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REVIEW ARTICLE

The importance of the vascular endothelial barrier in the immune-inflammatory response induced by radiotherapy

Running title:
The endothelium as a checkpoint of immune response in radiotherapy

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ABSTRACT

Altered by ionizing radiation, the vascular network is considered as a prime target to limit normal tissue damage and improve tumour control in radiotherapy. Irradiation damages and/or activates endothelial cells, which then participate in the recruitment of circulating cells, especially by overexpressing cell adhesion molecules, but also by other as yet unknown mechanisms. Radiation-induced lesions are associated with infiltration of immune-inflammatory cells from the blood and/or the lymph circulation. Damaged cells from the tissues and immune-inflammatory resident cells release factors that attract cells from the circulation, leading to the restoration of tissue balance by fighting against infection, elimination of damaged cells and healing of the injured area. In normal tissues that surround the tumours, the development of an immune-inflammatory reaction in response to radiation-induced tissue injury can turn out to be chronic and deleterious for the organ concerned, potentially leading to fibrosis and/or necrosis of the irradiated area. Similarly, tumours can elicit an immune-inflammation reaction, which can be initialized and amplified by cancer therapy such as radiotherapy, although immune checkpoints often allow many cancers to be protected by inhibiting the T cell signal. Herein, we have explored the involvement of vascular endothelium in the fate of healthy tissues and tumours undergoing radiotherapy. This review also covers current investigations that take advantage of the radiation-induced response of the vasculature to spare healthy tissue and/or target tumours better.

INTRODUCTION

All tissues can be damaged by ionizing radiation beyond a dose-volume threshold.[1] The damage results from the initial deposition of energy within the tissue. The processes of radiation injury begin at once after the irradiation, but some of the clinical and histological signs, such as necrosis and fibrosis, become visible weeks, months, or even years after a therapeutic treatment.[1,2] Damage is characterized by cell death, phenotypic changes, immune-inflammatory cell infiltration, and vascular and tissue remodelling, which can lead to chronic injury and ultimately necrosis.

Radiotherapy (RT) is used to treat a variety of cancers, as well as benign tumours, in more than half of patients with tumours.[3] Despite great advances in radiation dose delivery techniques, the therapeutic index of RT is still limited by normal tissue injury in organs at risk and by the radiation resistance of some tumours.[4] New approaches to optimize the response of normal tissue and tumours thus remain essential for improving the outcome of RT, by increasing the likelihood of cancer cure or by decreasing normal tissue toxicity, or both.[5,6]

In the vessels, the endothelium is a key cell compartment for the response to ionizing radiation of healthy tissue and tumours, and represents a promising target to improve the differential effect of RT in the future.[6,7] Following radiation exposure, the global endothelial cell response covers a wide range of molecular changes with a global expression pattern of modifications.[8,9] Changes occur at the transcriptional, translational and post-translational levels and impact cell phenotype as well as the microenvironment by production and secretion of soluble factors such as reactive oxygen and nitrogen species (respectively ROS and RNS), chemokines, cytokines and growth factors. These radiation-induced dynamic modifications of molecular networks may control the endothelial cell phenotype and govern recruitment of immune cells.

Ionizing radiation induces an inflammatory response in organs[10] and tumours[11] characterized by immune-inflammatory cell infiltration. Vascular endothelium plays an integrative role in the tissue response following stress and controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis and activation of leukocytes in the periphery.[11,12] The development of this inflammatory response is regulated by a complex process that involves leukocyte-endothelium interactions composed of activation, rolling, adhesion and transmigration in the surrounding tissue[13] (**Figure 1**). On the other hand, engaging the immune system for optimal anti-cancer therapy is an attractive contemporary concept.[14] Promising current strategies generate an effective immune response to destroy the tumour in combination with RT.[15] In this context, control of the adaptive immune response by the tumour endothelium is a crucial process.

THE VASCULAR ENDOTHELIUM

A healthy endothelium is in a well-balanced state between pro- and anti-oxidants, vasodilators and vasoconstrictors, pro- and anti-inflammatory molecules, and pro- and anti-thrombotic signals. It provides key functions in angiogenic and inflammatory processes, which are activated and finely regulated when required. As can happen in the case of inflammation, breakdown of this complex

balance leads to a diseased or pathological endothelium displaying pro-oxidant, vasoconstrictor, pro-inflammatory and pro-thrombotic properties.

Role in immune-inflammatory cell recruitment

The endothelium is able to activate a global molecular program in physiological conditions or in response to stress and serves as a key checkpoint to control the immune response. The adhesion of leukocytes to the vascular endothelium is one of the major characteristics of inflammation. This process involves glycoproteins which allow the adhesion of circulating leukocytes in the blood stream to endothelial cells. The objective of this adhesion is the passage of leukocytes from the bloodstream to the injured site. The endothelial cell-leukocyte interaction is the result of a large number of physical (shear forces), chemical (weak interactions and impairment of nitric oxide [NO] production) and biological (proteins and specific glycoproteins, substrates and cytokines) factors. Cell recruitment takes place through a cascade of seven events consisting of capture (or tethering), rolling, slow rolling, arrest, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular transmigration[13] (**Figure 1**). The multiplicity of molecular choices for each of the stages provides a great diversity of very specific combinations of leukocyte recruitment that makes it possible to adapt recruitment to the type of tissue and injury.[13,16]

Cell adhesion molecules (CAMs) and activation of integrins are at the heart of the adhesion process following selectin ligand recognition. Modulation of adhesion occurs through the action of chemical and biological factors released by injured tissues, endothelial cells, leukocytes and innate lymphoid cells. For instance, the degranulation of activated mast cells releases histamine, leukotrienes and platelet activating factor (PAF). Histamine then activates selectins, which allows the capture of leukocytes and the initiation of rolling. Subsequently, leukotrienes and PAF participate in the activation of integrins by increasing their avidity and expression, thus helping to maintain rolling and firm adhesion. On the other hand, cytokines and chemokines induce the transcription of endothelial CAMs such as selectins, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Activated endothelial cells also produce excess ROS, which results in the activation of oxidative stress-sensitive transcription factors such as the nuclear factor-kappa B (NF-κB) or the activator protein 1 (AP-1). Interestingly, AP-1 also directs endothelial cells towards a pro-adhesive leukocyte phenotype in an inflammatory environment.[17] Conversely, NO, prostaglandin I2 (PGI2) and adenosine are endogenous anti-adhesive factors.[17]

Importance of the endothelial glycome in endothelial-immune cell interactions

Modifications of the glycan profile, whose entire spectrum defines the glycome, alter the function of proteins and participate in cellular functions, notably adhesion and cellular communication in endothelial cells. Selectins are specialized protein receptors for leukocyte glycoconjugates that mediate tethering and rolling of leukocytes under flow in inflamed vascular beds.[18] Endothelial cells have many glycoconjugates on their surface that are regulated in an inflammatory context and are involved in interactions with circulating cells (**Figure 1**).[19] These glycan structures can thus be considered as entry points for the immune system. In addition, the endothelial glycocalyx, a

covering composed of a layer of polysaccharides covalently bound to the lipids and proteins of the membrane, also plays an important role in the immune-inflammatory response, especially via its role in the leukocyte-endothelial interaction.[20] Following an acute or chronic injury, pro-inflammatory stimuli, such as TNF-α, induce shedding of glycosaminoglycans (GAGs), thereby decreasing the width and size of the endothelial glycocalyx.[21] This allows greater accessibility of endothelial glycoprotein epitopes, on which circulating leukocytes roll and to which they adhere.

RADIOBIOLOGY AND RADIOPATHOLOGY OF THE VASCULAR ENDOTHELIUM

Ionizing radiation has multiple effects on the endothelium, which is thought to contribute to the initiation and progression of radiation-induced damage to healthy tissues following RT.[22] Among the main effects are radiation-induced endothelial cell death, loss of thromboresistance, cell activation and secretion of soluble factors such as cytokines and growth factors.[23] Vessels of normal tissues surrounding the tumours and of the tumours themselves are both impacted by ionizing radiation during RT. In most cases, normal and pathological tissues comprise genetically normal endothelial cells. In tumours, however, vascular cells undergo the influence of the tumour environment and display different phenotypes, and sometimes different genotypes, from those of endothelial cells from normal tissues. **Figure 2** shows a schematic view of the effects of ionizing radiation on the vascular endothelium, which are detailed below.

