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**XXXVIII CICLO DEL DOTTORATO DI RICERCA IN
BIOMEDICINA MOLECOLARE**

**Effects of mechanical stretch on lung tissue:
endothelial and epithelial proliferative dynamics in a
model of ventilator-induced lung injury and repair**

Settore scientifico-disciplinare: **MED10/Malattie dell'apparato respiratorio**

**DOTTORANDA
GIULIA COLUSSI**

**COORDINATORE
PROF. ALESSANDRO TOSSI**

**SUPERVISORE DI TESI
PROF.SSA SERENA ZACCHIGNA**

**CO-SUPERVISORE DI TESI
PROF. MARCO CONFALONIERI**

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ABSTRACT	3
1.INTRODUCTION	4
1.1 Clinical background: acute respiratory failure, ARDS and mechanical ventilation	4
1.1.1 Acute respiratory failure and ARDS: the global burden	4
1.1.2 Acute pathophysiology of ARDS: failure of the alveolar–capillary barrier	5
1.1.3 Mechanical ventilation in ARDS: life support and VILI (Ventilator-Induced Lung Injury)	6
1.1.4 Mechanical power: an integrative metric of injurious potential	6
1.1.5 Diffuse alveolar damage: the histological hallmark	7
1.1.6 Vascular histopathology	8
1.2. Ventilator-Induced Lung Injury and its experimental models	8
1.2.1 The four classical mechanisms of VILI	8
1.2.2 Regional inhomogeneity: the baby lung and stress concentration	10
1.2.3 Cellular stress failure	10
1.2.4 Rodent in vivo models of VILI	10
1.2.5 Ex vivo models	11
1.2.6 Cellular responses to VILI: injury, death and senescence	11
1.3 Alveolar repair	12
1.3.1 Pulmonary endothelial heterogeneity	12
1.3.2 AT2 proliferative repair after ALI and VILI	13
1.3.3 Endothelial proliferative repair	14
1.3.4 Endothelial-epithelial crosstalk	15
2. AIM OF THE THESIS	16
3. METHODS	17
3.1 In Vivo murine model of Ventilator-Induced Lung Injury (VILI)	17
3.1.1 Animals and experimental groups	17
3.1.2 Anaesthesia, orotracheal intubation and mechanical ventilation	17
3.1.3 Endpoint procedures and sample collection	18
3.2 Recovery Cohort for the EdU Proliferation Time-Course	20
3.3 Tissue sampling and histological analysis	20
3.3.1 Histological scoring framework	20
3.4 Isolation of Primary Alveolar Type II Cells and Pulmonary Endothelial Cells	22
3.4.1. Reagents and Equipment	22
3.4.2 Lung Harvest, Cannulation and Enzymatic Digestion	23
3.4.3 Mechanical Dissociation and Filtration	24
3.4.4 Depletion of Fibroblasts by Differential Plating (Pre-plating)	24
3.4.5 Immunomagnetic Purification of AT2 Cells	25
3.4.6 Immunomagnetic Purification of Pulmonary Endothelial Cells	25
3.4.7 Matrigel Coating of Silicone Stretch Membranes	25
3.5 In Vitro Cyclic Mechanical Stretch System	26

3.5.1 Device and Culture Format	26
3.5.2 Stretch Protocol Parameters	27
3.6 Immunofluorescence	27
3.6.1 Tissue Preparation and Antigen Retrieval	27
3.6.2 Blocking and Primary Antibody Incubation	27
3.6.3 EdU Detection (Click Chemistry)	28
3.6.4 Secondary Antibody, Nuclear Counterstain and Mounting	28
3.6.5 Cell-Type Assignment and Quantification	28
3.6.6 Application to the Ex Vivo Stretch System	28
3.7 Statistical methods	28
4. RESULTS	30
4.1 Establishing a mouse ventilator-induced lung injury (VILI) model	30
4.1.1 Ventilatory parameters for the Ventilator-Induced Lung Injury (VILI) model	30
4.1.2. Histology scores for different domains and recapitulatory composite score	30
4.1.3 Histological injury scores increase progressively with ventilatory stretch magnitude	31
4.2 From modelling stretch-related damage to modelling recovery	36
4.2.1 Anaesthesia protocol improvements for recovery model	36
4.3 In Vivo Proliferative Response After VILI	38
4.3.1. Experimental design	38
4.3.2 Endothelial cells proliferate soon after injury	39
4.3.3 Epithelia type 2 cells proliferate after endothelial cells	40
4.4 Ex-Vivo mechanical stretch model	41
4.1 AT2 monoculture Ex-Vivo mechanical stretch	42
4.4.2 Endothelial cell monoculture ex-vivo mechanical stretch	42
4.4.3 EC and AT2 co-culture Ex-Vivo mechanical stretch	43
5. DISCUSSION	45
5.1 Modelling Ventilator-Induced Lung Injury (VILI)	45
5.2 Distinct temporal dynamics of epithelial and endothelial proliferation In Vivo	46
5.3 Endothelial cells show intrinsic mechanosensitivity, while AT2 proliferation appears context-dependent	47
5.4 Methodological Considerations and Limitations	48
6. CONCLUSION	50
7. REFERENCES	51

ABSTRACT

This thesis investigates the cellular and physiological consequences of mechanical stretch on the murine lung through an integrated in vivo–ex vivo experimental strategy. First, an in vivo murine model of ventilator-induced lung injury (VILI) was established, using increasing tidal volumes as the injury mechanism, serving as a surrogate for increasing mechanical stretch exposure of the lung tissue. Four groups of escalating ventilation were compared: non-ventilated, lung-protective ventilation (7 $\mu\text{L/g}$), and injurious ventilation (15 $\mu\text{L/g}$ and 21 $\mu\text{L/g}$). Lung injury in this model was quantified using a multi-domain histological scoring system applied to sections stained with haematoxylin and eosin (H&E) and CD45 immunohistochemistry (IHC), enabling objective assessment of the lung injury sustained and characterisation of its component domains (inflammatory, structural, and according to the American Thoracic Society score).

The second part of the in vivo project examined recovery from lung injury at five timepoints, designed to capture the proliferative dynamics of the two principal cell types under study, namely endothelial cells (EC) and alveolar type II (AT2) epithelial cells. In this cohort, pulmonary endothelial cell proliferation peaked early, around day 2 post-injury, remaining significant up to day 7 and declining to baseline by day 15. AT2 cell proliferation instead emerged later, peaking around day 7, consistent with a dependence on the preceding endothelial response, as suggested by recent literature.

In support of this hypothesis, a series of ex vivo experiments were performed. Primary alveolar type II epithelial cells and pulmonary microvascular endothelial cells were isolated from murine lung tissue and used for subsequent mechanotransduction studies. A mechanical stretch system employing silicone membranes was used, subjecting cells to defined regimens of cyclic mechanical stretch by means of a dedicated mechanobiology platform (MechanoCulture FX), in monoculture and co-culture configurations. In this ex vivo model, the endothelial cell population appeared to proliferate in response to mechanical stretch, whereas AT2 cells were found to be non-proliferative.

Collectively, these findings support the hypothesis that the pulmonary endothelium plays a relevant role as a mechanosensitive population promoting lung regeneration following VILI.

1.INTRODUCTION

1.1 Clinical background: acute respiratory failure, ARDS and mechanical ventilation

1.1.1 Acute respiratory failure and ARDS: the global burden

Acute respiratory failure (ARF) represents one of the most prevalent, feared, and potentially lethal conditions that lead to admission in intensive care. ARF has multiple possible aetiologies, and all of them can converge upon a common pathophysiological endpoint: the acute respiratory distress syndrome (ARDS). First described in 1967 by Ashbaugh and colleagues ¹ as a clinical syndrome of refractory hypoxaemia, bilateral pulmonary infiltrates, and reduced lung compliance, ARDS has undergone successive extensive study and definition. For a long time, the Berlin Definition, established by international consensus in 2012, was used to characterise ARDS by the following criteria ²:

1. acute onset (within one week of a known clinical insult or new/worsening respiratory symptoms)
2. bilateral opacities on chest radiography or computed tomography (not fully explained by effusion, lobar collapse, or nodules)
3. respiratory failure not fully explained by cardiac failure or fluid overload
4. ratio of arterial partial pressure of oxygen to inspired oxygen fraction ($\text{PaO}_2/\text{FiO}_2$) below 300 mmHg on a minimum of 5 cmH_2O positive end-expiratory pressure (PEEP)

Severity was stratified into mild (200–300 mmHg), moderate (100–200 mmHg), and severe (<100 mmHg) categories based on the $\text{PaO}_2/\text{FiO}_2$ ratio. The Berlin stratification carried meaningful prognostic weight: a crude hospital mortality of approximately 27% in mild disease to 45% in the severe category, with a corresponding increase in median duration of mechanical ventilation in survivors ³. The Berlin Definition has itself recently undergone formal revision: in 2024, a new global definition of ARDS was proposed, in particular to frame the expanding use of high-flow nasal oxygen, the growing reliance on pulse oximetry over arterial blood gas sampling, the incorporation of point-of-care ultrasound as a chest-imaging modality, and the need for diagnostic applicability in resource-limited settings ⁴. For this thesis purpose, the relevance of the murine model relies on directly controlled ventilator parameters, and the new definition explicitly retained the Berlin conceptual model of diffuse, permeability-driven inflammatory lung injury, reinforcing the continued validity of diffuse alveolar damage as the translational anchor linking bench-level VILI models, including the present one, to clinical ARDS.

A large-scale international prevalence study demonstrated that ARDS accounted for 10.4% of all ICU admissions and 23.4% of patients receiving mechanical ventilation, with a crude hospital

mortality approximating 40% ⁵. Despite incremental improvements in supportive care and the evolving approach for lung-protective ventilation, prone positioning, and conservative fluid strategies, crude mortality has remained high and no pharmacological intervention tested in randomised controlled trials (RCTs) has reproduced the survival benefit achieved by reducing tidal volume in the landmark ARDSNet study ⁶.

The recent research landscape on ARDS has been characterised by increasing methodological sophistication: phenotype-enriched trial design, translational mechanistic investigation, and the emergence of single-cell and spatial-transcriptomic approaches to alveolar biology. The burden of ARDS extends substantially beyond the acute hospital stay, with long-term sequelae for survivors and compromised functional outcomes, underscoring the inadequacy of the reparative response that follows.⁷ This aspect further motivates the cellular and mechanobiological enquiries pursued in this thesis.

1.1.2 Acute pathophysiology of ARDS: failure of the alveolar–capillary barrier

Under physiological conditions, pulmonary gas exchange is possible thanks to an exquisitely refined tissue architecture. The alveolar wall is lined predominantly by thin epithelial alveolar type 1 (AT1) cells apposed across the underlying capillary endothelium with in-between apposition of minimal interstitial matrix. This anatomical peculiarity minimises the diffusion distance for oxygen and carbon dioxide whilst providing a selective structural barrier. Barrier integrity is maintained by an integrated programme of paracellular sealing and active vasoregulatory signals that maintain a non-adhesive, anti-inflammatory luminal phenotype under physiological conditions ⁸. Concurrently, alveolar type 2 (AT2) cells synthesise and secrete surfactant, a complex mixture of phospholipids and hydrophobic proteins, that reduces alveolar surface tension to prevent end-expiratory collapse, and moreover contributes to innate immune defence.

The transition from this homeostatic state to the diffuse alveolar damage (DAD) of ARDS is initiated by recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by pattern-recognition receptors on resident alveolar macrophages, dendritic cells, and epithelial cells. Signalling cascades rapidly up-regulate chemokine secretion driving the transendothelial migration of neutrophils that defines the early exudative phase of ARDS, followed by inflammatory cytokines release. Collectively, these effectors degrade tight-junction and adherens-junction proteins on both the epithelial and endothelial membranes, worsening the alveolo-capillary barrier failure ⁹. Protein-rich pulmonary oedema, a distinctive clinical feature of ARDS, floods the alveolar compartment diluting and inactivating surfactant, hyaline membranes form from precipitated plasma proteins, and gas exchange becomes critically compromised.

Epithelial AT1 cells, although providing the vast majority of the alveolar surface area, are terminally differentiated and incapable of self-renewal. Their depletion during ARDS injury represents therefore an irreversible cellular loss. The ultimate clinical trajectory of ARDS is determined by the balance between injurious processes and the ability of AT2-mediated re-epithelialisation and endothelial barrier restoration as part of the reparative process ¹⁰.

1.1.3 Mechanical ventilation in ARDS: life support and VILI (Ventilator-Induced Lung Injury)

Mechanical ventilation (MV) is the cornerstone of supportive care in ARDS and for most patients the only life-sustaining intervention to correct hypoxaemia and hypercapnia that would otherwise be fatal. This therapeutic necessity conceals a fundamental paradox: the process of ventilating an already-injured lung may substantially amplify the original injury through a mechanism called ventilator-induced lung injury (VILI) ¹¹.

In 1998 Amato and colleagues published a small trial in the New England Journal of Medicine that gave birth to the now widespread concept of protective ventilation. Interestingly, the cornerstone of protective ventilation is low tidal-volume ventilation, as confirmed by the ARDSNet trial published in the same journal the year after ⁶. Both trials reported a significant reduction in mortality in the group randomised to receive mechanical ventilation with low tidal volumes (6ml/kg of predicted body weight - PBW) when compared with traditional tidal volumes of 12 ml/kg PBW, which represented standard practice. Of note the ARDSnet trial was interrupted after the fourth interim analysis (861 patients) because of an already significantly lower mortality in the low-volume group ⁶. This survival benefit has not been matched by any pharmacological intervention tested in ARDS to date, supporting the study of the effects of mechanical stretch on lung parenchyma to achieve a deeper understanding of the cellular mechanisms of lung injury and to foster the development of new therapeutic strategies. Moreover, despite the advances, lung-protective ventilation remains an injury-reduction strategy rather than a reparative one.

1.1.4 Mechanical power: an integrative metric of injurious potential

The recognition that tidal volume, whilst a pivotal determinant of VILI, does not fully capture the injurious potential of mechanical ventilation prompted the development of a more integrative metric: mechanical power (MP). Defined as the energy delivered to the respiratory system per unit time (expressed in joules per minute) the concept of mechanical power incorporates all the major ventilatory parameters that contribute to energy load on the lung during ventilation: tidal volume, respiratory rate, driving pressure, PEEP, and respiratory system compliance ¹². The mechanobiological significance of MP lies in its acknowledgement that injury is not merely a function of the magnitude of the mechanical stimulus, i.e. the volume, but also of the rate and of the intrinsic characteristics of the lung.

