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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

1
2 Altered by ionizing radiation, the vascular network is considered as a prime target to limit normal
3 tissue damage and improve tumour control in radiotherapy. Irradiation damages and/or activates
4 endothelial cells, which then participate in the recruitment of circulating cells, especially by
5 overexpressing cell adhesion molecules, but also by other as yet unknown mechanisms. Radiation-
6 induced lesions are associated with infiltration of immune-inflammatory cells from the blood and/or
7 the lymph circulation. Damaged cells from the tissues and immune-inflammatory resident cells
8 release factors that attract cells from the circulation, leading to the restoration of tissue balance by
9 fighting against infection, elimination of damaged cells and healing of the injured area. In normal
10 tissues that surround the tumours, the development of an immune-inflammatory reaction in
11 response to radiation-induced tissue injury can turn out to be chronic and deleterious for the organ
12 concerned, potentially leading to fibrosis and/or necrosis of the irradiated area. Similarly, tumours
13 can elicit an immune-inflammation reaction, which can be initialized and amplified by cancer
14 therapy such as radiotherapy, although immune checkpoints often allow many cancers to be
15 protected by inhibiting the T cell signal. Herein, we have explored the involvement of vascular
16 endothelium in the fate of healthy tissues and tumours undergoing radiotherapy. This review also
17 covers current investigations that take advantage of the radiation-induced response of the
18 vasculature to spare healthy tissue and/or target tumours better.
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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 organs¹⁰ and tumours¹¹ 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 tissue¹³ (**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

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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 transmigration¹³ (**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- κ B) 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

1 covering composed of a layer of polysaccharides covalently bound to the lipids and proteins of the
2 membrane, also plays an important role in the immune-inflammatory response, especially via its
3 role in the leukocyte-endothelial interaction.²⁰ Following an acute or chronic injury, pro-
4 inflammatory stimuli, such as TNF- α , induce shedding of glycosaminoglycans (GAGs), thereby
5 decreasing the width and size of the endothelial glycocalyx.²¹ This allows greater accessibility of
6 endothelial glycoprotein epitopes, on which circulating leukocytes roll and to which they adhere.
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10 RADIOBIOLOGY AND RADIOPATHOLOGY OF THE VASCULAR 11 ENDOTHELIUM 12 13

14 Ionizing radiation has multiple effects on the endothelium, which is thought to contribute to the
15 initiation and progression of radiation-induced damage to healthy tissues following RT.²² Among the
16 main effects are radiation-induced endothelial cell death, loss of thromboresistance, cell activation
17 and secretion of soluble factors such as cytokines and growth factors.²³ Vessels of normal tissues
18 surrounding the tumours and of the tumours themselves are both impacted by ionizing radiation
19 during RT. In most cases, normal and pathological tissues comprise genetically normal endothelial
20 cells. In tumours, however, vascular cells undergo the influence of the tumour environment and
21 display different phenotypes, and sometimes different genotypes, from those of endothelial cells
22 from normal tissues. **Figure 2** shows a schematic view of the effects of ionizing radiation on the
23 vascular endothelium, which are detailed below.
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30 Differential effects of various forms of radiotherapy on endothelial damage 31

32 External beam RT using photon radiation, and especially X-rays, is used in almost all RT treatments.
33 However, particular indications can use high linear transfer energy (LET) particle RT with protons or
34 carbon ions (hadrontherapy), as well as internal RT with radioactive sources of gamma-rays
35 (brachytherapy) and systemic RT using targeted radionuclide therapy (radioimmunotherapy).
36 Although not investigated in depth, the effects of gamma- and X-rays on endothelium are supposed
37 to be similar because of their equal relative biological effectiveness (RBE). On the other hand, it is
38 conceivable that differences in endothelial cell response may occur as a function of dose rate,
39 energy spectrum and fractionation schedule. More likely, radioimmunotherapy and hadrontherapy
40 may have very different effects on endothelium compared to photon radiation, because of their
41 high LET radiation properties. However, most data are available on the effects of low LET radiation
42 and data on high LET radiation are scarce. For example, the impact of carbon and Fe ion irradiation
43 on the response of endothelial cells has been compared to X-ray irradiation, showing a differential
44 effect of high LET radiation compared to photon radiation.^{24,25} Interestingly, C-ions seemed to be
45 more effective in damaging endothelial cells than X-rays shortly after exposure, but late damage
46 was not found at the doses used (0.25-1.5 Gy).²⁵ On the other hand, Fe ions did not significantly
47 induce inflammation, in contrast to X-rays, but the radiation impact on gene and protein expression
48 was more marked and longer lasting for Fe ions than for X-rays.²⁴
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59 Endothelial cell apoptosis and senescence 60 61 62 63 64 65

