Radiation-induced bystander signalling in cancer therapy

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Abstract | Our understanding of how radiation kills normal and tumour cells has been based on an intimate knowledge of the direct induction of DNA damage and its cellular consequences. What has become clear is that, as well as responses to direct DNA damage, cell–cell signalling — known as the bystander effect — mediated through gap junctions and inflammatory responses may have an important role in the response of cells and tissues to radiation exposure and also chemotherapy agents. This Review outlines the key aspects of radiation-induced intercellular signalling and assesses its relevance for existing and future radiation-based therapies.

Photodynamic therapy
A therapeutic approach in which photosensitive chemicals are taken up into tumours and then activated by laser or other light source to produce damaging free radicals

Ganciclovir
A synthetic analogue of 2′-deoxyguanosine that becomes phosphorylated when taken up by cells and is incorporated into DNA by DNA polymerase and leads to chain termination of the replicating DNA strands.

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When ionizing radiation interacts with biological material, energy is deposited and chemical bonds are broken. In cells, the basic components of proteins, lipids and nucleic acids can all be damaged. However, a key consequence is that direct damage occurs to DNA within the nucleus, producing a range of lesions of which DNA double strand breaks (DSBs) have a pivotal role in determining whether cells survive radiation exposure[1]. If DNA damage is not correctly repaired two direct consequences can occur. Residual or unrepaired damage leads directly to chromosomal aberrations, loss of genetic material and cell death. Also, unrepaired or incorrectly repaired (misrepaired) damage can lead to mutations that might result in carcinogenesis or cell death (FIG. 1). The mechanisms underpinning DNA damage and repair processing in irradiated cells have been extensively studied since the discovery of DNA as the genetic material by Watson and Crick over 50 years ago. This has included exhaustive study of the DNA damage sensing and signalling pathways underpinning the DNA damage response that is present in cellular systems[2]. What is clear from these efforts is that cells have multiple and complex processes for sensing and repairing changes to their genomes to enable future propagation and stability. A series of sensor, transducer and effector proteins give cells important choices in response to radiation-induced DNA damage, such as DNA repair, cell cycle delay and cell death (apoptosis)[3].

Evidence now shows that, as well as these direct DNA damage-dependent effects, irradiated cells also send signals to their neighbours. These non-irradiated cells respond to signals produced by neighbouring irradiated cells by what has been termed a bystander effect (extensively reviewed in REFS 4–7). The term bystander effect is not new and has been observed in response to a range of other insults including ultraviolet radiation[8], photodynamic therapy[9], heat[10] and chemotherapy agents[11]. Its observation in response to chemotherapy agents underpins its considerable importance in gene therapy regimens, in which not all tumour cells are targeted and indirect killing of non-targeted cells is required to ensure maximal tumour cell kill[12]. For example, the archetypal gene therapy model is the herpes simplex virus-thymidine kinase (HSV-TK) system. In this system the *HSV-TK* gene is transfected into cells and these are incubated with the non-toxic agent ganciclovir, which is converted to a toxic analogue that diffuses and kills neighbouring cells[13]. This bystander effect involves direct cell–cell communication through gap junctional intercellular communication (GJIC) and requires expression and surface location of CX43 (also known as GJC1) gap junctions[14]. By contrast, the bystander effect mediated by the thymidine phosphorylase–5′-deoxy-5-fluorouridine suicide gene system involves a factor released into the medium that is independent of GJIC[15]. So, the paradigm of a bystander response after radiation treatment is not new in other fields; in essence it is a manifestation of intercellular signalling that is either specific or non-specific in its mode of action.

Radiation-induced bystander responses have been observed in a range of cell types, tissue models and *in vivo*. Although the majority of the evidence for bystander effects has come from cellular studies, a range of other responses have been classified as bystander effects in the literature. In humans, in response to radiotherapy, longer-range effects occurring within or between tissues have also been reported and have been termed abscopal,

REVIEWS

At a glance

- Radiation-induced bystander responses are defined as the response of cells to their neighbours being irradiated. These have been observed in a range of cell types and measured for a range of end points.

- Long-range, abscopal (out-of-field) effects have also been observed after the clinical use of radiation.

- The main mechanisms involve direct cell–cell communication by gap junction intercellular communication and release of factors into the medium.

- Bystander signalling has a key role in increasing the effectiveness of gene therapy approaches in which common mechanisms involving cytokine signalling and the production of reactive oxygen and nitrogen species have been used to maximize effectiveness.

- With the development of suitable strategies, radiation-induced bystander responses may be used to enhance tumour cell kill or protect normal tissues from the damaging consequences of radiation exposure.

out-of-field or distant bystander responses[16]. Radiation-induced bystander responses have been observed, not just from studies with external beam irradiation, but also from approaches using targeted radioisotopes. Several key questions emerge from these observations in terms of their relevance to cancer therapy. First, does an understanding of bystander mechanisms highlight new potential targets for cancer therapies and, if so, can this be modulated to either increase tumour cell killing or protect normal tissues? Second, do these bystander responses, especially after low-dose irradiation, contribute to increased carcinogenic risks associated with radiation exposure?

The bystander responses

A simple definition of a radiation-induced bystander response is one in which 'a cell that responds to the fact that its neighbours have been irradiated'. This definition has been expanded by many to include effects related to the production of clastogenic factors and longer-range abscopal effects studied in whole organisms, which are described below.

