *et al.* ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. *Sci. Immunol.* **1,** (2016).
58. Ingram, J. P. *et al.* ZBP1/DAI Drives RIPK3-Mediated Cell Death Induced by IFNs in the Absence of RIPK1. *J. Immunol.* **203,** 1348–1355 (2019).
59. Aarreberg, L. D. *et al.* Interleukin-1β Induces mtDNA Release to Activate Innate Immune Signaling via cGAS-STING. *Mol. Cell* **74,** 801–815.e6 (2019).
60. Di Micco, A. *et al.* AIM2 inflammasome is activated by pharmacological disruption of nuclear envelope integrity. *Proc. Natl. Acad. Sci. U.S.A.* **113,** E4671–80 (2016).
61. Takaoka, A. *et al.* DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **448,** 501–505 (2007).
62. Ishii, K. J. *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* **451,** 725–729 (2008).
63. Wang, Y. *et al.* Inflammasome Activation Triggers Caspase-1-Mediated Cleavage of cGAS to Regulate Responses to DNA Virus Infection. *Immunity* **46,** 393–404 (2017).
64. Banerjee, I. *et al.* Gasdermin D Restrains Type I Interferon Response to Cytosolic DNA by Disrupting Ionic Homeostasis. *Immunity* **49,** 413–426.e5 (2018).
65. Ning, X. *et al.* Apoptotic Caspases Suppress Type I Interferon Production via the Cleavage of cGAS, MAVS, and IRF3. *Mol. Cell* **74,** 19–31.e7 (2019).
66. Rodriguez-Ruiz, M. E. *et al.* Apoptotic caspases inhibit abscopal responses to radiation and identify a new prognostic biomarker for breast cancer patients. *Oncoimmunology* **8,** e1655964 (2019).
67. Widau, R. C. *et al.* RIG-I-like receptor LGP2 protects tumor cells from ionizing radiation. *Proc. Natl. Acad. Sci. U.S.A.* **111,** E484–91 (2014).
68. Ranoa, D. R. E. *et al.* Cancer therapies activate RIG-I-like receptor pathway through endogenous non-coding RNAs. *Oncotarget* **7,** 26496–26515 (2016).
69. Cañadas, I. *et al.* Tumor innate immunity primed by specific interferon-stimulated endogenous retroviruses. *Nat. Med.* **24,** 1143–1150 (2018).
70. Ablasser, A. *et al.* RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat. Immunol.* **10,** 1065–1072 (2009).
71. Chiu, Y.-H., Macmillan, J. B. & Chen, Z. J. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* **138,** 576–591 (2009).
72. Parisien, J.-P. *et al.* RNA sensor LGP2 inhibits TRAF ubiquitin ligase to negatively regulate innate immune signaling. *EMBO Rep.* **19,** 1193 (2018).
73. Glück, S. *et al.* Innate immune sensing of cytosolic chromatin fragments through cGAS promotes senescence. *Nat. Cell Biol.* **19,** 1061–1070 (2017).

74. Crasta, K. *et al.* DNA breaks and chromosome pulverization from errors in mitosis. *Nature* **482,** 53–58 (2012).
75. Liu, S. *et al.* Nuclear envelope assembly defects link mitotic errors to chromothripsis. *Nature* **561,** 551–555 (2018).

This study identifies defective nuclear envelope assembly in micronuclei is due to spindle microtubules.

76. Hatch, E. M., Fischer, A. H., Deerinck, T. J. & Hetzer, M. W. Catastrophic Nuclear Envelope Collapse in Cancer Cell Micronuclei. *Cell* **154,** 47–60 (2013).
77. Willan, J. *et al.* ESCRT-III is necessary for the integrity of the nuclear envelope in micronuclei but is aberrant at ruptured micronuclear envelopes generating damage. *Oncogenesis* **8,** 29–14 (2019).
78. Paglin, S., Delohery, T., Erlandson, R. & Yahalom, J. Radiation-induced micronuclei formation in human breast cancer cells: dependence on serum and cell cycle distribution. *Biochem. Biophys. Res. Commun.* **237,** 678–684 (1997).
79. Tsai, M.-H. *et al.* Gene expression profiling of breast, prostate, and glioma cells following single versus fractionated doses of radiation. *Cancer Res.* **67,** 3845–3852 (2007).
80. Brzostek-Racine, S., Gordon, C., Van Scoy, S. & Reich, N. C. The DNA damage response induces IFN. *J. Immunol.* **187,** 5336–5345 (2011).
81. Liu, H. *et al.* Nuclear cGAS suppresses DNA repair and promotes tumorigenesis. *Nature* **563,** 131–136 (2018).
82. Jiang, H. *et al.* Chromatin-bound cGAS is an inhibitor of DNA repair and hence accelerates genome destabilization and cell death. *EMBO J.* **498,** e102718 (2019).
83. Bartsch, K. *et al.* Absence of RNase H2 triggers generation of immunogenic micronuclei removed by autophagy. *Hum. Mol. Genet.* **26,** 3960–3972 (2017).
84. Gui, X. *et al.* Autophagy induction via STING trafficking is a primordial function of the cGAS pathway. *Nature* **567,** 262–266 (2019).