Differential effects of various forms of radiotherapy on endothelial damage

External beam RT using photon radiation, and especially X-rays, is used in almost all RT treatments. However, particular indications can use high linear transfer energy (LET) particle RT with protons or carbon ions (hadrontherapy), as well as internal RT with radioactive sources of gamma-rays (brachytherapy) and systemic RT using targeted radionuclide therapy (radioimmunotherapy). Although not investigated in depth, the effects of gamma- and X-rays on endothelium are supposed to be similar because of their equal relative biological effectiveness (RBE). On the other hand, it is conceivable that differences in endothelial cell response may occur as a function of dose rate, energy spectrum and fractionation schedule. More likely, radioimmunotherapy and hadrontherapy may have very different effects on endothelium compared to photon radiation, because of their high LET radiation properties. However, most data are available on the effects of low LET radiation and data on high LET radiation are scarce. For example, the impact of carbon and Fe ion irradiation on the response of endothelial cells has been compared to X-ray irradiation, showing a differential effect of high LET radiation compared to photon radiation.[24,25] Interestingly, C-ions seemed to be more effective in damaging endothelial cells than X-rays shortly after exposure, but late damage was not found at the doses used (0.25-1.5 Gy).[25] On the other hand, Fe ions did not significantly induce inflammation, in contrast to X-rays, but the radiation impact on gene and protein expression was more marked and longer lasting for Fe ions than for X-rays.[24]

Endothelial cell apoptosis and senescence

Apoptosis is a frequent cell death process after irradiation.[26] Exposure of healthy tissues to a high dose of ionizing radiation (≥10 Gy) induces an acute endothelial reaction characterized by a rapid wave of endothelial apoptosis.[22,27] Yet, the key role and the existence itself of radiation-induced endothelial apoptosis in normal tissue lesions remain controversial. Endothelial apoptosis has been shown to be the primary event responsible for gastrointestinal syndrome after whole-body irradiation at 15 Gy in mice.[28] Despite contradictory results, which have been discussed elsewhere,[29] the participation of endothelial apoptosis in the deleterious tissue effects after irradiation is still widely accepted.

Endothelial cells also experience stress-induced premature senescence *in vitro* and likely *in vivo*.[30] Senescent irradiated endothelial cells can accumulate in tissues, promoting age- and therapy-related disorders, including cardiovascular diseases. Through secretion of many inflammatory mediators and extracellular proteases, called the senescence-associated secretory phenotype (SASP), the senescent phenotype causes chronic inflammation and disruption of tissue structure and function. The surviving irradiated cells likely participate in the development of a dysfunctional vascular phenotype. In the early phase, this is marked by excessive secretion of pro-inflammatory cytokines, increased recruitment of circulating cells such as platelets and lymphocytes, activation of the coagulation system, and increased vascular permeability.[22] In the late phase, a collapse of the vessels is observed, associated with a reduction in the thickness of the basal membrane and the persistence of a pro-coagulating, pro-inflammatory and potentially senescent endothelial phenotype.[31]

Endothelial activation

Ionizing radiation triggers changes in the phenotype of endothelial cells that lead to endothelial activation and then potentially to endothelial dysfunction. Endothelial activation is defined as the endothelial expression of adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin.[32] It is usually triggered by pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α or interleukin (IL)-6. Loss of NO also leads to increased endothelial cell activation, which can then cause endothelial impairment, including dysfunction of vascular tone and chronic activation of the coagulation and immune-inflammatory systems. Radiation-induced endothelial activation results in increased expression of adhesion proteins such as VCAM-1, ICAM-1, PECAM-1, E-selectin and P-selectin which participate in the recruitment of circulating cells.[33-36] Irradiation leads to increased platelet-endothelial interactions both *in vitro* and *in vivo*[36,37] by a mechanism that involves PECAM-1. Recently, like chronic inflammation induced by TNF-α or in the context of atherosclerosis,[38,39] it has been shown that irradiation modifies the glycosylation pattern of endothelial cells, causing an increase in monocyte adhesion.[40] *In vivo*, an increase in endothelial expression of ICAM-1 and VCAM-1 has been shown in a model of intestinal inflammation induced by radiation.[41] Interestingly, ICAM-1 knock-out mice exhibited less severe pulmonary and intestinal inflammation than wild-type mice,[42] suggesting that cell infiltration could be deleterious in this context. In human subjects, the endothelium can be activated by therapeutic radiation[43,44] through activation of NF-κB, which is likely essential in the genesis of cardiovascular disease induced by RT.[45,46] These studies have

overall demonstrated that the increase in expression of adhesion molecules by endothelial cells after irradiation plays a decisive role in the recruitment of circulating cells and thus in the radiation-induced inflammation of the tissue and/or the tumour, with a potential deleterious effect on normal tissues.

Endothelial cell-derived extracellular vesicles

Cell-derived extracellular vesicles (EVs), comprising exosomes and microvesicles, have emerged as important mediators of a new mode of intercellular communication.[47,48] Like all cells, endothelial cells are able to secrete extracellular vesicles. These endothelial vesicles have been shown to play a variety of roles in the human body, ranging from contributing to cardiovascular disease to promoting endothelial cell survival.[49] Ionising radiation has been shown to induce production of extracellular vesicles by both primary and tumour cells.[50-54] Endothelial injury by ionising radiation elicits the release of microparticles, in correlation with apoptosis and ROS formation, which can be inhibited by antioxidative treatment.[55] It is now crucial to clarify the role of these endothelial microparticles in the context of irradiation as they may act locally or far from the irradiation site as mediators of angiogenesis, inflammation, coagulation or injury, certainly through their protein and/or microRNA content.[56]

Disruption of the endothelial cell-cell barrier after radiotherapy

RT is known to induce vascular permeability. Several studies are concordant with an increase of albumin leakage after single-dose irradiation from 2 Gy to 30 Gy or more. The increase in permeability is transitory, varies from one day to one week after RT and penetration depth from vessels varies by a factor 1.5 to 6, depending on the dose.[57,58] In microvascular endothelial cells, cytoskeletal changes mediated by RhoA-guanosine triphosphatase and Rho kinase are implicated in the redistribution of basal VE-cadherin and intercellular junctions.[59] Interestingly, mast cells may contribute to cutaneous microvascular hyperpermeability, as shown in an animal model of localized irradiation.[58] Fractionated RT has also been shown to increase vascular permeability, but not as a result of a lack of junction proteins. In contrast, ICAM-1 and Zo-1, which are involved in the endothelial barrier, are more expressed after fractionated RT.[60,61] It should be noted that microvascular permeability induced by fractionated RT seems to be more durable than that induced by single-dose RT.[62,63]

Endothelial tight junctions form the impermeable blood-brain barrier (BBB). In BBB models, microvascular permeability is increased in a dose-dependent manner after single-dose irradiation.[64] This increase is not directly explained by a change in tight junction protein expression or by basal lamina lesion, since the expressions of Zo-1 and the endothelial barrier antigen are not affected at doses below 20 Gy.[64,65] The increase in permeability could be explained by changes in the cytoskeleton of endothelial cells. In a clinical context of fractionated RT for the treatment of brain tumours, the radiation-induced disruption of the BBB has been widely demonstrated.[66-68]

Radiotherapy and vascular changes in tumours

RT significantly affects the state and function of blood vessels. The severity of vascular damages depends on the RT protocol used, *i.e.* the number of fractions, the dose rate and the total dose of radiation applied.[69] In solid tumours, the overproduction of pro-angiogenic molecules in response to RT results in the dilatation of microvessels, the disruption of the endothelial lining, more branches, and overall a very irregular blood vessel architecture. At the cell level, the blood capillaries are incompletely matured, the perivascular cells are absent or detached, the basement membrane is absent or abnormally thick and endothelial cell junctions are absent. This impaired vascularization is associated with deregulation of tissue oxygenation which finally leads to hypoxic areas in the tumour. As a consequence, the lack of oxygen lowers the amount of radiation-induced ROS, ultimately leading to decreased RT efficiency.[70]

Observations that transplanting cancer cells into a previously irradiated site in mice resulted in slower growth of the subsequent tumour led to the concept of "tumour bed effect",[71] especially following large doses of radiation (10 to 20 Gy and more). Suggestions were made that vascular damage could impact the ability to regrow after irradiation. More recently, it was demonstrated that microvascular damage, *i.e.* endothelial cell apoptosis, regulates the response of tumour cells to radiation at the clinically relevant dose range.[72,73] However, the involvement of endothelial cells in the tumour response remains controversial.[74] In SCID mice, in which the *scid* mutation radiosensitizes endothelial cells, tumour growth was not affected by radiation compared with wild-type mice, suggesting that the vasculature did not play a significant role in tumour response to radiation in this model.[75] Also, a specific deletion of *atm* in endothelial cells made them more sensitive to ionizing radiation in sarcomas,[76] but failed to enhance sarcoma eradication, unlike specific deletion of *atm* in tumour cells which increased sarcoma eradication.[77] These studies have strongly suggested that tumour cells, rather than endothelial cells, are critical targets for sarcoma eradication by RT delivered in high single dose, like doses delivered in stereotactic body radiation therapy (SBRT), although an enhanced growth delay was observed in the tumours with the more sensitive endothelial cells.

