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Modeling Radiation Induced Lung Injury: Lessons Learned from Whole Thorax Irradiation

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Abstract

Models of thoracic irradiation have been developed as clinicians and scientists have attempted to decipher the events that led up to the pulmonary toxicity seen in human subjects following radiation treatment. The most common model is that of whole thorax irradiation (WTI), applied in a single dose. Mice, particularly the C57BL/6J strain, has been frequently used in these investigations, and has greatly informed our current understanding of the initiation and progression of radiation induced lung injury (RILI).

Purpose: In this review, we highlight the sequential progression and dynamic nature of RILI, focusing primarily on the vast array of information that has been gleaned from murine model. Ample evidence indicates a wide array of biological responses that can be seen following irradiation, including DNA damage, oxidative stress, cellular senescence and inflammation, all triggered by the initial exposure to ionizing radiation (IR) and heterogeneously maintained throughout the temporal progression of injury, which manifests as acute pneumonitis and later fibrosis. It appears that the early responses of specific cell types may promote further injury, disrupting the microenvironment and preventing a return to homeostasis, although the exact mechanisms driving these responses remains somewhat unclear.

Conclusion: Attempts to either prevent or treat RILI in preclinical models have shown some success by targeting these disparate radiobiological processes. As our understanding of the dynamic cellular responses to radiation improves through the use of such models, so does the likelihood of preventing or treating RILI.

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Keywords

Whole Thorax Irradiation; Radiation Induced Lung Injury; Pneumonitis; Fibrosis; Murine Model

Rationale for the Use of Whole Thorax Irradiation Models:

Radiation induced lung injury (RILI) was first noted in the early to mid-20th century in breast and lung cancer patients receiving radiotherapy, with the first published accounts of intrathoracic changes in human subjects following treatment being reported in 1923 (Groover et al. 1923). Because of the doses and volumes used, it was not uncommon for patients to experience side effects from thoracic radiotherapy. Despite these effects, radiation therapy continues to be one of the primary modalities in lung cancer treatment, particularly for non-small cell lung cancer, but its application is limited by the potential for normal tissue injury.

Clinically, the pathogenesis of radiation induced lung injury has been well described (Rubin and Casarett 1968; Gross 1981; Fajardo et al. 2001). The period immediately following radiation therapy is often asymptomatic, and has classically been defined as a “latent period” in which no major pathology is evident (Rubin and Casarett 1968). Subsequently, between 2 and 6 months following treatment that the first consequential pathology manifests, an acute response known as radiation pneumonitis, (RP, or ARP). RP itself is a clinical indicator of pulmonary radiation toxicity, and left untreated, is a potentially life-threatening event (Hernando et al. 2001). The second, distinctive pathological finding known as radiation induced pulmonary fibrosis (RIPF) is a restrictive lung disease which generally evolves between 6 and 24 months post-treatment (McDonald, Rubin, Constine, et al. 1995) and has been shown to progress for up to 2 years, during which time, permanent deterioration of lung function may occur (McDonald, Rubin, Phillips, et al. 1995). Pulmonary radiation toxicity is volume dependent, and the fibrotic region is largely limited to the radiation portal. However, there is considerable patient heterogeneity, with some patients appearing asymptomatic, while others develop chronic respiratory insufficiency (Fajardo et al. 2001). In the most recent decades, refinements in clinicians’ ability to shape the radiation field, application of stereotactic body radiotherapy (SBRT) and altered fractionation protocols have managed to reduce the volume of exposed normal tissue while applying smaller dose fractions rather than a large single dose, thereby reducing the risk of pulmonary toxicity and their relative frequency (Williams and Newhauser 2018). However, despite these improvements, pulmonary radiation toxicity still exists and radiation oncologists must balance the need to deliver an effective dosing regimen to the appropriate volume of tissue, while minimizing radiation toxicity in the surrounding normal tissue (Emami et al. 1991). In fact, it has been estimated that that approximately 43% of lung cancer patients receiving a dose of 27.5 Gy or greater still develop some form of RILI (Graves et al. 2010). Furthermore, there are few approved or efficacious treatments for RILI; corticosteroids remain the standard of care for RP, despite the inherent drawbacks of their use, and some newer agents, such as Pirfenidone and Nintedanib, used to treat idiopathic pulmonary fibrosis, may slow the progression of disease (Spagnolo et al. 2015; Rangarajan et al. 2016).

The morbidity and mortality associated with RILI provide a strong rationale for the need for animal models of RILI, with mouse studies using whole thorax irradiation (WTI) being the most common. Because of this, the majority of WTI models referenced in this review (supplementary table 1) relate to findings in murine models, with a select few examples drawn from other species or hemi-thorax irradiation, and all of the WTI models discussed rely on photon irradiation from either X ray, ^{137}Cs , or ^{60}Co sources. Rats, pigs and non-human primates (NHP) have also been used recently to model RILI and develop similar pathological responses, although there are considerably fewer data available for pig and NHP models (supplementary table 2). While it can be easily argued that the use of WTI has limited application to the clinic, until the recent advent of small animal irradiators, the ability to accurately deliver small fields was not available to bench researchers (Verhaegen et al. 2011).

With respect to the choice of model species, a key consideration in selection is its ability to accurately recapitulate the pathogenesis of pulmonary radiation injury in humans. Mice are frequently selected to model RILI because in addition to the broad advantages of working with mouse models, they also, and importantly, display the clinically relevant outcomes of RP and fibrosis over a similar dose range to that which is seen in humans (Hopewell et al. 2000). While there has been considerable interest in how these responses differ by strain (Jackson et al. 2011; Jackson et al. 2014; Paun et al. 2015; Dabjan et al. 2016), their associated pathology has been well described by a number of groups (Travis 1980; Down and Steel 1983; Coggle et al. 1986; Sharplin and Franko 1989; Morgan and Breit 1995). Due to the strain variation in these responses, researchers need to carefully consider whether or not their strain of choice best reflects the outcome to be investigated. For example, CBA/J, C3H/HeJ and C57L/J develop RP earlier and at lower doses than the C57BL/6 (Jackson et al. 2011) and, because of this, the C3H and CBA strains have been commonly used to examine the acute effects of RILI. However, following RP, C3H and CBA strains do not appear to go on to develop contracted fibrosis (Sharplin and Franko 1989), limiting their usefulness in examining the late response and full progression of RILI.

The C57BL/6J strain has become the most common choice for modeling RILI in recent years (Dabjan et al. 2016), and is therefore the major focus of this review. In addition to the fact that these well characterized mice are readily available, relatively inexpensive, and the background most commonly utilized for genetic modification, when used to model radiation lung late effects, the C57BL/6J develops a relatively consistent fibrotic response (Williams, Johnston, et al. 2010) and is less likely to succumb to acute RP following a single dose exposure, which makes them a good choice for studying the full progression of injury. Perhaps it is for these reasons that this strain is the focus of many radiation based medical countermeasures studies (Williams, Brown, et al. 2010). In fact, both the C57BL/6J and C57L/J strains exhibit progression through the same, classically defined phases of RILI that are observed clinically, i.e. the immediate, pneumonitic and fibrotic response periods (Figure 1A). Interestingly the C57L/J demonstrate a temporal progression following doses similar to the average range observed in humans (Jackson et al. 2011) and, for this reason, have recently become a popular strain of choice. While there is some debate as to which strain is best suited for modeling the various aspects of RILI (Dabjan et al. 2016), the poor breeding, limited availability, elevated cost, and high mortality seen during the pneumonitic phase of

the C57L/J strain has supported the continued utilization of the C57BL/6J model. Therefore, it is the murine model, and the C57BL/6J strain in particular, that informs much of the discussion regarding our understanding of the mechanisms underlying the progression of RILI.