A key characteristic of the bystander responses, in contrast to direct irradiation effects, is the dose–response relationship (FIG. 2). Instead of an increased response with increasing radiation dose, the bystander response becomes saturated at relatively low doses (typically less than 1 Gy)[17,18]. This saturation means that above a certain dose no additional effect occurs and in practice means that, for any given endpoint, not every cell responds with a bystander effect. Also, it has been proposed that a binary mode of action may occur, with a simple on–off response, the probability of which increases with radiation dose[19]. It should be emphasized, however, that bystander responses can have a significant role even after high doses (>10 Gy)[20]. Interestingly, in many models after low-dose exposure, bystander responses have been almost equally effective as the direct response, suggesting that at least under some conditions bystander responses could predominate in overall effectiveness[21].

Bystander responses have been observed in most cell types, including lymphocytes[22], fibroblasts[23], endothelial cells[24] and tumour cells[25]. They have also been observed

for a range of end points and there has been considerable debate as to whether these can be termed damaging or protective bystander responses[26]. These include damage-mediated end points such as DNA damage[27], mutations[28], transformation[29] and cell death[30] and also protective responses involving terminal differentiation[31], apoptosis[32] (removal of damaged cells) and radioadaptive responses[33]. To some extent the debate over damaging or protective bystander responses is a moot point, as many of the studies have been done in specific cell models which have, in many cases, limited options or routes for coping with stress-mediated signalling. For example, our own work has extensively studied the response of Chinese hamster V79 cells to bystander signals[19,21]. In response to a stress signal, these cells can attempt DNA repair and, if this is unsuccessful, they accumulate mutations leading to chromosome aberrations and ultimately to cell death.

Molecular and cellular bystander mechanisms. Radiation-induced bystander responses have two main mechanisms of action, which mirror gene therapy bystander effects (FIG. 3). For cells in direct contact, bystander signalling can occur through GJIC. Gap junctions are multimeric protein channels between cells that allow transmission of signalling molecules[34]. Key to these structures are the connexion proteins, which are formed as individual hemi-channels on separated cells merge to form gap junctions as cells physically interact with each other. Typically these pores can allow molecules of up to 1,000–1,500 Da to pass through. Key ions and metabolites that are known to be transmitted through GJIC include ions such as Ca^{2+}, nucleotides, peptides and other secondary messengers[35].

Much of the early work in characterizing the role of GJIC was performed with confluent cell monolayers that were irradiated under situations in which not every cell was exposed. Individual charged particles deposit energy across cells in the form of tracks consisting of closely spaced ionizations and excitations. As the dose delivered to a cell population is reduced, the number of tracks crossing each cell is reduced until not every cell is irradiated. Using this approach, Little and colleagues showed that, under conditions in which only 1% of a cell population was irradiated, 30% showed chromosomal changes in the form of sister chromatid exchanges[17]. Further work in primary human fibroblasts showed that inhibitors of GJIC using inhibitors such as lindane prevented this signalling in bystander cells[36,37]. Importantly, GJIC is only available as a signalling option in normal cells and is generally downregulated in tumour cells in which it is a key phenotypical change in carcinogenesis[38].

The second route by which bystander responses are mediated is through the release of soluble factors from cells that have been irradiated. These factors can be transferred through cell culture medium from irradiated to non-irradiated cells[30]. These factors have been extensively studied (reviewed in REF. 39) and they have been postulated to be between 1,000 and 10,000 kDa in size and include lipid peroxide products[40], inosine nucleotides[41] and cytokines such as tumour necrosis factor-α

Clastogenic factor
A species that can break chromosomes.

Sister chromatid exchanges
Exchange of chromosomal material between the chromatids of a chromosome.

Lindane
A neurotoxin that can inhibit gap-junctional intercellular communication.

(TNFα)[42], but underlying their actions is the involvement of reactive oxygen species (ROS) such as superoxide radicals. The first report of soluble factors released after radiation exposure was in 1922: serum from irradiated animals was shown to stimulate the growth of lymphoid cells in suspension whereas control serum caused cell degradation[43]. The effect was evident 1–2 hours after irradiation but was not observed in serum isolated 17 hours after exposure. Further studies in the 1950s and 1960s reported these clastogenic factors in blood samples from radiotherapy patients[44] and more recent work has reported these factors were present in blood samples from individuals exposed to radiation from the Chernobyl nuclear power plant accident[45].

The mechanisms of bystander signalling are now starting to be elucidated at the molecular level and several key molecules are known to have major roles in some systems (FIG. 3). Not surprisingly, these are central factors involved in stress responses and cell–cell signalling, which are not generally specific to radiation exposure. A range of studies have shown clear evidence for a key role for cytokines, including interleukin 6 (IL-6)[46], IL-8 (REF. 47), transforming growth factor-β1 (TGFβ1)[48] and TNFα[49], ROS[50] and reactive nitrogen species[25,51]. Many aspects of bystander-mediated signalling and response have close parallels to inflammatory responses. For example, recent studies have shown that macrophages, which are key mediators of the inflammatory response *in vivo*, produce persistent increased levels of oxidative stress after radiation exposure under bystander conditions[52]. At the molecular level, cyclooxygenase 2 (COX2,

also known as PTGS2)-dependent signalling has been shown to be a central player in cellular inflammatory responses and also mediates bystander signalling. The activation of the MAPK pathways is crucial for the action of COX2. Downstream signalling leads to transcription factor activation, including inducible nitric oxide (NO) synthase (iNOS, also known as NOS2) activation leading to the production of reactive nitrogen species. Inhibition of these pathways in bystander cells leads to inactivation of bystander responses, suggesting a potential route for modulating these responses in a clinical setting[49]. For example, it may be beneficial to activate bystander responses to increase tumour cell killing, or to prevent them in order to protect normal tissues from additional damage.