1 Apoptosis is a frequent cell death process after irradiation.²⁶ Exposure of healthy tissues to a high
2 dose of ionizing radiation (≥ 10 Gy) induces an acute endothelial reaction characterized by a rapid
3 wave of endothelial apoptosis.^{22,27} Yet, the key role and the existence itself of radiation-induced
4 endothelial apoptosis in normal tissue lesions remain controversial. Endothelial apoptosis has been
5 shown to be the primary event responsible for gastrointestinal syndrome after whole-body
6 irradiation at 15 Gy in mice.²⁸ Despite contradictory results, which have been discussed elsewhere,
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11 Endothelial cells also experience stress-induced premature senescence *in vitro* and likely *in vivo*.³⁰
12 Senescent irradiated endothelial cells can accumulate in tissues, promoting age- and therapy-
13 related disorders, including cardiovascular diseases. Through secretion of many inflammatory
14 mediators and extracellular proteases, called the senescence-associated secretory phenotype (SASP),
15 the senescent phenotype causes chronic inflammation and disruption of tissue structure and
16 function. The surviving irradiated cells likely participate in the development of a dysfunctional
17 vascular phenotype. In the early phase, this is marked by excessive secretion of pro-inflammatory
18 cytokines, increased recruitment of circulating cells such as platelets and lymphocytes, activation
19 of the coagulation system, and increased vascular permeability.²² In the late phase, a collapse of
20 the vessels is observed, associated with a reduction in the thickness of the basal membrane and the
21 persistence of a pro-coagulating, pro-inflammatory and potentially senescent endothelial
22 phenotype.³¹

31 Endothelial activation

33 Ionizing radiation triggers changes in the phenotype of endothelial cells that lead to endothelial
34 activation and then potentially to endothelial dysfunction. Endothelial activation is defined as the
35 endothelial expression of adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin.³² It is usually
36 triggered by pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α or interleukin (IL)-6.
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1 overall demonstrated that the increase in expression of adhesion molecules by endothelial cells
2 after irradiation plays a decisive role in the recruitment of circulating cells and thus in the
3 radiation-induced inflammation of the tissue and/or the tumour, with a potential deleterious effect
4 on normal tissues.
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6 Endothelial cell-derived extracellular vesicles

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

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28 RT is known to induce vascular permeability. Several studies are concordant with an increase of
29 albumin leakage after single-dose irradiation from 2 Gy to 30 Gy or more. The increase in
30 permeability is transitory, varies from one day to one week after RT and penetration depth from
31 vessels varies by a factor 1.5 to 6, depending on the dose.^{57,58} In microvascular endothelial cells,
32 cytoskeletal changes mediated by RhoA-guanosine triphosphatase and Rho kinase are implicated in
33 the redistribution of basal VE-cadherin and intercellular junctions.⁵⁹ Interestingly, mast cells may
34 contribute to cutaneous microvascular hyperpermeability, as shown in an animal model of localized
35 irradiation.⁵⁸ Fractionated RT has also been shown to increase vascular permeability, but not as a
36 result of a lack of junction proteins. In contrast, ICAM-1 and Zo-1, which are involved in the
37 endothelial barrier, are more expressed after fractionated RT.^{60,61} It should be noted that
38 microvascular permeability induced by fractionated RT seems to be more durable than that induced
39 by single-dose RT.^{62,63}
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46 Endothelial tight junctions form the impermeable blood-brain barrier (BBB). In BBB models,
47 microvascular permeability is increased in a dose-dependent manner after single-dose irradiation.⁶⁴
48 This increase is not directly explained by a change in tight junction protein expression or by basal
49 lamina lesion, since the expressions of Zo-1 and the endothelial barrier antigen are not affected at
50 doses below 20 Gy.^{64,65} The increase in permeability could be explained by changes in the
51 cytoskeleton of endothelial cells. In a clinical context of fractionated RT for the treatment of brain
52 tumours, the radiation-induced disruption of the BBB has been widely demonstrated.⁶⁶⁻⁶⁸
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58 Radiotherapy and vascular changes in tumours

