Ex vivo and in vivo immunomodulation in acute lung injury and lung transplantation

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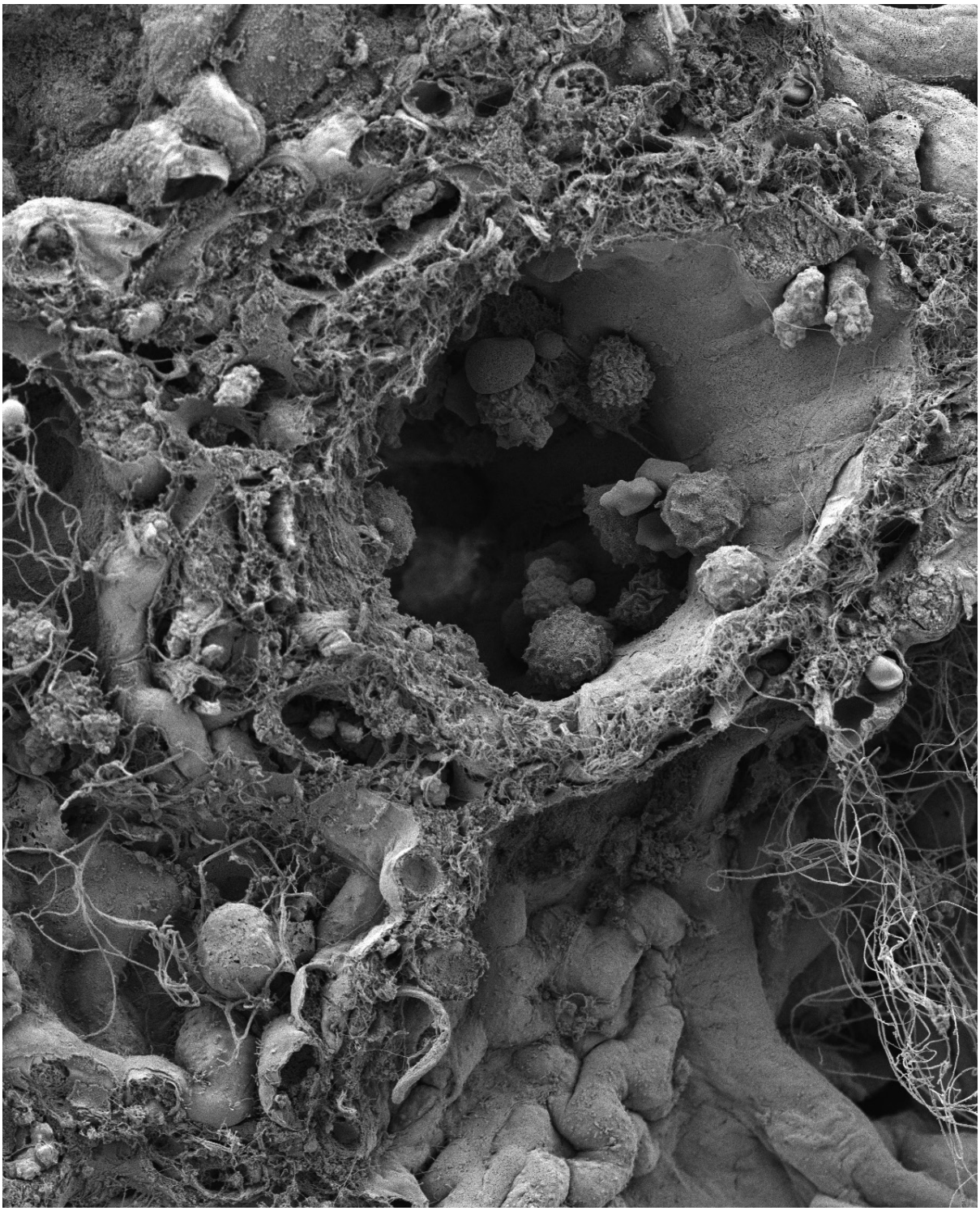

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Ex vivo and *in vivo* immunomodulation
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LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University. To be publicly defended on May 21st 2026, at 08.30 in Belfragesalen, BMC, Sölvegatan 19, 221 84 Lund, Sweden

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Abstract:

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Ex vivo lung perfusion (EVLP) has increased donor lung utilization by allowing functional assessment and short-term preservation of donor lungs outside the body. Apart from enabling lung ventilation and perfusion ex vivo, EVLP also provides a platform to test therapeutic interventions aimed at regenerating injured organs prior to transplantation. Targeting inflammatory pathways or promoting tissue repair during EVLP represents a promising strategy to rehabilitate marginal donor lungs.

This doctoral thesis investigated regenerative and immunomodulatory therapeutic strategies to restore donor lungs unsuitable for transplantation, with the goal of expanding the donor pool and improving post-transplant outcomes. In addition, the work aimed to increase the understanding of the molecular and immunological processes occurring during lung injury and lung transplantation and how these might be modulated by regenerative therapies. To address these objectives, several approaches were examined, including cytokine adsorption during lung transplantation in human patients, neutrophil extracellular trap removal during ex vivo lung perfusion, and mesenchymal stromal cell therapies to restore injured lungs and mitigate lung injury and graft dysfunction in porcine models.

Key words: lung transplantation; donor lung injury; ex vivo lung perfusion; primary graft dysfunction; cytokine adsorption; neutrophil extracellular traps; mesenchymal stromal cell therapy

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Cover image © by Nicholas B. Bèchet, depicting alveoli in a pig lung damaged by aspiration, with infiltration of various immune cells as well as neutrophil extracellular traps, visible as long fibres. The image was captured using scanning electron microscopy (SEM) at the Lund University Bioimaging Center.

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Für Dich, Papa, und deine unentwegte Unterstützung

And to all the people in my life who believe in me, even when I do not

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List of papers included in the thesis

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Lindstedt S, Niroomand A, **Mittendorfer M**, Hirdman G, Hyllén S, Pierre L, Olm F. Nothing but NETs: Cytokine adsorption correlates with lower circulating nucleosomes and is associated with decreased primary graft dysfunction. J Heart Lung Transplant. 2023 Oct;42(10):1358-1362. doi: 10.1016/j.healun.2023.06.011. Epub 2023 Jun 20. PMID: 37348689.

Paper II

Mittendorfer M, Pierre L, Huzevka T, Schofield J, Abrams ST, Wang G, Toh CH, Bèchet NB, Caprnja I, Kjellberg G, Aswani A, Olm F, Lindstedt S. Restoring discarded porcine lungs by ex vivo removal of neutrophil extracellular traps. J Heart Lung Transplant. 2024 Dec;43(12):1919-1929. doi: 10.1016/j.healun.2024.07.007. Epub 2024 Jul 20. PMID: 39038563.

Paper III

Mittendorfer M, Olm F, Edström D, Niroomand A, Bèchet NB, Hirdman G, Ghaidan H, Bodén E, Oeller M, Schallmoser K, Kjellberg G, Stenlo M, Scheding S, Hyllén S, Lindstedt, S. Repeated doses of mesenchymal stromal cells from two distinct sources regenerated discarded donor lungs and improved outcomes in porcine transplantation. 2026. (manuscript)

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Mittendorfer M, Olm F, Huzevka T, Bèchet NB, Wang Q, Gu R, Svereus F, Edström D, Janson EB, Hirdman G, Scheding S, Hyllén S, Lindstedt S. Early Allogeneic Stromal (Stem) Cell Therapy Mitigates Aspiration-Induced Lung Injury: Closed-System Automated Cell Manufacturing Using the CliniMACS Prodigy. 2026. (manuscript)

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Bechet NB, Celik A, **Mittendorfer M**, Wang Q, Huzevka T, Kjellberg G, Boden E, Hirdman G, Pierre L, Niroomand A, Olm F, McCully JD, Lindstedt S. Xenotransplantation of mitochondria: A novel strategy to alleviate ischemia-reperfusion injury during ex vivo lung perfusion. J Heart Lung Transplant. 2025 Mar;44(3):448-459. doi: 10.1016/j.healun.2024.10.033. Epub 2024 Nov 12. PMID: 39536924.

Hirdman G, Stenlo M, Bèchet NB, Niroomand A, **Mittendorfer M**, Wang Q, Edström D, Ghaidan H, Kjellström S, Pierre L, Olm F, Hyllén S, Lindstedt S. Unraveling Molecular and Functional Responses Across 3 Lung Injury Models to Expand the Donor Lung Pool. Transplantation. 2025 Jul 1;109(7):1166-1174. doi: 10.1097/TP.0000000000005353. Epub 2025 Feb 19. PMID: 39969856.

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Review papers

Huwyler F, Pfister M, Phuyal D, Dean YE, **Mittendorfer M**, Saemann L, Rasel H, Stoerzer S, Binz J, Tabatabaei B, Szabo G, Lindstedt S, Gharb BB, Tibbitt MW, Clavien P-A. Time as a Therapeutic Ally: The Promise of Long-Term Solid Organ and Tissue Perfusion. 2026. (in press)

Olm F, Kortleven P, **Mittendorfer M**, Carlon MS, Ceulemans LJ, Lindstedt M. Cell and Gene Therapy in Lung Transplantation: From Experimental Promise to Clinical Reality. 2026. (under review)

Abstract

Lung transplantation (LTx) remains the only curative treatment for patients with end-stage lung disease, yet long-term outcomes remain inferior to those of other solid organ transplantations, with a 5-year survival below 70%. Major contributors to this limited outcome include primary graft dysfunction (PGD), the leading cause of early mortality, chronic lung allograft dysfunction (CLAD), and low utilization of available donor lungs. Currently, only about 20% of potential donor lungs are used for transplantation, as many are declined due to factors such as infection or inflammation, contusion, size mismatch, or aspiration. Aspiration of gastric contents can lead to acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), a contributor to the development of PGD. Thus, strategies to both expand the donor pool and improve post-transplant outcomes are urgently needed.

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Popular science summaries

Popular summary in English

Many people have lung diseases so severe that they will die within two years after reaching the end-stage of the disease if nothing is done. There is no cure and the only way for these patients to survive is to give them new lungs. In a procedure called lung transplantation, the diseased lungs are removed and replaced with healthy lungs from a deceased organ donor. A major problem in lung transplantation is that there are too few donor lungs compared to the number of patients who need them.

Another problem is that many donor lungs cannot be used. In fact, only about 20% of donated lungs are suitable for transplantation, often because the lungs have been damaged by infection, inflammation, or stomach contents entering the lungs. To address this, scientists are working on ways to improve donor lungs before transplantation. One method is to keep the lungs alive outside the body using a machine that provides oxygen and nutrients. This technique, called *ex vivo* lung perfusion (EVLP), makes it possible to test lung function and has already increased the number of lungs available for transplantation. However, EVLP alone is sometimes not enough to repair the damage, so additional treatments are needed.

In my PhD work, we tested both filtering out harmful proteins produced by the immune system and adding stem cells to the blood circulation. Most of these studies were performed in pigs, as they are a highly relevant model for human lung transplantation. Filtering these harmful proteins restored damaged donor lungs, while stem cells instead release small molecules that have big effects and also supported recovery.

Unfortunately, even when a transplant is successful, problems can still occur after transplantation. Some patients develop complications within 3 days after surgery, while others develop long-term lung damage years later. As a result, many patients do not survive more than 6 years after a lung transplantation. We therefore also tried giving stem cells to pigs after transplantation and found that this helped prevent early complications.

The ultimate goal of my PhD research work is to make more donor lungs usable and to help patients live longer after transplantation.

Popular summary in Swedish

Många människor som lider av lungsjukdomar i sitt slutskede är så allvarligt sjuka att de riskerar att dö inom två år om ingenting görs. Det finns i nuläget inget botemedel, och det enda sättet för dessa patienter att överleva är att få nya lungor. Vid en operation som kallas lungtransplantation tas de sjuka lungorna bort och ersätts med friska lungor från en avliden organdonator. Ett stort problem inom lungtransplantation är att det finns alldeles för få donatorlungor jämfört med antalet patienter som behöver nya lungor.

Ett ytterligare problem är att många donatorlungor inte kan användas. Faktum är att endast omkring 20 % av de donerade lungorna är lämpliga för transplantation. Detta beror på att lungorna ofta har skadats före donationen, till exempel av infektion, inflammation eller av så kallad aspiration, vilket innebär att maginnehåll har kommit ner i lungorna. Forskare arbetar därför på att utveckla metoder för att förbättra skadade donatorslungor inför en transplantation. En metod går ut på att hålla lungorna vid liv utanför kroppen med hjälp av en maskin som tillför syre och näring. Denna teknik, som kallas ex vivo lung perfusion (EVLP), gör det möjligt att testa lungornas funktion innan de transplanteras och har redan ökat antalet lungor som kan användas. Men ibland räcker EVLP inte till för att reparera skadorna helt, och därför behövs ytterligare behandlingsmetoder.

I min avhandling undersökte vi både filtrering av skadliga proteiner som bildas av immunförsvaret och tillförsel av stamceller till blodcirkulationen. De flesta av dessa studier genomfördes på grisar, eftersom grisars lungor är väldigt lika människolungor. Både att filtrera bort dessa skadliga proteiner och att ge stamceller, som frisätter små molekyler som aktivt hjälper lungorna att laga sig själva, hade god effekt.

Tyvärr kan problem uppstå även när transplantationen är framgångsrik. Vissa patienter kan utveckla komplikationer redan tre dygn efter en transplantation. Andra patienter får långvariga lungskador först flera år senare. Som en följd överlever många patienter inte mer än sex år efter en lungtransplantation. Därför undersökte vi om det skulle hjälpa att ge stamceller även efter transplantation och såg att detta kunde förebygga de tidiga komplikationerna.

Det övergripande målet med mitt forskningsarbete är att göra fler donatorslungor användbara för transplantation, hjälpa patienter att leva längre efter en transplantation och med färre komplikationer.

Popular summary in German

Viele Menschen leiden an Lungenerkrankungen, die im Endstadium innerhalb von zwei Jahren zum Tod führen, wenn nichts unternommen wird. Es gibt keine Heilung, und die einzige Möglichkeit für diese Patientinnen und Patienten, zu überleben, ist, eine neue Lunge zu bekommen. Bei einem Eingriff, der Lungentransplantation genannt wird, werden die kranken Lungen entfernt und durch gesunde Lungen eines verstorbenen Organspenders ersetzt. Ein großes Problem bei der Lungentransplantation ist, dass es zu wenige Spenderlungen im Vergleich zur Anzahl der Patientinnen und Patienten gibt, die eine neue Lunge benötigen.

Ein weiteres Problem ist, dass sehr viele Spenderlungen nicht verwendet werden können. Tatsächlich sind nur etwa 20 % der gespendeten Lungen für eine Transplantation geeignet. Das liegt daran, dass die Lungen bereits vor der Spende geschädigt wurden, zum Beispiel durch Infektionen, Entzündungen oder dadurch, dass Mageninhalt in die Lunge gelangt ist. Um dieses Problem zu lösen, arbeiten Forscherinnen und Forscher an Methoden, um Spenderlungen vor der Transplantation zu verbessern. Eine dieser Methoden besteht darin, die Lungen außerhalb des Körpers mithilfe einer Maschine am Leben zu erhalten, die sie mit Sauerstoff und Nährstoffen versorgt. Diese Technik, die „Ex vivo Lungenperfusion (EVLP)“ genannt wird, ermöglicht es, die Lungenfunktion zu testen und hat bereits dazu beigetragen, mehr Lungen für Transplantationen verfügbar zu machen. Allerdings reicht EVLP allein manchmal nicht aus, um die Schäden vollständig zu reparieren, sodass zusätzliche Behandlungen notwendig sind.

In meiner Doktorarbeit haben wir sowohl das Herausfiltern schädlicher Proteine des Immunsystems als auch die Gabe von Stammzellen in den Blutkreislauf untersucht. Die meisten dieser Studien wurden an Schweinen durchgeführt, da Schweine ein sehr gut geeignetes Modell für die menschliche Lungentransplantation darstellen. Das Entfernen dieser schädlichen Proteine konnte die Qualität beschädigter Spenderlungen verbessern. Die Wirkung der Stammzellen, die kleine Moleküle freisetzen (Botenstoffe), unterstützte ebenfalls die Erholung der Lunge.

Leider können auch nach einer erfolgreichen Transplantation weiterhin Probleme auftreten. Manche Patientinnen und Patienten entwickeln bereits innerhalb von drei Tagen nach der Operation Komplikationen, während andere erst Jahre später langfristige Lungenschäden entwickeln. Infolgedessen überleben viele Patientinnen und Patienten nicht länger als sechs Jahre nach einer Lungentransplantation. Deswegen haben wir auch untersucht, ob die Gabe von Stammzellen nach der Transplantation helfen kann, und konnten zeigen, dass dadurch frühe Komplikationen verhindert werden können.

Das übergeordnete Ziel meiner Forschungsarbeit ist es, mehr Spenderlungen nutzbar zu machen und die Überlebensdauer der Patientinnen und Patienten nach einer Lungentransplantation zu verbessern.

Most common abbreviations

ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
ATMP	Advanced Therapy Medicinal Product
BVI	Bronchial-vascular Interface
CLAD	Chronic Lung Allograft Dysfunction
ECD	Extended Criteria Donor
ECLS	Extracorporeal Life Support
EVLP	Ex Vivo Lung Perfusion
FiO_2	Fraction of Inspired Oxygen
GMP	Good Manufacturing Practice
IRI	Ischemia Reperfusion Injury
LPS	Lipopolysaccharide
LTx	Lung Transplantation
MSC	Mesenchymal stromal (stem) cell
NET	Neutrophil Extracellular Trap
PaO_2	Partial pressure of Oxygen
PGD	Primary Graft Dysfunction
PVR	Pulmonary Vascular Resistance
SCD	Standard Criteria Donor
SEM	Scanning Electron Microscopy
SVR	Systemic Vascular Resistance

Introduction

The lung

The lung is part of the respiratory system, which can be broadly divided into the upper and lower respiratory tracts (Fig. 1). The upper respiratory tract warms and humidifies inhaled air and prevents larger particles or foreign bodies from reaching the lungs through mechanisms such as nasal filtration and the cough reflex. In contrast, the lower respiratory tract is home to one of the most essential functions of the human body- gas exchange[1].

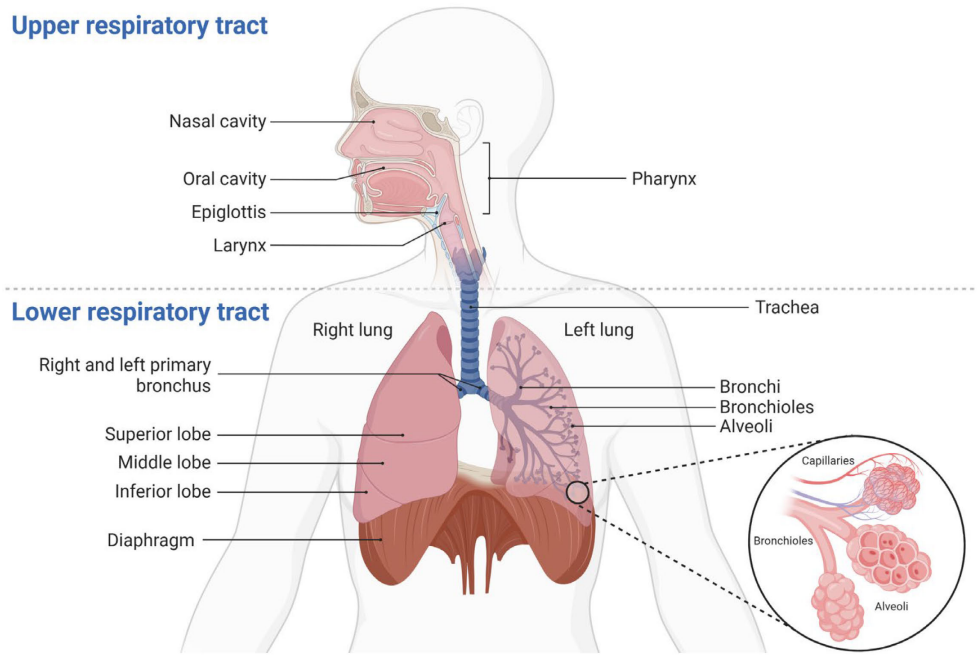

Figure 1: The respiratory system.
Schematic overview of the respiratory system with the upper and lower respiratory tract and with a close-up of the terminal bronchioles, in which the alveoli reside. Figure created with BioRender.

The primary role of the lung is to supply the body with oxygen and expel carbon dioxide. This process is called gas exchange and happens at a thin blood-gas barrier in the alveoli, termed the alveolar-capillary membrane. The alveoli, home to the alveolar-capillary membrane, is located in the terminal bronchioles, which represent the smallest and most distal airways in the tracheobronchial tree. This figurative tree is composed of the trachea and 23 so-called generations of bronchi and bronchioles. Based on the pioneering work by Ewald Weibel[2], who coined the term lung morphometry, the quantitative morphological study of the lung, the alveolar surface area reaches an impressive area of 130m^2. The capillary surface area, in turn, is estimated at 115m^2. Converting these numbers into everyday-life objects, this would be a rather sizeable apartment with at least three rooms, on the scale of a larger city in southern Sweden.

Apart from the gastrointestinal tract, the lung is the only organ continuously exposed to the external environment and therefore relies on a range of immunological defence and clearance mechanisms[3,4]. Considering all the diverse functions of the lung, ranging from air conduction, pathogen clearance and gas exchange within highly specialized alveolar structures, the lung is a complex organ composed of multiple cell and tissue types. A recent study utilized single-cell sequencing to map all the different cell types in the healthy human lung and identified 58 different cell populations, including 15 epithelial, 9 endothelial, 9 stromal, and 25 immune cell populations[5]. Like other organ systems, the lung, with all 58 cell populations and distinct functions, operates under tightly regulated homeostasis and disruption of this balance leads to pathological changes[4]. When these pathological changes progress beyond the point of effective treatment, patients may ultimately become candidates for lung transplantation.

Lung transplantation

According to the most recent numbers from the International Society of Heart and Lung Transplantation (ISHLT) Transplant registry, Idiopathic Pulmonary Fibrosis (IPF) is the most common indication for lung transplantation with 27% of all cases[6]. IPF is tightly followed by Chronic Obstructive Pulmonary Disease (COPD) at 24% and cystic fibrosis (CF) at 10%, for which the number of patients needing a transplant has decreased with the advent of new, effective medication. IPF can be characterized as a restrictive and COPD as an obstructive lung disorder with distinct pathological mechanisms, and both are classified as chronic, progressive diseases and are still lacking cures[7].

