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The Tumour Microenvironment after Radiotherapy: Mechanisms of Resistance and Recurrence

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Abstract

Radiotherapy plays a central part in curing cancer. For decades, most research on improving treatment outcomes has focussed on modulating radiation-induced biological effects on cancer cells. Recently, we have better understood that components within the tumour microenvironment have pivotal roles in determining treatment outcomes. In this Review, we describe vascular, stromal and immunological changes induced in the tumour microenvironment by irradiation and discuss how they may promote radioresistance and tumour recurrence. Subsequently, we highlight how this knowledge is guiding the development of new treatment paradigms in which biologically targeted agents will be combined with radiotherapy.

Introduction

Radiotherapy is used to treat up to 50% of cancer patients and to manage 40% of patients who are cured¹. For decades, research into improving outcomes from radiotherapy focussed almost entirely on the cancer cell itself, ignoring complex biological interactions between the tumour and the stroma in which it grows – the so-called tumour microenvironment (TME). As a result, classical radiobiology largely failed to appreciate that the effects of radiotherapy on the TME, and the responses that are triggered within it, may be critical in determining the success or failure of therapy. Moreover, pre-clinical studies in some tumour models have suggested that radiotherapy-induced changes in the TME might, in fact, promote tumour invasion and spread in certain situations – even though decades of clinical experience have failed to show clear proof that radiotherapy promotes invasion and metastasis in patients. Thus, attempts to combine radiotherapy with new biologically-targeted modalities were often predicated on their potential to enhance radiotherapy-induced cancer cell death, rather than their potential to re-engineer biological processes within the TME².

Over the past two decades, this narrow radiobiological view has shifted to recognise the central importance of the TME^{3–5}. The initial formulation of the hallmarks of cancer described cancers as complex tissues containing multiple cell types participating in heterotypic interactions with one another⁶. At around the same time, evidence that an irradiated stroma might favour tumour growth emerged with the observation that COMMA-D cells [G], which are cells that exhibit several characteristics of normal mammary epithelial

cells and are rarely tumorigenic, formed large tumours when implanted into pre-irradiated fat pads of syngeneic hosts⁷. Since then, a significant body of work has shown that radiation oncologists must take account of the TME, not only its ability to promote radioresistance and recurrence, but also as a legitimate therapeutic target in its own right.

Whilst a detailed explanation of the current state of understanding of the radiobiological model relating to radiotherapy has been reviewed elsewhere⁸, in this Review, we focus on mechanisms of radioresistance mediated by the tumour stroma and explore how these can be targeted to improve radiotherapy responses. We briefly discuss early and late radiotherapy-mediated effects on normal tissue, as normal tissue toxicity limits the dose of radiation that can be used in cancer treatment. With respect to tumours, we address the effects of radiotherapy on hypoxia, fibrotic responses and immune activation within the TME to understand how they may confer initial resistance or promote subsequent loco-regional or distant recurrence (Figure 1). At all stages, we will emphasise the potential for developing novel, mechanism-based, targeted therapies that will exert favourable effects on the TME.

Effects of radiotherapy on the TME

Effects on the vasculature

Possibly the best studied components of the TME with respect to radiation are endothelial cells and the tumour vasculature. Radiation induces endothelial cell dysfunction, characterised by increased permeability, detachment from the underlying basement membrane and apoptosis^{9, 10}. High single-fraction doses (8–16 Gy) have been linked to up-regulation of acid sphingomyelinase (ASMase), which induces endothelial cell apoptosis¹¹. Endothelial cell dysfunction and apoptosis contribute to post-irradiation inflammation and fibrosis. Within vessels, irradiation generates a pro-thrombotic state characterised by platelet aggregation, microthrombus formation and increased adhesion of inflammatory cells to endothelial cells with subsequent diapedesis into the perivascular space¹².

Structurally, irradiation of the vasculature causes dose-dependent destruction of blood vessels, especially affecting microvasculature¹³. This reduced vascular density increases the distances between functioning vessels, meaning that some parenchymal tissues are not perfused. Over time irradiated blood vessels become thicker — primarily due to thickening of the intimal layer — and are prone to atherosclerosis [G]^{14–17}. Other late morphological changes can occur including development of thrombosis, fibrosis, and medial necrosis [G]. Larger single-fraction radiation doses (15–20 Gy) may permanently reduce blood flow, suggesting irreversible changes to the vascular architecture¹⁸. Early and late responses of normal tissue to irradiation are dose-limiting factors in radiotherapy, affecting therapeutic efficacy as well as the quality of life of cancer patients (Figure 2).

Understanding the effect of radiation on the functional status of the tumour microvasculature is important for maximising the efficacy of radiotherapy. Radiotherapy-induced changes in the tumour vasculature remain dependent on total dose and fraction size, as well as the type, location and stage of the tumour itself¹⁹. Depending on how they are derived (i.e. through angiogenesis, vasculogenesis or vessel co-option [G]), tumour vessels may lack basement membrane and pericyte coverage rendering them more permeable and leaky and more

radiosensitive than vessels in the surrounding normal tissue²⁰. Endothelial cells within the TME are characterised by rapid proliferation rates, contributing to their inherent radiosensitivity. Each endothelial cell supports the growth of around 2,000 cancer cells²¹, and their response to radiotherapy — including mechanisms of recovery and survival — have been the focus of research since they were first postulated as a critical target in tumour control. Similar to high dose irradiation of normal tissues¹¹, single, large radiation doses have been shown to induce ASMAse-dependent microvascular damage in fibrosarcoma and melanoma xenograft models in the first 6 hours with subsequent tumour cell death²².

Radiation-induced vascular damage potentiates tumour hypoxia and triggers immune responses through increased production of cytokines/chemokines that induce immune cell recruitment. Subsequent tumour revascularisation occurs via hypoxia-inducible factor-1 α (HIF-1 α)-dependent and -independent recruitment of bone marrow-derived cells (BMDCs)^{23–27}.

Effects on the stroma

Chronic inflammation is the main driver of fibrosis, in which persistent immune responses occur alongside tissue remodelling and repair processes. Within normal tissues, infiltration of inflammatory cells triggers myofibroblast transformation, resulting in sustained production of growth factors (such as transforming growth factor β (TGF- β) and connective tissue growth factor (CTGF)^{28, 29}), proteolytic enzymes and angiogenic and/or fibrogenic cytokines that ultimately leads to excess deposition of components of the extracellular matrix (ECM) and scarring of the involved tissues³⁰. The inflammatory processes that arise immediately after radiotherapy are not the same as those that appear decades later (Figure 2). Although the triggers in wound healing, normal tissue fibrosis and radiation fibrosis differ, the pathophysiological processes that ensue show some common features that implicate the immune system, the activation of stromal cells (such as myofibroblasts) and self-propagating signalling networks that result in a progressive phenotype³¹ (Box 1). As with radiotherapy-induced microvascular injury [G], prevention of fibroproliferative processes is important for maintaining normal organ function and, thus, improving quality of life following radiotherapy.

Cancer-associated fibroblasts (CAFs) are a heterogeneous cell population that comprise the majority of the cells within the stroma in many carcinomas. CAFs often have a spindle-shaped, mesenchymal morphology and are classically identified as either fibroblast activation protein (FAP) and fibroblast specific protein (FSP)-positive fibroblasts or α -smooth muscle actin (sma)-positive myofibroblasts, depending on their origin³². They secrete ECM proteins (such as tenascin C (TN-C) and collagen I), cytokines (e.g. hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and chemokine (C-X-C motif) ligand 12 (CXCL12)), and matrix remodelling enzymes (such as matrix metalloproteinases (MMPs))^{33, 34}. Unlike myofibroblasts that arise in response to inflammation or wound healing, CAFs may be resistant to apoptosis and irreversibly activated³⁵. The heterogeneous nature of CAFs varies according to tumour type and stage of disease progression, and may also determine whether they exhibit tumour-promoting or inhibiting roles³⁶. TGF- β is a potent activator of fibroblasts and fibrocytes^{37–39} and is also a key CAF-secreted factor,

along with CXCL12, that participates in autocrine signalling loops during tumour progression⁴⁰. As well as cytokine-mediated activation, CAFs can obtain an activated phenotype through interaction with other cells in the TME, such as leukocytes and macrophages⁴¹.

Therapeutic irradiation of tumours will affect CAFs, but few detailed studies have been performed. Moreover, very few studies have used CAFs freshly isolated from human tumours. Hellevik *et al.* demonstrated that CAFs isolated from non-small cell lung cancer (NSCLC) samples survived ablative radiation but lost their tumour-invasive capability⁴². They proposed that radiation impeded CAF mobility by stabilising focal contacts through increased integrin ($\alpha 2$, $\beta 1$ and $\alpha 5$) expression; formation of integrin-mediated attachments may facilitate CAF survival. Indeed, a number of preclinical studies have implicated $\beta 1$ integrin in tumour survival following radiotherapy^{43–45}. More recently, it was proposed that $\beta 1$ integrin signalling in pancreatic cancer cells is required for stromal-mediated radioprotection⁴⁶. Malignancies with a pronounced desmoplastic reaction [G] may therefore be inherently radioresistant due, in part, to integrin-mediated interactions between cancer cells, stromal cells and the surrounding ECM. In addition, integrin $\alpha 11\beta 1$ has been shown to induce myofibroblast activation⁴⁷ and integrin $\alpha V\beta 6$ was reported to be upregulated in irradiated lungs immediately prior to the onset of fibrosis⁴⁸, suggesting that integrins may also be involved in the development of radiation fibrosis.

Effects on the immune system

Tumours evolve to evade immune recognition⁴⁹, such that immune escape is now considered a ‘hallmark of cancer’⁵⁰. This, combined with other intrinsic and extrinsic survival-pressures such as resistance to hypoxia and rapid growth, likely results in the observed state of chronic inflammation^{51–53} and adaptive immunity suppression. This modulation is maintained by altered cytokine signalling (tumour necrosis alpha (TNF- α), interleukin (IL)-1 β , IL-6, IL-10 and TGF- β ^{54, 55}) and the recruitment of locally suppressive immune cells (TAMs⁵⁶, myeloid derived suppressor cells (MDSCs) and T_{regs}[G]). After radiotherapy, a relative increase in these locally suppressive cell types, which are less radiosensitive than other lymphocyte subsets^{57, 58}, is counterbalanced by recruitment of circulating immune cells and increased antigen exposure and presentation. These changes profoundly affect the immune compartment of the TME, potentially re-priming it for an adaptive immune response.

After radiotherapy, inflammatory signals occur through activation of cell survival pathways and stimulation of the innate immune system [G]. Both direct and indirect radiation effects [G] trigger inflammatory cytokine signalling (IL-1, TNF- α) and immune cell recruitment (for example, by endothelial cell expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin). These events are principally co-ordinated by the intracellular actions of reactive oxygen species (ROS) and NF- κ B⁵⁹. ROS also affect TNF signalling pathways⁶⁰ and can activate NF- κ B, leading to further TNF- α production. The resulting cellular stress and death may also stimulate an immune response through the generation of damage-associated molecular patterns (DAMPs) [G] and their corresponding pattern recognition receptors (PRRs)⁵⁹.