As a consequence of endothelial sensitivity to radiation, the irradiated tumour microenvironment (TME) undergoes changes in vasculature and oxygenation rates. For large single doses over 10 Gy, microvascular density is impaired at least in a transitory manner. The decrease in vessel density corresponds to an alteration of blood perfusion and to an increase in hypoxic zones as shown in different tumour models.[78-81] Nevertheless, for repeated doses, the radiation schedule seems to matter in a more important way. A vascular density reduction is also observed but is not systematically followed by an increase in tumour hypoxia. In a clinically relevant pattern of repeated doses from 2 to 15 Gy per fraction, the microvascular density reduction resembles vascular normalization rather than destruction. Unlike using large single doses, repeated RT leads to transitory or durable hypoxia reduction in pulmonary, prostate, and head and neck cancer models.[78,80,82]

On the other hand, fractionated RT impacts pericyte coverage of blood vessels and blood vessel functionality in the TME. Pericytes are helpful to stabilize microvessels and regulate their permeability in healthy tissues.[83] They are less frequent and more loosely associated with

endothelial cells in tumours. Also, pericytes are involved in tumour hypoxia reduction[84] and cancer metastatic diffusion.[85] Interestingly, pericytes are also involved in transmigration and trafficking of immune cells into tumours.[86] Several studies show that after repeated doses from 2 to 12 Gy, pericyte coverage and blood perfusion are improved despite microvascular density reduction.[61,78,80,87] As a consequence, immune infiltrate and immunotherapy efficacy should be enhanced.[88] Tumour blood perfusion is likely multifactorial and dependent on the quality of blood supply as well as cell density and metabolism.[89] The consequences of single-dose RT on pericyte coverage remain unclear and deserve to be better known. After 12 Gy, pericyte coverage and perfusion decreases in a neuroblastoma model[79] and after 20 Gy in a fibrosarcoma model,[81] whereas it increases after 12 or 14 Gy in pulmonary tumour models.[80,90]

Radiation-induced vascular changes in normal tissues

Endothelial apoptosis, increased vascular permeability, cell activation and recruitment of inflammatory cells as well as activation of the coagulation system[91] are all early phenomena contributing to the induction and progression of radiation-induced tissue damage. Late vascular lesions, such as fibrosis and luminal reduction, also generate areas of tissue hypoxia that contribute to and amplify radiation-induced healing.[91] On the morphological level, the vascular lesions are different depending on the size of the vessels. Microvasculature is considered to be the most radiosensitive part of the vasculature. Capillary ruptures and dilatations, hypertrophy and detachment of endothelial cells from the basal lamina as well as thrombosis are observed.[91] In the case of large vessels, it is very difficult to distinguish, at the morphological level, the vascular lesions of conventional atherosclerosis from radiation-induced vascular lesions. The latter are characterized by vascular fibrosis with luminal reductions, excessive extracellular matrix deposition in the media and adventitia, neointimal hyperplasia and thrombus formation.[92] The histological similarities between radiation-induced vascular lesions and atherosclerotic lesions suggest that similar initiation and progression mechanisms could be involved.[93] At the endothelial cell surface, thrombomodulin downregulation, observed very early after irradiation, persists chronically in both rats and humans.[94-96] The endothelium is in a chronic procoagulant state, which may contribute to the long-term persistence of deleterious effects of irradiation. The induction of ICAM-1 expression in irradiated endothelial cells and an increase in neutrophil adhesion are maintained more than ten days after irradiation, suggesting a pro-inflammatory endothelial cell phenotype that persists over time.[97] *In vivo* studies also suggest that vascular damage contributes to radiation-induced fibrosis. In a pulmonary radiation-induced fibrosis model in rats, significant hypoxia associated with severe fibrosis six months after irradiation could result from damage to endothelial cells, interstitial oedema and vascular dysfunction.[98] Recently, it has been shown that endothelial hypoxia-inducible factor (HIF)-1α deletion confers resistance to radiation-induced enteritis, whereas similar deletion in intestinal epithelium does not, suggesting new functions of endothelial hypoxia-inducible factor-1α-signalling pathways as mediators of mucosal inflammatory processes.[99] On the other hand, it has been demonstrated *in vivo* that the endothelium is directly involved in the progression of radiation-

induced enteritis by using a mouse model harbouring an endothelium-specific deletion of the *serpin 1* gene, which encodes the plasminogen activator inhibitor-type 1 (PAI-1).[100]

IMMUNOLOGICAL CONSEQUENCES OF RADIOTHERAPY

RT has immune-inflammatory effects on both tumours and healthy tissues. **Figure 3** summarizes these effects, which are discussed below.

Consequences in tumours and healthy tissues

Radiation induces changes to the tumour cell immunophenotype and immunogenicity by damaging DNA and membranes, and by production of cytoplasmic ROS, which activate many transcription factors and signalling pathways.[101] RT promotes the presentation of tumour antigens by tumour cells by increasing the cell pool of specific antigens and by stimulating the expression of the major type 1 histocompatibility complex (MHC class 1).[102] It also generates immunogenic cell death, i.e. the dispersion of immune-stimulating tumour antigens from dying cells into the surrounding milieu,[103] through the release of damage-associated molecular patterns (DAMPs).[104,105] The activated dendritic cells then present the tumour antigens to the naive T lymphocytes in the lymph nodes allowing the formation of cytotoxic T lymphocytes specific for the tumour antigen.[106] RT facilitates the recruitment of these effector T lymphocytes by generating the secretion of the chemokine CXCL16 and endothelial expression of the cell adhesion ICAM-1, VCAM-1 and E –selectin.[97,107] These processes finally trigger an immune response, which is accompanied by a pro-inflammatory reaction, with release of IL-1, IL-2, IL-6, IL-12, interferon (INF)-α and IFN-β, and TNF-α, which are involved in amplification of the anti-tumour immune response.[105]

In normal tissues injured by radiation, release of DAMPs and secretion of cytokines and chemokines also activate the immune system. This phase moves to an acute inflammatory phase characterized by an activated pro-inflammatory response and vascular leakage. In this phase, the recruitment of diverse immune cells of myeloid and lymphoid origin, which is associated with a perpetual cytokine/chemokine cascade,[108,109] leads to various degrees of inflammation and symptoms. Lymphocyte subpopulations such as T_H1, T_H17, and possibly innate lymphoid cells, can contribute to inflammation, while T_{reg} could be needed to control damaging and excessive pro-inflammatory responses. An excessive response, sustained by activation, proliferation of these cells and cytokine secretion can then shape the microenvironment of normal tissues towards the development of severe inflammation such as, for example, severe pneumonitis in the case of irradiation of the lung.[110] Mitotic cell death occurs later and can subsequently trigger tissue hypoxia leading to the release of DAMPs and cytokines/chemokines from resident cells thereby modifying the microenvironment in the tissue. These changes impact the tissue-resident immune cells that then release cytokines. Finally, epithelial-to-mesenchymal and endothelial-to-mesenchymal transitions, recently found to be induced by irradiation,[111,112] mesenchymal stem cell differentiation, and the altered microenvironment contribute to myofibroblast activation and collagen deposition, which ultimately leads to fibrosis.