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

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

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

38 On the other hand, fractionated RT impacts pericyte coverage of blood vessels and blood vessel
39 functionality in the TME. Pericytes are helpful to stabilize microvessels and regulate their
40 permeability in healthy tissues.⁸³ They are less frequent and more loosely associated with
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endothelial cells in tumours. Also, pericytes are involved in tumour hypoxia reduction⁸⁴ 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 model⁷⁹ 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 system⁹¹ 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)-1 α deletion confers resistance to radiation-induced enteritis, whereas similar deletion in intestinal epithelium does not, suggesting new functions of endothelial hypoxia-inducible factor-1 α -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-

1 induced enteritis by using a mouse model harbouring an endothelium-specific deletion of the *serpin*
2 *1* gene, which encodes the plasminogen activator inhibitor-type 1 (PAI-1).¹⁰⁰
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4 IMMUNOLOGICAL CONSEQUENCES OF RADIOTHERAPY

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

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11 Radiation induces changes to the tumour cell immunophenotype and immunogenicity by damaging
12 DNA and membranes, and by production of cytoplasmic ROS, which activate many transcription
13 factors and signalling pathways.¹⁰¹ RT promotes the presentation of tumour antigens by tumour cells
14 by increasing the cell pool of specific antigens and by stimulating the expression of the major type 1
15 histocompatibility complex (MHC class 1).¹⁰² It also generates immunogenic cell death, i.e. the
16 dispersion of immune-stimulating tumour antigens from dying cells into the surrounding milieu,¹⁰³
17 through the release of damage-associated molecular patterns (DAMPs).^{104,105} The activated dendritic
18 cells then present the tumour antigens to the naive T lymphocytes in the lymph nodes allowing the
19 formation of cytotoxic T lymphocytes specific for the tumour antigen.¹⁰⁶ RT facilitates the
20 recruitment of these effector T lymphocytes by generating the secretion of the chemokine CXCL16
21 and endothelial expression of the cell adhesion ICAM-1, VCAM-1 and E-selectin.^{97,107} These
22 processes finally trigger an immune response, which is accompanied by a pro-inflammatory
23 reaction, with release of IL-1, IL-2, IL-6, IL-12, interferon (INF)- α and IFN- β , and TNF- α , which are
24 involved in amplification of the anti-tumour immune response.¹⁰⁵
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28 In normal tissues injured by radiation, release of DAMPs and secretion of cytokines and chemokines
29 also activate the immune system. This phase moves to an acute inflammatory phase characterized
30 by an activated pro-inflammatory response and vascular leakage. In this phase, the recruitment of
31 diverse immune cells of myeloid and lymphoid origin, which is associated with a perpetual
32 cytokine/chemokine cascade,^{108,109} leads to various degrees of inflammation and symptoms.
33 Lymphocyte subpopulations such as T_H1, T_H17, and possibly innate lymphoid cells, can contribute to
34 inflammation, while T_{reg} could be needed to control damaging and excessive pro-inflammatory
35 responses. An excessive response, sustained by activation, proliferation of these cells and cytokine
36 secretion can then shape the microenvironment of normal tissues towards the development of
37 severe inflammation such as, for example, severe pneumonitis in the case of irradiation of the
38 lung.¹¹⁰ Mitotic cell death occurs later and can subsequently trigger tissue hypoxia leading to the
39 release of DAMPs and cytokines/chemokines from resident cells thereby modifying the
40 microenvironment in the tissue. These changes impact the tissue-resident immune cells that then
41 release cytokines. Finally, epithelial-to-mesenchymal and endothelial-to-mesenchymal transitions,
42 recently found to be induced by irradiation,^{111,112} mesenchymal stem cell differentiation, and the
43 altered microenvironment contribute to myofibroblast activation and collagen deposition, which
44 ultimately leads to fibrosis.
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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 VEGF. Lastly, RT increases the

