The importance of hypoxia in radiotherapy for the immune response, metastatic potential and FLASH-RT

Eui Jung Moon*, Kristoffer Petersson, and Monica M. Olcina

Purpose: Hypoxia (low oxygen) is a common feature of solid tumors that has been intensely studied for more than six decades. Here we review the importance of hypoxia to radiotherapy with a particular focus on the contribution of hypoxia to immune responses, metastatic potential and FLASH radiotherapy, active areas of research by leading women in the field.

Conclusion: Although hypoxia-driven metastasis and immunosuppression can negatively impact clinical outcome, understanding these processes can also provide tumor-specific vulnerabilities that may be therapeutically exploited. The different oxygen tensions present in tumors and normal tissues may underpin the beneficial FLASH sparing effect seen in normal tissue and represents a perfect example of advances in the field that can leverage tumor hypoxia to improve future radiotherapy treatments.

Introduction – The historical importance of hypoxia to radiation responses

Tumors are often found under hypoxic (low oxygen) conditions due to the imbalance between oxygen consumption and supply. The chaotic vasculature and abnormal mitochondrial function are unable to meet the metabolic demands of the growing tumor, despite the induction of angiogenesis (LaGory and Giaccia 2016). As a consequence, pockets of nutrient and oxygen deprivation coupled to high lactate and extracellular acidity form within tumors. Importantly, these microenvironmental features can profoundly impact tumor biology and treatment response (Bertout et al. 2008; Hanahan and Coussens 2012; Yoshimura et al. 2013). Tumor hypoxia is significantly associated with poor patient survival and radiation outcomes (Nordsmark et al. 1996; Höckel and Vaupel 2001; Overgaard 2011; Sorensen and Horsman 2020; Matulevicuči et al. 2021). Extensive studies by Nordsmark et al., highlighted the prognostic value of oxygenation in cervix and head and neck cancer patients using an oxygen electrode (Nordsmark et al. 1996; Nordsmark et al. 2001; Nordsmark and Overgaard 2004; Nordsmark et al. 2005; Nordsmark et al. 2006). Women researchers in the field have also made important contributions to the development of hypoxia gene expression signatures and imaging methods, which have once again highlighted how common hypoxia is in solid tumors e.g. West, Buffa and Lyng (Lyng et al. 2001; Winter et al. 2007; Buffa et al. 2010; Eustace et al. 2013; Harris et al. 2015; Hillestad et al. 2020; Sorensen and Horsman 2020). While work by Denekamp et al., led to clinical trials of accelerated radiotherapy with carbogen and nicotinamide (ARCON), recent studies including those led by Koritzinsky and Papandreu suggested metabolic inhibitors such as metformin, papaverine, and atovaquone as radiation sensitizers (Zackrisson et al. 1994; Bernier et al. 2000; Zannella et al. 2013; Ashton et al. 2016; Benej et al. 2018). Targeting hypoxic tumor cells by using prodrugs specifically activated in the reductive environment of hypoxic tumors has also been extensively investigated including with important contributions from McKeown, Robson, McCarthy, Williams, Hammond, and Pedley (McCarthy et al. 2003; McErlane et al. 2005; Dearling et al. 2007; McKeown et al. 2007; Cowen et al. 2008; O’Rourke et al. 2008; O’Connor et al. 2016; Mistry et al. 2017; Mehibel et al. 2021; Skwarska et al. 2021).

Hypoxia leads to cellular stress responses that allow tumor cells to survive and often thrive under what would be considered harsh living conditions for untransformed cells (Giaccia 1996). Severe hypoxia (often termed radiobiological hypoxia when below 10 mmHg) is particularly relevant for tumor radiation responses due to the requirement for oxygen to form free radicals and to induce the downstream DNA breaks and lethality (Hammond et al. 2014; Hall and Giaccia 2019). In fact, there is a rapid change in radiosensitivity as oxygen concentrations increase with 0.5% O₂.

