The immunological consequences of radiation-induced DNA damage

Anna C Wilkins[1,2], Emmanuel C. Patin[2], Kevin J Harrington[1,2] and Alan A Melcher[1,2]

[1] *The Royal Marsden NHS Foundation Trust, Fulham Road, London SW3 6JJ, United Kingdom*.

[2] *Division of Radiotherapy and Imaging, The Institute of Cancer Research, 123 Old Brompton Road, London SW7 3RP, United Kingdom*.

Correspondence to: Anna Wilkins, Division of Radiotherapy and Imaging, The Institute of Cancer Research, 123 Old Brompton Road, London SW7 3RP. Tel: 00442073528133. E-mail: Anna.wilkins@icr.ac.uk

Running title: The immunological consequences of radiation-induced DNA damage

The authors declare that they have no conflict of interest.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/path.5232

Abstract

Historically, our understanding of the cytotoxicity of radiation has centred on tumour cell-autonomous mechanisms of cell death. Here, tumour cell death occurs when a threshold number of radiation-induced non-reparable double-stranded DNA breaks is exceeded. However, in recent years, the importance of immune mechanisms of cell death has been increasingly recognised, as well as the impact of radiotherapy on non-malignant cellular components of the tumour microenvironment. Conserved anti-viral pathways that detect foreign nucleic acid in the cytosol and drive downstream interferon responses via the cGAS/STING pathway are key components of the immune response to radiation-induced DNA damage. In pre-clinical models, acute induction of a type 1 interferon response is important for both direct and abscopal tumour responses to radiation. Inhibitors of the DNA damage response show promise in augmenting this inflammatory interferon response. However, a substantial proportion of tumours show chronic interferon signalling prior to radiotherapy which paradoxically drives immunosuppression. This chronic interferon signalling leads to treatment resistance, and heterotypic interactions between stromal fibroblasts and tumour cells contribute to an aggressive tumour phenotype. The effect of radiotherapy on myeloid cell populations, particularly tumour-associated macrophages, has an additional impact on the immune tumour microenvironment. It is not yet clear how the above pre-clinical findings translate into a human context. Human tumours show greater intra-tumoural

genomic heterogeneity and more variable levels of chromosomal instability than experimental murine models. High quality translational studies of immunological changes occurring during radiotherapy that incorporate intrinsic tumour biology will enable a better understanding of the immunological consequences of radiation-induced DNA damage in patients.

Key words: radiation-induced DNA damage response, interferon response, abscopal response, stromal fibroblasts, macrophages

Introduction

Patients who are systemically immunosuppressed, either pathologically or iatrogenically, can show inferior responses to radiotherapy [1]. In addition, loco-regionally delivered radiotherapy can trigger tumour responses outside the radiation field which are, at least in part, immunologically-mediated [2]. These observations suggest an important link between clinical responses to radiotherapy and the immune system. Until recently, it was widely believed that the cytotoxicity of radiotherapy was entirely tumour cell-autonomous and mediated by radiation-induced DNA damage (RIDD). According to this model, inflicting breaks in nuclear DNA above an unspecified threshold that the cell is incapable of repairing would lead to cell death, typically via apoptosis or mitotic catastrophe [3]. Latterly, non-tumour cell-autonomous, immunological aspects of radiation-induced cell death have been seen as increasingly important, alongside a greater understanding of the profound effects of radiotherapy on the wider tumour microenvironment.

Radiotherapy has both immunostimulatory and immunosuppressive effects [4]. The balance between these two effects depends on the intrinsic biology of individual tumours and their associated microenvironments, as well as the physical characteristics of the delivered radiation. A central component of this balance is the relationship between the RIDD response and the subsequent immune reaction. Radiotherapy can induce immunogenic cell death, characterised by tumour cell surface expression of calreticulin and release of

danger-associated molecular patterns (DAMPs), including ATP and high-mobility group protein B1 [5]. Conserved anti-viral pathways that detect foreign nucleic acid in the cytosol and drive downstream interferon responses are key components of the immune response to radiotherapy. This review will discuss our current understanding of the immunological consequences of RIDD, with particular focus on the role of conserved interferon responses. Whilst the review focuses on RIDD, the immunological consequences of DNA damage that we describe are of relevance beyond radiotherapy. We will also discuss some of the key effects of radiotherapy on components of the tumour microenvironment.

RIDD can drive production of type 1 interferon and a subsequent anti-tumour CD8+ T cell response

Pre-clinical work has demonstrated that the efficacy of radiotherapy in immunocompetent melanoma murine models relies upon induction of type 1 interferons, which stimulate both innate and adaptive anti-tumour immune responses [6]. Ablative radiotherapy increased intra-tumoural interferon-β and the therapeutic efficacy of radiotherapy was abrogated in mice lacking the IFNα/β receptor 1. Tumour-infiltrating CD45+ haematopoietic cells (predominantly dendritic cells) were the main source of the type 1 interferon. Subsequent tumour rejection required CD8+ T cells, and the associated expansion of antigen-specific CD8+ T cells was driven by interferon-β.

Further work has shown that the acute induction of type 1 interferon following radiotherapy is predominantly driven by cytosolic double-stranded DNA (dsDNA) that arises as a consequence of RIDD [7]. Cytosolic dsDNA is "sensed" by the cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS), which increases levels of the downstream adaptor stimulator of interferon genes (STING). STING drives increased production of type 1 interferon which leads to activation of BATF3+ dendritic cells and subsequent priming of tumour-specific CD8+ T cells [6, 7] as shown in Figure 1.

Whilst antigen-presenting cells, such as conventional dendritic cells, are likely to be the main source of type 1 interferon, intrinsic cancer cell production has also been demonstrated [8]. *In vitro* irradiation of TSA1 and 4T1 mouse breast cancer cells and MCA38 mouse colorectal cancer cells, in the absence of stroma, showed that generation of interferon-stimulated gene products can be a tumour cell-autonomous response independent of antigen-presenting cells. In addition, delivery of cytosolic DNA from irradiated tumour cells to antigen-presenting cells via exosomes has also been recently reported [9]. This study specifically identified dsDNA within the exosome, as opposed to dsDNA on its external surface, as the key factor inducing downstream interferon production. Furthermore, exosomes derived from tumours treated with radiotherapy were able to induce protective anti-tumour immunity when injected subcutaneously as a vaccination strategy. This immune response included induction of tumour-specific T cells, demonstrating the potent immunogenicity of such exosomes.