The experimental foundation for the clinical relevance of mechanical power derives mainly from a rat model of experimental acute respiratory distress syndrome in which anaesthetized, mechanically ventilated animals were exposed to combinations of tidal volume and respiratory rate that yielded low versus high mechanical power ¹³, with further support from rodent and porcine models in which reciprocal respiratory-rate/tidal-volume combinations and an internally-referenced mechanical-power ratio were associated with histological VILI ^{14,15}. These animal data indicate that the mechanical-power concept can translate across species and provide conceptual support for the reciprocal tidal-volume/respiratory-rate adjustments used in the murine ventilation protocol of the present thesis.

Comparison between VILI models is confined to the relative structure of the mechanical power gradient while the absolute thresholds appear to be different in different species, consistent with the relative-MP logic validated in the porcine model described above¹⁵.

1.1.5 Diffuse alveolar damage: the histological hallmark

The pathological correlate of ARDS is diffuse alveolar damage (DAD), a pattern of alveolar injury that constitutes its canonical histological substrate, although not entirely specific to ARDS, and against which the cellular events of injury and repair must be understood. DAD evolves through a sequence of overlapping phases that reflect the dynamic interplay between ongoing injury, inflammation, and the endogenous reparative response.

Diffuse alveolar damage is characterised at low magnification by airspace flooding with proteinaceous oedema fluid and erythrocytes (exudative phase of ARDS), and at high magnification by the deposition of hyaline membranes, the infiltration of the alveolar interstitium and airspace by neutrophils and macrophages, interstitial oedema, that reflects the concurrent microvascular injury. Most significantly from the perspective of subsequent repair, there is extensive loss of AT1 cells together with the loss of normal alveolar architecture; conversely AT2 cells begin to proliferate and to populate the denuded alveolar surfaces during the later phases of ARDS (proliferative phase of ARDS), a cellular response that constitutes the histological evidence for progenitor activation that has been repeatedly documented in DAD biopsies and post-mortem material ^{16,17}.

The proliferative phase, predominating between approximately days seven and fourteen, is characterised by AT2 cell hyperplasia and migration of fibroblast and myofibroblast into the alveolar interstitium and early collagen deposition. Partial re-epithelialisation restores some barrier function, and the inflammatory exudate begins to resolve, though areas of residual consolidation persist. The co-ordinated behaviour of epithelial and vascular elements during this phase determines the extent of functional recovery: successful re-epithelialisation requires

concurrent restoration of the alveolar capillary bed to provide oxygen and trophic signals to regenerating epithelium.

A significant proportion of ARDS patients who die show fibrotic changes on post-mortem examination even after relatively brief disease courses, indicating that the transition from inflammation to fibroproliferation can occur rapidly. Clinically, the fibrotic phase correlates with failure to wean from mechanical ventilation, progressive radiological opacification, and reduced respiratory system compliance.

ARDS patients will possibly undergo the transition from exudative to proliferative DAD during their ICU stay, meaning that their lungs are simultaneously experiencing the last stages of acute injury and the first stages of repair throughout the duration of ventilatory support. The identification of the cellular and molecular determinants of AT2 cell and endothelial cell behaviour during this window therefore carries direct clinical relevance.

1.1.6 Vascular histopathology

The pulmonary capillary bed undergoes characteristic pathological changes in VILI and ARDS that are linked to the alveolar epithelial injury described above. In the exudative phase, pulmonary capillaries exhibit congestion, microthrombus formation within alveolar capillaries and small venules, endothelial cell swelling and focal endothelial cell necrosis. Recent evidence demonstrates that alveolar capillaries are formed by two intermingled cell types with complex morphologies and distinct functions. The first cell type, called 'aerocyte' (aCap), specialises in gas exchange while the other cell type, termed gCap ('general' capillary), is specialized to regulate vascular tone and functions as a progenitor cell in capillary homeostasis and repair ¹⁸. The histological recognition of endothelial heterogeneity within what was classically regarded as a morphologically uniform cell population reflects a fundamental revision of the conceptual framework for alveolar vascular repair.

The injurious and reparative responses to ARDS and mechanical ventilation are cellular processes, enacted by identifiable cell types, with AT2 epithelial cells and pulmonary capillary endothelial cells exerting a leading role in these processes. A deeper understanding of the effects of mechanical stretch on these cell types and how it is translated into injury or repair signalling at the cellular and molecular level appears to be important for developing strategies to reduce VILI and promote alveolar recovery.

1.2. Ventilator-Induced Lung Injury and its experimental models

1.2.1 The four classical mechanisms of VILI

Ventilator-induced lung injury (VILI) is determined by four conceptually distinct but mechanistically interdependent mechanisms: barotrauma, volutrauma, atelectrauma, and biotrauma. In contemporary practice, elements of all four mechanisms operate simultaneously in most ventilated patients, and lung-protective ventilation strategies must be designed to address each simultaneously.

Barotrauma refers to macroscopic lung injury resulting from the application of excessive airway pressures that exceed the structural tolerances of alveolar walls causing gas dissection along broncho-vascular bundles and fascia that represent the clinical manifestations of pneumothorax, pneumomediastinum, subcutaneous emphysema, and pulmonary interstitial emphysema ¹¹.

Volutrauma describes the injury attributable to excessive volumetric distension of the alveoli. The conceptual distinction between barotrauma and volutrauma, which may at first appear superimposable, was established through a series of experimental studies by Dreyfuss and colleagues in the 1980s ¹⁹. Using a rat model, they demonstrated that high-volume ventilation, if guaranteed to develop low-pressure by strapping the thorax to limit wall excursion, produced significantly less lung injury than high-volume ventilation without restraint ²⁰. This elegantly dissociated pressure from volume as the primary determinant of injury, establishing the deformation of the alveolar wall beyond its elastic limit (overdistension) as the key pathological event.

Atelectrauma arises from the repeated cyclic opening and collapse of alveolar units during each ventilatory cycle. In a lung with regional heterogeneity dependent lung regions are susceptible to derecruitment at end-expiration, particularly in the absence of sufficient positive end-expiratory pressure (PEEP). Upon reinflation, these alveoli are subject to enormous shear stresses at the interface between aerated and collapsed regions. PEEP is the primary therapeutic intervention targeting atelectrauma, functioning by maintaining a positive end-expiratory distending pressure that prevents alveolar derecruitment between breaths and thereby eliminates the cyclic shear injury ¹¹.

Biotrauma describes the biological and inflammatory dimension of VILI. Of importance, it is distinct from the purely mechanical processes described above. Regardless of the initiating mechanical stimulus the consequence at the cellular level is the activation of mechanosensitive signalling cascades, the release of damage-associated molecular patterns (DAMPs), and the induction of a robust pulmonary inflammatory response characterised by the elaboration of proinflammatory cytokines ²¹. Neutrophil recruitment to the lung, a hallmark of ARDS histopathology, is amplified by VILI through both cytokine-mediated chemotaxis and the physical disruption of the alveolar-capillary barrier, which facilitates the entry of circulating immune cells into the alveolar space.

1.2.2 Regional inhomogeneity: the baby lung and stress concentration

The concept of the "baby lung" was developed by Gattinoni and colleagues arising from studies that analysed the computed tomographic images of patients with ARDS²². In ARDS, a large portion of the lung parenchyma is consolidated, flooded, or atelectatic and thus does not participate in ventilation. The aerated fraction is represented by a small volume of the lung tissue, called for this reason the "baby lung". When a tidal volume is delivered during each breath, it is distributed almost entirely to this small aerated fraction, producing volumetric strains per unit of tissue that are far in excess of those predicted. The baby lung concept explains why a tidal volume of 6 ml/kg predicted body weight may still produce regional volutrauma, hence VILI, in the setting of severe ARDS. This observation provides part of the rationale for ultra-lung-protective ventilation strategies employing tidal volumes below 4 ml/kg, with or without extracorporeal carbon dioxide removal to manage the hypercapnia.

Emerging work has further complicated the static conception of VILI as a progressive injury process by demonstrating that injury and repair are concurrent, not sequential. Bates and colleagues have proposed that VILI should be conceptualised as a dynamic balance between ongoing epithelial cell damage from cyclic mechanical forces and active cellular recovery¹⁰. For this reason, interventions that promote cellular repair, in addition to those that limit injury, may contribute to the preservation of lung function during mechanical ventilation, especially of the diseased lung.

1.2.3 Cellular stress failure

The signalling consequences of sub-lethal cyclic stretch have been characterised in detail using in vitro stretch systems. Yamamoto and colleagues demonstrated that cyclic mechanical stretch applied to alveolar epithelial cells induces the secretion of IL-8 and TGF-beta1 through a protein kinase C (PKC)-dependent pathway²³. Notably, the same group demonstrated that mechanical stretch simultaneously induces the release of hepatocyte growth factor (HGF) from alveolar epithelial cells which is a mitogen for AT2 cells and hence a driver of alveolar repair²⁴. This simultaneous release of both proinflammatory (IL-8, TGF-beta1) and pro-regenerative (HGF) signals from the same mechanical stimulus serves as an example of the complexity of the cellular response to stretch: the alveolus is not simply a passive structure that fails under excessive load but has an active signalling role that can lead to both injury and regeneration.

1.2.4 Rodent in vivo models of VILI

The in vivo murine and rodent model of VILI are widely used to investigating the mechanistic basis of ventilator-induced lung injury, providing a physiologically intact system in which the complexity of inflammatory, cellular, and structural responses can be studied. VILI models more

often involve mechanical ventilation with large tidal volumes, exploiting the known mechanism of volutrauma to produce lung injury within a defined experimental window. In rats and mice high-tidal-volume ventilation (20 to 40 μ l/g in mice; 25 to 40 μ l/g in rats) reliably produces the main characteristics of ARDS-like lung injury: alveolar flooding with protein-rich oedema, neutrophilic inflammatory cell recruitment to both the alveolar space and the interstitium, disruption of the alveolar-capillary barrier and the histopathological features of diffuse alveolar damage (DAD)^{25,26}.

1.2.5 Ex vivo models

En vitro stretch systems have similarly revealed the amplitude-dependence and temporal dynamics proliferative responses of pulmonary microvascular endothelial cells. Birukova and colleagues demonstrated that 5% cyclic stretch enhances endothelial barrier integrity through Rac1/cortactin-dependent cytoskeletal remodelling, whilst 18% stretch produces barrier disruption hypothesizing a biphasic strain-magnitude dependent endothelial barrier response to cyclic stretch²⁷. Lai and colleagues applied a systems biology approach to analyse the transcriptomic and proteomic responses of human lung microvascular endothelial cells to 10% and 20% cyclic stretch over multiple time points, revealing that the net proliferative response to stretch is determined by the balance between pro-growth and growth-inhibitory pathways that evolve over hours²⁸.

The development of micro-physiological systems and organoid technologies represents an important methodological advance beyond conventional two-dimensional monoculture stretch systems. Niu and colleagues developed a biomimetic alveolus-on-chip incorporating a polyurethane membrane and an AT2-endothelial co-culture system, demonstrating synergistic proliferative effects when both cell types were co-cultured under cyclic stretch compared with either monoculture alone²⁹. The organ-chip platform of Hajari and colleagues, incorporating patient-derived AT2 cells, extends these advances to a clinically relevant cellular context, providing a bridge between the controlled mechanobiology of in vitro stretch experiments and the heterogeneous cellular reality of the human lung³⁰.

1.2.6 Cellular responses to VILI: injury, death and senescence

Distinct AT2 death programmes operate in VILI, each shaping the inflammatory microenvironment for subsequent repair. Apoptosis is caspase-mediated, anti-inflammatory, and proceeds via orderly clearance. Necroptosis, driven by RIPK3/MLKL pore formation following DAMP release, is lytic and pro-inflammatory. Pyroptosis proceeds through NLRP3/caspase-1 activation, gasdermin D pore formation, and IL-1 β /IL-18 release. Ferroptosis is a caspase-independent death driven by iron-dependent lipid peroxidation. These pathways co-occur and share regulatory nodes, but the mode of death determines repair capacity: necroptosis and

pyroptosis impair progenitor proliferation, whereas apoptosis is comparatively permissive of repair ³¹.

Endothelial injury is universal and early in VILI, contributing to barrier failure and oedema. Zhu et al. showed that Neutrophil Extracellular Traps (NETs) endothelial proliferation by inhibiting PLK1 and causing G2/M arrest finding a direct link between neutrophilic inflammation and impaired vascular repair ³². Price & Garcia argue against treating endothelial activation as a binary "positive" or "negative" state. Instead, they support the idea that the same molecular programme sits on a continuum where the functional consequence depends on two variables: its degree (how strongly the endothelium is activated) and its duration (how long that activation state is sustained) ³³. At physiological or moderate levels, this activation is described as adaptive, conversely when activation is dysregulated it becomes pathological. The "razor's edge" framing is meant to capture that there isn't a clean threshold separating a protective endothelial response from a harmful one and the outcome (repair versus prolonged injury) depends on where on that continuum, and for how long, the endothelium sits.

1.3 Alveolar repair

1.3.1 Pulmonary endothelial heterogeneity

For many decades the pulmonary endothelium was conceptualised as a relatively homogeneous continuous lining constituting a passive barrier separating blood from parenchyma. This view has been thoroughly dismantled by the application of single-cell RNA sequencing to the lung, which has revealed an intricate landscape of endothelial subtypes defined by distinct transcriptional identities, anatomical positions, and functional specialisations. Of note, the work of Gillich and colleagues, who used scRNA-seq combined with three-dimensional electron microscopy to define two molecularly and morphologically distinct capillary endothelial cell types in the murine lung ¹⁸. The first, called aerocytes, are highly specialised for gas exchange and leukocyte trafficking and have specific morphology: they are large, extremely thin, form transcellular holes traversed by erythrocytes, and express carbonic anhydrase IV along with endothelin receptor B. Aerocytes appear to be lung-specific, with no clear homologue in other organs, and their unique architecture reflects extreme functional adaptation to respiratory physiology ¹⁸. The second population, named general capillary endothelial cells (gCap), expresses apelin receptor, ficolin-3, and glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1; gCap cells regulate vascular tone, are positioned throughout the capillary bed, and critically and function as a progenitor population ¹⁸.

Niethamer and colleagues further demonstrated that highly proliferative endothelial cell populations emerge following influenza-induced acute lung injury and contribute to alveolar revascularisation ³⁴. More recently, James and colleagues used scRNA-seq combined with time

and trajectory analyses to define five distinct gCap subpopulations in the normal lung ³⁵. Among these, a Flotillin-1 (Flot1)-positive mitotically active subpopulation located at the arterial-capillary interface was identified as the proliferative "root" of the gCap population. This subpopulation expands markedly under hypoxic conditions and is established during postnatal lung maturation as a FoxM1-positive progenitor pool ³⁵.