KEYWORDS
FLASH; immune system; metastasis; cancer; women in research; ultra-high dose rate; tumor microenvironment

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representing a relative halfway in radiosensitivity (Hall and Giaccia 2019). Furthermore, the cellular stress response mounted in conditions of radiobiological hypoxia can impact radiation responses and may offer potential therapeutic targets (Wilson and Hay 2011; Hasvold et al. 2016). For example, studies from Hammond, Pires, Leszczynska, Olcina and Foskolou have demonstrated that radiobiological hypoxia will lead to replication stress and the induction of a DNA damage response (DDR) (Hammond et al. 2002; Pires et al. 2010; Olcina et al. 2013; Foskolou et al. 2017). Inhibiting DDR members can sensitize hypoxic cells to radiotherapy (RT) (Fokas et al. 2012; Pires et al. 2012; Weber and Ryan 2015; Dillon et al. 2019). Furthermore, the unfolded protein response (UPR) is also induced under these conditions and the study of crosstalk between UPR and DDR signaling could provide a source of future therapeutic targets to improve RT (Wouters and Koritzinsky 2008; Ramachandran et al. 2021).

A key driver of the biological response under hypoxic conditions is hypoxia-inducible factor (HIF), a heterodimeric transcription factor. While the HIF\(\alpha\) subunit is constitutively active, the hydroxylation of HIF\(\alpha\) leads to its proteasomal degradation under oxic conditions (Wang GL and Semenza 1993; Maxwell et al. 1999). Under hypoxia, hydroxylation is inhibited and stabilized HIF\(\alpha\) forms a heterodimer with HIF\(\beta\) to transactivate its target genes. HIF activates complex biological responses including altered metabolism, apoptosis, autophagy, metastasis and angiogenesis, as demonstrated by female scientists including Simon, Bertout Papandreou, Chan, Rankin, Carroll and Ashcroft (Carroll and Ashcroft 2006; Chen and Giaccia 2007; Bertout et al. 2008; Mijaljica et al. 2010; Krock et al. 2011; Greer et al. 2012; Yang et al. 2012; Rankin and Giaccia 2016).

Previous studies investigating the role of tumor hypoxia on radiation responses have heavily focused on the biological consequences at the level of the tumor cell itself. However, it is becoming increasingly recognized that hypoxia and radiation also have a systemic impact on blood circulation, circulating tumor cells and immune processes (Doedens et al. 2010; Martin et al. 2014; Palazon et al. 2014; Olcina et al. 2019; Matulevicute et al. 2021).

In this review we highlight the contribution of women leaders in the hypoxia and radiation fields with respect to three key areas that are or have the potential to impact clinical outcome: radiation-induced metastasis, immune responses, and FLASH-RT.

**Radiation-induced tumor invasion and metastasis and its crosstalk with hypoxia and redox pathways**

Radiation treatment is given as standard-of-care to enhance local tumor control. External radiation is also applied to control local recurrence or to treat bone or brain metastases, which results in better patient survival. However, local-regional failure or micrometastasis after radiation indicate that there are systemic effects supporting the survival of subsets of tumor cells to become resistant and metastatic. Historically, tumor bed effects indicate that pre-irradiating normal tissues delays uptake and growth of the primary tumor, suggesting that radiation-induced changes to the microenvironment affect tumor cell growth (Stenstrom et al. 1955). Interestingly, although limiting the primary tumor growth, many studies observed that treating normal tissues or primary tumors with radiation promoted metastatic features of tumor cells (von Essen 1991). Early studies using a mouse xenograft model with subcutaneously injected mammary carcinoma, indicated that radiation (4–10 Gy) induced lung metastasis (Kaplan and Murphy 1949). A subsequent study further suggested that when mouse legs were pre-irradiated (30 Gy) before tumor cell inoculation, lung metastasis was increased while primary tumor growth was delayed (Milas et al. 1987). Although these studies represent radiation treatment to primary tumors versus normal tissues, they suggest systemic radiation responses might result in enhanced dissemination of tumor cells.

In addition to the effects of radiation to tumor cell recruitment to distant organs, tumor self-seeding effects were also observed in mouse breast cancer models by Vilalta and Rafat (discussed in further detail below) (Vilalta et al. 2014; Rafat et al. 2018). The self-seeding effect, referring to circulating tumor cells (CTC) colonizing the site of origin in a breast cancer model. Vilata and colleagues demonstrated that radiation can induce the self-seeding effect through the secretion of GM-CSF using both in vitro and in vivo models (Vilalta et al. 2014). Following this study, Rafat from the same group, further demonstrated that irradiation of normal tissues also recruited tumor cells from a distant site (Rafat et al. 2018). Although these two studies involved immunocompromised mice, they suggest that secretion factors from irradiated tissues recruit tumor cells from other sites to cause a local recurrence.

