The involvement of the NLRP3 inflammasome in donor lung injury

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Abstract

Lung transplantation remains the only feasible treatment option for people with end-stage pulmonary disease who do not respond to other therapeutic strategies. However, the demand for donor lungs greatly outweighs the availability of organ supply, which results in waiting list mortality. Lungs are extremely susceptible to damage within the donor environment, and only 20% of donor lungs meet the criteria for standard transplantation. This has led to increased use of marginal donor lungs, which in turn increases the risk of complications such as rejection and primary graft dysfunction. Ex vivo lung perfusion (EVLP) is used to evaluate, and also to improve pulmonary function in these marginal donor lungs. Interleukin-1β (IL-1β), a potent pro-inflammatory cytokine has been identified as a prognostic indicator of non-recovery in human EVLPs. The release of IL-1β is dependent on the assembly of inflammasome, a large multiprotein complex. The hypothesis of these studies was that inhibiting the NLRP3 inflammasome formation would reduce the release of active IL-1β thereby decreasing the inflammatory response during EVLP, thus potentially encouraging organ recovery. NLRP3 inflammasome inhibitor was introduced into porcine ex vivo organ perfusion systems of healthy lungs and damaged kidneys. Organ function was assessed along with the inflammatory profile of the organs in the control and treatment groups. Furthermore, the levels of other inflammasome-related inflammatory mediators IL-1β, pro-IL-1β, caspase-1 and IL-18 were measured in human EVLP perfusate and bronchoalveolar lavage samples from lungs that were transplanted and lungs that were discarded. Lastly, the concentrations of these mediators were assessed in plasma samples of lung transplant recipients immediately before and up to 72 hours post-operatively, and correlated the values with pulmonary oxygenation capacity as an indicator of lung function. Whilst a decrease in the extracellular inflammasome particles in the porcine ex vivo perfusions occurred, there were no differences between the control and the inhibitor groups in terms of their cytokine profiles, cellular outflow or organ function. This demonstrated that despite the inhibitor therapy reducing inflammasome activity, it did not convey protection or promote organ recovery. In the perfusate of human lungs that did not recondition during EVLP and were rejected for transplant, a significant increase in pro-IL-1β, the inactive precursor molecule expressed intracellularly, was detected. This suggests that inflammasome inhibition therapy alone may not protect donor lungs from injury or promote recovery, and that pro-IL-1β is released following cell death rather than actively secreted as part of a specific inflammatory process.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Dedication

I could not have completed this thesis without the everlasting support and love from my fiancé Kam, friends Kadri, Raili and Kristina, as well as my mum Helle, and I dedicate this work to them.

Additionally, I am grateful for having had the opportunity to work on ex vivo organ perfusion in Dr James Fildes’ group at the University of Manchester, collaborating nationally and internationally with world-class experts in the field. I am also appreciative to the whole group for the continuous support and advice I’ve received throughout the course. I have learnt a lot during my years working here, academically and otherwise, and I am thankful for all the lessons and the people I have met. I am also incredibly appreciative to Merlin and Lindsay who sparked my love for research and who have helped me to become a better scientist. I hope the future brings you everything you deserve.
Preface

This report is written in the standard Doctor of Philosophy thesis format and describes the experiments conducted and data analysed over the course of three years at the University of Manchester. As the author of this thesis I have a background in science, having completed a BSc degree in Biology with Human Psychology in 2010 with Upper Second class honours. I subsequently volunteered in a research group in order to gain more experience in a laboratory setting, where I worked in the area or stem cells and regenerative medicine. Following that I successfully completed an MSc degree in Biomedical Science in 2013 with a Distinction, where my dissertation project focused on chronic obstructive pulmonary disease and I became interested in lung inflammation. I was then awarded the current Cystic Fibrosis Trust funded PhD position. In the three years of working on this degree, I have gained invaluable experience in these diverse laboratory-, clinical- and pre-clinical settings. I have learnt flow cytometry staining-, immunohistochemistry-, fluorescent in situ hybridisation-, polymerase chain reaction, as well as various reactivity assay techniques. I have collected patient samples in a clinical setting with great appreciation for the collaborative work that clinicians and scientists are doing. I have also learnt about the ex vivo organ perfusion models utilising various organs and how these can be utilised in both for clinical transplantation and scientific research. Collaborative work was undertaken with several groups at the University of Manchester, other UK universities and abroad, as well as clinical staff at University Hospital South Manchester. Collaborators at University of Manchester include Dr Gloria Lopez-Gastejon and Dr David Brough. The porcine EVLPs were conducted in Igelösa, Sweden with the support of Prof Stig Steen's research group. The work on the DEVELOP-UK clinical trial was conducted in collaboration with Prof Andrew Fisher and his team from the University of Newcastle. The assessment of inflammasome particles was carried out with the advice from Dr Pablo Pelegrin and his team at Murcia’s BioHealth Research Institute, in Spain.
## Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AKI</td>
<td>acute kidney injury</td>
</tr>
<tr>
<td>ALI</td>
<td>acute lung injury</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin conjugate</td>
</tr>
<tr>
<td>AR</td>
<td>acute rejection</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated Speck-like protein with a caspase-recruitment domain</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BOS</td>
<td>bronchiolitis obliterans syndrome</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CM</td>
<td>classical monocytes</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemotactic cytokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>chemotactic cytokine receptor</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger associated molecular pattern</td>
</tr>
<tr>
<td>DBD</td>
<td>donation after brain death</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DCD</td>
<td>donation after cardiac death</td>
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<td>DEVELOP-UK</td>
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DMSO – dimethyl sulfoxide
ECMO – extracorporeal membrane oxygenation
EDTA – ethylenediaminetetraacetic acid
EVLP – *ex vivo* lung perfusion
EVNP – *ex vivo* normothermic kidney perfusion
FCS – foetal calf serum
FiO₂ – fraction of inspired oxygen
FITC – fluorescein isothiocyanate conjugate
G-CSF – granulocyte colony-stimulating factor
GM-CSF – granulocyte macrophage colony-stimulating factor
GTN – glyceryl trinitrate
HMGB – high mobility group box
HRP – horseradish peroxidase
ICAM – intracellular adhesion molecule
ICU – intensive care unit
IFN - interferon
IL – interleukin
IPF – idiopathic pulmonary fibrosis
IQR – interquartile range
ISHLT – International Society for Heart and Lung Transplantation
LA – left atrium
LPS – lipopolysaccharide
L Tx – lung transplant
MCCIR – Manchester Collaborative Centre for Inflammation Research
MCP – monocyte chemotactic protein
MHC – major histocompatibility complex

NBC – novel boron compound

NCM – non-classical monocytes

NF-κB – nuclear factor κB

NHS – National Health Service

NIHR – National Institute for Health Research

NK – natural killer

NLR – Nucleotide binding and Leucine rich repeat Receptor

NLRP3 – NLR pyrin domain-containing type 3

NPO – neurogenic pulmonary oedema

NSAID – non-steroidal anti-inflammatory drugs

OCS – Organ Care System

PA – pulmonary artery

PaCO₂ – partial pressure of arterial carbon dioxide

PAMP - pathogen associated molecular pattern

PaO₂ – partial pressure of arterial oxygen

PAP – pulmonary arterial pressure

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

pCO₂ – partial pressure of carbon dioxide

PGD – primary graft dysfunction

PE – phycoerythrin conjugate

PE-Cy7 – phycoerythrin: Cy-7 tandem conjugate

PEEP – positive end-expiratory pressure

P/F – PaO₂/FiO₂ ratio
PGD – primary graft dysfunction

PhD – Doctor of Philosophy

PMA - phorbol myristate acetate

pO$_2$ – partial pressure of oxygen

PVR – pulmonary vascular resistance

RBC – red blood cells

RBF – renal blood flow

RPMI - Roswell Park Memorial Institute medium

ROS - reactive oxygen species

SD – standard deviation

sVCAM – soluble vascular cell adhesion molecule

THAM – tromethamine

TLR – toll-like receptor

TNF – tumour necrosis factor

UHSM – University Hospital South Manchester

UK – United Kingdom

USA – United States of America
Chapter 1: Introduction
1.1 Lung transplantation – an overview

Respiratory disease remains one of the leading causes of death worldwide [1]. In the United Kingdom (UK) the estimated direct and indirect economic burden of respiratory illness is £11.1 billion per year [2]. Current therapeutic strategies for severe respiratory disease only provide symptomatic rather than prognostic improvement. Lung transplantation (LTx) remains the only treatment in cases of end-stage lung disease that are refractory to maximal medical therapy, such as medicines or breathing devices. The LTx process involves surgical removal of the patient’s severely diseased organs and replacement with healthy lungs from a suitable donor. If successful, the procedure is associated with promising post-transplant outcomes, with full recovery and improvements in the patient’s quality of life normally taking approximately 3 to 6 months [3]. However, lung transplantation is limited by a scarcity of donor lungs and waiting lists for potential recipients are long. As lungs are easily damaged resulting from the inflammatory conditions of the donor environment before and during the retrieval process, approximately 80% of the donor organs are deemed unsuitable for transplantation. As such, the demand for lung transplants greatly outweighs the supply of donor lungs. In the UK 167 lung transplants were performed from April 2016 to March 2017 between the five centres in Newcastle, Cambridge, London, Birmingham and Manchester [4]. The issues surrounding LTx are well recognised and much effort is put into addressing them through different strategies, so the overall numbers of transplants are on a steady increase. One of the most recent technological advancements to address the limited supply of suitable lungs is to remove them from the donor and to assess them using normothermic ex vivo lung perfusion (EVLP). However, as this can still be considered new to clinical practice, much is yet to be learned about the specific mechanisms of the procedure.

Still, at the moment the demand for donor organs far outweighs the supply, and even though a successful LTx is a life-saving operation, there are further limitations that need to be considered. The main concepts of LTx, as well as short- and long-term complications, and how clinical work and science, along with EVLP, have advanced in this area, will be discussed in the following chapter, along with the aims and objectives of this thesis.

1.1.1 Types of lung transplantation

There are several types of lung transplant procedures that are utilised according to the suitability and need of the recipient, and the availability of donors. During a single lung transplant only one of the lungs of the patient is removed and replaced with a single lung from the donor. This procedure is undertaken when there is no risk of harm from the remaining lung to affect the new organ. This means that this operation is suitable for
patients with lung fibrosis or emphysema, but not for patients who suffer from an infection in the lungs or cystic fibrosis (CF). These conditions have the potential to compromise, spread to and thereby damage the new organ further endangering the recipient. If suitable recipients can be found and prepared in time, two single lung transplants can be achieved from one donor. A bilateral- or double lung transplant involves the removal of both lungs from the patient and their replacement with donor lungs. It is the most common type of lung transplant performed, and utilised to treat patients with CF and chronic obstructive pulmonary disease (COPD). In patients with pulmonary hypertension where both the heart and the lungs are diseased, a combined heart-lung transplant will be performed where the two donor lungs are transplanted together with the heart as one block. Due to the particularly limited availability of donor organs this complicated process was only performed 6 times in 2015/2016 [5] and 2 times in 2016/2017 [4] in the UK. Occasionally lungs from living donors can be utilised for transplant [6]. In this case however, two donors are required for one recipient. The left and the right lower lobes are taken from different donors, and replace the lobes in the recipient. It is mainly used in young patients with CF who have consenting relatives willing to donate. The living donor transplant has the advantages of having the operation done electively at a chosen time, as well as the donor organs being less damaged during the retrieval and processing. Additionally it is estimated that patients who receive genetically related transplants may have fewer post-transplant complications [7]. It is usually more difficult to match paediatric CF patients with a donor, so a living donor LTx may save the recipients from the high mortality rates of staying in the transplant waiting list [8]. However, this type of lung transplant has not been carried out in the UK since the period of 2007/2008 [4, 5].

1.1.2 Donor lung selection and procurement
The International Society for Heart and Lung Transplantation (ISHLT) has defined standard donor criteria that determine whether or not donor lungs can be accepted for transplantation (Table 1.1). However, these guidelines for the ideal donor were drawn up when LTx first became more common in general practice over 30 years ago [9]. The criteria remain relatively conservative to ensure the best possible outcome and safety of the recipients, but in doing so, many donor lungs are rejected. In practice, most transplant centres utilise extended criteria donors, which leads to a considerable variation in the donor lung criteria being used in practice.

When donor lungs become available, the donor and recipient are first matched by blood group and size of the organ [10]. A chest radiograph is taken to confirm the absence of serious parenchymal abnormalities, and a bronchoscopy is performed to exclude the
presence of major infections [10]. Finally, the organ’s gaseous exchange capacity is evaluated through an oxygen challenge. Lung compliance and oedema are assessed by the retrieval surgeon [10]. Upon procurement the lungs are flushed with a cold solution (such as Perfadex, XVIVO, Sweden) with added heparin, and stored on ice for transportation to the recipient hospital [10]. Cold static storage has been the standard practice for organ transplantation for a long time, as keeping the tissues in hypothermia will slow down the metabolic processes and cellular damage. However, for standard lung transplants, the optimal cold ischemic time between cooling down the graft at procurement and restoring the blood supply in the recipient is suggested to be 4-6 hours. In practice this period may occasionally be extended when the risk of staying in the waiting list outweighs the added mortality risk of receiving lungs with increased ischemic time. However, in most cases this means that donor lungs have to be transplanted within a certain timeframe or else they become unusable even if the initial parameters were favourable. In smaller countries this may not be an issue but in countries where distances are longer, such as the United States or Canada, potentially matched donor and recipient may just be geographically too far apart for a successful transplant. Extending the acceptable ischemic timeframe, without compromising the integrity of the organs, would therefore benefit the availability of matched donor lungs, especially in these countries.
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<td>Age &lt; 55 years</td>
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<td>ABO compatibility</td>
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<tr>
<td>Clear chest X-ray</td>
</tr>
<tr>
<td>PaO$_2$ &gt; 40kPa (300 mmHg) at FiO$_2$ = 1.0, PEEP = 5 cm H$_2$O</td>
</tr>
<tr>
<td>No purulent secretions at bronchoscopy</td>
</tr>
<tr>
<td>No aspiration or sepsis</td>
</tr>
<tr>
<td>No thoracic trauma</td>
</tr>
<tr>
<td>No previous thoracic operation</td>
</tr>
<tr>
<td>Negative sputum gram stain</td>
</tr>
<tr>
<td>Smoking &lt; 20 pack-years</td>
</tr>
</tbody>
</table>

(Number of cigarette packs smoked per day multiplied by the number of years smoked)
1.1.3 Donation after brain death

In most cases, lungs are procured for transplantation from donations after brain death (DBD), as the cessation of neurological function is equivalent to the legal definition of death in many countries [12], but the donor organs remain operative due to cardiac function and intensive care unit (ICU) support. Pulmonary dysfunction in these patients is most commonly related to aspiration, pneumonia, contusion and ventilator-induced injuries, but the onset of brain death can also result in organ damage [13]. The irreversible damage to the brain stem and loss of its function induces neurogenic pulmonary oedema (NPO) and inflammatory acute lung injury (ALI) resulting from central nervous system injury or stimulation, haemodynamic changes, and systemic release of pro-inflammatory mediators (Figure 1.1) [13].

NPO is caused by the ‘sympathetic storm’ that occurs immediately after injury to the brain stem, but can also take place before the patient can be considered as a potential organ donor. The ‘sympathetic storm’ is an unmediated hypothalamic stimulation of the sympathetic nervous system, leading to significant increases of adrenaline and noradrenaline in the circulation, extreme systemic hypertension, tachycardia and arrhythmias [14]. As vasoconstriction increases the cardiac afterload, it causes augmented left ventricle and atrial pressures. Blood is then diverted from the periphery to the core, which in turn increases the pulmonary blood volume and pulmonary artery pressure. The hypertensive period, lasting only a few minutes, is followed by a systemic hypotensive plateau, vasodilation and gradual decrease in arterial blood pressure [14]. Collectively, these changes result in a substantial escalation of pulmonary capillary pressure, which can damage the epithelial structure of the lung, and together with elevated hydrostatic pressure, lead to the formation of pulmonary oedema [13]. As α-adrenergic receptors are responsible for vasoconstriction, stimulating them can increase the permeability of pulmonary capillaries, and blocking them can prevent the formation of NPO [13]. The haemodynamic alterations due to α-adrenergic stimulation can further damage the lung tissue when changes in the blood flow cause rapid variations in the degree of shear stress. This leads to the activation of endothelial cells and generation of reactive oxygen species (ROS), the induction of the nuclear factor (NF)-κB pathway, and up-regulation of cytokines and adhesion molecules [14].

Furthermore, brain death is accompanied by a systemic release of pro-inflammatory factors and cytokines, whilst inflammatory responses take place in all organs that can be considered for transplantation. In the lung, elevated levels of interleukin-1β (IL-1β), IL-2, IL-6, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) have been observed and correlated to the development of ALI [14]. The presence of these cytokines and the
expression of endothelial adhesion molecules means that the lungs are in an immunologically activated state, which can then result in decreased function, histological damage, increased risk of primary graft dysfunction (PGD) and acute rejection (AR) after transplantation [15, 16]. In addition, the epithelial and endothelial cells in the lungs secrete IL-8, a potent chemoattractant and activator of neutrophils, which in turn mediates the expression of neutrophil integrins CD11β and CD18 [14]. This results in neutrophils adhering to and migrating into the interstitium and alveolar spaces of the lung, and causing tissue damage through the release of ROS and proteolytic enzymes [14]. PGD and higher rates of early mortality after lung transplant have been associated with increased levels of IL-8 in donor bronchoalveolar lavage (BAL) [17-19]. De Parrot et al. also demonstrated that IL-8 concentrations in donor lung tissue increased over time after reperfusion and can be predictive of early PGD [20]. They also found that the IL-8 levels after 2 hours of reperfusion correlated with lung function, the mean airway pressure and the length of ICU stay. Their results further demonstrated that increased concentrations of IL-18 were associated with longer ischemic time [20].

In addition to the sympathetic storm, there are also significant haemodynamic disturbances. This leads to changes in capillary-alveolar exchange, increased hydrostatic pressure and organ ischemia, all of which contribute to the formation of NPO and ALI. Moreover, there is a shift from aerobic to anaerobic metabolism. This results in exhaustion of energy stores, lactic acidosis, and the release of lipases, proteases, endonucleases and ROS [14], all of which can potentially lead to further damage of the pulmonary tissue.

Nonetheless, DBD donors are easier to manage in a sense that they are most commonly already at a hospital setting being monitored and receiving the attention of the medical staff, and more planning can be put into the organ procurement process. However, it can also be that after declared death, the donor management becomes sub-optimal compared to patient care, and thus any physiological damage can have greater impact on the donor organs.
Figure 1.1 Summary of cellular and molecular pathways in brain death. These lead to neurogenic pulmonary oedema and acute lung injury formation. Purple – stimulation of $\alpha$-adrenergic caused by the catecholamine storm; green – haemodynamic changes; red – inflammatory changes. Adapted from Avolantis et al. [14].
1.1.4 Donation after cardiac death

All of the aforementioned risks to organ damage associated with DBD lungs - the possibility of oedema formation and increased number of immune cells present in the donor lung - decrease the number of donor lungs meeting the required criteria for transplantation. As an alternative method to combat this issue, donations after cardiac/circulatory death (DCD) donors have recently been utilised [21, 22].

Despite the haemodynamic imbalances that occur following cardiac/circulatory death, the absence of the pro-inflammatory cytokine storm that is triggered by brain death, and therefore the avoidance of potential formation of NPO and ALI, could prove advantageous [23]. Kang and colleagues studied pre- and post-transplant lung tissues and found that DCD donor lungs expressed fewer inflammatory features compared to DBD patients [24]. They identified major gene expression features and listed significantly altered genes, and concluded that in DBD donors genes that control innate immunity, intracellular signalling, cytokine interactions, cell communication and apoptosis were up-regulated. DCD lungs are most vulnerable to injury at the time between cardiac arrest and/or withdrawal from life support therapies, and cold preservation flush as the lungs can potentially be damaged by the unavoidable warm ischemia, and therefore be at risk of hypotension and aspiration [25]. During the warm ischemic period, it is well documented that rapid cellular stress and diffuse tissue damage can occur, impairing subsequent organ function [26].

Five categories of non-heart beating donors have been described by the Maastricht classification divided into controlled and uncontrolled DCD (Table 1.2) [27]. Life support for the patients within the controlled categories III and IV is stopped during a planned procedure and organ procurement is commenced in a controlled environment. This has led to most cardiac death donations being accepted for transplant from controlled DCD patients. The use of DCD has been increasing but is still not a common practice globally. In the United States it has been reported to account for 10% of all donations, whilst in Japan it remains the main source of organs as their national legislation has only recently adopted the concept of brain death [28]. In Europe, DCD donations are becoming more accepted but are only used in a few countries, such as Sweden, United Kingdom, Italy, Spain, Denmark and France at present [28]. This could be due to the lack of knowledge about the outcomes of DCD transplants with only a limited number of studies and published data supporting the strategy to date. The wider adoption of DCD is also complicated by legislation in countries prohibiting donations from patients declared deceased by circulatory or respiratory criteria (although allowing type IV DCD) [28]. A lack of practical experience and technical expertise is also one of the main issues faced by most centres when adopting methods for DCD. This results in a vicious cycle where research is not
carried out because of the limited amount of surgeries performed, and there are fewer transplants because of lack of knowledge about the outcome of using DCD donors.

There is currently no overall consensus about the safety of DCD transplantation, especially within the uncontrolled categories, due to the limited number of cases available. However, Cypel and colleagues report a 97% 30-day and 89% 1-year survival rate for DCD lung transplants, which is comparable to that of DBD [25]. Others have demonstrated similar findings for the short- and mid-term outcomes of DCD lung transplants, and concluded that the use of DCD for human lung transplants is associated with acceptable early clinical results [29, 30]. De Oliveira et al. studied the long-term outcome of DCD lung transplants 5 years after LTx and determined that the patient and graft survival rates were equivalent to those of DBD donors [21]. However, the number of DCD transplants performed in all of these studies is small and the results require further investigation for more definitive conclusions.

These studies show huge potential for the utilisation of DCD organs, and indicate that authorised centres should therefore establish precise strategies and protocols by which these donors should be managed. Hospitals and healthcare professionals should also receive appropriate consultations and training needed to provide sufficient knowledge and experience to utilise the organs from these potential donors. As mentioned previously, research has suggested that changes in legislation should be implemented if necessary to allow transplants from DCD to increase the numbers of available organs. If these studies are to be considered, uncontrolled DCD could notably increase the number of donor organs available, although this still requires a significant amount of work to be undertaken in terms of organisation and organ preservation. It is paramount to maintain the quality of organs ready to be transplanted whilst the donor criteria are being expanded and this is also where the use of EVLP can prove to be invaluable in terms of assessing the organ function but potentially altering the immunogenicity of the donor lungs [31].
### Table 1.2 The Maastricht classifications of donors after circulatory death
(adapted from Longnus et al. [27])

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Dead on arrival to the hospital</td>
<td>Uncontrolled</td>
</tr>
<tr>
<td>II</td>
<td>Unsuccessful resuscitation</td>
<td>Uncontrolled</td>
</tr>
<tr>
<td>III</td>
<td>Anticipated cardiac arrest but does not meet the criteria for brain death</td>
<td>Controlled</td>
</tr>
<tr>
<td>IV</td>
<td>Cardiac arrest after brain death</td>
<td>Controlled</td>
</tr>
<tr>
<td>V</td>
<td>Cardiac arrest at the hospital</td>
<td>Uncontrolled</td>
</tr>
</tbody>
</table>
1.1.5 Organ Transplantation 2020 strategy

Following from the success of the 2008 strategy in the UK, whereby the number of donors increased by 50% between 2007/8 and 2012/13, the National Health Service (NHS) has put forward a plan to further improve on these numbers by 2020 [32].

Firstly, it is the matter of the general public's attitude and willingness to consent to organ donation. It is estimated that currently in the UK this consent is given in 57% of cases, which is one of the lowest in the Western world [32]. On 1st December 2015 Wales implemented a new soft opt-out system making it easier for people to become organ donors [33]. This means that if a person has not registered their decision with the NHS, either as a willing donor or to opt out, it would be assumed that they do not object to donation, and a deemed consent would be implied. Within one year of passing the law, 39 organs were transplanted from donors with deemed consent in Wales [34]. This demonstrates clear benefits of the new approach and can be used as an example for other regions and countries to consider executing similar legislature. However, more definite conclusions can be drawn after more long-term observations [35]. France has adopted the same system as of 1st January 2017 [36] and the UK government has launched a consultation on introducing ‘opt-out’ consent for organ donation in England [37]. At the same time it is essential that the public attitude towards organ donation be changes through education and open discussions, and it becomes part of a standard way of thinking, especially in the communities where organ donation is not the cultural norm. Equally, it is necessary to invest in and train specialist staff that can offer appropriate care and support to the organ donors and their families to address any concerns people would have at such a difficult time. The NHS Blood and Transplant and the UK Health Departments aim to increase the consent for organ transplantations to >80% with their 2020 strategy.

Despite this, even with patient or family consent, not all organs from every donor can be utilised. In the UK only 30% of hearts and lungs from consented DBD donors, and 7% from DCD donors, are transplanted [32]. The 2020 plan proposes several approaches to increase these numbers by 5%. In many cases in the UK end-of-life patient care is limited before brain-stem death can be diagnosed, even when the individuals had stated their consent to donate [32]. As discussed previously, lungs are highly susceptible to damage and without proper care they can become damaged. Timely testing for and confirmation of brain-stem death is needed, otherwise organ donations may not be possible. Increasing the number of DBD donations should be supported by developing systems and training programmes that sustain clinical expertise. Likewise, having appropriately trained clinical staff readily available is required to increase the utilisation of DCD donors. The 2020
strategy also suggests reviewing new techniques and technologies for preservation and assessment that can improve the quality of the procured organs, which is where the true potential of *ex vivo* lung perfusion can be utilised [32].

### 1.1.6 Immunosuppression

The early attempts in LTx were unsuccessful mostly due to insufficient immunosuppression and complications with bronchial anastomosis [38]. The invention of cyclosporine in the 1970s led to improvements in both of these aspects when it replaced corticosteroids as the main method for immunosuppression [39]. It was found that the use of corticosteroids weakened the bronchial anastomosis and that cyclosporine was a superior immunosuppressant [38]. As a result, adequate immunosuppression has proved to be essential for the success of LTx.

The lung is in constant contact with the external environment and has consequently developed its own local immune network: the bronchus-associated lymphoid tissue, which is populated with lymphocytes and macrophages, which in the case of a transplant, would be transferred to the recipient. The lung is also in contact with the entire cardiac output and therefore represents a huge area of interaction between the donor and recipient cells [40]. Triple immunosuppression is used through a combination of calcineurin inhibitors (cyclosporine A, tacrolimus), cell cycle inhibitors (azathioprine) and glucocorticoids (prednisolone) [40]. Recently, new mammalian target rapamycin inhibitors and anti-CD25 antibodies have also been used in these combinations [41], but no studies have yet demonstrated superiority of one drug over the other [40]. The major shortcoming of these immunosuppressive therapies is that the patient is required to take them for the rest of their life. As a result, patients receiving organ transplants have to carefully balance the use of immunosuppressants to avoid graft rejection, whilst avoiding infections and increased risk of cancer. They need to carefully monitor their lung function every day at home, and attend regular visits to the hospital where clinicians can carry out more specific radiological, chemical and microbiological tests. Usually, lung function improves after the transplant and then remains at a steady state after 3 to 6 months [40]. In the case of a sudden decrease in organ function, or the development of coughing, mucus, or breathlessness, patients need to contact the transplant centre to investigate the symptoms to avoid the risk of complications [40].

### 1.1.7 Complications after lung transplant

The process of LTx has several stages at which serious injury to the donor organ can occur. These include brain death of the donor, the procurement process (including explantation and preservation), cold ischemic storage and transport to the recipient site, implantation and ischemia-reperfusion injury, and postoperative care of the patient [42]. Organ damage
derived from these can have significant impact on the outcome and complications of the transplant and the recovery of the patient.

1.1.7.1 Primary graft dysfunction

PGD is the most common cause of mortality in the first 30 days after lung transplant and results from ischemia-reperfusion injury [42, 43]. It is a symptom of ALI that occurs within the first 72 hours after transplantation [42]. PGD is characterised by pulmonary oedema and alveolar damage that clinically manifests in the patient as progressive hypoxemia and pulmonary infiltrates without other distinguishable causes on chest radiographs [42, 44, 45]. It has also been known under the labels of re-implantation response and oedema, reperfusion oedema, non-cardiogenic pulmonary oedema, early graft dysfunction, primary graft failure, and post-transplant acute respiratory distress syndrome, but the classification was officially standardised in 2005 with the term of PGD [42, 44, 46]. According to this taxonomy, PGD is assessed at 0-, 24-, 48- and 72-hour time points after transplantation, and is divided into 4 categories according to severity: Grades 0-3. The specifications for each category are given in Table 1.3. Radiographic infiltrates can be abnormal substances such as blood, pus or protein in tissues, interstitium or alveoli. The fraction of inspired oxygen (FiO₂) is the percentage of oxygen in the space being measured; in normal air FiO₂ = 0.21 [47]. PaO₂/FiO₂ (P/F) ratio signifies the ratio of partial pressure of arterial oxygen and fraction of inspired oxygen, and is clinically commonly used as a marker for hypoxia. The incidence of severe Grade 3 PGD in the first 72 hours after transplant has been reported to be approximately 30% [42] and thereby has a significant impact on the length of post-transplant care needed, including prolonged time on mechanical ventilation, ICU- and hospital stay, increased cost as well as both short- and long-term mortality [48-51]. Christie and colleagues also found that 12 months after transplant, the 6-minute walking distance scores were higher in patients without PGD compared to 72-hour Grade 3 PGD patients [52]. This demonstrates the significant impact that PGD has to the short- and long-term success of the transplant, organ function and mortality rates. It is therefore essential to understand the ways by which the risks of PGD can be reduced or alleviated. Remarkably, studies that have compared transplantation of EVLP reconditioned lungs to standard transplants have found that in addition to increasing the number of acceptable organs, the procedure also potentially reduces the risk of severe PGD [53-59].
Table 1.3 The ISHLT defined categories for primary graft dysfunction (adapted from Suzuki et al. and Snell et al. [42, 45])

<table>
<thead>
<tr>
<th>Grade</th>
<th>Radiographic infiltrates</th>
<th>PaO$_2$/FiO$_2$ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No</td>
<td>Any</td>
</tr>
<tr>
<td>1</td>
<td>Yes</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>27–40</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>&lt;27</td>
</tr>
</tbody>
</table>
1.1.7.2 Rejection

Graft rejection affects approximately 55% of the LTx patients within 1 year post-transplant, which is more than any other solid organ [60]. 1-year survival rate is reported to be 80% and, decreasing to 50% at 5 years [60] but many patients live more than 10, or occasionally more than 20 years after LTx. Rejection is defined as inflammatory infiltration of mononuclear cells into the perivascular or peribronchial areas of the lung tissue [60]. The severity of AR is classed between 0 (none) and 4 (severe) based on the key features of these infiltrates (Table 1.4). Although the symptoms can be treated, rejection acts as a major risk factor for bronchiolitis obliterans syndrome (BOS). The alloimmune response is primarily driven by recipient T cells that recognise donor major histocompatibility complexes (MHC) [61]. This recognition process can occur via two pathways. Donor dendritic cells (DCs) present the donor peptide to the recipient T cells directly in association with MHC on the cell surface [61]. Indirect allore cognition may occur, in which recipient DCs extravasate into the graft and then acquire, process and present alloantigen to recipient T cells in the local lymph nodes [62]. Both donor and recipient monocytes can potentially differentiate into DCs and amplify the immune response thereby impairing early graft function [63]. In addition to non-self antigens present in the recipient, endogenous tissue damage caused by the operation and exogenous infections can further augment the immune response. It has been speculated that in some cases the perivascular infiltrate associated with AR can also be predominantly tolerogenic, in which case the risk of extensive immunosuppression may outweigh its benefits [64]. It is therefore essential to elaborate on the cellular mechanisms occurring in the transplanted lung and EVLP can provide an excellent platform to do so.
**Table 1.4 Grading scheme and key features of acute rejection**
(adapted from Martinu et al. [60])

<table>
<thead>
<tr>
<th>Grade</th>
<th>Meaning</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Normal lung parenchyma</td>
</tr>
<tr>
<td>1</td>
<td>Minimal</td>
<td>Modest amount of perivascular mononuclear cells</td>
</tr>
<tr>
<td>2</td>
<td>Mild</td>
<td>More frequent perivascular mononuclear cells; eosinophils present</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>Dense perivascular infiltrates, also in interstitial space; can involve endothelialitis, eosinophils and neutrophils</td>
</tr>
<tr>
<td>4</td>
<td>Severe</td>
<td>Diffuse perivascular, interstitial and air-space infiltrate with lung injury; can involve neutrophils</td>
</tr>
</tbody>
</table>
1.1.7.3 Bronchiolitis obliterans syndrome

Additionally, both PGD and AR have been linked with BOS, which in turn has been negatively correlated with long-term survival rates after LTx [65]. BOS is the clinical manifestation of obliterative bronchiolitis that occurs in patients who have undergone lung or haematopoietic stem cell transplantation [66]. BOS is accompanied by a progressive decline in lung function with elevated levels of peribronchial and perivascular lymphocyte and neutrophil infiltrates [67]. These neutrophils release cytokines, including IL-8, and proteases that further contribute to sustained inflammation and destruction of the small airways [67]. The obliteration of alveoli and fibrotic narrowing of the bronchioles results in gradual airflow obstruction, often accompanied by chronic lower respiratory tract infections and symptoms such as dyspnoea and non-productive coughing [68]. BOS results most likely from frequent episodes of rejection, but the underlying pathogenesis of the condition, diagnosis and treatment methods are poorly understood [67, 69].

1.1.7.4 Other complications

Infections are the leading cause of mortality within 1 year of lung transplant [65]. This is often due to the extensive immunosuppression and the fact that the lungs are constantly exposed to the environment. Additionally, there is a lack of cough reflex in the transplanted organ causing a reduction of mucociliary clearance due to denervation and disturbance of the lymphatic circulation [40]. With extensive immunosuppression transplant patients are also exposed to a higher risk of several types of cancer compared to the general population [70].

Taken together, it is essential that new strategies are established to combat the high mortality rates of staying on the LTx waiting list, as well as the post-transplant complications of patient care. *Ex vivo* organ perfusion technologies have become more prevalent and warrant further investigation in terms of the specific mechanisms by which they can improve on the numbers and potentially the quality of donor organs. The following paragraphs will further describe the EVLP protocols currently in clinical and laboratory use.
1.2 EVLP Method

Approximately one third of patients needing new lungs die while waiting for their transplant [26]. In order to increase the number of available donor lungs several methods have been considered and tested, including donor management [71], expansion of current donor criteria [72, 73] or using lungs from living donors [8, 74]. However, using suboptimal lungs can increase the risk of acute rejection, infection and other complications post-operatively [75]. The standard procurement protocol requires the lungs to be preserved in hypothermic conditions with decreased metabolic activity, which does not allow meaningful organ evaluation. Normothermic EVLP has been established to simulate the natural in vivo environment of the lungs by perfusion and ventilation, and to evaluate and preserve the organ [76].