Micronuclei arising from cell cycle progression through mitosis in cells harbouring double-strand breaks, for example following radiotherapy, can also drive acute production of type 1 interferons via the cGAS/STING pathway [10]. This alternative mechanism of acute interferon production was elucidated by identification of a biphasic DNA damage-induced inflammatory response in irradiated cells. This included a delayed-onset response occurring days after DNA damage, alongside the rapid response occurring within minutes to hours discussed earlier. In irradiated MCF1A cells, and following disruption of the micronuclear envelope, cGAS localised to micronuclei post-mitosis. Robust activation of interferon-stimulated genes (*e.g.* STAT1) followed, although this effect was lost if mitotic progression was blocked, or if cGAS or STING were knocked down. An independent set of experiments validated the above mechanism: at 6 days after irradiation at 10Gy, an enhanced green fluorescent protein (eGFP) reporter driven by the IFNβ1 promoter showed increased activity specifically in cells with micronuclei [10].

Both cytosolic dsDNA and micronuclear DNA are likely to be important in driving acute interferon production and the subsequent anti-tumour inflammatory response following radiation. A substantial body of evidence from immunocompetent murine models indicates that the combination of radiotherapy plus immune checkpoint blockade (ICB) with anti-CTLA4 [11], anti-PD-1 [12], or both [13], can enhance both direct and abscopal responses to radiotherapy. Acute induction of the STING pathway is of particular importance

for the abscopal response and both tumour-intrinsic and exosomal-mediated mechanisms of acute interferon production are likely to contribute to this effect (Figure 1).

Recent work has shed light on important associations between the presence of micronuclei and chromosomal instability in the form of chromothripsis [14]. However, the precise mechanism of cell death in cells with micronuclei remains unclear and is likely to have important immunological consequences beyond the interferon responses discussed above. Apoptotic cell death, mitotic catastrophe or a combination of both may occur in irradiated cells with micronuclei [15]. Historically, apoptosis has been considered to have predominantly anti-inflammatory consequences. More recently, the phagocytic clearance of apoptotic cells by efferocytosis has been shown to increase immunosuppressive cytokines and leukocytes [16]. However, these anti-inflammatory effects are likely to be counterbalanced by the highly pro-inflammatory pre-apoptotic fragmentation and sensing of dsDNA [17].

Optimising acute induction of type 1 interferons in a therapeutic context

Optimising fraction size and total dose of radiotherapy

Optimal acute induction of type 1 interferons is dependent on both the fraction size and the total dose of radiation [11]. In a series of experiments using TSA1

and MCA38 breast and colorectal mouse tumour models respectively, the combination of radiotherapy, dosed at 24 Gy in 3 fractions or 30 Gy in 5 fractions, plus CTLA4 blockade, yielded anti-tumour responses in both the directly-irradiated tumour and a second non-irradiated tumour on the contralateral flank (*i.e*. an abscopal response). However, the abscopal response was lost when a single ablative dose of 20 Gy was applied in combination with CTLA4 blockade.

The mechanistic basis of such radiation dose-dependent abscopal responses centres on induction of the cytoplasmic DNA exonuclease, Trex1, which degrades cytosolic dsDNA. Activation of Trex1 is thought to occur when supra-threshold levels of cytosolic dsDNA are reached [8]. In the above murine models, a short-lived but substantial induction of Trex1 was seen after a single 20 Gy fraction, but not after the other fractionation schedules, and this dose-schedule was associated with attenuation of the type 1 interferon response. The additional relevance of total dose of radiation was also shown experimentally; here, 24 Gy in 3 fractions generated much stronger abscopal responses than 8 Gy in a single fraction [8]. As yet, these intriguing preclinical findings on fraction size and total dose await human validation. Nevertheless, a large number of translational clinical trials, encompassing different fraction sizes and ICB, are underway, which should help address this question [18].

Addition of DNA damage response inhibitors (DDRi) to enhance the immunogenicity of radiation

Addition of specific DNA damage response inhibitors (DDRi) to radiotherapy may increase cytosolic DNA and thereby augment the interferon responses described above. AZD6738 is an ATR inhibitor (ATRi) that has shown radiosensitising effects preclinically [19]. It is currently being evaluated in early phase clinical trials, including the PATRIOT study (NCT02223923), which includes an arm in which AZD6738 is combined with palliative radiation [20]. AZD6738 has been evaluated in combination with radiotherapy in mouse models of K-ras mutant cancer. Here, the ATRi reduced radiation-induced surface expression of PD-L1 on tumour cells and also significantly reduced tumour infiltration of regulatory T cells. Enhanced anti-tumour CD8+ activity was seen as a result of these effects [21].

Further evaluation of AZD6738 in an immunocompetent mouse model of HPV-driven malignancy also showed that AZD6738 potentiated radiation-induced inflammatory changes in the tumour microenvironment [22]. An increase in pattern recognition receptors sensing cytoplasmic nucleic acid and interferon-stimulated genes was seen with addition of ATRi to radiotherapy, compared to radiotherapy alone. Further transcript-level data indicated increased antigen processing and presentation with ATRi plus radiotherapy versus radiotherapy alone. The combination of radiotherapy and ATRi also enhanced the intratumoural myeloid cell infiltrate. Although this myeloid infiltrate included a

mixture of immunostimulatory and immunosuppressive cell populations, this study indicates the therapeutic potential of combining radiotherapy with DDRi and established or novel immunomodulatory agents.

The above data indicate considerable potential for DDRi, particularly ATRi, plus radiation to augment the type 1 interferon response to radiation. It will be exciting to see whether other DDRi, for example PARP inhibitors, increase quantities of cytosolic DNA and thereby augment the downstream interferon response.

