Understanding Reperfusion and the Donor and Recipient Immune Response in Lung Transplantation

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

YEAR OF SUBMISSION
2018

ALEXANDRA BALL

School of Biological Sciences
Division of Infection, Immunity and Respiratory Medicine
CONTENTS

List of Tables .................................................................................................................. 6
List of Figures ..................................................................................................................... 7
List of Abbreviations ........................................................................................................ 9
Abstract ............................................................................................................................. 12
Declaration ......................................................................................................................... 13
Copyright Statement ......................................................................................................... 14
Acknowledgements ........................................................................................................... 15
Thesis Submission ............................................................................................................. 16

CHAPTER 1: Introduction to Lung Transplantation ...................................................... 18
  1.1 History of Lung Transplantation ............................................................................. 19
  1.2 Organ Donation ....................................................................................................... 21
    1.2.1 Donation after Brain Death .......................................................................... 21
    1.2.2 Donation after Cardiac Death ....................................................................... 24
    1.2.3 Donor Organ Management ........................................................................... 26
    1.2.4 Extended Donor Criteria for Transplantation .............................................. 28
  1.3 Complications of Lung Transplantation ................................................................ 32
    1.3.1 Ischaemia-Reperfusion Injury ....................................................................... 32
    1.3.2 Primary Graft Dysfunction .......................................................................... 36
    1.3.3 Rejection ......................................................................................................... 40
  1.4 Ex-Vivo Lung Perfusion .......................................................................................... 52
    1.4.1 The History of EVLP .................................................................................... 52
    1.4.2 The Technique of EVLP .............................................................................. 54
    1.4.3 The Three Protocols of EVLP ...................................................................... 56
    1.4.4 The Clinical Experience of EVLP ................................................................. 59
    1.4.5 The Science of EVLP .................................................................................... 61
  1.5 Summary ..................................................................................................................... 64

CHAPTER 2: Characterisation of the Inflammatory Signalling Profile of the
Donor Lung following EVLP and Standard Transplant .............................................. 65
  2.1 Abstract ..................................................................................................................... 66
  2.2 Introduction ............................................................................................................... 67
4.3 Methods ........................................................................................................... 106
  4.3.1 Ethical Approval......................................................................................... 106
  4.3.2 Donor Organ Procurement....................................................................... 106
  4.3.3 Ex-Vivo Lung Perfusion ........................................................................... 107
  4.3.4 Cellular Migration During Standard EVLP ............................................. 108
  4.3.5 The Effect of a Continual Reperfusion Flush on Cellular Migration ...... 108
  4.3.6 Sample Collection and Processing ........................................................... 112
  4.3.7 Flow Cytometry ...................................................................................... 112
  4.3.8 Quantitative PCR .................................................................................... 115
  4.3.9 Statistical Analysis .................................................................................. 116
4.4 Results ............................................................................................................. 117
  4.4.1 Cellular Migration During Standard EVLP ............................................. 117
  4.4.2 The Effect of a Continual Reperfusion Flush on Cellular Migration ...... 121
4.5 Discussion ......................................................................................................... 132

CHAPTER 5: Is Circulating Cell-Free mtDNA During EVLP A Biomarker of Lung Function? ................................................................................................. 137
5.1 Abstract ......................................................................................................... 138
5.2 Introduction .................................................................................................... 139
5.3 Methods ........................................................................................................ 140
  5.3.1 DEVELOP-UK Study ............................................................................. 140
  5.3.2 Porcine Injury Model ............................................................................. 142
  5.3.3 Quantitative PCR ................................................................................... 144
  5.3.4 Luminex® Analysis .............................................................................. 146
  5.3.5 Statistical Analysis ................................................................................. 146
5.4 Results ............................................................................................................. 147
  5.4.1 DEVELOP-UK Study ............................................................................. 147
  5.4.2 Porcine Injury Model ............................................................................. 148
5.5 Discussion ....................................................................................................... 155

CHAPTER 6: Is Cell-Free mtDNA a Biomarker of Graft Dysfunction Post Lung Transplantation? ................................................................................. 158
6.1 Abstract ......................................................................................................... 159
6.2 Introduction .................................................................................................... 160
6.3 Methods ........................................................................................................ 161
  6.3.1 Ethical Approval....................................................................................... 161
  6.3.2 Inclusion and Exclusion Criteria ............................................................. 161
List of Tables

Table 1. 1: The Modified Maastricht Classification of DCD ........................................... 25
Table 1. 2: Standard Donor Organ Selection Criteria ...................................................... 29
Table 1. 3: The ISHLT Grading System for PGD ............................................................... 37
Table 1. 4: The Histological Grading System for Acute Cellular Rejection ...................... 44
Table 1. 5: The Grading System for BOS ....................................................................... 47
Table 1. 6: The Three Protocols of EVLP ....................................................................... 57

Table 3. 1: Antibody Cocktails to Identify Leukocyte Populations ................................. 90
Table 3. 2: Lung Ischaemic Times ..................................................................................... 94

Table 4. 1: The Toronto EVLP Protocol ....................................................................... 108
Table 4. 2: Antibody Cocktails to Identify Leukocyte Populations .................................. 113

Table 5. 1: The Concentration of Cytokines During EVLP ............................................. 153

Table 6. 1: The ISHLT Grading System for PGD ............................................................... 163
Table 6. 2: The Grading System for BOS ....................................................................... 164
Table 6. 3: Donor and Recipient Variables of the Recruited Patients .............................. 166
Table 6. 4: Primary and Secondary End-Points of Recruited Patients ............................. 167
Table 6. 5: Concentration of mtDNA and PGD Scores at Defined Time-Points .......... 169
Table 6. 6: Concentration of mtDNA at Defined Time-Points and Rejection Grade ...... 171
Table 6. 7: Concentration of mtDNA Pre-Transplant According to Recipient Diagnosis 173

Table 7. 1: Overview of mtDNA Concentration Pre- and Post-Transplantation ............ 186
List of Figures

Figure 1. 1: The Total Number of Adult Lung Transplants ........................................ 20
Figure 1. 2: Direct Allorecognition ........................................................................ 41
Figure 1. 3: Indirect Allorecognition ....................................................................... 42
Figure 1. 4: Semi-Direct Allorecognition ................................................................. 43
Figure 1. 5: The EVLP Circuit .............................................................................. 55

Figure 2. 1: Schematic Diagram of the Porcine EVLP Transplant Model ................. 70
Figure 2. 2: Protein Expression in Transplanted Lung Tissue ................................... 75
Figure 2. 3: Expression of Apoptosis Related Proteins in Transplanted Lung Tissue .... 76
Figure 2. 4: Concentration of Cell-Free gDNA Following Lung Transplant ............. 76
Figure 2. 5: Concentration of Cell-free mtDNA Following Lung Transplantation ..... 77
Figure 2. 6: Concentration of mtDNA and gDNA Following EVLP Transplant .......... 78
Figure 2. 7: Concentration of mtDNA and gDNA Following Standard Transplant .... 78
Figure 2. 8: The Pathways of Apoptosis ................................................................. 80
Figure 2. 9: The Activation and Inhibition of Apoptotic and Cell Survival Signalling Pathways ........................................................................................................................................ 82

Figure 3. 1: Unusable Porcine Lungs ...................................................................... 94
Figure 3. 2: Total Number of Viable and Non-Viable CD45+ Cells ......................... 95
Figure 3. 3: Total Number of Individual Leukocyte Populations ............................. 96
Figure 3. 4: Total Number of T cell Subtypes ......................................................... 97
Figure 3. 5: Total Number of Monocyte Subtypes ............................................... 98
Figure 3. 6: Total Quantity of Cytokines ............................................................... 99
Figure 3. 7: Total Quantity of mtDNA ................................................................. 99

Figure 4. 1: Diagrammatic Representation of the Recirculating Perfusion Circuit ...... 110
Figure 4. 2: Diagrammatic Representation of the Non-Recirculating Perfusion Circuit 111
Figure 4. 3: Migration of CD45+ Cells During EVLP ............................................ 117
Figure 4. 4: Migration of Total Cell Populations During EVLP .............................. 118
Figure 4. 5: Migration of T and B Cell Populations During EVLP ......................... 119
Figure 4. 6: Migration of Macrophages and Monocytes During EVLP ................... 119
Figure 4. 7: Migration of NK Cell Populations During EVLP ............................... 120
Figure 4. 8: Migration of Granulocyte Cell Populations During EVLP .................. 120
Figure 4. 9: Total Number of CD45+ Cells in the Collected Perfusate .................. 121
Figure 4. 10: Total Number of CD45+ Cells in the Individual Perfusate Collection Volumes ........................................................................................................................................ 122
Figure 4. 11: Migration of CD45+ Cells into the Perfusate During Recirculation .... 122
Figure 4. 12: Total Number of Cell Populations in the Collected Perfusate .......... 123
Figure 4. 13: Number of Granulocytes in the Individual Perfusate Collection Volume .... 124
Figure 4. 14: Migration of Granulocytes into the Perfusate During Recirculation ..........124
Figure 4. 15: Number of T Cells in the Individual Perfusate Collection Volumes ..........125
Figure 4. 16: Migration of T Cells into the Perfusate During Recirculation...............126
Figure 4. 17: Number of Monocytes in the Individual Perfusate Collection Volumes ......127
Figure 4. 18: Migration of Monocytes into the Perfusate During Recirculation ..........127
Figure 4. 19: Number of Macrophages in the Individual Perfusate Collection Volumes ..128
Figure 4. 20: Migration of Macrophages into the Perfusate During Recirculation ..........128
Figure 4. 21: Number of NK cells in the Individual Perfusate Collection Volumes .......129
Figure 4. 22: Migration of NK Cells into the Perfusate During Recirculation ..........129
Figure 4. 23: Number of B Cells in the Individual Perfusate Collection Volumes .........130
Figure 4. 24: Migration of B Cells into the Perfusate During Recirculation ..........130
Figure 4. 25: Concentration of Cell-Free mtDNA in the Collected Perfusate ............131
Figure 4. 26: Concentration of Cell-Free mtDNA in the Perfusate During Recirculation .131

Figure 5. 1: Concentration of mtDNA During EVLP According to Transplantation ..........147
Figure 5. 2: Concentration of mtDNA During EVLP According to Outcome ................148
Figure 5. 3: Oxygenation Capacity During EVLP .................................................149
Figure 5. 4: Pulmonary Vascular Resistance During EVLP ......................................150
Figure 5. 5: Lung Compliance During EVLP ..........................................................150
Figure 5. 6: Concentration of mtDNA During Standard EVLP and Following Injury ......151
Figure 5. 7: Cytokine Concentration During Control EVLP ......................................152
Figure 5. 8: Cytokine Concentration During Injury EVLP .........................................152
Figure 5. 9: Concentration of Individual Cytokines in Control and Injury EVLP ..........154

Figure 6. 1: Levels of mtDNA Pre- and Post-Transplantation ..................................169
Figure 6. 2: Concentration of mtDNA and PGD Scores at Defined Time-Points ..........170
Figure 6. 3: Concentration of mtDNA at Defined Time-Points and Rejection Grade ......171
Figure 6. 4: Concentration of mtDNA According to BOS Grade ..............................172
Figure 6. 5: Trendline of mtDNA Concentration According to BOS Grade ...............172
Figure 6. 6: Concentration of mtDNA According to Donor Smoking History ..........174
Figure 6. 7: Trendline of mtDNA Concentration According to Donor Smoking History ..174

Figure 7. 1: Diseased Abattoir Porcine Lungs .........................................................189
Figure 7. 2: Oedematous Porcine Lungs on the EVLP Circuit ..................................190
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-MP</td>
<td>6-mercaptopurine</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>A1AT</td>
<td>Alpha-1 antitrypsin</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>AMR</td>
<td>Antibody mediated rejection</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ATG</td>
<td>Anti-thymocyte globulin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncho-alveolar lavage</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BOS</td>
<td>Bronchiolitis obliterans syndrome</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CLAD</td>
<td>Chronic lung allograft dysfunction</td>
</tr>
<tr>
<td>CNI</td>
<td>Calcineurin inhibitor</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSP</td>
<td>Cold static preservation</td>
</tr>
<tr>
<td>CXR</td>
<td>Chest X-ray</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DBD</td>
<td>Donation after brain death</td>
</tr>
<tr>
<td>DCD</td>
<td>Donation after cardiac death</td>
</tr>
<tr>
<td>DEVELOP-UK</td>
<td>Donor EVLP in the United Kingdom</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
</tr>
<tr>
<td>DSA</td>
<td>Donor specific antibodies</td>
</tr>
<tr>
<td>ECD</td>
<td>Extended criteria donor</td>
</tr>
<tr>
<td>ECMO</td>
<td>Extracorporeal membrane oxygenation</td>
</tr>
<tr>
<td>ET</td>
<td>Endotracheal</td>
</tr>
<tr>
<td>EVLP</td>
<td>Ex-vivo lung perfusion</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>FKBP₁₂</td>
<td>12kDa FK506 binding protein</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Ht</td>
<td>Haematocrit</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptosis</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>ISHLT</td>
<td>International society for heart and lung transplantation</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischaemic reperfusion injury</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LA</td>
<td>Left atrium</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen associated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMF</td>
<td>Mycophenolate mofetil</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MV</td>
<td>Minute volume</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHBD</td>
<td>Non-heart beating donor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NPO</td>
<td>Neurogenic pulmonary oedema</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OB</td>
<td>Obliterative bronchiolitis</td>
</tr>
<tr>
<td>OCS</td>
<td>Organ care system</td>
</tr>
<tr>
<td>PA</td>
<td>Pulmonary artery</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of arterial oxygenation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end-expiratory pressure</td>
</tr>
<tr>
<td>PGD</td>
<td>Primary graft dysfunction</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTX3</td>
<td>Pentraxin-3</td>
</tr>
<tr>
<td>PV</td>
<td>Pulmonary vein</td>
</tr>
<tr>
<td>PVR</td>
<td>Pulmonary vascular resistance</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end-products</td>
</tr>
<tr>
<td>RAS</td>
<td>Restrictive allograft dysfunction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RR</td>
<td>Respiratory rate</td>
</tr>
<tr>
<td>SAG</td>
<td>Self-antigen</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SO</td>
<td>Superoxide</td>
</tr>
<tr>
<td>sRAGE</td>
<td>Soluble RAGE</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>T3</td>
<td>Tri-iodothyronine</td>
</tr>
<tr>
<td>TBI</td>
<td>Total body irradiation</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>VC</td>
<td>Vital capacity</td>
</tr>
</tbody>
</table>
Abstract

Lung transplantation remains limited by a lack of donor organs available for transplant and a reduced median survival secondary to allograft rejection and graft dysfunction. Upon donor death, a catecholamine and cytokine storm ensues, resulting in inflammatory cell infiltration within the lung. Reperfusion of such immunologically primed donor lungs can then drive a heightened recipient immune response. Despite this, the donor immune compartment is often ignored. The aim of this PhD was to characterise the effects of reperfusion upon the donor immune compartment and the recipient response, to identify potential biomarkers and avenues for manipulation. A porcine model was used to determine whether EVLP could alter the inflammatory signalling profile of the donor lung upon transplantation. Indeed, a global up-regulation of cell-survival proteins was observed within EVLP transplanted lungs, yet not in lungs that underwent standard transplant. Increased levels of cell-free mtDNA were also detected in the recipient circulation following standard transplant. The capability of EVLP to facilitate the mobilisation of inflammatory mediators from the lung prior to transplantation was explored. A secondary preservation flush was performed to characterise the immune content of the donor lung following cold ischaemic storage. The migratory pattern of donor leukocytes during EVLP was also elucidated, to determine whether a continual non-recirculating perfusion flush could reduce the donor leukocyte burden prior to transplantation. In addition, cell-free levels of mtDNA were also quantified during human and porcine EVLP, and in the recipient circulation post human lung transplantation. These levels were then correlated with functional and clinical outcomes to determine whether mtDNA is a biomarker of lung function. The study demonstrated that the donor immune compartment is comprised of a large repertoire of donor leukocytes that readily migrate from the lung upon immediate revascularisation. A continual flush with non-recirculating perfusate facilitated the removal of 9 billion donor leukocytes, yet upon perfusate recirculation, donor leukocyte migration continued. The overwhelming propensity of the donor leukocyte repertoire to mobilise upon revascularisation demonstrates the need to consider donor leukocyte depletion prior to transplantation. Furthermore, cell-free mtDNA was observed to correlate with injury on the EVLP circuit, and the incidence of chronic graft dysfunction post-transplantation, highlighting its potential to serve as a biomarker in lung transplantation.
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.
Copyright Statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and she has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DoculInfo.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University’s policy on Presentation of Theses.
Acknowledgements

Firstly I would like to thank my supervisors, Dr James Fildes and Professor Nizar Yonan, for providing me with the opportunity to work as a clinical research fellow and complete this research project with their support. In addition to the tremendous support he has provided during my research career, I am also extremely grateful to Professor Yonan for the on-going advice and guidance he has given to me during my professional clinical career.

A special thank you must also be extended to the Fildes Research Group, in particular, Rebecca Edge, Kavit Amin, William Critchley, John Stone and Timothy Entwistle. They have all been invaluable to my research career, providing support and advice whenever needed. Specifically, I would like to thank Rebecca Edge for going above and beyond to help me complete this research project.

I must also extend my gratitude to Professor Stig Steen and Trygve Sjoberg, and their research group at Igelosa, Lund. The majority of my animal experiments were completed in Lund, and without their surgical expertise I would not have been able to complete my work. In addition, they have been on hand to offer advice and support, and have always welcomed me into their research facility.

I would like to thank the Transplant Unit at Wythenshawe Hospital, for all of their help and support in facilitating my research. From the administration staff to the nurses, transplant coordinators and doctors, they have all contributed in helping me to complete my PhD.

Having gone back in to clinical work when writing up my thesis, I am also extremely grateful to Dr Kwong, my current clinical supervisor. He has provided me with overwhelming support to ensure that I completed this thesis and for that I am extremely grateful.

Finally, I would like to say a special thank you to my family for supporting me throughout, but in particular over these past few months. Thank you especially to my husband Simon, and my mum, who have provided me with unwavering support and advice.
Thesis Submission

This thesis has been written in the alternative format, and has therefore been incorporated into separate chapters that are in a format suitable for submission for publication in a peer-reviewed journal. It has been submitted in this format as each chapter outlines an experimental aim with subsequent results, all of which combine to characterise the effects of reperfusion upon the donor lung compartment and recipient circulation, to determine whether donor leukocyte transfer can be manipulated upon reperfusion on an EVLP circuit, and to identify circulating biomarkers of graft dysfunction.

A summary of each chapter and my individual contribution to the work presented is outlined below:

Chapter 1:
This is an introductory chapter to outline the current up-to-date literature in the field of lung transplantation, with particular reference to the complications of transplant and ex-vivo lung perfusion, as these areas were explored in my own work. I have performed an extensive literature review and have written the chapter without any collaboration.

Chapter 2:
In this chapter a porcine model of EVLP transplant was used to characterise the inflammatory signalling profile of the lung following standard and EVLP transplant and delineate the immediate impact on the recipient circulation. Professor Stig Steen and his research team in Lund, Sweden carried out the porcine transplant model. His team therefore performed all surgical procedures, including donor lung explantation, EVLP and subsequent transplantation. I then processed the collected samples for analysis and collated the results. A statistician checked statistical analysis and I have then written the chapter without any collaboration.

Chapter 3:
In this chapter, a secondary preservation flush was performed on abattoir porcine lungs following standard donor organ procurement and cold ischaemic storage to characterise the immune content that initially migrates from the donor lung upon reperfusion. The experimental concept and design was formulated by myself. Following which, I carried out all of the procedures involved including donor organ retrieval, procurement and subsequent flush. I went on to process and analyse all samples and collated the results. A statistician checked statistical analysis and I have then written the chapter without any collaboration.

Chapter 4:
In this chapter, a two-part study was performed using a porcine model of EVLP. In the first part, the EVLP circuit was used to map the temporal kinetics of cellular diapedesis during perfusion. In the second part, the EVLP circuit was modified to evaluate the impact of continual reperfusion with a non-circulating perfusate on cellular migration. The porcine model of EVLP
was performed in Lund, Sweden and Stig Steen and his team performed all surgical procedures. The experimental design and subsequent EVLP process was formulated and performed in collaboration with XVIVO. I analysed all samples and collated the results. Statistical analysis was checked by a statistician and I have then written the chapter without any collaboration.

Chapter 5:
In this chapter, a two-part study was performed to evaluate whether levels of cell-free mtDNA correlated with graft function. In the first part of this study cell-free mtDNA was quantified in the perfusate samples from n=20 EVLPs performed as part of the DEVELOP-UK study and correlated with transplant outcome. In the second part of this study, a porcine EVLP model was then used to determine whether increased levels of cell-free mtDNA were observed following induced injury on the EVLP circuit. DEVELOP-UK samples were collected as part of the study, and sent for analysis. I then processed the samples for quantification of DNA and collated the results. The porcine model of EVLP was performed in Lund, Sweden and therefore Stig Steen and his team performed all surgical procedures, and I collected and processed all of the samples for analysis. I have then collated all of the results. Statistical analysis was checked by a statistician and I have then written the chapter without any collaboration.

Chapter 6:
In this chapter, a clinical study of human transplant was performed to determine whether increased levels of plasma cell-free mtDNA post-transplantation were associated with inferior outcomes, and specifically PGD, and therefore can it serve as a reliable biomarker of graft dysfunction. I completed the application for ethical approval and was the lead investigator in the study. All elements of the clinical study were performed by myself, including patient recruitment, data collection and subsequent analysis. Results were collated and statistical analysis was performed by a statistician. I have subsequently written the chapter without any collaboration.

Chapter 7:
In this chapter I have provided a summary of the findings from all of the individual chapters and completed an over-riding discussion. The limitations of this thesis and plan for future work have also been outlined.
CHAPTER 1

Introduction to Lung Transplantation
Chronic respiratory diseases are a leading cause of morbidity and mortality worldwide, and pose a significant socio-economic burden. However current pharmacological treatments provide symptomatic benefit rather than disease regression. Over the past 60 years, lung transplantation has emerged as an established treatment for patients with end-stage lung disease to improve morbidity and mortality.

1.1 History of Lung Transplantation

The pioneering animal work of Vladimir Demikhov in the 1940s demonstrated the technical feasibility of performing intrathoracic transplantation. Although his work was predominantly in heart transplantation, he successfully performed the first lung transplantation in dogs in 1947 (1). James Hardy then went on to perform the first human lung transplantation in 1963, at the University of Mississippi Medical Centre. He performed a left single lung transplant on a 58 year-old convicted murderer, who had a background of left sided lung cancer and emphysema, with recurrent pneumonia unresponsive to antibiotics, and underlying renal impairment. He died 18 days post-procedure secondary to renal failure (2). There were further attempts at lung transplantation over subsequent years. However, success was thwarted by surgical complications – specifically anastomotic healing – and rejection, with survival limited to days. The advent of cardio-pulmonary bypass in cardiothoracic surgery, and the emergence of Ciclosporin A as a superior immunosuppressive medication, paved the way for Bruce Reitz et al to perform the first successful heart-lung transplantation on a 45 year-old female with primary pulmonary hypertension. Despite two episodes of acute rejection, she responded well to treatment and ultimately survived five years post-transplantation (3). Surgical techniques continued to improve, and Joel Cooper performed the first successful single lung transplantation in a 58 year-old male with advanced pulmonary fibrosis, with the recipient surviving for 6 years (4). This success led Cooper’s team performing the first double lung transplant in a 42 year-old female with chronic obstructive pulmonary disease (5). She went on to live for fifteen years and died of unrelated causes.

Since then, with improved surgical techniques including bronchial anastomotic omentopexy, and a greater understanding of transplant rejection and immunosuppressive regimes, lung transplantation has been on a gradual increased trend. The registry of the International Society for Heart and Lung Transplantation (ISHLT) recently reported that in 2015, 4122 adult lung transplantations were performed worldwide, a stark contrast to the 5 performed in 1985 (6).
Figure 1.1: The Total Number of Adult Lung Transplants

The total number and types of adult lung transplants reported to the ISHLT registry between 1985 and 2015 (6)

Yet, despite the increasing annual activity of lung transplantation worldwide, it remains limited by the lack of donor organs available and, a reduced median survival when compared to other solid organ transplantations (6-8).
1.2 Organ Donation

Although there has been a sustained increase in transplant activity, there has been a concurrent rise in the incidence of chronic respiratory disease, and as such, an increase in the need for transplantation. The demand for transplantation significantly outweighs the supply of donor organs available, resulting in high waiting list mortality (9). In the UK, there were 378 patients on the active waiting list for lung and heart-lung transplant in 2016/2017, with a median waiting list time of 255 days and a waiting list mortality of 9% (10).

The problem of donor organ shortage is two-fold. Firstly, there is a shortage of organs donated at the time of death, with families often refusing or over-riding the consent for organ donation on behalf of the deceased (10). Secondly, of the limited organs donated for transplant, only 20% are actually utilised, with the remainder being deemed unsuitable for transplantation in accordance with the acceptance criteria (10, 11). Donor lungs therefore have the lowest utilisation rate in comparison to other solid organ transplants, including the liver, kidney and heart (10).

At the time of retrieval, the donor lung is often injured, secondary to a myriad of pathophysiological processes occurring within the donor, prior to, upon, and after death (12, 13). Accurate evaluation of functionality is therefore difficult, and potentially usable organs are often rejected for transplantation (14).

1.2.1 Donation after Brain Death

The conventional donors for lung transplantation have been those who have died from brain stem death – so called donation after brain death (DBD) donors. DBD donors are normally healthy patients who have died secondary to a catastrophic intracranial injury. Prior to brain death being confirmed, the lungs are at risk of infection, aspiration and ventilator associated injury. Furthermore, upon brain death, the haemodynamic, hormonal and metabolic compromise that ensues, can cause additional lung injury, and render it unsuitable for transplantation (13, 15).

Brain injury results in intracranial oedema and a subsequent rise in intracranial pressure (ICP). If an adequate cerebral perfusion pressure cannot be maintained, rostro-caudal ischaemia ensues, the effects of which have been demonstrated in various animal models (16, 17). Pontine ischaemia leads to the Cushing response of hypertension and bradycardia – a reflex response to improve cerebral perfusion via mixed sympathetic and vagal stimulation (18). As ischaemia progresses to the medulla oblongata and the vagal motor nucleus, there is an unopposed sympathetic drive, initiating a catecholamine storm, with marked hypertension, tachycardia and peripheral vasoconstriction (16, 17, 19-21). Ischaemia extends to the spinal cord with herniation and irreversible loss of the brainstem motor nuclei. Sympathetic outflow is then lost, with consequential loss of vasomotor tone, and subsequent peripheral vasodilatation.
and systemic hypotension (22). Catecholamine levels also fall, further exacerbating the hypotension (20).

During this haemodynamic compromise, there is also disruption of the hypothalamic-pituitary axis, yet the effect on hormonal levels appears to be somewhat variable. Animal studies have demonstrated a decline in multiple pituitary hormones, including vasopressin, thyroxine, triiodothyronine (T3), adrenocorticotrophic hormone (ACTH), cortisol, insulin and glucagon (19, 23). In humans, however, although vasopressin deficiency and resultant diabetes insipidus have been found in over 75% of DBDs, the remaining hormones are inconsistently found at normal or low levels (24-26).

Ultimately, the haemodynamic and hormonal compromise result in organ hypoperfusion and inhibition of mitochondrial function, with a subsequent switch from aerobic to anaerobic metabolism (27, 28). Lactate and fatty acids accumulate in the plasma, adenosine triphosphate (ATP) synthesis is reduced, and energy stores are depleted, leading to compromised cellular metabolism (13, 27, 28).

This cascade of events can result in donor lung injury via two distinct processes – the development of neurogenic pulmonary oedema (NPO), and an acute lung injury (ALI) secondary to inflammatory cell infiltration (13, 15).

**Neurogenic Pulmonary Oedema**

NPO is the process of oedema formation in the lungs secondary to a significant insult to the central nervous system. It was initially reported as a complication of epileptic seizures, whereby its presence was associated with a high mortality rate (29). The process has since been observed following various forms of intracranial insult (30-33). It is an immediate, acute event, having been found during autopsies of Vietnam combat casualties who died instantly following head trauma, and in patients who suffered sudden death secondary to aneurysmal subarachnoid haemorrhage (30, 34).

The pathogenesis of NPO is thought to be secondary to cardiopulmonary haemodynamics and sympathetic stimulation. Upon brain death, the unopposed sympathetic drive leads to marked peripheral vasoconstriction. The increase in systemic vascular resistance, results in an increase in left ventricle (LV) afterload, with a concomitant decrease in LV output and increase in LV pressure. Left atrial (LA) pressure therefore increases with a subsequent rise in the pulmonary capillary pressure. Additionally, the peripheral vasoconstriction leads to increased blood flow in the pulmonary circulation, with an increase in pulmonary artery (PA) and capillary pressures. The two-fold augmentation in pulmonary capillary pressure results in increased hydrostatic pressure and subsequent oedema formation. This is exacerbated further due to the haemodynamic sheer stress damaging the capillary endothelium, diminishing the integrity of the capillary/alveolar membrane. This so-called blast and stress theory has been demonstrated in various experimental animal models (35-38). Novitzky *et al* confirmed, in Chacma baboons,
that following the haemodynamic changes upon brain death, the cardiothoracic circulating volume increased from 24% to 72% of the total circulating blood volume (37). The resultant rise in intra-pulmonary pressure then leads to oedema directly and indirectly, through damage to the anatomical integrity of the pulmonary endothelium (37, 38). Yet the hydrostatic blast and stress theory has come into question with NPO occurring in the presence of raised ICP and normal pulmonary pressures in experimental cat models (39, 40). It has since been proposed that sympathetic stimulation can directly increase pulmonary capillary permeability, as evidenced in experimental sheep models, whereby alpha(α)-adrenergic blockade can then stop the process (41, 42). However, the degree of oedema observed is not to the extent of that seen following significant intracranial insult, suggesting that the mechanism may occur in parallel to the haemodynamic process. It has been demonstrated that α-adrenergic blockade and spinal cord transection can prevent the haemodynamic compromise and NPO seen upon brain death, however beta(β)-blockade and adrenalectomy cannot (15, 43). This highlights the importance of sympathetic stimulation, and specifically noradrenaline, in the pathogenesis of NPO. It appears to drive the haemodynamic compromise seen upon brain injury, in addition to its direct effect on pulmonary capillary permeability. The latter of which may well be the result of increased sympathetic discharge serving as a catalyst for the release of pro-inflammatory mediators.

**Inflammatory Cell Infiltration**

Following brain stem death, there is also an initiation of an inflammatory, immune cascade, with a rise in the circulating levels of pro-inflammatory cytokines (15). The direct initiating mechanism remains unclear and may well be multifactorial, secondary to brain ischaemia and disruption of the blood brain barrier, the cytokine storm, haemodynamic endothelial shear stress, and resultant organ hypoperfusion.

In an experimental rat model, Takada et al demonstrated that following brain death, there is an up-regulation of interleukin (IL)-1, IL-2, IL-6, tumour necrosis factor (TNF)-α and interferon-gamma (IFN-γ) in peripheral organs (44). Skrabal et al demonstrated that upon brain death in pigs, there is an increase in the plasma cytokine levels of TNF-α, IL-6 and IL-1β, along with an organ specific up-regulation of pro-inflammatory cytokines, with increased levels of IL-6, IL-1β, TNF-α, monocyte chemoattractant protein (MCP)-1 and transforming growth factor (TGF)-β seen in the lungs, with TNF-α being specific to the lungs (45). Avlonitis et al have echoed these findings in rats, however they also report an increase in the neutrophil expression of the β2-integrins CD11b/CD18 in the serum, along with elevation of IL-1β and TNF-α in broncho-alveolar lavage (BAL) samples (43).

In humans, the importance of these cytokines and adhesion molecules in the pathogenesis of ALI and acute respiratory distress syndrome (ARDS) is well documented (46). Therefore the inflammatory cascade seen upon brain death may mirror that which is seen in the development of ALI. Alveolar macrophages release IL-1β and TNF-α, secondary to activation of the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (47). These cytokines stimulate the release of further pro-inflammatory cytokines, including IL-6 and
IL-8, and activate endothelial cells to express the adhesion molecules, P-selectin, E-selectin and intercellular adhesion molecule (ICAM)-1 (46, 47). IL-8 mediates neutrophil chemotaxis, and upon interaction between the neutrophil L-selectin and the endothelial selectins, the neutrophils weakly adhere to the endothelium, rolling along the surface (48). Neutrophils finally arrest and firmly adhere to the endothelial surface through the interaction between neutrophil β2-integrins and ICAM-1 (47, 48). They then migrate into the lung tissue across the epithelial membrane, either paracellularly or intracellularly, where they release oxidants and proteolytic enzymes along their way and upon arrival, causing diffuse alveolar injury (48). Damage to type I and type II epithelial cells leads to abnormal alveolar fluid transport and reduced surfactant production by type II cells, with resultant oedema formation and alveolar collapse (49).

Increased plasma levels of IL-1β, TNF-α, IL-6 and IL-8 have been associated with increased morbidity and mortality in patients with ALI and ARDS (50, 51). Additionally, elevated levels of IL-8 in BAL samples have been found in patients who later developed ARDS (52). This is interesting, given that in DBD donors, increased circulating levels IL-1β, TNF-α and IL-6 have been found, along with elevated levels of IL-8 and IL-6 in BAL samples (53-55). Furthermore, Fisher et al have demonstrated that increased levels of IL-8 in the donor BAL correlates with the development of primary graft dysfunction (PGD) post-transplantation (56).

This pro-inflammatory cell infiltration may not only cause direct lung injury, but it may also prime the graft for subsequent rejection upon transplantation. In experimental rat models, in addition to the increased expression of pro-inflammatory cytokines in the kidneys upon brain death, there is also an up-regulation of major histocompatibility complex (MHC) Class I and Class II (44, 57-59). MHC Classes I and II are responsible for presenting antigens to T cells, which then initiate an inflammatory response, and in the context of transplantation, are critical to rejection. Indeed, Pratschke et al demonstrated that upon transplantation of DBD kidneys in a rat transplant model, there is accelerated rejection and subsequent renal failure, leading to increased animal death (58).

Lungs from DBD donors are therefore susceptible to significant injury that can result in them not being used for transplant. If they are utilised for transplant, they are then vulnerable to further injury that is associated with increased morbidity and mortality. In view of this, increased efforts to optimise the donor organ pool, without compromising long-term survival, have led to a review of donor organ management, the acceptance criteria for transplantation, and the use of non-conventional non-heart beating donors (NHBDs).

1.2.2 Donation after Cardiac Death

In the initial era of transplantation, all organs were from donation after cardiac death (DCD) donors, whereby organs were retrieved immediately following cardiorespiratory arrest. Following increased knowledge of brain death and the advent of brain stem testing however, organ retrieval switched to heart beating donors, who had been declared brain dead.
More recently, given the degree of injury that donor lungs can sustain during brain death, and the subsequent reduction in utilisation for transplantation, there has been increased interest in the use of DCD donors. Although considerable haemodynamic changes can occur upon cardiac death, there is often absence of the pro-inflammatory cytokine storm that can occur upon brain death, potentially limiting the degree of donor lung injury. Furthermore, ischaemic heart disease has become one of the leading causes of death in the Western World, with a high incidence of cardiac arrest, and as such DCD donors can potentially serve as a large donor organ pool (60).

There has previously been a reluctance to utilise organs from DCD donors, due to the increased risk and unpredictable length of warm ischaemic injury, following cardiorespiratory arrest. In addition, there are the ethical concerns regarding confirmation of death following circulatory death, with the potential for a return of spontaneous circulation and on-going debate regarding underlying cerebral activity (61). In 1995, there was an international meeting in Maastricht concerning the use of NHBDs which led to a classification system to help identify potential DCD donors (62). It categorises DCD donors into two groups: controlled and uncontrolled. Uncontrolled DCDs describe organ retrievals following unexpected and irreversible cardiorespiratory arrest, whereas controlled DCDs are from retrievals following death secondary to the withdrawal of essential, life-sustaining treatments (62). It further sub-categorises the groups, according to the clinical circumstances in which they occur.

**Table 1. 1: The Modified Maastricht Classification of DCD**

A classification system for the identification of potential DCD donors. ICU = intensive care unit

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Type of DCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Dead on arrival</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</td>
<td>Controlled</td>
</tr>
<tr>
<td>IV</td>
<td>Cardiac arrest in a brain dead donor</td>
<td>Controlled</td>
</tr>
<tr>
<td>V</td>
<td>Unexpected cardiac arrest in an ICU patient</td>
<td>Uncontrolled</td>
</tr>
</tbody>
</table>

Organ procurement is only permitted once a patient has been certified as dead, to ensure that the retrieval procedures are not contributory to patient death. According to the Uniform Determination of Death Act (UDDA), a person is declared dead if there is irreversible cessation of either cardiorespiratory function or complete brain function. Given the uncertainty regarding the irreversible nature of initial cardiorespiratory arrest, it has been previously agreed that there should be a minimum “hands-off” period of two to five minutes, whereby no interventions are made to the patient to ensure that there are no signs of cardiorespiratory effort (63). Observing such a period is thought to then ensure that there is no possibility of a spontaneous return of circulation. There have been some case reports however, in which there has been a return of spontaneous circulation and neurological function in the five to ten minutes after cardiac arrest,
termed the Lazarus phenomenon (64, 65). For this reason, in most centres a ‘hands-off’ period is observed for five to ten minutes. During this time the organs are not perfused and as such are at risk of warm ischaemic damage. Furthermore, in uncontrolled DCDs, this period of warm ischaemia can often be longer and even more unpredictable. This has led to reluctance to use such donors. However, it has been increasingly demonstrated that their use does not compromise long-term outcomes.

In experimental pig models, lungs from DCD donors were shown not only to be viable, but also appeared less susceptible to reperfusion injury despite periods of prolonged warm ischaemia (66, 67). Indeed of all the solid organs, the lungs are perhaps the least vulnerable to ischaemic injury, given that they do not rely solely upon vascular perfusion for oxygenation. Following death, oxygen remains in the lungs and can be used for cellular respiration, by means of ventilation with the external environment (68). This permits their use for several hours after death. Furthermore, they are not exposed to the complex pathophysiological processes that occur upon brain death.

Clinically, there has been comparable patient survival and incidence of bronchiolitis obliterans syndrome (BOS) in recipients of DBD and DCD lungs, with the latter trending towards earlier extubation, with improved lung function and reduced infections post-operatively (69-71). Although one single centre has reported inferior outcomes using DCD lungs when compared to other centres, their results are similar to national averages for lung transplantation (72). Moreover, recent multi-centre reviews and meta-analyses have reported excellent, comparable outcomes using DCD lungs, confirming them to be safe and effective donors (73-75). Yet clinical experience has predominantly been with controlled DCD donors, primarily due to the ethical implications and prolonged nature of warm ischaemia associated with uncontrolled DCDs. Recently however, transplantation using uncontrolled DCD lungs following a period of assessment using ex-vivo lung perfusion (EVLP) has also been shown to be successful and has led to increased efforts to explore the feasibility of this donor organ group (76, 77).

DCD donors therefore provide an alternative source for donor lungs, helping to improve the scarcity of those available for transplantation, with the potential to be superior to the conventional DBD donors. Despite this however, the unpredictable nature of cardiac death with the limited time for organ assessment remains a major caveat for their use. As such, this donor organ cohort remains relatively under-utilised, with further work required in this area, to optimise their use.

1.2.3 Donor Organ Management

The importance of donor organ management has been demonstrated in recent years, not only to increase the yield of organs at the time of retrieval but also to optimise graft function and limit complications post-transplantation. The aim of donor management is to reduce and potentially
reverse the injury to donor organs by attenuating the pathophysiological processes observed upon donor death.

Strict fluid resuscitation, bronchoscopy and regular bronchial toilet, antimicrobial therapy, and protective, low volume pressure-controlled ventilation, with periods of higher positive end expiratory pressure (PEEP) can limit donor lung injury and improve functionality in organs initially deemed unacceptable (78-80). In recent years, more invasive monitoring of cardiopulmonary haemodynamics with PA catheterization has helped to guide fluid resuscitation and the need for vasopressor support, enhancing cardiovascular support and limiting extravascular lung water, and maximizing donor organ yield (80, 81).

Hormonal resuscitation with vasopressin, insulin, steroids and T3 has also been advocated to improve donor organ yield and graft function post-transplantation (80-82). Triple hormonal resuscitation of DBD donors with methylprednisolone, vasopressin and T3 has been shown to increase the yield of donor hearts, and result in improved graft function post-transplantation (83). In lung transplantation however, the routine standardised use of hormones remains controversial, with their individual benefits being questioned (84).

In donor organ management, vasopressin use is dependent upon donor haemodynamics. Pennefather et al have previously demonstrated that DBD donors treated with vasopressin have improved haemodynamics with a reduced need for inotropic support (85). Chen et al further advocated its use proposing that even in the absence of diabetes insipidus, haemodynamically unstable DBD donors have baroreflex dysfunction with impaired vasopressin secretion (86). Therefore it clearly has a beneficial role in the haemodynamically unstable donor, potentially limiting over-aggressive fluid resuscitation and high doses of inotropes (81, 82).

In a multivariate retrospective analysis, donor corticosteroid administration was found to be an independent predictor of successful donor lung procurement (87). It has been demonstrated that high dose methylprednisolone during donor organ management, improves donor lung oxygenation and reduces extravascular lung water (80, 88). Furthermore, corticosteroids reduce pro-inflammatory cytokines in the circulation and donor lung tissue, ameliorating lung injury both before and after transplantation (89). Consequently, it has become routine practice to administer methylprednisolone during donor management.

Insulin is routinely given in intensive care units (ICUs) to maintain normoglycaemia, and such glycaemic control is important in donor management. In addition it has been postulated to have anti-inflammatory properties, potentially attenuating the cytokine storm upon brain death (15). However, the benefit of its routine use in donor organ management has yet to be seen (84).

T3 therapy remains somewhat controversial. Novitzky et al have demonstrated in various animal models and human studies that T3 treatment promotes aerobic metabolism, replenishing energy stores and conferring haemodynamic stability, particularly in the context of heart
transplantation (23, 27, 90, 91). Certainly, in some retrospective human analyses of donor organ management T3 treatment is associated with an increased thoracic donor organ yield, and perhaps superior graft function (84, 92). Yet in other reviews, T3 therapy confers no additional benefit, and as such is not routinely indicated (24, 93, 94). Given the controversy surrounding its therapeutic value, its use is often limited to donors with significant haemodynamic compromise, whereby it has shown to be of value (81, 82).

Dopamine may also have a potential role in donor organ management, to improve graft function and reduce its immunogenicity upon transplantation. Dopamine can inhibit cytokine production and the expression of adhesion molecules, and as such, reduce neutrophil chemotaxis and T cell proliferation (95). It has been shown to be beneficial in rat models of brain death, whereby it is associated with haemodynamic stabilization, as well as a reduction in cellular infiltration within both renal and lung grafts (96, 97). In such lung grafts, there is reduced reperfusion injury following prolonged cold storage (97). The pre-conditioning effect of dopamine is thought to be secondary to the production of enzymes such as heme-oxygenase that may protect against ischaemic reperfusion injury (IRI) upon transplantation (98). This could lead to reduced organ dysfunction and improved outcomes post-transplantation. In renal transplantation, dopamine administration has been associated with improved graft function and survival post-transplantation (99).