The source of endothelial progenitors for post-injury repair is not confined to the gCap compartment. Wong and colleagues employed a pulmonary venous endothelium specific Cre model to demonstrate that venous endothelial cells can function as a source of capillary repair. Following both influenza infection and hyperoxia injury, venous-derived cells differentiated into gCap and aerocyte phenotypes, expanding the pool of potential progenitor sources beyond the capillary bed and suggesting that repair capacity may be distributed across the vascular tree ³⁶.

The relevance of these findings is demonstrated by the conservation of the aerocyte/gCap dichotomy across species. Schupp and colleagues constructed a comprehensive human lung endothelial cell atlas confirming the existence of homologous populations in human tissue ³⁷, and the subsequent integrated multi-centre human lung atlas further supported their conservation in humans and provided reference transcriptomes at population scale ³⁸. The parallel murine endothelial single-cell atlas from Kalucka and colleagues provided a pan-organ endothelial reference, confirming the lung specialisation of aerocytes ³⁹.

A further dimension of endothelial heterogeneity concerns the dependence of EC identity upon epithelial signalling. Ellis and colleagues demonstrated that epithelial VEGFA is required for specification of an aerocyte-like endothelial population during alveologenesis ⁴⁰. This finding has important implications for injury: destruction or dysfunction of alveolar epithelial cells may deprive adjacent endothelial cells of their identity-maintaining paracrine signals, driving endothelia de-differentiation. The dependence of endothelial phenotype on epithelial context illustrates the deeply integrated nature of the alveolar unit.

1.3.2 AT2 proliferative repair after ALI and VILI

The restoration of alveolar epithelial integrity following acute lung injury proceeds through a stereotyped but regulated sequence of events. In the initial phase, AT1 cell loss creates denuded regions of basal lamina. Surviving AT2 cells rapidly flatten to cover this exposed surface, providing a temporary but insufficient barrier. In the second phase, surviving AT2 cells re-enter the cell cycle and undergo proliferative expansion, replacing lost cells and building the numerical reserve required for repair. In the third phase, a proportion of the expanded AT2 population transdifferentiates into AT1 cells, restoring the thin gas-exchange epithelium required for normal lung function. The fidelity of this final step is the critical determinant of functional recovery.

Yang and colleagues characterised the kinetics of this response in a rat acute lung injury model, establishing that AEC2 proliferation peaks several days post-injury, with HGF/c-Met signalling identified as a key paracrine driver of this proliferative response ⁴¹. This timeline is consistent with the days-to-weeks recovery period observed clinically following ARDS.

In models directly relevant to mechanical ventilation, Lin and colleagues performed murine VILI studies demonstrating that lung-recruited monocytes produce VEGF in a time-dependent manner following injurious ventilation, and that this monocyte-derived VEGF production correlates temporally and spatially with AT2 cell EdU incorporation at days 7 and 14 post-VILI ⁴². This establishes an inflammatory cell-to-epithelial paracrine axis as a driver of proliferative repair following mechanical injury — and, importantly, it implicates the same VEGF signalling molecule that governs EC-AT2 crosstalk in the angiocrine programme.

The AT2 proliferative response is not uniform across the entire AT2 pool but operates through molecularly distinct subpopulations. Zhang and colleagues identified a specific AT2 subpopulation that responds to CTGF/CCN2 with selective proliferation, detectable by sequential EdU and BrdU labelling, and mechanistically distinct from the bulk AT2 proliferative response ⁴³. Given that CTGF is a downstream target of YAP/TAZ in mechanically stimulated EC, this finding creates a plausible mechanistic link between EC mechanical activation, CTGF secretion, and selective AT2 subpopulation expansion.

1.3.3 Endothelial proliferative repair

The pulmonary endothelium recovers from acute injury through two temporally distinct mechanisms. The first is junctional reannealing and the second mechanism is genuine endothelial cell proliferation.

The master regulator of this proliferative programme is FoxM1, a member of the Forkhead box (Fox) family of transcription factors whose expression is tightly cell-cycle-coupled: low or absent in quiescent G0 cells, rising through S phase, and peaking at G2/M. FoxM1 directly transactivates genes encoding the machinery of mitosis and is thus an essential coordinator of cell cycle progression rather than merely an indicator of proliferative activity.

The importance of FoxM1 in EC repair was established by Huang and colleagues, who demonstrated that transgenic overexpression of FoxM1 specifically in EC led to markedly increased BrdU incorporation in pulmonary endothelial cells following polymicrobial sepsis in mice, and to improved vascular integrity and organ function ⁴⁴. This proof-of-concept established that augmenting the EC proliferative programme is sufficient to improve recovery from inflammatory lung injury.

The importance of FoxM1-mediated EC regeneration across the lifespan has been further explored by Huang and colleagues, who used EC-specific lineage tracing to confirm that EC proliferation is required for complete vascular repair following inflammatory injury in young mice

⁴⁵.

An important counter-regulatory mechanism has been described by Zhu and colleagues, who demonstrated that neutrophil extracellular traps (NETs) generated in the inflammatory milieu of VILI impair EC proliferative repair by inhibiting PLK1, a direct FoxM1 transcriptional target and essential mitotic kinase ³². PLK1 inhibition by NET-associated components caused G2/M cell cycle arrest in pulmonary EC, effectively preventing completion of mitosis and blocking new EC production. This finding identifies the neutrophil-dominated inflammatory microenvironment of early VILI as an active suppressor of the endothelial regenerative response, creating a mechanistic basis for the clinical observation that the severity of the initial inflammatory response is a negative predictor of recovery.

1.3.4 Endothelial-epithelial crosstalk

The concept that endothelial cells function not merely as passive barriers but as active instructive secretory compartments has transformed understanding of organ homeostasis and repair ⁴⁶. In the alveolus, the anatomical intimacy of EC and AT2 cells provides the structural substrate for highly efficient paracrine signalling.

The bidirectional character of this crosstalk was established by Ellis and colleagues, who demonstrated that epithelial VEGFA instructs EC identity during alveologenesis ⁴⁰.

A mechanistically compelling link between EC mechanical activation and AT2 proliferation is provided by the CTGF/CCN2 axis. YAP/TAZ nuclear translocation in mechanically stimulated EC drives transcriptional upregulation of CTGF, which is secreted and acts on adjacent AT2 cells to promote cell cycle entry in a specific subpopulation⁴⁷. This creates the following mechanobiological sequence in which the EC functions as an intermediate transducer converting mechanical information into a paracrine mitogenic signal: stretch → EC YAP activation → CTGF secretion → AT2 proliferation.

The synergistic importance of both mechanical stimulation and EC presence for AT2 proliferation has been demonstrated in an engineered alveolus-on-chip model by Niu and colleagues, who showed that AT2 cells proliferate more robustly when co-cultured with endothelial cells in the presence of cyclic mechanical stretch than under either condition alone ²⁹.

2. AIM OF THE THESIS

This thesis aims to investigate the role of mechanical stretch in driving proliferative responses of pulmonary endothelial cells (EC) and alveolar type 2 (AT2) cells in the context of ventilator-induced lung injury (VILI) and repair. Using a murine VILI model alongside an ex vivo cyclic stretch bioreactor system, the work pursues three integrated aims.

The first aim seeks to characterise a model that keeps the main histological characteristics of VILI and is also suitable for the study of the recovery that happens in the first weeks after injury.

The second leverages the developed model to investigate the temporal kinetics of EC and AT2 cell proliferation in vivo across the post-injury recovery time-course.

The third aims to investigate whether cyclic mechanical stretch, applied in an ex vivo setting, can drive proliferation in EC and AT2 cells respectively, and whether the two cell types differ in their response when exposed to mechanical stretch.

Collectively, these aims are designed to further understand whether and how the pulmonary endothelium and epithelium could contribute to alveolar repair following mechanical injury.

3. METHODS

3.1 In Vivo murine model of Ventilator-Induced Lung Injury (VILI)

3.1.1 Animals and experimental groups

Adult C57BL/6 mice were randomly allocated to one of four experimental groups:

- 1 non-ventilated (NV) control group
- 2 protective ventilation (PV) group, ventilated with a low tidal volume (7 μ l/g) and high respiratory rate designed to approximate lung-protective ventilation strategies
- 3 intermediate-injury group (IV- injurious ventilation), ventilated with a moderate tidal volume of 15 μ l/g (IV15)
- 4 high-injury group, ventilated with a markedly elevated tidal volume of 21 μ l/g (IV21)

3.1.2 Anaesthesia, orotracheal intubation and mechanical ventilation

Anaesthesia was induced by intraperitoneal injection of a ketamine/xylazine combination (75/5 mg/kg). Anaesthetic depth was maintained throughout the protocol with supplemental intraperitoneal boluses of ketamine/xylazine administered approximately hourly, titrated to the absence of a withdrawal reflex. Body temperature was monitored continuously and maintained at 37–38 °C using a heating pad/lamp to prevent anaesthesia-associated hypothermia.

Following induction, animals in the three ventilated groups underwent orotracheal intubation under direct visualization. Mice were secured to surgical panel and the superior incisors were hooked and tied with a suture to the border to help straighten the curvature between the trachea and larynx and allow visualization of the oropharyngeal cavity. A pointed light source with a flexible arm was centred in the ventral part of the mouse's neck ideally above the trachea just below the vocal cords. The tongue was uplifted with forceps to expose the oropharyngeal cavity and the larynx, once adequate visualization was achieved (as shown in Figure 3.1) the trachea was intubated directly with a 22-G intravenous catheter over a tip-blunted needle modified so that it never reaches the tip of the catheter to avoid traumatism to the mouse's airway.

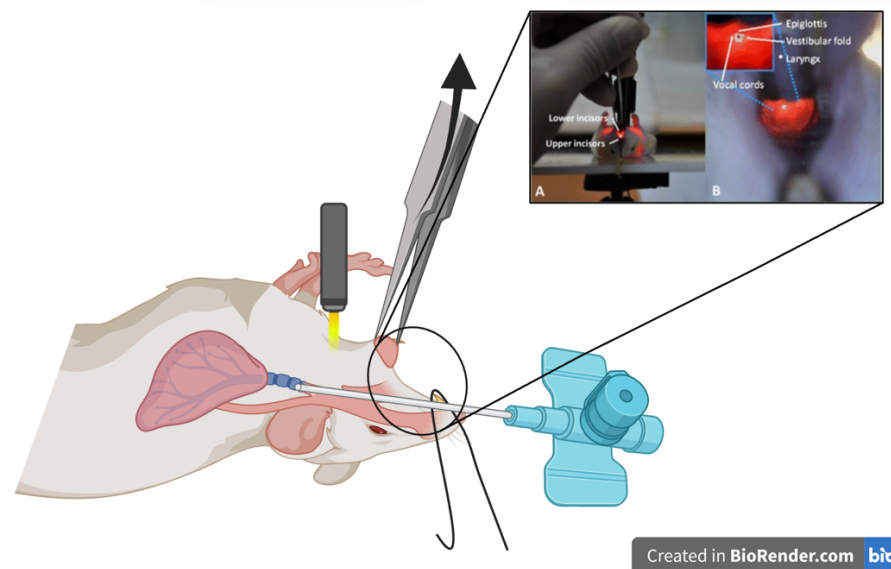


Figure 3.1. Orotracheal intubation in the mouse via transillumination of the larynx. In the rectangle a view of the exposed oral cavity obtained by lifting the tongue (panel A) and a view of the transilluminated glottis in panel B from Su et al.⁴⁸

Correct tracheal placement of the cannula was confirmed by visible bilateral chest excursion synchronous with the ventilator cycle. Animals were then connected to a small-animal mechanical ventilator (Minivent, HSE) and ventilated at zero end-expiratory pressure (ZEEP) with an inspired oxygen fraction (FiO_2) of 0.21 (room air) for the duration of the protocol, as detailed in Table 3.1.

Tidal volume (VT) and respiratory rate (RR) were set according to group assignment (Table 3.1). Ventilation was maintained for the full duration of the experiment (4 hours) with continuous clinical monitoring of bilateral ventilation, adequate sedation, and perfusion per protocol.

To maintain adequate hydration during anaesthesia, boluses of warmed NaCl 0.9% of 0.1 ml were administered intraperitoneally at one-hour intervals, alongside supplemental anaesthetic boluses, in all ventilated groups.

3.1.3 Endpoint procedures and sample collection

At the end of the ventilation period, the following procedures were performed under terminal anaesthesia:

- 1 Bronchoalveolar lavage (BALF) collection. The lungs were lavaged with 0.6 ml PBS, administered and withdrawn three times (total instilled volume $\approx$ 1.8 ml), to recover BALF. Recovered BALF was centrifuged and the supernatant stored at -80°C .

- 2 Pulmonary vascular perfusion. The pulmonary vasculature was perfused via the right ventricle with PBS/0.9% NaCl to clear the circulation prior to tissue harvest.
- 3 Macroscopic lung harvest. Lungs were excised and separated by lobe (right superior, middle, inferior, and post-caval lobes; left lung as a single lobe) and fixed in formalin.

Table 3.1. Ventilation and procedural parameters across the four primary experimental groups.

Parameter	Non-ventilated (control)	Protective ventilation (PV)	Injurious ventilation, 15 µl/g	Injurious ventilation, 21 µl/g
Mechanical ventilation	None (spontaneous)	Yes	Yes	Yes
Tidal volume (VT)	Spontaneous	7 µl/g	15 µl/g	21 µl/g
Respiratory rate (RR)	Spontaneous	160 breaths/min	120 breaths/min	80 breaths/min
FiO ₂	0.21 (room air)	0.21	0.21	0.21
PEEP	—	ZEEP (0 cmH ₂ O)	ZEEP (0 cmH ₂ O)	ZEEP (0 cmH ₂ O)
I:E ratio	—	1:1	1:1	1:1
Anaesthesia induction	—	Ketamine/xylazine 75/5 mg/kg IP	Ketamine/xylazine 75/5 mg/kg IP	Ketamine/xylazine 75/5 mg/kg IP
Anaesthesia maintenance	—	Supplemental IP boluses (~hourly)	Supplemental IP boluses (~hourly)	Supplemental IP boluses (~hourly)
Airway management	None (no intubation)	Orotracheal intubation	Orotracheal intubation	Orotracheal intubation
Fluid support	Free water access	0.9% NaCl, ~0.1 ml IP / 1 h (warmed)	0.9% NaCl, ~0.1 ml IP / 1 h (warmed)	0.9% NaCl, ~0.1 ml IP / 1 h (warmed)
Body temperature	37–38 °C	37–38 °C	37–38 °C	37–38 °C

Parameter	Non-ventilated (control)	Protective ventilation (PV)	Injurious ventilation, 15 μ l/g	Injurious ventilation, 21 μ l/g
Duration of ventilation	Equivalent observation period	4 h	4 h	4 h

3.2 Recovery Cohort for the EdU Proliferation Time-Course

The intermediate-injury ventilation protocol (IV15, 15 μ l/g) was selected for this cohort specifically because it reliably produced substantial, graded histological injury while maximising the likelihood of successful extubation and survival to the later recovery time points.