The temporal nature of RILI: Immediate and early responses trigger dynamic radiobiological processes that promote late effects

Although the lung is known to demonstrate both acute and late responses to ionizing radiation (IR), clinically they do not always appear in a consequential fashion, with some patients presenting with fibrosis in the absence of a preceding RP (Fajardo et al. 2001). As a result, there has been some debate as to whether acute responses are required for the development of late effects (McDonald, Rubin, Phillips, et al. 1995) and the level of dependence of later outcomes on initial events remains uncertain. However, evidence gained over the past 2 decades of research, utilizing well described and characterized animal models, supports the assertion that immediate and acute responses to irradiation either promote or permit the pathogenesis of RILI. Moreover, it is thought that these outcomes are dependent, at least in part, on the radiosensitivity and/or response of the various cell types present in the lung at the time of irradiation. A number of downstream responses to radiation injury have been proposed as contributing to the pathogenesis of RILI, these processes acting dynamically, and often in concert, allow or promote the outcomes that are observed long after the initial exposure (Figure 2). Beyond immediate induction of cell death, delayed cell death, reduced proliferative capacity, loss of progenitor populations, and cellular senescence have been well described (Hall and Giaccia 2012), and these processes may act as contributors to both the acute and delayed outcomes. In addition, inflammatory signaling associated with canonical wound healing can transition into chronic inflammation and a persistent mobilization of activated immune cells, capable of producing reactive oxygen species (ROS) that generate oxidative stress and can cause DNA damage. The concept that radiation can create a state of sustained or repeated induction of DNA damage in exposed tissues, which in turn contributes to dysregulated wound healing, chronic inflammation and senescence, has recently drawn increasing scrutiny and is the subject of several reviews which examine the interplay of these complex phenomena (Stone et al. 2003; Richardson 2009; Yarnold and Brotons 2010; Ding et al. 2013; Morgan and Sowa 2015). Several groups have been able to experimentally demonstrate, to varying degrees, associations between these processes in the progression of pulmonary radiation injury in rodent WTI models (Fleckenstein et al. 2007; Zhang et al. 2012; Mahmood et al. 2014; Beach et al. 2017). Indeed, a number of interventions targeting these various processes appear to either delay or prevent the late fibrotic response when administered during the immediate and/or acute periods of RILI (Table 1).

A misconception put to rest: There is no “latent” period in RILI

The asymptomatic days and weeks preceding the onset of RP has classically been referred to as a “latent” period, a term which is easily misconstrued and could be taken to mean that cells and tissues are non-responsive at this time. However, it is now well known that despite the lack of symptomology, cells and tissues mount a rapid injury response. IR exerts

immediate cellular genotoxicity via the formation of single and double strand DNA lesions, capable of causing immediate cell death or functionally impairing surviving cells. In addition, an injury response induced by the downstream consequences of cell loss and tissue damage rapidly initiates cellular signaling processes mediated through the release of damage associated molecular patterns (DAMPs) and pro-inflammatory cytokines. Using WTI models, numerous investigators have shown that, in the hours to weeks following irradiation, changes in numerous cytokine and chemokine profiles occur in both C57BL/6J and C3H/HeJ mouse strains (Rubin et al. 1995; Johnston et al. 1996), indicating that the activation of cellular responses to radiation is both rapid and robust. The transcriptional profile of a multitude of pro-fibrotic and pro-inflammatory cytokines, chemokines and mitogens is altered; for example, $Tnf-\alpha$, $Tgf-\beta$, $Il-6$, $Cxcl1$, $Il-1\alpha$, $Il-1\beta$, $Mcp-1$, $Vegf$, $Ctgf$, and $Fgf2$ are induced in a temporal manner that follows the progression of the radiation response (Rubin et al. 1995; Johnston et al. 1996; Johnston et al. 1998; Kalash et al. 2013; Kalash et al. 2014; Chung et al. 2016; Sternlicht et al. 2018). Indeed, as a result of these and similar findings, the term “perpetual cascade of cytokines” (Rubin et al. 1995) was coined to describe the immediate and persistent alterations in message expression following irradiation, i.e. during the “latent period” which is, in actuality, a period of injury response (Westbury and Yarnold 2012).

An evolving understanding of immediate and acute cellular responses

Classically, 4 cell types were considered to be “targets” of pulmonary radiation: type 2 pneumocytes, alveolar macrophages, endothelial cells and fibroblasts (Rubin et al. 1992). This concept has continued to evolve, and it is now generally accepted that complex intercellular signaling between these and other populations (Figure 3) produce the observed normal tissue responses and it is becoming increasingly clear that it is the responses of these critical lung cell types, rather than their loss, that contributes to acute and chronic pneumopathies, providing an explanation for the delayed appearance of the final outcomes (Rodemann and Blaese 2007; Yarnold and Brotons 2010; Westbury and Yarnold 2012; Ding et al. 2013).

Pulmonary epithelial cell populations perform essential homeostatic functions such as maintaining efficient gas exchange and host defense, in part through the production of inflammatory mediators, surfactant and secretoglobins. Type 2 alveolar epithelium (AEC2s) and club cells are particularly crucial to maintaining the local environment; the depletion of club cells (Perl et al. 2011) or injury to alveolar epithelial cells (Sisson et al. 2010) has been shown to be sufficient to induce focal fibrosis. Therefore, while at this time, the majority of data generated in WTI models has been focused on the activation of pro-inflammatory and pro-fibrotic signals, we cannot rule out the possibility that these responses are secondary to the damage induced in the epithelium. The concept that radiation exposure disrupts the delicate homeostatic balance between parenchymal and immune cells, preventing return to a quiescent state and creating conditions conducive to chronic immune dysregulation is the subject of further discussion.

In vitro, studies using irradiated human bronchial epithelial cells (HBEC) have shown radiation induced activation and production of the cytokines GM-CSF, $GRO\alpha$, $IL-1\alpha$, $IL-8$,

MIF and Serpine E1 (Werner et al. 2015). Similarly, in C57BL/6J mice exposed to WTI, epithelial cells surviving the initial radiation induced cell death respond with continued cytokine signaling, contributing to TNF- α , IL-1 α , IL-6, and TGF- β output in both the immediate and acute periods of RILI (Rübe et al. 2000; Rübe et al. 2005). Experimentally, the robust expression of inflammatory cytokines in these cells appears to be associated with DNA damage and the presence of oxidative stress, and there is further evidence linking this response to cellular senescence.

