LOW DOSE RADIATION RESPONSE IN THE LUNGS AND SPLEEN

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IN THE LUNGS AND SPLEEN

Ву

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Lay Abstract

Diagnostic procedures such as computed tomography (CT) and X-ray imaging are a common part of intensive and critical care medicine. Some physicians are concerned that this exposure to diagnostic radiation may negatively affect the health of their patients, who are prone to infection and who often need a machine to breathe for them. In order for doctors to make informed decisions, the possible effects of these levels of radiation must be understood. To improve this understanding, this study looked at the short-term effects of X-ray doses on key organs affected by critical illness, the lungs, and the spleen, which is an important organ of the immune system that helps fight infection.

Using an animal model, doses of X-rays in the range of diagnostic radiation (0-200 mGy) were examined and no significant effect on lung health was found. However, the highest dose of X-rays tested, which is greater than that expected for a single CT scan, did have an effect on cells from the spleen. Spleen cells are designed to multiply when they detect various types of infection, so that there are more immune cells to fight that infection. The cells from animals that were given the highest dose of X-rays didn't multiply as much in response to infective stimulus as those from animals that received lower doses, or no X-rays at all.

Overall, it seems that diagnostic radiation doesn't have an effect in the lungs, but very high diagnostic doses could slightly affect a patient's ability to fight infection. It is important to remember that patients in critical care are very sick, so doctors have good reason to use diagnostic tools available to them. Missing a diagnosis has major and immediate consequences, which must be balanced against the potential small risks of using radiation to make that diagnosis.

Abstract

Patients in the intensive and critical care unit frequently undergo diagnostic radiology procedures such as computed tomography (CT) and X-ray imaging. As these patients often require respiratory assistance and are vulnerable to infection, it is important to understand the potential acute effects of these procedures on the lungs and immune system. The aim of this study was to determine the acute effects of a single clinically relevant low-dose X-ray exposure in order to establish baseline responses in markers of lung injury and immune function in a rodent model.

Male Sprague-Dawley rats (200-250 g) were irradiated with 0, 2, 20 or 200 mGy whole-body X-rays in an XRAD 320 irradiator. Markers of lung injury and immune activation in the lungs and spleen were evaluated 0.5, 4, and 24 h post-irradiation to examine the acute stages of the physiological and immunological response. Intratrachaeal lipopolysaccharide (LPS) exposure was used as a positive control model of acute lung injury. Lung injury endpoints included respiratory mechanics, pulmonary oedema, arterial blood oxygenation, histological analysis, and cellular and proteinaceous infiltrate *via* bronchoalveolar lavage. Immunological measures in the spleen focused on splenocyte proliferation, using the MTS assay and differential cell counts before and after stimulation with LPS or concanavalin A (Con A), as compared to unstimulated cultures.

Splenocyte proliferation in response to Con A, but not LPS, was significantly decreased after 200 mGy *in* vivo X-irradiation (repeated measures two-way ANOVA with LSD post-hoc, p=0.024). There was a non-significant trend towards increased lung tissue resistance after 200 mGy, with no significant effect on pulmonary oedema, cellular or proteinaceous infiltrate, nor other aspects of respiratory mechanics (two-way ANOVA with LSD post-hoc, p>0.05).

A clear understanding of these immunological and physiological effects informs the responsible use of medical diagnostic procedures in modern medicine. Establishment of this model for the elucidation of acute immune effects of lowdose radiation will facilitate future work evaluating these parameters in disease models, mimicking patients in intensive care.

Acknowledgements

"Science is a collaborative effort. The combined results of several people working together is often much more effective than could be that of an individual scientist working alone." - John Bardeen (Nobel Prize in Physics 1956, 1972)

My thesis would not be complete without the combined efforts of so many individuals. Each and every one of you has contributed to this accomplishment.

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List of Abbreviations and Symbols

BAL	bronchoalveolar lavage
Con A	concanavalin A
G _{tis}	tissue resistance
H&E	haematoxylin and eosin
H _{tis}	tissue elastance
ICCU	Intensive and Critical Care Unit
ICRP	International Commission on Radiation Protection
IR	ionizing radiation
LDIR	low dose ionizing radiation
LPS	lipopolysaccharide
OD	optical density
PBS	phosphate-buffered saline
PEEP	positive-end expiratory pressure
Raw	airways resistance
SDS	sodium dodecyl sulphate

Declaration of Academic Achievement

I, Stacy Muise, am the sole author of this manuscript and conducted the original research contained herein. However, I recognize the invaluable contributions of my fellow researchers to the research process:

Assoc. Prof. Dixon, as my immediate supervisor, was involved at every step of the research process, including experimental design, implementation, data analysis and interpretation, and editing of this manuscript. Prof. Douglas Boreham and Assoc. Prof. Antony Hooker were instrumental in project design and contributed to editing of the manuscript. James McEvoy aided in the optimization and follow-through of the splenocyte proliferation protocol. Tara Bouchier assisted with the routine implementation of experimental protocols. Prof. Andrew Bersten and Dr. Shailesh Bihari provided insightful advice throughout, especially for the interpretation of respiratory mechanics results.

1 Introduction

lonizing radiation is, and has always been, all around us. Natural sources include cosmic rays, radioisotopes in food and water, radon gas, and external terrestrial radiation [1]. The typical annual dose in North America from such sources is about 2.4 mSv, but ranges from 1 to 13 mSv, with certain populations exceeding 20 mSv per year. The additional dose from artificial sources is overwhelmingly determined by medical diagnostic exposures, which vary greatly according to individual health needs [2]. As the prevalence of such diagnostic procedures has escalated in recent decades [3], investigating possible risks of these exposures is of increasing importance.

Often in conversations surrounding the health risks of radiation exposure, the risks of *avoiding* such exposures are minimized or completely ignored. For example, targeted rather than whole-body computed tomography (CT) scanning resulted in higher mortality among trauma patients, according to a recent meta-analysis [4]. This difference was attributed to early identification of underlying injuries among patients undergoing whole-body CT. Furthermore, the National Lung Screening Trial Research Team found that low-dose CT screening reduced lung-cancer mortality by 20% compared to simple chest radiography, due to a 3-fold increase in positive findings [5]. These cases highlight the benefits of higher-dose scans, and the harm that can result from dose reduction strategies. Radiological procedures remain a key tool in diagnostic medicine.

1.1 Critical care medicine

Patients in the intensive and critical care unit (ICCU) require a high level of medical supervision and intervention, including invasive monitoring, regular diagnostic

procedures, and organ support. Given their precarious health status, the risks and benefits of these actions must be carefully weighed.

A common form of organ support provided in the ICCU is mechanical ventilation, with a third of ICCU patients requiring mechanical ventilation at some point in their stay [6]. Respiration is essential for life; therefore, the pulmonary function of patients is of utmost importance. Due to the invasive nature of care, ICCUs bear a disproportionate rate of nosocomial infection relative to other wards [7]. By far the most common sites of infection are the lungs and respiratory tract. Pneumonia in particular is strongly associated with mechanical ventilation. Thus, it is important to understand the possible effect of procedures on respiratory and immune health.

1.1.1 Measuring respiratory function

Respiratory mechanics are a highly sensitive measure of lung function. Using a computer-controlled ventilator, a range of frequencies are applied to the lungs to measure lung impedance from the airways and tissues. The data from this forced oscillation technique are fitted to the constant phase model:

$$Z = R_{aw} + jI + (G_{tis} - j \cdot H_{tis})/(2\pi f)^{\alpha}$$

where Z is lung impedance, R_{aw} is airway resistance, G_{tis} is tissue resistance, H_{tis} is tissue elastance, I is inertance, *j* is the imaginary unit, f is frequency and $\alpha = (2/\pi)arc \tan (H_{tis}/G_{tis})$ [8]. Inertance is negligible and is therefore not reported.