Anaesthesia was obtained with Medetomidine/Ketamine, the advantage being that Medetomidine has a reversal agent (atipamezole). The ventilation protocol was the same as previously described.

At the end of the 4 hours of ventilation, sedation was reversed and mice were extubated as soon as an appropriate breathing pattern was present. During recovery heart rate and visual assessment of peripheral perfusion.

EdU was administered by intraperitoneal injection on the day of ventilation (after anaesthesia induction) and at four-day intervals thereafter, and animals were sacrificed at Day 1, Day 2, Day 3, Day 7, and Day 15 post-ventilation, for a total of five recovery time points.

3.3 Tissue sampling and histological analysis

Following completion of the assigned ventilation or recovery protocol, animals were euthanised and lungs were fixed by standardised, fixed-volume intratracheal instillation of PBS to standardise alveolar geometry. Vascular perfusion was performed prior to fixation to clear intravascular blood, ensuring that any haemorrhage identified on histological sections reflected true extravasation into the interstitial or alveolar compartment rather than retained intravascular content. Lungs were sampled by lobe per animal (R1–R4 and L, reflecting murine lung anatomy), processed to paraffin, fixed, cut and mounted to slides.

Serial sections from each region were stained with H&E for assessment of and inflammatory architecture, and with CD45 IHC for assessment of leucocyte infiltration. All sections were imaged at 4 $\times$, 10 $\times$, 20 $\times$ and 40 $\times$ according to the requirements of the individual scoring instrument described below.

3.3.1 Histological scoring framework

Lung injury was quantified using four complementary scoring instruments (ATS, modified-ATS, structural and inflammatory), each derived from the same underlying field-level component grades according to a distinct formula and rationale (Table 3.3.1), and then combined together forming a Composite score. All scores were normalised to a 0–1 range to facilitate comparison across instruments and across animals.

Table 3.3.1 Histological scoring instruments and formulae

Score	Instrument & stain	Formula	Components
ATS score (2011)	Full ATS weighted score (H&E, 40×)	$[(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)] / (N \times 100)$	A = alveolar neutrophils, B = interstitial neutrophils, C = hyaline membranes, D = proteinaceous airspace debris, E = septal thickening; each 0–2 per field, N fields. Range 0–1.
Modified-ATS score	Restricted ATS subscale (H&E, 40×)	$[(14 \times P_{\text{int}}) + (2 \times S)] / (N \times 32)$	P_int = interstitial neutrophils, S = septal thickening; same relative weighting (7:1) as the original ATS formula. Range 0–1.
Structural score	Unweighted structural injury (H&E, 4×)	$\Sigma(O + Hg + R + At + Ov) / (N \times 10)$	O = oedema, Hg = haemorrhage, R = rupture, At = atelectasis, Ov = overdistension; each 0–2 per field, equal weight. Range 0–1.
Inflammatory score	Total CD45 ⁺ leucocyte density, quadrant-graded (IHC, 40×)	Field score = mean of 4 quadrant grades / 3; animal score = mean field score across all qualifying 40× fields	Each 40× field divided into four equal quadrants (TL/TR/BL/BR); each quadrant's total pooled CD45 ⁺ count (no PMN/Mφ/Lym subtyping)

Instrument		Formula	Components
Score	& stain		
			graded 0–3 against a four-tier density scale (0 = 0–5 cells; 1 = 6–10; 2 = 11–20; 3 = >20 cells), adapted from Heinemann et al. (2018). Range 0–1.
Composite score	Domain-representative composite	(Modified-ATS + Structural + Inflammatory) / n_domains_available	Mean of the most representative subscore from each of three non-overlapping injury domains. Range 0–1.

3.4 Isolation of Primary Alveolar Type II Cells and Pulmonary Endothelial Cells

3.4.1. Reagents and Equipment

The following reagents and equipment were prepared in advance of each isolation: Dispase (4 ml per animal), pre-warmed to 37 °C, DNase I recombinant; 1000 IU in 10 ml DMEM, pre-warmed to 37 °C, Enzymatic digestion mix “DPA”: DMEM (no FBS, no penicillin/streptomycin) supplemented with three enzyme components (Enzyme D, Enzyme P, Enzyme A) at the volumes shown in Table 3.4.1; prepared fresh and used at roughly 1 ml per lung, cell-isolation buffer (PEB): 5 ml 5% BSA (sterile) + 200 µl 0.5 M EDTA + PBS to 50 ml, sterile-filtered (0.22 µm) and stored at 4 °C; prepared fresh on each occasion of use.

Culture plates were pre-coated with fibronectin/gelatin (F/G; 2 h at 37 °C) to coat both plastic and silicone membrane plates. Surgical instruments, ethanol for instrument sterilisation, anaesthetic and intraperitoneal injection syringes, a 22G tracheal cannula, a tissue chopper blade and disc.

The following were used: FBS (15 ml, to quench enzymatic reactions) and warmed culture media: DMEM with 10% FBS and penicillin/streptomycin, PneumaCult (for AT2 culture), and EGM-2 (for EC culture).

Table 3.4.1 Composition of the enzymatic digestion mix (“DPA”) at three preparation volumes.

Component	2.5 mL	5 mL	10 mL
DMEM (no FBS, no P/S)	2.35 mL	4.7 mL	9.4 mL
Enzyme D	100 μ L	200 μ L	400 μ L
Enzyme P	25 μ L	50 μ L	100 μ L
Enzyme A	18 μ L	36 μ L	72 μ L

3.4.2 Lung Harvest, Cannulation and Enzymatic Digestion

Mice were euthanized by intraperitoneal injection of ketamine and the following surgery was performed.

The thoracic cavity was opened just below the costal margin and the diaphragm incised. Because diaphragmatic puncture rapidly compromises cardiac output, lung perfusion was performed promptly thereafter (step 1 shown in figure 3.2). The pulmonary vasculature was perfused via the right ventricle (pulmonary artery) with 10 ml cold PBS, delivered slowly and steadily; perfusion was repeated with a further 10 ml if the lungs did not turn uniformly white, indicating incomplete clearance of intravascular blood.

The trachea was exposed by removing the overlying superficial muscles. A small incision was made in the trachea midway between two cartilage rings, positioned at approximately the mid-point of the trachea. A 22G cannula was inserted gently through the incision until it reached proximity but did not pass the carina. The lungs were lavaged with 10 ml PBS, instilled via the cannula so that both lungs were observed to inflate symmetrically during this step and then completely removed. Keeping the cannula stable in the trachea, the PBS syringe was removed and replaced with a syringe containing 1 ml dispase, which was instilled slowly and steadily. Before withdrawing the cannula, the trachea was closed with fine forceps or ligated with suture to prevent efflux of the instilled dispase (step 2 as shown in figure 3.2).

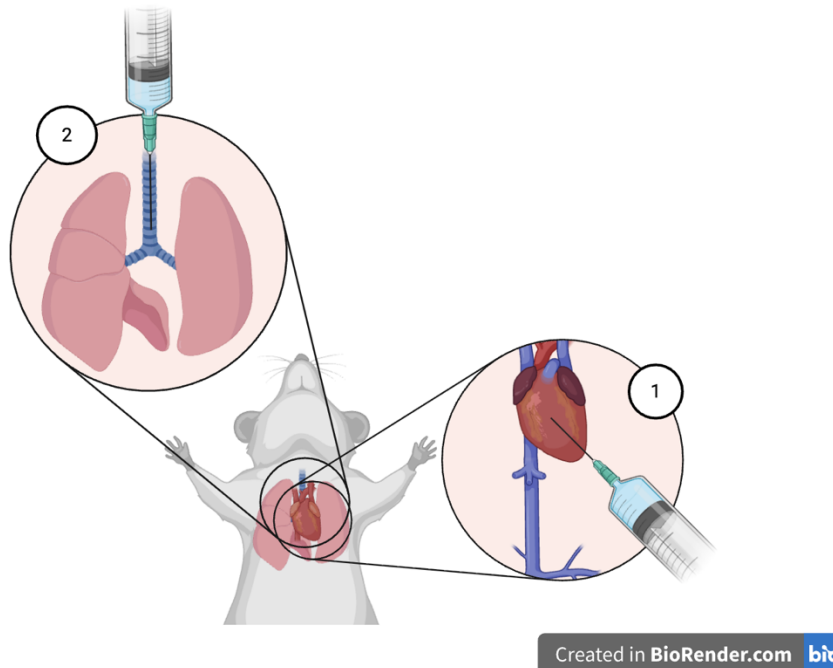


Figure 3.2. Step 1: *perfuse the lung with 10 mL PBS via the pulmonary artery. Step 2: inject 2 mL dispase through the trachea.*

The lungs were then excised without opening the trachea (easily achieved by beginning the dissection from the dorsal aspect) and transferred into 3 ml dispase in a 50 ml tube for 30 minutes at room temperature (precision in this step is of critical importance for digestion consistency).

After 30 minutes, the distal lung tissue was cut away and mechanically finely chopped. The digested tissue was then incubated with DNase (10 ml DMEM + 100 μ l DNase stock) for 10 minutes at 37 °C. At the end of digestion, the enzymatic reaction was quenched with 1 ml FBS.

3.4.3 Mechanical Dissociation and Filtration

The digested tissue suspension was pipetted vigorously (approximately 20 strokes) until little or no intact tissue remained visible, then sequentially passed through 100 μ m and 40 μ m cell strainers, each pre-wetted with 1 ml FBS and rinsed with a further 2 ml FBS to maximise cell recovery and minimise loss to the filter membrane. The filtered suspension was centrifuged at 400 $\times$ g for 10 minutes.

3.4.4 Depletion of Fibroblasts by Differential Plating (Pre-plating)

The cell pellet was resuspended in 8 ml DMEM containing 10% FBS and penicillin/streptomycin and plated onto two 100 mm primary (tissue-culture-treated) dishes for 30 minutes at 37 °C, exploiting the more rapid adherence of contaminating fibroblasts relative to epithelial and

endothelial cells. The supernatant, containing the non-adherent epithelial/endothelial-enriched fraction, was then gently collected and re-plated onto a fresh dish, with the original dish washed gently with 2 ml medium to recover residual non-adherent cells without dislodging the adherent fibroblasts. This pre-plating step was repeated for a total of two to three cycles, after which the non-adherent fraction was centrifuged at $400 \times g$ for 10 minutes and carried forward into the immunomagnetic purification steps described below.

3.4.5 Immunomagnetic Purification of AT2 Cells

The fibroblast-depleted cell pellet was resuspended in 30 μ l of a biotinylated antibody cocktail directed against T-cell and dendritic-cell surface markers (Dynabeads Mouse Untouched T-cell and DC Enrichment kits) plus 100 μ l PEB, and incubated on ice for 20 minutes with gentle mixing every 2–3 minutes. During this incubation, the corresponding Dynabeads (40 μ l) were washed three times in 500 μ l PEB on a magnetic separator. The antibody-labelled cell suspension was then diluted with 3 ml PEB and centrifuged at $400 \times g$ for 10 minutes; the resulting pellet was resuspended in 700 μ l PEB and combined with the pre-washed beads (resuspended in a further 700 μ l PEB), and the combined suspension incubated on ice for 15 minutes. Bead-bound T cells and dendritic cells were then captured on a magnet and discarded, and the unbound (T-cell/DC-depleted) supernatant, enriched for AT2 cells, was recovered and centrifuged at $400 \times g$ for 10 minutes.

The resulting cell pellet was plated onto dishes pre-coated with Matrigel. AT2 cells adhere to this substrate over approximately 48 hours; plates were left undisturbed during this period, as cells dislodged before stable attachment do not survive.

3.4.6 Immunomagnetic Purification of Pulmonary Endothelial Cells

A parallel purification strategy was used to isolate CD31-expressing pulmonary endothelial cells from the same (or an equivalent) lung digest, following pre-plating as described in the methods section 3.4.4. After the final pre-plating step, contaminating erythrocytes were lysed (RBC lysis) and the suspension centrifuged at $400 \times g$ for 10 minutes.

The cell pellet was resuspended in PEB containing anti-CD31 MicroBeads (Miltenyi Biotec), again at 1:20 (200 μ l PEB + 20 μ l beads per mouse), and incubated on ice for 20 minutes with gentle agitation every 2–3 minutes to positively label endothelial cells. The suspension was applied to a fresh MACS column pre-equilibrated with PEB, washed with 5 ml PEB and CD31-positive endothelial cells were eluted from the column by flushing with 1–2 ml EGM-2 under gentle positive pressure. Eluted cells were counted and seeded directly in EGM-2.

3.4.7 Matrigel Coating of Silicone Stretch Membranes

Silicone culture membranes used in the MechanoCulture FX stretch system were coated with basement-membrane extract (Matrigel, MG) to provide a substrate more representative of the native alveolar basement membrane and compatible with the mechanical properties of the silicone wells.

An aliquot of concentrated Matrigel (stored at -20°C) was thawed on ice for approximately 15–20 minutes. Matrigel was never thawed at room temperature, as this promotes premature gelation. Once thawed, the Matrigel was diluted by transferring a small volume of cold coating medium into the Matrigel tube, then transferring the entire diluted volume back into the 15 ml Falcon tube containing the remaining coating medium. The diluted Matrigel solution was mixed by gentle inversion of the tube three times (vortexing was avoided, to prevent bubble formation and premature gelation). Wells or silicone membranes were coated with the diluted Matrigel solution and placed in the incubator for at least 30 minutes, and ideally 1 hour; incubation was not extended beyond 2 hours. Coated membranes were washed once with PBS immediately before cell seeding. Cells were added directly in their culture medium following the PBS wash.

Coated wells were not permitted to dry out between the coating and seeding steps, as this causes the Matrigel layer to crystallise and lose its substrate properties; wells were kept moist throughout handling.

3.5 In Vitro Cyclic Mechanical Stretch System

3.5.1 Device and Culture Format

Cyclic mechanical stretch was applied to primary AT2 cells and pulmonary endothelial cells using the MechanoCulture FX system (CellScale Biomaterials Testing, Waterloo, Canada), a bench-top mechanobiology platform that allows uniaxial cyclic stretching of up to 16 independent culture wells.

The system uses sterile, single-use silicone culture plates in which each well measures 8 mm $\times$ 8 mm and is bounded by a thin (0.25 mm), optically transparent base membrane with optical properties similar to a glass coverslip, permitting high-quality brightfield and fluorescence imaging of adherent cells during or after stretch. For the monolayer culture experiments described in this thesis, cells were seeded directly onto the Matrigel-coated membrane as an adherent monolayer. Wells are top-accessible when loaded into the device, permitting cell seeding and medium exchange without removal of the plate from the instrument.