Single organ perfusion, i.e. the preservation of an organ by saturation with a protective agent outside the body, was first attempted in 1849 and further developed in the first half of the 20th century [77]. Initial attempts to develop the lung model in 1970 failed because of the inability to maintain the air-liquid barrier within the pulmonary tissue, which led to the development of oedema and increased pulmonary vascular resistance [78]. The pioneers in the field who overcame this limitation were Prof Stig Steen and colleagues who developed the modern EVLP perfusion circuit to evaluate lung function outside of the donor environment [76]. Their initial lung transplant experiments using EVLP were done on a porcine model, due to the physiological similarity in size and function to human lungs [79]. Steen and colleagues successfully preformed the first human lung transplant from a DCD donor using EVLP in 2000 [22]. In 2005 they performed a successful LTx of a non-acceptable donor lung after reconditioning ex vivo [80].

The technique is now being used to evaluate sub-optimal donor lungs [75, 81]. EVLP has been shown to improve initial lung evaluation parameters and thereby increase the safety of transplanting marginal donor organs. Improved parameters include reduced lung oedema, enhanced gaseous exchange and possible reduction of graft immunogenicity leading to comparable survival rates as with standard lung transplants [22, 76, 80, 82, 83].

A substantial amount of work is being carried out to delineate the clinical outcomes related to EVLP following LTx; however the EVLP model can also be used as an invaluable research tool. Little is known about the precise cellular and molecular mechanisms that regulate the reconditioning process during the procedure. Understanding this phenomenon could provide a fundamental basis to develop a platform to study novel drugs and treatments in the lung that can further aid transplantation, but also provide insight into lung disease in general.
The technique has gained global popularity with large-scale clinical trials currently underway in Europe, Canada, United States and Brazil [84]. The primary and secondary end points of these studies include the assessment of primary graft dysfunction and mortality rates after transplantation as measures for early and mid-term outcome. Similar studies have already been performed, which demonstrate the promising potential of EVLP [47, 53-59, 82, 83, 85-87]. Likewise, A Study of Donor Ex Vivo Lung Perfusion in United Kingdom (DEVELOP-UK) was a multi-centre clinical trial carried out in the UK aiming to assess the non-inferiority of EVLP to standard lung transplantation in terms of cost effectiveness and safety.

1.2.1 Protocols for EVLP

In short, the clinical EVLP circuit consists of an organ chamber where the lungs are placed after procurement, a ventilator connected to the trachea, a pulmonary arterial cannula connecting the vasculature to the circuit, a pump that circulates the blood replacement perfusate, a heater cooler unit that regulates the perfusate temperature, a membrane oxygenator (de)oxygcnates the perfusate, a leucocyte filter, and a series of pressure and temperature sensors (Figure 1.2) [22]. The EVLP protocol is divided into the following phases: priming of the circuit, connecting the lungs, reconditioning, evaluation and cooling [22]. Even though slight variation in the protocols utilised by different groups can be found, the main principles remain the same.

In the priming phase 1.5-2 L of high-albumin isotonic solution, usually Steen Solution™ (XVIVO, Sweden) is added to the circuit. Two different protocols may be utilised in the clinic, which primarily differ in the composition of their perfusate being either cellular or acellular. In the cellular Lund protocol, developed by Prof Stig Steen and colleagues, red blood cells (RBC) are added to the perfusate to reach a haematocrit of 10-15% [22, 47], whereas no RBC are added according to the acellular Toronto method, developed by Dr Shaf Keshavjee and his group [55]. Heparin, antibiotics and antifungals are added to prevent clot formation and to treat infections in the circuit. The perfusate is buffered to pH 7.35-7.45 to resemble the normal physiological values in the blood. If needed, the alkalising agent tromethamine (THAM) is added to the perfusate. In human EVLP, glucose levels can be manipulated with glucose and insulin, and are kept between 5-20 mmol in order to supply energy to the cells to keep them functioning under normothermic conditions.

After a blood gas analysis confirms that the perfusate meets the set criteria to begin the EVLP, lungs are connected to the circuit via the pulmonary artery (PA) cannula. An endotracheal tube is inserted to the trachea and connected to the ventilator. In the acellular Toronto protocol, the left atrium (LA) is also cannulated to keep the vessels from
kinking and obstructing the perfusate flow [55], or it can be left open to let the fluid pour directly back into the reservoir in the organ chamber for recirculation, as per the Lund protocol [22]. The organ chamber is kept covered to maintain humidity and the temperature more uniform. If possible, the lungs are stored prior to EVLP in a semi-inflated state with the trachea clamped to prevent the development of atelectasis. A gaseous mixture of O$_2$, N$_2$ and CO$_2$ is administered to the perfusate to provide necessary oxygenation to the pulmonary tissue, but also to balance the pH levels of the perfusate.

The reconditioning phase is started with low perfusate flows to gradually warm up the lungs. The flow rate is pressure controlled with the PA pressure kept below 20 mmHg. At 32°C, protective ventilation is initiated and recruitment manoeuvres can be performed to alleviate any atelectasis formation [22]. As the lungs warm up and vasodilation occurs, the flow rates will increase. The Lund protocol aims to achieve 100% of estimated cardiac output of the donor (70 mL/kg donor weight), whereas the acellular Toronto protocol aims for 40% of that [55]. Once the lungs achieve stable flow and ventilation at 37°C, the evaluation phase can be initiated.

The lungs are evaluated on their capacity for gaseous exchange. A gas mixture of nitrogen, oxygen and carbon dioxide is connected to the oxygenator to deoxygenate the perfusate so that it mimics venous blood [22], which then allows the lungs’ oxygenation ability to be assessed. The values that indicate a successful reconditioning phase are partial pressure of arterial CO$_2$ (PaCO$_2$) < 3.2 kPa (45 mmHg) and PaO$_2$ > 40 kPa (300 mmHg) with FiO$_2$=0.5. If required, bronchoscopy and suction of the airways can also be performed. Oedema formation is evaluated by a collapse test when the lungs are disconnected from the ventilator and their deflation is observed. Normal lungs collapse and become globally atelectatic, whereas oedematous areas remain unchanged. If the lungs are approved at the evaluation stage, they are accepted for transplantation and re-cooled until the procedure. If the values do not meet the transplant criteria, reconditioning and evaluation phases can be repeated [22, 47, 55].

It is not currently clear how long the lungs can be connected to the circuit before any damage can be deemed irreversible and no further improvements can be expected. The Swedish group and others have kept the EVLP periods short with no more than 4 hours of perfusion on the circuit. However, the Toronto group argue that the perfusion period can be safely prolonged for lung maintenance for at least 12, possibly even 24 hours [88], although no definitive conclusions have yet been reached. However, the Canadian group has had success in keeping porcine lungs viable for 12 hours on the EVLP circuit [89]. Using this method the EVLP circuit can also be utilised as a platform for delivering therapeutics that may help to recondition the lungs. Prolonged perfusion periods may also
benefit LTx in areas where distances are long and a prolonged perfusion might give more chances for suitable donors and recipients to be matched.
Lungs are placed on an organ chamber with a reservoir and connected via a pulmonary artery cannula. Perfusate exits the lungs through the left atrium and collects into the reservoir. The roller pump circulates perfusate from the organ chamber reservoir into the oxygenator. The heater/cooler regulates perfusate temperature by circulating water around the oxygenator, and the gas cylinder provides a gaseous mixture to oxygenate/deoxygenate the perfusate through the oxygenator. The leucocyte filter removes circulating white blood cells until it becomes saturated. The pressure within the pulmonary artery and the temperature of both the perfusate and lungs peripherally are monitored with probes in real-time whilst the organ is attached to the circuit.
1.2.2 EVLP systems currently available

Steen and colleagues engineered the mechanical parts of the EVLP system, as well as developing a buffered, albumin-rich extracellular solution that would be used as lung perfusate in the EVLP circuit, called Steen Solution™ (XVIVO Perfusion, Sweden). This system was commercially available as the older LS1, or the newer LS2 model (Figure 1.3 A). Vivoline Medical was first to make this system commercially available, but XVIVO Perfusion has recently taken over the company. The Lund protocol uses Steen Solution™ infused with RBC as perfusate, in which RBCs carry the oxygen molecules around the lung tissue. The mechanical setup incorporates a roller pump for perfusate circulation, and an open atrium from which the perfusate drains back into the organ chamber to be recirculated.

Steen Solution™ provides the lung tissue with optimal colloid osmotic pressure that keeps the fluid within the intravascular space, and delivers necessary nutrients to the cells within the organ to maintain lung viability [76]. Steen Solution™ is now globally utilised as the perfusate both in research and clinical settings, and other groups have developed variations of Steen's original EVLP circuit [88]. A group in Brazil demonstrated that using their locally produced preservation solution LPDnac yielded similar results to that of the Steen Solution™, and suggested that its use would reduce costs in their centres [90]. Likewise, a group in the UK demonstrated similar results when utilising Papworth-Blood solution, a donor lung preservation solution with limited clinical usage at present, as the perfusate for a porcine model of cellular EVLP [91]. It is important to note that however effective certain therapeutic strategies are, if their cost is too high, they will not be accessible to every centre. This can become a problem in countries where LTx is not very prevalent and an expensive procedure such as EVLP may not be affordable compared to the costs of standard transplantation. Shafaghi et al. estimated the cost of a standard LTx and EVLP in Iran, where the procedure is not covered by insurance and only 14 lung transplants were carried out in 2014, to be approximately 22,800 USD and 15,000 USD respectively [92]. As such, developing new models or components to existing ones can further propagate the use of current ones.

Steen's group developed EVLP for short-term evaluation of the donor lungs, however a prolonged perfusion period might be beneficial when considering novel therapies that require longer time to demonstrate effects. Erasmus et al. trialled the perfusion period for 6 hours to test lung preservation, recovery and repair under prolonged normothermic conditions [93]. Similarly to Steen's group, they used a porcine model but encountered increased pulmonary vascular resistance (PVR) and airway pressures [93]. Cypel et al. were the first to successfully carry out a long-term normothermic EVLP [88] in both pigs
and humans, and concluded that their acellular perfusion model can be used to safely assess, maintain and treat injured donor lungs. Cypel et al. use the Toronto protocol with the XPS perfusion system, first developed by XVIVO Perfusion (Sweden) (Figure 1.3 B). Contrary to the Lund team, they perfuse the lungs with acellular perfusate of Steen Solution™, which can be argued to be logistically simpler in a clinical setting, and to avoid the issue of short lifespan of RBCs in the circuit [94]. In the Lund protocol that is intended to last for a maximum of 4 hours, the damage and lysis of RBC is not a particular concern, but in prolonged perfusion this can lead to an increase of lactate and potassium from the dying cells, and further inflammatory response during EVLP. Cypel and colleagues therefore conclude that for extended EVLP the use of acellular perfusate the benefits outweigh using a cellular protocol [88]. Similarly, Pego-Fernandes et al. also utilise the XPS circuit with acellular perfusate, arguing that the circulating RBC may undergo mechanical damage and thereby add to the ischemia-reperfusion injury [95, 96]. However, more recently Spratt and colleagues described 24-hour cellular porcine EVLP with normalised lung haemodynamics and compliance at the end of the perfusions [97], demonstrating that the established protocols are continually being tested and altered. The fundamental distinction between the acellular and cellular EVLP protocols remains the ability of gaseous exchange assessment of the lungs. Using RBC in the perfusate means that those cells bind oxygen molecules and in the evaluation phase of the EVLP, the true oxygenation capacity of the lungs can be determined. For the acellular protocol, the gaseous exchange of the lungs may be lower than according to the blood gas readings, as there are no RBCs to bind the molecules that are simply diffused in the perfusate.

The Toronto protocol also differs from Steen’s by having a LA cannula fitted to maintain a constant positive left atrial pressure of 3-5 mmHg [88]. At the start of the perfusion, the LA cannula is connected to the circuit and a slow retrograde flow is used to de-air the PA cannula. As the perfusion is initiated with anterograde flow, the LA cannula helps to keep the pulmonary veins straight and open [88]. It has been reported that the EVLP protocol with closed LA reduces the development of oedema and improves lung physiology [89]. However, even in the Lund protocol the LA pressure is not zero as the perfusate outflow is pouring out of the LA as a continuous flume. Regardless of these criticisms however, the acellular Toronto protocol remains a popular EVLP method in clinic as well as research, with successful outcomes in both areas. In a recent study Becker et al. demonstrate that even in prolonged 12-hour EVLP both protocols can be deemed suitable with similar outcomes [98]. Recently, Nilsson and colleagues compared the cellular and acellular EVLP methods in a porcine model and demonstrated no differences in lung function, inflammatory response, ischemia-reperfusion injury or histopathological differences.
between the two groups [99], in agreement with the findings of a previous study by Roman et al. [91].

Whereas the LS1, LS2 and XPS systems are designed for clinical or research use at a specific location, the Organ Care System (OCS) Lung by Transmedics (United States) (Figure 1.3 C) is the world’s only portable lung perfusion system, currently in clinical use in Europe and Australia but not commercially available in the USA [100]. This system also uses an RBC-based cellular perfusate with OCS Solution, a roller pump, an open atrium, and a bellow-type ventilator. The need for a portable and compact normothermic EVLP system stems from the predication that cold ischemic time is detrimental to the damage inflicted to the donor lungs. As such it enables lungs to be perfused and ventilated between the donor and recipient sites reducing the time of ischemic injury. Warnecke and colleagues investigated 12 patients who received donor lungs preserved with the OCS Lung system [101]. The lungs were connected to the system for 3-10 hours. They assessed PGD, hospital stay and 30-day survival rates, and found that lungs preserved with the OSC Lung resulted in successful LTx [101]. This served as a pilot study to the international INSPIRE trial that is currently underway comparing the OSC Lung perfusion device to the standard cold flush and storage of donor lungs. However, as it was a pilot study, Warnecke and colleagues did not include a control group and concluded that they had a selection bias, but the group has declared they have addressed both issues when designing the INSPIRE trial [101]. EXPAND Lung trial utilising the OCS system is recruiting patients to further delineate on the findings of the pilot trial. The study aims to assess the mobile OCS Lung device in preserving and evaluating suboptimal donor lungs, either from donors older than 55 years, DCD donors, lungs with ischemic time more than 6 hours, and/or donor PaO$_2$/FiO$_2$ < 40 kPa (300 mmHg) [102]. Zeriouh and colleagues at the Harefield Hospital in the UK also demonstrated that OSC perfusion of 14 lungs prior to LTx resulted in excellent survival rates and superior early lung function to standard transplantation, suggesting the feasibility and safety of this method [103]. Similarly, Luc et al. report from a single-centre pilot study that the portable OCS can be utilised to objectively evaluate DCD lungs for transplantation with encouraging post-LTx outcomes [87]. In a recent publication, a porcine model utilising the OCS Lung perfusion system assessed different perfusate combinations in a 24-hour EVLP [104]. The authors concluded that the use of autologous donor whole blood enabled a 24-hour preservation period using the system, whilst also being superior to protocols with added RBC or acellular dextran-albumin solution [104]. They observed that after 6 hours of perfusion the lungs in the acellular group did not meet the standards for transplantation because of increasing vascular resistance, oedema formation and worsening compliance. After 24 hours the whole blood
group demonstrated superiority over the RBC group in terms of pulmonary arterial pressures and PaO$_2$/FiO$_2$ ratios [104].

Nevertheless, in a clinical setting it currently remains undetermined whether this mobile perfusion system is superior to the other EVLP systems, and both the cellular Lund and acellular Toronto protocols are currently used in clinics. A study in a porcine model by Mulloy and colleagues concluded that even after 60 minutes of warm ischemia, combining EVLP with cold-static preservation (4 hours at 4°C), rather than EVLP without cold storage, demonstrated the best reconditioning of lung function and lowest levels of pro-inflammatory cytokines at the end of the procedure [105]. Therefore, even if the OCS system can be considered superior in terms of logistics, its effects are not yet fully understood. As a number of trials are conducted on these different systems in larger cohorts, more will be understood on the particular benefits or shortcomings of the specific devices and protocols.
Figure 1.3 Different EVLP systems produced available at the moment
A - LS2 Perfusion System, used for the Lund protocol; B - and XPS Perfusion System, used for the Toronto protocol (both XVIVO Perfusion, Sweden). C - Transportable Organ Care System, used in the INSPIRE and EXPAND Lung trials (TransMedics, USA).
1.2.3 EVLP, removal of donor immune cells, and cytokine profile

The cellular EVLP circuit includes a leucocyte filter that has been shown to accumulate donor white blood cells during the perfusion procedure [106]. Even though the circuit utilises packed RBCs, the circulating perfusate still contains donor leucocytes [31, 106]. As previously mentioned, the immune cell repertoire in the lungs is substantial, and therefore the mechanical removal of these cells may reduce immunogenicity in the recipient once the organ is transplanted.

Our group has previously studied this in both porcine models and humans [31, 106]. In human studies, perfusate samples and leucocyte filters were collected from 7 explanted lungs undergoing EVLP. The cell populations were characterised by flow cytometry and it was found that there was a rapid diapedesis of passenger leucocytes into the perfusate during EVLP [31]. Interestingly, it was also observed that 80% of these cells were non-classical monocytes (NCM) that normally constitute only approximately 10% of the monocyte population [107]. To characterise these cells, alveolar monocytes were isolated and differentiated into DCs, and their secretory profile demonstrated a high expression of inflammatory cytokines IL-2 and IFN-γ, as well as a low expression of IL-10 [31]. The migration and differentiation patterns of monocytes were also investigated using an air-liquid interface model, and it was found that there was a rapid NCM diapedesis into the alveoli. It was demonstrated that compared to classical monocytes (CM) these NCM expressed higher levels of surface CD86, which indicates a polarisation towards an antigen-presenting phenotype. In transplants, donor-derived DCs that migrate to recipient T cell regions provide a co-stimulatory signal to T cells, and secrete inflammatory cytokines that are essential for T-cell survival, contributing to the development of AR. It can thereby be suggested that during EVLP the removal of these passenger monocytes that are involved in recipient immune responses could potentially benefit all donor lungs.

However, the accumulation of passenger cells leads to saturation of the leucocyte filters in the EVLP circuit. Leading on from this, it has also been argued that there is no objective evidence that leucocyte filters improve EVLP outcomes when this saturation happens [108]. This can, however, potentially be addressed by using multiple filters during one procedure. Moreover, in the study by our group, only the leucocyte filters from short interval EVLP were utilised, whereas different patterns of cell migration may potentially be found during a 12-hour procedure, where the use of multiple filters could be more advantageous.

In addition to the leucocyte filter, another group tested a cytokine-removing absorbent membrane in a 12-hour porcine EVLP model [109]. Kakishita and colleagues noted significantly higher levels of the pro-inflammatory cytokines IL-8 and TNF-α in the
perfusate of the control group compared to the membrane group, but detected no differences in lung function between the groups during EVLP [109]. They suggested that either those particular cytokines did not play a crucial role in deterioration of organ function, or that the increased levels of IL-8 had no effect because of the use of acellular perfusate without a notable number of neutrophils. Another explanation for the lack of improved lung function could be that the addition of the membrane also removed anti-inflammatory and protective compounds, such as IL-10 or albumin. However, these were not measured in the study and the results remain unknown. Similarly, Iskander and colleagues demonstrated reduced cytokine expression, tissue myeloperoxidase activity and microscopic lung injury with decreased pulmonary oedema and electrolyte imbalance, when utilising a cytokine filtration device in 12-hour porcine EVLP [110]. Overall, if any success is anticipated from the addition of another filter to the EVLP circuit, it needs to be particularly selective to only remove cytokines and compounds that can induce lung injury. Additionally, if the pulmonary tissue itself produces these cytokines, the possibility remains that they will still be secreted post-transplantation into the recipient. This could be investigated through the comparison of the cytokine levels in the pulmonary tissue compared to the data from the perfusate.

Sadaria et al. investigated the cytokine profile of 7 human lungs undergoing normothermic EVLP with biopsies taken at 1, 6 and 12 hours after the start of perfusion [111]. All of the lungs were successfully reconditioned and met the traditional transplant criteria after EVLP. They found that there was a significant up-regulation of the pro-inflammatory cytokines IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1) and granulocyte colony-stimulating factor (G-CSF), and a down-regulation of granulocyte macrophage colony-stimulating factor (GM-CSF). IL-1β, IL-4, IL-7, IL-12, IFN-γ, macrophage inflammatory protein-1β, and TNF-α were also detected but their levels remained unchanged. The authors speculate that the down-regulation of GM-CSF leads to decreased macrophage production and possible reduction of ischemia-reperfusion injury [111]. In this study, the increase in cytokine levels was only significant at the 12-hour time point, with the exception of IL-6 and GM-CSF, which were significantly elevated at 6 hours compared to 1 hour. Even though they reported no loss of pulmonary function or histologic injury by the end of their experiments, the cytokine levels might drastically change with a prolonged procedure. The 6-hour time point compared to 12-hour one demonstrated more significant changes in cytokine levels than 1-hour in relation to the 6-hour time point. It would be interesting to observe any fluctuations in cytokine expression at even longer intervals. In this instance however, short-term EVLP had minimal effect on the cytokines studied. It is also possible that mild elevations in concentrations of these particular cytokines have no adverse effects on the pulmonary function or histologic condition of the
organ. The absence of a control group (i.e. lungs not undergoing EVLP), and samples taken before the start of the procedure during cold ischemic time, are considerable limitations to this study. It is therefore unknown how the cytokine levels changed in the first hour of EVLP, when the perfusion had washed out the levels initially produced. As with the Kakishita et al. study, samples from the tissue could have been compared to the perfusate samples at each time point to more accurately map out the patterns of cytokine activity in the circuit.

1.2.4 EVLP as a platform for drug delivery

In addition to mechanical removal of donor cells, EVLP can also be used to deliver immunomodulatory compounds into the lung tissue. Even though it is currently unclear which cellular processes accompany the reconditioning phases of the procedure, future studies in this area can greatly benefit the way unsuitable donor lungs can be recovered.

Cypel and colleagues examined the possible outcomes of gene therapy combined with the EVLP platform by studying the effects of human IL-10 on lung repair. IL-10 is a known anti-inflammatory cytokine involved in immunoregulation and inflammation. It is associated with the production of regulatory T cell cytokines. IL-10 downregulates Th1, Th2 and allergic responses by inhibiting inflammatory DC and macrophage activity [112]. As such, understanding the regulation of IL-10 has been of great interest in developing therapeutic strategies in disease. Cypel et al. utilised 10 explanted human lungs unsuitable for transplantation and delivered an adenoviral vector encoding for human IL-10 or a placebo to the lungs. After 12 hours of normothermic acellular EVLP, this strategy resulted in significant improvements in lung function of the treated lungs compared to controls, accompanied with a shift from a pro-inflammatory to an anti-inflammatory cytokine profile, and a recovery of vascular barrier integrity [113]. They concluded that treatment of donor lungs with IL-10 could be potentially used to recover injured organs and render them suitable for transplantation. They have followed this up with a porcine LTx model after lungs on the EVLP circuit were treated with IL-10 gene therapy [114]. The authors demonstrated safety of the treatment with an improved post-transplant outcome in terms of gaseous exchange and decreased production of IFN-γ by the lymphocytes [114].

In a study by Emaminia et al., EVLP was used as a platform to deliver an adenosine A_{2A} agonist to pig lungs, which were subject to 14 hours of cold storage [115]. They hypothesised that because adenosine has been known to protect the lung against ischemia-reperfusion injury, administration of its A_{2A} agonist after a significant period of cold ischemia would attenuate the acute inflammatory response and improve pulmonary function. The drug was added to the EVLP circuit at priming and after 3 hours of perfusion. They found that combining EVLP with administration of adenosine A_{2A} agonist
significantly enhanced lung function, assessed by declined oedema formation and mean airway pressures, and decreased levels of IFN-γ. Other inflammatory cytokines including IL-1β, IL-6 and IL-8 were also reduced in the adenosine agonist group, but the results were not statistically significant [115]. The authors do, however, suggest that the use of the XPS system with acellular perfusate could mean that important sources of inflammatory cytokine production were excluded, and that because adenosine A<sub>2A</sub> agonist is a fast-acting drug, prolonged perfusion had no additional benefits.

Similarly, another group investigated the administration of pulmonary surfactant via EVLP circuit in porcine models [116, 117]. The physiological pulmonary surfactant system decreases the surface tension of alveoli and prevents their collapse. During acute lung injury, the surfactant system is damaged and active surfactant is necessary to regularise gaseous exchange, compliance and alveolar stability [117]. In the earlier study, Inci and colleagues induced lung injury in pig lungs with a betaine-HCl/pepsin mixture. They administered surfactant Curosurf with intrabronchial lavage and found that the treatment group demonstrated lower pulmonary vascular resistance and lessened oedema formation [116]. Subsequently, the same group tested Category III DCD lung reconditioning on EVLP system with diluted surfactant lavage also in a porcine model [117]. Corresponding with their previous findings, the surfactant treated group showed better oxygenation, lower pulmonary vascular resistance and lower oedema formation. They also measured lower concentrations of IL-1β and IL-6 in both the serum and BAL of the surfactant group. Additionally, the percentage of neutrophils in BAL was significantly diminished in the surfactant group. Neutrophil infiltration into the lung tissue was also decreased in the treated group compared to controls at the end of the experiment. The authors suggested that surfactant lavage delivers the required amount of active surfactant into alveoli and thereby normalises their surface tension and function, while partially removing alveolar and airway debris [117]. In agreement with previously mentioned studies, Inci et al. found improved lung reconditioning and function in the groups with lower inflammatory cytokine levels. Recently, Nakajima et al. demonstrated that lung lavage followed by surfactant administration during porcine EVLP decreased IL-1β, IL-6, IL-8 and phospholipase A<sub>2</sub> contributing to improved post-LTx lung function [118].

Numerous other therapeutic agents have been investigated utilising the EVLP model. Mordant and colleagues studied human mesenchymal stem cells (MSCs) in a porcine EVLP model and concluded that intravenous delivery was superior to the intratracheal route with an optimal tolerated dose of 150 million MSCs [119]. This therapy was associated with a decreased concentration of pro-inflammatory IL-8 in the perfusate [119]. In a recent study, Hamid and colleagues demonstrated that aspirin alleviates neutrophil-
dependent alveolar damage in a lipopolysaccharide (LPS)-induced model of pulmonary injury in healthy volunteers but also in EVLP of human lungs rejected for transplant [120]. They described decreased neutrophilia in the BAL of the perused lungs that had aspirin intervention, which adds to the body of evidence that aspirin may be considered an effective treatment for acute respiratory distress syndrome (ARDS), a severe form of ALI [120]. Lin et al. administered α1-anti-trypsin to porcine lungs undergoing a 12-hour treatment of normothermic acellular EVLP and demonstrated reduced pulmonary oedema, inflammation and cell death in response to the drug [121]. Antioxidant treatment has also been studied in the context of EVLP and recently, Yamada and colleagues administered nebulised N-acetylcysteine, a glutathione precursor, during porcine EVLP and assessed the post-LTx lung function [122]. They demonstrated trends of improved oxygenation with inhibited myeloperoxidase activity and NF-κB activation. Cosgun and colleagues administered trimetazidine, an antianginal agent, intravenously during 4-hour porcine EVLP [123]. They described inhibited neutrophil activation, lipid peroxidation and alveocapillary membrane permeability in the transplanted lung resulting in protection against ischemia-reperfusion injury. Another study investigated the benefits of the addition of anti-thrombosis drug alteplase into the preservation flush in a DCD porcine EVLP model [124]. The authors demonstrated no significant improvement in the lungs following the intervention; however they concluded that DCD lungs are a safe and effective method of increasing the donor pool.

Evidently, there is a great interest in the research delineating on the reconditioning mechanics of the lungs undergoing ex vivo perfusion. However, not all marginal lungs recondition during EVLP, therefore inter-patient variability is an important factor to consider when testing new therapeutics. Andreasson and colleagues demonstrated that the perfusate from the lungs that did not recover during EVLP contained significantly higher levels of IL-1β and IL-8 compared to the lungs that did [125]. This suggests that certain cytokines, such as IL-1β and IL-8, can be used as a predictor of organ repair in EVLP, but also that inhibiting the IL-1β levels can be used as potential therapeutic intervention. Further research is warranted to investigate the specific mechanisms by which these pro-inflammatory cytokines are released and if their production can be manipulated in order to improve on the quality of EVLP lungs.
1.3 Interleukin-1\(\beta\) and the Inflammasome

1.3.1 Synthesis and secretion of IL-1\(\beta\)

IL-1\(\beta\) is a potent pro-inflammatory pyrogen, belonging to the interleukin 1 family of cytokines. It has a variety of effects on various cell types and therefore its production and secretion mechanisms are tightly regulated within the cell. It is most commonly secreted by blood monocytes, tissue macrophages and dendritic cells, but even airway epithelial cells have been demonstrated to release small amounts upon stimulation and after interactions with leucocytes [126]. IL-1\(\beta\) is synthesised in response to pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) binding to toll-like receptors (TLR) (Figure 1.4). This first signal initiates the NF-k\(\beta\) pathway activation in the nucleus leading to the production of non-active form of pro-IL-1\(\beta\) (molecular weight 31 kDa) as well as the Nucleotide binding and Leucine rich repeat Receptor (NLR) family particles [127]. The inactive pro-IL-1\(\beta\) remains intracellular and only becomes activated when cleaved by caspase-1 into mature IL-1\(\beta\) (molecular weight of 17 kDa) that is then secreted by the cell. A secondary cellular stress signal initiates the assembly of the inflammasomes - large multi-protein complexes that facilitate the activation of IL-1\(\beta\). There are several members in the inflammasome family but the NLR pyrin domain-containing type 3 (NLRP3) inflammasome is the most widely studied form and can be activated by numerous danger signals. It is formed by oligomerisation of NLRP3 particles with apoptosis-associated Speck-like protein with a caspase-recruitment domain (ASC) adaptor molecules, and pro-caspase-1 [128]. The latter is then cleaved into its active form of caspase-1, which in turn cleaves pro-IL-1\(\beta\) into the mature IL-1\(\beta\) that can subsequently be secreted (Figure 1.5). Extracellular IL-1\(\beta\) binds to the IL-1 receptor and exacerbates inflammation. In addition, extracellularly secreted ASC and NLRP3 inflammasome specks have been demonstrated to amplify pro-inflammatory responses in neighbouring cells [128].

Similarly, activation of IL-18, another cytokine of the IL-1 family, requires the assembly of the inflammasome complex and cleavage by caspase-1. However, contrary to pro-IL-1\(\beta\), the inactive pro-IL-18 is also consistently intracellularly present, does not necessitate the first stimulatory signal to be synthesised, and it can be expressed extracellularly in its uncleaved form [129]. IL-18 induces the secretion of IFN-\(\gamma\) and T helper type 1 immune responses. IL-18 binding protein (IL-18BP) blocks IL-18 by binding to the cytokine at a significantly higher affinity than the IL-18 receptor, and dissociating at a low rate [130]. The serum levels of IL-18BP in healthy adults are augmented compared to the levels of IL-18 itself [131]. These inflammasome-dependant cytokines then attract neutrophils and monocytes and cause inflammation-related cell death, called pyroptosis. Similarly to
necrosis, this pro-inflammatory form of cell death involves disintegration of the cellular membrane and the release of intracellular contents into extracellular space [132]. However, similarly to apoptosis, nuclear condensation and DNA fragmentation occur during pyroptosis [132].

Different groups have quantified the inflammasome particles in human serum samples and concluded that activation of inflammasomes leads to the release of extracellular inflammasome particles from macrophages that further amplify the inflammatory response [128, 133]. Thus blocking this specific part of the IL-1β secretion pathway may lead to mechanisms of manipulating inflammatory signalling.
Figure 1.4 NLRP3 inflammasome activation and secretion of mature IL-1β.

Signal 1: Pro-IL-1β and NLRP3 particles are synthesised upon TLR activation by PAMPs and DAMPs. Signal 2: potassium efflux, lysosomal damage or activation of ROS trigger the assembly of NLRP3, ASC and pro-caspase-1 that form the inflammasome. Active caspase-1 then cleaves pro-IL-1β into its active form of IL-1β, which is secreted by the cell. Adapted from Stutz et al. [134].
Figure 1.5 Structure of NLRP3 inflammasome.

NLRP3 particles are synthesised upon stress signals received by the cell. They then bind to ASC adapter molecules that recruit pro-caspase-1 into the oligomerising complex. The pro-caspase-1 is then self-cleaved into caspase-1 that subsequently cleaves pro-IL-1β and pro-IL-18 into their biologically active forms.
1.3.2 IL-1β and inflammasome targeting therapies

IL-1β is associated with several inflammation-related diseases such as sepsis, rheumatoid arthritis, gout, sickle cell anaemia, familial Mediterranean fever, cryopyrin-associated periodic syndrome, and others, but also notably lung injury [135, 136]. In mice, bleomycin induces fibrotic pulmonary damage by increased IL-1β levels and neutrophillic inflammation, which can be reversed by IL-1 receptor antagonist therapy [137]. Additionally, asbestos and silica particles can activate inflammasome complexes and promote IL-1β production and lung injury [138]. More recently, inflammasome has been demonstrated to be involved in inflammatory processes in clinical pulmonary disease. The NLRP3 inflammasome is proposed to mediate ventilator-induced lung injury [139], adenosine triphosphate (ATP) released in lung epithelial cell injury has been shown to augment pulmonary fibrosis [140] and inflammasome-related cytokines to be contributing to the development of ARDS [141]. Thus increasing interest in inflammasome-specific targeted therapies has become more prevalent.

IL-1β targeted therapies have been successful in some diseases, for example canakinumab, a monoclonal IL-1β antibody, rilonacept, an IL-1 blocker, and anakinra, an IL-1 receptor antagonist, have been recognised as effective treatments and are widely used [136, 142]. In a rat EVLP and LTx model caspase-1 inhibition together with leucocyte removal from the circuit resulted in marked improvement in the lung quality [143]. Whilst it is possible to inhibit the mature IL-1β and observe improvements in certain conditions, novel, targeted and more effective potential therapies are under investigation. As IL-1β activation requires inflammasome oligomerisation, it could be postulated that this offers an innovative and specific therapeutic target. Indeed, the inflammasome inhibition therapies have been gaining major interest in treating IL-1β related diseases. In EVLP where a portion of the suboptimal-for-transplantation lungs do not recover, and that express augmented levels of IL-1β, inflammasome inhibition therapy might yield a novel and exciting form of therapy.
1.4 Aims and objectives

Given that IL-1β has been liked to organ recoverability on the EVLP circuit, the aim of this thesis was to investigate the involvement of IL-1β and NLRP3 inflammasome activation pathways in irreversible lung injury. The hypothesis was that these specific inflammatory pathways would be predictive of organ recovery and function. The aim was to investigate whether these pathways could be manipulated, and if novel inflammasome-targeted therapies delivered via the perfusion system would encourage organ recovery. The aim of the following chapters was to evaluate the existing understanding of the cellular processes underlying ex vivo organ perfusion, and to assess the potential of this model as a tool to manipulate immune responses occurring in the lung.

Firstly, the objective was to assess if inflammasome inhibitor therapy introduced to healthy porcine lung on the EVLP system changes the overall inflammatory response, specifically IL-1β and inflammasome levels, and organ function over a short-period perfusion.

Secondly, inflammasome inhibitor therapy was introduced to a porcine failing organ perfusion model to assess the inflammatory profile and recoverability of those organs.

Thirdly, the IL-1β-associated molecules in clinical human EVLP were measured, distinguishing between recoverable versus non-recoverable organs, to further elucidate the IL-1β activation pathway.

Finally, the involvement of the IL-1β release pathway was investigated in the context of lung transplantation. The correlation between early lung function and plasma levels of IL-1β-related molecules in LTx recipients was examined in order to assess whether lung injury post-transplant would demonstrate similar patterns in organ recoverability to the ex vivo perfusion system.
Chapter 2: Methods
In order to provide a clear overview and to deliver main chapters that are easier to follow for the reader, all methods undertaken for the work in this thesis are collectively presented in the following chapter. Here all methods will be discussed in more depth and detail than in the individual chapters that follow thereafter. A number of these methods have been repeated for different parts of this research and hence would become repetitive in the chapters below.

