

RADIATION INDUCED LUNG INJURY IN NON-HUMAN PRIMATES

BY

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DEDICATION

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ABBREVIATIONS

Acute radiation syndrome	ARS
Acyl-CoA wax Alcohol Acyltransferase 2	AWAT2
Albumin	ALB
Alpha smooth muscle actin	α -SMA
Alveolar type II pneumocytes	ATII
Angiotensin convertase enzyme	ACE
Angiotensinogen	AGT
Ankyrin Repeat Domain 7	ANKRD7
Ataxia telangiectasia-mutated genes	ATM
Ataxia-telangiectasia related genes	ATR
ATPase H ⁺ /K ⁺ Transporting Non-Gastric Alpha2 Subunit	ATP12A
Average lung density	ALD
BMP/Retinoic Acid Inducible Neural Specific 1	BRINP1
Bronchoalveolar lavage	BAL
C-C Motif Chemokine Ligand 11	CCL11
C-C Motif Chemokine Ligand 2	CCL2
Chronic obstructive pulmonary disease	COPD
Claudin-10	CLDN 10
Computed tomography	CT
Cyclin Dependent Kinase Inhibitor 1A	CDKN1A
Cytokinesis-block micronucleus	CBMN

Damage-associated molecular pattern molecules	DAMPs
Delayed effects of acute radiation exposure	DEARE
Dendritic cells-specific transmembrane protein	DCSTAMP
Differentially expressed genes	DEGs
Digital imaging and communications	DICOM
DNA damage response	DDR
DNA dependent-protein kinase	DNA-PK
Double-stranded breaks	DSBs
Epidermal growth factor	EGF
Epiregulin	EREG
Epithelial-mesenchymal transition	EMT
Estrogen Related Receptor Gamma	ESSRG
Extracellular matrix	ECM
Family With Sequence Similarity 107 Member A	FAM107A
Fas Apoptotic Inhibitory Molecule 2	FAIM2
Gap Junction Protein Beta 2	GJB2
Gap Junction Protein Beta 2	GJB2
Ground-glass opacities	GGO
Group IID secretory phospholipase A2	PLA2G2D
Growth Hormone 1	GH1
Hematoxylin and eosin	H&E
Hounsfield units	HU
Hyperdense volume as a percent of total volume	PCHV/TV

Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4	HCN4
Idiopathic pulmonary fibrosis	IPF
Immunohistochemistry	IHC
Ingenuity pathway analysis	IPA
Interleukin 1 Beta	IL1B
Interleukin-1 alpha	ILA1
Leucine Rich Repeat Neuronal 4	LRRN4
Lipoprotein(a)	LPA
Low linear energy transfer	LET
Lung fibrosis	LF
Masson's trichrome	MST
matrix metalloproteinase-3	MMP3
Medical countermeasures	Mcm
Membrane Spanning 4-Domains A15	MS4A15
Monocyte chemoattractant protein-1	MCP-1
Necropsy	Nx
Non-human primate	NHP
Paraformaldehyde	PFA
Percent change in hyper-dense lung volume	PCHV
Phenylethanolamine N-Methyltransferase	PNMT
Platelet-derived Growth Factor	PDGF

Potassium voltage-gated channel subfamily H member 5	KCNH5
Rac GTPase activating protein 1	RACGAP1
Radiation pneumonitis	RP
Regions of interests	ROI
Renin angiotensin system	RAS
Retinoid X receptor	RXRG
Secretoglobin Family 3A Member 1	SCGB3A1
Serine protease inhibitor Kazal-type 1	SPINK1
Serpin Family A Member 3	SERPINA3
Signal Transducer And Activator Of Transcription 1	pSTAT1
Simian Immunodeficiency Virus	SIV
Simian retrovirus	SRV
Simian-T-lymphotropic Virus	STLV
Single-fraction stereotactic body radiation therapy	SF-SBRT
Solute carrier family 6 member 4	SLC6A4
superoxide dismutase	SOD
TOX High Mobility Group Box Family Member 3)	TOX3
Transforming growth factor beta 1	TGF-beta1
Usual interstitial pneumonia	UIP
Vascular endothelial growth factor	VEGF
Visiopharm Integrator System	VIS
Wake Forest School of Medicine	WSFM

Whole thorax lung irradiation

WTLI

ABSTRACT

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Radiation-induced lung injury (RILI) is a progressive disease; an early acute exudative phase occurs in about 4-13 weeks post-irradiation, followed by the proliferative phase (3-9 months). Both phases are clinically classified as radiation pneumonitis (RP). Severe injury and persistent damage may lead to a late phase of irreversible lung fibrosis (LF).

The overall aim of this study was to understand the differential susceptibility to RILI in response to a single dose of 10 Gy thorax irradiation and to define the pathogenesis and molecular changes occurring at early and late phase of RILI in a controlled study of nonhuman primates (NHPs).

To achieve this goal, animals were investigated longitudinally over a long time-span of up to 8 months. The work presented in this thesis involves two separate studies which utilized 17 NHPs exposed to a single dose of 10 Gy whole-thorax lung irradiation (WTLI) and compared to three non-irradiated controls. Post- WTLI exposure, animals were categorized as non-survivors, reaching a predefined clinical endpoint based on higher respiratory rates, and survivors surviving to the end of the study (8 months).

In Chapter 2, our results revealed that non-survivors developed more severe RILI relative to survivors. However, irradiation induced significant lung fibrosis in both the irradiated groups (survivors and non-survivors) with an abundance of M2-like macrophages. Transcriptional profiling of lung tissues differentiated survivors, non-

survivors, and controls. This work highlights new molecular targets at the mRNA and protein level.

Chapter 3 was a follow-up study that expanded on longitudinal quantitative CT analysis focussed on threshold-based summary statistics obtained from whole lung CT scans. The entire segmented lungs' histograms were extracted to measure lung average density, hyper-dense lung volume (PCHV), hyper-dense lung volume as a percent of entire lung volume (PCHV/TV). All three density parameters showed a significant increase in lung injury in non-survivors relative to survivors. However, PCHV was the most sensitive parameter that detected a significant increase in radiodense volume, at time points when differences were not detected by the other two parameters. In conclusion, our studies provide strong clinical and molecular data that provide valuable insight into the evolution of RILI to fibrosis in a NHP model.

CHAPTER ONE

INTRODUCTION

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Background

Acute exposure to high doses of ionizing radiation in accidental or war-like situations could cause multi-organ system failure due to serious injury to radio-sensitive organs like the gastrointestinal tract, bone marrow, and lung. Lungs are the major target organ during thoracic exposures and in survivors of acute gastrointestinal and bone marrow sub-syndromes (1). Radiation-induced lung injury (RILI) is a well-known effect of acute radiation exposure (2). In clinical radiotherapy, RILI is the most common side effect associated with thoracic radiation therapy and limits the radiation dose to target cancer. Around two-thirds of lung cancer patients receive radiation therapy along with chemotherapy during the course of the disease. These high-energy beams/particles such as X-rays, gamma rays, electron beams or proton, are used to target lung cancer cells but they can significantly damage surrounding healthy tissue leading to the cause of death in as many as 50% of cancer patients due to severe radiation pneumonitis and incurable lung fibrosis (3). Techniques like stereotactic body radiotherapy (SBRT) use high doses of ionizing irradiation in one or a few fractions to effectively treat the tumor. Over 50% of SBRT patients develop some degree of radiation-induced lung injury as the most common side-effect, although the incidence of severe pneumonitis (>grade 2) is relatively low (1-9%) (4). In two studies, up to 28% of patients requiring re-irradiation (SBRT) developed severe radiation pneumonitis ($\geq$ Grade 3) (5, 6) which is associated with high mortality rate, up to 50% (3). RILI is a progressive condition that broadly exists in two phases, an early phase of radiation pneumonitis (RP), which includes inflammation, edema, and the

proliferation of epithelial and endothelial cells, and a late phase of irreversible lung fibrosis (LF) with the accumulation of fibroblasts, myofibroblasts and extracellular matrix remodeling (7).

The reported incidence of RILI in clinical studies varies significantly (5-50%) and to a degree is attributed to fractionation, dosimetric, clinical and biological parameters (8-10). Only patients with severe pneumonitis (usually > grade 2) are symptomatic and symptoms usually manifest as shortness of breath and/or coughing while others exhibit only radiographic evidence of RP (7). Tapering dose corticosteroid treatment is frequently used to mitigate symptoms of RP however, relapse after discontinuation of corticosteroid therapy is common (11). Incidence of symptomatic RP is generally lower (5-15%), probably due to under-reporting. However, radiographically, incidence of RP was detected as high as 43% (10, 12). Once RILI progresses to fibrosis, there is no established FDA approved treatment for it. Therefore, in order to develop medical countermeasures for RILI, it is very important to understand the progression of this disease from acute to fibrotic stage in a well-controlled and human-relevant animal model.

Mechanistic sequence of events post irradiation

Various signaling mechanisms and target molecules have been proposed to study RILI. According to the most accepted mechanism, ionizing radiation can cause direct DNA damage or interact with tissue water to generate highly reactive free radical species that include reactive oxygen species (ROS). The radiobiology of the effect also depends upon the type of irradiation e.g., high or low linear energy

transfer (LET). Low LET ionizing radiation like x-rays, gamma rays and electrons, mostly results in indirect DNA damage, while high LET (e.g. heavy charged particle exposure) is a major cause of direct DNA damage that mostly results in double-stranded breaks (DSBs) (13). After radiation exposure, the chemical reactions start within fractions of seconds (0.01 ps) followed by ROS/RNS production. Antioxidant or detoxifying enzymes are upregulated to enhance cellular defense mechanisms in response to radiation injury. However, a high dose of ionizing irradiation significantly disrupts cellular redox balance due to increased production of ROS, and the progress in cellular damage occurs in minutes to hours. Excessive ROS/RNS production causes oxidative damage to biomolecules like DNA, protein, and lipid and results in the activation of a pro-inflammatory response.

The lung is a complex organ and consists of over 40 different cell types. Cells with rapid turnover are usually more sensitive to ionizing radiation which includes alveolar epithelial and bronchial epithelial cells in the lungs. It is known that Alveolar Cells Type I and Type II (ATI and ATII) are major target cells of a high dose of ionizing radiation, and surviving ATII pneumocytes proliferate as a recovery response to loss of ATI and ATII (14, 15). Nucleic acids are a radio-sensitive target and damage to DNA due to irradiation generated ROS/RNS is a well-accepted critical mechanism (16, 17). DNA double-strand breaks (DSBs) are correlated with lethality (18) and can either lead to cell death by apoptosis or by necrosis (19). However, radiation-induced damage is only an initiating event in a cascade process and DSBs can be efficiently repaired by various DNA repair mechanisms (18). The DNA damage response (DDR) following irradiation results

in cell cycle arrest as evidenced by the increased gene and protein expression of TP53 dependent or related genes such as CDKN1A/p21, GADD45, MDM2, and others (20) and activated DNA repair sensing enzymes such as DNA dependent-protein kinase (DNA-PK), ataxia telangiectasia-mutated genes (ATM), ataxia-telangiectasia related genes (ATR), and others (21). Cell cycle arrest could be a protective response to irradiation in order to allow sufficient time for repair. Studies have shown that deletion of cell cycle arrest related proteins (p53, p21) exacerbates radiation injury (22, 23). However, radiation-induced senescent cells express p21 associated with progressive radiation inducing pulmonary fibrosis (15, 24). A senolytic drug (ABT-263) reversed radiation-induced persistent fibrosis in a mouse model exposed to a single dose of 17 Gy to right thorax. The drug selectively targeted senescent type II pneumocytes in vitro suggesting the important role of senescent cells in radiation- induced lung fibrosis (25). Even though most DNA damage gets repaired, incorrect repair and telomeric DNA tracts which are resistant to repair, can trigger persistent DNA damage (26).

Following injury and apoptotic death to multiple cell populations of the lungs, damage-associated molecular pattern molecules (DAMPs) are released from dying/apoptotic cells and trigger the pro-inflammatory response. The response includes cytokine production, vascular leakage, edema, and recruitment of a variety of hematopoietic and non-hematopoietic cells including neutrophils, foamy macrophages, eosinophils, and lymphocytes into the alveolar spaces and the interstitium. Neutrophils are usually first responders followed by macrophages and lymphocytes (27). Irradiation also disturbs lipid metabolism in the irradiated lung

leading to an increasing number of foamy lipid-filled macrophages in response to increased accumulation of surfactant lipid (28).

Increased levels of cytokines such as macrophage colony-stimulating factor (M-CSF) and monocyte chemoattractant protein-1 (MCP-1/CCL2) were observed in mice exposed to a single high dose (12.5Gy) of thoracic irradiation (29, 30). Macrophages and monocytes secrete CCL2, which can further recruit macrophages to the site of injury post-irradiation through the CCL2/CCR2 axis (21). These findings indicate that the CCL2/CCR2 axis has a vital role in developing radiation-induced lung fibrosis (29). Macrophages are highly plastic in nature and are capable of changing their phenotype depending upon the stimulus or microenvironment around them. A pro-inflammatory milieu causes classical activation of macrophages (M1) that produce inflammatory cytokines/chemokines/ROS/RNS intermediates (TNF- α , IL1- β , IL-12, CCL2). The M1 phenotype is also associated with an increase in the production of matrix-degrading molecules (MMP2, MMP-9). Radiation injury activates M1 macrophages leading to the initial stage of radiation pneumonitis (31). The polarization of macrophages from a pro-inflammatory phenotype (M1) to a pro-fibrotic phenotype (M2) is central to the control of inflammation and fibrosis (32). M2 macrophages control inflammation, but if the insult persists, M2 macrophages release pro-fibrotic cytokines (TGF β , PDGF, VEGF, etc.) that stimulate resident fibroblasts, fibrocytes, epithelial and endothelial cells to acquire a myofibroblast-like phenotype which produces extracellular matrix leading to fibrosis (31). Clinically, the interplay of microenvironment with factors like dose, fractionation, existing

interstitial disease, and other treatments such as chemotherapy or immunotherapy is very complex. Therefore, adequate well-controlled studies are needed to establish the role of macrophages in RILI and subsequent development of LF to further their role as a therapeutic target (33).

TGF-beta is a key regulator of fibrosis and is expressed by multiple cell types e.g. epithelial cells, fibroblasts, and macrophages. It has an important role in the transformation of fibroblasts to myofibroblasts and induces extracellular matrix (ECM) production via canonical and non-canonical pathways (34, 35). In a mouse model, therapeutic intervention using an inhibitor of the TGF- β type I receptor kinase (ALK5)/EW-7197 alleviated bleomycin-induced fibrosis and reduced the expression of the TGF-beta/smad signaling pathway (36).

In chronic lung conditions including lung fibrosis, epithelial cells undergo epithelial-mesenchymal transition (EMT) and produce ECM in a TGF-beta dependent manner (37). In an *in vitro* experiment, primary human alveolar type II pneumocytes underwent EMT upon TGF-beta stimulation as evidenced by increase in vimentin. Vimentin signal reduced when the TGF-beta type I inhibitor was used (38). The involvement of ATII in EMT was shown in a mouse model (FVB/N mice) exposed to a high dose of thoracic irradiation (12 Gy, 30 Gy and f24 Gy) where pro-SP-c and α -SMA was colocalized suggesting conversion of ATII cells to a mesenchymal phenotype (39).

Pathology and clinical manifestation

The initial acute response to ionizing radiation is classified as radiation pneumonitis (RP). The early acute exudative response occurs about 4-12 weeks after the completion of radiotherapy and in the case of severe injury, it progresses to the proliferative phase in about 3-9 months. Clinically and radiologically, the exudative and proliferative response corresponds to RP. Common clinical symptoms of RP include are dyspnea, cough, with or without low-grade fever, and chest discomfort (11, 40). Clinically, radiation fibrosis can present with dyspnoea, persistent dry cough, or signs of cor pulmonale (affected right ventricle due to pulmonary resistance/hypertension due to fibrosis) (40).

Histologically, RP is characterized by early changes (exudative phase) of degeneration in alveolar epithelial and endothelial cells followed by disruption of the microvasculature, and thrombosis leading to leakage of proteinaceous fluid into interstitium and alveolar spaces (edema). Sloughed epithelial cells, foamy macrophages, and fibrin-rich fluid form hyaline membranes within alveoli. In the proliferative phase, the frequency of cellular atypia and hyperplasia of type II pneumocytes increases. During the progressive organization, thickening of alveolar septa occurs due to proliferation of fibroblasts, infiltration by immune cells, and deposition of collagen fibrils (40). Severe injury leads to irreversible fibrosis which is characterized by extracellular matrix remodeling and marked septal thickening due to excessive deposition of collagen associated with basement membranes of endothelial and epithelial cells (27).

CT Imaging

Radiographically, chest CT images are sensitive and detect early RP changes as ground-glass opacities (GGO) a few weeks post-irradiation. Usually, these opacities resolve but if the injury is severe enough these opacities may progress to patchy appearances and eventually lead to linear scarring with consolidation and well-defined areas of volume loss, traction bronchiectasis, and pleural thickening of the affected side, clinically recognized as radiation fibrosis. Although these changes usually correspond to the radiation field, a few studies have also reported out-of-field RP (7, 41). Both small and large RILI animal models have been used to correlate CT density changes to histological findings (42-44). Pleural and pericardial effusions associated with RILI are often diagnosed on CT scans (45), however biology and cytopathology of radiation induced pleural effusion is not completely understood (46). Clinically, risk factors associated with pleural and pericardial effusions include radiation dose, the volume of lung exposed, presence of other lung diseases, and concurrent treatment (47-49). Single-dose exposure NHP studies (Whole thorax irradiation/WTI) and partial body exposure with marginal bone marrow sparing/PBI/5BM) frequently reported pleural and cardiac effusion and correlated the incidences with increasing radiation doses (50-52).

Risk factors

Dose: Accidental non-uniform or partial body exposure to a high single dose of ionizing irradiation could involve the entire lung or a large volume of the lung leading to RILI. The incidence of RP in NHPs exposed to a single 10 Gy dose of irradiation to the thorax is 63% which is close to the incidence of RP in humans given a large single dose of 9.5 Gy (52%) (52, 53). In the clinical scenario, cases

of severe RP are usually associated with higher dose and radiation exposure to larger lung volumes (7). Cancer treatment modalities like SBRT utilize larger doses and are fractionated, which is effective to target cancer but also increases the dose to surrounding normal tissue often resulting in RP. Regardless of treatment modality of radiotherapy, dose-related factors like total dose, mean lung dose, and volume of lung receiving 20 Gy (V20) are most commonly used to predict RILI (10, 54, 55). However, 7-16% of patients with low predicted risk (based on dose-related parameters) still develop severe RILI and 46-71% of patients do not experience RP despite high predicted risk (56) suggesting a significant role of other confounding factors and co-morbidity. Whole thorax irradiation (WTLI) animal models exposed to a single high dose are most commonly utilized to study RILI. These models increase our understanding of RILI regarding its pathogenesis and disease progression and also identify new biomarker and targets (57).

Sex: Most clinical studies reveal no association between sex and risk of RP. Hence, sex is usually not considered as an important criterion while prescribing radiotherapy (58). Sex did not affect the radiation dose-response relationship in a nonhuman primate model of WTLI (50). However, female mice appear more sensitive to RILI relative to males (59).

Age: Age could be an independent predictor of RILI, but some studies have not found any association between age and RILI (60, 61). Others have shown an association between advanced age (>65 years old) and the severity of RP (58, 62). However, limited data is available on the effect of age on the final outcome of LF (56). The overall incidence of RP in children receiving RT to the lung as an

adjacent tissue is lower (2-9%) and less severe than those of adults (63, 64). Chronic cough (>10%), dyspnea (>15%), lung fibrosis (5-30%), and pleurisy (>6%) were commonly reported as long-term outcomes in children receiving whole lung radiotherapy (e.g., metastatic Wilm's or Ewings), mediastinal and total body irradiation for pediatric cancer (56, 65, 66).

Regional differences: Several studies have shown spatial differences in lung radiosensitivity. In humans, lower lung lobes are considered radio-sensitive relative to upper lung lobes due to better ventilation and blood flow (due to gravity) suggesting that lower lobes are more prone to irradiation injury and injury to the upper lobe might be less symptomatic (67-69). Rodents studies have also indicated increased radio-sensitivity of lower/base lung regions relative to upper lung sub-volumes possibly either due to the concentration of target cells at the base relative to the apex (70).

Animal models

Various animal models have been used to study acute and delayed effects of radiation injury, however, a great deal of diversity in the initiation and progression of RILI has been reported both in humans and animal models in terms of clinical symptoms, pathological lesions, and genetic contributions to susceptibility (71, 72).

Mice are a well-characterized animal model to study RILI (73) and several mouse strains have been widely used to study both the acute or late effects of RILI; however very few mice strains justify being used as a favorable model to study the early phase of RILI and late phase of established LF(71). FVB/N mice exposed to

single high dose (12 and 13Gy) or fractionated (24Gy) dose of whole thoracic irradiation developed histologic evidence of RP 2-5months post-irradiation that showed markedly thickened alveolar septa, alveolar foamy macrophages, other inflammatory cell infiltration, ATII hyperplasia, collagen deposition, and regional fibrosis (39). In another mouse study, the mean survival of B6 mice (12.5 ± 1.1 weeks) was longer relative to C3H mice (11.7 weeks) after 18 Gy WTI. This disparity was attributed to a differential apoptotic response to radiation that spared the inflammatory response in the lungs of B6 mice relative to C3H mice (74).

Many mouse models like BALB/c are well recognized for radiosensitivity, however, they develop early pleural effusion post-irradiation that can cause respiratory distress contributing to early mortality which interferes with the direct assessment of RILI. Pleural effusions are rarely reported in humans after thoracic irradiation. *Jackson et.al* (2011) found that only three mouse strains (CBA, C3H, and C57L) out of seven studied met the requirement of producing clinically relevant models to study the early progression of RILI (71).

Among rodents, rats have also been used for long term (up to 28 weeks) lung radiation studies (75-77). A rat strain (WAG/ RijCmcr) exposed to a range of radiation doses (8-13 Gy) to the thorax, developed alveolar granulomatous lesions (giant cell and macrophages containing cholesterol clefts) along with fibrosis at around 21 weeks (78). The clinical implication of granuloma is not clear however these lesions have also been reported in humans after irradiation (79).