Several weeks following exposure, approaching the pneumonitic period, delayed cell death becomes apparent. Under normal conditions, club cells line the bronchioles and can differentiate into ciliated and AEC2 cells in response to injury (Hogan et al. 2014). Findings in a WTI C57BL/6J model, demonstrated that the number of club cells is reduced by 8 weeks post irradiation, an effect that endured for the remainder of the time course examined (Manning et al. 2013). Indeed, this cell type has proven to be radiosensitive, even at relatively low doses, as demonstrated in *ex vivo* irradiation experiments in which a significant dose dependent decrease in colony forming ability was observed at doses as low as 1 Gy X-irradiation (Farin et al. 2015), possibly explaining their persistent decrease. Similarly, in C57BL/6Ncr mice, AEC2 cells are also persistently reduced in number, beginning 2 weeks following single dose or fractionated WTI (Citrin et al. 2013). This raises the possibility that the remaining epithelial cells, which normally function to suppress inflammation and fibrosis, are unable to maintain tissue equilibrium. For example, production of PGE₂ by club and AEC2 cells suppresses both collagen production and proliferation of fibroblasts under homeostatic conditions (Bozyk and Moore 2011), and therefore it would be of great interest to determine if PGE₂ is decreased in lung tissue or type 2 cells following irradiation. While a number of researchers have promoted the idea the pro-inflammatory-profibrotic milieu as a key factor in the radiation response (Rübe et al. 2005; Schaeue et al. 2012) a close examination of the temporal production of anti-inflammatory and anti-fibrotic cytokines as part of the radiation response of epithelial cells may yield further insight into the progression of RILI and reveal additional potential targets for the treatment of this injury.

Endothelial cells are also responsive to radiation, again becoming activated during the immediate injury period and producing inflammatory cytokines/chemokines, such as IL-6 (Gaugler et al. 1997), MCP-1, and IL-8 (Baselet et al. 2017), as well as the mitogens, FGF and PDGF (Witte et al. 1989). The expression of ICAM-1 is also upregulated, which allows for leukocyte adhesion and infiltration (Gaugler et al. 1997). These responses, which occur in tandem with inflammatory epithelial and immune cell signaling, may help promote the cumulative and pronounced recruitment of inflammatory cells observed during the acute response. Radiation is toxic to vascular tissues and can cause appreciable endothelial cell loss, both immediately and in a delayed fashion. Vessel density has been observed to significantly decrease within a month of WTI, and appeared to be persistent up to a year post-radiation in a WTI WAG/Rij/MCW rat model (Ghosh et al. 2009). Experiments utilizing bovine aortic endothelial cells (BAEC) or human umbilical vein endothelial cells (HUVEC) have indicated a level of radiosensitivity similar to that observed in epithelial cells. In a clonogenic assay, at 14 days post exposure, the surviving fraction of BAEC was reduced in a dose dependent manner, with a nearly 40% reduction in proliferation observed

at a dose of 1.2 Gy X-irradiation, and nearly 100% of cells losing proliferative capacity following 8 Gy (Panganiban et al. 2013). In these cells, apoptosis was shown to be induced in 10–30% of cells exposed to 10–30 Gy, with the majority of the surviving cells displaying a senescent phenotype. This study was consistent with earlier findings, which had demonstrated nearly 90% of surviving cells exposed to 8 Gy become senescent (Igarashi et al. 2007). Interestingly, the senescent endothelial phenotype has been shown to persist for at least 20 weeks *in vitro* (Oh et al. 2001), suggesting that global endothelial functions, such as angiogenesis and oxygen transport, may be chronically reduced, leading to a failure to expand the vasculature with growth, and hypoxia.

Radiation is also harmful to the immune compartment in the lung. The primary immune cell type present in the lung under homeostatic conditions is the macrophage (Hunninghake et al. 1981). A rapid depletion of resident pulmonary macrophages occurs even after sub-lethal doses of lung irradiation, lasts for several weeks and is thought to be due to the loss of radiosensitive macrophage precursors (Gross and Balis 1978). Alveolar macrophages (AMs) experience the greatest cell loss, which can be detected within 3 days post WTI exposure in C57BL/6J and C3H/HeJ mice (Groves et al. 2015; de Leve et al. 2017). The duration of this effect appears to be dose dependent, ranging from 16 to 60 days before recovery to control values following exposure to 2 – 10 Gy, respectively (Peel and Coggle 1980; Chiang et al. 2005). During this time period, however, cell loss is paralleled by immune cell influx (Groves et al. 2015; de Leve et al. 2017). Importantly, the cellular injury experienced by these major resident lung cell populations results in the early release of potent cytokines, known to stimulate the immune system to attract inflammatory cells from the circulation to the site of injury. These cells play a crucial role in both wound healing and fibrotic processes, and it is the dysregulation of innate and adaptive immune responses which have been hypothesized by some investigators to play a major role in the progression of RILI to RIPF (McBride et al. 2004; Schaeue and McBride 2010). Chronic inflammation promotes chemotaxis, fibroblast proliferation, and collagen synthesis, and it is widely accepted that a perpetual state of persistent injury and inflammation underlies many forms of pulmonary fibrosis. Yet it remains unclear how, or if, these important pro-inflammatory and pro-fibrotic signals, many of which promote fibroblast activation, are maintained by parenchymal cells in this radiation model.

Pneumonitis: A tipping point in the progression of injury

In the C57BL/6J WTI model it appears that the threshold dose for pathogenesis falls at approximately 10 Gy (Manning et al. 2013; Zhou et al. 2017) and following the initial radiation responses, RP is observed between 8–16 weeks of exposure (Figure 1A). RP is a life threatening outcome in humans and, in mouse models, is characterized by a buildup of a macrophage rich infiltrate, lymphocytes, fibrin and debris in the alveolar space (Travis 1980), accompanied by interstitial edema and monocyte accumulation in the alveolar septa (Sharplin and Franko 1989). During this period, infiltrating immune cells are likely to encounter a significantly altered microenvironment in which fewer epithelial cells are present and resident alveolar macrophages are greatly reduced in number; this may allow for the further dysregulation of the immune response. Because this pathology is of immense clinical importance, C3H and CBA strains, which present with a discrete pneumonitic

response that closely resembles observations in humans, have classically been interrogated (Jackson et al. 2011). These studies have provided valuable insights into differential strain responses with respect to the progression of injury; for example, in contrast to C57BL/6NHsd, C3H/HeNHsd do not demonstrate an increased transcriptional abundance of mitogens, such as Tgf- β , Ctgf, and Fgf1, as injury progresses following RP (Kalash et al. 2014), although the underlying mechanisms have not yet been fully elucidated. While the progression to injury, or lack thereof, is clearly linked to genetic background, a great deal of work remains to determine how, or if, genetic differences affect the responses of the cell types contributing to the development of fibrosis.

In fibrosis prone strains, C57BL/6J among them, the pneumonitic phase is followed by interstitial thickening, collagen deposition and fibrotic remodeling. In mice, these lesions begin primarily on the periphery of the lung and culminate in the development of pulmonary fibrosis (Sharplin and Franko 1989); this is a progressive, un-resolving disease associated with the accumulation of extracellular matrix (ECM) proteins, such as collagen and hydroxyproline, and begins in the lung at around 24 weeks (Coggle et al. 1986) (Figure 1B). In the irradiated pulmonary microenvironment, secreted cytokines and mitogens, in addition to ROS and possibly cellular senescence, share a common characteristic in that they are capable of eliciting a response in pulmonary fibroblasts, notably a transition from relatively quiescent to an activated, secretory state (Citrin et al. 2017) in response to a variety of signaling pathways (Figure 3). A key mediator in this process is TGF- β (Martin et al. 2000), which appears to undergo transient increases in many WTI experiments and, indeed, its genetic overexpression is sufficient to induce pulmonary fibrosis (Sime et al. 1997). TGF- β itself is known to have potent effects on fibroblasts, primarily via Smad signaling (Moses et al. 2016), and its profibrotic effects potentially include enhanced infiltration or proliferation of preexisting fibroblasts, generation of terminally differentiated, contractile myofibroblasts, increased ECM synthesis, and inhibition of collagenolysis (Morikawa et al. 2016). Myofibroblasts, which are biochemically responsible for the pathogenic accumulation of matrix proteins characteristic of fibrosis (Rodemann and Blaese 2007) are widely considered to be “bad actors” in fibrotic disease; as a result, many cytokines which trigger activation of normally quiescent fibroblasts have been a target of interest in mitigating pulmonary late effects (Table 1).