All cell-contacting components of the system are autoclavable, and the device is designed to operate inside a standard tissue-culture incubator, maintaining physiological temperature, humidity and gas exchange throughout the stretch protocol. Stretch waveforms — including ramp (constant-velocity) and sinusoidal patterns — together with stretch magnitude, frequency,

hold and rest durations, and cycle (repetition) counts, are user-programmable via the manufacturer's software and executed by an on-board controller.

3.5.2 Stretch Protocol Parameters

Cyclic stretch magnitudes were selected to model physiological and pathological regimens of alveolar deformation as characterised in the published mechanobiology literature, in which physiological tidal strain is typically in the range of 5–12% linear or equibiaxial strain and pathological, injurious strain regimens are typically modelled at magnitudes of approximately 15–30% strain, depending on the experimental system.

Standard condition: 5% linear strain at 0.25 Hz, applied as a displacement of approximately 0.5 mm per cycle, for a total duration of 24 hours, with a 2-second extension/2-second return duty cycle.

Comparator (increased-frequency) condition: the same 0.5 mm displacement ($\approx 6\%$ linear strain), but with the duty cycle shortened to 1-second extension/1-second return, doubling the cycling frequency at the same linear displacement (≈ 0.5 Hz), for the same total duration of 24 hours.

EdU was present in the culture medium only during the stretch phase, to label cells proliferating in direct temporal association with the mechanical stimulus rather than during the preceding 72-hour adherence period.

3.6 Immunofluorescence

3.6.1 Tissue Preparation and Antigen Retrieval

Paraffin sections were deparaffinised (60 °C oven, 4 h, followed by two 15-minute xylene washes) and rehydrated through a descending ethanol series (100%, 95%, 70%, 50%, 4 minutes each), followed by two 5-minute washes in distilled water. Antigen retrieval was performed by heat-induced epitope retrieval in citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0; prepared from 2.94 g tri-sodium citrate dihydrate per litre of distilled water, pH-adjusted with HCl), brought to boiling in a microwave and maintained at approximately 60% microwave power for 30 minutes, followed by cooling to room temperature on ice for 1 hour and washing in PBS.

Sections were permeabilised in 0.1% Triton X-100 in PBS for 20 minutes, and a hydrophobic barrier was drawn around each section using a Pap pen before sections were placed in a humidified chamber for all subsequent incubation steps.

3.6.2 Blocking and Primary Antibody Incubation

Sections were blocked for at least 1 hour at room temperature in 5% BSA in 0.1% Triton X-100 (2.5 g BSA per 500 mL of 0.1% Triton/PBS), with the blocking serum selected according to the host species of the secondary antibody to be used. Primary antibodies were applied directly (without an intervening wash) in blocking solution and incubated overnight at 4 °C. Endothelial cells were identified using an antibody against ERG (ETS-related gene), a nuclear endothelial-lineage transcription factor; alveolar type II (AT2) cells were identified using an antibody against pro-surfactant protein C (pro-SPC), a cytoplasmic AT2 marker (used at 1:300 in blocking solution in the related static-culture AT2 staining protocol; abcam ab90716, rabbit).

3.6.3 EdU Detection (Click Chemistry)

Following primary antibody incubation and washing, EdU was detected using a Click-iT-based reaction cocktail (Click-iT reaction buffer, CuSO₄, an Alexa Fluor azide, and reaction buffer additive, combined per kit instructions immediately before use). Sections were washed in PBS, incubated with the freshly prepared reaction cocktail for 30 minutes protected from light, and washed a further three times in PBS.

3.6.4 Secondary Antibody, Nuclear Counterstain and Mounting

Secondary antibodies were applied at 1:500 in blocking solution for 2 hours at room temperature, protected from light. Nuclei were counterstained with Hoechst or DAPI (1:5000 in PBS, 15 minutes), and sections were mounted using Fluoromount G or Mowiol and allowed to dry fully, protected from light, before imaging.

3.6.5 Cell-Type Assignment and Quantification

Endothelial cells were assigned by EdU⁺/ERG⁺ co-localisation against the DAPI nuclear stain; AT2 cells were assigned by restricting EdU⁺/DAPI⁺ nuclei to those located within a pro-SPC⁺ cell. For each animal and region, the proportion of EdU⁺ cells within each lineage-marker-positive population were calculated as the primary proliferation readout.

3.6.6 Application to the Ex Vivo Stretch System

The same EdU click-chemistry detection chemistry was used for the ex vivo cyclic stretch experiments, with EdU present in the culture medium only during the 24-hour stretch phase. Following stretch, silicone-membrane cultures were fixed, permeabilised, and stained following the same general sequence as the paraffin-section protocol above, substituting fixation in 4% PFA for 15 minutes at room temperature for the deparaffinisation/antigen-retrieval steps used for fixed paraffin tissue.

3.7 Statistical methods

Three distinct statistical approaches were used across the experimental components of this thesis:

Histological injury scores were treated primarily descriptively (group means and SD). Exploratory analysis was performed with Anova.

The in vivo EdU proliferation time-course was analysed by Anova (Nested) across the recovery time-course (NV/Day1/Day2/Day3/Day7/Day15). The ex vivo stretch experiments were analysed using an unpaired t-test with Welch's correction for unequal variances for single-replicate comparisons between control and stretch conditions, and a nested t-test, treating individual culture wells as nested within independent experimental replicates. Unless otherwise specified, the experimental unit for in vivo histological and proliferation analyses is the animal (multi-field measurements were averaged per animal prior to statistical comparison), whereas ex vivo stretch analyses were performed at the level of individual culture wells, nested where indicated within independent experimental replicates.

All analyses and graphs were performed in GraphPad Prism (version 10).

4. RESULTS

4.1 Establishing a mouse ventilator-induced lung injury (VILI) model

The first part of the project aimed to create a mouse lung injury model that could be used for recovery studies afterwards, focusing on the effect of stretching on lung parenchyma. For this reason, a Ventilator-Induced Lung Injury (VILI) model was chosen, and two different injurious volumes were compared, to isolate the effects of different magnitudes of lung stretch.

4.1.1 Ventilatory parameters for the Ventilator-Induced Lung Injury (VILI) model

Based on existing literature, two levels of injurious ventilation chosen were 15 $\mu\text{l/g}$ (IV15) and 21 $\mu\text{l/g}$ (IV21), the two VILI experimental groups were compared with a non-ventilated group and a protective ventilation group mimicking physiologic ventilation, giving the following four experimental groups:

1. Not ventilated (NV; n = 2)
2. Protective ventilation group with volume of 7 $\mu\text{l/g}$ (PV; n = 2)
3. Injurious ventilation group with volume of 15 $\mu\text{l/g}$ (IV15; n = 2)
4. Injurious ventilation group with volume of 21 $\mu\text{l/g}$ (IV21; n = 2)

All ventilated animals were sedated, intubated and mechanically ventilated for 4 hours as described in detail in the Methods section (Table 3.1). In the ventilated groups, I:E was always 1:1, no PEEP (Positive End Expiratory Pressure) was applied; tidal volume (TV) and respiratory rate (RR) were varied reciprocally by experimental design across the four groups:

- A. Protective ventilation group. TV= 7 $\mu\text{l/g}$, RR= 160bpm
- B. Moderate injurious ventilation group. TV= 15 $\mu\text{l/g}$, RR= 120bpm
- C. Severe injurious ventilation group. TV= 21 $\mu\text{l/g}$, RR= 80bpm

No oxygen was given (FiO_2 = 21%) to avoid oxygen-related damage in line with the stretch-injury model.

4.1.2. Histology scores for different domains and recapitulatory composite score

Lung slides were stained for haematoxylin and eosin (H&E) and for CD45 immunohistochemistry (DAB chromogen, haematoxylin counterstain), and sections were scored blind to group identity using an histological framework created following the “Update on the Features and

Measurements of Experimental Acute Lung Injury in Animals: An Official American Thoracic Society Workshop Report”⁴⁹ of 2022 that updated the 2011 Workshop Report “Features and Measurements of Experimental Acute Lung Injury in Animals” of the same scientific society⁵⁰. Accordingly, lung injury was quantified using four related sub-scores that address separately the general histology, structural and inflammatory domains:

1. ATS score (2011): quantifies alveolar neutrophils, interstitial neutrophils, hyaline membranes, proteinaceous airspace debris, septal thickening, as per the Matute-Bello et al.⁵⁰. Preserves direct comparability with the wider published ATS literature, using the original five-component weighted formula unmodified.
2. Modified-ATS score: restricted to the two ATS components not influenced by sample handling (interstitial neutrophils and septal thickening), with the same weighting given in the original score
3. Structural score: quantifies architectural derangement and loss of normal alveolar architecture (oedema, haemorrhage, rupture, atelectasis, overdistension), modified from Villar et al. in which a rat model of ventilator induced lung injury was scored against pulmonary edema, atelectasis, alveolar overdistension, vascular congestion, alveolar rupture, hemorrhage, fibrosis (0-4 per item)⁵¹
4. Inflammatory score: uses CD45 immunohistochemistry to quantify total leucocyte density. Quantification of CD45 immunostaining as a percentage of positive area per field has been validated in murine models of acute lung inflammation⁵² in the setting of LPS-induced ARDS
5. A composite score was then obtained in order to summarises overall histological severity, being calculated using the most representative subscore of each domain (modified-ATS, structural and inflammatory scores)

4.1.3 Histological injury scores increase progressively with ventilatory stretch magnitude

Across all histological scores, mean values increased progressively from the protective-ventilation group, which is comparable to the non-ventilated baseline, to the high-tidal-volume conditions (Figure 4.1). The modified-ATS score (restricted to non-sample-handling influenced components) rose from 0.451 (NV) to 0.406 (PV) to 0.750 (IV15) and 0.982 (IV21). The ATS score, as expected, was influenced by sample handling; although it kept the same trend, the absolute values were underestimated, increasing from 0.004 (NV) to 0.013 (PV) to 0.030 (IV15) and 0.034 (IV21). The structural score increased from 0.017 (NV) to 0.038 (PV) to 0.125 (IV15) and 0.233 (IV21). The inflammatory score rose from 0.426 (NV) to 0.476 (PV) to 0.750 (IV15) and 0.731 (IV21).

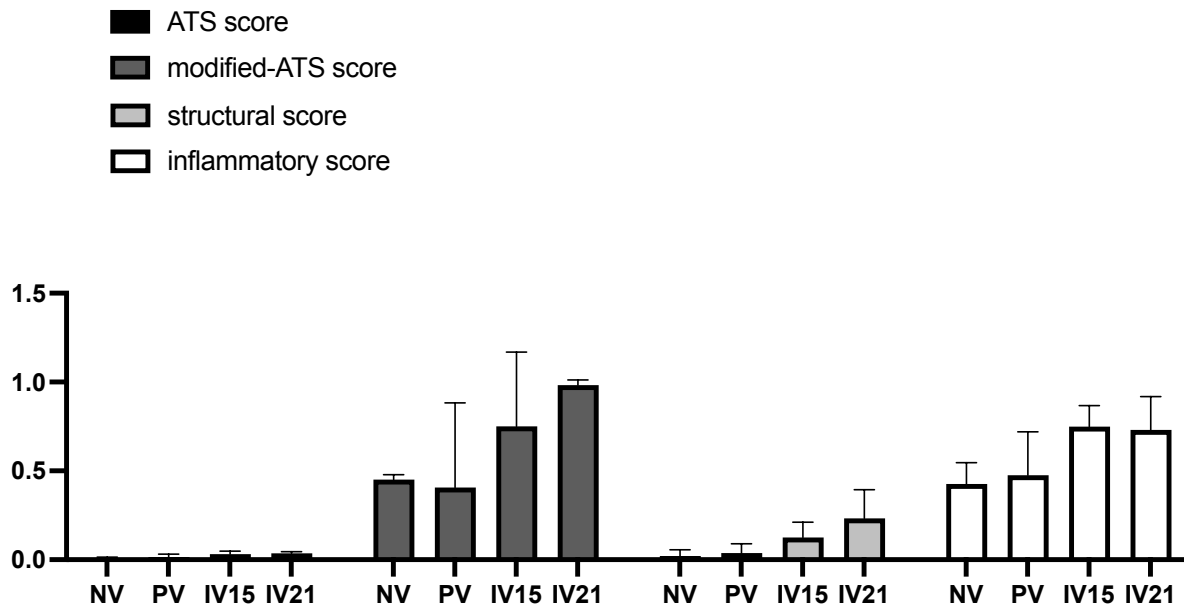


Figure 4.1. Mean histological scores (+/- SD) by subscore across the four ventilation groups across the four ventilation groups: NV= Not ventilated, PV=Protective Ventilation, IV15= Moderate Injurious ventilation with 15 μ l/g, IV21=Severe Injurious ventilation with 21 μ l/g. All four scores increase with increasing ventilatory stretch magnitude, while the protective-ventilation (PV) group shows scores similar to the non-ventilated (NV) control.

A Composite injury score, combining different injury domains in a unique index (modified-ATS, structural and inflammatory scores, but not the ATS score as it would represent duplicate values), showed the same increasing pattern with increased lung stretch with mechanical ventilation (Figure 4.2). It rose from a low baseline in NV and PV (0.18 and 0.37) to the highest values (0.89 and 0.85) in IV15 and IV21.

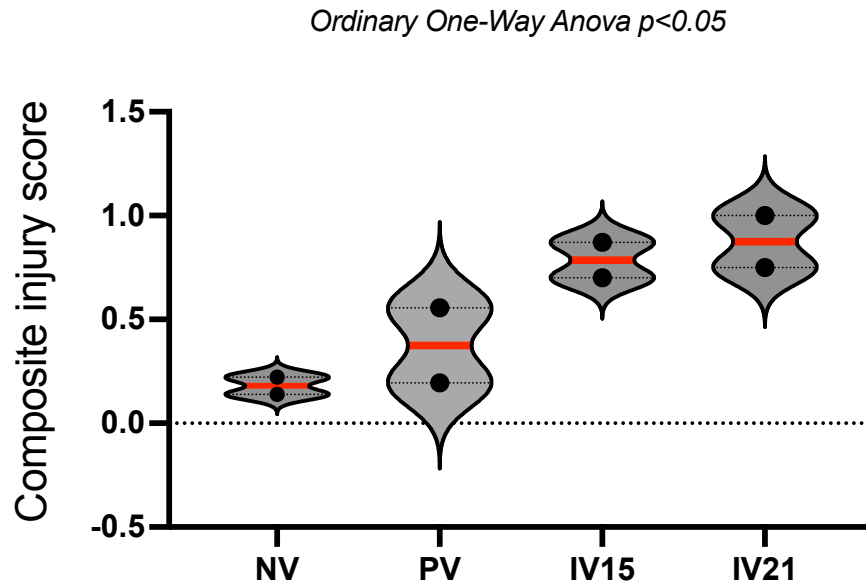


Figure 4.2. Violin plot of the composite injury score across the four ventilation groups: NV= Not ventilated, PV=Protective Ventilation, IV15= Moderate Injurious ventilation with 15 $\mu\text{l/g}$, IV21=Severe Injurious ventilation with 21 $\mu\text{l/g}$. The mean of the group is marked by a red line, each black dot represents the composite score per one subject, with the average score of the animal showed by the dashed line. Ordinary One-Way Anova $p < 0.05$.