Even though disease-specific indications for lung transplantation differ between conditions, some general criteria must be met according to ISHLT's consensus document from 2021[8]:

- >50% risk of death from lung disease within 2 years
- >80% likelihood of 5-year post-transplantation survival

Historical perspective

The year 2026 marks 63 years since the first human lung transplantation (LTx) was performed. In 1963, James Hardy pushed boundaries by being the first to transplant a human single left lung into a human patient[9,10]. The transplant was successful, but the patient died 18 days later from renal failure. With the second attempt only days later at another hospital, more than a dozen lung transplantation attempts followed over the next few years. While the surgeries were all reportedly successful, most of the patients died within the first three weeks after the transplant due to inadequacies in both surgical technique and post-operative immunosuppressive regimen at the time[10,11]. The first human single lung transplantation that resulted in longer survival than a few weeks was performed by Fritz Derom in 1968, after which the patient lived for 10 more months. After a downward trend in lung transplantations over the next ten years, the advent of modern lung transplantation was not until 1983, when Bruce Reitz and colleagues reported the first long-term survivor after a heart-lung transplantation (HLTx) in which both lungs and the heart were transplanted en bloc with improved surgical technique and use of immunosuppressive drugs. Since then, lung transplantation has advanced rapidly, showing progress due to better surgical techniques, complex immunosuppressive drug regimens, improved definitions of suitable recipients and donor management, bridging to transplant for critically ill patients, and, of course, expanded research in pre-clinical animal models[10,11].

However, several hurdles remain, limiting progress. Donor organ shortage resulting in waitlist mortality, ischemia-reperfusion injury leading to the development of primary graft dysfunction and chronic lung allograft dysfunction are some of the major contributors to early and late mortality after lung transplantation, respectively. All these factors need to be addressed for the field to advance.

Donor organ shortage

The Global Observatory on Donation and Transplantation (GODT) reports a 2% increase in transplant activity across all organs and a 6% increase in lung transplantation worldwide since 2023[12,13]. Nevertheless, only ≤10% of the global organ need is met. The GODT further reports 92% utilization across all organs when comparing deceased donors and transplanted organs. Despite this high utilization rate across all solid organs, lungs are severely lagging behind with utilization rates at 20-30%[14-16]. One measure to expand the donor pool for transplantation is the utilisation of extended criteria donors (ECD). ECD lungs are classified as grafts from donors with characteristics outside standard ideal criteria regarding for example donor age, PaO_2/FiO_2 ratio, smoking history and abnormalities on chest x-

ray[17,18]. Moreover, the implementation of *ex vivo* lung perfusion to further increase the use of ECD alongside standard criteria donors (SCD)[19,20] has further increased the number of available donor lungs. Similarly, inclusion of donation after cardiac death (DCD) donors into clinical practice alongside the more standard donation after brain death (DBD) donors has expanded the donor pool, even though Sweden is lagging behind other countries in the use of DCD lungs[12]. Despite general improvements, utilization numbers are increasing only marginally over time, and progress is urgently needed.

The most common causes of death in donors are head trauma: 39%, stroke: 35% and anoxia: 21%[6]. Head trauma and cerebrovascular incidents leading to unconsciousness often result in gastric aspiration, which describes the inhalation of gastric fluid and/or digested food particles into the airways[21].

Gastric aspiration

Together with neurogenic edema, also a result of traumatic brain or central nervous system (CNS) injury, as well as infection or inflammation and contusion, gastric aspiration is one of the most common reasons for declining an available donor lung[21,22]. According to the literature, aspiration accounts for at least 20% of lungs rejected for transplantation[14]. However, clinical experience from transplant surgeons and coordinators suggests that the true proportion may be substantially higher, with estimates ranging from 50% to 80%. Gastric aspiration leads to a strong pro-inflammatory response, including a large influx of neutrophils and pro-inflammatory chemokines into the alveolar spaces (Fig. 2), frequently progressing to acute respiratory distress syndrome (ARDS)[21,23].

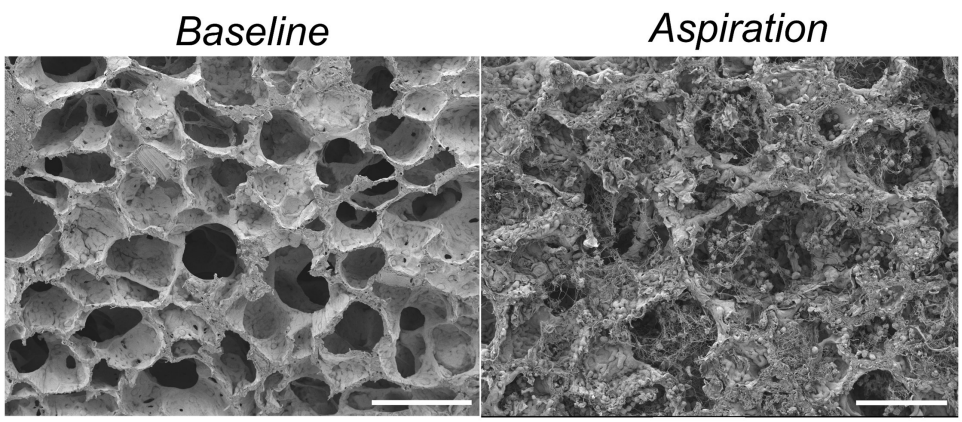

Figure 2: Representative images of the alveoli, comparing healthy lung tissue to lung tissue damaged by aspiration injury.
Scanning electron microscopy (SEM) images of alveoli in a healthy, porcine lung ('Baseline') compared to a lung injured by gastric aspiration ('Aspiration'). Scale bars: 100 µm. Adapted from paper III with permission, image credit Nicholas B. Bèchet.

Lung pathologies contributing to donor lung shortage

Several pathological processes contribute to both the limited availability of donor lungs and adverse outcomes after lung transplantation. Acute respiratory distress syndrome (ARDS) is a key factor, leading to the rejection of donor lungs and increasing susceptibility to ischemia-reperfusion injury (IRI). ARDS has also been proposed as a risk factor for primary graft dysfunction (PGD) in the recipient. In turn, patients who develop PGD are at increased risk of progressing to chronic lung allograft dysfunction (CLAD) later in the disease course.

Acute respiratory distress syndrome

ARDS is the most severe form of acute lung injury (ALI) and describes a severe inflammatory injury with a diffuse alveolar damage (DAD) pattern in the parenchymal tissue of the lung[24,25]. A strong pro-inflammatory cascade, damage to the alveolar-capillary membrane and disrupted alveolar fluid clearance leading to oedema and haemorrhage are pathological hallmarks of ARDS (Fig. 3). Furthermore, neutrophil infiltration and activation, including the release of neutrophil extracellular traps (NETs) as well as fibrin deposition, are strong characteristics[26-28].

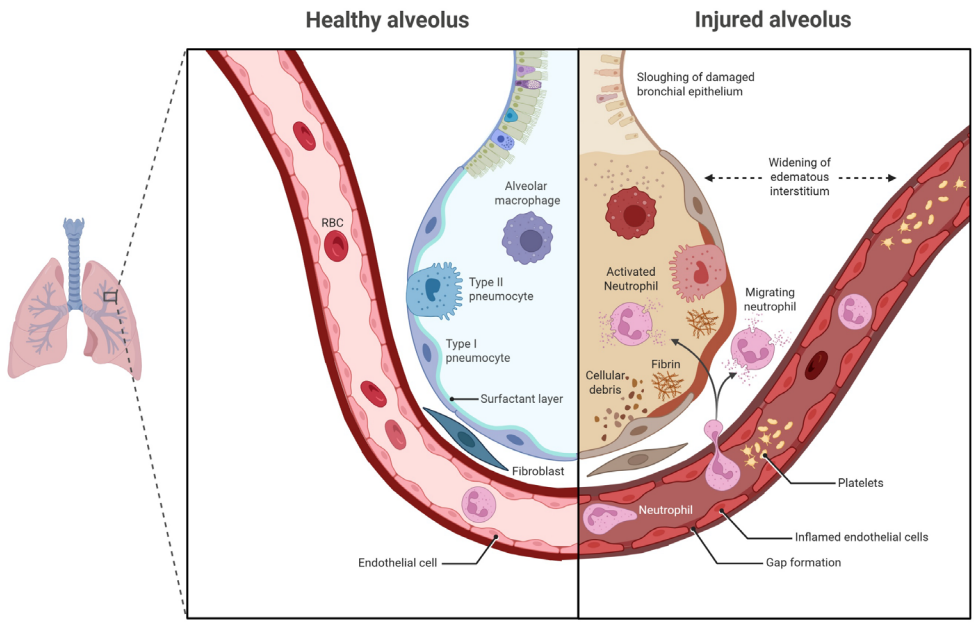

Figure 3: Cellular pathophysiology of acute respiratory distress syndrome (ARDS).
ARDS is characterized by extensive damage to the alveolar-capillary membrane, leading to alveolar edema and hemorrhage, neutrophil activation and infiltration, as well as a strong pro-inflammatory response. Figure created with BioRender.

In 2012, due to incoherencies in the clinical diagnosis of ARDS, the ARDS Definition Task Force convened to postulate 'The Berlin criteria' for the definition of ARDS[29]:

- Onset within one week of known clinical insult or worsening of respiratory symptoms
- Bilateral opacities visible upon chest imaging
- Oedema, not explainable by cardiac failure or fluid overload
- Oxygenation classified by PaO_2/FiO_2 ratio:
    - Mild: 200-300 mmHg
    - Moderate: 100-200 mmHg
    - Severe: ≤100 mmHg

Ischemia-reperfusion injury

IRI describes the state of cellular stress induced when blood flow is restored to tissues previously deprived of oxygen (ischemic). IRI most importantly leads to the generation of reactive oxygen species (ROS), which in turn results in the initiation of a pro-inflammatory cascade and increased cellular apoptosis[30]. During the donation process, IRI occurs naturally, since the lung undergoes a limited period of ischemic time from the point of organ retrieval to the point of implantation into a recipient (Fig. 4). Previous studies have investigated the effect of donor lung ischemic time on recipient outcome and found a positive correlation between increased ischemic time and worse outcome post-transplantation[31,32].

Lung pathologies affecting post-transplantation outcome

While the outcome after lung transplantation remains under the influence of many contributors such as rejection, postoperative infections, malignancies, and renal insufficiencies, early and late dysfunctions in the grafts leading to PGD and CLAD, remain the main culprits[33]. Despite the one-year survival after lung transplantation having steadily increased in the last 20 years to 88,4 % in 2023, the long-term outcome after lung transplantation is inferior compared to other solid organ transplants[33,34]. Comparing the outcome after lung transplantation with, for example kidney and liver, the lungs are severely lagging behind with a 5-year survival rate of less than 70% [33-36].

Primary graft dysfunction

The correlation between prolonged ischemic time and worse post-transplantation outcomes might be due to the development of PGD in these grafts. PGD occurs within 72h after lung transplantation and affects approximately 25% of recipients[37-39] (Fig. 4). PGD describes the injury resulting from IRI in the peri-operative period that is not resolved post-transplantation and is characterised by persistent lung oedema, impaired gas exchange and increased pulmonary vascular resistance

(PVR). At the molecular pathophysiological level, PGD presents as somewhat extended IRI, with characteristics similar to ARDS. Fluid build-up in the alveolar spaces and excessive inflammation leading to the release of damage-associated molecular patterns (DAMPs) and the prominent role of neutrophil activation are shared hallmarks across all pathologies, with the addition of increased complement activation in PGD[40,41]. To reach consensus on the clinical hallmarks of PGD, the ISHLT proposed definitions and a grading scale that are now used in standard care in the clinic[42,43]. PGD grades are assessed based on chest x-ray and PaO_2/FiO_2 ratios at four timepoints (0h, 24h, 48h and 72h) post-transplantation according to the following criteria:

- Grade 0: no opacities visible on chest x-ray, any PaO_2/FiO_2 ratio
- Grade 1: opacities visible on chest x-ray, PaO_2/FiO_2 ratio >300 mmHg
- Grade 2: opacities visible on chest x-ray, PaO_2/FiO_2 ratio 200-300 mmHg
- Grade 3: opacities visible on chest x-ray, PaO_2/FiO_2 ratio <200 mmHg

PGD is one of the main contributors to early mortality after lung transplantation, with PGD grade 3 displaying the most significant correlation to increased mortality[37,38,44].

Chronic lung allograft dysfunction

The development of PGD is not only the main reason for early mortality but has also shown a correlation with late mortality post-transplantation[37,38]. PGD grade 3, specifically, has shown a distinct correlation with the development of CLAD[37,45,46]. CLAD is defined by a persistent decline of ≥20% in forced expiratory volume in one second (FEV_1) compared to the patient's own baseline value, which is calculated as the mean of the two best FEV_1 measurements obtained post-transplantation[47,48]. CLAD occurs within weeks to months post-transplantation (Fig. 4) and can be subdivided into four phenotypes: bronchiolitis obliterans syndrome (BOS) as the most prevalent form, restrictive allograft syndrome (RAS), the mixed and the undefined phenotype[47].

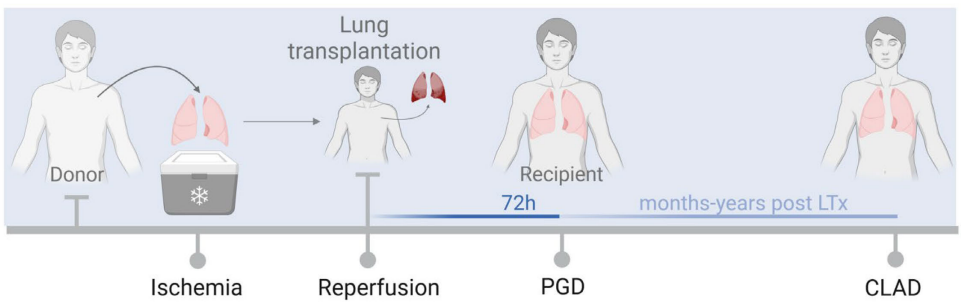

Figure 4: Timeline of ischemia-reperfusion injury, primary graft dysfunction and chronic lung allograft dysfunction. Schematic overview of at which point in the timeline of lung transplantation transplantation-related pathologies occur. Abbreviations: Primary graft dysfunction (PGD), Chronic lung allograft dysfunction (CLAD). Figure created with BioRender.

Ex vivo lung perfusion

Since post-transplantation deterioration of the implanted grafts is most often thought to result from the initial choice, preservation and handling of donor grafts, ischemic injury remains a major challenge in lung transplantation[32,49]. Short-term perfusion using *ex vivo* lung perfusion (EVLP) has emerged as a key strategy to mitigate ischemia-reperfusion injury and to evaluate declined donor lungs prior to transplantation to increase organ utilization[50-53]. The principle of EVLP is to maintain the donor lung grafts in their physiologic state by keeping them perfused and ventilated in a closed system *ex vivo* (Fig. 5). Normothermic perfusion with nutrient-rich solutions and mechanical ventilation, together with a deoxygenator, simulates the lung's normal conditions in the body. EVLP can aid in returning damaged donor lungs to their normal function and currently presents the most advantageous method to improve marginal donor lungs previously rejected for transplantation. EVLP is a novel technology that, although recent, has been safely introduced into clinical use worldwide[54-60].

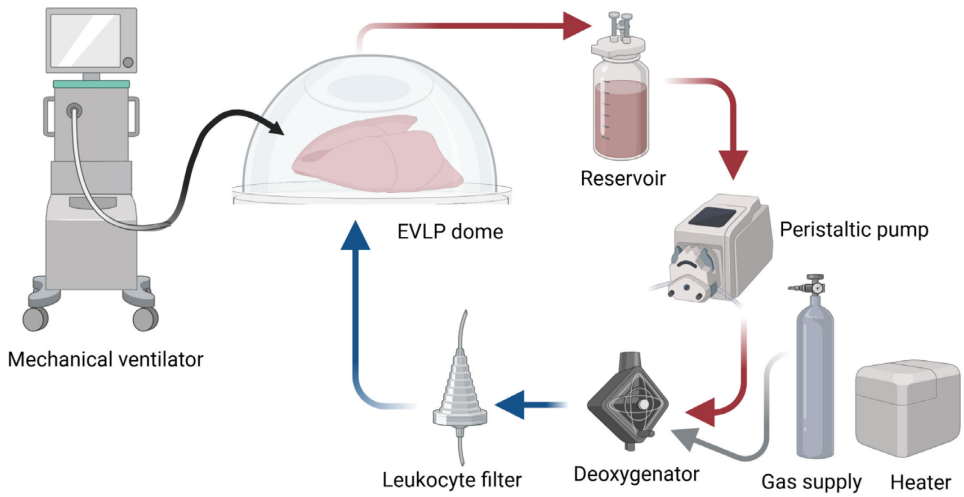

Figure 5: Schematic overview of the ex vivo lung perfusion setup utilized in the described studies.
A mechanical ventilator is connected directly to the explanted donor lungs in the EVLP dome, which are also connected to a set flow of perfusate, that is collected in a reservoir. The perfusate is then pumped via a peristaltic pump through a deoxygenator, connected to gas supply and a heater, passes a leukocyte filter and is ultimately pumped back into the donor lungs in the EVLP dome. Adapted from Ghaidan et al. 2022[61] with permission according to CC BY 4.0.

Historical perspective

Professor Stig Steen in Lund initially invented an *ex vivo* lung function assessment as a method to re-evaluate donor lungs from a non-heart-beating donor DCD *ex vivo*[62]. He then further developed the method in a porcine model of warm ischemia, proving its efficacy by transplanting the *ex vivo* conditioned lungs into recipient pigs, resulting in good lung function 24 hours after transplantation[63]. After its advent in Lund, EVLP was further developed and expanded by the Toronto Lung Transplantation Program[64,65].

Different EVLP protocols

Three main EVLP protocols are employed in clinical practice: the Lund protocol, the Toronto protocol and the Organ Care System (OCS) protocol[49,51-53]. The protocols differ in several key methodological parameters, including the use of cellular versus acellular perfusate, target perfusion flows, and whether the left atrium is kept open or cannulated during perfusion[66].

Briefly, the Lund protocol utilizes perfusate containing STEEN solution (XVIVO Perfusion AB) supplemented with red blood cells (RBC, cellular perfusate), a target perfusion flow rate of 100% of the cardiac output (CO) and an open left atrium. The Toronto protocol instead uses STEEN solution without supplemented RBCs (acellular perfusate), a target flow rate of 40% of CO and a closed atrium. The OCS protocol aligns with the Lund protocol in keeping the atrium open and using cellular perfusate supplemented with RBCs; however, it employs OCS solution instead. Within that protocol, the target flow rates are set to 2-2.5 L/min. Both STEEN solution and the OCS solution are high oncotic pressure solutions specifically designed to allow for keeping physiologic pressures and flows during perfusion[51-53].

Two different, main perfusion systems are used in these three protocols, each with protocol variations. The XPS system (XVIVO) is a stationary platform that can be operated with either cellular perfusate or acellular solution. In contrast, the Lung Organ Care System (OCS, TransMedics) is portable and exclusively utilizes a cellular perfusate. Both perfusion systems have equally shown clinical safety and efficacy in their respective randomized clinical trials (RCT)[67,68]. However, cellular perfusates have proven beneficial under experimental conditions[69,70].

Clinical EVLP

EVLP has facilitated the utilization of ECD lungs. Some lung transplantation centres report an expansion of the donor pool by more than 80% after EVLP implementation[15]. Several single- and multi-centre reports[54-60], including two RCTs[67,68], have demonstrated both the safety and clinical performance of EVLP in SCD[57,67] and ECD[19,20,56] lungs.

Although a substantial body of literature supports the safety, efficacy, and potential for extended preservation with EVLP, clinical implementation has progressed more slowly than scientific evidence would suggest. While EVLP is well established in many high-volume transplant centres (>32 lung transplantations and >15 EVLP cases per year), small-volume centres often struggle to maintain the necessary expertise and staffing for EVLP, with the associated on-call personnel costs being a negating factor[71-73]. One proposed solution is the establishment of centralized EVLP facilities serving multiple hospitals[74,75]. While this model has been successfully implemented in some regions, larger countries with substantial transport distances are still faced with logistical limitations, highlighting the need for new technologies enabling longer-term preservation of donor lungs.

Synthesis of findings from multiple clinical studies indicated a trend towards higher incidence of early PGD in recipients of EVLP-treated donor lungs compared to those receiving SCD lungs[76]. Importantly, analyses suggested that PGD following EVLP did not carry the same mortality risk as PGD observed after transplantation of non-EVLP-treated lungs[74]. These observations were true for lungs assessed at centralized EVLP centres in particular and led to the general hypothesis that EVLP-associated PGD may represent a distinct clinical phenotype with unique biological and prognostic characteristics[75,77].

EVLP as a platform to deliver therapeutic agents

EVLP is an ideal platform to deliver therapeutics to the lung *ex situ*. Because the lung is isolated in the circuit, potential systemic off-target effects can be completely avoided[50,78]. Furthermore, lung physiological parameters and potential treatment effects can be monitored in real-time while investigating new treatment strategies. Consequently, we and others have chosen to leverage the EVLP platform for assessing the effect of immunomodulatory interventions[78-80]. Selected works relevant to the thesis scope will be presented in the following sections.

Selected targets for immunomodulation and therapeutic intervention

Cytokine storm during LTx

IRI and the subsequent development of PGD both present with excessive infiltration of cytokines leading to a cytokine storm, which ties cytokine infiltration to worse outcomes after lung transplantation[81,82]. Several pre-clinical studies have investigated the use of cytokine (hemo)adsorption to improve lung function and reduce IRI during EVLP[61,83,84]. Furthermore, it was hypothesized that use of

cytokine hemoadsorption after lung transplantation would lead to early improved graft performance due to reduced inflammation as well as decreased development of PGD[61,84,85]. Due to the promising results observed in experimental, large animal models, cytokine adsorption was introduced into the clinic and early reports have been published. Interestingly, one report details the first usage of a hemoadsorption device within clinical EVLP to recondition marginal donor lungs and seemingly increased cytokine levels after treatment with cytokine hemoadsorption as compared to without[86]. However, the treated patients in-hospital mortality and 1-year death rate was significantly lower. Another report described the use of hemoadsorption after LTx for patients with extracorporeal membrane oxygenation (ECMO) in the intensive care unit (ICU) with a positive outcome on survival at 1-year post-LTx[87]. Furthermore, our research group in Lund pursued a pilot study of perioperative cytokine adsorption in 4 patients undergoing lung transplantation, which led to a significant improvement in patient outcome and prompted the approval of a large, nationwide clinical trial in Sweden (NCT05526950)[88,89]. The goal of the trial is to test the efficacy of cytokine adsorption in dampening the strong immune response observed during the reperfusion phase of lung transplantation and thereby improving post-LTx outcomes[89]. Interestingly, one of the immune cell types observed in connection with post-transplant deterioration was neutrophils and their derivatives, neutrophil extracellular traps.