Overall, the underlying mechanisms involved in the signalling of bystander responses to neighbouring cells and the cell signalling pathways in those cells are being elucidated. However, there is a significant paucity of data on the mechanisms of release of bystander signals from irradiated cells. Using microbeams to target radiation to specific subregions of cells, it was shown that direct DNA damage of an irradiated cell is not required to trigger a bystander response[53]. Energy deposited in the cytoplasm is capable of producing ROS, which can indirectly lead to nuclear DNA damage[54]. Therefore, it has been proposed that subcellular targets such as mitochondria could play an important part, either as direct targets for the production of bystander signals or as parts of a signal transduction mechanism[55,56]. This probably involves the direct release of ROS in response to direct irradiation or indirect triggering of cytochrome *c* release owing to changes in mitochondrial membrane permeability.
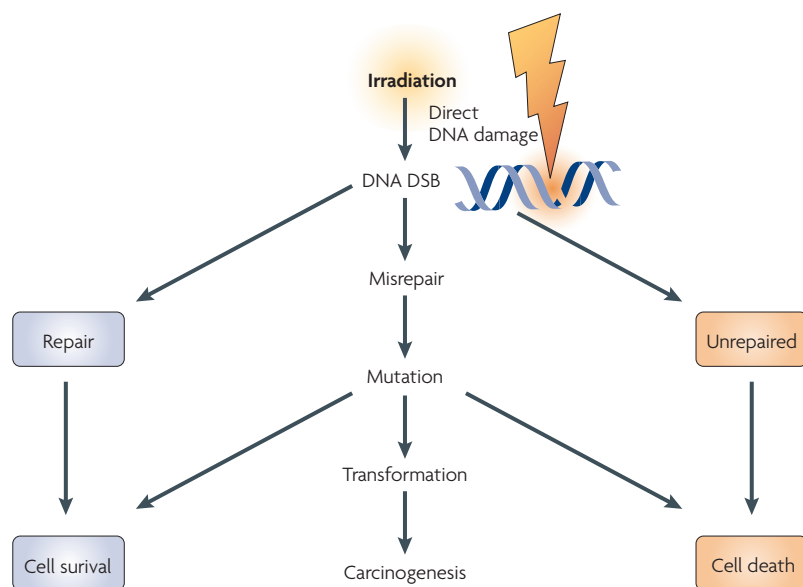
Long-range bystander effects. It is important to determine how the bystander responses manifest in more complex three-dimensional systems and *in vivo*. A natural progression from studies looking at bystander interactions between different cell types has been the use of intact tissue sections or reconstruct models for bystander signalling[57]. Some of the earliest work in this area has been done in urothelial models owing to their organized structures and ease of isolation from animal sources and from patients undergoing urology procedures. In pioneering work, Mothersill and colleagues used samples of both human and murine urothelium. Using a cell reporter system for measuring the bystander effect, sections of bladder were irradiated and medium was transferred from these to the reporter system. Significant changes in the survival of the reporter cells were observed[58]. Measurements *in vivo* from mice confirmed such changes in survival and provided evidence for a genetic component underlying these responses[59].

Belyakov and colleagues used a microbeam approach with both human and porcine urothelial samples. In a series of studies they reported that, as well as a damaging bystander response in locally irradiated sections of tissues, there was increased differentiation of cells after bystander signalling. This response was significantly greater than the number of damaged cells being produced in the population suggesting that, at least in
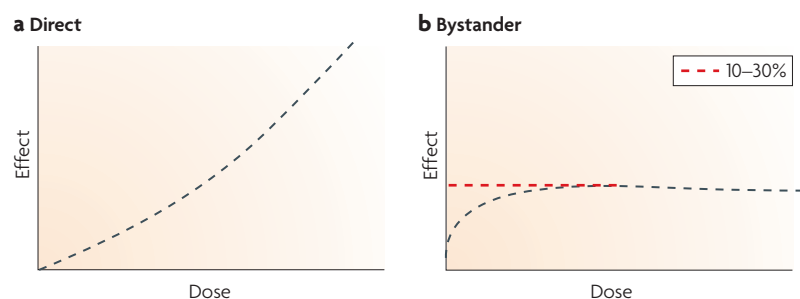

Figure 1 | **Direct DNA damage radiation model.** The schematic shows the standard model of DNA damage responses to radiation in biological systems, with direct DNA damage having a central role and the production of DNA double strand breaks (DSBs) leading to downstream biological consequences. Cells have complex pathways for sensing DNA damage and correctly repairing the DNA damage to survive the radiation exposure. If the DNA damage is not repaired, there is a high probability of cell death. DNA damage that is misrepaired can lead to mutations, increasing the probability of transformation and carcinogenesis.

a Direct

b Bystander

- - - 10–30%

Figure 2 | **Key aspects of radiation-induced bystander responses.** Typical dose response curves for direct (**a**) and bystander (**b**) responses are shown, highlighting the commonly observed saturation of response for bystander effects.

this model, a significant protective bystander response was occurring[31,60]. In further studies, sections of three-dimensional human skin reconstructs were locally irradiated with helium ions from a microbeam and then 72 hours later the skin was sectioned, and apoptotic and micronucleated cells were scored at different distances away from the irradiated plane. Significant numbers of damaged cells were observed up to 1 mm away from the targeted region[61]. More recently, these long-range bystander responses have also been extended to a lung reconstruct model in which levels of the phosphorylated histone variant γH2AX, which is used as a marker of DSBs, were found to be increased. Methylation and fractions of senescent cells were also observed to increase[62].