The PRR family includes Toll-like receptors (TLRs), nucleotide binding oligomerisation domain-like receptors (NLRs), RIG-I-like receptors and C-type lectin receptors⁶¹. The recognition of pathogen-associated molecular patterns (PAMPs) [G] links stimuli from pathogenic sources to an inflammatory response. There is an analogous pathway that responds to endogenous damage via DAMPs, for example after radiotherapy⁶². DAMPs can be subdivided into three groups: those exposed on the surface (e.g. calreticulin); passively released substances (e.g. high-mobility-group box 1 (HMGB1) proteins); and those actively secreted (e.g. adenosine triphosphate (ATP))⁶³. After tumour irradiation, a DAMP-PRR-mediated damage response may lead to immunogenic cell death (ICD) [G] of cancer cells, changing the predominant TME cytokine profile towards an immunostimulatory one⁶³, activating DCs and maturing them into effective antigen presenting cells (APCs)⁶⁴. ICD is recognised as important for triggering an effective anti-tumour immune response (Figure 3). However, the overall effects of radiotherapy-induced ICD responses are complex. Checkpoint inhibition by T_{regs}, whose expression of CTLA-4⁶⁵ affects the activation of CD8+ T-cells (Figure 3B), may still prevent the development of an effective adaptive immunity [G] to tumour cells. On the other hand, STAT-1, downstream of IFN- β signalling, drives chemokine expression (notably CXCL9 and CXCL10) which recruits CXCR3-positive T-cells⁶⁶. Radiotherapy-induced CXCL16 expression in the vasculature of the TME also recruits CXCR6-positive activated CD8+ T-cells which have displayed anti-tumour effects in a murine breast cancer model⁶⁷. Radiotherapy-induced effects on the TME therefore also lead to enhanced immunostimulatory T-cell recruitment^{68, 69}.

T-cell activation requires several signals: antigen in an appropriate major histocompatibility complex (MHC) binding to a T-cell receptor (TCR); co-stimulatory signals (e.g. from an APC); and background levels of cytokine stimulation. Negative feedback from up-regulation of CTLA-4 helps control this process by competitively inhibiting co-stimulatory signals from CD80 (also known as B7-1) and CD86 (B7-2) expressed on APCs to CD28 on T-cells⁶⁵. Thus, radiotherapy acts to prime the immune system against cancer cells via ICD, but potentially remains constrained by the enhanced actions of suppressive immune cells. Overcoming these effects to harness the potential of the immune system against cancer cells can generate beneficial local and abscopal effects [G]. Unlocking abscopal effects in particular is the subject of a plethora of active research after case reports suggested that localised RT combined with immunomodulation may unlock the anti-metastatic potential of the adaptive immune system^{70–75}.

In summary, complex reactions by the immune system to an irradiated TME are neither wholly immunostimulatory nor immunosuppressive. They comprise effects on cells intrinsic to the TME such as altered production of inflammatory cytokines, antigen exposure and DC priming as well as relative increases in radioresistant immunosuppressive macrophage and T-cell populations. Multifactorial signalling from both tumour ICD and radiotherapy-induced changes to endothelial cells also lead to an influx of circulating immune cells which are more immunostimulatory than those inherent to the TME. Therefore, radiotherapy is not a perfect mechanism for triggering ICD of cancer cells with subsequent effective adaptive responses. However, key events, such as the change in immunostimulatory cytokines, DC maturation/activation and T-cell recruitment and stimulation, mean that it does have the

potential to prime potent responses against tumour cells if important immunosuppressive effects can be overcome.

Targeting TME-mediated radioresistance

Many strategies have been proposed for overcoming radioresistance in tumour cells², but too little attention has been given to TME-mediated resistance mechanisms and how they might be circumvented. There are currently drugs which target various aspects of the TME being trialed as monotherapies or in combination with radiotherapy to treat cancer. For example, many drugs have shown efficacy against fibrosis but have so far not been tested for their ability to reduce radiotherapy-mediated late adverse effects in the TME or prevent tumour recurrence and metastasis. We hypothesise a number of these will improve the prognosis of patients after radiotherapy and also propose a number of immune and vascular targets that may show efficacy in radiosensitising the TME in future studies (Table 1).

Targeting hypoxia

Hypoxia is a key regulatory factor in tumour growth⁷⁶ and has long been known to play a crucial role in radiotherapy resistance⁷⁷ (Box 2). Most solid tumours contain zones where oxygen tensions fluctuate between almost anoxic (0% oxygen), hypoxic (1%/7.5 mmHg oxygen) and normoxic (8%/60 mmHg oxygen)⁷⁸. High tumour cell proliferation rates and the abnormal structure of tumour vasculature lead to both chronic, diffusion-limited hypoxia and acute, transient, perfusion-limited hypoxia^{79, 80}. Furthermore, changes in the TME as a result of radiotherapy can result in regions of cycling hypoxia⁸¹. Following radiotherapy, lack of oxygen reduces the production of reactive and cytotoxic species, such as ROS, and ultimately prevents irreparable DNA damage from occurring in cancer cells, preventing cancer cell death. Hypoxia also upregulates HIF-1 α which independently promotes radioresistance (see below)⁸². Radioresistance manifests in oxygen tensions <10 mmHg and becomes maximal around 0.5 mmHg. Brizel *et al.* reported post-radiotherapy disease-free survivals of 78% in patients whose tumours had median oxygen tensions of >10 versus 22% in patients with median oxygen tensions of <10 mmHg⁸³.

Therapeutic attempts to restore tumour oxygenation before or during radiotherapy have included red blood cell transfusion, erythropoietin administration, and hyperbaric oxygen (HBO) treatment⁸⁴. Despite HBO treatment having a therapeutic benefit in patients with head and neck squamous cell carcinoma (HNSCC)⁷⁷, inconsistent clinical trial data for other cancer types and huge logistical hurdles have impeded widespread uptake of this approach. Accelerated radiotherapy with carbogen and nicotinamide (ARCON) has since been developed to overcome tumour hypoxia and is currently being evaluated in Phase III clinical trials^{85–87}. As yet, despite promising pre-clinical studies, clinical trials have ended with conflicting and inconclusive results with respect to improving local tumour control. Similarly, clinical trials of early nitroimidazole derivatives which act as oxygen mimetics in the radiochemical process have been inconsistent and severe dose-limiting toxic effects have restricted their development^{88–90}. However, water-soluble compounds with improved toxicity profiles, such as doranidazole and nimorazole, have yielded survival benefits for NSCLC patients when used in combination with chemoradiotherapy in Phase I/II trials⁹¹

and with radiotherapy in supraglottic and pharyngeal tumours in a Phase III study⁹². Nimorazole in combination with radiotherapy is a standard-of-care treatment in Denmark and a phase III trial assessing this combination is currently recruiting in the UK⁹³. Finally, a recent development in nanotechnology allows oxygen to be generated in hypoxic tumour regions by intratumoural injection of bioreactive albumin-MnO₂ nanoparticles⁹⁴. This therapy has been shown to improve tumour response to radiotherapy in preclinical studies⁹⁴.

Irradiated tumours have elevated expression of HIF-1 (Box 2)⁹⁵ and high tumour HIF-1 activity is an independent predictor of poor prognosis after radiotherapy^{96, 97}. Furthermore, markers of both HIF-1 and HIF-2 hypoxia-response pathways are strongly associated with radiotherapy failure in head and neck cancer⁹⁸. A number of drugs have been designed to inhibit HIF-1 activity and may affect radiosensitivity. Acriflavine directly binds the HIF-1 α subunit, thereby inhibiting HIF-1 dimerisation and transcriptional activity⁹⁹. Acriflavine has been shown in preclinical studies to downregulate mRNA expression of *VEGF*, *CXCL12* and stromal cell factor (*SCF*) and reduce tumour vasculature⁹⁹. Another HIF-1 inhibitor, YC-1, improved treatment outcome when administered after radiation¹⁰⁰. Stabilisation of the HIF-1 α subunit can also occur through increased levels of TGF- β in the TME. TGF- β has an important role in maintaining normal vascular homeostasis and has been shown to stimulate angiogenesis in irradiated environments¹⁰¹. However, as TGF- β can promote both pro- and anti-angiogenic effects¹⁰², strategies that target TGF- β directly are likely to be challenging.

As previously discussed, tumours contain abnormal vessels that contribute to tumour hypoxia. Vessel normalisation to increase radiotherapy response and reduce tumour recurrence and metastasis is currently being investigated¹⁰³ (Figure 4). VEGF-A is the principal angiogenic molecule implicated in development of abnormal vasculature in the TME¹⁰⁴. Pharmacological inhibition of VEGF has been achieved with an anti-VEGF antibody (bevacizumab), a VEGF-trap molecule (aflibercept, which targets all VEGF molecules and placental growth factor, PlGF), a VEGF receptor (VEGFR)-inhibiting antibody (DC101), and receptor tyrosine kinase inhibitors (TKIs, sunitinib, sorafenib, pazopanib)¹⁰⁵. Although disappointing results have been observed for anti-VEGF monotherapy, treated tumours were sensitised to chemotherapy and radiotherapy due to better drug delivery and reduced hypoxia, respectively¹⁰⁶. TKIs have also been shown to contribute to vessel normalisation in many preclinical models and synergise with radiation in orthotopic models of GBM, melanoma, and colon, lung, and head and neck cancer⁸⁴. Anti-VEGF effects are somewhat transient, however, and targeting the downstream molecules PI3K and mTOR (using NVP-BEZ235 and NVP-BGT226) in combination with radiotherapy could prove to be more efficacious through durable vessel normalisation¹⁰⁷. Vessel normalisation occurs during a specific time-window following different anti-angiogenic treatments and this translates into optimal tumour growth delay when radiotherapy is scheduled accordingly^{108, 109}. Similarly, the dose of anti-angiogenic compounds used in combination therapy needs to be carefully considered. Carmeliet and Jain proposed that higher doses of anti-angiogenic therapy could be harmful as a result of normal tissue toxicity and complete vessel destruction, which may paradoxically increase tumour hypoxia¹¹⁰.

Two endogenous angiogenesis inhibitors, angiostatin and endostatin have synergistic anti-tumour effects in combination with radiotherapy⁸⁴. Likewise, TNP-470, the synthetic analogue of a naturally occurring antibiotic, arrests endothelial cell growth and improves tumour oxygenation and response to radiotherapy^{111–113}. Angiostatin and TNP-470 are no longer in clinical trials, due to poor toxicity profiles, lack of efficacy, and the development of more effective anti-angiogenic drugs. Endostar, however, a more potent and stable version of endostatin¹¹⁴, improves radiation responses in preclinical cancer models through inhibition of VEGF, TGF- β , HIF-1 α and bFGF^{115, 116} and is currently being combined with radiotherapy in Phase II clinical trials.