Airways resistance is the resistance to the flow of air through the airways of the lungs [9]. The most influential factor on this parameter is the radius of the airways. If the airways become constricted, for example in asthma or anaphylactic shock, then the resistance to airflow increases by the fourth power of the radius, greatly increasing lung impedance. This metric is generally an indicator of a hypersensitivity response [10].

Tissue resistance and elastance, by contrast, indicate how difficult it is to expand the lungs to accommodate a volume of air [9]. Tissue resistance is a measure of energy dissipated in the tissues, as opposed to that stored as elastic energy during inspiration [8]. Elastance is the elastic recoil component of lung expansion. It is the inverse of compliance, which refers to how easily the lungs change shape [9]. Increased elastance corresponds to less compliant lungs, meaning they are more difficult to inflate, usually indicating a decrease in surfactant function or an increase in tissue stiffness due to fibrotic or structural changes.

The role of surfactant is to decrease the surface tension on the inner surface of alveoli [11]. The thin layer of water that facilitates gas exchange at the alveolar surface exerts a net inward force due to surface tension. When surfactant is decreased, degraded or inactivated, the surface tension increases, increasing the force required to inflate the alveoli.

1.1.2 Immune function - the spleen

The spleen plays a key role in the immune response to infection, especially with polysaccharide-encapsulated bacteria [12,13]. Located in the left abdomen adjacent to the stomach, the spleen is the largest secondary lymphoid organ. In addition to its innate and adaptive immune functions, it also removes old red blood cells and foreign material from the circulation [14]. These dual functions are reflected in the distinct structural regions of the spleen. The 'red pulp' acts as the blood filter, with specialized macrophages phagocytosing apoptotic cells and other debris. The 'white pulp' contains a variety of T- and B-cells, macrophages, dendritic cells, and plasma cells. The interface of these two regions, the marginal zone, allows for efficient screening for and clearance of blood-borne pathogens

[15]. Marginal zone macrophages target pathogens based on pattern recognition, whereas marginal zone B cells and dendritic cells target antigens, triggering an adaptive response.

1.1.3 Critical care medicine and radiation

ICCU patients are regularly subject to diagnostic imaging procedures such as chest X-rays and CT scans [16]. Some physicians have raised concerns regarding the risks of radiation exposure, resulting in efforts to reduce the dose by refinement of technique or replacement with non-radiographic alternatives [16–18]. Although gains have been made in dose reduction while maintaining image quality, the radiation cannot be completely eliminated, and non-radiographic alternatives are not always available or practicable. For example, CT is the recommended modality for evaluating lung disease, chest and abdominal traumas, and tube or catheter placement, all common in the ICCU [19].

The level of radiation exposure from diagnostic imaging is highly dependent on the nature of the procedure. A simple chest X-ray, which ICCU patients used to receive daily [20] and is still the most common radiographic procedure [18], results in an average dose of 0.02 mGy [21]. Doses from CT imaging range from about 2-15 mGy, depending on target region of the body and image resolution required [2]. Maximum doses can exceed 100 mGy for fluoroscopy and angiography, which use live CT imaging to guide the internal placement of medical tools [22].

The International Commission on Radiation Protection (ICRP) does not recommend a limit on medical radiation exposure [23]. It is left to the discretion of the doctor to determine whether the benefit of the procedure outweighs the risk of adverse effects due to radiation exposure. In order to make such decisions, physicians must have accurate information regarding the risks of radiation exposure, which are still under contention at such low doses (<100 mGy).

1.2 Effects of diagnostic-level radiation

Deterministic effects of radiation exposure are those that occur predictably in response to increasing doses, such as skin erythema [23]. Stochastic effects are those that are increasingly *likely* to occur with increasing dose, such as cancer.

The most common risk model for cancer is the linear no threshold (LNT) model, in which a unit increase of radiation incurs a linearly proportional increase in the risk of developing cancer, with no threshold below which risk is not increased [23]. This relationship was established based on results at high doses, and extrapolated down to low doses, in the absence of definitive data for that region. Although this risk depends on cancer type, target organ, age, sex, and other risk factors, the overall estimated risk of cancer is 5.5% per Sv [23]. However, the ICRP has recognized that this estimate should not be applied for doses below 100 mGy.

There is much debate regarding the actual risk relationship at low doses, defined variously as doses below about 100 to 1000 mGy [23–25]. Alternative models include: supralinearity, in which risk is greater than expected at low doses; a threshold model, in which there's a threshold below which there is no increased risk, and; hormesis, in which low doses actually decrease risk of stochastic effects (**Figure 1**) (22,25,26).

Phenomena associated with the hormesis model include cancer prevention or even treatment with low dose ionizing radiation (LDIR) [28–30], successful treatment of inflammatory disorders with LDIR [31–34], and adaptive responses, in which LDIR moderates the effects of high dose radiation or other stressors [35]. Other reports seem to contradict these findings, showing low-dose hypersensitivity [26], bystander cell damage [36], or simply linearly increasing risk in alignment with high dose relationships [37].

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Figure 1. Risk models of radiation exposure. From [38].

1.2.1 Lungs

The lungs are a highly radiosensitive organ. One of the major dose-limiting side effects of radiotherapy in the region of the thorax is pneumonitis, or inflammation of the lungs [39]. Prolonged pneumonitis can lead to fibrotic tissue repair through increased collagen and elastin production, increasing the stiffness of the lungs and therefore work of breathing, and decreasing oxygenation capacity. However, the risk of developing such inflammation after diagnostic-level doses (<100 mGy), has not yet been investigated.

Most low-dose research in the lung has focused on cancer as an end point, which may also indirectly indicate immunological changes. For example, DNA damage, the precursor to cancer increased in lungs after single and repeated 100 mGy doses of photons [40]. However, 100-1000 mGy gamma-irradiation reduced lung tumour incidence in mice, an effect attributed to enhanced anticancer immunity [41].

Few groups have examined molecular or immunological changes in the lungs in response to low doses of radiation. In vitro 50 mGy gamma-irradiation stimulated proliferation of human lung fibroblasts 24-48 hours post-IR, possibly through the ERK pathway, but also in the presence of p38 activation 2-10 hours post-IR [42]. In vivo 500 mGy y-rays induced increased apoptosis in the lungs of C57BL/6 mice [43]. Physiological changes in these mice were accompanied by dose-dependent alterations in gene or protein expression in the lungs of adult but not neonatal mice, notably IL-1B, IL-1R2, CKCR2, and IL-6. Apoptosis was moderately increased in the lungs of female C57BL/6 mice 3 days after 200 mGy whole-body y-irradiation [44]. Characterization of gene expression across multiple organs in the same model revealed that most overexpression occurred consistently across all organs tested, but protein tyrosine kinase and platelet membrane glycoprotein lib were specific to both the lungs and spleen. The interpretation of this finding is difficult, as protein tyrosine kinases are a broad class of enzymes that translate a variety of cell signals across the membrane [45], and the particular type was not indicated by the researchers. The glycoprotein's function is to bind fibrinogen or other factors for adhesion [46], which may imply platelet recruitment.

Overall, it seems there may be some subtle molecular changes occurring in the lungs in response to low dose radiation, though the health consequences of these results are unclear. Much more research is required, given the paucity of studies examining the effects of clinically-relevant doses on the lungs.