Rodents have a shorter life span and most mouse studies (83%) are terminated at 6 months of age and do not address long-term effects of radiation-induced lung injury (80). The subgross lung anatomy and pathology of rodents differs from that of human regarding lobularity, airway branching, the relative thickness of the septa and pleura and blood supply (81).

The NHP model emulates most closely the heterogeneous nature of human populations and serves as a strong model to study RP and its late outcome LF. Rhesus macaques have been used as a model to study acute radiation syndrome (ARS) and delayed effects of acute radiation exposure (DEARE) (52, 82) and for the development of medical countermeasures (MCMs) against RILI (2, 50, 83, 84). The FDA animal rule (2002) for the development of radiation countermeasures has certain criteria in using animal models that include a thorough understanding of the pathophysiology of toxicity/radiation and its reduction by the drug in a well-characterized animal model for predicting human response (85, 86). The whole thorax lung irradiation (WTLI) NHP model is a well-established animal model to study RILI (50, 52, 83). Another recommended NHP model to study the early and late effects of radiation is partial body exposure (PBI) with marginal bone marrow sparing (PBI/5BM) (51). This model represents survivors of ARS whose spared bone marrow can restore the acute effects of radiation to the hemopoietic system and allows to study delayed effect of radiation to organs like lung, kidney, and gastrointestinal system (82).

Other studied large animal RILI models include sheep and pig. In sheep, histologic features of RP observed 37 days after the fractionated radiotherapy (30 Gy total

dose) to a defined unilateral lung lobe were similar to those noted in rodent models. Grossly, they observed dark red discolored lung regions with firmer underlying parenchyma. Microscopically, in addition to RP features mentioned previously, occasional intra-alveolar fibrin (fibrillar material) and atypia were noticed (87) similar to those noticed in humans (67). In another study, sub-volumes of pigs' lungs were exposed to a single dose of 12.5 Gy irradiation, and their CT findings significantly correlated with lung pathology (88).

In summary, despite advanced radiotherapy modalities, toxicity to normal lung tissue remains a significant dose-limiting factor. Besides dose-related risk factors, patient-related factors, co-morbidity, pre-existing lung disease, and concurrent therapy are major unavoidable confounders in clinical studies. These confounders not only obscure the understanding of the exact cause and course of RILI but can significantly modulate or potentiate the outcome of the disease. RILI can be challenging to distinguish from tumor progression. In a retrospective clinical study, 24.5% of patients receiving SBRT for lung cancer treatment, tumor progression was misdiagnosed in place of RILI (62), which supports the importance of well-controlled RILI studies in human-relevant animal models. Well-established RILI models have not only improved the understanding of RILI, but also have identified molecular targets and biomarkers in local and systemic fluids with clinical application potential. A WTLI NHP study analyzed peripheral blood lymphocytes (PBL) from whole blood of irradiated animals (10 Gy) and observed significant micronuclei induction (radiation specific biomarker assay; cytokinesis-block micronucleus assay, CBMN) in irradiated animals that persisted to their endpoints

(84). This study indicates the potential role of peripheral blood biomarkers to track the persistent damage due to RILI.

An extensive study on RILI models has proposed several mechanisms and has identified that a variety of lung cell populations is affected by irradiation (15). However, a unifying concept on the cell-specific role in induction and progression of RILI, mechanisms that drive these changes, and interindividual variations in radio-responsiveness is still unclear and yet remains to be elucidated. Further well control longitudinal studies in human-relevant animal models are needed that can address these gaps. Besides, imaging plays a significant role in diagnosing the disease and has been used as quantitative imaging biomarkers of RILI (89). Quantitative and qualitative CT findings not only can diagnose RILI but have the potential to differentiate comorbidity and lesion type (4, 90).

Research Summary

The purpose of the present work is to understand the intrinsic basis for varying individual responses to the same dose of irradiation. To achieve this goal, we characterized and assessed RILI longitudinally in a human-relevant NHP model (rhesus macaque) exposed to a single high dose of ionizing radiation to the thorax. To achieve these aims, our multidisciplinary approach included clinical, pathological, and molecular analysis. Animals for the present research work were derived from prior two studies of superoxide dismutase (SOD) mimetic radiation mitigators, in which the mitigator did not provide a clear benefit. These animals were exposed to a single 10 Gy whole-thorax dose of ionization radiation at 4

Gy/min using a 6 MV linear accelerator. Three saline-treated and sham-irradiated control animals derived from another study were compared to the chest irradiated animals.

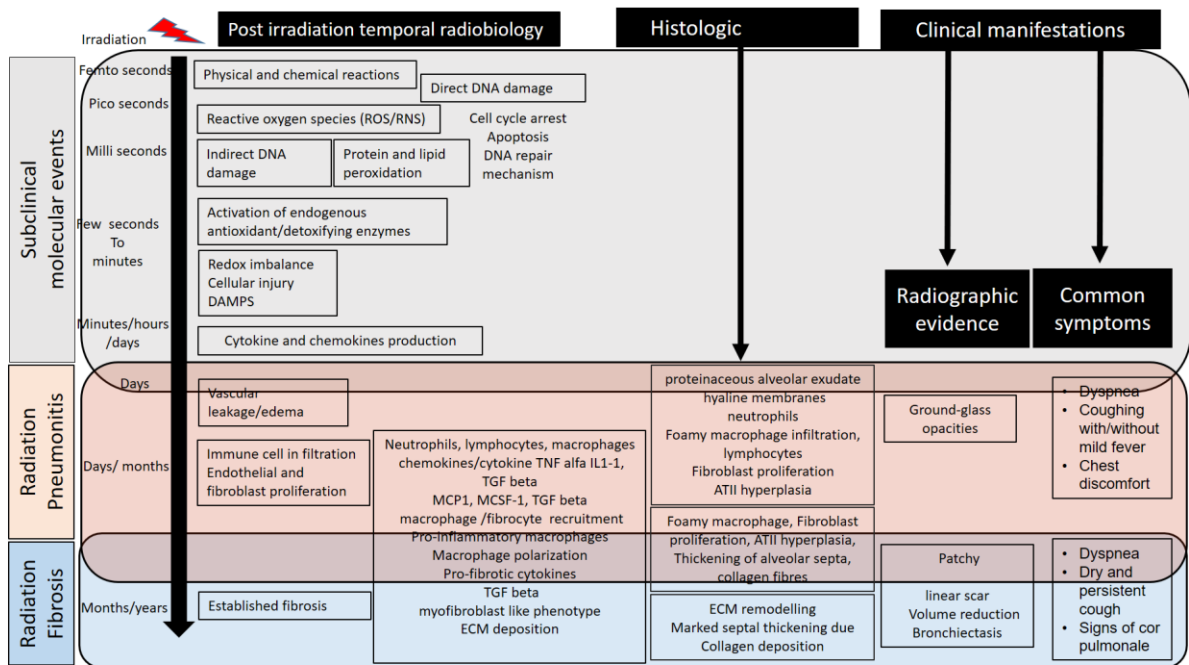
Chapter 2 is focused on understanding the pathogenesis and progression of RILI in the NHP model and a basis for investigating underlying mechanisms in RILI. To address this aim, clinically, we analyzed parameters like respiratory rates and computed tomographic scans. The pathological assessment included gold standards like histopathological scoring and molecular analysis comprised of transcriptomic approaches like RNA sequencing of frozen lung tissues and analysis of relevant proteins utilizing immunohistochemistry, immunoassays in local bronchoalveolar (BAL) and systemic fluids.

Chapter 3 represents a follow-up study, in which we expanded our analysis on quantitative assessment of lung density over time which involved extracting threshold-based summary statistics from whole lung CT scans. To measure and evaluate changes in the density distribution of RILI over time, we utilized lung CT scans of rhesus macaques from the same study presented in Chapter 2.

In summary, we demonstrate an understanding of RILI in the early proliferative phase of RP and late phase of fibrogenesis and remodeling. Our data support the role of previously proposed mechanisms (TGF beta and macrophage polarization) and provide insight into new potential pathways. Molecular techniques like RNA sequencing highlight the role of top differentially expressed transcripts (SERPINA3, ATP12A, GJB2, CLDN10, TOX3, LPA) as possible biomarkers and

potential therapeutic targets of RILI. We also provide evidence that threshold-based CT measurement indices of radiodensity in RILI can be a good surrogate to histopathology.

Summary figure:



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CHAPTER TWO

Clinico-pathological and transcriptomic analysis of radiation-induced lung injury in non-human primates

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Data Availability Statement for this Work

“Research data are stored in an institutional repository and will be shared upon request to the corresponding author.”

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Clinico-pathological and transcriptomic analysis of radiation-induced lung injury in non-human primates

Radiation-induced lung injury

Abstract

Purpose: Radiation-induced lung injury (RILI) is a progressive condition, with an early phase, radiation pneumonitis (RP), and a late phase, lung fibrosis (LF). RILI may occur after partial-body ionizing radiation exposures or by internal radioisotope exposure, with wide individual variability in timing and extent of lung injury. This study aims to provide new insights into the pathogenesis and progression of RILI in the non-human primate (NHP) rhesus macaque model.

Methods and Materials: We used an integrative approach to understand RILI and its evolution at clinical and molecular levels in seventeen NHPs exposed to 10 Gy of whole thorax irradiation in comparison to three sham-irradiated control NHPs. Clinically, we monitored respiratory rates, CT scans, plasma cytokine levels, and bronchoalveolar lavage (BAL) over 8 months and lung samples collected at necropsy for molecular and histopathological analyses using RNA sequencing and immunohistochemistry.

Results: Elevated respiratory rates, greater CT density and more severe pneumonitis with increased macrophage content were associated with early mortality. Radiation-induced LF included polarization of macrophages towards the M2-like phenotype, TGF- β signaling, expression of CDKN1A/p21 in epithelial cells, and expression of α -SMA in lung stroma. RNA sequencing analysis of lung

tissue revealed SERPINA3, ATP12A, GJB2 and CLDN 10, TOX3, and LPA as top dysregulated transcripts in irradiated animals. In addition to transcriptomic data, we observed increased protein expression of SERPINA3, TGF-beta1, CCL2, CCL11, in BAL and plasma samples.

Conclusions: Our combined clinical, imaging, histological, and transcriptomic analysis provide new insights into the early and late phases of RILI, and highlights possible biomarkers and potential therapeutic targets of RILI. TGF-beta activation and macrophage polarization appear to be key mechanisms involved in RILI.

Key words: Radiation induced lung injury, RNA sequencing, CT scans, Histopathology, Bronchoalveolar lavage, fibrosis

Introduction

High doses of ionizing radiation exposure to thorax during accidental or clinical events can disrupt normal lung biology substantially, leading to radiation induced lung injury (RILI). The lung is a radiosensitive organ and RILI may occur at single doses of $\geq 9.5\text{Gy}$. For fractionated therapeutic exposures mean lung dose ($\geq 35.5\text{Gy}$), volume of lung exposed to $\geq 20\text{Gy}$ (V_{20}) and higher total dose ($\geq 45\text{Gy}$) are consistent predictors of radiation pneumonitis (RP) (1-6). Non-uniform and partial body exposure are likely scenarios that could lead to significant lung damage (7). RILI is a progressive condition which has two phases, an early phase of radiation pneumonitis (RP), which includes inflammation, edema and the proliferation of epithelial and endothelial cells, and a late phase of irreversible lung fibrosis (LF) with the accumulation of fibroblasts, myofibroblasts and extracellular matrix (ECM) remodeling. Persistent RILI leads to fibrosis, for which there is no FDA approved treatment (8). Thus, RP and LF represent important clinical challenges for diagnosis, prognosis, and treatment.

Macrophages play an important role in instigating radiation-induced pro-inflammatory and pro-fibrotic responses (9). However, their role in RILI and subsequent development of LF is not fully understood (10). TGF-beta is a major regulator of fibrosis (11) and is the most potent inducer of ECM synthesis. TGF-beta promotes the development of myofibroblasts which can arise from resident fibroblasts, circulating fibrocytes or via epithelial-mesenchymal transition (EMT) (12). We hypothesize that macrophage polarization and increased TGF-beta are important triggers for the progression of LF in the RILI NHP model. Our data

provide new insights into the pathogenesis and progression of RILI in the NHP model and a basis for underlying investigating mechanisms in RILI.

Methods

Animals and study design

For the present study, we utilized seventeen rhesus macaques (median age 4.76 years and median weight 6.0 kg), given a single 10 Gy whole-thorax dose of ionization radiation at 4 Gy/min using a 6 MV linear accelerator (Supplementary methods) and three sham-irradiated, saline-treated controls (median age 2.75 years and median weight 3.6Kg). These animals were derived from prior studies of whole thorax irradiation (13) (Supplementary figure 2). Our goal was to understand the intrinsic basis for varying individual responses to the same dose of irradiation. Animals were studied for 8 months to assess clinical, pathologic, imaging, and molecular outcomes contributing to survival. Animals developing respiratory rates of >80 breaths per minute (clinical endpoint) were euthanized and categorized as non-survivors. Animals surviving to the 35 weeks/8 months experimental endpoint were categorized as survivors and were euthanized at the end of the study. Clinical observations, BAL cytology/fluid samples and CT scans were taken at baseline and post-irradiation at 2-month intervals throughout the study. After euthanasia, detailed necropsies were immediately performed and lung tissue was collected for histopathologic and transcriptomic analysis. For transcriptomic analysis (RNA seq), eight saline-treated irradiated animals were compared to the 3 sham-irradiated, saline-treated controls. Additional details are

provided in the supplementary methods; and a schematic of the experimental design is shown in supplementary figure 1 A.

Study approval

The protocol was approved by the Wake Forest University Institutional Animal Care and Use Committee. Wake Forest is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures in this study were performed at Wake Forest University, in accordance with the Animal Welfare Act and other applicable federal regulations. All animals were screened for measles, Simian retrovirus (SRV), Filoviruses, Simian Immunodeficiency Virus (SIV), Simian-T-lymphotropic Virus (STLV) and tuberculosis and were quarantined under Centers for Disease Control guidelines prior to inclusion in the study. Supportive fluid therapy, analgesics (for example, ketoprofen), and symptomatic care was given as needed, based on clinical pathology abnormalities and clinical signs.

CT Image analysis

CT scans were done prior to irradiation and every two months thereafter until the end of the study using a Toshiba Aquilion 32 slice scanner with a voxel resolution of 0.5 x 0.625 x 0.625 mm. Two animals reached their predefined euthanasia endpoint before the first 2-month CT scan, hence fifteen monkeys were utilized for CT analysis. We utilized Mimics software (Materialise mimics innovation suite, Belgium, version 21.0.0.406) to quantify the percent volume of lung occupied by radio-opaque lung tissue and created a three-dimensional reconstruction of

abnormally dense lung tissue along with surrounding healthy tissue, using a modification of our previously published method (13) to determine the percentage change in abnormally dense lung tissue by survival status, corrected for baseline. Additional details of CT procedures are described in the supplementary methods.

Histopathological assessment, histochemical staining, and immunohistochemistry

At necropsy, 6 lung regions were consistently sampled from each animal, consisting of immediately-adjacent fixed and frozen samples. Fixed samples were preserved in neutral 4% paraformaldehyde (PFA), embedded in paraffin blocks, and sectioned at 4 microns. An automated immunohistochemistry (IHC) stainer, the Bond RX (Leica Biosystems, Buffalo Grove, IL) was used to stain slides. Sections were stained with hematoxylin and eosin (H&E), Masson's trichrome (MST), and IHC staining was done for myofibroblasts (alpha smooth muscle actin, α -SMA, Abcam, catalog # ab5694); macrophages (CD163, Thermo scientific catalog # MA1-82342, and HAM56, sigma/Millipore, catalog # 279M-16); the M1 macrophage specific transcription factor pSTAT1 (Cell Signaling Technologies, catalog # 9167); the M2 macrophage specific transcription factor CMAF-1 (Abcam, catalog # ab199424); the cell cycle arrest marker p21 (Abcam, catalog # ab109520); an epithelial marker (pancytokeratin, Dako / Agilent, catalog # M3515); and the type II pneumocyte marker SPC (Abcam, catalog # ab90716. Other details and a list of immunohistochemical markers are described in supplementary methods and supplementary table 1.

Stained slides were scanned at 20x magnification on an Olympus VS120 scanner. H&E stained slides were used for histopathological assessment of pneumonitis severity. The histologic scoring criteria for pneumonitis are shown in supplementary figure 4. Images of MST-stained slides were analyzed using Visiopharm Integrator System (VIS), version 6.5.0.2303 (Visiopharm, Horsholm, Denmark) image analysis software. The percentage collagen area in the section was determined as percent collagen = (Blue area/total area) x100. Quantification details of fibrosis (MST), macrophage (HAM 56), M1 macrophages (CD163+pSTAT 1) and M2 macrophages (CD163 +CMAF-1) are described in the supplementary methods.

Cytokine analysis

Plasma and BAL supernatant samples from irradiated monkeys were assessed for 29 cytokines using the Monkey Cytokine Magnetic 29-Plex Panel (LPC0005M, Thermo Fisher), TGF-beta 1,2 and 3 using U-PLEX TGF- β Combo NHP kit (catalog # K15243K) and Meso Quickplex SQ120 (Meso Scale Diagnostics LLC, USA), SERPINA3 using Monkey Alpha1 Antichymotrypsin (AACT) ELISA Kit (MBS7209859, MyBioSource) following the manufacturer's instructions (additional details are provided in supplementary methods).

RNA sequencing

Snap frozen lung samples (immediately adjacent to the fixed samples) were selected from 3 lung regions from 8 irradiated animals and 2 regions from 3 control animals for RNA extraction and sequencing. Sequencing was done using an

illumina Next seq 500 NGS (Illumina, San Diego, CA). Further details of RNA extraction, sequencing methods, and data analysis are described in the supplementary methods. Significant Differentially expressed genes (DEGs) were conservatively defined as log2fold change ratios $\geq \pm 1$ and $p < 0.05$ after adjustment for false discovery (Benjamini-Hochberg). NHP lung RNA-Seq data will be deposited in the NCBI's Gene Expression Omnibus database.

Statistical Analysis

All data were plotted, and descriptive analysis was done to assess normality and skew using GraphPad Prism 8. Statistical significance was determined using paired or unpaired student T-test tests or Mann-Whitney U test depending on sample distribution. For grouped or multivariate analysis, one-way or two-way ANOVA or Mixed-effects model (restricted or residual maximum likelihood (REML)) matching tests were done, followed by post-tests (Tukey's or Wilcoxon Signed Rank test). The strength of correlations was determined by Pearson's test. For all experiments, a p-value < 0.05 was considered statistically significant.

Results

Survival

The cumulative percent mortality at the study's end was 52.9% (9/17). Non-survivors reached a predefined euthanasia end point, having respiratory rates of > 80 respirations per minute and were euthanized between 1.5 month (7.4 weeks) and 6 months (24.8 weeks) after irradiation (Supplementary figure 1, B-D).

CT imaging reveals greater lung injury in non- survivors relative to survivors

Increased CT density was a feature of RILI in both survivors and non-survivors. Figures 1, A and B show CT images of a representative non-survivor and a survivor over the course of the study. Figure 1, C, and D show three-dimensional reconstructions of abnormally dense radio-opaque lung tissue (gold) and surrounding normal lung tissue (blue), from the same two animals. The distribution of the lesions was patchy, and surprisingly the location of opacities changed over time. The percent injured lung volume increased significantly in both non-survivors (Figure 1E) and survivors (Figure 1F) post irradiation relative to baseline. However, the percentage of injured lung volume was significantly greater in non-survivors relative to survivors by 4-months post-irradiation (Figure 1G). There was a significant positive correlation between respiratory rates and percent injured lung volume on CT scans, primarily driven by non-survivors (Supplementary figure 3, A and B).

Histopathological evaluation shows more severe pneumonitis in non-survivors

The average histological pneumonitis score of each animal is shown in figure 2 A, where each bar represents one animal and dots represent 6 different regions of the lung. Monkeys at their clinical endpoints (non-survivors) developed moderate to severe pneumonitis. Animals euthanized at their experimental end points (survivors) had only minimal to mild pneumonitis by the study's end ($p < 0.05$). Figure 2, C-E shows images of H&E stained sections representative of a control

non-irradiated lung, a non-survivor lung with a typical histologic pattern of RP, and a survivor's lung, respectively. A significant positive correlation was observed between lung weights and pneumonitis scores (Figure 2F) indicating the influence of pneumonitis on increased lung weights. HAM56 staining revealed greater lung area occupied by macrophages at early clinical end points (non-survivors) relative to later end points (survivors) (Figure 2, G-J). Together, these observations show that non-survivors developed significant pneumonitis predominated by macrophages, which was either not present or waned over time in survivors.

We observed a positive and significant correlation between the abnormally radiodense lung volume (quantified on the last CT scans corresponding to their end points) and the histopathologic pneumonitis scores in non-survivors (Supplementary figure 3C). This correlation was not significant for survivors (Supplementary figure 3D). These findings indicate that the inflammatory infiltrate is the major contributor to abnormally radiodense lung volume in non-survivors.

Irradiation induced significant fibrosis

Masson's trichrome staining identified areas of collagen accumulation and fibrosis as early as 7.4 weeks post irradiation (Figure 3A). Fibrosis was significantly greater in irradiated animals, relative to non-irradiated controls (Figure 3B), but did not differ between survivors and non-survivors. Representative Masson's trichrome stained lung sections are shown in figure 3C, 3D and supplementary figure 5B.

M2-like macrophages predominate in irradiated lungs

Inflammatory M1-like macrophages coexpressing CD163 and PSTAT1 were only significantly elevated in non-survivors ($p < 0.05$) (Figure 3E). M2 like (pro-fibrotic) macrophages coexpressing CD163 and CMAF were significantly higher in both irradiated groups ($p = 0.0021$), and more abundant in non-survivors relative to both controls and survivors (both $p = 0.001$) (Figure 3F). A representative lung section from a non-survivor (thirteen-week post-irradiation) shows lung macrophages (CD163), negative for the M1 specific transcription factor (PSTAT1; Figure 3G) depicted in figure 3G whereas macrophages in the corresponding serial section are positive for the M2 specific transcription factor (CMAF; Figure 3H).

Transcriptional profiling of lung tissues in survivors, non-survivors, and controls

Unsupervised Principal Components Analysis of RNA seq clustering data revealed three different gene expression patterns in controls, non-survivors and survivors (Figure 4A).