Blocking vasculogenesis may also improve radiotherapy-mediated local tumour control and prevent recurrence¹¹⁷ (Figure 4). In their preclinical study, Kioi *et al.* abrogated BMDC-mediated tumour vasculogenesis through inhibition of HIF-1 activity using NSC-3475425. Likewise, administration of AMD3100, which inhibits the interaction between CXCL12 and CXCR4, prevented blood flow returning to irradiated tumours and completely inhibited recurrence for up to 100 days. A similar effect was observed with a CXCR4 neutralising antibody. Another preclinical study has implicated Angiopoietin 2 (ANG-2; a molecule proposed to compensate for VEGF inhibition by recruiting perivascular BMDCs to support the weak tumour vasculature) in tumour vasculogenesis. Burrell *et al.* demonstrated that treatment with an inhibitor of both ANG-1 and ANG-2, AMG386, prevented the influx of perivascular BMDCs to the central vasculature and it was hypothesised that ANG-2 inhibition may overcome resistance to anti-VEGF therapies and prevent tumour recurrence following RT¹¹⁸.

Finally, as with CAF-associated integrins, the role of integrins in mediating cell-ECM attachment is important for endothelial cell survival and migration. Early studies revealed that radiotherapy up-regulated integrin $\alpha v \beta 3$ expression in endothelial cells and concurrent treatment with an $\alpha v \beta 3$ antagonist could enhance radiotherapy-mediated anti-angiogenic and anti-tumour responses in xenograft models¹¹⁹. Integrin inhibitors have been developed (cilengitide against $\alpha v \beta 3 / \alpha v \beta 5$, vitaxin against $\alpha v \beta 3$, and volociximab against $\alpha 5 \beta 1$) and shown efficacy alone or in combination with radiotherapy in preclinical and clinical studies. Cilengitide showed encouraging activity in a phase II trial¹²⁰, but has not improved outcomes for patients with GBM in a phase III trial with chemoradiotherapy¹²¹. Nevertheless, further trials are ongoing (Supplementary Table). Likewise, volociximab and vitaxin have anti-angiogenic activity but data on their radiosensitising capabilities are lacking^{122–124}.

In conclusion, direct and indirect therapeutics to reduce tumour hypoxia have been developed and show preclinical promise in combination with radiotherapy. However, changes in the TME during therapy and complex issues relating to drug dose and schedule mean that we do not yet know how best to employ these agents for clinical benefit.

Targeting the stroma

Inhibition of RT-induced inflammatory responses may reduce long-term fibrotic processes that could be important if complete eradication of local tumour cells is not achieved. Up-regulation of pro-inflammatory NF- κ B post-irradiation is often associated with increased

IL-1 β , IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and COX-2 in the TME¹²⁵. Therefore, developing inhibitors that interfere with this inflammatory network has been a focus of radiosensitisation approaches. Many natural and synthetic anti-inflammatory compounds have proven efficacious in tumour radiosensitisation¹²⁵. However, their effects have been mediated through anti-angiogenic, anti-tumour cell survival, or decreased BMDC mobilisation mechanisms rather than through inhibition of fibrotic processes. It must also be borne in mind that attempts to reduce inflammation as a means of preventing tumour-promoting fibrotic reactions may have deleterious knock-on consequences for the beneficial effects of inflammation on anti-tumour immunity, which will be discussed later.

TGF- β signalling is augmented by radiotherapy¹²⁶ and is a key inducer of stromal activation in the TME. Within the context of the post-radiotherapy TME, direct modulation of TGF- β is complex. Although it has been shown to directly mediate tumour suppression in irradiated environments¹²⁷, inhibition of TGF- β in conjunction with radiotherapy has also delayed tumour growth and prevented radiotherapy-mediated increases in circulating levels of TGF- β , thereby reducing cancer metastasis in preclinical models^{128, 129}. Although these studies mainly focussed on TGF- β -mediated effects on the tumour cells, the increased levels of circulating TGF- β observed following irradiation may be a consequence of CAF activation and could also influence tumour progression through further modulation of CAF activity. So far, TGF- β inhibition, in isolation, has not proven effective in the clinic¹³⁰. The broad and often opposing activities of TGF- β can pose problems in targeting strategies and more targeted inhibition of downstream molecules may be more beneficial. Indeed, preclinical studies of a TGF- β R1 inhibitor, SD-208, showed reduced tumour growth and decreased fibrosis in the TME¹³¹. There is, therefore, a rationale for combining it with radiotherapy in order to prevent radiation-induced activation of CAFs.

Molecules secreted by CAFs, such as HGF and TN-C, could be promising selective targets in the tumour stroma to negate radiotherapy-induced fibrotic phenotypes that may facilitate radioresistance (Figure 4). Monoclonal antibodies against HGF and MET (also known as hepatocyte growth factor receptor, HGFR) and MET small molecule inhibitors have shown efficacy in clinical trials¹³². Preclinical studies have demonstrated that MET inhibition enhances radiotherapy responses and prevents tumour cell invasion mediated by CAF-derived HGF secretion following irradiation^{133, 134}. However, HGF and MET inhibitors have yet to be combined with radiotherapy in the clinic. Alternatively, high stromal expression of TN-C, as a result of CAF activation, can be targeted with the ¹³¹I-labelled anti-TN-C antibodies 81C6 and F16SIP. A phase II clinical trial demonstrated some success for combining 81C6 with radiotherapy^{135, 136} and a phase III trial has since been proposed¹³⁷.

A number of agents with anti-fibrotic actions may impact on radiotherapy-mediated inflammation and fibrosis in the TME. Imatinib, nilotinib and dasatinib inhibit TGF- β and PDGF signalling and, hence, collagen synthesis¹³⁸. They are currently being evaluated in clinical trials for the treatment of fibrosis. Other inhibitors affecting growth factor signalling include BIBF1000 and BIBF1120, which target PDGFR, VEGFR and bFGFR. These molecules have some efficacy in treating idiopathic pulmonary fibrosis^{139, 140} and may

modulate post-irradiation changes in the TME. Hedgehog (Hh) signalling also regulates fibrosis¹⁴¹ and has been implicated in the desmoplastic reaction in pancreatic ductal adenocarcinoma¹⁴². Attempts to target this pathway have led to the development of inhibitors (vismodegib, saridegib, sonidegib) of the downstream molecule smoothed (SMO). Monotherapy preclinical and clinical trials had encouraging outcomes^{143–145}, and results of early studies combining vismodegib with radiotherapy¹⁴⁶ are also promising. Phase II clinical trials of radiotherapy plus Hh inhibition are ongoing (Supplementary Table).

Targeting growth factors involved in the activated networks in the TME has led to the development of suramin, which inhibits PDGF, EGF, TGF- β , FGF-2 and IGF signalling¹⁴⁷. Suramin has exhibited anti-proliferative effects against many advanced cancers¹⁴⁸. Although suramin did not improve survival when combined with cranial radiotherapy in patients with glioblastoma¹⁴⁹, the combination is yet to be tested in cancers in which an activated stroma is common, such as breast and pancreatic cancer. Interestingly, suramin was recently shown to inhibit heparanase enzymes, thereby reducing tissue damage and fibrosis in a rat model of hepatocellular carcinoma¹⁵⁰. Heparanase is involved in ECM breakdown and regulates the bioavailability of growth factors and cytokines in the TME¹⁵¹. Most importantly, heparanase has been shown to augment radiation-induced cancer cell invasion¹⁵². An inhibitor of heparanase, SST0001, has shown potential in preclinical studies where it reduced the spread of pancreatic cancers in combination with radiotherapy¹⁵². Other heparanase inhibitors, PG545, M402 and PI-88, have also displayed anti-tumour activity in murine models and have entered clinical trials^{153–155}. The activity of heparanase is also regulated indirectly by MMPs¹⁵¹. Many MMP inhibitors, including marimastat, prinomastat, batimastat and tanomastat, have been evaluated in clinical trials for the treatment of various cancers, mostly with disappointing results¹⁵⁶. The broad-spectrum activity of MMP inhibitors may explain their failure in monotherapy clinical trials. Preclinical studies with more specific MMP inhibition have demonstrated some improvement in the efficacy of radiotherapy (e.g. MMP-2 inhibition has been shown to abrogate radiotherapy-induced tumour invasion and progression^{157–160}), indicating further research is required.

CAFs are generally genetically more stable than tumour cells, making them good therapeutic targets. However, they display functional duality, making the balance between tumour inhibition and promotion very delicate. For example, a recent report by Özdemir and colleagues demonstrated that CAF depletion in a mouse model of pancreatic cancer may actually accelerate cancer progression¹⁶¹. This suggests that care needs to be taken when targeting CAFs specifically and blocking downstream mediators of CAF activation may prove to be more efficacious. Whether desmoplasia provides a growth disadvantage for cancer cells or promotes their dissemination is also still under debate. Nevertheless, promising results from trials involving inhibitors of ECM turnover suggest agents developed for the treatment of fibrotic diseases may enhance radiotherapy-mediated tumour growth delay and reduce subsequent TME changes that could facilitate tumour cell migration and cancer spread.

Immune modulation

Much of the current interest in combining immunomodulation and radiotherapy lies in strategies to overcome the persistent suppression of adaptive immune responses by the tumour and its microenvironment. Of particular interest is the promise of the abscopal effect, whereby localised radiotherapy acts as the catalyst for systemic anti-tumour immunoreactivity^{74, 75}.

Whilst radiotherapy does lead to ICD, some tumours exhibit accelerated tumour repopulation by paracrine caspase 3-dependent PGE₂-mediated signalling¹⁶². Signalling from surviving and recruited MDSCs and TAMS also suppress T-cell function through antagonistic cytokine signals, such as IL-6, IL-10 and TGF- β ¹⁶³. Their relevance is highlighted in a phase I/II clinical trial combining radiotherapy and a primed DC vaccine against high-risk soft-tissue sarcoma. A 52.9% rate of tumour-specific immune responses was reported, but non-responders had significantly higher levels of tumour MDSCs prior to immunisation¹⁶⁴.

Manipulation of the TLR response can increase activation of DCs, triggering an effective adaptive immune response after radiotherapy¹⁶⁵. Direct TLR manipulation is currently being trialled using the TLR7 agonist, imiquimod, which is approved for basal cell skin carcinomas and melanomas. Imiquimod is being used in conjunction with local radiotherapy as a topical treatment against breast cancer metastasis with promising preclinical data¹⁶⁶. Using cancer vaccines comprising TLR agonists with tumour antigens to stimulate DCs and T-cell responses has been effective in preclinical models and has been translated clinically¹⁶⁷. GM-CSF has also been used in the clinic, combined with radiotherapy, to treat patients with metastatic disease across multiple cancer types (lung, thyroid, eccrine [G], cervical and breast cancer)¹⁶⁸. In that study, patients treated with chemotherapy received radiotherapy (35 Gy in 10 fractions) delivered to a metastatic site followed by daily subcutaneous injections of GM-CSF for 14 days after the first week of radiotherapy. A systemic effect was seen in 27% of patients, with a corresponding improvement in survival in those who experienced abscopal effects (median overall survival of 25 months versus 10 months).