Novel immunomodulatory agents in combination with radiotherapy

Blockade of Chemokine Receptor Type 2 (CCR2) represents an exciting example of a novel immunomodulatory strategy used alongside ablative radiotherapy [23]. CCR2 is a receptor for Monocyte Chemoattractant Proteins 1, 3 and 5 (CCL2, CCL7 and CCL12, respectively) and is expressed on the surface of a subset of monocytic myeloid-derived suppressor cells (mMDSCs). These $CCR2^{+}Ly6C^{hi}$ mMDSC cells are important mediators of radioresistance via immunosuppressive effects, which include negative regulation of tumour-specific CD8+ T cell responses [24]. Liang *et al*. demonstrated that the murine anti-CCR2 monoclonal antibody MC-21, plus stereotactic radiotherapy given at a dose of 20 Gy, significantly improved tumour rejection and substantially increased the CD8+/CD4+FoxP3+ (Treg) ratio in MC38 colorectal and Lewis

lung cancer murine models [24]. Tumour rejection was augmented with further addition of the STING agonist cGAMP to anti-CCR2 and ablative RT.

Intriguingly, the above study also showed that innate DNA sensing via STING, and the subsequent type 1 interferon response discussed earlier, play a key role in the recruitment of mMDSCs. This demonstrates the complexities of type 1 interferon signalling in tumour immunology, as well as how induction of STING can be a double-edged sword with opposing anti-tumour and pro-tumour immunological consequences.

The differential effects of acute versus chronic interferon signalling.

Acute induction of type 1 interferon to drive activation of dendritic cells and subsequent CD8+ T cell priming is central to the generation of anti-tumour immune responses. However, chronic interferon signalling present within tumours prior to any treatment can promote an entirely different tumour phenotype, in which interferon paradoxically has a predominantly immunosuppressive effect [25] (Figure 2). Benci *et al*. explored the mechanisms of PD-L1-independent resistance to radiotherapy plus anti-CTLA4 in murine melanoma models. Here, chronic type I and II interferon production led to a multitude of STAT1-driven epigenetic and transcriptomic modifications [25]. The consequences of such modifications included induction of multiple T cell inhibitor receptor (TCIR) ligands, including PD-L1, TNFRSF14, LGALS9, MHCII

and CD86, as well as T cell exhaustion. Inhibition of interferon signalling by knockout of IFNA, IFNGR or STAT1, or use of the JAK1/2 inhibitor Ruxolitinib, plus dual ICB inhibition, led to expansion and re-invigoration of distinct populations of exhausted T cells. The specific population showing most expansion was PD-1^{high}TCIR^{high}T cells, which also showed an increase in markers of improved function including Ki67 and Granzyme B [25].

Both of the interferon stimultated genes (ISGs), IFIT1 and Mx1, showed a tight association with increased STAT1 expression. To demonstrate the clinical relevance of chronic interferon signalling, the expression of both genes was analysed in a recently published cohort of melanoma patients receiving PD-1 inhibition [25]. The computational modelling strategy incorporated ISG expression and the rates of non-synonymous single nucleotide variations (nsSNV), to account for the known effect of varying neo-antigen load. The key findings were that lower IFIT1 and Mx1 expression and higher rates of nsSNV correlated with increased response to treatment with anti-PD-1.

The precise relevance of such chronic interferon signalling to radioresponsiveness is not entirely clear. A substantial body of evidence indicates that upregulation of ISGs in tumours predicts a radioresistant phenotype. An experimentally-derived interferon-related DNA damage resistance signature (IRDS), including STAT1, ISG15 and IFIT1, was developed by repeated irradiation to a xenograft of the radiosensitive cell line SCC-61 to generate a radioresistant tumour [26]. The resulting ISG-enriched signature [27]

has subsequently been evaluated in different *in vitro* cell systems and xenograft models. Upregulation of IRDS genes has consistently been demonstrated during fractionated radiotherapy [28, 29] and constitutive expression of STAT1 and other ISGs has repeatedly predicted radioresistance [30]. In patients, analysis of gene expression databases has shown constitutive expression of ISGs in a substantial proportion of patients with head and neck, prostate, breast, lung and cervical cancers and high grade gliomas [31-34]. In breast cancer, an IRDS-based 7-gene classifier, including STAT1, was evaluated in 295 patients treated with adjuvant radiotherapy [27]. Patients with high expression of the signature (IRDS+) showed a significantly greater rate of loco-regional failure at ten years post-radiotherapy.

Collectively, these studies indicate that constitutive STAT1/interferon signalling drives aggressive and radioresistant tumour phenotypes. Measurement of ISGs, such as Mx1 and IFIT1, may help identify such phenotypes [25]. It is clear that the impact of chronic interferon signalling in driving unfavourable tumour biology is substantial (Figure 2). However, it is much less clear how tumours driven by chronic immunosuppressive interferon production can be manipulated to enable the beneficial anti-tumour effects of acute interferon induction during radiotherapy to predominate.

The role of stromal fibroblasts in chronic interferon signalling

A multi-faceted heterotypic interaction between cancer-associated fibroblasts (CAFs) and tumour cells has been elucidated in recent years (Figure 3). This complex interaction provides a mechanistic explanation for some of the aggressive tumour behaviour associated with upregulation of ISGs discussed above. A key aspect of the heterotypic interaction is the exosomal transfer of double-stranded RNA (dsRNA) from CAFs to tumour cells, where it binds the pattern recognition receptor retinoic acid-inducible gene-I-like receptor (RIG-I) and drives production of ISGs [35]. Intriguingly, the specific dsRNA acting as a DAMP within exosomes has recently been identified as the long non-coding RNA, RN7SL1 [36]. RN7SL1 exists endogenously in the cytosol, yet it is upregulated and unshielded in exosomes by an extensive transcriptional program in CAFs, including NOTCH1 and MYC pathway signalling.