2.1 Porcine EVLP model

A porcine EVLP model was utilised to study the effects of inflammasome inhibition in healthy organs in order to establish if this therapy would impact on lung function and cytokine release (Figure 1.2). The study was designed in collaboration with Prof Stig Steen's group from the University of Lund and was performed at their laboratory in Igelösa, Sweden. The Ethical Committee of the University of Lund granted approval for the study. The animals were treated in accordance to the ‘Principles of Laboratory Animal Care’ by the National Society of Medical Research, and the ‘Guide for the Care and Use of Laboratory Animals’ by the National Institutes of Health, 1985.

2.1.1 Organ procurement

Organ procurement and EVLP were performed based on the previously published protocol by the Lund group [76] after inducing circulatory death with minimal warm ischemia. Healthy domestic pigs (n=10) ranging from 35-75kg (median weight 35 kg) were used in this set of experiments. To retrieve the lungs, pigs were sedated via an initial muscle injection consisting of 0.5 mg atropine (Kabi Pharmacia, Sweden), 100 mg xylasin (Bayer, Sweden) and 20 mg ketamine (Parke-Davis, USA) per kg of bodyweight, followed by intravenous medication of fentanyl (Lilly, France) 4 μg/kg bodyweight and midazolam (Roche, Switzerland) 0.4 mg/kg bodyweight. Once sedated, the animals underwent a tracheotomy and an endotracheal tube was introduced, after which pressure-controlled ventilation with a Siemens Servo ventilator 300 (Siemens-Elema, Sweden) was started. A sternotomy was subsequently performed; an injection of 500 iU/kg bodyweight of heparin (Leo Pharma, Sweden) was given and the superior vena cava was incised. The blood that was collecting within the chest cavity was suctioned to be cell saved in order to add autologous packed RBCs to each EVLP circuit. The pulmonary artery was then cannulated and the lungs were flushed in situ with 2 L 8°C Perfadex solution (XVIVO Perfusion, Sweden) with added calcium chloride (1 mmol/L) (Vitrolife AB, Sweden) and glyceryl trinitrate (GTN) (5 mg/L) (Meda, Sweden). The heart-lung block was then dissected, with the lungs kept in a semi-inflated state, and placed into cold saline, and kept at 8°C.
2.1.2 Priming

The circuit was primed with 2 L of Steen Solution™ (XVIVO Perfusion, Sweden), 10 000 iU of heparin and approximately 0.5 L of packed red blood cells to reach the target haematocrit of 10-15%. The temperature of the circuit was initially set to 15°C in order for the lungs to be gradually rewarmed. The pH of the perfusate was adjusted to 7.35-7.45 with THAM (Kabi, Sweden). Blood gas analysis was performed using the Radiometer 800 device (Radiometer Ltd, UK) to check that the haematocrit, base excess, electrolytes, pH, pCO2 and pO2 levels matched physiological parameters. In the priming phase 40 mg of flufenamic acid (Sigma-Aldrich, UK), a potent and specific NLRP3 inflammasome inhibitor was added to the circuit prior to connecting the lungs (n=5). The dosage concentration was calculated based on previous work of collaborators of this group who used flufenamic acid in in vivo murine experiments [144]. Flufenamic acid was dissolved in 8 mL of 25% cyclodextrin (Sigma-Aldrich, UK) in normal 0.9% saline (Baxter Healthcare, UK). The pH of the solution was buffered to 7.9 with 0.2 M NaOH (Sigma-Aldrich, UK) solution. In the control group (n=5) only the carrier, 25% cyclodextrin in saline with matched pH, was added to the circuit. The EVLPs were performed for 2 hours as described below.

In n=6 cases only the right single lung was connected to the EVLP circuit. The readings and results from the EVLP were normalised to be comparable to the double-lung EVLPs with the premise of the right lung constituting 60% of the total lung capacity and size. Within this cohort, n=2 were assigned to the inhibitor group and n=4 to the control group.

2.1.3 Reconditioning

The lungs were connected to an EVLP system with an integrated roller pump, membrane oxygenator, heater/cooler unit, leucocyte filter and pressure and temperature probes (Figure 2.1). This marked the start of the perfusion. The lungs underwent gradual pressure-controlled reperfusion and rewarming to achieve a target flow rate of 70 mL/kg bodyweight/min and a temperature of 37°C. A gaseous mixture of nitrogen, oxygen and carbon dioxide was connected to the circuit in order to provide the lungs with necessary oxygen, and balance the perfusate pH and blood gas values. Protective volume-controlled ventilation was started at 32°C with full ventilation achieved at 37°C. Recommended maximum ventilation settings were 100 mL/kg donor body weight/min, 50% FiO2, PEEP of 5 cmH2O and frequency of 7-15/min. Blood gases were regularly checked throughout the perfusion and corrected to maintain physiological parameters.

2.1.4 Evaluation and end of the perfusions

The evaluation phase was initiated when the pO2 was greater than 30 kPa (225 mmHg), the lungs had stabilised and it was deemed suitable by the surgical team. At the start of the evaluation phase, oxygen was disconnected from the EVLP circuit, and replaced with a
special gas mixture of 93% nitrogen and 7% carbon dioxide to allow the evaluation of the gaseous exchange of the lungs at ventilation with FiO₂ of 50%. This meant that the perfusate in the circuit was deoxygenated via the oxygenator, and as such the pO₂ and pCO₂ levels measured after 5 minutes, were reflecting the lungs’ oxygenation capacity. Both venous (perfusate entering the lungs) and arterial (perfusate exiting the lungs) samples were taken for evaluation. Lung function was determined by haemodynamic analysis and gaseous exchange. The lungs were deemed acceptable for a theoretical transplant if the blood gas analysis demonstrated: pO₂ > 20 kPa (150 mmHg) and pCO₂ < 6 kPa (45 mmHg) at ventilation with FiO₂ 50%.

Oxygen uptake was calculated by the following equation:

\[ O₂ \text{ uptake (mL/min)} = CO \times Hb \times 1.4 \times (sₐO₂ - sᵥO₂) \times 0.01 \]

CO = cardiac output, in this case the perfusate flow rate (L/min)
Hb = haemoglobin concentration (g/L) (1 g haemoglobin binds 1.4 mL O₂ when 100% saturated)
sₐO₂ = saturation of arterial oxygen (%)
sᵥO₂ = saturation of venous oxygen (%)

After the evaluation, oxygen was re-connected to the circuit and the lungs were returned to reconditioning parameters. N=8 EVLPs were completed at 120 minutes in normothermia when the lungs were in the reconditioning phase. In n=2 cases a cooling phase was initiated with steadily decreasing temperature and flow rates before the 120-minute time point, where the final flow, temperature, pulmonary arterial pressure (PAP) and PVR values were recorded prior to the cooling (marking the 'End' time point reading). For the cooling phase the trachea was clamped with the lungs semi-inflated at 32°C, and at 15°C the topical cooling kit was placed on top of the lungs. Final perfusate samples were collected at the 2-hour time point before circulation was stopped.

2.1.5 Sample collection

Perfusate sample were collected at baseline (at the end of the Priming phase without the lungs connected), and at 60- and at 120-minutes from the start of the perfusion. All samples were centrifuged consecutively at 300 g and 1000 g for 10 minutes each at 4°C. The slower speed was used for the plasma samples that were going to be used to measure the inflammasome particles, as at higher speeds a loss of these particles of interest could have occurred. Samples spun at the higher speed were used for cytokine analysis in the Luminex experiments. All of the plasma samples was stored at -80°C.
Figure 2.1 Porcine lungs on the EVLP circuit

Porcine lungs connected to the ex vivo lung perfusion circuit and perfused according to the Lund protocol at the Igelösa laboratory, Sweden. The trachea (yellow arrow) was connected to a ventilator, whereas the pulmonary artery (green arrow) was connected to the EVLP circuit.
2.2 Porcine ex vivo kidney perfusion model

The aim was to utilise EVLP to also study inflammasome inhibition in a lung injury model to investigate its effects on organ recovery. However, in the absence of a working large animal model for lung injury available at the time of the experiments, a porcine ex vivo normothermic perfusion (EVNP) kidney model was utilised instead (Figure 2.2). The experiments were conducted at the perfusion laboratory in the University of Manchester, utilising Landrace pigs from a local abattoir. The animals, with a mean weight of 80 kg, were culled according to the regulations of the Schedule 1 of the Home Office Act of 1986 at the local abattoir, where organ procurement and preservation were conducted.
Figure 2.2 Schematic representation of EVNP circuit.

The kidney is placed into an organ chamber with a reservoir and connected via a renal artery cannula. Perfusate exits the kidney through the renal vein and collects into the reservoir. The centrifugal pump circulates perfusate from the organ chamber reservoir into the oxygenator. The heater/cooler regulates perfusate temperature by circulating water around the oxygenator where the gas cylinder provides carbogen to oxygenate the perfusate. The pressure and the temperature of the perfusate are monitored with probes in real-time before it enters the renal artery, whilst the organ is attached to the circuit. The perfusate is kept at 38°C from the start of the perfusion.
2.2.1 Organ procurement

N=10 pigs were rendered unconscious by electrocution, and culled by exsanguination. Approximately 3 L of whole blood was collected from each animal, mixed with 25,000 iU unfractionated heparin (Fannin, UK) and stored on ice for later cell saving. The abdomen of the animal was then opened by midline incision; the kidneys were excised en-bloc, inspected for any visible damage, i.e. cysts or lacerations, and placed over ice. For each individual kidney the renal artery and ureter were isolated and cannulated, the renal vein was left open. 20 mL of GTN (Hameln Pharmaceuticals, UK) was flushed through kidneys for vasodilation. The organs were then flushed with 1 L of 4°C Soltran preservation solution (Baxter Healthcare, UK) supplemented with 10,000 iU of unfractionated heparin at a hydrostatic pressure of 100 cmH₂O until the effluent from the renal veins was running clear and the kidney was uniformly blanched. The organs were then placed into ice-cold Soltran solution for a standardised period of 2 hours of static cold storage. The organs were transported to the perfusion laboratory at the University of Manchester. After 2 hours of cold ischemia, the kidneys were flushed with 10 mL of GTN and 200 mL 4°C Ringer's isotonic solution to remove the residual preservation solution. The kidney was weighed, and placed onto an EVNP circuit. Ringer's isotonic solution was prepared in the laboratory prior to each organ procurement and perfusion by dissolving 8.6 g NaCl (Fisher Scientific, UK), 0.33 g CaCl (Sigma-Aldrich, UK) and 0.3 g KCl (Sigma-Aldrich, UK) in 1 L distilled H₂O.

2.2.2 Priming

The EVNP circuit was designed and assembled using a reservoir, a centrifugal pump, a membrane oxygenator (Haemonetics, US) and a heater-cooler system, with connected syringe driver and temperature- and pressure probes. The perfusion procedure was established based on previously available and published protocol [145]. The EVNP system was filled with 350 mL of Ringer's solution, 25 mL of 10% mannitol (Sigma-Aldrich, UK), 20 mL of 8.4% sodium bicarbonate (Sigma-Aldrich, UK), 8 mg dexamethasone (Hameln, UK) and 10,000 iU unfractioned heparin. The membrane oxygenator of the EVNP circuit was connected to a carbogen (mixture of 95% oxygen and 5% carbon dioxide) (BOC, UK) supply and infused into the perfusate at a rate of 0.5 L/min. A mix of 50 mL Nutriflex (B. Braun, Germany) and 10 mL epoprostanol (Sandoz, UK) with 40 iU insulin (Novo Nordisk, Denmark) was set up on a syringe driver and infused into the circuit at 20 mL/hr. The perfusate temperature was set to 38°C from the start of the perfusion. The whole blood collected during organ procurement was washed with 0.9% normal saline using a Cell Saver 5+ Autologous Recovery System (Haemonetics, US), and leucocyte filtered (Haemonetics, US). Approximately 900 mL of filtered RBC was added to the perfusion circuit to reach a target haematocrit of 25-28%.
Novel boron compound (NBC) 6, a novel boron-based NLRP3 inflammasome-specific inhibitor, was used in the EVNP circuit, rather than flufenamic acid utilised in the EVLP experiments. 53 mg of the NBC6 drug was added to the circuit prior to connecting the kidneys (n=5). A top-up dose of 26.5 mg of NBC6 was added into the arterial line of the circuit every 2 hours in order to prevent the system being depleted of the drug. The drug concentrations were calculated based on previous work by this group’s collaborators at the University of Manchester who synthesised the drug in their laboratory [146]. 53 mg NBC6 was dissolved in 530 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK), 530 μL cremophor (Sigma-Aldrich, UK) and 9.54 mL phosphate buffered saline (PBS) (Sigma-Aldrich, UK). N=5 kidneys were allocated to the control group and were perfused without the drug. The study was not blinded.

2.2.3 Perfusion
The kidneys were connected to the EVNP circuit via the renal artery (Figure 2.3). The perfusate exited the organ via the open renal vein and collected in the reservoir. The perfusate flow rate was adjusted to maintain the renal arterial pressure at 75 mmHg. The organ chamber was covered to sustain consistent temperature and humidity. Urine was collected via the cannulated ureter, measured hourly and replaced with an equal amount of fresh Ringer’s solution to prevent loss of fluids from the system. Kidney function was determined by the renal flow rate, urine output, which were normalised according to the size of the organs, and serial blood gas values (Table 2.1).

2.2.4 Sample collection
Perfusate samples were taken at baseline before the kidneys were connected, and then at 30, 60, 180 and 360 minutes from the start of the perfusions. 500 μL of the perfusate sample was used fresh for flow cytometry. The rest of the samples were centrifuged consecutively at 300 g and 2000 g for 10 minutes each at 4°C. The plasma from the samples was stored at -80°C.
Figure 2.3 Porcine kidney on the EVNP circuit

Porcine kidney connected to the *ex vivo* kidney perfusion circuit and perfused with Ringer's solution with red blood cells at the perfusion laboratory at the University of Manchester. The ureter (yellow arrow) and renal artery (green arrow) were cannulated upon organ retrieval.
Table 2.1 Parameters for the porcine EVNP blood gas analysis with normal reference ranges and strategies to correct any deviations.

Adapted from this group’s Porcine *Ex Vivo* Kidney Perfusion (EVNP) Operating Procedure Summary of Guidelines Version 4.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Range</th>
<th>How to Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.35 – 7.45</td>
<td>If out of range, review CO₂ and HCO₃⁻ levels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- high CO₂ and low HCO₃⁻ can lower pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- low CO₂ and high HCO₃⁻ can raise pH</td>
</tr>
<tr>
<td>pO₂</td>
<td>75–100 mmHg</td>
<td>If low, increase oxygen flow rate</td>
</tr>
<tr>
<td></td>
<td>10–14 kPa</td>
<td>If high, no need to correct for the kidney circuit</td>
</tr>
<tr>
<td>pCO₂</td>
<td>35–45 mmHg</td>
<td>If low, increase carbogen flow rate</td>
</tr>
<tr>
<td></td>
<td>4.5-6 kPa</td>
<td>If high, reduce carbogen flow rate</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>22 - 28 mmol/L</td>
<td>If low, add 1 mmol/L NaHCO₃ per mmol/L out of range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>If high, ensure pCO₂ is correct</td>
</tr>
<tr>
<td>Base Excess</td>
<td>+/- 2 mmol/L</td>
<td>Correct HCO₃⁻ and pCO₂ and if remains out of range add 3mmol THAM per minus unit in Base Excess</td>
</tr>
<tr>
<td>Na⁺</td>
<td>135 – 145 mmol/L</td>
<td>If low, add 1mmol/L Na⁺ per mmol/L out of range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>If high, ensure adequate circulating volume and if not add more Ringer’s or packed RBCs</td>
</tr>
<tr>
<td>K⁺</td>
<td>3.5 – 8 mmol/L</td>
<td>If low, add 1mmol/L K⁺ per mmol/L out of range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- If high, add 10-20 iU Actrapid insulin +/- glucose dependent on level</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- If remains high reduce circuit pressure to reduce haemolysis</td>
</tr>
<tr>
<td>Glucose</td>
<td>5–20 mmol/L</td>
<td>If low, add 20 mL 5% glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>If high, add 10 iU Actrapid Insulin</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>25-28%</td>
<td>If low, add more packed RBCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>If high, add more Ringer’s solution</td>
</tr>
<tr>
<td>Lactate</td>
<td>&lt;4 mmol/L</td>
<td>If high, ensure no obstruction to perfusate flow</td>
</tr>
</tbody>
</table>
2.3 Luminex Analysis

The concentrations of cytokines and chemokines in EVLP and EVNP were assessed using the neat perfusate plasma samples that were centrifuged at 1000 \textit{g} or 2000 \textit{g} respectively. A commercially available porcine 13-plex (GM-CSF, IFN-\textgamma, IL-1\textalpha, IL-1\beta, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-\alpha) magnetic bead Luminex panel (Merck Millipore, USA) was utilised according to the manufacturer’s protocol guidelines. In brief, 200 \textmu L of assay buffer was added to the wells of the 96-well plate, which was incubated for 10 minutes and then the buffer discarded. 25 \textmu L of standards, controls, buffer, matrix solution and sample was added to the designated wells in duplicates. 25 \textmu L of beads was added to each well. The plate was incubated overnight at 8°C on a plate shaker. The following day the contents of the wells were discarded, while using a magnetic separation block (Merck Millipore, USA). 50 \textmu L of detection antibodies was added per well, the plate was covered with foil and incubated at room temperature for 2 hours on a plate shaker. Next, 50 \textmu L of streptavidin-phycoerythrin was added per well, the plate was covered with foil and incubated at room temperature for a further 30 minutes on a plate shaker. The plate was washed thrice, and 100 \textmu L of sheath fluid was then added into each well before reading it using a Bio-Plex 200 system (BioRad, UK).
2.4 Flow cytometry on cellular diapedesis in EVNP

Immunophenotyping of the EVNP perfusate samples was performed on a BD LSR II flow cytometer (BD Biosciences, UK). This was not available for the EVLP perfusate samples as the experiments were conducted in the laboratory in Sweden without access to a flow cytometer. The selection and titration of the antibodies, as well as setting up the cytometer parameters, was completed according to previous experimental designs by this group. The antibodies were divided between 4 panels depending on their fluorescence and cell types under consideration. 100 μL of EVNP perfusate was incubated with each pre-made antibody cocktail at 4°C for 30 minutes in the dark. Cells were then treated with 2 mL of RBC lysing solution (BD Biosciences, UK), incubated in the dark for 10 minutes at room temperature, washed with 1 mL of flow staining buffer - PBS with 2% foetal calf serum (FCS) (Sigma-Aldrich, UK) - and re-suspended in 300 μL of flow staining buffer. 20 μL of 123count eBeads (eBioscience Ltd, UK) was added to each sample tube to be used to calculate the number of cells per mL in sample. Samples were analysed for 3 min on the flow cytometer. Cell numbers were calculated in Microsoft Excel software (Microsoft, USA) according to the formula: total cell count = (cell count x eBeads volume x eBeads concentration) / (eBeads count x cell volume).

All gating strategies were performed using FlowJo version 10.0.6 (FlowJo LLC, USA). First, the counting beads were gated out of the total number of events. Then all cells were gated from sample debris based on the size according to forward scatter and side scatter. A panel of antibodies was utilised to characterize B cells (CD3-CD21+), helper T cells (CD3+CD4α+), cytotoxic T cells (CD3+CD8α+) (Figure 2.4); classical monocytes (CD14++CD163-), intermediate monocytes (CD14+CD163-), non-classical monocytes (CD14+CD163+) (Figure 2.5); natural killer (NK) cells (CD335+) (Figure 2.6); immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), and mature eosinophils/basophils (6D10-2B2+) (Figure 2.7). The antibodies utilised in this study were conjugated as follows: phycoerythrin: Cy-7 tandem conjugate (PE-Cy7) anti-CD3, Alexa Fluor 647 anti-CD8α (BD Bioscience, UK), PE anti-CD4α, fluorescein isothiocyanate conjugate (FITC) anti-CD21, FITC anti-CD14, PE CD163, Alexa Fluor 488 anti-CD335, FITC anti-2B2, PE anti-6D10 (Bio-Rad Laboratories, UK).
Figure 2.4 Gating strategy for B cells and T cells in the perfusate samples of porcine EVNP

Beads were gated out of the total event count and used for total cell calculations (A). All cells were selected by side scatter and forward scatter leaving out the debris (B). B cells were gated for being CD21+ and CD3- (C). CD3+ cells were selected for overall T cell count (D). Out of CD3+ cells, helper CD4+ T cells (E) and cytotoxic CD8+ T cells (F) were gated.
Figure 2.5 Gating strategy for monocytes in the perfusate samples of porcine EVNP. Beads were gated out of the total event count and used for total cell calculations (A). All cells were selected by side scatter and forward scatter leaving out the debris (B). Classical monocytes CD14++CD163- (C), intermediate monocytes CD14+CD163- (D), non-classical monocytes CD14+CD163+ (E) were selected.
Figure 2.6 Gating strategy for NK cells in the perfusate samples of porcine EVNP. Beads were gated out of the total event count and used for total cell calculations (A). All cells were selected by side scatter and forward scatter leaving out the debris (B). NK cells were gated for being CD335+ (C).
Figure 2.7 Gating strategy for neutrophils and eosinophils/basophils in the perfusate samples of porcine EVNP.

Beads were gated out of the total event count and used for total cell calculations (A). All cells were selected by side scatter and forward scatter leaving out the debris (B). Mature neutrophils were gated for being 6D10+2B2+, immature neutrophils 6D10+2B2- and mature eosinophils/basophils 6D10-2B2+ (C).
2.5 THP1 cell stimulation for inflammasome speck assessment

The initial experiments setting up flow cytometry parameters to assess the NLRP3 inflammasome particles were done using a THP1 human monocytic cell line (Figure 2.8). Supernatants were collected from either resting cells or stimulated cells in culture. The THP1 are derived from an acute leukaemia patient and it has been suggested that this cell line is suitable for studying novel molecular mechanisms in monocytes and macrophages [147]. The cells were cultured in T75 tissue culture flasks (Sigma-Aldrich, UK) in Roswell Park Memorial Institute medium (RPMI) medium (Sigma-Aldrich, UK) supplemented with 10% FCS (Sigma-Aldrich, UK). As the THP1 cells are non-adherent, the medium with the cells was firstly centrifuged at 500 g for 5 minutes at room temperature. The cells were then counted and seeded at 2 million cells per well in a 6-well tissue culture plate (Sigma-Aldrich, UK). 0.5 µM phorbol myristate acetate (PMA) was added into each well to stimulate cell adhesion. After 3 hours the medium with PMA was removed and replaced with fresh 10% FCS medium. Following an overnight incubation at 37°C, the cells were primed with 4-hour stimulation of 1 µg/mL LPS in 800 µL serum-supplemented medium, followed by 1-hour stimulation with 10 µM nigericin in serum-free RPMI medium. The supernatants were collected and the plates with cells discarded. The supernatants were then centrifuged for 5 minutes at 300 g at 4°C, stored at -20°C and transferred to -80°C the following day. These samples were used to set up flow cytometry experiments with staining for ASC+, NLRP3+ and ASC+NLRP3+ inflammasome specks, as stimulated THP1 cells would release increased levels of extracellular inflammasome particles.

2.5.1 Staining and gating strategy for ASC+ and NLRP3+ inflammasome particles in human samples

The staining and gating strategies for inflammasome particles were conducted following a previously published protocol by this group's collaborators in Spain [128]. 1 mL of collected supernatants from stimulated or unstimulated THP1 cells were incubated for 1 hour at room temperature on a plate shaker with 1 µg rabbit polyclonal allophycocyanin (APC)-conjugated anti-ASC antibody (Caltag Medsystems, UK) and 1µg primary NLRP3 mouse monoclonal anti-NLRP3 antibody (Caltag Medsystems, UK). The samples were then washed with 1 mL of staining buffer (PBS with 3% FCS and 0.01% sodium azide). 0.5 µg of secondary PE-conjugated anti-mouse goat antibody was added to 250 µL of staining buffer per sample, and the samples were incubated for 40 minutes at room temperature on a plate shaker. The samples were washed and re-suspended in 500 µL staining buffer. Sky Blue fluorescent beads of 0.84 μm size (Spherotech, USA) were ran along with the stained plasma samples. The initial gating was set according to the side and forward scatter including particles that were bigger than the fluorescent beads as ASC particles form
specks of 1 μm in size but these can further oligomerise. Gated events were presented as positive percentages of total events. The samples were run using BD FACSCanto II flow cytometer (BD Biosciences, UK) for 3 minutes and data was analysed in FlowJo version 10.0.6 (FlowJo LLC, USA). The same protocol was used to stain human plasma samples from the clinical EVLPs and post-LTx patients.

2.5.2 Staining and gating strategy for ASC+ inflammasome particles in porcine perfusate samples

As stated on the manufacturer’s website, the ASC and NLRP3 antibodies (Caltag Medsystems, UK) are reactive with human and mouse antigens. In order to investigate whether or not these antibodies were also compatible with porcine samples, they were first analysed on the online protein sequencing database Basic Local Alignment Search Tool (BLAST) (National Centre for Biotechnology Information, USA). The *mus musculus* and *homo sapiens* ASC proteins are 72% identical with 81% positive matches, and the *sus scrofa* and *homo sapiens* ASC proteins are 76% identical (87% positive matching). Likewise, the *mus musculus* and *homo sapiens* NLRP3 proteins demonstrate 81% identity and 88% positive matching, whereas these values are 83% and 88% for the *sus scrofa* and *homo sapiens* NLRP3 proteins respectively. The identity matching means that the amino acid sequences in the protein are exact matches, whereas the positive matching means that the amino acids are similar in size and shape in the corresponding sequences. This indicates that it was indeed feasible to use these specific antibodies to detect porcine ASC and NLRP3 particles. However, the preliminary work stimulating peripheral blood mononuclear cell (PBMC)-derived porcine monocytes demonstrated that whereas the ASC antibody was able to bind the porcine ASC particles, the NLRP3 antibody was binding non-specifically in the plasma from both control and stimulated cells. Hence, porcine plasma samples were only stained with the ASC antibodies.

The staining protocol followed the one for human samples with the exception of omitting the NLRP3 and secondary antibodies. 1 mL of perfusate supernatants that were previously centrifuged at 300 g for 10 min, were incubated for 90 minutes at room temperature on a plate shaker with 1 μg rabbit polyclonal APC-conjugated anti-ASC antibody (Caltag Medsystems, UK). The samples were washed with 1 mL and re-suspended in 500 μL staining buffer. The samples were read and recorded using BD FACSCanto II flow cytometer (BD Biosciences, Oxford, UK) for 3 minutes and data was analysed in FlowJo version 10.0.6 (FlowJo LLC, USA). The gating strategy for the porcine ASC particles followed that of the one used for human samples.
Figure 2.8 Gating strategy for ASC+ and NLRP3+ inflammasome particles.

Gating based on size bigger than 0.84 μm beads was applied as ASC+ inflammasome specks are 1 μm in size (A). Supernatant from cultured THP1 cell line monocytes left untreated and stained with antibodies for ASC (APC) and NLRP3 (PE) (B-C); supernatant from cultured THP1 cell line monocytes stimulated with 10 ng nigericin for 1 hour, and stained with antibodies for ASC and NLRP3 (D-E).
2.6 Clinical EVLP

Perfusate samples were analysed from EVLPs performed as part of the UK multi-centre clinical trial DEVELOP-UK (ISRCTN44922411), which evaluated the safety and cost-effectiveness of EVLP in the context of clinical lung transplantation. The sites were located at the lung transplant centres in Newcastle, Manchester, Birmingham, Harefield and Papworth hospitals. The perfusions were performed on lungs that had been assessed upon procurement but had not met the acceptability criteria for standard transplantation (Table 1.1) [148]. These perfusions were conducted utilising the Vivoline LS1 system (XVIVO Perfusion, Sweden) according to the protocol described by Fisher et al. [148]. Perfusate and BAL samples were available from n=20 clinical EVLP procedures, where n=10 lungs did not recover during perfusion and were discarded, and n=10 lungs reconditioned sufficiently to meet the acceptability criteria (Table 2.2), and were subsequently transplanted after the EVLP. Out of the 10 transplanted EVLP lungs n=7 recipients had good post-operative outcomes, whereas n=3 had poor post-operative outcomes (Figure 2.9). Perfusate samples were collected at 15- and 90 minutes and at the end of the perfusions (maximally 240 minutes but depending on the length of the EVLP), centrifuged at 180 g for 6 minutes at 4°C, and stored at -80°C for later analysis. BAL samples were collected pre- and post-EVLP. 120 mL of sterile saline was flushed through the bronchi and collected. The samples were then filtered through sterile gauze and centrifuged at 700 g for 10 minutes. The samples were aliquotted and stored at -80°C. Samples from all centres were collected and stored at Newcastle, the leading centre for the DEVELOP-UK trial, who then selected the n=20 EVLP perfusate samples for the study discussed here. The research team was blinded to the sample groups until analysis was completed.
Figure 2.9 Sample selection for clinical EVLPs

Schematic representation of n=20 EVLP procedures included in the analysis of perfusate and BAL samples from the DEVELOP-UK trial.
Table 2.2. Acceptability criteria for lung transplantation following EVLP in the DEVELOP-UK clinical trial
(adapted from the DEVELOP-UK trial protocol [148])

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
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<tbody>
<tr>
<td>Pulmonary arterial pressure &lt;20 mmHg</td>
</tr>
<tr>
<td>Perfusate flow of up to 70 mL/kg ideal bodyweight/min</td>
</tr>
<tr>
<td>Oxygen capacity &gt;40 kPa (300 mmHg) with selective pulmonary vein gases &gt;30 kPa (225 mmHg)</td>
</tr>
<tr>
<td>Peak airway pressure &lt;25 cmH₂O with adequate ventilation</td>
</tr>
<tr>
<td>Stable or improving lung compliance</td>
</tr>
<tr>
<td>Stable or decreasing lung resistance</td>
</tr>
<tr>
<td>No pulmonary oedema build-up in the endotracheal tube</td>
</tr>
<tr>
<td>Satisfactory assessment on inspection and palpitation</td>
</tr>
</tbody>
</table>
2.7 Post-lung transplantation blood samples

Blood samples were collected from n=7 patients undergoing lung transplantation at University Hospital South Manchester (UHSM), UK between October 2016 and March 2017. The recruitment criteria included patients between 18-80 years old, already entered or newly added to the lung transplant waiting list at UHSM, and able to give informed and signed consent to participate in the study. The samples were collected with the approval of the ethical committee for the study design to investigate markers of lung injury, as part of the study 14/NW/0260: Exploring the Mechanisms of Homeostasis and Disease Before and After Lung Transplant. All samples were taken at the time of routine clinical blood gas analyses by trained hospital staff without any extra risks for the study participants. Patients receiving single- or double lung transplants were included in the study.

The first sample, collected into 2x 4 mL purple-top ethylenediaminetetraacetic acid (EDTA) tubes (Becton Dickinson, US), was taken immediately prior to the lung transplant operation, at the anaesthetic room and marked the pre-operative time point. Samples were then collected at 12-, 24-, 48- and 72 hours post-reperfusion. These latter time points were then matched with the PaO$_2$/FiO$_2$ ratio recorded at the time of sample collection, which was considered as a representative of lung function for the purposes of this arm of the study.

The fresh samples were centrifuged for 10 minutes at 500 g at 4°C, aliquotted and stored at -80°C.
2.8 Enzyme linked immunosorbent assays (ELISA)

The levels of pro-IL-1β were determined by a commercially available Quantikine™ human pro-IL-1β ELISA kit (R&D Systems, USA) with a range of 23.4-1,500 pg/mL and sensitivity of 8.9 pg/mL. The capture antibody in the assay was specifically targeted towards the pro-sequence of the pro-IL-1β molecule and hence was able to differentiate from the active IL-1β. Perfusate and plasma samples were used neat as per manufacturer’s protocol. Briefly, 50 μL of assay diluent and 200 μL of standard or sample was added to the wells in duplicates, and incubated at room temperature for 90 minutes. The plate was washed thrice and 100 μL of human pro-IL-1β antiserum was added to the wells, and incubated at room temperature for 30 minutes. The plate was washed again and 100 μL of human pro-IL-1β conjugate was added to each well, followed by 30 minutes of incubation at room temperature. Again, the plate was washed and 200 μL of substrate solution was added to each well. The plate was incubated at room temperature for 20 minutes protected from light. Finally, 50 μL of stop solution was added to each well.

The levels of IL-1β in post-LTx plasma samples were determined with a commercially available human IL-1β Quantikine™ High Sensitivity ELISA kit (R&D Systems, USA) with the assay rage of 0.1-8 pg/mL and sensitivity of 0.063 pg/mL. The assay measured specifically the bioactive form of IL-1β with minimal (3.9%) cross-reactivity with the precursor pro-IL-1β, as stated on the official product datasheet. Plasma samples were used neat as per manufacturer’s protocol. Briefly, 50 μL of assay diluent and 100 μL of standard or sample was added to the wells in duplicates, and incubated at room temperature for 120 minutes on a plate shaker. The plate was washed thrice and 200 μL of human IL-1β high sensitivity conjugate was added to the wells, and incubated at room temperature for 60 minutes on a plate shaker. The plate was washed again and 200 μL of streptavidin horseradish peroxidase was added to each well, followed by 30 minutes of incubation at room temperature on a plate shaker. Again, the plate was washed and 200 μL of substrate solution was added to each well. The plate was incubated at room temperature for 30 minutes protected from light. Finally, 50 μL of stop solution was added to each well.

The levels of IL-18 were measured in the clinical EVLP perfusate samples using a commercially available PicoKine™ human IL-18 ELISA kit (BosterBio, USA) with the assay range of 15.6-1,000 pg/mL and sensitivity of <1 pg/mL. The samples were used neat as per manufacturer’s protocol. Briefly, 100 μL of sample diluent and 100 μL of standard or sample was added to the wells in duplicates, and incubated at 37°C for 90 minutes. The contents of the plate were discarded and 100 μL of biotinylated anti-human IL-18 antibody was added to the wells, and incubated at 37°C for 30 minutes. The plate was washed three times and 100 μL of avidin-biotin-peroxidase complex was added to each
well, followed by 30 minutes of incubation at 37°C. Again, the plate was washed and 90 μL of tetramethylbenzidine solution was added to each well. The plate was incubated at 37°C for 20 minutes protected from light. Finally, 100 μL of stop solution was added to each well.

The levels of IL-18 in the post-LTx plasma samples were measured using a commercially available Quantikine™ total human IL-18 ELISA kit (R&D Systems, USA) with a range of 15.6-1,000 pg/mL and sensitivity of 5.15 pg/mL. The assay could not distinguish between the pro-IL-18 precursor and its active form of IL-18, as advised per manufacturer. Plasma samples were used neat as per manufacturer’s protocol. Briefly, 50 μL of assay diluent and 50 μL of standard or sample was added to the wells in duplicates, and incubated at room temperature for 120 minutes on a plate shaker. The plate was washed thrice and 100 μL of human total IL-18 conjugate was added to the wells, and incubated at room temperature for 60 minutes on a plate shaker. The plate was washed again and 200 μL of streptavidin-HRP was added to each well, followed by 30 minutes of incubation at room temperature on a plate shaker. Again, the plate was washed and 200 μL of substrate solution was added to each well. The plate was incubated at room temperature for 20 minutes. Finally, 50 μL of stop solution was added to each well.