Mechanisms of RILI important to Pneumonitis and Fibrosis:

The driving factors that lead to the development of pneumopathy in the aftermath of lung radiation exposure appear to share a common feature in that they are the product of disrupted homeostasis in the pulmonary microenvironment and are associated with persistent or recurring injury. As such, the establishment of a dysregulated lung landscape may arise from a failure of parenchymal and resident immune cells to fully resolve the injury, which eventually deteriorates the pulmonary niche and results in a state of chronic dysfunction. The prevention of maladaptive responses by strategies which aim to normalize homeostasis within the niche, such as cell-cell interaction, cytokine release, production of metabolites, etc., holds promise for the preservation of lung functionality (Williams et al. 2016).

DNA damage and the P53 response

DNA damage is a primary outcome of IR, and it has been estimated that a 1 Gy exposure can result in approximately 1000 single strand breaks (SSBs) and 25–40 double strand breaks (DSBs) per cell (Olive 1998; Maier et al. 2016). Formation of SSBs and DSBs results from either direct or indirect ionizations, the latter involving a rapid intracellular burst of reactive oxygen species (Robbins and Zhao 2004; Azzam et al. 2012). These initial events are known to be extremely short lived, lasting only fractions of a second, but nonetheless are capable of inducing rapid cell death through multiple mechanisms, events which have been well described in textbooks (Hall and Giaccia 2012). However, since clonogenic assays have indicated the repair of non-lethal damage is largely completed within 1–2 days following exposure to ionizing radiation, the classical “target cell killing” hypothesis, which explains the cytotoxic properties of IR, fails to explain the means by which acute and later reactions occur in the months following exposure. Interestingly, experimental evidence has revealed that DNA damage can persist in the lung for days following single 1 Gy (Werner et al. 2017), or multiple 100 mGy exposures to total body irradiation (TBI) (Flockerzi et al. 2014). Other long-term experiments, which measured outcomes occurring during acute or late phases of RILI, have observed that DNA lesions are present weeks and months following a single WTI exposure (Fleckenstein et al. 2007; Zhang et al. 2012; Kunwar and Haston 2015). One explanation for these lesions is that both IR and oxidative stress can create unrepairable DNA lesions, known as DNA-SCARS (DNA segments with chromatin aberrations reinforcing senescence), and these have been detected in both alveolar and bronchial cells following TBI (Rodier et al. 2011). From WTI models, it appears that both persistent and newly generated DNA damage can be detected, with the new lesions possibly being created as a result of downstream generation of ROS and/or chronic inflammation (Figure 2).

The continuation of DNA damage, cell death, and senescence is dependent, at least in part, on the activation of tumor protein P53 mediated DNA damage responses (DDR). The response to DNA lesions is mediated through damage sensors ATM, ATR, and DNA-PKs. ATM is thought to be the “master regulator” of responses to DNA DSBs including, but not limited to, DNA repair, checkpoint activation, apoptosis, and senescence (Blackford and Jackson 2017). ATM initiates a series of phosphorylation steps, acting upon the signal transducer, CHEK2 kinase, which in turn phosphorylates P53, activating the DNA damage response (supplementary figure 1) (Sancar et al. 2004), known to be enhanced through phosphorylation and binding of TP53-binding protein 1 which is also localized to regions of DSBs (Iwabuchi et al. 1998). ATM also mediates the phosphorylation of the H2AX histone, producing the variant γ H2AX, a widely accepted indicator of DSBs (Rothkamm et al. 2015). For this reason, it is the co-localization of both γ H2AX and p53bp1 which is frequently interrogated to indicate the presence of DSBs (Schultz et al. 2000).

The mechanism by which P53 induces differing cellular outcomes such as apoptosis or senescence in irradiated tissue is an area of active research and in the context of RILI remains incompletely understood. As such, the multiple control mechanisms to determine cell fate appear to depend on cell type, cell state, level of damage and other environmental conditions (Gudkov and Komarova 2003). Given the heterogeneous nature of cellular

responses in RILI, which acts upon the multiple cell types represented within the lung, it is highly likely that the P53 mediated DDR leads to a range of outcomes in the tissue itself, in both a cell type and time dependent manner.

In radiosensitive cells, such as those of hematopoietic origin, P53 activity promotes apoptosis which can occur intrinsically through BAX/BCL2 family signaling, or extrinsically through the upregulation of FAS and TRAIL receptors (Gudkov and Komarova 2003; Helton and Chen 2007). The induction of apoptosis indicates that in these cell types, the role of P53 signaling is to maintain genetic fidelity, by rapidly removing DNA damaged cells from the pool of cycling cells.

In parenchymal cells, such as epithelia and endothelia, it appears that P53 signaling will allow for cell cycle arrest to promote DNA repair. Should repair be unsuccessful, cells will exit the cell cycle, through senescence, mitotic catastrophe or other means (Maier et al. 2016). Both cell cycle arrest and senescence can result from the p53 activation of cyclin-dependent kinase inhibitor CDKN1A (p21) (Campisi and d'Adda di Fagagna 2007; Helton and Chen 2007), this may partly explain the observation that pulmonary epithelia (Citrin et al. 2013; Pan et al. 2017), human diploid fibroblasts (Campisi and d'Adda di Fagagna 2007), and endothelial cells (Panganiban and Day 2013; Panganiban et al. 2013) are prone to senescence and not limited to apoptosis in the setting of radiation induced DNA damage, thus contributing to the delayed appearance of the acute response. Perhaps the observed epithelial senescence results in part from the formation of DNA SCARS, which can appear independently of ATM and/or ATR (Rodier et al. 2011), sustain P53 activity and maintain senescence growth arrest (Campisi 2011).

While there is little doubt that P53 is active in RILI, much work remains in elucidating the specific ways in which it can be manipulated in the various pulmonary cell types in order to prevent normal tissue injury. Conceivably, the properly timed application of antioxidants, or suppression of ROS producing immune cells is a more practical alternative to preventing persistent or later appearing DNA damage.

Oxidative Stress

There is ample evidence to indicate that oxidative stress, generated internally or through environmental exposure, plays an important role in pulmonary fibrotic diseases (Kliment and Oury 2010; Liu and Gaston Pravia 2010; Cheresch et al. 2013; Liu et al. 2013; Turgut et al. 2016) and, more specifically, in RIPF (Kang et al. 2003; Fleckenstein et al. 2007; Graves et al. 2010; Zhang et al. 2012; Ding et al. 2013; Mahmood et al. 2013). Direct detection of ROS and/or reactive nitrogen species (RNS) is difficult, however, especially in an *in vivo* setting, therefore such studies primarily rely upon the detection of products of oxidation, such as malondialdehyde (MDA) or 8-hydroxy-2'-deoxyguanosine (8-OHdG). These products are more stable, but limit the ability to identify the source(s) of ROS/RNS. That ROS themselves are capable of inducing DNA lesions is well established, however there are multiple possible causative mechanisms that might lead to increased oxidative stress beyond the initial radiation injury. Those pertinent to RILI include mitochondrial damage (Kim, Fiskum, et al. 2006; Azzam et al. 2012), disruption of intracellular metabolic oxidation/reduction reactions (Spitz et al. 2004; Yahyapour et al. 2018), and activation of

inflammatory cells (Stone et al. 2003; McBride et al. 2004; Schaue and McBride 2010; Azzam et al. 2012; Westbury and Yarnold 2012). Recent evidence indicates that oxidative stress can be immune cell driven and is likely to promote effector responses such as cellular senescence (Zhao and Robbins 2009), potentially perpetuating pro-inflammatory feedback (Figure 2).