Inter-animal heterogeneity was higher within the protective-ventilation group, possibly indicating that protective ventilation produced a more variable histological phenotype and reflecting regional heterogeneity in lung recruitment under this ventilatory mode at zero end-expiratory pressure (ZEEP).

The constituent features of the histology indexes showed that interstitial neutrophilia, septal thickening and intra-alveolar oedema each increased in a linear way across the ventilation gradient. Mean per-field neutrophil scores rose from 1.00 (NV) to 1.71 (IV21), septal thickening from 0.22 to 1.71, and oedema from undetectable at baseline (0.00) to 1.43 at the highest tidal volume. Notably, alveolar rupture (0.14), atelectasis (0.29) and overt overdistension (0.43) were observed exclusively, or near-exclusively, in the IV21 group, indicating that the most severe structural features are distinctive of the higher-threshold level of stretch, that causes severe injury.

CD45⁺ leukocyte density followed the same graded pattern: CD45⁺ macrophage counts per field increased from 1.50 (NV) to a peak of 2.57 (IV15) before declining slightly to 2.22 (IV21), while CD45⁺ neutrophil density continued to rise across the full range (1.00 $\rightarrow$ 1.89). It is important to notice that a proportion of resident macrophages normally inhabits the alveoli, as shown in Figure 4.5, upper panels, consistently with the inflammatory score quantifications.

Representative low-power H&E sections (Figure 4.3) shows the progressive loss of normal alveolar architecture across the increasing stretch conditions (7 to 15 to 21 $\mu\text{l/g}$) represented by more compact and hypercellular parenchyma and narrowing of airspace in the alveoli.

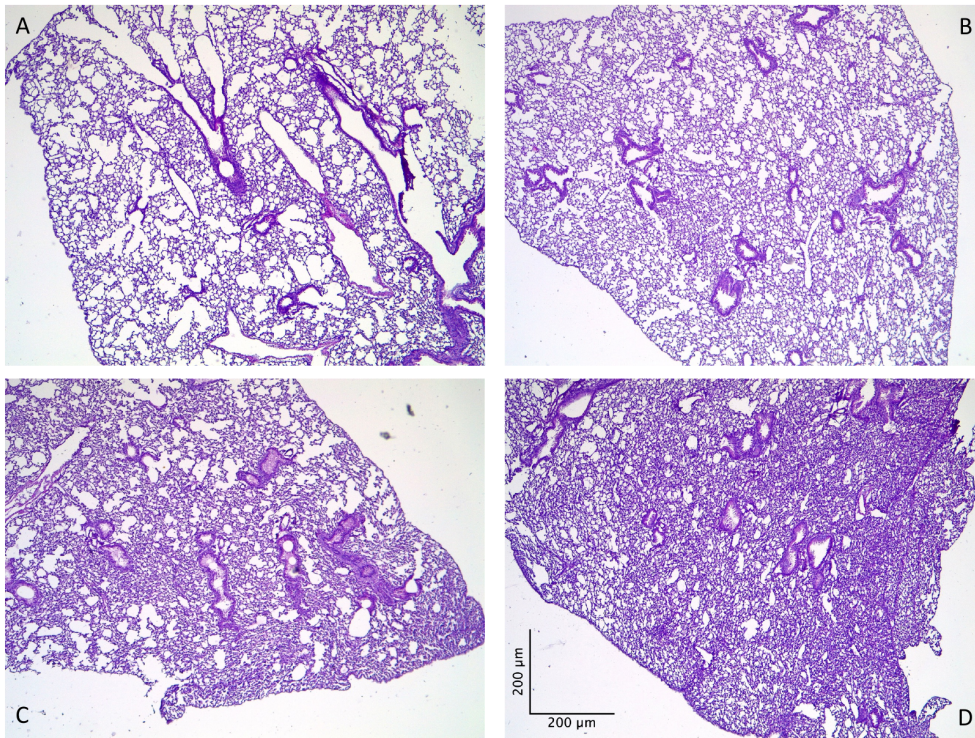


Figure 4.3. Representative low-power (40 $\times$; scale bar 200 μm) H&E-stained lung sections spanning the ventilatory stretch gradient (A: non-ventilated; B: protective-ventilation; C: volume-ventilated, 15 $\mu\text{l/g}$; D: volume-ventilated, 21 $\mu\text{l/g}$). Progressive loss of normal alveolar architecture and increasing parenchymal cellularity are apparent from A to D.

At higher magnification (Figure 4.4), it is possible to note thickened alveolar septa, with increased number of septal leucocytes, and, in the most severely affected regions, foci of proteinaceous interstitial oedema (marked with an asterisk).

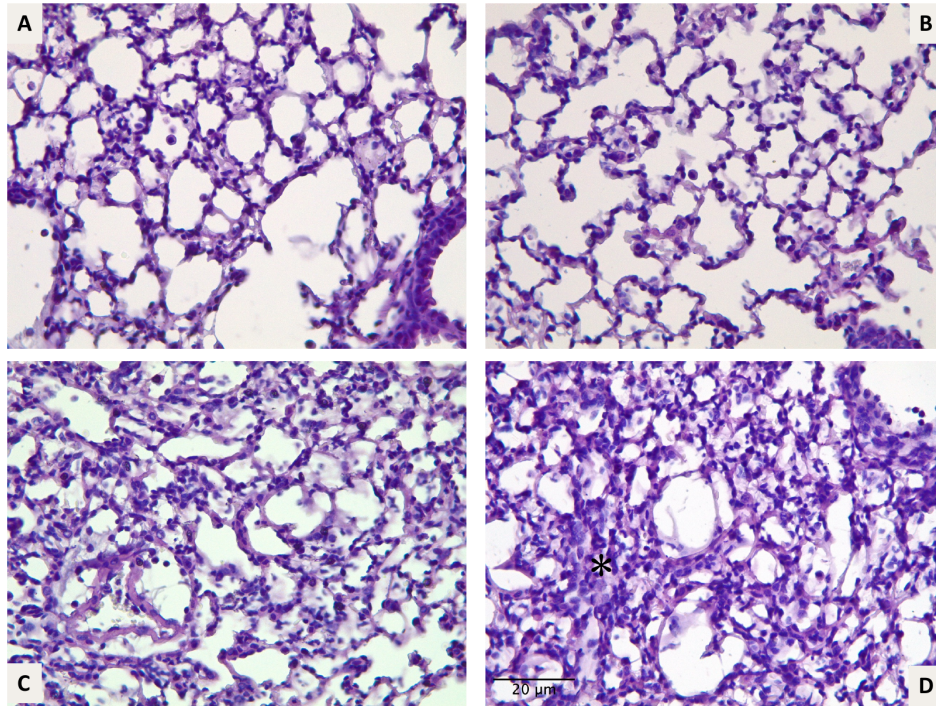


Figure 4.4. Representative high-power (40 $\times$; scale bar 20 μ m) H&E-stained fields from the same stretch-gradient as Figure 4.3. Septal thickening and leucocyte infiltration increase from A to D; the asterisk in panel D marks a focus of proteinaceous interstitial oedema.

Immunohistochemical staining for CD45 (Figure 4.5) shows a corresponding increase in brown (DAB-positive) leukocyte staining across the same stretch-gradient, both within thickened septa and free within the alveolar lumen, mirroring the rise in the quantitative CD45 inflammatory index.

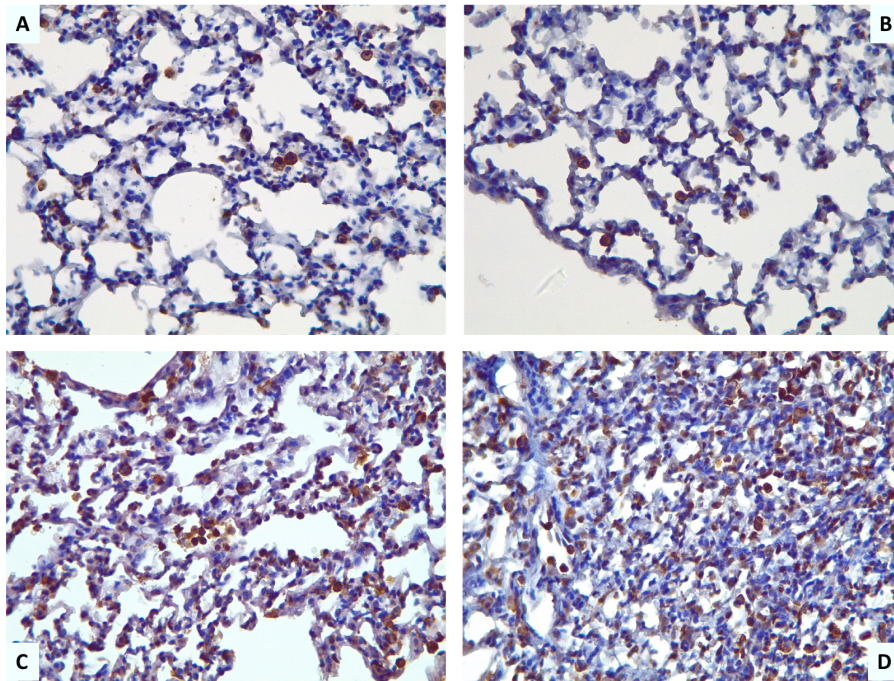


Figure 4.5. CD45 immunohistochemistry (brown, DAB chromogen; haematoxylin counterstain) across the same increasing volume conditions. CD45⁺ leukocyte density increases from A to D, both within the alveolar septa and free within the alveolar airspace. Of note, in panels A and B, the leukocytes are mostly represented by resident macrophages.

Taken together, the histological data demonstrate a clear, near-linear, magnitude-dependent relationship between ventilatory stretch and lung injury severity across the non-ventilated, protective-ventilation and high-volume (15 and 21 $\mu\text{l/g}$) conditions with the unweighted H&E score providing the most sensitive discrimination across the full injury range. These findings provide the histological foundation, and an *in vivo* injury-severity framework, for the following recovery experiments.

4.2 From modelling stretch-related damage to modelling recovery

4.2.1 Anaesthesia protocol improvements for recovery model

To enable long-term recovery rather than terminal endpoints, we refined our anaesthesia protocol. We replaced xylazine/ketamine with medetomidine (Domitor®)/ketamine, administered intraperitoneally, which allowed us to reverse anaesthesia with atipamezole (Antisedan®) and thereby shorten and improve post-operative recovery. To counteract the bradycardia associated with medetomidine, atropine was co-administered intraperitoneally, and haemodynamic parameters were monitored throughout the procedure to ensure cardiovascular

stability, including capillary refill time (CRT), assessed at the footpad by applying gentle digital pressure and timing the return of perfusion, with values exceeding 2 seconds considered indicative of impaired peripheral perfusion. Ophthalmic ointment was applied to both eyes to prevent corneal desiccation, and depth of anaesthesia was tightly controlled using a refined dosing plan combined with periodic reflex checks (toe-pinch) to confirm adequate anaesthesia while avoiding overdose, see scheme in Figure 4.6. To prevent intra- and post-operative hypothermia, animals were kept on warmed pads and received warm saline during surgery, and were placed in a warmed cage immediately after waking, with a warming lamp positioned at a safe distance to provide additional heat support while allowing the animal to move away from the heat source if desired. Following surgery, mice recovered in a warm, clean, quiet environment away from other animals and were continuously visually monitored, with physiological parameters checked at least every 15 minutes, until they regained an upright posture and were walking normally about the cage. Mice were monitored daily for survival and for signs of stress or discomfort, and received intraperitoneal injections of EdU every 3 days, allowing the analog to be available for incorporation into the DNA of cycling cells throughout the recovery period and thus mark cells in the proliferative phase

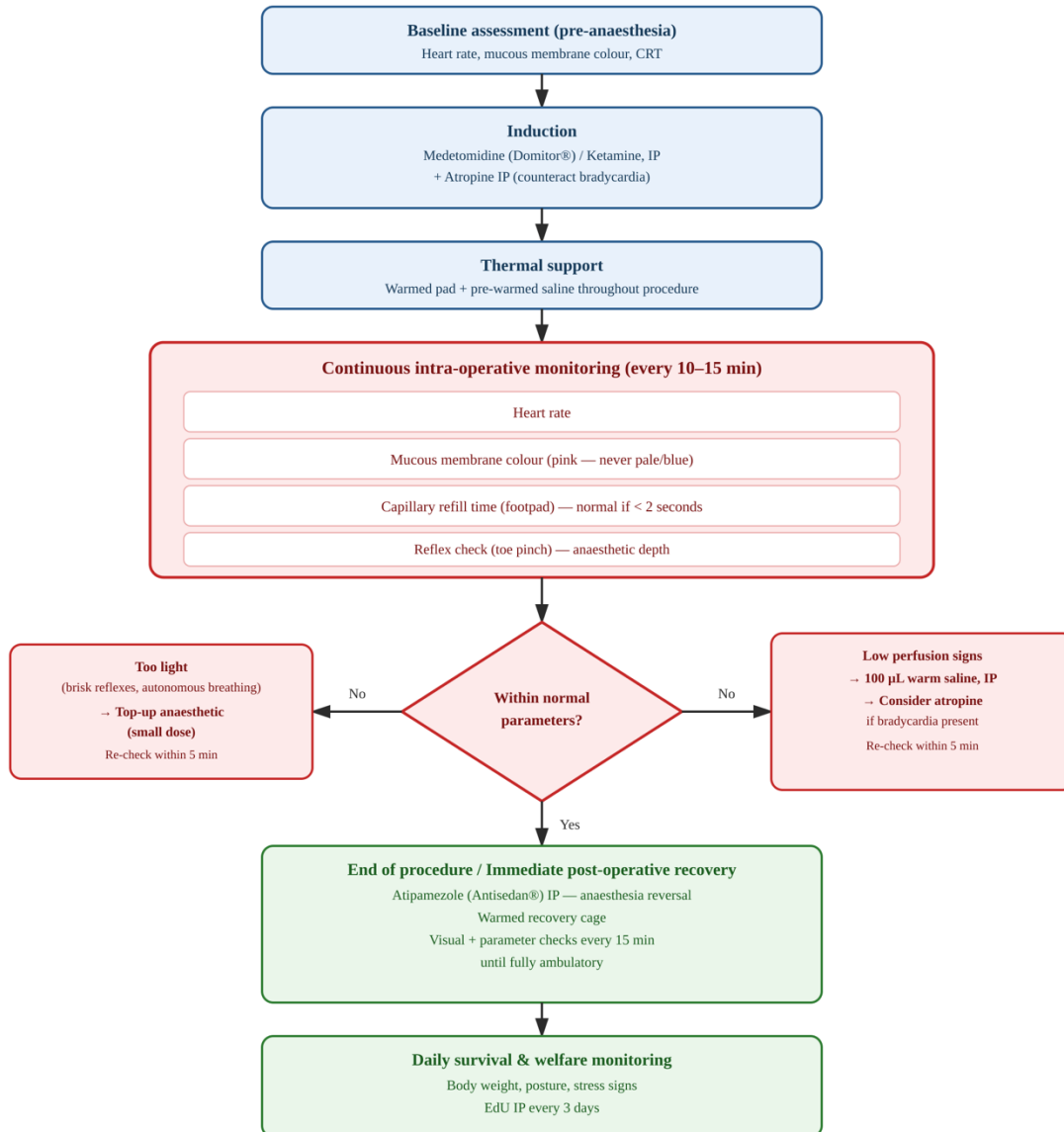


Figure 4.6. Protocol for the maintenance of anaesthesia developed to obtain recovery after injurious ventilation.