This paper reports how STING, independent of IFN signaling, can facilitate LC3 lipidation and autophagosome biogenesis. They provided evidence that suggests autophagy is a primordial function of STING.

85. de Oliveira Mann, C. C. *et al.* Modular Architecture of the STING C-Terminal Tail Allows Interferon and NF-κB Signaling Adaptation. *Cell Reports* **27,** 1165–1175.e5 (2019).
86. Kim, H., Sanchez, G. A. M. & Goldbach-Mansky, R. Insights from Mendelian Interferonopathies: Comparison of CANDLE, SAVI with AGS, Monogenic Lupus. *J. Mol. Med.* **94,** 1111–1127 (2016).
87. Härtlova, A. *et al.* DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity. *Immunity* **42,** 332–343 (2015).
88. Gul, E. *et al.* Type I IFN-related NETosis in ataxia telangiectasia and Artemis deficiency. *J. Allergy Clin. Immunol.* **142,** 246–257 (2018).
89. Gratia, M. *et al.* Bloom syndrome protein restrains innate immune sensing of micronuclei by cGAS. *J. Exp. Med.* **216,** 1199–1213 (2019).
90. Lord, C. J. & Ashworth, A. BRCAness revisited. *Nat. Rev. Cancer* **16,** 110–120 (2016).
91. Parkes, E. E. *et al.* Activation of STING-Dependent Innate Immune Signaling By S-Phase-Specific DNA Damage in Breast Cancer. *J. Natl. Cancer. Inst.* **109,** djw199 (2016).

This paper showed that a DNA damage response deficient breast cancer subtype corresponded to upregulation of interferon-related genes. This was STING dependent and corresponded to increased T cell infiltration and PD-L1 expression.

92. Heijink, A. M. *et al.* BRCA2 deficiency instigates cGAS-mediated inflammatory signaling and confers sensitivity to tumor necrosis factor-alpha-mediated cytotoxicity. *Nat. Commun.* **10,** 100–14 (2019).
93. Hafsi, H. *et al.* Combined ATR and DNA-PK Inhibition Radiosensitizes Tumor Cells Independently of Their p53 Status. *Front. Oncol.* **8,** 127–12 (2018).
94. McLaughlin, M. *et al.* HSP90 inhibition sensitizes head and neck cancer to platin-based chemoradiotherapy by modulation of the DNA damage response resulting in chromosomal fragmentation. *BMC Cancer* **17,** 86 (2017).
95. Barker, H. E. *et al.* CHK1 Inhibition Radiosensitizes Head and Neck Cancers to Paclitaxel-Based Chemoradiotherapy. *Mol. Cancer Ther.* **15,** 2042–2054 (2016).
96. Patel, P. *et al.* Enhancing direct cytotoxicity and response to immune checkpoint blockade following ionizing radiation with Wee1 kinase inhibition. *Oncoimmunology* **8,** e1638207 (2019).

This study shows Wee1 inhibition in combination with radiotherapy enhanced the cytolytic activity of T cells and enhanced the responsiveness of tumours to PD-1 checkpoint blockade.

97. Friedman, J. *et al.* Inhibition of WEE1 kinase and cell cycle checkpoint activation sensitizes head and neck cancers to natural killer cell therapies. *J. Immunother. Cancer* **6,** 59 (2018).
98. Zhang, Q. *et al.* Inhibition of ATM Increases Interferon Signaling and Sensitizes Pancreatic Cancer to Immune Checkpoint Blockade Therapy. *Cancer Res.* **79,** 3940–3951 (2019).

This article demonstrated ATM inhibition increased radiation induced type-I IFN signaling. ATM knockdown enhanced the tumour control and CD8 infiltration due to radiotherapy and anti-PD-L1.

99. Reisländer, T. *et al.* BRCA2 abrogation triggers innate immune responses potentiated by treatment with PARP inhibitors. *Nat. Commun.* **10,** 3143–13 (2019).
100. Chabanon, R. M. *et al.* PARP inhibition enhances tumor cell-intrinsic immunity in ERCC1-deficient non-small cell lung cancer. *J. Clin. Invest.* **129,** 1211–1228 (2019).