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1.2.2 Spleen

At high doses, ionizing radiation suppresses spleen function. Whole-body 1-7 Gy γ -irradiation of C57BL/6 mice induced a decline in the number of cells in the spleen, as well as the remaining cells' proliferative response to the mitogen phytohaemagglutinin A [47,48]. Single 1-5 Gy γ -irradiation of BALBc/3 mice reduced IFN- γ expression, inhibiting STAT1 phosphorylation and IRF-1 expression, with corresponding suppression of cell-mediated immunity [49]. Doses below 1 Gy, by contrast, have yielded less obviously damaging and more immunologically interesting results.

LDIR-induced rates of apoptosis in the spleen are somewhat contradictory. Gamma-irradiation of 500 mGy or greater seems to increase apoptosis 4-6 h postirradiation, sometimes persisting to 24 h [50], and in other cases resolving [43]. Also within 4 h, 10-50 mGy doses decreased apoptosis, with 100 mGy having no significant effect [50]. Analysis three days after 200 mGy γ -irradiation yields contradictory results. Immune cells from the spleen (splenocytes) stimulated with Con A after *in vivo* radiation were more apoptotic when sourced from BALBc/3 mice, but less apoptotic from C57BL/6 mice [51]. This is in direct contrast to increased apoptosis detected in the spleens of C57BL/6 mice after the same dose [44]. This variable response to 200 mGy requires further investigation with different animal models to elucidate the dynamics of spleen effects in this clinically-relevant dose range.

Furthermore, several groups have found that low doses of radiation actually increased the splenocyte proliferation response in both BALBc/3 and C57 mice [50–58]. This effect was often time- and/or dose-dependent, and somewhat inconsistent among studies.

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The distribution and function of remaining leukocytes has also varied among studies. Enhanced proliferation in response to low dose radiation in the spleen has been variously attributed to antigen-presenting cells [52,59–61], CD8⁺ cytotoxic T-cells [57], and CD4⁺ helper T-cells [62]. Other altered immune cell functions have also been identified, such as enhanced NK cell cytotoxicity [63,64] and macrophage phagocytic activity [57]. These changes are often accompanied by shifts in cell signalling patterns. Low doses downregulated most cytokines tested in C57BL/6 mice, with a shift away from Th1/2 responses, indicating an overall anti-inflammatory effect [50]. BALB/c mice X-irradiated with 100 and 200 mGy in single or 10 repeat doses increased splenocyte production of IL-2 and IFN- γ [65]. *In vivo* 75 mGy X-irradiation of Kunming mice decreased IL-10 from splenocytes, with increased CD-28 expression, indicating increased immune activation [59,66].

Much of the work examining spleen response to radiation has used highly inbred strains of mice, which are known to exhibit skewed immune responses [67]. For example, C57BL/6 mice are biased toward Th 1 responses, and BALB/c toward Th 2 [68]. These peculiarities restrict the applicability of results to humans. To address this, a broader variety of animal models should be used, preferably with less skewed immune responses, such as the outbred Sprague Dawley rat strain.

1.3 Research outline

Risk analyses of adverse effects from radiation usually focus on stochastic phenomena such as cancer induction. However, in the ICCU, acute effects must be adequately weighted, given the imminent risk of death. Therefore, this study focused on deterministic effects within a 24 h window post-irradiation. Due to the precarious respiratory health of many ICCU patients, as well as the high risk of infection, two key organs were investigated: the lungs and the spleen. The question to be addressed herein is whether clinically-relevant whole-body Xirradiation elicits biologically significant changes to pulmonary or immune function. The aim of this study was to establish a healthy animal model of lowdose X-ray exposure in order to establish baseline physiological effects and immune responses in the lungs and spleen. Sprague-Dawley rats were chosen as the model animal due to the reduced level of inbreeding compared to mouse models, thus reducing immunological peculiarities [67,69] that limit translation to humans. X-ray doses were based on expected exposure from diagnostic procedures, with 2 and 20 mGy representing the median range of CT scans [2], and 200 mGy mimicking possible maximum exposure from continuous imaging procedures such as fluoroscopy or angiograms [22].

The endpoints of this investigation included parameters of acute lung injury and immune response in the spleen. Acute lung injury was measured by blood oxygenation levels, pulmonary oedema, immune cell and protein infiltration, respiratory mechanics, and histological changes. The immune response in the spleen was evaluated based on the proliferation response of splenocytes cultured with or without stimulus.

It was hypothesized that doses of X-radiation up to 200 mGy would not induce acute lung injury, nor adversely affect splenocyte proliferation, and may even enhance splenocyte proliferation at some doses.

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2 Methods

2.1 Ethics

All animal procedures were approved by the Animal Welfare Committee of Flinders University in Adelaide, Australia, where the research took place. These studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose and the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

2.2 Animals

Male Sprague Dawley rats (200-250 g) were sourced from the University of Adelaide Laboratory Animal Services and the Flinders School of Medicine Animal Facility. Animals from the University of Adelaide were allowed a week to recover from transport before irradiation. See **Figure 2** for an overview of the following methods.

2.3 Irradiation

Rats were trained to be comfortable in a Perspex restraint to minimize stress during exposure. Training involved 5 min in the restraint, covered to limit light, followed by 10 min uncovered, in the irradiation room with the XRAD on standby, at least three days before irradiation. Froot Loops[™] were used as a positive reward. Rats were then irradiated using an XRAD 320 cabinet irradiator (Precision X-ray, USA) to deliver whole body doses of 2 mGy, 20 mGy, or 200 mGy (**Table 1**). Sham-irradiated control rats were placed in the cabinet irradiator for 15 min in standby mode, so that the noise and light levels were consistent for all rats. Dosimetry information was provided by Professor Eva Bezak, Medical Physicist and Professor in Medical Radiation, School of Health Sciences, University of South Australia. XRAD 320 calibration was performed by Dr. Thomas Hutten, medical physicist at the Royal Adelaide Hospital, using an ion chamber and confirmed with a thermoluminescent dosimeter wrapped in wet gauze to simulate an animal.

Dose (mGy)	2	20	200		
Time (s)	6	77	785		
<i>Filter</i> 0.8 mm Tin + 0.25 mm Copper + 1.5 mm Alum (HVL ≈ 3.7 mm Cu)					
FSD	62.5 cm (accounting for Perspex backscatter block)				
Shielding	0.6 cm Perspex (rat restraint)				
keV		200			
mA		1			

Table 1. XRAD settings for X-irradiation. All settings remained the same for each dose, except for time of irradiation.

Due to the nature of endpoint analysis, only one rat could be analysed at a time, therefore controls could not be implemented perfectly in parallel. They were instead distributed throughout the process, ensuring at least one of the four to five weekly rats was a negative control. Furthermore, the logistics of post-irradiation times restricted the randomization of subjects such that most 4 h rats were analysed in the afternoon, and most 0.5 and 24 h rats were analysed in the morning.

2.4 Positive controls

As a positive control indicative of biologically significant pulmonary outcomes detectable with the proposed methodology, a group of animals with endotoxininduced acute lung injury were analyzed alongside the irradiated treatment groups and sham-irradiated negative control groups. This endotoxin model of acute lung injury involves the intratracheal administration of lipopolysaccharide (LPS), a distinctive component of gramnegative bacteria that triggers a strong immune response [70]. The resulting inflammatory cascade leads to cellular injury, breakdown of the alveolocapillary barrier, influx of oedematous fluid, and impairment of surfactant production and function. Measurable outcomes of this model include decreased arterial blood oxygenation, increased lung tissue resistance and elastance, pulmonary oedema, immune cell recruitment to the lungs, and proteinaceous infiltrate in the alveoli [71].