In vivo evidence of abscopal bystander responses are limited and in humans rely on anecdotal evidence (reviewed in REF. 16). However, it is interesting to speculate as to the underlying mechanisms involved. It is also important to put this in the context of what we know about how normal tissues respond to radiation exposure. For radiotherapy, when looking at normal tissue complications, the key assumption has been that the degree of effect is related to the volume of tissue irradiated, although many studies have shown that the relationship is much more complex[63]. Khan *et al.*[64], found that when rat lung was partially irradiated micronucleus formation was observed in other non-irradiated areas of the lung, indicating DNA damage at these non-irradiated sites. Pre-injection of animals with Cu–Zn superoxide dismutase (SOD) or the NOS inhibitor L-NAME led to a reduced response in the shielded area, indicating the involvement of ROS and NO[65]. This was accompanied by waves of macrophage activation and production of cytokines, including IL-1α, IL-1β, IL-6, TNFα and TGFβ, lasting up to 16 weeks after irradiation[66]. In mouse models, activated macrophages and T cells were shown to radiosensitize tumour cells through two bystander effect mechanisms. First, activated immune cells secrete cytokines, leading to NOS2 induction and endogenous production of the radiosensitizing molecule NO inside tumour cells. Second, activated macrophages produce NO, which diffuses to and radiosensitizes bystander tumour cells[67]. If these responses are proved in humans, it may be necessary to incorporate directional and geometrical information into calculations of normal tissue complication probabilities for lung; these are currently not

considered in the conventional dose–volume histograms used for therapy[68]. In humans, abscopal events have also been observed in patients, involving bilateral pneumonitis after unilateral irradiation[69], and these may also involve inflammatory responses. The cytokine involvement in these responses *in vivo* agrees with analyses using *in vitro* cell culture-based bystander signalling. Indirect macrophage responses have been reported in irradiated tissues and they may be key drivers of bystander signalling at the tissue level[70].

Further evidence of long-range bystander responses has come from localized irradiations in mice: irradiation (1 Gy X-rays) of one side of the body led to epigenetic changes in the shielded non-exposed side 1 cm away. Differential changes in the activity of DNA methyltransferases at the non-irradiated site were observed, suggesting that epigenetic regulation was involved in the aetiology of bystander responses[71]. In a further study, cranial irradiation of a higher dose (20 Gy) was used to determine the extent of distant effects in the spleen, which also showed epigenetic effects[72]. Further work has reported sex-dependent differences in response in these studies[73] and also a potential role for microRNAs[74]. Recent work has also suggested that these long-range effects may have a role in oncogenesis. In a series of studies in which mice were irradiated with the head shielded, significant levels of DNA DSBs, apoptotic cells and evidence of carcinogenesis were found in the cerebellum of *Ptch1* mutant mice, which develop cerebellar tumours resembling human medulloblastoma. These long-range effects involved GJIC in the central nervous system[75].

Other long-range interactions have also been reported between normal tissue and tumours in mice. Camphausen *et al.* irradiated the legs of mice (five fractions of 10 Gy) that had tumours transplanted at the dorsal midline. They observed reduced tumour growth rates when the leg was irradiated, with tumour growth inhibition decreasing when the radiation dose was reduced to 12 fractions of 2 Gy. The response was prevented when the drug α-pifithrin, which blocks p53, was given to the mice[76].

Studies with internally deposited radioactive materials have also reported evidence for bystander effects *in vivo*. When hamsters were injected with the α-particle emitters $^{239}PuO_2$ or [^{230}Pu]plutonium citrate, which concentrate in the liver, the induction of chromosome aberrations was independent of large changes in the local dose homogeneity when this was altered by injecting a range of particle sizes but maintaining a constant total dose to the liver[77]. A similar response was observed when the induction of liver tumours was observed[78]. Thus the authors suggested that the liver was responding to the total energy and total dose to the liver, not to the numbers of cells traversed by an α-particle or the local dose distribution[79].

Relevance of bystander responses to therapy

From our understanding of the mechanisms that underpin bystander signalling and the growing evidence for their role *in vivo*, it is clearly of interest to consider the relevance of bystander responses to cancer therapy.

Bilateral pneumonitis
Inflammation of lung tissue in both lungs.

Radionuclide therapy
The use of radioisotopes tagged to molecules or proteins for treating cancer

Dose rate
The amount of dose delivered per unit time.

As defined earlier, the term bystander response is used in the field of gene therapy, in which the requirement to increase cell killing beyond cells that have taken up vectors expressing bioactive or chemotoxic agents is crucial to efficacy. Understanding radiation-induced bystander responses may therefore highlight potential new therapeutic approaches that invoke mechanisms related to cell–cell communication of damage-sensing signals and allow amplification of cell killing effects. For example, recently it was shown that NO-dependent signalling is required in tumour cells to undergo radiation-induced bystander responses[51]. This observation coincided with efforts to use gene therapy approaches to introduce NOS2 into cells for therapeutic gain[80] and also to use radiation-inducible promoters[81] to drive NOS2 expression. Production of NO will affect larger numbers of cells than those originally transfected, increasing effectiveness. Combining gene therapy with targeted radionuclide therapy might therefore increase cell killing owing to bystander responses[82]. Clearly, more mechanistic and preclinical information is required, particularly under conditions relevant to radiotherapy, before this can be fully developed, but it does offer a rationale for the development of new approaches based on bystander mechanisms.