This study showed that PARP inhibition in ERCC1-defective tumor cells generated micronuclei that activate cGAS-STING and downstream type-I IFN signaling.

101. Ghosh, R., Roy, S. & Franco, S. PARP1 depletion induces RIG-I-dependent signaling in human cancer cells. *PLoS ONE* **13,** e0194611 (2018).
102. Bhattacharya, S. *et al.* RAD51 interconnects between DNA replication, DNA repair and immunity. *Nucleic Acids Res.* **45,** 4590–4605 (2017).
103. Golden, E. B., Demaria, S., Schiff, P. B., Chachoua, A. & Formenti, S. C. An abscopal response to radiation and ipilimumab in a patient with metastatic non-small cell lung cancer. *Cancer Immunol. Res.* **1,** 365–372 (2013).
104. Golden, E. B. *et al.* Local radiotherapy and granulocyte-macrophage colony-stimulating factor to generate abscopal responses in patients with metastatic solid tumours: a proof-of-principle trial. *Lancet Oncol.* **16,** 795–803 (2015).
105. Dovedi, S. J. *et al.* Fractionated Radiation Therapy Stimulates Antitumor Immunity Mediated by Both Resident and Infiltrating Polyclonal T-cell Populations when Combined with PD-1 Blockade. *Clin. Cancer Res.* **23,** 5514–5526 (2017).
106. Garnett, C. T. *et al.* Sublethal irradiation of human tumor cells modulates phenotype resulting in enhanced killing by cytotoxic T lymphocytes. *Cancer Res.* **64,** 7985–7994 (2004).
107. Reits, E. A. *et al.* Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J. Exp. Med.* **203,** 1259–1271 (2006).
108. Sharma, A. *et al.* γ-Radiation promotes immunological recognition of cancer cells through increased expression of cancer-testis antigens in vitro and in vivo. *PLoS ONE* **6,** e28217 (2011).
109. Sharabi, A. B. *et al.* Stereotactic Radiation Therapy Augments Antigen-Specific PD-1-Mediated Antitumor Immune Responses via Cross-Presentation of Tumor Antigen. *Cancer Immunol. Res.* **3,** 345–355 (2015).
110. Rudqvist, N.-P. *et al.* Radiotherapy and CTLA-4 Blockade Shape the TCR Repertoire of Tumor-Infiltrating T Cells. *Cancer Immunol. Res.* **6,** 139–150 (2018).
111. Schaue, D. *et al.* T-cell responses to survivin in cancer patients undergoing radiation therapy. *Clinical Cancer Res.* **14,** 4883–4890 (2008).
112. Gulley, J. L. *et al.* Role of Antigen Spread and Distinctive Characteristics of Immunotherapy in Cancer Treatment. *J. Natl. Cancer Inst.* **109,** djw261 (2017).
113. Lhuillier, C., Rudqvist, N.-P., Elemento, O., Formenti, S. C. & Demaria, S. Radiation therapy and anti-tumor immunity: exposing immunogenic mutations to the immune system. *Genome Med.* **11,** 40–10 (2019).
114. Van Allen, E. M. *et al.* Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science* **350,** 207–211 (2015).
115. Alexandrov, L. B. *et al.* Signatures of mutational processes in human cancer. *Nature* **500,** 415–421 (2013).
116. Knijnenburg, T. A. *et al.* Genomic and Molecular Landscape of DNA Damage Repair Deficiency across The Cancer Genome Atlas. *Cell Reports* **23,** 239–254.e6 (2018).
117. Twyman-Saint Victor, C. *et al.* Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature* **520,** 373–377 (2015).
118. Burnette, B. & Weichselbaum, R. R. Radiation as an Immune Modulator. *Semin. in Radiat. Oncol.* **23,** 273–280 (2013).
119. Mondini, M. *et al.* Synergy of Radiotherapy and a Cancer Vaccine for the Treatment of HPV-Associated Head and Neck Cancer. *Mol. Cancer Ther.* **14,** 1336–1345 (2015).
120. Chang, M.-C. *et al.* Irradiation Enhances Abscopal Anti-tumor Effects of Antigen-Specific Immunotherapy through Regulating Tumor Microenvironment. *Mol. Ther.* **26,** 404-419 (2018).