The levels of caspase-1 were measured using a commercially available Quantikine™ human caspase-1 ELISA kit (R&D Systems, USA) with a detection range of 6.3-400 pg/mL and sensitivity of 1.24 pg/mL. The assay could not distinguish between pro-caspase-1 and mature caspase-1, as advised per manufacturer. Perfusate samples were diluted 20-fold and post-LTx plasma samples were used neat for caspase-1 analysis. The assay was run as per manufacturer’s protocol. Briefly, 50 μL of assay diluent and 100 μL of standard or sample was added to the wells in duplicates, and incubated at room temperature for 90 minutes. The plate was washed thrice and 100 μL of caspase-1 antiserum was added to the wells, and incubated at room temperature for 30 minutes. The plate was washed again and 100 μL of human caspase-1 conjugate was added to each well, followed by 30 minutes of incubation at room temperature. Again, the plate was washed and 200 μL of substrate solution was added to each well. The plate was incubated at room temperature for 20 minutes protected from light. Finally, 50 μL of stop solution was added to each well.

The optical density for all of the ELISA experiments was determined using the Infinite M200PRO microplate reader (Tecan, UK). The plates were read at 450 nm with wavelength correction at 540 nm. The results were analysed in Microsoft Office Excel (Microsoft, USA) to determine the compound concentrations.
2.9 Statistical Analysis

Statisticians from the UHSM and University of Manchester were consulted in regards to the data examination. Statistical analysis was carried out using SPSS software version 22 (IBM, USA) and Prism software version 7 (GraphPad Software, USA). As the sample sizes were small across the experiments, data was assessed for normality using descriptive statistics of skewness, kurtosis, means and medians. Approximate normality was also accepted. In order to test differences between specific time points, normally distributed data was analysed using independent T tests, while non-normal data was assessed using a Mann-Whitney U test. When data was mixed, non-parametric tests were always preferred due to the small number of replicates. Changes over time within- and between groups were evaluated with repeated measures analysis of variance (ANOVA) if all data was normally distributed. Within-group changes over time were assessed using Friedman Test for non-normal data. Associations between two parameters were assessed with Pearson’s correlation coefficient if the data was Gaussian, and Spearman’s correlation coefficient if the data was not normally distributed. Data was considered significantly different if $P \leq 0.05$. Normally distributed data is presented in text and in figures as means with standard deviations (SDs), whilst non-normal data is given as medians with interquartile ranges (IQRs). Graphs were drawn in Prism version 7 (GraphPad Software, USA).
Chapter 3: Porcine *ex vivo* lung perfusion and inflammasome inhibition with flufenamic acid
3.1 Abstract

EVLP is a technique used to evaluate and recondition marginal donor lungs that otherwise would be discarded for transplantation. IL-1β has been identified as a prognostic indicator of non-recovery during clinical EVLP. IL-1β is a potent pro-inflammatory cytokine that can contribute to acute tissue injury. Its secretion is dependent on intracellular inflammasome assembly, so the hypothesis of this study was that inflammasome inhibitor therapy could represent a novel strategy to improve lung reconditioning during EVLP. A double-blinded randomised study was performed using flufenamic acid as an NLRP3 inflammasome inhibitor during porcine EVLP. ASC+ inflammasome particles were measured as an indicator of inflammasome activity, and the cytokine profile of the organs were assessed. Whilst a decrease in inflammasome particles was demonstrated, there were no differences between the control and the inhibitor groups in terms of cytokine profile or organ function. As pulmonary function was good in all lungs prior to organ procurement, it suggests that NLRP3 inflammasome inhibition did not improve the course of perfusion or the inflammatory response of lungs from healthy pigs. Instead an injury model would be more appropriate to assess the potential benefits of this therapy on marginal organs.
3.2 Introduction

Recent evidence suggests that IL-1β represents a biomarker of pulmonary injury during EVLP that correlates with post-LTx outcome [125, 149]. Previous work by this group demonstrated that a broad range of pro- and anti-inflammatory cytokines, including IL-1β, increase during the course of EVLP using healthy porcine lungs [106]. The secretion of IL-1β is a tightly regulated process where the inflammasome, a multi-protein intracellular complex, plays a crucial role initiating the cleavage of its inactive pro-form into active IL-1β. A marked increase beyond physiological levels in the IL-1β concentrations during porcine EVLP [106] lead to the investigation of the effects that inflammasome inhibition may have on the overall inflammatory profile of the lungs, as well as how it may influence pulmonary function. IL-1β is a potent pro-inflammatory cytokine, and novel therapies targeting this inflammatory mediator pathway may improve lung reconditioning during perfusion. Therefore the hypothesis was proposed that inhibiting the formation of the inflammasome, which would inhibit the cleaving of pro-caspase-1 into active caspase-1, would in turn impair pro-IL-1β cleavage into active IL-1β. Inhibiting IL-1β during EVLP may thereby improve lung recovery and function. In order to achieve this, flufenamic acid was chosen as the inflammasome inhibitor of interest for this set of experiments. Flufenamic acid belongs to the fenamate class of non-steroidal anti-inflammatory drugs (NSAIDs), which have been used for decades. They are known for inhibiting cyclooxygenase (COX) and preventing the synthesis of prostaglandins. Recently, they have been demonstrated to selectively and reversibly inhibit the NLRP3 inflammasome activity by blocking the volume-regulated anion channels in the plasma membrane [144]. Therefore, the fenamates can be considered and repurposed as therapeutic agents for NLRP3 inflammasome or IL-1β associated diseases, which is the reasoning for utilising flufenamic acid in this set of EVLP experiments.
3.3 Methods

3.3.1 Porcine EVLP

The Ethical Committee of the University of Lund granted approval for the study. The animals were treated in accordance to the 'Principles of Laboratory Animal Care' by the National Society of Medical Research, and the 'Guide for the Care and Use of Laboratory Animals' by the National Institutes of Health 1985.

In brief (please refer to Chapter 2: Methods for detailed methodology), healthy laboratory pigs (n=10) were sedated and anesthetised as previously described [76]. Lungs were procured as per established guidelines, placed on a standard EVLP circuit and perfused following the cellular Lund protocol [76] for a maximum of 2 hours. Briefly, the circuit was primed with 2 L of Steen Solution™ (XVIVO Perfusion, Sweden), 10,000 iU of heparin (Leo Pharma, Sweden) and 500 mL of packed autologous RBC to reach the target haematocrit of 10-15%. The lungs underwent gradual pressure-controlled reperfusion and rewarming to achieve a target flow rate of 70 mL/kg bodyweight/min and a temperature of 37°C. Protective volume-controlled ventilation was started at 32°C with full ventilation achieved at 37°C. When these values were reached and stabilised, the lungs underwent evaluation on the EVLP circuit and were deemed acceptable if the blood gas analysis demonstrated 
\[ pO_2 > 20 \text{ kPa} \] and \[ pCO_2 < 6 \text{ kPa} \] at ventilation of 50% FiO₂. Lung function was determined by haemodynamic analysis and gaseous exchange. After evaluation, the reconditioning phase was re-established and continued until 120 minutes from the time of connecting the lungs to the circuit. In n=2 cases the cooling phase was initiated before the 2-hour time point and the last functional measurements were taken at the end of the reconditioning phase in normothermia, rather than from the hypothermic perfusion. As the perfusate was still circulating the lungs, the perfusate samples were still taken at 120 minutes.

3.3.2 NLRP3 inflammasome inhibitor therapy in porcine EVLP

The lungs (n=10) were randomised into two groups to receive either the inflammasome inhibitor drug or the carrier control. In the intervention group 40 mg of flufenamic acid (Sigma-Aldrich, UK), a potent and specific NLRP3 inflammasome inhibitor, was added to the circuit prior to connecting the lungs [144]. The dosage concentration was calculated based on previous work by this group's collaborators [144]. Flufenamic acid was dissolved in 8 mL of 25% cyclodextrin (Sigma-Aldrich, UK), in normal 0.9% saline (Baxter Healthcare, UK), as a carrier for the drug. In the control group the same volume of cyclodextrin in saline was added to the circuit. The study team were blinded as to what solution was added until all of the analyses were complete.
Perfusate samples were collected at baseline before the lungs were connected to the circuit, and at 60- and 120 minutes from the start of the perfusions. Samples were centrifuged at 300 g for the inflammasome particle experiments, and at 1000 g for the Luminex experiments, both for 10 minutes at 4°C, and stored at -80°C.

3.3.3 Flow cytometry on ASC+ inflammasome particles on porcine EVLP samples

The ASC+ inflammasome particles were measured in the serial EVLP perfusate samples via flow cytometry. 1 mL of the sample was incubated with 1 µg of anti-ASC antibody (Caltag Medsystems, UK) for 90 minutes on a plate shaker at room temperature. The samples were then washed with 1 mL and resuspended in 500 µL of staining buffer. The samples were read on the BD FACSCanto II flow cytometer (BD Biosciences, UK). The gating strategy was applied in FlowJo version 10.0.6 (FlowJo LLC, USA).

3.3.4 Luminex analysis on porcine EVLP samples

The concentrations of cytokines and a chemokine in the EVLP perfusate were assessed. A commercially available 13-plex magnetic bead panel (Merck Millipore, USA) consisting of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α was utilised according to the manufacturer’s protocol. The plate was read using a Bio-Plex 200 system (BioRad, UK).

3.3.5 Statistical analysis

All statistical analysis was carried out using SPSS software version 22 (IBM, USA) and Prism version 7 (GraphPad, USA). Data was assessed for normality with descriptive statistics of skewness, kurtosis, means and medians. Approximate normality was also accepted. Differences between individual time points were evaluated with an independent T test if the data was normally distributed, or Mann-Whitney U test if the data was not normally distributed. Within-group changes over time were assessed using repeated measures ANOVA if data was normal, and Friedman test if it was non-normal. Statistical differences on changes over time between groups were evaluated with repeated measures ANOVA if all data was normally distributed. Statistical differences were considered significant if P≤0.05. Normally distributed data is presented as means with standard deviations; otherwise it is presented as medians with interquartile ranges.
3.4 Results

3.4.1 Study Groups

There was a gas supply error at the priming stage in one of the treatment lungs. As such this dataset has been omitted from analysis. The data below includes n=5 control EVLPs versus n=4 treated EVLPs.

3.4.2 Functional parameters

None of the lung assessment parameters significantly differed between the control and the inhibitor treated groups during EVLP, and all lungs met the criteria for standard transplantation. All lungs warmed to normothermia within the first 20 minutes [mean time in the control group 15.8 (±4.32) min, and 18.5 (±3.87) min in the inhibitor group (P=0.363)] and maintained that temperature until the end of the perfusions (Figure 3.1 A).

The pulmonary arterial pressure (Figure 3.1 B) started at 10.9 (±4.75) mmHg in the control group, and in the inhibitor group the mean PAP was 11.31 (±5.49) mmHg (P=0.993). In both groups the PAP values were stable during the EVLPs. At the end of the EVLPs, PAP in the control group was stable at 15.1 (±0.13) mmHg, and in the inhibitor group it measured at 13.55 (±2.66) mmHg (P=0.341).

All lungs demonstrated a decrease in PVR as the flow rates increased. In the control group the perfusate flow rates started at 19.16 (±11.49) mL/kg/min and increased to 70.02 (±2.26) mL/kg/min at the end of the perfusion. In the inhibitor group these values were 15.23 (±6.01) mL/kg/min and 74.93 (±9.27) mL/kg/min respectively (P=0.676 at the start and P=0.754 at the end of the perfusions between groups). Full perfusate flow values were achieved within 30 minutes and remained at steady levels for the duration of the perfusions (Figure 3.1 C). At the beginning of the EVLPs, in the control group the mean PVR measured at 1,390.69 (±473.46) dyn·s²/cm⁵, and in the inhibitor group 1,310.06 (±380.97) dyn·s²/cm⁵ (P=0.791). These values, however, decreased rapidly and remained low in both groups throughout the perfusion (Figure 3.1 D). By the end of the perfusions, the mean PVR in the control group decreased to 359.89 (±84.69) dyn·s²/cm⁵, whilst in the inhibitor group it measured at 288.84 (±100.12) dyn·s²/cm⁵ (P=0.286). No statistically significant differences were found between the groups in any of the parameters at any of the individual time points.

During the evaluation phase, the mean arterial partial pressure of oxygen and carbon dioxide (pO₂ and pCO₂) values were recorded at FiO₂ of 50%. The mean pO₂ measured at 35.47 (±2.39) kPa in the control group, and at 31.12 (±3.79) kPa in the inhibitor group (P=0.073) (Figure 3.2 A). The mean pCO₂ in the control group measured at 3.56 (±0.29) kPa, and 3.86 (±0.78) kPa in the inhibitor group (P=0.44) (Figure 3.2 B). PaO₂/FiO₂ ratios
were therefore calculated at 70.94 (±4.79) kPa in the control group, and slightly decreased at 62.24 (±7.59) kPa in the inhibitor group (P=0.073) (Figure 3.2 C). The mean oxygen uptake in the control group measured at 39.97 (±2.85) mL/min, and 36.69 (±8.17) mL/min in the inhibitor group at evaluation phase (P=0.107) (Figure 3.2 D).
Figure 3.1 Functional parameters of porcine EVLP

Lungs in both the control and inhibitor groups reached normothermia by 30 minutes into the EVLP and remained stable until the end of the perfusion (A). Pulmonary arterial pressure was maintained at approximately 15 mmHg in both groups throughout the perfusion (B). Perfusate flow rates increased similarly in both groups and remained stable at approximately 70 mL/kg/min until the end of the EVLPs (C). Pulmonary vascular resistance decreased in both groups and remained stable until the end of the perfusions (D). N=5 in the control group and n=4 in the inhibitor group. Data presented as means with SDs, no statistically significant changes over time between groups (repeated measures ANOVA).
Figure 3.2 Blood gas analysis during the evaluation of porcine EVLP

pO\textsubscript{2} and pCO\textsubscript{2} at FiO\textsubscript{2} 50% demonstrated that all lungs were deemed suitable for a theoretical transplant by meeting the determined criteria of pO\textsubscript{2} > 20 kPa and pCO\textsubscript{2} < 6 kPa at ventilation of 50% FiO\textsubscript{2} (A, B). PaO\textsubscript{2}/FiO\textsubscript{2} ratio and oxygen uptake during evaluation further signified good pulmonary oxygenation capacity in both groups (C, D). N=5 in the control group and n=4 in the inhibitor group. Data presented as means with SDs, no statistically significant differences observed between the groups (independent T tests).
3.4.3 ASC+ inflammasome particles

The ASC+ inflammasome particles were measured as a percentage of total particles of the gated size (Figure 3.3). At the start of the perfusion there were similar levels of ASC+ specks in both groups. In the control group the mean ASC+ value was 2.96 (±2.67)% of all particles, and in the inhibitor group 3.47 (±2.94)% (P=0.79). At 60 minutes into the perfusion these had increased to 4.74 (±5.97)% in the control group, and decreased to 1.75 (±1.55)% in the inhibitor group (P=0.367). At the end of the EVLPs the levels of ASC+ particles measured at 4.77 (±5.2)% in the control group, and 1.11 (±0.89)% in the inhibitor group (P=0.212).

There were no statistically significant differences between the control and inhibitor groups at individual time points. When evaluating within group differences, there was no statistically significant change over time throughout the perfusion in the control group (P=0.549). However, in the inhibitor group, there was a significant decrease in ASC+ particles from the start of the EVLP compared to the end (P=0.039).
ASC+ inflammasome specks in porcine EVLP perfusate

ASC+ inflammasome specks increased in the control group (n=5) from the start of the perfusion until the end without any statistical significance (repeated measures ANOVA, P=0.549). There was a significant decrease of the inflammasome particles in the inhibitor group (n=4) during the EVLP (repeated measures ANOVA, P=0.039). However, there were no statistically significant differences between the individual time points of each group (independent T tests). Data presented as means with SDs.
3.4.4 Cytokine profile

The inflammatory profile was assessed during EVLP with a general trend of increasing levels of most cytokines in both groups exhibiting similar patterns. However, no statistically significant changes were found at any of the individual time points between groups.

3.4.4.1. Inflammasome-related cytokines IL-1β and IL-18

At the start of EVLP the median IL-1β (Figure 3.4 A) concentration was 0 (IQR=0-515) pg/mL in the control group, and 0 (IQR=0-7.5) pg/mL in the inhibitor group (P=0.73). At 60 minutes the median IL-1β concentration increased to 20 (IQR=5-2,170) pg/mL in the control group, and to 5 (IQR=0-17.5) pg/mL in the inhibitor group (P=0.268). At 120 minutes the median IL-1β concentration was 220 (IQR=40-2,350) pg/mL in the control group and 35 (IQR=7.5-430) pg/mL in the inhibitor group (P=0.413). When assessing within-group differences, there was a significant increase in the control group (P=0.039), whereas in the inhibitor group the change was non-significant (P=0.061) over the course of the perfusions.

The median levels of IL-18 (Figure 3.4 B) started in the control group at 0 (IQR=0-20) pg/mL and in the inhibitor group at 0 (IQR=0-720) pg/mL (P=1). IL-18 then increased to 40 (IQR=20-210) pg/mL in the control group, and 115 (IQR=22.5-507.5) pg/mL in the inhibitor group at 60 minutes (P=0.73). By the 120-minute time point there was a further increase to 140 (IQR=70-420) pg/mL in the control group and, and 250 (IQR=107.5-917.5) pg/mL in the inhibitor group (P=0.432). Conversely to the IL-1β levels, there was no statistically significant change in the control group for IL-18 (P=0.074), but a significant increase in the inhibitor group over time (P=0.038).
Figure 3.4 Inflammasome-related cytokines IL-1β and IL-18 concentrations in porcine EVLP perfusate

IL-1β increased significantly in the control group from the start of the EVLPs until the end, whereas this change was non-significant in the inhibitor group (Friedman tests, $P=0.039$ and $P=0.061$ respectively) (A). However, no statistical differences were observed between individual time points (Mann-Whitney U tests). Contrarily, IL-18 augmented significantly only in the inhibitor group from the start of the perfusion (Friedman test, $P=0.038$), with non-significant increase in the control group (Friedman test, $P=0.074$) (B). There were no statistically significant differences between individual time points (Mann-Whitney U tests). N=5 in the control group and n=4 in the inhibitor group. Data presented as medians with IQRs.
3.4.4.2 Cytokines detected in high concentrations in porcine EVLP

There was an overall trend towards an increase in the inflammatory cytokines IL-6, IFN-γ and TNF-α during EVLP (Figure 3.5).

At the start of perfusion there was no IL-6 (Figure 3.5 A) detected in the control group, and the median concentration in the inhibitor group was 0 (0-180) pg/mL (P=0.556). At 60 minutes there was a slight increase to 10 (IQR=0-20) pg/mL in the control group, whereas IL-6 measured at 0 (IQR=0-127.5) pg/mL in the inhibitor group (P=0.73). At 120 minutes however, the levels of IL-6 increased to 650 (IQR=180-1,650) pg/mL in the control group, and to 105 (IQR=30-1,327.5) pg/mL in the inhibitor group (P=0.556). Within-group analysis demonstrated a significant increase in the levels of IL-6 in both groups (P=0.022 and P=0.023 for the control and inhibitor groups respectively).

IFN-γ (Figure 3.5 B) was detected at the start of EVLP at 1,060 (IQR=660-2,800) pg/mL in the control group, and 1,115 (IQR=207.5-5,720) pg/mL in the inhibitor group (P=1). At the 60-minute time point it measured at 910 (IQR=455-2,295) pg/mL in the control, and 1,265 (IQR=477.5-5,247.5) pg/mL in the inhibitor group (P=1). There was a further increase at the end of the perfusion in the control group to a median concentration of 1,840 (IQR=1,405-6,350) pg/mL, whilst in the inhibitor group it measured at 1,060 (IQR=1,060-5,000) pg/mL (P=0.393). Over the course of the EVLP, the IFN-γ increased significantly in the control group (P=0.041), but the change was non-significant in the inhibitor group (P=0.717).

The levels of TNF-α (Figure 3.5 C) were negligible at the beginning of the perfusions in both groups, measuring at 0 (IQR=0-20) pg/mL in the control group, and 15 (IQR=0-30) pg/mL in the inhibitor group (P=0.556). At 60 minutes, the increase was more pronounced in the control group with TNF-α measuring at 1,980 (IQR=450-5,350) pg/mL, and at 350 (IQR=122.5-2,032.5) pg/mL in the inhibitor group (P=0.286). This trend continued to the 120-minute time point, when the median TNF-α measured at 4,540 (IQR=2,395-11,235) pg/mL and 2,925 (IQR=790-7,872.5) pg/mL in the control and inhibitor groups respectively (P=0.286). These values demonstrated that there was a significant increase in TNF-α within both the control (P=0.007) and inhibitor (P=0.018) groups over time.
Figure 3.5 High cytokine concentrations in the porcine EVLP perfusate: IL-6, IFN-γ and TNF-α

Over time there was a significant increase in the levels of IL-6 within both the control and inhibitor groups (Friedman tests, P=0.022 and P=0.023) (A). IFN-γ increased significantly over the course of the EVLPs in the control group (Friedman test, P=0.041) but remained stable in the inhibitor group (Friedman test, P=0.717) (B). TNF-α augmented significantly over time in both groups (Friedman tests, P=0.007 in the control, and P=0.018 in the inhibitor groups) (C). N=5 in the control group and n=4 in the inhibitor group. Data presented as medians with IQRs.
3.4.4.3 Cytokines detected in low concentrations in porcine EVLP

IL-1α, IL-1Ra, IL-2, IL-4, IL-8, IL-10, IL-12 and GM-CSF were present at low or undetectable concentrations throughout the perfusions.

No IL-1α (Figure 3.6 A) was detected in either the control or inhibitor groups at the baseline and 60-minute time points. At 120 minutes there was a marginal increase to 10 (IQR=0-15) pg/mL in the control group, but it measured at 0 (IQR=0-15) pg/mL in the inhibitor group (P=0.556). Within-group analysis demonstrated a statistically significant increase in the control group (P=0.05), but no significant change in the inhibitor group (P=0.368).

Equally, there was no IL-1Ra (Figure 3.6 B) detected in the inhibitor group at the start of the perfusion, whereas in the control group this was measured at 0 (IQR=0-10) pg/mL (P=0.413). At 60 minutes these measured at 10 (IQR=10-30) pg/mL in the control group, and 10 (IQR=2.5-25) pg/mL in the inhibitor group (P=0.556). At 120 minutes the IL-1Ra increased to 300 (IQR=35-540) pg/mL in the control group, and to 45 (IQR=25-192.5) pg/mL in the inhibitor group (P=0.286). Statistically there were significant changes in both groups (P=0.011 in the control, and P=0.022 in the inhibitor group).

IL-2 (Figure 3.6 C) was not detected in any of the samples in the control group for the duration of the perfusion in any of the EVLPs performed. Low levels were identified in some of the samples in the inhibitor group with the baseline IL-2 measuring at 0 (IQR=0-52.5) pg/mL (P=0.556), the 60 minute time point at 0 (IQR=0-22.5) pg/mL (P=0.556), and no IL-2 measured at 120 minutes (P=1). There were no statistically significant differences between any individual time points or over time within the inhibitor group (P=0.368).

Likewise, no IL-4 (Figure 3.6 D) was detected in the control group at baseline or at 60 minutes. In the inhibitor group it was present in some of the samples measuring at 0 (IQR=0-7.5) pg/mL (P=0.556) and 5 (IQR=0-10) pg/mL (P=0.286) respectively. At 120 minutes IL-4 measured at 0 (IQR=0-5) pg/mL in the control group, and at 10 (IQR=2.5-17.5) pg/mL in the inhibitor group (P=0.19). Changes over time were non-significant within each group (P=0.386 in both).
Figure 3.6 Low cytokine concentrations in the porcine EVLP perfusate: IL-1α, IL-1Ra, IL-2 and IL-4

IL-1α (A) was detected in the perfusate only at the 120-minute time point in both groups, significantly increasing only in the control group (Friedman test, P=0.05) and not the inhibitor group (Friedman test, P=0.368). Similarly, the IL-1Ra (B) significantly increased by 120 minutes in the control group (Friedman test, P=0.011) as well as the inhibitor group (Friedman test, P=0.022). IL-2 (C) was only found in the inhibitor group with the highest concentrations at the baseline decreasing over the course of the EVLPs (Friedman test, P=0.368). IL-4 (D) was not detected in the control group until 120 minutes (Friedman test, P=0.368), and in the inhibitor group it demonstrated an increasing trend (Friedman test, P=0.368). There were no statistically significant differences between any of the individual time points (Mann-Whitney U tests). N=5 in the control group and n=4 in the inhibitor group. Data is presented as medians with IQRs.
The mean IL-8 (Figure 3.7 A) measured at 20 (IQR=5-55) pg/mL in the control group, and at 15 (IQR=0-45) pg/mL in the inhibitor group at baseline (P=0.73). By 60 minutes there was a small rise in the IL-8 concentration in the control group to 70 (IQR=10-100) pg/mL, with a slight decrease to 10 (IQR=2.5-40) pg/mL in the inhibitor group (P=0.157). Similarly, there was a rise in IL-8 levels to 295 (IQR=92.5-505) pg/mL in the control group, and also to 65 (IQR=52.5-557.5) pg/mL in the inhibitor group (P=0.486) at the end of the EVLPs. However, statistically IL-8 increased significantly over time only in the inhibitor group (P=0.038), while the change remained non-significant in the control group (P=0.368).

At the start of the perfusion there was no IL-10 (Figure 3.7 B) in the perfusate in the control group. In the inhibitor group it measured at 0 (IQR=0-7.5) pg/mL (P=0.556). At 60 minutes there was a marginal increase to 20 (IQR=15-50) pg/mL in the control group, and to 5 (IQR=0-25) pg/mL in the inhibitor group (P=0.19). By 120 minutes a further increase had occurred bringing the IL-10 levels in the control group to 220 (IQR=45-385) pg/mL, and to 105 (IQR=17.5-215) pg/mL in the inhibitor group (P=0.413). The within group changes over time were significant in both groups with P=0.008 in the control group, and P=0.024 in the inhibitor group.

No IL-12 (Figure 3.7 C) was detected in either of the groups at the start of the perfusions. At 60 minutes it measured in the control group at 0 (IQR=0-5) pg/mL, and at 5 (IQR=0-17.5) pg/mL in the inhibitor group (P=0.413). At 120 minutes IL-12 measured at 60 (IQR=15-100) pg/mL in the control group, and 15 (IQR=10-87.5) pg/mL in the inhibitor group (P=0.556). The increases over time were statistically significant within both groups (P=0.023 in the control and P=0.037 in the inhibitor group).

The levels of GM-CSF (Figure 3.7 D) started at 0 (IQR=0-5) pg/mL in the control group and 0 (IQR=0-7.5) in the inhibitor group (P=1). These increased to 10 (IQR=0-25) pg/mL and 35 (IQR=30-55) pg/mL, respectively (P=0.063) by 60 minutes. By the 120-minute time point, there was a further rise in both groups with the mean GM-CSF measuring 30 (IQR=25-40) pg/mL in the control group, and 50 (IQR=10-90) pg/mL in the inhibitor group (P=0.413). The increase over time was significant within the control group (P=0.029), but non-significant in the inhibitor group (P=0.071).
Figure 3.7 Low cytokine concentrations in the porcine EVLP perfusate: IL-8, IL-10, IL-12 and GM-CSF

The levels of IL-8 (A) significantly increased over time in the inhibitor group (Friedman test, P=0.038), but no significance was noted in the control group (Friedman test, P=0.368). IL-10 (B) significantly increased in both the control (Friedman test, P=0.008) and inhibitor groups (Friedman test, P=0.024) by the 120-minute time point. IL-12 (C) significantly augmented in both the control (Friedman test, P=0.023) and the inhibitor (Friedman test, P=0.037) groups. There was a gradual rise in the concentrations of GM-CSF (D) in both groups throughout the perfusions, P=0.029 in the control group and P=0.071 in the inhibitor group, Friedman tests. There were no statistically significant differences between any of the individual time points (Mann-Whitney U tests). N=5 in the control group and n=4 in the inhibitor group. Data is presented as medians with IQRs.
3.5 Discussion

This study aimed to assess if the NLRP3 inflammasome inhibitor, flufenamic acid, could be used to improve lung reconditioning during EVLP. This hypothesis was based upon previous findings from the DEVELOP-UK trial, which demonstrated that IL-1β was a prognostic indicator of recoverability during EVLP [149]. In this trial lungs that did not recover and were discarded had significantly higher levels of IL-1β as early as 30 minutes from the start of the perfusion, which also correlated with post-transplant patient survival, indicating that it can be a potential biomarker for lung recoverability. Andreasson and colleagues have demonstrated that perfusate from discarded lungs significantly augments the expression of adhesion molecules including intracellular adhesion molecule-1 (ICAM-1) and E-selectin on endothelial cells, which in turn leads to an increased infiltration of neutrophils. These cells further amplify the inflammatory response and cause damage in the tissue. Plasma ICAM-1 levels have been previously linked with the development of PGD post-LTx [150]. Equally, another study demonstrated that increased levels of soluble vascular cell adhesion molecules (sVCAM-1) during clinical EVLP were significantly correlated with PGD development post-LTx [151]. Hashimoto et al. thereby hypothesise that the sVCAM-1 levels at 1 or 4 hours into EVLPs can be predictive of post-operative lung function [151]. However, Andreasson et al. also demonstrated that blocking IL-1β reversed the expression of ICAM-1 and E-selectin, further indicating a significant role for this cytokine in lung injury [149].

IL-1β is now being developed as a prognostic biomarker for lung recoverability during EVLP, and it could also be a potential point of therapeutic intervention for improving organ reconditioning. Blocking the activation of pro-IL-1β via inflammasome was investigated to determine whether it would have any effects on the reconditioning process during perfusion, or alter the inflammatory profile of the perfusate. The perfusion systems available at the time were utilised in collaboration with the researchers at Lund, Sweden. They provided the clinical expertise and established protocols in order to investigate the inflammasome inhibition using porcine EVLP. Porcine lungs are of similar size to humans, and the same clinical equipment can be used for the procedures. This means that any findings are more likely to be translatable to human lungs, as opposed to experimenting on small rodent models.

In this study there were no significant differences between the control and inflammasome inhibitor groups in terms of lung function. All lungs performed similarly during the perfusion process, warming up at similar rates, demonstrating decreasing pulmonary vascular pressures and increasing perfusate flow rates. During the evaluation phase, no significant differences were noted in terms of the oxygenation capacity of the lungs. The
PaO$_2$/FiO$_2$ ratio as well as oxygen uptake, were comparable in both groups. All lungs utilised in these experiments were however procured from healthy laboratory pigs and therefore had excellent function *in situ*. As such they differ from the marginal lungs that would presently be considered for clinical EVLPs. It is feasible that inflammasome inhibition therapy does not enhance lung function that is already optimal under the conditions of *ex vivo* perfusion.

The calculated value of oxygen uptake can provide a more accurate estimate for delivery of oxygen to the cells, which is also the reasoning for utilisation of cellular EVLP protocols being advantageous [152]. It has been shown that oxygen delivery is enhanced with the addition of red blood cells, which facilitate improved exchange of carbon dioxide and oxygen through haemoglobin at a cellular level, and with haemoglobin helping to maintain the buffering capacity of the solution [152]. It is also suggested that a cellular perfusate is beneficial for capillary recruitment and vascular distension [153], as it provides a more comparable model to evaluate how the lungs would function post-LTx. Therefore, the Lund method for EVLP represents the most accurate method currently available to test any therapeutics on the perfusion circuit. Even though it has been suggested that the addition of RBC to the perfusate complicates the procedure, and limits the length of time the lungs can be perfused as the RBC start to die [94], it can be argued that the benefits of a short-term cellular EVLP outweigh the disadvantages. Indeed, as the functional data demonstrates, even though there were no significant differences between the groups for organ function, this established method is highly reproducible and replicable, and can provide valid results.

The inflammatory profile between the two groups had limited differences and in general remained comparable between groups. As expected, ASC+ inflammasome particles significantly reduced over the course of the perfusion in the inhibitor group, while in the control group there was no statistically significant change. This indicates that flufenamic acid added to the circuit in the given concentration inhibited the activity of the inflammasome complex. Moreover, the higher inflammasome activity was also reflected in the significant increase of the levels of IL-1β in the control group over time, whereas this change was reduced in the inhibitor group. Together these observations suggest that flufenamic acid can be utilised to inhibit the activation of the inflammatory IL-1β, and can therefore be considered as a potential therapeutic agent for this purpose. Comparably but at much lower concentrations, IL-1α also increased significantly in the perfusate samples of the control but not of the inhibitor group. IL-1α is also produced by macrophages, monocytes and dendritic cells, but constitutively expressed in its bioactive form and can induce cellular activity at similar concentration range to IL-1β [130]. It can be cell
membrane-bound or it can be present in soluble form, both of which have been reported
to have differing biological functions [154, 155]. However, it has also been demonstrated
that inflammasome activation and IL-1β can induce the secretion of soluble IL-1α [154],
which then also corresponds to the results from the current study, where the
inflammasome inhibition is likely to have reduced the levels of IL-1α in the perfusate.

However, these effects for the other inflammasome-related cytokine IL-18 were not
observed, and the effect of flufenamic acid appeared to be reversed with significantly
increasing levels found in the inhibitor group, and not in the control lungs. The normal
serum levels of IL-18 in healthy adult subjects have been reported at approximately 60
pg/mL [156], which is considerably higher than the normal serum levels of IL-1β, often
reported as non-detectable [131, 156, 157]. The synthesis of IL-18 differs from that of IL-
1β in that the inactive pro-form of IL-18 is constitutively present even in resting cells. IL-
18 is also present in epithelial cells as well as blood monocytes. Compared to IL-1β,
significantly higher levels of IL-18 of more than 10 ng/mL [130], and often a co-
stimulatory signal, are needed in order to signal and activate other cells and drive the
inflammatory response [158]. IL-18 signalling together with IL-12 and IL-15 induces the
production of INF-γ [159]. However, the levels of INF-γ in the current study did not follow
the pattern of IL-18 concentrations. INF-γ remained at consistent levels in the inhibitor
group, but increased over time in the control group. It can be proposed that the secretion
of INF-γ was reduced in the inhibitor group because of a lack of a secondary signal, even
with excess IL-18 present. This does not explain the increased levels of IL-18 in the
inhibitor group, where different interpretations may be needed. However, the
concentrations of IL-18 were already augmented in the inhibitor group compared to the
controls at the start of the perfusions and there were no significant differences between
the individual time points due to the large intra-group variability.

At the same time, IL-8 also known as chemotactic cytokine ligand 8 (CXCL8), normally
present at approximately 30 pg/mL in healthy adults [156], was present at the end of the
2-hour EVLPs at 10-fold higher levels, and increased significantly in the inhibitor group,
but not in the control one. The alveolar macrophages and pulmonary epithelial cells have
been known to secrete the chemokine IL-8 and recruit inflammatory cells to the lungs
[160]. IL-8 secretion has been mainly associated with neutrophils, but after appropriate
stimulation can also be released by a variety of different cell types including monocytes,
endothelial cells, T cells, fibroblasts, tumour cells, epithelial cells, hepatocytes,
macrophages and keratinocytes [161]. The chemotactic cytokine receptor 1 (CXCR1) and
CXCR2 are also expressed in numerous cell types: monocytes, CD8+ T cells, mast cells,
basophils, NK cells and myeloid-derived suppressor cells [161]. It is the infiltration of
neutrophils into pulmonary tissue that has been associated with deteriorated outcomes post-transplantation in terms of allograft rejection and PGD [161]. There has been some evidence that blocking IL-8 activity can reduce post-LTx ischemia-reperfusion injury in a rat model [162]. Following from that a phase II clinical trial investigating the effects of repertaxin, a CXCR2 antagonist, on the PGD scores after lung transplantation was conducted (NCT00224406), but no data has been published on the results of that study. In the current experiments, the statistically significant change in the inhibitor group may be explained by the fact that the levels remained low in that group at the first two time points, but in the control group there was a steady rise both at 60- and 120 minutes, so the statistical P-value reflects simply the occurrence of change over time, rather than the scale of change. Between the individual time points there were no significant differences, even with a trend of reduced IL-8 in the inhibitor group compared to the controls.