Factors modulating bystander responses. For the use of radiation therapy, we know of several key factors that modify response and determine overall efficacy of treatment. These include repair, cell cycle distribution, repopulation, reoxygenation and individual radiosensitivity[1]. Underpinning these factors are modulators of response, which include radiation quality, dose rate and fractionation schedule[1]. An important consideration is what role these would have on bystander responses, but in many cases information is limited or completely lacking. However, some clues are emerging; for example, several groups have shown evidence for some of the key DNA damage response and repair processes occurring in bystander cells[83,84]. Importantly, there may be differential DNA damage responses in direct and bystander cells that could be modulated in future therapies[85]. For example, in directly irradiated cells the kinases ataxia–telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK) and ataxia–telangiectasia and Rad3-related (ATR) have key roles such that inhibition of these DNA damage sensors increases radiosensitivity. In bystander cells, ATR is important, with ATM acting downstream. In contrast to directly irradiated cells, inhibition of ATR or ATM prevents the killing of bystander cells[85].
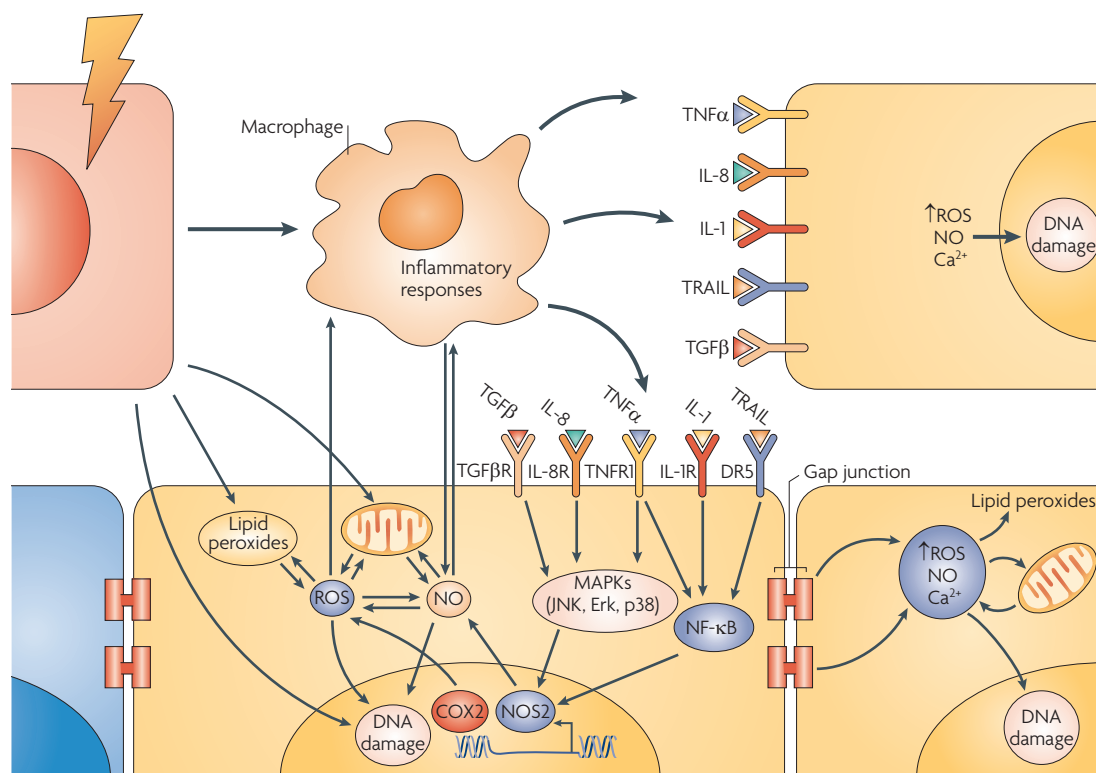

Figure 3 | **Key pathways affecting bystander signals.** Cells respond to direct radiation (red cell) by producing bystander responses through two key routes. One involves direct cell–cell communication through gap junctions and the second release of cytokine signals into the extracellular matrix. Not all cells respond (for example, the blue cell). *In vivo*, macrophages may be important mediators, which in response to radiation-induced tissue damage release bystander signals that affect non-irradiated cells (yellow cells). Some of the key pathways and mechanisms are now being elucidated, with roles for cytokine-mediated signalling, signal transduction through MAPKs and nuclear factor-κB (NF-κB) alongside the production of reactive oxygen and nitrogen species. COX2, cyclooxygenase 2; DR5, death receptor 5 (also known as TNFRSF10B); IL, interleukin; JNK, Jun N-terminal kinase; NO, nitric oxide; NOS2, NO synthase 2; ROS, reactive oxygen species; TGFβ, transforming growth factor-β; TGFβR, TGFβ receptor; TNFα, tumour necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand.
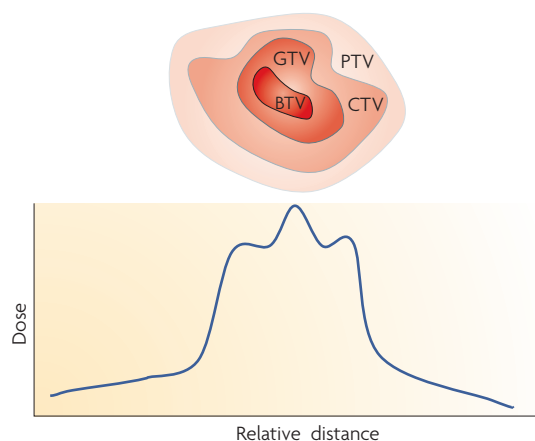
Figure 4 | **Defining tumours for external beam targeting.** For external beam radiotherapy it is usual to define the site of the tumour in terms of a gross tumour volume (GTV), which is where the tumour is located and includes a region of subclinical disease that is partially infiltrated by the tumour. Together this gives the clinical target volume (CTV), which is the volume in which there is a malignancy. The planning target volume (PTV) is then used to define the area that needs to be irradiated. In the future, it may be possible to define regions within the tumour, such as areas of hypoxia, and define a biological target volume (BTV) that could receive additional dose. A simplistic representation of the dose profile across the region is given, showing a low dose area with the PTV and an additional high dose area within the GTV, covering the BTV.