Mitochondria have long been identified as potential sources of oxidative stress following irradiation, and radiation induced mitochondrial dysfunction and increased ROS generation have been reported in various cells and tissues (Zhao and Robbins 2009). Early increases in cellular oxidative stress, apart from the oxidative burst caused by radiation itself, may be attributed to the fact that mitochondria are prone to radiation induced damage (Azzam et al. 2012), as they occupy a considerable volume of the cell (4–25%) (Leach et al. 2001), and the damage may persist due to their lack of repair capacity and protective histones (Kim, Fiskum, et al. 2006). It is reasonable to consider that disruption of either electron transport chain complexes, or mutations in mitochondrial or nucleic DNA encoding them, can increase ROS production (Kim, Chandrasekaran, et al. 2006). In fact, in the minutes following exposure, radiation has been shown to increase the percentage of non-phagocytic cells producing mitochondrial ROS and RNS, an event which is coupled with the loss of mitochondrial membrane potential (Leach et al. 2001).

Investigation into the sources of ROS following irradiation remains an area of active research, especially with respect to the development of interventions by which the inhibition of excess ROS can mitigate the fibrotic response following WTI. Perhaps some of the best evidence for the role of ROS in promoting fibrogenesis in WTI comes from the many experiments demonstrating the efficacy of antioxidants in attenuating RIPF (Table 1). The superoxide dismutases (SODs) play a crucial role in protection from oxidative stress, through the conversion of superoxide to less reactive hydrogen peroxide and water, which in turn can be further reduced by catalases or glutathione peroxidase to oxygen and water. Genetic overexpression of extracellular SOD (EC-SOD) has been shown to protect against RIPF in WTI transgenic mice (Kang et al. 2003); this was associated with decreased abundance of TGF- β and activated macrophages in the lung as compared to irradiated non-transgenic controls. Pharmacologic intervention using superoxide dismutase mimetics or similar agents, either alone or in combination with other drugs, have also been successful in ameliorating RIPF in WTI animal models such as mouse, and other animal species including rat and non-human primates (Machtay et al. 2006; Mahmood et al. 2013; Mahmood et al. 2014; Murigi et al. 2015; MacVittie et al. 2017). Some of these studies have demonstrated similar outcomes via a decrease in TGF- β abundance, numbers of activated macrophages, oxidative stress and DNA damage (as indicated by 8-OHdG), hydroxyproline and/or collagen abundance which are standard measures of fibrosis.

Elevated levels of angiotensin converting enzyme (ACE) have been detected in the bronchoalveolar lavage fluid of patients presenting with interstitial lung disease, including idiopathic pulmonary fibrosis (Specks et al. 1990), indicating the renin-angiotensin system as another interesting target in the prevention or treatment of RILI. This class of drugs has been shown to exert a protective effect on endothelia (Ward et al. 1988), which may operate through several mechanisms. Angiotensin 2 has pleiotropic properties, affecting several

factors associated with RILI, such as TGF- β production, mediating the release of pro-inflammatory cytokines and chemokines, and altering cell function through the generation of ROS via activation of nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase (NOX) (Robbins and Diz 2006). NOXes are active in the production of ROS in both phagocytic and non-phagocytic cells, therefore it is likely that these drugs act upon multiple cell types present in the lung, including macrophages and endothelia, and it should be noted that the ROS mitigating properties of these drugs are unlikely to be the result of any antioxidant capacity. In the Fisher and WAG/RijCmcr rat models of WTI, angiotensin converting enzyme inhibitors (ACEi), such as captopril and enalapril, appear to have a similar capacity to enhance survival and diminish RP, hydroxyproline and collagen production (Kma et al. 2012), TGF- β , oxidative stress, and numbers of activated macrophages present (Mahmood et al. 2014), although it is likely the effects of these drugs are exerted through different means than the SOD mimetics. Importantly, preclinical findings have led to retrospective studies of clinical data reporting the outcomes of thoracic radiation therapy, which have indicated a decreased incidence of RP in patients taking ACE inhibitors (Kharofa et al. 2012; Sun et al. 2018), and these findings should encourage further investigation into the potential use of ACE inhibitors for the prevention of late effects.

Cellular Senescence

Cellular senescence can occur through the direct incitement of DNA damage by radiation, or in response to oxidative stress (Lu and Finkel 2008; Munoz-Espin and Serrano 2014). Classically, cellular senescence is initiated and maintained by the p53/p21 and p16/Rb pathways (Coppe et al. 2008), which can act either independently or in a complementary fashion to produce an identical outcome - permanent cell cycle arrest (Salama et al. 2014). Interestingly, senescent cells are found in many forms of pulmonary fibrosis (Minagawa et al. 2011; Aoshiba et al. 2013; Chilosì et al. 2013) and is not limited to RPIF. Yet, in RPIF, increases in markers widely accepted to represent senescence, such as senescence associated beta-galactosidase (SA-bgal), p16 or p21, have been shown to increase within hours of radiation exposure and are present up to 30 weeks post treatment (Citrin et al. 2013; Panganiban and Day 2013; Pan et al. 2017).

The consequence of cellular senescence *in vivo* is not well understood at this time, but experiments performed *in vitro* have indicated that senescent cells may adopt a pro-inflammatory senescence associated secretory phenotype (SASP) (Coppe et al. 2008; Rodier et al. 2009; Freund et al. 2010). Interestingly enough, the SASP has been described to a great extent in irradiated human fetal lung fibroblasts, a finding which may have relevance to WTI models. At the transcriptional level, increases in IL-6, IL-8, MCP-1, IL-1 α , IL-1 β , and CXCL1 have been detected in X-ray induced senescence in human lung fibroblast cultures (Freund et al. 2010) and recapitulate the production of the same factors in the lungs of whole thorax irradiated C57BL/6 mice (Johnston et al. 1996; Johnston et al. 1998; Ao et al. 2009; Johnston et al. 2010) although the timing of these events do not always occur in conjunction with the appearance of senescent cells *in vivo*. Similar increases in cytokine expression have also been observed *in vitro* with X-ray induced senescent epithelial cells; these cells secreted significantly increased levels of IL-6, IL-8, MCP-1, IL-1 α , IL-1 β , GM-CSF, VEGF and IGFBP2 compared to unirradiated, pre-senescent controls (Coppe et al. 2008). Moreover, it

has been proposed that a population of senescent cells scattered throughout organs and tissues could act as a seed for systemic chronic inflammatory responses (Freund et al. 2010). In the C57BL/6Ncr WTI model, the number of senescent AEC2 cells was elevated for the (30 week) duration of the experiment, while the overall number of AEC2s present in the tissue was decreased (Citrin et al. 2013) indicating a diminished number of epithelial cells present to regulate the pulmonary microenvironment. This experiment also demonstrated a connection between senescence and oxidative stress, as administration of diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, appeared to prevent the overall loss of AEC2s, and reduce both the number of senescent AEC2 cells and collagen accumulation in the lung. A similar experiment, which reduced AEC2 senescence through the application of the anti-senolytic agent, ABT-263 (a Bcl-2/xl inhibitor), also showed evidence of a dampened fibrotic response in C57BL/6J mice (Pan et al. 2017) (Table 1).