The concentrations of the other cytokines quantified - IL-6, TNF-α, IL-1Ra, IL-2, IL-4, IL-10 and IL-12 - followed the same trends in both groups, with no changes in the inflammatory profile during the EVLPs in the presence or absence of flufenamic acid. The overall increase in the cytokines measured throughout the EVLPs has been previously described and can be considered a normal response to EVLP when leucocytes are activated by perfusion and the EVLP circuit itself [106, 125]. Despite this, lung function was similar between both groups throughout EVLP. The surgical team, also blinded to the study groups, noted no distinct differences in the macroscopic appearance of the lungs and deemed both groups suitable for transplantation following evaluation. On a molecular level, there was indeed a reduction in the inflammasome ASC+ particles as well as decreased cytokine IL-1β, which suggest a successful inhibition of the NLRP3 inflammasome by the flufenamic acid in a porcine EVLP model. However, no functional benefits accompanied this particular therapy in the set of experiments.

There are several limitations that should be addressed when discussing this study. Firstly, the sample size in both groups was small, which lead to considerable within-group variations. It is however exceptionally difficult to conduct large animal model ex vivo perfusions with the restrictions of funding, equipment and skilled personnel. The technology needed for the experiments is still relatively novel and not widely utilised with only certain centres throughout the world dedicated to EVLPs. Setting up a lung perfusion circuit at the laboratory in the University of Manchester was intended initially, but due to several difficulties occurring, both technical and related to organ supply, the solution at the time was to work in collaboration with the perfusion team in Lund. The number of perfusions available was therefore optimised according to their schedule and what was available. However, after discovering technical issues, n=1 EVLP became unusable for this
set of experiments. Data from the other EVLPs was analysed, but the statistical power of the results is low. Still, the data suggest evidence of successfully inhibiting the NLRP3 inflammasome with flufenamic acid even within the modest sample size. EVLP is a difficult technique, and problems are frequently encountered, especially in experimental systems. This is also the reason why there was a slight discrepancy in n=2 EVLPs, where the cooling phase of the perfusion was commenced before the initially drafted 120-minute time point. Upon analysing the data however, it was discovered that one of these belonged to the control group and the other to the inhibitor group, so it was not assumed that this alteration would have contributed to a significant change in the study results.

At the same time, no functional benefits were observed in the inhibitor group, which indicates that the inflammasome inhibitor therapy may not improve on any real or meaningful clinical outcomes. Here two aspects should be considered though. Firstly, the drug was introduced into the EVLP circuit only in one pre-determined dose, and no dose-response curves in other concentrations were tested. However, given the scarcity of the experimental materials available, this would not have been practical. After deliberating with a group in the University of Manchester who specialise in the inflammasome activity, flufenamic acid, a drug they had been working on [144], was chosen in the concentration calculated according to their experimental data. Nevertheless, other concentrations of the drugs could have perhaps revealed differing results on the perfused lungs. Secondly, the pharmacological intervention was evaluated only during the perfusions, and not in the post-transplant setting. It could have been interesting to examine, which group would have demonstrated improved post-LTx outcomes if the perfused lungs were actually transplanted and followed up for a period of time. Again, a huge caveat here is the cost and availability of the model, which prevents such elaborate experiments being conducted more frequently and in larger volumes. At the same time, it could also be considered wasteful to utilise such advanced technologies and skill for experiments that may still need further development. Additionally, it can be questioned if a longer perfusion period would have changed the outcomes of the therapy. However, no trends of differing function in either group over the course of the two hours were observed. All lungs in this set of experiments can be considered healthy and without significant damage, so any pharmacological effects that the flufenamic acid may have been undetected. As such, testing inflammasome inhibition in an injury model may further elucidate its therapeutic potential.

Additionally, further analysis could have been carried out on the collected samples post-perfusions to further investigate the cellular and molecular differences between the groups. Histology on the post-EVLP pulmonary tissue biopsies could have been used to
evaluate cell death, apoptosis or cellular infiltration into the tissue between the two groups. Ideally, fresh perfusate samples could have been collected for flow cytometry to map the cellular diapedesis profiles in the control and inhibitor groups. This was not an option at the time due to lack of equipment, and a reliable method for freeze-thawing the samples was not optimised. However, these experimental routes were not pursued further as no significant functional differences between the two groups during the two-hour perfusion period were observed.

In conclusion, inflammasome inhibitor therapy yielded no significant benefits when utilising healthy porcine lungs, but it did reduce the ASC+ inflammasome particles and limited the IL-1β release. Considering this and the apparent importance of IL-1β as a predictor of lung recoverability during EVLP, the inflammasome inhibition may still be able to encourage recovery in damaged donor organs. In order to investigate the effects of this therapy in more detail and to see whether or not it might improve function in marginal lungs, an injury model is essential to delineate the mechanisms of this strategy further.
Chapter 4: Porcine *ex vivo* kidney perfusion and inflammasome inhibition with NBC6
4.1 Abstract

Acute kidney injury (AKI) is a rapid loss of kidney function, which results in renal failure. Inflammasome-related cytokines IL-18 and IL-1β have been described as markers of organ injury and recoverability, as well as inflammation. Their secretion requires the formation of the inflammasome, a multiprotein complex that cleaves pro-IL-1β and pro-IL-18 into their mature forms. The associated extracellular inflammasome particles further amplify the inflammatory response. Therefore, inhibiting the inflammasome complex has the potential to reduce inflammation. The aim of this study was to investigate if inflammasome inhibition would prevent or protect from AKI using a porcine kidney ex vivo normothermic perfusion (EVNP) model. Porcine kidneys were randomised into two groups, control (n=5) and treatment (n=5) with NBC6 inflammasome inhibitor added to the circuit. Both groups were perfused for 6 hours and the course of renal failure was mapped using renal blood flow and urine production combined with blood gas analysis. Cell populations were characterised and inflammasome particles measured by flow cytometry, and the cytokine profile was established by Luminex. The mean renal blood flow and urine output were comparable between groups and both groups demonstrated similar patterns of cytokine release and cellular diapedesis during perfusion. There was an increase of IL-1β and IL-18 in both groups. However, during the EVNP there was a significantly lower level of inflammasome particles in the inhibitor group compared to the control (P=0.008). Despite the NBC6 treatment successfully inhibiting inflammasome activity, as indicated by the reduced level of inflammasome particles in the EVNP kidney circuit, it did not convey protection or prevent AKI onset. This suggests that in this EVNP AKI model, inflammasome inhibition has no effect on organ recoverability.
4.2 Introduction

For this set of experiments normothermic porcine ex vivo kidney perfusion model was utilised instead of EVLP. Inflammasome inhibition during EVLP of healthy lungs significantly reduced inflammasome activation and impaired IL-1β secretion. As a follow-up to this study, it was intended to test the drug in injured lungs in order to more closely resemble the clinical scenario of donor lung evaluation and reconditioning using EVLP. However, significant obstacles in developing a reproducible EVLP injury model were encountered. At the time this laboratory group established an acute renal injury perfusion model, so it was decided that an evaluation of inflammasome inhibition should be performed in this system while the EVLP injury model was in development.

Kidney transplantation faces the same caveats as lung transplantation: there is a shortage of donor organs compared to the number of people on the waiting list for kidney transplants [163]. This leads to an increased usage of DCD donors and marginal organs in kidney transplantation [164]. Similarly, EVNP has been developed in order to evaluate marginal kidneys and to extend the availability of donor organs [165]. It has also become evident that perfusion allows for a more accurate assessment of suboptimal organs but may also improve the donor kidney function [166, 167]. There is further evidence that EVNP can alter the immune content of the donor kidneys prior to transplantation, and thereby potentially improve post-transplant outcomes [168]. Taken together, this implies that the EVNP model operates on the same principles and can be an appropriate alternative to an EVLP system in terms of evaluating the contribution of the inflammasome inhibition to organ function.

Acute kidney injury (AKI) in the donor, previously referred to as acute renal failure, is a rapid and progressive loss of electrolyte- and acid-base balance along with kidney function, which results in renal failure [169]. Acute tubular necrosis, resulting from ischemic or toxic injury, is one of the causes for the development of AKI [169]. Ischemia can lead to hypoperfusion that can initiate cellular damage leading to cell death [169]. Ischemia-reperfusion injury has been demonstrated to increase IL-1β levels in mice [170] and IL-18 has been suggested as a indicator for clinical AKI along with other biomarkers [171-173]. As described previously in this thesis, the release of these inflammatory cytokines is dependent on the formation of the intracellular multiprotein complex, the inflammasome. Therefore, inflammasome inhibition can potentially be used as a point of pharmacological intervention to reduce the release of these cytokines, moderate the inflammatory response and thereby potentially reduce injury in the organ.
The aim of this study was to investigate whether inhibiting the inflammasome activity could prevent or protect from AKI, improve renal function or alter the inflammatory profile using the porcine EVNP model.
4.3 Methods

4.3.1 Porcine EVNP

Kidneys were retrieved from n=10 Landrace pigs from a local abattoir. The animals were culled according to the regulations of Schedule 1 of the Home Office Scientific Act 1986. In brief, the pigs were rendered unconscious by electrocution and culled by exsanguination. The kidneys were procured and perfused as per protocols previously described [145, 168] and outlined in more detail here in Chapter 2: Methods of this thesis. The cellular EVNP was established for the duration of 360 minutes. Briefly, the circuit was primed with 350 mL of Ringer’s isotonic solution, 25 mL of 10% mannitol (Sigma-Aldrich, UK), 20 mL of 8.4% sodium bicarbonate (Sigma-Aldrich, UK), 8 mg of dexamethasone (Hameln, UK), 10,000 iU of unfractionated heparin (Fannin, UK) and approximately 900 mL of autologous leucocyte-filtered packed RBCs to reach the target haematocrit of 25-28%, based on the protocol previously published [145]. An inflow of carbogen (95% oxygen and 5% carbon dioxide) was connected to the circuit at a rate of 0.5 L/min. A continuous infusion of Nutriflex and epoprostanol at 20 mL/hr was connected to the arterial line of the circuit. The perfusate temperature was set to 38°C from the start of the perfusion. The kidneys were placed into the organ chamber onto a permeable stand draining into a venous reservoir. The kidneys were connected to the circuit via the renal artery while the renal vein was left open to drain into the reservoir. Urine was collected via the cannulated ureter, and replaced hourly with fresh Ringer’s solution. The pressure in the renal artery was raised to 75 mmHg and maintained at this level adjusting the perfusate flow levels. Hourly blood gas analysis was performed to maintain stable electrolyte balances within the reference ranges (Table 2.1). The course of renal failure was mapped through observational parameters of renal flow and urine production, as well as blood gas analysis on the electrolytes and pH levels.

4.3.2 NLRP3 inflammasome inhibition therapy in porcine EVNP

Initially, n=5 porcine kidney EVNP experiments were carried out, which constituted as the control group. Subsequently, another n=5 kidneys were perfused with a novel boron-based NLRP3 inflammasome-specific inhibitor NBC6, forming the inhibitor group. A different therapy compared to the EVLP experiments was utilised as NSAIDs have been related to nephrotoxicity [174, 175]. During the priming phase of each perfusion 53 mg of the drug was added to the circuit at baseline before the kidney was connected, and topped up with a 26.5 mg dose at the 2- and 4-hour time points via the renal artery. 53 mg NBC6 was dissolved in 530 μL of DMSO and 530 μL of cremophor, and added to 9.54 mL of PBS. The NBC6 concentrations were previously determined [146].
10 mL of perfusate was collected at baseline before the kidney was connected to the circuit, and at 30-, 60-, 180- and 360 minutes from the start of the perfusions. 500 μL of the perfusate was used fresh for flow cytometry on the cellular content. The rest of the samples were centrifuged at 300 g for the inflammasome particle experiments, and at 2000 g for the Luminex experiments, both for 10 minutes at 4°C, and stored at -80°C.

4.3.3 Flow cytometry on ASC+ inflammasome specks on porcine EVNP samples
The ASC+ inflammasome particles were measured in the serial EVNP perfusate samples at 0, 60, 180 and 360 minutes via flow cytometry. 1 mL of the sample was incubated with 1 μg of anti-ASC antibody (Caltag Medsystems, UK) for 90 minutes on a plate shaker at room temperature. The samples were then washed with 1 mL and re-suspended in 500 μL of staining buffer. The samples were read on the BD FACSCanto II flow cytometer (BD Biosciences, UK). The gating strategy was applied in FlowJo version 10.0.6 (FlowJo LLC, USA).

4.3.4 Luminex analysis on porcine EVNP samples
The levels of cytokines and a chemokine in the EVNP perfusate were assessed at the 0-, 60-, 180- and 360-minute time points. A commercially available 13-plex Luminex magnetic bead panel (Merck Millipore, USA) measuring GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α was utilised according to the manufacturer's protocol. The plate was read on a Bio-Plex 200 system (BioRad, UK).

4.3.5 Flow cytometry on cellular diapedesis on porcine EVNP samples
Cellular diapedesis was evaluated in the EVNP perfusate samples at 0-, 30-, 60-, 180-, and 360-minute time points. Perfusate samples were incubated with a panel of antibodies to characterise B cells (CD3-CD21+), T helper cells (CD3+CD4α+), cytotoxic T cells (CD3+CD8α+), classical monocytes (CD14++CD163-), intermediate monocytes (CD14+CD163-), non-classical monocytes (CD14+CD163+); NK cells (CD335+); mature neutrophils (6D10+2B2+), immature neutrophils (6D10+2B2-), and mature eosinophils/basophils (6D10-2B2+). The cells were then incubated in the dark for 10 minutes with 2 mL of RBC lysing buffer (BD Biosciences, UK) and finally washed and re-suspended in flow staining buffer with 20 μL 123count eBeads (eBioscience Ltc, UK). The samples were read on a BD LSR II flow cytometer (BD Biosciences, UK). All gating strategies were performed using FlowJo version 10.0.6 (FlowJo LLC, USA) and cell numbers were calculated in Microsoft Office Excel (Microsoft, USA).

4.3.6 Statistical analysis
Statistical analysis was carried out in the software SPSS version 22 (IBM, USA) and Prism
software version 7 (GraphPad, USA). Descriptive statistics of skewness, kurtosis, means and medians were utilised to assess data for normality. Approximate normality was also accepted. Differences between individual time points between groups were assessed with independent T tests if data was normally distributed, and with Mann-Whitney U test if the data was not normally distributed. As the number of replicates was small within both groups, non-parametric tests were preferred if there was a mix of normal and non-normal data within a group or between same time points. For normal data changes over time within and between groups were evaluated with repeated measures ANOVA. For non-normal data changes over time within groups were tested with Friedman test. Data was considered statistically significant if $P \leq 0.05$. Normally distributed data is presented as means with standard deviations; non-normally distributed data is given as medians with interquartile ranges.
4.4 Results

4.4.1 Functional parameters

Gradually declining renal function was observed in both groups. Renal perfusate flow or renal blood flow (RBF) rates and urine production did not differ between the control or inflammasome inhibitor groups over the 6-hour perfusion period (P=0.775 and P=0.671 respectively) (Figure 4.1). There were also no statistically significant differences between the two groups at any specific measured time points of 0, 60, 180 and 360 minutes for either of the parameters.

When looking at changes over time within the specific group, no statistically significant changes were observed in either of the groups for renal flow. In the start of the perfusion the renal flow measured at 25 (±9.3) mL/min/100 g of the weight of the kidney in the control group and 32.4 (±8.5) mL/min/100 g in the inhibitor group (P=0.225). There was a small increase in the RBF in both groups at 60 minutes where the mean renal blood flow measured at 48.3 (±22.1) mL/min/100 g in the control group, and at 53.3 (±19) mL/min/100 g in the inhibitor group (P=0.71). This was followed by a marginal decline at 180 minutes to 45 (±19.4) mL/min/100 g in the control group, and to 40.5 (±22.3) mL/min/100 g in the inhibitor group (P=0.74). At 360 minutes the renal blood flow measured at 53.7 (±44.8) mL/min/100 g in the control group, and 32.8 (±15.5) mL/min/100 g in the inhibitor group (P=0.37). Within group differences over time were statistically non-significant (P=0.175 for the control group, and P=0.079 for the inhibitor group).

However, in the control group urine production decreased significantly over time (P=0.001) but this was not the case in the inhibitor group (P=0.073). The changes between the two groups over time were statistically non-significant (P=0.671). Urine secretion was first measured at the 60-minute time point and then hourly until the end of the perfusion. At 60 minutes urine production was 117.5 (±43.5) mL/hr/100 g in the control group and 132.3 (±62.3) mL/hr/100 g in the inhibitor group (P=0.676). By 180 minutes there was a decline in the hourly urine production in both groups to 38.4 (±32.2) mL/min/100 g in the control group, and 40.8 (±29.2) mL/min/100 g in the inhibitor group (P=0.903). At 360 minutes it remained similar measuring a 38.7 (±35.4) mL/hr/100 g in the control group, and slightly increased at 46.3 (±45.9) mL/hr/100 g in the inhibitor group (P=0.776).
Figure 4.1. Functional parameters renal blood flow and urine output during the porcine EVNP

Renal perfusate flow rate (A) and urine output (B), adjusted for the weight of the kidneys, during ex vivo kidney perfusion did not differ between the control (n=5) and inflammasome inhibitor (n=5) groups over the 360 minutes of perfusion (repeated measures ANOVA, P=0.775 and P=0.671 respectively). Data is presented as means with SDs.
4.4.2 ASC+ inflammasome particles

The level of ASC+ inflammasome specks was similar in both groups at the start of the perfusion (Figure 4.2). However, these levels were significantly decreased in the inhibitor group compared to the control over the course of the 6-hour perfusion (P=0.008). However, within group changes over time were not significantly different in either group (P=0.196 in the control group, P=0.081 in the inhibitor group). When comparing individual time points between the two groups, a statistically significant difference was only observed at 180 minutes. At the start of the perfusion the ASC+ inflammasome particles measured at 3.64 (±2.65)% in the control group and 2.34 (±1.66)% in the inhibitor group (P=0.379). At the 60-minute and 180-minute time points these levels measured at 3.89 (±1.43)% and 3.82 (±1.89)% in the control group, and 2.1 (±1.37)% and 1.24 (±1.05)% in the inhibitor group respectively (P=0.077 at 60 minutes, and P=0.029 at 180 minutes). At the end of the perfusion the ASC+ inflammasome specks constituted 1.47 (±0.86)% in the control group, and 0.49 (±0.52)% in the inhibitor group (P=0.061).
ASC+ inflammasome specks in porcine EVNP perfusate

ASC+ inflammasome particles were reduced in the inhibitor group compared to the control group during porcine *ex vivo* kidney perfusion over 360 minutes (repeated measures ANOVA, *P*=0.008). At individual time points there was a significant difference between the two groups only at the 180-minute time point (independent *T* test, *P*=0.029). *N*=5 for both groups. Data is presented as means with SDs.
4.4.3 Cytokine profile

The cytokine profiles recorded during the EVNPs demonstrated similar patterns in both groups, where there was a statistically significant increase in the levels of IL-1β, IL-1Ra, IL-2, IL-6, IL-10, IL-12 and IL-18 during the perfusion. TNF-α increased until the 180-minute time point but then decreased by the end of the perfusion in both groups. No IL-4 was detected in either of the groups at any time point. IL-1α and IFN-γ increased significantly only in the inhibitor group, whereas IL-8 increased significantly only in the control group. GM-CSF demonstrated no changes over time in either of the groups. No statistical analysis was possible to conduct to compare changes between groups over time, as the data was mostly non-parametric, and as such these changes were analysed within the individual groups and at individual time points.

4.4.3.1 Inflammasome-related cytokines IL-1β and IL-18

No IL-1β (Figure 4.3 A) was detected in the control group at the beginning of the perfusions and the median level of IL-1β in the inhibitor group measured at 0 (IQR=0-90) pg/mL (P=0.69). At 60 minutes no IL-1β was detected in either of the groups. At 180-minutes there was an increase in IL-1β to 190 (IQR=80-265) pg/mL in the control group and 710 (IQR=510-1,230) pg/mL in the inhibitor group (P=0.008). At the end of the EVNP IL-1β in the control group measured at 2,120 (IQR=325-5,785) pg/mL, and in the inhibitor group at 2,060 (IQR=1,090-4,620) pg/mL (P=0.841). The increase in IL-1β was statistically significant within both groups (P=0.003 in the control group and P=0.002 in the inhibitor group).

At the beginning of the EVNP the levels of IL-18 (Figure 4.3 B) measured at 20 (IQR=20-55) pg/mL in the control group and at 0 (IQR=0-355) pg/mL in the inhibitor group (P=0.548). There was a slight increase in the IL-18 at 60 minutes to 90 (IQR=75-145) pg/mL in the control group, and to 90 (IQR=75-145) pg/mL in the inhibitor group (P=0.841). This trend continued to the 180-minute time point with the IL-18 measuring at 100 (IQR=75-165) pg/mL in the control group, and 120 (IQR=75-550) pg/mL in the inhibitor group (P=0.548). At 6 hours the IL-18 levels further increased to 150 (IQR=120-310) pg/mL in the control group and to 180 (IQR=135-495) pg/mL in the inhibitor group (P=0.841). The increase over time was significant both the control (P=0.005) and inhibitor (P=0.011) groups.
There was a statistically significant increase in both groups over time for IL-β (A) (Friedman tests, P=0.003 in the control group, P=0.002 in the inhibitor group) as well as IL-18 (B) (Friedman tests, P=0.005 in the control group and P=0.011 in the inhibitor group). There were no significant differences between individual time points between the groups for either of the cytokines except for IL-1β at 180 minutes when it was increased in the inhibitor group (Mann Whitney U test, P=0.008). N=5 for both groups. Data presented as medians with IQRs.

Figure 4.3 Inflammasome-related cytokines IL-1β and IL-18 in porcine EVNP perfusate
4.4.3.2 Cytokines detected in high concentrations in porcine EVNP

The IL-1Ra levels (Figure 4.4 A) in the control group started at 60 (IQR=20-85) pg/mL, and at 40 (15-90) pg/mL in the inhibitor group (P=0.841). There was a marginal increase at 60 minutes to 70 (IQR=15-90) pg/mL in the control group, and 80 (0-140) pg/mL in the inhibitor group (P=0.69). There was a more pronounced augmentation at the 180-minute time point, when IL-1Ra measured 460 (IQR=285-790) pg/mL in the control group and 510 (IQR=390-3,875) pg/mL in the inhibitor group (P=0.69). At 360 minutes the IL-1Ra measured at 1,010 (IQR=760-2,085) pg/mL in the control group and 1,320 (IQR=545-10,820) pg/mL in the inhibitor group (P=0.841). These changes were significant within both the control (P=0.03) and inhibitor (P=0.005) groups.

In the control group the IL-6 (Figure 4.4 B) measured at 0 (IQR=0-20) pg/mL, and in the inhibitor group at 60 (IQR=0-100) pg/mL (P=0.222) at the start of the perfusion. At the 60-minute time point IL-6 measured at 0 (IQR=0-160) pg/mL in the control group while increasing to 150 (IQR=75-165) pg/mL in the inhibitor group (P=0.31). This increase was more marked at 180 minutes with IL-6 measuring at 4,390 (IQR=2,550-7,590) pg/mL in the control group and at 9,020 (IQR=5,380-9,510) pg/mL in the inhibitor group (P=0.151), and at 360 minutes at 22,390 (IQR=11,430-23,245) pg/mL in the control group, and at 21,950 (IQR=18,550-26,870) pg/mL in the inhibitor group (P=0.69). The change over time was significant within both groups (P=0.002).

At the beginning of the perfusion IL-8 (Figure 4.4 C) measured at 30 (IQR=0-30) pg/mL in the control group and 0 (IQR=0-40) pg/mL in the inhibitor group (P=0.548). After 60 minutes the IL-8 levels measured in the control group at 0 (IQR=0-45) pg/mL, and at 60 (IQR=10-120) pg/mL in the inhibitor group (P=0.151). The median IL-8 increased to 1,130 (IQR=885-4,245) pg/mL in the control group, but decreased to 50 (IQR=0-16,445) pg/mL in the inhibitor group (P=0.548) at 180 minutes. At the end of the EVNP IL-8 had increased to 5,700 (IQR=1,965-34,990) pg/mL in the control group and to 14,980 (IQR=0-36,165) pg/mL in the inhibitor group (P=1). Statistically, this increase was significant only in the control group (P=0.025) and not in the inhibitor group (P=0.315).

No IFN-γ (Figure 4.4 D) was detected in the EVNP perfusate in either of the groups until 180 minutes into the perfusion when it measured at 0 (IQR=0-820) pg/mL in the control group and at 0 (IQR=0-535) pg/mL in the inhibitor group (P=0.905). By 360 minutes these levels measured at 0 (IQR=0-2,440) pg/mL in the control group, and at 1,480 (IQR=0-2,695) pg/mL in the inhibitor group (P=0.877). The IFN-γ levels demonstrated a statistically significant increase only within the inhibitor group (P=0.043), and not in the control group (P=0.145).
The levels of TNF-α (Figure 4.4 E) measured at 0 (IQR=0-35) pg/mL in the control group and 0 (IQR=0-90) pg/mL in the inhibitor group at the start of the perfusion (P=0.548), and augmented to 30 (IQR=0-150) pg/mL in the control group and to 170 (IQR=100-600) pg/mL in the inhibitor group at 60 minutes into the EVNP (P=0.095). At 180 minutes there was a further increase to 740 (IQR=400-4,825) pg/mL in the control group, and to 2,900 (IQR=1,270-4,990) pg/mL in the inhibitor group (P=0.421). However, the levels of TNF-α decreased by the 360-minute time point measuring at 110 (IQR=85-1,395) pg/mL and 620 (IQR=410-735) pg/mL in the groups respectively (P=0.151). The change over time was statistically significant in both groups (P=0.004 in both).
Figure 4.4 High levels of cytokines released during the porcine EVNP: IL-1Ra, IL-6, IL-8, IFN-γ and TNF-α.

There was an overall increase in the concentrations of IL-1Ra (A), IL-6 (B), IL-8 (C) and IFN-γ (D) over the course of the 360-minute porcine EVNPs. For TNF-α there was an increase until the 180-minute time point, but a subsequent decrease at the 360-minute time point. These changes over time were statistically significant within the control and inhibitor groups for IL-1Ra (P=0.03, P=0.005, respectively), IL-6 (P=0.002 for both) and TNF-α (P=0.004 for both). IL-8 increased significantly only in the control group (P=0.025), and IFN-γ only in the inhibitor group (P=0.043). N=5 for both groups. Data is presented as medians with IQRs, statistical analysis done using Friedman tests.
4.4.3.3 Cytokines detected in low concentrations in porcine EVNP

The median levels of IL-1α (Figure 4.5 A) were 0 (IQR=0-10) pg/mL in the control group and 10 (IQR=0-10) pg/mL in the inhibitor group at the start of the perfusion (P=0.69). At 60 minutes there was no IL-1α detected in either of the groups. At 180 minutes there was a marginal increase in the IL-1α to 10 (IQR=0-10) pg/mL in the control group, and to 20 (IQR=10-35) pg/mL in the inhibitor group (P=0.056). By the end of the perfusion IL-1α measured in the control group at 10 (IQR=5-85) pg/mL and at 30 (IQR=30-45) pg/mL in the inhibitor group (P=0.222). These changes over time were statistically significant only in the inhibitor group (P=0.003) but not in the control group (P=0.093).

No IL-2 (Figure 4.5 B) was detected at the start of the perfusion and at 60 minutes in either of the groups. At the 180-minute time point IL-2 was only present in some of the samples of the inhibitor group at 0 (IQR=0-30) pg/mL (P=0.69). By the end of the EVNP, the IL-2 had increased to 20 (IQR=0-30) pg/mL in the control group and to 30 (IQR=0-75) pg/mL in the inhibitor group (P=0.69). These changes were statistically significant in both the control (P=0.029) and inhibitor (P=0.043) groups.

Similarly, there was no IL-10 (Figure 4.5 C) detected at the beginning of the perfusion and at 60 minutes in either of the groups. At 180 minutes IL-10 had increased to 60 (IQR=45-80) pg/mL in the control group, and to 70 (IQR=50-155) pg/mL in the inhibitor group (P=0.548). At 360 minutes the IL-10 levels measured at 70 (IQR=65-95) pg/mL in the control group and at 110 (IQR=80-195) pg/mL in the inhibitor group (P=0.222). Statistically these increases over time were significant within both groups (P=0.003 for both).

The levels of IL-12 (Figure 4.5 D) started at 40 (IQR=15-45) pg/mL in the control group and at 40 (IQR=0-55) pg/mL in the inhibitor group (P=0.841), but at 60 minutes had decreased to 0 (IQR=0-20) pg/mL in both groups (P=1). There was a subsequent increase of the IL-12 levels at 180 minutes to 60 (IQR=0-140) pg/mL in the control group, and to 110 (70-135) pg/mL in the inhibitor group (P=0.151). By the end of the perfusion these had reached 110 (IQR=100-360) pg/mL in the control group and 230 (IQR=175-300) pg/mL in the inhibitor group (P=0.222). The changes over time were statistically significant in both the control (P=0.006) and the inhibitor (P=0.002) groups.

In the inhibitor group no GM-CSF (Figure 4.5 E) was measured at the beginning, at 180 minutes or the end of the perfusion. At 60 minutes GM-CSF was present in some of the samples and measured at 0 (IQR=0-40) pg/mL in the inhibitor group. In the control group these levels measured at 0 (IQR=0-15) pg/mL at the start of the perfusion (P=0.69), at 0 (IQR=0-25) pg/mL at 60 minutes (P=1), at 0 (IQR=0-30) pg/mL at 180 minutes (P=0.69),
and at 0 (IQR=0-95) pg/mL at the end of the perfusion (P=0.31). GM-CSF did not significantly change over time in either of the groups (P=0.154 in the control group and P=0.392 in the inhibitor group).
Figure 4.5 Low levels of cytokines released during the porcine EVNP: IL-1α, IL-2, IL-10, IL-12 and GM-CSF.

There was an overall increasing trend for the concentrations of IL-1α (A), IL-2 (B), IL-10 (C), IL-12 (D) and GM-CSF (E) during the 360-minute porcine EVNPs. Statistically these changes over time were significant for IL-1α only in the inhibitor group (P=0.003), but for IL-2 (P=0.029, P=0.043), IL-10 (P=0.003 for both) and IL-12 (P=0.006, P=0.002) both in the control and inhibitor groups, respectively. No significant change over time was noted in either of the groups for GM-CSF. N=5 for both groups. Data presented as medians with IQRs, statistical analysis done using Friedman tests.
4.4.4 Cellular diapedesis

Equally to the cytokine profiles, the overall cellular diapedesis during the EVNP followed similar patterns in both groups over the course of the perfusion. As most of the data was non-parametric, comparisons between groups over time were only possible for non-classical monocytes, where no statistically significant differences were observed (P=0.113). When looking at changes in cellular content of the perfusate over time within the two groups, similar patterns can be described for most cell types.

4.4.4.1 B cell and T cell diapedesis

The CD21+ B cells (Figure 4.6 A) measured at 4,479 (IQR=4,180-4,732) cells/mL in the control group and 5,447 (IQR=3,762-6,831) cells/mL in the inhibitor group at the beginning of the EVNP (P=0.151). These levels remained stable and comparable through the 30-, 60- and 180-minute time points in both groups (6,363 (IQR=5,115-6,819) cells/mL, 6,530 (IQR=6,360-7,237) cells/mL, 8,090 (IQR=6,593-8,829) cells/mL in the control group; and 6,255 (IQR=6,147-9,769) cells/mL, 4,909 (IQR=3,989-8,086) cells/mL, 5,247 (IQR=4,283-7,304) cells/mL in the inhibitor group; with P=0.841, P=0.69 and P=0.095 respectively). At the end of the perfusion the B cells measured at 9,545 (IQR=8,348-22,656) cells/mL in the control group and 12,957 (IQR=7,555-19,568) cells/mL in the inhibitor group (P=1). This increase over time was statistically significant in both the control (P=0.003) and inhibitor (P=0.015) groups.

Compared to B cells, the CD4+ helper T cells (Figure 4.6 B) were more scarce measuring at 2,073 (IQR=973-5,874) cells/mL in the control group and 2,805 (IQR=1,584-4,522) cells/mL in the inhibitor group at the start of the perfusion (P=0.841). There were slight fluctuations in the numbers of helper T cells in the perfusate samples throughout the EVNPs. At 30 minutes the helper T cells had increased to 3,997 (IQR=2,388-7,296) cells/mL in the control group, and 4,394 (IQR=3,807-7,873) cells/mL in the inhibitor group (P=0.548). There was a subsequent decrease at 60 minutes to 3,015 (IQR=2,420-7,309) cells/mL in the control group, and to 3,265 (IQR=1,434-6,345) cells/mL in the inhibitor group (P=0.841). At the 180-minute time point the helper T cells increased to 6,059 (IQR=2,443-9,483) cells/mL in the control group, but declined to 2,340 (IQR=1,573-6,139) cell/mL in the inhibitor group (P=0.31). By the end of the perfusions these measured at 2,982 (IQR=1,399-6,923) cells/mL in the control group and 3,495 (IQR=2,619-5,267) cell/mL in the inhibitor group (P=0.841). These changes over time were statistically non-significant in both the control group (P=0.26) and in the inhibitor group (P=0.126).
CD8+ cytotoxic T cells (Figure 4.6 C) measured at 4,280 (IQR=123-8,468) cells/mL and 479 (IQR=85-5,613) cells/mL in the control and inhibitor groups respectively at the start of the EVNP (P=0.421). However, there was a considerable increase by 30 minutes where the cytotoxic T cells measured at 30,473 (IQR=20,581-40,628) cells/mL and 39,437 (IQR=32,466-57,480) cells/mL in the control and inhibitor groups respectively (P=0.548). By the 60-minute time point these levels started to decrease measuring at 20,581 (IQR=18,942-41,303) cells/mL in the control group, and 26,729 (IQR=5,810-45,107) cells/mL in the inhibitor group (P=1). At the 180-minute time point the CD8+ T cells measured at 23,954 (IQR=19,853-42,848) cells/mL in the control group, and at 11,593 (IQR=7,594-32,403) cells/mL in the inhibitor group (P=0.151). At the end of the perfusions the cytotoxic T cells measured at 26,657 (IQR=12,485-30,935) cells/mL in the control group and at 21,589 (IQR=13,705-24,042) cells/mL in the inhibitor group (P=0.421). The changes over time within both groups were statistically significant (P=0.002 in the control group and P=0.001 in the inhibitor group).
Cellular diapedesis of B cells (A), CD4+ T cells (B) and CD8+ T cell (C) during porcine ex vivo kidney perfusion was similar between the control (n=5) and inflammasome inhibitor (n=5) groups during the 360 minutes of perfusion. There was a significant increase in the levels of B cells (P=0.003 in the control and P=0.015 inhibitor groups) and CD8+ T cells (P=0.002 in the control and P=0.001 in the inhibitor group), but not in the CD4+ T cells.