2.5 Surgery

The effects of X-ray exposure were evaluated 30 minutes, 4 hours, and 24 hours post-irradiation. At these times, rats were anaesthetized by i.p. injection of ketamine hydrochloride (100 mg/kg) and medatomidine (0.5 mg/kg). Body temperature was maintained at 37°C with a heating pad. Blood pressure was monitored with a Non-Invasive Blood Pressure Controller and Powerlab, (ADInstruments, AUS) to ensure that animals were sufficiently anaesthetised throughout the process.

2.5.1 Tracheotomy

After testing toe-pinch reflex to confirm anaesthesia, the trachea was exposed by sharp dissection. Surrounding connective tissue was freed by dissection, a small lateral incision was made, and a 16-gauge stainless steel cannula was inserted into the trachea, secured with a 3/0 suture (Dynek Pty Ltd).

2.5.2 Non-injurious ventilation

The lungs were ventilated using a computer-controlled flexiVent small animal mechanical ventilator (SCIREQ Scientific Respiratory Equipment, Montreal, Canada) with a tidal volume of 7 mL/kg, 2 cmH₂O positive end-expiratory pressure (PEEP), fraction of inspired oxygen of 1.0 on 100% oxygen, and respiration rate of 120 breaths/min.

2.5.3 Positive control group

Rats were treated identically to those in the radiation and sham groups with the exception that they were administered 15 mg/kg of LPS in 0.9% saline via the tracheal cannula in three 0.1 mL aliquots, five minutes apart, each followed immediately by a 3 mL air bolus and an induced sigh (2.5 x tidal volume, Vt) to facilitate the distribution of LPS throughout the lungs. Rats were mechanically ventilated for a further 30 min to allow the development of an immunological response before endpoint analysis (as below).

2.5.4 Respiratory mechanics

Rats were paralyzed by i.p. injection of pancuronium bromide (1 mL) to prevent spontaneous breathing during mechanics measurements. Respiratory impedance, an indication of lung function, was measured by applying low-frequency volume oscillations *via* the flexiVent small animal ventilator [8]. Before measurement of lung mechanics, a single sigh breath of 2.5x tidal volume (20 mL/kg) and 2 cmH₂O PEEP held for 6 s was delivered to normalize lung volume history. To measure impedance of the respiratory system, this was immediately followed by a forced oscillation of 16 s of interrupted ventilation during which 19 sine waves with multiple prime frequencies (0.25 to 19.125 Hz) and a peak-to-peak volume excursion of 1.0 mL above the end-expiratory lung volume were applied. The

volume displacement of the ventilator's piston and cylinder pressure signals were low-band pass filtered at 30 Hz and sampled at 128 Hz before acquisition. The data were then fitted to the constant phase model:

$$Z = R_{aw} + jI + (G_{tis} - j \cdot H_{tis})/(2\pi f)^{\alpha}$$

where Z is lung impedance, R_{aw} is airway resistance, G_{tis} is tissue resistance, H_{tis} is tissue elastance, I is inertance, *j* is the imaginary unit, f is frequency of the oscillation and $\alpha = (2/\pi)$ arc tan (H_{tis}/G_{tis}). Inertance, a measure of the pressure difference required to change flow rate, is negligible and is therefore not reported.

2.5.5 Blood collection

Immediately following the collection of lung mechanics data, a cardiac bleed was performed. The upper abdomen was opened by blunt dissection, just enough to access the heart from below. A 0.2 mL blood sample was taken from the left ventricle for analysis of pH, PaCO₂, and PaO₂ *via* a benchtop blood gas analyzer (ABL5, Radiometer, Denmark). A larger blood sample (5 mL) was also collected and stored in a 5 mL lithium-heparin Vacuette tube to prevent coagulation. This tube was centrifuged at 540 g (2400 rpm) for 10 min and the resultant plasma stored in four 500 µL aliquots at -80°C for potential cytokine analysis.

2.5.6 Lung harvest

Following blood sampling, mechanical ventilation was ceased and the trachea (with inserted tracheal cannula), lungs, and heart removed *en bloc*. The upper right lobe of the lung was tied off and the lungs degassed in ice-cold saline. A bronchoalveolar lavage (BAL) was performed on the remaining lobes at 2°C with three separate 32 mL/kg body weight volumes of 0.9% sodium chloride (NaCl), each instilled and withdrawn three times. The upper lobe was then resected and weighed for determination of wet weight. The middle and bottom right lobes were

resected, snap frozen in liquid nitrogen, and stored at -80°C for potential RNA and MPO activity analysis, respectively. The left lobes were fixed in 10% neutral buffered formalin at 20 cm H₂O for 2 h, submerged in formalin for 48h, and then stored in phosphate-buffered saline (PBS) with 0.01% sodium azide until paraffin embedding.

2.5.7 Spleen harvest

After removal of the lungs, the spleen was resected and cut into four parts. The posterior end was snap frozen in liquid nitrogen and stored at -80°C for subsequent RNA analysis. The next quarter was frozen in Tissue-Tek optimum cutting temperature (OCT) Compound (ProSciTech, AUS) by floating the mold on a thin layer of isopentane cooled to near-freezing by liquid nitrogen. The other middle quarter was fixed in formalin for 48 h before paraffin embedding. The final quarter was placed in ice cold PBS for immediate splenocyte isolation (see section 2.7.1 Splenocyte isolation).

2.6 Lung processing

2.6.1 BAL processing

The bronchoalevolar lavage (BAL) fluid was centrifuged at 626 g for 10 min. The supernatant was collected and stored at -80°C for potential determination of cytokine concentration and total lung lavage protein. Cell counts were performed on the resultant cell pellet by trypan blue exclusion light microscopy. The remainder was split for differential cell counts and potential RNA analysis.

2.6.2 BAL cell count

The cell pellet was resuspended in the remaining ~100 μ L of lavage fluid, and the volume of the resuspension measured. A 12 μ L aliquot was diluted two-fold in milliQ water, then two-fold again in 0.4% trypan blue solution (Sigma Aldrich, USA). A 10 μ L aliquot of this 4X diluted solution was loaded onto each side of a haemocytometer and viewed on a Leitz SM-LUX microscope (Ernst Leitz GmbH, Wetzlar, Germany) at a magnification of 40X. The average number of viable cells in a 1 mm² area was counted, and the concentration of cells in suspension calculated.

2.6.3 BAL cell smears

A cell smear was performed on the remaining resuspended BAL pellet for cell differential and immunohistochemistry. Cells were fixed in 4% paraformaldehyde at a concentration of 1×10^6 cells/mL and total volume of 250 µL. The mixture was incubated at room temperature for 20 min, then centrifuged for 30 s at max speed, 17 000g (14 000 rpm). The supernatant was discarded and the fixed cells rinsed twice with 250 µL milliQ water, centrifuging for 30s at max speed each time. The final cell pellet was then resuspended in 25 µL of milliQ water. The final cell suspension was smeared onto SuperFrost Plus slides, 5 µL each, and dried on low heat. Dry smears were stored in the dark at 4°C until staining.

2.6.4 BAL cells for RNA analysis

The remaining BAL cells were collected by centrifugation for 5 min at 540g (2500 rpm). The supernatant was discarded and the cell pellet covered with 100 μ L of RNAlater (Sigma Aldrich, USA). Covered cell pellets were left overnight at 4°C and then stored at -80°C for potential RNA extraction.