A total of 2644 genes were differentially expressed (upregulated or downregulated) in non-survivors versus control and 680 genes in survivors versus controls. Significant differentially expressed genes (DEGs) were conservatively defined as $\log_2$ fold change ratios $\geq \pm 1$ and $p < 0.05$ after adjustment for false discovery. Out of the 2644 DEGs of non-survivors, 2202 differed from controls only in non-survivors and 238 out of 680 DEGs differed from controls only in survivors. 422 DEGs were shared between non-survivors and survivors (Figure 4B). Ingenuity pathway analysis (IPA) of non-survivors specific DEGs (2202) revealed hepatic

fibrosis as top canonical pathway with TGF-beta as main upstream regulator (Figure 4, B and C); top up-regulated transcripts included MMP3, DCSTAMP, GH1, PLA2G2D and EREG and the most downregulated transcripts included SLC6A4, MS4A15, SEC14LC3, SCGB3A1 AND AWAT2 (Figure 4 D). Cell division and cell cycle related pathways were prominent in IPA analysis of survivor specific DEGs (238). Their top upregulated transcripts were ALB, ANKRD7, SPINK1, HCN4 and FAIM2 and downregulated transcripts involved KCN5, BRINP1, ESSRG, NUF2 and RACGAP1 (Figure 4 E).

A unique subset of 101 genes out of shared 422 DEGs (Figure 5 A) between survivor and non-survivors, showed significant differential expression in all three groups (control, survivors and non-survivors). These common 101 genes differentially affected in non-survivors, survivors and controls, and were used to identify common target molecules and mechanisms underlying RILI leading to fibrosis. IPA analysis of these common 101 DEGs reflected functions associated with cancer, organismal injury, cellular movements, immune cell trafficking and inflammatory responses (Supplementary figure 6 A). The top upregulated genes among the 101 DEGs included SERPINA3, ATP12A, LRRN4, GJB2 and CLDN 10 and the most down-regulated genes were PNMT, TOX3, FAM107A, RXRG and LPA (Figure 5, B and C). Angiotensinogen (AGT), ILA1, IL1B and TGF-beta1 were major upstream regulators (Figure 5D). Associated target transcripts of TGF-beta1 upstream regulators (SERPINA3, GJB2, CCL2, CCL11 and CDKN1A/p21) revealed by IPA are shown in the supplementary figure 6 B and supplementary table 2.

Cytokine analysis revealed significantly increased levels of CCL2 and CCL11 in plasma at 2.3, 3.4, 4.3 weeks and at necropsy (Nx) (Figure 6, A and B) and a significant increase in TGF - beta1 in plasma at necropsy in the irradiated group relative to baseline (Figure 6C). We also observed increased levels of SERPINA3 protein in BAL fluid at 9.4 weeks post irradiation (Figure 6D), and increased TGF-beta1 relative to baseline (Figure 6E). Analysis of BAL cells revealed a significant increase in eosinophil percentage at 9.4 weeks post-irradiation relative to baseline, irrespective of survival status (Supplementary figure 7B).

Cell cycle arrest in a variety of lung cells, including alveolar type II pneumocytes (ATII)

In our study, irradiation induced significant fibrosis and TGF-beta expression. Ingenuity pathway analysis of the 101 DEGs also revealed CDKN1A /p21 among the top upregulated target molecules of TGF-beta (Figure 5 D and Supplementary figure 6B). We observed abundant CDKN1A/p21 staining in epithelial and alveolar type 2 (SPC) cells in non-survivors and survivors in comparison to non-irradiated controls (Figure 6, F and G), indicating cell cycle inhibition. Alpha smooth muscle actin (α -SMA) staining was also identified in irradiated animals in contrast to controls indicating a pro-fibrotic myofibroblast phenotype (Figure 6H).

Discussion:

In this study, we provide the first long term *in vivo* characterization of RILI in the NHP model in conjunction with quantitative CT analysis. Our findings are consistent with prior work (2), and in addition we assessed gene expression

patterns in lung tissue at the RNA level and confirmed the protein expression of target molecules in bronchoalveolar lavage and plasma samples to expand our understanding of the time course of disease in RILI. We observed 52.9% cumulative percent mortality at the experimental end point which is similar to a previous report of 62.5% in an established whole thorax lung irradiation (WTLI) NHP model (2). Non-survivors developed more severe pneumonitis as evidenced by increased CT opacities and higher respiratory rates, and the degree of injury on CT corresponded to histopathological pneumonitis scoring. Previous studies in humans have suggested that post radiotherapy, severe RP is symptomatic and often includes dyspnea and coughing with or without mild fever and chest discomfort (14). Our data shows that even survivors with no elevated respiratory rates (Supplementary figure 1, C and D) have CT evidence of lung injury (Figure 1F).

Histological data shows severe pneumonitis predominated by macrophage infiltration in the animals euthanized at their clinical endpoints (non-survivors, 7-24 weeks) relative to animals euthanized at a later experimental endpoint (survivors, 35 weeks). Both survivors and non-survivors, however, developed significant fibrosis relative to controls (Figure 3B). Similar histologic evidence of increased fibrosis has been reported in other NHP model studies (2). The mild pneumonitis accompanied by significant fibrosis in survivors at their experimental endpoints is likely due to persistent injury (15), and their CT scans show evidence of pneumonitis in the same time frame as non-survivors. Similarly, in human patients, the establishment of a fibrotic phase from pneumonitis using computed

tomographic scans (CT) has been reported between 9 months and 2 years after the completion of radiotherapy (14). In our study, the predominance of M2-like macrophages in both survivors and non-survivors could be an important contributor to activation of TGF- β pathways, along with other stressed cell types expressing p21 (16).

Our RNA sequencing data highlight non-survival specific, survival specific, and common gene signatures which differ via survival status. Observed upregulation of MMP3 in non-survivor-specific genes (2202) has also been reported in idiopathic pulmonary fibrosis (IPF) and in bleomycin induced lung fibrosis suggesting a role in extracellular matrix remodelling and epithelial to mesenchymal transition (EMT) in RILI. Increased serum levels of MMP3 are associated with shortened survival time in IPF patients (17). Another upregulated gene DCSTAMP (Dendritic cells-specific transmembrane protein) has been previously shown to promote macrophage infiltration and fusion through the CCL2 chemotaxis (18). We also observed increased expression of epiregulin (EREG) in non-survivors. Epiregulin belongs to epidermal growth factor (EGF) family and has an important role in inflammation, wound healing, tissue repair cell growth regulation in acute lung injury (19, 20). Our results showed downregulation of solute carrier family 6 member 4 (SLC6A4) in non-survivor's lung. This gene encodes for serotonin neurotransmitter transporter, also known as 5HTT/SERT which play important role in uptake of extracellular 5-HT. Changes in serotonin levels have been observed in various pulmonary disorders including pulmonary edema and fibrosis (21, 22). These gene expression pattern in non-survivors indicate striking ongoing

inflammatory and pro-fibrotic processes leading to increased mortality. Several DEGs specific to survivors (SPINK1, FAIM2, KCNH5, NUF2 and RACGAP1), highlighted processes related to cell cycle regulation, growth, proliferation, invasion and migration, indicating more repair-oriented response to the same radiation insult.

Among the 101 common DEGs, SERPINA3 was the top upregulated transcript both in non-survivors and survivors (Figure 5, B and C) and we observed elevated levels of SERPINA3 in BAL of the irradiated group at 9.4 weeks post irradiation relative to baseline (Figure 6D) which could be an early indicator for extracellular matrix remodeling. Hence, SERPINA3 is a potential biomarker of extracellular matrix remodeling locally (BAL) in irradiated lungs. Our findings are in line with previous studies showing SERPINA3 is a biomarker of extracellular matrix remodeling in various diseases (23), including idiopathic pulmonary fibrosis (24), SERPINA3 is also a urinary biomarker of acute renal injury in the chronic progression of renal fibrosis (25). The second top upregulated gene was ATP12A (ATPase H⁺/K⁺ Transporting Non-Gastric Alpha2 Subunit) whose upregulation is associated with cystic fibrosis (26). Other upregulated genes included LRRN4 (Leucine rich repeat neuronal 4), GJB2/Connexin 26 (Gap Junction Protein Beta 2) and CLDN10: (Claudin-10), their increased expression has been observed in acute, chronic lung injury and in EMT (27-30). Key genes among downregulated genes were TOX3 and LPA (lipoprotein A). Down regulation of the TOX3 gene is associated with increased EMT (31). While LPA is an independent risk factor for cardiovascular disease, its plasma levels were reduced in patients affected by

chronic obstructive pulmonary disease (COPD) compared to healthy individuals (32).

Our IPA analysis of 101 DEGs also indicated angiotensinogen (AGT) and TGF-beta as key upstream regulators. Dysregulation of renin angiotensin system (RAS) in RILI is well known and a number of investigations have studied various drugs that target RAS (33). Our observation of TGF-beta as a key factor in RILI, provides further evidence that TGF-beta signalling has an important role in the early and late phases of RILI. We validated the corresponding protein expression in BAL, plasma, and tissues collected at necropsy. Most of the aforementioned genes described in non-survivors (2202), survivors (238) and in the unique set of common 101 DEGs have been implicated in general lung injury, inflammation, EMT, fibrotic processes like IPF, bleomycin induced fibrosis and renal fibrosis. Their dysregulation in irradiated lungs indicates their likely role as therapeutic targets and biomarkers of early and late RILI.

Previous studies have reported that TGF-beta 1 induces the expression of CCL11 (eotaxin1) in human airway fibroblasts (34), and eotaxin 1 promotes the recruitment of eosinophils and subsequent development of fibrosis in bleomycin induced pulmonary injury (35). We observed elevated plasma protein levels of CCL11 (Figure 6B) and an Increased percentage of eosinophils in BAL cytopins (Supplementary figure 7B). Similarly, human studies have reported increased eosinophils in blood and BAL post-radiotherapy (36, 37). Radiation may produce eosinophilic pneumonitis in the early proliferative and fibrogenesis phase of RILI, and cytokines like TGF-beta (38) and TNF alfa (39) are chemotactic for

eosinophils. Cytokines, CCL2 and CCL11 were most relevant to transcriptomic analysis and were most significantly elevated among other measured 29 cytokines (Supplementary table 4).

Stress signals induced by TGF-beta can cause cell cycle arrest by stabilizing p21 in a Smad dependent (40, 41) or Smad independent manner (42, 43). Our observation of positive immunostaining for CDKN1A/p21 in multiple cell types including epithelial cells (Figure 6, F-H), is consistent with the finding of a heterogeneous cell population of senescent cells in radiation-injured lungs in mice (44). Cell cycle arrest due to sustained injury may contribute to the progression of fibrosis via releasing pro-fibrotic cytokines from senescent cells which is a feature of the cellular senescence in RILI (16, 45, 46). TGF-beta has an important role in the transformation of various cell types (resident fibroblast, circulating fibrocytes, EMT, Endothelial EMT) to myofibroblasts, which are associated with excessive extracellular matrix deposition and fibrosis. We believe this is the case in our study, as supported by positive α -SMA staining and fibrosis data.

Significant differences exist between this experimental model of RILI in rhesus monkey and RILI in humans. In, particular, we have used a single dose (10 Gy) of irradiation to the whole thorax whereas in humans, only a targeted volume of lung is exposed and doses are usually fractionated. However, the 10 Gy dose used in our study is higher than the single large estimated dose of 9.3Gy in a human retrospective study which caused RP in ~50% of patients (1, 47). The overall objective of the current study was to understand individual differences in the course of RILI and to find new molecular targets in animals exposed to a single

high thoracic dose of ionization radiation which mimics the scenario of accidental non-uniform or partial body exposure to irradiation (7). However, this information can be applied to a clinical situation like single-fraction stereotactic body radiation therapy (SF-SBRT), where a single high dose is prescribed to target a tumor. Although SF-SBRT is highly focussed, there is some likelihood of developing lung toxicity in the surrounding tissue. However, being a relatively young field, literature regarding SF-SBRT safety and efficacy is rare. In a situation like the COVID-19 pandemic, a preference for SF-SBRT relative to multiple fractions is growing especially in the patients with inoperable lung tumors (48, 49). In a clinical trial, incidence of RP to a single dose of 10 Gy and a fractionated total dose of 12 Gy (total body irradiation) was comparable (50, 51). Clinical studies of RILI are hampered by comorbidities like COVID-19 and concurrent chemotherapy. Studies in NHP WTLI irradiation models offer an opportunity to explore possible translatable biomarkers and molecular targets in the absence of such confounders.

This study did not address the effects of sex or age on RILI. Female C57BL/6 mice are marginally more sensitive to RILI than males (52). Human and NHP studies have not shown an influence of gender in radio-responsiveness, but data are limited (53, 54). Human studies show that older patients are at greater risk to develop RILI post-irradiation (53). In our study, the median age of non-survivors was 3.76 years at irradiation whereas the median age of survivors was 5.7 years at irradiation; both of these are "adolescent" ages in rhesus monkeys, which have a typical lifespan of 30-35 years. The median bodyweight of non-survivors (5.95 Kg) and survivors (7.17Kg) at irradiation were not significantly different. The age

and body weight distribution of studied animals is shown in supplementary table 3. Future studies with more advanced aged animals will likely show an effect of age.

In addition to the lung, the radiation field in this study included the distal trachea, esophagus, thymus, heart, and other adjacent tissues, including the superior portion of the liver and stomach. However, histologic examination of trachea, esophagus, thymus, and liver in-field and out-of-field did not show any evidence of cellular injury, inflammation or fibrosis aside from thymic degeneration in animals dying early (data not shown). The co-irradiation of lung and heart can cause cardiac and pulmonary injury via different mechanisms. In general, the heart is less sensitive to doses <15Gy as compare to lungs early after irradiation (55). Hence, the 10Gy dose likely did not cause major heart toxicity in short term experimentation. However, short and long term effects of radiation on heart cannot be disregarded and require further investigation. Post irradiation changes in heart pathology and heart function are part of an ongoing separate study.

A possible confounder in this study is the inclusion of SOD mimetic drug treated animals. We did not see any effect of SOD mimetic treatment on study outcomes, however, its effect at molecular levels cannot be overlooked. Therefore, for RNA expression studies we used only non-drug-treated irradiated and unirradiated animals. We believe these data provide useful and new insights to the understanding of RILI.

Conclusion: Our data enhances understanding of RILI in non-survivors and survivors in a NHP model and supports the role of TGF-beta activation and

macrophage polarization in RILI. RNA sequencing highlights the role of SERPINA3, ATP12A, GJB2, CLDN10, TOX3, LPA as possible biomarkers and potential therapeutic targets of RILI. Their differential expression in non-survivors and survivors correlated with the severity of the disease. The observed upregulation of TGF-beta 1 extends prior reports by showing the increased expression of its associated target transcripts (SERPINA3, CCL11, CCL2, p21) at the mRNA and protein level indicating not only the role of active TGF-beta signaling but also the role of these molecules in the early proliferative phase of RP (non-survivors) and late phase of fibrogenesis and remodeling (survivors).

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Figures and Legends

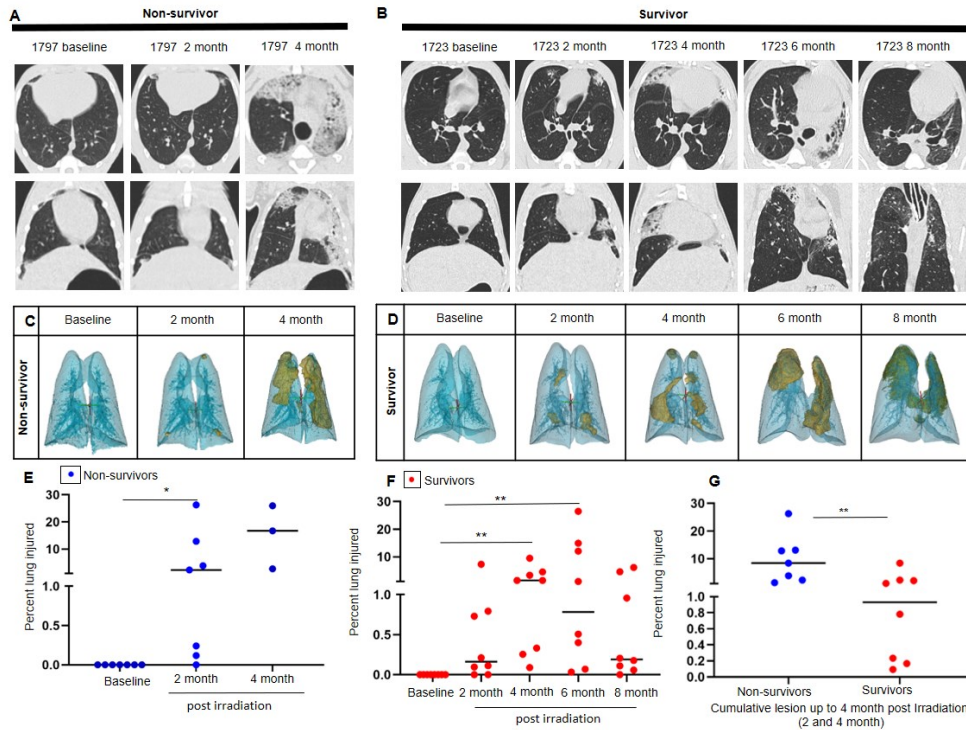


Figure 1. Post-irradiation lung injury assessment using computed tomography (CT).

Computed tomographic images (axial and coronal view) of a representative non-survivor (**A**) and survivor (**B**) at baseline and at the interval of 2-months until clinical end point (4 months) or experimental end point (8 months). (**C**) and (**D**) Three-dimensional reconstruction of lung injury (gold) of the same representative non-survivor and survivor using Mimics software. (**E**) Percent injured lung volume of non-survivors at 2 months (n=7) and 4 months (n=3) relative to baseline. Results are expressed as the median and statistical significance was determined by Wilcoxon Signed Rank test. (**F**) Percent injured lung volume of survivors at 2, 4, 6, and 8 months (n=8) relative to baseline. Results are expressed as the median and statistical significance was determined by Friedman test with Dunn's multiple comparison post test. (**G**) Cumulative injured lung volume in survivors and non-survivors at 4 months. Results are expressed as the median. Statistical significance was determined by Mann Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for all data.

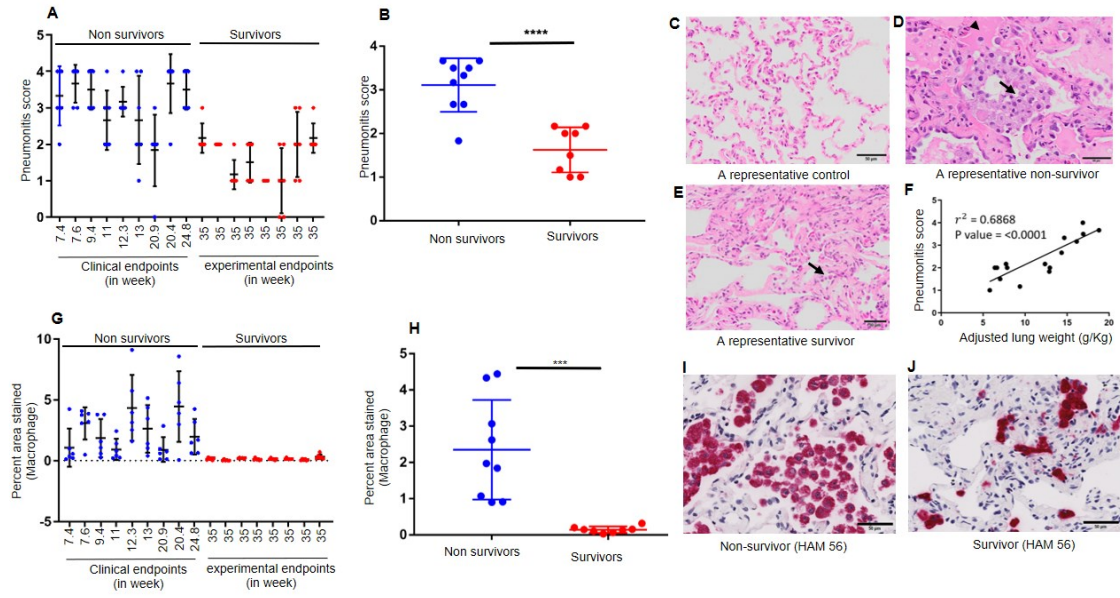


Figure 2. More severe pneumonitis dominated by macrophages in non survivors relative to survivors at necropsy. (A) Pneumonitis scores of individual non-survivors (blue dots) and survivors (red dots). Dots represent 6 different lung regions in each animal. (B) Statistically significant differences in pneumonitis scores between non-survivors (n=9) and survivors (n=8). (C) An H&E stained photomicrograph of a control non-irradiated lung, (D) a representative non-survivor, and (E) a representative survivor demonstrating infiltration of immune cells (arrow) and edema fluid (arrow head) within the alveolar lumina. (F) A positive correlation between adjusted lung weights and pneumonitis scores of all irradiated animals. (G) Macrophage staining (HAM56, red stain) of individual non-survivors (blue dots) and survivors (red dots). The dots for each animal represent 6 different lung regions. (H) Macrophage staining (percent area stained) between non-survivors (n=9) and survivors (n=8). Results are expressed as the mean $\pm$ SD and statistical significance was determined by unpaired t test. P value criteria * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for all data. (I) and (J) Representative images of a 13-week post irradiation non-survivor and a survivor, respectively stained with the macrophage marker, HAM56 on the lung obtained at necropsy. Scale bars are 50 μ m.