Data presented as medians with IQRs, statistical analysis done using Friedman tests.
4.4.4.2 Monocyte diapedesis

At the start of the EVNPs the classical monocytes (Figure 4.7 A) were present at more modest levels than the B- and T cells measuring at 713 (IQR=340-1,936) cells/mL in the control group and 1,169 (IQR=925-2,278) cells/mL in the inhibitor group (P=0.421). 30 minutes later these figures increased to 3,928 (IQR=2,711-5,004) cells/mL in the control group and 2,436 (IQR=1,600-3,122) cells/mL in the inhibitor group (P=0.056). At 60 minutes the numbers of classical monocytes increased to a further 5,749 (IQR=3,904-7,869) cells/mL in the control group and 2,879 (IQR=2,253-5,772) cells/mL in the inhibitor group (P=0.151). However, by 180 minutes these numbers had declined to 1,944 (IQR=1,447-2,496) cells/mL and 1,476 (IQR=1,090-2,336) cells/mL in the control and inhibitor groups respectively (P=0.841). By the 360-minute time point the classical monocytes measured at 1,305 (IQR=1,120-1,956) cells/mL in the control group and at 1,712 (IQR=1,163-2,129) pg/mL in the inhibitor group (P=0.548). A statistically significant change over the course of the perfusions was observed for the levels of classical monocytes in the control group (P=0.002) but not in the inhibitor group (P=0.118).

Compared to the classical monocytes the numbers of intermediate monocytes (Figure 4.7 B) started higher at 3,540 (IQR=3,509-4,938) cells/mL in the control group and at 5,533 (IQR=4,457-5,778) cells/mL in the inhibitor group (P=0.151). These levels demonstrated more pronounced fluctuations in the inhibitor group, whilst remaining more stable in the control group during the perfusions. At 30 minutes there was an increase in the numbers of intermediate monocytes in both the control and inhibitor groups, measuring at 6,767 (IQR=4,320-10,830) cells/mL and 7,413 (IQR=6,312-12,949) cells/mL respectively (P=0.31). At the 60-minute time point these measured at 6,505 (IQR=4,605-9,178) cells/mL in the control group, and 10,050 (IQR=7,785-11,450) cells/mL in the inhibitor group (P=0.151). At 180 minutes the intermediate monocytes remained at a stable level of 6,533 (IQR=4,466-9,928) cells/mL in the control group, but decreased to 7,124 (IQR=6,519-7,835) cells/mL in the inhibitor group (P=0.548). At 360 minutes these levels increased to 9,263 (IQR=4,658-14,032) cells/mL in the control group, and 14,726 (IQR=7,213-17,938) cells/mL in the inhibitor group (P=0.421). The levels of intermediate monocytes did not significantly change over time in the control group (P=0.364) or in the inhibitor group (P=0.051).

In comparison to the intermediate monocytes, there were fewer non-classical monocytes (Figure 4.7 C) measured at the start of the perfusion with 2,290 ±983 cells/mL in the control group and 2,807 ±1,176 cells/mL in the inhibitor group (P=0.472). These levels remained stable in the control group but demonstrated more noticeable changes in the inhibitor group. At the 30-minute time point the non-classical monocytes measured at
2,388 (±834) cells/mL in the control group, and at 2,692 (±473) cells/mL in the inhibitor group (P=0.498). At 60 minutes there was a statistically significant difference between the two groups, with the non-classical monocytes measuring at 1,960 (±566) cells/mL in the control group, and 3,206 (±642) cells/mL in the inhibitor group (P=0.012). This significant difference was not observed at the 180-minute time point, when these cells measured at 2,279 (±855) cells/mL in the control group, and 2,647 (±743) cells/mL in the inhibitor group (P=0.488). There was a further increase and a significant difference in the numbers of non-classical monocytes at 360 minutes with 2,539 (±628) cells/mL in the control group, and 4,166 (±1,129) cells/mL in the inhibitor group (P=0.023). In general, the change over time in the levels of non-classical monocytes was not significant within the control group (P=0.265), but was so in the inhibitor group (P=0.002). However, between group differences over time were not statistically significant (P=0.113).
Figure 4.7 Monocyte diapedesis during porcine EVNP

The changes over time in cellular diapedesis of classical monocytes (A) during porcine ex vivo kidney perfusion were statistically significant only in the control group (Friedman test, \( P=0.002 \)) \( (n=5) \) but not in the inhibitor group (Friedman test, \( P=0.118 \)) \( (n=5) \). For intermediate monocytes (B) these changes were non-significant in both groups (Friedman tests, \( P=0.364 \) in the control and \( P=0.051 \) in the inhibitor group). For non-classical monocytes (C; data presented as means and SDs) the change over time was statistically significant only within the inhibitor and not in the control group (\( P=0.364 \) and \( P=0.265 \) respectively), or between groups (\( P=0.113 \)), all tested by repeated measures ANOVA. Also, significant differences were noted at 60 and 360 minutes between groups (independent T tests, \( P=0.012 \) and \( P=0.023 \) respectively). Data presented as medians with IQRs unless otherwise stated.
4.4.4.3 Diapedesis of other cell types

At the start of the perfusion there were 5,457 (IQR=4,481-9,923) mature neutrophils (Figure 4.8 A) per mL in the perfusate samples of the control group, and 11,489 (IQR=7,293-15,876) cells/mL in the inhibitor group (P=0.095). These levels increased at 30 minutes measuring 7,214 (IQR=2,825-13,412) cells/mL in the control group, and 11,535 (IQR=5,358-26,418) cells/mL in the inhibitor group (P=0.421). At the 60-minute time point there was a decrease in the number of mature neutrophils in the control group at 6,550 (IQR=2,340-31,057) cells/mL, and in the inhibitor group to 7,385 (IQR=6,351-14,539) cells/mL (P=0.421). There was a further augmentation by the 180-minute time point in the number of mature neutrophils in the control group measuring at 15,055 (IQR=6,591-47,770) cells/mL, and in the inhibitor group at 9,431 (IQR=7,162-12,668) cells/mL (P=0.421). At 360 minutes these values measured at 29,302 (IQR=12,377-46,193) cells/mL in the control group, and 20,567 (IQR=8,834-48,094) cells/mL in the inhibitor group (P=1). The changes over time in the number of mature neutrophils did not significantly change over time within either the control group (P=0.075) or the inhibitor group (P=0.218).

There were fewer immature neutrophils (Figure 4.8 B) present in both groups compared to mature neutrophils, measuring at 1,225 (IQR=757-1,936) cell/mL in the control group and 1,666 (IQR=1,106-2,309) cells/mL in the inhibitor group at the beginning of the EVNPs (P=0.31). These levels measured at 30 minutes in the control group at 965 (IQR=549-2,176) cells/mL, and slightly increased in the inhibitor group measuring at 2,463 (IQR=698-4,336) cells/mL (P=0.421). At 60 minutes the numbers of immature neutrophils measured at 1,152 (IQR=419-4,573) cells/mL in the control group, and decreased to 1,755 (IQR=837-2,229) cells/mL in the inhibitor group (P=0.69). There was an increase to 2,935 (IQR=691-7,058) cells/mL in the control group, and to 1,880 (IQR=990-2,696) cells/mL in the inhibitor group at 180 minutes into the perfusions (P=0.69). At the end of the perfusion the median number of immature neutrophils measured at 5,281 (IQR=2,728-7,382) cell/mL in the control group and at 3,147 (IQR=1,789-9,167) cell/mL in the inhibitor group (P=1). However, similarly to the mature neutrophils, the changes over time within either group were statistically non-significant (P=0.086 for the control group and P=0.406 for the inhibitor group).

The baseline mature eosinophils/basophils (Figure 4.8 C) measured at 3,949 (IQR=2,636-5,898) cells/mL in the control group and 5,389 (IQR=3,183-6,450) cells/mL in the inhibitor group (P=0.548). There was a decrease in these numbers by the 30-minute time point in the control group to 2,154 (IQR=969-6,652) cells/mL, and an increase in the inhibitor group to 6,999 (IQR=2,010-14,967) cells/mL (P=0.31). At 60 minutes the levels
of mature eosinophils/basophils measured at 1,752 (IQR=644-9,273) cells/mL in the control group, and at 5,269 (IQR=2,663-9,114) cells/mL in the inhibitor group (P=0.31), with a following increase to 9,614 (IQR=1,799-16,585) cells/mL in the control group and decrease to 4,493 (IQR=3,095-7,198) cells/mL in the inhibitor group (P=0.69) at 180 minutes. By the end of the perfusions these levels measured at 7,647 (IQR=2,656-27,686) cells/mL in the control group and 5,421 (IQR=2,850-17,307) cells/mL in the inhibitor group (P=1). Again, comparably to neutrophils, statistically the numbers of mature eosinophils/basophils during EVNPs did not significantly change over time within the control group (P=0.171) or the inhibitor group (P=0.663).

The NK cells (Figure 4.8 D) measured at the start of the perfusions at 4,719 (IQR=3,454-10,642) cells/mL in the control group and 6,714 (IQR=3,803-10,297) cells/mL in the inhibitor group (P=1). At 30 minutes there was a notable increase in both groups with the NK cells measuring at 22,873 (IQR=13,307-32,363) cells/mL in the control group, and 19,726 (IQR=15,725-34,407) in the inhibitor group (P=0.841). The numbers of NK cells increased at the 60-minute time point to 24,585 (IQR=10,914-27,299) cells/mL in the control group, and slightly decreased to 19,115 (IQR=11,207-33,434) cells/mL in the inhibitor group (P=0.841). By 180 minutes there as a decline to 17,520 (IQR=11,172-22,576) cells/mL in the control group and to 18,997 (IQR=10,394-21,379) cells/mL in the inhibitor group (P=1). After 360 minutes of perfusion, the number of NK cells increased to 34,101 (IQR=25,689-43,240) cells/mL in the control group, and to 23,153 (IQR=16,565-31,079) cells/mL in the inhibitor group (P=0.151). These changes over time for the NK cells were statistically significant within both the control (P=0.007) and the inhibitor (P=0.02) groups.
Cellular diapedesis of mature neutrophils (A), immature neutrophils (B), eosinophils/basophils (C), and NK cells (D) during porcine ex vivo kidney perfusion was similar between the control (n=5) and inflammasome inhibitor (n=5) groups during the 360 minutes of perfusion. Statistically there were no significant changes over time in either of the groups for mature neutrophils, immature neutrophils, and eosinophils/basophils, but the change in the number of NK cells over time was statistically significant within both groups (P=0.007 in the control group, P=0.02 in the inhibitor group). All analysed by Friedman tests. Data presented as medians with IQRs.
Figure 4.9 Porcine kidneys before and after 6 hours of EVNP

Porcine kidneys on the *ex vivo* normothermic perfusion circuit at the start of the perfusion (A) and at the end of the 360-minute perfusion (B).
4.5 Discussion

An EVNP model was utilised to characterise the cell populations and cytokines released during the ex vivo porcine model for AKI with or without an inflammasome inhibitor. It was hypothesised that inhibiting the inflammasome would potentially alter the inflammatory response during acute injury and organ function. These experiments followed on from perfusing porcine lungs with an inflammasome inhibitor, where the organs could be classed as sufficiently healthy from the start of the procedures and therefore the therapy may not have had any additional functional benefits during EVLP. In order to investigate inflammasome inhibition in damaged organs, a porcine ex vivo normothermic kidney perfusion model was utilised, where the organ function was deteriorating over the course of 6 hours. As the principles between the perfusion setups of both organs are comparable, the results from these EVNP experiments may also be transferrable to EVLP injury models.

However, instead of utilising the NSAID flufenamic acid for inflammasome inhibition as was conducted in the lung perfusion experiments, a novel inflammasome inhibitor was used instead. NSAIDs have been linked to nephrotoxicity [174, 175] and therefore these EVNP experiments were conducted using an alternative pharmacological agent. Contrarily to flufenamic acid, already an established and prescribed drug, the NBC6 is a novel boron-based NLRP3 inflammasome inhibitor, developed and synthesised at the University of Manchester. Besides MCC950 (also known as CRIID3 or CP-456,773) that is also currently in development, there are no new NLRP3 inflammasome specific inhibitors available despite the increasing interest of the inflammasome as a therapeutic target [176]. Baldwin et al. demonstrated that the NBC6 drug surpressed the NLRP3 inflammasome related inflammation in vitro and in vivo, whilst showing no toxicity in kidney HEK293 or liver HepG2 cell lines up to 24 hours [146]. Additionally, they describe how the NBC6 drug has no off-target and potentially harmful effects on the Ca\(^{2+}\) channels, and the drug's activity was irreversible for the duration of their three-hour experiments [146]. They also contributed to the experimental design of this EVNP study, advising on the delivery and dosage concentrations, and hence it was judged as appropriate to progress with this particular method of inflammasome inhibition for this set of experiments.

A progressive decline in kidney function was observed over the course of the EVNP, characterised by low renal perfusate flow rates as well as visual observations of the organ and perfusate colour and appearance. Renal blood flow and urine output did not statistically differ between the control and inflammasome inhibitor groups during the course of 6 hours of perfusion. RBF was marginally higher in the inhibitor group compared to the controls at the start of the perfusions, but decreased at the two later time points,
whereas it remained more stable in the control group throughout the EVNPs. It has been suggested that the minimal rate of renal blood flow should not fall below 50 mL/min/100g for the kidney to be adequately perfused [177]. This means that during these experiments, organ damage was initiated by under-perfusing the kidneys, which then did not receive the appropriate amount of nutrients and oxygen to all areas of the organ. The kidneys suffered progressive ischemic injury, leading to interrupted cellular metabolism and further damage to the cells, resulting in cell death by apoptosis or necrosis. As the renal arterial pressure was kept constant during the EVNP, the low flow rates also indicate increased resistance and constricting blood vessels. Therefore, kidneys that suffered hypoperfusion over the course of 6 hours were considered suggestive of severe renal injury. These patterns did not differ between the control and inflammasome inhibitor groups.

Creatinine clearance was not tracked in this set of experiments, but urine production was measured as an additional indicator for organ function. Despite the low RBF rates, urine production started from the beginning of the perfusions, and remained comparable between both groups. It was elevated at the first hour of perfusion compared to the later time points. Urine production can indicate good kidney function, but it can also be stimulated, e.g. with the addition of mannitol to the circuit [178], and therefore has to be considered together with the blood flow rates. Therefore together with the prolonged renal hypoperfusion, it could be concluded that functionally there was no difference between the kidneys in the control or inhibitor groups. It was also evident from the visual macroscopic inspection of the kidneys (Figure 4.9), where dark speckles started to appear on the cortex of the organ by the end of the perfusion, as well as the dark red colour of the cellular perfusate, despite being oxygenated through the membrane oxygenator.

However, over time there was a significant reduction in ASC+ inflammasome particles in the EVNP perfusate in the inflammasome inhibitor group. Similarly to the EVLP experiments discussed in the previous chapter of the thesis, the activation of the NLRP3 inflammasome was indeed successfully inhibited during ex vivo kidney perfusion. Encouragingly, the direct effect of the drug was noticeable at the concentrations and the patterns introduced to the EVNP circuit. However, this did not change the overall inflammatory response or function of the kidneys.

The cytokine profiles of both groups were comparable with similar trends of cytokine secretion observed. This was the case for inflammasome-related IL-1β and IL-18, as well as other cytokines and chemokine measured. IL-1β was present at a considerably higher level at the end of the perfusion compared to IL-18, which suggest indeed a highly inflammatory environment and can further indicate organ damage. Interestingly, even
though the only individual time point where the ASC+ inflammasome particles were significantly reduced in the inhibitor group compared to the control one, was also the only individual time point where IL-1β was significantly higher in the inhibitor group compared to the control. This can indicate that in case of sustained and progressive damage to the organ, IL-1β activation is not only an inflammasome-dependant mechanism, but other pathways may be responsible for facilitating the same response. Extracellular pro-IL-1β can be cleaved and thereby activated by a number of proteases [179-181]. Neutrophil elastase, cathepsin G, proteinase 3 released by neutrophils can process the inactive pro-IL-1β at a slightly altered cleavage site compared to caspase-1 [179]. Additionally, chymase, released by mast cells, has also been shown to activate IL-1β [181]. However, this also results in a weaker IL-1β signalling and inflammatory response initiated than compared to IL-1β cleavage with caspase-1. This can suggest that inflammasome inhibition may need to be supplemented with inhibiting other proteases if these are prevalent in the specific disease model or condition.

As a model for a failing kidney, the pro-inflammatory response in terms of the cytokine profile as well as the immune cell content was intensifying throughout the period of 6 hours of perfusion. Leucocytes were found in the perfusate samples even at baseline when the kidneys were not yet connected to the circuit, despite leucocyte-filtering the packed RBCs in the priming stage. This demonstrated the questionable efficacy of using leucocyte filters for large animal organ perfusion, when a large volume of blood is utilised. The patterns of cellular diapedesis during the 6-hour porcine EVNP were also similar between the control and inhibitor groups. The most abundant cell types present in the perfusate were mature neutrophils, NK cells and CD8+ cytotoxic T cells. For the majority of cell types there was an overall trend of increase in the perfusate from the beginning of the EVNPs until the end. However, classical monocytes demonstrated distinct patterns in both groups, where there was a considerable increase at the 30- and 60-minute time points, followed by substantial decrease to the baseline levels at the 180- and 360-minute marks. Similar but more gradual patterns were observed in both groups for cytotoxic T cells, where the numbers in the perfusate started to decline after the 30-minute time point. This can suggest either that these cells migrated back to the kidney from the circulating perfusate, they were caught on the tubing and other parts of the circuit itself, or significant cell death occurred specifically in these cell populations. Due to the flow cytometry panel established at the time not including a live/dead cell marker, the latter cannot be confirmed from this set of experiments. The former explanation can explain the increasing inflammatory environment in the hypoperfused and damaged organ potentially signalling to recruit more immune cells to infiltrate the tissue. The increasing cellular content in the perfusate over the course of perfusion also signifies the extent of inflammatory capacity of
the kidney with a notable reservoir of immune cells. Interestingly, the cellular output in this porcine EVNP model differed from the healthy porcine kidney perfusion experiments also conducted by this group [168]. There substantially larger numbers of B cells, T cells and NK cells were identified in the perfusate, whilst the levels of mature neutrophils were higher in the experiments described in this thesis. This can suggest that hypoperfusion damage in the kidney will result in neutrophil activation and efflux. The importance of different cell types in experimental models of AKI has been investigated previously, however the specific roles for each of these remain to be further explored [182]. Nevertheless, it is clear that the kidney presents as a significant source for cellular and molecular immune content that can be released into circulation or be recruited into the organ, or in transplantation setting – into the recipient.

A limitation of this study was that it was not randomised or blinded. As the control and inflammasome inhibitor perfusions were carried out whilst knowing, which group the kidneys belonged to, it can be argued that bias was not eliminated and groups could have been treated differently. While this concern cannot be overlooked, given the lack of difference between groups, bias is unlikely. It is also a limitation that the control group was not perfused with the carrier of the NBC6 drug, as that would have been necessary for a direct comparison for the effects of the drug itself as an intervention.

The sourcing for the porcine kidneys utilised in this study was novel – the organs were procured not from laboratory animals, but from abattoir pigs. On one hand, this is a major ethical advantage when scientific research can utilise animals that are going to be culled for human consumption. This is in line with the 'Three Rs' of animal welfare principles: replacement, reduction and refinement [183]. Laboratory animals were replaced and not brought in specifically for these experiments and an alternative source for the kidneys was established. Additionally, the study did not require ethical approval for the use of laboratory animals and official training for the staff for handling laboratory animals. This also considerably reduced the cost of purchase and upkeep for the pigs required for the experiments. The animals were culled in accordance with standard abattoir protocols and the Home Office Act of 1986 Schedule 1 regulations, and were not treated any differently than they would have been, had the study not taken place. However, there are also caveats that accompany this form of sourcing the animals for research and that must be taken into consideration. The animals that are brought into abattoirs present a largely heterogeneous population. Even when the pigs are the same breed and have similar size, the farms where the animals are sourced from may have different conditions for raising them. For this study it was not possible to control for or even track an exact recorded history of how many pigs were raised together, in what size pens, what they were fed and more
importantly what drugs, antibiotics and supplements they were given. It became evident that not all of the pigs culled at the abattoir were healthy, and therefore any pre-existing conditions would also be difficult to document. Compared to a laboratory setting that is controlled and regulated to a much higher standard in terms of animal welfare, the abattoir is a very stressful place for the animals, which may be considered as a variable that can skew the quality of organs retrieved and thereby the study results. Nevertheless, the benefits of utilising porcine kidneys from the abattoir outweigh the disadvantages and as such this group has since carried out a number of studies with these as an alternative to laboratory animals.

In conclusion, in an ex vivo AKI model NLRP3 inflammasome inhibition does not impact on the decline in renal function or inflammatory response. The elevated levels of IL-1β in both groups demonstrate that NLRP3 inflammasome inhibition specifically does not have an effect on the systemic inflammation in the kidneys and the inflammatory cytokines are secreted via alternative pathways. It can be postulated that these results are translatable to an ex vivo lung injury model, whereby inflammasome inhibition would potentially not improve the function of marginal organs. Subsequently it can be suggested that inflammasome-related cytokines may not be a suitable point of therapeutic intervention, but rather manifestations of irreversible organ damage, and potentially also activated via inflammasome-independent pathways. Therefore, other IL-1β-related inflammatory mediators warrant further investigation in the context of organ recoverability during ex vivo perfusions.
Chapter 5: Inflammasome-related inflammatory mediators in clinical EVLP
5.1 Abstract

Even though IL-1β has been identified as a prognostic indicator of non-recovery during clinical EVLP, no functional benefits of inflammasome inhibition were observed in porcine ex vivo perfusion models, for either healthy or damaged organs. Therefore other inflammatory mediators linked with IL-1β secretion were investigated in clinical EVLP setting. Perfusate and BAL samples were collected from marginal lungs that successfully reconditioned and were used for transplantation (n=10), and lungs that failed to recover and were discarded (n=10). The inactive intracellular precursor molecule, pro-IL-1β, as well as NLRP3 inflammasome particles, pro-IL-1β cleavage enzyme caspase-1, and IL-18 were quantified and compared between the two groups. A significant increase in pro-IL-1β concentration was observed in human lungs that did not recondition during EVLP compared to those that successfully reconditioned and were used for transplantation. No differences were described for the other IL-1β-related compounds between the transplanted and discarded groups. It can be hypothesised that pro-IL-1β is passively released following cellular necrosis of the donor lung, rather than actively secreted as mature IL-1β after inflammasome activation. However, given the large quantities of caspase-1 in the perfusate, subsequent pro-IL-1β activation is still likely to occur.
5.2 Introduction

Recent clinical evidence indicates that increased levels of IL-1β are correlated with worsening lung function during EVLP and early graft function post-transplant [125, 184]. Having perceived no remarkable changes with the addition of NLRP3 inflammasome inhibitors to the porcine ex vivo perfusion models, except for decreased levels of inflammasome particles in the perfusate, the IL-1β release pathway was investigated in more detail in the human setting.

Even with recent research demonstrating a direct role for IL-1β in mediating lung injury [139-141], its specific role during EVLP remains unclear. As described previously, IL-1β affects a wide range of cells. It is synthesised in response to cellular stress signals as intracellular inactive pro-IL-1β, which is cleaved by caspase-1 into mature IL-1β enabling its extracellular secretion [127]. Similar mode of activation occurs for IL-18, another IL-1 family inflammatory cytokine. Activated IL-1β and IL-18 are potent cytokines that further the inflammatory response and drive recruitment of neutrophils and monocytes, inducing inflammation-related cell death referred to as pyroptosis.

In this chapter, perfusate and BAL samples collected as part of the National Institute for Health Research (NIHR) funded DEVELOP-UK trial were analysed. This was a prospective clinical trial involving all UK lung transplant centres. It was hypothesised that specifically measuring different components of the IL-1 cytokine family activation pathway, such as inflammasome specks, pro-IL-1β, IL-18 and caspase-1, in lungs that reconditioned and lungs that did not recover during EVLP, may further elucidate the findings connecting IL-1β to lung injury.
5.3 Methods

5.3.1 Sample selection
All EVLPs were conducted according to the protocol described in Chapter 2: Methods section of this thesis. Ethics was in place (REC 11/NE/0342) for this national multi-centre study. Perfusate and BAL samples were collected from all UK lung transplant centres and stored centrally at the Freeman Hospital, Newcastle. Perfusate and BAL samples were requested from n=20 EVLPs with an equal division from lungs that functioned well and were transplanted, versus a group that did not recondition during EVLP and were declined for transplantation. The sample selection remained blinded until analysis was completed when the grouping was revealed as shown in Figure 2.9. In brief, for n=10 procedures lung function did not recover sufficiently to enable safe transplantation, whereas for n=10 procedures lung function recovered sufficiently to enable transplantation. The latter group was further separated as n=7 lungs transplanted with good post-LTx outcomes, and n=3 lungs transplanted but the recipients died within 90 days. Perfusate samples were analysed at 15- and 90-minutes from the start of, and finally at the end of perfusions. BAL was collected before and after EVLPs.

5.3.2 Flow cytometry on inflammasome specks on clinical EVLP samples
ASC+, NLRP3+ and double-positive ASC+NLRP3+ inflammasome specks were measured in the perfusate and BAL samples using flow cytometry as described in a published protocol [128] as well as the previous chapters of this thesis with specifics given in Chapter 2: Methods. In brief, 1 mL of the sample was incubated for 1 hour at room temperature on a plate shaker with 1 µg anti-ASC antibody (Caltag Medsystems, UK) and 1µg anti-NLRP3 antibody (Caltag Medsystems, UK). The samples were washed with 1 mL of staining buffer. 0.5 µg of secondary antibody was added to 250 µL of staining buffer per sample. Samples were incubated for another 40 minutes at room temperature on a plate shaker. Finally, the samples were washed and re-suspended in 500 µL staining buffer. The samples were read using BD FACSCanto II flow cytometer (BD Biosciences, UK) and data was analysed in FlowJo version 10.0.6 (FlowJo LLC, USA).

5.3.3 ELISAs
Concentrations of pro-IL-1β, caspase-1, and IL-18 were measured with commercially available ELISA kits (the former two from R&DSystems, USA, and the latter from BosterBio, USA) according to the manufacturers’ protocols described in more detail in Chapter 2 of this thesis. Specific concentrations for the compounds were calculated in Microsoft Office Excel (Microsoft, USA).
5.3.4 Statistical analysis

Statistical analysis was completed in SPSS software version 22 (IBM, USA) and Prism software version 7 (GraphPad Software, USA). Data was assessed for normality according to descriptive statistics of mean, median, kurtosis and skewness. Approximate normality was also accepted. Normally distributed data was further analysed with independent T tests for comparisons between specific time points, and with repeated measures ANOVA for changes over time within groups and between groups. Non-normal data was analysed with Mann-Whitney U test when comparing individual time points, and with Friedman’s test when assessing changes over time. Log transformation was utilised to evaluate changes over time between groups when raw data was not normally distributed. Normal data is presented as means and SDs; non-normal data is presented as medians with IQRs.
5.4 Results

5.4.1 Inflammasome particles in clinical EVLP

NLRP3 inflammasome particles were measured in perfusate and BAL samples. However, due to the small quantities of samples available, BAL was analysed from n=6 lungs for the discarded group and n=4 lungs for the LTx group without further dividing these groups into successful and unsuccessful LTx ones. In the perfusate samples there was an overall trend of decreased inflammasome specks in the LTx group compared to the discarded group. The same was observed for the BAL samples, except for the pre-EVLP ASC+ particles.

ASC+ inflammasome specks in the perfusate samples (Figure 5.1 A) measured at 9.17 (IQR=4.72-16.78)% of particles measured in the discarded group, and at 6.02 (IQR=2.86-7.92)% in the LTx group 15 minutes into the clinical EVLPs (P=0.182). At the 90-minute time point there was a slight increase to 12.2 (IQR=4.12-24.3)% in the discarded group, and to 10.6 (IQR=5.26-17.15)% in the LTx group (P=0.661). By the end of perfusion there was a further increase in ASC+ inflammasome particles in the discarded group to 12.5 (IQR=5.42-21.9)% and a decrease in the LTx group to 8.9 (IQR=4.23-16.9)% (P=0.529). The change over time was not statistically significant in either the discarded group (P=0.163) or the LTx group (P=0.182). ASC+ particles in the pre-EVLP BAL (Figure 5.1 B) measured at 10.8 (IQR=4.02-18.85)% in the discarded group, and 13.5 (IQR=2.21-31.43)% in the LTx group (P=1). Post-EVLP ASC+ particles measured at 10.03 (IQR=1.52-29.8)% in the BAL of the discarded group, and 5.97 (IQR=0.8-21.53)% in the BAL of the LTx group (P=0.476). Changes over time within groups were not significant in the discarded group (P=0.655) or in the LTx group (P=0.317).

The NLRP3+ particles (Figure 5.1 C) were present at lower levels in the perfusate samples of both groups, measuring at 3.08 (±0.68)% in the discarded group, and at 2.62 (±0.43)% in the LTx group (P=0.1) 15 minutes into the EVLP. After 90 minutes there was an increase in the NLRP3+ specks in the discarded group to 3.35 (±1.36)% and a decrease to 2.38 (±0.17)% in the LTx group (P=0.052). By the end of the EVLPs these levels measured at 3.41 (±1.54)% in the discarded group, and at 2.8 (±0.67)% in the LTx group (P=0.271). Changes over time in the NLRP3+ particles between groups were statistically not significant (P=0.096), similarly to changes over time within each group (P=0.562 in the discarded group and P=0.179 in the LTx group). The levels of NLRP3+ inflammasome particles in the pre-EVLP BAL samples (Figure 5.1 D) were increased in the discarded group measuring at 15.23 (±13.32)% but remained low in the LTx group measuring at 1.86 (±1.51)% (P=0.088). In the post-EVLP BAL samples the NLRP3+ specks had decreased in the discarded group to 4.85 (±3.62)% and increased to 3.27 (±1.14)% in the
LTx group (P=0.431). There was no statistically significant difference in the change over time of the NLRP3+ particles between the two groups (P=0.102), or within groups: P=0.125 in the discarded and P=0.239 in the LTx group.

The levels of double-positive ASC+NLRP3+ inflammasome particles in the perfusate samples (Figure 5.1 E) were higher in the discarded group at 6 (±3.86)% compared to the LTx group at 3.47 (±1.6)% 15 minutes into the EVLPs (P=0.081). This was followed by a small increase in both groups where these specks measured at 6.64 (±5.03)% in the discarded group, and at 4.4 (±1.46)% in the LTx group (P=0.206) at 90 minutes after the start of the perfusions. By the end of the EVLPs the double-positive inflammasome specks increased to 9.12 (±5.6)% in the discarded group, and to 7.03 (±4.25)% in the LTx group (P=0.359). The change over time in the double-positive ASC+NLRP3+ inflammasome particles in the perfusate samples within both the discarded and the LTx group was not statistically significant (P=0.146, P=0.074 respectively). Between group changes over time were also not statistically significant (P=0.099). Similarly to the NLRP3+ specks, in the pre-EVLP BAL samples the ASC+NLRP3+ inflammasome particles (Figure 5.1 F) measured higher at 22.18 (±15.67)% in the discarded group, compared to 12.44 (±7.64)% in the LTx group (P=0.268). Post-EVLP there was a decrease in the discarded group to 12.88 (±5.76)% and in the LTx group to 9.3 (±5.17)% in these double-positive particles (P=0.347). These changes over time in the BAL samples were not statistically significant between the two groups (P=0.256), within the discarded (P=0.187) or within the LTx group (P=0.574).

When the LTx group was divided into successful and unsuccessful transplants, the levels of inflammasome particles were comparable in the perfusate samples between the two groups. At 15 minutes the ASC+ specks (Figure 5.2 A) measured at 5.48 (IQR=2.24-7.74)% in the successful LTx group and 7.1 (IQR=3.13-23.5)% in the unsuccessful LTx group. At 90 minutes these had increased to 10.2 (IQR=4.6-19.6)% in the successful LTx group, and to 10.6 (IQR=8.45-14.6)% in the unsuccessful LTx group. At the end of the perfusion there was a further increase in the successful LTx group to 13.4 (IQR=4.42-21.1)% but a decrease in the unsuccessful LTx group to 8.17 (IQR=0.58-9.62)%. The NLRP3+ inflammasome particles (Figure 5.2 B) were present at lower levels measuring at the start of the EVLPs at 2.66 (IQR=2.47-3.19)% in the successful LTx group and at 2.43 (IQR=1.81-2.78)% in the unsuccessful LTx group. At 90 minutes and at the end of the EVLP the NLRP3+ particles measured at 2.46 (IQR=2.27-2.57)% and 2.38 (IQR=2.23-3.31)% in the successful LTx group, and at 2.32 (IQR=2.18-2.43)% and 3.28 (IQR=3.28-3.44)% in the unsuccessful LTx group respectively. The double-positive ASC+NLRP3+ inflammasome specks (Figure 5.2 C) started at 3.47 (IQR=2.17-3.92)% and increased to 4.64 (IQR=2.79-
6.02\% at 90 minutes and to 4.82 (IQR=4.14-12.5)\% at the end of the perfusions in the successful LTx group. Similar trends were found in the unsuccessful LTx group where these particles measured at 2.43 (IQR=2.4-6.83)\%, 3.74 (IQR=2.8-6.09)\% and 6.49 (IQR=2.09-13.7)\% at the respective time points. No meaningful statistical analysis was applied to these values, as there were an insufficient number of replicates in the unsuccessful LTx group.
Figure 5.1 Inflammasome particles in clinical EVLP samples

In the clinical EVLP perfusate samples there was a general trend of reduced ASC+ (A; data presented as medians with IQRs and analysed by Friedman test), NLRP3+ (C) and ASC+NLRP3+ (E) particles in the lungs that were transplanted (n=10) compared to the discarded lungs (n=10). The ASC+ specks post-EVLP, and NLRP3+ specks (D) as well as the ASC+NLRP3+ specks (F) were reduced in the LTx group both pre-EVLP and post-EVLP. For BAL samples n=6 in discarded and n=4 in LTx group. No statistically significant changes were observed. Data presented as means with SDs and analysed by repeated measures ANOVA unless otherwise stated.
Figure 5.2 Inflammasome particles in clinical EVLP perfusate samples of transplanted lungs

The levels of NLRP3 inflammasome particles were comparable in the clinical EVLP perfusate samples between the successfully transplanted (n=7) and the unsuccessfully transplanted (n=3) groups. These levels were the highest for the ASC+ and ASC+NLRP3+ inflammasome particles (A, C), and lower for the NLRP3+ specks (B). Data presented as medians with IQRs. No statistical analysis was conducted as n=3 in the unsuccessful LTx group.
5.4.2 Caspase-1 in clinical EVLP

Similarly to the sample analysis for the inflammasome particles, the issue of sufficient sample volume was encountered when measuring caspase-1 in the EVLP perfusate and BAL. As such the number of replicates for each time point for both perfusate and BAL alters for this variable.

Caspase-1 was high in both groups in the perfusate throughout the clinical EVLPs as well as the BAL samples. At the start of the perfusions, the mean caspase-1 concentration in the perfusate (Figure 5.3 A) was 1,188.52 (±1,051.52) pg/mL in the discarded group and 654.81 (±426.57) pg/mL in the transplanted group (P=0.214). At 90 minutes caspase-1 increased to 1,671.85 (±1452.08) pg/mL in the perfusate samples of the discarded group, and to 1,561.72 (±943.53) pg/mL in the LTx group (P=0.851). By the end of EVLP caspase-1 measured at 1,558.4 (±965.79) pg/mL in the perfusate of the lungs that did not recondition, and at 1,572.81 (±1,057.35) pg/mL in the LTx group (P=0.978). Despite no significant differences over the course of the EVLPs between groups (P=0.633), there was significant increase in the levels of caspase-1 within both the discarded (P=0.004) and the LTx group (P=0.003) individually.