The drive to improve and optimise donor lung function, has led to widened interest in therapies that can modulate the pathophysiological processes observed following donor death. More recently, for example, a role for β-adrenergic agonists to treat pulmonary oedema and improve alveolar fluid clearance, thereby increasing donor yield, has been postulated (100). In clinical practice however, they were of no benefit, with no improvement in oxygenation and adverse tachycardia in the donor (101). Alternative therapies are continually being reviewed. Furthermore, the role of the donor lung compartment in the induction of graft dysfunction and rejection post-transplantation, has led to donor management becoming a potential platform for pre-conditioning strategies.

1.2.4 Extended Donor Criteria for Transplantation

Lung transplantation has continued to evolve, however its on-going growth remains hampered by the scarcity of donor organs available for transplantation. Although improved donor organ management has been shown to limit donor lung injury and improve organ yield, and the use of DCD donors has increased the donor organ pool, the problem still remains. Over recent years this has led to a re-evaluation of the selection criteria used for transplantation, and the determination of variables that define a suitable donor.

Established standard donor lung criteria, used to assess the suitability of an organ for transplant, have been based on historic opinion and experience rather than evidence, and remain somewhat subjective, with the decision to transplant ultimately resting with the operating
surgeon. The ideal donor for lung transplantation has previously been defined as one who is aged between 20-45 years with; no smoking history, a partial pressure of arterial oxygenation (PaO₂) to fraction of inspired oxygen (FiO₂) ratio of > 350mmHg, a clear chest X-ray (CXR), no positive micro-organisms on gram stain, and a clear bronchoscopy (102). In the initial years of transplantation, increased selectivity may have been appropriate. However, as transplantation has progressed through the years, such stringent criteria have considerably limited the donor organ pool. Initially, this led to flexibility in donor age and smoking history, accepting donors up to 55 years of age and with a history of tobacco use of up to 20 pack years (103). The standard criteria used to determine donor lung suitability was subsequently amended, and split into two stages: the first stage based upon preliminary criteria from the donor, and the second stage based upon clinical evaluation by the retrieval team, as outlined in Table 1.2 (103).

Table 1.2: Standard Donor Organ Selection Criteria

Clinical criteria to determine the suitability of donor organs for transplant (103)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preliminary</td>
<td>Donor age &lt; 55 years</td>
</tr>
<tr>
<td></td>
<td>ABO blood group compatibility</td>
</tr>
<tr>
<td></td>
<td>CXR clear</td>
</tr>
<tr>
<td></td>
<td>Smoking history ≤ 20 pack-years</td>
</tr>
<tr>
<td></td>
<td>No history of significant trauma (blunt/penetrating)</td>
</tr>
<tr>
<td></td>
<td>No history of aspiration or sepsis</td>
</tr>
<tr>
<td></td>
<td>No prior cardiac or pulmonary operation</td>
</tr>
<tr>
<td></td>
<td>Gram stain and culture negative</td>
</tr>
<tr>
<td></td>
<td>PaO₂ ≥ 300mmHg on FiO₂ of 1 and a PEEP of 5cmH2O</td>
</tr>
<tr>
<td></td>
<td>Adequate size match</td>
</tr>
<tr>
<td>Evaluation</td>
<td>CXR and PaO₂ remain stable/no worse</td>
</tr>
<tr>
<td></td>
<td>Bronchoscopy clear with no aspiration or mass</td>
</tr>
<tr>
<td></td>
<td>Visual and manual inspection reveals</td>
</tr>
<tr>
<td></td>
<td>1) satisfactory parenchyma</td>
</tr>
<tr>
<td></td>
<td>2) no adhesions or masses</td>
</tr>
<tr>
<td></td>
<td>3) no evidence of trauma</td>
</tr>
</tbody>
</table>

However the accuracy of such criteria in determining the degree of lung injury and hence their suitability for transplantation has been questioned, with many lungs being deemed unusable when on functional evaluation they are considered suitable for transplantation (104-106). Furthermore, it has been demonstrated that lungs that do not fulfil the standard criteria can be successfully transplanted (107-115).
Kron et al were the first group to transplant lungs that were initially deemed unsuitable, with good post-operative outcomes (107). Shumway et al further demonstrated that liberalisation of the standard criteria, accepting donors up to the age of 60 years with, a positive smoking history with no evidence of underlying lung disease, a lower oxygenation and a degree of infiltration on CXR, had no adverse effects on outcomes (108). This has led to an increasing number of centres transplanting donor lungs that do not fulfil the standard criteria – so called marginal or extended criteria donor (ECD) lungs. In the most part results have been positive, with many centres reporting no difference in the short and intermediate terms when using standard donors and ECDs, with comparable ventilation times, hospital stays, complication rates, and 1-year survival (109-111, 113). Sommer et al also reported a lower incidence of PGD using ECDs (114). More recently however, Somers et al documented their 10-year experience of ECDs and reported an increased incidence of PGD and longer hospital stay, but long-term outcomes including survival and incidence of BOS, were comparable (115).

Nonetheless, there is on-going debate regarding the safety of ECDs, and their potential impact upon both short- and long- term outcomes. Pierre et al reported an increase in 30-day mortality following ECD transplantation, highlighting that caution was needed when extending certain criteria, particularly CXR infiltration and bronchoscopy findings (112). The subjectivity of such criteria makes it difficult to draw a line between what is acceptable and what is too much, and as such relies heavily upon the retrieval team. It has therefore been argued that only objective criteria should be extended (110). However, upon review of donor variables and their impact upon survival outcomes, donor age over than 50 years, PaO₂ to FiO₂ ratio < 300mmHg, CXR infiltration and bronchoscopy findings with evidence of sepsis have no impact upon mortality (116, 117). Nevertheless, although individual donor variables may not be independent predictors of outcome, the cumulative effect of using multiple extended criteria can lead to adverse results (117). Such is the case with donor age and ischaemic time; individually, increased donor age and prolonged ischaemic time have not been shown to influence survival, however together, they are associated with decreased intermediate and long-term survival (118-120).

Therefore although ECDs can successfully be used in lung transplantation, caution has to be exercised to ensure their use does not compromise outcomes. The development of a donor lung scoring system may have a role in determining the eligibility of a donor. Indeed, when applied to retrospective data, they have been shown to be reflective of acceptance, with higher scores predicting mortality (121). The subjective nature of donor lung assessment and utilisation has long been recognised, with a high degree of variation between centres. A validated scoring system may help to stratify the risk associated with individual donors, particularly those with multiple extended criteria, therefore providing guidance as to their suitability.

As the demand for lung transplantation continues to grow, there is increased pressure to improve donor organ supply and reduce waiting list mortality. Although improved donor management and the use of DCDs and ECDs may help to bridge the demand, they must not
compromise morbidity and mortality. As mentioned, the major caveats to lung transplantation are the lack of donor organs available for transplantation and the poor long-term survival. In addressing one issue however, we must not exacerbate the other.
1.3 Complications of Lung Transplantation

The median survival following lung transplantation is 6 years, significantly less than other solid organ transplants (6, 8). Although survival is associated with the indication for transplantation, the leading cause of death remains to be graft failure, as a result of acute rejection, IRI and PGD, and chronic lung allograft dysfunction (CLAD) (6).

1.3.1 Ischaemia-Reperfusion Injury

During standard donor procurement, the lungs are flushed with a low potassium dextran preservation solution and then undergo cold static preservation (CSP), whereby they are stored on ice prior to transplantation. Upon transplantation, the lungs are removed from the ice and implanted into the recipient, following which blood flow is re-established by the recipient circulation. IRI encompasses a variety of pathophysiological processes that occur whilst the lung is stored in a cold ischaemic state, and are then exacerbated upon reperfusion. These processes result in the production of reactive oxygen species (ROS), endothelial dysfunction, altered ion transport, complement activation, the release of pro-inflammatory mediators and mitochondrial dysfunction (122-124). Cellular death and dysfunction ensues, with increased capillary permeability, alveolar infiltration and ALI. The initial cellular injury and dysfunction is reversible, however it can progress to irreversible damage with cellular death and organ dysfunction, if the duration of ischaemia is prolonged or the reperfusion injury is of sufficient magnitude (124).

CSP is essential to decrease the cellular metabolic rate, thus reducing energy requirements and maintaining organ viability (123). However, this period of hypothermic ischaemia leads to an alteration in blood flow and a reduction in oxygen supply. The reduction in haemodynamic shear stress leads to activation of endothelial cells and the upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NF-kB (123). NADPH oxidase catalyses the reduction of oxygen to superoxide (SO) anion and hydrogen peroxide, highly unstable ROS that induce lipid peroxidation, increased cellular permeability and cell death (122-124). Additionally, NF-kB promotes the production of pro-inflammatory cytokines and up-regulation of adhesion molecules. Furthermore, ischaemia also results in leukocyte activation, further enhancing the release pro-inflammatory cytokines and up-regulation of adhesion molecules, and stimulating leukocyte-endothelial interactions (123).

In the lungs, alveolar oxygen can maintain cellular respiration and therefore delay hypoxic injury. When PaO$_2$ falls below 7mmHg hypoxic injury ensues, with an alteration of cellular metabolism, anaerobic glycolysis, ATP depletion and disturbed ion distribution (122, 123). Anaerobic glycolysis leads to an accumulation of lactate and fatty acids, with a subsequent fall in cellular pH (124, 125). There is resultant activation of the Na$^+$/H$^+$ pump to increase H$^+$ excretion in exchange for Na$^+$, with the latter accumulating in the cell (124). This is further compounded by the reduced activity of the Na$^+$/K$^+$ exchange pump secondary to hypothermia.
and a fall in ATP, leading to further intracellular Na\(^+\) accumulation, water accumulation, impaired cytoskeletal organization and subsequent cellular swelling and rupture (122, 123). Additionally, there is an increase in intracellular Ca\(^{2+}\) secondary to increased cellular influx in exchange for Na\(^+\) along the Na\(^+\)/Ca\(^{2+}\) channel, coupled with a decreased re-uptake and increased release by the endoplasmic reticulum (123, 124). The resultant calcium overload leads to further cellular damage through activation of calcium dependent proteases. It can also potentiate the effects of reperfusion injury through its activity upon the mitochondria and opening of mitochondrial permeability transition pores (MPTPs) (124, 125).

Reperfusion is essential to re-establish blood flow to facilitate the delivery of oxygen and nutrients and removal of cellular by-products, thereby maintaining cellular metabolism. However it can also be detrimental, driving pathological processes that ultimately result in reperfusion injury. The generation of ROS with their subsequent downstream effects, has long been recognised as the instigator of injury progression, with scavengers of these radicals conferring protection (126).

Upon reperfusion, platelets and leukocytes are delivered to the tissues with the release of further pro-inflammatory cytokines (125). Activated leukocytes form adhesive interactions with the endothelium, leading to the migration and infiltration of neutrophils, T cells and monocytes into the tissues whereby they release ROS and hydrolytic enzymes that are deleterious to cellular function (123, 125). The process is biphasic, with the early phase dependent upon donor-derived cells, predominantly macrophages, and the later phase secondary to the recruitment of recipient cells.

During lung ischaemia, there is a build-up of the ATP degradation product hypoxanthine, and xanthine oxidase, which upon reperfusion and the re-introduction of molecular oxygen, promote the production of ROS, specifically SO and hydrogen peroxide (123). Inhibitors of xanthine oxidase, such as allopurinol, have been shown to block this process (123).

ROS cause lipid peroxidation, DNA oxidation and activation of matrix metalloproteinases (MMPs) and other proteases that cleave proteins and receptors (123-125). In the mitochondria, upon reperfusion and restoration of a normal pH, and in the presence of increased intracellular calcium, they cause opening of the MPTPs (124, 125). The resultant increase in cellular permeability with loss of the membrane potential and uncoupling of the electron transport chain depletes ATP levels, promoting further ROS release, and eventually leads to cell swelling and rupture (125). Certainly, the role of the mitochondria and their dysfunction, appears to be critical to the development of reperfusion injury (125).

The generation of ROS also leads to the initiation and exacerbation of inflammatory and pro-thrombotic cascades, with further production of pro-inflammatory cytokines and up-regulation of adhesion molecules, activation and deposition of complement and increased production of platelet activating factor (PAF) (122-124). The combined effects of cellular swelling, with
increased leukocyte adhesion and migration, platelet aggregation and formation of microthrombi, can lead to microvascular dysfunction, limiting blood flow and propagating further ischaemia and release of ROS (122, 123).

Ultimately these processes converge to cause cell death through necrosis, necroptosis, apoptosis and autophagy (123, 124). Necrosis and necroptosis lead to cell rupture and release of pro-inflammatory mediators, evoking an inflammatory response that can cause further damage to parenchymal cells. Apoptosis and autophagy however, are regulated processes, whereby cellular contents are digested, and therefore do not elicit an inflammatory response (124). The contribution of these processes to graft dysfunction remains unclear, however apoptosis has not been shown to occur during ischaemia, but rather during reperfusion, where it appears to be associated with increased recoverability (123). Indeed on analysis of lung tissue biopsy samples, Fischer et al found no evidence of apoptosis following both warm and cold ischaemia, however upon reperfusion, apoptotic activity increases in a time-dependent manner, with no significant impact upon lung function (127). A rat model of IRI echoed these findings, additionally demonstrating that necrosis occurs during both ischaemia and reperfusion, with the percentage of necrotic cells during reperfusion correlating with graft dysfunction (128). The deleterious effects of necrosis and the role of apoptosis in limiting cellular dysfunction is further evidenced in models of brain ischaemia, whereby upon blocking apoptosis, there is a reduction in cellular recovery and a subsequent increase in the release of pro-inflammatory mediators and eventual necrosis (129). Yet more recently, in animal models of IRI, administration of alpha-1-antitrypsin (A1AT) has been shown to reduce apoptosis and is associated with improved lung function (130, 131). Therefore perhaps there is a threshold limit of apoptosis, whereby at lower levels it is beneficial, however over-activity accentuates graft dysfunction.

The reparative effects of apoptosis may be enhanced when the levels of ROS fall. At lower levels, ROS can promote cellular proliferation and migration through activation of growth factors essential for tissue repair and remodelling (125). Although these processes are essential for organ recovery, enhanced cellular proliferation can then lead to fibrosis and organ dysfunction (125). Indeed, IRI following lung transplantation has been implicated in the development of later organ dysfunction, manifested as BOS (132).

**Mediators of IRI**

IRI in the lung has been demonstrated to be biphasic, with the early acute phase mediated by ROS, and the later sub-acute phase mediated by neutrophil sequestration (133-135). The early phase appears to peak within 30 minutes of reperfusion and is thought to be secondary to activation of donor-derived alveolar macrophages that release pro-inflammatory mediators and ROS during ischaemia and upon initial reperfusion (133-135). Indeed in animal models, the early injury is reduced on inhibition of macrophages during reperfusion (135). Toll-like receptors (TLRs) on their surface are activated and initiate the activation of mitogen-associated protein kinases (MAPKs) and NF-κB, which then mediate the production of inflammatory mediators. TLR signalling has been shown to be crucial for the development of IRI specifically via TLR-4.
The later phase of IRI peaks around 4 hours of reperfusion, with progressive intrapulmonary recipient neutrophil recruitment and activation leading to injury (133, 134). Myeloperoxidase is a marker of neutrophil sequestration and has been shown to increase during reperfusion (133). Subsequent inhibition of neutrophil recruitment prior to reperfusion reduces the later phase injury (133, 138). Previously, Zwacka et al demonstrated in a mouse model of liver IRI that this late phase neutrophil response is mediated by CD4+ cells, and not CD8+ cells (138). These findings are also shown to be true in IRI in the lung, whereby CD4+ T cells accumulate upon reperfusion and initiate neutrophil chemotaxis (139).

The release of pro-inflammatory cytokines also appears to play a role in this biphasic response. In human and animal studies of IRI in the lung, levels of TNF-α, IFN-γ, IL-1β, IL-8, IL-10, IL-12 and IL-18 are increased during ischaemia and early reperfusion but then fall, with only IL-8 increasing upon reperfusion (134, 140, 141). During ischaemia, activated donor macrophages release TNF-α, IFN-γ, IL-1β, IL-8, IL-12 and IL-18. Upon reperfusion, these pro-inflammatory cytokines mediate the recruitment and activation recipient T cells and neutrophils (123). Specifically TNF-α and IL-1β appear to localize to activated alveolar macrophages, where they are co-stimulatory, enhancing recipient cell chemotaxis and pro-inflammatory cytokine release (142, 143). IL-8 is known to be a powerful neutrophil chemo-attractant, and its importance in the development of IRI has been demonstrated in various animal models (141, 144). IL-8 levels have been shown to increase upon reperfusion, with their rise correlating with a decline in lung function, and increased length of ICU stay (140). IL-17 also appears to be increased during IRI and is crucial to its development, with its release being mediated by CD4+ natural killer T (NKT) cells (145). Conversely, higher levels of the anti-inflammatory cytokine IL-10, during reperfusion, have been shown to be beneficial, with lower levels seen in older donors, perhaps explaining their susceptibility to IRI (140).

**Therapeutic Interventions**

To limit the detrimental effects of IRI, various therapies and techniques have been evaluated for their role during CSP and upon reperfusion.

During IRI, endogenous nitric oxide (NO) is reduced, secondary to increased destruction by oxygen-free radicals and reduced activity of endothelial NO synthase (123). The imbalance between SO and NO is thought to play a role in the development of IRI due to the decreased activity of NO upon endothelial cells, with NO normally reducing smooth muscle tone and preventing platelet aggregation (122). Dong et al demonstrated that in rat lungs, exogenous administration of inhaled NO during ischaemia and reperfusion, is associated with reduced IRI and improved graft function (146). However Eppinger et al have previously demonstrated disparate effects following the administration of inhaled NO upon lung reperfusion, with it exacerbating the early phase of IRI but reducing the later phase (147). Indeed, increased NO synthesis upon reperfusion in rabbit lungs has been implicated in the development of IRI (148). There has therefore been conflicting success with the use of exogenous NO in IRI, however it
has been shown to be beneficial in its most severe form, PGD, whereby it is associated with improved oxygenation and graft function (149).

Endothelial dysfunction is seen in IRI, with increased permeability, leukocyte adhesion and platelet aggregation (124). Prostaglandin E1 (PGE-1) administration during CSP has been shown to decrease endothelial dysfunction by stimulating cyclic 3,5 adenosine monophosphate-dependent protein kinases, leading to improved graft function (150). Other animal studies have also demonstrated the beneficial effects of PGE-1 administration during CSP and reperfusion, with improved graft function not related to endothelial function, but rather a shift to an anti-inflammatory profile within the lung (151, 152).

Phospholipase A2 induces the production of PAF in IRI. It is then released by a number of cells, initiating a pro-inflammatory cascade via up-regulation of cell adhesion molecules, platelet aggregation and cytokine release. Administration of PAF antagonists during CSP and reperfusion has been shown to improve graft function in various models of IRI (153-156).

Other mediators have been tested in various animal models and have been shown to ameliorate IRI secondary to an attenuated inflammatory response. They include adenosine A2A receptor antagonists (157, 158), A1AT (130, 131), N-acetylcysteine (159) and Diannexin (160). However to date, the only universal treatments used during donor organ procurement to limit the degree of IRI, are low potassium dextran solutions and methylprednisolone. Although various other treatments have been evaluated in animal studies, their clinical use has not yet been proven.

IRI is therefore an important pathophysiological process that occurs during lung transplantation. The degree of IRI and the subsequent cellular and organ dysfunction, is known to be associated with adverse donor variables and increased ischaemic times, initiating inflammatory cascades that are further exacerbated upon reperfusion. The injury process is known to be multifactorial as outlined above, however the individual contribution and importance of each of these remains unknown and poorly understood. In most cases, the injury is mild and self-limiting however in its most severe form, IRI results in PGD.

1.3.2 Primary Graft Dysfunction

PGD is a syndrome of acute allograft dysfunction with features similar to ALI and ARDs, that occurs in the first 72 hours post lung transplantation and the aetiology of which is multifactorial (161, 162). Its prevalence has previously been quoted as occurring in 15-57% of recipients post lung transplantation, however this was due to varied definitions (163). As a result, the ISHLT released a consensus statement in 2005, defining PGD as the development of non-cardiogenic pulmonary oedema and infiltrates on CXR, with or without a reduced PaO₂ to FiO₂ ratio of less than 300mmHg (163). The degree of oxygen impairment is then used to grade the severity of PGD (Table 1.3).
Table 1.3: The ISHLT Grading System for PGD

A grading system to determine the severity of PGD based upon CXR infiltration and oxygenation capacity (163)

<table>
<thead>
<tr>
<th>PGD Grade</th>
<th>PaO$_2$/FiO$_2$ ratio (mmHg)</th>
<th>Bilateral CXR infiltrates (consistent with pulmonary oedema)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt; 300</td>
<td>Absent</td>
</tr>
<tr>
<td>1</td>
<td>&gt; 300</td>
<td>Present</td>
</tr>
<tr>
<td>2</td>
<td>200-300</td>
<td>Present</td>
</tr>
<tr>
<td>3</td>
<td>&lt; 200</td>
<td>Present</td>
</tr>
</tbody>
</table>

The grading system remains valid today, being universally used to determine the presence of PGD at 4 specific time-points post-transplantation – 0 hours, 24 hours, 48 hours and 72 hours, whereby 0 hours corresponds to the first 6 hours of lung re-perfusion (162). Therefore using this standardized definition of PGD, current figures demonstrate its incidence to be approximately 30% in lung transplant recipients, with grade 3 PGD occurring in 15-20% (164). It is associated with increased morbidity and mortality, with various studies reporting an increase in duration of mechanical ventilation, length of hospital stay, and 30-day and 1-year mortality in patients with PGD (161, 165-167). Furthermore, survivors of PGD are shown to have a prolonged recovery, with increased severity correlating with reduced lung function and development of BOS (164, 166, 168).

Thus the high incidence of PGD and its association with increased morbidity and mortality has led to further research into its development and potential avoidance, including identification of risk factors and therapeutic targets.

**Risk Factors for PGD**

Various donor, recipient and operative risk factors have been implicated in the development of PGD, including increased donor age, female donor sex, African-American donor, donor head injury, donor smoking, decreased donor lung size, increased recipient body mass index (BMI), recipient diagnosis of sarcoidosis, idiopathic pulmonary fibrosis (IPF) or pulmonary arterial hypertension (PAH), prolonged ischaemic time, the use of cardiopulmonary bypass, increased transfusion of blood products and increased oxygenation upon reperfusion (164, 169-171). However, using multivariate models in a study of 1255 patients, Diamond et al identified that the only independent risk factors for the development grade 3 PGD were donor smoking, increased recipient BMI, recipient sarcoidosis or PAH, the use of cardiopulmonary bypass and increased FiO$_2$ during lung reperfusion (172). More recently Shah et al used objective variables to stratify patients according to their risk of development of grade 3 PGD, with low risk recipients having a normal BMI, underlying chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF), and absent or mild pulmonary hypertension, with all other patients considered higher risk (173). Interestingly, the addition of donor smoking to the high-risk group is associated with further increased risk, however it is not in the low-risk group (173).
A history of cigarette smoking is therefore thought to be the only definite donor risk factor for the development of PGD (164). It has also been associated with the development of ARDs in critically unwell patients, and as such it clearly has a role in the pathogenesis of ALI (174). Interestingly, increased donor age is not a definite predictor of PGD, conflicting with previous data (164). Indeed Baldwin et al reported that an increase in donor age of 55 to 64 years was not associated with an increased risk of PGD upon transplantation (175).

Although Hayes et al demonstrated that pulmonary hypertension in IPF recipients did not affect post-transplant outcomes (176), other studies have associated recipient PAH with the development of PGD (169, 172, 177). Consequently, the ISHLT working group associated recipient PAH, along with increased recipient BMI, with an increased risk of PGD (164).

More recently, genetic risk factors have been implicated in the development of PGD. Pentraxin-3 (PTX3) is an inflammatory mediator produced by antigen presenting cells (APCs) and is involved in innate immunity (178). Diamond et al found increased levels of PTX3 in the plasma of IPF recipients who went on to develop PGD (179). Further work by his group later demonstrated genetic polymorphisms in PTX3 increased the risk of PGD (180). Genetic variations in PGE2 have also been associated with the development of PGD (178, 181).

**Pathogenesis of PGD**

The development of PGD has long been attributed to IRI, however prior donor lung injury has also been implicated (182). The pathogenesis remains poorly understood. Ultimately, injured cells release damage associated molecular patterns (DAMPs), which along with pathogen associated molecular patterns (PAMPs), bind to pattern recognition receptors (PRRs), the most important of which are TLRs. Their interaction stimulates the release of inflammatory mediators through activation of MAPKs and NF-κB (182). TLR signalling therefore plays an important role in the development of PGD, as it does in IRI (182).

The hallmark of PGD is endothelial and epithelial cell dysfunction. The receptor for advanced glycation end products (RAGE) is expressed on alveolar epithelium. In lung injury, RAGE expression increases and mediates inflammatory responses via NF-κB (183, 184). Elevated soluble RAGE (sRAGE) levels have previously been seen in IRI in mouse models, whereby RAGE blockade then attenuates injury through decreased NF-κB activation and IL-8 production (185). Increased plasma and BAL levels of sRAGE have since been demonstrated to be associated with the development of PGD (186, 187). Furthermore, Pottecher et al have recently demonstrated that immediate increases in levels of sRAGE along with increased extravascular lung water are early predictors of PGD (188).

Pro-inflammatory and anti-inflammatory cytokines are released during the pathogenesis of PGD. The inflammatory response is similar to that seen in IRI, whereby there is a biphasic release of inflammatory mediators (182). Intrapulmonary neutrophil sequestration is important
for the development of PGD. Activated macrophages release inflammatory mediators via TLR signalling, with TNF-α, IL-1β, IL-8 and IL-17 promoting leukocyte and specifically neutrophil recruitment (182). The importance of IL-17 has been extensively demonstrated, whereby it drives neutrophil recruitment and infiltration into the lung, following its production by RAGE activated NKT cells (145, 189, 190). Activated neutrophils then initiate further inflammatory and immune cascades implicated in the development of ALI and subsequent PGD. Bharat et al demonstrated that in the early post-transplant period, recipients with PGD have higher circulating levels of IFN-γ, IL-1β, IL-2, IL-12 and MCP-1 (191). These cytokines then up-regulate the expression of human leukocyte antigen (HLA) class II on the donor allograft, increasing donor specific alloimmunity (191). Additionally, PGD has been associated with the early development of donor specific antibodies (DSA), which can promote the later development of BOS (192). Furthermore, recent studies using rat models have demonstrated that preformed antibodies to collagen type V can lead to PGD via IL-17 (193). Therefore there is a clear association between PGD and the innate immune response, with enhanced alloimmunity predisposing the graft to chronic dysfunction.

**Treatment of PGD**

Protective ventilation and fluid restriction are the hallmarks of PGD treatment, to limit further alveolar damage and promote recovery (194). Pulmonary vasodilators such as inhaled NO and prostaglandins have been extensively studied in animal models of IRI, and have also been shown to have a role in PGD.

In 2014, Sommer et al reported their experience of using C1 esterase inhibitor in patients with signs of severe PGD in theatre. Survival outcomes were improved when compared to patients to patients who developed grade 3 PGD within 72 hours who were not treated, however were lower when compared to normal controls (195). Yet despite acceptable outcomes, there has been a lack of further research into the use the C1 esterase inhibitors in PGD.

There is emerging evidence to suggest mesenchymal stem cells (MSCs) are a potential therapy for PGD. It has been demonstrated in animal models of IRI, that administration of intravenous (IV) MSCs confers protection against IRI, with a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines (196, 197). Additionally, intra-bronchial administration of multi-potent adult progenitor cells into human lungs rejected for transplantation is associated with decreased inflammation secondary to cold ischaemia (198). MSCs have also been shown to have a role in ARDS and recently, there has been a multicentre phase I clinical trial demonstrating that a single infusion of allogeneic bone marrow derived MSCs is well tolerated in patients with ARDS (199). Therefore the clinical use of MSCs in ARDS may help to pave the way for their use in transplantation in the prevention and treatment of PGD.

Other novel treatments used in IRI have also been postulated to have a role in the treatment of PGD, including PAF antagonists, however to date, there has been no clinical evidence to support their use.
PGD is therefore a prevalent complication following lung transplantation, associated with increased short-term and long-term morbidity and mortality. Although risk factors implicated in its development have been clearly defined, further work is required to better understand the pathophysiological processes involved in IRI and PGD. Furthermore, the identification of early biomarkers of dysfunction would facilitate the refinement of donor organ management and the development of targeted therapies.

1.3.3 Rejection

One of the major limitations to successful transplantation is rejection. Indeed historically, the success of solid organ transplantation was thwarted by its incidence. It was not until the work of Medawar et al on skin homografts that we developed a greater understanding of the rejection process and the role of the immune system (200). This paved the way for the first successful solid organ transplantation of a kidney between identical twins (201), and led to on-going research into the pathways of rejection and the role of immunosuppression in transplantation.

Despite advances in our knowledge of the immune system, rejection remains problematic and is a major contributor to reduced median survival post adult lung transplantation (6). It is responsible for 3.6% of deaths in the first 30-days post-transplantation, and 1.8% from 1 month to 1 year, with a third of recipients being treated for at least one episode of acute rejection within that first year post lung transplantation (202).

Clinically, there are three categories of pulmonary rejection, based upon onset, presentation and histological features: hyper-acute rejection, acute rejection and chronic rejection. Hyper-acute rejection arises early, following reperfusion of the transplanted lung. It is mediated through pre-existing antibodies, and is therefore a form of humoral rejection, ultimately leading to extensive graft infiltration with diffuse alveolar oedema (203). Acute rejection has traditionally been thought to arise secondary to cellular allorecognition alone, however there is increasing evidence to suggest that humoral immunity also plays a role (204). Chronic rejection remains poorly understand despite being one of the leading causes of long-term mortality (205). It is thought to be initiated by inflammatory and alloimmune processes and has been linked to both cellular and humoral rejection (206).

**Acute Cellular Rejection**

Cellular rejection is driven by allorecognition, whereby the immune system recognises and distinguishes between self and non-self antigens from individuals of the same species, and initiates an adaptive immune response. This alloimmune response is therefore dependent upon T cells and their recognition of foreign MHC.

The MHC is a group of cell surface proteins that bind antigenic peptides and present them to T cells. In humans, the MHC is also referred to as the HLA complex and is encoded by genes
located on the short arm of chromosome 6 (207). The MHC is divided into 3 classes that are encoded by different HLA genes (208). MHC class III is associated with the complement system whereas MHC class I and class II are important in antigen presentation with unique structures and functions.

MHC Class I are expressed on nearly all nucleated cells, where they present endogenous cytosolic peptides to CD8+ T cells. The HLAs corresponding to MHC Class I are HLA-A, HLA-B and HLA-C (209). MHC Class II are expressed only on APCs, namely B cells, dendritic cells (DCs) and monocytes, where they present exogenous extracellular peptides to CD4+ T cells. The HLAs corresponding to MHC Class II are HLA-DP, HLA-DQ and HLA-DR (209).

Individuals inherit two different HLA haplotypes, one from each parent, which are then co-dominantly expressed on each cell. Coupled with this, the MHC repertoire is polygenic and the genes are highly polymorphic, with multiple different alleles existing (210). The multiple HLA loci and the variety of alleles that can exist leads to uniquely diverse MHC classes, which although protective to unwanted foreign invasion, creates a considerable barrier to transplantation through allorecognition (211).

In cellular rejection, three pathways of allorecognition have been described: the direct, indirect and more recently, semi-direct pathways (204, 209, 212). Despite the initiating pathway, upon activation, recipient T cells clonally expand and differentiate, migrating to the lung allograft and inducing injury and dysfunction (204, 209).

Direct Allorecognition

In the direct pathway, passenger donor APCs present allogeneic MHC directly to recipient CD4+ and CD8+ T cells (213). Given that recipient APCs are not required for antigen processing and presentation, this pathway is thought to occur in the early period post-transplantation, independent of the indirect pathway (214-216). Furthermore, the alloimmune response is heightened by the increased reactivity of T cells to alloantigens (217).

![Figure 1. 2: Direct Allorecognition](image)

*In the direct pathway, donor peptides are presented to recipient T cells by donor MHC on donor APCs.*
Donor-derived APCs are critical for the direct response, and this has been highlighted in a rat model of renal transplant whereby immunogenicity is lost on depletion of passenger DCs and restored upon injection of donor cells (218). Donor APCs traffic to recipient lymph nodes whereby they initiate the direct alloresponse (212, 219). Indeed, in a mouse model of cardiac transplantation, upon removal of secondary lymphoid tissue there is failure of an alloimmune response (220).

Within the lung, direct allorecognition is of particular relevance given that it has its own intrinsic immune compartment. Bronchus associated lymphoid tissue contain APCs, namely macrophages and DCs, that highly express MHC (221). It has already been highlighted that upon donor death and subsequent lung procurement, the lungs undergo a myriad of pathophysiological processes that lead to inflammatory cell infiltration and up-regulation of MHC within the graft (12, 15, 43). These processes can lead to increased graft immunogenicity via direct allorecognition.

**Indirect Allorecognition**

In the indirect pathway, recipient APCs present allogeneic MHC to predominantly CD4\(^+\) T cells following internalization and processing (216, 222). Various animal models have demonstrated the role of the indirect pathway in allorecognition (223, 224). Previously, it has been thought to be associated with delayed chronic rejection however in a study of human heart transplant, indirect allorecognition also contributed to early acute rejection (225).

*Figure 1. 3: Indirect Allorecognition*

In the indirect pathway, allogeneic material, shed from donor APCs, is phagocytosed by recipient APCs, and the processed donor peptides are then presented to recipient T cells by recipient MHC on the recipient APCs.

Indirect allorecognition has also been shown to play a role in chronic rejection and graft dysfunction (212). In a renal transplant model, cultured T cells from patients with chronic graft dysfunction were primed to respond to donor MHC allopeptides, in a process driven by the indirect pathway (226). This finding was also observed in rat models of skin grafts (227).
**Semi-direct Allorecognition**

In the semi-direct pathway, donor APCs transfer intact allogeneic MHC complexes to recipient APCs which then present them to CD8<sup>+</sup> T cells, as well as simultaneously presenting allogeneic MHC peptides from phagocytosed extracellular material to CD4<sup>+</sup> T cells (212, 228). Thus the same recipient APC initiates a CD4<sup>+</sup> and CD8<sup>+</sup> T cell alloresponse (228). The transfer of donor MHC to recipient APCs is thought to be via direct contact with donor APCs or via the release of MHC containing exosomes from donor APCs (229-231).

![Figure 1.4: Semi-Direct Allorecognition](image)

In the semi-direct pathway, donor peptide is presented to recipient T cells by donor MHC on recipient APCs. The donor MHCs can be transferred by donor APCs directly by cell to cell contact between recipient and donor APCs (A). Alternatively donor APCs can release small exosomes containing intact donor MHC (B), which then fuse with the membrane of recipient APCs (C).

**Diagnosis of Acute Cellular Rejection**

Clinically, patients may be asymptomatic or present with non-specific features of increased dyspnoea, dry cough, low-grade fever or fatigues, the degree of which may correlate with the severity of the rejection (232). There may be a decline in the forced expiratory volume in one second (FEV<sub>1</sub>), mid-forced expiratory volume and vital capacity (VC) on pulmonary function tests however again, these findings can be non-specific (233). Additionally, although radiological examinations may help to differentiate from other aetiologies, features that may be seen in rejection are often non-diagnostic (204, 209). For these reasons, the histological features from a minimum of five trans-bronchial biopsy samples are used to diagnose and grade acute cellular rejection (203).

The histological features of rejection are of perivascular or interstitial mononuclear lymphocytic infiltration, with or without airway involvement (203). The extent of perivascular infiltration is used to determine the severity of rejection and a universal grading system has been formulated...
based upon these findings (Table 1.4) (203). In addition, the degree of airway inflammation is graded, based upon the bronchiolar submucosal infiltration, however this may be challenging dependent upon whether bronchial tissue is present in the samples (203). Caution should also be exercised upon interpretation of these airways changes as they are non-specific, and are often seen in acute infection (204).

Table 1. 4: The Histological Grading System for Acute Cellular Rejection
Classification system for the grading of acute rejection based upon histological appearance (203)

<table>
<thead>
<tr>
<th>Category</th>
<th>Grade</th>
<th>Meaning</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-GRADE: Perivascular Inflammation</td>
<td>0</td>
<td>None</td>
<td>Normal lung parenchyma</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Minimal</td>
<td>Scattered, inconspicuous small mononuclear perivascular infiltrates; no eosinophils or endothelialitis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild</td>
<td>More frequent, prominent perivascular infiltrates; eosinophils and endothelialitis may be present</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moderate</td>
<td>Dense perivascular infiltrates with extension into the interstitial space; eosinophils and neutrophils common</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Severe</td>
<td>Diffuse perivascular, interstitial and airspace infiltrates with alveolar damage and endothelialitis; intra-alveolar neutrophils may be present</td>
</tr>
<tr>
<td>B-GRADE: Airway Inflammation</td>
<td>0</td>
<td>None</td>
<td>No evidence of bronchiolar inflammation</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Low grade</td>
<td>Infrequent, scattered or single layer mononuclear cells in bronchiolar submucosa</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>High grade</td>
<td>Increased infiltrates of larger and activated lymphocytes in bronchiolar submucosa; can involve eosinophils and plasmacytoid cells</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>Ungradable</td>
<td>No bronchiolar tissue available</td>
</tr>
</tbody>
</table>

The diagnosis of acute cellular rejection is therefore dependent upon an invasive bronchoscopic examination with trans-bronchial sampling. Given that such techniques can be associated with risks, other diagnostic markers have been postulated. Studies investigating the immunological profiles of BAL samples have correlated levels of IL-17 (234) and IL-15 (235) with the presence of acute rejection, however such techniques remain invasive and may well be cost-ineffective. Potential serological biomarkers of rejection have been investigated in other solid-organ transplants, including gene profiling (236) and ATP levels (237). Specifically in lung
transplantation, it has been postulated that serum levels of hepatocyte growth factor may be predictive of acute rejection (238). However despite these findings, histological diagnosis remains the gold standard for acute rejection, with no other validated diagnostic methods.

**Humoral Rejection**

Antibody-mediated rejection (AMR) has emerged as an important clinical entity in lung transplantation, and results from the production DSA to HLA by B cells and plasma cells (239, 240). The process is well recognized in renal and cardiac transplantation, however it remains poorly understood in the context of lung transplantation (241, 242). This led to the production of a consensus report by the ISHLT, defining AMR and identifying key diagnostic criteria (239).

DSA may be pre-formed in sensitized patients, secondary to a history of blood transfusions, pregnancy or previous transplantation, or develop de novo post-transplantation (239). In renal transplantation, their presence has been associated with inferior graft function and increased mortality post-transplantation (243, 244). Similar findings have been observed in lung transplantation, whereby pre-formed antibodies are not only associated with increased waiting list times, but also increased AMR and reduced 1-year survival (245, 246). In addition, de novo DSA are also associated with inferior outcomes, increased rejection and the development of CLAD (247-249).

More recently, a role for autoantibodies against epithelial antigens such as Collagen-V has emerged in AMR that is not associated with the presence of DSA (239). So-called tissue self-antigens (SAGs) have been strongly implicated in development of CLAD (250).

In pulmonary AMR, B cells and plasma cells produce DSA against HLA antigens, forming an antibody-antigen complex that mediates an immune response via both complement-dependent and complement-independent pathways (209, 239). In the complement-dependent pathway, the antibody-antigen complex activates the complement cascade with complement deposition, subsequent endothelial injury and a pro-inflammatory response (239, 251, 252). In the complement-independent pathway, the antibody-antigen complex can act directly on endothelial cells to induce increased gene expression and proliferation, and on leukocytes via the Fc receptor portion of the antibody to induce pro-inflammatory cytokine production (239, 251, 253).

**Diagnosis of AMR**

The diagnosis of pulmonary AMR is challenging secondary to the lack of definitive and specific diagnostic features, the variable presence of DSAs, and the lack of correlation with graft dysfunction (239). The histopathological features of AMR are relatively non-specific and can include neutrophil margination, capillaritis and arteritis (203). The identification of the complement product C4d on capillaries by immunohistochemistry is also non-specific, and its absence does not rule out AMR (239). Thus, the diagnosis cannot solely rely upon the presence of an individual feature, but rather must involve the assessment of various features in determining the likelihood of AMR.
The ISHLT have subsequently published a consensus opinion of the definition and diagnostic criteria for pulmonary AMR, whereby it encompasses clinical, serological, pathological and serological findings (239). Clinical AMR is defined as the presence of allograft dysfunction, and at least one of the following criteria: positive DSA, positive histopathology suggestive of AMR and positive C4d staining. It is further categorized into definite, probable and possible based upon whether the allograft dysfunction is associated with the presence of 3, 2, or 1 additional criteria, respectively (239). Sub-clinical AMR is defined as the presence of histologic features suggestive of AMR on surveillance trans-bronchial biopsies, in the absence of allograft dysfunction with or without DSA or positive C4d staining.

**Chronic Rejection**

The pathogenesis of chronic rejection remains poorly understood. Historically it has been recognized as a progressive decline in FEV$_1$ with an obstructive picture on pulmonary function tests, and given the histological features of obliterative bronchiolitis (OB), has been termed as BOS (254). More recently however, a restrictive picture of chronic rejection with associated fibrosis has been described, and termed as restrictive allograft syndrome (RAS) (255). This has led to the umbrella term of CLAD being used to describe chronic rejection, encompassing both BOS and RAS phenotypes (205).

**Bronchiolitis Obliterans Syndrome**

The presence of OB in patients with obstructive lung function post lung transplantation was first described by Burke et al in 1984 (256). Over the coming years, these features became increasingly observed in lung transplant recipients, and it eventually led to the term BOS being adopted to identify this syndrome (254). OB is characterised by lymphocytic infiltration of the bronchioles, leading to peribronchiolar fibrosis and constriction of the bronchiolar lumen (257). Histological identification of these features is difficult however, as trans-bronchial lung biopsies are often inadequate for bronchiolar assessment with lung biopsies being superior but clinically impractical (254). Therefore a $\geq 20\%$ decline in FEV$_1$ from post-transplant baseline, that is persistent over a minimum of 3 weeks, is used to diagnose BOS (254). The degree of this decline is then used to grade severity (Table 1.5) (258).
Table 1: The Grading System for BOS

<table>
<thead>
<tr>
<th>BOS Grade</th>
<th>Spirometry Results (% of Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>FEV₁ &gt; 90% and FEF₂₅-₇₅% &gt; 75%</td>
</tr>
<tr>
<td>0p</td>
<td>FEV₁ 81-90% and/or FEF₂₅-₇₅% ≤ 75%</td>
</tr>
<tr>
<td>1</td>
<td>FEV₁ 66-80%</td>
</tr>
<tr>
<td>2</td>
<td>FEV₁ 51-65%</td>
</tr>
<tr>
<td>3</td>
<td>FEV₁ ≤ 50%</td>
</tr>
</tbody>
</table>

The pathogenesis remains unclear, however it appears to be driven by an alloimmune response to specific triggers that results in chronic rejection. Previous episodes of acute cellular rejection, particularly grade A2 or above, have been found to be risk factors for the late development of BOS (259-262). Studies also suggest a role for minimal A1 rejection (263-265). Additionally, the presence of DSA has correlated with incidence of BOS (266-268). Further risk factors have also been identified including: PGD (191, 269-271), gastro-oesophageal reflux disease (272-274), bacterial infection (275-277), fungal infection (278-280) and viral infection (281, 282) specifically cytomegalovirus (CMV) infection (283-285). It is thought that these triggers, whether immune or inflammatory, activate an innate and adaptive immune response with pro-inflammatory cytokine release, cellular infiltration and progressive aberrant fibrosis (258). Treatment is therefore focused upon minimizing these risk factors and attenuating the immune response.