There are four mechanisms, which could explain how radiation promotes tumor metastasis: 1) direct radiation effect on tumor cells, 2) radiation effects in distant sites to prime metastatic niches, 3) release of tumor cells into the blood circulation, and 4) increased time for tumor cell release into the bloodstream due to a radiation-induced tumor growth delay (von Essen 1991). According to our current understanding, these mechanisms do not seem to separately affect radiation-induced tumor metastasis but rather interplay to select more aggressive and resistant tumor cells. In this part of the review, we will focus on radiation-induced metastasis through expression of tumorigenic factors with a particular focus on those impacted by hypoxia and redox pathways.

It has been speculated that destruction of vasculature is the main cause of tumor cell release into the circulation after radiation treatment. Martin and colleagues detected the presence of CTCs expressing a mesenchymal marker (vimentin) in non-small cell lung cancer patients after radiation (Martin et al. 2014). However, before the release of
tumor cells, multiple steps are required including hypoxia-mediated angiogenesis and invasion/migration of tumor cells in their primary sites (Sundahl et al. 2018). Radiation effects on hypoxia and angiogenesis were initially demonstrated using a mouse dorsal window chamber model (Moeller et al. 2004). This study found that radiation treatment (5, 10, 15 Gy) enhanced tumor hypoxia and HIF-1 expression through ROS production; and activation of HIF-1 pathways led to enhanced angiogenesis. A study by Williams and colleagues further demonstrated that HIF-1 is a crucial factor for radioresistance using a mouse xenograft model with a HIF-1 deficient hepatoma cell line (Williams et al. 2005). While these studies focused on the role of HIF-1 in radiation resistance, it was further demonstrated that radiation induces tumor hypoxia and angiogenesis, which results in tumor metastasis (Rofstad et al. 2005). This study found that expression of well-known pro-angiogenic factors, IL-8 or urokinase plasminogen activator surface receptor (uPAR) were elevated in primary tumors, which were pre-irradiated 24 hours before tumor inoculation. Their expression, specifically IL-8, was correlated with tumor hypoxia and microvessel density, indicating that hypoxia-induced IL-8 promotes angiogenesis. Inhibition of IL-8 or uPAR decreased tumor metastasis, while it had no effect on primary tumor growth. These data demonstrated that the hypoxic microenvironment in the pre-irradiated tumor bed governed tumor metastatic potential by inducing IL-8 as an angiogenic factor, or uPAR as an invasive factor. A study by Bouchard and colleagues additionally identified two inflammatory factors, IL-6 and cyclooxygenase-2 (COX-2) as pro-migratory factors in mouse mammary carcinoma grown in pre-irradiated mammary fat pad (Bouchard et al. 2013). Interestingly, while spontaneous metastasis was increased in pre-irradiated mice, tail vein injection did not show significant differences in lung nodule formation between non-irradiated and pre-irradiated mice, confirming that radiation-induced microenvironmental changes contribute to the tumor metastasis. Her subsequent studies further identified that pre-irradiation-mediated lung metastasis was observed specifically in triple negative breast (TNB) cancer models and was dependent on membrane type-1 matrix metalloproteinase (MT1-MMP), which degrades extracellular matrix, by activating MMP2 and MMP9, pro-invasive markers (Bouchard et al. 2016, 2017). Bouchard and colleagues also showed that chloroquine treatment inhibits radiation-induced metastasis, and this treatment might be used to enhance disease-free survival of TNB cancer patients. The study by Riekkilä and her group reported that radiation-induced elevation of MMP1 and MMP2 (TIMP1 and TIMP2), two essential matrix metalloproteinases, in breast cancer patients who had surgery and radiation (Riekkilä et al. 2000). Experimental studies by Wild-Bode and colleagues using in vitro and in vivo models also showed that increased MMPs lead to tumor metastasis (Wild-Bode et al. 2001).