In irradiated normal tissues, the recruitment of immune cells has a dual effect. Resolution of inflammation and repair progression are concomitant with late mitotic cell death, which results from the initial damage, hypoxia and release of DAMPs, cytokines and growth factors, representing the chronic phase of radiation-induced injury of many normal tissues. These environmental changes may contribute to immunomodulation. For example, different populations of lymphocytes such as T_H2, T_H9, T_{reg} and possibly innate lymphoid cells display both anti-inflammatory and pro-fibrotic effects, thereby potentially promoting the induction of pathologic myofibroblasts and fibrosis.[110] It is believed that lymphocytes play a complex role in radiation diseases in which, depending on the disease stage and the environmental conditions, specific subpopulations of lymphocytes could exert beneficial or adverse effects.[110] For instance, in the case of radiation-induced pulmonary fibrosis, a disturbed balance between tissue inflammation and repair processes, with an involvement of lymphocytes, may participate in the development of the syndrome as described for other fibrotic diseases.[113] A question is whether immune cells contribute directly to radiation diseases or only modulate disease progression. In addition, it is not yet established if innate lymphoid cells also contribute to radiation-induced late injuries. These questions have been nicely explored in several reviews that we recommend as further reading in the context of radiation-induced pulmonary acute and late effects.[110,113,114]

Influence of the adaptive immune response by the tumour endothelium in the context of radiotherapy

Normal endothelial cells participate in adaptive immune responses by recruiting circulating T cells into inflamed tissues.[115] In contrast, tumour endothelial cells play a role in immune cell exclusion and inhibition of lymphocyte activation,[116] causing the development of intratumoral immunosuppression, which is involved in tumour escape from immunity and conventional cancer therapies.[14,117] Mechanisms underlying this dysfunction involve the absence or expression at low levels of ICAM-1, VCAM-1 and E-selectin on tumour vasculature, despite an inflammatory environment.[118] This lack of response to inflammatory stimuli, called endothelial anergy,[119] may be due at least in part to angiogenic factors such as vascular endothelial growth factor-A (VEGF-A), VEGF-C, VEGF-D and basic fibroblast growth factor (bFGF),[120,121] which are expressed in response to tissue hypoxia through activation of the HIF pathway. Therefore, approaches that improve the recruitment and activation of lymphocytes by tumour endothelial cells are being investigated.[14,118,122] On the other hand, RT is immunostimulatory and has the potential to enhance anti-tumour response by the immune system.[123] For instance, in this way, vascular normalization by VEGFR2 blockade has been shown to cause an increase in oxygenation able to govern brain tumour response to radiation by restoring anti-tumour T-cell activity.[124] Also, low doses of irradiation in the range of a fraction dose of conventional RT have been shown to cause aberrant vascular system normalization and efficient recruitment of tumour-specific T cells in human pancreatic carcinomas, as well as T-cell-induced rejection of tumours and prolonged survival in spontaneous and xenograft tumour models.[125] These effects were in part related to the suppression of the production of angiogenic, immunosuppressive and tumour growth factors such as VEFG. Lastly, RT increases the

expression of adhesion molecules on tumour endothelial cells, including ICAM-1, VCAM-1 and E-selectin,[35,125-127] which helps to normalize the tumour vasculature and makes it easier to infiltrate.

Radiation-induced abscopal effects and their cross-talk with vascular remodelling

Tumour regression may occur in lesions far from the radiation field of the tumour site by a systemic effect (abscopal effect).[126] This effect is currently being observed in clinics and could be the result of immunogenic cell death, which triggers tumour vaccination.[127] It requires efficient infiltration of the primary tumour by immune cells, subsequent extravasation of these cells so they can act away from the irradiated site, and ultimately infiltration of the secondary tumour by the educated immune cells. Many barriers, including the vascular barrier, prevent radiation-induced *in situ* tumour vaccination,[128] which explains why only a few distant responses in non-irradiated sites have been reported.[129] In solid tumours, poor vascularization may impair this process by decreasing leukocyte infiltration and effector function, otherwise leading to hypoxia, which can contribute to immune tolerance by regulating immunosuppressive cell populations.[130-135] On the other hand, RT induces a pro-inflammatory environment which can enhance lymphocyte infiltration and adaptive immune system activation by normalizing the vasculature.[136] This infiltration may be caused by upregulation of ICAM-1 correlated with RT.[33]

Alteration of microbiota following radiotherapy and its impact on vascular and immune systems

The microbiome plays a critical role in the maintenance of vascular health and the development of vascular disease.[137] Changes in intestinal microbiota composition after RT have been reported in several studies.[138] The major changes were reduced diversity of Firmicutes and Bacteroidetes and increase of Proteobacteria. In a preclinical model of radiation proctitis, recent findings have demonstrated that local radiation treatment induces microbial dysbiosis.[139] Remarkably, this radiation-induced dysbiotic microbiota was able to transmit inflammatory susceptibility and to render germ-free recipient mice more susceptible to ionizing radiation through a host cytokine induction. Conversely, faecal microbiota transplantation from healthy mice to total body irradiated mice improved gastrointestinal tract function and epithelial integrity of the small intestine, and facilitated angiogenesis.[140] These two studies suggest that the microbiota may be manipulated to improve or prevent radiation-induced tissue damage.

Evidence suggests that interactions between the enteric microbiota and the innate immune system are important in modulating the response to radiation,[141] especially through triggering of innate immune receptors by the microbiota.[142] After exposure to ionizing radiation, the integrity of the intestinal barrier is decreased, allowing intestinal bacteria and their components (pathogen-associated molecular patterns, PAMPs) to translocate to the lamina propria where they are recognized by Toll-like receptors (TLRs) in host antigen-presenting cells (APCs).[143,144] Activated APCs then secrete proinflammatory cytokines and prime donor T cells. On the other hand, endothelial apoptosis has been shown to be lower in germ-free than in conventionally raised mice.[145] The microbiota-associated enhancement of endothelial radiosensitivity did not require mature

lymphocytes since Rag1 knockout mice did not show differences in the development of lethal radiation enteropathy.[145]

Little is known about the effect of microbiota on the regulation of tumour response to RT.[142] Yet, recently, the influence of the gut microbiome composition on the response to anti-programmed cell death protein (PD)-1 immunotherapy in cancer patients was described in three outstanding articles.[146-148] These studies suggest that the microbiota should be considered when assessing therapeutic intervention. In the same way that the gut microbiota affects the immune response induced by immunogenic cell death in chemotherapy and immunotherapy,[149-151] it can therefore be assumed that the gut microbiota also plays a role in the immunostimulatory effects of RT.

ENDOTHELIAL-ORIENTED STRATEGIES FOR THERAPEUTIC GAIN IN RADIATION ONCOLOGY

Endothelium-based strategies for therapeutic gain in radiation oncology attempt either to increase injuries in the tumour vascular system or to protect the vasculature of normal tissue from radiation injuries, or ideally both. Many studies have attempted to preserve the endothelial barrier of normal tissues by protecting endothelial cells from death, or by inhibiting vascular inflammation and endothelial cell activation. Apart from approaches that aim to radiosensitize tumours by directly targeting cancer cells, the manipulation of the TME based on the tumour vasculature and on the immune system has aroused great interest. The different endothelial-oriented strategies to improve RT are detailed below and illustrated in **Figure 4** for normal tissues and in **Figure 5** for tumours.

Endothelial-oriented strategies to spare normal tissue

Preventing endothelial cytotoxicity

Among effective modifiers of radiation-induced endothelial cytotoxicity *in vivo* and *in vitro*, bFGF has generated great interest in the protection of endothelial cells from apoptosis.[28,152,153] Administration of bFGF increases the endothelial expression of thrombomodulin and is effective in reducing fibrosis, for instance within the urinary bladder.[154] Without going through bFGF, direct activation of the thrombomodulin-activated protein C (TM-APC) pathway has beneficial biological effects on the vasculature because of its anti-inflammatory, cytoprotective, antifibrinolytic, antioxidant and anticoagulant properties.[155] By using a pharmacological strategy to activate this pathway, mitigation of radiation toxicity was achieved in a relevant model of RT in which a loop of rat small bowel was exposed to nine daily doses of 5 Gy.[156] On the other hand, the targeting of CD47, a thrombospondin-1 receptor, has demonstrated that inhibiting CD47 signalling maintains the viability of normal tissues following irradiation, likely through radioprotection of endothelial cells, while increasing the radiosensitivity of tumours.[157] Also, an angiopoietin-1 mimic significantly reduces skin radiation toxicity, potentially by increasing survival and function of irradiated endothelial cells through tyrosine kinase with immunoglobulin and EGF homology domains 2 (Tie2) receptor activation.[158] In addition, the use of a ceramide-targeting antibody to prevent ceramide platform formation in endothelial cells protects against gastrointestinal syndrome.[159] Also, a

strategy that targets PAI-1 to prevent endothelial cell death has been proposed to mitigate the severity of intestinal radiation injury.[160] This work showed a promising temporary protection against early lethality in a mouse model of radiation-induced enteropathy.

Decreasing coagulopathy

A radiation-induced decrease in endothelial thrombomodulin-1 leads to an increase in thrombin, which results in activation of blood clotting. In liver, lung and heart, the lumen of central veins becomes blocked by fibrillar material resulting in obstruction of irradiated vessels with platelet aggregates.[161] Therefore, there has been great interest in modulating blood coagulation as a strategy to reduce radiation toxicity of normal tissue. Increased platelet adherence to irradiated endothelial monolayers can be blocked by anti-von Willebrand factor (vWF) antibodies.[162] The use of anticoagulants such as heparin has been investigated, but the results to date have been mostly insignificant and generally inconsistent.[163] In contrast, the TM–APC pathway is a hopeful target for preventing or treating radiation toxicity in normal tissues using strategies aimed at restoring or preserving endothelial TM or replacing PC.[156,164,165] Also, thrombin inhibition has been investigated as a strategy to minimize the side-effects of RT. The recombinant thrombin inhibitor hirudin has shown a protective effect against small bowel radiation toxicity in a model of localized small bowel radiation in rats.[166] However, the authors felt that targeting specific thrombin functions may be superior to global thrombin inhibition. On the other hand, the administration of the thrombin peptide TP508 has been shown to activate endothelial cells and stem cells to revascularize and regenerate tissues in a whole body irradiation model,[167,168] suggesting a possible use to limit RT side-effects.