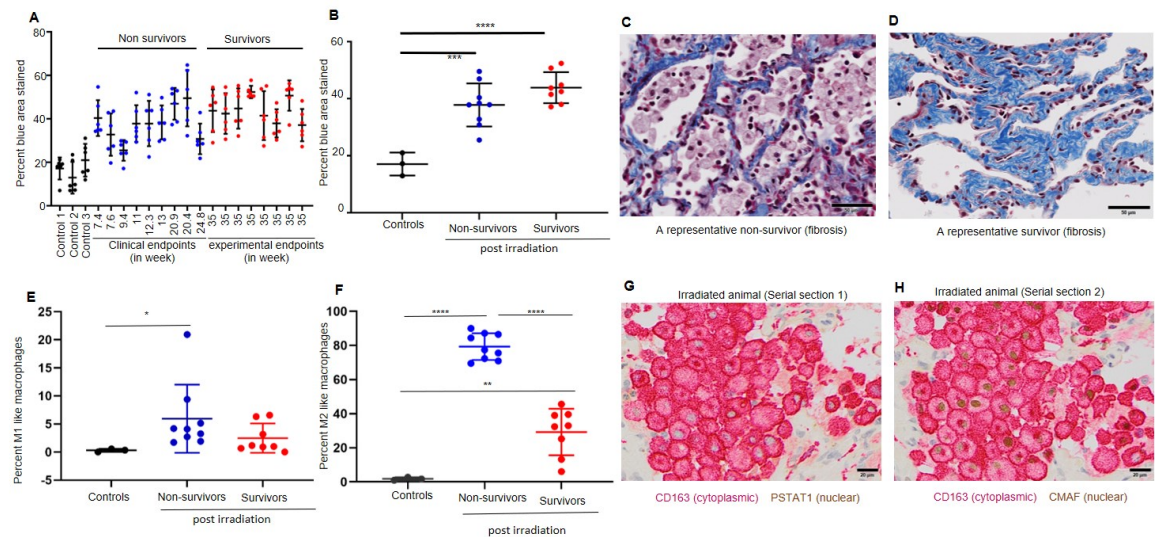


Figure 3. Quantitative assessment of fibrosis and pro-fibrotic macrophage (M2) phenotype in irradiated lungs. (A) Quantification of fibrosis as a percent area stained utilizing Masson's trichrome staining in individual controls (black bars), non-survivors (blue bars) and survivors (red bars). Six dots for each animal represent 6 different lung regions. (B) Average fibrotic area expressed as the mean $\pm$ SD between controls (n=3), non-survivors (n=9) and survivors (n=9). Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons post test. (C) and (D) Representative images of a post irradiation non-survivor and a survivor stained with Masson's trichrome stain. Scale bars are 50 μ m. (E) and (F) Quantification of M1 like (CD163+PSTAT1) and M2 like (CD163+CMAF) macrophages performed on serial lung sections of non-irradiated controls (n=3) and irradiated monkeys (non-survivors, n=9 and survivors, n=8) obtained at necropsy. Results for percent M1 macrophages are expressed as median $\pm$ IQR and statistical significance was determined by Mann Whitney test. For M2, results are expressed as the mean $\pm$ SD and statistical significance was determined by unpaired t test. (G) Double immunostaining for macrophage marker, CD163 (red) and M1 specific transcription factor, PSTAT1 (brown) in thirteen-week post-irradiated lung section. (H) Double immunostaining for macrophage marker, CD163 (red) and M2 specific transcription factor, CMAF (brown) in a representative non-survivor post-irradiated serial section of same lung. P value criteria * P < 0.05; ** P < 0.01; *** P < 0.001 for all data. Scale bars are 50 μ m.

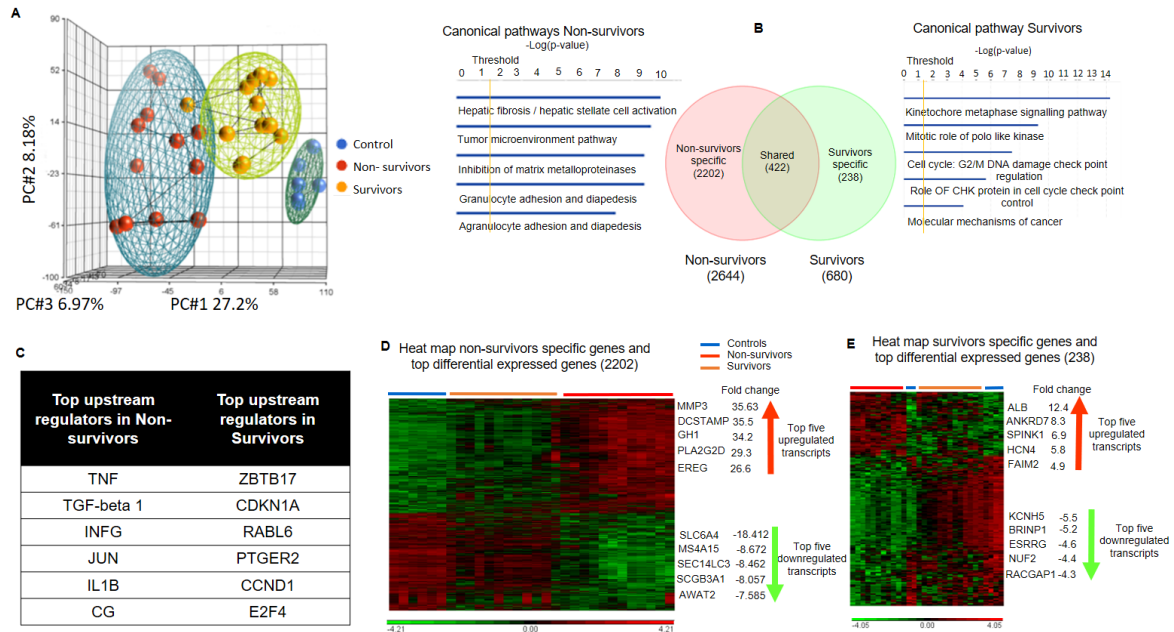


Figure 4. Survivors and non-survivor's specific lung transcriptomic analysis (RNA sequencing). (A) Unsupervised principal component analysis of primate lung RNA sequencing data shows clear clustering into controls, non-survivors and survivors (blue = controls, red = non-survivors, yellow = survivors). (B) Venn diagram of differentially expressed transcripts by group comparisons. Values indicate number of transcripts with significant differential expression (P value < 0.05) across that contrast; non-survivors versus controls (2644), survivors versus controls (680), non-survivor specific (2202), survivor specific (238) and shared transcripts (422), along with canonical pathways elicited by non-survivor and survivor specific differentially expressed genes (DEGs) using ingenuity pathway analysis. (C) Upstream regulators (using Ingenuity pathway analysis) of non-survivor and survivor specific DEGs illustrated by the Venn diagram. (D) and (E) shows hierarchical clustering combined with heat map of non-survivor and survivor specific DEGs shows clear differentiation between groups, listed with their top five upregulated and down regulated transcripts. Significant differentially expressed genes (DEGs) were conservatively defined as $\log_2$ fold change ratios $\geq \pm 1$ and $p < 0.05$ after adjustment for false discovery.

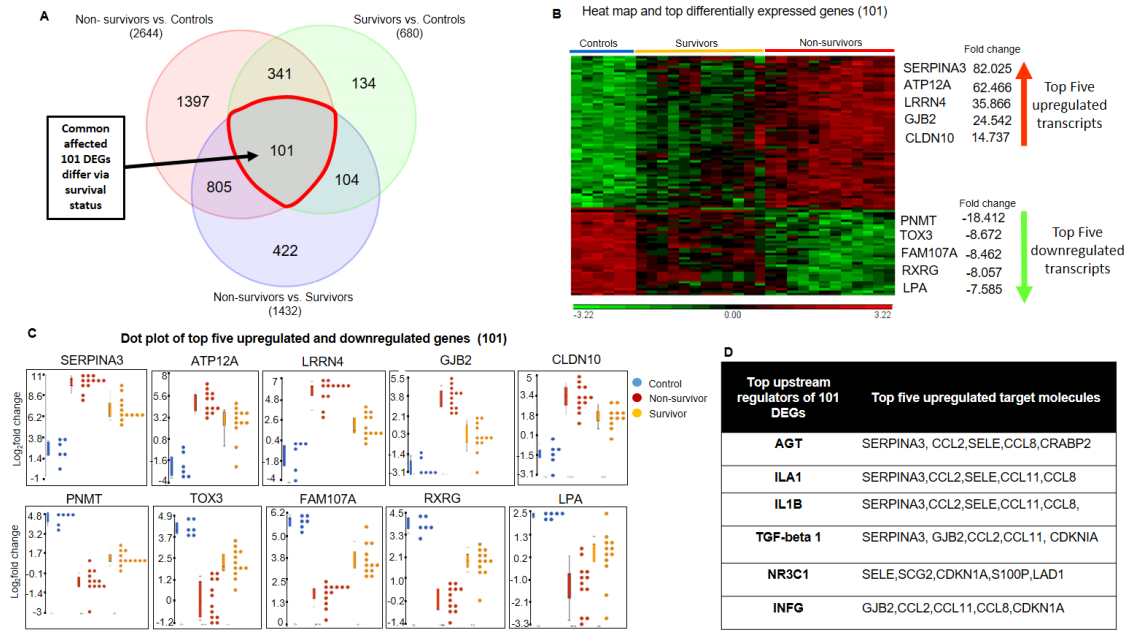


Figure 5. Transcriptomic analysis of 101 DEGs, a subset of shared genes. (A) Venn diagram of differentially expressed transcripts by group comparisons highlights 101 DEGs, a unique subset of shared genes. **(B)** Hierarchical clustering heat map shows clear differentiation between control, non-survivors and survivors, listed with their top five upregulated and down regulated transcripts. **(C)** Dot plots of top five upregulated and down regulated genes, showing differing patterns by outcome. **(D)** Upstream regulators (using Ingenuity pathway analysis) and top target transcripts. Significant differentially expressed genes (DEGs) were conservatively defined as $\log_2$ fold change ratios $\geq \pm 1$ and $p < 0.05$ after adjustment for false discovery.

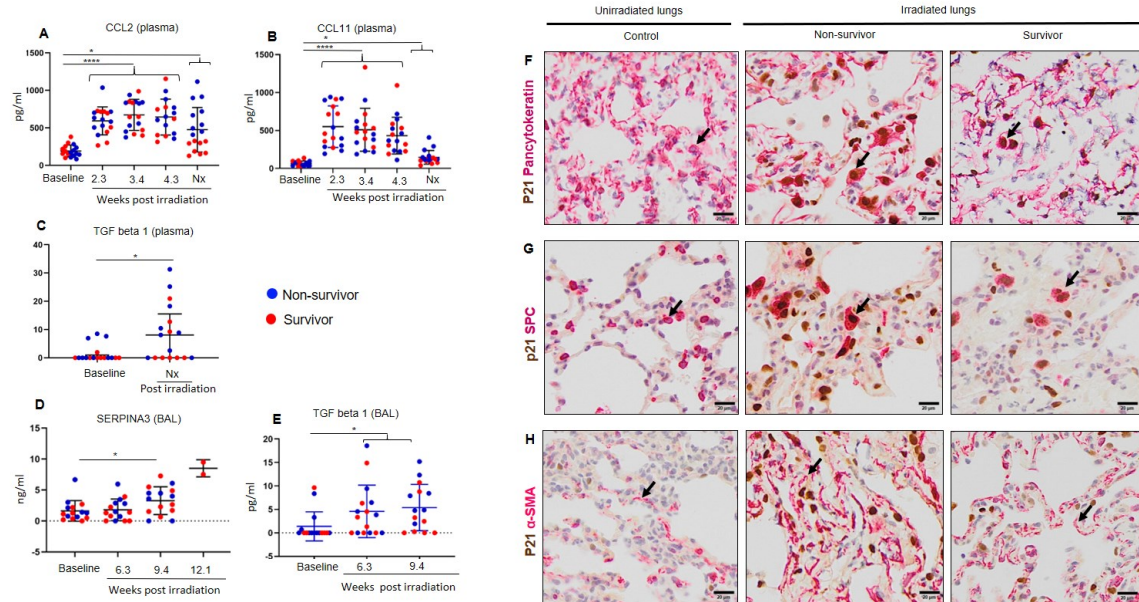


Figure 6. Protein expression of TGF-beta1 and its associated transcripts. (A) and (B) Mean plasma values of CCL2 and CCL11 measured at 2.3 weeks (n=17), 3.4weeks (n=17), 4.3 week (n=16) and at necropsy (Nx, n=16) post irradiation relative to baseline (n=17). Results are expressed as the mean $\pm$ SD and statistical significance was determined mixed effect analysis and RM one way-ANOVA and Tukey's multiple comparison post-test. **(C)** TGF-beta 1 levels measured in plasma at necropsy (n=17) relative to baseline (n=17). Results are expressed as the median $\pm$ IQR and Wilcoxon matched-pairs signed rank test was used for statistical significance **(D)** Mean SERPINA3 levels detected via ELISA in bronchoalveolar lavage (BAL) fluid (n=15) collected at 3.6 weeks 9.4 week and 12.1 weeks (n=2) post irradiation relative to baseline. Results are expressed as the mean $\pm$ SD and statistical significance was determined by t-test. **(E)** TGF-beta 1 levels assessed on BAL fluid collected at 3.6 weeks (n=16), 9.4 week (n=15) post irradiation relative to baseline (n=16). Results are expressed as the median $\pm$ IQR and Wilcoxon matched-pairs signed rank test was used for statistical significance **(F)** Upper panel, double staining (arrow) for epithelial marker, cytokeratin (cytoplasmic) and cell cycle arrest marker, p21 (nuclear), **(G)** middle panel, double staining (arrow) for alveolar type II pneumocyte marker SPC (cytoplasmic) and p21 (nuclear), **(H)** Lower panel, smooth muscle actin, α -SMA (cytoplasmic, arrow) and p21 (nuclear) using representative lung sections of a control, non-survivor and survivor. Scale bars are 50 μ m.

Supplementary material

Supplementary methods

Irradiation

All the animals to be irradiated were sedated with ketamine (10-15 mg/kg), anesthetized with isoflurane in 100% O₂, and exposed to 10 Gy of 6 MV X-rays from a clinical linear accelerator (Varian Medical Systems, Palo Alto, CA), using AP-PA parallel-opposed fields (field size 13.0 cm (4,0 cm x 9 cm) long x 12 cm wide), with the isocenter at the thorax midplane and a nominal dose rate of 4Gy/min. Each anesthetized animal was placed in the supine position for irradiation, lightly restrained with arms up over the head, with the central axis of the anterior beam at the xiphoid. The radiation field included all lung lobes, the distal trachea, esophagus, heart, thymus, and other adjacent tissues, including the superior portion of the liver and stomach. An electronic portal imager (digital x-ray image detector) was used to confirm the anatomical position and the AP radiation field placement for each animal, immediately prior to irradiation. The animal position was adjusted if needed, and then re-imaged to confirm the final position. The linear accelerator was then rotated to the PA position and the same x-ray imaging was performed to confirm the position. The PA (posterior) field was first irradiated at 6 MV and then the linear accelerator was rotated back to the anterior position and the AP field was irradiated. Both the PA and then AP fields were delivered, 5Gy each, within about 1 minute of each other, for a total of 10Gy to the midplane. Monitor units, the numerical value set on the linear accelerator needed to deliver the correct dose of 5 Gy, were calculated for each field, based on the

average AP mid-plane depth along the central axis (xyphoid). The two-dimensional dose calculation algorithm used considered the thorax as homogeneous, unit-density tissue (water equivalent) and did not account for tissue heterogeneity, such as lung vs muscle or bone. Three groups of size for MU calculations were determined, based on the grouping of average anatomical AP diameters within ± 4 mm for each group (less than 2% variation in dose). A beam modulator called a “wedge” was used in the AP beam to provide dose homogeneity for the AP beam in the coronal plane at midplane depth, compensating for the slope of the chest from xiphoid (thicker) to the sternum-clavicle junction (thinner). This wedge angle ranged from 10 to 30 degrees and was determined for each animal based on AP diameters measured at the sternum-clavicle junction, the mid-sternum and the xiphoid. MU calculations for the anterior beams incorporated the wedge correction factor. The clinical linear accelerator used was maintained with routine quality control procedures for its operating characteristics, including dose calibration. All radiation dose delivery parameters were set and captured using an electronic database, for validation of the beam geometry and monitor units delivered during irradiations.

Animals

Seventeen male rhesus macaques of Chinese origin, aged 3.76-11.25 years (median age 4.76 years) ranging from 4.2 to 10.3 kg in weight (median weight 6.0 kg) were obtained from Primate Products (Immokalee, FL), and given a single 10 Gy whole-thorax dose of radiation at 4 Gy/min using a 6 MV linear accelerator. Originally, this study was conducted to evaluate the mitigating effect of a

superoxide dismutase (SOD) mimetic drug on chest irradiated monkeys compared to control/saline treated irradiated monkey. Eight of the 17 irradiated animals were given a SOD mimetic at 0.125mg/kg, twice daily by subcutaneous injection throughout the study and 9 were given saline for the duration of the study. As there were no statistically significant differences in survival or histologic or fibrosis related to SOD mimetic drug treatment (Supplementary figure 2), these data were combined. Only the saline-treated irradiated animals were used for transcriptomic analysis (RNA seq), in comparison to 3 sham-irradiated, saline-treated controls (median age 2.75 years and median weight 3.6Kg) obtained from a prior study (1). Further treatment based and endpoint details of the studied animals are provided in the supplementary table 3.

CT procedure, image acquisition and broncho-alveolar lavage (BAL) collection

For these procedures, the animals were sedated with ketamine and medetomidine, intubated, and anesthesia was induced and maintained with isoflurane. The lungs were fully inflated by manual pressure to the rebreathing bag for approximately 10-20 seconds during the scan to avoid motion artifact. Images were acquired on a Toshiba Aquilion 32 slice CT scanner with parameter settings: $V_{\max}=120$ kV, $I_{\max}=100$ mA, field of view (FOV) = 320 mm, matrix size = 512 x 512, and slice thickness = 0.5 mm, giving voxel dimensions of 0.625 x 0.625 x 0.5 mm³ using a pediatric protocol. Images were obtained in digital imaging and communications (DICOM) format. Using Mimics software (Materialise mimics innovation suite, Leuven, Belgium, version 21.0.0.406), automatic thresholding, region growing,

morphology, and Boolean operations and manual editing were utilized to create masks representing each volume. Normal healthy lung measured in pre irradiation scans was found to range in intensity from -1024 Hounsfield units (HU) to -750 HU.

For BAL collection, 40-60 ml of saline was infused through the endotracheal tube and BAL fluid was slowly aspirated back. The volume and color of aspirated BAL were recorded. Cytospins were made and the remainder of BAL fluid was snap-frozen. Samples were kept in a container on ice at 4°C during processing and transport. BAL samples were collected at 6.3 weeks, 9.4 weeks post irradiation. To avoid complication of BAL procedure in the animals reaching clinical endpoint, only limited samples were obtained at 12.1 weeks post-irradiation.

Histopathological assessment (Pneumonitis scoring)

A histopathological assessment was done on hematoxylin and eosin (H&E) stained lungs sections by a board-certified veterinary pathologist (NK). Six regions per lung from each animal were scored for the severity of pneumonitis based on cellular infiltration by macrophages, neutrophils, and other inflammatory cell types (Supplementary figure 4).

Histochemical staining and immunohistochemistry

Pulmonary lesions were photographed at necropsy, lungs were weighed, and six regions of lung (left cranial/middle/caudal, right cranial/middle/caudal) were snap-frozen in liquid nitrogen, with parallel samples fixed in 4% paraformaldehyde and changed to 70% ethanol after 24 hours, trimmed and embedded in paraffin.

Paraffin embedded tissue samples were sectioned at 4 microns stained using an automated IHC stainer, the Bond RX (Leica Biosystems, Buffalo Grove, IL). The instrument dewaxed (Leica Biosystems, Dewax, cat# AR9222) the sections, rinsed using Bond Wash Solution (cat# AR9590) and applied antigen retrieval solutions as programmed. The sections were blocked with Leica Biosystems, Powervision IHC/ISH Super Blocking (cat# PV6122) for 20 minutes. The primary antibodies, all of which were diluted in Leica Biosystems, Primary Antibody Diluent, (cat# AR9352) were applied for 15 to 30 minutes. Each antibody was optimized. A list of antibodies and blocking agent used are shown in supplementary table 1. The alkaline phosphatase (AP)-linked compact polymer (Leica's Polymer Refine Red Detection Kit, catalog # DS9390) and Fast Red chromogen or Leica's Polymer Refine Detection Kit (catalog # DS9800) that uses a polymeric HRP linker and DAB chromogen detection systems, were used to visualize the primary antibody. Hematoxylin was used as a counterstain. Slides were dehydrated and cleared using 1 change - 95% ethanol, 4 changes -100% ethanols, and 4 changes of p-xylene. Permount was used for coverslipping.

IHC stained slides were scanned (at 20x objective magnification) on an Olympus VS120 scanner and quantification of digitalized slide images was done using image analysis software Visiopharm (VIS 6.5.0.2303).

Serial lung sections of lungs were stained with Masson's trichrome (MST) to assess fibrosis, imaged and analyzed using Visiopharm software. The image analysis application protocol package (APP) for fibrosis quantification was created using Visiopharm software. The APP was optimized using pre-processing filters to

differentiate stained tissue, air space, and other tissue subtypes. For MST quantification, large airways (bronchi) were manually selected out and then the batch process was run using the APP. The percentage collagen area in the section was determined as percent collagen = (Blue area/total area) x100.

Immunohistochemically, Lung sections were stained using the human alveolar macrophage marker HAM56 (Dako, Carpinteria, CA, USA). The histologic percentage of the lung parenchyma consisting of macrophages staining for the HAM56 macrophage marker was measured on 6 sections from each animal. Entire sections were measured using computer-assisted color image analysis (Image Pro Plus, v. 5.1, Media Cybernetics, Silver Spring, MD).

M1 and M2 quantification was done using cell counts by a trained observer blinded to treatment groups, in serial sections. Sections were co-stained with nuclear stains pSTAT1 to identify M1 macrophages, and CMAF to identify M2 macrophages. Macrophages (CD163-positive in cytoplasm) were counted in randomly distributed counting frames including 20-25% area of the entire section. At least 200 macrophages were counted, including only cells with the nucleus in the plane of section. The percentages of M1 and M2- like cells were quantified as percent M1-like cells= (Total pSTAT 1 positive CD163 cells/Total CD163 cells) X 100, or percent M2-like cells = (Total CMAF positive CD163 cells/Total CD 163 cells) X 100.

Other IHC markers included CDKN1A/p21, pan-cytokeratin, surfactant protein C (SPC) and α -SMA in order to identify senescent cells, epithelial cells, type II pneumocytes, and smooth muscle cells, respectively (Supplementary table 1).

Single randomly selected sections of lung per animal in 4 survivors and 4 non-survivors were dual stained for cytokeratin with p21, or SPC with p21 to determine whether these cell types were senescent in radiation induced damage.