121. Golden, E. B. *et al.* Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death. *Oncoimmunology* **3,** e28518 (2014).
122. Chao, M. P. *et al.* Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci. Transl. Med.* **2,** 63ra94 (2010).
123. Apetoh, L. *et al.* Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat. Med.* **13,** 1050–1059 (2007).
124. Ghiringhelli, F. *et al.* Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat. Med.* **15,** 1170–1178 (2009).
125. Matsumura, S. *et al.* Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. *J. Immunol.* **181,** 3099–3107 (2008).
126. Spranger, S., Dai, D., Horton, B. & Gajewski, T. F. Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell* **31,** 711–723.e4 (2017).
127. Ruocco, M. G. *et al.* Suppressing T cell motility induced by anti-CTLA-4 monotherapy improves antitumor effects. *J. Clin. Invest.* **122,** 3718–3730 (2012).
128. Marciscano, A. E. *et al.* Elective Nodal Irradiation Attenuates the Combinatorial Efficacy of Stereotactic Radiation Therapy and Immunotherapy. *Clin. Cancer Res.* **24,** 5058–5071 (2018).

This study reveals how elective nodal irradiation can reduce the efficacy of radiotherapy and immunotherapy combinations.

129. Mondini, M. *et al.* CCR2-Dependent Recruitment of Tregs and Monocytes Following Radiotherapy Is Associated with TNFα-Mediated Resistance. *Cancer Immunol. Res.* **7,** 376–387 (2019).
130. O'Garra, A., Vieira, P. L., Vieira, P. & Goldfeld, A. E. IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. *J. Clin. Invest.* **114,** 1372–1378 (2004).
131. de Leve, S., Wirsdörfer, F. & Jendrossek, V. Targeting the Immunomodulatory CD73/Adenosine System to Improve the Therapeutic Gain of Radiotherapy. *Front. Immunol.* **10,** 698 (2019).
132. Bos, P. D., Plitas, G., Rudra, D., Lee, S. Y. & Rudensky, A. Y. Transient regulatory T cell ablation deters oncogene-driven breast cancer and enhances radiotherapy. *J. Exp. Med.* **210,** 2435–2466 (2013).
133. Connolly, K. A. *et al.* Increasing the efficacy of radiotherapy by modulating the CCR2/CCR5 chemokine axes. *Oncotarget* **7,** 86522–86535 (2016).
134. Liang, H. *et al.* Host STING-dependent MDSC mobilization drives extrinsic radiation resistance. *Nat. Commun.* **8,** 1736–10 (2017).
135. Kalbasi, A. *et al.* Tumor-Derived CCL2 Mediates Resistance to Radiotherapy in Pancreatic Ductal Adenocarcinoma. *Clin. Cancer Res.* **23,** 137–148 (2017).
136. Vendetti, F. P. *et al.* ATR kinase inhibitor AZD6738 potentiates CD8+ T cell-dependent antitumor activity following radiation. *J. Clin. Invest.* **128,** 3926-3940 (2018).

This study profiles the impact of ATR inhibition on different T cell populations and PD-L1 expression in combination with radiotherapy.

137. Tang, H. *et al.* PD-L1 on host cells is essential for PD-L1 blockade-mediated tumor regression. *J. Clin. Invest.* **128,** 580–588 (2018).
138. Lin, H. *et al.* Host expression of PD-L1 determines efficacy of PD-L1 pathway blockade-mediated tumor regression. *J. Clin. Invest.* **128,** 805–815 (2018).
139. Vanpouille-Box, C. *et al.* TGFβ Is a Master Regulator of Radiation Therapy-Induced Antitumor Immunity. *Cancer Res.* **75,** 2232–2242 (2015).
140. Rodríguez-Ruiz, M. E. *et al.* TGFβ Blockade Enhances Radiotherapy Abscopal Efficacy Effects in Combination with Anti-PD1 and Anti-CD137 Immunostimulatory Monoclonal Antibodies. *Mol. Cancer Ther.* **18,** 621–631 (2019).
141. Burnette, B. C. *et al.* The efficacy of radiotherapy relies upon induction of type I interferon-dependent innate and adaptive immunity. *Cancer Res.* **71,** 2488–2496 (2011).
142. Weichselbaum, R. R. *et al.* An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 18490–18495 (2008).
143. Boelens, M. C. *et al.* Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. *Cell* **159,** 499–513 (2014).