Targeting premature endothelial senescence

Approaches to target endothelial cell senescence to prevent, mitigate and treat radiation-induced cardiovascular diseases are under investigation.[31] The different approaches rely on three main strategies: (i) preventing endothelial cells from becoming senescent using antioxidants to scavenge ROS[169] or inhibitors of the IGF1/PI3K/Akt/mTOR pathway that activate radiation-induced endothelial senescence;[170] (ii) suppression of SASP to prevent most of the deleterious effects of senescent cells,[171] for instance by using RNAi or JAK inhibitors to target the JAK pathway,[172] an upstream regulatory pathway of SASP; and (iii) clearing senescent endothelial cells with senolytic drugs to selectively kill senescent cells.[173] Clearance of senescent cells in mice by ABT263, a Bcl-2/xL-specific inhibitor, effectively cleared senescent cells in several tissues, including senescent bone marrow hematopoietic stem cells and muscle stem cells, and suppressed SASP in the lungs.[174] Nevertheless, inhibiting the induction of senescence could be detrimental by increasing tumorigenesis and decreasing tumour response to RT. Moreover, since senescent cells persist *in vivo*, their elimination or death may ultimately be worse with deleterious effects on the irradiated organ, despite their dysfunction and their key role in radiation-induced diseases, as discussed previously.[175]

Modulation of the immune-inflammatory response

A number of studies suggest that the recruitment of immune cells may become detrimental to healthy tissues when chronic and unresolved,[22] and possibly participates in the initiation and/or the

development of acute and late adverse tissue effects in the course of RT. Thus, strategies that target normal tissue-associated endothelial cells would be of interest to impair their ability to recruit immune cells. In such a strategy, the question of the timing of the modulation should be explored in depth. Many studies have aimed to identify strategies to antagonize adhesion molecule function, by either preventing interactions with receptors or inhibiting NF-κB signalling, to limit the inflammatory response. Hitherto, no clinical strategy has emerged that controls inflammation by targeting expression of the NF-κB-dependent endothelial adhesion molecule. However, mechanisms independent of the NF-κB pathway may also regulate adhesion molecule function, including post-transcriptional regulation by IL-19[176] and post-translational modification by N-linked sugars.[39] Since leukocyte trafficking in inflammation is actually governed by protein-glycan interaction,[19] understanding of these complex interactions raises great hopes for the emergence of new therapeutic targets to treat inflammatory diseases such as atherosclerosis, intestinal bowel disease and thus also for radiation diseases.[40]

Endothelial-oriented strategies to target tumours

Increasing injury to tumour vasculature

The strategy of destroying the tumour vasculature has long been considered of interest in enhancing the potential of RT, although a poorly vascularized tumour may then prove to be more resistant to ionizing radiation due to lower oxygen content. Using anti-VEGF antibodies (bevacizumab), anti-angiogenic therapies have an antivascular effect in human tumours, but are still far from being effective in monotherapy.[177] On the other hand, the benefit of the combination of anti-angiogenic therapy with other conventional treatments such as RT or chemo-RT has been reported in numerous studies.[177,178] However, combined therapy was found to be associated with increased severe toxicity, indicating the need to improve timing and dose delivery in the future.[178,179] Radiation-induced damage to endothelial cells has been proposed to explain the therapeutic advantage of SBRT, but the targets that allow this enhanced therapeutic response are still the subject of debate.[29] In particular, the aforementioned study of Moding et al. reported cancer cells as primary targets of SBRT rather than endothelial cells.[77] In the same way, differences in the radiosensitivity of endothelial cells in the tumour and the normal tissues could explain the differential effect of flash-irradiation with a dose rate above 100 Gy/s.[180,181]

Cancer immunotherapy

The tumour immune response largely involves the vascular system, which allows the transport of immune cells to the tumour. Radiation facilitates the trafficking, homing and extravasation of effector cytotoxic $CD8^+$ T cells into the tumour, resulting in radiation-induced immunogenic cell death.[182] The complex reactions of the immune system in an irradiated TME are both immunostimulatory and immunosuppressive.[104] Immunostimulatory effects come from the recruitment of circulating immune cells due to the production of cytokines and other proinflammatory factors by the tumour and its microenvironment, antigen exposure and dendritic cell priming as well as activation of the endothelial cells present in the tumour. In contrast, tumours and their microenvironment contain immunosuppressive immune cells like tumour

associated macrophages (TAMs), which resemble the alternatively activated M2 macrophage, and T_{Reg} cells that have immunosuppressive and tolerizing effects. If radiation-induced immunosuppressive effects could be overcome and immunostimulatory effects enhanced, or both, RT would promote strong responses against tumour cells. A strategy to improve the recruitment of immune cells in tumours to enhance tumour cell death would be to manipulate the tumour-associated endothelial cells. In this way, Wilson et al. have shown that the delivery of miR-103 in tumour-bearing mice leads to decreased angiogenesis and tumour growth by radiosensitization of tumour cells.[183] Also, a strategy that consists in increasing the ability of endothelial cells to adhere to circulating cells would also allow immune infiltration of the tumours to promote tumour cell death. However, to date, the molecular targets and the molecules to test have yet to be discovered.

Combining immunotherapy, anti-angiogenic therapy and radiotherapy

A therapeutic perspective might consider the combination of immune checkpoint inhibitors, angiogenic inhibitors and RT, although this warrants further investigation.[184] On the one hand, an immunotherapy-antiangiogenic combination has given promising results.[185,186] In such therapy, the immunostimulatory effects of inhibitors of immune checkpoint regulators, such as antibodies against PD-1 or its ligand PD-ligand 1 (PD-L1), or cytotoxic lymphocyte-associated antigen-4 (CTLA-4), are combined with antibodies against VEGF, or its receptor VEGFR, which have immunosuppressive properties through inhibition of dendritic cell maturation in addition to their angiogenic properties. On the other hand, as discussed previously, RT has immunosuppressive effects and can be combined with antiangiogenic therapy to increase efficacy. The combination of these three therapies is certainly an innovative approach with potential clinical benefits.

Opening the endothelium for drug delivery

Local tumour irradiation (≥ 15 Gy) has recently been shown to substantially improve the delivery of therapeutic nanoparticles in a preclinical study with a benefit in tumour control.[187] Interestingly, TAM, which can serve as a nanoparticle drug depot,[188] increased in the vicinity of the microvasculature after therapeutic priming of the TME by irradiation, which finally increased therapeutic nanoparticle delivery. In this model, radiation initiated a vascular burst of TAM extravasation through a cascade of changes to the tumour vasculature and microenvironment, leading to increased uptake of the drug in neighbouring tumour cells. Vessel thickening, tortuous vascular branching and perivascular TAM localization were induced by radiation, all of which helped to enhance vessel permeability, allowing selective therapeutic nanoparticle delivery through TAM and improving tumour killing. These findings show that TAM were beneficial for drug delivery and, since RT stimulates an increase in TAM relative to the tumour,[125,187,189] open new interesting perspectives for combined RT and therapeutic nanoparticle treatment with broad applicability.

Since the BBB can be disrupted by RT, the modulation of BBB permeability by SBRT has been proposed to enhance drug delivery to the brain.[190] For instance, the idea of combining the use of RT to open the BBB with intravenous injection of nanoparticles is an interesting new concept for the treatment of brain tumours. Nanoparticles are a potential tool in the treatment of cancer because of their low toxicity and their ability to increase vascular sensitivity to radiation.[191] They

significantly increase the DNA damage to blood vessels of the brain induced by ionizing radiation. It has therefore been proposed that low doses of irradiation could locally increase the response of endothelial cells and thereby increase the permeability of the BBB with limited toxicity. Given the increased accuracy of RT, it would theoretically be possible to disrupt the BBB in strategic locations in the tumour bed prior to the administration of chemotherapy to increase its distribution and efficacy. However, it is not known which dose or dose schedule will best achieve the desired results or when permeability peaks after RT.