Effective T-cell activation requires both antigen presentation and co-stimulation from an appropriate APC. Several of the steps that regulate this process, including CTLA-4, OX40, PD-1 and CD137, are likely to represent important targets for immunomodulation after radiotherapy-induced immune priming. Ipilimumab (anti-CTLA-4 monoclonal antibody) is approved in advanced-stage melanoma, having improved survival in phase III trials^{169, 170}. In murine studies, unilateral radiotherapy and systemic anti-CTLA-4 blockade delayed the growth of a contralateral, unirradiated tumour¹⁷¹. Interestingly, this abscopal response was dependent on radiation dose-fractionation with 24 Gy delivered in 3 fractions appearing to be optimal¹⁷¹. This suggests there may be preferred fractionation schemes for effective immune priming, although the evidence for this is limited at the moment (see also table 2) and may well differ according to therapeutic agent and tumour histology. Scheduling of immunological therapy relative to radiotherapy is also likely to be important, with an initial period of tolerance seen after radiotherapy (Table 2). Indeed, there is some early evidence that an interval of around 1 month between radiotherapy and immunomodulation may be

optimal for synergy (Table 2). Anecdotal clinical reports of radiation-induced abscopal responses have been published and results from ongoing clinical trials are eagerly awaited (Supplementary Table).

OX40 signalling co-stimulates activation of several T-cell subtypes (e.g. CD4+ and CD8+). It mediates positive feedback, potentiating activation and, thus, polarising the T-cell response. In combination with radiotherapy, where CD8+ priming/activation already occurs, activation of OX40 signalling might be particularly effective. In a murine model, OX40 agonistic antibodies improved local control after surgery or radiotherapy¹⁷². It is also thought that OX40 can help prime low affinity T-cells and reverse T-cell tolerance. As such, activating OX40 signalling might work synergistically with CTLA-4 blockade to activate T-cells post-radiotherapy¹⁷³. T-cell co-stimulating proteins, such as CD137 (4-1BB)¹⁷⁴, also represent promising targets.

Induced CD-8+ T-cell exhaustion is a radioresistance mechanism (Figure 4). This has been characterised in a murine model of lymphocytic chorio-meningitis virus-13 (LCMV-13) infection in comparison to normal, uninfected T-cells¹⁷⁵. Similarities between virally-induced T-cell exhaustion and ineffective T-cells in melanoma have been studied¹⁷⁶. Markedly different gene expression was seen in cancer-infiltrating CD8+ T-cells and those in the circulation, with patterns similar to the exhaustion profiling seen in the LCMV-13 murine model. Modulation of these exhaustion pathways by targeting programmed death-1 (PD-1), T-cell immunoglobulin mucin-3 (TIM3)¹⁷⁷, or lymphocyte activation gene-3 (LAG3)¹⁷⁸ expression represents an exciting area for combination with radiotherapy. Thus far, of these exhaustion pathways, the PD-1 receptor ligand PD-L1/PD-1 axis has been the best studied in the context of radiotherapy and immune-modulation. Expression of PD-1 on T-cells in the TME is suggestive of their exhaustion and results in ineffective T-cell-mediated immune responses. Upregulation of PD-L1 on tumour cells after irradiation¹⁷⁹ also represents a mechanism of radioresistance. Blockade of the PD-L1/PD-1 axis in combination with radiotherapy is effective at the primary tumour site and in generating abscopal responses in AT-3 mammary tumours¹⁸⁰. Early clinical results using this strategy against incurable metastatic breast cancer also look promising¹⁸¹.

Oncolytic viruses preferentially infect and replicate in tumour cells, leading to cell death¹⁸². They are also recognised as potent immune therapeutics¹⁸³. When combined with radiotherapy, they have demonstrated synergy in preclinical models^{184, 185}. In early clinical trials of radiotherapy and oncolytic virus combinations, we reported a tolerable toxicity profile that does not overlap with radiotherapy-induced side-effects¹⁸⁶. In the TME, oncolytic virus infection triggers a PAMP/DAMP-related PRR signal^{162, 187} and could potentially play a role in ICD, immunomodulation and T-cell activation. This viral response may represent another method of overcoming the TME's suppression of adaptive immune responses.

Viruses can also be used to deliver therapeutic genes that exert immunomodulatory effects. Examples include the over-expression of chemokines (CCL3 and CCL5), immune cytokines (GM-CSF, IL-2, IL-4, IL-12) and T-cell co-stimulators (CD40L, CD80, 4-1BBL). Talimogene laherparepvec (T-Vec) is a replication-competent GM-CSF-expressing herpes

simplex virus that has been tested in a phase I clinical trial in combination with chemoradiotherapy in head and neck cancer^{186, 188}. The combination of viral oncolysis, immune stimulation and immunomodulation may represent a powerful approach that is worthy of further evaluation.

Conclusions

Radiotherapy leads to the activation of an interconnected series of processes in the TME: inflammation, cycling hypoxia, immunomodulation, revascularisation, CAF co-ordinated ECM remodelling, and fibrosis. These changes occur in the TME in an interconnected fashion (Figure 5). This is highlighted by the overlapping and widespread effects of several factors - especially growth factors TGF- β , VEGF and PDGF; cytokines TNF- α , CXCL12, IL-1, IL-2, IL-6 and IL-10; and transcription factors NF- κ B and HIF-1 (Figure 1).

Against this background, emerging therapeutic strategies have aimed to tip the balance of the TME-radiotherapy response towards more effective cancer therapy, whilst minimising adverse effects (Figure 4). Hypoxia directly affects initial radiosensitivity, immune responses and promotes the development of neovasculature. It also stimulates ongoing CAF activity. Strategies currently under development to increase tumour oxygenation, for example by modulating oxygen availability or by normalising tumour vasculature, demonstrate great potential. Furthermore, targeting HIF-1 could have TME-wide benefits, particularly through its modulation of TGF- β signalling. Related strategies to prevent BMDC-mediated tumour revascularisation by using CXCL12 inhibitors also appear to be very promising and are now in early clinical trials (Supplementary Table). Integrins, central to the survival and repopulation of both ECs and CAFs after radiotherapy, are also important emerging targets (Table 1).

Targeting drivers of the initial inflammatory response, such as NF- κ B, COX-2 and TNF- α , have been shown to decrease radiotherapy-mediated side-effects in surrounding normal tissues without improving tumour responses. Moreover, targeting inflammation may counteract the anti-tumour benefits of immunomodulation. Therefore, in this regard, emphasis should be placed on reducing late radiation effects on ECM structure rather than the initial inflammatory response. Abrogating excess ECM production by specifically targeting CAFs with anti-FAP antibodies has, so far, not shown efficacy in cancer trials. Indeed, depleting the CAF population may prove detrimental in some tumour types. It may be more beneficial to prevent ECM remodelling events in the TME, either through prevention of ECM cross-linking or breakdown. Many molecules impeding ECM remodelling, such as inhibitors of growth factors, their receptor kinases, hedgehog signalling and matrix enzymes, are currently being used to treat fibrotic disorders and may show efficacy in preventing the development of a radiotherapy-mediated invasive environment (Table 1). Possibly the most exciting development in modulation of the tumour ECM is the generation of heparanase-targeted therapies which aim to prevent tumour cell dissemination by modulating ECM remodelling and reducing availability of growth factors and cytokines in the TME.

With respect to immune modulation, the most promising areas of development are in generating an effective adaptive response both to treat cancer locally and abscopally. The strategies involved can be summarised as: increasing ICD, and both numbers and activation levels of DCs, using a combination of cytokines, growth factors and TLR stimuli; appropriate recruitment, activation of T-cells with primed DCs; and the abrogation of inhibitory signalling (CTLA-4) and T-cell exhaustion (PD-L1/PD-1). A number of these approaches are now in clinical development both as single-agents and combined with pre-existing chemotherapy and other TME modulators (Supplementary Table). New strategies are also emerging rapidly and warrant investigation (Table 1): chemokine recruitment of anti-tumour T-cell subgroups (for example using CXCL16), targeting T-cell exhaustion markers (such as TIM3 and LAG3), and oncolytic immunotherapies (including gene therapy to deliver immunomodulation).

Investigation of the TME-radiotherapy response has led to the development of an exciting array of novel treatments. Given the timing and interplay of events in the response of the TME to radiotherapy, it will be a significant challenge to develop rational radiotherapy-drug combinations in this field. Nonetheless, we believe that carefully designed combinations of radiotherapy with vascular normalisation, immunomodulation and anti-fibrotic therapy will prove effective in maximising therapeutic benefits and minimising both unwanted off-target effects and the possibility of metastasis and recurrence. While many questions remain, we believe that the door is beginning to open on the synergistic potential that will be the future of cancer therapy.

Supplementary Materials

Refer to Web version on PubMed Central for supplementary material.

Biographies

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Glossary

Adaptive immunity

a carefully regulated, adaptable, specific immune response comprising humoral- and cell-mediated components. It is capable of both systemic actions and immunological memory to specific stimuli. It is triggered by known antigens or by appropriate antigen presentation from the innate immune system.

Atherosclerosis

thickening of the artery wall as a result of white blood cell invasion and accumulation.

Abscopal effects

irradiation of tumour cells or the adjacent extracellular matrix (ECM) can induce biologically relevant changes in distant cells, which may or may not have been irradiated themselves. These are distinguished from bystander (which refers to changes affecting nearby unirradiated cells) and cohort effects (which refer to changes affecting off-target irradiated cells).

COMMA-D cells

an epithelial cell line derived from the mammary tissue of a mid-pregnant BALB/c mouse, which exhibits many characteristics distinctive of normal mammary epithelial cells.

Damage-associated molecular patterns (DAMPs)

stimuli released by stressed, dying or injured cells that may trigger an inflammatory response by activation of a number of pattern recognition receptors (PRRs).

Desmoplastic reaction

a stromal reaction that can be induced by tissue injury, wound repair or cancer growth. Increased ECM and growth factor production and secretion result in the formation of scar-like fibrotic tissue.

Direct and Indirect effects

radiation damage can be divided into direct effects, where the damage is a result of the ionising radiation itself, and indirect effects, which refer to the resultant changes in cellular pathways as a result of radiation for example as a result of ROS.

Eccrine (tumour)

tumour type from secretory sweat glands.

Immune tolerisation

The recognition of “self” and “non-self” during antigen presentation is important and carefully regulated to prevent autoimmunity, tolerisation is the process of recognising antigens as “self”.

Immunogenic cell death

cell death that triggers an immune reaction by DAMP/PRR signalling. This may occur as a result of different types of tissue damage, for example RT or chemotherapy.

Innate immune system

the initial immune response which occurs in a generic fashion to inflammatory stimuli, comprising complement activation and immune cell recruitment and activation. It coordinates the activation of the adaptive immune system by antigen presentation.

Medial necrosis

necrosis of the middle portion of vessel walls (anatomically called the tunica media).

Microvascular injury

injury to the fine network of blood vessels and capillaries resulting in changed patterns of blood flow.