Neutrophil extracellular traps

Neutrophil extracellular traps or NETs were first described and the term coined by Brinkmann et al. in 2004[90]. NETs are web-like structures that are released by activated neutrophils (Fig. 6A-D). In their normal, regulated state, NETs trap and kill pathogens and are needed for a healthy, functioning immune system, but when haemostasis becomes dysregulated, they become highly pathogenic[28,91]. NETs are mainly comprised of DNA in the form of decondensed chromatin, histones, the enzymes neutrophil elastase (NE), myeloperoxidase (MPO) and other granule proteins (Fig. 7a-b)[91,92]. NE and MPO are major drivers of NET formation, with NE actively degrading histones to facilitate chromatin decondensation and aided in the process by MPO[93]. The primary process by which NETs are released is a novel cell death mechanism called NETosis[94]. Since the discovery of NETosis in 2007, two distinct forms have been described in the literature[95]. The initially described NETosis involved a programmed form of cell death involving the emptying of cytosolic components by membrane rupture and is now known as non-vital ("suicidal") or lytic NETosis. The later discovered vital, non-lytic NETosis, in turn, describes a more rapid process by which NETs are expelled from the neutrophil by vesicular transport without membrane rupture, thereby not inherently leading to cell death[96].

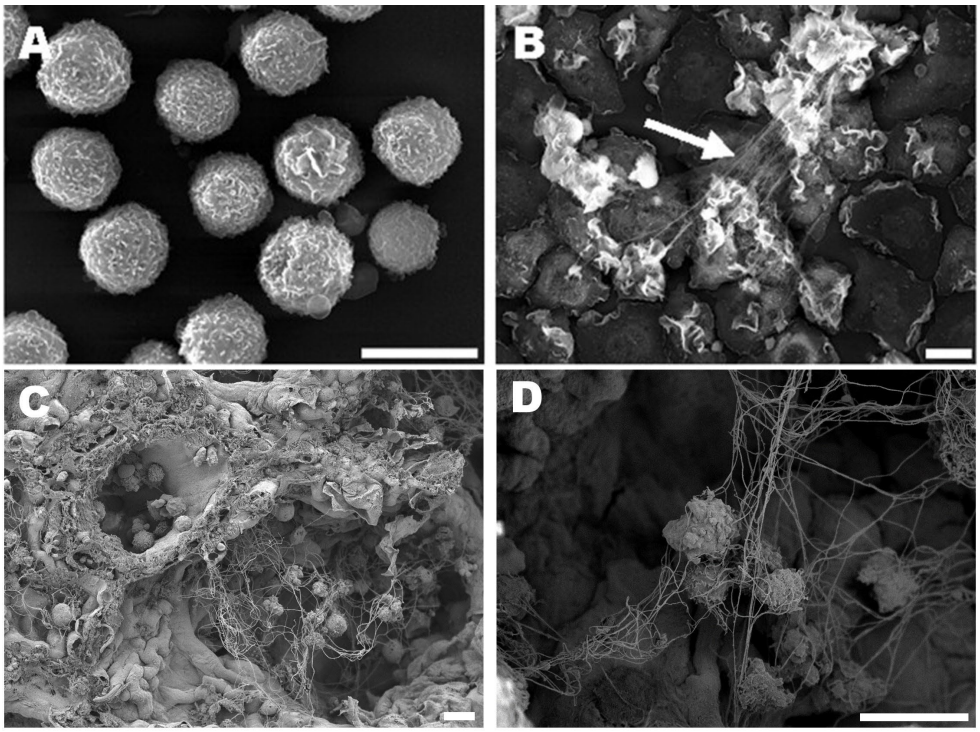

Figure 6: Neutrophil extracellular traps through the lens.
Neutrophil extracellular traps (NETs) were captured by Scanning Electron Microscopy (SEM). In the top row (A,B) are images adapted from the original first description of NETs in the literature by Brinkmann et al in 2004[90]. In the lower row (C, D) are images of porcine lung tissue captured at the Lund University Bioimaging Centre (LBIC), captured by and copyrighted by Nicholas B. Bechet. **A)** Non-activated neutrophils without NETs. **B)** Upon activation, neutrophils form NETs (indicated by arrow). **C)** Porcine alveoli damaged by aspiration- induced acute lung injury displaying activated immune cells, including NETs. **D)** Close-up of activated neutrophils and protruding NETs. Scale bars: 10 µm.

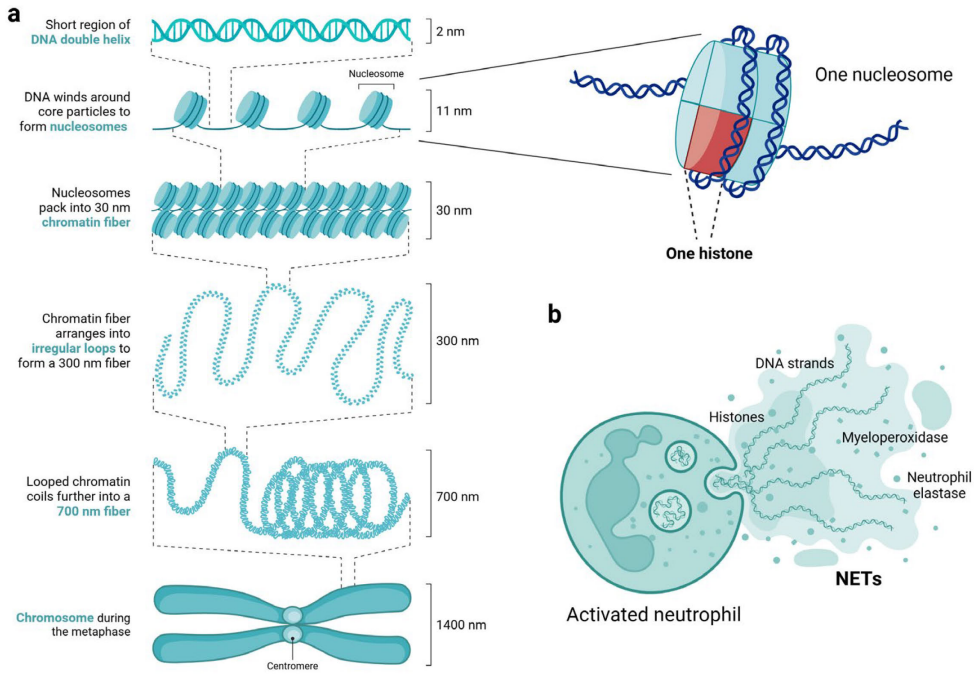

Figure 7: Chromatin, histones and neutrophil extracellular traps (NETs).
a) Schematic overview over the different stages/layers of DNA packaging, from chromosomes down to histones. **b)** An activated neutrophil in the process of expelling NETs, including some of the components of NETs. Figure created with BioRender.

NETs in damaged donor lungs and LTx

NETs are known to be involved in the pathogenesis of ARDS, but have also been implicated as key drivers of aspiration-induced acute lung injury, specifically[26,79,97]. Furthermore, excessive NET formation has been linked to worse outcomes after lung transplantation, both in pre-clinical models[98] and in the clinic[99]. Furthermore, elevated levels of NETs in EVLP perfusate have been correlated with worse outcomes after lung transplantation in these patients[100].

Another therapy modality sharing the common denominator of immunomodulation with cytokine adsorption and neutrophil extracellular traps removal, is cell therapy utilising mesenchymal stromal (stem) cells (MSCs) from different tissue sources.

Mesenchymal stromal (stem) cells

In donor organ regeneration and lung transplantation, all pathologies are multidimensional, with multiple branches of the immune system being involved. Thus, developing therapeutic strategies that effectively target multiple pathological

pathways simultaneously would be highly beneficial. One such strategy is the use of mesenchymal stromal cells (MSCs). MSCs are multipotent, non-hematopoietic stem cells[101]. They are not defined by the expression of a single cell marker, but rather by a collection of several surface markers. Even though they partly share the same space as, for example, HSCs in the bone marrow, MSCs differ from hematopoietic stem cells in their expression of surface markers[101]. MSCs were first discovered in 1966 by Friedenstein et al., who described spindle-like cells resembling fibroblasts with the ability to differentiate into osteoblasts[102]. Caplan et al. first coined the term mesenchymal stem cell in 1991[103] and over the next decade, the true 'stemness' of MSCs was proven in their ability to differentiate into osteogenic, adipogenic and chondrogenic cells[104,105].

Since the discovery of multipotent MSCs, their cultivation has attracted interest from numerous laboratories worldwide. Importantly, small differences in cell expansion and cultivation protocols and practices might lead to large differences in MSC properties. Thus, thanks to the rapidly expanding interest in the therapeutic potential of MSCs, the International Society for Cellular Therapy (ISCT) proposed minimal criteria for the definition of multipotent mesenchymal stromal cells in 2006 that are still widely used today[106]. The criteria consist of three characteristics:

- Adherence to tissue culture plastic
- Specific expression profile of surface markers:
    - POSITIVE (≥95% of population positive) for CD105, CD73 and CD90
    - NEGATIVE (≤2% of population) positive for CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR
- Differentiation into osteoblasts, adipocytes, chondroblasts *in vitro*

MSCs and the immune system

MSCs are potent immunomodulators, interacting with various components of the innate and adaptive immune systems, such as monocytes and macrophages, neutrophils, the complement system and T-cells[107-109]. Nevertheless, MSCs inherently exhibit low immunogenicity, making them ideal candidates for therapeutic intervention in contexts where tissue repair is needed[110]. Scientists have elucidated that MSCs exert their effects mainly via the secretion of immunomodulatory signalling molecules and are thought to adapt their secretome to the environment in which they find themselves. Despite this, the exact mechanisms for context-specific tissue repair remain the topic of many *in vitro, in vivo* and clinical studies as well as numerous literature reviews[101,111-113].

MSC sources

Mesenchymal stromal cells can be derived from different sources, such as for example adipose tissue, umbilical cord, amniotic fluid and bone marrow[101] (Fig. 9). Despite the requirements for stemness remaining the same, MSCs isolated from different sources may vary in efficiency. Previous *in vitro* studies have juxtaposed bone marrow-derived MSCs (BM-MSCs) to adipose-tissue-derived multipotent stromal cells (AT-MSCs[114]) or human umbilical cord perivascular cells (HUCPVCs[115]). AT-MSCS and HUCPVCs were shown to have slightly higher immunomodulatory and proliferative capacities than BM-MSCs. In the context of graft-versus-host disease, AT-MSCs and MSCs isolated from Wharton's jelly were found to more efficiently suppress pro-inflammatory T-cells as compared to BM-MSCs[116]. Importantly, all reported studies were performed *in vitro*, and studies investigating whether the difference in efficacy is maintained *in vivo* remain scarce. Some of these MSC types have yet to be investigated in vivo, and there is currently a lack of studies directly comparing them. BM-MSCs remain a widely used source due to their high proliferative capacity, availability, and the fact that their safe use in humans has been established in clinical trials[117].

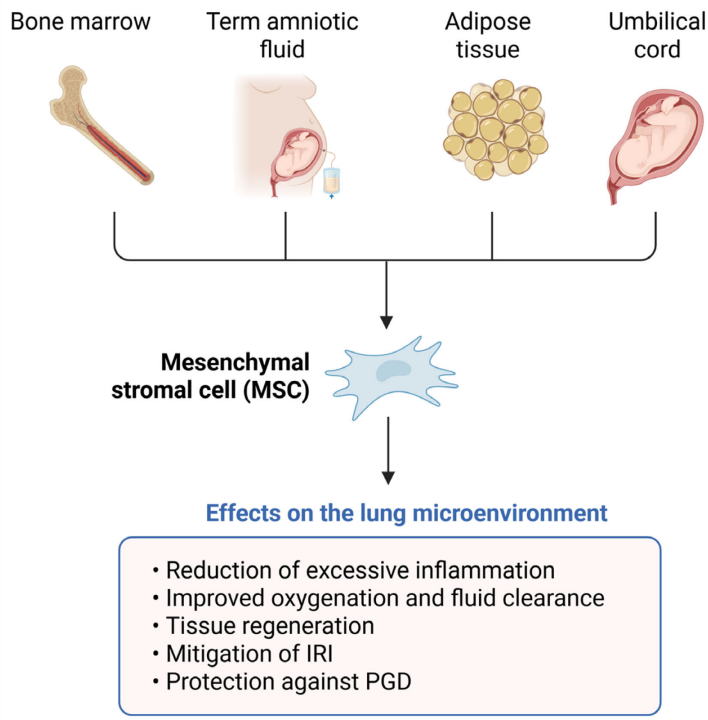

Figure 8: Mesenchymal stromal cell (MSC) sources.
Overview of selected sources from which MSCs can be isolated and a selection of effects that studies have shown on the lung microenvironment. Abbreviations: Ischemia-reperfusion injury (IRI), primary graft dysfunction (PGD). Figure created with BioRender

MSCs as a treatment modality

Due to their immunomodulatory capacities, combined with low immunogenicity, MSCs have gained widespread interest in the field of organ regeneration and transplantation[78,80,118,119]. Within the field of lung transplantation, several studies report the use of MSCs to treat ARDS, regenerate damaged donor lungs during EVLP or mitigate the development of PGD and improve the outcome of lung transplantation.

Clinical studies investigating MSC therapy

The safety and feasibility of using MSCs as therapeutic interventions has been evaluated in several clinical trials. The SafeCell meta-analysis reviewed the outcome of cell therapy in a total of 1012 participants with clinical diagnoses ranging from cardiovascular, neurological and oncological/haematological conditions to healthy volunteers[117]. The systematic review did not find any correlation between stem cell therapy and acute infusional toxicity, organ system complications, infection, death or malignancy[117].

For the treatment of ARDS, the double-blind, randomised, placebo-controlled START, REALIST and STAT trials all show clinical safety, with no significant differences in neither mortality, nor physiological improvements[120-123]. Some smaller clinical studies also reported a decrease in biomarkers related to ARDS pathogenesis[124].

Looking at lung transplantation specifically, most clinical trials have focused on the mitigation of CLAD, displaying that MSC therapy is clinically feasible and safe in CLAD patients[125,126] and might slow down CLAD progression as observed in some patients[127]. One recent study investigated MSC therapy to mitigate the development of PGD post-transplantation, showing clinical safety but no difference in PGD grades 72 hours after transplantation[128].

MSCs in experimental settings

Even though treatment with MSCs shows clinical safety along an array of human pathologies and mild beneficial effects, several questions remain. The majority of models used in experimental settings remain limited to small animals such as mice and rats. In the case of larger animal models or even rejected human donor lungs being employed, studies were most often limited to EVLP, without implantation of the treated grafts, to investigate whether they would sustain transplantation[78,80,119]. Furthermore, clear implementation strategies towards an off-the-shelf therapy that might be used in the clinic in the future are lacking. However, a few recent translational studies are easing the path towards clinical implementation. In a previous study conducted in our research group, we investigated TAF-MSCs as a treatment option to ameliorate porcine LPS-induced ARDS following transplantation and found that repeated-dose TAF-MSCs improved lung function

and decreased inflammatory regulators associated with neutrophil activity[129]. A selection of the most recent experimental studies pursued is presented in Table 1.

Table 1: Selected experimental studies investigating mesenchymal stromal (stem) cell (MSC) therapy in the field of lung transplantation, published 2020-2026.
Studies published before 2020 have been the subject of extensive reviews [78,80]. Abbreviations: Ex vivo lung perfusion (EVLP), Acute respiratory distress syndrome (ARDS).

Reference	Study Type	MSC source	Key concept
Nykänen et al., 2021[130]	Human EVLP	Human umbilical cord MSCs with augmented human anti-inflammatory hIL-10 production ($MSCs^{IL\text{-}10}$)	MSCs with augmented anti-inflammatory IL-10 production to regenerate damaged donor lungs
Shimoyama et al., 2022[131]	Rat transplantation	Adipose tissue-derived MSCs (ADMSCs)	MSCs for immunosuppression after transplantation
Edström et al., 2023[132]	Porcine ARDS	Human integrin α10β1-selected adipose tissue-derived MSCs (integrin α10β1-MSCs)	MSCs to treat sepsis-induced ARDS
Edström et al., 2024[129]	Porcine EVLP + transplantation	Human amniotic fluid-derived MSCs	MSCs to mitigate donor lung injury and PGD post-transplantation
Nykänen et al., 2024[133]	Porcine EVLP + transplantation	Human umbilical cord MSCs with augmented human anti-inflammatory hIL-10 production ($MSCs^{IL\text{-}10}$)	MSCs with augmented anti-inflammatory IL-10 production for immunomodulation post-transplantation

MSC derivatives, non-MSC cell therapies, and cell-free approaches

Even though the multipotent properties of mesenchymal stromal cells (MSCs) are beneficial for the treatment of several pathologies relevant to lung transplantation, in some cases, a more targeted approach toward specific immune cell types or mechanisms may be preferred (Table 2). In this context, the use of cell-derived products such as extracellular vesicles (EVs) and exosomes may offer advantages, as they can be generated directly from the specific cell type of interest[78,134]. Furthermore, the use of MSCs as living entities raises certain biosafety concerns, particularly regarding their long-term behaviour in vivo and the potential risk of malignant effects, similar to those described for pluripotent stem cells[134].

Both cellular and cell-free therapeutics in the context of organ regeneration can be defined as Advanced Therapy Medicinal Products (ATMPs)[118,146]. Another ATMP strategy involves gene therapy, using viral vectors or clustered regularly interspaced palindromic repeat (CRISPR)-based systems to modulate specific pathways in donor lungs during EVLP[53,147]. Adenoviral delivery of human IL-10 during EVLP has been reported to enhance its expression and was associated with improved post-transplant lung function in porcine models, while subsequent studies suggested that CRISPR-mediated activation of IL1RN and IL-10 could be induced and maintained after transplantation[148,149]. In a separate approach, lentiviral shRNA targeting swine leukocyte antigen enabled genetic modification of donor lungs and was associated with prolonged graft survival for several years without immunosuppression in a miniature swine model[150,151].

Table 2: Cellular and cell-free therapeutics for the treatment of pathologies related to lung transplantation.
Selected, recent studies within the field of lung transplantation and MSC-derived or cell-free therapies. Abbreviations: T-regulatory cells (Tregs), Ex vivo lung perfusion (EVLP), Mesenchymal stromal cells (MSCs), Extracellular vesicles (EVs), Cellular vesicles (CVs).

Category	Reference	Study Type	MSC source	Key concept
Immune cell therapies				
Tregs	Miyamoto et al., 2022[135]	Rat EVLP + transplant OR human rejected donor EVLP	Allogeneic rat Tregs or frozen allogeneic human Tregs	T-regulatory cells for immunosuppression post-transplantation
Tregs	Takahagi et al., 2025[136]	Rat EVLP + transplantation	Allogeneic polyclonal rat regulatory T cells (Tregs)	Combination therapy with Tregs and standard immunosuppression to mitigate rejection
T cells	Pierucci et al., 2016[137]	Case report transplantation	Autologous CMV-specific CD8+ T cells	T-cell therapy for the treatment of ganciclovir-resistent CMV
Cell-derived or cell-free therapies				
MSCs + MSC-EVs	Stone et al., 2017[138]	Mouse EVLP	Human umbilical cord-derived MSCs or their derived EVs	MSCs or MSC-derived EVs for pre-conditioning DCD lungs
MSC-EVs	Lonati et al., 2019[139]	Rat EVLP	Human bone marrow-derived MSC-derived EVs	EVs for the mitigation of IRI
MSC-EVs	Yang et al., 2025[140]	Rat Transplantation	Human umbilical cord mesenchymal stromal cell-derived extracellular vesicles (hucMSC-EVs) or hucMSC with encapsulated miR-146a (EVs-miR-146a)	EVs for the mitigation of IRI post-transplantation
MSC-EVs	Wang et al., 2026[141]	Rat Transplantation	Human adipose-derived MSC-derived EVs	EVs for the mitigation of IRI post-transplantation
Neutrophil-CVs	Yuan et al., 2025[142]	Rat EVLP+ Transplantation	Human promyelocytic leukemia cells (HL60) neutrophil-derived ROS-responsive cellular vesicles (SOD2-Fer-1@CVs)	Neutrophil-CVs to mitigate IRI and PGD post-transplantation
Cell-derived exosomes	Ribeiro et al., 2025[143]	Porcine EVLP	Human lung spheroid cell-exosomes	Exosomes to reduce IRI in DCD lungs
Mitochondria	Cloer et al., 2023[144]	Porcine EVLP + rejected human donor EVLP	Frozen mitochondria isolated from porcine heart tissue	Mitochondria for the mitigation of IRI
Mitochondria	Bechet al., 2025[145]	Porcine EVLP	Freshly isolated mitochondria from murine liver or porcine muscle tissue	Mitochondria for the mitigation of IRI

Aims of the thesis

General aim

This doctoral thesis aims to investigate novel regenerative and immunomodulatory therapeutic strategies to restore donor lungs that were initially deemed unsuitable for transplantation, with the goal of expanding the donor pool and improving post-transplant outcomes, particularly by reducing the incidence and severity of primary graft dysfunction. In addition, this work sought to advance the understanding of the molecular and immunological processes occurring before, during, and after lung transplantation and how these might be modulated by regenerative therapies.

Specific aims

In **paper I**, we investigated whether treatment with a cytokine adsorber coupled to ECLS during lung transplantation could decrease circulating nucleosomes and mitigate the development of acute rejection and PGD in a pilot cohort of human patients.

In **paper II**, we sought to improve lung function and salvage injured lungs by applying NET removal during EVLP to porcine donor lungs damaged by aspiration-induced acute lung injury.

In **paper III**, we investigated whether MSCs from bone marrow or full-term amniotic fluid could restore severely injured donor lungs and prevent primary graft dysfunction in a porcine transplantation model, and whether these effects could be sustained with postoperative dosing.

In **paper IV**, we examined the efficacy of MSC therapy in mitigating early ARDS development in a porcine model of aspiration-induced acute lung injury. MSCs manufactured in a fully compliant GMP system were compared to standard cell culture manufacturing, including a dose-response investigation.

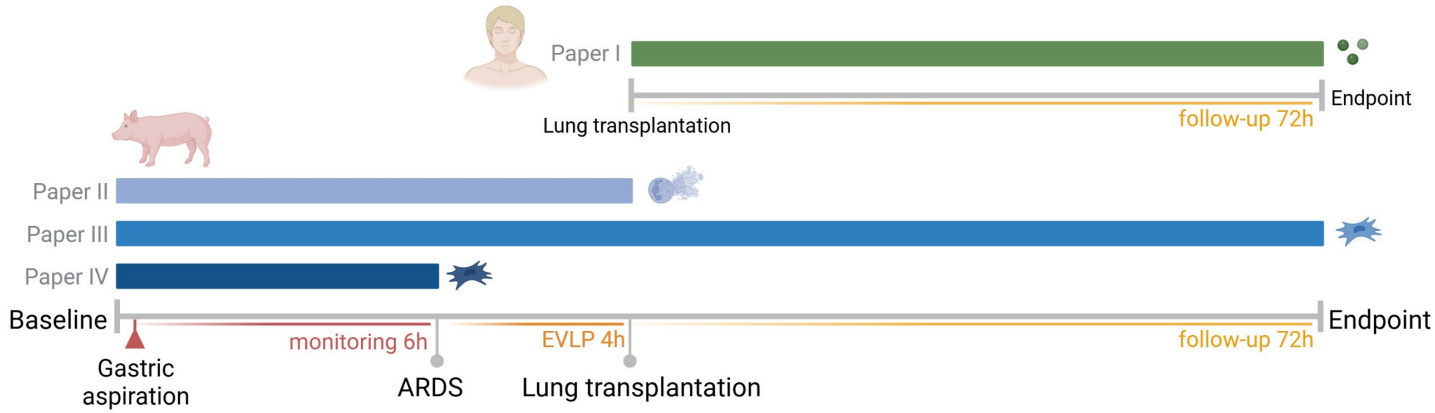

Figure 9: Schematic overview of the experimental timelines of the four papers included in this thesis.
Different coloured bars represent the experimental timelines of the different studies and icons describe the species in which the studies were performed. Small icons to the right of each bar depict the type of immunomodulatory therapy used in the specific papers. Cytokines (green): cytokine adsorption; Neutrophil extracellular trap (NET, light blue): NET removal; Mesenchymal stem cell (MSC, medium and dark blue): MSC therapy. Abbreviations: Acute respiratory distress syndrome (ARDS), Ex vivo lung perfusion (EVLP)

Summary of studies included in the thesis

Paper I

Background and results

Paper I is based on the positive findings from a study previously performed in our research group. In a porcine model of lung transplantation, we demonstrated that cytokine adsorption before LTx, during EVLP and post-LTx improved lung function and lowered the incidence of PGD in a porcine lung transplantation model[61]. The positive results from this study facilitated the procurement of the necessary ethical permission to pursue a pilot study in human patients and laid the foundation for paper I[88].