Restrictive Allograft Dysfunction

A form of CLAD similar to BOS but with a restrictive decline in lung function has also been reported, and subsequently termed RAS (255). Pakhale et al described a form of restrictive CLAD in lung transplant recipients, associated with fibrotic lung change in a predominantly upper lobe distribution, with features of interstitial fibrosis and occasionally OB on biopsy (286). Sato et al went on to define a group of patients with a progressive decline in total lung capacity (TLC) ≥10% from baseline accompanied by a ≥20% decline in FEV₁ from baseline as having RAS (255). Such patients also demonstrated features of upper lobe fibrosis with diffuse alveolar damage and extensive interstitial and pleural fibrosis with or without OB (255). It has since become established that in patients with RAS, the histological features evolve from diffuse alveolar damage to pleuroparenchymal fibroelastosis as the disease progresses (287).

RAS is therefore characterised by a progressive and restrictive decline in lung function with a decrease in FEV₁, forced vital capacity (FVC) and TLC, persistent parenchymal and sub-parenchymal...
fibrosis on computed tomography imaging, and pleuroparenchymal fibroelastosis with or without OB on histology (288). To date however, there is no internationally approved definition or diagnostic criteria for RAS (288).

The pathogenesis is thought to be similar to that observed in BOS, with features of chronic rejection (255). However RAS is a much more progressive disease associated with worse survival outcomes, with median survival after diagnosis being limited to 6 to 18 months, in comparison to BOS which is 3 to 5 years (289). The risk factors for development of RAS remain somewhat obscure, however they are thought to be similar to those associated with BOS (288). Although the subsequent alloimmune response is thought to be more pronounced with a more significant inflammatory injury (288).

Although BOS and RAS have been described as separate entities, there is also significant overlap between them. Patients can often present with features of both, and BOS may develop into RAS at a later stage (205). CLAD has therefore been developed as an umbrella term to describe specific phenotypes of graft dysfunction, including those associated with chronic rejection, BOS and RAS (205). Nonetheless, the exact pathophysiological processes underlying these phenotypes require further work to elucidate specific therapeutic targets.

**Immunosuppression**

Immunosuppression therapies and regimes have evolved over the years, as our understanding of the immune system has improved. This has led to significant advances in the immunosuppression regimes adopted in transplantation.

**The History of Immunosuppression**

Following the Cold War, there was increased interest in the effects of radiation exposure, where it was revealed to cause significant bone marrow suppression. It was subsequently used at sub-lethal levels to treat mice with leukaemia, following which normal bone marrow was infused (290). The use of IV bone marrow infusions in humans, following treatment with total body irradiation (TBI) or chemotherapy, was also trialled, and although the infusion process was safe, successful engraftment was limited (291). However in siblings, and particularly identical twins, positive results were achieved following TBI (292, 293). Additionally in skin grafts, radiation induced bone marrow suppression followed by IV infusion of donor bone marrow cells, was shown to induce chimerism with the potential induction of tolerance (294).

The relative success of TBI in bone marrow transplantation led to its use in other solid organ transplantation and Merrill *et al* reported the first successful homologous renal transplant between non-identical twins following the use of radiation (295). Further renal transplants were successfully performed using TBI, with steroids used an adjunct to therapy (296, 297). However the advent of chemical immunosuppression was a major breakthrough in transplantation success, whereby successful donor engraftment was achieved without significant immune-compromise.
The first chemical immunosuppressant described was azathioprine, a purine analogue derived from 6-mercaptopurine (6-MP), that inhibits purine synthesis with subsequent inhibition of DNA replication and lymphocyte proliferation (298). 6-MP was initially used in the treatment of malignancies, and in a rabbit model it was shown to attenuate the immune response to bovine albumin (299). It was subsequently associated with improved survival in skin homografts (300). Therefore in a canine model of kidney transplant, Calne investigated the effect of 6-MP on rejection and found that it was associated with increased survival (301). Subsequently, there was increased work on less toxic derivatives of 6-MP and this led to the use of azathioprine, whereby in combination with prednisolone it was associated with reduced graft rejection and improved tolerance in renal transplantation (302). It was not until the 1970s however, upon the discovery of ciclosporin as a potent immunosuppressant that there was a significant evolution in transplant immunosuppression with improved outcomes (303).

Ciclosporin is an anti-fungal drug that was found to be toxic in rats, however facilitated successful skin grafts (303). Ciclosporin inhibits calcineurin, a serine threonine phosphatase that activates T cell transcription factors, up-regulating cytokine expression, specifically IL-2, which in turn stimulates T cell proliferation and differentiation. In lymphocytes, ciclosporin binds to the cytosolic protein cyclophilin, and the subsequent ciclosporin/cyclophilin complex inhibits calcineurin (304). Its immunosuppressive potential was first assessed in renal transplant patients, whereby its use was associated with improved outcomes (305). Although successful in reducing rejection, it was also associated with toxic side effects, particularly to the brain and kidney, with increased incidences of infection and lymphoma, and this led to people advocating for its clinical use to cease (306). Nonetheless, its significant benefits in transplantation were extensively demonstrated, particularly when combined with prednisolone, and therefore its use continued (307).

Further research over the years has led to the development of other immunosuppressive agents, including tacrolimus, mammalian target of rapamycin (mTOR) inhibitors and mycophenolate (308). Chemical immunosuppression has therefore become the mainstay of successful outcomes in transplantation. In the context of lung transplantation, immunosuppression regimes have become universally established and can be divided into induction and maintenance therapy.

**Induction Immunosuppression**

Induction therapy is aimed at depleting the recipient T cell population in the early period post-transplantation through administration of antibody therapy (308). The commonest agents used are polyclonal anti-thymocyte globulins (ATGs) and monoclonal antibodies directed against specific leukocyte surface molecules, such as IL-2 (309).

Polyclonal ATGs are produced by inoculating horses or rabbits with human thymocytes, to produce gamma (γ) immunoglobulins against human lymphocytes (309). Upon interaction, they
bind and deplete lymphocytes via induction of complement-mediated lysis and opsonisation (310). Induction therapy with polyclonal ATGs is associated with a reduced incidence of acute rejection and BOS (311). However ATGs use has declined over recent years, with more than 80% of patients who have induction therapy now receiving monoclonal IL-2 antibodies, such as basiliximab (6).

Monoclonal IL-2 antibodies are chimeric human-murine antibodies that bind to CD25 on the α-chain of the IL-2 receptor of activated T cells, inhibiting cellular proliferation and differentiation (308). They therefore attenuate allore cognition and the alloimmune response. Use of such monoclonal antibodies is associated with fewer side effects and improved tolerance.

Induction therapy with either ATG or IL-2 monoclonal antibodies has been proven to improve survival outcomes post-transplantation, with a reduced incidence of acute rejection (312). Therefore they are increasingly used in the initial post-operative period.

**Maintenance Immunosuppression**

Following induction therapy, transplant recipients are established on maintenance therapy consisting of a triple regime of calcineurin inhibitors (CNIs), cell-cycle inhibitors and prednisolone. mTOR inhibitors are also occasionally used post-transplantation, usually upon withdrawal or reduction of the CNIs (309).

Ciclosporin was the first CNI described and its use has revolutionized the world of transplantation. Since then another CNI, tacrolimus has been identified. Tacrolimus is a macrolide antibiotic that binds to the immunophilin 12kDa FK506 binding protein (FKBP12). The tacrolimus-FKBP12 binding complex then binds to a different site on calcineurin, to inhibit T cell proliferation and differentiation via reduced expression of IL-2 (313). It was first shown to be beneficial in liver, kidney and pancreas transplantation, whereby rejection despite ciclosporin therapy, responded to a switch to tacrolimus (314). It has since become an established CNI agent used in lung transplantation, with similar efficacy. Both drugs are associated with renal toxicity, hypertension, dyslipidaemia, neurotoxicity and diabetes. Ciclosporin is associated with more pronounced hypertension and dyslipidaemia, as well as additional hypertrichosis and gingival hypertrophy, whereas tacrolimus is associated with more neurotoxicity and diabetes, as well as additional alopecia (315). The choice of agent used is dependent upon the transplant physician and the most acceptable side effect profile.

Azathioprine was the first cell-cycle inhibitor described in transplantation, and was the first drug therapy used in renal transplantation. Mycophenolate mofetil (MMF) has since emerged as an additional anti-metabolite following initial success in animal models of heart and kidney transplant, and then human renal transplant (316). Mycophenolic acid is the active component of MMF and inhibits inosine monophosphate dehydrogenase, an enzyme required for the de novo synthesis of purines (309). Although other cells have salvage purine synthesis pathways,
lymphocytes do not and as such rely heavily on de novo synthesis of purines, therefore inhibition leads to reduced lymphocyte proliferation (309). Myelosuppression is the main side effect of cell-cycle inhibitors, in addition gastro-intestinal disturbance is also associated with MMF (309).

In addition to maintenance therapy, prednisolone is also used in induction therapy and the treatment of acute rejection. It is therefore essential to the success of transplantation. It is a glucocorticoid and as such its lipophilic structure enables passage through cellular membranes, where it binds to glucocorticoid receptors. This complex ultimately translocates to the nucleus where it then binds to specific DNA binding sites, resulting in altered gene transcription with a reduced anti-inflammatory and pronounced regulatory response (308). In the context of maintenance therapy, it is the only drug consistently used, with the choice of CNI and cell-cycle inhibitor varying between centres due to the conflicting data regarding the superior use of specific regimes (308).

mTOR inhibitors are the most recent development in immunosuppressive therapy. mTOR is a serine/threonine protein kinase that regulates cell growth and proliferation via DNA and protein synthesis. mTOR inhibitors such as rapamycin, bind to the FKBP12 immunophilin, and this complex inhibits the activity of mTOR, causing cellular arrest and reduced proliferation (308). Although there was initial interest in using this drug as an early post-operative immunosuppressant, its anti-fibroproliferative activity negatively impacted wound and specifically anastamotic healing thereby restricting its use (309). As such, it is predominantly used as a second line immunosuppressant to replace or enable reduction in CNIs, secondary to intolerable side effects namely nephrotoxicity (309).

Although immunosuppression has facilitated successful transplantation, it is also associated with a number of side effects that can adversely affect the recipient and can result in increased morbidity. Furthermore, it can lead to the development of infection and post-transplant lymphoproliferative disease, which can impact upon survival, with infection also being one of the leading causes of mortality post-transplantation (6).

Therefore although immunosuppression has advanced over the years, lung transplantation remains limited by the incidence of rejection. Furthermore, immunosuppressive therapies are associated with increased morbidity and require careful monitoring. As such, there is scope for further research. Interestingly, current therapies are aimed at reducing the recipient immune response, and the donor immune compartment remains relatively ignored. However it could potentially serve as an important therapeutic target. Recently models of EVLP have been used in transplantation to evaluate donor organ function, however they could also provide a platform for donor organ immunomodulation.
1.4 Ex-Vivo Lung Perfusion

EVLP has emerged as a technique to enable the extended evaluation of donor organs to determine their suitability for transplantation (317). Lungs are removed from the donor environment and placed on an ex-vivo circuit that mimics the normal physiological in-vivo environment. Upon connection to the circuit, they undergo gradual controlled normothermic reperfusion with protective ventilation, thereby facilitating viability (317). This is in contrast to CSP, where lungs are cooled to preserve organ integrity by reducing metabolic activity whilst they are transported in an ischaemic state (318). The EVLP circuit therefore provides a controlled and stable environment to facilitate lung recovery and assessment, without causing additional insult. The reconditioning capability of the circuit has led to the increased utilisation of donor organs thereby optimising the donor organ pool. More recently however, there has been increased interest in its capability to provide a platform to facilitate donor organ manipulation.

1.4.1 The History of EVLP

The concept of ex-vivo perfusion was first established in 1935, when Carrel and Lindbergh described cat and rabbit models of ex-vivo normothermic perfusion in various organs, including the thyroid, ovary, kidney and heart (319). Jirsch et al later went on to develop models of ex-vivo organ perfusion to assess lung physiology, however success was thwarted by the development of lung oedema (320).

In the 1990s, Steen et al performed extensive work in animal models to optimise organ preservation prior to lung transplantation, where they determined the feasibility of exposing donor lungs to extended warm ischaemia and developed extracorporeal lung assist models (321-325). In 2001, his group then went on to successfully perform the first human lung transplantation from a DCD donor, with lungs that had undergone prior functional assessment using an ex-vivo perfusion circuit (326). Steen went on to describe how this ex-vivo perfusion can effectively assess donor lungs prior to transplantation using a porcine model of EVLP transplant (66).

The emergence of pulmonary oedema secondary to a loss of vascular integrity had hindered previous attempts at EVLP. However Steen overcame this problem through the development of a hyper-oncotic preservation solution to optimise osmotic pressure, which was perfused in a controlled manner (66). The solution was referred to as STEEN Solution™.

There are therefore three key principles that underpin successful EVLP, without the advent of additional injury (66, 327, 328):

i) The use of a buffered, hyper-oncotic perfusate, to restore normal electrolyte balance and facilitate the delivery of nutrients essential for cellular viability.
Additionally, the high colloid-osmotic potential promotes alveolar fluid reabsorption (66, 327).

ii) Controlled, gradual perfusion with low mean PA pressure, to reduce hydrostatic pressure and avoid disruption of the alveolar/capillary membrane barrier secondary to haemodynamic sheer stress (329, 330). In the early period during re-perfusion endothelial permeability has been shown to be increased, heightening the risk of oedema formation (331).

iii) Controlled, protective ventilation with reduced tidal volumes, to avoid baro-trauma and the subsequent activation of inflammatory signalling pathways that ultimately lead to ALI (332). Reduced ventilation can abrogate these changes, preserving lung integrity and reducing reperfusion injury (333).

Steen et al demonstrated in their porcine model of EVLP transplant, that EVLP enables the accurate functional assessment of lungs from DCD donors without oedema formation. Furthermore upon transplantation of such lungs, post-operative lung function and gas exchange was comparable to in the donor prior to cardiac arrest (66). The latter suggested the potential of the EVLP circuit to facilitate a reconditioning process within the donor lungs and therefore allowing the use of sub-optimal lungs. His findings were then supported by other groups who reported the success of EVLP to facilitate donor lung assessment, in particular ECD lungs, using both animal models (334, 335) and human models (336).

In 2005, Steen et al went on to perform the first human lung transplantation using a lung that was initially deemed unacceptable upon evaluation in the donor (337). The donor lungs were harvested and underwent standard organ preservation prior to being placed on the ex-vivo circuit for further evaluation. The left lung was successfully reconditioned with improvement in oxygenation, and was successfully transplanted into a 70-year old male recipient with underlying COPD (337). His group then went on to further demonstrate the reconditioning capability of EVLP by utilising the technique on 6 donor lungs that had been declined for transplantation secondary to a PaO$_2$ < 300mmHg (327). Using the EVLP model, the lungs underwent controlled and gradual normothermic reperfusion with hyperosmolar STEEN Solution™, mixed with red blood cells to a haematocrit (Ht) of 15% (327). During EVLP, the lung oxygenation improved from an average PaO$_2$ of 203mmHg in the donor to 428mmHg following reperfusion on the EVLP circuit (327). His work therefore revealed the potential of EVLP to not only serve as a tool to facilitate the extended evaluation of donor lungs, but also as a platform to recondition marginal donor lungs.

The reconditioning potential of EVLP led to further interest by other groups as to whether extended ex-vivo normothermic preservation is associated with improved lung function and recovery. Through manipulation of the EVLP technique previously described by Steen et al, Cypel et al demonstrated in a pig and human model that 12 hours of extended normothermic preservation using an acellular STEEN Solution™, was associated with adequate lung function and reduced oedema formation (338). Additionally, on transplantation and subsequent perfusion
of the porcine lungs, lung function was maintained and histology preserved (338). Their findings supported that EVLP can enable accurate assessment of donor lungs. In addition, they demonstrated that prolonged acellular EVLP could facilitate the extended assessment and preservation of donor lungs, with the capability of using it as a platform for therapeutic manipulation.

Erasmus et al previously demonstrated that EVLP was superior to preservation with topical cooling using a porcine model (339). Following this, using a porcine transplant model to compare the effects of EVLP following a prolonged period of cold ischaemia with prolonged CSP alone, Cypel et al went on to demonstrate the superiority of EVLP in maintaining the alveolar/capillary membrane and preserving metabolic activity following a prolonged period of ischaemia, with improved lung function upon transplantation (340). The superiority of EVLP to preserve donor lungs led Warnecke et al to modify the ex-vivo circuit further, creating a mobile circuit, to be used immediately following donor lung harvest, thereby minimizing ischaemic time (341).

EVLP has therefore evolved over recent years, to facilitate and optimise lung preservation and achieve superior post-operative outcomes. In recent years, it has also been used as a research tool, to investigate lung physiology and the impact of pharmacological therapies.

1.4.2 The Technique of EVLP

The EVLP system comprises of an organ perfusion chamber attached to a ventilator and a modified cardio-pulmonary circuit, consisting of a venous reservoir, a pump, an oxygenator with a built-in heat exchanger and a leukocyte filter (66). Temperature and pressure probes are also incorporated into the circuit. The pump circulates perfusate through the oxygenator and heat exchanger before entering the specialised organ perfusion chamber via the leukocyte filter. From here, it then drains into the venous reservoir before being re-circulated (66).

Following standard donor organ procurement, lungs are prepared for EVLP either immediately or following a period of CSP. The PA is cannulated and the LA is either left open with free drainage from the pulmonary veins (PVs), or closed with a funnel shaped cannula secured to the LA cuff to maintain an LA pressure of 3-5mmHg (66, 327, 338). An endotracheal (ET) tube is then inserted and clamped whilst the lungs are in a semi-inflated state (66, 327).
Prior to placing the lungs in the organ perfusion chamber, the circuit is primed with 2 litres of supplemented perfusate. The perfusate may be cellular with the addition of leukocyte-depleted red cells to achieve a target Ht, or acellular without the addition of red cells (66, 327, 338). The pH and glucose are adjusted to physiological levels using isotonic trometamol and glucose, respectively. In addition, a gas mixture of oxygen ($\text{O}_2$), nitrogen ($\text{N}_2$) and carbon dioxide ($\text{CO}_2$) is supplied to the membrane oxygenator and adjusted to resemble mixed venous blood (327). The partial pressure of CO$_2$ is maintained between 35 to 45mmHg, with abnormal levels associated with impaired alveolar fluid absorption secondary to endothelial injury (342).

Following adequate priming of the circuit, the donor lungs are placed in the organ perfusion chamber and connected to the cardio-pulmonary bypass circuit and ventilator via the PA cannula and ET tube respectively. Air is removed from the circuit and flow is then initiated through the pump, initially at low pressure (66). Perfusate circulates through the pulmonary circulation via the PA cannula, with the PV effluent draining into the venous reservoir via the LA, for re-circulation. The lungs are gradually re-warmed to 37°C by increasing the temperature of the perfusate. Concurrently, the pump flow rate is gradually increased to a maximum flow rate, whilst maintaining a PA pressure $< 20$mmHg. Protective ventilation is commenced when the temperature of the perfusate in the LA reaches at least 32°C, and is gradually increased to
reach maximum ventilation at 37°C. Recruitment manoeuvres with an increase in PEEP can then be performed to reduce atelectasis (66).

When maximum flow and ventilation is reached, the lungs can be assessed to determine their suitability for transplantation. If suitable, perfusate flow and ventilation is reduced with core cooling. Perfusion is then discontinued and the lungs clamped in a semi-inflated state prior to transplantation (66).

The fundamental technique of EVLP is to achieve gradual, controlled normothermic reperfusion with protective ventilation, to maintain epithelial integrity and limit oedema formation. Since Steen described the process of EVLP that he adopted to perform the first human lung transplant using EVLP lungs, other groups have since adapted his protocol with clinical success. To date, there are three main protocols that are followed for EVLP perfusion: the original Lund protocol (66, 327), the Toronto protocol (338) and the Organ Care System (OCS) protocol (341).

1.4.3 The Three Protocols of EVLP

Steen et al successfully demonstrated that EVLP is a viable technique to enable the extended evaluation of donor lungs prior to transplant, through controlled reperfusion with a unique hyperosmolar perfusate, and a delayed and protective ventilation strategy (326, 337). The technique used has latterly been adopted as the Lund protocol and has since formed the basis of both the Toronto and OCS protocols. All three protocols therefore share the fundamental principles of EVLP, however they differ in how they achieve them, in relation to timing, perfusate composition, and reperfusion and ventilation strategy (317).
Table 1.6: The Three Protocols of EVLP
Comparison of the three protocols used in EVLP, in relation to perfusate composition, flow rates and ventilation. RBC = red blood cells; Ht = haematocrit; CO = cardiac output

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>LUND Protocol</th>
<th>TORONTO protocol</th>
<th>OCS protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timing</td>
<td>Delayed following CSP</td>
<td>Delayed following CSP</td>
<td>Immediate</td>
</tr>
<tr>
<td>Per fusate</td>
<td>STEEN Solution™ + leukocyte depleted RBCs</td>
<td>STEEN Solution™</td>
<td>OCS solution + leukocyte depleted RBCs</td>
</tr>
<tr>
<td>Target Ht. (%)</td>
<td>15</td>
<td>0</td>
<td>15-25</td>
</tr>
<tr>
<td>Target flow</td>
<td>100% CO</td>
<td>40% CO</td>
<td>2.0-2.5 l/min</td>
</tr>
<tr>
<td>PAP (mmHg)</td>
<td>≤ 20</td>
<td>10-15</td>
<td>≤ 20</td>
</tr>
<tr>
<td>LA</td>
<td>Open</td>
<td>Closed</td>
<td>Open</td>
</tr>
<tr>
<td>Start temp (°C)</td>
<td>32</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>Tidal Volume (ml/kg/bw)</td>
<td>5-7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>RR (bpm)</td>
<td>20</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>PEEP</td>
<td>5</td>
<td>5</td>
<td>5-7</td>
</tr>
<tr>
<td>FiO₂ (%)</td>
<td>50</td>
<td>21</td>
<td>12</td>
</tr>
</tbody>
</table>

The Lund and Toronto protocols adopt a delayed EVLP strategy, whereby the lungs undergo standard donor organ procurement followed by CSP prior to EVLP usually being performed at the recipient hospital (343, 344). The OCS protocol aims to limit ischaemic exposure, thereby focusing upon immediate EVLP following organ procurement with a brief preservation flush (341). The concept of reducing ischaemia, is to maintain metabolic viability, preserve cellular function and therefore optimise graft function. Nevertheless, Mulloy et al found delayed EVLP to be superior to both immediate EVLP and CSP, with improved oxygenation and pulmonary haemodynamics, and a reduced inflammatory profile and preserved integrity on histological examination (345). They postulated that the inflammatory response initiated during warm ischaemia and donor death is attenuated by cold storage with a reduction in signalling pathways (345).

The EVLP perfusate used in both the Lund and Toronto protocol is STEEN Solution™, a buffered, extracellular low-potassium dextran solution with the addition of albumin. The dextran protects the endothelium, reducing complement activation and platelet aggregation by coating the vasculature and plastic surfaces (66). Albumin further increases the oncotic potential, thereby optimising vascular water re-absorption. The need for hyperosmolar perfusion solutions has been demonstrated in animal models, whereby perfusion with normal saline solution is associated with oedema formation (346, 347). Furthermore, Chang et al have previously demonstrated in a rabbit model of EVLP, that the addition of albumin resulted in reduced oedema (348). However other studies have demonstrated that albumin is not a crucial
component provided hyper-osmolarity is achieved (349). The OCS protocol therefore utilises a hyper-osmolar low-potassium dextran solution without the addition of albumin with no reported adverse effects (341).

The addition of red cells to the perfusate continues to be debated. The Lund and OCS protocols supplement the perfusate with leukocyte-depleted red cells to achieve a target Ht, whereas the Toronto protocol does not (341, 343, 344). Steen et al have previously advocated the use of red cells due to their buffering capabilities and improved oxygenation. With the latter, the addition of haemoglobin improves the oxygen carrying capacity of the perfusate and oxygenation is reflective of the physiological oxygen-dissociation curve, facilitating a reliable assessment of lung function (350). In an acellular perfusate, Henry's Law is adopted whereby the oxygen that dissolves into the perfusate is directly proportional to its partial pressure and as such the relationship becomes linear and is not reflective of normal physiology (350). Furthermore, red cells promote vascular distension and therefore improve microvascular haemodynamics, which has been proven to be protective in IRI (148). However their presence can also be detrimental, particularly in more prolonged perfusion, whereby there is mechanical haemolysis and increased pulmonary vascular resistance (PVR) secondary to free haemoglobin scavenging NO (339). Thus acellular perfusate may be superior in more prolonged EVLP, whereas for short-term assessment, cellular EVLP may be more physiologically reflective of lung function.

There has also been much debate regarding the optimal flow rate during EVLP. The Lund protocol advocates a target flow rate of 100% of the estimated cardiac output, whilst maintaining the PA pressure < 20mmHg (343). However the Toronto protocol has a target flow rate of 40% of the estimated cardiac output, with PA pressures maintained at 10 to 15mmHg (344). Unlike the Toronto and Lund protocols, whereby flow rate is calculated from the estimated cardiac output, the OCS protocol adopts a set maximum flow rate of 2.5L/min, whilst maintaining the PA pressure < 20mmHg (341). Lower flow rates are associated with reduced endothelial shear stress and therefore reduced oedema formation (330). Yet the flow must be adequate enough to effectively perfuse the lung vasculature. In a rabbit model of EVLP, Sasaki et al demonstrated that optimal perfusion pressure is 10 to 15mmHg, with extremes of pressure resulting in inferior perfusion of the vascular beds and subsequent reduced lung function (351). However reduced flow rates are not representative of the normal physiological lung conditions and therefore may not reflect the functional capability of the lungs upon transplantation, whereby they are exposed to higher perfusion pressures. Nevertheless, the Toronto protocol has been shown to adequately perfuse the pulmonary vasculature during EVLP, with no adverse features post-transplantation (344).

The Toronto group also advocates closure of the LA by suturing a funnel shaped cannula to the LA cuff, maintaining an LA pressure of 3-5mmHg (344). This is in contrast to the Lund and OCS protocols, whereby the LA cuff is kept open therefore maintaining a pressure of 0mmHg (341, 343). It has previously been established that pulmonary haemodynamics, particularly LA pressure, can impact upon airway mechanics (352). An increased LA pressure maintains
venous afterload and can prevent PV collapse during the cyclical pressure changes throughout normal ventilation thereby reducing PVR. Indeed, it has been demonstrated that reduced LA pressure is associated with ventilator-associated lung injury (353).

Finally, the protocols all differ in their ventilation strategies. All protocols advocate protective ventilation with a maximum tidal volume of 5-7ml/kg and a PEEP of 5cmH\textsubscript{2}O. The Lund and Toronto protocols commence ventilation at 32°C, whereas the OCS protocol commences ventilation at 34°C. The respiratory rate and the Fi\textsubscript{O\textsubscript{2}} during EVLP are different between all groups as outlined in Table 1.6 (343, 344).

The early success of EVLP has led to its increased use in lung transplantation to facilitate the utilisation of marginal donors and ECDs, thereby optimising the donor organ pool. As the clinical experience of EVLP increases, its impact upon long-term outcomes is continually evaluated.

1.4.4 The Clinical Experience of EVLP

Following on from their initial success, the Lund group went on to perform the first 6 lung transplants using EVLP reconditioned lungs that did not originally fulfil the criteria for transplantation (354). After a period of EVLP, oxygenation and lung function improved, rendering them suitable for transplantation. Following transplantation, all recipients were alive at 3 months, with 2 patients subsequently dying from sepsis and rejection. At 24 months, the 4 remaining recipients were well with no evidence of BOS (354). In a comparative study of post-operative outcomes using these lungs and 21 standard double lung transplants, there was no significant difference in the length of ICU and hospital stay between the two groups (355). Their results not only confirmed the capability of EVLP to serve as a reliable tool for the extended evaluation of donor lungs, but also demonstrated its ability to facilitate a reconditioning process. Furthermore, successful transplantation could be achieved using EVLP reconditioned lungs with outcomes comparable to standard transplantation, thereby optimising the donor organ pool and reducing the waiting list for transplantation.

Having established an acellular, modified EVLP protocol (338), Cypel et al went on to demonstrate the safety and efficacy of transplanting EVLP-reconditioned lungs using their Toronto protocol compared with standard transplantation (344). In the first prospective trial of EVLP, of the 20 lungs that underwent successful reconditioning with subsequent transplantation, there was no significant difference in mechanical ventilation, ICU and hospital stay, and 30-day mortality (344). Interestingly, although not statistically significant, there was a lower incidence of PGD seen in the EVLP transplanted lungs compared to standard transplantation (344). They went on to publish their experience of 50 EVLP lung transplantations compared with 267 standard transplantations and again reported comparable short-term outcomes, including 1 year mortality, with a non-significant, reduced incidence of PGD seen in the EVLP group (356).
Following the success of the Lund and Toronto group, centres across the world began to use EVLP to recondition marginal donor lungs. Initial results from Wallinder et al had demonstrated that they could successfully use EVLP to recondition marginal donors that were initially deemed unsuitable for transplantation and upon transplantation, short-term outcomes were similar to those following standard lung transplant (357). They have since gone on to publish their single-centre experience over a 4-year period, comparing 27 EVLP lung transplants following 32 EVLPs with 145 standard conventional transplants (358). They reported comparable short-term post-operative outcomes and although 1-year survival tended to be improved in the EVLP group, this was not significant. Overall survival, re-transplantation rate and freedom from rejection were similar between groups (358). Analogous results have been published by other worldwide centres including, the Harefield group (359), the Vienna group (360), and the Milan group (361), whereby outcomes following transplant with EVLP-reconditioned lungs were associated with good post-operative outcomes and survival, comparable to that of standard lung transplantation. Although the Toronto group have shown a preponderance to a lower incidence of PGD following transplantation with EVLP-reconditioned lungs (344, 356), other centres have failed to demonstrate any significant difference, with PGD occurrence comparable between EVLP and standard transplant groups (358-362). However Boffini et al did report a superior recovery from PGD in EVLP transplanted lungs (362).

Conversely, a recent multi-centre observational study of donor EVLP in the United Kingdom (DEVELOP-UK) was stopped prematurely secondary to an increased incidence of early grade 3 PGD following transplantation with EVLP-reconditioned lungs compared to standard transplant, with an increased need for extra-corporeal membrane oxygenation (ECMO) and prolonged ventilation with longer ICU stays (363). However incidence of PGD at 72 hours was comparable between EVLP and standard transplant groups, as was length of hospital stay, incidence of infection and rejection, and 1-year survival (363). The study however had many limitations. Firstly they used a hybrid protocol combining aspects of both the Lund and Toronto techniques, the safety and efficacy of which had not previously been tested. Secondly, the protocol was changed mid-point through the study, switching to a cellular perfusate. Finally, recruitment into the EVLP group was poor with only 18 transplants being performed using EVLP-reconditioned lungs, compared to 184 in the standard transplant group. Their results should therefore be interpreted with caution, and further long-term, multi-centre studies are needed to truly evaluate the impact of EVLP upon medium and long-term morbidity. The NOVEL trial is a multi-centre study currently on-going in the United States, whereby outcomes following EVLP and standard transplant are being analysed, with early data showing comparable post-operative and 1-year survival, thereby supporting the safety of EVLP (364).

In addition to delayed EVLP, some centres have also adopted the OCS protocol, whereby immediate EVLP is performed following organ procurement and continued during transportation to reduce the need for CSP, with the potential to preserve and optimise lung function prior to transplantation. Warnecke et al were the first group to transplant lungs that had undergone immediate reperfusion using the OCS (341). 12 lung transplantations were subsequently
performed, with improvement in oxygenation in all lungs perfused on the OCS and no immediate complications following transplantation with 100% 30-day survival and hospital discharge (341). The Harefield group has since published promising data from their experience of using the OCS for immediate reperfusion and preservation compared to standard CSP (365). Over a 7-year period, 308 lungs were transplanted following standard CSP compared to 14 lungs that were transplanted following perfusion and preservation using the OCS. Recipients of lungs in the OCS group had significantly better post-operative FEV1 at 3-months and 6-months, with survival and incidence of BOS comparable between both groups (365). More recently, the use of OCS to preserve DCD lungs has been shown to be superior to topical cooling, with a reduced incidence of PGD and similar post-operative and 1-year outcomes (366).

Currently, there are two on-going multi-centre studies evaluating the use of the OCS prior to transplantation, not only as a preservation tool compared to standard CSP in standard donor organs (INSPIRE trial), but also as a reconditioning tool for marginal donors (the EXPAND trial) (317).

Therefore since the clinical emergence of EVLP in 2001, it has been shown to facilitate the extended evaluation and potential reconditioning of donor organs, which upon transplantation have outcomes comparable to those seen in standard transplantation. Its use has led to a 20-30% increase in transplant activity, thereby optimising use of the donor organ pool (356, 361, 367). More recently, there has also been increased interest in the use of EVLP as a platform to improve graft quality prior to transplantation. The Vienna group has recently published data on the use of EVLP in standard donor lungs prior to transplantation (368). In a prospective study, 80 donor lungs were randomly selected to undergo standard transplantation following CSP or EVLP following CSP and prior to transplantation. Although lungs in the EVLP group were exposed to a significantly higher cold ischaemic time, although not significant, the incidence of PGD and ECMO use was lower in the EVLP group with comparable mid-term outcomes (368).

Therefore as the role of EVLP continues to expand, further work is required to fully elucidate its potential to improve graft quality prior to transplantation. Furthermore, the cellular and molecular processes that underpin successful EVLP are yet to be fully understood.

1.4.5 The Science of EVLP

The concept of EVLP is to provide a physiologically stable ex-vivo environment to safely evaluate and recondition donor organs prior to transplantation. Clinically, it has been shown to achieve this by the use of a hyperosmolar oncotic perfusate to promote flow through the lung vasculature without the development of pulmonary oedema, a leukocyte filter to filter and remove donor-derived cells including clots and leukocytes that could potentiate an inflammatory response, and a ventilator to facilitate recruitment manoeuvres and eliminate atelectasis (66). Additionally, the circuit can also provide a platform to deliver therapeutic agents, specifically anti-microbials to reduce the bacterial load prior to lung transplantation (369),
methylprednisolone to reduce inflammation and maintain endothelial function (308, 328), and heparin to reduce clot formation (328).

It has already been discussed that following reperfusion of donor lungs after a period of ischaemia, there is an inflammatory response within the lungs that can result in endothelial dysfunction and ALI (122-124). The controlled and gradual nature of reperfusion during EVLP has been shown to adequately reperfuse the pulmonary vasculature without the formation of pulmonary oedema (66, 327, 338). More recently, the cellular and molecular processes that occur during EVLP have been explored to fully understand the potential of EVLP. Indeed Cypel et al demonstrated that EVLP prevents on-going damage to the alveolar epithelial tight junctions following a period of ischaemia secondary to preservation of metabolic pathways and protein synthesis (340).

In a human model of EVLP whereby marginal lungs underwent successful reconditioning, there were no histological features of ALI however an up-regulation of IL-6, IL-8, g-CSF and MCP-1 was observed in the lung tissue (370). This is interesting given that these cytokines have been implicated in the development of ALI and IRI (46, 47, 122, 123, 140). Perhaps mild elevation in these cytokines can be tolerated and the EVLP circuit provides a platform to wash them out of the lung. Furthermore, a non-specific inflammatory cytokine storm during EVLP has also been observed in porcine models of successful EVLP transplant (371).

More recently, Andreasson et al have analysed the cytokine profiles in perfusate samples during successful and unsuccessful reconditioning of marginal donors using EVLP, to determine whether there is a correlation between cytokine expression and graft function (372, 373). Higher levels of IL-8 and IL-1β were found in the perfusate of lungs that did not successfully recondition, and in those that did, IL-1β levels negatively correlated with post-transplant oxygenation within the first 24 hours (372). They have also demonstrated that the levels of IL-1β and TNF-α within the first 30 minutes of EVLP, in lungs that were successfully reconditioned and transplanted, correlated with hospital survival (373). IL-1β therefore consistently appears to be an important marker of successful EVLP. Indeed, increased levels of IL-1β have been associated with an up-regulation of ICAM-1 in the lung vasculature, and in vitro it has been shown to increase neutrophil adhesion (373). It is therefore apparent that during EVLP, there may be activation of immune pathways that can have a detrimental effect upon graft function and IL-1β signalling appears to be an important aspect of this process.

The attenuation of the donor immune response during EVLP could also have an impact upon the incidence of acute rejection post-transplantation. Clinically to date, EVLP reconditioning has not been shown to reduce the incidence of acute rejection (374), however further interrogation of the EVLP process has demonstrated its potential to. In a human model of EVLP, Stone et al revealed that non-classical monocytes rapidly diapedese from the donor lung within 30 minutes of perfusion and form 80% of the donor leukocyte population (375). On analysis in vitro, these cells readily differentiate into dendritic cells and therefore their removal during EVLP can
potentially reduce allore cognition in the recipient upon transplantation (375). Further work by Stone et al, using a porcine model of EVLP transplant, confirmed the rapid diapedesis of cells from the donor lung upon reperfusion within the recipient and demonstrated that prior EVLP reduced that transfer (371). Furthermore, the reduced leukocyte transfer that was observed following EVLP was associated with reduced T cell infiltration and acute rejection within the recipient (371). Perhaps therefore, EVLP should not be used as a tool to recondition only marginal donor lungs but all donor lungs, whereby the true benefit of reduced leukocyte transfer can be observed.

The immune profile of the donor lung is therefore essential to graft function, and it has been demonstrated that through manipulation on the EVLP circuit and delivery of therapeutic agents, there can be further attenuation of the inflammatory response. A porcine study of EVLP previously demonstrated that the addition of an absorbent membrane into circuit was associated with reduced levels of TNF-α and IL-8, however this did not correlate with any improvement in graft function (376). More recently however, Iskender et al have demonstrated in another porcine model of EVLP that use of a similar device was associated with a reduced level of circulating cytokines and this correlated with improved pulmonary haemodynamics and airway mechanics (377). Furthermore, there was less pulmonary oedema and electrolyte imbalance with reduced anaerobic glycolysis and MPO activity (377).

In murine models of EVLP, the delivery of an adenosine A2A receptor agonist into the circuit has been shown to attenuate the inflammatory profile of the lung with reduced levels of pro-inflammatory cytokines, including IL-17, correlating with improved graft function (157, 158, 378). EVLP delivery of the anti-inflammatory cytokine IL-10 using viral vectors, has also been shown to reduce IRI following transplantation via an attenuated immune response (379), specifically via reduction in IL-17 signalling (380). There has also been increased interest in the use of MSCs over recent years, with positive results observed upon EVLP administration (196, 197).

The importance of the donor immune compartment prior to transplantation, and its role in the development of IRI, PGD and rejection has been recognized. Having initially been discovered as a platform to facilitate optimal lung evaluation, there is emerging evidence that EVLP may also confer long-term benefits post-transplantation, with a reduced incidence of PGD (356, 366) and potentially acute rejection (371). The ability of EVLP to attenuate the inflammatory profile of the donor lung prior to transplantation therefore has far-reaching consequences, and as a research tool it can now serve as a viable platform to fully elucidate the donor immune compartment, the effects of reperfusion and potential attenuation (381, 382).
1.5 Summary

Lung transplantation remains to be the mainstay of treatment for patients with end-stage lung disease, refractory to medical therapy. Yet, despite its evolution over the years, success remains limited by the lack of donor organs available for transplantation and a high incidence of morbidity and mortality secondary to rejection and graft dysfunction post-transplantation.

Prior to transplantation, donor lungs are subjected to the deleterious effects of donor death and subsequent ischaemia, whereby an inflammatory response is an initiated. These immunologically primed and injured lungs are susceptible to further injury upon reperfusion with the recipient circulation, whereby PGD and acute rejection can ensue. However our current assessment of the suitability of donor lungs for transplantation is not only subjective, but also objective measures such as oxygenation, may not accurately correlate with the functionality and recoverability of the lung upon transplantation. Furthermore, the donor lung compartment remains relatively ignored upon transplantation, with immunosuppressive regimes targeting the recipient immune response.

The advent of EVLP over recent years has enabled the extended evaluation of donor organs, in a stable environment outside the donor. It has therefore optimised donor organ utilisation by facilitating the use of marginal donors that have undergone successful reconditioning. Additionally, it has provided a platform to manipulate and treat the donor lung prior to transplantation through the delivery of therapeutic agents. Interestingly, there is also emerging clinical data to suggest its use is associated with a reduced incidence of PGD and rejection upon transplantation.

Reperfusion of the donor lung vasculature and the subsequent recipient immune response can drive the processes of PGD and acute rejection. Immunological characterisation of the optimal donor lung however, has yet to be established. Understanding the immediate effect of donor lung reperfusion upon the donor lung compartment and the recipient circulation may therefore identify therapeutic targets and biomarkers of graft dysfunction. Furthermore, it may also facilitate optimization of the donor lung prior to transplantation, translating into improved clinical outcomes post-transplantation.

This PhD will therefore examine the hypothesis that reperfusion of the donor lung leads to an immediate cellular migration, altering the inflammatory signalling profile of the donor and the recipient, and directly impacting upon clinical outcome. The aims will be to characterise the effects of reperfusion upon the donor lung compartment and recipient circulation, to determine whether donor leukocyte transfer can be manipulated upon reperfusion on an EVLP circuit, and to identify circulating biomarkers of graft dysfunction.
CHAPTER 2

Characterisation of the Inflammatory Signalling Profile of the Donor Lung following EVLP and Standard Transplant
2.1 Abstract

Background: PGD and acute rejection represent major caveats to successful lung transplantation. Reducing inflammation and the immunogenicity of donor lungs prior to transplantation may improve outcomes. Evidence exists that EVLP can alter the donor lung environment, although the mechanisms remain unclear. This study aimed to characterise the inflammatory signalling profile of the lung following standard and EVLP transplant and delineate the immediate impact on the recipient circulation.

Methods: Female recipient pigs (n=12) were randomised to undergo left single lung transplantation from male donors either after 2 hours of CSP or following 3 hours of EVLP. The relative expression of 44 phosphokinases and 35 apoptosis related molecules were profiled within the donor lung at 24 hours post-transplantation. Circulating levels of free genomic and mitochondrial DNA were also quantified as markers of cell death.

Results: A global profile of mitochondrial salvage and cell survival was observed in the EVLP lung tissue compared to standard transplant. This included up-regulation of AKT and PRAS40 pathways with increases in associated downstream pro-survival signalling molecules, including ERK1/2, and FAK. There was up-regulation of the anti-apoptotic proteins BCL-2, HSP-70, LIVIN and PON2 with down-regulation of apoptosis inducing mitochondrial associated molecules, including clusterin, cytochrome C and HTRA2/Omi. In the early post-operative period, there were also significantly lower levels of circulating mitochondrial DNA in pigs receiving EVLP lungs compared to standard transplant (p=0.016). Genomic DNA did not differ between groups.