2.7 Spleen processing

2.7.1 Splenocyte isolation

The anterior quarter of the spleen was homogenized in phosphate-buffered saline (PBS) using the plunger of a 3 mL syringe and a 70 μ m cell strainer (Corning, USA). The resultant cell suspension was centrifuged for 10 min at 540 *g* (2400 rpm) and the supernatant discarded. The cell pellet was continuously pipetted up and down for three minutes with 750 μ L of 0.8% M (0.15 M) ammonium chloride (pH = 5) to lyse the erythrocytes. The solution was neutralized by addition of PBS to 15 mL, centrifuged for 10 min at 540 *g*, and the cell pellet rinsed twice with 15 mL of fresh PBS, centrifuging for 10 min at 540 *g* each time. The final pellet was resuspended in 10 mL of RPMI media with 25 mM HEPES and NaHCO₃ (Sigma Aldrich, USA, cat#R5886), 2 mM L-alanyl-glutamine (Sigma Aldrich, USA), and 100 U Penstrep (Sigma Aldrich, USA). The concentration of cells was counted using a haemocytometer and adjusted to 2 x 10⁶ cells/mL with complete media.

2.7.2 Splenocyte culture and proliferation assay

Two 96-well cell culture plates (Greiner Bio-One, USA) were prepared with six replicates each of 50 μ L complete media, media with 40 μ g/mL of lipopolysaccharide (2X LPS; Sigma Aldrich, USA, cat#L2880), and media with 4 μ g/mL of concanavalin A (2X Con A; Sigma Aldrich, USA, cat#C2010). To each of these wells, 50 μ L of the 2 x 10⁶ cells/mL suspension was added, for a final concentration of 1 x 10⁶ cells/mL in each, and 20 μ g/mL and 2 μ g/mL of LPS and Con A, respectively. Six additional wells with 100 μ L of media were used as plate blanks, and the whole array surrounded with a moat of 100 μ L PBS to prevent evaporation effects. An additional 6-well plate was prepared in a similar manner, adding 500 μ L of cells to 500 μ L each of media, 2X LPS, and 2X Con A in one well

each. These plates were then incubated at 37°C and 5% CO₂, one 96-well plate for 1 h and the other two plates for 48 h.

After their respective incubation periods, the proliferation of cells in the 96-well plates was evaluated using the MTS Assay Kit (Promega, USA). Each well received 20 μ L of MTS reagent, and the entire plate was incubated at 37°C in 5% CO₂. After 30 min and 60 min, the absorbance at 490 nm was measured with a spectrophotometer (VersaMax, Molecular Devices, USA). Data were trimmed to reduce CVs below 10 before statistical analysis by repeated measures two-way ANOVA. The within-subjects factors in this analysis were the incubation time (1 h vs 48 h) and the presence or absence of stimulus (LPS or Con A vs no stimulus). LPS and Con A were analysed separately against the control cells. The between-subjects factors were radiation dose (0, 2, 20, 200 mGy) and time post-irradiation (0.5, 4, 24 h).

2.8 Sample analysis

2.8.1 Pulmonary oedema

The previously weighed (wet) upper lobes were dried overnight in a freeze dryer, then weighed to obtain the dry weight for calculation of the ratio of wet to dry weights, an indication of pulmonary oedema. Wet and dry lobe mass to body mass ratios were also calculated.

2.8.2 Paraffin embedding and sectioning of left lung lobe and spleen

The spleen and left lung lobes were dehydrated by sequential submersion in milliQ water (30 min) then 70%, 80%, 90%, 100%, and again 100% ethanol (1 h each). The tissues were then cleared in chloroform overnight for embedding the next day. The left lobe was separated from the rest of the organs and equilibrated in molten
paraplast (Leica Biosystems, Germany) before infiltration under vacuum for two 45 minute periods, changing the wax each time. Samples were embedded in moulds using the Tissue-Tek paraffin embedding system and stored at room temperature until sectioning. Paraffin-embedded tissues were cut into 5 μ m sections using a microtome and collected on SuperFrost Plus slides (Thermo Fisher Scientific, USA), then dried overnight at 37°C and stored at room temperature until staining.

2.8.3 Histology

Lung sections were stained with haematoxylin and eosin (H&E) and imaged by brightfield microscopy for histological analysis. Scoring was performed of pulmonary inflammatory cell infiltration and alveolar wall thickening using a semiquantitative score (0-3; 0= 0-25%, 1 = 25-50%, 2 = 50-75%, 3 = 75-100%) on blinded sections [72].

2.8.4 Differential cell counts

Both BAL and splenocyte smears were stained with Quick Dip (Thermo Fisher Scientific, USA) for differential cell counts. Slides were dipped repeatedly in absolute methanol for 20 s, Fixative for 5 s, Solution A (Eosin Yellowish) for 15 s, and Solution B (Methylene Azure B) for 5 s, then rinsed thoroughly in deionized water before air drying. Stained slides were dehydrated by two brief dips in ethanol, then submerged in xylene twice for two minutes each before mounting with DPEX.

2.8.5 BAL protein content

The protein content of BAL supernatants was determined using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, USA). A standard curve of 3.91 to 250

 μ g/mL BSA was created by two-fold dilutions in 2% sodium dodecyl sulphate (SDS), 0.9% saline solution. Samples were diluted 1:10 with 10% SDS and 0.9% saline, for a final concentration of 2% SDS in all samples and standards, including blanks. Only 50 μ L of each sample or standard and 50 μ L of working reagent were combined in the 96-well plate (Corning, USA), for a total volume of 100 μ L. Plates were incubated at 37°C and 5% CO₂ for 1 h before reading at 560 nm on a spectrophotometer (VersaMax, Molecular Devices, USA).

2.9 Statistics

Respiratory outcomes were analyzed by two-way ANOVA with LSD post-hoc analysis using IBM SPSS Statistics v.23, with dose and time as fixed factors. Splenocyte proliferation was analyzed by repeated measures ANOVA with LSD post-hoc analysis, with dose and time as between-subjects factors and stimulus and incubation time as within-subject factors.



Figure 2. Flowchart of methods outlining tissues examined, samples produced, techniques used, and corresponding outcomes measured. FFPE: Formalin fixed, paraffin embedded. MTS: a tetrazolium compound used for colourimetric cell viability assays.

3 Results

A total of 95 rats weighing 253±25 g contributed to the following results.

- 3.1 Lungs
- 3.1.1 Oxygenation

There was no significant change in arterial blood oxygenation with dose or time among the irradiated groups, which varied around 500-600 mmHg after ventilation with 100% oxygen (**Figure 3**). The LPS group had significantly lower oxygenation than all irradiated groups (p<0.05), less than 400 mmHg.



Figure 3. Arterial blood oxygenation in X-irradiated or LPS-exposed rats. Blood samples were drawn 0.5, 4, or 24 h post-exposure. Rats were ventilated with 100% oxygen for approximately 30 min prior to sampling. *Two-way ANOVA with LSD post-hoc, p<0.05; n=4-7; mean±SD.

3.1.2 Respiratory mechanics

Irradiation had no significant effect on airways resistance or tissue elastance. There was a trend towards increasing tissue resistance with increasing X-ray dose, although this was not quite significant at the 0.05 level with the current sample size (**Figure 4**; two-way ANOVA, p = 0.11; LSD post-hoc, p = 0.039). The LPS positive control group did not have increased airways resistance relative to the negative controls, but did have increased tissue resistance and elastance (**Figure 5**, two-way ANOVA with LSD post-hoc, p < 0.05).



Figure 4. Airways resistance (R_{aw}) in the lungs after X-irradiation or LPS exposure, as measured by a computer-controlled small animal ventilator using the forced oscillation technique. No statistical differences were found (two-way ANOVA with LSD post-hoc, p>0.05; n=4-7; mean±SD).



Figure 5. A) Tissue resistance (Gtis) and **B)** tissue elastance (Htis) of the lungs after X-irradiation or LPS exposure, as measured by a computer-controlled small animal ventilator using the forced oscillation technique. *Two-way ANOVA with LSD posthoc, p<0.05; n=5-6; mean±SD.