Cytokine analysis

Plasma and BAL supernatant samples from irradiated monkeys were assessed for cytokine concentrations at baseline and throughout the study. Plasma was analyzed at baseline, 2.3 weeks, 3.4 weeks, 4.3 weeks post-irradiation and at necropsy. BAL was analyzed at baseline, 6.3 weeks, 9.4 weeks and 12.1 weeks post-irradiation. The samples were stored at -80C, thawed at the time of analysis and analyzed for 29 cytokines. The cytokines were measured and interpreted utilizing Luminex x-MAP (x = analyte, MAP = Multi-Analyte Profiling) technology which is a bead based multiplex immunoassay system in microplate format that allows simultaneous detection of multiple analytes in a single sample. For detection, a high throughput and multiplexed screening platform, Bio-phase 200 system (BioRad, CA, USA) was used. TGF-beta 1,2 and 3 were separately analyzed in BAL and plasma samples. The electrochemiluminescence of immunoassays were measured on multiplexing instrument, Meso Quickplex SQ120 (Meso Scale Diagnostics LLC, USA) according to the manufacturer's instructions. SERPINA3 was analyzed in plasma and BAL samples at baseline, 6.3 and 9.4 weeks post-irradiation and results were read on a microplate Reader (Synergy H1 Microplate reader, BioTek, Vermont).

RNA isolation

Total RNA was extracted from 40-80 mg frozen lung tissues from three different affected regions of the lung from eight irradiated animals (vehicle controls) and from two different randomly selected regions from three unirradiated control animals. Lung regions in the irradiated animals were chosen based on high fibrosis and pneumonitis scoring on histopathology from the same respective lung lobes. Tissue was placed into a 1.4 mm ceramic bead tube with 1 ml QIAzol lysis reagent and homogenized using a bead mill (Bead Ruptor 24, Omni International, Kennesaw, GA). The tissue sample tube was processed on the Bead Ruptor for 1 cycle at a speed of 5.5 m/s for 20 sec; and repeated up to six times until the sample was completely homogenized. All homogenized lysates were extracted for total RNA using the RNeasy Microarray Tissue Mini kit (Qiagen, Germantown, MD). Extracted RNA was DNase-treated and purified using the RNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA), then assessed for RNA quality using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA)

cDNA Library Preparation and Sequencing Methods

Total RNA from 30 samples was used to prepare cDNA libraries using the Illumina® TruSeq Stranded Total RNA with the Ribo-Zero Gold Preparation kit (Illumina Inc.) and the SciClone NGS Work Station (Perkin Elmer). RIN values for the RNA samples ranged from 7.9 to 9.5. Briefly, 750 ng of total RNA was rRNA depleted followed by enzymatic fragmentation, reverse-transcription and double-stranded cDNA purification using AMPure XP magnetic beads. The cDNA was end-repaired, 3' adenylated, with Illumina sequencing adaptors ligated onto the

fragment ends, and the stranded libraries were pre-amplified with PCR. The library size distribution was validated and quality inspected on a Bioanalyzer DNA 1000 chip (Agilent Technologies, USA). The quantity of each cDNA library was measured using Qubit 3.0 (Thermo Fisher, USA). Two library pools were formed, each containing 15 libraries. Library pools were sequenced to a target read depth of 25M reads per library using single-end 76 cycle sequencing with the High Output 75-cycle kit (Illumina Inc., San Diego, CA) on the Illumina NextSeq 500.

RNA Data Analysis

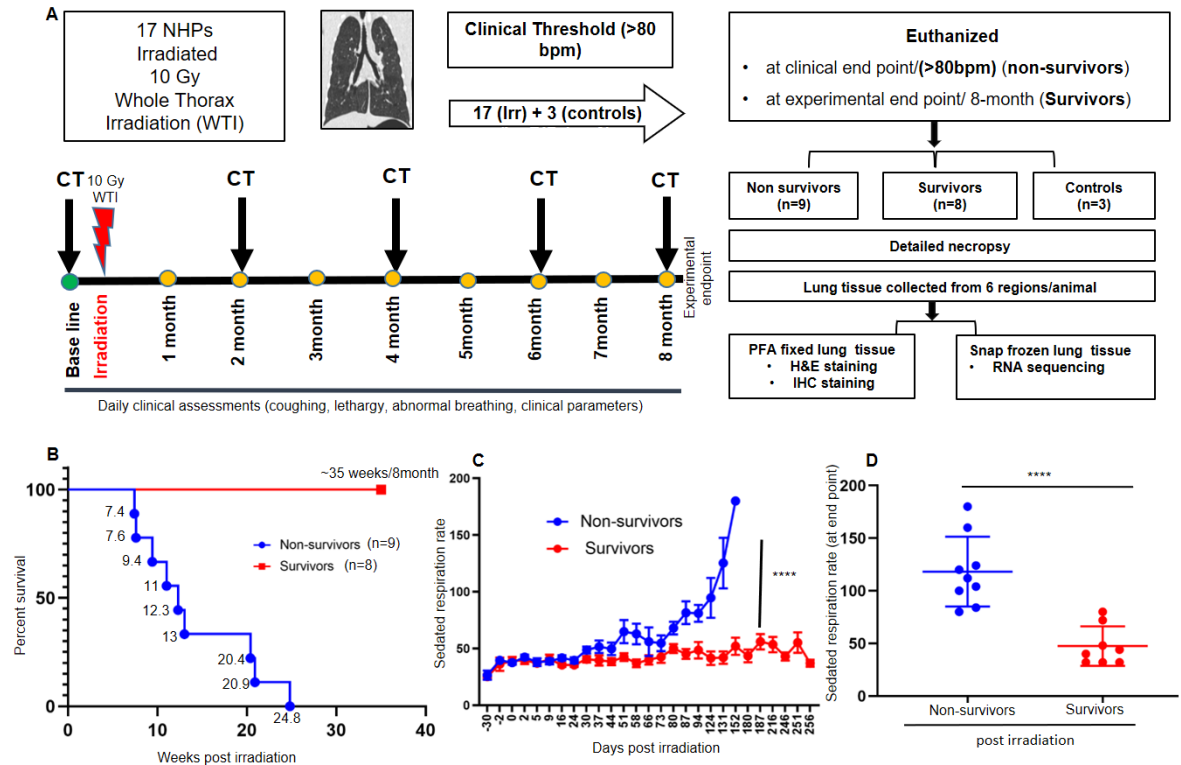
Raw read quality was assessed by FASTQC analysis (Babraham Bioinformatics). Uniquely mapped reads ranged from 21M-36M reads per sample. Reads with >Q20 quality score were aligned to the Ensembl *Macaca mulatta* genome build Mmul_1 using the STAR sequence aligner(2) and gene counts were extracted using feature Counts software(3). Differentially expressed genes (DEGs) were analyzed using limma-voom(4, 5). Significant DEGs were conservatively defined as log₂fold change ratios $\geq \pm 1$ and $p < 0.05$ after adjustment for false discovery (Benjamini-Hochberg). We used a visualization tool, heat map combined with hierarchical clustering to analyse the differential expression of the genes (cut off of log₂ fold change -1 and +1 and adjusted $p > 0.05\%$) in irradiated non-survivors and survivors relative to unirradiated control lungs. Hierarchical clustering analysis, heat maps, Venn diagrams, and descriptive plots (dot plots) were generated using Partek® Genomics Suite™ Software (version 7.0, Partek Inc., St Louis, Missouri, USA). Genes identities were preliminarily mapped in Ingenuity Pathway Analysis (IPA, QIAGEN Inc., version 52912811) using Ensembl

identification numbers for the rhesus macaque. Ensembl IDs which did not correspond to an identified gene were recorded and excluded from pathway analysis. Differentially expressed genes were analyzed by causal network analysis and the upstream regulator tool of ingenuity pathway analysis software (IPA).

References:

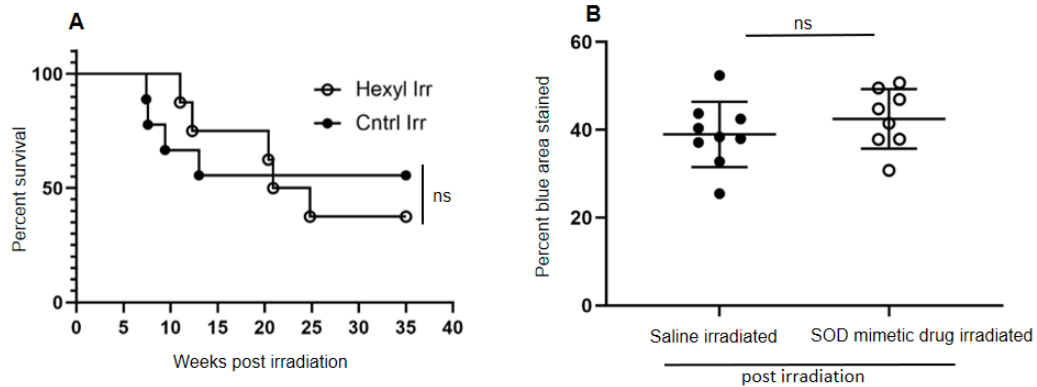
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Supplementary Figures

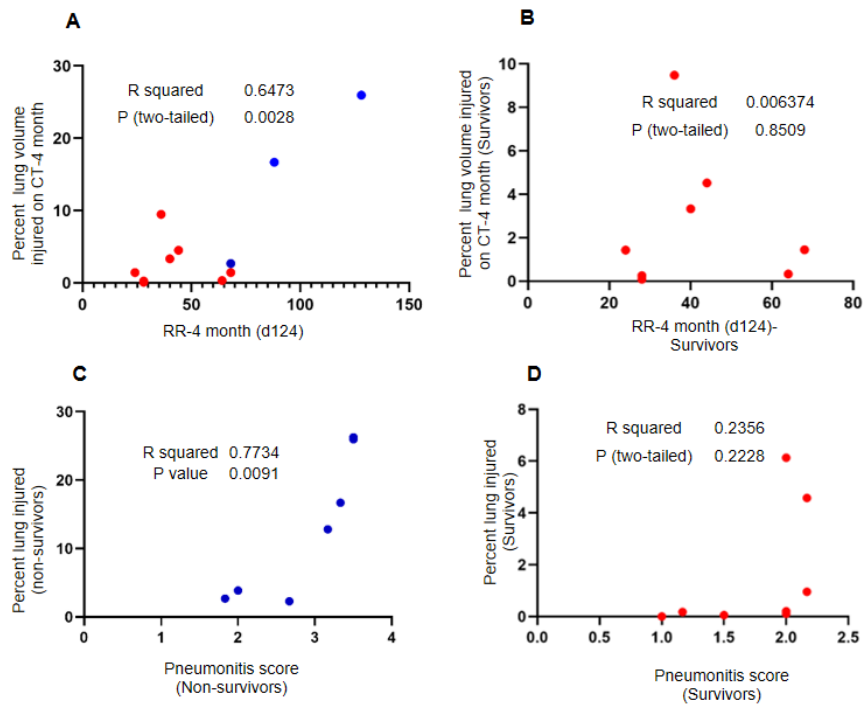


Supplementary figure 1. Experimental design, survival curve and respiratory rates.

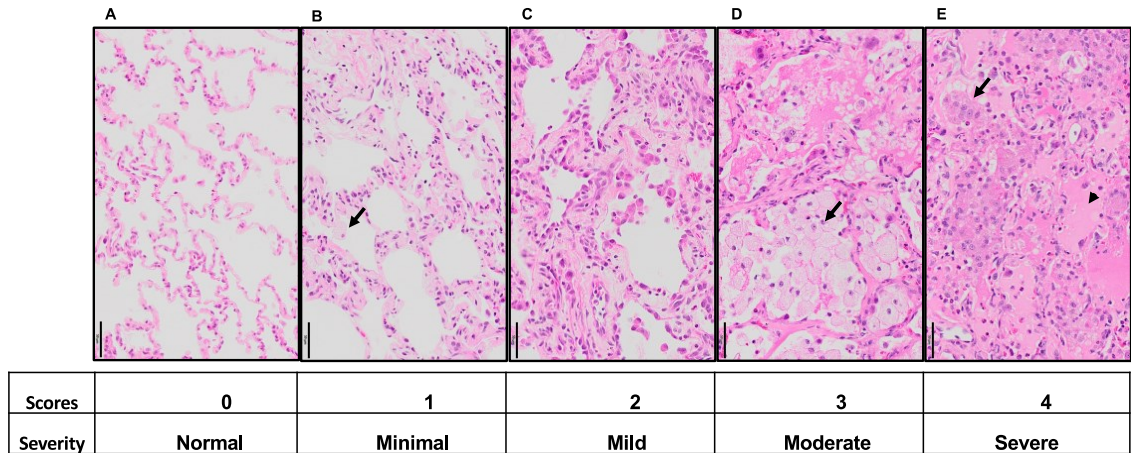
(A) Study design. **(B)** Kaplan-Meier Survival curve of 17 irradiated monkeys. The blue line represents non-survivors (n=9) and blue dots correspond to their clinical end points in weeks. The red line represents survivors (n=8) which were euthanized at the 8-month (experimental end point). **(C)** Sedated respiratory rates over time show a significant difference (Chi square test of trend) between survivors and non-survivors. **(D)** Sedated respiratory rates of non-survivors and survivors at their terminal end points. Results are expressed as the mean $\pm$ SD and statistical significance was determined by unpaired t test. P value criteria * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



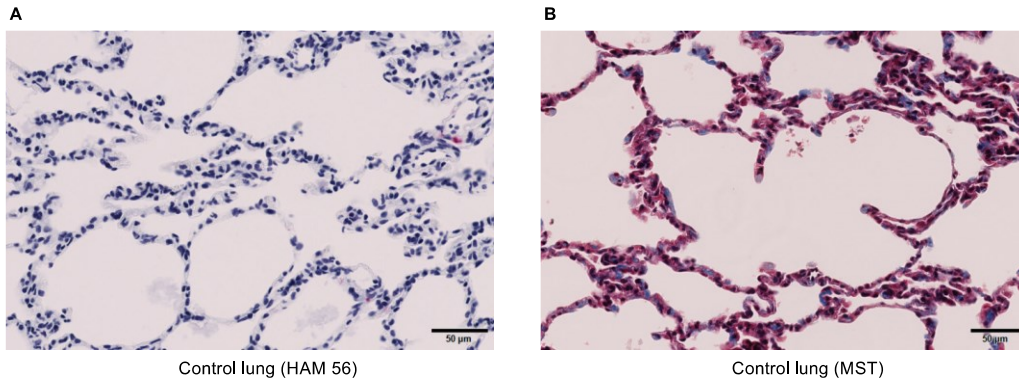
Supplementary figure 2. No effect of superoxide dismutase (SOD) mimetic drug on survival and lung fibrosis. (A) Kaplan-Meier Survival curve of 8 SOD mimetic drug treated and 9 saline treated irradiated monkeys. statistical significance was determined by Log-rank (Mantel-Cox) test. (B) Average percent fibrotic area (Masson's trichrome staining) expressed as the mean $\pm$ SD between 8 SOD mimetic drug treated (n=8) and 9 saline treated (n=9) irradiated monkeys. Statistical significance was determined by unpaired t test. P value criteria $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ for all data.



Supplementary figure 3. Relationships between histologic pneumonitis score, respiratory rate, and % lung injured on CT in irradiated subjects. (A) Pearson correlation analysis of CT- lesions and respiration rates at 4-month in all irradiated animals (n=11). (B) Pearson correlation analysis of CT lesions and respiration rates at 4-month in survivors (n=8). (C) Pearson correlation analysis of pneumonitis scores (at necropsy) and percent injured volume at last CT scans of non-survivors (n=7). (D) Pearson correlation analysis of histologic pneumonitis score at necropsy and percent injured volume at last CT scans of survivors (n=7). P value criteria * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for all data.

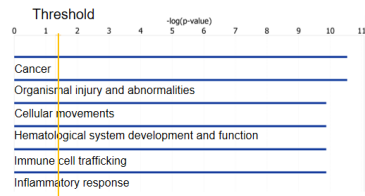


Supplementary figure 4. Histologic scoring of pneumonitis on hematoxylin and eosin (H&E) stained lung sections assessed by a certified pathologist. (A) A healthy normal unirradiated lung (score 0). **(B)** A minimally affected lung (score 1) demonstrates few intra-alveolar macrophages (arrow) and increased thickness of alveolar septa. **(C)** Demonstrates clearly increased infiltration of inflammatory cells (Score 2). **(D&E)** Depicts moderately (score 3) to severely (score 4) affected lung with increased intra- alveolar inflammatory cells (macrophages, arrow) and proteinaceous edema fluid (arrowhead). Scale bars are 50 μ m.

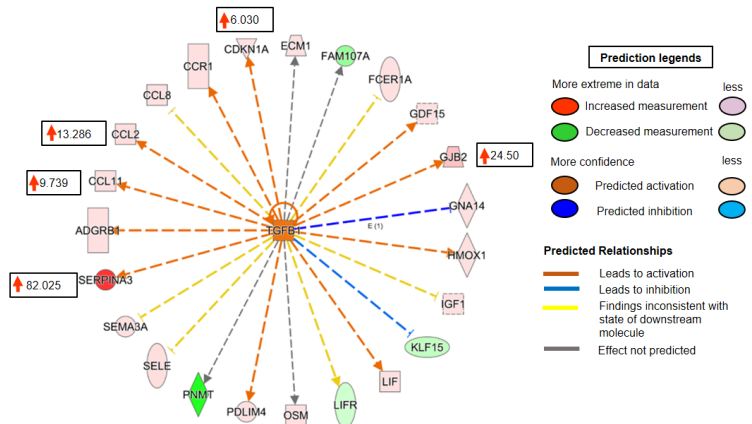


Supplementary figure 5. Positive controls for macrophage staining (HAM 56) and fibrosis (MST). (A) A healthy normal unirradiated lung stained for HAM 56. (B) A healthy normal unirradiated lung stained with Massons trichrome staining (MST). Scale bars are 50 µm.

A Disease and function (101 DEGs)

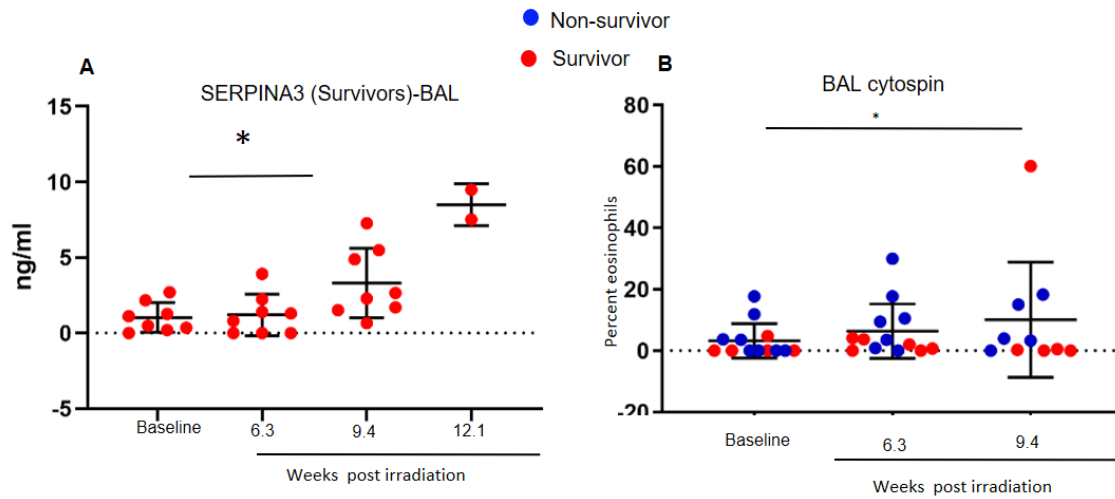


B Target molecules of TGF-beta upstream regulator (101 DEGs)



Supplementary figure 6. Ingenuity Pathway Analysis (IPA) of common 101 DEGs.

(A) 101 DEGs reflected functions associated with cancer, organismal injury, cellular movements, immune cell trafficking and inflammatory responses. (B) Associated target transcripts of TGF-beta1 upstream regulators (SERPINA3, GJB2, CCL2, CCL11 and CDKN1A/p21).



Supplementary figure 7. (A) Mean SERPINA3 levels detected via ELISA in bronchoalveolar lavage (BAL) fluid of survivors relative to baseline (n=8). Results are expressed as the mean $\pm$ SD and statistical significance was determined by t-test. **(B)** Median values of percent eosinophils (differential count on BAL cytospins) at 6.3 weeks (n=14) and 9.4 weeks (n=10) post irradiation relative to baseline (n=14). Results are expressed as the median $\pm$ IQR and Wilcoxon matched-pairs signed rank test was used for statistical significance. P value criteria * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for all data

Supplementary Tables

Supplementary table 1. Lists of antibodies used for immunohistochemistry.

Antibody	Tissue/cell specificity	Cellular localization	Company	Catalogue number	Antigen Retrieval	Blocking Solution	Antibody Dilution	Detection
α -SMA (alpha smooth muscle actin)	Myofibroblast	Cytoplasm	Abcam	ab5694	Citrate buffer pH 6 / 30 min	IHC / ISH Super Blocking /Leica	1:200	polymeric Alkaline phosphatase (AP) / Fast Red
CD163	Macrophage	Cytoplasm	Thermo-scientific	MA1-82342	Citrate buffer pH 6 / 20 min	IHC / ISH Super Blocking /Leica	1:800	polymeric Alkaline phosphatase (AP) / Fast Red
pSTAT1	M1 specific Transcription factor	Nuclear	Cell Signaling Technologies	9167	EDTA buffer pH 9 / 20 min	3% Hydrogen Peroxide	1:25	polymeric HRP / DAB
CMAF-1	M2 specific Transcription factor	Nuclear	Abcam	ab199424	EDTA buffer pH 9 / 20 min	IHC / ISH Super Blocking /Leica	1:50	polymeric Alkaline phosphatase (AP) / Fast Red
p21	Cell cycle arrest marker	Nuclear	Abcam	ab109520	EDTA buffer pH 9 / 20 min	IHC / ISH Super Blocking /Leica	1:50	polymeric Alkaline phosphatase (AP) / Fast Red
Pan cytokeratin	Epithelial marker	Cytoplasmic and membranous	Dako / Agilent	M3515	Proteinase K / 20 min	3% Hydrogen Peroxide	1:400	polymeric HRP / DAB
SPC (Pro-surfactant protein C)	Type II pneumocytes	Cytoplasm	Abcam	ab90716	EDTA buffer pH 9 / 20 min	IHC / ISH Super Blocking /Leica	1:500	polymeric Alkaline phosphatase (AP) / Fast Red
HAM56	Macrophage	Cytoplasm	Sigma/Millipore	279M-16	EDTA buffer pH 9 / 20 min	IHC / ISH Super Blocking /Leica	1:25	polymeric Alkaline phosphatase (AP) / Fast Red

Supplementary table 2. Target transcripts of TGF-beta upstream regulator in dataset (101 common gene list) revealed by Ingenuity Pathway Analysis (IPA).