The levels of caspase-1 in the BAL samples (Figure 5.3 B) were higher compared to the perfusate samples. In the discarded group pre-EVLP caspase-1 measured at 2,394.3 (IQR=1,552.62-3,050.38) pg/mL, and similarly at 2,289.9 (IQR=1,070.5-3,238.7) pg/mL in the LTx group (P=0.805). These concentrations decreased in the post-BAL in the discarded group to 1,843.82 (IQR=1,549.96-3,613.7) pg/mL, and in the LTx group to 1,175.8 (IQR=806.19-1,863.8) pg/mL (P=0.108). The change over time was not significant within the discarded group (P=0.125) or within the LTx group (P=0.301).

When the LTx group was again further divided into successful and unsuccessful LTx groups depending on the 90-day survival of the recipients, the caspase-1 levels in the perfusate samples were similar in both groups, but demonstrated a decreasing trend in the post-EVLP BAL samples in the successful LTx group. In the perfusate samples (Figure 5.3 C) of the unsuccessful LTx group the caspase-1 measured 474.19 (IQR=253.66-694.72) pg/mL, 1,298.5 (IQR=627.9-2,334.2) pg/mL and 1,271.9 (IQR=820.84-1,722.9) pg/mL, and in the successful LTx group it measured 602.3 (IQR=291.78-1,187.5) pg/mL, 1,537.5 (IQR=499.33-2,215.2) pg/mL and 1,846 (IQR=480.62-2,128.3) pg/mL at 15 minutes, 90 minutes and at the end of the EVLPs respectively. The caspase-1 levels in the BAL samples (Figure 5.3 D) pre-EVLP measured 2,289.9 (IQR=1,070.5-3,238.7) pg/mL in the unsuccessful LTx group and 2,157.1 (IQR=1,234.5-4,039.3) pg/mL in the successful LTx group. Post-EVLP the BAL caspase-1 decreased in the unsuccessful LTx group to 2,052.55 (IQR=1675-2,430.1) pg/mL, and to 974.19 (IQR=733.14-1,210.8) pg/mL in the successful
LTx group. However, no meaningful statistical analysis could be done because of the small number of replicates.
Figure 5.3 Caspase 1 in clinical EVLP samples
Concentrations of caspase-1 in human EVLP perfusate samples at 15-, 90 minutes and the end of perfusion (A; data presented as means with SDs) and in pre-EVLP and post-EVLP bronchoalveolar lavage (BAL) samples (B) for marginal lungs that did not recondition and were discarded, and lungs that recovered sufficiently to meet the criteria for lung transplantation (LTx). The levels of caspase-1 in the perfusate increased significantly over time (repeated measures ANOVA, P=0.004 in the discarded and P=0.003 in the LTx group). No significant changes over time within groups were observed for caspase-1 in the BAL samples (Friedman test, P=0.125 in the discarded group and P=0.301 in the LTx group). When the LTx group was further divided into successful and unsuccessful groups depending on the 90-day survival of the recipients, the caspase-1 levels in the perfusate samples were similar in both groups (C), but demonstrated a decreasing trend in the post-EVLP BAL samples in the successful LTx group (D). No statistical analysis was conducted for (C-D) as n=3 in the unsuccessful LTx group. Data presented as medians with IQRs unless otherwise stated.
5.4.3 Pro-IL-1β in clinical EVLP

The perfusate levels of pro-IL-1β (Figure 5.4 A) demonstrated differing patterns between the two groups over the course of the EVLP. In the discarded group pro-IL-1β measured at 7.86 (IQR=0-53.87) pg/mL at the start of the perfusion, in the LTx group the median values measured at 0 (IQR=0-2.85) pg/mL (P=0.165). After 90 minutes of perfusion the pro-IL-1β levels increased to 31.34 (IQR=5.9-152.28) pg/mL in the discarded group, and to 4.85 (IQR=0.75-29.27) pg/mL in the LTx group (P=0.133). By the end of the EVLPs these levels measured at 82.18 (IQR=45.32-238.83) pg/mL in the discarded group, and at 21.11 (IQR=4.68-41.82) pg/mL in the LTx group (P=0.007). The levels of pro-IL-1β increased significantly over time in both groups, however this change was more pronounced in the lungs that did not recondition (P<0.0001) compared to the ones that were transplanted (P=0.002). When raw pro-IL-1β concentration values were logged, the data could be considered normally distributed and the change over time between groups was also found to be statistically significant (P=0.025).

Similar trends were observed in the pre- and post-EVLP BAL samples (Figure 5.4 B). Before the perfusions the pro-IL-1β measured at 37.21 (IQR=0-172.65) pg/mL in the discarded group, and at 4.98 (IQR=0-99.5) pg/mL in the LTx group (P=0.72). In the discarded group these levels increased to 118.83 (IQR=3.13-388.72) pg/mL, and to 6.59 (IQR=0-297.86) pg/mL in the transplanted group in the post-EVLP BAL samples (P=0.4). The change over time was not statistically significant within either group (P=0.739 in the discarded and P=0.257 in the LTx group) or between groups (P=0.471).

When dividing the LTx group into successful transplants and unsuccessful transplants, defined by 90-day post-transplant survival, further trends for differences between the two groups could be demonstrated. In the successful transplant group the perfusate levels of pro-IL-1β (Figure 5.4 C) measured at 0 (IQR=0-0.23) pg/mL, whereas in the unsuccessful transplant group they were 4.21 (IQR=0-8.64) pg/mL 15 minutes after the start of the perfusion. At the 90-minute time point the pro-IL-1β measured at 1.93 (IQR=0-10.78) pg/mL in the successfully transplanted group, and 29.96 (IQR=10.12-33.8) pg/mL in the unsuccessfully transplanted lungs. At the end of the EVLP these values increased to 20.9 (IQR=0-24.34) pg/mL in the successfully transplanted group, and to 55.42 (IQR=16.32-87.07) pg/mL in the unsuccessful transplant group. Similar results were observed in the BAL samples (Figure 5.4 D) where the pre-EVLP pro-IL-1β measured at a higher 126.46 (IQR=0-1,077.7) pg/mL in the unsuccessful LTx group, and at a lower 2.49 (IQR=0-43.1) pg/mL in the successful LTx group. There was an increase in the concentrations of pro-IL-1β in the post-EVLP BAL samples of the unsuccessful LTx group measuring at 514.18 (IQR=16.06-934.83) pg/mL, and a decrease in the successful LTx group measuring at 1.18
(IQR=0-25.33) pg/mL. However, no meaningful statistical comparisons could be calculated as the unsuccessful transplant group only consisted of n=3 replicates.
Figure 5.4 Pro-IL-1β in clinical EVLP samples

Concentrations of pro-IL-1β in human EVLP perfusate samples at 15-, 90 minutes and the end of perfusion (A), as well as pre-EVLP and post-EVLP bronchoalveolar lavage (BAL) samples (B), for marginal lungs that did not recondition and were discarded (Discarded) (n=10), and lungs that recovered sufficiently to meet the criteria for lung transplantation (LTx) (n=10). There was a significant increase over time in the levels of pro-IL-1β in the perfusate of the lungs that were discarded compared to LTx (repeated measures ANOVA, P=0.025), as well as within individual groups (Friedman tests, P<0.0001 in the discarded and P=0.002 in the LTx group). Additionally, at the end of the perfusions, pro-IL-1β was significantly elevated in the discarded group (Mann-Whitney U test, P=0.007). These differences were not significant for the BAL samples (Friedman test, P=0.739 in the discarded and P=0.257 in the LTx group). When the LTx group was divided into successful (n=7) and unsuccessful (n=3) groups depending on the 90-day survival of the recipients, the pro-IL-1β levels were higher in the unsuccessful LTx group both in the perfusate and the BAL samples (C-D). No statistical analysis was conducted for those however as n=3 in the unsuccessful LTx group. Data presented as medians with IQRs.
5.4.4 IL-18 in clinical EVLP

The levels of IL-18 increased in the perfusate samples of both groups over time, but were marginally elevated throughout the perfusions where lungs did not recover (Figure 5.5 A). At the start of the perfusions, the IL-18 concentration in the discarded group was 46.46 (±28.81) pg/mL, and in the transplanted group was 29.5 (±21.81) pg/mL (P=0.161). At 90 minutes levels of IL-18 increased to 100.15 (±57.02) pg/mL in the discarded group, and to 59.96 (±40.6) pg/mL in the LTx group (P=0.143). By the end of EVLP the concentrations of IL-18 increased to a further 133.04 (±63.83) pg/mL in the discarded lungs, and to 89.23 (±72.1) pg/mL in the reconditioned lungs (P=0.181). The increase over time in the levels of IL-18 was statistically significant in the discarded group (P=0.001) as well as the LTx group (P=0.009). However, the changes over time between groups were not significant (P=0.1).

The IL-18 levels in the pre-EVLP BAL samples (Figure 5.5 B) were comparable in both groups measuring 68.55 (IQR=47.48-237.37) pg/mL in the discarded group, and 75.09 (IQR=43.93-146.91) pg/mL in the LTx group (P=0.931). The IL-18 increased in the post-EVLP BAL samples of the discarded group to 90.69 (IQR=37.68-151.09) pg/mL, and decreased in the LTx group to 61.78 (IQR=28.15-84.99) pg/mL (P=0.133). The changes over time in IL-18 concentrations in BAL samples within both of the groups were not statistically significant (P=0.317 in both).

Similarly to caspase-1, when the LTx group was further divided into successful and unsuccessful groups depending on the 90-day survival, the IL-18 levels in the perfusate samples were comparable in both groups, but demonstrated a decreasing trend in the post-EVLP BAL samples in the successful LTx group. In the perfusate (Figure 5.5 C) these levels measured at 31.32 (IQR=15.24-43.55) pg/mL, 60.73 (IQR=34.57-92.02) pg/mL and 56.7 (IQR=48.14-157.12) pg/mL in the unsuccessful LTx group, compared to 20.46 (IQR=10.28-33.65) pg/mL, 46.93 (IQR=18.41-112.24) pg/mL and 64.28 (IQR=25.75-164.79) pg/mL in the successful LTx group at the respective time points. In the BAL samples (Figure 5.5 D) the IL-18 measured at 84.82 (IQR=56.95-130.99) pg/mL and 90.01 (IQR=79.97-107.42) pg/mL in the unsuccessful LTx group, and at 64.08 (IQR=33.76-216.48) pg/mL and 32.33 (IQR=26.66-62.88) pg/mL in the successful LTx group before and after the EVLPs respectively. However, no statistical analysis could be completed as n=3 for the unsuccessful LTx group.
Figure 5.5 IL-18 in clinical EVLP samples

Concentrations of IL-18 in human EVLP perfusate samples at 15-, 90 minutes and the end of perfusion (A), and in the pre-EVLP and post-EVLP bronchoalveolar lavage (BAL) samples (B) for marginal lungs that did not recondition and were discarded (n=10), and lungs that recovered sufficiently to meet the criteria for lung transplantation (LTx) (n=10). The levels of IL-18 in the perfusate samples did not significantly change over time between the two groups (repeated measures ANOVA, P=0.1; data presented as means with SDs). However, there was significant increase over time in the levels of IL-18 in the perfusate samples in both groups when considered individually (repeated measures ANOVA, P=0.001 in discarded and P=0.009 in LTx group). These changes were not statistically significant in the BAL samples (Friedman test, P=0.317 in both groups). When the LTx group was divided into successful (n=7) and unsuccessful (n=3) groups depending on the 90-day survival, the IL-18 levels in the perfusate samples were similar in both (C), but demonstrated a decreasing trend in the post-EVLP BAL samples in the successful LTx group (D). No statistical analysis was conducted for those however as n=3 in the unsuccessful LTx group. Data presented as medians with IQRs, unless otherwise stated.
5.5 Discussion

In the sets of experiments discussed in previous chapters of this thesis, NLRP3 inflammasome inhibition did not impact upon the function or inflammatory profile of healthy porcine lungs or failing kidneys. As a result, the inflammasome pathway was investigated in more detail during EVLP in humans.

Andreasson et al. quantified IL-1β with a panel of other inflammatory mediators in samples collected as part of the DEVELOP-UK trial, including from the 20 EVLPs analysed in the current chapter [125, 184]. Their group reported that IL-1β was a prognostic indicator of recoverability of the donor lungs during EVLP. Therefore the NLRP3 inflammasome particles and caspase-1 were measured in this cohort, as these are involved in cleaving the inactive precursor molecule pro-IL-1β into the active form. The concentrations of pro-IL-1β and another inflammasome-related cytokine IL-18 correlated to lung injury [141, 185] were also measured. The levels of the inflammasome specks and caspase-1 demonstrated similar patterns of change over time in the serial perfusate samples of both lungs that were discarded and lungs that were transplanted after EVLP. However, the increase over time in pro-IL-1β was significantly more pronounced in the discarded group compared to the transplanted lungs. Similar patterns were observed for the perfusate IL-18 levels but without statistically significant differences between groups.

Conventionally, pro-IL-1β is not present in the extracellular space as it is an intracellular peptide, synthesised upon stress signals and only actively secreted in its mature form [127]. However, the levels of pro-IL-1β mirrored the levels of mature IL-1β described in the DEVELOP-UK trial [125, 184]. The inactive pro-form of the cytokine was abundant in the discarded group as early as 15 minutes after the start of perfusion and steadily increased until the end. This was in contrast to the successful EVLP group where pro-IL-1β remained low. Even more intriguingly, patients who were transplanted with lungs that had elevated pro-IL-1β levels during EVLP had the poorest post-transplant outcomes in terms of 90-day mortality. Furthermore, pro-IL-1β was higher than mature IL-1β, which was previously measured by the Newcastle group [125]. This indicates that the cells were producing pro-IL-1β in response to received stress signals but were dying before mature IL-1β could be cleaved and secreted, releasing the pro-form of the cytokine into the perfusate upon cell death. Based on this evidence it can be hypothesised that cellular injury has already occurred to the lungs prior to EVLP, where cell membranes are ruptured and intracellular contents are released into the extracellular space (i.e. in the case of EVLP, the perfusate) – a hallmark of necrosis or pyroptosis [132].
IL-18 increased significantly in both groups during EVLP but with no statistically significant differences between the discarded and LTx groups. The general trends were similar to those of pro-IL-1β where the levels were higher in the discarded group without statistical significance. By contrast, these trends for the perfusate IL-18 levels were similar between the successful and unsuccessful LTx groups. However, it was not possible to distinguish between the pro-IL-18 and the mature IL-18 due to a lack of technical resources, hence it cannot be determined whether the concentrations of IL-18 truly emulate the pro-IL-1β and can also be considered as markers for cellular damage. Similarly to pro-IL-1β, higher pro-IL-18 could further signify cell membrane rupture if it is released into the extracellular space, even though contrarily to pro-IL-1β, it is constitutively expressed within the cell, rather than only after cellular stimulation.

In contrast, caspase-1 levels expressed similar patterns of change over time in the perfusate and BAL samples in both groups. However again, it was not possible to distinguish between pro-caspase-1 and caspase-1 in these samples. The presence of NLRP3 inflammasome particles within all samples indicates that even the inactive pro-caspase-1 may be cleaved within the perfusate into its active form. Given that high concentrations of caspase-1 were detectable in all perfusate samples irrespective of EVLP outcome, it is likely that extracellular pro-IL-1β as well as pro-IL-18 can be cleaved in the perfusate. This enables active IL-1β and IL-18 to initiate downstream signalling pathways including the upregulation of endothelial adhesion molecules. In addition, as mentioned in previous chapters, studies have also demonstrated that a number of proteases are able to cleave pro-IL-1β into its active form, which means that caspase-1 is not solely responsible for IL-1β cleavage. In turn this can also suggest that even with inhibiting the NLRP3 inflammasome specifically, pro-IL-1β can be cleaved and activated in the perfusate [179]. Inhibiting the mature IL-1β in the perfusate may therefore affect the subsequent cellular interactions and processes. However, if cellular death, pyroptosis or necrosis, of the lung is occurring as suggested by the presence of intracellular pro-IL-1β in perfusate, regulating those specific cytokines may not yield further benefits in terms of improving lung reconditioning during EVLP. Currently there are no quantifiable necrosis-specific biomarkers available, but it can be presumed that finding intracellular particles in the extracellular space marks inflammatory and uncontrolled cell death. Similarly, Hashimoto et al. [186] have recently correlated cell death related proteins in EVLP perfusate with worse post-transplant PGD scores. They measured the levels of M30 as a marker of epithelial apoptosis, and high mobility group box 1 (HMGB1) as a marker for inflammation and cell death, and suggested that these biomarkers could be utilised for improved donor organ selection. Correspondingly, Hsin and colleagues [187] described the metabolic profile of 50 clinical EVLP perfusate samples. They suggested that the metabolic profile
differs in the EVLP lungs that are transplanted compared to the discarded ones, but also in the LTx lungs where recipients develop grade 3 PGD post-transplant as opposed to those who do not. Even more interestingly they demonstrate differences in a number of metabolites that are components of the cell membrane integrity, which can be argued to comply with the model of cellular injury in the lungs proposed here. Therefore pro-IL-1β can be considered as one of the molecules of interest as a marker of cellular damage and death, and it warrants further research in lung function.

Again, one of the main limitations of this study, similarly to the large animal experimental ex vivo perfusion models, was the small sample size. EVLP is a relatively novel clinical technique; it was therefore invaluable to have the opportunity to work with serial perfusate and BAL samples from the DEVELOP-UK clinical trial. The samples were collected across five sites in the UK that were taking part in the study, and as such the amount of work that went into obtaining and processing these samples has to be appreciated and noted. Years of work were put into setting up and establishing this multi-centre trial, and the perfusions performed between April 2012 and July 2014 [184]. It is challenging to gain access to any human samples but to do so for EVLP, that is still in its infancy, is especially rewarding and can provide researchers with invaluable knowledge. On the other hand, it means that the assays and experiments conducted on these samples had to be carefully considered and calculated to utilise the available amounts of each sample as efficiently as possible. There was also no room for errors or repeats for the assays, and it would have been difficult to conduct follow-up experiments to the ones initially run with the same sets of samples.

After completing the initial analysis on n=20 clinical EVLP samples, it was planned to extend the study to the full n=52 lungs recruited into the trial. However, issues with available sample material as well as timings with this PhD project prevented this to be examined further. In addition to this and contrarily to the porcine experiments, the clinical EVLP samples were not available in larger volumes and multiple aliquots. As mentioned, this meant that the experimental design and protocols was extremely limited. As the various inflammasome-related compounds in this model of pulmonary injury were characterised, it was not feasible to carry out any functional assays to investigate the effects these may yield on the different cell types of the lung. Additionally, sufficient quantities of the samples were not available to investigate cell death markers in more detail, which would have been interesting to compare to the levels of pro-IL-1β as a marker for cellular injury. Still, these results are intriguing and future studies on lung injury and/or transplantation should consider this pathway in more detail.
In conclusion, even though IL-1β is predictive of lung recoverability as well as post-transplantation outcome, it can be argued that inhibiting its release would not yield any benefits for lung reconditioning as a whole, as damage to the organ has already occurred. The data indicates that therapeutic interventions to control or reverse cell death either in the donor environment, or more realistically during preservation, may be of benefit. Such strategies warrant further investigation in order to maximise donor lung availability for transplantation.
Chapter 6: Inflammasome-related mediators in early post lung transplant plasma samples
6.1 Abstract

After investigating the role of IL-1β and pro-IL-1β in the reconditioning processes and injury of donor lungs undergoing EVLP, it was decided to investigate the importance of these inflammatory mediators in post-lung transplantation recovery processes. Plasma samples were collected from n=7 LTx recipients immediately before and at 12, 24, 48 and 72 hours post-transplant. The NLRP3 inflammasome-related molecules were measured in these samples and correlated with the corresponding PaO$_2$/FiO$_2$ ratio scores as indicators of lung function. No statistically significant associations were found between these values, despite IL-18 demonstrating a trend of negative correlation with the P/F ratio at 72 hours post-LTx. However, these results are limited for the small sample size and increased numbers may further elucidate these specific trends.
6.2 Introduction

Lung transplant recipients are at high risk of serious complications and death in the early post-operative period. It has been estimated that 10-30% of lung transplant recipients develop primary graft dysfunction (PGD), one of the leading causes of early death post-transplant [188]. PGD is characterised by reduced lung compliance, rising pulmonary pressures and in severe cases pulmonary oedema [188, 189]. It is defined by persistent hypoxemia and the presence of bilateral pulmonary infiltrates at 6, 24, 48 and 72 hours after reperfusion, graded 0-3 by increased severity (Table 1.3) [42, 189]. Grade 3 PGD has been linked to prolonged ICU- and hospital stays, duration of mechanical ventilation as well as augmented short- and long-term mortality rates, and worsened quality of life at later stages compared to patients with lower PGD [50, 190, 191]. The underlying pathophysiology leading to the development of PGD is poorly understood. However, activation of neutrophils and alveolar macrophages, the recruitment of leucocytes and their interaction with endothelium, and the loss of epithelial integrity have been suggested to have a major role in the progression of the condition [189]. Understanding these in more detail may lead to novel therapeutic approaches for treatment and prevention of PGD that currently are lacking.

As previously discussed, no lung injury specific biomarkers have been defined but Cantu et al. have described inflammasome-related innate immune signalling pathways that are involved in the development of PGD [192]. Considering these findings, as well as the previously observed differences in the levels of pro-IL-1β between the lungs that had recovered during EVLP and been subsequently transplanted, compared to those that had not reconditioned and been discarded, the inflammasome- and IL-1β-related proteins were assessed immediately following lung transplantation. This study aimed to investigate the involvement of these mediators in the early post-LTx setting to determine whether any of these could be considered as potential biomarkers for pulmonary injury at this period, irrelevant of EVLP. Finding such markers could potentially further the understanding of the development of pulmonary injury post-LTx and thereby lead to improved therapeutic strategies and patient outcomes. Equally, such strategies could then potentially be applied to managing lung injury in organ donors.

Therefore the levels of inflammasome-associated products were evaluated in plasma samples pre- and up to 72 hours post-LTx, correlating these to early post-operative outcomes. The aim of the study was to assess if inflammasome pathway products could be associated with early post-operative lung function.
6.3 Methods

6.3.1 Sample collection
Blood samples were collected from n=7 patients undergoing lung transplantation at UHSM between October 2016 and March 2017. The recruitment criteria included patients between 18-80 years old, already entered or newly added to the lung transplant waiting list at UHSM, and able to give informed consent to participate in the study. Patients receiving single- or double lung transplants were included in the study. The study was conducted with the approval of the ethical committee to investigate markers of lung injury as part of the study 14/NW/0260: Exploring the Mechanisms of Homeostasis and Disease Before and After Lung Transplant. All samples were taken at the time of routine clinical blood gas analyses by trained hospital staff without any extra risks for the study participants.

The baseline sample was taken immediately prior to the lung transplant operation at the anaesthetic room and marked the pre-transplant time point. Samples were then collected at 12-, 24-, 48- and 72 hours post-reperfusion. These latter time points were then matched with the PaO₂/FiO₂ ratio recorded at the time of sample collection, which was considered as a representative of lung function for the purposes of this arm of the study.

All samples were collected into 2x 4 mL purple-top EDTA tubes and centrifuged for 10 minutes at 500 g at 4°C, aliquotted and stored at -80°C.

6.3.2 Flow cytometry on inflammasome specks on post-LTx plasma samples
ASC+, NLRP3+ and double-positive ASC+NLRP3+ inflammasome particles were measured in the post-LTx plasma samples using flow cytometry as described in the previous chapters of this thesis. Briefly, 1 mL of the sample was incubated for 1 hour at room temperature on a plate shaker with 1 µg anti-ASC antibody (Caltag Medsystems, UK) and 1 µg anti-NLRP3 (Caltag Medsystems, UK). The samples were then washed with 1 mL of staining buffer. 0.5 µg of secondary antibody was added per sample, followed by a 40-minute incubation period at room temperature on a plate shaker. The samples were washed and re-suspended in 500 µL staining buffer and processed for 3 minutes on the BD FACSCanto II flow cytometer (BD Biosciences, UK). Data was analysed in FlowJo version 10.0.6 (FlowJo LLC, USA).

6.3.3 ELISAs
Commercially available Quantikine® ELISA kits were utilised to measure IL-1β, pro-IL-1β, IL-18 and caspase-1 (R&DSystems, USA) according to the manufacturer’s protocols. The specifics of these protocols are given in Chapter 2: Methods of this thesis in more detail.
6.3.4 Statistical analysis

Statistical analysis was carried out using SPSS software version 22 (IBM, USA) and Prism version 7 (GraphPad, USA). Data was tested for normality by assessing descriptive statistics of skewness, kurtosis, medians and means. Approximate normality was also accepted. If normality was observed, parametric tests were utilised, and data is presented as means with standard deviations. If the data was not normally distributed, non-parametric tests were used for analysis and the data is presented as medians with interquartile ranges. Changes over time were evaluated with repeated measures ANOVA if all data was normally distributed, or with Friedman Test if it was not. Correlations between two parameters were assessed with Pearson’s correlation coefficient if the data met the requirements for normal distribution, and with Spearman's correlation coefficient if the data was non-normal. Results were considered significantly different if P≤0.05.
6.4 Results

Out of the n=7 patients, n=4 received a double-lung transplant and n=3 received a single-right-lung transplant. All of the double-lung transplant recipients were female and the single-lung recipients were male. The mean age of the LTx recipients was 53.4 (±13.7) years with the youngest patient being 27, and the oldest 64 years old. The most common diagnosis for the recipients was idiopathic pulmonary fibrosis (IPF) (n=4), followed by non-specific interstitial pneumonia (n=2) and cystic fibrosis (n=1).

The mean PaO$_2$/FiO$_2$ ratio (Figure 6.1) was recorded at 40.22 (±15.1) kPa at 12 hours post-LTx, but decreased to 35.03 (±10.7) kPa at the 24-hour time point. At 48 hours it measured at 39.24 (±10.21) kPa, increasing again to 42.21 (±9.56) kPa at the 72-hour time point. The change over time of the PaO$_2$/FiO$_2$ ratio was not statistically significant (P=0.164).
Figure 6.1 PaO$_2$/FiO$_2$ ratio in post-LTx plasma samples

PaO$_2$/FiO$_2$ ratio at 12, 24, 48 and 72 hours post lung transplant did not significantly change over time (repeated measures ANOVA, P=0.164) (n=7). Data presented as means with SDs.
Inflammasome particles remained at stable levels throughout the first 72 hours post-transplant. At baseline, ASC+ specks (Figure 6.2 A) measured at 0.46 (±0.3)% of the total particles. There was a marginal increase at 12 hours post-transplantation to 0.55 (±0.28)% at 24- and 48-hour time points to 0.48 (±0.28)% and 0.45 (±0.24)% respectively. At 72 hours post-LTx ASC+ inflammasome particles increased to 0.74 (±0.36)%. The change over time was not statistically significant for the ASC+ specks (P=0.242).

NLRP3+ specks (Figure 6.2 B) were slightly higher at 1.02 (IQR=0.39-1.45)% at baseline and 0.97 (IQR=0.66-1)% at the 12-hour time point. There was a small decrease at the 24-hour time point where the NLRP3+ particles measured at 0.87 (IQR=0.35-1.21)% and at the 48-hour time point at 0.86 (IQR=0.73-1.07)% at 72 hours post-transplant there was an increase in the NLRP3+ specks to 1.24 (IQR=0.91-1.51)% at 72 hours post-transplant. These changes over time were not statistically significant (P=0.622).

The levels of double-positive ASC+NLRP3+ inflammasome specks (Figure 6.2 C) measured at 0.96 (IQR=0.7-2.08)% at baseline, 1.19 (IQR=1-2.46)% at 12 hours, 1.14 (IQR=0.81-1.29)% at 24 hours, 1.29 (IQR=1.14-1.68)% at 48 hours, and 1.34 (IQR=1.03-1.87)% at 72 hours post-transplant. These changes over time were not statistically significant (P=0.376).
Figure 6.2 NLRP3 inflammasome particles in post-LTx plasma samples

The levels of ASC+ (A; data presented as means and SDs, repeated measures ANOVA, P=0.242), NLRP3+ (B; data presented as medians with IQRs, Friedman test, P=0.622) and ASC+NLRP3+ (C; data presented as medians with IQRs, Friedman test, P=0.376) inflammasome particles did not significantly change over time pre-operatively and up until 72 hours post-transplant (n=7).
In contrast to inflammasome particles, there was more variation in the levels of measured cytokines over the course of the 72 hours post-transplant. IL-1β (Figure 6.3 A) measured at the baseline time point at 0.37 (IQR=0.3-0.73) pg/mL but increased to 0.72 (IQR=0.37-1.08) pg/mL 12 hours post-LTx. However, these levels decreased to 0.2 (IQR=0.12-0.26) pg/mL and 0.07 (IQR=0-0.22) pg/mL at 24- and 48 hours post-transplant respectively, followed by an increase to 0.13 (IQR=0-0.25) pg/mL at 72 hours post-LTx. The changes over time in the levels of IL-1β were statistically significant (P=0.008). For n=1 patient samples, the IL-1β concentrations measured higher than the detection range of the kit utilised and the outlier was omitted from this analysis.

No pro-IL-1β (Figure 6.3 B) was detected in the baseline plasma samples for any of the patients. At 12 hours post-LTx pro-IL-1β measured at 18.5 (±7.69) pg/mL, decreasing at the 24-hour time point to 15.89 (±6.09) pg/mL. There was an increase to 24.49 (±8.98) pg/mL at the 48-hour time point, followed by a further increase to 32.3 (±18.47) pg/mL at the 72-hour time point. The change over time in the levels of pro-IL-1β in the plasma samples post-transplant was statistically significant (P<0.0001).

The levels of caspase-1 (Figure 6.3 C) started at 73.24 (±27.38) pg/mL at the baseline time point and amplified to 143.87 (±41.81) pg/mL at the 12-hour time point post-LTx. These concentrations then decreased to 99.29 (±32.5) pg/mL at 24 hours post-transplant, to 77.12 (±27.24) pg/mL at 48 hours, and to 70.75 (±21.28) pg/mL at 72 hours post-LTx. The changes over time in the levels of caspase-1 were statistically significant (P<0.0001).

The levels of IL-18 (Figure 6.3 D) were higher than those of the other inflammatory mediators measured, starting at 229.47 (±124.04) pg/mL at baseline but remaining stable at 236.57 (±97.51) pg/mL at 12 hours post-LTx. There was an increase in the levels of IL-18 at the 24-hour time point to 436.48 (±256.92) pg/mL, followed by a decrease to 386.24 (±184.89) pg/mL and to 336.88 (±102.83) pg/mL at the 48- and 72-hour time points post-transplant respectively. The change over time in the levels of IL-18 was not statistically significant (P=0.082).
Figure 6.3 Cytokines and mediators in post-LTx plasma samples

The changes over time in the levels of IL-1β (n=6) (A; data presented as medians with IQRs, Friedman test, P=0.008), pro-IL-1β (B, repeated measures ANOVA, P<0.0001) and caspase-1 (C, repeated measures ANOVA, P<0.0001) before and up to 72 hours after LTx were statistically significant (n=7 for the latter two). The concentrations of IL-18 (n=7) (D, repeated measures ANOVA, P=0.082) did not significantly change over the same period. Data presented as means and SDs unless otherwise stated.
At 12 hours (Figure 6.4) there was no statistically significant correlation between PaO$_2$/FiO$_2$ ratio and ASC+ ($r=-0.44$, $P=0.323$), NLRP3+ ($\rho=0.536$, $P=0.238$) and ASC+NLRP3+ ($\rho=0.357$, $P=0.444$) inflammasome particles, IL-1β ($\rho=-0.543$, $P=0.297$), pro-IL-1β ($r=-0.406$, $P=0.366$), caspase-1 ($r=-0.518$, $P=0.234$) and IL-18 ($r=0.058$, $P=0.902$).

Equally, at the 24-hour time point (Figure 6.5) there were no significant associations between PaO$_2$/FiO$_2$ ratio and ASC+ ($r=-0.069$, $P=0.884$), NLRP3+ ($\rho=0.643$, $P=0.139$) and ASC+NLRP3+ ($\rho=0.321$, $P=0.498$) inflammasome particles, IL-1β ($\rho=0.029$, $P=1$), pro-IL-1β ($r=0.078$, $P=0.867$), caspase-1 ($r=-0.647$, $P=0.117$) and IL-18 ($r=-0.581$, $P=0.171$).

Similar trends were observed at 48 hours post-transplant (Figure 6.6) with no significant correlations between PaO$_2$/FiO$_2$ ratio and ASC+ ($r=0.336$, $P=0.461$), NLRP3+ ($\rho=0.571$, $P=0.2$) and ASC+NLRP3+ ($\rho=0.5$, $P=0.267$) inflammasome particles, IL-1β ($\rho=0.03$, $P=1$), pro-IL-1β ($r=-0.155$, $P=0.74$), caspase-1 ($r=0.138$, $P=0.768$) and IL-18 ($r=-0.232$, $P=0.617$).

Finally, no statistically significant associations were demonstrated at 72 hours post-LTx (Figure 6.7) between PaO$_2$/FiO$_2$ ratio and ASC+ ($r=-0.224$, $P=0.629$), NLRP3+ ($\rho=-0.198$, $P=0.67$) and ASC+NLRP3+ ($\rho=-0.536$, $P=0.236$) inflammasome particles, IL-1β ($\rho=0.406$, $P=0.433$), pro-IL-1β ($r=0.185$, $P=0.691$), caspase-1 ($r=-0.273$, $P=0.554$) and IL-18 ($r=-0.705$, $P=0.077$).
Figure 6.4 Correlation between PaO$_2$/FiO$_2$ ratio and inflammasome-related inflammatory mediators in plasma samples at 12 hours post-LTx

There was no statistically significant correlation between the PaO$_2$/FiO$_2$ ratio values and the plasma levels of ASC+ (A), NLRP3+ (B), ASC+NLRP3+ (C) inflammasome particles, IL-1β (D), pro-IL-1β (E), caspase-1 (F) or IL-18 (G) at the 12-hour time point post-transplant. Analysis was done using Pearson's (A, E, F, G) or Spearman's (B, C, D) correlation coefficient.
Figure 6.5 Correlation between PaO$_2$/FiO$_2$ ratio and inflammasome-related inflammatory mediators in plasma samples at 24 hours post-LTx
There was no statistically significant correlation between the PaO$_2$/FiO$_2$ ratio values and the plasma levels of ASC+ (A), NLRP3+ (B), ASC+NLRP3+ (C) inflammasome particles, IL-1β (D), pro-IL-1β (E), caspase-1 (F) or IL-18 (G) at the 24-hour time point post-transplant. Analysis was done using Pearson's (A, E, F, G) or Spearman's (B, C, D) correlation coefficient.
Figure 6.6 Correlation between PaO$_2$/FiO$_2$ ratio and inflammasome-related inflammatory mediators in plasma samples at 48 hours post-LTx

There was no statistically significant correlation between the PaO$_2$/FiO$_2$ ratio values and the plasma levels of ASC+ (A), NLRP3+ (B), ASC+NLRP3+ (C) inflammasome particles, IL-1β (D), pro-IL-1β (E), caspase-1 (F) or IL-18 (G) at the 48-hour time point post-transplant. Analysis was done using Pearson’s (A, E, F, G) or Spearman’s (B, C, D) correlation coefficient.
Figure 6.7 Correlation between PaO₂/FiO₂ ratio and inflammasome-related inflammatory mediators in plasma samples at 72 hours post-LTx

There was no statistically significant correlation between the PaO₂/FiO₂ ratio values and the plasma levels of ASC+ (A), NLRP3+ (B), ASC+NLRP3+ (C) inflammasome particles, IL-1β (D), pro-IL-1β (E), caspase-1 (F) or IL-18 (G) at the 72-hour time point post-transplant. Analysis was done using Pearson’s (A, E, F, G) or Spearman’s (B, C, D) correlation coefficient.
6.5 Discussion

For this single-centre study n=7 patients were recruited and tracked immediately before and up to 72 hours following lung transplantation. The plasma concentrations of NLRP3-inflammasome particles, IL-1β, pro-IL-1β, caspase-1, and IL-18 were correlated with the P/F ratio values in order to assess if these molecules were associated with decreased oxygenation capacity of the lungs post-LTx.