Production of reactive oxygen species (ROS) impacts radiation-induced cell killing through indirect DNA damage and altered expression of tumor progression factors including HIF-1 and Transcription growth factor β (TGFβ) (Barcellos-Hoff and Dix 1996; Moeller et al. 2004; Shimura et al. 2018). The work by Barcellos-Hoff and colleagues showed that radiation-induced ROS are responsible for enhanced TGFβ expression (Barcellos-Hoff and Dix 1996). Her later studies also highlighted the role of the tumor microenvironment in metastasis and radiation responses (Ilä-Bochaca et al. 2014; Qiang et al. 2016). TGFβ is a major cytokine known for its role in normal tissue fibrosis, which was supported by studies from female scientists Martin and Rube (Martin et al. 1997; Rube et al. 2000). Rube and her colleagues performed radiation dose and time dependent studies using non-tumor bearing C57BL/6 mice and showed that TGFβ expression reached the maximum values at two and four weeks after radiation. However, TGFβ can also be induced as early as 6 hours after radiation, indicating its effect on early radiation responses. The role of TGFβ in tumor metastasis has been extensively studied in a variety of tumor models including breast and colon cancers (Padua et al. 2008; Calon et al. 2012). Its involvement in endothelial-to-mesenchymal transition (EMT) pathways and metastasis, through regulation of two hypoxia-regulated genes, angiopoietin-like-4 (ANGPTL4) and IL11, identified TGFβ as a therapeutic target (Zhang et al. 2012; Moon et al. 2021). Biswas and her colleagues reported that in MMTV/PyVmT, a transgenic breast cancer mouse model, radiation at 10 Gy induced TGFβ levels in plasma while CTC and lung metastasis were also increased (Biswas et al. 2007).

Interestingly, thoracic radiation promoted dissemination of primary tumor cells grown in the mouse mammary fat pad to the mouse lung, suggesting that radiation also amends the tissue microenvironment to ‘recruit’ tumor cells. In this study, inhibition of TGFβ signaling, using a conditional TGFβRII knockout animal model or TGFβ antagonizing antibody, decreased radiation-induced metastasis and the number of CTC in circulation, confirming that TGFβ is a driving factor of radiation-induced metastasis (Biswas et al. 2007). Subsequent studies have also reported that blocking TGFβ in combination with anti-PD-1, anti-CD137 and irradiation can improve CD8 T cell infiltration into non-irradiated lesions and enhances abscopal responses (tumor eradication outside the irradiation field) (Rodriguez-Ruiz et al. 2019). More recently a combination of radiation with a bifunctional fusion protein simultaneously inhibiting TGFβ and PD-L1 was shown to result in reduced RT-induced fibrosis and spontaneous lung metastasis, suggesting the beneficial effects of combining targeted and systemic treatments (Lan et al. 2021).

While ROS are also known to induce tumor invasion and metastasis after radiation (Kambach et al. 2014; Gu et al. 2015), regulation of redox pathways might also reduce the radiation-mediated metastatic features of cancer. NRF2 is a basic leucine zipper (bZIP) protein, which is well known for its regulation of antioxidant responses (Moon and Giaccia 2015). Although BACH1 was known as a negative regulator of redox pathways via competition with NRF2, a recent study provided significant insights indicating that NRF2 and BACH1 interact to promote tumor metastasis (Lignitto et al. 2019). Although extensive studies by Rosner and Lee have...
identified the important role of BACH1 in tumor metastasis, the function of BACH1 in radiation responses has not been reported (Sun et al. 2013; Lee et al. 2014, 2019). Studies suggest the importance of NRF2 in tumor biology and radiation resistance (McDonald et al. 2010; Jeong et al. 2017). Given that ROS might play a major role in promoting radiation-induced metastasis through upregulation of metastatic factors, it will be important to understand the impact of NRF2 or BACH1 pathways on tumor progression after radiotherapy. A recent study by Moon and colleagues determined that MAFF, an indispensable binding partner of NRF2 and BACH1, is more responsible for hypoxia regulation and tumor metastasis (Moon et al. 2021). Unpublished data by Moon also suggested that MAFF is highly induced by radiation, indicating its potential role in radiation and radiation-induced metastasis. Therefore, a better understanding of regulation of antioxidant responses could provide therapeutic targets to improve patient outcomes, including in the metastatic setting.

