Assessing the Impact of Ex Vivo Perfusion on Graft Immunogenicity

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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
2016
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Abstract

Whilst the major caveat to the success of organ transplantation remains the severe lack of donor organs, rejection is still a primary confounding factor to transplant outcomes. This is an allospecific response that occurs when the recipient immune system recognises conserved proteins on donor-derived cells as ‘non-self’. Currently, all immunosuppressive regimes target the recipient immune response, ignoring the large donor immune repertoire despite these cells playing a central role in acute rejection. This is likely as a result of a lack of understanding of the temporal migration of the donor compartment and its contribution to the inflammatory cascade that ensues. The development of ex vivo perfusion provides the opportunity to assess this in isolation, with no confounding factors. Furthermore, inducing the mobilisation of passenger leukocytes on an ex vivo circuit allows their removal prior to transplantation. Reducing the inflammatory burden of donor organs has the potential to impact on the clinical outcome of patients, manifesting as a reduction in the incidence or severity of acute rejection.

The aim of this PhD thesis was to characterise the donor immune compartment of lungs and kidneys, to assess the impact of ex vivo perfusion on this, and determine the post-transplant impact of removing a proportion of these cells. For this purpose, donor lungs were perfused using ex vivo lung perfusion (EVLP) and the immune compartment characterised. A comparison of EVLP versus standard transplanted lungs was performed using a porcine transplant model. Clinical parameters were recorded and a histological assessment of cellular infiltration was performed to diagnose the incidence of acute rejection. To determine if these results were translatable to other organs, a porcine model of kidney ex vivo perfusion was established. In both models, a significant efflux of donor leukocytes was observed and inflammatory mediators detected. In a transplant model of EVLP, reducing the transfer of these passenger leukocytes translated into improved clinical outcomes, manifesting as a lower incidence of acute rejection, for animals receiving EVLP lungs compared to a standard transplant. Similar benefit is likely to occur following transplantation of perfused kidneys.

This study describes for the first time the contribution of donor organs to the inflammatory processes that ensued following transplantation. It is clear that this untargeted population is of significant importance in clinical outcomes. Immunomodulatory strategies to alter the donor immune environment prior to transplantation therefore warrant development.
Declaration

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

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Acknowledgements

First and foremost I would like to thank my supervisor, Dr James Fildes, for giving me the opportunity to undertake this research. This project would not have been possible without his guidance and continuous support, which has been invaluable. I would also like to thank Professor Nizar Yonan for his continual input into the running of this study.

Part of this study took place in collaboration with Professor Stig Steen’s group in Lund, Sweden. I am grateful to them for taking the time out of their busy schedule to perform the transplant models described within this thesis. Their expertise and continual support has also guided me along the way and I feel privileged to have been able to work with them. I would also like to thank another collaborator, Professor Marc Clancy, who has been equally as integral to the development of this project.

My gratitude also goes to my colleagues for the help, support and encouragement provided by them. All members of the research team have been fundamental in this study. However, special thanks should go to William Critchley and Alexandra Ball for their support and advice throughout this project.

Finally, I would like to say a special thank you to my family and friends who have endlessly supported me throughout this process. In particular I would like to thank my mum and dad who have always encouraged me to pursue a career that I enjoy and to do the best that I can. Their financial support over the 4 years has also allowed me to focus on my research without having to have any additional stresses.
Abbreviations

APCs - Antigen presenting cells
AR - Acute rejection
ATP - Adenosine 5′-triphosphate
BAL - Bronchoalveolar lavage
BSA - Bovine serum albumin
CIT - Cold ischaemic time
CMV - Cytomegalovirus
CNI - Calcineurin inhibitor
CPB - Cardiopulmonary bypass
CXCL-8 - C-X-C Motif Chemokine Ligand 8
CXCR - C-X-C chemokine receptor
DAMPs - Damage-associated molecular patterns
DBD - Donation after brain death
DCs - Dendritic cells
DCD - Donation after circulatory death
DGF - Delayed graft function
EBV - Epstein-Barr virus
ECD - Expanded criteria donors
EDTA - Ethylenediaminetetraacetic acid
ELISA - Enzyme-linked immunosorbent assay
EVHP – Ex vivo heart preservation
EVNP – Ex vivo normothermic preservation
EVOP - Ex vivo organ perfusion
EVLP – Ex vivo lung perfusion
**FiO₂** - Fraction of inspired oxygen