Pathogen-associated molecular patterns (PAMPs)

signalling from pathogens by particular stimuli that can be recognised by immune cells leading to an inflammatory response.

Pleiotropic cytokines

Cytokines whose actions are context dependent with relation to a particular process or pathway, that is to say in some circumstances they may be stimulatory in others inhibitory.

TAMS

Tumour associated macrophages are macrophages associated within the tumour microenvironment, they are largely immunosuppressive and resemble the alternatively activated “M2” macrophage.

T_{reg} cells

Regulatory T-cells are a T-cell subset that exert immunosuppressive and tolerising effects and have an important role in cancer immune editing, the maintenance of a permissive cancer microenvironment and in preventing effective adaptive immune recognition of tumour cells.

Vessel co-option

a mechanism by which tumours obtain a blood supply by hijacking the existing vasculature.

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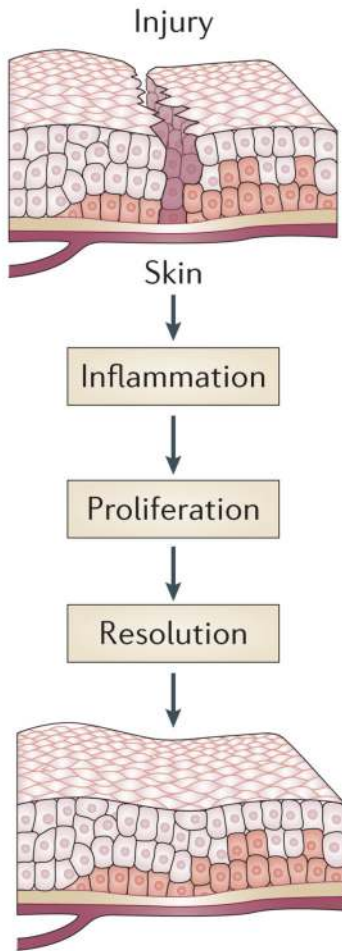
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Box 1

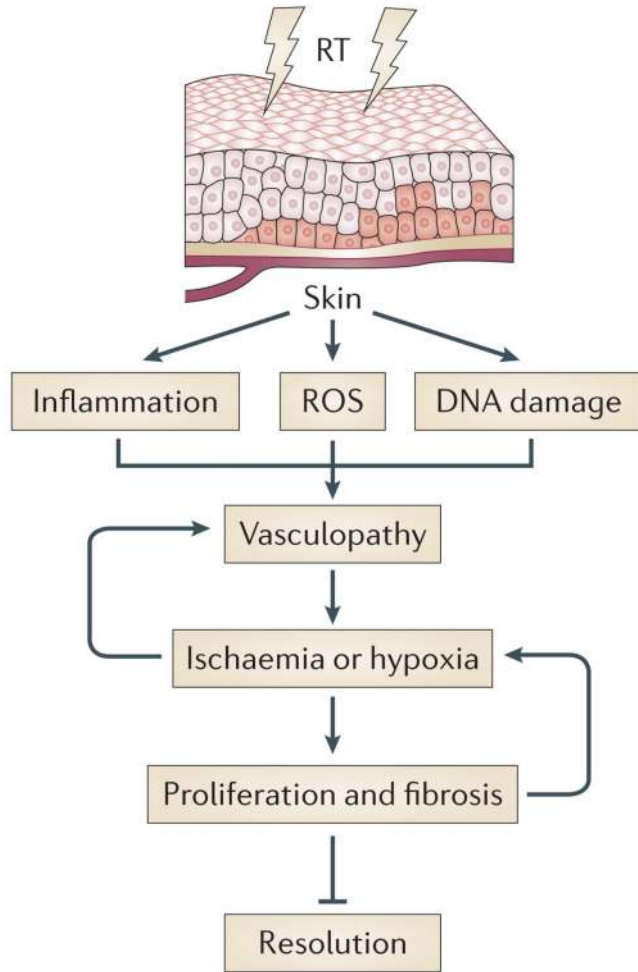
Normal wound healing versus radiation fibrosis

The normal phases of wound healing progress from the injurious stimulus to inflammation, proliferation and finally resolution. By contrast, radiation generates reactive oxygen species (ROS), DNA damage and inflammation as the early stimuli that mediate activation of a dysregulated proliferative phase. Unlike wound healing, radiation exerts a field effect on the vascular compartment. Endothelial dysfunction leads to a progressive vasculopathy characterised by impaired gaseous exchange and the development of tissue hypoxia, which drive an uncontrolled proliferative stage that replaces the normal proliferative phase of wound healing. This may represent a poorly coordinated haemostatic response aiming to preserve tissue oxygenation. The progressive and perpetuating (blue arrows represent positive feedback loops) proliferative phase prevents tissue resolution and results in the development of late adverse effects.

Normal wound healing

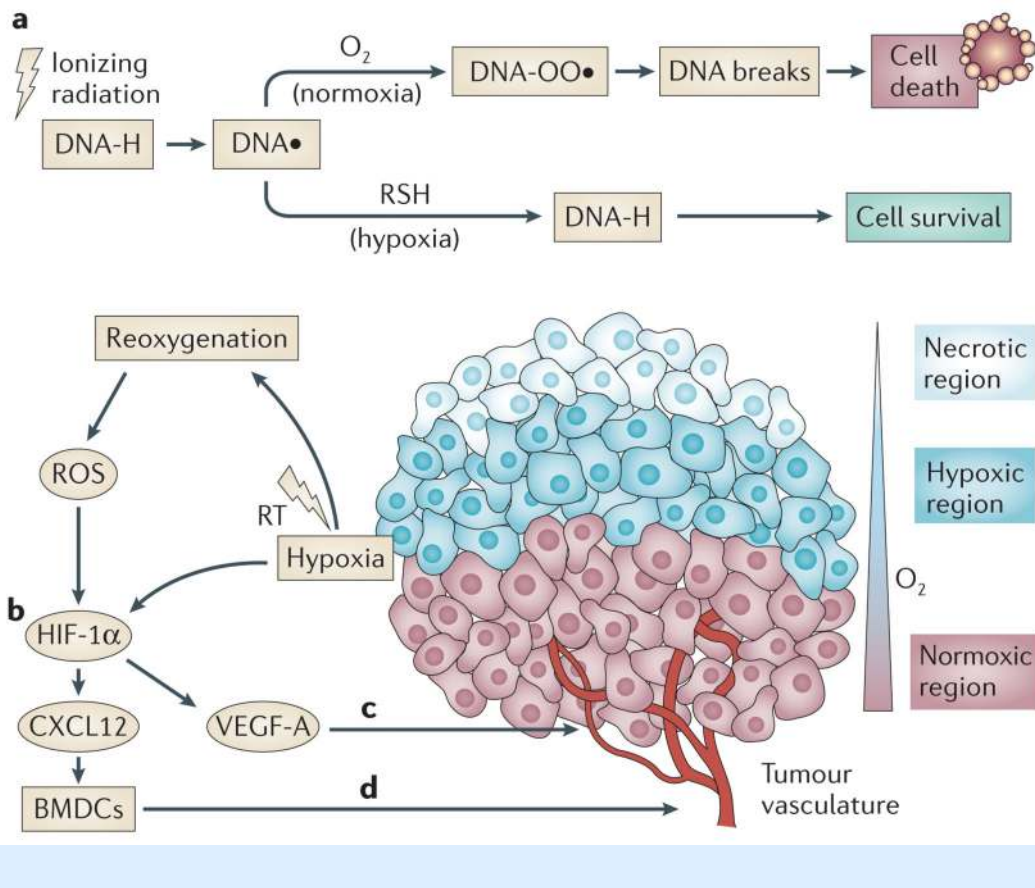


Radiation-induced fibrosis



Box 2**Hypoxia - mediated mechanisms of radioresistance in the tumour microenvironment (TME)**

- a) Ionizing radiation produces DNA radicals (DNA •) which are usually fixed by O₂ (in normoxic regions) and causes DNA damage that can lead to cell death if unrepaired. However, under hypoxia DNA damage is reduced (mainly by –SH containing compounds) leading to cell survival. Modified with permission from Ref 189.
- b) HIF-1 α is stabilised under hypoxic conditions leading to up-regulation of genes involved in cell survival. Radiotherapy causes tumour reoxygenation, an increase in reactive oxygen species (ROS), and contributes to HIF-1 α stabilisation.
- c) Hypoxia increases VEGF-A production leading to the formation of abnormal vessels, which also contribute to tumour hypoxia.
- d) Vasculogenesis aids tumour recurrence following radiotherapy and can be driven by HIF-1 α - mediated BMDC recruitment.



Key points

- Radiotherapy is a common treatment option for cancer patients. However, many aspects of the tumour microenvironment (TME) can render a tumour resistant to radiotherapy *de novo* or lead it to recur with a worse prognosis following therapy.
- Normal tissue toxicity limits the dose of radiotherapy that can be delivered safely.
- Combination strategies are required in order to achieve better tumour control.
- Radiotherapy-mediated immunogenic cell death (ICD) can elicit an immune response, but anti-tumour immunity may be limited due to the presence of radioresistant suppressor cell types in the TME. Combining radiotherapy and immunomodulatory treatments may overcome adaptive immune suppression and has great promise both locally in the primary tumour and abscopally.
- Hypoxia plays a crucial role in radioresistance due to reduced oxygen-mediated fixation of DNA damage and hypoxia induced factor-1 α (HIF-1 α)-mediated cell survival. Attempts to increase oxygen delivery, normalise tumour vessels, inhibit HIF-1 α , and prevent the recruitment of bone marrow derived cells (BMDCs) required for vasculogenesis are all being tested to reduce tumour hypoxia, improve radiotherapy responses and prevent tumour recurrence after therapy.
- Tumour irradiation induces a wound healing response characterised by inflammation, cancer-associated fibroblast (CAF) modulation and ECM remodelling, which may facilitate tumour recurrence. Targeting the initial inflammatory response may counteract attempts to boost the immune-mediated anti-tumour response following radiotherapy. Therefore, reducing ECM remodelling by inhibiting growth factors, receptor kinases, or matrix enzymes may be more effective in preventing the post-irradiation stiffening of the TME that could facilitate tumour spread.
- Careful scheduling of tumour reoxygenation strategies with radiotherapy will be required to maximise tumour control. Subsequent inclusion of immunomodulatory and anti-fibrotic treatments should be considered to maximise therapeutic benefits and prevent post-irradiation tumour recurrence and metastasis.