If breast cancer cells are separated from CAFs by a transwell filter, which enables exosomal transfer but does not permit cell-to-cell contact, upregulation of tumour cell ISGs is seen, but the radioresistance observed in ISG-high tumours does not occur [35]. This lack of radio-resistance, despite paracrine signalling via exosomal dsRNA as described above, can be explained by further juxtacrine signalling in which NOTCH3 in breast cancer cells binds JAG1 on CAFs via direct cell-to-cell contact. The juxtacrine signalling drives an expansion of treatment-resistant $CD44^{+}CD24^{low+}$ cells with tumour-initiating properties. The paracrine and juxtacrine pathways ultimately converge because STAT1 enhances the transcriptional response to increased NOTCH3 signalling

[35]. The stromal fibroblasts or CAFs, therefore, drive both reduced tumour cell death and increased tumour growth.

Boelens *et al*. (30) carried out extensive expression profiling of human breast tumours as clinical qualification of the mechanisms identified in experimental models. Amongst other findings, they showed that breast tumour NOTCH3 and stromal JAG1 are important regulators of NOTCH target genes, and that NOTCH3 and STAT1 localise to sites of tumour-stroma interaction. Additionally, high IRDS/STAT1 and NOTCH3 identify patients with radio- and chemo-resistance – this gene expression pattern is particularly common in basal and claudin-low subtypes of breast cancer, which are known to be enriched when cancer stem cell-like features are present. Both NOTCH3 and the IRDS (including STAT1) may prove to be useful predictive biomarkers to guide treatment with agents that block NOTCH activation, such as gamma secretase inhibitors [35].

The importance of myeloid cell populations in the response to radiotherapy

Myeloid populations - for example, tumour-associated macrophages (TAMs) - are often abundant in the tumour microenvironment and are an attractive target for anticancer therapies, including combinations of radiotherapy and systemic agents [37-39]. Macrophages display significant plasticity, but are ordinarily

classified between classically-activated (M1) and alternatively-activated macrophages (M2). Response to numerous inflammatory stimuli dictates the polarisation of macrophages towards M1 or M2 phenotypes [40, 41]. M1 macrophages are endowed with anti-tumoural activities and act mainly as a driver of a protective T_H1 immune response, whereas M2 macrophages are responsible for tumour growth and resistance to anticancer therapies [42].

In tumours, macrophages usually possess a deleterious M2 phenotype which promotes angiogenesis, suppression of anti-tumour T cell responses, and metastatic dissemination. Consequently, high numbers of TAMs are generally associated with poor prognosis and lower survival rates in cancer patients [43, 44]. Several reports have demonstrated an increase in macrophage infiltration in tumours following radiotherapy which may limit treatment efficacy. However, studies of polarisation of recruited TAMs following radiotherapy have yielded conflicting results, depending on the tumour model, radiation fraction size and total dose, and the host's genetic background [45].

The RIDD response pathway is likely to be important for the activation of macrophages towards a pro-inflammatory M1-like phenotype [46]. Wu *et al*. showed that NOX2-dependent reactive oxygen species (ROS) production following radiotherapy induced ATM phosphorylation, which subsequently led to IRF5 expression and pro-inflammatory responses in macrophages. These data suggest that systemic agents that modulate the RIDD response may favourably influence macrophage function to improve anti-tumour responses, as well as

enhancing the post-radiotherapy acute type 1 interferon response discussed earlier.

The molecular mechanisms regulating macrophage infiltration and activation following radiotherapy in tumours have been extensively investigated. Findings indicate that there is potential for therapeutically beneficial immune-modulation using agents that may synergise with DDRi. In a xenograft model of non-small cell lung carcinoma, interleukin-6 (IL-6) induced the recruitment of macrophages in irradiated tumours via CCL2/CCL5 secretion [47]. Colony-stimulating factor-1 (CSF-1) was also proposed as an important factor in recruiting macrophages in mouse mammary tumours [48]. In addition, neutralisation of cytokines that are important for M2 polarisation (IL-4 and IL-13) enabled reduction of tumour growth. Tumours originating from the 4T1 breast cancer cell line show higher proportions of $iNOS^+$ TAMs (M1) following inhibition of matrix metalloproteinase 14 [49]. M1 macrophage recruitment, together with reduction of anti-inflammatory TGF-β, improved radiotherapy-mediated tumour control.

More research is required to understand fully the molecular determinants of TAM polarisation following radiotherapy and optimise drug combinations to exploit the potential of macrophage reprogramming post-radiotherapy. Possible synergistic drug combinations include DDRi in combination with other immunomodulatory agents. Such combinations may enable both the suppression of deleterious macrophages and the promotion of beneficial anti-tumour macrophages following radiotherapy.

How do immunogenic properties of radiotherapy established in pre-clinical models apply in a human context?

Key differences between human tumours and murine model systems

The mechanistic studies explained above are, by necessity, conducted in experimental murine models and it is unclear how these findings translate into a human context. The radiotherapy schedules used in the above studies are reasonably representative of schedules used in the clinic. However, there are important biological differences between primary and secondary human tumours and the above model systems. The time period over which branched evolution operates within human tumours does not apply to murine models. This means that intra-tumoural genomic heterogeneity is likely to be much greater in human tumours than in commonly used murine models. Furthermore, the biological mechanisms enabling successful metastasis of human tumours do not occur in the bilateral flank models typically used to evaluate the abscopal response in murine tumour systems. As a consequence, the genetic and phenotypic variation between human primary and metastatic tumours is likely to be considerably greater than in the equivalent murine models.

Chromosomal instability

Human tumours are likely to exhibit a greater degree of chromosomal instability (CIN) than their murine equivalents and CIN is thought to occur to a larger extent in metastases than in primary tumours [50]. Recent data suggest that increased cytosolic DNA and cGAS/STING signalling occur in untreated cancer cells with high CIN, and such signalling is involved in metastatic progression. Cancer cells with high CIN, and those isolated from metastases, have higher numbers of micronuclei and greater quantities of cytosolic DNA than primary tumours with low CIN. Additionally, if micronuclear envelope incompetence is suppressed, quantities of cytosolic DNA are reduced in high CIN cells. The increased cytosolic DNA in high CIN cells can activate non-canonical NFκB signalling in a STING-dependent, yet MYC and TBK1-independent, manner. Such signalling can be important for metastatic progression, as tumour dissemination was reduced in models of high CIN depleted of STING [50]. This diversion away from a STING-dependent inflammatory response towards non-canonical signalling in tumours with high CIN may impact how effectively an acute interferon response, and subsequent abscopal response, is generated following radiotherapy.