**FISH** - Fluorescent *in situ* hybridisation

**GAPDH** - Glyceraldehyde 3-phosphate dehydrogenase

**gDNA** – Genomic DNA

**GFR** - Glomerular filtration rate

**GM-CSF** - Granulocyte-macrophage colony-stimulating factor

**GTN** - Glyceryl trinitrate

**HLA** - Human leukocyte antigen

**Hs-CRP** - High sensitivity C-reactive protein

**IFN-γ** - Interferon-gamma

**IL** - Interleukin

**IRI** - Ischaemia-reperfusion injury

**IRR** - Intra-renal resistance

**mφ** - Macrophages

**MAP** - Mean arterial pressure

**MHC** - Major histocompatibility complex

**MPAP** - Mean pulmonary artery pressure

**mtDNA** - Mitochondrial DNA

**mTOR** - Mammalian target of Rapamycin

**NC3Rs** - National Centre for the Replacement, Refinement and Reduction of Animals in Research guidelines

**NK** - Natural killer

**PA** - Pulmonary artery

**PaO₂** - Partial pressure arterial oxygen

**PBS** - Phosphate-buffered saline

**PCO₂** - Partial pressure of carbon dioxide

**PD-1** - Programmed cell death protein 1
PD-L1 - Programmed death ligand 1
PEEP - Positive end-expiratory pressure
PRR - Pattern recognition receptors
PTLD - Post-transplant lymphoproliferative disorder
qPCR - Quantitative PCR
RBF - Renal blood flow
ROS - Reactive oxygen species
SCS - Static cold storage
SEM - Standard error of the mean
TBS - Tris-buffered saline
TLRs - Toll-like receptors
TNF-α - Tumour necrosis factor alpha
Treg - Regulatory T cells
UO - Urine output
WI - Warm ischaemic
Summary of Chapters

The work presented within this thesis is written using the alternative format and as such is incorporated into several journal style chapters. This format was chosen due to the complex nature of the methods used throughout this study, meaning that writing the methods in the traditional format would become confusing for the reader. Instead, each chapter in the thesis is self-contained and allows the procedures to be separated and the nature of the study can be explained in logical chapters. An overall introduction of the topic has been included detailing the relevant background to the topic and current knowledge in this field. However, the contents of the introduction may be repeated in several chapters to describe the main issue that is being addressed in each study.

In chapter 2 the aim of the experiment was to characterise the donor lung immune content using ex vivo lung perfusion (EVLP). Using this method we describe the temporal kinetics of passenger leukocytes following revascularisation and provide a detailed evaluation of the effect of normothermic perfusion on the immune burden of the donor lung.

Chapter 3 focuses on the effect of reducing passenger leukocyte transfer using EVLP. A porcine transplant model was utilised, with pigs receiving either a standard lung transplant or a transplant following EVLP. The main aim of this chapter was to determine if removing passenger leukocytes via EVLP confers benefit post-transplantation using clinically relevant outcomes measures.

In chapter 4, a model of ex vivo normothermic perfusion of kidneys was trialled using currently established clinical protocols. The aim of this chapter was to establish a clinically relevant porcine model of this protocol for research purposes. A description of the problems encountered is included.

In chapter 5 the focus was to develop a clinically relevant protocol of ex vivo normothermic perfusion that allowed prolonged perfusion of porcine kidneys. The aim of this was to determine if ex vivo normothermic perfusion offers similar clinical benefit via a reduction in the immune burden of donor kidneys as described with EVLP. A detailed description of the protocol established and the validation of this is included, followed by the experimental results.
Section 6 describes the effect of a secondary preservation flush on the immune compartment of donor kidneys. The aim of this was to determine if performing a second preservation flush of donor kidneys immediately prior to transplantation could also reduce graft immunogenicity via a removal of passenger leukocytes.

Finally, an overall discussion drawing together all of the work described, as well as the avenue for future work, is incorporated into section 7.
Section 1 – Introduction
1.1 History of Organ Transplantation
Since the first successful kidney transplant in 1954, organ transplantation has remained the mainstay treatment option for patients with end stage organ failure, refractory to maximal treatment. However, one of the major limitations to successful transplantation then, as is still true today, is the risk of rejection due to the immune response that ensues following transplant. Medawar’s work to understand the rejection of skin grafts pioneered the understanding of the role of the immune system (1). This allowed Joseph Murray and his team to perform the first successful (defined as lasting more than 6 months) solid organ transplant between identical twins (2). Success in kidney transplantation then paved the way for liver, heart and lung transplantation.