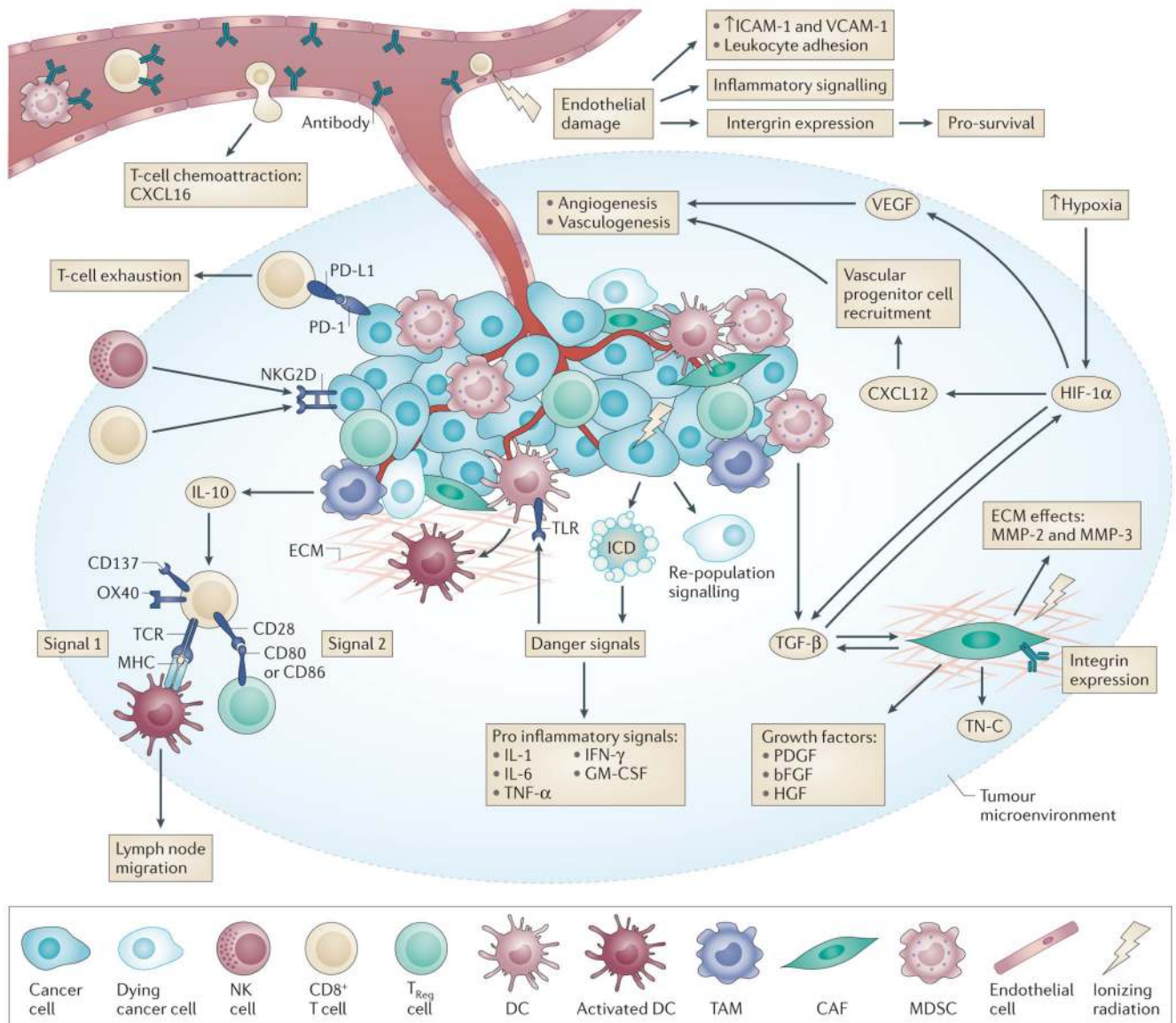


Figure 1. Radiation effects on the tumour microenvironment (TME)

Ionizing radiation damage leads to effects on numerous cell types within the TME. Tumour endothelial cells are sensitive to radiation, and their death initiates the inflammation cascade. Damage also leads to increased ICAM and VCAM expression and increased attraction of innate immune cells. Upregulation of integrins on endothelial cells leads to increased survival, which acts as a method of radioresistance. Vascular depletion potentiates the effects of hypoxia leading to HIF-1 signalling and to pro-angiogenic stimuli through VEGF and pro-vasculogenic stimuli through CXCL12. CAF activation following radiation leads to altered growth factor secretion and release of numerous modulators of the ECM and cytokines. TGF- β signalling is complex and pleiotropic — directly affecting tumour cells and CAFs, driving HIF-1 signalling and reducing the activation of T-cells and dendritic cells (DCs). Within the immune compartment, increased tumour cell antigen availability and increased antigen processing by higher mTOR levels combine with a DAMP-related TLR

response and increased pro-inflammatory cytokine signalling to activate DCs and thus T-cells; activated DCs also migrate to proximal lymph nodes. This signalling is often still blocked by high T_{reg} CTLA-4 inhibition of co-stimulation within the TME. Whilst radiation also upregulates NKG2D signals on tumour cells which allow direct cytotoxic effects by NK cells and CD8+ T-cells, other tumour escape mechanisms such as PD-L1 signalling and MDSC derived IL-10 immunosuppression remain intact.

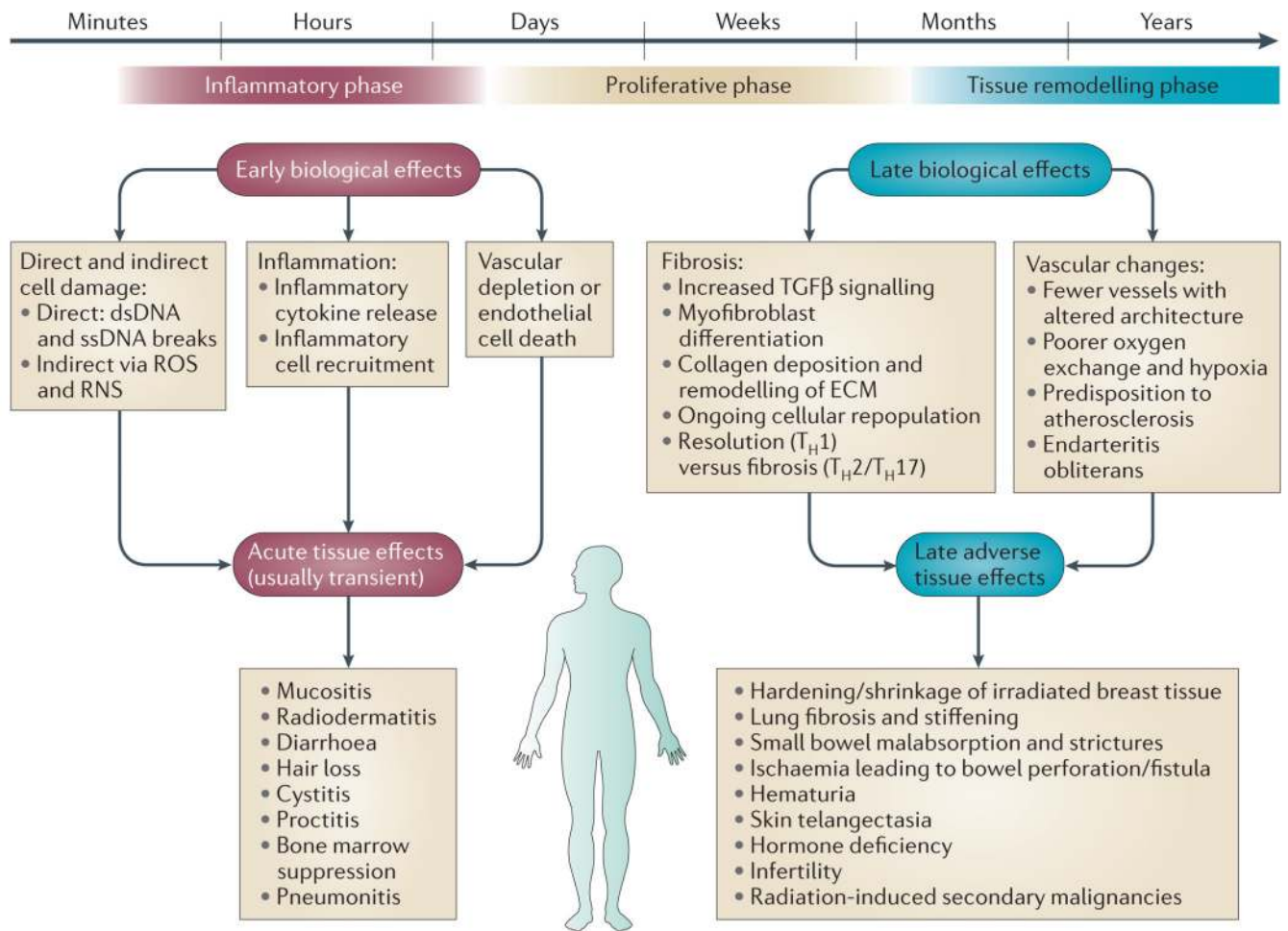
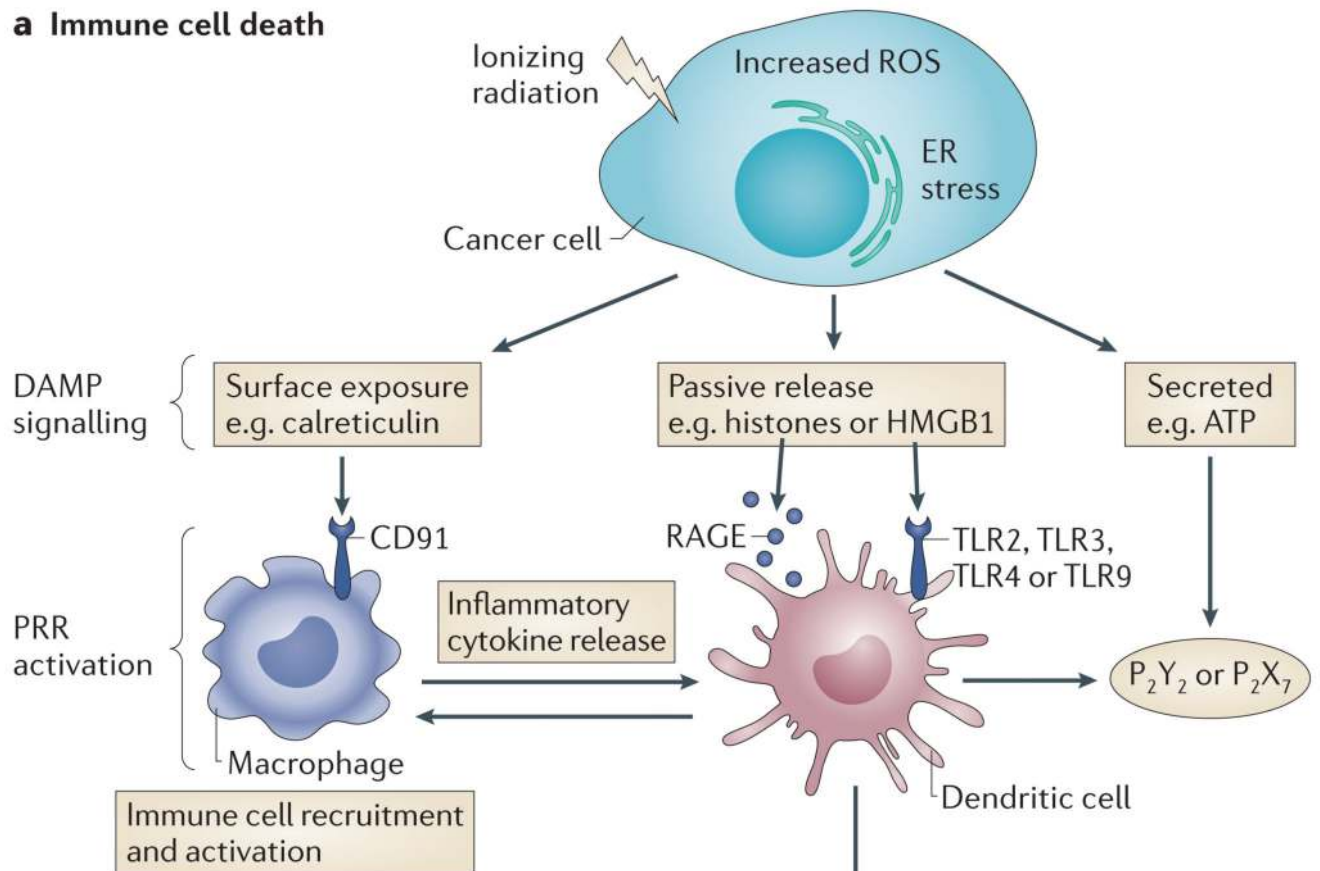
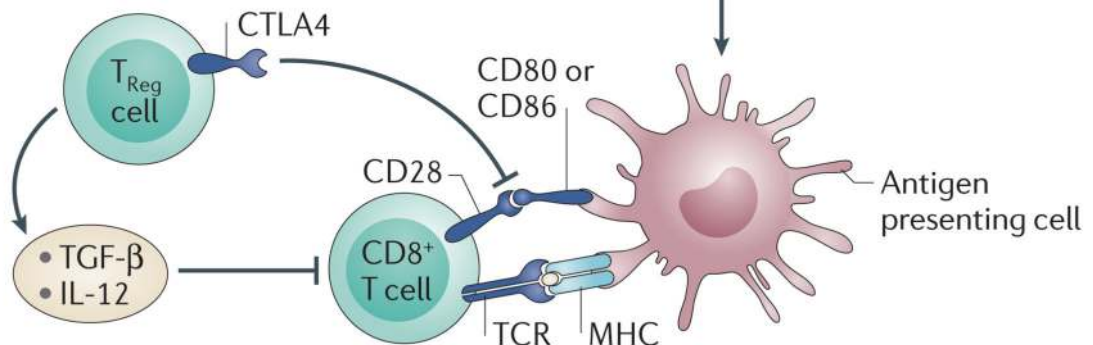