The DNA exonuclease Trex1, discussed earlier in the context of radiotherapy fraction sizes, also has a role in human chromothripsis. Chromothripsis or chromosome shattering is thought to occur as a single event during which a high number of focal copy number alterations are generated. Trex1 has a specific role in the resolution of anaphase bridges following telomere fusion,

which is an important mechanism for chromothripsis [51]. This illustrates how key proteins linking the DDR and immune response have much wider roles, including the regulation of chromosomal stability. The cGAS/STING pathway is increasingly recognised to have diverse context-dependent components, and an improved understanding of the complexities of this pathway will be necessary for the development of successful human trials of immunotherapy/radiation combinations.

Generation of anti-tumour CD8+ T cell responses

Radiation-induced cell lysis causes release of tumour neo-antigens that are taken up by antigen presenting cells in the surrounding tumour microenvironment. The subsequent sustainment of an anti-tumour immune response likely requires generation of CD8+ T memory cells that recognise these tumour neo-antigens [52]. A CD8+ T cell response to clonal neo-antigens, rather than sub-clonal neo-antigens, is thought to be important [53]. The considerable genomic diversity of human tumours may limit the effectiveness of CD8+ T cell responses following irradiation of a single site, with failure to engage neo-antigens that are private to other non-irradiated tumour sites. However, it is also possible that 'epitope spreading' occurs in which a strong response to one epitope supports generation of responses to other less immunogenic neo-epitopes [54]. It has been suggested that radiotherapy may

increase sub-clonal neoantigens, potentially leading to T cell exhaustion [53]. However, to our knowledge this has not been shown to date, in either experimental models or patients receiving radiotherapy.

The evolution of T cell receptor (TCR) clones during radiotherapy, in both human and murine models, is a particularly exciting area of current research. In a study of radiotherapy combined with CTLA4 blockade in the 4T1 breast cancer mouse model, clonally-expanded tumour-specific T cells formed the majority of the CD8+ T cell population [55]. Deep sequencing of TCR-β showed that radiotherapy broadened the TCR repertoire, as has also been reported in other murine tumour models [12, 13]. In contrast, anti-CTLA4 treatment increased TCR clonality and, therefore, focussed the TCR repertoire. These TCR dynamics, driven by radiation-induced inflammatory changes, may be important for the observed tumour rejection in these models.

Early findings from a human trial of radiotherapy plus ipilimumab in patients with non-small cell lung cancer also show a diversification and intensification of TCR clones during radiotherapy [56]. In this study, the persistent expansion of tumour-specific T cell clones in peripheral blood correlated with partial or complete response to treatment. A particularly interesting finding was seen in a patient showing a complete response to radiation and ipilimumab. Here, expansion of two tumour-specific T cell clones recognising epitopes within the gene *KPNA2* was seen. *KPNA2* is known to be upregulated by radiotherapy. Importantly, an increase in serum interferon-β from baseline was significantly

correlated with a radiological response to radiotherapy plus ipilimumab. Together, these findings suggest that radiation may reveal immunogenic mutations by increasing their expression thus enabling antigen presentation, production of IFN-β and a subsequent T cell response. These exciting early findings require confirmation in other larger patient cohorts together with much more research, to understand how radiotherapy plus immunomodulatory combinations impact T cell clonal evolution in human tumours.

Conclusion

There is a wealth of pre-clinical data indicating that RIDD and the immune response can be exploited for therapeutic benefit. Radiotherapy and immunotherapy as an effective combination has already been demonstrated in the randomised placebo-controlled phase III PACIFIC trial of the PD-L1 inhibitor durvalumab after chemo-radiotherapy, in locally-advanced lung cancer [57]. Here, the added overall benefit in terms of median time to death or distant metastases of the addition of durvalumab was just over twelve months [58]. There is tremendous potential for this substantial survival benefit to be realised in other tumour sites, together with further improvements in survival in lung cancer. However, there is still a lot that we do not understand about the relationship between radiotherapy and the immune response, particularly in a human context. Currently, there is an urgent need for high quality human

studies with translational endpoints that profile longitudinal changes occurring during radiotherapy alone, and with radiation and ICB (or other immunomodulatory treatments including chemotherapy) combinations. Such studies ideally need to incorporate measures of CIN, stromal fibroblast biology and interferon signalling at baseline, and during radiotherapy. These translational studies should further our understanding of the evolution of tumours, their associated microenvironments and TCR clones during radiotherapy, paving the way for therapeutic gains for patients.

Acknowledgements

We acknowledge funding to the NIHR Biomedical Research Centre at The Royal Marsden NHS Foundation Trust and The Institute of Cancer Research, London. We thank Simon and Victoria Roulstone for their help with image production.

Statement of author contributions

AW and EP drafted the manuscript. KH and AM provided critical review of the manuscript. All authors agreed the final version for submission.

List of abbreviations

APC, antigen presenting cell;

ATRi, ATR inhibitor;

CAF, cancer-associated fibroblast;

CCR2, chemokine receptor type 2;

cGAS, cyclic guanosine monophosphate–adenosine monophosphate synthase;

CIN, chromosomal instability;

CSF-1, colony-stimulating factor-1;

DAMP, danger-associated molecular pattern;

DDR, DNA damage response;

DDRi, DNA damage response inhibitor;

dsDNA, double-stranded DNA;

dsRNA, double-stranded RNA;

ICB, immune checkpoint blockade;

IRDS, interferon-related DNA damage resistance signature;

ISG, interferon-stimulated gene;

mMSDCs, monocytic myeloid-derived suppressor cells;

nsSNV, non-synonymous single nucleotide variation;

RIDD, radiation-induced DNA damage;

RIG-1, retinoic acid-inducible gene-I-like receptor;

ROS, reactive oxygen species;

STING, stimulator of interferon genes;

TAM, tumour-associated macrophage;

TCIR, T cell inhibitor receptor;

TCR, T cell receptor

Figure Legends

Figure 1. Radiation-induced acute production of type 1 interferon.
Following tumour cell irradiation, dsDNA is present in the cytosol where it can stimulate the dsDNA sensor cGAS and the downstream STING pathway (1). Exosomal transfer of dsDNA to antigen presenting cells (APC) can also occur leading to cGAS/STING pathway signalling within the APC (2). Micronuclei arising in the daughter cells of irradiated tumour cells can also release dsDNA which stimulates cGAS and drives signalling via STING (3). Increased signalling via the cGAS/STING pathway leads to activation of BATF3+ dendritic cells and subsequent priming of tumour-specific T cells. APC: antigen presenting cell; dsDNA: double stranded DNA; IFN: interferon; STING, stimulator of interferon genes.