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

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

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

26 Alteration of microbiota following radiotherapy and its impact on vascular and 27 immune systems

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

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

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

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

33 Approaches to target endothelial cell senescence to prevent, mitigate and treat radiation-induced
34 cardiovascular diseases are under investigation.³¹ The different approaches rely on three main
35 strategies: (i) preventing endothelial cells from becoming senescent using antioxidants to scavenge
36 ROS¹⁶⁹ or inhibitors of the IGF1/PI3K/Akt/mTOR pathway that activate radiation-induced endothelial
37 senescence;¹⁷⁰ (ii) suppression of SASP to prevent most of the deleterious effects of senescent
38 cells,¹⁷¹ for instance by using RNAi or JAK inhibitors to target the JAK pathway,¹⁷² an upstream
39 regulatory pathway of SASP; and (iii) clearing senescent endothelial cells with senolytic drugs to
40 selectively kill senescent cells.¹⁷³ Clearance of senescent cells in mice by ABT263, a Bcl-2/xL-
41 specific inhibitor, effectively cleared senescent cells in several tissues, including senescent bone
42 marrow hematopoietic stem cells and muscle stem cells, and suppressed SASP in the lungs.¹⁷⁴
43 Nevertheless, inhibiting the induction of senescence could be detrimental by increasing
44 tumorigenesis and decreasing tumour response to RT. Moreover, since senescent cells persist *in vivo*,
45 their elimination or death may ultimately be worse with deleterious effects on the irradiated organ,
46 despite their dysfunction and their key role in radiation-induced diseases, as discussed previously.¹⁷⁵
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57 *Modulation of the immune-inflammatory response*

58 A number of studies suggest that the recruitment of immune cells may become detrimental to
59 healthy tissues when chronic and unresolved,²² and possibly participates in the initiation and/or the
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1 development of acute and late adverse tissue effects in the course of RT. Thus, strategies that
2 target normal tissue-associated endothelial cells would be of interest to impair their ability to
3 recruit immune cells. In such a strategy, the question of the timing of the modulation should be
4 explored in depth. Many studies have aimed to identify strategies to antagonize adhesion molecule
5 function, by either preventing interactions with receptors or inhibiting NF- κ B signalling, to limit the
6 inflammatory response. Hitherto, no clinical strategy has emerged that controls inflammation by
7 targeting expression of the NF- κ B-dependent endothelial adhesion molecule. However, mechanisms
8 independent of the NF- κ B pathway may also regulate adhesion molecule function, including post-
9 transcriptional regulation by IL-19¹⁷⁶ and post-translational modification by N-linked sugars.³⁹ Since
10 leukocyte trafficking in inflammation is actually governed by protein-glycan interaction,¹⁹
11 understanding of these complex interactions raises great hopes for the emergence of new
12 therapeutic targets to treat inflammatory diseases such as atherosclerosis, intestinal bowel disease
13 and thus also for radiation diseases.⁴⁰

20 Endothelial-oriented strategies to target tumours

22 *Increasing injury to tumour vasculature*

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

46 *Cancer immunotherapy*

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

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

15 *Combining immunotherapy, anti-angiogenic therapy and radiotherapy*

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

33 *Opening the endothelium for drug delivery*

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

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

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

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

1
2 **Figure 1.** The leukocyte adhesion cascade is triggered by a pro-inflammatory stimulus. Injury
3 activates endothelial cells, allows production of free radicals, and damages tissue and tissue-
4 resident immune cells leading to the release of cytokines, chemokines and growth factors, which
5 then attract leukocytes from the circulation. Circulating leukocytes undertake a seven-step process
6 of capture, rolling, slow rolling and activation, arrest and adhesion strengthening, intravascular
7 crawling and finally transmigration to reach sites of inflammation. Each step of this process is
8 controlled by various adhesion molecules at the surface of the endothelium. All of these proteins
9 are glycosylated, a post-translational modification process that may be regulated during
10 inflammation. During this cascade of events, leukocytes are also activated by interactions with
11 cytokines, chemokines and growth factors sequestered by the glycosaminoglycans of the
12 endothelial glycocalyx.
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18 **Figure 2.** Ionizing radiation injures the vascular endothelium. DNA damage and ceramide production
19 lead to cell death, stress-induced premature senescence, cell activation mainly characterized by
20 the overexpression of adhesion molecules, and disruption of the endothelial barrier. Endothelial
21 activation promotes a pro-thrombotic and pro-inflammatory phenotype that ultimately leads to
22 thrombosis and recruitment of leukocytes. Irradiated endothelial cells can also undergo an
23 endothelial-to-mesenchymal transition that potentially contributes to fibrosis via a final
24 differentiation into activated myofibroblasts capable of secreting collagens.
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29 **Figure 3.** Immune-inflammatory effects of radiation exposure on normal tissues, tumours and
30 tumour microenvironment. Ionizing radiation causes tumour and normal cell damage, cell death and
31 release of damage-associated molecular patterns (DAMPs), which act as pro-inflammatory signals.
32 Radiation activates cells that then release cytokines/chemokines. Activated endothelial cells
33 acquire a pro-inflammatory phenotype which promotes the leukocyte adhesion cascade. These
34 initial responses finally lead to the recruitment and activation of diverse immune cells, which can
35 then also participate in the abscopal effect of radiotherapy. Delayed mitotic death and proliferation
36 of immune cells in the tissue then cause environmental changes that ultimately contribute to
37 necrosis or fibrosis. Irradiation of the microbiota of the intestinal tract can also influence the
38 responses of normal tissues and tumours through immune-vascular crosstalk.
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45 **Figure 4.** Potential endothelial-oriented strategies to spare normal tissue by targeting cytotoxicity,
46 coagulation, activation of the immune-inflammatory response and senescence.
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49 **Figure 5.** Endothelial-oriented strategies to enhance tumour control by targeting the
50 immunostimulatory and immunosuppressive reactions, inducing cell death and increasing vascular
51 permeability to allow immune cell infiltration.
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Figure 1