Figure 2. Biological effects and normal tissue toxicity after radiotherapy

Early biological events cause acute tissue effects, which are usually transient and normally resolve within three months of completing treatment. They also result in more protracted biological effects which can manifest in tissues as late biological effects and secondary malignancies. Higher radiation dose per fraction seems to increase the severity of late adverse effects. Abbreviations: dsDNA, double strand DNA; ECM, extracellular matrix; ssDNA, single strand DNA; TGF- β , transforming growth factor β ; TH2/TH17, T-helper cells (classified by the interleukin they principally secrete).

a Immune cell death**b Immunosuppression tolerance****Figure 3. Immunogenic cell death (ICD) of cancer cells and immune tolerisation**

a) Radiotherapy damage to cancer cells leads to cell stress mediated by reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress; this in turn results in DAMP signalling. Three main types of DAMP signalling are now recognised - surface exposure, passive release and active secretion⁵⁷. The degree of different DAMP release appears to vary with different types of cell stress so that cell stress, apoptosis and necrosis likely result in a different pattern of DAMP signalling. Multiple DAMP signal activation appears to be necessary to achieve effective ICD in cancer cells to cause an adaptive immune response.

DAMP signals through multiple PRR types, e.g. HSP90 or calreticulin surface exposure leads to phagocytosis mediated by CD91 (expressed on a variety of innate immune cells); HGMB1 release activates TLR2 and TLR4 which in turn lead to MyD88 signalling, DC activation and increased production of inflammatory cytokines; ATP secretion activates purinergic receptors P2Y2 and P2X7 which have wide ranging immunostimulatory actions across DCs, NK cells, macrophages and T-cells. Effective ICD of cancer cells therefore increases the background inflammatory signalling and generates activated DCs to act as APCs.

b) Immune tolerisation [G]. APCs present processed antigen on MHC I receptors to CD8+ T-cells via their corresponding TCRs. Co-stimulation is required to achieve T-cell activation. This can be achieved by the interaction of CD80 (also known as B7.1) and CD86 (also known as B7.2) on the APC with CD28 on the CD8+ T-cell. T_{reg} cells express CTLA-4, which has a higher affinity for B7.1 and B7.2 than CD28 and thus effectively inactivates the co-stimulatory signal leading to ineffective T-cell activation. Overcoming this process with appropriate immunomodulation allows for effective exposure of cancer cells to the immune system and the exciting prospect of novel cancer treatments.

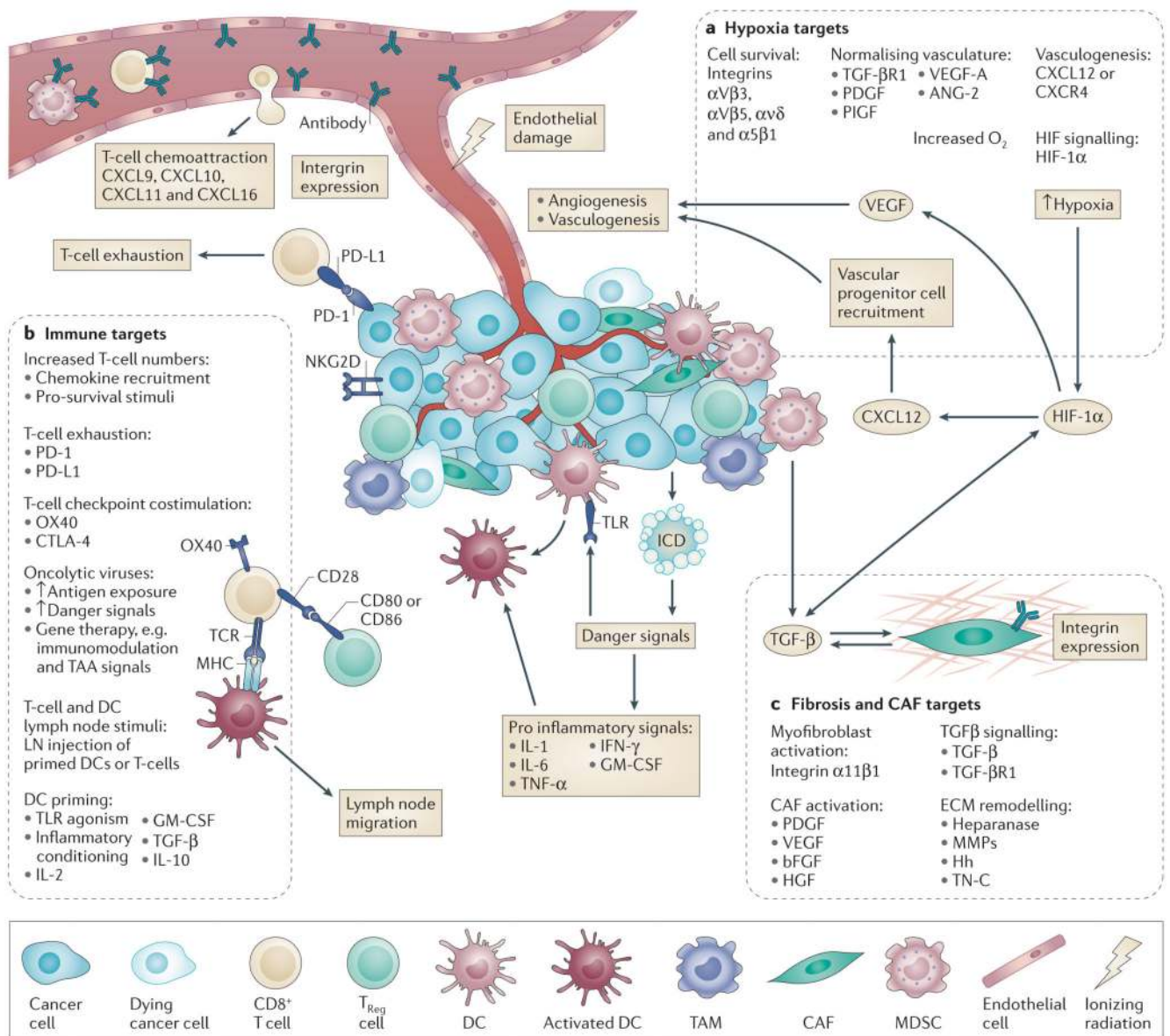


Figure 4. Targets for radiosensitisation

After radiotherapy, there are numerous potential targets within the tumour microenvironment (TME) whose modulation may lead to radiosensitisation. These are categorised broadly by area of biological effect — a) hypoxia, b) fibrosis and CAF related, and c) immune — and their actions portrayed in relation to the effects of radiotherapy on the TME summarised in Figure 1. Ongoing clinical trials addressing these in combination with radiotherapy are addressed in the Supplementary Table.

a) Hypoxia targets can be further subdivided into those that affect endothelial cell survival (for example, targeting integrins¹²⁰), that normalise vasculature (for example, antagonising VEGF¹⁰⁶), that prevent vasculogenesis (for example, targeting CXCL12 recruitment of endothelial progenitor cells²⁵), that alter oxygen delivery (for example, ARCON therapy^{85–87}), or that alter HIF-1 α signalling (for example, the HIF-1 α inhibitor YC-1100).

b) Fibrosis targets can be sub-categorised into those that affect stromal activation (for example, by inhibition of growth factors by suramin¹⁴⁸), that affect ECM remodelling (for example, targeting TN-C with the antibody 81C6^{135, 136}), or that affect TGF- β signalling (for example, by targeting its receptor TGF- β R1 with the inhibitor SD-208¹³¹).

c) Immune targets can be divided into those that prime dendritic cells (for example, the TLR7 agonist imiquimod¹⁶⁶), that affect T-cell checkpoint co-stimulation (for example, the anti-CTLA-4 antibody ipilimumab^{74, 75, 171}), that affect T-cell exhaustion (for example, the anti-PD-1 antibody Pembrolizumab¹⁸¹) or that affect T-cell recruitment (for example, targeting the chemokine CXCL16). Oncoviruses are also an immunomodulatory stimulus and can be used to affect multiple aspects of the immune pathway in the irradiated TME (for example, GM-CSF-expressing herpes simplex virus^{186, 188}).