Figure 2. Chronic interferon stimulation involving heterotypic interaction between tumour cells and cancer-associated fibroblasts. Chronic interferon signalling leads to T cell exhaustion via STAT1-driven epigenetic and transcriptomic modifications which induce multiple T cell inhibitor receptor ligands and lead to T cell exhaustion. Inhibition of interferon signalling by knockout of IFNA, IFNGR or STAT1, or use of the JAK1/2 inhibitor ruxolitinib, plus dual ICB inhibition, leads to expansion and re-invigoration of exhausted T cells. ICB: immune checkpoint blockade; IFNAR: interferon alpha/beta receptor; IFNG: interferon-gamma; IFNGR: interferon-gamma receptor; ISGs: interferon-stimulated genes, MHC: major histocompatibility complex.

Figure 3. The heterotypic interaction between cancer-associated fibroblasts and tumour cells contributes to chronic interferon signalling. Paracrine signalling involves exosomal transfer of dsRNA from cancer-

associated fibroblasts to tumour cells, where it binds RIG-I and drives production of ISGs. Juxtacrine signalling in which NOTCH3 on tumour cells binds JAG1 on CAFs via direct cell-to-cell contact drives expansion of radioresistant tumour-initiating cells. dsRNA: double stranded RNA; IRDS: interferon-related DNA damage resistance signature; ISG: interferon-stimulated genes; NICD: NOTCH intracellular domain; RIG-I: retinoic acid-inducible gene-I-like receptors.

References

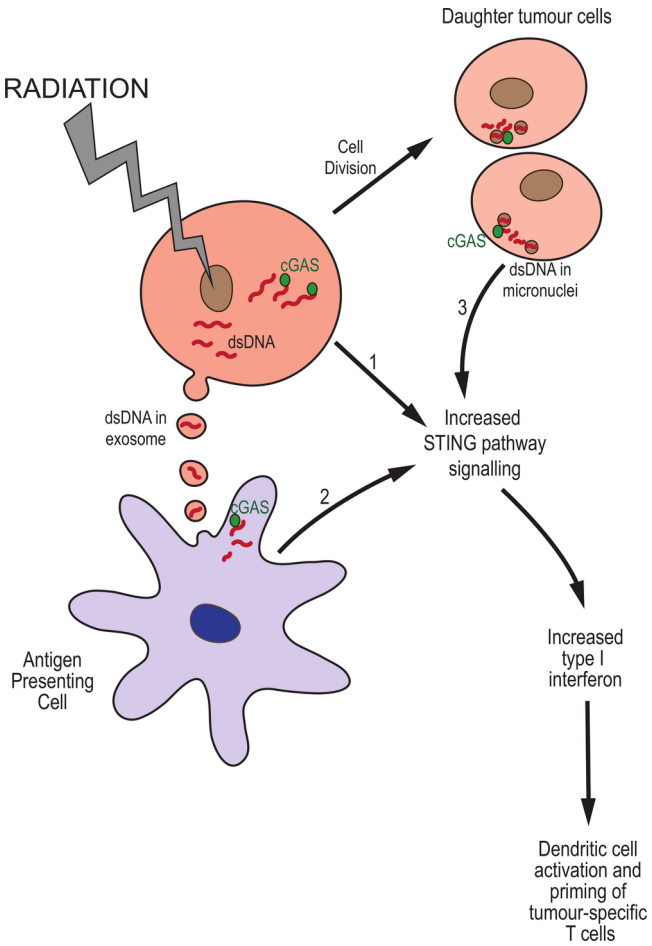
1. Tseng YD, Nguyen MH, Baker K, *et al*. Effect of patient immune status on the efficacy of radiation therapy and recurrence-free survival among 805 patients with Merkel cell carcinoma. *Int J Radiat Oncol Biol Phys* 2018; **102**: 330-339.
2. Postow MA, Callahan MK, Barker CA, *et al*. Immunologic correlates of the abscopal effect in a patient with melanoma. *N Engl J Med* 2012; **366**: 925-931.
3. Warters RL, Hofer KG, Harris CR, *et al*. Radionuclide toxicity in cultured mammalian cells: Elucidation of the primary site of radiation damage. *Curr Top Radiat Res Q* 1978; **12**: 389-407.
4. Weichselbaum RR, Liang H, Deng L, *et al*. Radiotherapy and immunotherapy: A beneficial liaison? *Nat Rev Clin Oncol* 2017; **14**: 365-379.
5. Golden EB, Frances D, Pellicciotta I, *et al*. Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death. *Oncoimmunology* 2014; **3**: e28518.
6. Burnette BC, Liang H, Lee Y, *et al*. The efficacy of radiotherapy relies upon induction of type I interferon-dependent innate and adaptive immunity. *Cancer Res* 2011; **71**: 2488-2496.
7. Deng L, Liang H, Xu M, *et al*. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity* 2014; **41**: 843-852.
8. Vanpouille-Box C, Alard A, Aryankalayil MJ, *et al*. DNA exonuclease Trex1 regulates radiotherapy-induced tumour immunogenicity. *Nat Commun* 2017; **8**: 15618.
9. Diamond JM, Vanpouille-Box C, Spada S, *et al*. Exosomes shuttle Trex1-sensitive IFN-stimulatory dsDNA from irradiated cancer cells to DCs. *Cancer Immunol Res* 2018; **6**: 910-920.