Genes in dataset	Prediction	Findings	Expr Fold Change
SERPINA3	Activated	Upregulates (3)	82.025
GJB2	Activated	Upregulates (1)	24.542
CCL2	Activated	Upregulates (13)	13.286
SELE	Inhibited	Downregulates (8)	12.985
CCL11	Activated	Upregulates (6)	9.739
CCL8	Inhibited	Downregulates (1)	8.489
ECM1	Affected	Regulates (2)	6.656
CDKN1A	Activated	Upregulates (143)	6.03
CCR1	Activated	Upregulates (4)	5.032
OSM	Affected	Regulates (1)	5.029
ADGRB1	Activated	Upregulates (1)	4.994
HMOX1	Activated	Upregulates (19)	4.96
FCER1A	Inhibited	Downregulates (1)	4.39
PDLIM4	Activated	Upregulates (2)	4.353
GNA14	Inhibited	Downregulates (1)	3.981
IGF1	Inhibited	Downregulates (9)	3.419
SEMA3A	Inhibited	Downregulates (2)	3.264
LIFR	Inhibited	Upregulates (1)	-3.894
KLF15	Activated	Downregulates (2)	-5.105
FAM107A	Affected	Regulates (1)	-8.462

Supplementary table 3. Distribution of SOD mimetic drug treated and saline treated animals in the study. Lung samples from grey highlighted animals (n=11) were utilized for RNA sequencing.

Animal #	Irradiation	Endpoint	Treatment	Age (yrs)	BW (Kg)
1	10 Gy	Non-survivor	SOD-mimetic drug	3.76	5.95
2	10 Gy	Non-survivor	SOD-mimetic drug	3.76	4.85
3	10 Gy	Non-survivor	SOD-mimetic drug	5.76	9.45
4	10 Gy	Non-survivor	SOD-mimetic drug	3.76	5.8
5	10 Gy	Non-survivor	SOD-mimetic drug	3.76	6.2
6	10 Gy	Non-survivor	Saline treated	3.76	4.85
7	10 Gy	Non-survivor	Saline treated	4.76	5.75
8	10 Gy	Non-survivor	Saline treated	4.76	7.35
9	10 Gy	Non-survivor	Saline treated	4.76	5.6
10	10 Gy	Survivor	SOD-mimetic drug	9.25	8.75
11	10 Gy	Survivor	SOD-mimetic drug	11.25	12.6
12	10 Gy	Survivor	SOD-mimetic drug	4.76	4.9
13	10 Gy	Survivor	Saline treated	4.76	5.6
14	10 Gy	Survivor	Saline treated	4.76	4.35
15	10 Gy	Survivor	Saline treated	5.76	7.05
16	10 Gy	Survivor	Saline treated	5.76	9.85
17	10 Gy	Survivor	Saline treated	9.25	8.3
Control 1	Sham-irradiated	Control	Saline treated	2.75	3.6
Control 2	Sham-irradiated	Control	Saline treated	2.83	4.5
Control 3	Sham-irradiated	Control	Saline treated	2.5	3.45

Supplementary table 4. Statistical significance of plasma cytokines measured using the multiplex immunoassay (Magnetic 29-Plex Panel) at 2.3 weeks, 3.4weeks, 4.3 week and at necropsy post irradiation relative to baseline. P value criteria ns (≥ 0.05), **P* (0.01 to 0.05); ***P* (0.001 to 0.01); ****P* (0.0001 to 0.001), *****P* (< 0.0001).

	Cytokines	2.3 (wks)	3.4 (wks)	4.3 (wks)	Nx (Necropsy)	Statistical significance	Post-tests	Animals (n)
1	MCP 1	****	****	****	*	Mixed effect model (REML)	Tukey's multiple comparisons test	17
2	Eotaxin	****	****	****	*	RM one-way ANOVA	Tukey's multiple comparisons test	17
3	IL-1beta	****	***	*	undetectable	Friedman test	Dunn's multiple comparison	17
4	IL-1ra	****	**	**	***	Friedman test	Dunn's multiple comparison	16
5	IL-2	ns	ns	ns	Ns	Friedman test	Dunn's multiple comparison	17
6	IL-4	***	**	**	undetectable	Friedman test	Dunn's multiple comparison	17
7	IL-5	****	**	**	undetectable	Friedman test	Dunn's multiple comparison	17
8	IL-6	ns	ns	ns	undetectable	Friedman test	Dunn's multiple comparison	17
9	IL-17A	****	**	**	undetectable	Friedman test	Dunn's multiple comparison	17
10	IL-8	**	*	*	undetectable	Friedman test	Dunn's multiple comparison	17
11	IL-10	ns	ns	ns	undetectable	Friedman test	Dunn's multiple comparison	17
12	l-Tac	****	****	****	Ns	Friedman test	Dunn's multiple comparison	17

13	IL-12	***	***	****	Ns	Friedman test	Dunn's multiple comparison	17
14	IL-15	***	**	ns	undetectable	Friedman test	Dunn's multiple comparison	17
15	MIG	ns	ns	ns	undetectable	Friedman test	Dunn's multiple comparison	17
16	FGF basic	*	**	**	Ns	Friedman test	Dunn's multiple comparison	17
17	G-CSF/CSF-3	ns	ns	ns	undetectable	Friedman test	Dunn's multiple comparison	17
18	GM-CSF	*	ns	ns	undetectable	Friedman test	Dunn's multiple comparison	17
19	IFN-g	ns	ns	ns	Ns	Friedman test	Dunn's multiple comparison	17
20	IP-10	***	**	****	Ns	Friedman test	Dunn's multiple comparison	16
21	MDC/CCL22	ns	ns	ns	ns	Friedman test	Dunn's multiple comparison	17
22	MIP-1alpha	*	ns	ns	undetectable	Friedman test	Dunn's multiple comparison	17
23	MIF	**	ns	ns	Ns	Friedman test	Dunn's multiple comparison	15
24	EGF	*	****	****	undetectable	Friedman test	Dunn's multiple comparison	17
25	RANTES	ns	ns	ns	**	Friedman test	Dunn's multiple comparison	16
26	TNF-a	****	*	*	undetectable	Friedman test	Dunn's multiple comparison	17
27	HGF	ns	ns	ns	*	Friedman test	Dunn's multiple comparison	17
28	VEGF	*	**	*	undetectable	Friedman test	Dunn's multiple comparison	17
29	MIP-1Beta	*	**	*	undetectable	Friedman test	Dunn's multiple comparison	17

CHAPTER THREE

Quantitative assessment and comparative analysis of longitudinal lung CT scans
of chest-irradiated nonhuman primates

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(*Radiation Research*).

Abstract

Computed tomography (CT) imaging has been used to diagnose radiation-induced lung injury for decades. However, histogram-based quantitative tools have rarely been applied to assess lung abnormality due to RILI. Here, we used first-order summary statistics to derive and assess threshold measures extracted from whole lung histograms of CT radiodensity in rhesus macaques. For the present study, CT scans of animals exposed to 10 Gy of whole thorax irradiation were utilized from a previous study spanning 2-9 months post-irradiation. These animals were grouped into survivors and non-survivors based on their clinical and experimental endpoints. We quantified the change in lung attenuation post-irradiation relative to baseline using three density parameters; ALD (average lung density), PCHV (percent change in hyper-dense lung volume), PCHV/TV (hyperdense volume as a percent of total volume) at 2-month intervals over time and compared each parameter between the two irradiated groups (non-survivors and survivors). We also correlated our results with histological findings. All of the three indices (ALD, PCHV, and PCHV/TV) obtained from density histograms showed a significant increase in lung injury in non-survivors relative to survivors, with PCHV relatively more sensitive to detect early RILI changes. We observed a significant positive correlation between histologic pneumonitis scores and each of the 3 CT measurements, indicating that CT density is useful as a surrogate for histologic disease severity in RILI.

Introduction

Survivors of accidental acute radiation exposure, like the Chernobyl nuclear accident, developed significant lung injury, contributing to mortality and morbidity (1). Clinically, injury to normal healthy lung tissue is a common outcome following radiotherapy, which targets lung cancer, breast cancer, or other thoracic malignancies. Based on symptoms and radiographic appearance, the early phase of radiation-induced lung injury (RILI) is clinically recognized as radiation pneumonitis (RP) that may or may not progress to its late outcome, known as radiation fibrosis (RF). The incidence of RP associated with radiotherapy is highly variable. It is governed by several factors like mean lung dose, the volume of lung exposed to radiation, patient-related factors like age or pre-existing lung disease, and concurrent therapies. In general, 10-35% of the patients receiving radiotherapy for the treatment of lung cancer, thoracic malignancies like Hodgkin and non-Hodgkin lymphoma, and breast cancer develop radiation pneumonitis. And the incidence is usually high (>15%) in patients requiring re-irradiation for treating tumor relapse (2-7). RP is usually treated with corticosteroids. However, there is no FDA-approved treatment for RF.

Individual responses to irradiation are extremely unpredictable (8) and the mechanisms underlying variable radio-responsiveness are unclear. Even advanced clinical models based on total dose, mean lung dose and volume of lung receiving 20 Gy (V20) cannot accurately predict the risk of RILI (RILI) (9). Computed tomographic (CT) scans are a sensitive means to detect early lung changes and have been used as a diagnostic tool to detect RILI (10-12). The early

phase of RP appears as ground-glass opacities (GGO) that may progress to patchy lesions (the so-called proliferative phase) and eventually progress to scarring with consolidation (RF) (13). In humans, CT can provide a definitive diagnosis of particular interstitial lung disease like idiopathic pulmonary fibrosis (IPF) based on the presence of radiographic usual interstitial pneumonia (UIP) patterns (reticular opacities, traction bronchiectasis, and honeycombing/clustered cystic airspaces, less extensive GGO) without requiring biopsy. However, heterogeneity in disease presentation poses challenges in diagnosis (14). Quantitative CT methods are a useful non-invasive tool that can assist in diagnosing and longitudinal management by tracking lung disease progression (including RILI) in human and animal models(15-21). Some studies have used basic CT density-based measurement parameters like average lung density or attenuation (22-24), skewness, and kurtosis (25) to quantify lung pathology. However, the potential of non-invasive CT quantitative tools like increased density to measure early pathological changes has been scarcely explored (22).

A great deal of information can be obtained from CT data that ranges from threshold-based measurement, first-order statistics, and higher-order textural analysis, which uses spatial information and textural characteristics of regions of interests (ROI) (26). First-order summary statistics based on threshold measures (count of voxels above or below a particular value) are obtained from a whole lung histogram or an ROI (17).

To measure and evaluate changes in the density distribution of RILI over time, we utilized lung CT scans of 15 rhesus macaques from a previous study (Chapter 2)

which were exposed to a single 10 Gy dose of ionizing radiation to the thorax. In this follow-up study, our focus was on quantitative assessment of lung density over time which involved extracting threshold-based summary statistics from whole lung CT scans.

Methods

Animals: Fifteen irradiated animals for the present study were derived from previous study (Chapter 3, supplementary figure 1). Briefly, 17 young adult male rhesus macaques were exposed to a single 10 Gy dose of whole thorax irradiation. Two of animals were euthanized less than 2 months after irradiation, and were excluded from the present study. After irradiation, the animals were studied for 8 months (experimental endpoint). Nine animals reached their clinical endpoint early which was based on a predefined criterion, respiratory rates >80 breaths/minute (non-survivors), whereas eight animals survived to the experimental endpoint (survivors). Computed tomographic (CT) scans of these animals were performed at baseline and then every 2 months post irradiation until their endpoints. Median age and median weight of the remaining 15 animals were 4.7 years (range=7.5, 95% CI=96.48%) and 6.2 kg (range=6.1, 95% CI=96.48%) respectively. The protocol for this study was approved by the Wake Forest School of Medicine (WFSM) Institutional Animal Care and Use Committee. WFSM is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures in this study were performed at WFSM, in accordance with the Animal Welfare Act and other applicable federal regulations.

Irradiation: All the animals to be irradiated were sedated with ketamine (10-15 mg/kg), anesthetized with isoflurane in 100% O₂, and exposed to 10 Gy of 6 MV X-rays from a clinical linear accelerator (Varian Medical Systems, Palo Alto, CA), using AP-PA parallel-opposed fields (field size 13.0 cm (4,0 cm x 9 cm) long x 12 cm wide), with the isocenter at the thorax midplane and a nominal dose rate of 4Gy/min. Both the PA and then AP fields were delivered, 5Gy each, within about 1 minute of each other, for a total of 10Gy to the midplane. The radiation field included all lung lobes, the distal trachea, esophagus, heart, thymus, and other adjacent tissues, including the superior portion of the liver and stomach.

Chest CT scans and image acquisition:

For the CT procedure animals were sedated with ketamine and medetomidine and maintained on isoflurane anesthesia. A consistent breath hold technique (10-20 seconds) was used to minimize motion artifact due to breathing. Images were acquired using a Toshiba Aquilion 32 slice CT scanner with parameter settings: $V_{\max}=120$ kV, $I_{\max}=100$ mA, field of view (FOV) = 320 mm, matrix size = 512 x 512, and slice thickness = 0.5 mm, giving voxel dimensions of 0.625 x 0.625 x 0.5 mm³ using a human pediatric protocol. The radiation dose delivered by each CT scan was less than 0.02 Gy. Image files were obtained in digital imaging and communications (DICOM) format.

Lung segmentation

Mimics software (Materialise Mimics Innovation Suite, Leuven, Belgium, version 21.0.0.406) was used for analysis of lung scans. We created lung masks with the

lower threshold range of -1020 Hounsfield units (HU) and maximum threshold range of 220 HU at baseline and post-irradiation times. This range excluded aerated regions (-1024 HU), and included normal lung (usually -500 to -1020) and regions of soft-tissue density representing inflammation and fibrosis (-600 to 200 HU) (16, 27). Right and left lungs were manually segmented comprising all slices using the Mimics multiple slice edit tool. Manual segmentation allowed the inclusion of peripheral radio-dense regions like pleural adhesions. Anatomical structures like trachea, bronchi and major pulmonary vessels were excluded by manual segmentation.

Quantification of CT data

The grey values (HU) of all voxels in the lung masks created on Mimics were exported as text files. The text files generated from Mimics were directly imported to data management software (Tableau 2020.3, Seattle, WA) to analyze the large datasets through a visual interface to create a master sheet of HU values from text files. Left and right lung HU values were stacked/ merged into one lung data set (with the retained identity of left and right lung) for each scan. A histogram analysis of voxel density (HU) of entire segmented lungs was performed for each animal at baseline and each post-irradiation timepoint. Next, histogram data of each animal was exported to excel files for further quantitative assessment of the average lung density and hyper-dense lung volume. Baseline voxel densities were evaluated for each animal to determine the HU threshold at which 5% of the voxels were greater density than the threshold; this allowed baseline correction for inter-animal

variation. Hyper-dense voxels on subsequent scans were defined for each animal to be voxels that had HU values greater than the 5% threshold value determined at baseline. Percent change in hyper-dense lung volume from baseline was calculated as $PCHV = [(V_n - V_b)/V_b] \times 100$ and hyper-dense lung volume as a percentage of entire lung volume was calculated as $PCHV/TV = [V_n/\text{Total volume}] \times 100$, where V_b is the volume of lung above 5% cutoff value at baseline and V_n is volume of lung above 5% cutoff value (determined at baseline) at subsequent time points. Similar measures of hyper-dense volume are described in previous studies (15, 28). TV is total lung volume which is equal to total voxels in ROI $\times 0.195$ (Each voxel is $0.625\text{mm} \times 0.625\text{mm} \times 0.5\text{mm} = 0.195 \text{ mm}^3$).

Histopathological assessment (Pneumonitis scoring)

Average histopathological pneumonitis scores evaluated on hematoxylin and eosin (H&E) stained lung sections of 15 animals were obtained from the previous study (Chapter 2, Figure 2). These lung sections from each animal were scored for the severity of pneumonitis based on cellular infiltration by macrophages, neutrophils, and other inflammatory cell types.

Statistical analysis

GraphPad Prism 8 was used for descriptive analysis to assess normality and skew. Paired or unpaired student T-test and one-way ANOVA followed by post tests, were used to assess the difference between the groups. When means were not normally distributed, the difference from one time point to another in matching groups was assessed by a non-parametric statistical test (Wilcoxon signed-rank

test). The strength of correlations was determined by Pearson's test. For all experiments, a p-value <0.05 was considered statistically significant.

Results

Onset of RILI over time and its quantitative assessment using histograms

Thorax exposure to high dose ionizing irradiation induced significant lung injury as evidenced by the chest CT scans post irradiation (previous publication/Chapter 2). Non-survivors and survivors both developed lung injury; however, the extend of injury varied over time. Figures 1 A and D show representative coronal and axial CT images of a non-survivor and a survivor over time. The hyper-dense or radio-opaque brighter regions relative to black aerated background in the irradiated lungs indicate RILI that includes pneumonitis and/or fibrosis. To quantify the entire lung average density and to understand the density distribution of hyper-dense radio-opaque regions, we extracted from histograms of the entire segmented lungs. Figure 1 shows examples of manually segmented lung CT images where normal radiodense anatomical structure were excluded.

We observed a change in the shape of the lung density histogram post-irradiation. There was a decrease in the percent voxels which fell in the normal lung range (-1020 to -400HU) and an increase in the percentage of voxels that fell in the inflammation or fibrosis range (-200 to 220 HU) representing radiodense affected lung (Figure 1B and E).

Higher average lung density correlated to gross lung injury

Non-survivors had higher average lung density in comparison to survivors at 4 months post-irradiation ($P < 0.01$), (Figure 2 A) which was consistent with our gross observation of marked pneumonitis at necropsy. Representative gross images of a control, non-survivor and a survivor lung are shown in Figure 2 D-F. Relative to baseline, non-survivors exhibited an increasing trend of average lung density (Figure 2B) and survivors showed significant increases at 2 and 4 months post irradiation (Figure 2C).

Quantitative assessment of hyper-dense lung volume

We quantified the number of voxels above the 5% baseline cutoff value at each post-irradiation interval for survivors and non-survivors. Figure 3 A and B shows overlapping histograms of a representative non-survivor and a survivor where histograms (inset) reflect changes in hyper-dense lung volume. Similar to average density, we observed a considerably higher ($P < 0.05$) percent change in hyper-density parameters, PCHV and PCHV/TV for non-survivors relative to survivors at 4 months (Figure 4, A and D). Relative to baseline, non-survivors showed a significant increase in PCHV at 2-month ($P < 0.05$) (Figure 4 B). However, PCHV for survivors was greater at all post-irradiation times compared to baseline ($P < 0.01$) (Figure 4 C). Density parameter PCHV/TV for non-survivors was only increased at 4-month ($P < 0.01$) post-irradiation relative to baseline. For survivors, PCHV/TV was higher at 2-month and 4-month post irradiation in comparison to baseline (Figure, 4 E and F).

Correlation between average lung density, hyper-dense volume and histological pneumonitis scoring

To determine the relationship between different types of CT quantification measures (average lung density, PCHV, PCHV/TV) and histologic pneumonitis scores, we compared the histologic pneumonitis scores with their corresponding CT scan closest to the time of necropsy. We observed a positive and significant correlation between all quantification measures and histologic pneumonitis. The correlation was strongest between PCHV/TV and histology ($r=0.89$), followed by PCHV and histology (0.85) and average lung density and histology (0.76) (all $p<0.0001$; Figure 5, A-C). We also observed a positive correlation between baseline average lung density and average density at necropsy (Figure 5D).

Discussion:

Many measurement indices can be extracted from lung histograms. Histogram derived densitometric parameters like mean lung density is most commonly used to assess the degree of structural lung abnormality (22, 29). These structural abnormalities vary from decreased radio-opacity (emphysema) to increased radio-opacity (inflammation and fibrosis) (30). We utilized histogram-based measurement parameters; average total lung density, PCHV, and PCHV/TV. Average lung density captured the mean signal of all the voxels in segmented lung within a defined threshold range (-1020 to 220 HU). In two longitudinal CT scan analysis studies, rhesus macaques were infected with viruses, and quantitative measure PCHV correlated well with qualitative lung pathology assessed by radiologist. In this study, PCHV was least affected by the variability due to breath-holding techniques compared to normal lung volume over time (15, 28). We calculated all three density parameters before and after irradiation to assess the

change over time and compared them between two irradiated groups (non-survivors and survivors).

In our study, all three indices (AVG lung density, PCHV, PCHV/TV) obtained from density histogram showed a significant increase in lung injury in non-survivors relative to survivors. These animals were euthanized early as they reached their clinical endpoint and their necropsy findings revealed significant pneumonitis and lung fibrosis (Chapter 3, Figure 2 and 3). A positive correlation between RP and CT injury measurement indices on corresponding lung scans indicates that all of the non-invasive CT quantification methods can be used to interpret the severity of in-vivo lung pathology (pneumonitis). Previously, small and large RILI animal models have also reported histological association to CT findings (16, 31).

Survivors showed a significant increase in average lung density and PCHV/TV at 2 and 4 months post-irradiation compared to baseline. This was sooner than the increase in lung density seen in non-survivors. On the other hand, density parameter PCHV was significantly greater in survivors at all time points than baseline. However, we did not observe a positive correlation between PCHV and pneumonitis scoring in survivors (data not shown). This can be explained by minimal pneumonitis in survivors at the late phase of lung injury (experimental endpoint) and less change in radiodensity parameters at the late time point. Previously (Chapter 2), we reported significant fibrosis in survivors and non-survivors. One possible reason for the lack of correlation between histology and CT at later times could be the heterogeneous nature of lesions that include

radiodense areas like fibrosis and low density attenuated regions, e.g., emphysema that could average out each other (17).

In our study, all three measurement indices (average lung density, PCHV, PCHV/TV) showed a significant increase in RILI. However, PCHV was the most sensitive parameter that detected a significant difference in radiodense volume at two months post-irradiation for non-survivors and 6 and 8 months post-irradiation for survivors, not detected by the other two parameters (average lung density and PCTV/TV).