CONCLUSIONS

In the light of current knowledge, the vascular endothelium can be considered as a principal checkpoint for radiation-induced inflammatory and immunity processes following radiation exposure in both normal tissues and tumours. The endothelium could therefore be an ideal target compartment for improving the therapeutic index of RT. Future studies should focus on endothelial molecular targets of both normal tissues and tumours, but with opposite objectives : in normal tissues, therapeutic strategies will aim to modulate immune-inflammatory cell entry, at times which will have to be precisely determined for each treatment, whereas in tumours, treatments associated with RT and other treatments will aim to open the endothelium barrier so as to enhance the immune response.

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FIGURE LEGENDS

Figure 1. The leukocyte adhesion cascade is triggered by a pro-inflammatory stimulus. Injury activates endothelial cells, allows production of free radicals, and damages tissue and tissue-resident immune cells leading to the release of cytokines, chemokines and growth factors, which then attract leukocytes from the circulation. Circulating leukocytes undertake a seven-step process of capture, rolling, slow rolling and activation, arrest and adhesion strengthening, intravascular crawling and finally transmigration to reach sites of inflammation. Each step of this process is controlled by various adhesion molecules at the surface of the endothelium. All of these proteins are glycosylated, a post-translational modification process that may be regulated during inflammation. During this cascade of events, leukocytes are also activated by interactions with cytokines, chemokines and growth factors sequestrated by the glycosaminoglycans of the endothelial glycocalyx.

Figure 2. Ionizing radiation injures the vascular endothelium. DNA damage and ceramide production lead to cell death, stress-induced premature senescence, cell activation mainly characterized by the overexpression of adhesion molecules, and disruption of the endothelial barrier. Endothelial activation promotes a pro-thrombotic and pro-inflammatory phenotype that ultimately leads to thrombosis and recruitment of leukocytes. Irradiated endothelial cells can also undergo an endothelial-to-mesenchymal transition that potentially contributes to fibrosis via a final differentiation into activated myofibroblasts capable of secreting collagens.

Figure 3. Immune-inflammatory effects of radiation exposure on normal tissues, tumours and tumour microenvironment. Ionizing radiation causes tumour and normal cell damage, cell death and release of damage-associated molecular patterns (DAMPS), which act as pro-inflammatory signals. Radiation activates cells that then release cytokines/chemokines. Activated endothelial cells acquire a pro-inflammatory phenotype which promotes the leukocyte adhesion cascade. These initial responses finally lead to the recruitment and activation of diverse immune cells, which can then also participate in the abscopal effect of radiotherapy. Delayed mitotic death and proliferation of immune cells in the tissue then cause environmental changes that ultimately contribute to necrosis or fibrosis. Irradiation of the microbiota of the intestinal tract can also influence the responses of normal tissues and tumours through immune-vascular crosstalk.

Figure 4. Potential endothelial-oriented strategies to spare normal tissue by targeting cytotoxicity, coagulation, activation of the immune-inflammatory response and senescence.

Figure 5. Endothelial-oriented strategies to enhance tumour control by targeting the immunostimulatory and immunosuppressive reactions, inducing cell death and increasing vascular permeability to allow immune cell infiltration.

Figure 1

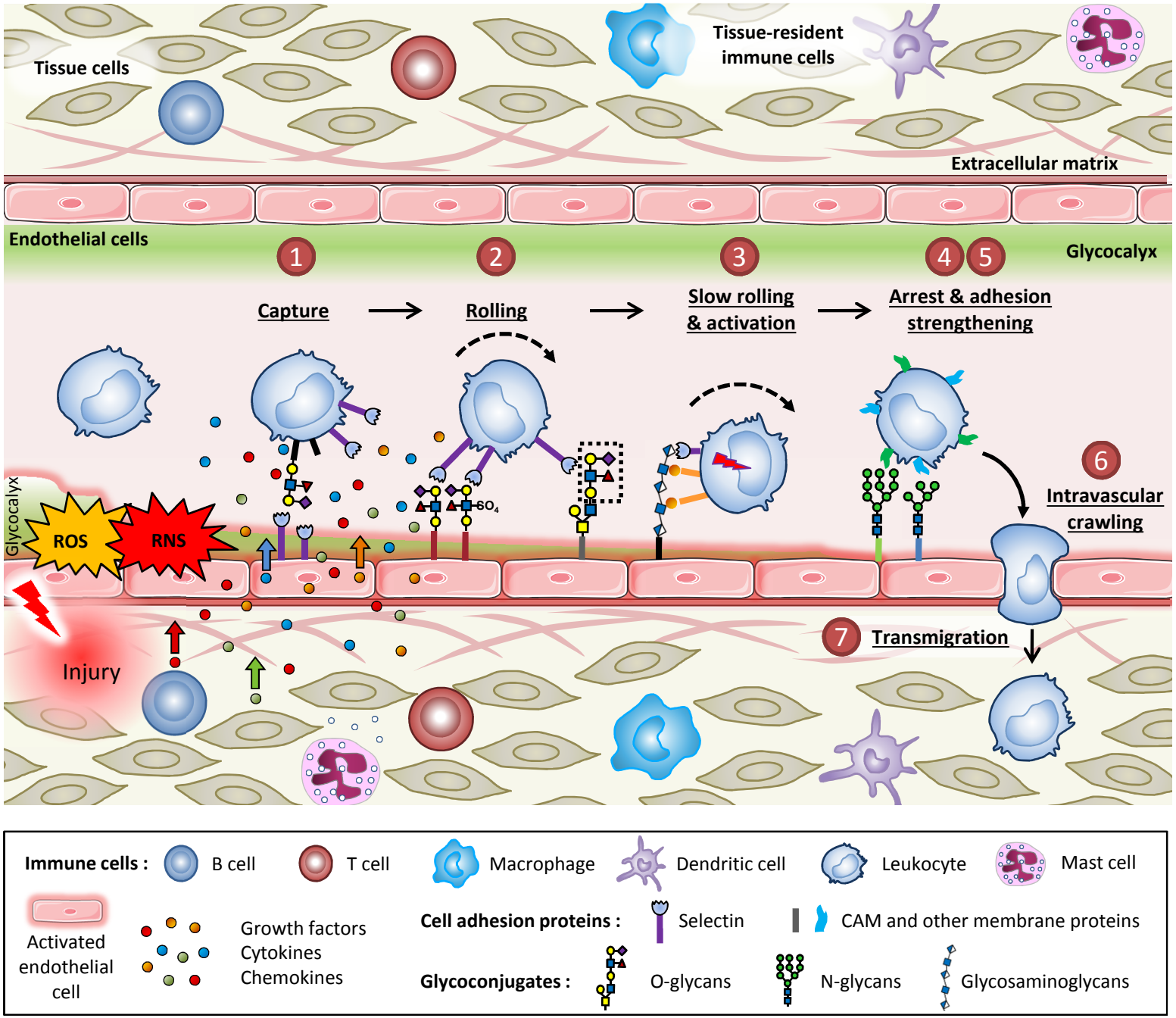

Tissue cells
Tissue-resident immune cells
Extracellular matrix
Endothelial cells
Glycocalyx
1
2
3
4
5
Capture
Rolling
Slow rolling & activation
Arrest & adhesion strengthening
6
Intravascular crawling
7
Transmigration
Glycocalyx
ROS
RNS
Injury
Immune cells :
B cell
T cell
Macrophage
Dendritic cell
Leukocyte
Mast cell
Activated endothelial cell
Growth factors
Cytokines
Chemokines
Cell adhesion proteins :
Selectin
CAM and other membrane proteins
Glycoconjugates :
O-glycans
N-glycans
Glycosaminoglycans