3.1.3 Oedema

There was no pulmonary oedema in any of the X-irradiated groups, based on lung wet-to-dry weight ratio (**Figure 6**). Both wet-to-body weight and dry-to-body weight ratio were also unchanged in all groups (**Figure 7**). The positive control group had significantly elevated mean wet-to-dry weight ratio relative to all other groups (two-way ANOVA, p<0.05). This difference was reflected in mean wet-to-body weight ratio, but also mean dry-to-body weight ratio.



Figure 6. Pulmonary oedema after X-irradiation or LPS exposure, determined as the ratio of wet to dry weight of the upper right lung lobe. *Two-way ANOVA with LSD post-hoc, p<0.05; n=5-10; mean±SD.



Figure 7. Wet upper lobe to body weight and dry upper lobe to body weight ratios after X-irradiation or LPS exposure. *Two-way ANOVA with LSD post-hoc, p<0.05; n=5-10; mean±SD.

3.1.4 BAL cells (numbers and differentials)

The number of cells retrieved in the bronchoalveolar lavage (BAL) was quite variable and showed no significant relationship with dose nor time, even in the LPS control group (**Figure 8**). Over 99% of these cells were macrophages, with the occasional lymphocyte (**Figure 9**; data not shown).

3.1.5 BAL fluid – proteinaceous infiltrate

The protein content in BAL fluid from LPS-exposed rats was significantly elevated compared to all other groups (**Figure 10**). There was no significant difference between any of the irradiated or sham-irradiated groups, though there is great variability in the data.







Figure 9. Bronchoalveolar lavage (BAL) cells from a representative rat, demonstrating >99% macrophages as consistently found across all groups.



Figure 10. Protein concentration in the BAL fluid from rats lavaged 0.5, 4, or 24 h post-irradiation, and 0.5 h after LPS exposure for the positive controls. *Two-way ANOVA with LSD post-hoc, p<0.05; n=5-8; mean±SD.

3.1.6 Histology

Histological analysis of lung tissue indicates no adverse effects of X-irradiation (**Figure 11**). Lung tissue from the positive control group, by contrast, exhibit some cellular infiltrate and increased thickness of alveolar walls (**Figure 11 B**).



Figure 11. Images of H&E stained lung sections from rats that were **A**) shamirradiated, **B**) LPS-instilled (positive control), **C**) 200 mGy-irradiated (4h post-IR) and **D**) 200 mGy-irradiated (24 h post-IR). Note the thickening of the alveolar walls and greater cellular infiltrate in the positive control compared to the rest.

3.2 Spleen

3.2.1 Proliferation

MTS absorbance values of splenocyte cultures incubated for 1 h were relatively consistent among groups, around 0.100 (**Figure 12 A**), which corresponds to about 1.7 x 10^6 cells/mL (see **Figure 14** in Appendix). After 48 h, unstimulated cultures

consistently dropped to about half of initial values (**Figure 12 B**). LPS-stimulated cultures generally increased slightly, to 0.100-0.150 (1.7-2.6 x 10^6 cells/mL), whereas Con A-stimulated cultures generally increased more, to about 0.15-0.25 (2.6-4.3 x 10^6 cells/mL). However, Con A-stimulated cultures from 200 mGy-irradiated rats did not reach this same level of absorbance, remaining barely above baseline levels (0.12-0.15).

Two-way repeated measures ANOVA comparing stimulated to unstimulated cultures at 1 h and 48 h indicates that splenocytes from 200 mGy-irradiated rats exhibit a smaller increase in absorbance after Con A stimulation than splenocytes from unirradiated rats (with LSD post-hoc, using log-transformed data, p = 0.024). The same significance was not observed for LPS stimulation (p>0.05).

3.2.2 Cell differentials

Initial splenocyte differential cell counts did not yield any notable differences in population distribution between macrophages and lymphocytes. The proportion of macrophages was relatively consistent in the seeding cultures before incubation (15-20%). The cells retrieved after incubation exhibited great variability in proportions, though lymphocytes were consistently the majority (60-90%).

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Figure 12. Optical density (OD; absorbance values) of splenocytes cultured without stimulus or incubated with LPS or Con A for **A**) 1h and **B**) 48h. Absorbance was measured after an additional 60 min incubation with MTS, a colourimetric assay indicative of viable cell counts. Absorbance values with Con A stimulus were lower for 200 mGy than sham-irradiation, based on individual log₁₀ transformed data; repeated measures at two incubation times (1 h vs 48 h) and with or without mitogen (Con A vs no stim); two-way ANOVA (time post-IR and dose of X-rays) with LSD post-hoc analysis, p=0.024; mean±SD).



Figure 13. Optical density (OD; absorbance values) summarized by **A**) radiation dose and **B**) post-irradiation after 48h incubation with unstimulated, LPS-stimulated, or Con A-stimulated cultures. The baseline is the combined mean absorbance of all cultures after 1 h, to show relative change over time. *Statistical differences based on individual log₁₀ transformed data; repeated measures at two incubation times (1 h vs 48 h) and with or without mitogen (Con A vs no stim or LPS vs no stim) with two-way ANOVA (time post-IR and dose of X-rays) with LSD post-hoc analysis, p=0.024; mean±SD).

4 Discussion

4.1 Splenocyte proliferation

Splenocytes from 200 mGy-irradiated rats had a significantly reduced proliferative response to Con A stimulation compared to all other X-ray doses, as indicated by the lower absorbance values in the MTS assay. This effect has previously been detected in rats 4 h after 500 mGy, but not 250 mGy X-rays [58]. A mouse study found a similar decrease in proliferation after only 40 mGy *in vivo* γ -irradiation [52].

The increase in absorbance after incubation was smaller in magnitude in the LPS cultures than Con A cultures over the 48 h timeframe, and no effect of radiation dose was detected. There are precedents for irradiation altering proliferative responses to Con A but not LPS [55,56]. This is likely due to the different cell types stimulated; Con A is generally recognized as a T-cell mitogen and LPS as a B-cell mitogen. Therefore, radiation may only affect T-cell and not B-cell proliferative responses.

Unstimulated cells in this study had reduced absorbance between 1 and 48 h, indicating that cell death outcompeted any proliferative capacity in these conditions.

This model failed to reproduce the immune enhancement often found after LDIR. For example, Con A-induced proliferation of splenic macrophages from Xirradiated C57BL/6 mice increased 4h after a 20 mGy dose (by 70% over sham controls) [52]; doses below 100 mGy increased plaque formation, with a slight increase in unscheduled DNA synthesis occurring 24 h after a 50 mGy exposure [54]. No such increases were found in the current model after 2 or 20 mGy. Kojima and colleagues noted increased splenocyte proliferation 2-6 h after 500 mGy γ irradiation [53] though this is in direct contrast to the decrease found by Ishii and colleagues 4 h after the same dose of X-rays [58].

Nearly all of the above experiments used γ-irradiation rather than X- irradiation, except for Liu and colleagues [54,59,66] and Ishii and colleagues [58]. Dose rates ranged from 2 up to 1100 mGy/min, with 12.5 mGy/min [54] the most similar to these experiments, which used 15.6 mGy/min. Most also used tritiated thymidine incorporation to measure proliferation, whereas the current model used the MTS assay. The uptake of tritiated thymidine into cultured cells is a direct indication of DNA synthesis, whereas the MTS assay indirectly measures the concentration of viable cells at a given time point based on metabolism of a substrate.

Overall, it seems that doses up to 20 mGy had no effect on the spleen in this model. A single dose of 200 mGy decreased the splenocyte proliferative response to T-cell stimulus, though a role for apoptosis cannot yet be ruled out.