Pathologically elevated levels of NETs have been clearly connected to lung injury and worse outcomes in experimental studies of lung transplantation[98]. Interestingly, one study reported increased levels of NET in donor lung EVLP perfusate of LTx patients who would go on to need longer times on the ventilator in the ICU[100]. There is thus a strong rationale for investigating treatments that could remove NETs from the circulation and thereby improve the outcome for patients after LTx. In this study, we hypothesized that perioperative cytokine adsorption would decrease circulating nucleosomes as markers of NETs and hamper the development of signs of rejection after human lung transplantation (Fig. 9).

Four patients undergoing bilateral lung transplantation at Lund University Hospital were randomized to either receive treatment with a cytokine adsorber during extracorporeal life support (ECLS) or ECLS only, without cytokine adsorption (Fig. 10a). Blood samples were collected from all patients before, at 12 hours, and at 72 hours post-LTx.

Perioperative cytokine adsorption reduced circulating leukocytes and NET-related markers post-LTx

Circulating cell-free nucleosomes, total leukocytes (white blood cells, WBC), lymphocytes and neutrophils were assayed in all patients including five healthy control subjects. None of the assayed markers differed significantly between neither treated and non-treated patients pre-operatively nor the healthy control subjects. At

12 hours post-transplantation, circulating H3.1 nucleosomes were significantly increased in non-treated patients, both compared to treated patients and healthy controls (Fig. 10b). 72 hours post transplantation, circulating nucleosome levels had dropped but were still higher in the non-treated as compared to the treated patients. Total WBCs as a marker for increased immune system function were elevated in non-treated as compared to treated patients (Fig. 10c, left). Similarly to circulating nucleosome levels, WBC levels had decreased at 72 hours post transplantation. Lymphocyte counts instead displayed decreased values at 12h post-operatively, with a slight increase at 72 hours (Fig. 10c, middle). However, lymphocyte counts appeared higher in untreated patients than in treated patients. Total neutrophil counts displayed the same trend as total WBCs (Fig. 10c, right). None of the differences seen between differential blood cell counts reached statistical significance.

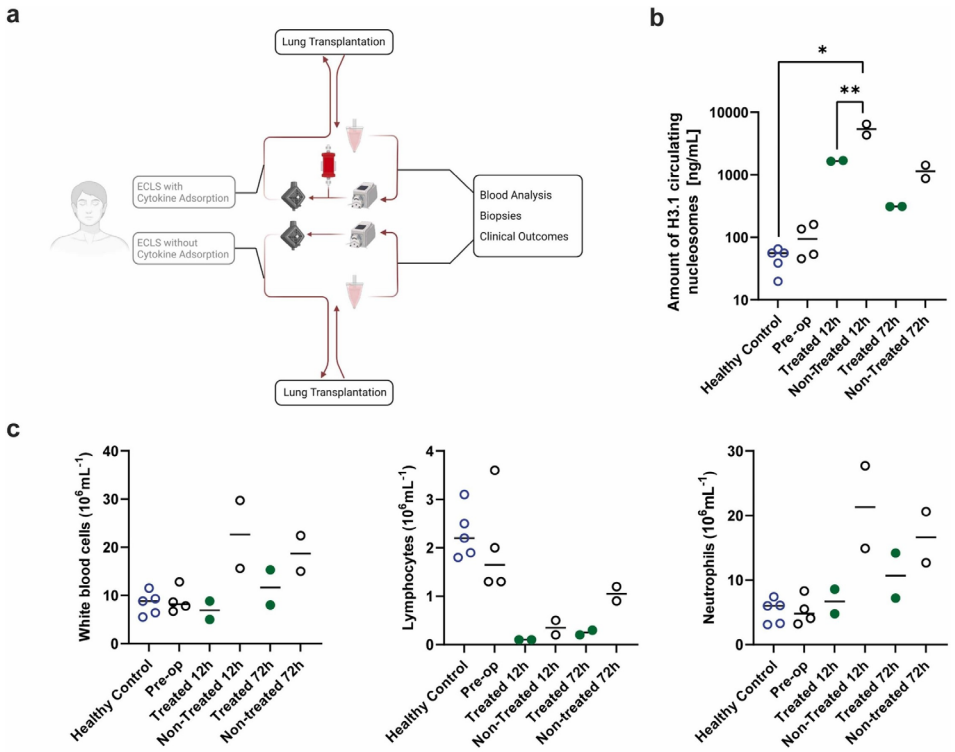

Figure 10: ECLS coupled with a cytokine adsorber reduced circulating nucleosomes and leukocytes in LTx patients.
Use of cytokine adsorption during transplantation and resulting circulating nucleosome and differential blood cell counts. **a)** Four transplant recipients received either treatment intraoperatively with a cytokine adsorber in line with extracorporeal life support (ECLS) or ECLS only. Recipients were then followed for 3 months. Created with biorender.com. **b)** Circulating nucleosomes were measured from the transplant recipients as well as from healthy controls. Recipients were evaluated preoperatively (pre-op), and at 12 and 72 hours following the transplant procedure. **c)** Peripheral blood cell counts were measured from the transplant recipients as well as from healthy controls. Recipients were evaluated pre-op, and at 12 and 72 hours following the transplant procedure. Adapted from [88] with permission under the CC BY 4.0 license.

Citrullinated nucleosome histone H3, a key mediator in NET release, was additionally assessed as a marker of NET formation. The findings were consistent with the H3.1 nucleosome analysis, demonstrating that concentrations were significantly elevated 12 hours after LTx in non-treated patients (554.9±295.5 ng/mL) compared to preoperative levels (73.4±134.9 ng/mL), healthy controls (3.5±4.0 ng/mL, p=0.0219), and treated patients 72 hours after transplantation (69.3±33.0 ng/mL). Furthermore, cytokine levels were assayed in plasma, exhibiting low IL-6 and IL-8 concentrations in healthy controls and in all patients prior to transplantation. Levels of both cytokines increased 12 hours post-LTx, but by 72 hours post-transplantation, IL-6 levels had declined while IL-8 levels were unchanged. Although cytokine patterns tended to favour the treated group, the differences observed between groups were not statistically significant.

Patients treated with a cytokine adsorption device together with ECLS did not develop any signs of rejection or PGD

None of the treated patients developed any signs of PGD in the 72 hours following transplantation or signs of acute rejection up to three months post-operatively (Table 3). Adversely, both patients without cytokine adsorption developed PGD grade 1 and 3, respectively, as well as acute cellular rejection, detected in transbronchial biopsies (Fig. 11). One month after transplantation, one of the patients demonstrated histological grade A1 acute cellular rejection along with low-grade (B1R) lymphocytic bronchiolitis, without identification of donor-specific antibodies. Three months post transplantation, the histological score remained unchanged (A1 B1R 0DSA). The second non-treated patient exhibited A2 rejection at one month, which improved to A1 rejection by the 3-month assessment.

To conclude, this brief communication reports that perioperative treatment with a cytokine adsorption device during lung transplantation decreased the incidence of PGD as compared to patients transplanted without treatment. The positive results in this pilot case series led to approval of a national study to explore cytokine adsorption in a large national cohort as a treatment option to reduce the incidence of PGD after lung transplantation.

Table 3: Selected patient characteristics before and after lung transplantation.
Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; IPF, idiopathic pulmonary fibrosis; PGD, primary graft dysfunction. Data are shown as a range of values. Table adapted from [88] under the CC BY 4.0 license.

Patient Characteristics	Treated transplant (n=2)	Nontreated transplant (n=2)
Diagnosis		
COPD	2	1
IPF	0	1
Age (years)	59–60	47–66
Sex (female)	1	2
BMI	20.9–21.2	25.7–27.1
Smoking history	2	1
History of comorbidities		
Hypertension	1	0
Diabetes mellitus	0	0
Pulmonary hypertension	0	0
Postoperative dialysis	0	0
PGD		
Grade 0	2	0
Grade 1	0	1
Grade 2	0	0
Grade 3	0	1
Time on mechanical ventilation (hours)	6–24	12–72
Time in ICU (days)	7–8	6–10
Acute rejection at 1 month	0	A1 B1R 0DSA, A2
Acute rejection at 3 months	0	A1 B1R 0DSA, A1

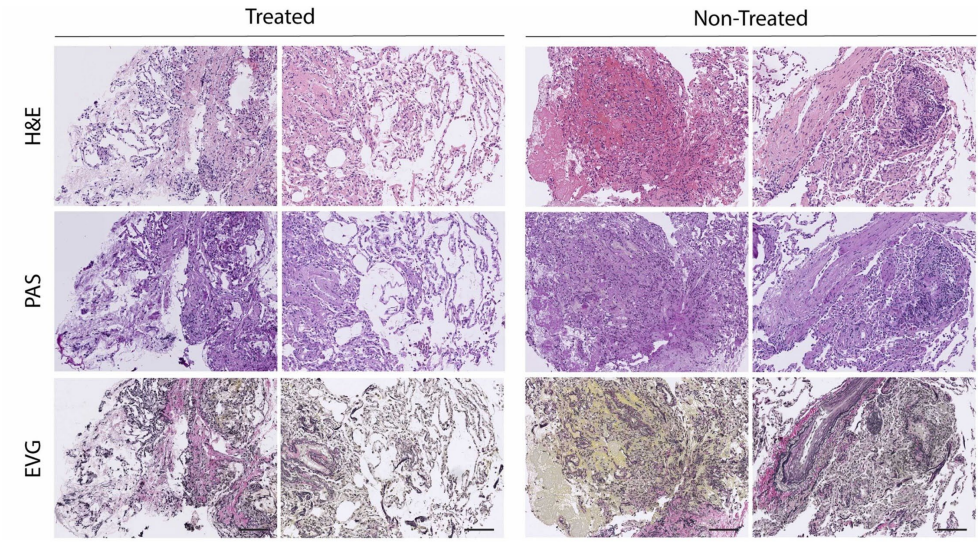

Figure 11: Perioperative cytokine adsorption reduced signs of rejection in LTx patients.
Representative histopathological images of transbronchial biopsies stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and elastin van Gieson (EVG) for treated (n=2) and nontreated (n=2) patients. Images are presented at 10× magnification, scale bars represent 1 mm. Adapted from [88] under the CC BY 4.0 license.

Paper II

Background and results

The under-utilization of available donor lungs remains a critical problem within lung transplantation[152]. One of the main reasons for donor lungs being discarded is the development of acute lung injury as a result of the aspiration of gastric contents prior to donation[153]. Neutrophil extracellular traps have been explicitly linked to the development and severity of ALI and acute respiratory distress syndrome, as the most severe form of ALI[26,28,154]. Furthermore, increased levels of NETs have been linked to worse outcomes after lung transplantation[98-100,155]. The removal of NETs from donor lung grafts therefore merits investigation to make more lungs available for transplantation and improve outcomes. While previous studies have already investigated the removal of NETs using DNAse or other NET-specific therapies[26,154,156], the use of such therapeutics often leads to disruptive off-target effects due to the inflammatory nature of fragmented NET components[28,155,157,158]. Due to these reasons, we hypothesized that the use of a highly selective bead-based filter connected in line with an EVLP circuit would reduce NETs in the circulation of porcine donor lungs damaged by aspiration and improve lung function[79] (Fig.8).

Acute lung injury was induced by gastric content instillation in 12 pigs under anaesthesia. The lungs were explanted and placed on EVLP for four hours. One group (n=6) received EVLP coupled with a neutrophil extracellular trap (NET) removal device and the other group (n=6) received EVLP treatment only.

Acute lung injury was induced according to the Berlin criteria

To start with, acute lung injury was confirmed in all animals according to the Berlin definition of ARDS as defined by PaO_2/FiO_2 ratios of less than 300 mmHg[29] (Fig. 12a). Over the course of lung injury induction, no significant differences in PaO_2/FiO_2 ratio between treatment groups could be observed (Fig. 12b). Furthermore, bilateral infiltrates on chest x-ray were analysed and retrospective histological examination was performed (Fig. 12c-d). Standard Hematoxylin&Eosin staining revealed typical signs of lung injury including infiltration of immune cells and oedema (Fig. 12d). Immunohistochemical staining for citrullinated histone H3 confirmed the relevance of NETs in the pathology of the lung injury model, as evidenced by higher numbers in tissue at confirmed ARDS compared to Baseline (Fig. 12e).

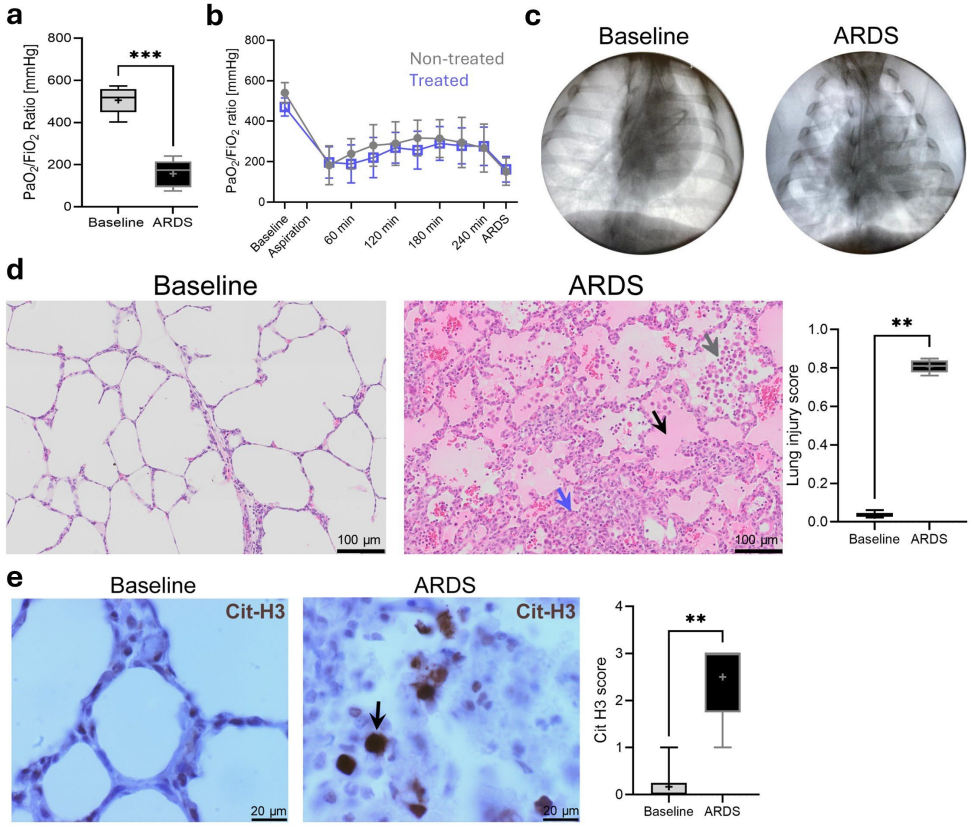

Figure 12: Induction of lung injury in porcine donor lungs according to Berlin criteria.
a) PaO2/FiO2 ratio in all pigs at baseline and after confirmation of ARDS. ***p<0.001 (two-sided Wilcoxon test) **b)** Comparison of PaO_2/FiO_2 ratio of treated (blue squares) and non-treated pigs (grey full circles) over time from baseline (before instillation of gastric contents) until confirmation of ARDS. **c)** Representative chest x-rays before induction of ARDS (Baseline) and after ARDS was confirmed (ARDS). **d)** Representative images of haematoxylin and eosin-stained histology sections of lung tissue at baseline (left) vs at ARDS (middle), with arrows indicating thickening of alveolar walls (blue arrow), pulmonary oedema (black arrow) and infiltration of immune cells into alveolar spaces (grey arrow). Scale bars 100 µm. Lung injury scores (right) comparing healthy lung tissue at baseline to tissue after ARDS was confirmed. Boxplots: median lines, plus at the mean and whiskers indicating minimum and maximum. **p<0.01 (two-sided by Mann-Whitney test). **e)** Representative images and quantification of citrullinated (Cit)-H3 in tissue identified by immunohistochemistry, indicated by brown staining and black arrow. Scale bars 20 µm. Adapted from [79] under the CC BY 4.0 license.

Four hours of NET removal treatment in combination with EVLP improved lung function and reduced inflammation in treated grafts

After four hours of EVLP, the grafts that had received NET removal treatment displayed significantly improved lung function compared with non-treated lung grafts, most notably by an increase in PaO_2/FiO_2 ratio. Five of six grafts in the treated group thus reached the 300mmHg threshold for re-acceptability into the donor pool after NET removal (Fig. 13a). The profound inflammation seen at confirmation of ARDS was significantly reduced after NET removal treatment as displayed in H&E-stained tissue sections (Fig. 13b), levels of pro-inflammatory

cytokines in plasma and BALF (Fig. 13c-d) as well as by immunohistochemical staining of pro-inflammatory marker AIF-1 (Fig. 13e). The decrease in inflammatory cells was accompanied by decreased oedema in the lung as assessed by wet/dry ratio (Fig. 13f).

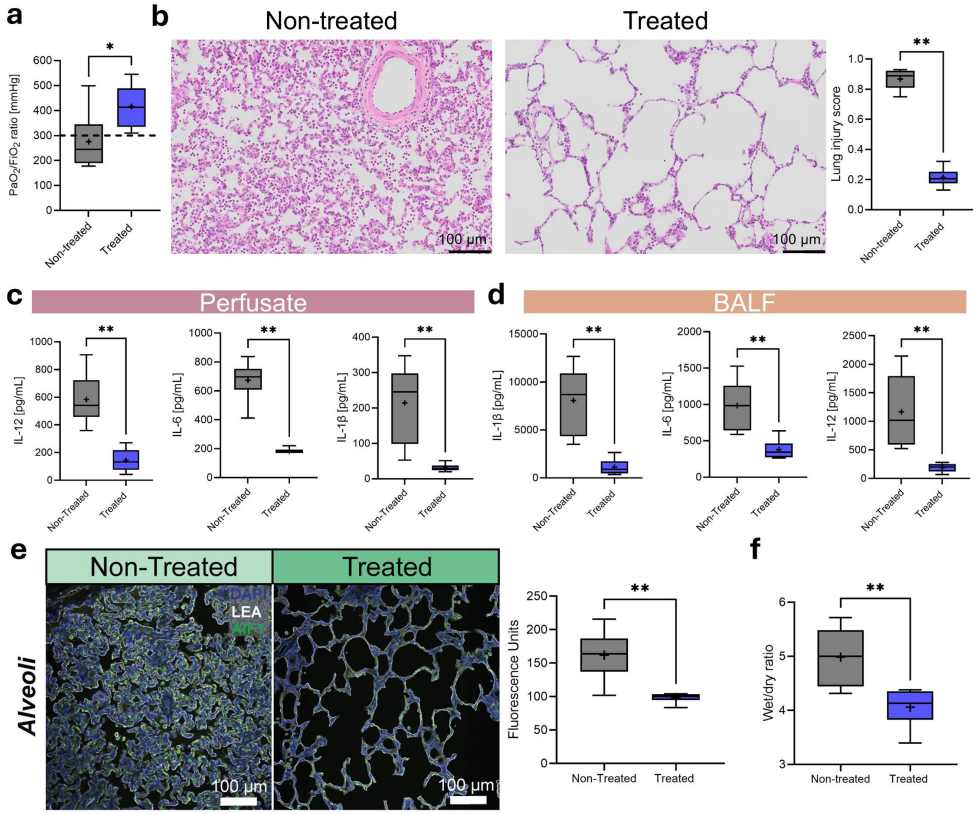

Figure 13: NET removal during 4 hours EVLP significantly improved lung function and decreased signs of inflammation in donor grafts.
a) PaO_2/FiO_2 ratios for treated and non-treated lungs after 4 hours of EVLP. The black dotted line at 300 mmHg indicates the clinical threshold for acceptance of lungs for transplantation. **b)** Representative haematoxylin and eosin (H&E) stained lung sections from the non-treated and treated group. Scale bars: 100 µm. Cytokine levels in **c)** EVLP perfusate and **d)** BALF, comparing treated to non-treated grafts **e)** Representative images (left) and quantification (right) of fluorescent staining of allograft inflammatory factor-1 (AIF-1, green), 4′,6-diamidino-2-phenylindole (DAPI, in blue) and Lycopersicon Esculentum lectin (LEA, in white) comparing non-treated to treated grafts. **f)** Wet/Dry ratio of lung tissue after 4h of EVLP for non-treated and treated lungs. Boxplots: median lines, a plus at the mean and whiskers indicating minimum and maximum. *p<0.05, **p<0.01 (two-sided Mann-Whitney test). Adapted from [79] under the CC BY 4.0 license.

NETs-specific markers and cell-free DNA were reduced after four hours of treatment

Next, we were interested in assessing the efficiency and specificity of the treatment column in removing NETs in particular, both from the lung grafts locally but also the circulating perfusate in the EVLP circuit. The citrullination of histone H3 is a strong indicator of NET formation and has developed as a commonly used marker for NETs over a wide range of disciplines[159-161]. In our study, citrullinated H3 was significantly downregulated in lung tissue as observed by immunohistochemical staining as well as in BALF and circulating perfusate (Fig. 14a-c). Additionally, cell-free DNA as a marker of fragmented DNA that could indicate the mediation of toxic effects, was significantly downregulated in the treated lungs as compared to non-treated lungs (Fig. 14d).

In conclusion, the results from this study suggest that NET removal during EVLP is feasible and effective and could be a valuable tool for reintroducing previously rejected donor lungs damaged by aspiration back into the donor lung pool.