For radiotherapy, fractionation of the dose delivered to a tumour has long been used to improve the differential effect of killing tumour cells relative to normal cells, as splitting the dose allows time for normal cells to repair damage between fractions[1]. A similar effect occurs when the dose rate is reduced[1]. However, almost all studies of bystander responses have used relatively high-dose-rate, single-fraction exposures. One study has looked at the effect of repeated addition of medium from irradiated cells to bystander cells and also the effect of repeated dose exposures to the cells producing bystander signals as a way of mimicking fractionated exposures using a bystander signal. The authors reported that fractionated bystander treatments removed the conventional dose sparing that is observed after fractionated radiations due to the gap between fractions allowing time for DNA repair[86]. Another study has recently reported differences after changing the dose rate, albeit over a limited range[87].

With the significant advances in delivery of external beam radiotherapy, in particular three-dimensional conformal radiotherapy (3DCRT), it is now possible to deliver radiation to tumours with high physical reproducibility[88]. 3DCRT first involves the identification of the gross tumour volume (FIG. 4), representing the visible tumour (usually identified clinically by cross-sectional imaging by a computed tomography scan, for example). By adding a margin to encompass microscopic tumour extension, the gross tumour volume becomes the clinical target volume. The planning target volume is then used

to define the area that should be irradiated. The planning target volume is the clinical target volume plus a margin to accommodate for organ motion and deformation as well as errors in treatment set up. Radiotherapy treatment plans are assessed according to the homogeneity of dose to the planning target volume as well as by the dose to the organs at risk. These criteria are measured using dose–volume histograms. Considerable clinical data now exists for correlating dose–volume histogram criteria with radiation toxicity outcomes[89]. Currently, little consideration is given to the potential of bystander effects in the aetiology of toxicity.

Intensity-modulated radiation therapy (IMRT) is an evolution of 3DCRT in which modulation of the beam intensity in time and space permits shaping of the high-dose radiation volume to conform to complex tumours[90]. This technique results in the organs at risk being exposed to a lower dose. It could be hypothesized that a larger volume of irradiated tissue could have a higher risk of a significant bystander effect. Optimal targeting of the tumour can lead to steep dose gradients in the region of the planning target volume. In the future it may also be possible to apply dose escalation to specific areas within a tumour — for example, those that contain hypoxic or radioresistant regions — which would lead to an improved therapeutic outcome but limit the dose to surrounding normal tissue. This involves the delineation of a biological target volume for delivering additional dose to a tumour[91] (FIG. 4), which would generate significant dose gradients within the tumour. The benefits of the complex dose distributions made possible by the use of IMRT can only be fully realized by image-guided radiation therapy. This technology uses a variety of techniques to image target volumes while the patient is being treated on a linear accelerator. By reducing or eliminating set-up error, and ensuring the precision and reproducibility of radiation delivery, image-guided radiation therapy technology enables a reduction in the margins that are added to the clinical target volume to create the planning target volume[92]. This degree of precision is particularly important when in-field boost volumes such as biological target volume are being treated using IMRT.

Little is known about radiation response within dose gradients and whether bystander responses are involved. In recent elegant work[93], Suchowerska and colleagues have started to address the effectiveness of dose gradients. They have measured survival in cells exposed to a 6 MV X-ray intensity-modulated beam, in a three compartment flask such that cells could be irradiated separately in the three sections or share the same medium. They exposed the flasks to a modulated dose defined by a wedge placed in the beam path. Cells that could communicate (that is, shared the same medium) gave poorer survival than predicted after low-dose exposure, but higher than expected survival after high dose exposure. This effect was abolished when communication was prevented, suggesting significant modulation of response in situations in which dose gradients are present. In further studies they compared the effects of a uniform field with those when 25% of a flask was exposed either as a single region or as three

Intensity-modulated radiation therapy
IMRT. An advanced mode of radiotherapy that uses multiple modulated beams in which the intensity is varied to allow maximal conformation of the beam delivery to the tumour in three dimensions.

Linear accelerator
A device for the acceleration of subatomic particles that can produce electron beams for radiotherapy.

parallel stripes. Survival was dependent on a complex interplay between the fraction of shielded cells and the dose to the exposed areas, again indicative of bystander signalling having a significant effect[94].

Other examples of localized dose delivery are proton and other heavy ion particle therapies that provide the ability to deliver high radiation doses to precise volumes with resultant high dose gradients, although nothing is known regarding the role of bystander responses in the clinical setting[95]. Studies in animal models have used microplanar X-ray microbeams as a way of differentially targeting brain tumours with high dose while sparing normal tissue[96] and have shown significant evidence for bystander responses[97].

The second issue related to the potential role of bystander responses is related to radiation protection and carcinogenesis. In most external beam therapies, multiple beams are delivered from different directions to maximize the dose to the tumour and minimize the exposure to normal tissues. Two consequences of this require discussion with respect to bystander responses. A consequence of the use of IMRT is that a greater volume of normal tissue is exposed to low-dose radiation than in conventional radiotherapy. This is largely a consequence of the use of multiple beams, as well as the increased time required to deliver the prescribed dose. Newer evolutions of modulated beam therapy technology that involve rotational delivery of radiation including Tomotherapy (Tomotherapy, Inc.), Rapid Arc (Varian Medical Systems) and volumetric modulated-arc therapy (VMAT; Elekta) all result in larger irradiated volumes than conventional 3DCRT or finite field IMRT. It has been postulated that this large volume of low-dose exposure may lead in the longer term to a 2–3-fold increase in secondary cancer rates. It is clearly of relevance to determine whether these changes in the delivery of radiation have consequences for patient outcomes[98].