Figure 2

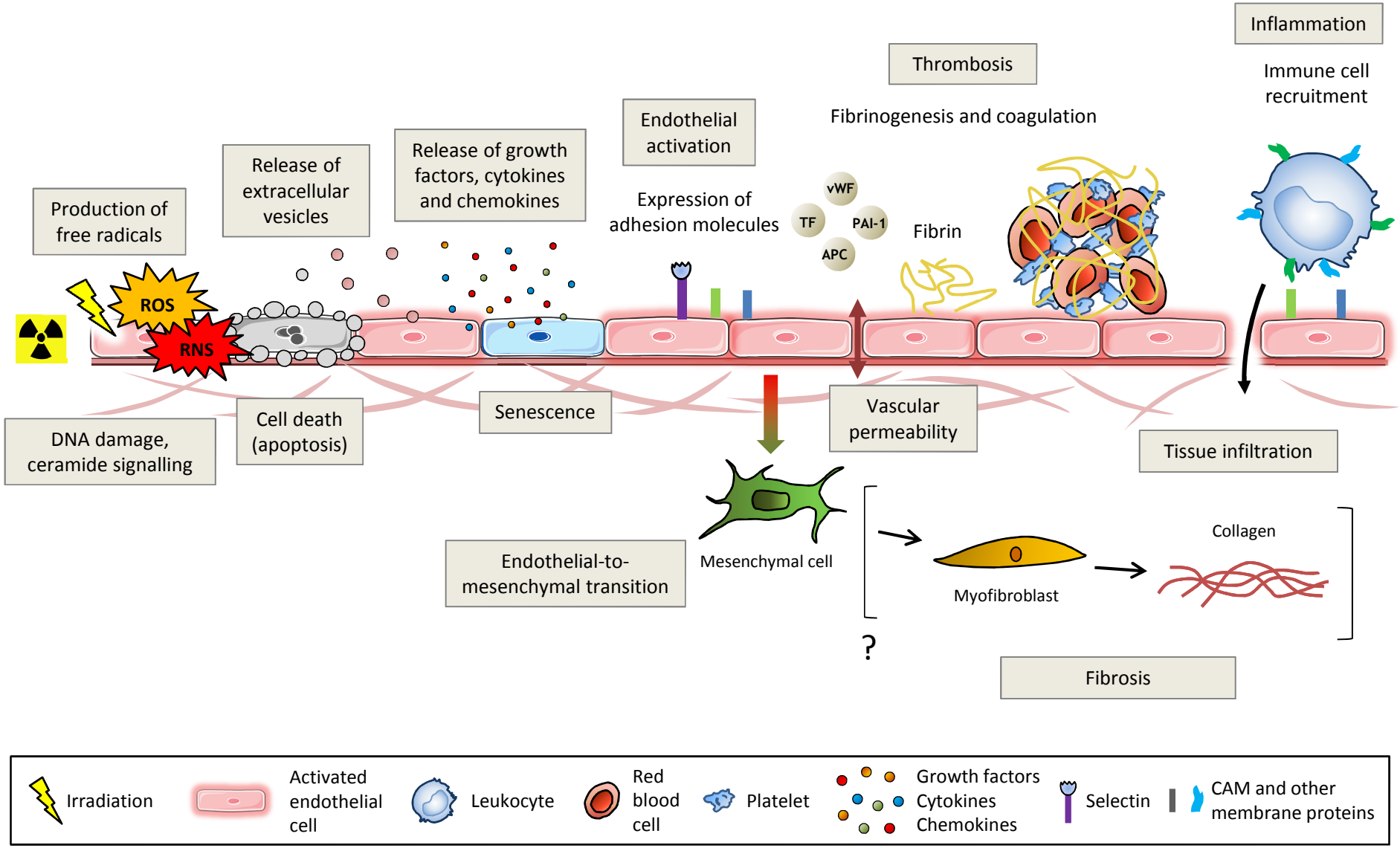

Inflammation
Immune cell recruitment
Thrombosis
Fibrinogenesis and coagulation
Endothelial activation
Expression of adhesion molecules
Release of growth factors, cytokines and chemokines
Release of extracellular vesicles
Production of free radicals
vWF
TF
PAI-1
APC
Fibrin
ROS
RNS
DNA damage, ceramide signalling
Cell death (apoptosis)
Senescence
Vascular permeability
Tissue infiltration
Endothelial-to-mesenchymal transition
Mesenchymal cell
Myofibroblast
Collagen
?
Fibrosis
Irradiation
Activated endothelial cell
Leukocyte
Red blood cell
Platelet
Growth factors
Cytokines
Chemokines
Selectin
CAM and other membrane proteins

Figure 3

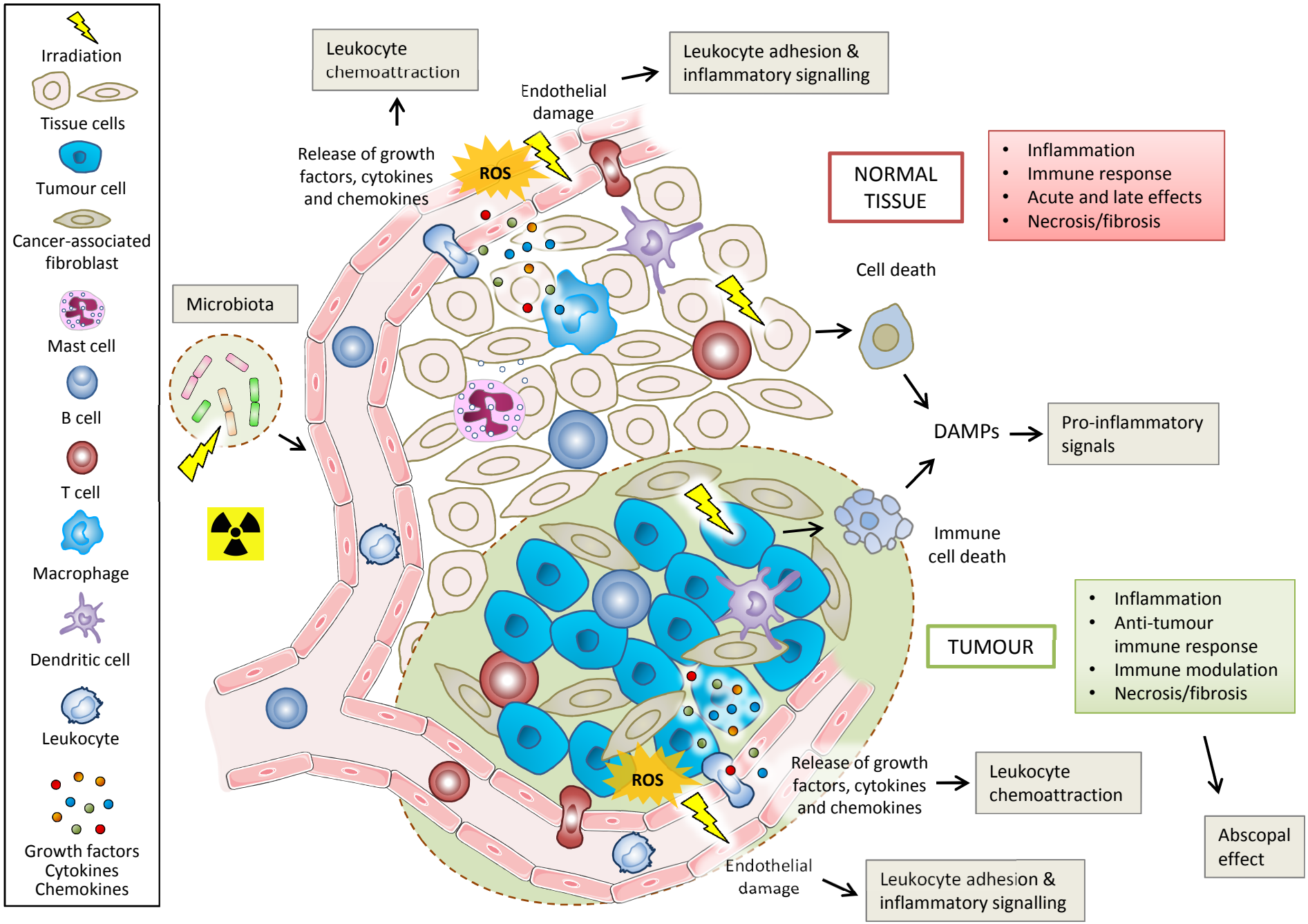

Irradiation
Tissue cells
Tumour cell
Cancer-associated fibroblast
Mast cell
B cell
T cell
Macrophage
Dendritic cell
Leukocyte
Growth factors
Cytokines
Chemokines
Leukocyte chemoattraction
Endothelial damage
Leukocyte adhesion & inflammatory signalling
Release of growth factors, cytokines and chemokines
ROS
NORMAL TISSUE
Inflammation
Immune response
Acute and late effects
Necrosis/fibrosis
Cell death
Microbiota
DAMPs
Pro-inflammatory signals
Immune cell death
TUMOUR
Inflammation
Anti-tumour immune response
Immune modulation
Necrosis/fibrosis
ROS
Release of growth factors, cytokines and chemokines
Leukocyte chemoattraction
Abscopal effect
Endothelial damage
Leukocyte adhesion & inflammatory signalling