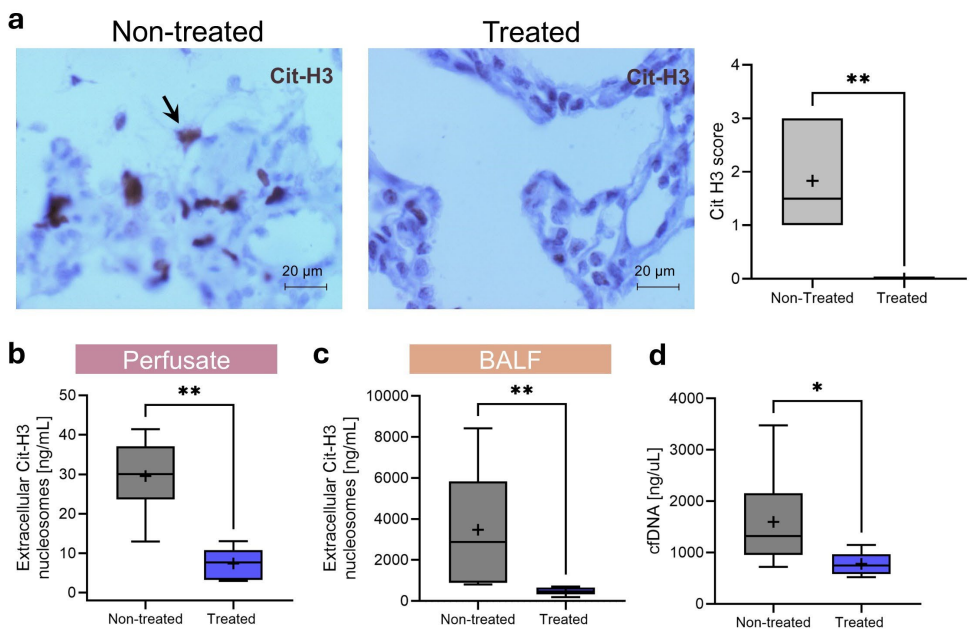

Figure 14: Citrullinated H3 nucleosomes and cell-free DNA are diminished after treatment with a NET removal device.
a) Cit-H3 in tissue identified by immunohistochemistry, representative images (left) with Cit-H3 in brown, indicated by a black arrow, scale bars: 20 µm. Quantitative scoring (right). Levels of extracellular citrullinated (Cit) H3 nucleosomes quantified by ELISA in **b)** perfusate and in **c)** bronchoalveolar lavage fluid (BALF). **d)** Levels of cell-free DNA (cfDNA) in perfusate. Boxplots: median lines, a plus at the mean and whiskers indicating minimum and maximum. *p<0.05, **p<0.01 (two-sided Mann-Whitney test). Adapted from [79] under the CC BY 4.0 license.

Paper III

Background and results

After observing that regeneration of aspiration-damaged donor lungs was possible by using a NETs removal device coupled to EVLP in paper II, we sought to investigate other promising regenerative therapies for their efficacy in restoring lung function in aspiration-damaged donor lungs. Mesenchymal stromal (stem) cells (MSCs) are widely studied for their immunomodulatory properties, combined with low immunogenicity[107-109,119]. Although early-phase clinical trials of MSCs for the improvement of outcome after lung transplantation in human patients exist, results are heterogeneous and highlight the need for further, more detailed studies in translational animal models[125,127,128,162]. Using MSCs from two distinct sources, we hypothesized that MSC therapy, particularly with repeated-dosing regimens, would improve graft performance, prevent PGD, and reveal source-specific mechanisms of action.

Lung injury was induced in 24 pigs by instillation of gastric content as described in paper II and confirmed, after which the injured lungs were explanted and placed on EVLP (Fig. 9). During EVLP, the lungs were either treated with one dose of bone marrow-derived mesenchymal stromal cells (BM-MSCs, n=12), one dose of term-amniotic fluid-derived lung-specific mesenchymal stromal cells (TAF-MSCs, n=6) or EVLP only (n=6, non-treated). The left lungs of all grafts were then transplanted into 24 healthy recipients, and the animals were monitored for 72 hours. The lungs that had received BM-MSCs during EVLP were subdivided into two groups, where one group received two more doses of BM-MSCs post-LTx (BM-MSCs repeated, n=6) and the other received no further doses (BM-MSCs single, n=6). The group that had received TAF-MSCs during EVLP continued to get two more doses of TAF-MSCs (TAF-MSCs repeated).

Gastric aspiration reproducibly led to the development of ARDS in all donors

Aspiration of acidic gastric contents induced acute lung injury, meeting the Berlin definition of ARDS and displaying a marked increase in PVR and SVR, a decline in pulmonary compliance as well as the characteristic decline in oxygenation (Fig. 15a). PaO_2/FiO_2 ratios decreased from 521.0±44.3 at Baseline to 193.7±57.2 at confirmation of ARDS and histological analysis confirmed extensive lung injury with immune cell infiltration and pulmonary oedema (Fig. 15b).

This functional and structural impairment was accompanied by a pronounced inflammatory response, including significant elevations of IL-1β and IL-6 in both plasma and BALF (Fig. 15c), and TUNEL staining demonstrated a significant increase in apoptotic cells following aspiration (Fig. 15d).

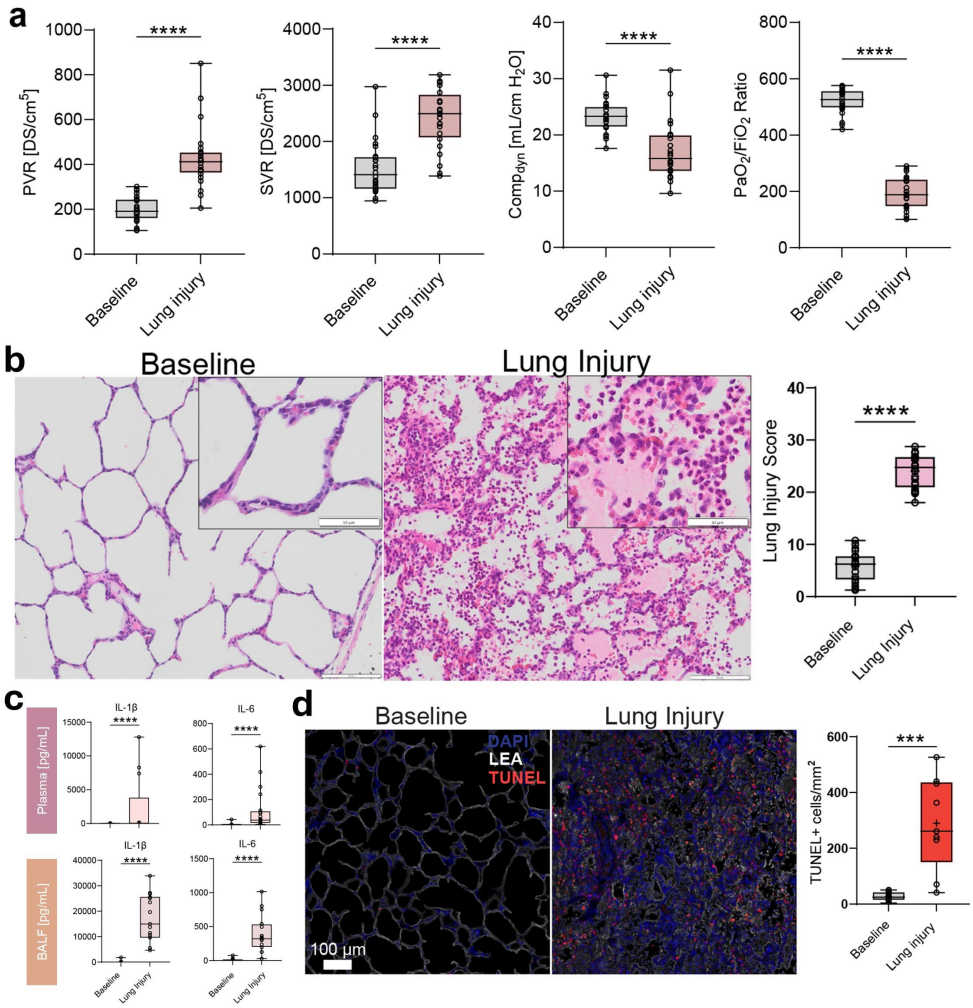

Figure 15: Induction of aspiration-induced acute lung injury.
a) Selected hemodynamic and respiratory parameters at baseline (light grey) and after lung injury (dark pink), including pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), dynamic compliance ($Comp_{dyn}$), and PaO_2/FiO_2 ratio. **b)** Representative haematoxylin and eosin (H&E) stained lung sections at baseline and at lung injury, scale bars: 100 µm (overview); 50 µm (callout). Quantification of histological lung injury scoring (right). **c)** Cytokine IL1β and IL-6 levels at baseline and lung injury in plasma (upper) and bronchoalveolar lavage fluid (BALF, lower). **d)** Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, red), 4′,6-diamidino-2-phenylindole (DAPI, blue), and Lycopersicon esculentum lectin (LEA), white, at baseline (left) and at confirmed lung injury (middle). Scale bar represents 100 µm. Quantification of $TUNEL^+$ cells/mm^2 (right). ***p<0.001, ****p<0.0001 (Student t-test or Mann-Whitney U-test). Boxplots: centre line, median; box, interquartile range; whiskers, min-max. Adapted from paper III with permission.

Ex vivo lung perfusion combined with MSC treatment reduced immune cell infiltration

After EVLP, both groups of animals given repeated doses of MSCs, whether BM-MSCs or TAF-MSCs, showed significantly improved lung function compared to the non-treated animals, which remained severely impaired. Other clinically relevant

parameters, such as PVR and dynamic compliance, were also improved in relation to both the single-dose BM-MSC-treated and non-treated groups (Fig. 16a). These improvements were paralleled by reduced inflammatory signalling, with significant reductions in IL-1β for both MSC groups and IL-6, most notably in BALF in the TAF-MSC group (Fig. 16b). Histologically, MSC-treated grafts exhibited significantly lower injury scores and reduced immune cell infiltration (Fig. 16c), and TUNEL staining confirmed markedly decreased apoptosis in both treatment groups (Fig. 16d).

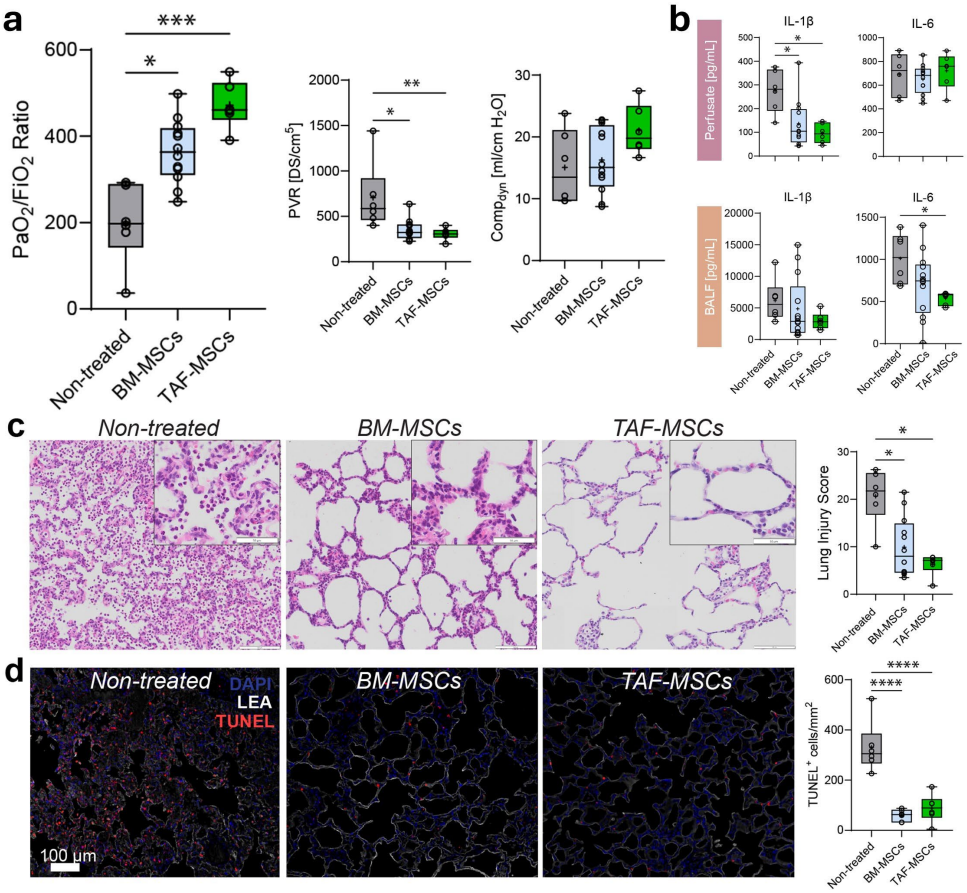

Figure 16: MSC treatment during *ex vivo* lung perfusion improved lung function and morphology.
All lungs were evaluated at the end of EVLP. Non-treated grafts received placebo (grey), while treatment groups received a single dose of bone marrow-derived MSCs (BM-MSCs; light blue) or term amniotic fluid-derived MSCs (TAF-MSCs; green). **a)** Selective hemodynamic and ventilatory parameters, including pulmonary vascular resistance (PVR), dynamic compliance ($Comp_{dyn}$), and ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (PaO_2/FiO_2). **b)** Cytokine IL1β and IL-6 levels in EVLP perfusate (upper) and bronchoalveolar lavage fluid (BALF, lower). **c)** Representative haematoxylin and eosin (H&E) lung sections, scale bars: 100 µm (overview); 50 µm (callout). Quantification of histological lung injury scoring (right). **d)** Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, red), 4′,6-diamidino-2-phenylindole (DAPI, blue) and Lycopersicon esculentum lectin (LEA, white) staining of lung tissue with quantification of $TUNEL^+$ cells/mm^2 (right). Scale bar: 100 µm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (one-way ANOVA or Kruskal-Wallis test). Boxplots: centre line, median; box, interquartile range; whiskers, min-max. Adapted from paper III with permission.

Repeated MSC treatment of either origin hampered PGD development post-transplantation

Following transplantation, repeated-dose groups maintained significantly higher PVR and overall improved ventilatory parameters (PIP, $PaCO_2$, dynamic compliance) and PaO_2/FiO_2 ratios compared with non-treated animals (Fig. 17a). As measured by standard clinical criteria, none of the animals in the repeated-dose MSC groups developed PGD, whereas 5 animals each in the non-treated and single-dose BM-MSC treatment groups developed PGD grades 2-3 (Fig. 17b). Inflammatory cytokines IL-1β and IL-6 were not significantly changed (Fig. 17c-d), but overall inflammation, as measured by influx of immune cells in the grafts, was significantly reduced in repeatedly treated lungs (Fig. 17e). Non-treated lungs also displayed the highest number of TUNEL-positive cells compared to all treatment groups (Fig. 17f).

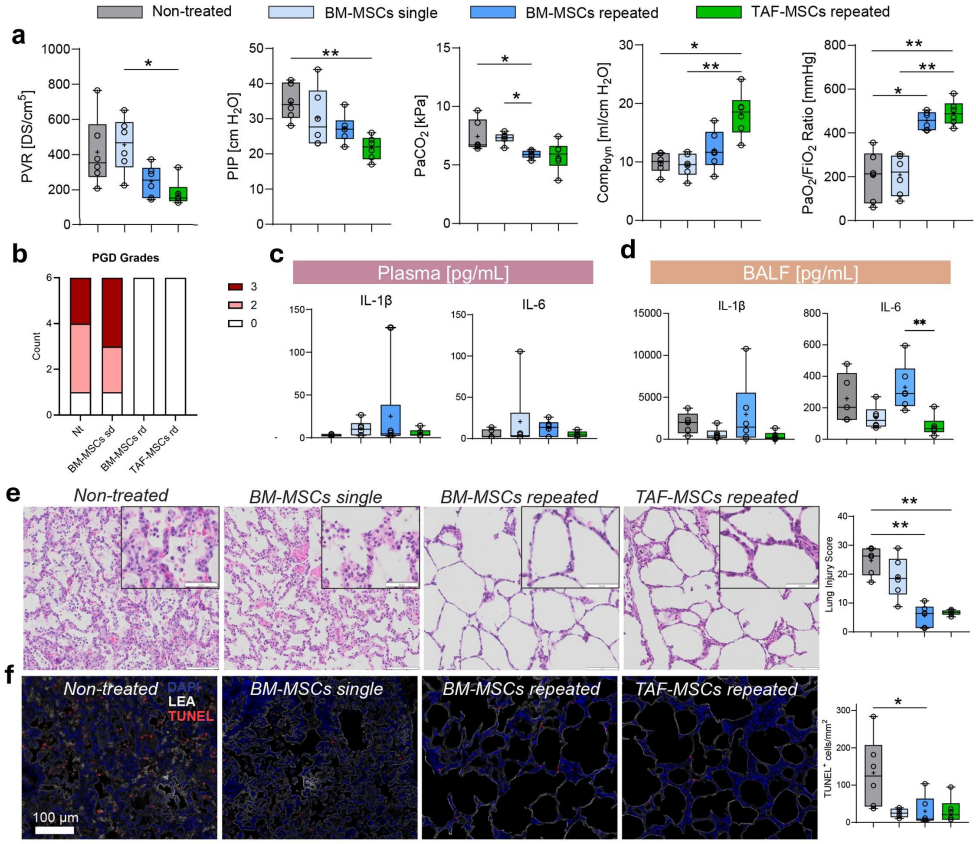

Figure 17: Repeated MSC treatment reduced primary graft dysfunction post transplantation.
Endpoints were compared across the four different groups: non-treated (grey), BM-MSCs single dose (sd, light blue), BM-MSCs repeated dose (rd, dark blue), and TAF-MSCs repeated dose (rd, green). **a)** Selected hemodynamic and ventilatory parameters, including pulmonary vascular resistance (PVR), peak inspiratory pressure (PIP), partial pressure of carbon dioxide ($PaCO_2$), dynamic compliance ($Comp_{dyn}$), ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (PaO_2/FiO_2). **b)** Comparison of PGD grades at endpoint. Cytokine IL1β and IL-6 levels in **c)** plasma and **d)** bronchoalveolar lavage fluid (BALF). **e)** Representative haematoxylin and eosin (H&E) stained lung sections, scale bars: 100 µm (overview); 50 µm (callout). Quantification of histological lung injury scoring (right). **f)** Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, red), 4′,6-diamidino-2-phenylindole (DAPI, blue), and Lycopersicon esculentum lectin (LEA, white) staining of lung tissue with quantification of $TUNEL^+$ cells/mm^2 (right). Scale bar: 100 µm. *p<0.05, **p<0.01 (one-way ANOVA or Kruskal-Wallis), Boxplots: centre line, median; box, interquartile range; whiskers, min-max. Adapted from paper III with permission.

Proteomic analysis of lung tissue three days post-transplantation revealed significantly different immune signatures

To determine whether the differences between treatment groups were mirrored at the proteomic level, mass spectrometry was performed on endpoint whole-tissue samples. A total of 8,965 proteins were identified, of which 1,227 were differentially expressed between groups. Hierarchical clustering demonstrated clear separation between non-treated and MSC-treated grafts (Fig. 18a). Compared with non-treated lungs, TAF-MSC treatment resulted in 203 downregulated and 68 upregulated proteins. In the BM-MSC single-dose group, 289 proteins were downregulated and 279 upregulated, whereas repeated BM-MSC treatment led to 148 downregulated and 62 upregulated proteins (Fig. 18b).

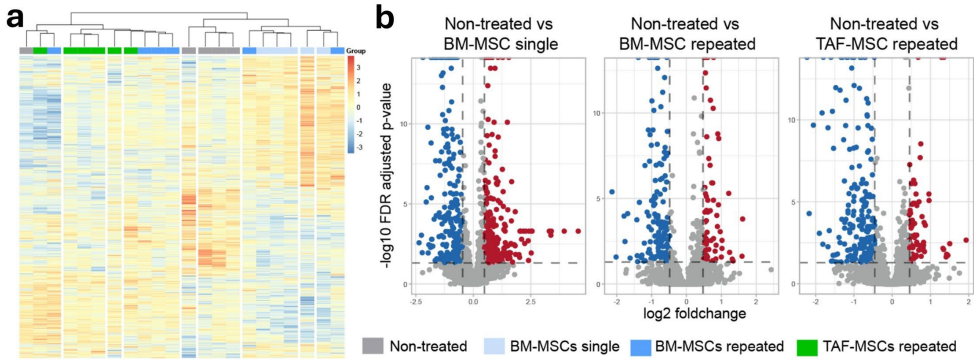

Figure 18: Mass spectrometric analysis of whole tissue at endpoint revealed differential protein expression between groups.
a) Heatmap and hierarchical clustering of protein expression across individual samples from treated and non-treated groups. **b)** Volcano plots of differential protein expression comparing non-treated to TAF-MSCs (left), BM-MSC single (middle), and to BM-MSC repeated (right) with significantly overexpressed proteins in red and underexpressed in blue. Adapted from paper III with permission.

Across all treatment groups, proteins associated with monocytes, neutrophils, leukocyte trafficking, and both the classical and alternative complement pathways were consistently downregulated, while markers of tissue regeneration were upregulated (Fig. 19a-d).

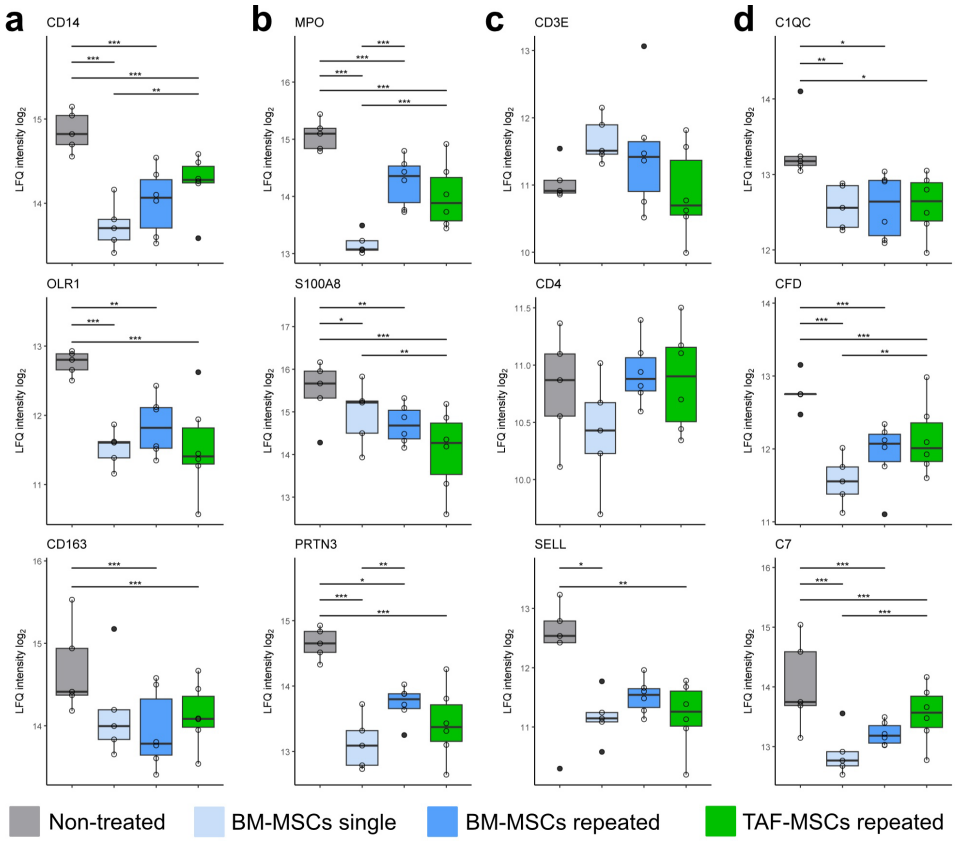

Figure 19: Mass spectrometry (MS) of whole tissue at endpoint revealed downregulation of inflammatory markers across treatment groups.
Quantification of selected protein expression levels associated with **a)** monocytes **b)** neutrophils **c)** lymphocytes and **d)** the complement cascade. All MS values are presented as log2 fold change in intensity, including Oxidized low density lipoprotein receptor (OLR1), Myeloperoxidase (MPO), S100 calcium binding protein A8 (S100A8), Proteinase 3 (PRTN3), CD3 epsilon chain (CD3E), L-Selectin (SELL), complement C1q C chain (C1QC), Complement Factor D (CFD), Complement C7 (C7). for proteomic data. *p<0.05, **p<0.01, ***p<0.001 FDR-corrected p-values (q-values). Boxplots: centre line, median; box, interquartile range; whiskers, min-max. Adapted from paper III with permission.