1.2 Fundamentals of Transplant Immunology
One of the fundamental properties of the immune system is its ability to recognise foreign antigens and respond to danger and damage. The human immune system removes as many as 20 billion cells a day, many of which are cancerous, with no adverse events. However, when tissue becomes stressed or injured, damage-associated molecular patterns (DAMPs) are released and ligate to Toll-like receptors (TLRs). Ultimately this leads to the activation of the immune system and an augmented immune response ensues. In the context of transplantation, this is coupled with the presence of genetically encoded polymorphisms within the major histocompatibility complex (MHC) that are recognised by recipient lymphocytes. In humans, this is typically referred to as the human leukocyte antigen (HLA). MHC are a set of cell surface proteins first described in 1936 that are responsible for antigen presentation to naïve T cells (3). The MHC is encoded for by a set of closely linked genes located on the short arm of chromosome 6 known as the HLA genes (4). Traditionally these genes are subcategorised into Class I and Class II based upon their structure and function. MHC Class I are present on nearly all nucleated cells in the body and are composed of an α chain, non-covalently bound to a β2 microglobulin. The α chain contains three domains, referred to as α1, α2 and α3. The peptide binding groove, where antigen is held for presentation to T cells, is formed by the α1 and α2 domains, (3, 5). The main function of this class of MHC is presenting intracellular peptides that have been degraded and transported to the cell surface. These antigens are presented to cytotoxic T cells, leading to destruction of infected cells. MHC Class II are structurally similar to Class I, although there are two α and two β chains that have
transmembrane regions and cytoplasmic tails, and as such they have the ability to deliver intracellular signals. In addition, MHC Class II are only present on the cell surface of professional antigen presenting cells (APCs) and present exogenous antigen that originate extracellularly (3, 5).

MHC molecules have distinct properties that make it very difficult for foreign antigens to evade the host immune system. Individuals possess several subsets of Class I and Class II MHC genes with different peptide-binding specificities. For example, the α chain of the Class I MHC is encoded for by HLA regions A, B and C, with an individual inheriting one allele of Class I from each parent. These alleles are co-dominantly expressed meaning that both maternal and paternal proteins are expressed. In addition, these genes are highly polymorphic and therefore multiple alleles exist within the general population. The domains that encode the peptide-binding groove can differ from allele to allele by up to 20 amino acid sequences (6). Whilst this generates a diverse range of antigen specificity, it is this principle that is central within transplantation, with recipient leukocytes recognising donor MHC as foreign. Indeed, graft survival post-transplantation is influenced by the compatibility between donor and recipient HLA (6, 7).

T cells cannot directly recognise foreign antigen in the same way B cells can; instead antigens need to be directly presented via the MHC to the T cell receptor. Peptide fragments of both self and ‘non-self’ (or foreign) origin are presented to the T cell receptor on the T cell surface within the peptide binding groove of an MHC molecule. In this way, T cells can distinguish between self and non-self and respond appropriately. Interaction of foreign antigens with naïve T cells leads to T cell activation and an immune response ensues (8). APCs can internalise extracellular antigens, typically via phagocytosis, where degradation and transport to the cell surface then occurs. Antigen presentation in this case is to helper T cells, which ultimately induce antibody production and leukocyte recruitment (8). It is these fundamental immunological principles that contribute to rejection following transplantation.

### 1.2.1 Direct and Indirect Pathways

The process of organ transplantation involves the replacement of a diseased organ with a healthy organ, typically from a deceased donor. During this process endogenous ligands are released due to tissue injury incurred during the process of death, organ retrieval and
transplantation itself. Following transplantation, these stimulate conserved pattern recognition receptors (PRRs) (such as TLRs) on a range of donor and recipient leukocytes, including dendritic cells (DCs) (9). Indeed, innate immune cells of recipient origin, including macrophages (mφ) and neutrophils, infiltrate the graft early and release pro-inflammatory cytokines (10). In addition, donor organs contain a large reservoir of leukocytes, including professional APCs that express MHC on their surface (11). These cells are transplanted along with the organ, meaning a large immunological burden is transferred from the donor to the recipient. Donor-derived leukocytes that have been primed will extravasate from the graft into the recipient circulation and home to local lymph nodes. Recipient T and B cells that recognise genetically encoded polymorphisms within the donor MHC as non-self on these cells induce graft-specific immune responses. Ultimately, this results in an alloresponse to the graft, the initiation of acute rejection (AR) and a loss of graft function.