In summary, radiation effects are beneficial to primary tumors by promoting local control. However, multiple studies suggest that local and systemic effects allow tumor cells to invade, release, and to metastasize through hypoxia and ROS-mediated factors, which could lead to loco-regional failure or recurrence of tumor (Figure 1). However, despite numerous preclinical studies, the effect of radiation on metastasis induction is still controversial (Sundahl et al. 2018). Although the mechanisms underlying this phenomenon are poorly understood, it seems clear that a more effective treatment strategy is required to modulate tumor hypoxia and redox status of tumors. Also, further investigation into the role of radiation-induced systemic changes including immune modulation is required.

**Hypoxia and radiation-induced immune responses**

Immune regulation by hypoxia occurs in a number of pathological settings including in response to infections and in the tumor microenvironment (Palazon et al. 2014; LaGory and Giaccia 2016). Paradoxically, while in response to infections, movement of immune cells from the oxygen-rich blood to the hypoxic infected tissue contributes to host immune responses, the hypoxic tumor microenvironment is generally considered immunosuppressive (D’Ignazio et al. 2016).

While the effects of hypoxia mainly promote immune escape and an overall immunosuppressive environment, radiation can potentiate immune recognition and tumor clearance (Figure 2). Increased antigen release following radiotherapy and immunogenic cell death can enhance immune infiltration and may activate both local and distant site (non-radiated) anti-tumor immunity (Apetoh et al. 2007; Obeid et al. 2007; Ma et al. 2011; Demaria et al. 2015; Vaes et al. 2021; Zhu et al. 2021). Although radiation can also result in suppression of host anti-tumor immunity, its immune-stimulatory effects have sparked renewed interest in radiotherapy, including its potential in combination with immune checkpoint inhibitors (Wirsdorfer et al. 2014; Pilones et al. 2015; Eckert et al. 2019). When considering radiotherapy as a means of boosting anti-tumor immunity it is important to bear in mind that, as mentioned above, most solid tumors are hypoxic and therefore likely under some degree of immunosuppression (Chouaib et al. 2017; Eckert et al. 2019).

For example, tumor hypoxia is associated with recruitment of immunosuppressive cell types such as myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs) as reported by several studies including Chak-Lui Wong, Jendrossek and colleagues (Yan et al. 2011; Chiu et al. 2017; Westendorf et al. 2017; Jayaprakash et al. 2018). While the exact role of hypoxia in Treg biology remains controversial, HIF-2α but not HIF-1α was recently identified as critical for Treg function, with Treg selective knockout of HIF-2α rendering mice resistant to tumor growth and metastasis (in colorectal and melanoma models, respectively) (Hsu et al. 2020). HIF-driven secretion of chemokines by tumor cells can also contribute to immunosuppression via MDSC recruitment to the TME (Chiu et al. 2016). Furthermore, HIF affects MDSC differentiation into tumor associated macrophages (TAMs) (Corzo et al. 2010; Chiu et al. 2016). HIF-1α also upregulates immune checkpoint factors such as PD-L1 on MDSCs, macrophages, dendritic cells, and tumor cells (Noman et al. 2014). Murthy and Lord recently found that hypoxia inhibits tumor cell expression of IFN-γ-dependent chemokines CXCL9, CXCL10 and MHC Class I. Additionally, T-cells cultured in hypoxia (0.5% O₂) following antigenic stimulation, display reduced proliferation and IFN-γ production. Interestingly, the effects on IFN-γ production by T-cells appear HIF-1α independent and can be rescued by reoxygenation (into 21% O₂) (Murthy et al. 2019). Hypoxia can therefore impact immune modulation through both direct effects on immune cells as well as indirectly through the effect that hypoxic tumor cells have on immune cell recruitment and function.
While hypoxia-mediated immunosuppression can be a challenge for effective RT treatment, it also offers an Achilles’ heel that can be targeted. Indeed, studying hypoxia-induced expression of proteins associated with reduced immune infiltration can serve as a means of identifying therapeutic targets to combine with immune checkpoint blockade and RT. A good example of this is the work carried out by Le, Kuo et al., on galectin 1, a carbohydrate binding protein secreted by hypoxic tumor cells (Le et al. 2005; Kuo and Le 2014). Work from Le and her group has shown that Gal1 expression is inversely correlated with lymphocyte marker CD3 in head and neck (H&N) patients; and that Gal1 and CD3 are predictors of overall survival (Le et al. 2005). Subsequently, Le, Nambiar et al., elegantly showed that tumor Gal 1 reprograms the tumor endothelium to express PD-L1 and galectin 9 thereby preventing T-cell migration. Blocking Gal1 together with anti-PD1 therapy improves tumor response including in combination with radiotherapy in H&N cancer models (Nambiar et al. 2019).