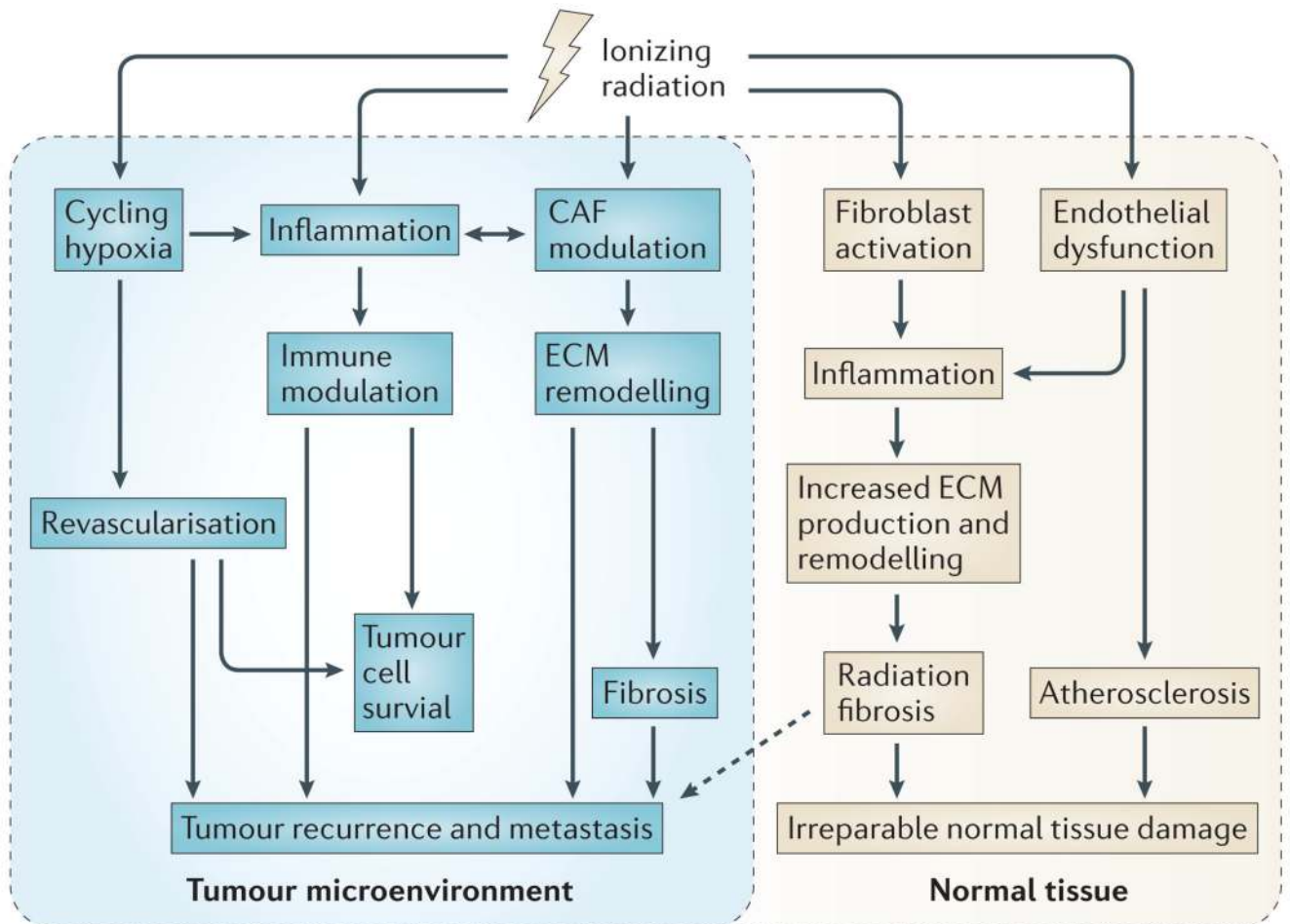


Figure 5. The interconnected radiotherapy-mediated changes in the tumour microenvironment (TME)

Hypoxic regions within the TME may initially affect tumour radiosensitivity. Radiotherapy elicits an inflammatory response in the TME as well as contributing to cycling hypoxia and modulation of CAF activity. These responses are interconnected with immunomodulation, revascularisation, ECM remodelling, and radiation fibrosis that may impact tumour cell survival and/or tumour recurrence and metastasis. Similar processes may occur in the surrounding normal tissue that could result in irreparable damage to critical organs. These events must all be taken into account when combining radiotherapy with therapies to modulate the immune response as well as reducing hypoxia and ECM remodelling and fibrosis in carefully timed treatment strategies.

Table 1

Current and future TME targets for radiosensitisation

Resistance mechanism	Drugs	Targets	Mode of action
Immune response			
	Ipilimumab	CTLA-4;	T-cell activation
	Imiquimod	TLR7;	DC activation
	Oncolytic viruses	Tumour cells	Activate immune response
	Future inhibitors	IL-6, IL-10	T-cell activation
		PD-1, PD-L1, TIM3, LAG3;	Prevent T-cell exhaustion
	Future agonists	GM-CSF, CXCL16, OX40, CD40L, CD80, CD137 (4-1BBL);	T-cell recruitment and activation
		CCL3, CCL5, IL-2, IL-4, IL-12, IRX-2;	Activate immune response
Hypoxia			
	Nitroimidazole derivatives (i.e. Nimorazole)	Hypoxic cells	Reduce tumour hypoxia
	Bioreactive albumin-MnO ₂ nanoparticles	Hypoxic cells	Reduce tumour hypoxia
	Acridine, YC-1	HIF-1 α ;	Reduce hypoxia response pathways
	Aflibercept	all VEGF molecules and PlGF;	Vessel normalisation
	AMG386	ANG-1, ANG-2;	Inhibit pBMDC recruitment
	Endostar	VEGF, TGF- β , HIF-1 α , bFGF;	Inhibit angiogenesis
	AMD3100	CXCL12, CXCR4;	Inhibit BMDC recruitment and vasculogenesis
	Integrin inhibitors (i.e. Cilengitide, Vitaxin and Volociximab)	Integrins α v β 3, α v β 5, α 5 β 1	Inhibit angiogenesis
	Future inhibitors	Integrins α 6 β 1, α 6 β 4;	Reduce EC survival and inhibit angiogenesis
	Future inhibitors	PlGF, ANG-2;	Vessel normalisation and overcome resistance to anti-VEGF therapies
Fibrotic processes			
	BIBF1000, BIBF1120	PDGF, VEGF, bFGF receptors;	Reduce GF signalling and TME remodelling; fibrosis
	Imatinib, Nilotinib, Dasatinib	TGF- β , PDGF	GF signalling; collagen synthesis
	Vismodegib, Saredigib, Sonedegib	SMO;	Reduce HH signalling; fibrosis
	Suramin	PDGF, EGF, TGF- β , FGF-2 and IGF receptors and heparanase enzymes;	Reduce GF signalling and TME remodelling; fibrosis
	ST0001, PG545, M402, PI-88	Heparanase;	Inhibit TME remodelling
	SD-208	TGF- β R1;	Inhibit TGF- β signalling
	Simtuzumab	LOXL2;	Reduce TME remodelling; liver fibrosis
	81C6, F16SIP	TN-C;	Reduce CAF-mediated TME remodelling

Resistance mechanism	Drugs	Targets	Mode of action
	Future inhibitors	HGF, CTGF, MMP-2, MMP-3, Integrins $\alpha 11\beta 1$, $\alpha v\beta 6$ and $\alpha 3\beta 1$;	TME activation and remodeling; radiation-mediated fibrosis

Abbreviations: ANG-1/-2, angiopoetin 1 and 2; bFGF, basic fibroblast growth factor; BMDC, bone marrow-derived cells; CTGF, connective tissue growth factor; CTLA-4, cytotoxic T-lymphocyte associated protein 4; DC, dendritic cells; EGF, epithelial growth factor; FGF-2, fibroblast growth factor 2; GF, growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; HH, hedgehog; HIF-1 α , hypoxia inducible factor 1 α ; IGF, insulin growth factor; IRX2, iroquois homeobox 2; LAG3, lymphocyte activation gene 3; LOXL2, lysyl oxidase-like 2; MMP-2/-3, matrix metalloproteinase 2 and 3; pBMDC, perivascular bone marrow-derived cells; PD-1, programmed death 1; PD-L1, programmed death ligand 1; PDGF, platelet-derived growth factor; PIGF, placental growth factor; SMO, smoothened; TGF- β , transforming growth factor β ; TGF- β R1, transforming growth factor β receptor 1; TIM3, T-cell immunoglobulin mucin 3; TLR7, toll-like receptor 7; TME, tumour microenvironment; TN-C, tenascin C; VEGF, vascular endothelial growth factor

Table 2

Fractionation of radiotherapy and Timing of immunomodulation

Study type	Cancer type	Intervention	Outcome	Reference
<i>Fractionation</i>				
Preclinical	Murine glioma	RT: 10 Gy in 1 fraction Immunomodulation: Anti-PD-1	Longer survival seen (53 days (RT and anti-PD-1) vs. 25 days (control), 27 days (anti-PD-1) or 28 days (RT))	Zeng et al, 2013190
Preclinical	Murine breast cancer	RT: 20 Gy in 1 fraction, 24 Gy in 3 fractions or 30 Gy in 5 fractions Immunomodulation: Anti-CTLA-4	Best response seen in 24 Gy in 3 fractions	Dewan et al, 2009171
Preclinical	Murine breast cancer	RT: 12 Gy in 1 fraction, 24 Gy in 2 fractions Immunomodulation: Anti-CTLA-4	CD8+ T-cell anti-tumour immunity demonstrated	Demaria et al, 2005191
Clinical	Hepatoma patients	RT: 8 Gy in 1 fraction Immunomodulation: DC vaccine	2 partial responses, 4 minor ones	Chi et al, 2005192
Clinical	Prostate cancer	RT: 70 Gy in 30 fractions Immunomodulation: IL-2 and GM-CSF	PSA specific T-cells increased	Gulley et al, 2005193
Clinical	MF	RT: 9-18 Gy in 9 fractions Immunomodulation: Injected TLR9 agonist	5/15 responses	Kim et al, 2010194
<i>Timing</i>				
Clinical	MM or RCC	RT: 60 Gy in 3 fractions Immunomodulation: IL-2	8/12 responses	Seung et al, 2012195
Preclinical	Murine Prostate Ca expressing HA	RT: 15 Gy 1 fraction Immunomodulation: CD4+ T-cells primed against HA (given at points on a time-course after RT)	Tolerance seen between days 3 and 16. Normal T-cell response by day 33.	Harris et al, 200855
Clinical case report	MM	RT: 28.5 Gy in 3 fractions Immunomodulation: Ipilimumab (anti-CTLA-4) at 1 month after RT	Complete response	Postow et al, 200974
Clinical case report	MM	RT: 54 Gy in 3 fractions Immunomodulation: Ipilimumab	Complete response	Hiniker et al, 201254

Abbreviations: CTLA-4, cytotoxic T-lymphocyte associated protein 4; DC, dendritic cell; MM, malignant melanoma; MF, mycosis fungoides; PD-1, programmed death 1; PD-L1, programmed death ligand 1; PSA, prostate specific antigen; RCC, renal cell carcinoma; TLR, toll-like receptor; RT, radiotherapy;