144. Erdal, E., Haider, S., Rehwinkel, J., Harris, A. L. & McHugh, P. J. A prosurvival DNA damage-induced cytoplasmic interferon response is mediated by end resection factors and is limited by Trex1. *Genes & Development* **31,** 353–369 (2017).
145. Post, A. E. M. *et al.* Interferon-Stimulated Genes Are Involved in Cross-resistance to Radiotherapy in Tamoxifen-Resistant Breast Cancer. *Clin. Cancer Res.* **24,** 3397–3408 (2018).
146. Benci, J. L. *et al.* Opposing Functions of Interferon Coordinate Adaptive and Innate Immune Responses to Cancer Immune Checkpoint Blockade. *Cell* **178,** 933–948.e14 (2019).
147. Jiang, P. *et al.* Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat. Med.* **24,** 1550–1558 (2018).
148. Stout-Delgado, H. W., Getachew, Y., Miller, B. C. & Thiele, D. L. Intrahepatic lymphocyte expression of dipeptidyl peptidase I-processed granzyme B and perforin induces hepatocyte expression of serine proteinase inhibitor 6 (Serpinb9/SPI-6). *J. Immunol.* **179,** 6561–6567 (2007).
149. Chen, J. *et al.* Type I IFN protects cancer cells from CD8+ T cell-mediated cytotoxicity after radiation. *J. Clin. Invest.* **8,** 26496 (2019).
150. Dillon, M. T. *et al.* PATRIOT: A phase I study to assess the tolerability, safety and biological effects of a specific ataxia telangiectasia and Rad3-related (ATR) inhibitor (AZD6738) as a single agent and in combination with palliative radiation therapy in patients with solid tumours. *Clin. Transl. Radiat. Oncol.* **12,** 16–20 (2018).
151. Demaria, S. *et al.* Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. *Clin. Cancer Res.* **11,** 728–734 (2005).
152. Hanoteau, A. *et al.* Tumor microenvironment modulation enhances immunologic benefit of chemoradiotherapy. *J. Immunother. Cancer* **7,** 10 (2019).
153. Kroon, P. *et al.* Radiotherapy and Cisplatin Increase Immunotherapy Efficacy by Enabling Local and Systemic Intratumoral T-cell Activity. *Cancer Immunol. Res.* **7,** 670–682 (2019).
154. Luo, R. *et al.* Cisplatin facilitates radiation-induced abscopal effects in conjunction with PD-1 checkpoint blockade through CXCR3/CXCL10-mediated T cell recruitment. *Clin. Cancer Res.* **25,** 7243-7255 (2019).
155. Lecavalier-Barsoum, M. *et al.* Targeting CXCL12/CXCR4 and myeloid cells to improve the therapeutic ratio in patient-derived cervical cancer models treated with radio-chemotherapy. *Br. J. Cancer* **121,** 249–256 (2019).
156. Kim, Y. H. *et al.* In situ vaccination against mycosis fungoides by intratumoral injection of a TLR9 agonist combined with radiation: a phase 1/2 study. *Blood* **119,** 355–363 (2012).
157. Frank, M. J. *et al.* In Situ Vaccination with a TLR9 Agonist and Local Low-Dose Radiation Induces Systemic Responses in Untreated Indolent Lymphoma. *Cancer Discov.* **8,** 1258–1269 (2018).
158. Gulen, M. F. *et al.* Signalling strength determines proapoptotic functions of STING. *Nat. Commun.* **8,** 427–10 (2017).
159. Baird, J. R. *et al.* Radiotherapy Combined with Novel STING-Targeting Oligonucleotides Results in Regression of Established Tumors. *Cancer Res.* **76,** 50–61 (2016).
160. Arina, A. *et al.* Tumor-reprogrammed resident T cells resist radiation to control tumors. *Nat. Commun.* **10,** 3959–13 (2019).

This study shows that tumor resident T cells are more resistant to radiotherapy than those in circulation or lymphoid tissue.

161. Khan, A. A. *et al.* Genetically modified lentiviruses that preserve microvascular function protect against late radiation damage in normal tissues. *Sci. Transl. Med.* **10,** eaar2041 (2018).
162. Huang, Y. *et al.* Improving immune-vascular crosstalk for cancer immunotherapy. *Nat. Rev. Immunol.* **18,** 195–203 (2018).
163. Kalluri, R. The biology and function of fibroblasts in cancer. *Nat. Rev. Cancer* **16,** 582–598 (2016).
164. Al-Assar, O. *et al.* Contextual regulation of pancreatic cancer stem cell phenotype and radioresistance by pancreatic stellate cells. *Radiother. Oncol.* **111,** 243–251 (2014).
165. Sun, Y. *et al.* Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. *Nat. Med.* **18,** 1359–1368 (2012).
166. Park, C. C., Zhang, H. J., Yao, E. S., Park, C. J. & Bissell, M. J. Beta1 integrin inhibition dramatically enhances radiotherapy efficacy in human breast cancer xenografts. *Cancer Res.* **68,** 4398–4405 (2008).