4.1.1 Cell differentials

Previous work has identified a role for low-dose-irradiated antigen-presenting cells (macrophages), which induced T-cell proliferation [59]. Preliminary cell differentials in the present study indicate that macrophages comprised 15-20% of splenocytes prior to incubation, the remainder of which were lymphocytes. Post-incubation differentials varied greatly, with 10-40% macrophages and no consistent trend in macrophage proportion in relation to time or dose.

Variability in cell types after incubation can likely be attributed to the retrieval method employed. Macrophages are more adherent than lymphocytes, therefore any inconsistency in the use of the cell scraper to remove adherent cells could greatly affect the proportion of macrophages represented. A common alternative would be to use trypsin to lift adherent cells for collection. However, trypsin does not selectively cleave only cell surface proteins that contribute to adhesion, but rather can affect all exposed proteins, including common targets of cell type or activation state identification [73]. Furthermore, trypsin has been shown to alter protein expression, downregulating growth and metabolism pathways while upregulating apoptosis, which could affect the proliferation assay. A better alternative would be to prevent adhesion in the first place. There are options for reducing the adherent properties of plasticware (*e.g.* 'Ultra-Low Attachment' products from Corning). Incubating cultures on a cell shaker would also prevent macrophages from remaining in close contact with the plastic surface, decreasing the likelihood of successful adhesion.

In the future, analysis of splenocytes by flow cytometry rather than differential stains and immunofluorescence could be performed. These latter methods are highly labour intensive, requiring visual analysis of hundreds of cells per target outcome per subject. For example, to identify just the types of T-cells that proliferated in splenocyte cultures would require three targets to differentiate cytotoxic, helper, and regulatory T-cells. If each of these targets were analyzed at four dose levels, at three time points, in response to three different stimuli, with six subjects per group, a total of 216 slides would be required, with multiple fields of view from each, for each of the three targets. Essentially, over 200 000 individual cells would need to be visually evaluated for fluorescence. Twice, since proper protocol calls for the process to be repeated by different operators. Flow cytometry would allow much faster analysis of a much greater number of cells, which also results in more statistically robust values.

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4.2 Lung injury

4.2.1 Positive control

Lung injury in the positive control group was confirmed by several factors. Decreased arterial blood oxygenation indicates that the main function of the lungs, gas exchange, was compromised. Increased tissue resistance and elastance indicated increased work of breathing. These are classic indications of the alveolar response to LPS exposure, in which inflammation and damage of alveolar tissue interferes in normal tissue dynamics and function [70,74]. Airways resistance remained unaffected, as expected, since this parameter is generally associated with bronchoconstriction, which is not expected in LPS-induced acute lung injury.

Another indication of alveolar inflammation was pulmonary oedema [75], as indicated by the increased wet-to-dry weight ratio of the upper lung lobes from LPS-exposed rats. The wet-to-body weight ratio was also increased, as expected, due to the accumulation of oedematous fluid in the alveolar or interstitial space [74,75]. However, there was also an increase in dry-to-body weight ratio, which usually indicates an influx of protein, cellular recruitment, and/or structural remodelling of the tissues.

This is corroborated by an increase in protein in the BAL fluid, as well as increased cellular infiltrate and alveolar wall thickness based on histology scoring. However, there was no significant increase of cells in the bronchoalveolar lavage. Variability in the data suggests that this measure was not well executed in the lab, which would mask any real effect, if present. Differentials cell counts yielded a notable increase in neutrophil presence within the BAL cell population, but not quite to the degree previously found in this model [71]. This is likely due to the reduced time to post-instillation analysis (0.5 h) as compared to previous studies (2 h) [71],

suggesting the full magnitude and spectrum of effects has not yet developed. It was not possible to match the 4 h and 24 h time points of the irradiated animals due to the high mortality rate of this model within the first 4 hours of disease development.

4.2.2 X-irradiation

By contrast, the lungs of irradiated rats exhibited very little, if any, physiological or immunological effects. Doses of 2 and 20 mGy induced no significant changes in oxygenation, respiratory mechanics, pulmonary oedema, cellular or proteinaceous infiltrate, or alveolar wall thickening.

The only possible result after 200 mGy X-irradiation was a trend in increasing tissue resistance relative to sham-irradiated rats, consistent across all time points (Two-way ANOVA p = 0.11, LSD post-hoc, p=0.024). Although this was not statistically significant, it may be biologically interesting and warrants further discussion.

Tissue resistance is a measure of energy dissipation in the lungs during expansion [8]. If there were an increase in tissue resistance, it could be caused by a few factors, such as changes in surfactant, tissue stiffness, or lung heterogeneity.

Surfactant, as previously mentioned, decreases surface tension in the alveoli, reducing the force required to inflate the lungs [76]. If the production or degradation of surfactant is altered, then the resistance to stretch could increase. However, this would usually also affect the elastance, which does not exhibit the same trend of increasing with dose.

Tissue stiffness is often related to fibrogenesis, in which collagen and other extracellular matrix components are overproduced [77]. These structural proteins

reduce the compliance of lung tissue, increasing the work of breathing. However, this process is not expected to manifest within the first 24 h [78].

Increased tissue resistance can also indicate increased lung heterogeneity, a function of both the airways resistance and the tissue elastance [79]. Regions of the lung that are more difficult to inflate due to increased local airways resistance are the last regions to inflate upon inspiration, but more importantly also the last to deflate upon expiration, leaving residual pressure in the alveoli fed by these airways [80]. This issue is exacerbated if the tissue elastance of the alveoli is decreased, as there is less elastic force pushing air out through the constricted airways. After expiration, the pressure differential among regions causes air to move from these high pressure regions to other lower pressure regions, a process known as pendelluft. This inefficiency in the system is measured as increased tissue resistance. This possible mechanism could be investigated via in-depth analysis of impedance values.

However, it is important to note that this trend of increasing tissue resistance was not significant at the 0.05 level. If it is not a chance finding, it represents a small change in the overall function of the lung. In order to further explore this possible effect, it would be interesting to measure tissue resistance after higher doses of X-rays, such as 500 mGy, after which pulmonary oedema (based on wet weight) has been observed [81]. It is also conceivable that this could be a transient effect, or just the beginning of a future cascade, thus it would also be interesting to reexamine this metric at later times post-irradiation.

As this is the first investigation of respiratory mechanics after diagnostic-level radiation, there is very little literature with which to compare pulmonary outcomes. The few studies examining the effects of doses below 200 mGy in the lungs focused on apoptosis [43], gene expression [44], or cell signalling [43], which

have not yet been explored in this current model. Future work will allow comparison of these aspects of the low-dose radiation response.

Overall, it seems that single clinically-relevant doses of X-rays do not significantly affect the lungs in this healthy animal model. Although there was a trend in increasing tissue resistance with dose, this was not significant at the 0.05 level, and did not translate into reduced lung function, as indicated by the normality of arterial blood oxygenation. There was also no measurable immunological response that might underpin abnormal mechanics, although molecular measures of inflammation have yet to be characterized. Possible long term effects related to this result could be examined by extending this model beyond the first 24 h.

4.3 Study limitations

As with all studies, there are some limitations in the experimental design, implementation, and interpretation of this research.

True randomization of animals was limited by the logistics of assessment time and use of the XRAD cabinet irradiator. For example, nearly all 4 h groups were assessed in the afternoon, and nearly all 0.5 or 24 h time points in the morning. There is some suggestion of diurnal variation potentially affecting the response to radiation, although no such effect was observed in this study.