Conclusion: EVLP alters the inflammatory signalling profile of the donor lung prior to transplantation, with a global cell survival and anti-apoptotic signature.
2.2 Introduction

Transplantation is the mainstay of treatment for patients with end-stage lung disease refractory to medical therapy, yet its median survival remains limited due to the high incidence of PGD and allograft rejection (6). Inflammation within the donor, cold ischaemic time and IRI, have all been implicated in the development of PGD and allograft rejection (12, 123, 172). Upon death, a catecholamine and cytokine storm occurs within the donor that results in an inflammatory cell infiltration in the lungs, with subsequent up-regulation of MHCs (15). The lungs then undergo a preservation flush and are stored on ice for transport prior to implantation within the recipient, as per standard donor organ procurement. This ischaemic insult leads to the generation of ROS, with dysfunctional mitochondrial respiration that, in association with altered cellular metabolism, results in cell injury (123). The resident donor-derived leukocyte compartment is activated, secreting pro-inflammatory cytokines and chemokines. This is exacerbated upon reperfusion, with further ROS production and activation of MMPs and other proteases (125, 135). Cell death ensues via necrosis, necroptosis and apoptosis, with the release of pro-inflammatory DAMPs (135). This cascade of events causes cellular injury within the lungs, manifesting as PGD following transplantation. Furthermore, the inflamed and immune activated environment within the donor lung promotes allograft rejection, driving recipient allorecognition through donor leukocyte activation and up-regulation of MHC class II expression on donor tissue (12, 214).

EVLP was initially developed as a technique to enable the extended evaluation of donor lungs prior to transplantation (326), however it has since been used as a reconditioning tool for marginal donors (327). The physiological preservation of donor lungs using EVLP may reduce the impact of IRI, and has been shown to be associated with a reduced incidence of PGD post-transplantation (344, 356). Furthermore, it has been demonstrated that EVLP can alter the donor immune compartment via reduced passenger leukocyte transfer, thereby reducing recipient T cell recruitment following transplantation (371). However the effect of reperfusion and EVLP on the cellular and molecular pathways within the donor lung remains poorly understood.

A porcine model of EVLP transplant was therefore used, to characterise the inflammatory signalling profile of the donor lung following standard and EVLP transplantation, and delineate the immediate effect within the recipient circulation.
2.3 Methods

2.3.1 Ethical Approval

The ethics board at the University of Lund approved this study. All animals were treated in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the “Principles of Laboratory Animal Care” developed by the National Society for Medical Research.

2.3.2 Animal Model

Twenty-four Swedish outdoor free-range domestic pigs (n=12 male donors versus n=12 female recipients) with a mean weight of 63kg +/- 2kg were used in this experiment. All pigs were sedated and anaesthetised as previously described in the literature (66). Briefly, the pigs were sedated, using intramuscular ketamine (Ketalor, Parke-Davis, Morris Plains, NJ) and Xylasin® (Rompun, Bayer, Gothenburg, Sweden), and placed in the supine position. Induction anaesthesia was then achieved using intravenous sodium thiopental (Pentothal; Abbot, North Chicago, IL) and atropine (Atropine; Kabi Pharmacia, Uppsala, Sweden). Following this, intravenous pancuronium bromide (Pavulon, Organon Teknika, Boxel, The Netherlands) was given and a tracheostomy (Portex® #8, Hythe, Kent, England) was performed. The tracheal tube was connected to a Siemens Servo ventilator 300 (Siemens – Elema AB, Solna, Sweden) and volume and pressure controlled ventilation was commenced at 10L/min with a respiratory rate (RR) of 18 breaths per minute, PEEP of 8cm H₂O, a maximum inspiratory pressure of 30cm H₂O, and a FiO₂ of 0.5. Maintenance anaesthesia was achieved by a continuous infusion of ketamine, midazolam (Dormicum, Rochel, Basel, Switzerland) and pancuronium, in 500ml 5% dextrose. The PA was instrumented with a Swan-Ganz catheter and baseline pulmonary haemodynamics were assessed. Additionally, blood gases were recorded.

2.3.3 Donor Organ Procurement

Male donors (n=12) underwent median sternotomy and ventricular fibrillation was induced following electrical stimulation. The ventilator was stopped and the lungs were permitted to collapse. The heart-lung block was then explanted, using the technique previously described (321). Briefly, the PA and aortic arch were cannulated with distal ligation of the brachiocephalic and left subclavian arteries. The superior and inferior vena cavae were ligated, and the ascending and descending aorta were clamped. A small cut was made in the right atrium and the LA appendage. The PA was then perfused with 2L of 4°C Perfadex® (XVIVO Perfusion AB, Gothenburg, Sweden) and the heart-lung block was excised. The left lung was harvested at the level of the carina and the orifices of the left PVs. The lung was then submerged in cold (6-8°C) Perfadex® for a period of 2 hours. The lungs were then randomly assigned to either standard (n=6) or EVLP (n=6) groups. The standard group was transplanted immediately following CSP.
In the EVLP group, the lungs underwent CSP followed by 3 hours of EVLP prior to transplantation.

### 2.3.4 Ex-Vivo Lung Perfusion

EVLP was performed for 3 hours on the six donor lungs selected. The technique followed the previously described Steen protocol (66), using the Vivoline® LS1 system (Vivoline® Medical AB, Lund, Sweden). Briefly, following removal from Perfadex®, the lung was inspected and any visible clots in the PA and LA were removed. The PA was then cannulated with a pressure probe attached. The LA appendage was left open and a temperature probe was sutured between the PV orifices. The trachea was cleared of secretions and a size 9mm ET tube was inserted. The lung was then placed in the organ perfusion chamber, and the PA cannula was connected to the circuit. Following the removal of air, the lungs underwent controlled cellular reperfusion via the PA, with gradual re-warming and protective volume controlled ventilation. Flow through the PA was initially commenced at 100ml/minute, which then gradually increased as the temperature of the perfusion fluid exiting the LA gradually increased. The PA pressure never exceeded 20mmHg. When the temperature reached 32°C, volume controlled ventilation was commenced, initially at 1L/min with a FiO₂ of 0.5. Full ventilation was achieved at 37°C with a ventilator minute volume (MV) less than 2.5 x the perfusion flow, and a PEEP of 5-8cmH₂O. At full ventilation, recruitment manoeuvres were performed by temporarily increasing the PEEP to reduce lung atelectasis. After 3 hours, perfusion flow and ventilation were reduced whilst the lung underwent core cooling to 20°C. Perfusion and ventilation were then discontinued, and the PA cannula and ET tube were removed, with the lung clamped in a semi-inflated state. The lung remained immersed in the perfusion chamber with topical cooling until transplantation.

### 2.3.5 Left Single Lung Transplantation

Female recipient pigs (n=12) were randomised to receive a left single lung transplantation using either standard or EVLP lungs. All transplantations were male to female to determine the presence of donor-derived DNA via detection of the Y chromosome. The female pigs were anaesthetized and sedated as outlined above. Left lung transplantation was performed, followed by right pneumonectomy within 1 hour using the technique previously described (321). Briefly, the internal jugular veins and aorta were cannulated and a right thoracotomy was performed. The pulmonary ligaments were cut with diathermy and the hilum was accessed via the pericardium. A left thoracotomy was then performed. Near the level of the carina, the left main bronchus was occluded, a vascular clamp was placed on the proximal PA and another clamp was placed intra-pericardially across the LA, incorporating the left pulmonary vein stumps. A left pneumonectomy was then performed and the left donor lung implanted. The bronchial anastomosis was initially formed, with a running 4/0 Prolene® (Ethicon, Somerville, NJ) suture. An atrial cuff was formed and the atrial anastomosis was sutured with a running 6/0 Prolene® suture. The bronchus was then unclamped and ventilation commenced, following which the PA and then the LA clamps were removed. A pressure probe was placed in the LA and a flow
probe was placed around the PA. Once blood flow through the transplanted lung was stable, the
left thoracotomy was closed and the pigs were placed with their right side up. A right
pneumonectomy was then performed using a TA™-90 surgical stapler (United States Surgical
Corp, Norwalk, CT), via the right thoracotomy, which was subsequently closed. The pig was
then turned with their left side up and ventilation and fluid resuscitation were maintained for 24
hours. At 24 hours, the pigs were sacrificed following a final assessment of pulmonary
haemodynamics and gas exchange.

![Schematic Diagram of the Porcine EVLP Transplant Model](image)

**Figure 2.1: Schematic Diagram of the Porcine EVLP Transplant Model**

*n=12* left single lungs were explanted from male pigs, as per standard donor organ procurement
guidelines, and underwent 2hrs of CSP. The lungs were then randomised into two groups and
transplanted into *n=12* female recipients either immediately (*n=6*), or following 3 hours of EVLP
(*n=6*).

### 2.3.6 Sample Collection and Storage

Following transplantation, 20ml samples of peripheral blood were taken at sequential time
points – 0 hours, 6 hours, 12 hours and 24 hours. At 24 hours, lung tissue biopsy samples were
taken. Following venesection, all peripheral blood samples were centrifuged at 500g for 10
minutes at 4°C and plasma supernatant aliquots were stored at -80°C. The lung biopsy samples
taken at 24hrs post-transplant were snap-frozen in liquid nitrogen and also stored at -80°C.

### 2.3.7 Proteomic Profiling

A human phosphokinase proteome profiler and a human apoptosis antibody array kit (R&D
Systems, Minneapolis, USA) were used to examine the relative expression of 35 apoptosis-
related proteins and the phosphorylation of 43 kinases in the transplanted lung tissue at 24
hours. Using a BLAST search, homology of over 90% was determined between porcine and
human proteins, and therefore cross-reactivity was predicted in 29 of the proteins.
Proteomic profiling was performed according to manufacturer’s guidelines and using the reagents provided. Briefly, the porcine lung tissue was thawed and then homogenised in lysis buffer, to create a lung tissue lysate. Adjustment of the final volume was achieved through the addition of array buffer.

Array buffer was added to the wells of the multi-dish being used following which, the membranes were added for each sample and the multi-dish was incubated for 1-hour on a rocking platform shaker. The array buffer serves as a block buffer. Array buffer was then aspirated from the wells of the multi-dish and the tissue lysate samples were added to the membranes. Following incubation overnight on a rocking platform at 2-8°C, the membranes were carefully removed from the multi-dish and washed 3 times in wash buffer. Diluted detection antibodies were added to the wells of the multi-dish and the corresponding membranes, following careful removal from its wash container, were returned to multi-dish. There was a further 2-hour incubation at room temperature on a rocking platform. The membranes were then carefully removed once again and washed in wash buffer for a further three washes. Dilute Streptavidin-HRP was then added to each well of the multi-dish and again, the corresponding membranes were returned to the multi-dish wells. The multi-dish was incubated on a rocking platform for 30-minutes at room temperature. The membranes were then removed and again washed in wash buffer 3 times. The membranes were then placed on the plastic sheet protector and Chemi-Reagent mix was pipetted evenly onto each set of membranes to ensure complete coverage. Excess Chemi-Reagent mix was removed, and the membranes were covered with plastic wrap, extending around the underlying plastic sheet. The covered membranes were then placed in an autoradiography film cassette and exposed to X-ray film. Chemilluminescence was used to visualise the membranes with a Bio-Rad ChemiDoc™ MP imaging system (Bio Rad Hertfordshire, UK). Pixel density analysis was then performed using ImageLab™ software, and normalised as per the manufacturer’s guidelines. Differential expression was only accepted when there was more than a 10% change in pixel density between the two groups.

2.3.8 Quantitative PCR

Quantitative PCR (qPCR) was performed to determine the amount of cell-free genomic DNA (gDNA) and mitochondrial DNA (mtDNA) present in the plasma samples collected during the first 24 hours post-transplant.

**Plasma DNA extraction**

All plasma samples from the recipient pigs, collected at the four time-points post-transplantation, were thawed. DNA was then extracted using the QIAamp® DNA Mini and Blood Mini Kit (QIAGEN, Manchester, UK), according to the manufacturer’s protocol and using the materials provided. Briefly, each plasma sample was added to a microcentrifuge tube containing QIAGEN protease. Buffer AL was then added to each sample and the tube pulse-vortexed for 15 seconds. The samples were then incubated at 56°C for 10 minutes. Following incubation, ethanol was added to the samples, which were then mixed again by pulse-vortexing for 15
seconds. Each sample mixture was added to separate QIAamp® Mini spin columns with collection tubes and centrifuged at 6000g for 1 minute. The QIAamp® Mini spin columns were then placed into new collection tubes, and the old collection tubes containing filtrate were discarded. Buffer AW1 was then added to the QIAamp® Mini spin columns and centrifuged for 6000g for 1 minute. Again, the QIAamp® Mini spin columns were placed into clean collection tubes, and the old tubes discarded. Buffer AW2 was then added to the QIAamp® Mini spin columns and centrifuged at 20,000g for 3 minutes. The QIAamp® Mini spin columns were placed into clean and labelled microcentrifuge tubes, and the collection tubes containing the filtrate were again discarded. Buffer AE was added to the QIAamp® Mini spin columns and incubated at room temperature for 1 minute, and then centrifuged at 6000g for 1 minute. The QIAamp® Mini spin columns were then discarded, leaving the extracted DNA samples in the labelled microcentrifuge tubes. If the samples were not analysed immediately, they were stored at -20°C.

**Primer Selection**

To identify DNA, primers were designed using the Primer Express™ Software v3.0.1 (Life Technologies, Paisley, UK), and their homology to other genes was then assessed using BLAST. Primer sets were synthesized and de-salted by Sigma Aldrich, and prior to use were re-suspended in nuclease-free water (Ambion, USA) to adjust their concentration to 150nM.

To identify cell-free gDNA, forward and reverse primers to the housekeeping gene, glyceraldehyde-3-phosphate (GAPDH) were used:

- **GAPDH Forward**
  5’ TGCTCCTCCCGTTGCA

- **GAPDH Reverse**
  5’ GGCTTACCTGGAATGCA

To identify the Y chromosome, to determine whether the gDNA was donor or recipient-derived, primers specific for the sex-determining-Y (SRY) region were used:

- **SRY Forward**
  5’ CAAGTGGCTGGATGCAAGT

- **SRY Reverse**
  5’ TCGAAGATTTCGCGGCTTTT

To identify cell-free mtDNA, forward and reverse primers specific to Cytochrome B were used:

- **Cytochrome B Forward**
  5’ ACACATCAGACACAACAACA

- **Cytochrome B Reverse**
  5’ GTAGCGAATAACTCATCCGTA

**Standard Curve**

To enable quantification of DNA in the unknown samples, standard curves were created comprising of known gDNA and mtDNA concentrations. The standard curves were also used to determine the efficiency of the primers to detect their target DNA. Stock concentrations of both
gDNA and mtDNA were therefore extracted from porcine blood and tissue, respectively. For mtDNA, intact mitochondria were initially isolated from tissue prior to DNA extraction.

**Mitochondrial Isolation**
Mitochondria were isolated from porcine lung tissue using a mitochondrial isolation kit (ScienCell™, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, porcine lung tissue was thawed and 0.1g weighed. The weighed tissue was then washed twice with ice-cold phosphate buffered saline (PBS) (ScienCell™, Carlsbad, CA). Mitochondrial isolation buffer was added to the sample, following which homogenization was achieved using a Polytron tissue disruptor. The homogenate was then added to an Eppendorf tube and centrifuged at 1000g for 5 minutes at 4°C. The resultant supernatant was transferred to a new Eppendorf and centrifuged at 10,000g for 20 minutes at 4°C. The supernatant was discarded and the mitochondrial pellet was then re-suspended in mitochondrial isolation buffer and placed on ice prior to DNA extraction.

DNA was extracted from the re-suspended mitochondrial pellet and the porcine blood sample as outlined above. Following extraction, the stock DNA concentration was quantified using Nanodrop™ 2000 (Thermofisher Scientific, Waltham, MA, USA), from which serial dilutions were performed to achieve a relative standard curve consisting of 25ng/µl, 10ng/µl, 5ng/µl, 1ng/µl and 0.5ng/µl.

**Quantitative PCR**
All of the standard curve concentrations and the extracted plasma DNA samples were then prepared for qPCR. Each standard curve and plasma DNA sample was mixed with Power SYBR™ green PCR master mix (Life Technologies, Paisley, UK), nuclease-free water, forward primer and reverse primer. Each mixture was then split between 4 individual wells (20µl per well) of a MicroAmp™ Optical 384-Well Reaction Plate, to produce quadruplet samples. An optical adhesive film was then used to seal the plate and following a brief centrifuge at 500g for 2 minutes, qPCR was performed using a QuantStudio™ 12K Flex System. The thermal profile used was 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and finally 60°C for 1 minute. The fluorescence of each well was read and then a melt curve from 60°C to 95°C was created for analysis. The number of signals required for the fluorescent signal to cross the automated threshold was calculated and given as a C_T value.

If there was amplification in the blank sample or if there were multiple peaks in the melt curve, results were discarded and the plate was repeated, as this would indicate primer dimer formation. The standard curve was then analysed to determine the efficiency of the PCR amplifications, by creating a semi-log regression line plot. A slope of -3.32 indicates 100% efficiency with an R^2 value of 1. Regression line plots with R^2 values > 0.95 were accepted.

Using the standard curve calculation and the sample C_T values, DNA was then quantified for each sample. If the C_T value was > 40, this was indicative of minimal amounts of target nucleic
acid being present in the sample, therefore levels could not be quantified and cell-free DNA was deemed undetectable.

2.3.9 Statistical Analysis

Statistical analysis was performed using SPSS® v.22.0. Data normality was determined by using the Shapiro-Wilk test, and normally distributed data was expressed as mean ± standard deviation (SD). For direct comparisons between EVLP and standard samples obtained at a single time-point, either independent samples T-test or the Mann Whitney U test were used, dependent on data distribution. GraphPad® Prism v.7.0 was utilised to formulate graphs.
2.4 Results

2.4.1 Proteome Profile

A total of 29 phosphokinases were analysed, and the expression of 19 altered by over 10% between the EVLP and standard transplant lung tissue (Figure 2.2). There was a general up-regulation of protein expression observed in the EVLP transplanted lung tissue, with a differential expression of over 20% in AMPKalpha1 (43.89%), Hck (35.71%), Fgr (33.73%), PDGFRbeta (29.64%), STAT5a/b (27.39%), Lck (23.92%), STAT6 (22.01%), Fak (21.76%) and ERK1/2 (20.39%).

In addition, 34 out of 35 proteins associated with cell death and survival, were detected in the lung tissue samples (Figure 2.3). Within the EVLP transplanted tissue, there was a marked down-regulation of clusterin (28.98%), cytochrome C (19.54%) and BCLx (14.86%), and to a lesser degree BAX (11.23%), and HTRA2/OMI (11.05%). In addition, there was a marked up-regulation of HSP70 (23.17%), Pro-Caspase 3 (17.08%), Trail R1/DR4 (15.64%), Trail R2/DR5 (12.62%) and BCL2 (11.08%).
The percentage change in expression of apoptosis-related proteins in EVLP transplanted lung tissue compared to standard transplantation.

2.4.2 Quantitative PCR

There was no statistical difference in the circulating levels of cell-free gDNA at all time-points post-transplant, between pigs transplanted with EVLP re-conditioned lungs and those receiving standard lungs (Figure 2.4).

Figure 2. 3: Expression of Apoptosis Related Proteins in Transplanted Lung Tissue
The percentage change in expression of apoptosis-related proteins in EVLP transplanted lung tissue compared to standard transplantation.

Figure 2. 4: Concentration of Cell-Free gDNA Following Lung Transplant
Circulating concentration of cell-free gDNA in the first 24 hours following EVLP and standard transplantation.
Additionally, no Y chromosome was detected in the DNA. There was less circulating cell-free mtDNA within the first 24 hours in pigs receiving EVLP re-conditioned lungs than those receiving standard lungs (Figure 2.5). This was only significant ($p=0.016$) however, in the early post-operative period (0 hours – within minutes of cross-clamp removal and reperfusion) (Figure 2.5). After this time-point, in the EVLP group, concentrations peaked at 6 hours before declining over time. Conversely, in the standard group, concentrations steadily increased in the first 12 hours post-transplant before rapidly declining to levels similar to the EVLP group (Figure 2.5).

**Figure 2.5: Concentration of Cell-free mtDNA Following Lung Transplantation**

Circulating concentration of cell-free mtDNA in the first 24 hours following EVLP and standard transplantation.
The levels of circulating cell-free mtDNA in the peripheral blood were statistically higher than the levels of cell-free gDNA at all time-points, following both EVLP (Figure 2.6) and standard transplant (Figure 2.7).

**Figure 2.6: Concentration of mtDNA and gDNA Following EVLP Transplant**
Comparison of circulating levels of cell-free mtDNA and gDNA in the circulation 24 hours post EVLP transplantation

**Figure 2.7: Concentration of mtDNA and gDNA Following Standard Transplant**
Comparison of circulating levels of cell-free mtDNA and gDNA in the circulation 24 hours post standard transplantation


2.5 Discussion

Lung transplantation remains limited by the high incidence of morbidity and mortality secondary to rejection and graft dysfunction post-transplantation (6). Donor lungs are exposed to a myriad of pathophysiological processes that occur upon donor death and during ischaemic storage, with resultant activation of inflammatory cascades (13, 123). They are then transplanted in this immune activated state, where upon reperfusion within the recipient, it can drive further inflammatory infiltration with subsequent graft dysfunction (123). In addition to facilitating the extended evaluation of donor lungs (66), EVLP can also be used to recondition sub-optimal donor lungs and potentially reduce graft inflammation prior to transplantation, ameliorating graft dysfunction post-transplantation (327, 344, 356). However, the cellular and molecular mechanisms that underpin this process remain poorly understood.

Using the porcine transplant model, it has been identified that following 24 hours of transplantation with EVLP lungs, there is a universal up-regulation of protein expression within the lung tissue, suggesting a complex interaction between wide ranging pathways involved in cell signalling. Such universal up-regulation is not seen within the standard transplanted lung tissue. Furthermore, the expression of proteins involved in cell death is notably different following EVLP and standard transplantation.

Apoptosis is a programmed form of cell death that can occur via the intrinsic or extrinsic pathways, both of which converge on the common execution pathway (383). Pathway initiation and subsequent down-stream progression is dependent upon interaction with various signalling proteins (Figure 2.8).
Figure 2.8: The Pathways of Apoptosis

Diagrammatic representation of the pathways involved in cellular apoptosis, whereby the black arrows indicate activation and the red blunted lines indicate inhibition. IAPs = inhibitors of apoptosis. The intrinsic pathway is mediated via the mitochondria, which upon opening of the MPTP, stimulates the release of cytochrome C, SMAC/Diablo and HtrA2/Omi. Cytochrome C stimulates the formation of an apoptosome, which then cleaves caspase 9 to activate the common pathway, via cleavage of caspase 3. The extrinsic pathway is mediated by transmembrane death receptors (DRs); Fas receptor (FasR), TNF receptor 1 (TNFR), DR3 and DR4. Upon activation by their specific ligands – Fas ligand (FasL), TNFα, Apo3 ligand, and Apo2 ligand respectively – there is recruitment and binding of the cytoplasmic death receptor adapter proteins, FADD, RIP and TRADD, and pro-caspase 8, to form the death inducing signalling complex (DISC), leading to cleavage of caspase 8, which then activates the common pathway, via cleavage of caspase 3. In the common execution pathway, activated caspases 3, 6 and 7 act on the nucleus to trigger cell death via apoptosis (383).

In the EVLP transplanted lung tissue, AKT and PRAS40 were up-regulated, both of which regulate processes involved in cellular proliferation and survival, and inhibit apoptosis. AKT inhibits the pro-apoptotic Bcl-2 proteins, Bax and Bak, and along with PRAS40, Bad, thereby inhibiting the activation of apoptotic initiator proteins (384). In addition, through downstream binding and phosphorylation of FoxO, AKT inhibits the activity of FoxO transcription factors that normally induce the expression of the pro-apoptotic Bcl-2 family proteins and death receptor ligands (384).

Other pro-survival signalling proteins were up-regulated in EVLP transplanted tissue including, ERK1/2, FAK, PDGFRβ, STAT5/6 and members of the Src family of protein tyrosine kinases.
Fgr, Hck and Lck. This broad group of proteins promotes cellular proliferation through increased protein transcription within the nucleus. An up-regulation of AMPK, EGFR and JNK was also observed. EGFR can inhibit AKT activity and therefore promote apoptosis. AMPK and JNK can be involved in both pro-survival and pro-apoptotic signalling pathways. Therefore although AMPK can also inhibit AKT activity and JNK can induce the transcription of pro-apoptotic genes including p53 and the cleavage of Bid, thereby promoting apoptosis, they can also signal the transcription of proteins involved in cell survival and proliferation (385). Given that there was an up-regulation of proteins directly associated with cell survival signalling pathways, it would suggest they have a pro-survival, rather than pro-apoptotic role, with the pro-apoptotic activity appearing to be counter-balanced by the increased expression of pro-survival proteins.

Interestingly, in the EVLP transplanted tissue, there was also an increased expression of death receptors (DR) 4 and 5, which drive extrinsic apoptosis pathway activation. There was however, a concomitant decrease in the intrinsic pathway initiator proteins downstream of DR4 and DR5, including cytchrome C, HtrA2/Omi and, to a lesser degree, SMAC/Diablo. The decrease in initiator proteins correlate with increases in the anti-apoptotic proteins Bcl-2 and PON2, that inhibit their release, and a down-regulation of Bax, which normally promotes the opening of the MPTP with subsequent initiator protein release (386). Furthermore, there was an increase in the expression of pro-caspase 3 in EVLP lung tissue, but no increase in its active form, caspase 3. This may be due to inhibition of Apaf-1, by HSP and LIVIN, which prevent downstream activation to caspase 3. Additionally there was a down-regulation of clusterin, which is associated with the clearance of cellular debris and apoptosis. This would suggest increased downstream pro-survival signalling and a reduction in caspase 3 mediated cell death. Therefore collectively this proteomic profile is indicative of mitochondrial salvage and cell survival in the EVLP transplanted lung tissue, following 24 hours of transplantation (Figure 2.9).
Figure 2.9: The Activation and Inhibition of Apoptotic and Cell Survival Signalling Pathways
The picture demonstrates how various proteins can act upon the pathways of apoptosis, to be inhibitory, represented again by a blunted red line, or stimulatory, represented by a black arrow. In addition, pro-survival pathways and their associated proteins, that counteract the effects of apoptosis, are highlighted. The proteomic data demonstrates an up-regulation and down-regulation of various proteins in the EVLP transplanted tissue, that are either directly involved in the pathways of apoptosis, or that can indirectly augment or inhibit them. In the picture, the proteins that are up-regulated are highlighted, and those that are down-regulated are surrounded by red, dashed lines.

Circulating levels of cell-free gDNA and mtDNA were then quantified, as surrogate markers of cell death, in the recipient circulation post-transplantation. gDNA was selected not only as a marker of cell death but also to distinguish between donor (♂) and recipient (♀) via identification of Y chromosomal products. mtDNA was selected as a marker of cell membrane rupture and therefore necrosis. Surprisingly however, there was no difference in cell-free gDNA in the recipient circulation of both groups post-transplant. Furthermore there was no detectable Y chromosome, demonstrating that the cell-free gDNA that was present was not donor-derived but of recipient origin. However, immediately following reperfusion of the donor lung, the EVLP group had significantly lower levels of circulating cell-free mtDNA. In addition, the level of cell-free mtDNA in the circulation was significantly greater than cell-free gDNA at all time-points in the first 24 hours post-transplant in both groups. This is interesting given that cell-free mtDNA is a powerful immune activator that can act as a DAMP (387). As mtDNA is of bacterial origin, it is high in unmethylated CpG repeats and can therefore provoke similar responses to bacterial...
DNA. Free intracellular fragments can autonomously activate highly specific endosomal TLRs and initiate an amplified immune response, with activation of MAPKs, the release of pro-inflammatory cytokines, and subsequent neutrophil recruitment and degranulation (387, 388). Additionally, cell-free mtDNA can act as a second-messenger molecule, activating the inflammasome, with downstream activation of IL-1β and IL-18 (385, 388). In the context of transplantation, increased inflammatory signalling will lead to augmentation of the recipient immune system as well as activation of passenger donor immune cells. The subsequent inflammatory infiltration within the graft can then promote graft dysfunction and increased immunogenicity.

High levels of circulating cell-free mtDNA are associated with increased ICU mortality, poor clinical outcome following trauma, and increased severity of sepsis (387, 389). Therefore, if EVLP is associated with reduced levels of circulating cell-free mtDNA following transplantation, this may attenuate the recipient immune response and improve clinical outcome. The cause of increased cell-free mtDNA post-transplantation however, remains unknown, and the mechanism by which it is released from cells remains poorly understood, clearly warranting further investigation.

In this porcine transplant model, it has been demonstrated that EVLP alters the signalling profile of the donor lung following transplantation, up-regulating cell survival signalling pathways, and down-regulating mitochondria associated cell death molecules. Furthermore, EVLP significantly reduces cell-free mtDNA within the peripheral circulation, immediately following reperfusion. Collectively this data indicates that EVLP appears to exert beneficial effects on the donor lung. If EVLP can reduce inflammation and immunogenicity of the donor lung prior to transplantation, it may have the potential to reduce graft dysfunction and allograft rejection post-transplantation and therefore improve long-term clinical outcomes.
CHAPTER 3

Characterisation of the Immediate Inflammatory Response of the Donor Lung Upon A Secondary Preservation Flush
3.1 Abstract

**Background:** Allograft rejection limits long-term survival following lung transplantation. The driving force behind allorecognition and subsequent rejection is the presentation of donor leukocytes to the recipient circulation. Despite this, the immediate passenger leukocyte transfer from the donor lung upon revascularisation has not yet been fully explored. The immune content that initially migrates from the donor lung upon reperfusion with a secondary flush, following cold preservation, was therefore characterised.

**Methods:** A 1L post-preservation flush was performed in n=6 porcine lungs following donor organ procurement and CSP. The first 500ml effluent from the LA was collected. Flow cytometry, Luminex® analysis and qPCR were then used to quantify cell populations, cytokine concentrations and cell-free DNA, respectively.

**Results:** A total of 1.47x10^9 (±3.4x10^8) viable leukocytes were identified within the first 500ml of effluent. T cells were the dominant cell population, representing 69% of the total mobilised leukocytes, of which <0.01% were regulatory T cells. IL-18 was the most abundant cytokine, with a mean total quantity of 21634(±9949)pg within the 500ml venous effluent. In addition, there was a mean total quantity of 8130(±4124)ng of cell-free mtDNA within the 500ml effluent.

**Conclusion:** Following a 1L post-preservation flush, there was an abundance of leukocytes and cytokines along with damage-associated molecular patterns within the initial 500ml effluent. Such a pro-inflammatory donor load can enhance alloantigen presentation and drive the recipient alloimmune response. A secondary, post-preservation flush may therefore be an effective method for reducing the immune burden of the donor lung prior to transplantation.
3.2 Introduction

Lung transplantation is associated with a high incidence of acute and chronic allograft rejection, occurring in up-to 30% of patients within the first year post-transplantation - the highest rate in comparison to other solid organ transplants (6, 8, 209). Allorecognition is fundamental to graft rejection, and represents the process whereby donor antigens are recognised by recipient T cells as foreign. This occurs via three main pathways, the direct, semi-direct and indirect pathway (204). The direct pathway requires presentation of intact MHC alloantigens on donor-derived APCs to recipient T cells. The semi-direct pathway refers to the transfer of donor MHC peptides to recipient APCs, which then present allopeptides to recipient CD4+ and CD8+ T cells (231). Conversely, in the indirect pathway, MHC alloantigen presentation is via recipient APCs, following internal processing (216). Although all presentation pathways activate the recipient immune system to initiate an alloimmune response, it is the direct pathway that is thought to drive allorecognition and acute graft rejection in the early post-operative period, and can occur independently of indirect allorecognition (214).

Passenger leukocytes are critical for activation of this recipient alloimmune response and their removal prevents rejection (218). Despite this, current immunosuppressive therapies target the recipient immune system and the effector T cell response, with no focus on the donor immune compartment (390). This is of particular relevance within lung transplantation given that the lung has its own intrinsic immune compartment with bronchus associated lymphoid tissue consisting of lymphocytes and professional APCs including macrophages and dendritic cells that highly express MHC (221). Following donor death, the systemic catecholamine and cytokine storm that ensues, results in an inflammatory cell infiltration within the lung and a subsequent up-regulation of MHC expression (12, 13). It is in this inflamed and immunologically activated state that the lungs are then transplanted into the recipient, whereby upon revascularisation the donor immune compartment drives allorecognition which ultimately leads to graft infiltration and destruction.

The donor immune compartment within the lung is therefore critical to the development of rejection and graft dysfunction post-transplantation, and is a potential focus for manipulation. Our group have previously demonstrated that the lung possesses a large repertoire of leukocytes that mobilise upon revascularisation (371). In this study, removal of these cells prior to transplantation resulted in a lower incidence of acute rejection (371).

EVLP alters the inflammatory signalling profile of the donor lung following transplantation, and is associated with reduced levels of circulating cell-free mtDNA, particularly on initial reperfusion. Upon donor death and following ischaemic storage there is a marked inflammatory infiltrate within the immunologically primed donor lung. Upon transplantation and reperfusion within the recipient, the initial migration of donor cells and other inflammatory mediators will activate the recipient immune system to drive an inflammatory response, which can be detrimental to the graft. Removal of these cells prior to transplantation diminishes donor leukocyte transfer and
has the potential to reduce graft inflammation and the incidence of acute rejection via loss of direct presentation following transplantation.

Using a porcine model, the effluent following reperfusion of the donor lung after CSP was analysed to characterise the immediate migration of donor leukocytes and inflammatory mediators from the donor vasculature following donor death and ischaemic storage.
3.3 Methods

3.3.1 Donor Lung Procurement and Storage

Lungs were retrieved from n=6 Landrace pigs with a mean dry weight of 80kg. Briefly, following veterinary inspection the pigs were sacrificed via electrical stunning and exsanguination under EU Council Regulation (EC) No. 1099/2009, in keeping with Schedule 1 of the Home Office Scientific Act 1986. Following this a midline incision was performed and the lungs were mobilised and excised with the heart en bloc, at the level of the trachea and descending aorta. Once removed, the organs were immediately placed on ice and inspected for any evidence of disease or injury, which would deem them unusable. If acceptable, the trachea was clamped, the PA was identified and clamped and the PVs were identified and clamped at the level of the LA. The heart was then removed at the LA appendage, leaving an LA cuff and the PVs clamp was removed. The PA was then cannulated with a 14G PA cannula and infused with 20mls glyceryl trinitrate (Hameln, Germany-distributed by Gloucester, UK) and 10,000iU unfractionated heparin sodium (Fannin, UK). The lungs were then flushed, as per standard lung preservation with 2L of a low potassium-dextran solution at 4°C. The PA cannula and clamp were then removed, and a retrograde cannula was sequentially placed into the four main PVs. A retrograde flush of 200mls of 4°C low potassium-dextran was performed via each of the PVs. The perfusion pressure did not exceed 20cmH2O throughout the anterograde and retrograde flushes, and any visible clots were removed from the vasculature using forceps. Following preservation flush, the lungs were fully submerged in a bag of 4°C low potassium-dextran solution and placed on ice for storage and transport. The ischaemic times were documented, with the warm ischaemic time defined as the time from death until start of the preservation flush, and the cold ischaemic time as the time from preservation flush until removal from ice.

3.3.2 Secondary Preservation Flush

Following 2 hours of CSP, the lungs were taken off ice and an LA cannula was sutured onto the LA cuff using 4/0 Prolene® monofilament (Ethicon, Somerville, NJ). The LA cannula was connected to a collecting circuit with labelled measuring cylinders. The PA was re-cannulated with an XVIVO Lung Cannula Set™ (XVIVO, Gothenburg, Sweden) and connected to a giving set. 1L of room temperature STEEN Solution™ (XVIVO, Gothenburg, Sweden) was then infused via the PA under gravity at 20cm and the first 500mls of effluent from the LA was collected. STEEN Solution™ was used as a physiologically relevant cell-free plasma mimic to provide optimal colloid osmotic pressure and avoid oedema formation.

3.3.3 Sample Collection and Storage

The effluent samples were mixed thoroughly and 1ml was transferred to a Falcon™ tube, and centrifuged at 2000g for 2 minutes at 4°C. The supernatants were stored at -80°C in 250µl
 aliquots for later cytokine and DNA analysis. The cell pellets were then immediately re-
suspended in flow staining buffer for immediate flow cytometry.

3.3.4 Flow Cytometry

Leukocytes were immunophenotyped using a BD™ LSR II flow cytometer (Becton Dickinson, UK). Cell viability was determined using Zombie UV™ fixable viability dye (Biolegend, UK) and monoclonal anti-CD45+ was used as a pan-leukocyte marker. A specific antibody panel (Table 3.1) was then used to identify the following individual leukocyte populations; immature neutrophils (6D10^+2B2^−), mature neutrophils (6D10^−2B2^+), mature eosinophils and basophils (6D10^−2B2^+), total T cells (CD3^+), double positive/memory T cells (CD3^+CD4^+CD8^−), cytotoxic T cells (CD3^+CD8^+CD4^−), helper T cells (CD3^+CD8^−CD4^+), gamma-delta T cells (γδ^+), B cells (CD3^−CD21^+), natural killer (NK) cells (CD335^+), total monocytes (CD14^+), classical monocytes (CD14^+CD163^−) and non-classical monocytes (CD14^−CD163^+). In addition, the presence of regulatory T (Tregs) cells (CD3^+CD4^+CD25^+FoxP3^+) was confirmed via the expression of intracellular FoxP3 in T cells from the effluent of n=2 lungs.
Table 3.1: Antibody Cocktails to Identify Leukocyte Populations
The composition of each antibody cocktail used to determine specific leukocyte populations. PE-Cy7-conjugated anti-CD3ε, Alexa Fluor 647-conjugated anti-CD8α and PE-conjugated anti-γδ were all purchased from BD™ Bioscience (Oxford, UK), with the remaining antibodies purchased from AbD Serotec (Kidlington, UK).

<table>
<thead>
<tr>
<th>Tube</th>
<th>Antibody Cocktail</th>
<th>Cell Populations Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FITC-conjugated mouse anti-2B2</td>
<td>Immature neutrophils</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated mouse anti-6D10</td>
<td>Mature neutrophils</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7-conjugated anti-CD80</td>
<td>Mature eosinophils and basophils</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PE-Cy7-conjugated anti-CD3ε</td>
<td>Total T cells</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 647-conjugated anti-CD8α</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated mouse anti-CD4</td>
<td>Helper T cells</td>
</tr>
<tr>
<td></td>
<td>FITC-conjugated mouse anti-CD21</td>
<td>Memory T cells</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td>B cells</td>
</tr>
<tr>
<td>3</td>
<td>Alexa Fluor 488-conjugated anti-CD335</td>
<td>Gamma delta T cells</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated anti-γδ</td>
<td>NK cells</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>FITC-conjugated anti-CD14</td>
<td>Total monocytes</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated mouse anti-CD163</td>
<td>Classical monocytes</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7-conjugated anti-CD80</td>
<td>Non-classical monocytes</td>
</tr>
<tr>
<td></td>
<td>APC-conjugated anti-CD203a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td></td>
</tr>
</tbody>
</table>

Briefly, following re-suspension in flow staining buffer, the suspension was then divided into 4 separate flow tubes, labelled 1 to 4. The corresponding specific antibody cocktail was then added to each tube to identify specific cell types (Table 3.1). Following the addition of viability dye (Biolegend, UK), the tubes were incubated in the dark for 30 minutes at 4°C. Red cell lysis buffer (BD™ Biosciences, UK) was then added to each tube, and following a further 10 minute incubation in the dark at room temperature, flow-staining buffer was added to each tube and all were centrifuged at 400g for 5 minutes. The supernatants were discarded and the individual cell pellets were re-suspended in flow-staining buffer, and 123count™ eBeads (eBioscience, UK) were added to determine absolute cell counts. Samples were then analysed on the flow cytometer and gating was performed using FlowJo® v.10 software. Briefly, leukocyte populations were identified and gated as CD45+ cells and the viability of cells was confirmed. Granulocytes were identified according to their positive staining, or lack of, for 6D10 and 2B2. Total T cells were identified by their positive staining for CD3 and gated, and from this population, cytotoxic and helper T cells were identified according to their positive staining for
CD8 and CD4 respectively. B cells were gated from the CD3⁻ population if they stained positive for CD21. NK cells and gamma-delta T cells were determined by their positive staining to CD35 and γδ respectively. Monocytes were then identified by their positive staining to CD14 and gated, from which the classical and non-classical populations were determined according to their negative or positive staining to CD163, respectively. In addition, the presence of Treg cells (CD3⁺CD4⁺CD25⁺FoxP3⁺) was confirmed via the expression of intracellular FoxP3 in T cells from the effluent of n=2 lungs.

3.3.5 Luminex® Analysis

The concentration of chemokines and cytokines in the venous effluent was determined using a porcine specific magnetic bead-based multiplex assay (Merck Millipore, Billerica, MA, USA). The assay was used to quantify the concentration of 13 chemokines and cytokines – granulocyte-macrophage colony-stimulating factor (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.

The samples were thawed and then processed as per the manufacturer’s protocol, using the materials provided. Briefly, assay buffer was added to each well of the plate, and following sealing, it was placed on a plate shaker for 10 minutes at room temperature. The seal was then removed and the plate inverted to decant the buffer. Each of the standards and controls were added into their corresponding wells in duplicates. Assay buffer and serum matrix solution were added to the sample wells. Each sample was then added to their corresponding well, in duplicates. The mixed antibody-immobilised beads were then added to each well, and the plate was sealed. After being sealed and wrapped in foil, the plate was incubated overnight on a plate shaker at 4°C. Following incubation, the well contents were removed and the plate was washed 3 times. After detection antibodies were added into each well, the plate was sealed and covered with foil and incubated for a further 2 hours at room temperature on a plate shaker. The plate was then uncovered and Streptavidin-Phycocerythrin was added to each well, and the plate was then re-sealed and covered with foil prior to incubation for 30 minutes at room temperature, again on a plate shaker. Following incubation, the plate was uncovered and the well contents removed, and the plate was then washed 3 times. Sheath fluid was added to the wells and the plate was placed onto a plate shaker for 5 minutes, prior to being analysed using the Bio-Plex® 200 system (Bio Rad, Hertfordshire, UK).

3.3.6 Quantitative PCR

qPCR was used to measure the amount cell-free gDNA and mtDNA present in the 500ml effluent using the supernatant samples stored. The technique used has previously been described in detail in Chapter 2. The DNA was initially extracted from the stored plasma effluent samples, using a QIAamp® DNA Mini and Blood Mini Kit (Qiagen, Manchester, UK) as per the manufacturer’s guidelines. Briefly, the plasma samples were mixed with Buffer AL and incubated at 56°C for 10 minutes. Following a series of centrifuge and buffer wash steps,
extracted DNA was then eluted through the QIAamp® Mini spin column and collected in labelled microcentrifuge tubes. If the extracted DNA samples were not analysed immediately, they were stored at -20°C.