Upregulated expression of PCK and elastin indicated ongoing airway and vascular remodelling

To investigate whether the marked differences in immune cell infiltration correlated with specific spatial niches in the lung, extensive spatial imaging was performed. Lung tissue sections from baseline, at confirmation of ARDS, EVLP end, and post-transplantation were stained for pan-cytokeratin (PCK), which marks macrophages, basophils, naïve T cells, and plasmacytoid dendritic cells (Fig. 20a). PCK-positive cells increased after aspiration, peaked in untreated EVLP samples, and remained elevated in untreated and single-dose transplant samples. In contrast, repeated treatment with BM- or TAF-derived MSCs reduced immune cell infiltration to levels comparable to baseline. Given this marked immune accumulation, structural

changes in the airways and vasculature were assessed using PCK and elastin staining (Fig. 20b-d). Both markers were significantly upregulated after injury and remained elevated in untreated EVLP and transplant endpoint samples, indicating ongoing airway and vascular remodelling. These changes were attenuated in lungs repeatedly treated with MSCs, consistent with reduced immune infiltration and injury (Fig. 20e-h).

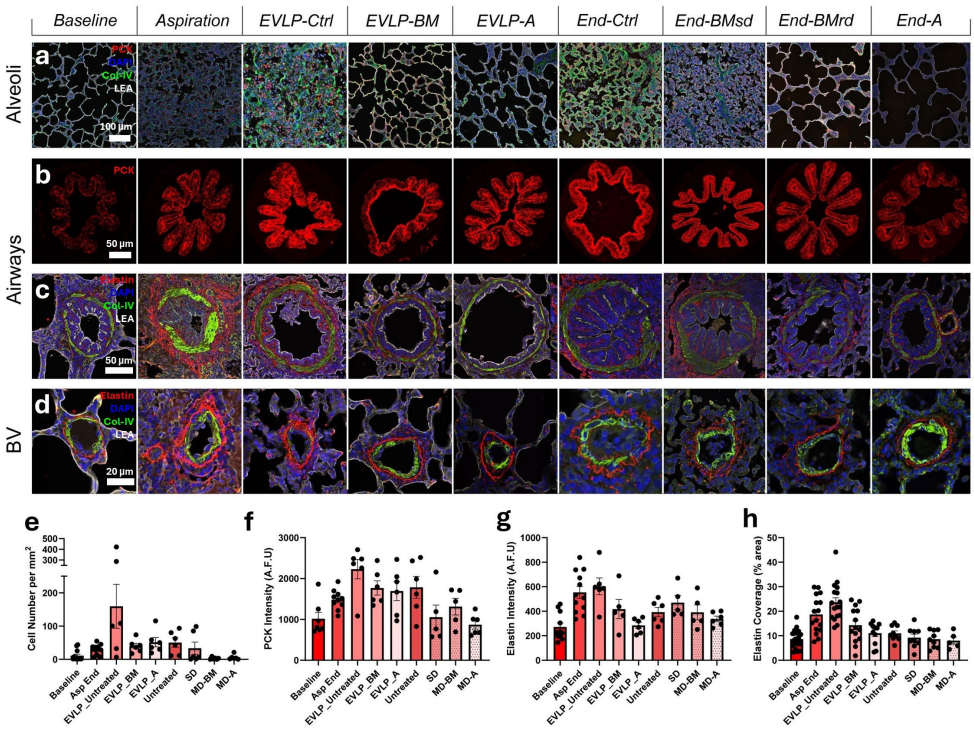

Figure 20: MSC treatment mitigated pathophysiological structural changes and immune cell infiltration. Representative immunofluorescence images showing pan-cytokeratin (PCK, red) staining in lung tissue at baseline, lung injury, EVLP and transplantation endpoint in **a)** alveoli and **b)** airways. Representative immunofluorescence images showing elastin (red) staining in lung tissue at baseline, lung injury, EVLP and transplantation endpoint in **c)** airways and **d)** blood vessels. 4′,6-diamidino-2-phenylindole (DAPI, blue), Collagen IV (Col-IV, green), and Lycopersicon esculentum Lectin (LEA, white), scale bars: 100 µm, 50 µm, and 20 µm respectively. **e)** Quantification of PCK+ cells per mm^2 tissue. **f)**-Quantification of PCK fluorescence intensity in airways. **g)** Quantification of elastin fluorescence intensity around airways. **h)** Quantification of elastin coverage around blood vessels. Ctrl= non-treated; BM= bone marrow-derived MSCs; A= term amniotic fluid-derived MSCs; SD=single dose; RD/MD=repeated dose.

The bronchial-vascular interface emerged as a specialised immunological niche

Large field-of-view imaging identified pronounced elastin expression at the bronchial-vascular interface (BVI), the junction between airways and blood vessels (Fig. 21a). To further characterize immune infiltration and the BVI, ultra-high content MACSima™ imaging was performed on endpoint samples from all groups, enabling subcellular analysis of approximately 1.07 million segmented cells (Fig. 21a-d), revealing increased CD3, CD4, and CD163 cell populations in non-treated and BM-MSC single-dose grafts (Fig. 21e-g).

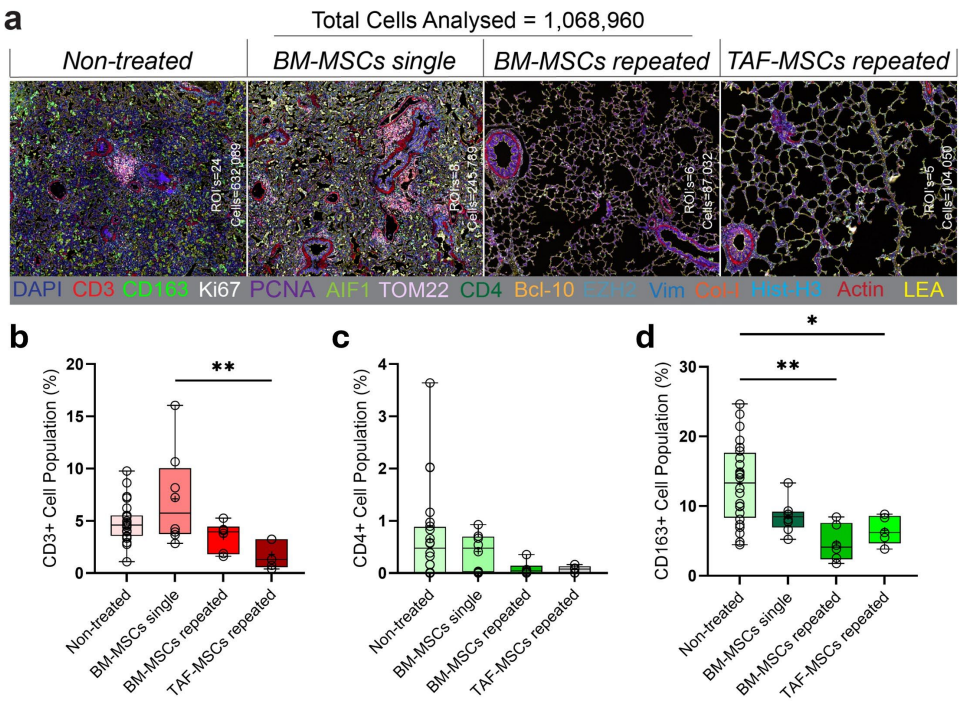

Figure 21: High-resolution spatial multiplex imaging highlighted the bronchial-vascular interface (BVI) as an immunological niche.
a) 15-plex ultra-high content imaging of transplant end samples from all treatment groups. Total cells segmented and analysed=1,068,960. ROI=region of interest. Quantification of **b)** $CD3^+$, **c)** $CD4^+$, and **d)** $CD163^+$ cell populations (% of whole population) in different ROIs. *p<0.05, **p<0.01 (one-way ANOVA or Kruskal-Wallis), Boxplots: centre line, median; box, interquartile range; whiskers, min-max. Adapted from paper III with permission.

Spatial analysis of the BVI in non-treated grafts demonstrated compartmentalization of immune cells (Fig. 22a-f), with $CD163^+$ macrophages predominantly localized to alveolar regions, while $CD3^+$ and $PCNA^+$ cells preferentially accumulated at bronchial and vascular sites (Fig. 22g-i).

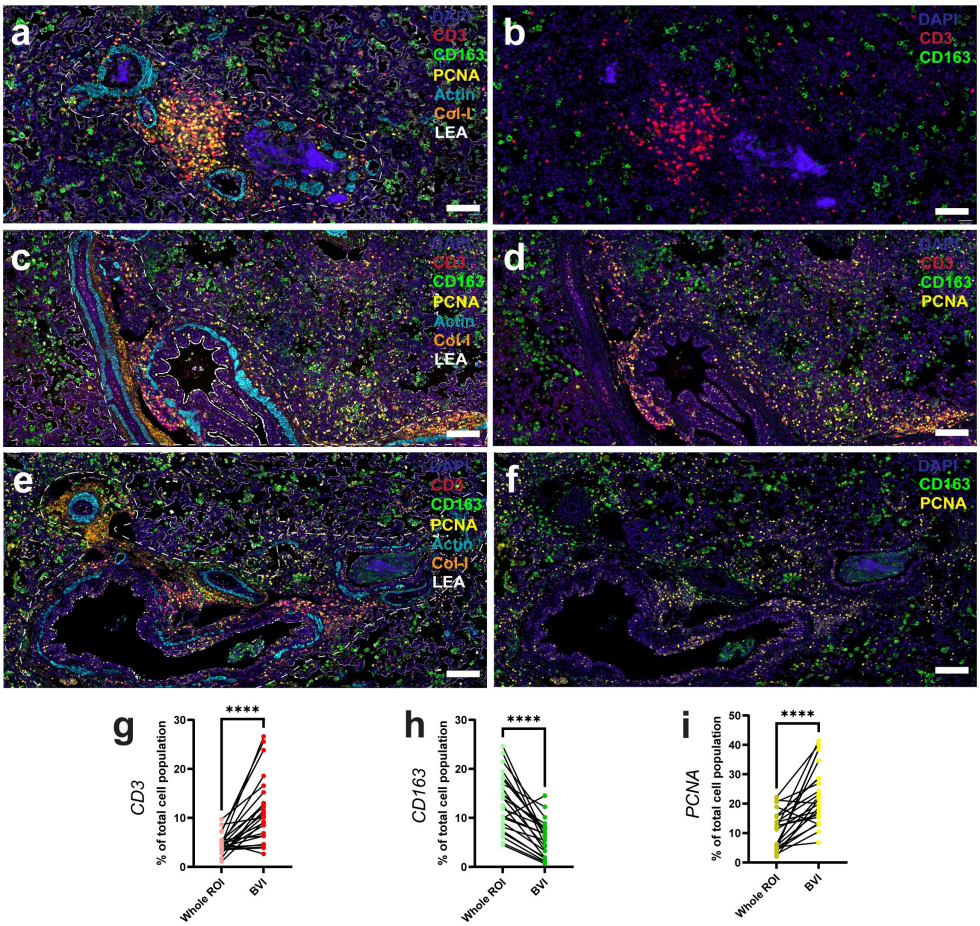

Figure 22: MACSima™ imaging of the BVI revealed the conglomeration of distinct immunological populations. a-f)-Representative images of the bronchial-vascular interface (BVI) from 3 untreated transplant-end samples showing polarised immune cell invasion in airways vs alveoli. Quantification of selected immune cell populations in the BVI compared to the whole field of view (FOV). **g)** CD3, **h)** CD163 and **i)** proliferating cell nuclear antigen PCNA. 4′,6-diamidino-2-phenylindole (DAPI), Collagen I (Col-I), Lycopersicon esculentum lectin (LEA). Scale bars: 100µm; ****p<0.0001 (paired T-test), Boxplots: centre line, median; box, interquartile range; whiskers, min-max. Adapted from paper III with permission.

Laser-capture microdissection confirmed distinct immunological profiles observed by MACSima imaging

Histology confirmed the BVI as a site of increased immune cell infiltration in non-treated grafts at endpoint (Fig. 23a). To further characterize the BVI, laser capture microdissection followed by mass spectrometry was performed on alveolar and BVI compartments, revealing a separation between regions based on proteomic profiling (Fig. 23b). Region-specific immune signatures in non-treated lungs were characterized by higher expression of neutrophil-associated proteins, complement components, and macrophage markers in alveolar areas (Fig. 23c). These results

support the BVI as a distinct inflammatory niche and indicate that repeated MSC therapy attenuates compartment-specific immune activation.

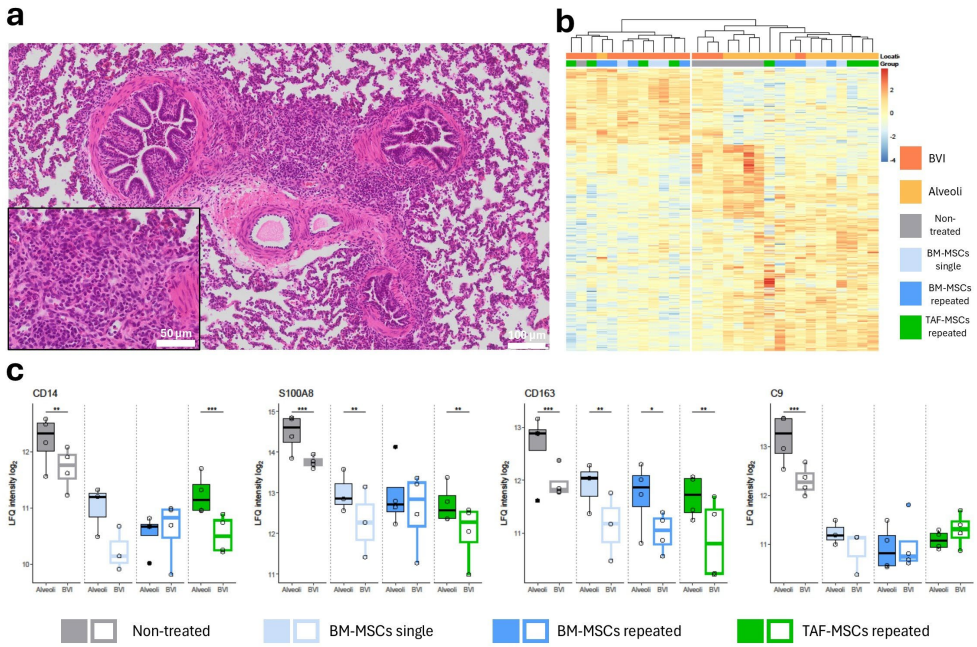

Figure 23: Laser-capture microdissection and mass spectrometry of the BVI confirmed the distinct subpopulations observed by tissue imaging.
a) Representative image of the bronchial-vascular interface (BVI) in Hematoxylin& Eosin-stained lung tissue in lung injury. Scale bar: 100µm (overview), 50µm (callout). **b)** Heatmap and hierarchical clustering of protein expression in BVI (dark orange) and alveoli (light orange) across treated and non-treated groups. **c)** Quantification of selected proteins associated with monocytes (CD14, CD163), neutrophils (S100 calcium binding protein A8 (S100A8)) and the complement system (Complement C9 (C9)). *p<0.05, **<0.01, ***p<0.001 (FDR corrected p-values (q-values) for proteomic data). Boxplots: centre line, median; box, interquartile range; whiskers, min-max.

To conclude, treatment with repeated dose of MSCs shows potential in improving damaged lungs on EVLP and reducing both the incidence of PGD as well as markers connected to a worse outcome after LTx.

Paper IV

Background and results

As a development of paper III, a study was conducted to investigate whether BM-MSCs can mitigate early ARDS onset in healthy pigs following gastric aspiration. Two different dosages of MSCs were compared, as well as BM-MSCs manufactured on a large scale manually or automated, in a Good Manufacturing Practice (GMP) setting. All MSCs were sourced from bone marrow, but one part was isolated and expanded in-house, while the other part was isolated and expanded by means of the CliniMACS Prodigy system, courtesy of Miltenyi Biotech in Germany. To test the MSC treatment, the same porcine model of gastric aspiration-induced lung injury as in papers II and III was utilised (Fig. 9). The pigs were stratified to the following groups: placebo group received no MSCs (n=10), GMP low received treatment with low dose of GMP-manufactured MSCs (n=6), GMP high received treatment with high dose of GMP-manufactured MSCs (n=6), in-house low received treatment with low dose of in-house manufactured MSCs (n=3) and in-house high received treatment with high dose of in-house manufactured MSCs (n=3).

MSC treatment improved ventilatory parameters after gastric aspiration

To start with, hemodynamic and ventilatory parameters were proportionately similar across all treatment groups before aspiration was induced (Table 4). MSC treatment was administered 30 minutes and one hour after gastric aspiration, and pigs were closely monitored for 6 hours. At the endpoint of the experiment, intravenous administration of MSCs had significantly improved pulmonary physiology compared with placebo. All MSC-treated groups, irrespective of cell manufacturing process or dose, showed significantly higher PaO_2/FiO_2 ratios, with a significant overall treatment effect of p=0.0028 (Fig. 24a). Overall, PIP was decreased and compliance was increased across treatment groups, indicating a favourable treatment effect on lung elasticity (Fig. 24b upper panel). In line with these results, MV and $PaCO_2$ were generally lower in treated lungs than in placebo-treated lungs (Fig. 24b, lower panel). No adverse effects were observed after either MSC treatment.

Table 4: Clinically relevant parameters measured at baseline across all treatment groups.
All parameters are presented as mean ± standard deviation (SD) values: oxygen saturation (Sat, %), systolic blood pressure (SBP, mmHg), diastolic blood pressure (DBP, mmHg), mean arterial pressure (MAP, mmHg), central venous pressure (CVP, mmHg); systolic pulmonary pressure (SPAP, mmHg), diastolic pulmonary pressure (DPAP, mmHg), mean pulmonary pressure (MPP, mmHg), pulmonary artery wedge pressure (PAWP, mmHg), cardiac output (CO, L/min), cardiac index (CI, L/min/m^2), systemic vascular resistance (SVR, dynes*s*cm^{-5}), pulmonary vascular resistance (PVR, dynes*s*cm^{-5}), systemic vascular resistance index (SVRI, dynes*s*cm^{-5}*m^2), pulmonary vascular resistance index (PVRI, dynes*s*cm^{-5}*m^2), pH, lactate (mmol/L), base excess (BE, mmol/L), peak inspiratory pressure (PIP, cmH_2O), peak end expiratory pressure (PEEP, cmH_2O), respiratory rate (RR, breaths/min), dynamic compliance ($Comp_{dyn}$, mL/ cmH_2O). Statistical differences were assessed using an ordinary one-way ANOVA or Kruskal-Wallis test in case of non-normal data distribution with a significance level of $p<0.05$ as the threshold.