Unfortunately, to date there is no direct evidence from WTI models of senescent cells producing pro-inflammatory or profibrotic cytokines and chemokines, and further complicating the role of senescent cells in RILI are findings that multiple cell types, such as fibroblasts and epithelia, exhibit a similar, but not identical SASP. Even within the same cell type, SASP can be further influenced by environmental conditions, such as exposure to lower or higher concentrations of oxygen during incubation (Coppe et al. 2008), making it difficult to draw parallels between the responses observed in WTI and SASP observed *in vitro*. It is reasonable to consider that the SASP may differ in response to the activation, or the lack thereof, of certain senescence associated pathways, induced through multiple mechanisms (Campisi and d'Adda di Fagagna 2007). In ATM depleted cells, upregulation of IL-6 seen in many senescent cells is lacking, similar to observations in cells exposed to low dose irradiation (Rodier et al. 2009). An additional example is the TGF- β treatment of human bronchial epithelial cells (HBECs) in culture, which has been shown to induce a p21 dependent cellular senescence, independent of p53. Similar to X-ray induced senescence, HBECs secretion of IL-1 β following irradiation is increased, and exposure of fibroblasts to conditioned media collected from senescent HBEC cultures was sufficient to increase collagen secretion and induce terminal differentiation to myofibroblasts (Minagawa et al. 2011). However, the connection between TGF- β and senescent cells in WTI has not yet been fully explored. Despite these current limitations, identification of senescent cells, which are easily detectable in RILI, has the potential to serve as a prognostic indicator of fibrosis in experimental models.

Inflammation

The direct consequences of lung radiation exposure, DNA damage, induction of ROS and cell death, result in the release of DAMPS which function as danger signals (McBride et al. 2004), and instigate canonical wound healing programs. Inflammation, initiated as a result of radiation induced injury, is a significant component of the biological events that occur in the aftermath of the exposure, contributing to each phase of the radiation response in the lung.

Within hours of WTI (Rübe et al. 2005), production of pro-inflammatory cytokines and chemokines by the pulmonary epithelium of C57BL/6 and C3H/HeJ mice (Rubin et al.

1995; Johnston et al. 1996; Johnston et al. 1998) stimulates the immune system to recruit inflammatory cells from the circulation to the site of injury. The first cells to arrive into the irradiated lung are neutrophils, which promote local inflammation through the release of TNF- α , IL-1, IL-6, and ROS, (Vallée et al. 2017). In C57BL/6J mice exposed to 15 Gy WTI, this influx can be observed as early as 6 hours following exposure (Paun et al. 2015). Migration of monocytes from the circulation follows (de Leve et al. 2017), the extent of which has been shown to correlate with dose in the C3H/HeN strain (Hong et al. 2003).

Inflammation as a dynamic process in the progression of RILI

Although the precise nature of the development of the acute and late outcomes differ between C57BL/6J and C3H/HeN strains (Chiang et al. 2005), early and persistent dysregulation of the pulmonary microenvironment is thought to disrupt immune homeostasis and thus promote the development of chronic inflammation, which can in turn initiate and sustain subsequent pathogenic outcomes. Indeed, at the molecular level, pro-inflammatory cytokines are released in a temporal pattern that suggests that they may function to mediate these slowly developing processes. As such, although the increase in pro-inflammatory cytokines occurring immediately in the hours following exposure is transient, it appears that the inflammatory response fails to fully resolve and is followed by subsequent releases of pro-inflammatory cytokines that can be observed over the course of the following weeks preceding the onset of acute RP, further contributing to the dysregulation of the tissue microenvironment (Johnston et al. 1996; Rübe et al. 2005).

Macrophage driven immunomodulation has long been thought to be one of the major mechanisms by which the delayed outcomes ensue (Rubin et al. 1995). In addition, the temporal progression of complex cytokine signaling pathways now appears to both result in, and contribute to, the inappropriate polarization of macrophage phenotypes. Therefore, macrophage behavior evolves over the course of the radiation response and, in turn, may affect and mediate the development of later outcomes. For example, iNOS expression, which is induced by pro-inflammatory stimuli to produce nitric oxide, and in turn ROS, is increased in macrophages at the onset of RP, while clusters of alternatively activated Arginase-1 expressing macrophages are observed in the lung later during the fibrotic phase of the C57BL/6J WTI response (Zhang et al. 2011; Groves et al. 2016). The transition to this phenotype can lead to the activation of pro-fibrotic signaling pathways which contribute to the development of RIPF (de Leve et al. 2017).

The specific ontology of resident and infiltrating macrophage subpopulations may define the nature of their response to radiation exposure. Alveolar and interstitial macrophages, which are seeded in the lung during embryogenesis, perform vital physiological functions that are seminal in maintaining a state of quiescence and are an essential component of the innate immune system, adapting to the microenvironmental fluctuations that occur during injury, inflammation and disease in order to resolve pro-inflammatory signaling and reestablish tissue homeostasis (Ginhoux and Jung 2014). Alveolar macrophages (AMs) are long lived and self-renew through local proliferation. In contrast, infiltrating macrophages arise from bone marrow precursors, are monocyte derived, and can not only contribute to interstitial macrophage (IM) populations but, importantly, expand the lung macrophage pool during

inflammatory conditions. This population responds to microenvironmental cues governing them to participate in either the initiation or resolution of the inflammatory response, before egressing from the tissue in a return to homeostasis, leaving the remaining resident macrophage populations to renew through local proliferation (Yona and Jung 2010; Ortega-Gómez et al. 2013). While the majority of resident lung macrophages are maintained independently of monocyte-derived populations in a healthy environment (Ginhoux et al. 2016), the genotoxic effects of radiation exposure appear to impair the ability of this population to self-maintain through local proliferation, and thus monocyte-derived macrophages that have migrated from the circulation are retained in the lung to repopulate the depleted macrophage pools (Hashimoto et al. 2013). Resident and infiltrating macrophage populations differ in their responsiveness to inflammatory stimuli, and therefore the retention of monocyte-derived infiltrating macrophages in the lung following radiation injury enables this non-resident population to continue to respond inflammatory signaling and contribute to further dysregulation. Findings that chemokine receptors which promote immune cell infiltration, including CCR2, were upregulated in fibrosis sensitive (C57BL/6) but not resistant (C3H/HeJ) WTI mice provide further indication that infiltrating macrophages may play a role in either establishing or maintaining a pro-fibrotic microenvironment (Johnston et al. 1998; Johnston et al. 2002). Further evidence that this immune population may drive fibrotic processes is provided by depletion experiments in which WTI chimeric mice were provided bone marrow lacking CCR2, and thus CCR2-mediated immune cell infiltration was prevented. Following whole lung irradiation, not only was the infiltration of monocyte-derived macrophages into the lungs impaired, the development of pulmonary fibrosis was ameliorated (Groves et al. 2018). Likewise, in C57BL/6J mice exposed to WTI, preventing the recruitment of bone marrow-derived IMs reduced pulmonary fibrosis, but depletion of resident AMs did not (Table 1) (Meziani et al. 2018). The pro-fibrotic role of infiltrating macrophages is also supported in other lung fibrosis models. For example, CCR+ macrophage populations are also implicated in fibrotic remodeling following targeted alveolar epithelial injury (Osterholzer et al. 2013), bleomycin exposure (Misharin et al. 2017) and silica instillation (Lebrun et al. 2017). Although the mechanism by which this population drives the development of a pro-fibrotic environment has not been elucidated, these models may provide some insights. For example, transcriptomic profiling of infiltrating monocyte-derived macrophages showed that their differentiation and phenotype evolved over the course of the response to bleomycin, expressing pro-fibrotic genes that drove fibrosis development, while their depletion had the opposite effect. Conversely, this study also found that tissue resident AMs may play a protective role in this process (Misharin et al. 2017). Moreover, in models of pulmonary fibrosis induced by silica (Lebrun et al. 2017) and targeted AEC2 depletion (Osterholzer et al. 2013), CCR2+ monocytes expressing Tgf- β and Colla1 were found to drive collagen production, while loss of CCR2 prevented fibrosis.