Primers were designed using the Primer Express™ Software v3.0.1 (Life Technologies, Paisley, UK) and their homology to other genes assessed using BLAST. To identify gDNA and mtDNA, primers to GAPDH and Cytochrome B were used respectively, as in Chapter 2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH Forward</td>
<td>5' TGCTCCTCCCCGTTCGA 3'</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>5' GGCTTTACCTGGCAATGCA 3'</td>
</tr>
<tr>
<td>Cytochrome B Forward</td>
<td>5' ACACATCAGACACAAACA 3'</td>
</tr>
<tr>
<td>Cytochrome B Reverse</td>
<td>5' GTAGCGAATAACTCATCGTAA 3'</td>
</tr>
</tbody>
</table>

Both primer sets were synthesized and de-salted by Sigma Aldrich. Prior to use, they were re-suspended in nuclease-free water (Ambion, USA) to adjust their concentration to 25nM. gDNA and mtDNA standard curve samples were then extracted from porcine blood and tissue, respectively. To extract mtDNA, mitochondria were initially isolated from porcine lung tissue as described in Chapter 2. Briefly, 0.1g of porcine lung tissue was washed in PBS, and homogenized. The suspension then underwent centrifugation at low and high speed, and the mitochondrial pellet was re-suspended in mitochondrial isolation buffer. mtDNA was then extracted from the isolated mitochondria and gDNA was extracted from porcine blood, as previously described. The concentrations were then quantified using Nanodrop™ 2000 (Thermofisher Scientific, Waltham, MA, USA), and serial dilutions were performed to achieve a relative standard curve consisting of 25ng/µl, 10ng/µl, 5ng/µl, 1ng/µl and 0.5ng/µl. The standard curve was then used to determine the efficiency of the primers to detect their target DNA and enable quantification of the DNA in the unknown samples following PCR.

qPCR was performed using a QuantStudio™12K Flex System with Power SYBR™ green PCR master mix (Life Technologies, Paisley, UK), as previously described in Chapter 2. Briefly, extracted effluent DNA samples and standard curve samples were separately mixed with Power SYBR™ green, nuclease free water and forward and reverse primers, and then added to four wells of a MicroAmp™ Optical 384-Reaction plate, in quadruplet samples. The plate was then sealed and following a brief centrifuge step, qPCR was performed, as previously described.

If there was any amplification in the blank sample or if there were multiple peaks in the melt curve, results were discarded and the plate was repeated, as this would indicate primer dimer formation. The standard curve was then analysed to determine the efficiency of the PCR amplifications, by creating a semi-log regression line plot. A slope of -3.32 indicates 100% efficiency with an R² value of 1. Regression line plots with R² values > 0.95 were accepted.

Using the standard curve calculation and the sample C_T values, DNA was then quantified for each sample. If the C_T value was > 40, this was indicative of minimal amounts of target nucleic
acid being present in the sample, therefore levels could not be quantified and cell-free DNA was deemed undetectable.

### 3.3.7 Statistical Analysis

Statistical analysis was performed using SPSS® v.22.0. Data normality was determined, and normally distributed data was expressed using mean ± SD. GraphPad® Prism v.7.0 was utilised to formulate graphs.
3.4 Results

n=6 lungs appeared healthy with no evidence of injury or lacerations. Lungs with any evidence of injury or disease were discarded and not used in this study (Figure 3.1).

![Figure 3.1: Unusable Porcine Lungs](image)

*Abattoir porcine lungs not used in the secondary preservation flush due to the presence of disease*

Ischaemic times were comparable between all lung flushes (Table 3.2). The time from death to harvest was comparable between all pigs with a mean time of 3.83 minutes (range 2-6 minutes). The mean warm ischaemic time, defined as the time of death to the start of the primary preservation flush, was 10.17 minutes (range 7-17 minutes). The mean cold ischaemic time, defined as the start of the primary preservation flush to removal of the lungs from ice and start of the secondary preservation flush, was 145.33 minutes (range 141-149 minutes).

**Table 3.2: Lung Ischaemic Times**

*Mean calculated ischaemic times for the retrieval and preservation of all porcine donor lungs, prior to the secondary preservation flush*

<table>
<thead>
<tr>
<th>Lung Flush</th>
<th>Retrieval Time (mins)</th>
<th>Warm Ischaemic Time (mins)</th>
<th>Cold Ischaemic Time (mins)</th>
<th>Total Ischaemic Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>17</td>
<td>149</td>
<td>166</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>7</td>
<td>148</td>
<td>155</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>9</td>
<td>142</td>
<td>151</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>12</td>
<td>141</td>
<td>153</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>8</td>
<td>149</td>
<td>157</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>8</td>
<td>143</td>
<td>151</td>
</tr>
</tbody>
</table>
3.4.1 Flow Cytometry

Within the first 500ml of effluent following the secondary preservation flush a total of $1.47 \times 10^9$ ($\pm 3.4 \times 10^8$) viable CD45$^+$ leukocytes were identified, with an additional $1.44 \times 10^8$ ($\pm 6.46 \times 10^7$) non-viable CD45$^+$ cells (Figure 3.2).

![Figure 3.2: Total Number of Viable and Non-Viable CD45+ Cells](image)

*Figure 3.2: Total Number of Viable and Non-Viable CD45+ Cells*

*Total number of CD45$^+$leukocytes in the 500ml LA Venous Effluent*

Of the viable CD45$^+$ leukocytes, CD3$^+$ T cells were the dominant cell population with a mean number of $1.01 \times 10^9$ ($\pm 2.82 \times 10^8$) cells, representing 69% of the total mobilised leukocytes (Figure 3.3).

---

95
Of the CD3+ T cells identified, cytotoxic T cells (CD8+CD4−) were highest in number with $3.02 \times 10^8 \pm 1.03 \times 10^8$ cells, followed by gamma-delta T cells with $2.36 \times 10^8 \pm 7.48 \times 10^7$ cells (Figure 3.4). The number of helper T cells (CD8−CD4+) and memory T cells (CD8+CD4+) were similar with $9.97 \times 10^7 \pm 3.09 \times 10^7$ cells and $1.1 \times 10^8 \pm 6.31 \times 10^7$ cells respectively (Figure 3.4). Additionally, in n=2 lungs, Tregs were identified in $<0.01\%$ of the total T cell population.
Figure 3.4: Total Number of T cell Subtypes
The total number of double positive T cells, cytotoxic T cells, helper T cells and gamma delta T cells in the 500ml LA Venous Effluent. Double positive T cells = memory T cells

Monocytes and NK cells were also identified in high numbers with $3.9 \times 10^8 (\pm 3.1 \times 10^8)$ cells and $1.64 \times 10^8 (\pm 2.99 \times 10^7)$ cells respectively (Figure 3.3). The monocytes identified were predominantly classical rather than non-classical with $2.94 \times 10^8 (\pm 2.59 \times 10^8)$ cells versus $9.63 \times 10^7 (\pm 1.09 \times 10^8)$ cells, with a significant variation in classical numbers (Figure 3.5). Granulocytes and B cells were found in comparatively lower numbers (Figure 3.3).
3.4.2 Luminex® Analysis

The mean total quantities of 13 cytokines and chemokines were determined in the 500ml venous effluents (Figure 3.6). Of the 13 tested, only 7 were consistently detectable: IL-18, IL-10, IL-1Ra, IL-1β, GM-CSF, IL-8 and TNF-α. IFN-γ, IL-1α, IL-4, and IL-12 were not detected. IL-2 and IL-6 were not detected in the venous effluents of n=5 lungs, however the total quantity of IL-2 in the 500ml venous effluent of n=1 was 31770pg, and the total quantity of IL-6 in the 500ml venous effluent of another lung was 465960pg. Given that these values markedly deviated from the rest of the group, they were omitted from the data set.

The most predominant cytokine was IL-18. The mean total quantity of IL-18 in the 500ml venous effluent of n=5 lungs was 21634(±9949)pg, however in the 500ml effluent of n=1 lung, the total was 397125pg. Given the marked deviation from the rest of the group, the value was omitted from the data set. The total quantity of IL-10 within the 500ml venous effluent was also high at 15441(±3312)pg. IL-1Ra, IL-1β, GM-CSF and IL-8 were all found in similar total quantities in the 500ml venous effluent, at 9487(±9433)pg, 9437(±2165)pg, 8628(±4450)pg and 7728(±2882)pg respectively. TNF-α was detectable at a lower total quantity of 1737(± 2693)pg in the 500ml venous effluent.
3.4.3 Quantitative PCR

Cell-free gDNA was not detected in any of the venous effluent samples. In contrast, cell-free mtDNA was detected in all samples, with a mean total quantity of 8130 (± 4124) ng in the 500ml venous effluent (Figure 3.7).

*Figure 3. 6: Total Quantity of Cytokines*
*The total quantities of cytokines in the 500ml LA Venous Effluent*

*Figure 3. 7: Total Quantity of mtDNA*
*The total quantity of cell-free mtDNA in the 500ml LA Venous Effluent*
3.5 Discussion

Despite the frequency of lung transplantation increasing worldwide, median survival remains limited secondary to rejection and primary graft dysfunction (6). It has previously been demonstrated in ex vivo perfusion models that upon reperfusion of the lungs there is a marked diapedesis of leukocytes from the donor lung into the recipient circulation (371). This donor-derived leukocyte milieu can drive allore cognition within the recipient, with subsequent rejection and graft dysfunction (214, 216). Yet in current practice, the donor immune compartment is somewhat overlooked, with the recipient immune system remaining to be the main therapeutic target (390).

In this study, the immediate effluent from the donor lungs upon reperfusion following CSP was characterised, to quantify the immediate inflammatory burden of the donor lung upon revascularisation. There was a significant mobilisation of donor leukocytes that would otherwise enter the recipient circulation upon reperfusion. In a 1L flush, on average, there were 1.5 billion leukocytes present in the first 500ml of effluent from the LA. Given that approximately 3.5L blood circulates through the lungs every minute, this suggests that upon revascularisation, billions of donor leukocytes can rapidly enter the recipient circulation following transplantation. These cells have the potential to up-regulate MHC expression, directly present alloantigens, and drive the recipient alloimmune T cell response, thereby increasing the risk of rejection (204, 214).

Within the mobilised leukocyte repertoire, there was a predominance of T cells, specifically cytotoxic and gamma-delta T cells. These cells are known to secrete high levels of cytotoxins and pro-inflammatory cytokines that can activate further inflammatory cascades within the recipient, with graft infiltration, neutrophil recruitment and cell death. Alloantigen presentation is heightened via up-regulation of MHC, as is the alloimmune response. Furthermore, expression of intracellular FoxP3 was present in <0.01% of the T cell population, demonstrating the T cells were not regulatory in nature and therefore their presence would not confer benefit through T cell anergy and immune tolerance. Removal of these cells is therefore likely to be beneficial.

Monocytes were also a predominant cell population within the effluent. Activated monocytes are known to secrete pro-inflammatory cytokines, thereby driving an inflammatory response. Additionally, they readily differentiate into macrophages and dendritic cells, and are involved in antigen presentation to effector T cells. Upon donor lung revascularisation, monocytes can therefore drive allore cognition and graft infiltration, and as such their role in transplant rejection and graft dysfunction is crucial (216, 218). The classical phenotype was present at higher concentrations than the non-classical phenotype, which is interesting given that previous studies have shown non-classical monocytes to be the major cell population that migrates from the lung during EVLP (375). The phenotypical functions of monocyte subsets remain heavily debated in relation to their phagocytic and inflammatory properties, with some studies demonstrating the non-classical subset to be more likely to display inflammatory attributes than
the classical subset (391). Furthermore, there is a smaller intermediate subset that appears to be transitional between the classical and non-classical subset (391). The discrepancy in the predominant monocyte subset in the donor effluent of this study and following reperfusion via EVLP may therefore be attributed to a constant flux in this intermediate subset to classical and non-classical subsets. The area however clearly warrants further exploration.

In addition to the abundant donor leukocytes within the effluent, there was also a high quantity of pro-inflammatory cytokines, including IL-18, IL-1β, GM-CSF and to a lesser degree, TNF-α. It has previously been demonstrated that a number of these cytokines can be found within the lungs during cold ischaemia and reperfusion, and have been implicated in the development of IRI (123). They are known to initiate an inflammatory cascade that can promote leukocyte infiltration and neutrophil sequestration with cellular damage and ultimately graft dysfunction. In addition, the potent chemokine IL-8 was also measured in high volumes within the venous effluent. It induces chemotaxis, primarily of neutrophils, and phagocytosis, and has been found to correlate with the severity of IRI and degree of graft dysfunction post-transplantation (56, 140).

Interestingly the quantities of the anti-inflammatory cytokines IL-10 and IL-1Ra were high. The relevance of this remains unclear however, especially given the predominant pro-inflammatory environment observed, whereby there is also a lack of Tregs. Therefore although they are present and may reflect the adaptive nature of the donor immune system to limit graft dysfunction, their anti-inflammatory properties are likely overwhelmed by the significant pro-inflammatory milieu.

High levels of cell-free mtDNA were also identified within the venous effluent, further promoting a pro-inflammatory environment. mtDNA is a potent immune activator that can act as a DAMP to initiate downstream pro-inflammatory signalling via TLR-9 and the inflammasome (387-389). Activation of the inflammasome ultimately leads to cleavage of the pro-inflammatory cytokines IL-18 and IL-1β. Indeed, both of these were found in high concentrations within the venous effluent and could perhaps be a consequence of cell-free mtDNA associated inflammasome activation. Interestingly cell-free gDNA was not detected in the effluent to suggest that the cell-free mtDNA present was a result of cell death. Its presence may well reflect ischaemic injury, given that mtDNA is known to be exquisitely sensitive to oxidative damage, more so than gDNA (388). Upon ischaemic compromise and exposure to extracellular ROS, mtDNA can become damaged and intracellular fragments may then accumulate and escape from cells. In the porcine lung transplant model, following EVLP and standard transplantation, there were high levels of cell-free mtDNA in the recipient circulation, however levels were lower following EVLP transplantation, and this was significant upon immediate reperfusion. Although this may well reflect the protective reperfusion of EVLP associated with reduced IRI, it may also be indicative of the ‘wash-out’ effect of EVLP, whereby inflammatory mediators that have accumulated within the donor lung are washed out of the lung and into the EVLP circuit prior to transplantation.
This study demonstrates that following donor lung procurement and preservation, there is a predominant pro-inflammatory population within the donor lung that rapidly migrates into the circulation upon reperfusion. The transfer of such a pro-inflammatory donor load to the recipient circulation is likely to drive alloimmune responsiveness and promote allore cognition. In this study, the secondary preservation flush was used to characterise the immediate donor lung effluent. It may however serve as a simple, cost-effective procedure that can be performed immediately prior to transplantation, to effectively remove the high concentration of donor leukocytes, inflammatory cytokines and DAMPs that have accumulated within the donor lung during ischaemic storage.

In summary, the donor lung compartment is an inflammatory, immunologically primed environment that has largely been ignored in the field of transplantation. Upon revascularisation, there is significant donor leukocyte migration from the lung that can drive the recipient alloimmune response. Despite this, it remains to be ignored, with immunosuppression focused upon the effector T cell response. EVLP has provided a platform for further interrogation of the donor lung, facilitating characterisation of the inflammatory compartment and cellular diapedesis following reperfusion, and identifying the potential for manipulation.
CHAPTER 4

Characterisation of the Immediate Cellular Migration During EVLP and the Impact of A Continual Reperfusion Flush
4.1 Abstract

**Background:** EVLP has emerged as a technique to facilitate donor lung evaluation. More recently it has also been used as a platform to manipulate the donor immune compartment prior to transplantation. PGD and allograft rejection are major caveats to long-term survival post lung transplantation, and the donor immune compartment is central to their development. The temporal kinetics of leukocyte diapedesis from the donor lung during EVLP was therefore determined, following which the impact of an initial continual flush with a non-circulating perfusate was evaluated.

**Methods:** Standard EVLP following the Toronto protocol was performed in n=3 double lungs for 2 hours and perfusate samples were collected at regular time-points. In the second part of the study, n=5 single lungs were placed on a modified EVLP circuit and underwent 2 hours of EVLP following the Toronto protocol, however in the first 40 minutes of reperfusion, the perfusate was continually replaced following a single circulation through the lung. After 40 minutes, the remaining perfusate was then recirculated and perfusion continued for a further 80 minutes. The non-circulating perfusate was collected in 500ml collection volumes, and following recirculation regular perfusate samples were taken until the end of perfusion. Flow cytometry was used to characterise the cell populations within all of the perfusate samples collected. qPCR was used to quantify cell-free mtDNA concentration.

**Results:** Donor leukocytes rapidly diapedesed into the EVLP circuit with a peak concentration of $6.74 \times 10^6 \pm 2.31 \times 10^6$ cells/ml within 30 to 60 minutes of perfusion. T cells and classical monocytes were the dominant migratory cell populations. During the second phase of perfusion (continual reperfusion with a non-circulating perfusate), $9.32 \times 10^9 \pm 5.49 \times 10^9$ donor leukocytes were removed from the EVLP circuit, with dominant cell populations of immature neutrophils and memory T cells.

**Conclusion:** The donor immune compartment comprises a large repertoire of marginal donor leukocytes that have a propensity to diapedese into the circulation upon reperfusion. EVLP can effectively be used to manipulate this donor immune compartment prior to transplantation, through modification of the EVLP circuit to facilitate reperfusion with a non-recirculating perfusate.
4.2 Introduction

Over recent years, EVLP has emerged as a technique to enable the extended evaluation of donor organs prior to transplantation (327). The process involves removing lungs from the suboptimal donor environment and placing them on an ex-vivo circuit that mimics the normal physiological in vivo environment. This controlled and stable setting provides a platform for lung recovery and assessment, and has led to an increase in the utilisation of organs donated for transplantation (327). More recently however, there has been increased interest in its capability to attenuate the donor immune compartment prior to transplantation.

PGD and allograft rejection are major caveats to successful lung transplantation (6). It is well known that upon donor death, the haemodynamic compromise that ensues triggers a catecholamine and cytokine storm with resultant inflammatory cell infiltration within the lung (13). The subsequent period of ischaemia further promotes the production of pro-inflammatory mediators, with up-regulation of adhesion molecules. It is in this inflamed and immune-activated state that the lungs are then transplanted into a recipient, whereby revascularisation can drive a myriad of pathophysiological processes leading to endothelial dysfunction and ALI, and ultimately IRI and PGD (123). Furthermore, the immunologically primed lung can promote allograft rejection, with up-regulation of MHC expression and increased allore cognition (216).

The immune compartment of the donor lung can therefore contribute to graft function post-transplantation. Indeed, porcine models of EVLP have demonstrated that upon reperfusion, there is a marked migration of passenger leukocytes from the donor lung, and removal of these cells prior to transplantation is associated with a reduced incidence of rejection (371). A secondary preservation flush following CSP (Chapter 3) demonstrated the pro-inflammatory environment of the donor lung prior to transplantation, and the significant migration of inflammatory mediators upon reperfusion.

In this two-part study, a porcine model of EVLP was used to map the temporal kinetics of cellular diapedesis during perfusion. In the second part of the study, the EVLP circuit was modified to evaluate the impact of continual reperfusion with a non-circulating perfusate.
4.3 Methods

In the first part of this study, a porcine model of EVLP was used to map the temporal kinetics of cellular diapedesis during standard EVLP. In the second part of this study, the porcine EVLP circuit was modified to determine whether initial perfusion with a non-recirculating perfusate could reduce subsequent cellular migration upon standard EVLP.

4.3.1 Ethical Approval

The ethics board at the University of Lund approved this study. All animals were treated in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the “Principles of Laboratory Animal Care” developed by the National Society for Medical Research.

4.3.2 Donor Organ Procurement

Eight Swedish outdoor free-range domestic pigs with a mean weight of 35kg were used in the two experiments. In experiment 1, n=3 pigs were used, and in experiment 2, n=5 pigs were used.

All pigs were sedated and anaesthetised, as previously described in chapter 2 and based upon prior literature (66). Briefly, the pigs were sedated using intramuscular ketamine (Ketalor, Parke-Davis, Morris Plains, NJ) and Xylasins® (Rompun, Bayer, Gothenburg, Sweden), and placed in the supine position. Anaesthesia was then achieved using intravenous sodium thiopental (Abbot, North Chicago, IL) and atropine (Kabi Pharmacia, Uppsala, Sweden). A median sternotomy was performed and ventricular fibrillation was induced following electrical stimulation, rendering the pigs dead.

The heart-lung block was then explanted, using the technique previously described in chapter 2 and based upon prior literature (321). The PA and aortic arch were cannulated, with distal ligation of the brachiocephalic and left subclavian arteries. The superior and inferior vena cavae were then ligated, and the ascending and descending aorta were clamped. A small cut was made in the right atrium and the LA appendage. The PA was then perfused with 2L of 4°C Perfadex® (XIVO Perfusion AB, Gothenburg, Sweden), and the heart-lung block was excised. In experiment 1, a double lung was used, whereby the lungs were harvested at the LA appendage, the main PA, and the trachea, distal to the carina. In experiment 2, a single left lung was utilised, being harvested at the level of the carina and the orifices of the PVs. The lungs were then submerged in cold (6-8°C) Perfadex® for a period of 120 minutes.
4.3.3 Ex-Vivo Lung Perfusion

EVLP was performed using the XVIVO XPS™ (XVIVO Perfusion AB, Gothenburg, Sweden). In order to map donor leukocyte transfer without the influence of revascularisation with red cells, an acellular perfusate with STEEN Solution™ (XVIVO, Gothenburg, Sweden) was used. As such, the Toronto protocol was followed, as previously described in the literature, whereby the LA was closed and a target flow of 40% of the estimated cardiac output was achieved (338).

Briefly, the circuit was primed with 1500ml of STEEN Solution™ and 10,000iU of unfractionated heparin (Fannin, UK). Following 120 minutes of cold ischaemia, the lung(s) was removed from the cold Perfadex® and prepared for EVLP. Firstly the PA and PVs were inspected and any visible clots were removed. A specially designed funnel-shaped LA cannula (XVIVO, Gothenburg, Sweden), with a built-in pressure catheter was then sutured to the LA cuff using Prolene® 4/0 monofilament (Ethicon, Somerville, NJ) to create a closed circuit. The PA was subsequently cannulated with a specialised PA cannula (XVIVO, Gothenburg, Sweden) with a built-in pressure catheter. Prior to connection onto the circuit a retrograde flush with 500ml of Perfadex® was performed under gravity drainage to ensure the PA and LA anastomoses were secure. The trachea was cleared of secretions and intubated with a 9mm ET tube and the lung(s) placed in the organ perfusion chamber. The LA cannula was connected to the circuit and the PA was de-aired by a slow retrograde flow at 100ml/min. Following de-airing, the PA cannula was then connected to the circuit and anterograde flow was commenced at room temperature, at a rate of approximately 10% of the target flow. Over the next 20 minutes, the perfusate temperature was incrementally increased to 32°C, during which time the perfusate flow was also incrementally increased to 20% of the target calculated flow (as outlined in Table 4.1). At 32°C, volume-controlled ventilation was commenced and the EVLP gas mixture (6% O₂, 8% CO₂, 86% N₂) was started at an initial flow rate of 0.5L/min, to de-oxygenate the inflow perfusate and maintain a partial pressure of CO₂ of between 35-45mmHg. The tidal volume was fixed at 7ml/kg, the PEEP at 5cmH₂O and the RR at 7bpm, with a FiO₂ of 0.21. The temperature of the perfusate was then increased to 37°C, and the flow rate was increased to 30% of the target flow. Over the next 30 minutes, the perfusate flow was incrementally increased over 10 minute intervals to reach 100% of the target flow (Table 4.1). Throughout the procedure the PA pressure was maintained between 10-15mmHg and the LA pressure between 3-5mmHg.
Table 4.1: The Toronto EVLP Protocol
The Toronto protocol was followed whereby perfusate temperature was incrementally increased, with subsequent increases in flow rate and commencement of ventilation. The target flow was rate was 40% of the cardiac output, which in pigs is estimated at 100ml/kg (338).

<table>
<thead>
<tr>
<th>Toronto Protocol</th>
<th>Perfusion Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Perfusion Temp (°C)</td>
<td>20</td>
</tr>
</tbody>
</table>

REPERFUSION

<table>
<thead>
<tr>
<th></th>
<th>10</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>50</th>
<th>80</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Target Flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA pressure (mmHg)</td>
<td>10-15</td>
<td>10-15</td>
<td>10-15</td>
<td>10-15</td>
<td>10-15</td>
<td>10-15</td>
<td>10-15</td>
</tr>
<tr>
<td>LA pressure (mmHg)</td>
<td>3-5</td>
<td>3-5</td>
<td>3-5</td>
<td>3-5</td>
<td>3-5</td>
<td>3-5</td>
<td>3-5</td>
</tr>
</tbody>
</table>

VENTILATION

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tidal Volume (mL/kg)</td>
<td></td>
<td></td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>PEEP (cmH2O)</td>
<td></td>
<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Respiratory Rate (bpm)</td>
<td></td>
<td></td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Gas Exchanger</td>
<td></td>
<td></td>
<td>On</td>
<td>On</td>
<td>On</td>
<td>On</td>
<td>On</td>
</tr>
</tbody>
</table>

Lung function was evaluated by determination of the oxygenation capacity and PVR. Oxygenation capacity was determined by the \( \Delta \) partial pressure of oxygen (PO\(_2\)) to FiO\(_2\) ratio, with \( \Delta PO_2 \) defined as LA perfusate PO\(_2\) – PA perfusate PO\(_2\). PVR calculated using the formula [(PA pressure – LA pressure)/PA flow] \times 80.

4.3.4 Cellular Migration During Standard EVLP

In this experiment (experiment 1), the temporal kinetics of cellular migration from the donor lung was mapped during standard EVLP. \( n=3 \) porcine double lungs were harvested and preserved as outlined above. Following 2 hours of CSP, the lungs were then prepared and placed on the XVIVO XPS™ and underwent 2 hours of standard EVLP. Perfusate samples were collected at baseline, prior to the lung being connected to the EVLP circuit, and then at 30-minute intervals throughout perfusion – i.e. 30 minutes, 60 minutes, 90 minutes and 120 minutes. Blood gas samples were also taken at these time-points and functional parameters including PA pressure and LA pressure were monitored throughout. All perfusate samples were collected from a sampling port distal to the LA cannula. Blood gas samples were also collected from this sampling port, as well as from a sampling port distal to oxygenator.

4.3.5 The Effect of a Continual Reperfusion Flush on Cellular Migration

In this experiment (experiment 2), the single left lung was placed on the EVLP circuit and initially perfused with a non-recirculating perfusate prior to standard EVLP, to determine the effect of a continual reperfusion flush on cellular migration. \( n=5 \) left single lungs were harvested.
and preserved as outlined above. Following 2 hours of CSP, the lungs were then prepared and placed on a modified XVIVO XPS™ (Figure 4.2). The lungs underwent 2 hours of EVLP as per the Toronto protocol, as outlined above. The perfusate was initially recirculated at the beginning of perfusion for technical purposes, to give the surgeon and operator sufficient time to ensure the perfusion was optimised. Following this, the inflow to the pump from the venous reservoir was closed, and the outflow from the venous reservoir was opened, to enable continual drainage of the perfusate following a single passage across the lung. A second venous replacement reservoir was filled with fresh STEEN Solution™ and connected to the inflow of the main circuit reservoir. Continuous ‘flush EVLP’ was performed for 40 minutes, followed by 80 minutes of standard EVLP (i.e. with re-circulation of the perfusate).

The modified circuit had two separate venous reservoirs, a standard LA venous reservoir, as well as an additional venous replacement reservoir. The standard venous reservoir collected perfusate from lungs via the LA cannula, and had two outflow ports: a circuit outflow port connected to the centrifugal pump within the EVLP circuit, and a 500ml collection outflow port controlled by a three-way tap. The venous replacement reservoir was a separate reservoir with a circuit outflow port connected to the centrifugal pump within the EVLP circuit. The additional venous replacement reservoir was initially clamped off from the EVLP circuit, as was the additional collection port from the LA venous reservoir (Figure 4.1).
**Figure 4.1: Diagrammatic Representation of the Recirculating Perfusion Circuit**

The left single lung is attached to a ventilator via the left main bronchus. Perfusate circulates through the centrifugal pump and into the deoxygenator, which is attached to a heat exchanger. It then flows into the lungs through the PA cannula, and drains back out through the LA cannula and into the LA venous reservoir. The LA venous reservoir has 2 outflow ports: one attached to the centrifugal pump and one attached to a 500mL collection system controlled by a 3-way tap.

During recirculation of the perfusate, this 3-way tap is closed and perfusate drains from the LA venous reservoir and through the centrifugal pump for recirculation through the deoxygenator and heat exchanger and back through the lungs. There is an additional venous replacement reservoir that is also attached to the centrifugal pump, however this is clamped off (Clamp A) when the perfusate is recirculating. There is a sampling port distal to the LA cannula where perfusate samples are taken for gas analysis and processing.

The circuit was primed with 1500ml of STEEN Solution™ and the lung was connected and EVLP commenced, as outlined above. After a short period of reperfusion to ensure the perfusion was optimised, the circuit outflow port of the LA venous reservoir was clamped off and the circuit outflow port of the venous replacement reservoir was unclamped (Figure 4.2). As perfusate was drained from the lungs and into the LA venous reservoir, now disconnected from the circuit, replacement STEEN Solution™ was added to circuit via the venous replacement reservoir in volumes of 500ml. As replacement STEEN Solution™ was added, perfusate in the LA venous reservoir was drained from the collection port into 500ml collection bottles via a 3-way tap, so as to maintain the LA pressure between 3-5mmHg.
Figure 4.2: Diagrammatic Representation of the Non-Recirculating Perfusion Circuit

As in Figure 4.1, the left single lung is attached to the ventilator via the left main bronchus. Perfusate circulates through the centrifugal pump and into the deoxygenator, which is attached to a heat exchanger. It then flows into the lungs through the PA cannula, and drains back out through the LA cannula and into the LA venous reservoir. In this circuit, the outflow port of the LA venous reservoir that is attached to the centrifugal pump is clamped off (Clamp B), and so perfusate no longer recirculates through the centrifugal pump. The outflow tract of the additional venous replacement reservoir that is also attached to the centrifugal pump, is now unclamped and fresh perfusate, is circulated via the centrifugal pump into the deoxygenator and heat exchanger and through the PA of the lungs. The perfusate is then drained from the lungs via the LA cannula and into the LA venous reservoir, which remains clamped off to the circuit. The perfusate in the LA venous reservoir is drained into 500mL collection bottles in a controlled manner via the 3-way tap to ensure that the LA pressure is maintained at 3-5mmHg. During the non-recirculation phase, the venous replacement reservoir is continually filled with 500mL of fresh perfusate to help facilitate LA pressure control, and ensure the circulating volume is maintained at 1500mL.

At 40 minutes, the circuit outflow port of the venous replacement reservoir and the collection port of the LA venous reservoir were both clamped, and the circuit outflow port of the LA venous reservoir was unclamped allowing re-circulation of perfusate (Figure 4.1). Standard EVLP with recirculation of the perfusate was then continued for a further 80 minutes.

Perfusate samples were collected at baseline, during continual circuit volume replacement and then during normal recirculation. During continual reperfusion, samples were collected from the
500ml collection bottles after thorough mixing, at cumulative collection volumes of 500ml, 1000ml, 2500ml, 5000ml, 7500ml and then from the final 500ml collection volume at the end of continual ‘flush EVLP’. Upon normal recirculation, perfusate samples were collected from the sampling port at the start of normal recirculation, then at 5 minutes, 10 minutes, 30 minutes, 60 minutes and 80 minutes. Throughout EVLP, functional parameters including LA and PA pressure were continually recorded and blood gases were taken at regular time intervals.

4.3.6 Sample Collection and Processing

All perfusate samples were collected in 10ml volumes. The samples were placed in labelled Falcon™ tubes and then stored in the fridge prior to processing at the end of each EVLP procedure.

Upon processing, 1ml of perfusate was decanted into separate flow tubes. The remaining 9ml samples were then centrifuged at 1000g for 10 minutes at 4°C. The supernatants were then separated into 5 x 1.8ml aliquots in labelled cryovials and stored at -80°C.

Flow tubes were then prepared for later analysis by flow cytometry. Briefly, samples were centrifuged at 1000g for 10 minutes at 4°C. The supernatants were then discarded and the remaining cell pellets were re-suspended in red cell lysis buffer (BD™ Biosciences, UK) and incubated in the dark for 10 minutes. Following incubation, 1ml PBS was added to each of the samples and centrifuged again at 1000g for 10 minutes at 4°C. The supernatants were discarded and the cell pellets were re-suspended in 1ml freeze-mix. Each of the samples were transferred to corresponding labelled cryovials, placed into a Mr. Frosty™ (Thermo Scientific™), and then stored at -80°C.

4.3.7 Flow Cytometry

The flow cytometry protocol used was similar to that described in detail in Chapter 3. Leukocytes were immunophenotyped using a BD™ LSR II flow cytometer (Becton Dickinson, UK). Cell viability was determined using Zombie UV™ fixable viability dye (Biolegend, UK) and monoclonal anti-CD45+ was used as a pan-leukocyte marker. A specific antibody panel (Table 4.1) was then used to identify the following individual leukocyte populations; immature neutrophils (6D10’2B2’), mature neutrophils (6D10’2B2’), mature eosinophils and basophils (6D10’2B2’), total T cells (CD3+), double positive/memory T cells (CD3+CD4+CD8+), cytotoxic T cells (CD3+CD8+CD4–), helper T cells (CD3+CD8–CD4+), B cells (CD3+CD21’), natural killer T (NKT) cells (CD3+CD56+), NK cells (CD3+CD56+), total macrophages (CD203a+), classical monocytes (CD14+CD163+), and non-classical monocytes (CD14+CD163–). In addition, the presence of Treg cells (CD3+CD4+CD25+FoxP3+) was confirmed via the expression of intracellular FoxP3 in T cells.
Table 4.2: Antibody Cocktails to Identify Leukocyte Populations

The composition of each antibody cocktail used to determine specific leukocyte populations. PE-Cy7-conjugated anti-CD3ε and Alexa Fluor 647-conjugated anti-CD8α were all purchased from BD™ Bioscience (Oxford, UK), with the remaining antibodies purchased from AbD Serotec (Kidlington, UK).

<table>
<thead>
<tr>
<th>Tube/Plate</th>
<th>Antibody Cocktail</th>
<th>Cell Populations Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FITC-conjugated mouse anti-2B2</td>
<td>Immature neutrophils</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated mouse anti-6D10</td>
<td>Mature neutrophils</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 700-conjugated anti-CD107a</td>
<td>Mature eosinophils and basophils</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7-conjugated anti-CD80</td>
<td>B cells</td>
</tr>
<tr>
<td></td>
<td>APC-Cy 7-conjugated anti-CD21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pacific Blue SIRP-a (anti-CD172a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>FITC-conjugated anti-CD14</td>
<td>Total macrophages</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated mouse anti-CD163</td>
<td>Classical monocytes</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7-conjugated anti-CD80</td>
<td>Non-classical monocytes</td>
</tr>
<tr>
<td></td>
<td>Pacific Blue SIRP-a (anti-CD172a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PE-conjugated Texas Red ant-CD203a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>APC-Cy 7-conjugated SLA-DR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PE-Cy7-conjugated anti-CD3ε</td>
<td>NKT cells</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 647-conjugated anti-CD8α</td>
<td>NK cells</td>
</tr>
<tr>
<td></td>
<td>APC-Cy 7-conjugated anti-CD56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PE-conjugated mouse anti-CD16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 700-conjugated anti-CD107a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pacific Blue SIRP-a (anti-CD172a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Alexa Fluor 700-conjugated anti-CD8</td>
<td>Total T cells</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7-conjugated anti-CD3ε</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td></td>
<td>FITC-conjugated mouse anti-CD4</td>
<td>Helper T cells</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated mouse anti-CD25</td>
<td>Memory T cells</td>
</tr>
<tr>
<td></td>
<td>APC-Cy 7-conjugated SLA-DR</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td></td>
</tr>
</tbody>
</table>
Briefly, the perfusate samples that had been stored for flow cytometry were removed from the -80°C freezer and rapidly thawed by immersing them in a water bath set to 37°C. Each sample was then immediately added to a corresponding labelled Falcon™ tube, containing a pre-warmed 50:50 mix of RPMI and FCS. Following re-suspension, samples were then centrifuged at 700g for 5 minutes and supernatants were discarded. The remaining cell pellets were re-suspended in PBS and each suspension was then aliquot into 4 corresponding wells in 4 x 96-well v-bottomed flow plates, labelled 1 to 4. Zombie UV™ viability dye (Biolegend, UK) was then added to each of the samples in the 96-well v-bottomed flow plates and incubated in the dark for 30 minutes at room temperature. Following incubation, flow-staining buffer was added to each of the sample wells, and the plate was then centrifuged at 500g for 2 minutes at 4°C and the supernatants were discarded. The specific antibody cocktail was added to each of the samples in the corresponding plate to identify specific cell types (Table 4.2). The plates were incubated in the dark for 15 minutes at room temperature. A wash step was then performed by adding flow-staining buffer to each sample and centrifuging the plates at 500g for 2 minutes at 4°C. The supernatants were discarded and the wash step was repeated.

For plates 1 to 3, a fixing solution was prepared using flow staining buffer and cell fix. The fixing solution was then added to each of the samples in plates 1 to 3 and mixed. The plates were then incubated in the dark for 15 minutes at 4°C. Following incubation, the plates were centrifuged at 500g for 2 minutes at 4°C and supernatants were discarded. Flow staining buffer was added to each of the remaining cell pellets and the plates were centrifuged again at 500g for 2 minutes at 4°C. Again, supernatants were discarded and the cell pellets were re-suspended in flow-staining buffer. Each of the re-suspended samples were then transferred to corresponding pre-labelled flow tubes and stored in the dark at 4°C until the samples were analysed on the flow cytometer.

For plate 4, following the second wash step the samples were re-suspended in fixation/permeabilisation solution and the plate was incubated in the dark for 40 minutes at 4°C. Following incubation, BD Perm/Wash™ buffer (BD™ Biosciences, UK) was added to each of the samples and the plate was centrifuged at 500g for 2 minutes at 4°C. The supernatants were discarded and the BD Perm/Wash™ buffer was again added to each of the samples, and the plates then centrifuged at 500g for 2 minutes at 4°C. Again, supernatants were discarded and the cell pellets were re-suspended in BD Perm/Wash™ buffer and APC-conjugated FoxP3 antibody was then added to each sample. The plate was incubated in the dark for 40 minutes at 4°C following which, BD Perm/Wash™ buffer was added to each sample and the plate was centrifuged at 500g for 2 minutes at 4°C. The supernatants were discarded and again the cell pellets were again re-suspended in BD Perm/Wash™ buffer. The plates were centrifuged again at 500g for 2 minutes at 4°C and supernatants were discarded. The cell pellets were then re-suspended in flow-staining buffer and each of the samples were transferred to corresponding pre-labelled flow tubes and stored in the dark at 4°C until the samples were analysed on the flow cytometer.
Upon analysis on the flow cytometer, the samples were removed from the dark and eBeads (eBioscience, UK) were added to each of the samples to determine absolute cell counts. The samples were then individually analysed on the flow cytometer. Subsequent gating was performed using FlowJo® v.10 software. Briefly, leukocyte populations were identified and gated as CD45+ cells and the viability of cells was confirmed. Granulocytes were identified according to their positive staining, or lack of, for 6D10 and 2B2. B cells were then identified according to their positive staining for CD21. Total macrophages were identified, by their positive staining for CD203a. Monocytes were then identified by their positive staining for CD14 and gated, from which the classical and non-classical populations were determined according to their negative or positive staining to CD163, respectively. NK cells were determined by their positive staining for CD56, and NKT cells by their positive staining for both CD56 and CD3. Total T cells were identified by their positive staining for CD3 and gated, and from this population, cytotoxic and helper T cells were identified according to their positive staining for CD8 and CD4 respectively. Double positive/memory T cells were also identified. Tregs were then identified, by their positive staining for CD3, CD4 CD25 and FoxP3.

### 4.3.8 Quantitative PCR

qPCR was utilised to quantify the amount of cell-free mtDNA in the perfusate samples collected and stored. The technique used has previously been described in detail in Chapter 2. Following thawing, cell-free DNA was initially extracted from the stored perfusate samples, using a QIAamp® DNA Mini and Blood Mini Kit (Qiagen, Manchester, UK) as per the manufacturer’s guidelines. Briefly, the perfusate samples were mixed with Buffer AL and incubated at 56°C for 10 minutes. Following a series of centrifuge and buffer wash steps, extracted cell-free DNA was then eluted through the QIAamp® Mini spin column, and collected in labelled microcentrifuge tubes. If the extracted cell-free DNA samples were not analysed immediately, they were stored at -20°C.

Primers were designed using the Primer Express™ Software v3.0.1 (Life Technologies, Paisley, UK) and their homology to other genes assessed using BLAST. To identify cell-free mtDNA, primers to Cytochrome B were used as in Chapter 2.

- **Cytochrome B Forward**: 5’ ACACATCAGACACAAACAA 3’
- **Cytochrome B Reverse**: 5’ GTAGCGAATAACTCATCCGTAA 3’

The primer sets were synthesized and de-salted by Sigma Aldrich. Prior to use, they were re-suspended in nuclease-free water (Ambion, USA) to adjust their concentration to 25nM. mtDNA standard curve samples were then extracted from porcine lung tissue, following initial isolation of mitochondria, as described in Chapter 2. Briefly, 0.1g of porcine lung tissue was washed in PBS and homogenized. The suspension then underwent centrifugation at low and high speed and the mitochondrial pellet was re-suspended in mitochondrial isolation buffer. mtDNA was
then extracted from the isolated mitochondria as described. The concentrations were then quantified using Nanodrop™ 2000 (Thermofisher Scientific, Waltham, MA, USA), and serial dilutions were performed to achieve a relative standard curve consisting of 20ng/µl, 10ng/µl, 5ng/µl, 2.5ng/µl, 1.25ng/µl and 0.65ng/µl. The standard curve was then used to determine the efficiency of the primers to detect their target DNA and enable quantification of the DNA in the unknown samples following PCR.

qPCR was performed using a QuantStudio™12K Flex System with Power SYBR™ green PCR master mix (Life Technologies, Paisley, UK), as previously described in Chapter 2. Briefly, extracted effluent DNA samples and standard curve samples were separately mixed with Power SYBR™ green, nuclease free water and forward and reverse primers and then added to four wells of a MicroAmp™ Optical 384-Reaction plate, in quadruplet samples. The plate was then sealed and following a brief centrifuge step, qPCR was performed as previously described.

If there was any amplification in the blank sample or if there were multiple peaks in the melt curve, results were discarded and the plate was repeated, as this would indicate primer dimer formation. The standard curve was then analysed to determine the efficiency of the PCR amplifications by creating a semi-log regression line plot. A slope of -3.32 indicates 100% efficiency with an R² value of 1. Regression line plots with R² values > 0.95 were accepted.

Using the standard curve calculation and the sample C_T values, DNA was then quantified for each sample. If the C_T value was > 40, this was indicative of minimal amounts of target nucleic acid being present in the sample, therefore levels could not be quantified and cell-free DNA was deemed undetectable.

4.3.9 Statistical Analysis

Statistical analysis was performed using SPSS® v.22.0. Data normality was determined and normally distributed data was then expressed using mean ± SD. Non-normally distributed data was expressed using median ± interquartile range. GraphPad® Prism v.7.0 was utilised to formulate graphs.
4.4 Results

4.4.1 Cellular Migration During Standard EVLP

n=3 double lungs successfully underwent EVLP following the Toronto protocol. Ischaemic times were comparable throughout, with a cold ischaemic time of 135 minutes in all lungs.

Functional parameters throughout EVLP were monitored and PA and LA pressures were maintained at 10-15mmHg and 3-5mmHg, respectively. The target flow was achieved in all three perfusions. The PVR decreased throughout EVLP from 832.24(±145.76) dynes/sec/cm$^2$ at 15 minutes, to 574.4(±95.91) dynes/sec/cm$^2$ at the end of perfusion. Lung compliance increased from 9.22(±4.35)L/kPa at 60 minutes to 14.03(±1.95)L/kPa at 120 minutes. Blood gases remained stable throughout, with a ΔPO$_2$/FiO$_2$ of 397.67 (±27.12)mmHg at the end of EVLP.