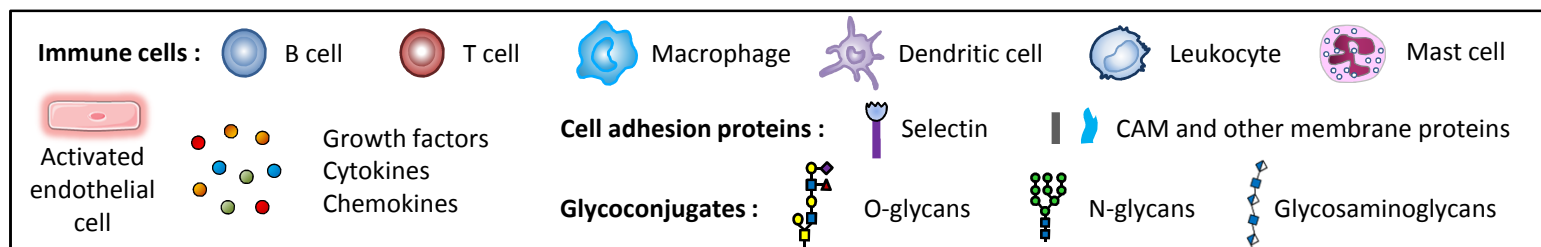
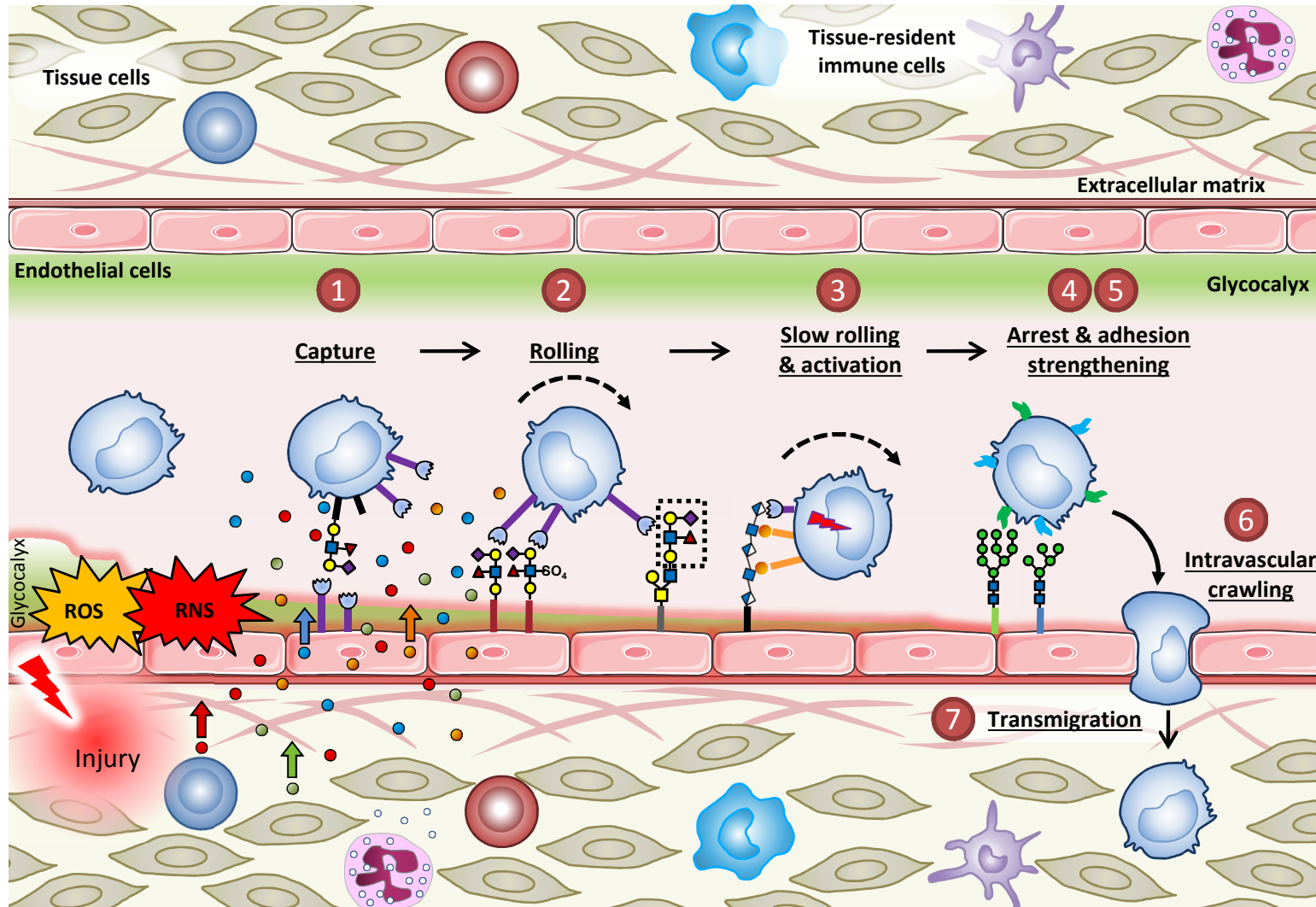