Macrophages and the microenvironment: the case for reciprocity

Macrophage plasticity and adaptability are major factors that govern the functioning of these cells in an irradiated tissue. Macrophages are exquisitely responsive to microenvironmental signals and the nature of their phenotype and activation state is largely dependent on the signals they encounter in their niche, hence their susceptibility to radiation injury. The

interplay between macrophages, epithelial cells and fibroblasts appears to be at the core of the mechanisms leading to the induction of pulmonary late effects (Rubin et al. 1992) and may provide an explanation for how early cellular events lead to the generation of later outcomes. The direct, initial damage of AEC2 cells by pulmonary irradiation causes dysregulated surfactant production and, as a result, disrupts surfactant clearance by AMs (Mühlfeld et al. 2017). This can lead to the formation of lipid-laden macrophages, known as foam cells, which are commonly observed in the fibrotic foci induced by radiation exposure (Almeida et al. 2013). Macrophage uptake of oxidized phospholipids, which may be generated as a result of radiation-induced oxidative stress, can result in foam cell transformation and cause these cells to acquire an alternatively-activated phenotype and produce increased levels of TGF- β (Romero et al. 2015). In addition, direct alterations of surfactant proteins by radiation can disrupt their multimeric structure and also affect macrophage responsiveness (Malaviya et al. 2014). For example, the lower molecular weight trimeric form of surfactant protein-D (SP-D) can bind to AMs and promote inflammatory responses (Yoshida and Whitsett 2006). Moreover, loss of this protein in SP-D $^{-/-}$ mice resulted in greater inflammatory mediator expression following radiation, suggesting that it plays a role in pulmonary radiation responses (Malaviya et al. 2014). Depletion of club cells and, therefore, loss of their major secretory protein, CCSP, by thoracic irradiation (Manning et al. 2013) can also contribute to dysregulation of the pulmonary microenvironment as this protein interacts with multiple components of the inflammatory and coagulation cascades, exerting an attenuating role on inflammation and macrophage activation (Kropski et al. 2009). CCSP $^{-/-}$ mice exhibit an exaggerated inflammatory response that is, in part, reliant on dysregulation of macrophage function (Snyder et al. 2010). Interestingly, *in vivo* depletion of club cells increased foam cell formation in silica models, suggesting CCSP may also play an attenuating role in macrophage activation, and as such the foam cells were also found to overexpress scavenger receptors which regulate phagocyte functions (Yatera et al. 2011). Radiation induced dysfunction of epithelial cells, therefore, has important implications regarding not only for parenchymal integrity, but also for inflammation severity and regulation of immune responses.

Macrophages are often observed intermingled with pro-fibrotic myofibroblasts and a close reciprocal interaction exists between these cell types (Wynn and Barron 2010). The pro-fibrotic functions of macrophages have been attributed to their production of mediators that directly induce fibroblast activation, namely PDGF and TGF- β (Murray et al. 2011). In C57BL/6J mice exposed to WTI, expression of these factors has been found to increase in the lung in an acute and prolonged manner and localize to areas of inflammatory cell accumulation and fibrosis (Rübe et al. 2000). Early indications of lung macrophages as sources of the pro-fibrotic signaling were gleaned from experiments in which AMs isolated from irradiated rabbits released increased amounts of TGF- β and also induced fibroblast proliferation (Rubin et al. 1992). Findings that blockage of the TGF- β mediator, CTGF, reduced M2 polarized macrophage accumulation and concurrently attenuated fibrotic remodeling in WTI exposed C57BL/6J mice also supports this concept (Bickelhaupt et al. 2017). IL-1, which is elevated in the lung following thoracic radiation (Johnston et al. 1996), also is a potent inducer of TGF- β . Chimera studies demonstrate that immune cells are the primary source of IL-1 β and the loss of this cytokine from bone marrow-derived infiltrating

immune cells ameliorate the radiation fibrotic response in a WTI model (Groves et al. 2018). Another mediator thought to promote fibroblast accumulation is PDGF, a potent fibroblast chemoattractant whose expression is observed in pulmonary macrophages from patients undergoing radiation therapy (Thornton et al. 1996). The chemotactic response of fibroblasts isolated from irradiated rats were found to be related to their increased binding affinity of the PDGF receptor (Tada et al. 2003). Redox signaling through immune driven pathways has also been shown to drive pro-fibrotic responses and may be a potential target for mediation of RIPF. TGF- β and other pro-fibrotic factors can activate the enzyme, NOX4, a significant source of ROS (Barnes and Gorin 2011). Findings that culture medium from pulmonary macrophages isolated from WTI-exposed mice induced myofibroblast differentiation and ROS production, a response that could be reduced with NOX inhibition (Chen et al. 2016), and that radiation-induced senescent and ROS-producing fibroblasts were found to stimulate migration of a monocytic cell line via a NOX4-dependent mechanism (Sakai et al. 2018) provides evidence for a role for NOX4 in a macrophage-fibroblast feedback loop as part of RIPF.

We have emphasized the role macrophages play in radiation responses, however they are by no means the sole immune cell impacted by and contributing to pulmonary outcomes. Lymphocytes, for example, are also effector cells that participate and shape the development of radiation pneumopathy. TH1 and TH17 populations, present in the tissue at the time of acute inflammation, can potentially contribute to inflammation, but are accompanied by Tregs, which can act to restrain excessive inflammation. This Treg population is predominant among lymphocytes during the late, fibrotic phase, where repair induction and immunomodulation can be contributed to by the anti-inflammatory and pro-fibrotic functions of this population (Wirsdorfer and Jendrossek 2016).

Overall, it appears evident that there is a close relationship between recruited immune cells, the altered microenvironment, and the effector cells responsible for RIPF. Indeed, it is clear that immune cells contribute to the mechanistic processes that lead to the pathogenesis of the acute and chronic outcomes of the radiation response. However, although some support the hypothesis that macrophages in particular function as master regulators of these processes, decades of assessing the use of anti-inflammatories have resulted in little clinical success. It may be that controlling, rather than inhibiting, the inflammation regulating potential of macrophages will prove more successful. Nonetheless, inflammatory cells continue to garner interest as potential targets for modulation, and a number of interventions which mediate the activity of these cells appear to be efficacious in delaying or preventing fibrosis (Table 1).