Flow Cytometry

Upon reperfusion, viable CD45$^+$ leukocytes diapedesed into the EVLP circuit throughout perfusion, peaking at 60 minutes, at a concentration of $6.74 \times 10^6$ (±$2.31 \times 10^6$) cells/ml. Following which the concentration within the circuit declined to $3.32 \times 10^6$ (±$2.12 \times 10^6$) cells/ml at 120 minutes, the end of EVLP (Figure 4.3).

![Figure 4.3: Migration of CD45+ Cells During EVLP](image)

Concentration of CD45$^+$ cells at regular time-points throughout EVLP

The dominant cell population migrating from the lung during reperfusion was T cells, with a peak of migration at 60 minutes. Macrophages and monocytes, and B cells also migrated from the lungs in high concentrations, with macrophages and monocytes peaking at 30 minutes, and B cells peaking between 30 and 60 minutes. NK cells and granulocytes peaked at lower
concentrations, with NK cells peaking between 60 and 90 minutes of reperfusion, and granulocytes at 60 minutes. From their peak, all cell populations decreased in concentration as perfusion continued, except B cells, which had a second peak in concentration at the end of perfusion (Figure 4.4).

Within the T cell population, CD8\(^+\) T cells were dominant, with a peak concentration of 2.54 x 10^5 (± 2.38 x 10^5) cells/ml at 60 minutes, falling to a concentration of 1.52 x 10^5 (± 1.46 x 10^5) cells/ml at the end of perfusion (Figure 4.5). CD4\(^+\) T cells migrated from the lungs at lower concentrations, peaking at 6.48 x 10^4 (± 4.29 x 10^4) cells/ml at 60 minutes. Migration of Tregs was minimal throughout perfusion, with a peak concentration of 163(±188) cells/ml. B cells diapedesed from the lungs in a similar pattern to CD8\(^+\) cells, peaking between 30 to 60 minutes at a concentration of 2.1 x 10^5 (±6.4 x 10^4) cells/ml. Unlike other cell populations, there was then a second peak in their concentration at the end of perfusion to 1.75 x 10^5(± 9.29 x 10^4) cells/ml (Figure 4.5).
Total macrophages migrated from the lung and peaked within the circuit at 60 minutes of perfusion at a concentration of $7.74 \times 10^4 \pm 3.43 \times 10^4$ cells/ml. Classical monocytes migrated from the lung at higher concentrations, peaking at $1.19 \times 10^5 \pm 6185$ cells/ml at 30 minutes. Non-classical monocytes diapedesed at lower concentrations, peaking at $2.07 \times 10^4 \pm 1.81 \times 10^4$ cells/ml, also at 30 minutes (Figure 4.6).

NKT cells were the dominant population of NK cells that migrated from the lung upon perfusion, peaking at a concentration of $8.47 \times 10^4 \pm 6.52 \times 10^4$ cells/ml at 90 minutes. NK cells diapedesed at lower concentrations, peaking earlier at 60 minutes, at a concentration of $3.42 \times 10^4 \pm 3.01 \times 10^4$ cells/ml (Figure 4.7).
Granulocytes diapedesed at lower concentrations from the lungs. Immature neutrophils were the dominant migratory granulocyte population, peaking at 30 minutes, at a concentration of $4.57 \times 10^4 \pm 2.75 \times 10^4$ cells/ml. Mature eosinophils and basophils and mature neutrophils migrated from the lung at lower concentrations, peaking at 60 minutes, at concentrations of $2.94 \times 10^4 \pm 2.62 \times 10^4$ cells/ml and $1.12 \times 10^4 \pm 1.24 \times 10^4$ cells/ml, respectively.
4.4.2 The Effect of a Continual Reperfusion Flush on Cellular Migration

n=5 left single lungs successfully underwent modified EVLP following the Toronto protocol, whereby for a 40 minute period the perfusate was not re-circulated but rather replenished with fresh perfusate. Prior to EVLP, the ischaemic times for all lungs were comparable throughout, with a warm ischaemic time of 18.8 (±2.86) minutes and a cold ischaemic time of 121.6 (±3.59) minutes.

Functional parameters were monitored throughout, and PA and LA pressures were maintained at 10-15mmHg and 3-5mmHg, respectively. The target flow was achieved in all five perfusions. The PVR in all five lungs decreased throughout EVLP. In the first 15 minutes it was initially high at 2330.16 (±863.75) dynes/sec/cm², however it reduced at 60 minutes to 831.94 (±260.65) dynes/sec/cm², and then again at 120 minutes to 630 (±168.54) dynes/sec/cm². The lung compliance increased from 60 minutes to 120 minutes, calculated as 12.57 (±1.36)L/kPa and 13.16 (±1.38)L/kPa, respectively. Blood gas analysis remained stable throughout perfusion, with a ΔPO₂ to FiO₂ ratio of 616 (±40.13)mmHg on evaluation at the end of EVLP.

During continual reperfusion, a mean volume of 9100 (±651.92)mls of perfusate was collected from the circuit.

Flow Cytometry
Flow cytometry was performed to quantify the cell populations present in each of the collected perfusate volumes during the continual reperfusion flush. The area under the curve was then used to calculate the total number of cells collected within the perfusate. A total of 9.32 x 10⁹ (±5.49 x 10⁹) CD45⁺ leukocytes were removed from the EVLP circuit during continual perfusion flush (Figure 4.9).

![Figure 4.9: Total Number of CD45⁺ Cells in the Collected Perfusate](image)
*The total number of CD45⁺ cells in the entire collected perfusate volume*
Within the collected perfusate, the number of total CD45\(^+\) peaked within the 2500ml perfusate collection volume, at a concentration of \(1.25 \times 10^6 \pm 1.02 \times 10^5\) cells/ml (Figure 4.11).

**Figure 4.10: Total Number of CD45\(^+\) Cells in the Individual Perfusate Collection Volumes**
The total number of CD45\(^+\) cells in each of the 500ml perfusate collections at the pre-specified cumulative collection volumes

Upon recirculation of perfusate, CD45\(^+\) leukocytes continued to migrate into the circuit with a peak concentration of \(1.14 \times 10^6 \pm 7.26 \times 10^5\) cells/ml within 10 minutes of recirculation. The concentration then fell to \(7.20 \times 10^5 \pm 5.16 \times 10^5\) cells/ml at the end of perfusion (Figure 4.11).

**Figure 4.11: Migration of CD45\(^+\) Cells into the Perfusate During Recirculation**
The concentration of CD45\(^+\) cells at regular time-points during recirculation in standard EVLP
Immature neutrophils were the predominant cell population removed from the circuit during the continual reperfusion flush, at a concentration of $1.07 \times 10^9 \pm 6.16 \times 10^8$ cells/9100mls (Figure 4.12).

**Figure 4.12: Total Number of Cell Populations in the Collected Perfusate**  
The total number of differing cell populations in the entire collected perfusate volumes

The number of immature neutrophils within the collected perfusate peaked in the 2500ml collection volume, at $1.69 \times 10^5 \pm 1.15 \times 10^5$ cells/ml, and then continued to be removed in high numbers (Figure 4.13).
Figure 4.13: Number of Granulocytes in the Individual Perfusate Collection Volume
The total number of granulocyte populations in each of the 500ml perfusate collections at the pre-specified cumulative collection volumes

Upon perfusate recirculation, immature neutrophils rapidly diapedesed from the lung, and their concentration within the circuit peaked within 10 minutes, at $1.27 \times 10^5 \pm 1.23 \times 10^5$ cells/ml. Their numbers then fell to a concentration of $7.01 \times 10^4 \pm 6.01 \times 10^4$ cells/ml at the end of EVLP (Figure 4.14).

Figure 4.14: Migration of Granulocytes into the Perfusate During Recirculation
The concentration of granulocyte populations at regular time-points during recirculation in standard EVLP

Mature neutrophils and mature eosinophils and basophils were removed from the circuit at lower concentrations during continual reperfusion flush, at $8.49 \times 10^7 \pm 5.99 \times 10^7$ cells/9100mls and $4.44 \times 10^7 \pm 2.84 \times 10^7$ cells/9100mls, respectively. Both had a peak concentration within the 2500ml perfusate collection volume at $1.52 \times 10^4 \pm 1.57 \times 10^4$ cells/ml.
and 6129(±5093) cells/ml, respectively. Upon recirculation, mature neutrophils migrated from the lungs at a lower concentration and remained stable throughout perfusion, with a peak concentration of 1.29 x 10^4 (± 1.76 x 10^4) cells/ml. Mature eosinophils and basophils also migrated from the lungs in smaller volumes, with a peak concentration of 7622(±5182) cells/ml within 10 minutes, before falling to 3676(±2780) cells/ml at the end of EVLP (Figure 4.14).

T cells were a dominant migratory population during EVLP (Figure 4.12). A total of 5.58 x 10^8 (± 3.99 x 10^5) CD4⁺ memory T cells (phenotyped via CD4⁺CD8⁺ expression) were removed during the continual reperfusion flush, with a peak concentration of 8.9 x 10^4 (± 7.49 x 10^4) cells/ml in the 5000ml collection volume (Figure 4.15).

Upon recirculation, CD4⁺ memory T cells continued to migrate from the lung, with a peak concentration of 8.12 x 10^4 (± 5.85 x 10^4) cells/ml within 5 minutes of recirculation. There was then a progressive decline in concentration to 3.59 x 10^4 (± 2.37 x 10^4) cells/ml at the end of EVLP (Figure 4.16).
The concentration of T cell populations at regular time-points during recirculation in standard EVLP.

2.82 x 10⁸ (± 2.57 x 10⁸) CD8⁺ T cells and 1.92 x 10⁸ (± 2.74 x 10⁸) CD4⁺ T cells were removed from the circuit during continual reperfusion flush (Figure 4.12). Both T cell subtypes peaked within the 2500ml perfusate collection volume at concentrations of 3.98 x 10⁴ (± 3.62 x 10⁴) cells/ml and 2.78 x 10⁴ (± 3.18 x 10⁴) cells/ml, respectively, and continued to be removed in high numbers (Figure 4.15). Upon perfusate recirculation, both T cell subtypes continued to migrate from the lung with CD8⁺ T cells peaking within 5 minutes to a concentration of 4.9 x 10⁴ (± 4.84 x 10⁴) cells/ml and CD4⁺ T cells peaking within the first 10 minutes to a concentration of 2.44 x 10⁴ (± 2.66 x 10⁴) cells/ml. Both concentrations then fell to 1.77 x 10⁴ (± 1.57 x 10⁴) cells/ml and 8470 (±5250) cells/ml at the end of perfusion. Tregs were one of the lowest concentrations of cells removed from the circuit during the continual reperfusion flush. A total of 1.58 x 10⁷ (± 2.93 x 10⁷) cells were present in the total collected perfusate, and upon recirculation, migration remained minimal, with a peak concentration of 1344 (± 2083) cells/ml within 10 minutes, before falling to less than 500 cells/ml.

Higher numbers of classical monocytes were removed from the circuit during the continual reperfusion flush than non-classical monocytes, with a concentration of 5.49 x 10⁸ (± 3.42 x 10⁸) cells within the collected perfusate, compared to 9.46 x 10⁷ (± 1.2 x 10⁸) non-classical monocytes/9100mls (Figure 4.12). The concentration of classical monocytes gradually increased in the perfusate collection volumes, peaking within the 2500ml and 5000ml collection volumes, at a concentration of 7.83 x 10⁴ (± 5.94 x 10⁴) cells/ml. Non-classical monocytes also progressively increased to a peak concentration of 1.7 x 10⁴ (± 2.48 x 10⁴) cells/mL in the 5000ml perfusate collection volume (Figure 4.17).
The total number of monocyte populations in each of the 500ml perfusate collections at the pre-specified cumulative collection volumes

Upon reperfusion, classical monocytes continued to migrate from the lung at a higher concentration than non-classical monocytes. Within the first 10 minutes of perfusate recirculation, the number of classical monocytes peaked within the circuit at $4.64 \times 10^4 \pm 2.12 \times 10^4$ cells/ml, before falling to $8495 \pm 6836$ cells/ml at the end of perfusion. Non-classical monocytes migrated from the lungs in small numbers with a delayed peak in concentration at 60 minutes, to $2.01 \times 10^4 \pm 3.68 \times 10^4$ cells/ml (Figure 4.18).

**Figure 4.17: Number of Monocytes in the Individual Perfusate Collection Volumes**
The concentration of monocyte populations at regular time-points during recirculation in standard EVLP.

**Figure 4.18: Migration of Monocytes into the Perfusate During Recirculation**
The concentration of monocyte populations at regular time-points during recirculation in standard EVLP.
During the continual reperfusion flush, a total of $1.85 \times 10^8 \pm 7.1 \times 10^7$ macrophages were removed from circuit. Their concentration peaked in the 5000ml perfusate collection volume with $2.7 \times 10^4 \pm 1.04 \times 10^4$ cells/ml (Figure 4.19).

![Figure 4.19: Number of Macrophages in the Individual Perfusate Collection Volumes](image)

The total number of macrophages in each of the 500ml perfusate collections at the pre-specified cumulative collection volumes

Upon recirculation, macrophages continued to migrate from the lungs, initially at a concentration of $2.19 \times 10^4 \pm 1.40 \times 10^4$ cells/ml, before progressively falling to $1.18 \times 10^4 \pm 6446$ cells/ml at the end of perfusion (Figure 4.20).

![Figure 4.20: Migration of Macrophages into the Perfusate During Recirculation](image)

The concentration of macrophages at regular time-points during recirculation in standard EVLP
During the continual reperfusion flush, NKT and NK cells were removed from the circuit at concentrations of $2.19 \times 10^8 \pm 2.5 \times 10^8$ cells/9100mls and $1.33 \times 10^8 \pm 1.62 \times 10^8$ cells/9100mls, respectively. NKT cells peaked within the 5000ml collection volume at $3.31 \times 10^8 \pm 4.66 \times 10^4$ cells/ml, whereas NK cells peaked within the 7500ml collection volume at $2.03 \times 10^5 \pm 2.63 \times 10^4$ cells/ml (Figure 4.21).

Upon recirculation, NKT cells continued to migrate from the lung at higher concentrations than NK cells, both peaking within 10 minutes at $3.40 \times 10^4 \pm 3.57 \times 10^4$ cells/ml and $1.79 \times 10^4 \pm 2.29 \times 10^4$ cells/ml respectively (Figure 4.22).

**Figure 4.21: Number of NK cells in the Individual Perfusate Collection Volumes**
The total number of NK cell populations in each of the 500ml perfusate collections at the pre-specified cumulative collection volumes

**Figure 4.22: Migration of NK Cells into the Perfusate During Recirculation**
The concentration of NK cell populations at regular time-points during recirculation in standard EVLP
B cells were the least migratory cell population upon reperfusion. During the continual reperfusion flush, \(1.81 \times 10^6 \pm 2.92 \times 10^6\) B cells were removed from the circuit, with a peak concentration of \(287(\pm 608)\) cells/ml in the 5000ml perfusate collection volume (Figure 4.23).

**Figure 4.23: Number of B Cells in the Individual Perfusate Collection Volumes**
The total number of B cells in each of the 500ml perfusate collections at the pre-specified cumulative collection volumes

Upon recirculation, B cells minimally diapeded from the lung, with a peak concentration of \(271(\pm 464)\) cells/ml, before falling to \(38(\pm 56)\) cells/ml at the end of perfusion (Figure 4.24).

**Figure 4.24: Migration of B Cells into the Perfusate During Recirculation**
The concentration of B cells at regular time-points during recirculation in standard EVLP
**mtDNA Quantification**

Cell-free mtDNA was quantified in the perfusate collection volumes and within the circuit upon recirculation. A total of $1.09 \times 10^5 \pm 4.3 \times 10^4$ ng of cell-free mtDNA was removed from the circuit during the continual reperfusion flush (Figure 4.25).

![Figure 4.25: Concentration of Cell-Free mtDNA in the Collected Perfusate](image)

*The total amount of cell-free mtDNA quantified in the entire perfusate collection volume*

Upon recirculation, cell-free mtDNA was present in the perfusate at all time-points, with a peak concentration of $23.56 \pm 14.63$ ng/ml (Figure 4.26).

![Figure 4.26: Concentration of Cell-Free mtDNA in the Perfusate During Recirculation](image)

*Levels of mtDNA within the perfusate, at regular time-points during recirculation in standard EVLP*
4.5 Discussion

The importance of the donor immune compartment prior to transplantation, and its role in the development of IRI, PGD and allograft rejection has long been recognised. Despite this, therapeutic targets continue to be directed towards the recipient, with the donor immune compartment relatively ignored (390). In recent years, EVLP has provided a stable environment to evaluate and recondition marginal donor lungs prior to transplantation. Furthermore it has been suggested that EVLP-reconditioned lungs are associated with a reduced incidence of PGD and rejection post-transplantation (356, 371). As such the capability of the EVLP circuit to attenuate the donor environment prior to transplantation has been met with interest.

Reperfusion of immunologically primed donor lungs can promote the release of pro-inflammatory mediators and the up-regulation of adhesion molecules, driving a heightened immune response with inflammatory cell infiltration and graft dysfunction (123). Additionally, the marked migration of donor leukocytes into the recipient circulation can further promote allorecognition and the alloimmune response with subsequent rejection. Indeed, porcine models of EVLP have demonstrated that removal of passenger leukocytes prior to transplantation is associated with a reduced incidence of rejection (371).

The secondary preservation flush (Chapter 3) in porcine lungs demonstrated that upon initial reperfusion following CSP, there was an immediate migration of pro-inflammatory mediators from the donor lung. In this study, porcine EVLP was therefore used as a model to characterise the donor immune compartment and map cellular diapedesis upon revascularisation, to determine whether a continual reperfusion flush can then attenuate the donor immune compartment prior to transplantation.

In the first part of this study, porcine double lungs underwent standard EVLP following two hours of cold ischaemia to evaluate immediate cellular diapedesis upon reperfusion. All lungs functioned well throughout EVLP, with comparable pulmonary haemodynamics and gas exchange. As such, the cellular diapedesis observed was considered to be that of a normal phenomenon during donor lung reperfusion, and not secondary to injury on the circuit.

Leukocytes rapidly migrated from the lung upon reperfusion, with a peak in migration between 30 to 60 minutes, at a concentration of 6.7 million cells/ml. Within a 1500ml circulating volume, during peak migration, a total 10.12 billion leukocytes therefore migrated from the lung and circulated within the closed EVLP circuit. However, in the context of transplantation, these leukocytes are not confined to a closed circuit, but instead can rapidly migrate into the recipient circulation upon revascularisation. Here, they can promote pro-inflammatory cascades with up-regulation of MHC and activation of the alloimmune response, facilitating allorecognition and graft rejection.
As in the secondary preservation flush in Chapter 3, in this study the predominant migratory population observed was also T cells, and specifically CD8+ cytotoxic T cells. These cells enhance cytotoxicity via the release of perforins and the activation of the caspase cascade. Additionally, they readily secrete pro-inflammatory cytokines, primarily TNF-α and IFN-γ, which further enhance the cytotoxicity of other leukocytes. These processes can therefore promote immune activation and induce inflammatory infiltration of the graft, with subsequent destruction and dysfunction (392). The proportion of Tregs within this migratory T cell population was again found to be low, as in the secondary preservation flush (Chapter 3). This would suggest that this inflammatory milieu is not regulatory in nature.

The increased diapedesis of B cells observed during standard EVLP is interesting, given that previous studies have demonstrated them to be a non-dominant population (371). B cells are integral to adaptive immunity, responsible for the production of antigen specific antibodies. They are capable of priming T cells, and driving inflammatory responses. Therefore their migration upon reperfusion could also potentiate the recipient alloimmune response and contribute to subsequent graft dysfunction.

Macrophages and monocytes were dominant cell populations within the circulating perfusate. These leukocytes are crucial to allograft rejection and graft dysfunction, with monocytes capable of rapidly differentiating into macrophages and dendritic cells, which then present alloantigen, driving direct allore cognition (216, 218). Classical monocytes were again found to the predominant monocyte subset despite previous studies reporting that non-classical monocytes are the major cell population that migrate from the lung during EVLP (375). In this study, the discrepancy may be explained by the type of EVLP protocol used. The original studies used the Lund protocol, where the target flow was 100% of the estimated cardiac output, with a Ht of 15-20%. Hypothetically, perfusion of the microcirculation may occur during the Lund protocol, and this may not be the case in the Toronto protocol, whereby the target flow was only 40% of the estimated cardiac output. Clearly this warrants analysis but is outside the remit of this thesis. Also as previously discussed, the phenotypical functions of monocyte subsets remain heavily debated, with recent recognition of an intermediate subset (391). Therefore the discrepancy between the dominant migratory subset upon reperfusion may well be reflective of a continual transitional state of intermediate monocytes differentiating into both classical and non-classical subsets.

NK cells and granulocytes also rapidly migrated from the lung during EVLP, however they were observed at lower concentrations within the perfusate, particularly granulocytes. NK cells are innate immune cells that have cytotoxic properties and can readily secrete pro-inflammatory cytokines. NKT cells are a distinct population of NK cells that express a unique T cell receptor. Upon activation, they can produce large numbers of cytokines that can interact with neighbouring leukocytes. Recipient-derived NK and NKT cells have been implicated in allograft rejection and graft dysfunction (393, 394). Given their capability to promote a pro-inflammatory cytotoxic environment, donor-derived cells therefore have the potential to further augment the
recipient immune response and also drive graft infiltration and destruction. Such infiltration can be enhanced by the presence of granulocytes, specifically neutrophils, which recognise PAMPs with resultant inflammatory cytokine secretion and cytotoxic degranulation.

It is therefore clear that the donor lung compartment possesses a large reservoir of pro-inflammatory leukocytes. During EVLP, these donor leukocytes rapidly diapedesed into the circuit and peaked within 30 to 60 minutes of reperfusion. This suggests that these leukocytes are marginal, and as such would have the capacity to rapidly migrate into the recipient circulation upon revascularisation, driving an immediate alloimmune response.

In the second part of this study, the impact of initially flushing the lung with a non-recirculating perfusate on cellular migration and graft function was evaluated. In the first part of the study, the peak of cellular migration was demonstrated to be within the first 30 to 60 minutes of EVLP. The lungs therefore underwent continual flushing with a non-recirculating perfusate for 40 minutes, before reconnecting the circuit and undergoing standard EVLP with perfusate recirculation. Throughout EVLP, with both a non-recirculating and recirculating perfusate, the lungs maintained their function with stable oxygenation capacity, lung compliance and pulmonary haemodynamics.

A total of 9.3 billion donor leukocytes were present in the collected perfusate during flush with non-recirculation, and as such were removed from the lung. Upon characterisation of that donor leukocyte population, immature neutrophils, memory T cells and classical monocytes were the dominant cell populations. Other T cell subsets, along with macrophages and NK cells were also observed in high numbers. Non-classical monocytes, mature granulocytes, B cells and Tregs were present at much lower concentrations.

The predominance of immature neutrophils and memory T cells is interesting, especially given that large populations of the former were not observed during standard EVLP in the first part of the study. Immature neutrophils have been detected in the circulation of septic patients with severe systemic inflammatory responses, whereby they are released prematurely from the bone marrow in response to overwhelming immune activation (395). Interestingly, it has been shown that they have a longer life span, with resistance to spontaneous apoptosis when compared with mature neutrophils (395). Their effector capabilities of cytokine release and phagocytosis are similar to mature neutrophils. In the context of lung transplantation, the haemodynamic compromise and subsequent catecholamine and cytokine storm upon donor death may promote the release of immature neutrophils from the donor bone marrow as a stress response. These cells then rapidly migrate to the acutely injured lung, where they remain resident, with their presence predominating due to their increased life span and resistance to apoptosis. Upon revascularisation, these cells can then rapidly diapedesed into the circulation where they mediate a range of immune cascades. It can therefore be inferred that their removal would confer benefit prior to transplantation. Following recirculation of the perfusate, immature neutrophils continued to diapedese from the lung with their circulating volume slowly decreasing in concentration.
Although immature neutrophils diapedesed from the lung during EVLP in the first part of this study (from n=3 lungs), they were not a dominant migratory population, and the cause of this discrepancy between their dominance in the collected perfusate during non-recirculation and subsequent recirculation is unclear and clearly warrants further work. It may be that their migration is dependent upon achieving an equilibrium between their concentration in the lung tissue and the circulating vasculature. Therefore through the continual replacement of perfusate, that equilibrium is never achieved, promoting on-going migration of the cells from the lung tissue. Upon recirculation, that equilibrium is gradually restored and perfusate concentration subsequently plateaus.

Memory T cells were the dominant T cell population within the collected perfusate during continual flush with non-recirculation. Increased circulating levels of memory T cells have previously been found in autoimmune and chronic inflammatory conditions, whereby they may possess both cytolytic and helper phenotypes (396). These cells can also regulate immune interactions and promote cytokine release (397, 398). Their presence within the perfusate during standard EVLP was not previously evaluated in the first part of the study, however it is clear that they possess an inflammatory capability.

Therefore continual reperfusion flush with non-recirculating of perfusate, effectively removed a broad spectrum of donor leukocytes that can mediate pro-inflammatory cascades, induce MHC up-regulation and enhance allorecognition. Interestingly, however, despite donor leukocyte depletion during continual flushing of the lung, leukocytes continued to diapedes from the lungs in high volumes upon subsequent recirculation of perfusate. This highlights the overwhelming propensity of donor leukocytes to migrate from the lung upon reperfusion. It would also suggest that upon transplantation and reperfusion of the donor lung, there is a continual and persistent diapedesis of donor leukocytes into the recipient circulation until stabilisation and depletion of the donor immune compartment occurs. A relative depletion of donor leukocytes prior to transplantation could therefore reduce the severity of cellular migration upon revascularisation within the recipient, attenuating the recipient alloresponse and reducing graft infiltration. This remains hypothetical, however the current study clearly demonstrates that a continual flush with a non-circulating perfusate effectively removes large populations of passenger leukocytes from the donor lung. It could therefore serve as a platform to achieve donor leukocyte depletion prior to transplantation if clinical benefit is demonstrated.

Interestingly, in addition to the cellular populations removed during the continual flush, cell-free mtDNA was also present at high concentrations within the collected perfusate. Furthermore, during the recirculation phase of the experiment, circulating levels continued to be observed within the perfusate. mtDNA is a potent immune activator, capable of mediating inflammatory cascades via TLR signalling and the inflammasome activation (387-389). The cause of mtDNA release into the circulating perfusate is unknown. However, having also been observed in the effluent of the secondary preservation flush (Chapter 3), it may well be reflective of ischaemic injury, given its sensitivity to oxidative damage (388). Despite this, following initial removal of
cell-free mtDNA during the flush stage of the experiment, cell-free mtDNA continued to be present during the recirculation phase, suggesting that its release is an active process that is on-going during reperfusion. Therefore although its removal from the circulating perfusate may confer benefit given its down-stream pro-inflammatory capabilities, its presence may provide a reliable indication of on-going oxidative damage and cellular injury. Yet within this study, its presence within the circuit did not correlate with objective evidence of lung injury via assessment of functional parameters, including oxygenation capacity and PVR. Clearly the mechanisms and relevance of mtDNA release from the donor lung warrants further investigation.

In summary, this study has demonstrated that the donor immune compartment comprises of a large population of marginal donor leukocytes that have a propensity to diapedese into the circulation upon reperfusion. Furthermore, EVLP can effectively be used to manipulate this donor immune compartment prior to transplantation. Modification of the EVLP circuit with a non-recirculating flush, whilst maintaining the principles of ex-vivo perfusion, can deplete the donor leukocyte population without impacting upon lung function during EVLP. Donor leukocyte depletion is likely to reduce passenger leukocyte transfer within the recipient upon revascularisation. In addition, the study has demonstrated that reperfusion of the donor lung is also associated with the release of cell-free mtDNA into the circulation, the relevance of which remains unclear. Should its presence be reflective of oxidative stress, it may well serve as a sensitive marker of IRI, with its release propagating further downstream inflammatory cascades and potentiating any lung injury. As such, the relationship between circulating cell-free mtDNA and lung reperfusion requires further exploration.
CHAPTER 5

Is Circulating Cell-Free mtDNA During EVLP A Biomarker of Lung Function?
5.1 Abstract

Background: EVLP has provided a platform to enable the extended evaluation and reconditioning of donor organs prior to transplantation, and as such has facilitated the use of marginal donor organs. However the assessment of lungs during EVLP remains somewhat subjective and continues to rely heavily on easily measurable parameters such as oxygenation. Prior studies have demonstrated that cell-free mtDNA is present within the perfusate during EVLP. This study aimed to evaluate whether levels of cell-free mtDNA correlated with graft function.

Methods: Cell-free mtDNA was quantified in the perfusate samples from n=20 EVLPs performed as part of the DEVELOP-UK study. Levels were then correlated with EVLP success and 12-month survival following transplantation. A porcine EVLP model was then used to determine whether increased levels of cell-free mtDNA were observed following injury.

Results: There was no significant difference between cell-free mtDNA levels during successful and unsuccessful human EVLP, however there was an increased variation in levels within the EVLP group that did not proceed to transplant. In the EVLPs that proceeded to transplant, increased cell-free mtDNA was observed in those that had inferior 12-month outcomes, although this was not significant. In the porcine EVLP model, there were increased levels of cell-free mtDNA within the perfusate following ischaemic lung injury, and this correlated with a pro-inflammatory cytokine profile. Interestingly, despite a concurrent rise in PVR and fall in lung compliance, oxygenation remained stable.

Conclusion: The current assessment of whether lungs are suitable for transplantation is somewhat subjective and relies upon easily measureable parameters that are not sensitive markers of lung injury. Circulating cell-free mtDNA was observed in the perfusate during EVLP, and levels were consistently higher in lungs that were injured. Furthermore, increased cell-free mtDNA during EVLP was associated with inferior outcomes following transplant. Cell-free mtDNA may therefore serve as a potential biomarker of lung function. However the level at which it is associated with injury has yet to be confirmed and clearly warrants further investigation.
5.2 Introduction

Lung transplantation remains limited by the shortage of donor organs available and the problem is two-fold. Firstly, there is a lack of donor organs donated for transplant, with many families refusing consent for organ donation at the time of death (10). Secondly, of those limited organs donated, only 20% are utilised, with the remainder deemed unsuitable for transplantation (10, 11). It has already been discussed that donor lungs are particularly susceptible to injury upon donor death and it is in this injured state when their suitability for transplantation is assessed. Steen et al established that EVLP is a viable technique to enable an extended functional evaluation of donor lungs prior to transplantation (66). Furthermore, he demonstrated that the procedure can facilitate a reconditioning process within the lungs, whereby functionality can improve and marginal donor lungs can recover whilst perfused on the circuit (337).

EVLP has since become an established technique in the field of lung transplantation, whereby it has optimised the use of donor organs donated for transplant and led to an increase in transplant activity (356, 360, 367). More recently, there is emerging evidence that EVLP can also improve long-term outcomes post-transplantation, with a reduced incidence of PGD (356, 366) and potentially rejection (371). However, the assessment of whether lungs are truly usable for transplantation following EVLP relies upon the assessment of oxygenation capacity and pulmonary haemodynamics, and can be somewhat subjective. The cellular and molecular processes that underpin successful EVLP are yet to be fully understood. Andreasson et al have previously analysed the cytokine profiles within the perfusate of successful and unsuccessful EVLP (372, 373). They found that higher levels of IL-1β and IL-8 correlated with unsuccessful reconditioning, with IL-1β also correlating with post-transplant outcomes (372, 373).

Prior studies have demonstrated that cell-free mtDNA is present in the circulating perfusate during EVLP. mtDNA is released from cells in response to damage and stress (399). It can then activate the innate immune system to mediate inflammatory cascades via TLR signalling. Activation of the inflammasome and the stimulator of interferon genes (STING) also occurs (387, 399). In rat models it has been shown to induce ALI (387, 400). Therefore its presence in the perfusate during EVLP may well be an indicator of lung injury, with its release initially being dependent upon cellular injury, with subsequent propagation of such injury through its activation of innate inflammatory signalling pathways.

In this study, perfusate samples were analysed from human EVLPs that were both successful and unsuccessful, based upon their use for transplantation. Cell-free mtDNA was quantified to determine whether levels correlated with EVLP success, in addition to 12-month survival post-transplantation. Following this, a porcine model of EVLP was then utilised to establish whether cell-free mtDNA is released as a direct response to ischaemic lung injury.
5.3 Methods

Two separate experiments were performed to determine whether cell-free mtDNA is a biomarker of lung injury during EVLP. In the first experiment, cell-free mtDNA was quantified in the perfusate samples from 20 randomly selected EVLPs, performed as part of the human DEVELOP-UK study. The second experiment was a porcine injury model, whereby cell-free mtDNA and cytokine secretion was analysed following induced ischaemic lung injury on an EVLP circuit.

5.3.1 DEVELOP-UK Study

The DEVELOP-UK study was a multi-centre study, designed to determine the clinical- and cost-effectiveness of EVLP to recondition marginal donor lungs for transplantation. The study was approved by the Health Research Authority in the UK, and was funded by the National Institute for Health Research Health Technology Assessment Programme and the UK Cystic Fibrosis Trust.

The study was an un-blinded and non-randomised observational study, to determine whether survival during the first 12 months after lung transplantation, was affected by using EVLP reconditioned organs, and therefore establish the non-inferiority of EVLP to standard transplant. In all patients consented to the study, clinical data was recorded for a period of 12 months following both standard and EVLP transplant. In addition to clinical data, perfusate samples were collected, processed and stored during each EVLP to facilitate mechanistic experimental studies.

Inclusion and Exclusion Criteria

Any patients aged over 18 years and on the active waiting list for first lung transplant could be included in the trial. Those under the age of 18 years or those listed for re-transplantation, heart-lung transplantation or live donor lobar transplant were excluded for selection.

Criteria for EVLP Assessment and Reconditioning

DBD and DCD lungs could be used in this study. Donor lungs fulfilling any of the following criteria could be considered for EVLP assessment:

i) For DCD donors, a warm ischaemic time of > 30 minutes but < 60 minutes
ii) CXR findings prohibitive to standard transplantation
iii) PO\textsubscript{2} < 35-40kPa and/or a selective PV gas < 30kPa on a FiO\textsubscript{2} of 1.0 with a PEEP of 8cmH\textsubscript{2}O
iv) A sustained peak airway pressure >30cmH\textsubscript{2}O
v) History of aspiration, with bronchoscopic inflammation/soiling of the airway, or recurrent secretions in the distal airway after adequate bronchial toilet
vi) Difficult to recruit atelectasis
vii) Unsatisfactory deflation test on disconnecting the ET tube
viii) Unsatisfactory palpation of the lungs, identifying undetermined masses, nodules or gross oedema

ix) Deterioration or cardiac arrest in the donor prior to retrieval with uncertainty over assessment

x) Logistical reasons that may extend donor lung ischaemic time > 10-12 hours

If any of these criteria were met, the lungs could be considered for EVLP assessment at the recipient hospital.

**Donor Lung Procurement**

A standardised donor organ procurement protocol was followed for all donor lungs. Briefly, an anterograde flush via the PA was performed using 60ml/kg of Perfadex® (XVIVO Perfusion AB, Gothenburg, Sweden), initially at room temperature and then 4°C. Following this, a retrograde flush of 200ml was performed via each PV.

**Ex-Vivo Lung Perfusion**

All perfusions were performed using the Vivoline® LS1 system (Vivoline® Medical AB, Lund, Sweden). From 1st April 2012 to 31st March 2013, the protocol for EVLP was a hybrid of components of the Lund and Toronto protocol. Briefly, the LA was left open, and the circuit was primed with 2L of acellular STEEN Solution™ (XVIVO, Gothenburg, Sweden). Upon connection to the circuit, controlled reperfusion was started 15°C, with a PA pressure maintained below 20mmHg. As the temperature was increased, the flow was gradually increased to a target of 40-60% of the cardiac output. At 32°C, volume controlled ventilation was commenced, with a FiO₂ of 0.5, to reach a tidal volume of 6-8ml/kg and a RR of 10-15bpm. Due to the incidence of adverse events, the protocol was subsequently changed and from 1st August 2013 to the end of the study, the Lund protocol was followed. Differing from the original protocol, the circuit was then primed with 2L of STEEN Solution™ supplemented with packed red cells, to reach a target Ht of 10-15%, and the target flow upon reperfusion was 100% of the cardiac output.

Evaluation was performed in both protocols at 37°C, when target flow and full ventilation was achieved. Prior to evaluation, oxygen was disconnected from the perfusion system and once de-oxygenation was confirmed on blood gas analysis, recruitment manoeuvres were performed. Following this, the FiO₂ was increased from 0.5 to 1.0, with a temporary increase in PEEP to 8cmH₂O, and blood gases were analysed. If upon evaluation the lungs met the following criteria for transplantation, they were then utilised for transplant:

i) PA pressure ≤ 20mmHg, whilst achieving stable perfusate flow

ii) Peak airway pressure < 25cmH₂O, whilst achieving adequate ventilation

iii) Oxygen capacity shown by deltaPO₂ > 40kPa (LA perfusate PO₂ - PA perfusate PO₂)/FiO₂

iv) Selective PV gas > 30kPa with a FiO₂ of 1.0 and a PEEP of 5 cmH₂O

v) Stable or improving lung compliance and, stable or falling lung resistance
vi) No pulmonary oedema in the ET tube
vii) Satisfactory assessment on inspection and palpation.

If the lungs were deemed unsuitable for transplantation, they could undergo further reconditioning and re-evaluation, for a maximum of 4 hours on the EVLP circuit. If the lungs were never deemed suitable for transplantation, perfusion was stopped and the lungs were discarded. If the lungs were deemed suitable for transplantation, oxygen was reconnected to the circuit, the tidal volume was reduced by 30% and the RR reduced to 8bpm. The temperature of the circuit was set to 32°C and when the temperature of the perfusate reached this, ventilation was stopped and the trachea was clamped, with the lungs partially inflated with a FiO₂ of 0.5. The perfusate was then cooled to 12°C, upon which the PA cannula was disconnected and a PA plug was inserted. The lungs were then topically cooled prior to transplantation.

**Sample Collection and Processing**

Perfusate samples were collected from all EVLPs performed, including those that did not proceed to transplant. All samples were taken from the sampling port of the Vivoline® machine. The first perfusate sample was taken prior to connecting the lung to the circuit. Subsequent perfusate samples were collected upon reperfusion, initially at 15 minutes, then at 30 minutes, and then for regular 30-minute intervals thereafter. A final perfusate sample was taken at the end of perfusion, prior to disconnection of the PA cannula.

The perfusate samples were stored on ice for the duration of the procedure and then at 4°C prior to processing. The samples were centrifuged at 180g for 6 minutes at 4°C to remove cellular debris. The supernatant was divided into 5 x 1ml aliquots and stored at -80°C. The samples were sent in batch on dry ice to the central centre. All samples were processed within 8 hours of collection.

Perfusate samples taken at 15 minutes, 90 minutes and at the end of EVLP were isolated from 20 randomly selected EVLPs, 10 of which were performed upon lungs that were subsequently transplanted, and 10 of which upon lungs that were discarded. The samples were then coded and sent for mtDNA analysis as part of this study. Following mtDNA quantification, the codes were then identified and the 12-month survival outcome from the lungs that were transplanted was revealed.

**5.3.2 Porcine Injury Model**

A porcine model of EVLP was utilised to quantify the amount of cell-free mtDNA in the EVLP circuit during standard EVLP and then following induced lung injury, to determine whether there the level of cell-free mtDNA was increased in response to ischaemic injury.
**Ethical Approval**

The ethics board at the University of Lund approved this study. All animals were treated in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the “Principles of Laboratory Animal Care” developed by the National Society for Medical Research.

**Donor Organ Procurement**

n=6 Swedish outdoor free-range domestic pigs, with a mean weight of 35kg, were used in this experiment. All pigs were sedated and anaesthetized, as previously described in chapter 2, and based upon prior literature (66). Briefly, the pigs were sedated, using intramuscular ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) and Xylasin® (Rompun, Bayer, Gothenburg, Sweden), and placed in the supine position. Anesthesia was then achieved using intravenous sodium thiopental (Abbot, North Chicago, IL) and atropine (Kabi Pharmacia, Uppsala, Sweden). A median sternotomy was then performed and ventricular fibrillation was induced following electrical stimulation, rendering the pigs dead.

The heart-lung block was explanted, using the technique previously described in chapter 2, and based upon prior literature (321). Briefly, the PA and aortic arch were cannulated, with distal ligation of the brachiocephalic and left subclavian arteries. The superior and inferior vena cavae were ligated and the ascending and descending aorta were clamped. A small cut was made in the right atrium and the LA appendage. The PA was then perfused with 2L of 4°C Perfadex® (XVIVO Perfusion AB, Gothenburg, Sweden), and the heart-lung block was excised. The left lung was then harvested at the level of the carina and the orifices of the PVs. The lung was then submerged in cold (6-8°C) Perfadex® for a period of 60 minutes.

**Ex-vivo Lung Perfusion**

All EVLPs were performed using a modified cardiopulmonary bypass circuit comprised of an oxygenator and heat exchanger, and venous reservoir, attached to an organ chamber and a Siemens Servo ventilator 300 (Siemens – Elema AB, Solna, Sweden). Following a 60 minute cold ischaemic time, the lung was removed from cold Perfadex® and prepared for EVLP. Visible clots were removed from the PA and PVs, and then a temperature probe was sutured in between the orifices of the two PVs. The PA was cannulated with a modified PA cannula, with built-in pressure probe, and an ET tube was inserted. The single left lung was then placed in the organ perfusion chamber and underwent EVLP following the Lund protocol, as described in detail in Chapter 2 and the literature (66). Briefly, following the removal of air, the lung underwent controlled cellular reperfusion via the PA, with gradual re-warming and protective volume controlled ventilation. Flow through the PA was initially commenced at 100ml/minute, which then gradually increased as the temperature of the perfusion fluid exiting the LA gradually increased. The PA pressure never exceeded 20mmHg. When the temperature reached 32°C, volume controlled ventilation was commenced, initially at 1L/min with a FiO₂ of 0.5. Full
ventilation was achieved at 37°C with a ventilator MV less than 2.5 x the perfusion flow, and a PEEP of 5cmH₂O.

In n=3 lungs, normal EVLP continued for 3 hours to establish the pattern of cell-free mtDNA and cytokine release in standard EVLP. In n=3 lungs, warm ischaemic injury was induced following 90 minutes of EVLP, whereby perfusion and ventilation was abruptly stopped and the lungs were disconnected from the perfusion circuit for a period of 30 minutes. Following this, the lungs were reconnected and maximum perfusion and ventilation was recommenced for a further 60 minutes. Throughout all EVLPs, lung function was assessed by continually monitoring pulmonary haemodynamics, including PA pressure, PA flow and PVR. Evaluation blood gases were performed at 90 minutes and 180 minutes, whereby oxygen was disconnected from the oxygenator to determine the oxygenation capacity of the lungs.

**Sample Collection and Storage**

Perfusate samples were collected from the LA appendage at regular time-points throughout all EVLPs – baseline, 60 minutes, 125 minutes and 180 minutes. All samples were collected in 10ml volumes, placed in labelled Falcon™ tubes and stored in the fridge prior to processing. Upon processing, the samples were centrifuged at 1000g for 10 minutes at 4°C. The cell pellets were discarded and the supernatants were divided into 1.8ml aliquots and stored in labelled cryovials at -80°C.