TAMs found in hypoxic regions are associated with an anti-inflammatory, pro-tumorigenic, and pro-angiogenic phenotype that could account for therapy resistance (Henze and Mazzone 2016). The effects of radiation on macrophage biology also support the idea that this cell population is detrimental to radiation response within the TME. The Muschel lab, for example, recently reported on the importance of macrophage programming in the TME following fractionated RT, with FGF2 playing an important role. Interestingly, treatment with FGF2 blocking antibody together with fractionated RT increases tumor growth delay and long-term survival in murine models (Im et al. 2020). Work from the Muschel group had also previously demonstrated that RT of murine colorectal or pancreatic xenografts induces colony-stimulating factor 1 (CSF-1), which is associated with an increase in TAMs with an immunosuppressive phenotype five days post RT. Depleting macrophages (with anti-CSF-1 antibody) and delivering RT resulted in tumor growth delay in a CD8 T cell-dependent manner and was associated with increased antigen priming. These data suggest that adaptive anti-tumor immune responses are limited by TAMs. Interestingly, addition of anti-PD-L1 antibody further enhanced the radiation response of macrophage depleted tumors (Jones et al. 2018). In support of these studies, the Ahn lab recently found that TAM depletion (with clodronate) attenuates tumor hypoxia and glycolysis while enhancing T-cell infiltration and PD-L1 expression. This study provides further rationale for combining TAM targeting strategies with anti-PD-L1 antibodies (Jeong et al. 2019).

Within the TME a survival strategy of the tumor cell involves hijacking those transcription factors that would normally drive inflammation and pathogen clearance (e.g. HIF and NF-κB) in a manner that instead allows tumor immune evasion (Jung et al. 2003; House et al. 2017). While the co-option of these pathways poses a barrier to effective cancer treatment, it also offers insights into particular tumor
vulnerabilities. For example, complement-mediated cytotoxicity has been proposed to contribute to tumor cell clearance (Roumenina et al. 2019). However, Olcina and colleagues demonstrated that within the TME, the cytotoxic effects of complement are compromised due to hypoxia and HIF-dependent expression of endogenous complement regulators such as CD55 in colorectal cancer cells (Olcina et al. 2018). HIF-dependent expression of endogenous complement regulators may therefore allow tumors to evade the cytotoxic effects of the complement system while benefiting from the tumor promoting properties of the pathway. Interestingly, increased CD55 expression has been associated with radioresistance and targeting different components of the complement system has been suggested to improve radiation response, albeit with somewhat conflicting results (Elvington et al. 2014; Surace et al. 2015; Leung et al. 2018; Olcina et al. 2020). Early studies from Elvington and her colleagues indicated that, a tumor targeted inhibitor (CR2-CrRy) that blocks all complement pathways at the level of C3 activation, improved response to fractionated RT (Elvington et al. 2014). However, subsequent reports by Surace, Van den Broek and colleagues, suggested that the positive effects of radiation on anti-tumor immune responses would be diminished if complement inhibition occurred together with primarily high single dose irradiation (20 Gy) (Surace et al. 2015). It is important to note that these two studies used different preclinical murine models, radiation schedules and modes of inhibiting complement, complicating the direct comparison of these two studies. Still, these studies do highlight the need to systematically investigate the contribution of immune-associated processes to radiation responses in single dose and fractionated radiation schedules, side-by-side and within the same murine models. Elegant work from Formenti, Demaria and Vanpouille-Box have demonstrated that different radiation regimens can indeed impact the outcome of anti-tumor immune radiation responses, including through regulation of intracellular innate immune processes (Pilones et al. 2015; Vanpouille-Box et al. 2017). Furthermore, when assessing tumor radiation responses in the context of different radiation schedules or novel modalities it will be important to consider the ever-increasing number of non-canonical functions reported for innate immune players (Dunphy et al. 2018; Bai et al. 2019; Olcina et al. 2020).