10. Harding SM, Benci JL, Irianto J, *et al*. Mitotic progression following DNA damage enables pattern recognition within micronuclei. *Nature* 2017; **548**: 466-470.
11. Dewan MZ, Galloway AE, Kawashima N, *et al*. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res* 2009; **15**: 5379-5388.
12. Dovedi SJ, Cheadle EJ, Popple AL, *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* 2017; **23**: 5514-5526.
13. Twyman-Saint Victor C, Rech A, Maity A *et al*. Radiation and dual checkpoint blockade activates non-redundant immune mechanisms in cancer. *Nature* 2015; **520**: 373-377.
14. Zhang CZ, Spektor A, Cornils H, *et al*. Chromothripsis from DNA damage in micronuclei. *Nature* 2015; **522**: 179-184.
15. Hintzsche H, Hemmann U, Poth A, *et al*. Fate of micronuclei and micronucleated cells. *Mutat Res* 2017; **771**: 85-98.
16. Werfel TA, Elion DL, Rahman B, *et al*. Treatment-induced tumor cell apoptosis and secondary necrosis drive tumor progression in the residual tumor microenvironment through MerTK and IDO-1. *Cancer Res* 2018; (in press).
17. Mackenzie KJ, Carroll P, Martin CA, *et al*. cGAS surveillance of micronuclei links genome instability to innate immunity. *Nature* 2017; **548**: 461-465.
18. Kordbacheh T, Honeychurch J, Blackhall F, *et al*. Radiotherapy and anti-PD-1/PD-L1 combinations in lung cancer: Building better translational research platforms. *Ann Oncol* 2018; **29**: 301-310.
19. Dillon MT, Barker HE, Pedersen M, *et al*. Radiosensitization by the ATR inhibitor AZD6738 through generation of acentric micronuclei. *Mol Cancer Ther* 2017; **16**: 25-34.
20. Dillon MT, Boylan Z, Smith D, *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* 2018; **12**: 16-20.
21. Vendetti FP, Karukonda P, Clump DA, *et al*. ATR kinase inhibitor Azd6738 potentiates CD8+ T cell-dependent antitumor activity following radiation. *J Clin Invest* 2018; **128**: 3926-40.
22. Dillon M T BK, Pederson M, Whittock H, Patin E, Smith H, Paget J, Patel R, Foo S, Bozhanova G, Campbell J, Ragulan C, Rontana E, Wilkins A, Sadanandam A, Melcher A A, McLaughlin M, Harrington K J. Atr Inhibition with Radiation Creates an Inflammatory Tumour Microenvironment. 2018.
23. Mack M, Cihak J, Simonis C, *et al*. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J Immunol* 2001; **166**: 4697-4704.

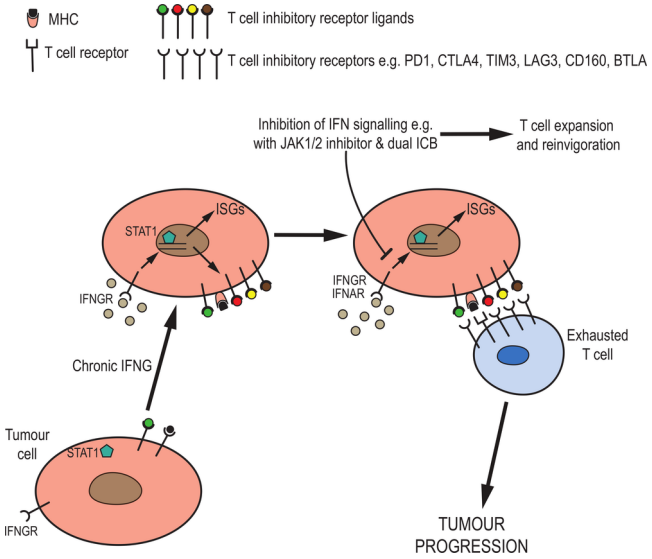
24. Liang H, Deng L, Hou Y, *et al*. Host STING-dependent MDSC mobilization drives extrinsic radiation resistance. *Nat Commun* 2017; **8**: 1736.
25. Benci JL, Xu B, Qiu Y, *et al*. Tumor interferon signaling regulates a multigenic resistance program to immune checkpoint blockade. *Cell* 2016; **167**: 1540-1554 e12.
26. Khodarev NN, Beckett M, Labay E, *et al*. STAT1 is overexpressed in tumors selected for radioresistance and confers protection from radiation in transduced sensitive cells. *Proc Natl Acad Sci U S A* 2004; **101**: 1714-1719.
27. Weichselbaum RR, Ishwaran H, Yoon T, *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* 2008; **105**: 18490-18495.
28. Tsai MH, Cook JA, Chandramouli GV, *et al*. Gene expression profiling of breast, prostate, and glioma cells following single versus fractionated doses of radiation. *Cancer Res* 2007; **67**: 3845-3852.
29. John-Aryankalayil M, Palayoor ST, Cerna D, *et al*. Fractionated radiation therapy can induce a molecular profile for therapeutic targeting. *Radiat Res* 2010; **174**: 446-458.
30. Khodarev NN, Minn AJ, Efimova EV, *et al*. Signal transducer and activator of transcription 1 regulates both cytotoxic and prosurvival functions in tumor cells. *Cancer Res* 2007; **67**: 9214-9220.
31. Khodarev NN, Roizman B, Weichselbaum RR. Molecular pathways: Interferon/Stat1 pathway: Role in the tumor resistance to genotoxic stress and aggressive growth. *Clin Cancer Res* 2012; **18**: 3015-3021.
32. Perou CM, Jeffrey SS, van de Rijn M, *et al*. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc Natl Acad Sci U S A* 1999; **96**: 9212-9217.
33. Chung CH, Bernard PS, Perou CM. Molecular portraits and the family tree of cancer. *Nat Genet* 2002; **32 Suppl**: 533-540.
34. Rajkumar T, Sabitha K, Vijayalakshmi N, *et al*. Identification and validation of genes involved in cervical tumourigenesis. *BMC Cancer* 2011; **11**: 80.
35. Boelens MC, Wu TJ, Nabet BY, *et al*. Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. *Cell* 2014; **159**: 499-513.
36. Nabet BY, Qiu Y, Shabason JE, *et al*. Exosome RNA unshielding couples stromal activation to pattern recognition receptor signaling in cancer. *Cell* 2017; **170**: 352-366 e13.
37. Nielsen SR, Schmid MC. Macrophages as key drivers of cancer progression and metastasis. *Mediators Inflamm* 2017; **2017**: 9624760.
38. van Ravenswaay Claasen HH, Kluin PM, Fleuren GJ. Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. *Lab Invest* 1992; **67**: 166-174.