Conclusion:

Models using WTI, particularly in the C57BL/6J strain, have revealed a multitude of promising targets in the prevention RILI, providing data which would otherwise be difficult, if not impossible to obtain in the clinical setting. These data have revealed patterns of recurring injury, which begin in the period immediately following exposure and appears to be maintained by interactions between the immune and parenchymal populations of cells as they respond to iterative waves of inflammation, oxidative stress and persistently generated DNA damage. Given the complex web of cells, signals, dysregulated responses and multiple

feedback loops involved in the development and progression of RILI, a poly-pharmaceutical approach is likely needed, targeted at resident and responding cells, their responses, and the dysregulated microenvironment as a whole. Timing will be critical in order to refrain from inadvertently disrupting beneficial responses working to re-establish the intricate homeostatic balance that is found in the lung. Given the limited success of reductionist approaches which target either a single process or cell type, it appears that a multipronged strategy to the prevention or treatment of RILI will provide the greatest opportunity to restore an irradiated lung to its functional normal state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1A:

Sequential periods of radiation induced lung injury in C57BL/6J mice. Modified from Travis (1980) and reprinted with permission from: Beach et. al., 2018. Recurrent DNA damage indicates persistent injury in progressive, radiation induced pulmonary fibrosis. 2018 International Journal of Radiation Biology, In Press

Figure 1B:

Pneumonitis and Fibrosis in C57BL/6J. H&E stained sections (A-F) indicate septal thickening, cellular infiltrate, and alveolar occlusion (F, arrows) during pneumonitis 16 weeks post exposure. Trichrome staining at 24 Wks (G-I) reveal the presence of large inflammatory cells and blue collagen fibers (I, arrows) characteristic of RIPF.

Figure 2:

Irradiation initiates a feed forward loop of biological processes which alters the pulmonary microenvironment and promotes acute and late periods of injury

Figure 3:
Proposed signaling dysfunction in the lung between resident and infiltrating cells during the progression of RILI

Table 1:

Intervention strategies mitigating effects of RILI in rodents. Animals were exposed to photon WTI in a single dose unless otherwise noted.

Drug	Rodent species/strain Exposure paradigm	Mechanism of Action	Result	Reference
ABT-263	C57BL/6J ♂ mice 17 Gy, hemi-thorax	Inhibition of Bcl2/x1, reduced numbers of senescent type 2 epithelial cells	Pneumonitis- not reported Fibrosis- slight reduction	Pan et al. 2017
AEOL 10150	CBA/J ♀ mice 14.6 Gy	Scavenging of ROS	Pneumonitis ↓ Fibrosis- not reported	Murigi et al. 2015
AEOL10150	C57BL/6J ♀ mice 15 Gy	Scavenging of ROS/RNS, decreased numbers of apoptotic cells	Pneumonitis- ↓ Fibrosis- not reported	Zhang et al. 2012
Amifostine	Fischer 344 ♀ rats 28 Gy, hemi-thorax	Scavenging of ROS, reduced active TGFβ	Pneumonitis- not reported Fibrosis- slight reduction	Vujaskovic et al. 2002
Anti CSF1R monoclonal antibody	C57BL/6J ♀ mice 16 Gy	CSF1R inhibition, reduced presence of bone marrow derived interstitial macrophages	Reduced lung tissue density Pneumonitis- not reported Fibrosis ↓	Meziani et al. 2018
Captopril Enalapril Fosinopril	WAG/RijCmcrr ♀ rats 13 Gy	Protected against radiation induced collagen deposition	Pneumonitis- captopril and enalapril reduced mortality Fibrosis- ↓ for all drugs	Kma et al. 2012
Captopril EUK-207 Alone or in combination	Sprague-Dawley ♀ rats 11 Gy	Scavenging of ROS, reduced oxidative stress, TGFβ, numbers of macrophages and hydroxyproline content	Pneumonitis- ↓ Fibrosis ↓ Greatest reductions occurred with combination treatment	Mahmood et al. 2014
Cerium oxide nanoparticles (CNP)	CBA/J ♀ mice 15 Gy	Scavenging of ROS/RNS, reduced inflammation, vascular damage and collagen accumulation	Pneumonitis ↓ Fibrosis ↓	Xu et al. 2016
Diphenyleneiodonium (DPI)	C57BL/6Ncr ♀ mice 5 Gy single dose 17.5 Gy single dose 5 daily fractions of 5 Gy 5 daily fractions of 6 Gy	NOX inhibition, reduced oxidative stress and cellular senescence	Pneumonitis- not reported Fibrosis- ↓	Citrin et al. 2013
EUK-207 Genistein Alone or in combination	Fischer ♀ rats 12 Gy	Inhibition of NFκB, scavenging of ROS, reduced oxidative stress, TGFβ, numbers of macrophages and collagen accumulation	Pneumonitis- not reported Fibrosis- ↓	Mahmood et al. 2013
FG 3019	C57BL/6J ♀ mice 20 Gy	CTGF antibody, abrogated M2 influx and myofibroblast abundance	Attenuated, prevented or reversed lung remodeling: Pneumonitis ↓ Fibrosis ↓	Bickelhaupt et al. 2017
Galunisertib + Imatinib or SU9518 (Sunitinib analog)	C57BL/6N ♀ mice 20 Gy	TGFβR1 inhibition in combination with PGDFR inhibition	Attenuated inflammation and septal thickening Pneumonitis ↓ Fibrosis ↓	Dadrich et al. 2016
Human recombinant TGFβ3	C57BL/6J ♀ mice 20 Gy	Decreased fibrocyte recruitment, increased percentage of tregs present	Reduced inflammation and delayed fibrosis Pneumonitis ↓ Fibrosis- slight reduction	Xu et al. 2014

Drug	Rodent species/strain Exposure paradigm	Mechanism of Action	Result	Reference
Lovastatin	C57BL/6J ♀ mice 15 Gy	Reduced presence of (ed1+) macrophages/monocytes and (cd3+) T cells	Pneumonitis- slight reduction Fibrosis ↓	Williams et al. 2004
Manganese superoxide dismutase-plasmid/liposome (Mn-SOD PL)	C57BL/6NHD ♀ mice 20 Gy	Decreased VCAM-1 and ICAM-1 expression in endothelial cells	Pneumonitis- not reported Fibrosis- not reported	Epperly et al. 2002
Neutralizing mouse IL-17A antibody	C57BL/6J ♂ mice 15 Gy	IL17A inhibition, reduced inflammation	Pneumonitis – slight reduction Fibrosis (reported at 16 weeks) - slight reduction	Wang et al. 2014
Polyethylene glycol conjugated Superoxide Dismutase (PEG-SOD) + PEG conjugated Catalase (PEG-CAT)	C57BL/6J ♀ mice 13.5 Gy	Scavenging of ROS by superoxide dismutase (SOD) mimetics	Pneumonitis- no reduction Fibrosis- ↓	Machtay et al. 2006
Recombinant truncated PAI-1 protein, rPAI-1 ₂₃	C57BL/6Ncr ♀ mice 5 daily fractions of 6 Gy	Reduced of fibrin accumulation and inflammatory/ senescence associated cytokines	Pneumonitis- not reported Fibrosis- slight reduction	Chung et al. 2016
SU9518 or SU11657 or Imatinib	C57BL/6J ♀ mice 20 Gy	PDGFR inhibitor reduced fibroblast proliferation/ fibrogenesis	Pneumonitis ↓ Fibrosis ↓	Abdollahi et al. 2005
Triptolide	C57BL/6J ♀ mice 15 Gy	Reduced NOX2/4 dependent production of ROS	Pneumonitis- not reported Fibrosis- slight reduction	Chen et al. 2016