Modulation of immune responses by hypoxia/HIF dependent mechanism can also occur through crosstalk with NF-κB, the main pro-inflammatory family of transcription factors (Karin 2006; Rius et al. 2008). Such crosstalk can occur in a number of cell types including macrophages, neutrophils and tumor cells themselves as elegantly reported by the Rocha lab (van Uden et al. 2008; D’Ignazio et al. 2016; 2020). Importantly, NF-κB is induced in response to hypoxia as well as ionizing radiation and is well-known to contribute to intrinsic tumor cell radioresistance, through modulation of pro-survival pathways (Criswell et al. 2003; D’Ignazio et al. 2016). Interestingly, NF-κB activation also occurs on radiosensitive normal tissues where NF-κB-driven pro-survival signaling can confer protection (Wang et al. 2004). Finding therapeutic approaches that maximize dynamic modulation of NF-κB signaling to prevent tumor cell radioresistance, while affording productive activation for enhanced anti-tumor and protective normal tissue responses could enhance the therapeutic index of radiotherapy.

Overall, the promise of improved local (and potentially systemic) tumor control by effective immune-modulation and RT combinations might be particularly appealing for hypoxic tumors that currently display immunosuppression, reduced local control and increased rates of distant metastasis.

Is radiation induced transient hypoxia responsible for the FLASH effect?

In recent years, pioneering studies mainly from the Vozenin lab have shown that FLASH irradiation, which is radiation delivered at ultra-high dose rates (>30–40 Gy/s), results in significantly less normal tissue toxicity compared to irradiation at conventional clinical dose rates (few Gy/min) (Favaudon et al. 2014; Loo et al. 2017; Montay-Gruel et al. 2017, 2018; Bourhis et al. 2019; Montay-Gruel et al. 2019; Simmons et al. 2019; Vozenin et al. 2019; Wilson et al. 2019; Alagband et al. 2020; Diffenderfer et al. 2020; Fouillade et al. 2020; Levy et al. 2020; Soto et al. 2020; Zhang et al. 2020). These studies have primarily been preclinical but a couple of veterinary clinical studies treating canine and feline patients have been reported by Konradsson et al. and Vozenin et al., respectively (Vozenin et al. 2019; Konradsson et al. 2021), as well as a first patient treated successfully with FLASH-RT (Bourhis et al. 2019). In addition to limiting toxicities, there have also been reports of FLASH irradiation maintaining the same tumor response as seen following conventional dose rate irradiation, e.g. the excellent work from the Rankin lab investigating the increase in therapeutic index for FLASH in abdominal radiotherapy in mice (Favaudon et al. 2014; Zlobinskaya et al. 2014; Bourhis et al. 2019; Levy et al. 2020; Montay-Gruel et al. 2021). The FLASH sparing effect has mainly been observed in vivo, though a few studies have shown an effect also in vitro (Buonanno et al. 2019; Montay-Gruel et al. 2019; Adrian et al. 2020; Fouillade et al. 2020; Adrian et al. 2021; Khan et al. 2021). The biological mechanisms responsible for this differential FLASH sparing effect between normal tissue and tumor tissue is not yet known but several hypotheses have been proposed (Wilson et al. 2019), e.g. radiochemical depletion of oxygen leading to transient hypoxia (Hendry et al. 1982; Hall and Brenner 1991; Vozenin et al. 2019), radical-radical interaction (Labarbe et al. 2020; Wardman 2020), and a modified immune response following FLASH relative to conventional dose rate irradiation (Durante et al. 2018; Jin et al. 2020).