4.3 In Vivo Proliferative Response After VILI

4.3.1. Experimental design

The intermediate high-volume condition of 15 µl/g was chosen to be used for further experiments because it appeared to reliably produce substantial, graded histological injury across all indexes while avoiding the most severe damage and structural complications. This approach

was preferred to optimize the chance of survival and to effectively extubate and monitor mice recovering after injurious ventilation, in accordance with the European directive (4 R's).

Following 4 hours of mechanical ventilation, mice were extubated and sedation was reversed, with recovery confirmed by heart rate and visual assessment of peripheral perfusion. EdU was administered on the day of ventilation and every 4 days thereafter, and animals were sacrificed at Day 1, 2, 3, 7 and 15.

Proliferating cells were identified by EdU incorporation and assigned to a cell type by co-staining: endothelial cells were identified using ERG (ETS-related gene), a nuclear endothelial marker, with EdU⁺/ERG⁺ co-localisation assessed against a DAPI nuclear stain; alveolar type II (AT2) cells were identified using pro-surfactant protein C (pro-SPC), a cytoplasmic AT2 marker, with EdU⁺/DAPI⁺ nuclei considered as proliferating only when located within a pro-SPC⁺ cell.

4.3.2 Endothelial cells proliferate soon after injury

Endothelial cells, in lungs recovering from VILI, show an almost immediate proliferative response rising on the first day after ventilation and peaking at Day 2-7 as shown in Figure 4.7. Proliferation slightly declined at Day 3 before rising again at Day 7 possibly reflecting a biphasic endothelial response. This could also be related to the small sample size. Finally, proliferation rate declines toward zero by Day 15. Nested One-Way anova was statistically significant. No different proliferation patterns were found between left and right lobes.

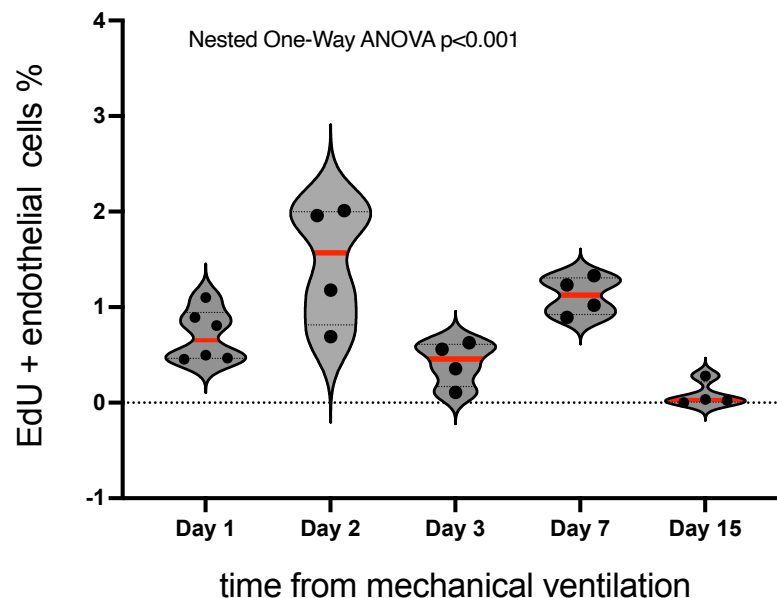


Figure 4.7. Percentage of EdU+ endothelial cells (EC) across the post-ventilation time-course. Animals $n=2$ per group, except from Day 1 timepoint $n=3$, the dot represent left and right lobes mean for each animal. Nested One-Way Anova was performed, $p<0.001$.

4.3.3 Epithelia type 2 cells proliferate after endothelial cells

AT2 proliferation remained almost negligible at Day 1 to Day 3, rising to a clear peak at Day 7 (mean 0.975%, SD 1.092%), before returning toward baseline by Day 15 (0.057%), see Figure 4.8 for detail. The Nested One-Way Anova was statistically significant ($p < 0.001$). No different proliferation patterns were found between left and right lobes.

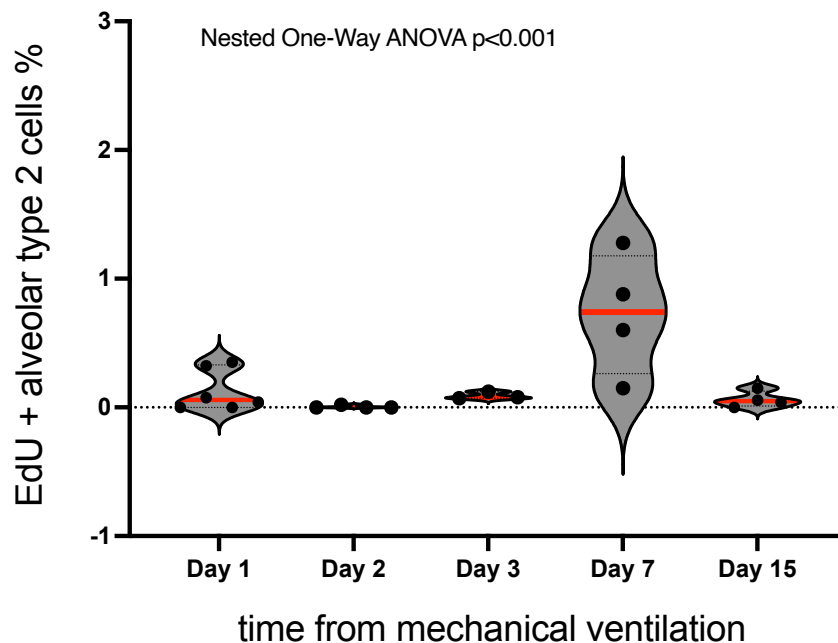


Figure 4.8. Percentage of EdU+ alveolar type 2 (AT2) cells across the post-ventilation time-course. Animals $n=2$ per group, except from Day 1 timepoint $n=3$, the dot represent left and right lobes mean for each animal. Nested One-Way Anova was performed, $p < 0.001$.

Endothelial cell proliferative response was notably faster in onset and larger in magnitude compared with alveolar type 2 cell proliferation, and this difference was sustained across the first days after ventilation.

By Day 7, however, the magnitude of alveolar type 2 cell proliferation approached that of endothelial cells, suggesting that the two populations converge on a broadly similar proliferative magnitude once the epithelial response is fully engaged, even though its onset remains delayed.

Plotting the animal-level mean EdU+ % for both cell types against time after ventilation (Figure 4.9) makes the differential kinetics visually apparent: the EC curve lies above the AT2 curve at almost every timepoint, peaks earlier (Day 2-7 for EC vs. Day 7 for AT2), and declines toward baseline over a similar overall time-frame.

This sequential pattern, with endothelial proliferation leading and epithelial proliferation following, could suggest a coordinated, temporally ordered regenerative response to mechanical lung injury, in which vascular repair precedes epithelial repair (Figure 4.9).

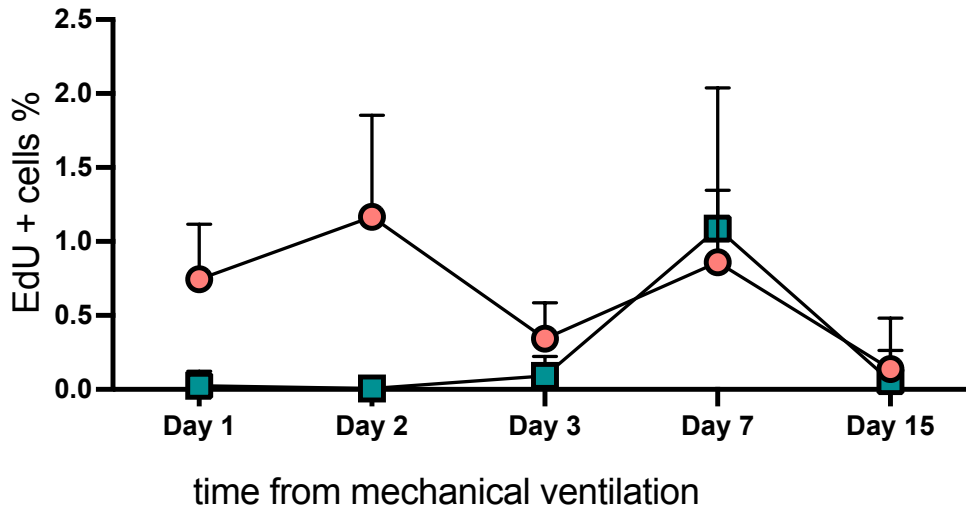


Figure 4.9. Mean EdU+ % (and SD) for AT2 (light green) and EC (light orange) cells across the post-ventilation time-course.

Lung tissue for the proliferation assay was sampled from the left lung and from the four regions of the right lung. A within-animal paired comparison between the left lung and the right lung showed no consistent difference between sides.

Both AT2 and EC cells show a proliferative response to mechanical ventilation that is almost absent in non-ventilated controls, with the endothelial response being faster in onset and detectable across a broader window (Day 1–Day 7). Both cell types return toward baseline proliferation by Day 15.

4.4 Ex-Vivo mechanical stretch model

To complement the in vivo findings an ex vivo stretch protocol was established. EC and AT2 cells were isolated from the lungs of 6-week-old mice using separate, cell-type-specific extraction protocols, then seeded onto Matrigel-coated silicone wells and allowed to adhere for 72 hours prior to stretch for 24 hours.

Mechanical stretch was applied using the MechanoCulture FX (MCFX-400, World Precision Instruments), a uniaxial stretch bioreactor fitted with a silicone 16-well plate. Cells were subjected to a displacement of 0.5 mm for 24 hours, with EdU present in the culture medium only during the stretch phase to label proliferating cells in response to mechanical stimulus. Following stretch, plates were fixed and stained, and the percentage of EdU⁺ cells was quantified.

The numerosity of the ex vivo experiments is limited due to limited survival of the extracted lung endothelial and epithelial cells on the coated silicon well.

4.1 AT2 monoculture Ex-Vivo mechanical stretch

In the mono-culture ex vivo stretch experiment, AT2 cells showed no detectable proliferative response to 24 hours of standard cyclic stretch (5% strain, 0.25 Hz), with EdU incorporation remaining at or near zero in both control and stretch conditions as shown in Figure 4.10.

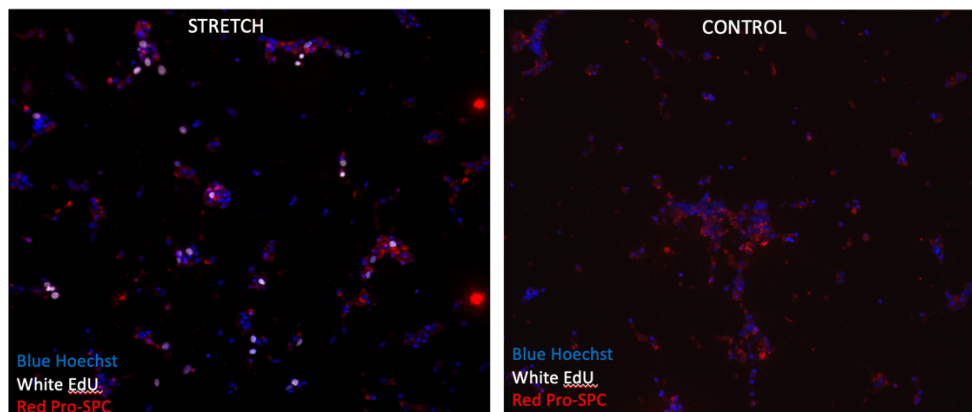


Figure 4.10. AT2 cells do not proliferate neither in the stretch nor in the control condition.

4.4.2 Endothelial cell monoculture ex-vivo mechanical stretch

Endothelial cells, by contrast, showed a trend to an increase in proliferation following stretch: $0.17 \pm 0.69\%$ EdU-positive cells in the control group versus $1.74 \pm 2.57\%$ in the stretch group (unpaired t-test with Welch's correction, $P = 0.03$). Most of both control and stretch wells showing no detectable EdU incorporation, and the group difference driven by a subset of strongly proliferative wells in the stretch condition (up to 7.7% EdU-positive cells in individual stretch wells, versus a maximum of 2.8% in any control well) suggesting that stretching could be a driver of proliferation in specific setting/geometry of the tissue.

The EC mono-culture stretch experiment was repeated, the nested analysis showed a significant increase in EC proliferation following stretch (control values ranging from 0–0.7%, mean $\approx 0.5\%$; stretch values ranging from 0.8–3.4%, mean $\approx 1.45\%$; nested t-test, $P = 0.0254$), shown in Figure 4.11.