Radionuclide-mediated responses. Another area in which bystander effects may have a significant role is with the use of therapeutic radionuclides. A range of studies have determined the role of bystander responses after treatments with various radionuclides both *in vitro* and *in vivo*. For experimental studies testing for radionuclide-induced bystander responses, it is an important challenge to ensure that no radioactivity is incorporated into cells that would otherwise be defined as bystander cells. This is especially crucial given the evidence from external radiation studies showing that bystander responses are essentially a low-dose phenomenon.

The earliest studies on radionuclide-induced bystander responses used ³H-labelled DNA produced by incubation of cells with [³H]thymidine. The mean energy of the β-rays is 5.67 keV and their mean range is 1 μm. Bishayee and colleagues compared the effectiveness of inactivation of radiolabelled cells in small multicellular spheroids typically of 1.6 mm diameter consisting of 4×10^6 V79 cells. They saw greater effectiveness (measured as loss of clonogenic survival) under conditions in which only 50% of the cells were labelled than was predicted from 100% labelled cells, which they concluded

was due to a bystander response. They also tested for a role for GJIC using lindane and found evidence for direct cell–cell communication in this model[99].

Bystander responses after radionuclide incorporation have also been reported *in vivo*. In a sophisticated protocol, human colon LS174T adenocarcinoma cells were pre-labelled with [¹²⁵I]uridine and injected subcutaneously into nude mice with a mixture of non-labelled cells. Under these conditions with 1:1 and 1:5 ratios of labelled to unlabelled cells, significant tumour regression derived from the unlabelled cells was observed[100]. In further studies, they compared the effects of ¹²⁵I-labelling with ¹²³I-labelling strategies in the same *in vivo* tumour model. They reported an inhibitory bystander response for ¹²⁵I-labelling, but a stimulatory bystander response was observed for ¹²³I-labelling, which was confirmed from *in vitro* studies. The reasons for these differences are unclear, as both radionuclides produced short range auger electron cascades. Interestingly, however, there are significant (~100-fold) differences in dose rate owing to the differences in half-life (¹²³I has a half-life of 13.3 hours and ¹²⁵I of 60.5 days).

These discrepancies for different radionuclides in effectively the same biological model are indicative of the need to more carefully compare different radionuclide-mediated bystander responses in comparison with external beam exposure. In a recent defining study, Boyd and colleagues[101] compared the effect of an external radiation-mediated bystander response with different radionuclide approaches. In particular, they compared three different halogenated analogues of *m*-iodobenzylguanidine (MIBG). MIBG is selectively taken up into cells expressing the noradrenaline transporter (*NAT*, also known as *SLC6A2*) gene. The authors compared the effectiveness of the β-emitter [¹³¹I]MIBG with the auger electron emitter [¹²³I]MIBG and the α-emitter [²¹¹At]*m*-astatobenzylguanidine ([²¹¹At]MABG) in two tumour lines transfected with *NAT*. For external beam irradiation followed by medium transfer onto non-irradiated cells a significant bystander response measured as a loss of clonogenic survival was observed. As found for other studies with external radiation approaches, the degree of bystander response increased at low dose and then saturated at ~60–70% survival in the two cell lines. This was in contrast to the studies with radionuclides for which, although bystander responses were detected, no saturation was observed. For [¹³¹I]MIBG, a significant bystander response was detected that increased in proportion to the activity added to the directly exposed cells, leading to killing of 70–80% of the bystander cells. By contrast, treatment of cells with either [¹²³I] MIBG or [²¹¹At]MABG led to an increased cell kill in recipient bystander cells up to a maximum of 35–70% but the effect decreased with increasing activity, leading to U-shaped response curves. These studies suggest there may be important linear energy transfer differences in the response of cells to bystander factors produced in response to radionuclide incorporation and that the types of bystander responses induced may be distinct from those observed after external radiation studies. One possibility is that the design of these studies may also be highlighting

Heavy ion particle therapy
The use of accelerated beams of high-atomic-mass elements (for example, carbon) for therapy.

Microplanar X-ray microbeams
Parallel beams of X-rays only a few micrometres across that are used for localized irradiation.

Auger electron cascades
Decay of radioactive isotopes by K-shell electron capture leads to the Auger effect, resulting in the loss of several orbital electrons.

important dose rate dependencies of bystander responses that have to date not been extensively explored with external radiation approaches.

It is important to speculate on the consequences of the observation of bystander responses after radionuclide treatments for therapy. Significant advances are being made in the use of targeted radionuclides. These include, for example, the ability to target small metastatic regions that are not accessible with conventional external beam approaches and the development of useful biological targeting strategies to give tumour cell specificity[102–104].

Earlier studies have predicted that the use of radionuclides that produce electrons that have relatively long ranges and interact with multiple cells would be beneficial owing to radiological crossfire. For example, studies in multicellular spheroids have shown that the effectiveness of [131I]MIBG is twice that observed in cell monolayer studies owing to significant crossfire from the long range of the β-rays[105]. If recent experimental studies are extrapolated into a tumour killing situation it becomes clear that a radiobiological bystander response as well as crossfire effects could be important in producing additional cell kill. Future therapies involving radionuclides need, *a priori,* to consider the effect of bystander responses in overall outcome. The suggestion that dose rate may be important needs to be further defined for both external beam and radionuclide exposures, as this may even affect our use

and development of brachytherapy approaches. To date we have bystander information on a limited range of radionuclides despite the large range of potential candidates for therapy[106]. We also do not know the consequences of low-dose exposure to radionuclides under conditions in which bystander responses may occur. If the robust bystander responses reported *in vitro* translate *in vivo*, this could affect the use of radionuclides for therapeutic and imaging approaches in the longer term. However, more study of the role of cell–cell communication in a range of biological contexts is required for this to be fully elucidated.