In a review of dose rate effects, including in vitro work carried out from the late 1950s, Hall and Brenner (referring to the behavior of clonogenic cell survival curves) concluded that: ‘If both the dose and instantaneous dose rate are sufficiently high, the rapid deposition of radiant energy consumes oxygen too quickly for diffusion to maintain an
adequate level of oxygenation, and dose-response curves obtained are those characteristic of hypoxia (Hall and Brenner 1991). A dose-response characteristic of hypoxia following irradiation at ultra-high dose rate has also previously been shown in vivo by Hendry et al. (Hendry et al. 1982). A recent in vitro study by Adrian et al. showed that the FLASH effect is modified by the oxygen concentration (Adrian et al. 2020), which was also recently shown in vivo; in mice by the Vozenin lab (Montay-Gruel et al. 2019) and by the Beyreuther lab in zebrafish embryos (Pavelke et al. 2021). Following FLASH irradiation, it is hypothesized that the physiological level of oxygen (physoxic) found in normal tissues decreases during the rapid dose delivery, creating an acute period of hypoxia in the irradiated tissue and consequently transient radioresistance. An effect that could be considered less important in tumors, with significant volumes of already hypoxic tissue (Wilson et al. 2019), and which was recently indicated by in vivo measurements of oxygen in tumor and normal tissue during ultra-high (300 Gy/s) and conventional dose rate (0.1 Gy/s) irradiation by Cao et al. (Cao et al. 2021). Also, Spitz et al. hypothesized that there are higher levels of redox-active iron (labile iron) in tumor than in normal tissue and that the tissues differ in their oxidative metabolism, with the more rapid removal and decay of the organic hydroperoxides and free radicals derived from peroxidation chain reactions in normal tissue, which could explain the differential effect seen between normal and tumor tissues (Spitz et al. 2019).

The impact of the oxygen consumption following FLASH irradiation has been modeled by several research groups, e.g. the Kirkby lab, and found to fit well to the available biological data (Pratx and Kapp 2019; Petersson et al. 2020; Zhou et al. 2020; Liew et al. 2021; Rothwell et al. 2021). However, studies that model and measure oxygen consumption in water from radiolysis either claim to support the hypothesis (Abolfath et al. 2020; Alanazi et al. 2021) or claim that the oxygen consumption is not a possible explanation for the FLASH effect, as the consumption is small for the amount of dose delivered in FLASH studies and that higher dose rate irradiation consume less oxygen than lower dose rate irradiation (Labarbe et al. 2020; Boscolo et al. 2021; Jansen et al. 2021). To illustrate both sides of this discussion, Figure 3 shows the oxygen consumption as measured in 5 ml cell media in T12.5 flasks, irradiated with 20 Gy at an ultra-high or conventional dose rate, and the clonogenic survival of cells exposed to such beams.

Our figure indicates that measurements of oxygen depletion in water (or media) are likely not adequate to describe the more complex situation that occurs in a cell, through which oxygen diffuses and is consumed differently (Weiss et al. 1974; Wardman 2020; Zhou et al. 2020; Lai et al. 2021). Furthermore, the reactions considered responsible for the depletion of oxygen in water, such as hydrated electrons ($e_{aq}^-$) and H$^*$ atoms reacting with O$_2$, are probably unlikely to occur to any significant extent in irradiated cells because of the high concentrations of competing scavengers (Wardman 2020). Consequently, such studies can neither prove or disprove the hypothesis that the FLASH effect is driven by oxygen depletion but can perhaps guide us in the right direction. As expected, oxygen diffusion profiles have been shown to be very different in vivo, where a circulatory system effectively hinders any reduction in oxygen concentration in the tissue irradiated with conventional dose rates, while it cannot hinder the reduction if the irradiation is at an ultra-high dose rate (Cao et al. 2021). From these studies, we can deduce that future in vitro studies on FLASH should predominantly be performed in a controlled oxygen environment (in physoxia or hypoxia), while in vivo studies looking at tumor response should include well-oxygenated as well as hypoxic tumor models. Likely, there is not one
simple explanation for the observed effect in vivo but rather a combination of several mechanisms, which result in the very promising FLASH effect.

Conclusion
Though the importance of hypoxia for RT has been studied since the 1950s, it is still being actively researched today and will likely continue to be a relevant research topic in the foreseeable future. In this review, we focused on three highly active fields of radiation research for which the leading researchers are women; metastasis, immune response, and FLASH-RT.

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