In addition to the correlations shown here between CT measurements and pathology, it will be important to also validate other biomarkers of RILI, and CT imaging could be useful in this regards. A previous study on peripheral blood lymphocytes (PBL) of 9 saline irradiated animals, including a portion of animals from the present study, has shown significant micronuclei induction (radiation specific biomarker assay; cytokinesis-block micronucleus (CBMN) assay) in non-survivors and survivors that continued to their endpoints (32). These findings indicate that radiation-specific damage in survivors persisted until late time points and appeared to correspond with PCHV findings.

We also found that baseline average CT values correlated significantly with average CT density close to necropsy times ($r=0.5166$, $p=0.048$), which indicates the possibility of predicting the severity of RP in visually normal-looking baseline lungs that have higher average lung density. Previous human studies have also correlated higher baseline lung density with risk of severe RP (33).

One of our study's strengths is the capability to correlate CT findings with histology, which is usually a limitation in human CT lung abnormality quantification studies (23). Besides strengths, our study also had few limitations. We carefully manually segmented lungs using multiple edit slice tools to include the entire lung and exclude hyper-dense anatomical structures like major airways and vessels. While manual segmentation is considered the gold standard, it is time-consuming and usually results in inter-observer variability. In our study, there was a lack of comparison to other segmentation methods. Another limitation of the study includes a lack of histological data corresponding to early CT densitometry parameters before the animals reached their endpoints. While acquiring CT scans, the same breath-hold technique was used for all animals. However, the lungs were not inflated to a precise pressure, and lung volume variability in an animal model is challenging to control, due to individual differences in lung compliance and size.

In this study, the radiation field included the heart and other organs (the distal trachea, esophagus, thymus, muscle, bone, spinal cord, and the superior portion of the liver and stomach) in addition to the lungs. We did complete post-mortem exams on all animals including histology, and did not observe any evidence of cellular injury, inflammation, or fibrosis aside from thymic degeneration in animals dying early (data not shown). However, we have previously reported regional thoracic osteopenia induced in this model (34), and it is likely that late bone and cardiac effects would be found years post-irradiation, based on our observations in total-body irradiated animals (35). Short-term heart toxicity due to 10 Gy

radiation is not likely as the heart is less sensitive to doses <15Gy than lungs early after irradiation (36). However, a separate study is going on to understand the short and long-term radiation-induced cardiac toxicity in this model.

Pleural effusion was not observed in this study, but can develop after radiotherapy either due to inflammatory or non-inflammatory processes. The exact cause of the radiotherapy-associated pleural effusion is not well understood (37). Risk factors for pleural and cardiac effusions include higher total dose and radiation field width resulting in large volume exposure (38). Pleural effusions developing at a later time (>1year) following radiotherapy have been attributed to constrictive pericarditis, leading to restriction of the superior vena cava or lymphatics (39, 40). A radiation dose-dependent increase in pleural effusion incidence has been reported in WTLI (Whole Thorax Lung Injury), an established RILI NHP model(41-43). The exact reason why our animals did not develop pleural effusion is uncertain. However, the critical difference responsible for the lack of pleural effusion could be attributed to variance in exposure protocols (e.g. higher dose rate in our studies) or lack of corticosteroid treatment in our work as opposed to other WTLI NHP studies.

One of the significant challenges in diagnosing RILI on CT scan is the simultaneous presence of other acute respiratory syndromes like viral infection, especially when both share common clinical and radiographic features (44-46). Patients undergoing radiotherapy are at high risk of developing COVID-19 infection, and the differential diagnosis of RILI and Covid-19 infection can be challenging (45). Radiomics and Machine learning methods applied to CT images might be a possible future method to distinguish between these different causes

of inflammatory lung disease (44). Human-relevant animal models like NHP provide a unique opportunity to examine the disease process histologically. We anticipate applying this information to higher-order textural analysis, which can use spatial information to differentiate RP from RF non-invasively on CT scans. Diagnosis of mixed pathologies like co-occurrence of ground-glass opacity, patchy lesions, nodules, and emphysema has always been challenging in clinical studies (14, 47). However, radiomics (26, 48) and deep learning methods (49) offer a base and future to assist the radiologist in differentiating complex lung disease and their quantification to predict and assess the severity of the disease.

In summary, all measurement indices of radiodensity showed a significant increase in lung injury in non-survivors relative to survivors, with PCHV relatively more sensitive to detect early RILI changes. We observed a significant positive correlation between histologic pneumonitis scores and all three measurement parameters; AVG lung density, PCHV, PCHV/TV, therefore, these measures may serve as surrogates for likely patterns of histopathology in future studies of the pathogenesis of RILI.

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Figures and legends

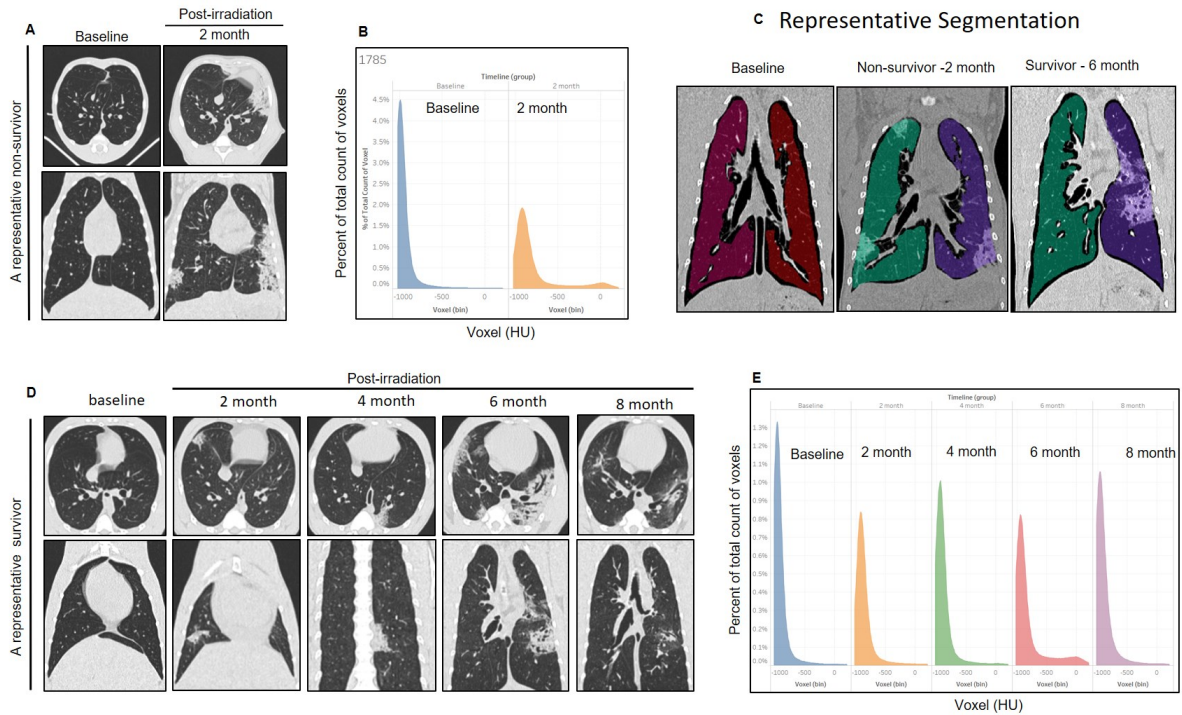


Figure 1. Histogram of entire segmented lung over time (0-8month post irradiation).

(A) Computed tomographic images (axial and coronal view) of a representative non-survivor at baseline and 2-month post-irradiation. (B) Histograms of the same non-survivor show the percentage of total voxels within the segmented lung at a particular voxel density (Hounsfield Units). (C) Example of manual lung segmentation; representative coronal images of baseline, a non-survivor and a survivor; the entire lung (left and right) was segmented excluding major airways and blood vessels. (D) Computed tomographic images (axial and coronal view) of a representative survivor over time. (E) Histograms of the same survivor show the percentage of total voxels in the segmented lung across a continuum of voxel density (HU).

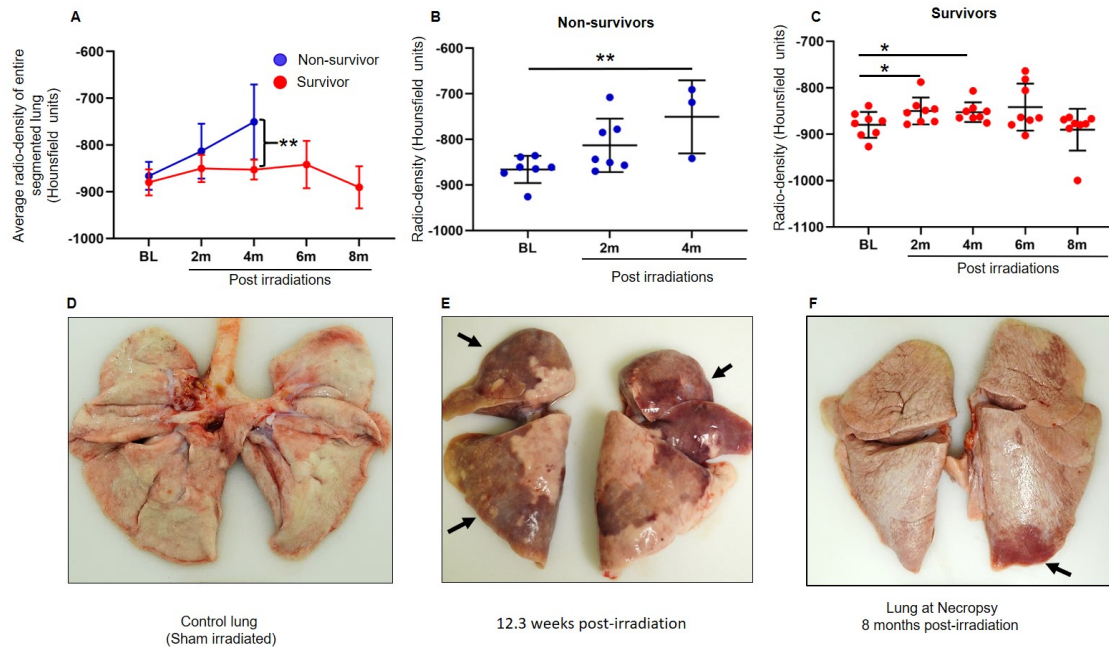


Figure 2. Average density of post-irradiation lung injury in non-survivors and survivors.

(A) Average lung density of survivors and non-survivors at baseline (BL) and post-irradiation timepoints. Each dot represents average radiodensity of the entire segmented lung of one animal. (B) Average lung density of non-survivors at baseline (BL, $n = 7$), 2 months ($n=7$), and 4 months ($n=3$). (C) Average lung radiodensity of survivors over time ($n = 8$). (D), (E) and (F) are representative gross images of a healthy normal lung, a post-irradiation non-survivor lung and a survivor lung at necropsy; red discolored regions (black arrows) represent radiation pneumonitis. Results are expressed as the mean $\pm$ SD; statistical significance was determined by unpaired t test. P value criteria $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ for all data.

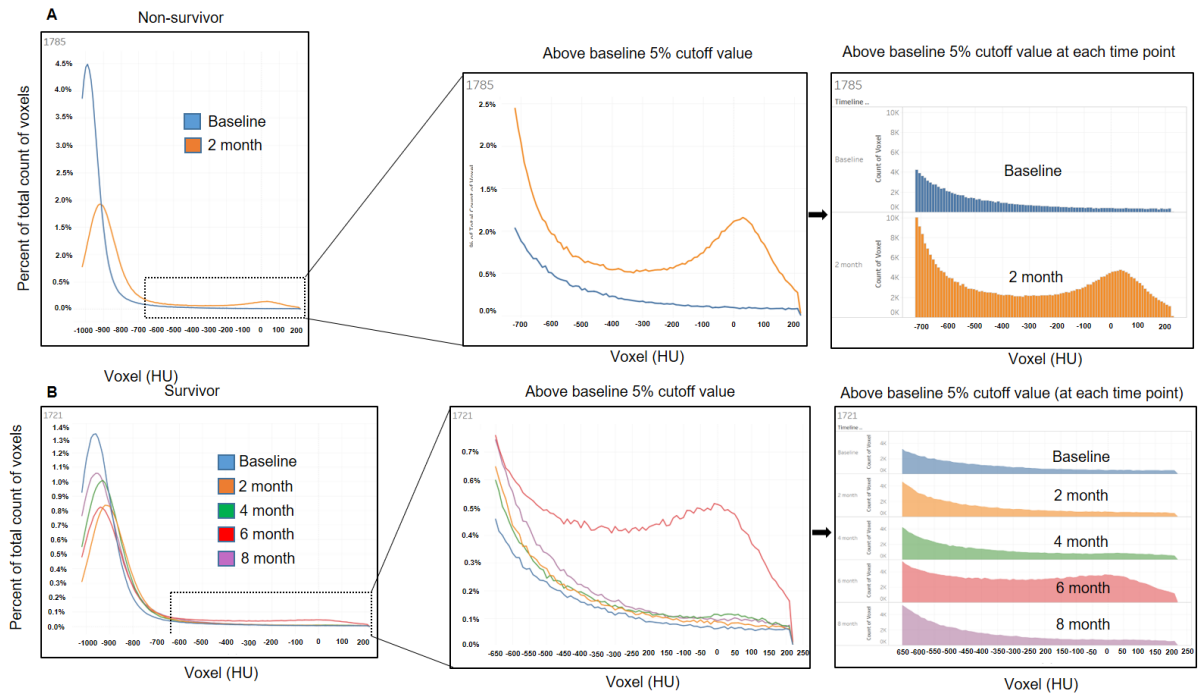


Figure 3. Histograms changes above 5 percent cutoff value.

(A) Overlapping histograms of a non-survivor at baseline and 2 months post irradiation (same animal as in figure 1) with projected histogram changes above 5% cutoff value determined at baseline (-720 HU). (B) Overlapping histograms of a survivor at baseline, 2, 4, 6 and 8-month post irradiation (same animal as in figure 1) with projected histogram changes above 5% cutoff value determined at baseline (-650 HU).

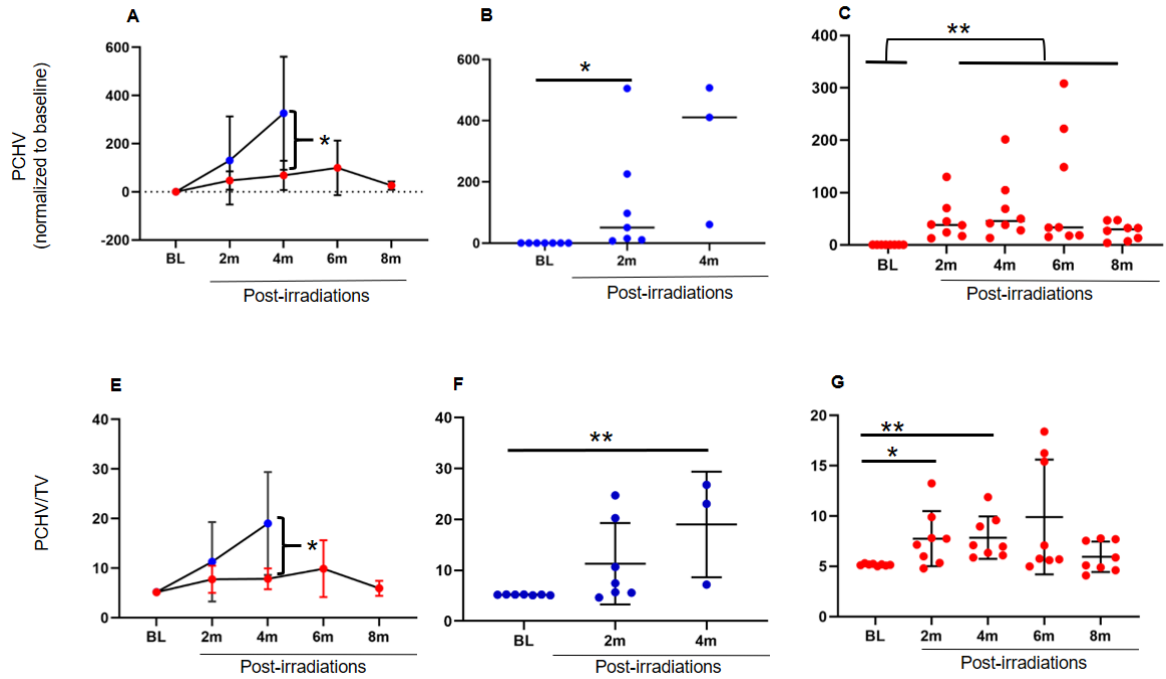


Figure 4. Quantitative measurement of hyperdense lung volume. (A) Post-irradiation percent change in hyperdense lung volume (PCHV) of survivors and non-survivors from baseline ($PCHV = [(V_n - V_b)/V_b] \times 100$). Results are expressed as the mean $\pm$ SD, Statistical significance was determined by unpaired t test. (B) Percent change in hyperdense lung volume of non-survivors from baseline (BL, $n=7$) at 2-month ($n=7$) and at 4-month ($n=3$); each dot represents an animal. (C) Percent change in hyperdense lung volume of survivors over time ($n=8$); each dot represents an animal. Results are expressed as the median, and the Wilcoxon signed rank test was used for statistical significance. (D) Hyperdense lung volume as a percent of entire segmented lung volume (PCHV/TV) of survivors and non-survivors (E) PCHV/TV of non-survivors at baseline (BL, $n=7$), 2-month ($n=7$) and at 4-month ($n=3$); each dot represents an animal. Results are expressed as the mean $\pm$ SD, Statistical significance was determined by unpaired t test. (F) PCHV/TV of survivors at baseline, 2-month, 4-month, 6-month and 8-month, ($n=8$); each dot represents one animal. Results are expressed as the mean $\pm$ SD, Statistical significance was determined by paired t test. P value criteria * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for all data.

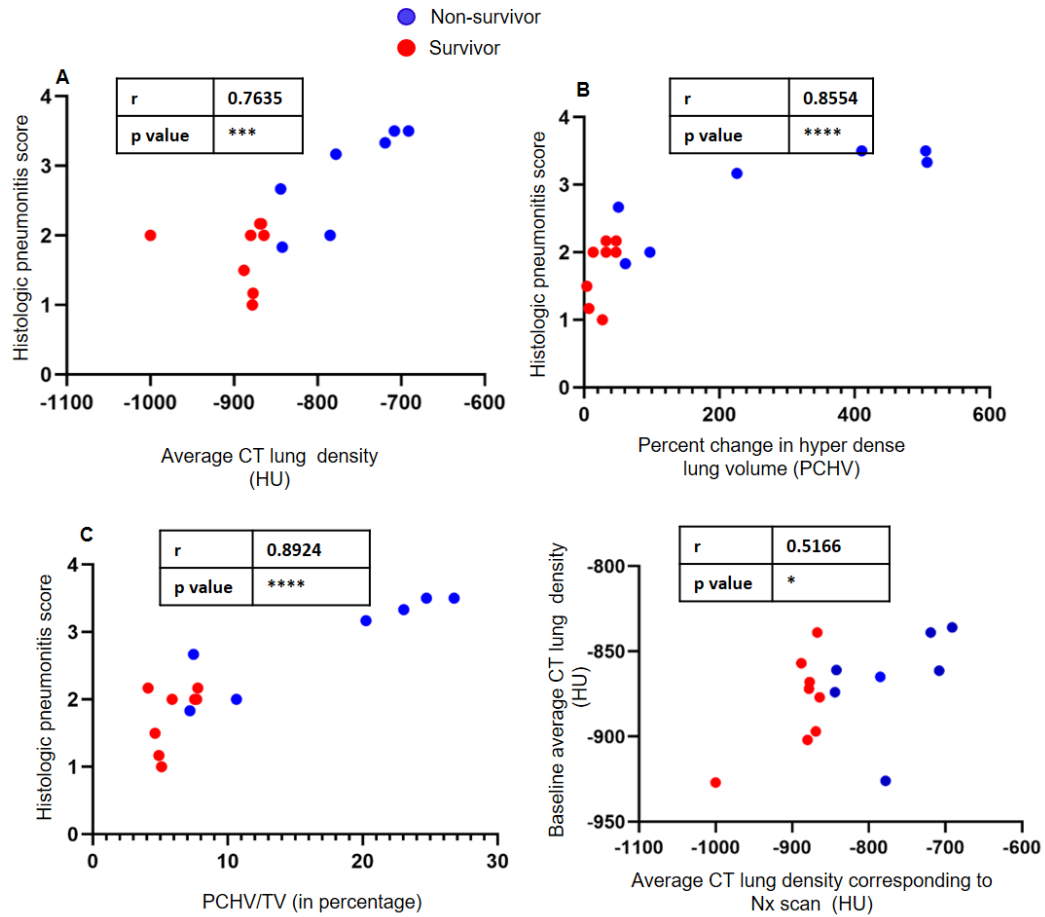


Figure 5. Correlation between histologic pneumonitis score, and average lung density, PCHV, PCHV/LV in irradiated subjects at their corresponding lung CT scans (last scans).

(A) Pearson correlation analysis of pneumonitis scores and average lung density of entire segmented lung (n=15). (B) Pearson correlation analysis of pneumonitis scores and PCHV (n=15) (C) Pearson correlation analysis of histologic pneumonitis score and PCLV/LV (n=15). (D) Pearson correlation analysis of baseline average density and final lung density scans (n=15). P value criteria $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$, $****P < 0.0001$ for all data.

CHAPTER FOUR

Summary and Future Directions

Priyanka Thakur, BVSc. & AH

Summary

Present work focused on longitudinally assessing RILI qualitatively and quantitatively in an established non-human primate model (rhesus macaque). A single dose of 10 Gy ionizing irradiation was given to the whole thorax. The 10 Gy dose used in our study is higher than the single large estimated dose of 9.3Gy in a human retrospective study which caused RP in ~50% of patients (1, 2). The relevance of this model lies in accidental/malicious non-uniform or partial body exposure to irradiation (3), leading to significant lung damage and mortality due to radiation pneumonitis (RP) and delayed effects of acute radiation exposure (DEARE) (4, 5). Whole thorax lung irradiation (WTLI) small and large animal models are commonly utilized to study radiotherapy-related normal lung tissue complications. However, human studies usually use fractionated radiotherapy to target a tumor within a defined volume. The single-dose WTLI model also has the potential to translate to single fraction radiotherapy (SF-SBRT) related normal lung tissue injury. This relatively young field utilizes a single high dose prescribed to target a tumor.