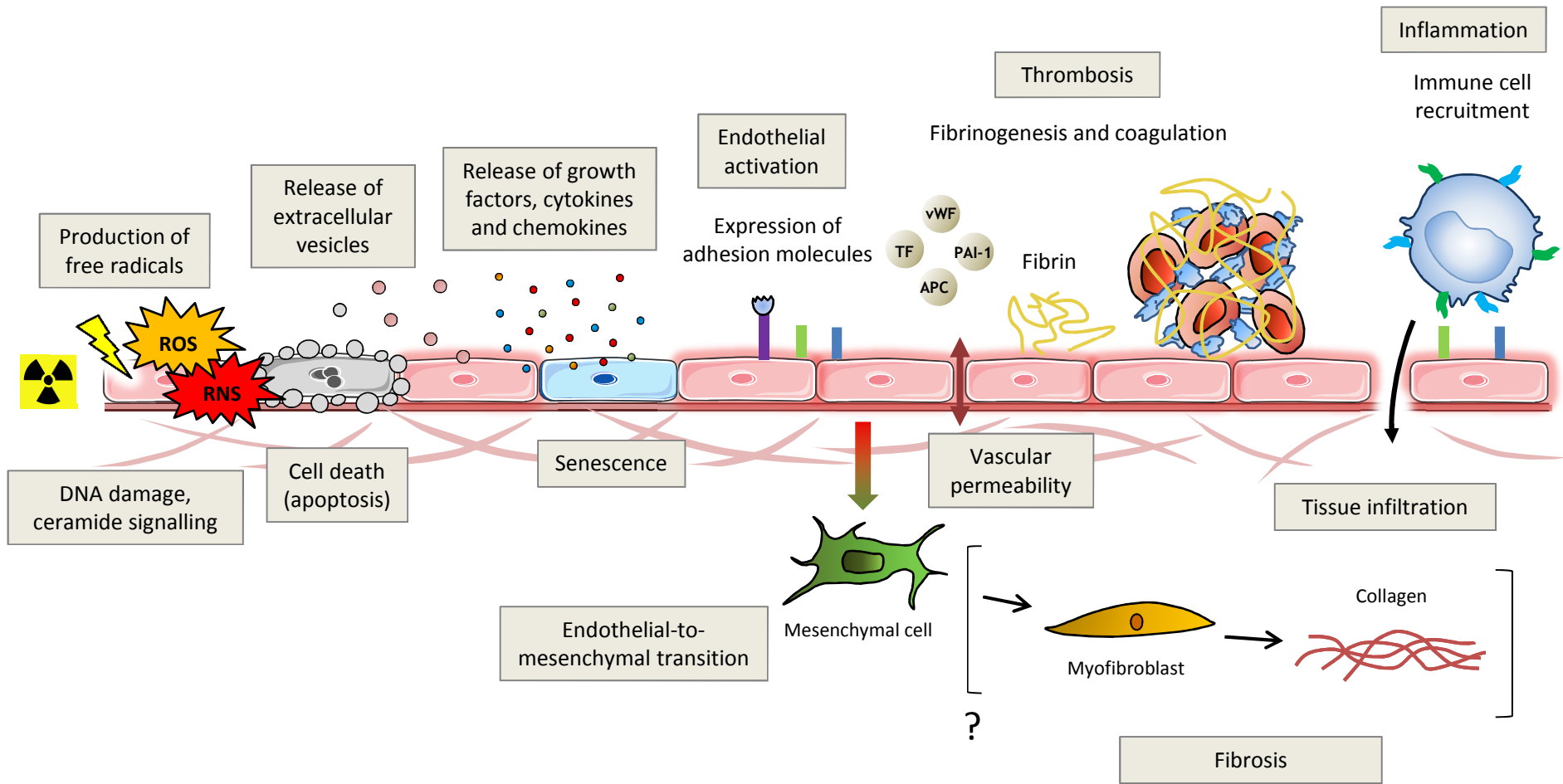