Previous studies have found correlations between different inflammatory compounds and post-LTx PGD scores. Christie and colleagues have reported augmented coagulation and impaired fibrinolysis [193], as well as higher levels of soluble receptor for advanced glycation end products [194] and P-selectin [195] correlating with PGD development following LTx. Hoffman et al. have previously measured plasma cytokines and chemokines in post-LTx samples and described elevated levels of pre-transplant MCP-1, and post-operative IL-6 associated with increased rates of PGD [16]. Diamond et al. demonstrated an association between IL-1-induced long pentraxin-3 levels and PGD in IPF patients [196]. However, these studies utilised samples from the same multi-centre cohort and in general this population has not been widely studied in more detail. The aim of the current study was to track and collect serial plasma samples from lung transplant recipients immediately prior and closely following the operation, and evaluate inflammasome products in those samples. The results described in this thesis form one arm of the study, and a variety of other inflammatory mediators and pathways were analysed and presented as part of a different project. As the inflammasome-related compounds were the main focus of the other chapters of this thesis, only these are discussed here.

Different patterns emerged for inflammatory mediators pre- and post-transplant. NLRP3-inflammasome particles did not alter throughout the timeline of this study. The concentrations of IL-1β were elevated pre-LTx and at 12 hours but decreased at the later time points, although this change was not statistically significant. Conversely, no pro-IL-1β was detected pre-transplant, but these levels significantly increased throughout the early post-operative period. The plasma concentrations of caspase-1 were the highest at 12-hours post-transplantation and steadily decreased thereafter to pre-operative levels. IL-18 peaked at 24-hours and decreased at latter time points. The P/F ratio values post-transplant remained stable but were lowest at 24 hours.

As a whole, a rise in inflammatory cytokines after invasive surgery such as lung transplantation is to be expected. The levels of IL-1β were increased in the earliest pre-LTx plasma samples compared to the latter 24-72-hour time points. As a potent pro-inflammatory cytokine, it has been found to be present in minimal levels at approximately
0.2 pg/mL in healthy individuals [197, 198]. However, patients on the waiting list for a lung transplant, even though well enough to undergo such a complex surgery, cannot be considered as fit as healthy individuals. As such, it is possible that these patients would have elevated levels of IL-1β prior to transplant. At the 12-hour time point concentrations were further increased as a likely result of the complex surgical procedure of the transplant with IL-1β being an early inflammatory mediator released upon cellular stress. The transplanted lungs are also expected to carry an inflammatory burden in the form of donor immune cell population, and secreted inflammatory cytokines in response to prior cellular death and damage as demonstrated by the EVLP models [31, 106]. At the later time points however, IL-1β was reduced to below pre-operative levels to those that could be considered normal. During such a short time-scale, it is unlikely to be a part of an inflammatory healing process, but it could also result from various post-operative therapies and medication that are delivered to the transplant recipients.

If the presence of pro-IL-1β is considered as a marker of cellular injury or death, it can be expected to be present after an extensive surgical procedure such as a lung transplant. These results can suggest that even with these processes occurring, the conversion of pro-IL-1β is regulated and does not result in augmented levels of mature IL-1β in the circulation. With nearly unchanged levels of NLRP3 inflammasome particles, even an increase in the caspase-1 levels did not culminate in conversion of pro-IL-1β into biologically active IL-1β. Hoffman et al. have previously assessed IL-1β levels as part of a cytokine panel in post-LTx patient plasma samples within a similar time frame that was investigated in this study [16]. They describe a markedly decreasing pattern post-LTx compared to the pre-LTx time point with IL-1β measuring approximately 80 pg/mL at first and then decreasing to approximately 20 pg/mL [16]. This is in stark contrast to the findings of the current study where these levels were considerably lower. However, they also state that in 67% of their samples from a cohort of n=50 patients, the IL-1β concentrations were below the 15 pg/mL detection limit of the assay kits utilised. Therefore their findings are not necessarily contradicting the results described here, and in fact the range of plasma IL-1β levels post-LTx may be more variable between individuals.

If the decrease in the plasma IL-1β concentrations can be considered a result of post-LTx medications, then IL-1β cannot reliably be utilised as a biomarker for lung injury post-transplant. It has been described that the early postoperative immunosuppressive treatment regime after LTx includes administration of cyclosporine, tacrolimus, methylprednisolone, azathioprine, mycophenolate mofetil, basiliximab and thymoglobulin [199]. Cyclosporine A has been shown to inhibit IL-1β production by PBMCs [200], and
both cyclosporine and prednisolone have been described to inhibit the IL-1β release in a mouse model [201]. Similarly, tacrolimus can suppress IL-1β levels in an in vitro human PBMC model [202] as well as in vivo mouse model [203, 204], with inhibiting properties also described for dexamethasone in a murine [203] and in human PBMC model [205, 206]. Some research has also suggested that methylprednisolone can inhibit IL-1β release when administered prior to a spinal cord injury in a rat model [207] and a porcine single-lung ventilation model [208]. Azathioprine and mycophenolate mofetil have previously not been found to inhibit cytokine production [209]. However, more recently the latter has been argued to suppress IL-1β comparably to dexamethasone in a murine lung injury model [210]. Even when differing therapeutic strategies are employed at different transplant centres, it is likely that IL-1β could be inhibited by such medications and would therefore not be an appropriate biomarker to utilise for lung injury following LTx. Moreover, a different combination of immunosuppressive protocols may yield varying outcomes, in addition to considering these effects on patients with different phenotypes [211]. One of the limitations of this study was the lack of investigation into the medication strategies of these particular transplant recipients; however the pharmacological variables should be considered in future analyses. Nevertheless there is evidence that the post-operative therapeutics may be responsible for the decreased levels of IL-1β in the plasma samples in these patients.

Whereas at 12 hours post-LTx most of the compounds measured in this study demonstrated a negative correlation to the P/F ratio, these trends were mostly stabilised or reversed at the later time points. None of these associations were found to be statistically significant, which is likely to be due to the small sample size utilised in the study. This should also be considered in combination with a broad inclusion criteria of patients with varying ages and aetiologies, making it difficult to control variables and eliminating confounding factors. It is possible that in this cohort data could have been polarised according to gender, as all double-lung recipients were female and single-lung recipients were male. The strongest negative correlation that was also closest to reaching statistical significance was IL-18 measured at 72 hours. Interestingly, the pattern of caspase-1 expression mirrored that of IL-18 by increasing after the transplant procedure and then reducing to pre-operative levels. Although as this occurred at the earlier time points, it suggests that caspase expression arises more rapidly. IL-18 activation comes as a delayed response to the stimulation of transplant surgery, but follows the increasing pattern of caspase-1. It can be suggested that the augmented caspase levels potentially drive the subsequent increase in the levels of IL-18 by cleaving and activating it also extracellularly. Other downstream inflammatory mediators, such as IFN-γ, may therefore also be upregulated. The increase in caspase-1 can also signify the occurrence of
pyroptosis, which in turn could also explain the significant augmentation in the levels of pro-IL-1β in the plasma.

The trend of increased IL-18 accompanying decreased oxygen exchange capability reflects the suggestions in literature, where this cytokine is linked to lung injury and reduced function [141, 185, 192, 212]. Cantu and colleagues analysed RNA from the BAL fluids from lung donors pre-procurement, and from recipients 1 hour post-reperfusion, and described an association between PGD scores and an increase in inflammasome-related genes, including IL-1β and NLRP3 [192]. Contrarily to the methodology of that study, the inflammatory mediators discussed here were measured in the LTx recipient plasma post-transplant, and not the BAL fluid. The systemic plasma levels of these mediators may not therefore be reflecting the inflammation of the lung specifically and may therefore also not be appropriate for describing biomarkers specifically for pulmonary injury. Libetta et al. reported that cyclosporine, mycophenolate mofetil and methylprednisolone therapies can also inhibit IL-18 secretion by PBMCs [213]. However, their study recruited patients several months after renal transplants with the treatment strategies also lasting for prolonged periods, contrarily to the study design presented here immediately after the LTx. Elsewhere, mycophenolate mofetil has been demonstrated to suppress IL-18 production in human in vitro model [214]. Even though no statistically significant correlations were established in this thesis, the findings of the current study may also be strengthened if the sample size was increased and more comparisons between lung function and IL-18 were made.

Having encountered difficulties compiling data for PGD scores for the recruited patient cohort, the available scale of PaO₂/FiO₂ ratio values were correlated instead to the measured levels of inflammatory mediators in the patient post-LTx plasma samples. The P/F ratio has been the most commonly used index for assessing oxygen exchange deficit in critically ill patients [215] and has also been included in the Berlin Definition to differentiate the grades of ALI and ARDS [216], as well as one of the two characteristics to diagnose PGD in lung transplant patients. However, there has been extensive criticism on the utilisation of P/F ratio to assess lung oxygenation status and injury levels [217, 218], and more recently the ISHLT have defined PGD categories where in the absence of pulmonary infiltrates, any P/F score can signify PGD grade 0 [189]. Even though the P/F ratio can give an estimate of the lungs’ oxygen exchange capacity, adjusting the ventilator settings can alter the readings. It is suggested that ideally the P/F ratio to be measured at a FiO₂ of 1 and a PEEP of 5 cmH₂O [189], but when the values were recorded for the current study, the FiO₂ varied between patients and time points, meaning this was not the case in clinical practice.
Additionally, correlating the levels of the inflammatory mediators solely to the P/F ratios of the patients may not be giving the full picture of patient condition post-transplant. PGD score could be considered as a more objective descriptive as it has been found to have significant impact on both short- and long-term outcomes of LTx recipients [189]. The initial aim of the study was to record PGD scores, however due to sample collection until the final months of this PhD, time restrictions became an issue to organise and blind at least two independent and experienced clinicians to grade the chest radiographs for allograft infiltrations and pulmonary oedema. It was however possible to access and record the data for each of the patient's P/F ratio scores for each time point. It was thus decided to correlate levels of the inflammatory compounds to these values instead as the P/F ratio was considered to be an appropriate measure to substitute the PGD scores for the purpose of the initial analysis.

As mentioned in the other chapters throughout this thesis, the small sample size can also be considered as one of the main limitations for this set of experiments. The n=7 LTx patients were included during the last six months of this PhD project, based on the previous findings in chapters 3-5. This depended on the rate of LTx surgeries undertaken and the patients consented to take part in the study. Increasing the sample size will therefore be a long-term project for a single-site investigation requiring considerable staff effort and resources. The number could also be increased if patients were recruited at multiple hospitals undertaking lung transplantations, which would entail additional work on the various planning and implementing stages of carrying out clinical trials. However, given the labour-intensity of doing this, a preliminary smaller study such as the one described here, can be utilised to guide larger studies in the future. Here inflammasome-related inflammatory mediators were investigated specifically, but other compounds of interest can also be studied to describe biomarkers related to patient recoverability post-LTx. Further studies looking at the patient outcome at later time points, such as 90-day or 1-year mortality rates, may also provide some insight whether or not the molecules described here will be predictive of transplant success rates. However, given the timeframe of the current study however, this was not a viable option at this stage.

Nevertheless, the study described here adds value to the current understanding of the inflammatory processes in the early post-LTx period. The nature of clinical transplant work means that the number of patients who could be recruited for the study in a given period of time is dependent upon the unpredictable number of surgeries performed. The erratic pattern and timings of lung transplants performed also complicate the sample acquiring procedures, where trained staff are required to be available at any time to coordinate the taking, as well as processing and storing of the blood samples.
Nevertheless, further overall work is warranted on the concept of closely following the recovery of lung transplant recipients directly before and after the operative period.

In conclusion, these results indicate that the inflammasome-associated mediators are not directly associated with early post-operative lung function. IL-1β was decreased at 24 hours post-LTx and had no association with lung oxygenation capacity. Only IL-18 demonstrated a trend of negative correlation with the 72-hour post-operative P/F ratio, whereas no associations were reported for the other inflammasome-related inflammatory mediators measured. Larger sample size and more detailed clinical investigations would benefit the validity of the findings in this study.
Chapter 7: Discussion
7.1 Background

One of the main limitations to successful LTx however is the shortage of organ donors, which leads to a high waiting list mortality. Numerous strategies have been attempted to increase the number of available donor organs, one of which has been expanding the acceptability criteria for lungs deemed suitable for transplantation. EVLP was first established in order to recruit DCD lungs for safe transplantation [22]. However, it has also become an intriguing platform for scientific research and drug delivery.

The work conducted for this thesis aimed to investigate the involvement of inflammasome-related inflammatory mediators in the development of lung injury. The basis for the hypothesis came from the observation that IL-1β may represent a potential biomarker of irreversible pulmonary injury in clinical EVLP [125, 184]. In the DEVELOP-UK trial the inflammatory profile of perfusate samples was mapped and increased levels of IL-1β were detected in the marginal lungs that did not recondition during EVLP, compared to lungs that were deemed acceptable and transplanted. IL-18 has also been suggested as a biomarker for lung injury [185, 219]. Previous work from this group has also demonstrated that levels of IL-1β increase during porcine EVLP [106], suggesting that it may be a point at which therapeutic intervention could improve organ function.

The conventional release of IL-1β and IL-18 is regulated by multifactorial intracellular mechanisms. Monocytes, macrophages and dendritic cells are the main cell types associated with the secretion of these potent pro-inflammatory cytokines, but other cell types, including epithelial cells, can also secrete them. Upon receiving stress signals, cells produce intracellular precursor molecules, pro-IL-1β and pro-IL-18. Assembly of the intracellular inflammasome complex occurs concurrently, which results in the conversion of pro-caspase-1 into active caspase-1. The active caspase-1 then cleaves the pro-cytokines into their bioactive forms of IL-1β and IL-18. These active cytokines are subsequently secreted and drive the inflammatory response further by activating neighbouring cells.

Numerous inflammasomes have been described but currently the NLRP3 inflammasome remains most widely investigated. There is interest in developing therapeutics to inhibit inflammasome formation and manipulate the inflammatory response. This formed the basis for the hypotheses underlying the studies described in this thesis, which aimed to investigate the involvement of inflammasome-related inflammatory mediators in lung injury in organ perfusion but also in transplant recipients post-LTx.
7.2 Inflammasome inhibition strategies in ex vivo perfusion

As demonstrated in Chapter 3, inflammasome inhibition therapy using flufenamic acid in a 2-hour EVLP circuit did not yield any functional benefits for healthy porcine lungs. Although the release of ASC+ inflammasome particles was successfully inhibited and there was limited IL-1β release during the EVLP, all the lungs in the control and the intervention group behaved comparably. The treated and control lungs warmed up within a similar time, demonstrated similar perfusate flow rates, arterial pressures, and oxygenation capabilities. Perfusate cytokine profiles were also comparable between groups and a general trend of increasing cytokine levels was observed as previously described [106]. In Chapter 4 inflammasome inhibition therapy was introduced to a failing porcine kidney EVNP model and a reduction in ASC+ inflammasome particles was detected in the treatment group. However, in this case the inflammasome-related cytokines IL-1β and IL-18 were not inhibited, and in fact at the 180-minute time point the levels of IL-1β were significantly higher in the inhibitor group compared to the control. This suggests that even successful NLRP3 inflammasome inhibition does not protect a failing organ by itself and other mechanisms are involved in driving the inflammatory response. In addition to the similar cytokine profiles seen in the two groups during EVNP, the patterns of cellular diapedesis were also comparable, implying that the inflammasome inhibition did not affect cellular mobilisation from the organs.

Even though the two studies used different inflammasome inhibitors and were performed in different organs due to technical restrictions, it is reasonable to hypothesise that similar findings would have been made in an induced lung injury EVLP model. Such results indicate that to convey a more notable change in the inflammatory profile expressed by an organ on the ex vivo perfusion circuit, NLRP3 inflammasome inhibition alone is not sufficient. Pro-IL-1β can be cleaved extracellularly by various proteases, which can be secreted by other cell types such as neutrophils. It was concluded from the experimental perfusion work that in these models, even when specifically reducing the levels of NLRP3-inflammasome particles, the inhibition therapy did not affect the organ. More work should therefore be conducted to investigate the specific benefits and modes of action of these drugs to best fit any clinical needs. It can also be suggested that inflammasome inhibitors may be more advantageous when combined with other therapeutics, which can then lead to improvements in organ function and therefore potential clinical benefits.

Alternatively, these therapies may be considered for perfusion procedures longer than the ones undertaken in these studies. As described in Chapter 1 there are other EVLP protocols available that can extend the perfusion period in order to facilitate more time for therapeutic interventions that may require longer periods to take effect. By terminating
the EVLPs at 2 hours and the EVNPs at 6 hours it was not possible to determine what the inflammasome inhibition effects would have been over a longer period. However, as there were no discernible differences between the control and treatment groups, it was not considered practicable to extend the perfusion times.
The work on this PhD project started via a Cystic Fibrosis Trust funded mechanistic arm of the NIHR funded DEVELOP-UK clinical trial. However, approximately 6 months after starting the work on this project, the DEVELOP-UK trial was closed down due to poor patient recruitment rates. As a result, no further clinical EVLP procedures were conducted at the UHSM site. All previously collected EVLP perfusate samples across the five participating centres were sent to the Newcastle group who were leading the project for the trial. Their initial analysis on the EVLP perfusate samples indicated significantly elevated levels of IL-1β in the marginal lungs that were not reconditioned during the perfusion compared to the transplanted ones [125, 184]. Collaboration was set up together with the Newcastle group in order to explore their findings further. As these were clinical human samples, resources were available to assess the inflammasome-regulated IL-1β release pathway in more detail and measure the levels of the different NLRP3 inflammasome particles, IL-18, caspase-1, and pro-IL-1β.

Although for this project samples were analysed from less than half of the EVLPs performed for the DEVELOP-UK trial, similar results to the Newcastle group with regards to active IL-1β levels were demonstrated. The levels of pro-IL-1β were also elevated in EVLP perfusate samples of the discarded lungs compared to marginal lungs that were reconditioned during the perfusion and transplanted. Intriguingly, the levels of pro-IL-1β were also elevated in the three lungs that were transplanted but where the patients had poor post-LTx outcomes. There was also a trend for increased IL-18 in the perfusate samples of the discarded lungs, but with no differences detected between good vs. poor post-LTx outcome groups. Caspase-1 was detected at the same levels in both groups demonstrating a potential mechanism for the precursor molecule to be activated during perfusions.

However, in these experiments, the precursor molecules for pro-caspase-1 and pro-IL-18 were not distinguishable from their mature forms, but it was possible to distinguish pro-IL-1β. This was achieved by utilising pro-IL-1β specific ELISA kits where the capture antibody binds the pro-part of the precursor cytokine. Unfortunately no such consumables are currently on the market for the other inflammatory mediators of interest. Pro-IL-1β is synthesised intracellularly and would conventionally not be found in the extracellular space. The levels of pro-IL-1β in the EVLP perfusate throughout the perfusions were considerably higher than mature IL-1β levels reported by the Newcastle group. Therefore, it was hypothesised that this can be considered as a biomarker for uncontrolled cellular damage and death, rather than an active form of inflammation. If this was the case - lung injury has occurred prior to the perfusion procedures - then the EVLP alone would not
actively repair the organs. Instead levels of pro-IL-1β discriminate between organs where injury or cell death has been minimal and lung reconditioning can occur to meet the transplantation criteria, and lungs where cellular damage is beyond recoverability.

Further studies can be conducted to confirm the association between pro-IL-1β and specific forms of cell death. It may also be beneficial to study pro-IL-1β and other markers of cellular damage, necrosis, or pyroptosis as biomarkers in EVLP, especially when considering the augmented levels of the IL-1β precursor in those lung transplants with poor outcomes. These findings correspond to recent findings from Hashimoto et al. [186] who quantified cell death related M30 and HMGB1 in human EVLP perfusate samples and correlated a higher level of these compounds with increased incidence of PGD. It can therefore be postulated that cellular injury and cellular death markers can be further considered as indicators for organ recoverability. Additional investigations should be conducted in order to specify biomarkers with most potential for appropriate utilisation in the clinical setting. Even though particular markers for these processes have yet to be established, it can be proposed that intracellular contents could be considered for this purpose, as they are not usually present in the extracellular space, since they would act as inflammatory DAMPs. Taking this further, biomarkers for cellular necrosis or damage could also be investigated in the donor population or during lung procurement. The pro-IL-1β perfusate concentrations were already elevated 15 minutes after the start of the EVLP, so hypothetically this could also be detected earlier in the lung procurement process, which could then be used as an indicator for cellular injury.
7.4 Biomarkers of lung injury in post-LTx

After observing increased pro-IL-1β levels during unsuccessful EVLP and postulating that this was due to the occurrence of cellular necrosis, it became of interest to investigate whether this pattern can be perceived in patients post-LTx. Serial plasma samples were collected from seven lung transplant recipients immediately prior to their operation and up to 72 hours post-operatively. The inflammasome-related inflammatory mediators were correlated in those samples with the patients’ post-LTx PaO2/FiO2 ratio scores as a representative for lung function.

Contrary to the predictions, there was no association between pro-IL-1β or any of the other mediators, and the P/F ratio in these samples. There was a negative correlation between the levels of IL-18 and P/F ratio at the 72-hour time point, but this was not statistically significant. IL-18 has been previously linked with the development of ARDS, with the circulating levels of the cytokine being associated with disease severity and mortality in the ICU [141]. Other inflammatory mediators were also quantified, but no correlations with the P/F ratio were observed. Clearly, there are inherent differences between the short-term clinical EVLP setting and the overall post-operative condition of recipients of standard lung transplants. These patients were tracked up for 72 hours, whereas the clinical perfusion sessions lasted for approximately 2 to 4 hours. The pro-IL-1β measured in the perfusate samples can be specifically associated with the lungs placed on the circuit, whereas the plasma samples reflect the systemic condition of the patient. This was also reflected on the lower levels of these compounds in the plasma samples compared to the more concentrated EVLP perfusate, except for IL-18. The LTx recipients were also given a variety of immunosuppressive medications. Even though certain drugs are incorporated into the EVLP priming protocol, it is assumed that due to the short length of the procedure, these do not substantially alter the inflammatory response in the circuit by themselves but are prophylactic. As discussed in Chapter 6, a range of therapeutic agents are administered to patients in the early post-LTx period, many of which have been associated with IL-1β inhibition. Considering this, it is not surprising that the levels of mature IL-1β were reduced by 24 hours post-LTx compared to earlier time points, after the administered therapeutics may have had time to effect the LTx recipient’s immune cells.

The systemic post-transplant levels of uncleaved pro-IL-1β were also higher compared to mature IL-1β, with an increasing trend even when active IL-1β was decreased. This could imply that even though pro-IL-1β is released, its conversion into the mature form is tightly regulated and therefore does not have an association with lung function. There was no change throughout the perioperative period in NLRP3-inflammasome particles but there
was a fluctuation in the levels of caspase-1 and IL-18. However, due to technical restrictions the precursor forms of the latter compounds were not distinguishable from the bioactive forms. As such it cannot be concluded if the initial increase followed by the decrease in caspase-1 and IL-18 occurred within the precursor molecules or the converted active forms. The apparently successful inhibition of IL-1β in these patients however may suggest that current therapeutic strategies are sufficient to inhibit this cytokine specifically and a further inflammasome inhibition for this purpose is not necessary.

In order to investigate these findings further it is essential to look at the levels of these inflammatory mediators in the context of other cytokines and biomarkers. It would also be beneficial to compare these to a more detailed medical record of the recipient, not only the P/F ratio scores.
7.5 Alternative research methods

7.5.1 Collaborations
Despite a few caveats, such as availability of compatible antibodies, reagents, and general expenses, the benefits of the porcine model outweigh its limitations, especially in studies set up as presented in this thesis. The swine genome was mapped in the past decade and it has become possible to gene-target pigs, which was previously one of the main limitations of the porcine models [220]. Such work has led to more extensive biomedical research specifically in pulmonary disease models for CF [221], COPD [222], pneumonia and ventilator-induced lung injury [223], as well as developing protocols for lung xenotransplantation from pigs [224]. Indeed, modern EVLP was built upon the pioneering research of Prof Stig Steen and colleagues, and their models are used extensively for EVLP research in different laboratories across the world. The cost of care and upkeep however is one of the main limitations of working on large animal models. At the same time, many scientists are now also moving towards limiting and minimising animal work, especially when those models are not easily translatable to humans. The work described in this thesis therefore aimed to maximise the resources available through collaborating with other groups, and finding alternative resources for other animal work experiments.

The EVLP experiments discussed in Chapter 3 were carried out with Steen and colleagues in their laboratory in Igelösa, Sweden. As Igelösa were conducting regular medical EVLP training sessions for clinical staff utilising their perfusion protocol, an opportunity arose to get involved in those sessions from a scientific perspective. The inflammasome-inhibitor therapy protocol was arranged so that it could accompany their training programme and it was possible to utilise those sessions to carry out the first set of experiments. The practicality of these EVLPs was thereby increased, and in doing so the number of animals culled solely for research was minimised. This highlights the benefits of inter-group collaborations and maintaining these working relationships, which in turn can benefit all parties involved by distributing costs and responsibilities, but also by leading to shared discoveries and potential publications.

7.5.2 Utilisation of abattoir animals
The experimental design for the EVNP part of the project from Chapter 4 was unique in that instead of utilising laboratory swine, it was possible for the group to gain access to surplus porcine organs through collaborating with a local abattoir. By taking this approach the number of animals bred specifically for laboratory experimentation was reduced. Taken together, it meant that this experimental design did not necessitate the culling of any animals solely for the purposes of the studies described here, and instead all work was
carried out in accordance with the three values of replacement, reduction and refinement of animals in research [183].

Collaborating with local abattoirs may prove beneficial for other groups in the future even though it requires reaching out and negotiating with local businesses outside of the academic setting. Multiple projects within the group were focused on different porcine organs, and it was often possible to coordinate the research methods to utilise one pig for several experiments. Similarly, after establishing a routine working relationship with the abattoir, there was interest from other groups at the University of Manchester who were also able to take samples from the same animal. The UK Home Office reported that pigs were utilised for 5,626 scientific procedures in 2016 [225]. Whilst this number is not as high as for some of the other species, such as rodents, birds, fish or sheep, it is still important to develop research methods that reduce the number of animals in the laboratories. Therefore combining the current work with that of the local abattoir effectively demonstrates how these alternatives can be successfully established with the potential of reducing the number of pigs used in laboratories and replacing them with the ones culled for industrial purposes. There is also the opportunity to do this for the sheep model, which is the most commonly used large animal in research with 48,095 experimental procedures performed in 2016 [225]. The health conditions of abattoir animals can considerably vary, especially when the environments in which they are kept are not as controlled and regulated as a laboratory setting. However, it can be a potential benefit for certain studies where a more heterogeneous population is more representative of human disease. In this case, even though an experimental EVLP model at the laboratory at the University of Manchester was not set up within the timeframe of this PhD project, a reproducible porcine kidney perfusion protocol was indeed developed from collaboration with a local abattoir.

7.5.3 Importance of novel research models

Although it would be considerably easier to increase the replicate numbers using in vitro- or small animal models, there is a lot to be gained from large animal models and especially human work. The purpose of these experiments described here has been to understand the underlying mechanisms of human disease. Whereas the basics on the molecular and cellular levels can be investigated with cell- or tissue culture work, or using genetically modified rodents, these fail to give a translational model for human conditions. Even though popular and widely utilised in the laboratory setting, lung injury or disease models in mice are not accurate representations of human lung conditions, such as CF [226]. Consequently, there are substantially fewer funding opportunities for the development of alternatives to well-known in vivo models and small animal experimentation is widely
regarded as the gold standard and first option for biomedical research [227] even with their known limitations. Others have claimed that eventually the refinement and technical advancements of in vitro and in silico models will offer cost-effective and viable alternatives for animal models [228, 229]. Even though at the moment animal models are still required for repeat dose toxicity and carcinogenicity of drugs as well as various behavioural studies [228], progress in finding these alternatives has been made in assessing inhalation toxicity [230] and organs-on-chips modelling in respiratory medicine [231].

This translational gap between animal research and clinical treatments for human use has led to an estimation that nearly half of animal studies fail to predict human outcomes [229, 232, 233]. The different animal ALI models include direct damage through intranasal or intratracheal administration of noxious material, such as LPS or bacteria, intra-tracheal administration of acid to mimic aspiration, or high tidal volume ventilation for replicating ventilator-induced lung injury [234]. Indirect pulmonary injury models include caecal ligation and puncture for inducing sepsis, intravenous administration of LPS or bacteria, or oleic acid to mimic the release of bone marrow oleic acid in ALI patients with long-bone fractures [234]. However, these models target only certain pathophysiological components of ALI, whereas in the clinical setting the pulmonary damage is a complex multifactorial process. The lack of successfully developed treatment strategies is also an indication that the current animal models lack in translational efficacy to human therapies.

From a more radical viewpoint it has even been argued that animal research as a whole can be potentially harmful for human health when its results inadequately predict therapeutic clinical outcomes [235]. Development of novel models for scientific research, which are more relevant to the clinical application, is therefore essential. Ex vivo perfusion may be a positive step, with abattoir animals a valid but under-used resource. Organ perfusion can be utilised to investigate the organ as a whole, or even in a multi-organ circuit, and using surplus organs from an alternative source such as an abattoir can reduce the number of animals in scientific research.
7.6 Limitations

One of the common limitations mentioned throughout the chapters of this thesis has been the small sample size in both the porcine work and using human clinical samples. Whereas this complicates the overall statistical analysis with the results less likely to be significant, it is essential not to undervalue the findings described here. Most journals expect researchers to demonstrate statistically significant differences in order to successfully publish their work. However, this approach has long been criticised and it has been suggested that P-values by themselves are not reliable enough to determine the trustworthiness of a clinical study, and that it is crucial to also consider the estimated effect size and estimated precision when interpreting research outcomes [236, 237]. It can be recognised that the work discussed here is limited by the small number of replicates in each study, but at the same time it offers suggestions for potential future work for the inflammasome-related inflammatory mediators, such as pro-IL-1β.

Another limiting factor working on porcine samples was the availability of compatible antibodies and assay kits specifically designed to use with the samples. There is a much larger selection accessible for human or mouse work, and unfortunately the reagents designed for porcine models are considerably more expensive and less varied. This limits the planning of investigative paths and study designs, in which detailed analysis is limited due to reagents rather than scientific imagination. This issue was encountered when attempting to measure the inflammasome particles in the porcine ex vivo perfusion experiments. Only the ASC-antibody, reported as compatible with murine- and human samples, was applied to the porcine perfusate, and not the NLRP3-antibody. Similar obstacles were confronted when searching for cytokine assay kits and antibodies for flow cytometry. As such, even though the findings regarding the levels of pro-IL-1β in the human study were intriguing, it was not possible to measure this specific cytokine precursor in the porcine samples. Similarly, there were no ELISA kits available that could distinguish between human caspase-1 and IL-18 and their precursors. Alternatively, Western blotting could have been utilised to detect the presence of and distinguish between the uncleaved and cleaved forms of these molecules. This can be further considered for future work in order to delineate these specific pathways.

Even though the utilisation of pigs as a large animal model in biomedical research is appropriate in terms of comparability to human anatomy, physiology, histology, biochemistry and immune responses, the lack of laboratory consumables sets out boundaries that researchers must consider when designing studies. Future developments in research protocols and available reagents can improve on the quality of porcine models.
used in science, which may lead to more relevant findings than mainly relying on rodent models.
7.7 Future work

The experiments described in this thesis were conducted within small sample sizes and cohorts. If these are expanded, the promising preliminary results could potentially be replicated and further confirmed. In clinical EVLP the results suggest that the levels of cellular injury and necrosis are crucially important to lung recoverability. This could be further confirmed by measuring other cell death markers in the perfusate or tissue samples of the lungs undergoing perfusion. As mentioned, if such markers can be identified and tested rapidly in a clinical setting, they could act as parameters to assist the clinical decision-making whether to proceed with a transplant or not. No cell death specific biomarkers have been identified as of yet, but ideally such biomarkers should be detectable in patient samples that can be easily obtained [238]. They should also be stable and accurate so that they can be used clinically. If the underlying cause for lung recoverability is indeed cellular injury and death, anti-necrotic or anti-apoptotic therapies may be beneficial prior to perfusions, perhaps during organ procurement or preservation, even if EVLP is not utilised.

Lung transplantation is a demanding but rewarding field from the clinical perspective and for researchers. The unpredictability and infrequency of the operations makes it challenging to establish clinical investigations. EVLP is not a routinely used clinical tool in the UK transplant centres, and it is difficult to set up these new systems as they require vast amounts of funding, training, and persistence to be implemented. These trials take long periods of time to recruit a sufficient number of study participants, but even then, there is the possibility of the research being terminated prematurely, as seen in the DEVELOP-UK trial. Any advancement, but especially in the clinical setting, can be met with scepticism, which in turn makes it trickier for it to be proven essential, which in turn drives the incredulity and doubt.

However, in order to utilise EVLP as a platform for investigating the mechanisms of lung injury and repair, more perfusions need to be conducted, especially on human lungs. Increasing the number of samples available for research will make any demonstrated results more reliable and valid. Future work should aim to re-establish EVLP as a clinical model in UK transplant centres. As demonstrated by the DEVELOP-UK trial, there is interest in it and there are many experts in the field capable of setting this up. Similarly, to investigate lung injury in LTx recipients and to get more reliable results, additional patients should be characterised in the early post-LTx period. A wider immune panel should be used to analyse plasma to provide a comprehensive view of the inflammatory profile of recovering patients.
From the work presented here IL-18 was the only compound that demonstrated a trend of correlation with the P/F ratio. Therefore, further work investigating the involvement of this cytokine may lead to a greater understanding of the mechanisms underlying patients’ recovery post-LTx. It may also aid the development of potential therapeutics. Additionally, a more detailed analysis of PGD scores, as well as donor and recipient medical histories, and therapeutic data, can be correlated with post-LTx cytokine profile. It is also likely that genetic inter-patient variability influences recipient recoverability and their inflammatory profile, so it may also be possible to modify more effective person-specific therapies.
7.8 Conclusion

The research incorporated in this thesis has been novel in delineating the specific mechanisms of the involvement of IL-1β in pulmonary injury. A state-of-the-art ex vivo perfusion model was utilised and samples were examined from pre-clinical and clinical settings in the donor lung. The same inflammasome-specific inflammatory mediators were quantified in the early post-operative period in lung transplant recipients, contributing to the general understanding of the lung injury in this specific timeframe, that as of yet has not been extensively studied.

In conclusion, despite that inflammasome-dependant IL-1β has been associated with lung recoverability during EVLP, no functional benefits or changes were observed in the inflammatory profile when inflammasome-inhibition therapy was introduced into an experimental porcine ex vivo perfusion circuit. However, increased pro-IL-1β was identified in the perfusate samples of human lungs that were discarded after EVLP compared to lungs that were transplanted, indicating the occurrence of inflammatory cell death. At the same time, in the early post-operative period for standard LTx recipients, only IL-18 demonstrated a trend of correlation with lung function at 72 hours post-transplant. It can therefore be suggested that NLRP3 inflammasome-related therapies, even when successfully and specifically inhibiting the inflammasome particles, may not be effective in protecting the lung against injury.


137. Piguet, P.F., et al., Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. Cytokine, 1993. 5(1): p. 57-61.