	Placebo	GMP low	GMP high	In-house low	In-house high	p-value
Sat (%)	99,5±1	99,3±0,8	100±0	99±1	99±1	0.1696
SBP (mmHg)	112,3±16,5	110±14,7	109,3±10,1	119,3±1,2	111±10,4	0.8669
DBP (mmHg)	81,9±11,6	82±20	75,2±7,5	88,3±2,1	73,7±14,2	0.5522
MAP (mmHg)	96,8±13,2	92,8±12,6	92±7,9	103,7±1,5	90,7±12,5	0.5607
CVP (mmHg)	8,7±2,5	9,5±2,9	6,5±3,3	6,7±1,2	9,3±2,1	0.2567
SPAP (mmHg)	26,7±5,5	27,3±2,8	26,7±4,8	28,7±3,1	18,3±15,9	0.7332
DPAP (mmHg)	17,1±4,1	17,5±3,8	14,7±2,4	16,3±3,2	7,3±8,7	0.2793
MPP (mmHg)	21,1±5,2	21,5±2,3	19,2±2,1	23±5,3	12±10,8	0.5748
PAWP (mmHg)	10,6±1,9	12±1,9	7,8±2,1	9,7±4,2	7,5±0,7	0.0240
CO (L/min)	3,3±0,7	3,5±0,5	3±0,7	3±0,2	3,1±0,5	0.6750
CI (L/min/m^2)	3±0,7	3,5±0,7	3,2±0,6	3,6±0,3	3,7±0,5	0.4237
SVR (DS/cm^5)	1890,2±571,2	1928,7±321,5	2387±756,6	2714,7±406,4	2093,5±890,2	0.1965
PVR (DS/cm^5)	253,4±131,6	227,5±62,5	331,8±123,8	348±63,1	314,5±82,7	0.1869
SVRI (DS/cm^5/m^2)	2215±558,6	1980,2±475,6	2115,2±603,5	2269±403,1	1779,5±755,9	0.7942
PVRI (DS/cm^5/m^2)	285,3±121,2	242±107	294,8±102,4	289±44,5	267±70,7	0.9174
pH	7,5±0,2	7,5±0	7,5±0	7,5±0	7,5±0	0.0856
sO2	98,6±1,2	99,8±0,4	100±0	100±0	100,3±0,6	0.0154
Lactate (mmol/L)	1,3±1,2	1,7±0,3	1,5±0,3	1,3±0,2	1,4±0,4	0.6557
BE (mmol/L)	5,3±1,9	5,3±2,3	3,8±1,9	5,5±1,5	5,7±4,4	0.6506
PIP (cmH_2O)	20,1±3,9	18±2,9	17±1,5	18,7±1,2	17±1,7	0.2717
PEEP (cmH_2O)	6,5±1,6	5±0	5±0	5,7±1,2	5±0	0.0561
RR (breaths/min)	25,2±2	21,7±4,6	21,8±4	24±6,9	21±2,6	0.1012
$Comp_{dyn}$ (mL/ cmH_2O)	19,6±3,3	22,4±6,5	19,2±3,4	18,1±0,6	17,2±1	0.3954

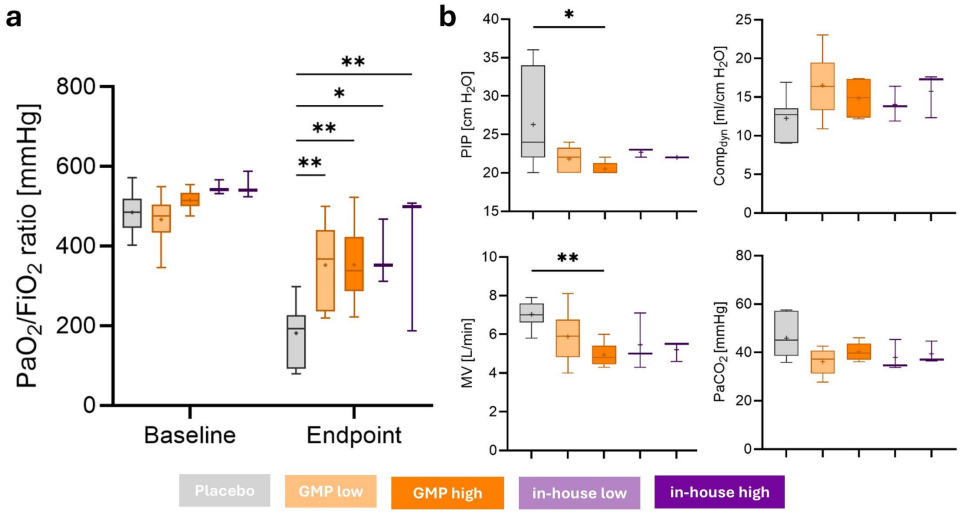

Figure 24: Administration of MSCs resulted in a significant improvement in ventilatory parameters. Placebo=no MSCs (grey), GMP low=low dose GMP-manufactured MSCs (light orange), GMP high=high dose GMP-manufactured MSCs (dark orange), in-house low=low dose in-house manufactured MSCs (light purple) and in-house high=high dose in-house manufactured MSCs (dark purple). **a)** PaO_2/FiO_2 ratio calculated from values obtained at baseline compared to experimental endpoint. **b)** Ventilatory parameters measured at the experimental endpoint: peak inspiratory pressure (PIP), dynamic compliance ($Comp_{dyn}$), minute volume (MV), partial pressure of carbon dioxide ($PaCO_2$). *p<0.05, **p<0.01 (ordinary one-way ANOVA or Kruskal-Wallis test). Box and whiskers plots (line at median; interquartile range; whiskers for min-max; plus sign for mean).

Histological and apoptotic staining revealed lessened lung injury and cell death in treated groups compared to placebo

After endpoint measurements had been recorded, all lungs were explanted and the tissue processed for downstream analysis. First off, standard histological assessment by H&E staining demonstrated lower lung injury scores in MSC-treated groups compared to placebo (Fig. 25a). Furthermore, TUNEL staining displayed lower amounts of apoptotic cells in treated grafts (Fig. 25b).

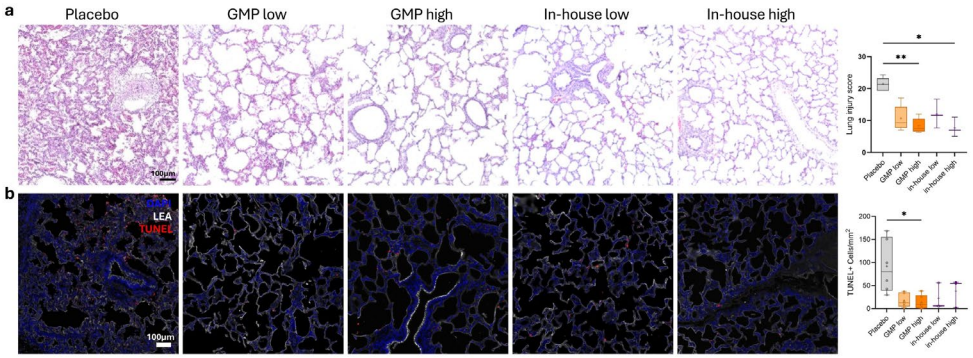

Figure 25: Treatment with either MSC regimen led to improved tissue morphology and reduced cell apoptosis. Placebo=no MSCs (grey), GMP low=low dose GMP-manufactured MSCs (light orange), GMP high=high dose GMP-manufactured MSCs (dark orange), in-house low=low dose in-house manufactured MSCs (light purple) and in-house high=high dose in-house manufactured MSCs (dark purple). Representative images of lung tissue stained with **a)** haematoxylin and eosin (H&E) and **b)** Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, red), 4′,6-diamidino-2-phenylindole (DAPI, blue), and Lycopersicon esculentum lectin (LEA, white). Respective quantification of lung injury score and of TUNEL+ cells/mm^2 in the right column. Scale bars: 100µm. *p<0.05, **p<0.01 (ordinary one-way ANOVA or Kruskal-Wallis test). Box and whiskers plots (line at median; interquartile range; whiskers for min-max; plus sign for mean).

MACSima™ imaging showed a distinct downregulation of JAK-1 in low-dose treated groups

To better understand the complex immunomodulatory effects of MSCs, high-resolution spatial imaging was performed using the MACSima™ platform (Fig. 26a). T-cell markers CD3 and CD4 were slightly up-regulated, while anti-inflammatory marker CD163 was slightly down-regulated across all treatment groups (Fig. 26b). The most pronounced differences between groups were observed in the enzyme JAK1, which was significantly downregulated in both GMP and in-house low-dose treatment groups compared to placebo (Fig. 26b).

To summarize, MSC administration showed no adverse events in a porcine model of acute respiratory distress syndrome and effectively mitigated the development of ARDS compared to placebo treatment.

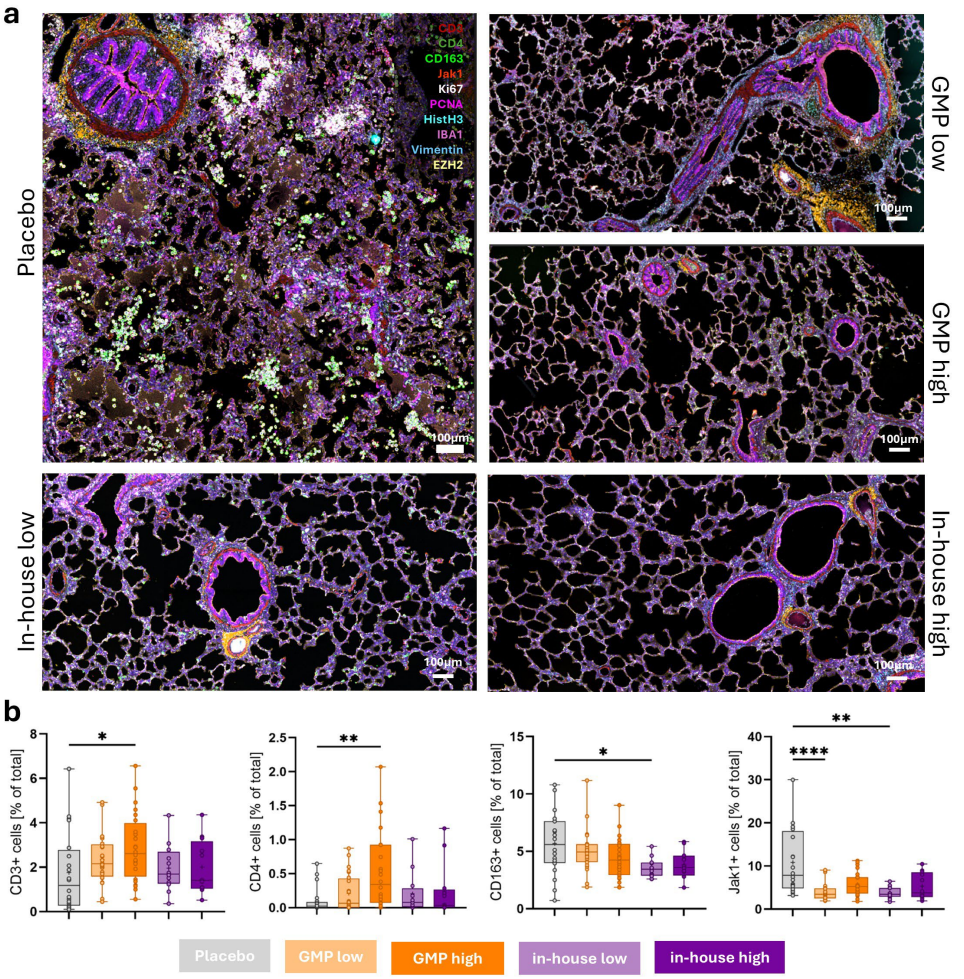

Figure 26: Leveraging the MACSima platform to better understand moelcular effects of MSC treatment. Placebo=no MSCs (grey), GMP low=low dose GMP-manufactured MSCs (light orange), GMP high=high dose GMP-manufactured MSCs (dark orange), in-house low=low dose in-house manufactured MSCs (light purple) and in-house high=high dose in-house manufactured MSCs (dark purple). **a)** Representative images of ultra-high content MACSima™ imaging of tissue obtained from all groups at experimental endpoint. Antigen Kiel 67 (Ki-67), Januskinase 1 (Jak1), Proliferating Cell Nuclear Antigen (PCNA), Histone H3 (HH3), Ionized calcium-binding adapter molecule 1 (IBA1), Enhancer of Zeste Homolog 2 (EZH2) Scale bars: 100µm. **b)** Quantification of selected cell populations (% of whole population) in different regions of interest (ROI) in lung tissue at the experimental endpoint. *p<0.05, **p<0.01, ****p<0.0001 (ordinary one-way ANOVA or Kruskal-Wallis test in case of non-normal data distribution). Box and whiskers plots (line at median; interquartile range; whiskers for min-max; plus sign for mean) with individual points.

Discussion

Preventing primary graft dysfunction remains a significant challenge in lung transplantation, especially since ischemia-reperfusion injury, one of the main culprits causing PGD, is unavoidable during the lung transplantation procedure. Consequently, mitigating and dampening IRI are key priorities in lung transplantation research. Targeting neutrophil extracellular traps and circulating nucleosomes, as reported in papers I and II, may represent an important therapeutic strategy for several reasons.

In **paper I**, cytokine adsorption coupled to an ECLS device peri-operatively reduced circulating nucleosomes after lung transplantation in a pilot cohort of human patients. Interestingly, cytokine-mediated signalling recruited neutrophils from the circulation and, in turn, their release of pro-inflammatory cytokines amplified tissue injury even further[163]. Prior studies have demonstrated that reperfusion-induced lung damage can be attenuated by decreasing neutrophil infiltration, supporting a protective role of neutrophil reduction[164]. Ischemic stress is thought to provoke NET-driven graft injury, where excessive NET formation contributes to microvascular damage. Moreover, elevated NET levels have been associated with worse outcomes in ARDS[28]. In paper I, the previously reported pathogenic role of NETs in early graft injury was further supported by markedly reduced levels of circulating H3.1 nucleosomes and citrullinated H3R8 nucleosomes, which serve as indirect markers of NET formation and NETosis, across all treated grafts. The reduction in NET-related markers was further associated with a decline in the incidence of both PGD and acute rejection. However, donor-derived immune responses during the perioperative period may have influenced systemic NET levels and warrant exploration in future studies.

In **paper II**, the positive effect of decreased nucleosomes observed in paper I was leveraged and further explored by focusing on the removal of NETs, specifically, from the bloodstream to improve porcine donor lungs damaged by aspiration. The results further build on the notion that removal of extracellular, circulating nucleosomes correlate with improved graft function in a lung transplantation setting. Upon putting a filtering device specific to histone protein H3.1, an avid binder of DNA, in line with an EVLP circuit, lung oxygenation improved significantly in treated grafts, as proven by a substantial increase in PaO_2/FiO_2 ratio. The explanation for the observed increase most likely can be found in the potential of NETs to cause inflammation. The previously described effects of the release of cytotoxic contents within NETs, such as microvascular and molecular damage as well as the induction of oedema, all contribute to the deterioration of lung oxygenation, also observed in this study[28,91,165]. Not only has the lung been described as a neutrophil depot, but NET release also serves as an inherent feed-forward mechanism for the release of more NETs, triggering a pro-inflammatory signalling cascade[27,166]. The triggering of this pro-inflammatory cascade is effectively

mitigated by using the NET removal column, as evidenced by a significant reduction in the pro-inflammatory cytokines IL-1β, IL-6, and IL-12, as well as the activated macrophage marker AIF-1, in treated porcine donor lungs (paper II). Elevations in these three specific biomarkers have previously been reported as predictors of worse outcomes after lung transplantation, with macrophage IL-1β-release having been directly connected to their interaction with NETs[167-171]. Histones, which comprise the majority of the protein content in NETs, are potent mediators of inflammation and have been implicated as drivers of NET-induced tissue damage in acute lung injury[165,172-174]. By targeting chromatin with histone-conjugated beads, the NET removal device likely improves lung function by eliminating circulating histones. As NETs can be generated via suicidal, vital, or inducible NETosis, producing distinct subtypes[91], our broad-spectrum strategy targets all forms without requiring pathway-specific differentiation. Moreover, in contrast to DNase, which degrades the NET-DNA backbone but does not prevent the injury effectual to NETs[174], our approach removes targeted NETs more comprehensively, including histones and neutrophil-derived proteins. This broad removal of NET-related products, which inherently cause cell damage, may reduce the risk of overall cellular and medicinal toxicity.

Severe PGD after lung transplantation has been correlated with elevated levels of NETs[44,99,100]. Interestingly, all grafts in paper II displayed varying degrees of interstitial oedema upon visual examination and clinically relevant parameters, which can be a supporting factor for the development of PGD. However, wet/dry ratios displayed significantly less oedema in treated as compared to non-treated lungs which is reportedly concomitant with a lower risk of PGD[42]. Since PGD phenotypes may reportedly vary between IRI lungs depending on IRI duration and whether the lungs were subjected to EVLP or not[74], the oedema in our study might likely be part of a distinct phenotype different to IRI and most likely contributed to by ARDS in the grafts.

As a result of the positive findings from the study conducted in paper II, a follow-up study was conducted in a porcine model of lung transplantation to test whether lungs treated with a NET removal device during EVLP could sustain transplantation (manuscript in preparation). The study showed that porcine lungs damaged by aspiration and treated with a NETs removal device could successfully be transplanted into healthy recipients and followed up for 3 days post transplantation with sustained good lung function. These observations were interesting and valuable, since the implantation of the treated lungs into an individual is an important factor in the translation of a regenerative therapy towards a clinical product that is often overlooked.

Building on the discovery that regeneration of donor lungs damaged by aspiration is possible, in **paper III,** we sought to determine whether regeneration can be achieved using MSC therapy as well. Since a vital point in lung transplantation research (and regenerative research in general) is to investigate whether the treated,

modified organ can sustain its function after implantation into a recipient, we extended the EVLP model from paper II into a porcine lung transplantation model previously established in our research group. Apart from the general efficacy of MSC therapy in this context, we assessed the function of MSCs from two different sources in regenerating damaged donor lungs as well as mitigating PGD post transplantation. We further aimed to elucidate some of the immunomodulatory mechanisms by which MSCs exert their effects.

Donor lung shortage remains the main limitation in lung transplantation, with aspiration injury comprising a substantial proportion of discarded grafts[6,17]. Aspiration-induced ARDS was confirmed in all animals and characterized by impaired gas exchange, elevated inflammatory cytokines, and severe histopathological injury. Although MSCs improved oxygenation during EVLP, as previously demonstrated in ischemia-reperfusion and LPS injury models[129,175,176], aspiration-induced injury is progressive, and single-dose therapy did not prevent post-transplant deterioration in our study. Repeated systemic dosing during the early post-transplant period resulted in persistent improvements in gas exchange, reduced inflammatory signalling, restored tissue morphology, and complete prevention of severe PGD. In contrast, most non-treated and single-dose treated animals developed severe PGD. Both BM-MSCs and TAF-MSCs demonstrated comparable efficacy, with a non-significant trend favouring TAF-MSCs. Although MSCs from different sources have been reported to exhibit varying immunomodulatory properties[114-116], our results indicate that the dosing strategy is the decisive factor for durable graft protection. These arguments are in line with previously published studies and underscore the need for continued peri- and post-transplant MSC therapy[175,177,178].

Mechanistically, both targeted high-resolution imaging and proteomic analyses demonstrated broad suppression of immune regulatory pathways in MSC-treated grafts. Proteins associated with monocytes, neutrophils, lymphocytes, and complement activation were markedly reduced. Both pro-inflammatory macrophage markers and overall macrophage infiltration decreased, consistent with global attenuation of immune recruitment. Neutrophil-related proteins and mediators of neutrophil extracellular trap formation were strongly reduced following repeated MSC treatment, indicating suppression of key drivers of acute lung injury[23,79,100,129]. Complement components from both the classical and alternative pathways, as well as terminal complex proteins, were also downregulated, in line with the known association between complement activation and PGD[40,179,180].

Spatial analyses identified the bronchial-vascular interface (BVI) as a region that merits investigation due to its distinct immunological profile. While the observation of this niche has been previously described in the lung, studies investigating its properties are lacking[181,182]. Meanwhile, the perivascular spaces, the equivalent immunological hotspot in the brain, have been the subject of more extensive studies

and have been shown to be sites of high immunological turnover and increased pathological interest[183]. We confirmed these observations in the non-treated grafts in paper III, as T-cell accumulation and proliferative activity were concentrated in the BVI, while macrophages predominated in alveolar regions, indicating compartment-specific immune pathology. Repeated MSC therapy markedly reduced this spatial immune activation and homogenized the inflammatory landscape. Proteomic profiling confirmed that immune-related proteins were preferentially enriched in alveolar regions of non-treated and single-dose treated grafts, whereas these compartmental differences were largely diminished after repeated MSC treatment. These findings prove the BVI as a site of immunological importance and indicate that normalization of regional differences in immune responses contributes to the prevention of PGD and might be part of the immunomodulatory repertoire of MSCs.

MSC therapy was well tolerated, and both cell sources demonstrated translational feasibility. While MSCs have been explored in ARDS and transplantation, repeated peri- and post-transplant dosing in a large-animal transplant model has not been evaluated previously. Although further clinical studies are required to optimise dosing and assess long-term outcomes, this work provides strong translational evidence that repeated MSC administration can rehabilitate severely injured donor lungs.

When considering translating MSC therapy into a viable clinical therapy, several factors need to be addressed[184]. Among others, the cell product needs to be reproducible to avoid batch and manufacturing variability. While being reproducible, the cell therapy also needs to be scalable to comply with demand as a future standard clinical therapy. To take the first step in that direction, in **paper IV**, we addressed these factors by comparing our in-house manufactured MSCs, used in paper III, to MSCs manufactured from the same source, bone marrow, but in an automated, closed GMP system. We further compared two different doses of either MSC type to assess dosing efficacy. The experimental model utilized was similar to the large animal studies performed in paper II and III, namely a porcine model of gastric aspiration-induced acute lung injury. In paper IV, however, MSCs were administered early after injury induction (30 and 60 minutes post-aspiration) to investigate whether ARDS could be mitigated by early administration of MSCs.

This early intravenous administration of MSCs following aspiration injury resulted in measurable improvements in lung function and a mitigation of progression toward severe ARDS and septic physiology. Across all cell sources and doses, animals receiving MSCs demonstrated higher PaO_2/FiO_2 ratios relative to placebo-treated controls, indicating more efficient pulmonary alveolar exchange. Because the PaO_2/FiO_2 ratio is central to defining ARDS severity, these results indicate that MSC therapy substantially reduced early impairment of gas exchange[29]. Improvements in pulmonary physiology were not limited to oxygenation. MSC-treated animals required lower minute ventilation to maintain carbon dioxide

clearance and exhibited reduced peak inspiratory pressures, consistent with improved pulmonary dynamics and reduced resistance, contrary to the known pathophysiology of ARDS[21]. Although changes in compliance did not reach statistical significance, the observed improvement is in line with reduced lung stiffness following injury. Histological analyses supported these physiological findings, showing lower injury scores and reduced apoptotic cell counts in treated groups, particularly at higher doses. No adverse effects were observed following MSC administration, and systemic parameters remained stable. In contrast to placebo-treated animals, which developed hemodynamic patterns consistent with developing sepsis despite standardized fluid management, MSC-treated animals maintained peripheral perfusion, stable systemic vascular resistance, and well-preserved urine output. These outcomes indicate that early MSC intervention may contribute to circulatory stabilization in addition to pulmonary protection.

This study also addressed key translational considerations. MSCs produced under GMP-compatible automated conditions performed comparably to cells expanded using conventional in-house protocols, suggesting that standardized manufacturing does not compromise efficacy. The data further point out the importance of early and repeated dosing. Acute lung injury involves sustained inflammatory activation, and a single intervention may be insufficient to counteract this process. The repeated administration strategy applied here likely contributed to the observed therapeutic effects and supports the concept that timing and dosing frequency are key determinants of MSC efficacy[185].

Spatially characterized protein analysis provided insight into local immune modulation within the injured lung. Markers of proliferation and tissue integrity remained comparable across groups, indicating preserved tissue morphology. In contrast, immune-associated markers differed between groups. Increased CD3 and CD4 expression in treated lungs, together with reduced CD163 and Jak1 levels, suggests selective modulation of the immunological niche in the lung rather than global immunosuppression[186]. Given the role of the JAK/STAT pathway in macrophage polarisation and inflammatory signalling, reduced Jak1 expression may reflect direct modulation of macrophage-driven inflammatory responses[187-189]. Notably, one research group reported increased levels of JAK-1 in human CLAD patients, validating interest in this marker in a lung transplantation setting as well[190].

Taken together, the observations from paper IV showed that MSC therapy mitigated ARDS progression and influenced local immune composition and signalling pathways during the early phase of aspiration-induced lung injury.

Limitations

In **paper I**, despite promising results, the sample size was relatively small and does not allow for drawing definitive conclusions about the larger population.

Additionally, a longer follow-up period would merit investigation, as CLAD typically develops later in the disease course.

Sample size considerations also apply to **papers II-IV**. Although these studies were adequately powered within the context of large animal models, further investigations are warranted to ensure that the treatment effect can be replicated in larger cohort studies. Moreover, while large animal models represent the closest approximation to human physiology, species differences must still be taken into account, and studies using discarded human donor lungs should be considered.

In **paper III**, BM-MSCs were not produced in a GMP-certified facility, but were manufactured under GMP-like conditions, which would be important for clinical translation in the future. Notably, the findings from Paper IV suggest that there are no significant differences between these manufacturing approaches.