**5.3.3 Quantitative PCR**

qPCR was performed to quantify the amount of cell-free mtDNA in the perfusate samples from both the DEVELOP-UK study and the porcine injury model. The technique used followed the same as that previously described in Chapter 2. Briefly, the perfusate samples were thawed and DNA was extracted from them using the QIAamp® DNA Mini and Blood Mini Kit (QIAGEN, Manchester, UK), according to the manufacturer's protocol and using the materials provided. The samples were mixed with Buffer AL and incubated at 56°C for 10 minutes. Following a series of centrifuge and buffer wash steps, extracted DNA was then eluted through the QIAamp® Mini spin column, and collected in labelled microcentrifuge tubes. If the extracted DNA samples were not analysed immediately, they were stored at -20°C.

Primers were designed using the Primer Express™ Software v3.0.1 (Life Technologies, Paisley, UK) and their homology to other genes assessed using BLAST. In both studies, to identify mtDNA, primers for Cytochrome B were used, as in Chapter 2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome B Forward</td>
<td>5’ ACACATCAGACACAACAACA 3’</td>
</tr>
<tr>
<td>Cytochrome B Reverse</td>
<td>5’ GTAGCGAATAACTCATCCGTAA 3’</td>
</tr>
</tbody>
</table>

In the DEVELOP-UK study, human primers were used, and in the porcine injury study, porcine primers were used.
Each of the primer sets were synthesized and de-salted by Sigma Aldrich. Prior to use, they were re-suspended in nuclease-free water (Ambion, USA) to adjust their concentration to 25nM. mtDNA standard curve samples were then extracted. In the DEVELOP-UK study, samples were extracted from human explanted lung tissue, retrieved from patients who had been consented for the lung study, “Exploring the mechanisms of homeostasis and disease before and after lung transplantation”, that will be discussed further in chapter 6. In the porcine injury model, samples were extracted from porcine lung tissue. To extract mtDNA, mitochondria were initially isolated from the relevant lung tissue, following the method previously described in chapter 2. Briefly, 0.1g of the explanted lung tissue was washed in PBS and homogenized. The suspension then underwent centrifugation at low and high speed, and the mitochondrial pellet was re-suspended in mitochondrial isolation buffer. mtDNA was then extracted from the isolated mitochondria and the concentration was quantified using Nanodrop™ 2000 (Thermofisher Scientific, Waltham, MA, USA). Serial dilutions were then performed to achieve a relative standard curve consisting of 20ng/µl, 10ng/µl, 5ng/µl, 2.5ng/µl, 1.25ng/µl and 0.625ng/µl. The standard curve was used to determine the efficiency of the Cytochrome B primers to detect their target human or porcine cell-free mtDNA, and enable quantification of the DNA in the unknown samples following PCR.

qPCR was then performed using a QuantStudio™12K Flex System with Power SYBR™ green PCR master mix (Life Technologies, Paisley, UK), as previously described in Chapter 2. Briefly, two MicroAmp™ Optical 384-Reaction plates were used, one for the DEVELOP-UK samples and one for the porcine injury model samples. The corresponding extracted perfusate DNA samples and standard curve samples were separately mixed with Power SYBR™ green, nuclease free water and their specific forward and reverse primers, and then added to four wells of the relevant MicroAmp™ Optical 384-Reaction plate, in quadruplet samples. The plates were then sealed and following a brief centrifugation step, qPCR was performed, as previously described.

If there was any amplification in the blank sample, or if there were multiple peaks in the melt curve, results were discarded and the plate was repeated, as this would indicate primer dimer formation. The standard curve was then analysed to determine the efficiency of the PCR amplifications, by creating a semi-log regression line plot. A slope of -3.32 indicates 100% efficiency with an R² value of 1. Regression line plots with R² values > 0.95 were accepted.

Using the standard curve calculation and the sample C_T values, cell-free mtDNA was then quantified for each perfusate sample. If the C_T value was > 40, this was indicative of minimal amounts of target nucleic acid being present in the sample, therefore levels could not be quantified and cell-free DNA was deemed undetectable.
5.3.4 Luminex® Analysis

The perfusate samples from the porcine injury model were analysed to determine the concentration of 13 chemokines and cytokines, using a porcine specific magnetic bead-based multiplex assay (Merck Millipore, Billerica, MA, USA) – 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.

The samples were thawed and then processed as per the manufacturer’s protocol using the materials provided, as described in Chapter 3. Briefly, assay buffer was added to each well of the plate, and following sealing, it was placed on a plate shaker for 10 minutes at room temperature. The seal was then removed and the plate inverted to decant the buffer. Each of the standards and controls were added into their corresponding wells in duplicates. Assay buffer and serum matrix solution were then added to the sample wells. Each sample was added to their corresponding well, in duplicates. The mixed antibody-immobilised beads were then added to each well, and the plate was sealed. After being sealed and wrapped in foil, the plate was incubated overnight on a plate shaker at 4°C. Following incubation, the well contents were removed and the plate was washed 3 times. After detection antibodies were added into each well, the plate was sealed and covered with foil and incubated for a further 2 hours at room temperature on a plate shaker. The plate was then uncovered and Streptavidin-Phycocerythrin was added to each well, and the plate was re-sealed and covered with foil, prior to incubation for 30 minutes at room temperature, again on a plate shaker. Following incubation, the plate was uncovered and the well contents removed, and the plate was washed 3 times. Sheath fluid was then added to the wells and the plate was placed onto a plate shaker for 5 minutes, prior to being analysed using the Bio-Plex® 200 system (Bio Rad, Hertfordshire, UK).

5.3.5 Statistical Analysis

Statistical analysis was performed using SPSS® v.22.0. Data normality was determined and normally distributed data was then expressed using mean ± SD. Non-normally distributed data was expressed using median and interquartile range. GraphPad® Prism v.7.0 was utilised to formulate graphs.
5.4 Results

5.4.1 DEVELOP-UK Study

Samples from n=20 double lung EVLPs were analysed. In n=6 of the EVLPs, the sample sets were incomplete, with n=5 missing the perfusate samples from 15 minutes, and n=1 missing the perfusate sample from the end of perfusion. In the n=10 EVLPs that resulted in transplantation, at 12 months, n=7 had 100% survival, n=3 had 100% mortality secondary to complications associated with lung transplantation.

mtDNA Quantification

In n=10 EVLPs, whereby the lungs were subsequently transplanted, the median (and interquartile range) of cell-free mtDNA concentration at 15 minutes, 90 minutes and at the end of perfusion was 29.96 (66.95)ng/ml, 39.96 (109.61)ng/ml and 183.54 (161.32)ng/ml, respectively. In n=10 EVLPs, whereby the lungs were discarded and not used for transplantation, the median of cell-free mtDNA at 15 minutes, 90 minutes and at the end of perfusion was 31.61 (60.73)ng/ml, 111.68 (119.87)ng/ml and 189.69 (387.23)ng/ml, respectively (Figure 5.1).

Statistical analysis was performed using the Mann-Whitney U test. At all time-points, there was no significant difference between the concentration of cell-free mtDNA in the EVLP circuit of lungs that were transplanted and in those that were not (p=0.529 at 15 minutes; p=0.079 at 90 minutes; p=0.661 at the end of perfusion).

Figure 5.1: Concentration of mtDNA During EVLP According to Transplantation

The concentration of cell-free mtDNA detected within the perfusate during EVLP of lungs that did and did not proceed to transplant. Tx = Lungs that were transplanted and No Tx = Lungs that were not transplanted
In the n=7 EVLPs that resulted in lung transplantation with a 100% 12 month survival, the median cell-free mtDNA concentrations were; 29.6 (77.67)ng/ml at 15 minutes, 35.21 (99.8)ng/ml at 90 minutes and 140.18 (170.89)ng/ml at the end of perfusion. In the n=3 EVLPs that resulted in lung transplantation with a 100% 12 month mortality, the median cell-free mtDNA concentrations were; 47.91 (56.37)ng/ml at 15 minutes, 119.01 (102.68)ng/ml at 90 minutes and 236.66 (203.1)ng/ml at the end of perfusion (Figure 5.2).

Statistical analysis was performed comparing the concentration of cell-free mtDNA during EVLP at all time-points between the three groups of, n=10 that were not transplanted, n=7 that were transplanted and had a 100% 12-month survival and n=3 that were transplanted and had a 100% 12-month mortality. Using the Kruskal-Wallis Test there was no significance in cell-free mtDNA concentration at all three time-points across the three groups (p=0.429 at 15 minutes, p =0.060 at 90 minutes, p = 0.369 at the end of perfusion).

5.4.2 Porcine Injury Model

n=6 single left lungs underwent EVLP. The ischaemic times were comparable between all lungs, with a mean warm ischaemic time of 12.68 (±1.21) minutes and a mean cold ischaemic time of 65.5 (±12.29) minutes. Lungs were then randomly selected to undergo control EVLP (n=3) or injury EVLP (n=3). In the injury group, at 90 minutes of standard EVLP there was a 30 minute period of warm ischaemia followed by a further 60 minutes of EVLP.
Functional parameters were monitored throughout EVLP, including oxygenation capacity, PVR and lung compliance. All were calculated using the following equations:

i) Oxygenation capacity = \( \frac{\text{LA PO}_2 - \text{PA PO}_2}{\text{FiO}_2} \)

ii) \( \text{PVR} = \frac{\text{PA pressure} - \text{LA pressure}}{\text{PA flow}} \times 80 \)

iii) Lung compliance = \( \frac{\text{Tidal Volume}}{\text{Peak airway pressure} - \text{PEEP}} \)

In the control group, pulmonary haemodynamics and oxygenation remained stable throughout. The mean oxygenation capacity was 459 (±36.87)mmHg at 90 minutes and 472.33 (±36.69)mmHg at 180 minutes (Figure 5.3). The PVR was calculated as 783.64 (±30.73) dynes/sec/cm\(^2\) in the first 15 minutes, increasing by 4% to 818.29 (±11.54) dynes/sec/cm\(^2\) at 90 minutes, where it then remained stable until the end of perfusion (Figure 5.4). The lung compliance was 17.72 (±1.98)L/kPa at 60 minutes and increased to 19.27 (±3.37)L/kPa at the end of perfusion (Figure 5.5).

![Figure 5.3: Oxygenation Capacity During EVLP](image)

*Figure 5.3: Oxygenation Capacity During EVLP*

The oxygenation capacity of the lungs at regular time-points during EVLP, calculated as \( \frac{\text{LA PO}_2 - \text{PA PO}_2}{\text{FiO}_2} \)

In the injury group, oxygenation capacity remained stable at 435.67 (±13.87)mmHg at 90 minutes, prior to injury, and 472.33 (±35.64)mmHg after injury and at the end of EVLP (Figure 5.3).
During the first 90 minutes of EVLP, the PVR decreased from 632.7 (±182.72) dynes/sec/cm$^2$ at 15 minutes, to 570.61 (±74.82) dynes/sec/cm$^2$ at 90 minutes. Following injury, there was then a 35% increase in the PVR to 770.65 (±110.34) dynes/sec/cm$^2$ at the end of EVLP (Figure 5.4). Lung compliance remained stable prior to injury, calculated as 19.12 (±2.98) L/kPa at 60 minutes and 19.2 (±1.22) L/kPa at 90 minutes. Following injury, there was then a 15% decrease in lung compliance to 16.29 (±4.89) L/kPa at the end of EVLP (Figure 5.5).

Figure 5.4: Pulmonary Vascular Resistance During EVLP
The vascular resistance of the lungs at regular time-points during EVLP, calculated as (PA pressure - LA pressure/PA flow)*80 where LA pressure = 0 in the Lund protocol

Figure 5.5: Lung Compliance During EVLP
The lung compliance at regular time-points during EVLP, calculated as Tidal Volume/(Peak airway pressure – PEEP)
**mtDNA Quantification**

Statistically, using a repeated measures general linear model, there was a significant change in cell-free mtDNA level over time during EVLP (p=0.042). Although there was no significant difference between the two groups at any time-point during EVLP (p=0.739), this may have been impacted by the low group numbers, as an increasing trend can be visualized within the injury model group. In both the control and injury groups, the concentration of cell-free mtDNA within the EVLP circuit at baseline, prior to connection of the lungs, was undetectable. At 60 minutes, the concentration of cell-free mtDNA was comparable between the two groups with a concentration of 17.16 (±29.73)ng/ml in the control group, and 19.9 (±34.46)ng/ml in the injury group. At 125 minutes, the concentration of cell-free mtDNA increased to 33.28 (±35.61)ng/ml in the control group and 43.44 (±38.96)ng/ml in the injury group, an increase of 93.9% and 118.3% from 60 minutes, respectively. At 180 minutes, the end of EVLP, the concentration of cell-free mtDNA in the control group fell to 21.7 (±20.02)ng/ml, yet remained 34.8% higher than at 60 minutes. In the injury group, the concentration of cell-free mtDNA continued to rise to 47.34 (±21.88)ng/ml at 180 minutes, an increase of 137.9% from the concentration at 60 minutes (Figure 5.6).

![mtDNA Concentration](image)

**Figure 5.6: Concentration of mtDNA During Standard EVLP and Following Injury**

*The concentration of cell-free mtDNA at regular time-points during EVLP. In the injury model, ischaemic injury was induced at 90 minutes for a period of 30 minutes.*

**Luminex® Analysis**

The concentration of 13 cytokines and chemokines were analysed in the perfusate samples collected during EVLP in both groups. The concentration of GM-CSF, IL-1α, IL-2 and IL-4 were undetectable in all of the perfusate samples collected. IFN-γ, IL-1β, IL-1Ra, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α were all detected in the perfusate samples during control EVLP (Figure 5.7) and injury EVLP (Figure 5.8).
The most predominant cytokine in the perfusate during both control and injury EVLP was TNF-α, followed by IFN-γ and IL-8. The remaining cytokines were detected at similar concentrations (Table 5.1). All of the cytokines detected increased in concentration throughout EVLP, and using a repeated measures general linear model, the difference in concentration over time was significant in all cytokines except IL-8 and IL-1RA (Table 5.1).
Table 5.1: The Concentration of Cytokines During EVLP

The mean (± SD) of all cytokines at regular time-points during EVLP. The general linear model assessed the difference in concentration of cytokines over all time-points during all EVLPs. TB=baseline

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control EVLP Group</th>
<th>Injury EVLP Group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB</td>
<td>T60</td>
<td>T125</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0</td>
<td>2.96±2.08</td>
<td>6.27±2.77</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.02±0.02</td>
<td>0.38±0.03</td>
<td>0.36±0.21</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.85±0.25</td>
<td>1.03±0.11</td>
<td>1.41±0.47</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0</td>
<td>0.01±0.01</td>
<td>0.12±0.09</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>0</td>
<td>0.01±0.01</td>
<td>0.29±0.22</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>0.01±0.01</td>
<td>0.76±0.4</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>0.04±0.03</td>
<td>0.27±0.15</td>
</tr>
<tr>
<td>IL-12</td>
<td>0</td>
<td>0</td>
<td>0.04±0.04</td>
</tr>
<tr>
<td>IL-18</td>
<td>0</td>
<td>0.06±0.04</td>
<td>0.17±0.03</td>
</tr>
</tbody>
</table>

Individual sample T tests were used to compare the individual cytokine concentrations between the control and injury EVLPs. Significance was only observed at 180 minutes in TNF-α and IL-1β, where p=0.045 and p=0.051, respectively (Figure 5.9).
Figure 5.9: Concentration of Individual Cytokines in Control and Injury EVLP

The concentration of individual cytokines at regular time-points during both control and injury EVLP.
5.5 Discussion

Since 2001 when Steen et al first reported their experience of using EVLP to assess donor lungs prior to transplantation, it has become an established technique in the field of lung transplantation (326). In addition to facilitating the use of marginal donor lungs, thereby optimising the donor organ pool and increasing transplant activity (356, 360, 367), more recently it has also been associated with superior post-transplant outcomes, with a reduced incidence of PGD (356, 366) and potentially rejection (371). The mechanism by which EVLP can impart such benefits remains poorly understood and has yet to be fully elucidated.

It has been observed that during EVLP, there is a non-specific cytokine storm (371). High levels of IL-1β and IL-8 have been shown to correlate with unsuccessful reconditioning, with IL-1β also appearing to correlate with inferior post-transplant outcomes (372, 373). In the previous study (Chapter 4), cell-free mtDNA was also present in the circulating perfusate during EVLP, the relevance of which is unknown.

mtDNA has been shown to be actively released from cells during injury and stress (399). Within the circulation, this cell-free mtDNA can then initiate immune cascades through TLR and STING signalling, and activation of the inflammasome (387, 399). In addition, increased levels of circulating cell-free mtDNA have previously been demonstrated to induce ALI in a rat model (387, 400). In view of this, one would expect that high circulating levels within the EVLP perfusate would be reflective of increased inflammation and lung injury. This study therefore aimed to elucidate whether levels of circulating cell-free mtDNA during EVLP correlated with successful reconditioning and post-transplant outcomes. A porcine model of EVLP was then used to determine whether cell-free mtDNA release was associated with ALI.

Within the perfusate samples from the DEVELOP-UK study, there was no significant difference in the level of circulating cell-free mtDNA at all three time-points, between the successful and unsuccessful EVLP groups. However, within the unsuccessful EVLP group, levels of cell-free mtDNA tended to be higher at 90 minutes and at the end of perfusion. The lack of significance may reflect the low numbers in the two groups, but also the degree of variability within the unsuccessful EVLP group. Interestingly, the range of cell-free mtDNA levels observed in the unsuccessful EVLP group was much greater than in the successful EVLP group, and perhaps suggests that not all lungs were truly non-transplantable. Our current assessment of whether lungs are acceptable for transplantation is somewhat subjective and is based upon oxygenation capacity and pulmonary haemodynamics. Yet such parameters may not accurately reflect lung function and the presence of injury. Indeed, Fisher et al have previously made this observation when they found no significant difference between the levels of neutrophils and cytokines in BAL samples collected from lungs that had been accepted and rejected for transplant, summising that the current selection criteria are poor discriminators of lung injury (106). In this study, in some of the perfusate samples collected from unsuccessful EVLPs low levels of circulating cell-free mtDNA were observed throughout, yet the lungs were deemed unsuitable.
for transplant according to current selection criteria. Therefore, although there was no significant difference between the levels of circulating cell-free mtDNA between the two EVLP groups, this may be more reflective of current selection criteria being ineffective, with poor sensitivity for lung function and therefore suitability for transplantation.

Interestingly when comparing the levels of cell-free mtDNA in the perfusates of successful EVLPs against 12-month survival, although not significant, cell-free mtDNA levels were much higher in the n=3 EVLPs that were associated with 12-month mortality. Lack of significance likely reflects the small numbers compared, however an increased trend of cell-free mtDNA was certainly observed in the EVLPs performed on lungs transplanted with poor outcomes. Again this highlights that perhaps the current selection criteria used to determine the suitability for transplantation are insensitive discriminators of lung function.

In the second part of this study, a porcine model of EVLP was used to determine whether a period of ischaemic injury was associated with an increase in cell-free mtDNA within the perfusate. In the control group, standard EVLP perfusion was performed for 180 minutes, during which time oxygenation capacity, lung compliance and pulmonary haemodynamics remained stable and fulfilled the criteria for transplantation. During EVLP, the levels of cell-free mtDNA within the perfusate steadily increased for 125 minutes. It then remained stable with a slight decline at 180 minutes. This is in contrast to the injury group, whereby following ischaemic injury, cell-free mtDNA levels progressively increased within the perfusate, with levels at 180 minutes being 138% higher than the levels seen before injury was induced. Following ischaemic injury, PVR was observed to increase and lung compliance to decrease. Therefore this would suggest that cell-free mtDNA was released following ischaemic injury and was reflective of ongoing injury. Interestingly, despite injury and the impact on pulmonary haemodynamics and lung compliance, the oxygenation capacity remained stable. This suggests that oxygenation capacity was not a sensitive indicator of lung function, and that perhaps it may decline late in lung injury.

Cytokine levels were assessed to further characterise the EVLP environment. In both the control and injury EVLP groups, a cytokine storm was observed with a significant change in levels over time. When comparing the levels between the two groups, TNF-α and IL-1β were found to be significantly higher in the EVLP injury group at 180 minutes. This is interesting as in previous human studies increased circulating levels of TNF-α and IL-1β have been associated with increased morbidity and mortality in patients with ALI and ARDS (50). More recently, it has also been demonstrated that higher IL-1β levels within the EVLP circuit correlated with tissue injury (372).

Following injury, although not significant, there were also increased levels of the pro-inflammatory cytokines IL-8, IFN-γ and IL-12 compared to in control EVLP, along with decreased levels of the anti-inflammatory cytokines IL-1Ra and IL-10. IL-6 has anti-inflammatory and pro-inflammatory properties. Its levels were observed to be lower following injury, suggesting that within the EVLP circuit, its anti-inflammatory properties predominate.
18 is known to initiate inflammatory immune cascades and levels were found in low concentrations in both control and injury EVLP. Therefore following injury, a pro-inflammatory environment was observed within the EVLP circuit that has the potential to negatively impact upon lung function, despite oxygenation remaining stable.

This study has demonstrated that circulating cell-free mtDNA within the perfusate could serve as a sensitive biomarker of inflammatory lung injury and a prognostic indicator for graft function during EVLP. In the porcine lung injury model, cell-free mtDNA levels were increased within the perfusate. Additionally, in human perfusate samples higher cell-free mtDNA levels were observed at the end of EVLP in lungs that were then associated with inferior 12-month survival. Furthermore, in the DEVELOP-UK study, the marked variation in cell-free mtDNA levels in lungs that were not utilised for transplantation highlights that our current selection criteria for transplantation are not sensitive measures of lung injury. Indeed, in the porcine model of EVLP injury, despite a significant rise in TNF-α and IL-1β levels within the circuit, coupled with a rise in other pro-inflammatory cytokines, oxygenation capacity remained stable, emphasising its relative disconnect from the inflammatory environment and potential lung injury observed. Therefore although oxygenation and pulmonary haemodynamics are easy to measure, this study suggests that they do not accurately reflect lung function. With this in mind, optimisation of the donor organ pool and improved transplant outcomes require the identification of objective and repeatable biomarkers of donor lung function prior to transplantation, and EVLP provides the platform to do this.

The findings of the study suggest that cell-free mtDNA is a potential biomarker of lung function, yet the level at which it is associated with injury has not been identified. Over recent years there has been increased interest in the role of circulating cell–free mtDNA in the pathogenesis of various diseases (399). Increased circulating levels have been found in patients with sepsis (401) and multi-organ dysfunction (402), and have been associated with increased mortality in ICU patients (389). Its role in transplantation however has not yet been explored and the area undoubtedly warrants further work.
CHAPTER 6

Is Cell-Free mtDNA a Biomarker of Graft Dysfunction Post Lung Transplantation?
6.1 Abstract

Background: Lung transplantation remains limited by a median survival of only 6 years secondary to the high incidence of rejection and graft dysfunction. Over recent years, there has been increased interest in the role of cell-free mtDNA as a biomarker of disease given that it is a potent immune activator that can mediate down-stream inflammatory processes. Indeed, it has since been associated with various autoimmune conditions, cardiac disease and cancer. Its role in lung transplantation has not yet been explored. This study aimed to determine whether increased levels of plasma cell-free mtDNA post-transplantation were associated with inferior outcomes, and specifically PGD, and therefore can it serve as a reliable biomarker of graft dysfunction.

Methods: n=14 patients were recruited into the study. Blood samples were collected pre-operatively and then at regular post-operative intervals until day 12 post-transplantation. Cell-free mtDNA was quantified in each sample using qPCR. CRP levels were recorded at all time-points correlating with mtDNA analysis. Clinical data was collected from all patients including PGD scores at 0hrs, 24hrs, 48hrs and 72hrs; length of mechanical ventilation, ICU stay and hospital stay; highest incidence of acute rejection in the first 6 months; incidence of BOS at 1-year; and mortality at 90-days and 1-year. Donor and recipient variables were recorded. Levels of cell-free mtDNA were then correlated with clinical outcomes, donor and recipient variables and levels of CRP.

Results: Circulating levels of cell-free mtDNA were observed in all patients pre- and post-transplant. Levels consistently declined from pre-transplant to cross-clamp release in all patients, suggesting that post-transplant levels were recipient-driven. There was a significant relationship between the levels of cell-free mtDNA observed over time and the incidence of BOS, with higher levels in patients who developed grade 2 BOS. There was a significant relationship between donor smoking history and cell-free mtDNA levels, with increased levels observed in the recipients of smoking donors. Although there was no significant relationship between levels of cell-free mtDNA and acute rejection, higher levels were observed at day 12 post-transplant, in patients who went on to develop acute rejection.

Conclusion: Cell-free mtDNA was present in all patients post-transplantation and appears to correlate with immune activity given its association with BOS. Its use as a potential biomarker in lung transplantation clearly warrants further investigation.
6.2 Introduction

Over recent years, there has been increased interest in the role of circulating cell-free mtDNA in the pathogenesis of disease. Cells are comprised of hundreds of mitochondria that are integral to their function due to their role in energy metabolism and calcium homeostasis (388). Each mitochondrion contains its own circular mtDNA genome that is extremely sensitive to oxidative damage, and it has been demonstrated that cellular injury can lead to the release of cell-free mtDNA into the circulation (388, 399). mtDNA is thought to be of bacterial origin and as such is high in unmethylated CpG repeats, thereby eliciting immune responses similar to bacteria (388). Within the circulation cell-free fragments can act as DAMPs, initiating downstream immune cascades via TLR and STING signalling and activation of the inflammasome (387, 399). Various studies have demonstrated the capability of polymorphonuclear leukocytes to actively release mtDNA in response to stress, where they can create nets of intertwined strands that trap invading microbes in a process called NETosis, in addition to being free within the circulation (403, 404).

Increased levels of circulating cell-free mtDNA have since been found in patients following trauma and sepsis (401) and in those with systemic inflammatory response syndrome (SIRS) and multi-organ dysfunction (387, 402). Increased levels have also been shown to correlate with increased morbidity and mortality in ICU patients (389). More recently, it has also been identified as a biomarker of disease, with increased levels observed in patients with rheumatoid arthritis (405), cardiomyopathy and ischaemic heart disease (406, 407), and cancer (408). However, its role in lung transplantation has yet to be established.

Through TLR signalling, mtDNA can activate MAPK, stimulating the release of pro-inflammatory cytokines with subsequent neutrophil recruitment and degranulation (387, 388). STING signalling also stimulates the release of interferons, amplifying the immune response (399). Additionally, cell-free mtDNA can act as potent, second-messenger molecule, activating the inflammasome, with downstream activation of IL-1β and IL-18 (385, 388). In the context of transplantation, increased inflammatory signalling may lead to augmentation of the recipient immune system, as well as the activation of passenger donor leukocytes. The subsequent inflammatory infiltration within the graft can then promote graft dysfunction and increased immunogenicity. In view of this, increased circulating levels of cell-free mtDNA following transplantation would be expected to be associated with increased morbidity and mortality.

On these grounds, a clinical study of lung transplantation was performed to determine whether levels of circulating cell-free mtDNA in the recipient post-transplantation correlated with clinical outcome, and specifically graft dysfunction.
6.3 Methods

A clinical study was developed, entitled “Exploring the mechanisms of homeostasis and disease before and after lung transplantation”, to determine whether levels of cell-free mtDNA post-transplantation correlated with graft function. The primary end-point was PGD grade. The secondary end-points were length of mechanical ventilation, ICU and hospital stay, incidence of rejection within the first 6 months, incidence of BOS at 1 year, and 90-day and 1-year mortality. Additionally, levels of C-reactive protein (CRP) were compared with levels of cell-free mtDNA, from samples taken at corresponding time-points.

6.3.1 Ethical Approval

The study was approved by the National Research Ethics Service (NRES) Committee North West – Liverpool Central (14/NW/0260).

6.3.2 Inclusion and Exclusion Criteria

Any patient on the active waiting list for first lung transplant or heart/lung transplant, aged between 16 - 70 years, were eligible for this study. Patients were excluded if they were awaiting re-transplantation or were unable to give informed written consent.

6.3.3 Sample Collection and Processing

Explanted tissue samples were collected at the time of transplant, where they were immediately snap frozen and stored at -80°C.

All patients had a baseline blood sample taken in the anaesthetic room prior to transplantation. Blood samples were then taken within 15 minutes of cross-clamp release, and then at the following time-points post cross-clamp release and reperfusion: 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, Day 4, Day 5, Day 6, Day 9 and Day 12. Note, in double lung transplant recipients, the time of the second and final cross-clamp release was used. Samples were taken until day 12, as all subjects uniformly remained as in-patients until this date.

Each blood sample was collected in 1 x EDTA Vacutainer and stored at 4°C prior to processing. Samples were processed within 2 hours of collection, at which point they were centrifuged at 500g for 10 minutes at 4°C. The plasma supernatants were then divided into 200µl aliquots and stored at -80°C prior to analysis.

6.3.4 Quantitative PCR

In the plasma samples, qPCR was performed to quantify cell-free mtDNA levels using the technique previously described in Chapter 2. Briefly, the plasma samples were thawed and
DNA extracted using the QIAamp® DNA Mini and Blood Mini Kit (QIAGEN, Manchester, UK), according to the manufacturer’s protocol and using the materials provided. The samples were mixed with Buffer AL and proteinase K and incubated at 56°C for 10 minutes. Following a series of centrifuge and buffer wash steps, extracted DNA was then eluted through the QIAamp® Mini spin column, and collected in labelled microcentrifuge tubes. If the extracted DNA samples were not analysed immediately, they were stored at -20°C.

Primers were designed using the Primer Express™ Software v3.0.1 (Life Technologies, Paisley, UK) and their homology to other genes assessed using BLAST. To identify mtDNA, human primers for Cytochrome B were used, as in Chapter 2.

| Cytochrome B Forward 5’ ACACATCAGACACAAACA 3’ |
| Cytochrome B Reverse 5’ GTAGCGAATAACTCTCATCCTGA 3’ |

The primer sets were synthesized and de-salted by Sigma Aldrich. Prior to use, they were re-suspended in nuclease-free water (Ambion, USA) to adjust their concentration to 25nM. mtDNA standard curve samples were then extracted. Samples were extracted from human explanted lung tissue, retrieved from patients consented for the study. Briefly, 0.1g of the explanted lung tissue was washed in PBS and homogenized. The suspension then underwent centrifugation at low and high speed, and the mitochondrial pellet was re-suspended in mitochondrial isolation buffer. mtDNA was then extracted from the isolated mitochondria and the concentration was quantified using a Nanodrop™ 2000 (Thermofisher Scientific, Waltham, MA, USA). Serial dilutions were subsequently performed to achieve a relative standard curve consisting of 20ng/µl, 10ng/µl, 5ng/µl, 2.5ng/µl, 1.25ng/µl and 0.625ng/µl. The standard curve was used to determine the efficiency of the Cytochrome B primers to detect their target human mtDNA, and enable quantification of the cell-free DNA in the unknown samples following PCR.

qPCR was then performed using a QuantStudio™12K Flex System with Power SYBR™ green PCR master mix (Life Technologies, Paisley, UK), as previously described in Chapter 2. Briefly extracted DNA from patient samples and standard curve samples were separately mixed with Power SYBR™ green, nuclease free water and corresponding forward and reverse primers, and then added in quadruplet to wells of a MicroAmp™ Optical 384-Reaction plate. Plates were then sealed and following a brief centrifuge step, qPCR was performed as previously described.

If there was any amplification in the blank sample or if there were multiple peaks in the melt curve, results were discarded and the plate repeated, as this would indicate primer dimer formation. The standard curve was then analysed to determine the efficiency of the PCR amplifications, by creating a semi-log regression line plot. A slope of -3.32 indicates 100% efficiency with an R² value of 1. Regression line plots with R² values > 0.95 were accepted.

Using the standard curve calculation and the sample C_T values, DNA was then quantified for each plasma sample. If the C_T value was > 40, this was indicative of minimal amounts of target
nucleic acid being present in the sample, therefore levels could not be quantified and cell-free DNA was deemed undetectable.

6.3.5 Clinical End-Points

The levels of cell-free mtDNA observed were correlated with clinical end-points collected prospectively throughout the study. The primary end point measured was the incidence of primary graft dysfunction. The secondary end-points were length of mechanical ventilation, ICU and hospital stay, highest incidence of acute rejection in the first 6 months, incidence of BOS at 1 year, and 90-day and 1-year mortality. In addition, donor and recipient variables were assessed to determine whether they influenced the mtDNA levels observed.

Primary End-Point

Primary graft dysfunction was measured and graded according to the ISHLT recommended guidelines at 0 hours, 24 hours, 48 hours and 72 hours (Table 6.1). 0 hours was defined as within 6 hours of cross-clamp release and reperfusion.

Table 6.1: The ISHLT Grading System for PGD

A grading system to determine the severity of PGD based upon CXR infiltration and oxygenation capacity (163). This grading system was used for the classification of patients with PGD in this study.

<table>
<thead>
<tr>
<th>PGD Grade</th>
<th>PaO$_2$:FiO$_2$ ratio (mmHg)</th>
<th>Bilateral CXR infiltrates (consistent with pulmonary oedema)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt; 300</td>
<td>Absent</td>
</tr>
<tr>
<td>1</td>
<td>&gt; 300</td>
<td>Present</td>
</tr>
<tr>
<td>2</td>
<td>200-300</td>
<td>Present</td>
</tr>
<tr>
<td>3</td>
<td>&lt; 200</td>
<td>Present</td>
</tr>
</tbody>
</table>

CXRs were performed and independently analysed by two respiratory physicians to determine the presence or absence of infiltrates. Both physicians knew the clinical background of each patient and as such were able to ensure that any CXR infiltration was not secondary to cardiogenic pulmonary oedema, pneumonia or hyperacute rejection. If there was discrepancy in the CXR interpretation between physicians, a third physician was used to verify the presence of infiltrates. Arterial blood gases were evaluated within 6 hours of all four time-points to determine the PaO$_2$ to FiO$_2$ ratio. The worst value was then used. Any patient requiring extra-corporeal membrane oxygenation was classified as grade 3. Any patient requiring NO with a FiO$_2$ > 0.5 for more than 48 hours were also classified as grade 3. If the patient was no longer mechanically ventilated and required a FiO$_2$ < 0.3, or were on nasal cannulae, the grade was dependent solely on the presence of CXR infiltration.
**Secondary End-Points**

The secondary end-points measured were:

i) Length of mechanical ventilation - defined as the time from intubation to the time of successful extubation, whereby the patient no longer required mechanical ventilatory support

ii) Length of ICU stay – defined as the time from the initial admission to the initial discharge from ICU. Re-admission was not included in this length of stay

iii) Length of hospital stay – defined as the time from admission for transplant to the date of discharge

iv) Highest incidence of acute rejection within the first 6 months – whereby acute rejection was graded from trans-bronchial biopsy samples assessed by the hospital histopathologist. The worst rejection grade within the first 6 months was used

v) Incidence of BOS at 1 year – whereby BOS was graded according to ISHLT guidelines, dependent upon deterioration from baseline lung function (Table 6.2). Baseline lung function was defined as the average of the two best FEV\textsubscript{1} values obtained when the patient was stable, and recorded greater than 3 weeks apart

vi) 90-day and 1-year mortality.

**Table 6. 2: The Grading System for BOS**

Patients were coded as having specific BOS grades according to this established diagnostic criteria. FEF\textsubscript{25-75%} is forced expiratory flow at 25-75% of vital capacity. Op is probable early BOS that does not yet fulfill the criteria for Grade 1. Baseline lung function is the average of 2 best values ≥ 3 weeks apart, when stable post-transplantation. The decline in lung function cannot be attributable to any other cause, such as infection and acute rejection.

<table>
<thead>
<tr>
<th>BOS Grade</th>
<th>Spirometry Results (% of Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>FEV\textsubscript{1} &gt; 90% and FEF\textsubscript{25-75%} &gt; 75%</td>
</tr>
<tr>
<td>0p</td>
<td>FEV\textsubscript{1} 81-90% and/or FEF\textsubscript{25-75%} ≤ 75%</td>
</tr>
<tr>
<td>1</td>
<td>FEV\textsubscript{1} 66-80%</td>
</tr>
<tr>
<td>2</td>
<td>FEV\textsubscript{1} 51-65%</td>
</tr>
<tr>
<td>3</td>
<td>FEV\textsubscript{1} ≤ 50%</td>
</tr>
</tbody>
</table>

**Donor and Recipient Variables**

To determine whether donor and recipient variables influenced the levels of cell-free mtDNA observed post-transplantation, the following donor and recipient variables were recorded for all patients and correlated with cell-free mtDNA levels:

i) Recipient diagnosis

ii) Type of transplant i.e. single, double or heart/lung

iii) Donor smoking and drug history, and ethnic origin

iv) Type of donor i.e. DCD or DBD

v) Total ischaemic time
**CRP Levels**

To determine whether there was a relationship between cell-free mtDNA and CRP levels, routine blood samples were taken at corresponding time-points and the CRP level analysed by the hospital biochemistry laboratory.

### 6.3.6 Statistical Analysis

Statistical analysis was performed using SPSS® v.22.0. Data normality was determined and normally distributed data was then expressed using mean ± SD. Non-normally distributed data was expressed using median and interquartile range. GraphPad® Prism v.7.0 was utilised to formulate graphs.
6.4 Results

Complete samples and clinical data sets were collected from n=14 patients.

6.4.1 Clinical End-Points

The donor and recipient variables are outlined in Table 6.3, and the clinical end-points in Table 6.4.

Table 6.3: Donor and Recipient Variables of the Recruited Patients

The donor and recipient variables of all the patients transplanted in this study. SLE = systemic lupus erythematosus; OB = obliterative bronchiolitis; HTN = hypertension; CF = cystic fibrosis; Hx = history and ischaemic time equates to total ischaemic time; Px = patient

<table>
<thead>
<tr>
<th>Px</th>
<th>Recipient Diagnosis</th>
<th>Type of Transplant</th>
<th>Type of Donor</th>
<th>Donor Smoke Hx</th>
<th>Donor Drug Hx</th>
<th>Donor Ethnicity</th>
<th>Ischaemic Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fibrosis</td>
<td>Double</td>
<td>DCD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>444</td>
</tr>
<tr>
<td>2</td>
<td>SLE with OB</td>
<td>Double</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>341</td>
</tr>
<tr>
<td>3</td>
<td>Emphysema</td>
<td>Double</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>376</td>
</tr>
<tr>
<td>4</td>
<td>Emphysema</td>
<td>Double</td>
<td>DBD</td>
<td>Yes</td>
<td>No</td>
<td>Caucasian</td>
<td>366</td>
</tr>
<tr>
<td>5</td>
<td>Fibrosis</td>
<td>Double</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>570</td>
</tr>
<tr>
<td>6</td>
<td>Pulmonary HTN</td>
<td>Heart/Lung</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>255</td>
</tr>
<tr>
<td>7</td>
<td>Emphysema</td>
<td>Double</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>700</td>
</tr>
<tr>
<td>8</td>
<td>Fibrosis</td>
<td>Single</td>
<td>DBD</td>
<td>Yes</td>
<td>No</td>
<td>Caucasian</td>
<td>257</td>
</tr>
<tr>
<td>9</td>
<td>Fibrosis</td>
<td>Single</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Asian</td>
<td>229</td>
</tr>
<tr>
<td>10</td>
<td>Emphysema</td>
<td>Double</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>297</td>
</tr>
<tr>
<td>11</td>
<td>CF</td>
<td>Double</td>
<td>DCD</td>
<td>Yes</td>
<td>No</td>
<td>Caucasian</td>
<td>242</td>
</tr>
<tr>
<td>12</td>
<td>Fibrosis</td>
<td>Double</td>
<td>DCD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>384</td>
</tr>
<tr>
<td>13</td>
<td>Fibrosis</td>
<td>Single</td>
<td>DCD</td>
<td>Yes</td>
<td>Yes</td>
<td>Caucasian</td>
<td>212</td>
</tr>
<tr>
<td>14</td>
<td>Emphysema</td>
<td>Double</td>
<td>DBD</td>
<td>No</td>
<td>No</td>
<td>Caucasian</td>
<td>196</td>
</tr>
</tbody>
</table>

Of the n=14 patients, the majority had a recipient diagnosis of pulmonary fibrosis (n=6) or emphysema (n=5). The remaining patients had either systemic lupus erythematosus with obliterative bronchiolitis, pulmonary hypertension or CF. n=10 patients received a double lung transplant, n=3 received a single lung transplant and n=1 received a combined heart/lung transplant. The majority of donors were DBD donors (n=10), compared with n=4 DCD donors. n=4 donors had a positive smoking history and n=1 of those also had a positive drug history. Donors were all of Caucasian ethnicity, except n=1, which was of Asian ethnicity. The mean total ischaemic time was 347.79±144.48 minutes, with a range of 196-700 minutes.
### Table 6.4: Primary and Secondary End-Points of Recruited Patients

The outcomes of all primary and secondary end-points for the patients transplanted in this study. Px. = patient; MV = mechanical ventilation; Hosp. = hospital and AR = acute rejection

<table>
<thead>
<tr>
<th>Px.</th>
<th>PGD Score (time in hrs)</th>
<th>Length (days)</th>
<th>Highest AR in 6/12</th>
<th>BOS Grade at 1 yr</th>
<th>90-day mortality</th>
<th>1 year mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>ICU stay</td>
<td>MV</td>
<td>Hosp. stay</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>9</td>
<td>3</td>
<td>24</td>
<td>A1</td>
<td>Alive</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>14</td>
<td>A0</td>
<td>Alive</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>101</td>
<td>A0</td>
<td>Alive</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>15</td>
<td>A1</td>
<td>Alive</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>44</td>
<td>36</td>
<td>64</td>
<td>A2</td>
<td>Alive</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>31</td>
<td>5</td>
<td>43</td>
<td>A2</td>
<td>Alive</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>21</td>
<td>15</td>
<td>37</td>
<td>A0</td>
<td>Alive</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>15</td>
<td>A2</td>
<td>Alive</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>21</td>
<td>A2</td>
<td>Alive</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>29</td>
<td>A1</td>
<td>Alive</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>37</td>
<td>A2</td>
<td>Alive</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>16</td>
<td>16</td>
<td>28</td>
<td>A1</td>
<td>Alive</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>28</td>
<td>A2</td>
<td>Alive</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>17</td>
<td>A2</td>
<td>Alive</td>
</tr>
</tbody>
</table>

n=8 patients had at least one PGD score ≥1 in the first 72 hours post-transplant. However only n=4 patients had a PGD score ≥1 at 72 hours. No patient had a PGD score ≥1 at all time-points within the first 72 hours, whereas n=5 patients had a PGD score of 0 at all time-points. Mean length of mechanical ventilation was 6.36 (±9.9) days, ICU stay 11.64 (±12.35) days and hospital stay 33.79 (±23.64) days. n= 3 patients had A0 rejection throughout the first 6 months of transplant, however n= 4 patients had at least one episode of A1 rejection and n=7 patients had at least one episode of A2 rejection. n=3 patients had grade 2 BOS at 1-year, with the remainder having no evidence of BOS. All patients were alive at 90 days and n=1 patient died within the first year.

### 6.4.2 mtDNA Quantification

Cell-free circulating mtDNA was detected in the plasma of all patients both pre- and post-transplantation. In all patients, the level of cell-free mtDNA pre-transplant was greater than the level detected at cross-clamp release.