39. Tsai CS, Chen FH, Wang CC, *et al*. Macrophages from irradiated tumors express higher levels of iNOS, Arginase-I and Cox-2, and promote tumor growth. *Int J Radiat Oncol Biol Phys* 2007; **68**: 499-507.
40. Shapouri-Moghaddam A, Mohammadian S, Vazini H, *et al*. Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol* 2018; **233**: 6425-6440.
41. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Rep* 2014; **6**: 13.
42. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; **8**: 958-969.
43. Zhang J, Yan Y, Yang Y, *et al*. High infiltration of tumor-associated macrophages influences poor prognosis in human gastric cancer patients, associates with the phenomenon of EMT. *Medicine (Baltimore)* 2016; **95**: e2636.
44. Zhao X, Qu J, Sun Y, *et al*. Prognostic significance of tumor-associated macrophages in breast cancer: A meta-analysis of the literature. *Oncotarget* 2017; **8**: 30576-30586.
45. Wu Q, Allouch A, Martins I, *et al*. Macrophage biology plays a central role during ionizing radiation-elicited tumor response. *Biomed J* 2017; **40**: 200-211.
46. Wu Q, Allouch A, Paoletti A, *et al*. Nox2-dependent ATM kinase activation dictates pro-inflammatory macrophage phenotype and improves effectiveness to radiation therapy. *Cell Death Differ* 2017; **24**: 1632-1644.
47. Wang X, Yang X, Tsai Y, *et al*. IL-6 mediates macrophage infiltration after irradiation via up-regulation of CCL2/CCL5 in non-small cell lung cancer. *Radiat Res* 2017; **187**: 50-59.
48. Shiao SL, Ruffell B, DeNardo DG, *et al*. Th2-polarized CD4(+) T cells and macrophages limit efficacy of radiotherapy. *Cancer Immunol Res* 2015; **3**: 518-525.
429. Ager EI, Kozin SV, Kirkpatrick ND, *et al*. Blockade of MMP14 activity in murine breast carcinomas: Implications for macrophages, vessels, and radiotherapy. *J Natl Cancer Inst* 2015; **107:** djv017.
50. Bakhoum SF, Ngo B, Laughney AM, *et al*. Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature* 2018; **553**: 467-472.
51. Maciejowski J, Li Y, Bosco N, *et al*. Chromothripsis and kataegis induced by telomere crisis. *Cell* 2015; **163**: 1641-1654.
52. Rizvi NA, Hellmann MD, Snyder A, *et al*. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015; **348**: 124-128.
53. McGranahan N, Furness AJ, Rosenthal R, *et al*. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science* 2016; **351**: 1463-1469.
54. Heckler M, Dougan SK. Unmasking pancreatic cancer: Epitope spreading after single antigen chimeric antigen receptor T-cell therapy in a human phase I trial. *Gastroenterology* 2018; **155**: 11-14.

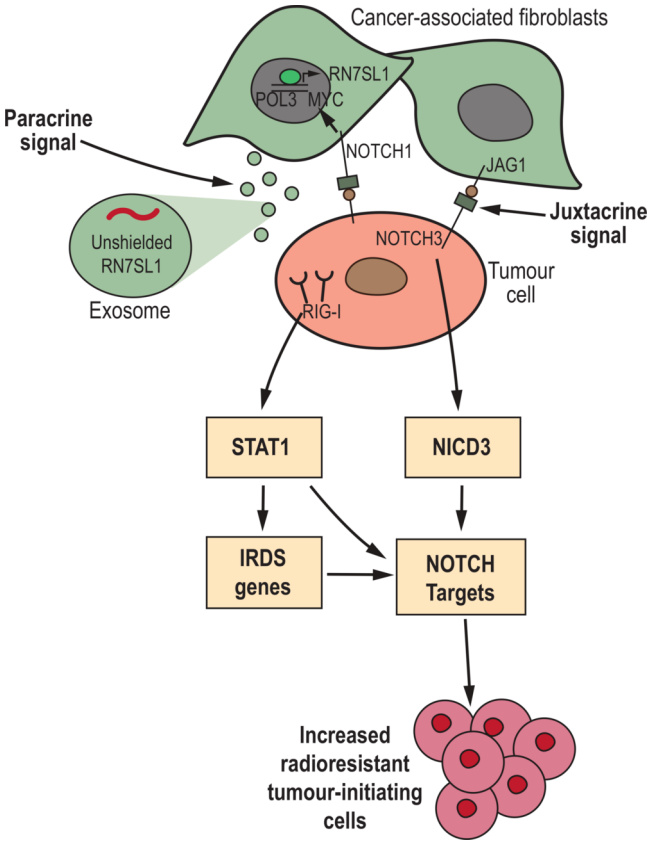
55. Rudqvist NP, Pilones KA, Lhuillier C, *et al*. Radiotherapy and CTLA-4 blockade shape the TCR repertoire of tumor-infiltrating T cells. *Cancer Immunol Res* 2018; **6**: 139-150.
56. Formenti SC, Rudqvist NP, Golden E, *et al*. Radiotherapy induces responses of lung cancer to CTLA-4 blockade. *Nat Med* 2018; **24:** 1845-1851.
57. Antonia SJ, Villegas A, Daniel D, *et al*. Durvalumab after chemoradiotherapy in stage III non-small-cell lung cancer. *N Engl J Med* 2017; **377:** 1919-1929.
58. Antonia SJ, Villegas A, Daniel D, *et al*. Overall survival with durvalumab after chemoradiotherapy in stage III NSCLC. *N Engl J Med* 2018; **379:** 2342-2350.



PATH_5232_PC_Figure1_TIFF .tif



PATH_5232_PC_Figure2_TIFF.tif



PATH_5232_PC_Figure3_TIFF.tif