Figure 2



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|-------------|----------------------------|-----------|----------------|----------|----------------|----------|---------------------------------|
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| Irradiation | Activated endothelial cell | Leukocyte | Red blood cell | Platelet | Growth factors | Selectin | CAM and other membrane proteins |
| | | | | | Cytokines | | |
| | | | | | Chemokines | | |

Figure 3

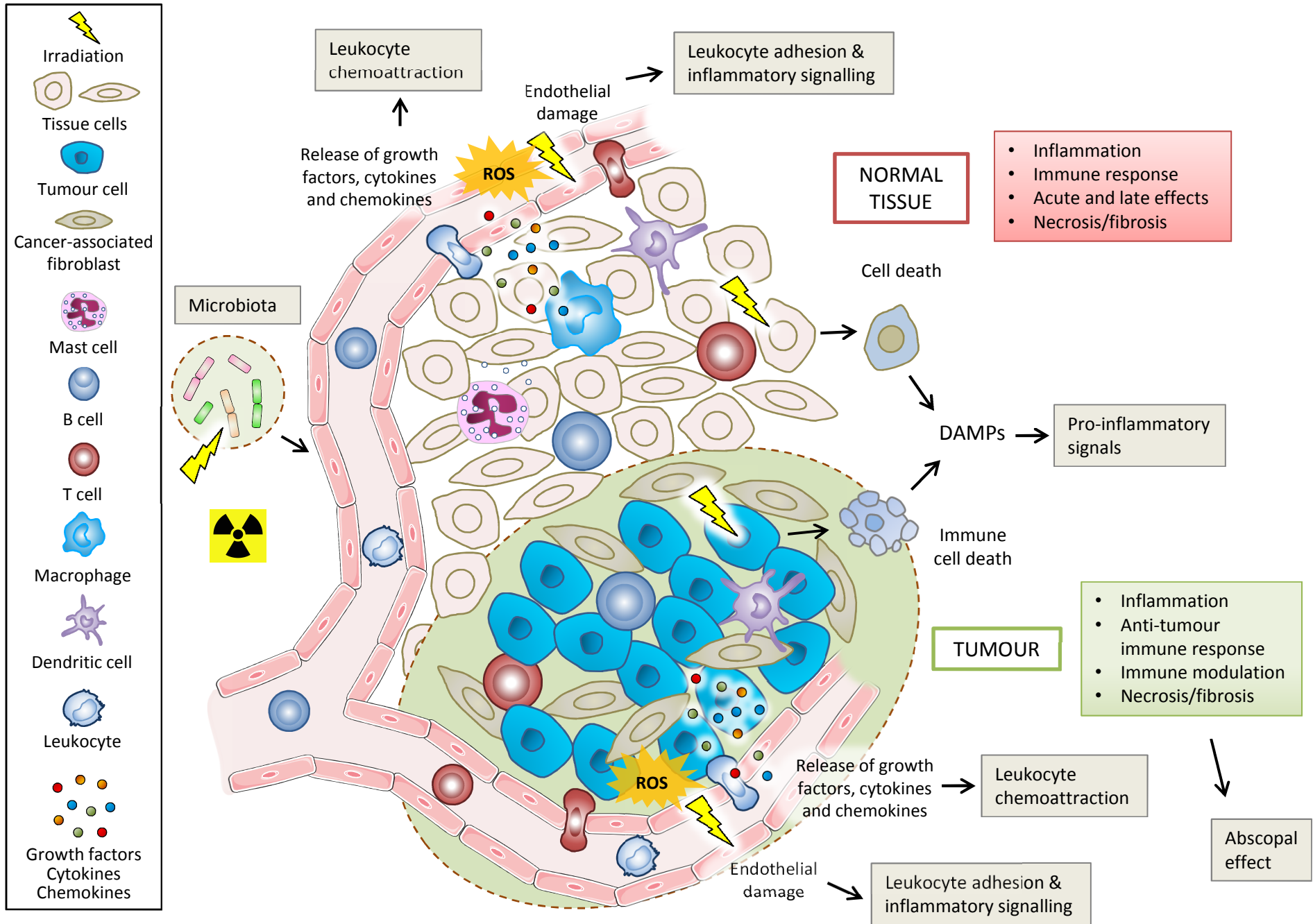


Figure 4

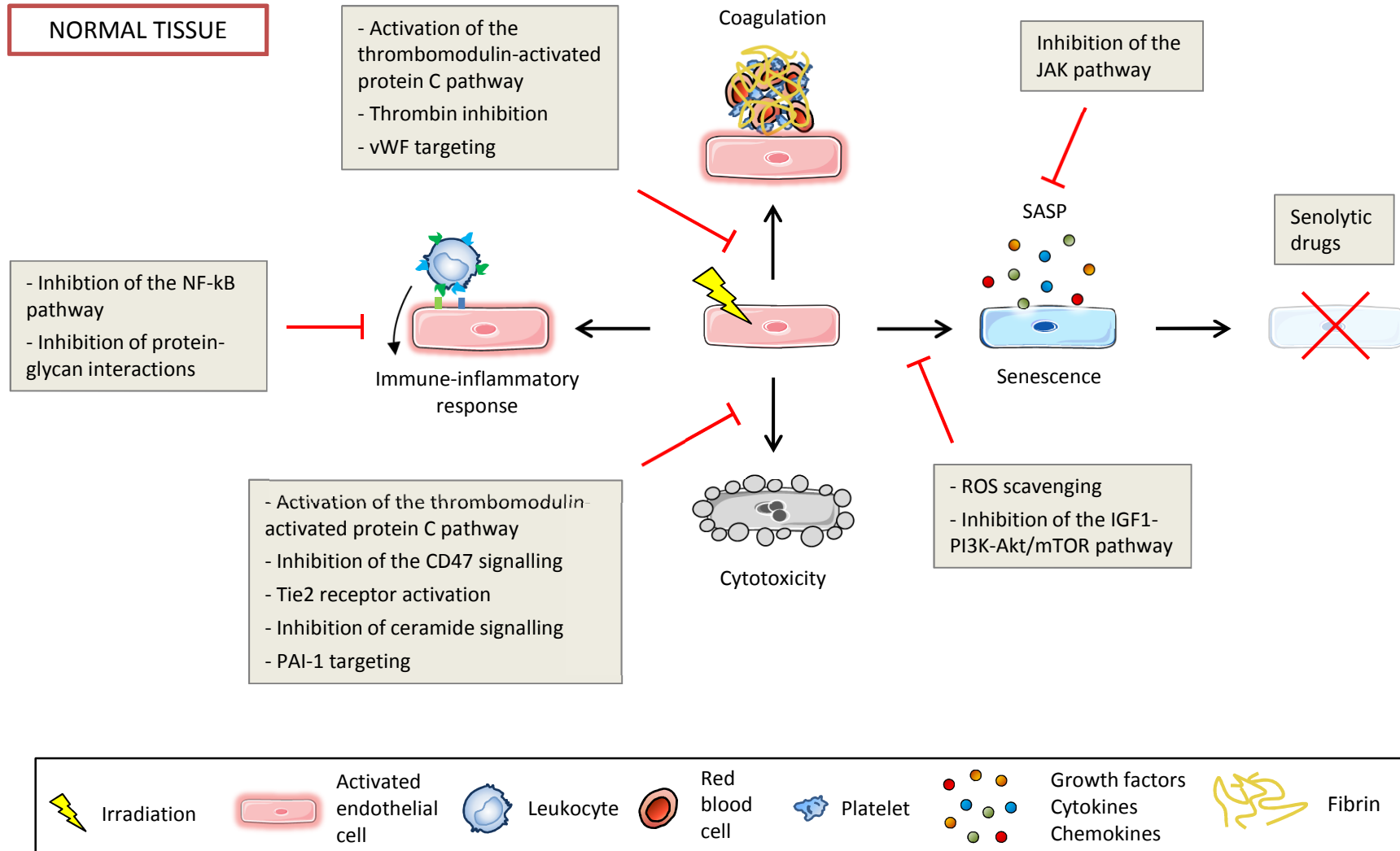


Figure 5