RILI is a progressive disease; the initial acute exudative phase occurs in about 4-13 weeks post-irradiation, followed by the proliferative phase (3-9 months). Both the phases are clinically classified as radiation pneumonitis (RP). Severe injury and persistent damage may lead to irreversible lung fibrosis (LF) (6, 7). Our main goal of the study was to understand the differential susceptibility to RILI in a controlled study where 17 NHPs were exposed to a single dose of 10 Gy thorax irradiation and compared to non-irradiated controls. In this longitudinal study,

animals were investigated over a long time-span of up to 8 months. Post-WTLI exposure, animals were categorized as non-survivors, reaching a predefined clinical endpoint based on higher respiratory rates, and survivors surviving to experimental endpoints (8 months). Hence throughout the study, comparisons between non-survivors and survivors were used to characterize them clinically, histologically, and at the molecular level. Chapter 2 used clinical, pathological, and transcriptomic analysis to understand the pathology and progression of RILI. Chapter 3 was a follow-up study that expanded on longitudinal quantitative CT analysis focussed on threshold-based summary statistics obtained from whole lung CT scans.

Major findings

Non-survivors developed severe RILI

Together, higher respiratory rates, longitudinal CT findings, and histological pneumonitis scores at necropsy show that non-survivors developed significant RILI, which was either not present or waned over time in survivors.

Irradiation induced significant fibrosis with an abundance of M2-like macrophages

Fibrosis and M2-like (pro-fibrotic) macrophages co-expressing CD163 and CMAF were significantly higher in both irradiated groups compared to non-irradiated controls. However, M2 specific macrophages were more abundant in non-survivors relative to both controls and survivors (both $p=0.001$). The predominance of macrophages in both survivors and non-survivors could be a pivotal contributor

to the activation of TGF- β pathways, together with other stressed cell types expressing p21 (8).

Survival specific and shared transcriptomic signature

RNA sequencing data highlighted non-survival-specific, survivor-specific, and common gene signatures, which differ via survival status. Top up-regulated and down-regulated transcripts in non-survivors were related to fibrosis, epithelial to mesenchymal transition (EMT), matrix remodeling (MMP3), macrophage infiltration and fusion (DCSTAMP), inflammation and wound healing in acute lung injury (EREG), pulmonary fibrosis (SLC6A4/5HTT/SERT) (9-14). Whereas several survivor-specific DEGs (SPINK1, FAIM2, KCNH5, NUF2, and RACGAP1) underlined processes linked to cell cycle regulation, growth, proliferation, invasion, and migration, indicating more repair-oriented responses to the same radiation insult.

Common DEGs which differ via survival status were comprised of transcripts whose upregulation is associated with extracellular matrix remodeling, pulmonary fibrosis (SERPINA3), cystic fibrosis (ATP12A), acute and chronic lung injury, EMT (LRRN4, GJB2 and CLDN 10) and EMT (TOX3) (15-23).

Relevant cytokine and protein expression

We reported increased plasma levels of CCL2, CCL11, and TGF - β 1. These cytokines were significantly elevated in plasma at both earlier and later time points. CCL2 secreted by macrophages is known to further recruit macrophages to the site of injury post-irradiation through the CCL2/CCR2 axis (24). CCL11 (eotaxin1)

promotes eosinophils' recruitment and subsequent development of fibrosis in bleomycin-induced pulmonary injury (25, 26). SERPINA3 protein and TGF-beta1 were elevated in BAL fluid at 1 to 2-months post-irradiation. Our findings indicate SERPINA3 as a biomarker of extracellular matrix remodeling in the irradiated lung, which is in line with previous studies showing SERPINA3 as a marker of extracellular matrix remodeling in various diseases, including idiopathic pulmonary fibrosis. Abundant CDKN1A/p21 staining in epithelial and alveolar type 2 (SPC) cells and positive alpha-smooth muscle actin (α -SMA) staining in non-survivors and survivors indicated cell cycle inhibition and a pro-fibrotic myofibroblast phenotype in irradiated lungs (8, 27, 28).

This study's impact lies in identifying new critical molecules that were differentially expressed in non-survivors and survivors. Hence, these new targets can help stratify individuals responding variably to the same radiotherapy treatment, and their distribution pattern may help manage or treat patients accordingly. However, identifying and detecting these key molecules concurrently in local (BAL) and systemic fluids (plasma) is needed for further validation.

Histogram-based CT quantitative measure using density parameters

All of the three density-based measurements extracted from the entire segmented lung histogram (average/AVG lung density, percent change in hyper-dense lung volume /PCHV, hyper-dense lung volume as a percent of entire lung volume/PCHV/TV) showed a significant increase in lung injury in non-survivors relative to survivors. However, PCHV was the most sensitive parameter that

detected a significant difference in radiodense volume at 2 months post-irradiation in non-survivors and 6 and 8 months post-irradiation in survivors, not detected by the other two parameters (AVG lung density and PCTV/TV). These three measurement parameters significantly correlated with histologic pneumonitis scores indicating their potential to serve as an excellent surrogate to histology. We also found that baseline average CT values correlated significantly with average CT density close to necropsy times ($r=0.5166$, $p=0.048$), indicating the possibility of predicting the severity of RP in visually normal-looking lungs which have higher baseline average lung density. Previous human studies have also correlated higher baseline lung density with severe RP risk (29).

Quantitative image features have prognostic value and the potential to predict clinical outcomes. Radiomics utilizes the full potential of medical images by extracting and analyzing enormous amounts of advanced quantitative features to provide prognostic, diagnostic, or predictive information. However, feature selection in radiomics is a crucial and challenging step that aims to involve features that optimally correlate to endpoints and each other (30, 31). The potential impact of histogram-based quantitative features (AVG lung density, PCHV, PCHV/TV) used in our study lies in their significant correlation to histopathology, which raises the possibility of including these features in future radiomics studies.

Limitations:

This study had a few limitations. The first limitation is that we did not address the effects of sex or age on RILI. Female C57BL/6 mice are marginally more sensitive to RILI than males. However, other studies have shown the least influence of

gender in radio-responsiveness (32-34). We included adolescent ages in rhesus monkeys, which have a typical lifespan of 30-35 years. Another limitation of the study is that whole thorax exposure included other organs in the radiation field. With the exception of the thymus, their histologic examination did not show any evidence of cellular injury, inflammation, or fibrosis. However, we have previously reported regional thoracic osteopenia induced in this mode (35). The heart was also in the radiation field, though short-term heart toxicity due to 10 Gy radiation is not likely as the heart is less sensitive to doses <15Gy than lungs early after irradiation (36). Based on our observations in total-body irradiated animals (37), it is likely that late bone and cardiac effects would be found years post-irradiation. Hence, post-irradiation changes in heart pathology and heart function are part of a separate ongoing study. A possible confounder in this study is the inclusion of SOD mimetic drug-treated animals. However, we did not see any effect of SOD mimetic treatment on observed secondary outcomes in our study cohort; however, its effect at molecular levels cannot be overlooked. Therefore, for RNA expression studies, only non-drug-treated irradiated and unirradiated animals were used. Our CT quantification study was not without limitations which included a lack of comparison to other segmentation methods and the possibility of lung volume variability due to the breath-hold technique. Although the same breath-hold technique was used for all animals while acquiring CT scans, the possibility of lung volume variability in an animal model is challenging to control due to individual differences in lung compliance and size.

Future directions

Molecular targets for potential future mechanistic studies

Besides SERPINA3, RNA sequencing of lung tissue has identified many dysregulated transcripts like ATP12A, GJB2, CLDN10, TOX3, and LPA/lipoprotein(a), which were differentially expressed between survivors and non-survivors. The LPA gene encodes for lipoprotein (a), an enigmatic class of protein with roles in wound healing, tissue repair, and vascular remodeling (38). LPA is of particular interest because, in humans, this gene is known to have a strong inherited component and is least affected by age, sex, diet, and environment (39). LPA gene is estimated to be accountable for 91% of the variance in lipoprotein's plasma levels (a) (40). Non-human primates and hedgehogs are the only two known species to naturally express this protein, making NHP a unique preclinical model for this protein (41-43).

Structurally, lipoprotein (a) is similar to low-density lipoprotein (LDL) attached to a glycoprotein called apoprotein (a) (41). Its elevated plasma levels in preclinical studies and clinical trials are associated with cardiovascular diseases, hence considered as an independent risk factor for coronary heart disease and stroke (44-46). However, the basal plasma concentration of lipoprotein (a) varies significantly with ethnicity; for example, Caucasians have low plasma levels compared to high levels in African Americans (46).

However, its role in pulmonary disease is conflicting. According to one clinical study, higher lipoprotein levels were associated with better pulmonary function

tests in community-dwelling older adults (47). Another study observed a decline in pulmonary function tests with elevated lipoprotein (a) levels (48). In a clinical study, patients affected by chronic obstructive pulmonary disease (COPD) reported low serum lipoprotein (a) levels relative to healthy individuals (38). Other than these studies, little information is available on the role of LPA in pulmonary disease, including RILI. In our study, we observed the downregulation of LPA in irradiated groups compared to non-irradiated controls. LPA was most downregulated in non-survivors relative to survivors, which also developed severe RILI, indicating disruption of a potential protective effect of LPA. Measuring lipoprotein (a) levels in plasma can further validate the reduced expression of the LPA gene. However, further, more profound studies are needed to understand better and evaluate the possible mechanisms responsible for the downregulation of LPA in RILI. This avenue of research could explain differential susceptibility and severity of RILI between survivors and non-survivors and may lead to a new opportunity for research for targeted interventions.

Our IPA analysis of common 101 DEGs also indicated angiotensinogen (AGT) as a critical upstream regulator. Apart from the well-established systemic renin-angiotensin system (RAS), which plays a critical role in circulatory homeostasis, local RAS within the lungs displays intrinsically high activity (49). Dysregulation of RAS in RILI is well known, and several investigations have studied various drugs that target RAS (50). In a rat study, angiotensin II expression was increased in a dose-dependent manner when lungs were exposed to 16 and 20 Gy (51). Angiotensin convertase enzyme (ACE) converts angiotensin I to angiotensin II

while ACE2 regulates angiotensin II degradation and hydrolysis. In turn, excess of Angiotensin II involves competitive inhibition, down-regulation, internalization, and degradation of ACE2 (52). ACE2 is highly expressed in the lungs and plays a crucial role in severe acute lung disease (53-55). ACE2 is also utilized by coronaviruses (SARS COVID-1 and COVID-19) to access target cells in the lungs (52, 56).

Briefly, a severe acute respiratory syndrome caused by coronaviruses, including the recent COVID-19 pandemic, share commonalities with RILI, not only in clinical symptoms and radiographic appearances but also in dysregulation of RAS. However, the multifaceted interplay of RAS is incompletely understood and requires further mechanistic insight (57).

Targeted exposure

Whole thorax irradiation (WTLI) models not only imitate the situation of accidental non-uniform or partial body exposure to irradiation (3) but also have provided us a great understanding of initiation and progression RILI (58). Using a multidisciplinary approach, our study in the WTLI NHP model has further extended the prior understanding by providing new insight into possible new molecular players. This study has increased our understanding of differential radio responsiveness to RILI, which would otherwise be difficult to tease apart in a clinical study where confounders' presence is unavoidable. However, clinically, this model's relevance would be weightier if a targeted volume of lung were exposed to single or multiple fractions using clinically relevant doses. Targeted field

exposure might help decipher the cause of radiographic changes beyond the radiation fields clinically associated with poor prognosis (59). Additionally, it can throw light on the differences in the mechanisms of pathogenesis and progression of RILI to a single fraction versus multiple fractionated doses.

Biological sampling and imaging

The relatively slow onset of RILI suggests significant subclinical damage occurring before the clinical symptoms appear. Plasma and broncho-alveolar lavage (BAL) samples collected at earlier time points can provide insight at the earliest cellular and molecular changes, determining the outcome. In our study, there was a disconnect between plasma and BAL sample collection time points. Future studies, including frequent earlier time points and matching plasma and BAL samples, will help understand the slow onset of the disease. Moreover, it will provide an opportunity for interventions and defining biomarkers (serum/plasma or BAL) to predict the onset and severity of the disease, therefore, offering a possibility to escalate radiation dose to target cancer effectively. In the present study, we utilized BAL samples for cytospin preparation and performed immunoassays to detect multiple analytes. Another possible extension of BAL utilization could be performing single-cell sequencing on BAL cellular pellets to get a deeper in-vivo insight into the disease process at the cellular level.

The role of quantitative thin-section CT analysis in evaluating diffuse lung diseases is growing as a viable tool that provide decision support to visual assessment and diagnosis (60). However, quantifying mixed pathologies like ground-glass opacity,

patchy lesions, nodules, and emphysema is challenging using basic histogram based threshold measures. Application of advanced higher-order textural analysis approaches like radiomics (61, 62) and deep learning methods (63) have been studied in diffuse interstitial disease like chronic obstructive pulmonary disease (COPD), usual interstitial lung disease (UIP) and idiopathic pulmonary fibrosis (IPF), however longitudinal studies related to RILI changes are scarce, and future research in this area can possibly decipher the complexity of mixed lesions like RP from RF non-invasively on CT scans. However, validation of these advanced approaches (radiomics and deep learning) in clinical practice is usually masked by many challenges which include variation in CT acquisition technique, segmentation methods, poor inspiration, quality of training data and lack of real-world assessment of outcomes (60).

Conclusion:

In conclusion, our clinical, pathological, and transcriptomic data improved the understanding of RILI in non-survivors and survivors in an NHP model. Our study supported the role of TGF-beta activation and macrophage polarization in RILI. RNA sequencing data highlighted non-survival-specific, survivor-specific, and common gene signatures, which differed by survival status. We identified new molecular targets for potential future mechanistic studies, including SERPINA3, ATP12A, GJB2, CLDN10, TOX3, and LPA. Their differential expression in non-survivors and survivors correlated with the severity of the disease. The observed upregulation of TGF-beta 1 extends prior reports by showing the increased expression of its associated target transcripts (SERPINA3, CCL11, CCL2, p21) at

the mRNA and protein level, indicating not only the role of active TGF-beta signaling but also the role of these molecules in the early proliferative phase of RP (non-survivors) and late phase of fibrogenesis and remodeling (survivors). We have shown that CT-based quantitative measurement indices of radiodensity, AVG lung density, PCHV, PCHV/TV extracted from the entire segmented lung histogram provide a unique opportunity to examine the disease process non-invasive manner and may serve as surrogates for likely patterns of histopathology in the future studies.

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BIOGRAPHICAL SKETCH

NAME: **Thakur, Priyanka**

POSITION TITLE: PhD Candidate in Molecular Medicine and Translational Sciences, Graduate Research Associate, Wake Forest University

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	END DATE MM/YYYY	FIELD OF STUDY
Dr. G.C. Negi College of Veterinary Sciences, Palampur, Himachal Pradesh	BVSc &AH	08/2008	Veterinary Medicine
Dr. G.C. Negi College of Veterinary Sciences, Palampur, North Carolina	MVSc	08/2010	Veterinary Pathology
Wake Forest Baptist Health, Wake Forest University, Winston Salem, NC	PHD	08/2021	Molecular Medicine and Translational Science

B. Positions and Honors

Positions and Employment

- 2011 - 2015 Research Assistant, Section of Hepatology/Gastroenterology,
Department of Medicine, Georgia Regents University, Augusta, GA
- 2015 - 2016 Graduate Research Assistant, Georgia Regents University, Augusta, GA
- 2016 - Present Graduate Research Associate, Molecular Medicine and Translational
Science, Wake Forest University, Winston Salem, NC

Other Experience and Professional Memberships

Laboratory Skills:

- Molecular biology: Multiplex immunoassays, ELISA, Immunohistochemistry, Geimsa staining, Wright Staining, Confocal Imaging, Blood biochemistry, Total leukocyte count, H&E staining, RNA and DNA isolation, Western blot,

Immunofluorescence, Serum Biochemistry, conventional and Real time PCR, Genotyping, and Cell culture, electron microscopy.

- Animals: Handling of lab animals (Mice, Rat, and Guinea pig) and genotyping.
- Computer analytical skills: Ingenuity pathway Analysis, Tableau, Mimics software (Materialise Mimics Innovation Suite), Visiopharm, Adobe Photoshop, Partek® Genomics Suite™ Software, Endnote, GraphPad, SPSS

Awards

- Scholar in training (SIT) travel award to attend the 2018 annual meeting of the Radiation Research Society, to be held at Chicago, Illinois (Sept 23-26).
- Student Travel Award to attend Society of Toxicological Pathology (STP) 38th Annual symposium, Raleigh, North Carolina, June 22–27, 2019.

C. Contribution to Science

- **Thakur P**, DeBo R, Dugan G, Bourland JD, Michalson K, Olson J, Register TC, Kock N, and Cline J. Clinico-pathological and transcriptomic analysis of radiation-induced lung injury in non-human primates. Under minor revision, **International Journal of Radiation Oncology, Biology and Physics**.
- **Thakur P**, Olson J, Dugan G, Bourland JD, and Cline J. Quantitative assessment and comparative analysis of longitudinal lung CT scans of chest-irradiated nonhuman primates. (manuscript in preparation, **Radiation Research**).
- Pathania R, Ramachandran S, Mariappan G, **Thakur P**, Shi H, Choi JH, Santhakumar Manicassamy, Kolhe R, Prasad PD, Sharma S, Ganapathy V, and Thangaraju M. Combined Inhibition of DNMT and HDAC Blocks the Tumorigenicity of Cancer Stem-like Cells and Attenuates Mammary Tumor Growth. **Cancer Research**, CAN-15-2249 Published June 2016

- **Thakur P**, Lamoke F, Chaffin J., Bartoli M, Lee J, Duncan MB. Dysplastic hepatocytes develop nuclear inclusions in a mouse model of viral hepatitis. **Plos one** (June16, 2014).

D. Posters/Presentation

- Sarvesh Kumar, Rajesh Kumar Asrani, **Priyanka Thakur** and Vipin Kumar Gupta. 'Comparative pathology of verminous pneumonia in cattle and goat.' Poster presented in Annual 1AVP conference, CL Davis Satellite Seminar and International Symposium on 'Philosophy of Disease Diagnosis Thru Morphological to bimolecular approaches' with Core Theme 'Diagnostic Pathology (2009).
- Srikar Kumar, **Priyanka Thakur** and Michael B. Duncan. 'TGF beta stimulates Heparanase 1 gene expression in hepatocellular carcinoma presented in summer.' 2011 medical scholar program in Georgia Regents University (Poster presentation).
- Abdul Rehman, **Priyanka Thakur** and Michael B. Duncan. 'Paracrine factors derived from hepatocellular carcinoma cells promote macrophage differentiation and pro-angiogenic gene expression.' 3rd International Conference on Stem Cells and Cancer (ICSCC-2012): Proliferation, Differentiation and Apoptosis. Poster presented In New Delhi, India.
- Grace Dougherty, Abdul Rehman, **Priyanka Thakur** and Michael B. Duncan. 'Paracrine regulation of macrophage phenotype by hepatocellular carcinoma cells is colony-stimulating factor 1 depended.' Summer 2012 Star program in Georgia Regents University (Poster presentation).
- Trey Forehand, **Priyanka Thakur** and Michael B. 'Type XVIII collagen is highly expressed in hepatocellular carcinoma.' Summer 2012 medical scholar program 2011 in Georgia Regents University (Poster presentation).
- James Mc Phail, **Priyanka Thakur** and Michael B. Duncan. 'Tgf beta 1 promotes talin – mediated focal adhesion assembly and hepatocellular carcinoma cell migration.' Summer 2013 star program in Georgia Regents University(Poster presentation).

- Abdul Rehman, **Priyanka Thakur**. 'Tumor associated macrophages show local proliferation in the microenvironment of Hepatocellular carcinoma. 'Abstract accepted for Digestive Disease week 2014'.
- Michael B. Duncan, **Priyanka Thakur**. 'Talin-1 regulate TGF b1- mediated focal adhesion assembly and hepatocellular carcinoma cell migration in vitro'. Abstract submitted for digestive disease week 2014.
- Ravirajsinh Jadeja, **Priyanka Thakur**, Sandeep Khurana, Michael B. Duncan. Type CXVIII collagen regulates cell survival and anti –oxidant response during elevated oxidative stress conditions, in vitro. Abstract accepted for AASLD 2014.
- Michael Duncan, Renumathy Dhanasekaran, **Priyanka Thakur**, Lewis R. Roberts. A potential role of type XVIII collagen as suppressor of hepato cellular carcinoma. Abstract accepted for AASLD 2014.
- **Priyanka Thakur**, BVSc, Greg Dugan, DVM; J. Daniel Bourland, PhD; John Olson MS, J. Mark Cline, DVM, PhD, DACVP, and Mary E. Sunday, MD, PhD. Single dose of gastrin-releasing peptide mAb 24 hours post-thoracic radiation extends survival and reduces pulmonary fibrosis in rhesus monkey survivors. Annual meeting of Radiation Research Society, Cancun, Mexico, 2017 (Abstract and Poster).
- **Priyanka Thakur**, BVSc, Ryne Debo, PhD, Greg Dugan, DVM, J. Daniel Bourland, J. Mark Cline, DVM, PhD, DACVP. Phenotypic characterization and transcriptomic analysis approach to understand the pathology and pathogenesis of radiation-induced lung pathology in non-human primates. Annual meeting of Radiation Research Society, Chicago, Illinois, 2018 (Abstract and Poster presented).
- **Priyanka Thakur**, BVSc, Ryne Debo, PhD, Greg Dugan, DVM, J. Daniel Bourland, Thomas C. Register PhD, Nancy D. Kock, DVM, PhD, DACVP, J. Mark Cline, DVM, PhD, DACVP. A Multidisciplinary Approach to Understand the Pathogenesis of Radiation Induced Lung Toxicity. 38th Annual symposium of Society of Toxicological Pathology (STP), Raleigh, North Carolina, 2019 (Abstract and Poster presented).

Conference/Workshop attended

- Society of Toxicological Pathology (STP), 38th Annual symposium, Raleigh, North Carolina, 2019 (Poster presented).

- Annual meeting of Radiation Research Society, Chicago, Illinois, 2018 (Poster presented).
- ACVP and ASVCP annual meeting in Atlanta, Georgia, 2014.
- International Symposium on “Quality Assurance in Pathology and Disease Diagnosis” and Satellite Seminar on “Descriptive Gross Microscopic Veterinary Pathology in Necropsy”, Biopsy Certification Examination” (C.L. Davis Foundation USA) (2008, India).
- Industry Awareness and Interaction (IAI) for student Program organized during the Indian Animal Industry Expo (2007).