Inconsistencies in cell counts may be due to the use of milliQ water for dilution before counting rather than saline. However, informal testing of these protocols against each other within samples did not seem to yield vastly different results. It is important also to recognize that bronchoalveolar lavage is not a trivial technique, which may have contributed to both cell count and protein concentration variability. In terms of splenocyte proliferation, absorbance results from the MTS assay would ideally have been confirmed with actual cell counts. When this was attempted, the variability of cell state made it difficult to perform proper counts. The time investment also did not fit within the logistics of the protocol.

As with many animal studies, the sample size is relatively small, about 4-8 in most outcomes. However, others have found substantial differences with such small numbers, and this should be sufficient to see at least a trend in the same direction. Without such a trend, decreasing standard deviation by increasing sample size may make the results more precise but isn't expected to generate a significant difference.

There were also particular differences between the animal population and the patient population in the ICU. The median age of ICU patients is often 65 or higher [ref]. By contrast, this study used juvenile rats, since the lung capacity is still proportional to body mass in this age range, which allows accurate respiratory mechanics measurement and consistent bronchoalveolar lavage volumes. ICU patients also tend to have multiple morbidities. This study used healthy animals as a model to establish a baseline onto which a variety of morbidities can be added. For example, future studies could address respiratory infection, septicemia, and/or chronic heart failure. These rats were also all male, which restricts inferences for female patients.

As with all animal studies, these results cannot be directly translated to human patients. Sprague-Dawley rats act only as a model of the biological effects of diagnostic radiation, and therefore care must be taken in translation to ICCU practice.

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4.4 Conclusion and future directions

In conclusion, X-ray doses of 2 and 20 mGy had no statistically significant effect on the lungs or spleen in this healthy animal model, based on the parameters evaluated. There was a trend toward increasing tissue elastance after 200 mGy, which may warrant further investigation. A single 200 mGy dose reduced splenocyte proliferation, contrary to some of the literature

As ICCU doctors, it is important to understand how these results may inform decisions regarding a patient's health. It is important to balance potential risks of radiodiagnostic procedures with the benefit of the information obtained.

This study was not an exhaustive examination of all possible effects of diagnosticimaging X-irradiation. It focused on a very acute window of time, select aspects of lung and immune health, and only a few single doses of radiation. Questions remain, some of which may be addressed by further analysis of existing tissues, and others that would require new experimental investigation.

4.4.1 Future use of existing tissues and data

To address some of the questions that remain, further analysis of existing tissues and data could be performed.

The possibility of tissue elastance increasing due to small airways resistance effects could be investigated by further analysis of the respiratory mechanics data. The impedance values given are amalgamated from all the individual oscillation frequencies, each of which reflect different sections of the lung. Low frequencies travel deeper into the lung than high frequencies, thus impedance values at low frequencies are indicative of issues in the small airways, which are further along the bronchial tree [82].

It is unclear whether the reduced proliferative response in splenocytes is due to decreased responsiveness or increased apoptosis. Therefore, the rate of apoptosis in the spleen will be determined using sections of the OCT-preserved samples. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) detects characteristic DNA fragmentation [83]. This should help clarify the reduced proliferation seen after 200 mGy irradiation.

To characterize the types of splenocytes that have proliferated in response to Con A stimulation, immunofluorescence microscopy of cell smears could identify a combination of cell surface markers, such as CD3, CD4, CD8, and Foxp3. This would allow comparison to previous studies that have identified certain subsets, such as cytotoxic or helper T-cells, as proliferative targets in the radiation response [50,59].

A broader look at gene expression in the lungs could identify key targets of future research. Illumina RNA sequencing of snap frozen lung tissue would yield a plethora of data, which could be combed to examine molecular responses relating to fibrosis, oxidative stress, DNA repair, collagen and surfactant production, cell signalling, cell cycle checkpoints, etc.

One of the directions this could lead would be which cytokines to look for in the plasma and BAL fluids, using enzyme-linked immunosorbent assay (ELISA).

4.4.2 Future projects

Future projects could explore a variety of unanswered questions.

An alternative immune organ to examine would be bone marrow (e.g. [84]Chun 2013). This is a complex environment containing a vast array of cells at varying stages of development, but would be fascinating to explore.

It would be interesting to extend these investigations to higher doses. For example, to determine whether 500 mGy further increases tissue resistance, or compare low doses to 4 Gy as a high radiation dose control for all effects measured.

Clinical projects would be tricky, as there are so many individual factors at play in ICCU patients. This would likely be better explored among the broader patient population. For example, CT angiography has been found to increase DNA double strand breaks, apoptosis, and DNA repair gene expression in circulating immune cells [85,86]. Blood samples like these could be further analyzed to examine immune function, such as activation and cell signalling.

To further elucidate the proliferative response, different types of splenocytes or macrophages could be isolated and irradiated *in vitro*.

The goal of this project was to establish an animal model of radiation exposure. This basis could be built upon in a variety of ways to address unhealthy patients, multiple radiodiagnostic procedures, and longer time frames.

4.4.2.1 Model of respiratory illness

Although this study focused on developing a healthy model of X-ray exposure, patients in the ICCU are not, in fact, healthy.

In addition to the disease or injury they presented with, nosocomial infections are common in the ICCU due to the invasive nature of care. For example, LPS-induced acute lung injury was used as a positive control model in this study, but could serve as the basis for a sick animal model, as could ventilator-induced lung injury.

An interesting next step would be to investigate how X-irradiation and LPS exposure interact. Intratracheal LPS instillation mimics respiratory infection,

whereas intraperitoneal LPS injection mimics sepsis, both of which are of concern in the ICCU [7]. Exposing animals to LPS before or after X-irradiation would mimic patients admitted with respiratory or systemic infections or those who develop them during their stay.

4.4.2.2 Multiple irradiation

Another distinct variable between this model and ICCU practice is the frequency of radiological procedures, with patients receiving on average 8.5 chest radiographs and 2.3 CT scans during their stay [87]. Physicians debate the utility of daily chest X-rays, expressing concerns about the effect of these procedures on their patients' health [18].

Dose rate and fractionation are known to modify the effect of the total dose, with lower dose rates and greater fractionation generally reducing the detrimental effects [88]. For example, 200 mGy, 400 mGy and 800 mGy γ-irradiation increased splenocyte response to Con A when administered in 40 mGy daily fractions [51,55– 57]. This regime failed to produce the same result when the daily dose was increased to 100 mGy [55].

A patient receiving chest X-rays (0.02 mGy) for one hundred consecutive days should not expect the same results as a patient receiving a single 2 mGy dose from a low-level CT scan. Likewise, a patient receiving 200 mGy total over the duration of their hospital stay, should not expect the same effect as if they'd received it in a single day, or in a single procedure.

4.4.2.3 Longer term effects

It is also important to note that this study focused on the first 24 h after irradiation. Evidently effects could still occur beyond this time frame, and many patients remain in critical care for extended periods of time.

Pulmonary fibrosis, which is correlated with increased mortality among mechanically ventilated patients [89], generally takes days or weeks to manifest following acute lung injury [78] or radiotherapy [90]. Molecular investigation of fibrotic pathways could be performed using existing tissues, or longer-term studies could look beyond the initiation stages, out to the onset of actual fibrosis.

Cancer induction would also require longer-term study, and is beyond the purview of the critical care medicine lab at University. The radiobiology lab at McMaster has previously found that a single CT scan can delay the onset of induced leukemia in mice [submitted for review], a result that would be interesting to repeat in other animal models.

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Figure 14. Splenocyte standard curve relating viable cell count to absorbance at 490 nm after a 60 min incubation with MTS. The equation of this relationship is a = 0.057c + 0.004, where a is absorbance and c is cell concentration. The corresponding inverse equation is a = 17.5c - 0.07.