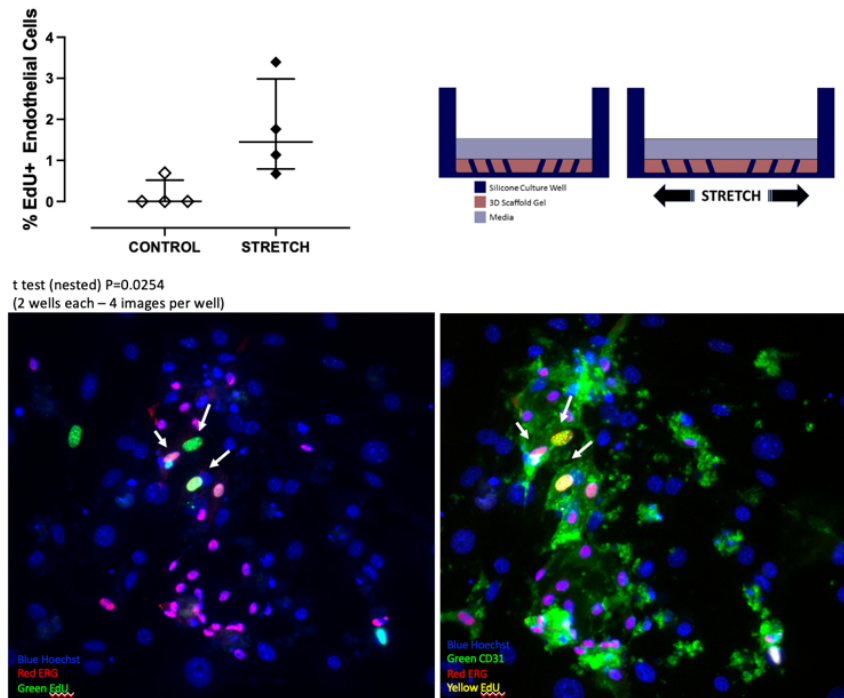


Figure 4.11. Endothelial cells proliferate more in the stretch group when compared to control (not stretched).

To determine whether the EC proliferative response to stretch scales with mechanical load, an additional ex vivo experiment was performed in which the stretch duty cycle was shortened from 2 seconds extension/2 seconds return to 1 second extension/1 second return doubling the cycling frequency at the same linear displacement. Under these intenser-load conditions, EC proliferation was reduced relative to the standard stretch condition, indicating that the relationship between mechanical stimulus and EC proliferation is not linear: a higher-frequency stretch regimen, rather than further enhancing the proliferative response, appeared to suppress it.

4.4.3 EC and AT2 co-culture Ex-Vivo mechanical stretch

When AT2 cells and EC were stretched together in co-culture, the overall pattern of proliferation was similar in character to the mono-culture experiments, with the majority of images in both control and stretch conditions showing no detectable EdU incorporation in either cell type, but with notable outlier responses. In the AT2 population, EdU incorporation was not significant across conditions. In the EC population, only some of the stretch images showed a proliferative response (Figure 4.12), which was not consistent throughout the wells.

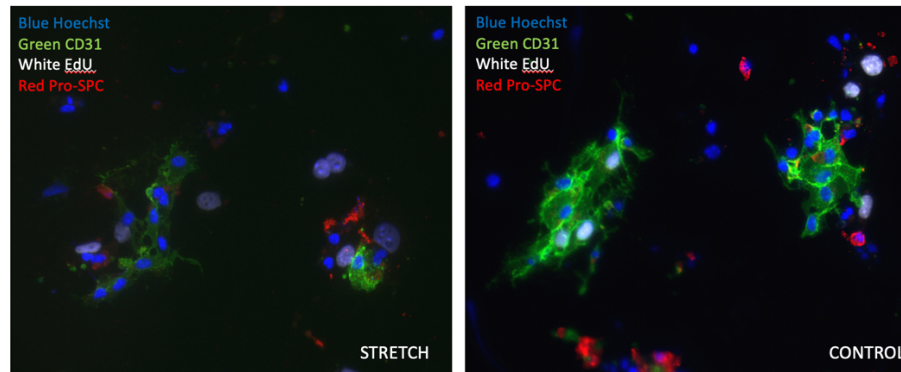


Figure 4.12. Grouped proliferation of endothelial cells, not consistent throughout the wells, in AT2 and EC coculture subjected to the stretch protocol (≈ 0.5 mm displacement, $\approx 5\%$ linear strain, 0.25 Hz). EdU was added to the culture medium only during the 24-hour stretch phase to restrict labelling to proliferation occurring in direct temporal association with the mechanical stimulus.

Across the preliminary ex vivo experiments a trend appears to show that EC, but not AT2 cells, show an intrinsic proliferative response to standard cyclic mechanical stretch in a loading-dependent manner, prompting this cellular type to be the mechano-sensitive population.

5. DISCUSSION

The presented results show that the four-group mouse ventilation protocol produces a graded, magnitude-dependent model of ventilator-induced lung injury (VILI) driven by mechanical stretch. Using the intermediate ventilation level to model recovery after injury, the alveolar epithelium and endothelium display different proliferative responses to mechanical stretch. Together, these findings position mechanical stretch magnitude as a possible candidate variable linking the in vivo histological injury phenotype to the ex vivo cellular proliferative phenotype, and motivate a closer examination of how graded ventilatory stretch translates into graded structural and inflammatory injury. They also generate hypotheses regarding the mechanisms underlying the divergence between the vascular and epithelial compartments in their regenerative response, although these observations alone do not allow conclusions to be drawn about the underlying biology. The sections that follow address each of these points in turn, before considering the methodological limitations of the model and the experiments it entails, and outlining directions for future work.

5.1 Modelling Ventilator-Induced Lung Injury (VILI)

The near-linear increase in all five histological subscores and the composite score across the non-ventilated, protective-ventilation (7 $\mu\text{l/g}$), and high-tidal-volume (15 and 21 $\mu\text{l/g}$) groups, together with the threshold-like emergence of alveolar rupture, atelectasis and overt overdistension exclusively in the IV21 group, is consistent with the broader principle that the transition from physiological to pathological mechanical stretch is qualitative rather than purely quantitative⁵³. The fact that these more severe features are absent below IV21 but appear once this threshold is crossed supports the hypothesis of the existence of a critical alveolar strain beyond which the parenchyma's viscoelastic and structural reserve is exhausted, precipitating mechanical failure of the alveolar wall (stress failure), rather than a graded worsening of the same lesions⁵⁴.

The fact that the protective ventilation group showed interindividual variability mirrors the heterogeneous regional recruitment patterns described for zero end-expiratory pressure (ZEEP) ventilation and is compatible with the concept that low tidal volume ventilation does not guarantee mechanical homogeneity at the alveolar level^{55,56}. Indeed, even protective tidal volumes are not distributed uniformly across the alveoli at zero end-expiratory pressure in particular, cyclical alveolar collapse and reopening (atelectrauma) can coexist with regional overdistension and collapse within the same lung.

The stepwise display of additional injury features at the highest tidal volume, like oedema, rupture, atelectasis and overdistension, appearing only once a critical stretch magnitude is

exceeded, is in line with the threshold-dependent engagement of distinct biophysical mechanisms (volutrauma, barotrauma, atelectrauma and biotrauma) that is thought to underlie clinical VILI ¹¹, and is consistent with the proposal that mechanical power, rather than tidal volume alone, best captures the injurious potential of a given ventilator strategy ^{12,55}. The graded histological response observed here is therefore fully consistent with a mechanical-power-based account of VILI severity, even though mechanical power itself was not directly computed as discussed in the limitations paragraph.

The CD45⁺ inflammatory data add further detail to this description. The relative plateau in leukocytes raises the possibility of a ceiling effect in their recruitment once stretch exceeds a certain magnitude. This would be consistent with the emerging view of macrophages as mechanosensitive cells whose recruitment and activation are themselves regulated by the biophysical properties of the injured tissue ⁵⁷; of note, the present plateau could reflect a limited sample size rather than a true biological ceiling. A biological ceiling in leukocyte recruitment would be consistent with a scenario in which the alveolar-capillary interface becomes saturated with adherent and transmigrated leukocytes once a maximal recruitable pool has been engaged, analogous to plateauing neutrophil responses described in other models of acute lung injury once chemokine gradients and endothelial adhesion-molecule expression reach saturating levels. Alternatively, the apparent plateau (and the slight decline in CD45⁺ macrophage density between IV15 and IV21) could reflect a shift in the balance between recruitment and local cell death or clearance at the highest stretch magnitude, since severely injured tissue may itself become less permissive to leukocyte survival and retention. Given the small group sizes, neither explanation can be distinguished from simple sampling variability, and this observation should be regarded as descriptive rather than confirmatory.

5.2 Distinct temporal dynamics of epithelial and endothelial proliferation In Vivo

The in vivo time-course data revealed a clear temporal dissociation between the EC and AT2 proliferative responses: EC proliferation rose sharply by Day 1, peaked at Day 2, and remained substantially elevated through Day 7, whereas AT2 proliferation remained negligible until Day 3 and did not peak until Day 7. This sequence, in which vascular proliferation precedes and substantially exceeds epithelial proliferation, is consistent with a growing body of evidence identifying the pulmonary capillary endothelium, rather than the epithelium, as the primary site of rapid proliferative repair following acute lung injury. Gillich et al. established that general capillary (gCap) cells serve as a proliferative progenitor population for capillary homeostasis and repair ¹⁸, and Niethamer et al. demonstrated that highly proliferative endothelial populations emerge specifically in an influenza-injury model ³⁴. More recently, James et al. identified a mitotically active Flot1⁺ gCap subpopulation that expands under hypoxic and injurious conditions ³⁵, and Wong et al. showed that venous endothelial cells also proliferate into the capillary bed

following injury ³⁶, collectively establishing a multi-source endothelial regenerative reservoir that would be well placed to support the rapid endothelial response that we observed. Taken together, these four lines of evidence converge on a model in which the capillary endothelium possesses multiple, at least partially redundant, proliferative sources that can be mobilized rapidly after injury, in contrast to the more restricted and slower-to-activate AT2 stem-cell compartment discussed below. This redundancy may itself help explain why the endothelial proliferative response in the present model was both faster in onset and larger in magnitude than the epithelial response: a system with several parallel proliferative sources would be expected to reach a detectable population-level signal more quickly than one relying on a single, more tightly regulated stem-cell pool.

The comparatively delayed and modest AT2 response is equally consistent with the existing literature on epithelial regeneration, in which AT2 proliferation and transdifferentiation toward AT1 cells is typically described as a slower process that becomes prominent only once acute injury has been established and partially resolved ⁵⁸. The five- to six-day lag observed here may reflect the time required for AT2 cells to transition from a quiescent to an activated proliferative state. The sequential, vascular-first then-epithelial pattern observed here also resonates with the proposal that epithelial-endothelial crosstalk, particularly through epithelial VEGFA signalling, is required to specify and maintain endothelial identity during alveolar repair ^{40,59}. This delay is compatible with the current understanding of the AT2 stem-cell niche, in which only a defined subpopulation of AT2 cells retains long-term self-renewal and injury-responsive proliferative capacity.

5.3 Endothelial cells show intrinsic mechanosensitivity, while AT2 proliferation appears context-dependent

Endothelial cells, but not alveolar type 2 cells, proliferated in response to cyclic stretch when cultured in isolation. This indicates that the endothelial proliferative response to cyclic stretch is, at least in part, cell-intrinsic and does not require paracrine input from other cell types. This is consistent with the well-established role of endothelial mechanosensors, including integrins, Piezo1, TRPV4 and VE-cadherin-associated junctional complexes, in directly transducing cyclic deformation into proliferative and barrier-regulatory signalling ^{9,60}. Endothelial Piezo1 has been shown to be directly activated by physiological cyclic alveolar stretch, triggering downstream signaling that stabilizes adherens junctions and limits stretch-induced vascular hyperpermeability. Its endothelium-specific deletion in mice exacerbates ventilator-induced barrier injury under high-tidal-volume ventilation ⁶⁰.

In marked contrast, alveolar type 2 cells in mono-culture showed no detectable proliferative response to the same stretch protocol. This pattern is most parsimoniously explained by a requirement for a paracrine signalling to unmask the alveolar type 2 proliferative response to mechanical stretch. This interpretation is consistent with, and supported by, the broader concept of an epithelial-endothelial mechanotransduction axis, and it raises the possibility that the alveolar epithelial mechanoresponse cannot be fully understood, or pharmacologically targeted, without accounting for the vascular compartment with which it is in continuous paracrine contact. This remains a hypothesis-generating inference drawn from a mono-culture-versus-co-culture contrast; direct perturbation experiments (e.g. blocking candidate paracrine mediators) would be required to directly demonstrate a paracrine mechanism.

5.4 Methodological Considerations and Limitations

Several methodological considerations and limitations should be acknowledged when interpreting the findings of this chapter. First, group sizes were modest throughout, in particular for the ex vivo experiments.

More importantly, the present study did not directly assess the molecular mechanosensing pathways implicated as mediators of the magnitude and context-dependent responses described above. The interpretations offered regarding underlying mechanism are therefore necessarily inferential.

In light of the limitations above, the suggested role of endothelial cells as primary mechanosensors and the hypothesised paracrine coupling between the two populations should be regarded as hypothesis-generating rather than as evidence of a causal relationship. The correlative, descriptive nature of the in vivo time-course data and the absence of direct mechanistic perturbation (e.g. pharmacological or genetic inhibition of candidate mechanosensors) mean that alternative explanations cannot be excluded. These include the possibility that the two proliferative programmes are driven independently by distinct upstream signals rather than by sequential cross-talk, that the apparent temporal dissociation reflects differences in assay sensitivity or EdU kinetics between cell types rather than true biological asynchrony, and that the ex vivo mono-culture findings do not fully recapitulate the co-culture or in vivo alveolar micro-environment, in which paracrine, mechanical and immune inputs are simultaneously present.

A further limitation specific to the framing of this work concerns mechanical power. Although the murine ventilation protocol was designed, in line with prior literature, to vary tidal volume and respiratory rate reciprocally, a direct calculation of mechanical power was not possible in this study. Correlating mechanical power with histological scoring and damage would have further strengthened the model's construct validity; however, doing so from the available data

would have required assumptions that were not well supported by the available airway pressure recordings, and mechanical power was therefore not calculated.

6. CONCLUSION

Taken together, these findings establish a graded, magnitude-dependent mouse model of ventilator-induced lung injury and offer a hypothesis-generating account of the cellular repair response that follows it. In vivo, the pulmonary vascular endothelium proliferates early and robustly after injury, while alveolar type 2 cell proliferation is delayed and comparatively modest, converging with the endothelial response only by Day 7. This temporal dissociation that is descriptive rather than mechanistically established. This pattern is compatible with, though not proof of, a model in which endothelial cells act early as mechanosensors: ex vivo, endothelial cells, but not alveolar type 2 cells, proliferate in response to cyclic stretch when cultured in isolation, suggesting that endothelial mechanosensitivity may be at least partly cell-intrinsic, whereas the epithelial response could depend on paracrine signaling from other cell types. A further, exploratory observation is that increasing stretch frequency reduced rather than enhanced endothelial proliferation. This raises the hypothesis-generating possibility that the relationship between mechanical stimulus and endothelial response is non-linear, and warrants dedicated confirmatory testing. None of these interpretations should be taken as established mechanism: given the modest sample sizes and the inferential nature of the associations described here, the present work is best understood as generating testable hypotheses about endothelial-epithelial coordination in VILI repair, rather than demonstrating the underlying mechanisms themselves.

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