Following cross-clamp release, there was a marked fluctuation in the level of circulating cell-free mtDNA in all patients within the first 72 hours post-transplantation. In some patients the levels of cell-free mtDNA then stabilized, whereas in others, fluctuation continued throughout the recorded time-period and varied between patients (Figure 6.1).
Figure 6.1: Levels of mtDNA Pre- and Post-Transplantation
The concentration of cell-free mtDNA quantified in the plasma of blood samples taken at regular time-points both pre- and post-transplantation for each patient

6.4.3 Relationship Between mtDNA and Primary End-Point

There were not enough PGD scores ≥1 to accurately determine whether there was a statistical relationship between levels of cell-free mtDNA and PGD scores. Therefore only descriptive statistics were assessed. The mean levels of cell-free mtDNA and the PGD scores at the corresponding time-points are documented in Table 6.5.

Table 6.5: Concentration of mtDNA and PGD Scores at Defined Time-Points
The mean (±SD) concentration of cell-free mtDNA quantified at the specific time-points correlating with the corresponding PGD scores. Blank cells represent time-points where n=0 were graded the corresponding PGD score.

<table>
<thead>
<tr>
<th>PGD score</th>
<th>Mean (± SD) mtDNA Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hours</td>
</tr>
<tr>
<td>0</td>
<td>1744.2(±938.2)</td>
</tr>
<tr>
<td>1</td>
<td>923.5(±664)</td>
</tr>
<tr>
<td>2</td>
<td>1909.4</td>
</tr>
</tbody>
</table>

No correlation was observed between the levels of cell-free mtDNA and the PGD scores (Figure 6.2).
Figure 6.2: Concentration of mtDNA and PGD Scores at Defined Time-Points
The mean (±SD) concentration of cell-free mtDNA quantified at the specific time-points correlating with the corresponding PGD scores.

6.4.4 Relationship Between mtDNA and Secondary End-Points

Length of Mechanical Ventilation, ICU Stay and Hospital Stay
There was no relationship between levels of cell-free mtDNA and length of mechanical ventilation, ICU stay and hospital stay.

Mortality at 90-days and 1-year
It was not possible to determine a relationship between 90-day and 1-year mortality and cell-free mtDNA levels, as all patients were alive at 90-days and only one patient did not survive to 1-year.

Highest Incidence of Acute Rejection
There was a marked variation over time in the levels of cell-free mtDNA in the grade of rejection groups. Therefore a GEE analysis was not performed as the results would be misleading. Subsequently, only descriptive statistics were performed. The mean levels of cell-free mtDNA at 6 hours and 12 days according to the highest rejection grade observed within the first 6 months are outlined in Table 6.6.
Table 6.6: Concentration of mtDNA at Defined Time-Points and Rejection Grade

Patients were split into rejection groups according to the highest grade of rejection diagnosed within the first 6 months. The mean (±SD) concentration of cell-free mtDNA quantified was determined at defined time-points for each rejection group and tabulated.

<table>
<thead>
<tr>
<th>Acute Rejection Grade</th>
<th>Mean (±SD) mtDNA Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hrs</td>
</tr>
<tr>
<td>A0</td>
<td>2047.2 (±1382.2)</td>
</tr>
<tr>
<td>A1</td>
<td>1527.6 (±843.3)</td>
</tr>
<tr>
<td>A2</td>
<td>1527.3 (±803.2)</td>
</tr>
</tbody>
</table>

In patients who did not develop rejection, the mean concentration of cell-free mtDNA was initially highest at 6 hours but then dropped to the lowest level at day 12. The reverse was true for patients who went on to develop A2 rejection (Figure 6.3).

Figure 6.3: Concentration of mtDNA at Defined Time-Points and Rejection Grade

The mean (±SD) concentration of cell-free mtDNA at defined time-points for patients with each rejection grade.

Incidence of BOS at 1 year

On GEE analysis, there was a relationship between the level of cell-free mtDNA and the incidence of BOS over time (GEE interaction term p<0.001). Specifically, the values appear to change by a different amount over time depending on BOS group (Figure 6.4 and Figure 6.5).
From 72 hours onwards, higher levels of cell-free mtDNA were observed in patients with grade 2 BOS compared to those with grade 0 BOS. On day 12 (288 hours), the concentration of cell-free mtDNA in patients with grade 0 BOS and grade 2 BOS was 1961.52 (±1201.05) and 2330.75 (±525.18), respectively.
6.4.5 Relationship Between mtDNA and Donor and Recipient Variables

Recipient Diagnosis
There were too few patients with different disease phenotypes to perform formal statistical analysis. The mean concentration of cell-free mtDNA pre-transplantation is outlined in Table 6.7.

Table 6.7: Concentration of mtDNA Pre-Transplant According to Recipient Diagnosis
The mean (±SD) concentration of cell-free mtDNA pre-transplantation dependent upon recipient diagnosis

<table>
<thead>
<tr>
<th>Recipient Diagnosis</th>
<th>n number</th>
<th>Mean Concentration of mtDNA ± SD (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrosis</td>
<td>6</td>
<td>2523.3 (±1440.3)</td>
</tr>
<tr>
<td>Systemic Lupus Erythematosus</td>
<td>1</td>
<td>380.6</td>
</tr>
<tr>
<td>Emphysema</td>
<td>5</td>
<td>2568.2 (±1368.5)</td>
</tr>
<tr>
<td>Pulmonary Hypertension</td>
<td>1</td>
<td>2867.3</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>1</td>
<td>3753.0</td>
</tr>
</tbody>
</table>

Type of Transplant
In the subject population, there was n=1 heart/lung transplant and n=3 single lung transplants, with the remainder double lung transplants. No formal statistical analysis could therefore be performed. The levels of cell-free mtDNA fluctuated across all three groups over time.

Donor Smoking and Drug History, and Ethnic Origin
It was not possible to determine a relationship between donor drug abuse and ethnic origin, as there was only n=1 donor with a drug history and n=1 donor that was non-caucasian.

On GEE analysis, there appeared to be a significant difference in how levels of cell-free mtDNA changed over time, according to whether patients had a smoking or non-smoking donor (GEE interaction term p<0.001) (Figure 6.6 and Figure 6.7).
Figure 6. 6: Concentration of mtDNA According to Donor Smoking History
The mean (±SD) concentration of cell-free mtDNA at all time-points post-transplantation dependent on donor smoking history.

Figure 6. 7: Trendline of mtDNA Concentration According to Donor Smoking History
Trendline of the mean concentration of cell-free mtDNA at all time-points post-transplantation dependent on donor smoking history.

**Type of Donor**
Due to the degree of variability in levels of cell-free mtDNA over time, in patients with DCD or DBD donors, in addition to the small sample size of DCD donors (n=4), formal statistical analysis was not performed.

**Total Ischaemic Time**
No relationship was observed between the levels of cell-free mtDNA and the total ischaemic time (p>0.05; Pearson Correlation).
6.4.6 Relationship Between mtDNA and CRP

There was no correlation between high levels of cell-free mtDNA and high levels of CRP between patients (p=0.064; Pearson Correlation). However, there was a weak negative correlation between CRP level and cell-free mtDNA level within patients (p=0.039; Parameter Estimates Table).
6.5 Discussion

mtDNA is known to be a potent DAMP, capable of mediating a plethora of downstream immune cascades via TLR and STING signalling and activation of the inflammasome (387, 399). TLR signalling results in MAPK activation and the release of pro-inflammatory cytokines with neutrophil recruitment (387, 388). Interferons are released secondary to STING signalling, and the inflammasome activation leads to the release of IL-1β and IL-18 (385, 388, 399). Although considered to be an adaptive response, such heightened immune activity may have detrimental consequences. Indeed, increased circulating levels of cell-free mtDNA have been found in patients following trauma and sepsis (401), and in those with SIRS and multi-organ dysfunction (387, 402), with increased levels correlating with increased morbidity and mortality in ICU (389).

Moreover, it has recently been implicated in the pathogenesis of inflammatory diseases, with increased levels observed in patients with rheumatoid arthritis (405), cardiomyopathy and ischaemic heart disease (406, 407), and cancer (408). However its role in lung transplantation has yet to be established.

It has already been established that following lung transplantation, there is a donor and recipient immune cell interaction that results in an inflammatory cell infiltration within the graft. Furthermore, heightened interaction can lead to increased allore cognition and subsequent acute rejection. Therefore the capability of cell-free mtDNA to increase inflammatory signalling, thereby augmenting the activation of passenger donor leukocytes and the recipient immune response, could have the potential to drive graft destruction and increase immunogenecity. This study was therefore performed to delineate the relationship between circulating levels of cell-free mtDNA and the incidence of PGD, and to determine whether increased levels are associated with inferior outcomes, including the incidence of acute rejection and BOS.

All patients were found to have cell-free mtDNA both pre- and post-transplantation. High levels of cell-free DNA have been found in patients with autoimmune diseases such as systemic lupus erythematosus, and levels correlate with disease activity (409). The role of cell-free DNA, and specifically mtDNA in chronic respiratory diseases, has yet to be established. However consistently high levels were observed in all patients with COPD and pulmonary fibrosis, suggesting that cell-free mtDNA may be reflective of chronic respiratory disease activity, and perhaps confirms the inflammatory component of these disease phenotypes. The highest level observed was in the patient with CF (n=1), however this patient was also the most unwell pre-transplant, having been admitted for treatment of sepsis with multi-organ dysfunction prior to transplantation. The high levels may therefore be in keeping with previous studies correlating cell-free mtDNA with sepsis and multi-organ dysfunction, rather than being representative of the recipient diagnosis.

Interestingly, all patients had significantly higher levels of cell-free mtDNA pre-transplant than upon cross-clamp release, which suggests that the subsequent post-transplant trend is recipient-driven. Following cross-clamp release and reperfusion, the levels of cell-free mtDNA
fluctuated considerably between patients, with no clear pattern observed. However the study was limited by the small sample size, and therefore the degree of normal heterogeneity between transplant recipients themselves may have influenced the fluctuating trends observed. Furthermore the lack of sufficient powering also limited statistical analysis.

Although no relationship was observed between the levels of cell-free mtDNA and incidence of graft dysfunction, analysis was limited by the lack of PGD scores >0. Furthermore, it may also highlight the subjective nature of PGD scoring systems, being reliant upon CXR interpretation, which may not accurately reflect organ dysfunction. To limit the degree of individual interpretive error, two independent physicians were used to corroborate CXR interpretation in this study, with 93% concordance. Yet still, the use of CXRs to diagnose graft dysfunction may be suboptimal. ARDS and ALI have been defined based upon PaO\textsubscript{2} to FiO\textsubscript{2} ratios alone, but in PGD dysfunction the initial diagnosis is based upon the presence of CXR infiltrates. Although infiltration is likely reflective of graft dysfunction, its presence may be indicative of more severe dysfunction, and as such, mild cases of graft dysfunction may not be accounted for. Such low levels of persistent graft dysfunction may in itself be pathogenic, with a prolonged inflammatory burden that may promote the development of chronic graft dysfunction. Indeed, recent studies have implicated persistent episodes of A1 rejection with the development of BOS (263-265), suggesting that persistent low-grade inflammation may be just as detrimental as single significant bouts. Therefore, although no correlation was observed in this study, further work is required to define the relationship between levels of cell-free mtDNA and PGD.

Interestingly, levels of cell-free mtDNA over time, were significantly associated with the incidence of BOS, with persistently higher levels of cell-free mtDNA observed from 72 hours up to day 12 in patients who went on to develop grade 2 BOS, compared to those who did not. The pathogenesis of BOS remains unclear, however it appears to be driven by a heightened alloimmune response to specific triggers. PGD and rejection have been implicated in its development, as well as infection, whereby the alloimmune response leads to cellular infiltration and aberrant fibrosis (258). Therefore, if levels of circulating cell-free mtDNA are reflective of immune activity, heightened and persistent elevation in the early post-operative period may suggest increased immune priming, paving the way for later aberrant activity. Furthermore, if levels of cell-free mtDNA post-transplantation can serve as a predictive biomarker of BOS, early attenuation of the immune response in patients with increased levels may lead to a reduction in the development of chronic graft dysfunction.

Although acute rejection has been implicated in the development of BOS, there was no significant relationship observed between the levels of cell-free mtDNA and the incidence of different rejection grades within the first 6 months. However, analysis was limited by the small sample size and the degree of fluctuation in levels over time between different rejection groups. Nonetheless, on review of cell-free mtDNA levels on day 12, mean plasma concentrations were higher in patients who developed more severe rejection episodes. This is interesting given that in the early reperfusion period at 6 hours, the reverse was true. As in BOS, it may be the
persistent and heightened elevation of cell-free mtDNA in the later post-operative period that is more pathogenic. This clearly requires further exploration, as if delayed or persistent elevation in levels of cell-free mtDNA are early predictors of rejection, it would facilitate pre-emptive intervention to limit its occurrence.

The use of smoking donors in lung transplantation has long been debated, and it remains to be a significant risk factor for the development of PGD (172). Bonser et al have previously demonstrated inferior survival outcomes in recipients of donors with a positive smoking history, and in particular, the incidence of graft failure after 90 days, including BOS, was higher in this cohort (410). Interestingly, in this study there was a significant relationship observed between how the levels of cell-free mtDNA changed over time, according to whether the donor had a positive or negative smoking history. Increased levels were persistently found in the plasma of recipients of lungs from smoking donors. Given the debate regarding whether smoking donors should be used in transplantation, this finding is intriguing as it may well indicate that smoking increases the inflammatory signalling of donor immune cells. Upon transplantation this could drive heightened immune responses with resultant cell injury and mtDNA release. Furthermore, this cell-free mtDNA can then continue to promote hyper-alloresponsiveness. This is of particular interest in the context of this study, given the association between increased levels of mtDNA and the incidence of BOS. The area therefore requires further exploration however, if smoking donors do promote heightened immune activity post-transplantation, a subsequent modified immunosuppressive regime may then facilitate their use.

Although this study was limited by the small study size and the subsequent impact upon statistical analysis, interesting trends were observed. Whilst results should undoubtedly be interpreted with caution, the role of cell-free mtDNA in lung transplantation clearly warrants further exploration. This study demonstrates that circulating cell-free mtDNA is present post-transplantation and can be observed at high levels. The association of increased levels with the development of BOS and acute rejection suggests that cell-free mtDNA may be a reliable indicator of immune activity. Additionally, high levels of cell-free mtDNA were not associated with high levels of CRP, suggesting that it is not simply a marker of generic inflammation. The marked fluctuation in the early post-transplant period was difficult to elucidate. Additionally, it may be that it is the delayed or persistent elevation in cell-free mtDNA in the later post-operative period that is more pathogenic. Furthermore, given the small sample size, no clear cut-off as to what is the acceptable range of circulating cell-free mtDNA was determined. Observationally, persistently lower levels appeared to be associated with more favourable outcomes. Indeed, patient 2, whose levels of cell-free mtDNA never peaked above 600ng/ml, had no evidence of PGD, with no episodes of acute rejection or evidence of BOS, and has remained well, with no hospital re-admissions and an FEV₁ of >100% predicted.

Therefore further studies need to be performed to determine whether cell-free mtDNA can serve as a predictive biomarker of graft function in lung transplantation. Moreover, if increased levels are associated with heightened immune activity, it has the potential to have significant
consequences in the field of lung transplantation. We know that lung transplantation remains limited by the high incidence of graft dysfunction and acute rejection, and current immunosuppressive regimes have remained unchanged for many years. Current practice is to treat lung transplant recipients with induction immunosuppression followed by maintenance therapy with a cocktail of a calcineurin inhibitor, cell-cycle inhibitor and a steroid, all of which are associated with significant side effects. Yet despite achieving adequate levels of the specific therapies, some patients still develop acute rejection, whilst others do not. This would suggest the approach to achieve generic target levels is not appropriate. If cell-free mtDNA can give an indication of the level of immune activity, immunosuppression regimes may then be attenuated according to individual patients and their immune response. Furthermore, if increased levels of cell-free mtDNA correlate with the incidence of acute rejection, plasma monitoring could abrogate the need for the invasive trans-bronchial biopsies required to diagnose acute rejection.
CHAPTER 7

Final Discussion
7.1 Discussion

Lung transplantation has evolved following the initial success of James Hardy, Bruce Reitz and Joel Cooper, and the procedure is now performed across multiple centres worldwide. It has become an established treatment for patients with end-stage chronic respiratory disease that has progressed despite maximal medical therapy. However, as the incidence of chronic respiratory diseases has increased, so has the need for lung transplantation. In 2015, 4122 lung transplants were performed worldwide, in stark contrast to the 5 performed 30 years earlier (6). Yet despite this increase in transplant activity, demand for donor organs has now exceeded supply, leading to increased waiting list times and subsequent mortality (10). Furthermore, the success of lung transplantation has remained limited by a median survival of only 6 years, secondary to a high incidence of graft dysfunction and allograft rejection (6).

It is well established that the donor lung is particularly susceptible to injury at the time of death, whereby the cytokine and catecholamine storm that ensues results in haemodynamic instability and metabolic compromise (16, 17, 19-21). There is a subsequent inflammatory cell infiltration, which primes the donor lung and increases MHC expression. This cascade of events can be detrimental to the graft in two ways. Firstly, it may impact upon lung function and oxygenation, potentially rendering the organ unsuitable for transplantation, which explains why only 20% of donated lungs are utilised. Secondly, should the donor lungs be accepted for transplantation, they are flushed with preservation solution and then undergo CSP prior to transplantation. This period of ischaemia contributes further to inflammation and also activates complement cascades. It is in this inflamed and immunologically primed state that donor lungs are transplanted into the recipient, whereby upon revascularisation the activated donor immune compartment drives allorecognition both locally within the graft and also systemically following migration into the recipient circulation. The series of events that ensue ultimately lead to recipient alloreactivity, T cell recruitment, graft dysfunction and rejection.

The donor immune compartment and its initial revascularisation within the recipient is therefore crucial to the development of graft dysfunction, rejection and subsequent failure post-transplantation. Yet in current practice, it is largely ignored, with immunosuppression therapy focused solely upon the recipient immune response. Furthermore, the criteria used to determine the suitability of an organ for transplant may not accurately reflect the functionality and recoverability of the lung post-transplantation. The advent of EVLP has not only facilitated the extended evaluation of donor organs prior to transplant, but it has also provided a platform by which the donor immune compartment and the effects of initial reperfusion can be further explored and potentially manipulated.

In light of this, the aim of this PhD was to characterise the donor immune compartment and to examine the hypothesis that reperfusion of the donor lung leads to cellular diapedesis, altering the signalling profile of the graft and directly impacting upon clinical outcome. Furthermore,
studies were performed to determine whether donor leukocyte transfer could be manipulated, and to identify measurable circulating biomarkers of graft dysfunction.

In the first part of this thesis, the immediate migratory pattern of leukocytes from the donor lung was characterised. Upon reperfusion of the donor lung, either via a secondary preservation flush (Chapter 3) or using EVLP (Chapter 4), there was a marked migration of donor leukocytes that would otherwise mobilise into the recipient circulation. As donor leukocytes can drive recipient alloimmune responses, their removal could be of benefit following transplantation.

Within the mobilised leukocyte repertoire, a predominance of T cells was observed, and specifically cytotoxic T cells. These cells are capable of secreting high levels of perforins and pro-inflammatory cytokines, activating inflammatory cascades within the recipient and promoting inflammatory cell infiltration and up-regulation of MHC within the graft. In the context of transplantation, this will drive the recruitment of recipient immune cells to the graft, including CD4+ and CD8+ T cells, which are considered the primary leukocytes responsible for graft rejection. Donor macrophages and monocytes also migrated from the lung following reperfusion. Macrophages and monocytes constitutively express MHC and can facilitate direct allorecognition by directly self-presenting donor MHC to recipient T cells. The peak of leukocyte migration was observed within the first 30 to 60 minutes of reperfusion, suggesting that these cells are of endothelial or marginal origin. Removal of these donor leukocytes is therefore feasible as a therapeutic intervention, and clearly this warrants further investigation. Indeed previous studies have demonstrated that removal of donor leukocytes reduces the incidence of acute rejection (218, 371). Additionally, within the porcine model of EVLP transplant (Chapter 2), an up-regulation of pro-survival signalling pathways and down-regulation of mitochondria associated cell death molecules was observed within the EVLP transplanted lung tissue. These findings suggest that EVLP not only facilitates the removal of donor leukocytes prior to transplantation, but also reduces graft immunogenicity via the induction of cell and mitochondrial survival pathways. Again this indicates that EVLP is more than an evaluation tool. Unfortunately it is very difficult to evaluate this in humans, as in order to fully elucidate the direct effect of leukocyte depletion post-transplant, a reduced immunosuppressive regime would be required. On these grounds further preclinical studies are necessary to assess if alterations to the molecular profile of the lung also contribute to an impeded recipient immune response to the graft.

Continual reperfusion with a non-recirculating perfusate (Chapter 4) effectively removed over 9 billion leukocytes that would otherwise have entered the recipient circulation and interacted with the recipient immune system to drive enhanced allorecognition. A broad spectrum of donor leukocytes were removed, with the dominant population being immature neutrophils, followed by memory T cells, monocytes, other T cell subsets, and NK cells. These leukocyte subsets are all capable of mediating a range of immune cascades and promoting cytokine release. The low concentration of Tregs further confirms that the donor lung compartment that mobilises during early reperfusion is pro-inflammatory rather than regulatory in nature. Given the traumatic
nature of reperfusion this is probably to be expected, as the lung has experienced death processes in the donor, cold storage and surgical intervention. The identification of large numbers of immature neutrophils within the continual flush perfusate also warrants further work. These cells would be expected within the airway, but given their immediate presence following reperfusion there are clearly significant populations in the endothelial or marginal spaces of the lung. Also, the discrepancy in immature neutrophils between flush EVLP compared to recirculating EVLP may be a response to a lower concentration of leukocytes in the former, which in turn may promote cellular migration. However, in a closed circuit, this margined pool of leukocytes can rapidly reach an equilibrium with the perfusate. This was highlighted in Chapter 4, where despite significant donor leukocyte removal during continual flushing, leukocytes continued to transfer from the lungs in high volumes upon perfusate recirculation in standard EVLP. This suggests that during reperfusion of the donor lung, there is a continual and persistent passage of donor leukocytes into the recipient circulation until stabilisation and depletion of the donor immune compartment occurs. Yet the inflammatory environment of the EVLP circuit could also promote this donor leukocyte efflux. The EVLP circuit is a closed environment comprising of plastic tubing that can activate immune cells. In addition, it has previously been demonstrated that a non-specific cytokine storm is observed during EVLP, with high concentrations of Th1 and Th2 cytokines and chemokines present within the perfusate (370, 371). The chemokines may promote chemotaxis and donor leukocyte migration. Furthermore, should the EVLP environment itself promote cellular diapedesis and immune cell activation, on-going exposure to EVLP may induce saturation, whereby the donor immune cells lose their effector functions. Therefore depleting the marginated pool of donor leukocytes prior to transplantation would result in a significant reduction in donor leukocyte migration within the recipient upon revascularisation. In addition, the donor leukocytes that do migrate to the recipient circulation may have a reduced capacity to induce a recipient immune response. Overall, this has the potential to reduce graft infiltration and allore cognition. However, this remains to be somewhat hypothetical and the clinical impact of donor leukocyte depletion by means of a continual non-recirculating flush needs further elucidation, as clearly a large repertoire of donor leukocytes can be removed using this technique. Moreover, the potential of the EVLP circuit to induce immune senescence warrants further exploration, by using in vitro models to assess the reactivity of donor cells within the perfusate.

In the second part of the thesis, the use of cell-free mtDNA as a potential biomarker of graft function in lung transplantation was evaluated. The importance of the donor immune compartment in the development of graft dysfunction and rejection has already been emphasised, yet the current practice in determining the suitability of organs for transplantation is somewhat suboptimal. At present lungs are accepted for transplantation based upon subjective assessment and objective evaluation of easily measurable parameters of oxygenation, and in the context of EVLP, pulmonary haemodynamics and compliance. However, these are poor discriminators of the functionality and recoverability of the donor lung upon transplantation, and the inflammatory burden that they possess. Indeed, a recent prospective analysis of the clinical risk factors for development of PGD post-transplantation removed donor oxygenation as a risk
factor (172). Data from this study (Chapter 5) also indicates that donor oxygenation is not a reliable indicator of lung function during evaluation. When ischaemic injury was induced on the EVLP circuit the value of objective parameters in the assessment of lung function and immune load were questioned. Although there was a slight increase in PVR and decrease in lung compliance in the injury group, they remained more favourable than in the control group, and oxygenation remained comparable between both. Yet there the inflammatory profile of the EVLP circuit replicated SIRS with increases in a broad spectrum of cytokines, most importantly IL-1β and TNF-α. These cytokines contribute to ALI, with persistent elevation associated with increased morbidity and mortality (50, 372). Therefore current donor lung assessment methodology does not accurately decipher the quality of the lung, and certainly does not identify the inflammatory burden prior to transplantation. The requirement for more reliable and objective parameters is therefore apparent.

Throughout the first part of this thesis, circulating cell-free mtDNA was persistently found during reperfusion of the donor lung, the relevance of which remains unknown. Cell-free mtDNA is a potent DAMP, initiating downstream immune cascades via TLR and STING signalling, and activation of the inflammasome (387, 399). Over recent years, it has been associated with trauma and sepsis (401), and has been implicated in the development of SIRS and multi-organ dysfunction (387, 402), as well as various diseases such as rheumatoid arthritis (405), cardiomyopathy and ischaemic heart disease (406, 407), and cancer (408). However, its role in lung transplantation has yet to be established.

Interestingly, higher levels of cell-free mtDNA were observed during human EVLP in lungs that were transplanted and associated with inferior outcomes, when compared to those with a superior 12-month survival (Chapter 5). This would suggest that cell-free mtDNA may be reflective of lung injury and has the potential to predict lung function post-transplantation. Indeed, upon induction of ischaemic injury within the porcine EVLP circuit, levels of cell-free mtDNA increased (Chapter 5). Importantly, the increase observed coincided with a significant increase in the pro-inflammatory cytokine IL-1β, which has recently been identified as a predictive biomarker of graft function on the EVLP circuit (373).

It has already been established that the current assessment modalities used to determine lung function and the suitability for transplant are suboptimal, therefore the need for reliable biomarkers are essential. Certainly, the failure to accurately identify truly unusable organs from those that are acceptable could explain the broad range of cell-free mtDNA levels observed in the EVLP perfusate of human lungs not accepted for transplantation. Therefore should cell-free mtDNA have the potential to serve as a reliable and easily measurable predictive biomarker of the inflammatory load and functionality of the lung on the EVLP circuit, it could facilitate a more accurate assessment of their suitability for transplantation. Inflamed and immunologically activated donor lungs will heighten the recipient alloimmune response upon transplantation and revascularisation, driving allore cognition and graft infiltration. Given that the high incidence of morbidity and mortality in lung transplantation is secondary to graft dysfunction and rejection,
identification of biomarkers that could predict such unfavourable outcomes will undoubtedly impact upon survival.

If cell-free mtDNA can serve as a reliable biomarker of lung function during EVLP, it may also have the potential to serve as a biomarker of lung function and potentially injury post-transplantation. In the clinical study of human transplantation (Chapter 6), cell-free mtDNA was detected in the plasma samples of all patients both pre- and post-transplantation, and levels were extremely variable between patients. Although circulating levels of cell-free mtDNA in the initial post-operative period were not shown to correlate with PGD score, there did appear to be a relationship between the level of circulating cell-free mtDNA and incidence of BOS over time. Additionally, increased levels of cell-free mtDNA were observed at day 12 in patients who developed higher rates of acute rejection within the first 6 months. The reason that this was identified in the day 12 sample is most likely to be arbitrary, as this day was selected to ensure that a uniformed ‘close to discharge’ sample was collected. However this is still interesting given that early graft dysfunction and inflammation have been implicated in the development of acute rejection, BOS and chronic graft dysfunction. The use of cell-free mtDNA as a prognostic tool still remains unclear, but the evidence from this study suggests a larger, powered evaluation of its use is warranted. However, before such a study is initiated it is important to determine the level at which circulating cell-free mtDNA becomes pathogenic or indicative of lung inflammation and dysfunction. Throughout this thesis, the levels of cell-free mtDNA observed within the donor pre-transplantation have been lower than those post-transplantation. Furthermore the levels observed within the EVLP circuit using porcine lungs were consistently lower than those during human EVLP, even after injury. Additionally higher peak levels were observed following human lung transplantation compared with porcine transplantation (Table 7.1).
Table 7.1: Overview of mtDNA Concentration Pre- and Post-Transplantation
The mean (±SD) concentration of cell-free mtDNA observed during the experiments performed throughout this thesis.

<table>
<thead>
<tr>
<th>Source of mtDNA</th>
<th>Experiment</th>
<th>mtDNA concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collected Perfusate Flush Effluent (total volume)</td>
<td>Secondary Preservation Flush (Chapter 3)</td>
<td>16.26(±8.25)</td>
</tr>
<tr>
<td></td>
<td>Continual Flush with Non-Recirculating Perfusate (Chapter 4)</td>
<td>11.47(±4.53)</td>
</tr>
<tr>
<td>Perfusate within the EVLP Circuit (peak level)</td>
<td>Porcine EVLP (Chapter 4)</td>
<td>23.56(±14.63)</td>
</tr>
<tr>
<td></td>
<td>Porcine Control EVLP (Chapter 5)</td>
<td>33.28(±35.61)</td>
</tr>
<tr>
<td></td>
<td>Porcine Injury EVLP (Chapter 5)</td>
<td>47.34(±21.88)</td>
</tr>
<tr>
<td></td>
<td>Human EVLP not Transplanted (Chapter 5)</td>
<td>189.69(±387.23)</td>
</tr>
<tr>
<td></td>
<td>Human EVLP Transplanted with Good Survival (Chapter 5)</td>
<td>140.18(±170.89)</td>
</tr>
<tr>
<td></td>
<td>Human EVLP Transplanted with Inferior Survival (Chapter 5)</td>
<td>236.66(±203.1)</td>
</tr>
<tr>
<td>Recipient Plasma Post-Transplant (range)</td>
<td>Porcine Standard Transplant in the first 24 hours (Chapter 2)</td>
<td>160-1570</td>
</tr>
<tr>
<td></td>
<td>Porcine EVLP Transplant in the first 24 hours (Chapter 2)</td>
<td>130-1250</td>
</tr>
<tr>
<td></td>
<td>Human Standard Transplant in the first 12 days (Chapter 6)</td>
<td>85-4122</td>
</tr>
</tbody>
</table>

The discrepancy in levels between human and porcine lungs may reflect the controlled nature of porcine donor death, whereby electrical stimulation induced ventricular fibrillation and warm ischaemic time was limited as a “hands off period” was not required. Subsequently, the myriad of pathological processes that occur within the donor prior to death and that can directly impact upon lung function are likely to be reduced in the controlled, laboratory environment. In light of this an obvious experiment is to quantify cell-free mtDNA (as well as cellular diapedesis and inflammation) from different categories of DBD and DCD donors. Our laboratory has therefore initiated this study in preclinical models, both in the donor during EVLP, and following reperfusion. This will only increase our understanding of the contribution of the donor to cell-free mtDNA burden. However the higher levels of circulating cell-free mtDNA observed in the post-transplant period suggests that mtDNA release is also likely to be recipient driven following transplantation. Circulating cell-free mtDNA may well reflect the degree of leukocyte activation, with increased levels correlating with increased and on-going alloimmune activity. Indeed, this would explain the relationship observed between cell-free mtDNA and the incidence of BOS and acute rejection. Additionally, the findings suggest that persistent or delayed elevation in cell-free mtDNA levels may be more pathognomonic in these conditions.

Therefore although the clinical study was limited by the small sample size, the preliminary data suggests that circulating cell-free mtDNA levels correlate with lung inflammation. As such, understanding the mechanism of its release and subsequent effector functions within the
context of lung transplantation could lead to the identification of novel therapeutic targets. Clearly the role of cell-free mtDNA in lung transplantation warrants further exploration, which was beyond the remit of this thesis.

**Summary**

Lung transplantation has evolved over the past 50 years to become the established treatment for patients with end-stage lung disease. Yet transplantation remains limited by a shortage of donor organs, a low conversion of these organs to transplantation, and a low median survival post-transplantation. This thesis has attempted to address these issues.

Firstly, the importance of the donor immune compartment and its initial reperfusion in the development of graft dysfunction and acute rejection has been highlighted by the findings of this thesis. Donor lungs possess a significant immune compartment dominated by populations of T cells, macrophages and monocytes. These leukocytes are likely to rapidly migrate into the recipient circulation and promote immune cell activation, driving graft infiltration and allorecognition. EVLP has already been demonstrated to reduce passenger leukocyte transfer post-transplantation with a subsequent reduction in acute rejection (371). In continuum of this, a single 1L secondary preservation flush promotes the removal of 1.5 billion leukocytes prior to transplantation (Chapter 3). A larger volume continual flush with a non-recirculating EVLP protocol depletes the donor organ of 9.3 billion leukocytes prior to transplantation. This is important, as despite its contribution to graft rejection, the donor immune compartment has historically been ignored as a therapeutic target due to the logistics and ethics of organ donation. The protocols developed in this thesis provide a clinically relevant tool to immunodeplete the lung prior to transplantation. Furthermore, given that current immunosuppressive regimes are associated with a plethora of serious side effects, donor immune manipulation prior to transplant is an avenue that should not and cannot be ignored.

Secondly, it has demonstrated that our current assessment of lung function and the usability of organs for transplant is sub-optimal. The definition of a “good donor lung” and an “unusable donor lung” has yet to be defined. However it is clear that oxygenation, pulmonary haemodynamics and subjective measures of injury and infection cannot provide us with reliable insight. Indeed this is reflected by the high incidence of morbidity and mortality post lung transplantation. The need for informative and easily measureable biomarkers is therefore essential to optimise benefit. In this thesis the use of cell-free mtDNA as a biomarker of lung function has subsequently been explored. Higher levels within EVLP were associated with worse outcomes post-transplant, and higher levels post-transplantation were associated with the development of acute rejection and BOS. Therefore the role of circulating cell-free mtDNA in lung transplantation and the levels at which its presence becomes pathogenic clearly warrants further work, especially given that cell-free mtDNA can be easily measured and repeated. Moreover, should its presence be associated with the development of rejection, it may abrogate the need for the current invasive measures required for diagnosis and facilitate the attenuation of patient-specific immunosuppressive regimes.
7.2 Limitations

The main limitation throughout this thesis was the small numbers used in each study and as such the lack of powering. Within the preclinical work this was a conscious decision to reduce the number of animals to an absolute minimum, in keeping with NC3Rs guidelines. As a consequence complete statistical analysis was limited in the majority of the studies and true significance could not be observed. Ideally, each study would have an increased n number with appropriate powering to facilitate improved statistical analysis however the difficulty in achieving this is outlined below.

Porcine Animal Studies

In the animal studies, numbers were limited predominantly by ethics and cost. In the early part of the thesis, the use of abattoir porcine lungs were explored, due to their lower costs, unlimited availability, and lack of requirement for ethical approval. Subsequently, for one year, attempts were made to create a porcine model of EVLP using abattoir lungs. However attempts were thwarted due to the high rate of infection and injury within abattoir lungs, and then the high incidence of pulmonary oedema upon reperfusion despite attempts at lowering vascular resistance.

In the majority of abattoirs, the mode of death is often by exsanguination, following initial electrical stunning or CO₂ poisoning to render them unconscious. The donor lungs therefore simulate DCDs. Standard abattoir practice is that following death by exsanguination, slaughtered pigs undergo a hot water wash (>70°C) +/- superficial blow torch for surface cleaning and hair removal as per hygiene laws. However, this can lead to hot water aspiration that can be deleterious to the lungs and lead to irreversible damage.

Upon death, an abattoir worker excised the heart-lung block from the thoracic cavity, to undergo standard donor organ procurement. However upon evaluation following excision, the lungs were often visibly diseased or injured (Figure 7.1).
Abattoir pigs are bred indoors in very large numbers. As such they are exposed to various diseases, specifically in the lungs. Furthermore, in the run up to slaughter, pigs are often starved to reduce vomiting and aspiration on death, and limit the possibility of meat contamination on gut puncture. The pigs can therefore be weak and sensitive to illness. Prior to their slaughter, a veterinary doctor normally examines the pigs to ensure there is no evidence of any gross disease. However, identification of lung disease is difficult. As such, it is often only identified on removal of the heart-lung block. Furthermore, in the presence of pleural disease, which is common, removal of the lungs from the thoracic cavity is difficult secondary to strong adhesions, and so they can be easily damaged during removal. In the absence of any gross disease, a large number of the lungs that were retrieved and placed on the EVLP circuit still displayed evidence of infection, with marked respiratory secretions and significant pulmonary infiltration upon dissection at the end of the procedure.

During EVLP, even in the absence of infection or injury, high PVR was observed in the porcine lung vasculature, which was not ameliorated by the use of vasodilators during procurement and perfusion. In the initial period, porcine lungs that had gone through the hot wash were used, however such high resistance was observed, and there was gross oedema formation upon reperfusion (Figure 7.2).
As a consequence only abattoirs that would omit this wash step and skin the pigs at the end of retrieval were used, which limited availability. Yet despite omitting the hot wash step, infection, injury and PVR remained problematic and the unpredictable nature of using abattoir porcine lungs led to the abandonment of their use for EVLP. It must be noted that porcine lungs were used for the secondary preservation flush in Chapter 3. However it took attending the abattoir weekly for 8 months to obtain 6 pairs of lungs suitable for the experiment. Therefore all other animal experiments were performed on laboratory pigs, with the subsequent implications of cost and availability limiting the numbers used.

**Human Studies**

In the clinical human study, although ethical approval for the use of explanted lung tissue and post-transplant samples was initially applied for at the start of the PhD, the focus of sampling changed following the findings observed throughout the thesis. Changes were therefore made to the sampling protocol, patient information, and consent forms causing a delay in ethical approval for the amendments. This led to delayed patient recruitment and subsequent sampling. Given the nature of transplantation, the study was also limited by the incidence of transplant. Furthermore, it was not technically feasible for one person to track all transplants performed with complete sample collection and processing. Time restrictions limited the number of patients in this study. However given the preliminary findings of this study, patient recruitment has continued and samples are now being collected as part of a larger study with increased follow-up.

In some studies, the findings and conclusions drawn could be deemed hypothetical. Specifically in the secondary preservation flush and continual flush studies, where a broad range of donor leukocytes were removed from the lung. Yet the impact of this post-transplantation can only be speculated based on previous studies and prior knowledge. To fully elucidate the impact of these techniques in altering the immune compartment and the recipient immune response requires a transplant model that was beyond the scope of this PhD. However, work is now underway to fully delineate this hypothesis.
7.3 Future Work

*Does Donor Leukocyte Depletion Impact Subsequent Immune Activation Upon Reperfusion, and Which Method is Best?*

This thesis demonstrated that upon secondary preservation flush and a prolonged continual flush with a non-recirculating perfusate, a large number of donor leukocytes were removed from the lung. To determine whether removal of these cells impacts subsequent immune activation and the inflammatory profile of the donor lung upon recipient reperfusion, a follow-up porcine EVLP study will be performed in the first instance. In addition, different techniques will be compared with standard practice. Porcine single left lungs will undergo standard donor procurement and following 2 hours of cold ischaemia, will be split into 6 groups: Group 1 will undergo immediate standard EVLP for 2 hours, Group 2 will undergo a 1L post-preservation flush prior to standard EVLP for 2 hours and Group 3 will undergo a 40 minute continual reperfusion flush with a non-circulating perfusate prior to standard EVLP for 2 hours. In the first 3 groups, following standard EVLP, the lungs will then be placed onto a different EVLP circuit where they will undergo perfusion with mismatched AO compatible blood. In the remaining groups, standard EVLP will not be performed, therefore following 2 hours of cold ischaemia, Group 4 will be immediately placed on an EVLP circuit for perfusion with mismatched blood, Group 5 will undergo a 1L post-preservation flush prior to mismatched perfusion and Group 6 will undergo a 40 minute continual reperfusion flush with a non-circulating perfusate prior to mismatched perfusion. Perfusate samples will then be processed for subsequent analysis by flow cytometry, Luminex® and DNA quantification. Additionally the lung tissue will be analysed to assess histological preservation and immune cell infiltration.

Should flushing the lung be associated with reduced immune cell activation and inflammatory signalling upon reperfusion with mismatched blood, compared to just standard EVLP and standard procurement, a porcine transplant study will then be performed. In this study, lungs will not be placed on an EVLP circuit to undergo perfusion with mismatched blood but will instead be transplanted into a recipient, who will then undergo a right pneumonectomy. Functionality will be assessed for a period of 24 hours with regular plasma sampling. At 24 hours the pigs will be sacrificed and the transplanted lung tissue will be analysed.

*Profiling the Injured Lung using EVLP*

In a porcine injury model, this thesis demonstrated that clinical parameters of lung function do not accurately correlate with the inflammatory signalling profile of the donor lung. In an extended model of porcine lung injury, this will be explored further.

Increased numbers of porcine lungs will undergo standard EVLP for 1 hour and will then be split into groups to undergo increasing lengths of ischaemic injury. Following injury, the lungs will be perfused for a further 2 hours on the EVLP circuit to determine the impact of injury on the inflammatory signalling profile of the lung. This will be correlated with clinical parameters of lung
function to determine whether there is a level at which the inflammatory changes impact upon clinical parameters.

**Do Cell-Free mtDNA Levels Correlate with Acute Rejection in Human Lung Transplantation?**

To determine whether a patient has acute rejection post-transplantation, the gold standard for diagnosis is to perform transbronchial biopsies. Such sampling is invasive and is associated with risks. Therefore if accurate circulating biomarkers of rejection can be identified, it will abrogate the need for such invasive procedures.

In this thesis, there was a trend towards increased cell-free mtDNA levels at day 12 post-transplantation and a higher incidence of acute rejection within the first 6 months. Therefore to determine whether circulating cell-free mtDNA is an accurate biomarker of rejection, cell-free mtDNA will be quantified in the plasma samples from patients undergoing transbronchial biopsies to diagnose acute rejection. Levels of cell-free mtDNA will then be correlated with acute rejection grades to determine if they are reliable biomarkers of acute rejection.

**Is Cell-Free mtDNA a Predictive Biomarker in Human Lung Transplantation?**

One of the main limiting factors of the human clinical study within this thesis was the small sample size, and given the degree of variability within the transplant population, it was difficult to perform statistical analysis. To fully elucidate whether cell-free mtDNA is a reliable biomarker of lung function post-transplantation, the study will be repeated with increased patient numbers. Given that the later elevations in cell-free mtDNA levels appeared to be related with higher incidences of acute rejection and BOS, sampling will occur daily until day 14, or if discharged prior to that the day of discharge, and then biweekly until the day of discharge. The same outcome measures will be assessed and statistical analysis performed.

Given that increased levels within the EVLP circuit also appeared to predict post-transplant outcomes, with increased levels observed in those with 1-year mortality, this area also warrants further work. Perfusate samples from human EVLP should be routinely collected and levels of cell-free mtDNA quantified. These levels should then be correlated with incidence of PGD, acute rejection and 1-year survival.
References


194


75. Loor GC, P; D’Ovidio, F; Edwards, L; Erasmus, M; Granville, A; Hertz, M et al. Comparison of Long-Term Survival Outcomes in Recipients of Lungs from Donation after Circulatory Death (DCD) and Donation After Brain Death (DBD). The Journal of Heart and Lung Transplantation. 2017;36(4):S144.