Concluding remarks and future directions

To conclude, the studies included in my PhD thesis identified three immunomodulation approaches with therapeutic potential in the context of lung transplantation and donor lung regeneration. In **paper I**, perioperative cytokine adsorption during lung transplantation was associated with a reduced incidence of PGD compared to standard treatment and is currently investigated in a national clinical trial. **Paper II** demonstrated that NET removal during EVLP may enable the reintroduction of donor lungs previously rejected due to aspiration-related injury into the donor pool. In **paper III**, repeated administration of MSCs during EVLP and post-transplantation showed potential to improve the condition of damaged donor lungs and reduce both PGD incidence and markers associated with poorer post-transplant outcomes. Finally, in **paper IV**, MSC administration was well tolerated in a porcine model of acute ARDS and effectively attenuated disease development compared to placebo. Taken together, these findings support a shift in lung transplantation from passive donor organ preservation towards a more active approach to restore and regenerate damaged lungs. In addition to mitigating and repairing injury, the strategies explored in this thesis aimed to improve organ quality, which may influence not only early outcomes such as PGD but also longer-term graft survival and recipient quality of life.

Importantly, these observations extend beyond the initial experimental work, as three out of the four studies have led to follow-up investigations, and one has progressed to an ongoing nationwide clinical trial. This highlights the translational potential of the work reported in this thesis and emphasises the value of large-animal models in bridging the gap between experimental proof of concept and clinical application. In this context, paper IV represents an important step towards clinical implementation by demonstrating the feasibility of GMP-compliant MSC

production using a fully automated cell manufacturing system, enabling the generation of a scalable and standardised cell product for future clinical studies. Future studies should aim to optimise the timing, dosing, and a potential combination of interventions, particularly in the context of EVLP and early post-transplantation management. In addition, a deeper understanding of the underlying mechanisms, for example through more detailed mechanistic and omics-based analyses, will be essential for developing more targeted approaches.

Finally, although this thesis work focuses on pathologies within lung transplantation, the underlying mechanisms are likely relevant to other forms of acute organ injury and repair. Combining cell-based and other immunomodulatory therapies with perfusion platforms and state-of-the-art analytical approaches may therefore contribute to the development of improved treatment strategies both within and beyond lung transplantation.

Main experimental methods

Patients

Ethical considerations

In **paper I**, 4 patients undergoing lung transplantation at Skåne University Hospital in Lund were randomised to receive either peri-operative treatment with a cytokine adsorption device coupled to extracorporeal life support (ECLS) or ECLS only. Ethical approval was obtained from the Swedish National Ethics Committee (Drn 2020-07115 and Drn 2020-01864). At the time of study inclusion, all patients were enrolled in an ongoing randomized pilot trial registered under NCT05242289.

All research pursued complied with the principles outlined in the Declaration of Helsinki of the World Medical Association[191]. Patients were informed both verbally and in writing about the study's purpose, procedures, and their right to withdraw at any time without affecting their clinical care and written informed consent was obtained from all participants prior to inclusion in the study. Study information and consent procedures were primarily provided by physicians involved in the clinical care of the patients, including the principal investigator, a transplant surgeon. All patient data was handled confidentially, processed and stored according to clinical guidelines and data protection regulations. All reported or published data were anonymized to protect patient privacy.

Patients undergoing lung transplantation constitute a potentially vulnerable population due to the severity of their underlying disease and reliance on specialized clinical care. To address this, measures were implemented to ensure that participation was entirely voluntary and that refusal to participate would not influence clinical treatment. The involvement of experienced clinicians in the consent process ensured that participants received accurate information and had opportunities to discuss the study with physicians possessing extensive clinical and scientific expertise. This approach was designed to support informed decision-making while upholding patient autonomy and welfare.

Patient characteristics and study criteria

Adult patients with end-stage lung disease listed for bilateral lung transplantation were eligible for inclusion. Individuals with a history of previous lung transplantation or prior solid organ or bone marrow transplantation were excluded. Donor lungs were obtained from donors after brain death who fulfilled established criteria for organ donation, and each graft was evaluated preoperatively and accepted for transplantation by the transplant team. Prior to transplantation, recipients underwent immunological risk assessment and showed no evidence of HLA sensitization, DQ mismatch, or other relevant immunological risk factors. Potential contributors to primary graft dysfunction (PGD), including smoking history, body mass index, and pulmonary arterial hypertension, were also assessed. In addition, five healthy volunteers served as controls. These individuals had no known medical conditions, were not taking any medications, and provided written informed consent. Two of the healthy controls were female, and their ages ranged from 25 to 31 years.

Post-transplantation follow-up

All recipients received a standard triple immunosuppressive regimen consisting of cyclosporine, prednisone, and mycophenolate mofetil (MMF), in addition to induction therapy with anti-thymocyte globulin (ATG). ATG was administered as a single intravenous infusion eight hours after transplantation at a dose of 1.5 mg/kg body weight (100 mg), together with hydrocortisone, clemastine, and paracetamol in accordance with institutional clinical protocols. At 72 hours post-transplantation, therapeutic drug monitoring confirmed stable cyclosporine concentrations between 200 and 300 ng/mL and MMF exposure levels of 40-50 mg/L/hour.

To monitor the development of PGD, chest radiographs were obtained on postoperative days 1, 2, and 3 and evaluated by a radiologist blinded to treatment allocation for the presence of pulmonary infiltrates. These findings were combined with arterial blood gas measurements to calculate the PaO_2/FiO_2 ratio, and the severity of PGD was graded according to the criteria established by the ISHLT[43]. For the evaluation of acute rejection, transbronchial biopsies were performed during routine follow-up at one and three months after transplantation using flexible bronchoscopy. Histological specimens were analysed and graded by a blinded pathologist in accordance with ISHLT classification guidelines.

Large animal model

Description of preclinical studies using local, farm-raised swine (*Sus scrofa domesticus*) in **papers II-IV**.

Ethical considerations

All porcine studies were approved by the local Ethics Committee for Animal Research at Lund University (Drn 5.2.18-4903/16 and Drn 5.2.18-8927/16). Animal handling and welfare monitoring was carried out according to the USA Principles of Laboratory Animal Care by the National Research Council in the Guide for the Care and Use of Laboratory Animals, National Academies Press (1996). An on-site veterinarian was present to further monitor experiments and ensure that all animal experiments were conducted in accordance with the approved ethical permit. The principal investigator listed in the permit was present throughout all experimental procedures, and all personnel involved were appropriately trained and licensed to work with porcine models.

During the experiments, animals were maintained under general anaesthesia throughout the procedures. Anaesthesia and physiological parameters were continuously monitored and managed by specialist anaesthesiologists with expertise in thoracic intensive care. The experimental design adhered to the principles of the 3Rs (*Replacement*, *Reduction*, and *Refinement*), as promoted by the National Centre for the Replacement, Refinement and Reduction of Animals in Research[192]. The animal disease model was specifically designed to maximize the scientific output of each experiment, thereby contributing to the principle of *Reduction*. Within the same experimental framework, the model enables investigation of treatment strategies for ARDS, functional rehabilitation of lungs declined for transplantation, and mechanisms related to rejection following lung transplantation. Efforts toward *Replacement* are pursued in the research group through complementary experimental approaches, such as the use of three-dimensional lung tissue slice models called precision-cut lung slices (PCLS) where applicable. However, in study II-IV, the aims of the research were to investigate the *in vivo* (clinically relevant) effects of the treatments, and the use of *in vitro* models would have therefore defeated the purpose. The principle of *Refinement* was addressed by ensuring appropriate housing and environmental enrichment for the animals and by providing continuous monitoring and specialized clinical care throughout the procedures to minimize pain and distress.

Animal preparation

In **paper II-IV**, the pigs included in the study were weighed and blood typed and for **paper III**, subsequently matched into donor-recipient pairs based on

compatibility. All pigs were stratified to their respective treatment groups and pre-medicated with ketamine and xylazine. After sedation, a peripheral intravenous catheter was placed in the ear vein, and pigs were intubated using a 7.5mm endotracheal tube. Following intubation, animals were mechanically ventilated with volume-controlled ventilation (VCV) and settings adjusted to maintain arterial carbon dioxide levels ($PaCO_2$) between 33 and 41 mmHg and tidal volumes maintained at 6-8 mL/kg. Hemodynamic monitoring was established by placing an arterial catheter in the right common carotid artery and a pulmonary artery catheter (Swan-Ganz CCOmbo V with Introflex by Edwards Lifesciences Services GmbH, Germany) via the right internal jugular vein. Furthermore, a urinary catheter was surgically inserted into the bladder. General anaesthesia was maintained throughout all studies using continuous infusions of midazolam, ketamine, fentanyl or buprenorphine. Arterial blood gas measurements were obtained throughout the experiments by drawing blood from the arterial catheter and analysing them with an ABL90 FLEX PLUS blood gas analyser (Radiometer Medical ApS, Denmark).

Induction of lung injury

In **paper II-IV**, lung injury was induced in fully anesthetized pigs by endotracheally instilling gastric contents into both lungs using a bronchoscope. All gastric contents were organically sourced from other pigs and frozen for later use after collection. Prior to instillation, gastric contents were thawed, homogenized, pooled, centrifuged, filtered, and finally set to a controlled pH of 2 using hydrochloric acid (HCl). Gastric contents at body temperature were then equally distributed into all lung lobes at a dose of 4ml/kg, divided into 2 mL/lung/kg. Administration was performed in a two-step process: the first dose represented 90% of the total volume, and the second, administered 1 hour later to prevent full hemodynamic deterioration of the pigs, comprised the remaining 10%. All pigs remained under anaesthesia over the course of injury induction, with hemodynamic and ventilatory measurements as well as arterial blood gases and blood samples being taken every hour throughout. In **paper III**, additional chest x-rays were obtained at baseline and hourly during the establishment of acute lung injury. After 6 hours, acute lung injury was confirmed in the form of ARDS by applying the Berlin criteria of ARDS [29]. PaO_2/FiO_2 ratios of $\leq$300 mmHg, bilateral infiltrates on chest x-rays (if applicable) and retrospective confirmation of pulmonary oedema, immune cell infiltration and haemorrhage were used to classify ARDS.

Pulmonary harvest

Once ARDS had been confirmed in all pigs in accordance with the Berlin criteria, a median sternotomy was carried out to access the thoracic cavity in pigs from **studies II and III**. The pulmonary artery was accessed through the right ventricle and

cannulated with a 28F cannula that was secured in place by using a purse-string suture. To isolate the pulmonary circulation, vascular clamps were applied to the superior vena cava, inferior vena cava, and ascending aorta, while the left atrium was opened and the inferior vena cava partially released to permit drainage. The pulmonary vasculature was then flushed antegrade with 4L of cold Perfadex® PLUS solution (XVIVO Perfusion, Sweden), maintaining a perfusion pressure below 20 mmHg. After completion of the flush, the lungs were removed *en* bloc following standard surgical harvesting techniques. The explanted lungs were subsequently submerged in cold Perfadex® solution and maintained under static cold storage at 4-8°C for approximately two hours. For study IV, the lungs were removed *en* bloc at the experimental endpoint without cannulation or flushing with Perfadex®.

Ex vivo lung perfusion

In **papers II and III**, ex vivo lung perfusion (EVLP) was carried out using a Vivoline LS1 system (XVIVO Perfusion) with the donor lungs maintained *en* bloc as previously described by our research group[61]. The circuit was primed with Steen™ Solution (XVIVO Perfusion) supplemented with red blood cells collected from the donor animal prior to injury induction to achieve a haematocrit of 15-20%. Perfusion flow was adjusted to approximately 40% of the donor's estimated cardiac output. Ventilation parameters were standardized with a tidal volume of 7 mL/kg donor body weight, a respiratory rate of 7 breaths per minute, a positive end-expiratory pressure (PEEP) of 5 cmH_2O, and an inspired oxygen fraction (FiO_2) of 21%. The lungs were perfused and ventilated for a total duration of four hours. Physiological and hemodynamic parameters, as well as arterial blood gases, were recorded at regular intervals during the perfusion period. Tissue biopsies, bronchoalveolar lavage fluid, and blood samples were collected for downstream analyses. If the perfusate volume in the reservoir dropped below 300 mL, additional Steen™ Solution was added to maintain circuit volume. At the end of the EVLP period, the lungs were cooled to approximately 10 °C for about 45 minutes prior to transplantation.

Left lung transplantation

In **paper III**, a single left lung transplantation was performed after EVLP as per Ghaidan et al.[61], following a protocol initially described by Mariscal et al.[193]. Briefly, following a left thoracotomy, the pulmonary hilum was exposed, carefully dissected and the left pulmonary artery, atrium, and main bronchus were individually isolated and clamped. Subsequently, the recipient's native left lung was removed by pneumonectomy and the donor lung was implanted. The bronchial anastomosis was constructed using interrupted polydioxanone sutures (PDS 4-0,

Ethicon, USA) and the anastomoses of the atrial cuff and pulmonary artery were completed with continuous polypropylene sutures (Prolene 5-0, Ethicon). Following completion of the bronchial anastomosis, bronchoscopy was performed to verify patency of the airway connection.

Post-transplantation follow-up

In **study III**, recipient animals were maintained under continuous general anaesthesia using infusions of ketamine, midazolam, fentanyl, or buprenorphine and rocuronium bromide for three days post-transplantation. Antibiotic prophylaxis consisted of intravenous imipenem (500 mg) three times daily and subcutaneous dihydrostreptomycin sulphate (0.1 mL/kg) once daily. Immunosuppression was maintained with oral tacrolimus (0.15 mg/kg) once and intravenous methylprednisolone sodium succinate (1 mg/kg) administered twice daily. Animals were monitored under mechanical ventilation with settings adjusted to ensure adequate oxygenation while minimizing ventilatory pressures, maintaining a PEEP of 5-10 cmH_2O and peak inspiratory pressures below 30 cmH_2O. At the end of the three-day follow-up period, the isolated function of the transplanted left lung was evaluated. Briefly, a right pneumonectomy, including the accessory lobe, was performed via mid-sternotomy after dissection of the pulmonary hilum. Hemodynamic parameters and blood gas measurements were recorded immediately prior to this procedure. Following pneumonectomy, recipients were observed for an additional four hours under one-lung ventilation, during which tidal volume and respiratory rate were adjusted to maintain peak airway pressures below 30 cmH_2O. Throughout the experiment, hemodynamic monitoring was performed using a Swan-Ganz catheter as described previously. Upon termination of the experiment at 72 hours post-transplantation, an additional chest x-ray was taken and PGD was graded based on the presence of opacities, as well as measured PaO_2/FiO_2 ratios adhering to the ISHLT guidelines[43].

Treatment methodology

Cytokine hemoadsorption

In **paper I**, patients undergoing lung transplantation for end-stage lung disease were treated with a cytokine hemoadsorption device (CytoSorb®, Germany). All transplant procedures were performed with intraoperative ECLS. In patients assigned to the treatment group, the treatment device was integrated into the ECLS circuit prior to its initiation, while patients in the non-treated group received intraoperative ECLS only.

NET removal

In **paper II**, a NucleoCapture column (Santersus AG, Switzerland) designed to bind and remove neutrophil extracellular traps (NETs), nucleosomes, and other circulating histone-DNA complexes was integrated into the EVLP setup in the treatment group. The column was connected to a Spectra Optia Apheresis System (Terumo BCT Inc., Belgium) operating at a perfusate flow rate of 80 mL/min. Both devices were incorporated into the EVLP circuit via a venovenous shunt originating from the reservoir.

MSC therapy

In **papers III and IV**, human bone marrow (BM) was obtained from healthy adult volunteers aged 20-25 years following informed consent, with procedures approved by the Swedish Ethical Review Authority at Lund University and performed at the Department of Haematology, Lund, Sweden. Mononuclear cells were isolated by density gradient centrifugation, enriched through plastic adherence, and expanded in a cell factory system to enable large-scale production. Bone marrow-derived mesenchymal stromal cells (BM-MSCs) were cultured under GMP-like, serum-free conditions using pooled human platelet lysate, as previously described, and cryopreserved at early passages until use.

In **paper III**, mesenchymal stromal cells derived from full-term amniotic fluid (TAF-MSCs) were isolated from consenting donors undergoing elective caesarean section according to established protocols[129]. A lung-specific MSC population was selected based on transcriptomic and phenotypic similarity to foetal lung-derived MSCs, followed by fluorescence-activated cell sorting to obtain the final cell product (AmnioPul-02, Amniotics AB, Sweden). The TAF-MSC product has previously been evaluated in a Phase 1b clinical trial for lower respiratory disease (NCT05348772), demonstrating a favourable safety profile without reported adverse events.

For **paper IV**, 4 bone marrow aspirates from Lund were processed as BM-MSCs above, but with all cell processing and expansion steps carried out using the automated GMP CliniMACS Prodigy® system in Germany (Miltenyi Biotec, Germany).

All MSCs used were characterized by flow cytometry for surface marker expression, evaluated for differentiation capacity, and assessed for immunomodulatory function according to the ISCT's guidelines[106], stored at -150 °C and thawed immediately prior to administration. After thawing, cells were washed by centrifugation and resuspended in phosphate-buffered saline (PBS) after cell concentration and viability had been determined.

Downstream analysis

Sampling

Blood samples were obtained from patients and pigs via the arterial catheter or perfusate from the EVLP circuit and collected in Ethylenediaminetetraacetic acid (EDTA) and/or citrate-containing tubes. A portion of the anticoagulated blood was used for differential cell counting using a Sysmex KX-21N automated haematology analyser (Sysmex, UK), providing measurements of white blood cells, lymphocytes, and neutrophils. The remaining sample was centrifuged to separate the plasma.

Bronchoalveolar lavage fluid (BALF) was obtained from pigs by using standard bronchoscopic techniques. BALF was aliquoted immediately, and live cell concentrations were determined using a NucleoCounter NC-250 system (Chemometec, Denmark).

All plasma and BALF aliquots were stored at -80 °C until analysis.

Molecular assays

Several different molecular assays were run on plasma, perfusate, BALF and homogenized tissue samples, mostly including standard enzyme-linked immunosorbent assay (ELISA), but also enzymatic assays. All assays were performed using pre-prepared kits purchased from established manufacturers and run according to the manufacturer's instructions.

In **paper I**, cytokine levels of IL-6 and IL-8 were measured using ProQuantum high-sensitivity immunoassays (Thermo Fisher Scientific, US). For **paper II-IV**, cytokine levels of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IFN-α, IFN-γ, and TNF-α were assayed using the multiplex kit Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel 1 (Thermo Fisher Scientific, US) and analysed using a Bioplex-200 system (BioRad, USA).

Although working with translational large-animal models such as the pig is highly scientifically rewarding, the search for molecular assays to detect specific targets in pigs remains challenging. The approach for the studies in this thesis was to adhere to pig-specific kits where available, and when not, kits targeting the human equivalent were purchased. In studies II-IV, only results from molecular assays that yielded usable scientific data were reported. However, the list of kits that did not yield usable results would probably be twice as long.

Tissue processing

Histopathological analysis

Lung tissue biopsies were collected at predefined time points and from randomized biopsy sites determined prior to the experiments. Samples were fixed in neutral-buffered 10% formalin at 4 °C, followed by dehydration through a graded ethanol series and clearing in xylene. Tissue samples were embedded in paraffin and, after paraffin hardening, 5-µm sections were prepared using a rotary microtome. Tissue sections were then mounted on glass slides, deparaffinized, and either stained with hematoxylin and eosin according to standard protocol or antibody-staining performed using standard immunohistochemical protocols. Slides were mounted and imaged using brightfield or fluorescence microscopy.

For standard hematoxylin and eosin staining, images were de-identified and randomized prior to blinded evaluation by independent observers experienced in porcine lung histology.

For fluorescent staining, evaluation was performed using pre-programmed scripts for fluorescence intensity with pre-determined cut-off values.

MACSima™

For **paper III and IV**, formalin-fixed, paraffin-embedded lung tissue sections (10 µm), prepared as described for histopathological analysis, were deparaffinized and rehydrated using standard protocols. Antigen retrieval was performed in TEC buffer, sections were pre-stained with 4′,6-diamidino-2-phenylindole (DAPI), washed in running buffer, and mounted in MACSwell™ 4 imaging frames. Multiplex imaging was carried out using the MACSima™ Imaging Platform (Miltenyi Biotec, Germany). A panel of directly conjugated antibodies was applied, including both pre-validated reagents from Miltenyi Biotec and externally sourced antibodies, labelled with FITC, PE, APC, Alexa Fluor 488, or Alexa Fluor 635. Staining parameters, including antibody dilution, incubation time, imaging cycle sequence, photobleaching, and exposure settings, were kept consistent across experiments. Image acquisition was performed using a 20× long working distance objective (NA 0.45), and data were analysed using MACS® iQ View software (Miltenyi Biotec).

Scanning electron microscopy (SEM)

For **paper III** and all other SEM images utilized throughout this thesis, lung tissue samples were embedded in 3% low-melting-point agarose and sectioned into 200 µm slices using a vibrating microtome. Samples were rinsed in 0.1 M Sørensen's buffer, fixed in 2% formaldehyde and 2% glutaraldehyde, and subsequently dehydrated through a graded ethanol series. Following critical point drying, specimen were mounted on aluminium stubs, sputter-coated with a Pt/Pd layer, and

imaged using a field emission scanning electron microscope (JSM-7800F FEG SEM; JEOL, Japan).

Proteomics

In **paper III**, lung tissue was processed using S-Trap–based protein extraction and analysed by data-independent acquisition on a TIMS TOF HT mass spectrometer. Raw data were processed with DIA-NN (v1.8.1) using a UniProt database, and normalization and differential expression analyses were performed in R using MS-DAP and MS-EmpiRe. Proteins were filtered based on detection criteria, and low-quality samples were excluded. Differential expression was defined using FDR-adjusted p-values and log2 fold-change thresholds. Laser-capture microdissection was used to isolate alveolar regions and the bronchial-vascular interface, which were analysed using the same proteomic workflow.

Statistical analysis

In **paper I**, statistical analyses were performed using the Kruskal-Wallis test with Dunn's post hoc correction for independent groups, and two-way ANOVA with Šídák's multiple comparisons test for repeated measures over time.

In **papers II-IV**, data are presented as mean ± standard deviation (SD) and tested for normality using the Shapiro-Wilk test. Parametric analyses included Student's t-test and one- or two-way ANOVA with Holm-Šídák or Tukey post hoc correction. Non-parametric comparisons were performed using Wilcoxon, Mann-Whitney U, Kruskal-Wallis, or Friedman tests with Dunn's correction where appropriate. Repeated measures were analysed using mixed-effects models with Šídák correction, and categorical variables were compared using the chi-squared test. Analyses were conducted in GraphPad Prism and R, with significance set at $p<0.05$.

Artificial Intelligence (AI) tools

During the writing process of this thesis, OpenAI's ChatGPT (GPT-5) and the AI-powered digital writing assistant Grammarly were utilized with the purpose of improving language grammar, clarity, and style as well as text readability and flow. All AI-generated suggestions were critically revised by this author, who takes full responsibility for the integrity of the written content of this thesis, scientific and non-scientific.

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