Implications for current and future therapies

For the future, further insight into the mechanisms underpinning bystander signalling is required if potential targets for therapy are going to be developed. Alongside this, a crucial appreciation of the relevance of bystander responses in radiation-induced carcinogenesis is required.

The current understanding of the role of radiation-induced bystander responses has to be seen in the context of our knowledge of how cells communicate and their integration in tissue level responses to localized and systemic therapies. For radiation-based therapies this means that molecular pathways and targets outside directly exposed fields could contribute to a therapeutic response. Future therapies will need to be optimized for tissue and tumour level responses to include differential effects that are mediated by intra- and inter-tissue signals if these are to affect treatment outcome. For targeted radionuclide approaches the modulation of bystander responses probably holds out the best opportunities in the near term (FIG. 5). This is not least because an analogy can be strongly made to the relevance of bystander responses in gene therapy. If the approaches taken in the gene therapy area are to be extrapolated to radionuclide therapies, further *in vivo* studies are needed. This requires not only further insight into molecular mechanisms and the highlighting of specific targets but also a greater appreciation of the interrelationships between specific activity, dose rate and radiological crossfire versus bystander responses. The observation of bystander responses after exposure to chemotherapy agents may also assist in the development of approaches for maximising radionuclide therapies and also enhancing external beam-mediated responses by maximizing their contribution to tumour cell kill.

For external beam therapies, the challenge is to define the physical ranges of bystander responses at the cellular, tissue and whole body levels and relate these to specific mechanisms. Development of strategies to maximize bystander responses in tumours and to minimize their expression in normal tissues will require further mechanistic information of potential molecular targets so that informed choices can be made. With the continuing developments in the delivery of radiotherapy and combined imaging, the possibility of dose-painting approaches urgently requires information on bystander signalling under conditions in which steep dose gradients exist, in particular IMRT, Tomotherapy and heavy ion particle therapy, to more carefully define their potential effect on both radiation effectiveness and radiation risk (FIG. 5).
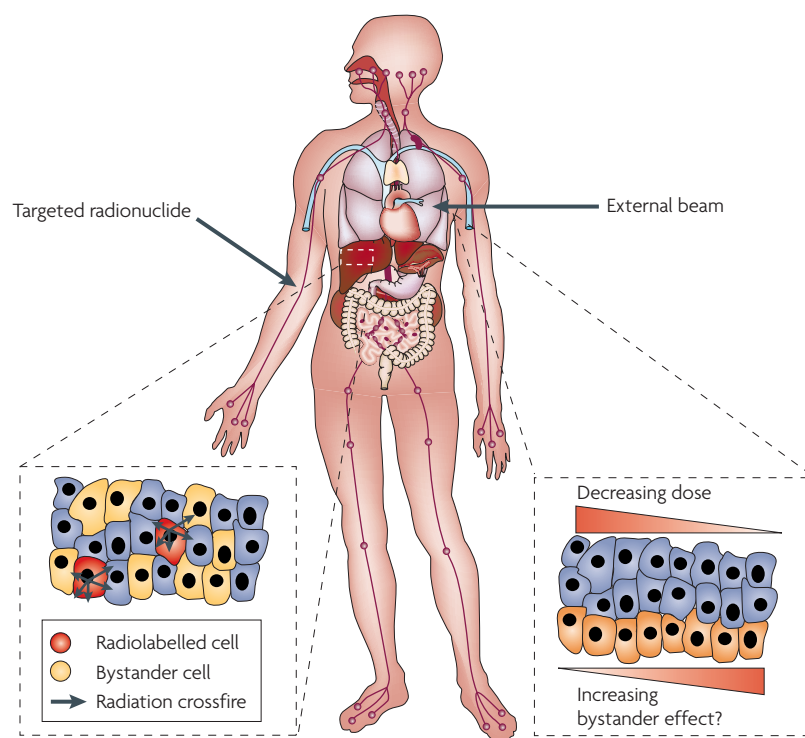

Figure 5 | **Treating cancer with radiation.** The figure shows the two potential routes by which bystander responses may affect clinical therapies. For external beam therapies (such as intensity-modulated radiotherapy), dose gradient-dependent responses may influence the effect. Tumour heterogeneity may also lead to non-linear responses within the treatment field and to longer-range, abscopal or systemic effects. For radionuclide approaches (such as those tagged to monoclonal antibodies), the signals from a few labelled cells may be amplified by bystander signals within tumours and may also have long-range, abscopal or systemic effects.

Linear no-threshold hypothesis
A model used for radiation protection that aims to describe the relationship between radiation dose and risk, a linear relationship that has no dose threshold for increased risk.

A key question is the role that intratumoural factors that may affect clinical response — such as hypoxia, proliferation and intrinsic radiosensitivity — affect intercellular and longer-range signalling mechanisms. As radiation is predominantly given to patients as part of multimodality therapies, the role of systemic responses resulting from these treatments and their effect on long-range effects is completely unknown.

The observation that bystander responses predominate after low-dose exposure implies that additional effects would be predicted. There has been considerable debate as to their relevance to radiation risk at low doses with some authors suggesting that they affect the current use of the linear no-threshold hypothesis for risk estimation[107]. This has led to the proposal that low-dose exposures may be considerably more active than previously thought and could, for example, affect secondary cancer rates after external beam therapies[98]. However, given the paucity of *in vivo* data for bystander responses in exposed individuals it is premature to make fundamental predictions, although recent data on carcinogenesis from animal models support an important role in this process. Extensive study is now required *in vivo* to quantify responses under relevant dose exposure scenarios, not just for environmental and occupational exposures but for clinically relevant dose and dose distributions at the tissue and whole-body level.

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