Figure 4

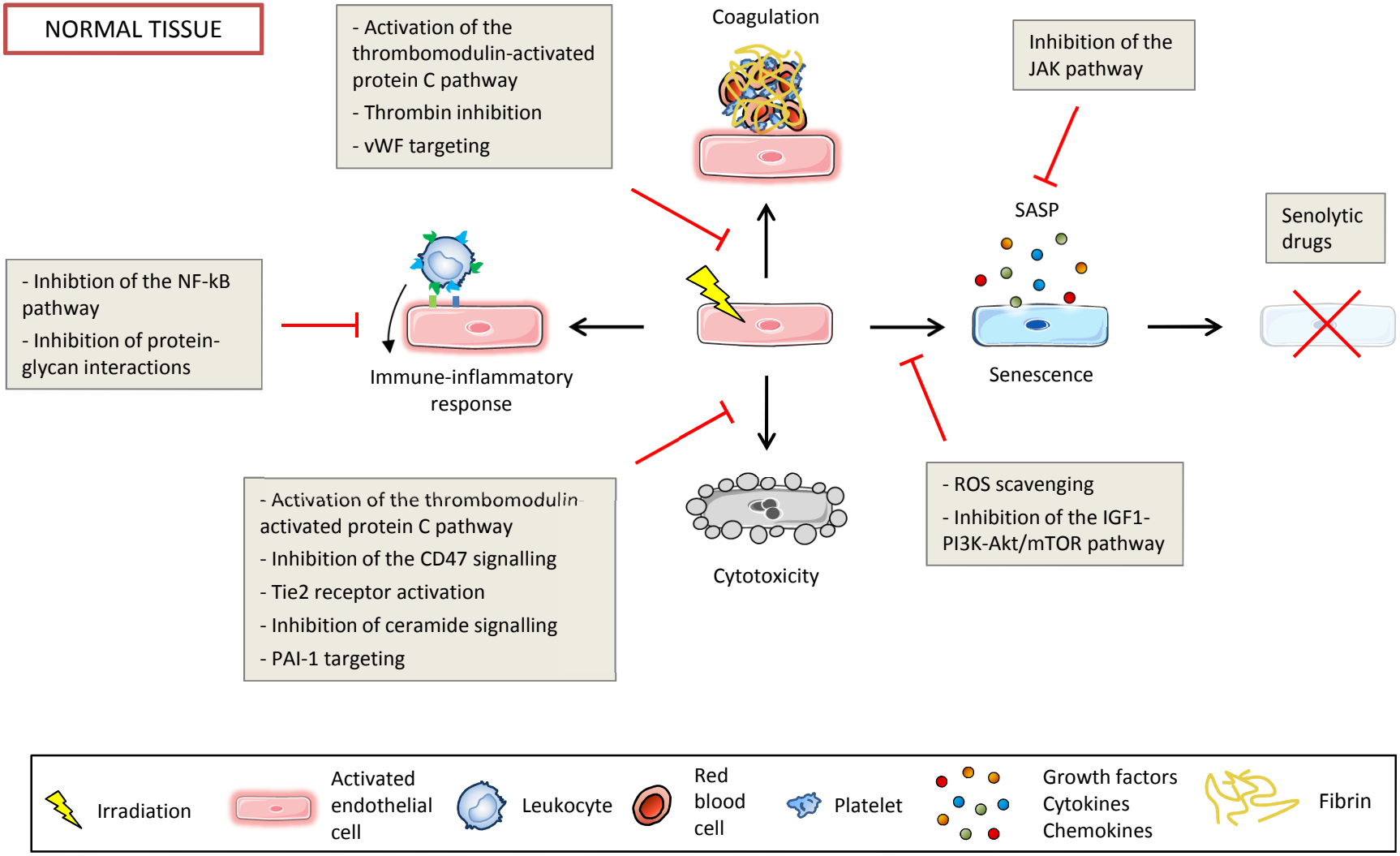
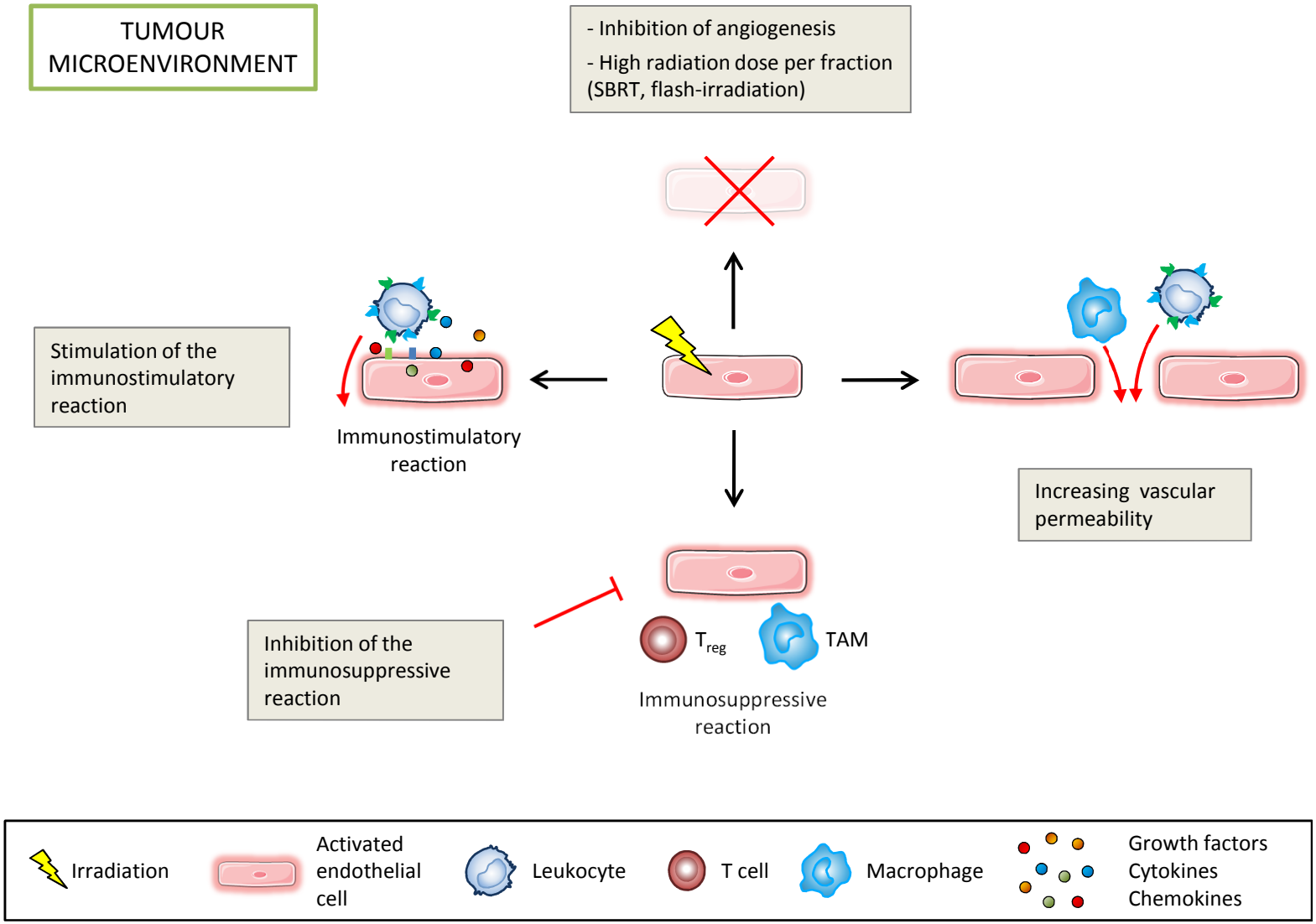

NORMAL TISSUE
- Activation of the thrombomodulin-activated protein C pathway
- Thrombin inhibition
- vWF targeting
Coagulation
Inhibition of the JAK pathway
SASP
Senolytic drugs
- Inhibtion of the NF-kB pathway
- Inhibition of protein-glycan interactions
Immune-inflammatory response
Senescence
- Activation of the thrombomodulin-activated protein C pathway
- Inhibition of the CD47 signalling
- Tie2 receptor activation
- Inhibition of ceramide signalling
- PAI-1 targeting
Cytotoxicity
- ROS scavenging
- Inhibition of the IGF1-PI3K-Akt/mTOR pathway
Irradiation
Activated endothelial cell
Leukocyte
Red blood cell
Platelet
Growth factors
Cytokines
Chemokines
Fibrin

Figure 5


TUMOUR MICROENVIRONMENT
- Inhibition of angiogenesis
- High radiation dose per fraction (SBRT, flash-irradiation)
Stimulation of the immunostimulatory reaction
Immunostimulatory reaction
Increasing vascular permeability
Inhibition of the immunosuppressive reaction
T_{reg}
TAM
Immunosuppressive reaction
Irradiation
Activated endothelial cell
Leukocyte
T cell
Macrophage
Growth factors
Cytokines
Chemokines