The introduction of immunosuppressive treatment has led to a reduction in the incidence of AR, although this still remains a significant problem and represents a major caveat to transplant success (12). Medawar and Gibson originally described rejection as an antibody mediated mechanism. However, in 1944 they established that this is actually a predominantly cellular (T cell mediated) response, although humoral (antibody mediated) mechanisms are involved (1, 13). T cell activation requires a co-ordination of signals involving the engagement of the T cell receptor by antigens presented via MHC, along with co-stimulatory signals that cause T cell activation and expansion. Interestingly, transplant rejection is unique as naïve T cells can be stimulated via two pathways (14). Firstly, recipient T cells may be activated directly by donor-derived leukocytes presenting donor-peptides (15). The recipient naïve T cells principally target the highly polymorphic MHC molecules expressed on all donor leukocytes, with APCs of donor origin having a high density of allo-MHC molecules. This pathway was described by Snell in 1957 and is classically referred to as the ‘direct’ pathway, as demonstrated in figure 1.1 (15-17). Experiments culturing lymphocytes from different individuals have demonstrated the strong T cell response that ensues to allogenic MHC molecules. In a typical immune response 1/ 300,000 T cells would respond, yet in response to foreign MHC 1/1000 to 1/50,000 T cells respond (18). It was initially thought that this pathway was solely responsible for allorecognition and AR. However, in the 1980s, it was postulated by Lechler et al that a second, ‘indirect’ pathway also existed and contributed to the rejection of the graft, as demonstrated in figure 1.2 (19). Here, recipient derived APCs infiltrate the donor organ via transendothelial migration and internalise donor MHC antigens.
Recipient APCs process donor antigens and present them via self-MHC to naïve recipient T cells in local lymph nodes, resulting in the maturation of effector T cells (20). It was the demonstration that donation of skin from MHC Class II knockout mice to MHC Class I knockout recipient mice was still rejected that certified the indirect pathway in rejection (21). Given that the donor cells lacked MHC Class II antigens, antigen recognition by recipient CD4+ T cells had to have been via donor antigen presented via recipient MHC Class II on APCs.

These two pathways are now accepted as the mechanism behind solid organ graft rejection; however, the relative contribution of each pathway to the rejection process remains unclear (16, 22). It is proposed that direct allorecognition is intrinsically involved in initiating AR, with chronic rejection being attributed to the indirect pathway as donor cell numbers diminish (23). However, evidence of donor chimerism is emerging, suggesting continued donor-recipient interactions occur (24). Despite this, all current immunosuppressive therapies solely target the recipient immune system, principally T cells, and completely ignore the donor compartment. In addition, advances in immunosuppressive drugs that spare patient morbidity and minimise graft rejection have been poor in recent times. This is partly due to a lack of new therapeutic targets. However, this is an area that is gaining renewed interest and clearly warrants further investigation.
Figure 1.1 - Schematic diagram representing the direct pathway of allore cognition

Donor APCs, including DCs rapidly migrate from the graft into the recipient circulation. These cells home to the local lymph nodes and present donor antigens via donor MHC to naïve recipient T cells. Allospecific recipient T cell populations clonally expand within the lymph node, enter the bloodstream and migrate into the donor organ where they cause damage to the graft, recruit other immune cells and ultimately induce AR.
Figure 1.2 - Schematic diagram representing the indirect pathway of allorecognition

Donor APCs, including DCs are transferred into recipients following solid organ transplantation. These cells shed proteins, including MHC, which are taken up by recipient DCs that have extravasated into the allograft. The donor peptide complex is processed and presented on the cell surface. These cells drain into blood vessels, where they home to the local lymph nodes and present donor antigens to naïve recipient T cells. Allospecific recipient T cell populations clonally expand within the lymph node, enter the bloodstream and migrate back to the allograft where they induce AR and damage to the graft.
1.2.2 Types of Rejection

Whilst all rejection is mediated by lymphocytes, the timing of rejection and the processes that occur are complex and can be sub-categorised depending on the clinical presentation of the symptoms and main cell types involved.

Hyperacute rejection:

Hyperacute rejection was the earliest type of rejection, typically occurring within the first 24 hours after transplantation. This occurs as a result of pre-existing antibodies within the recipient that recognise alloantigen present on graft endothelial cells, ABO mismatched blood or alloantigen shed from the graft itself. However, the incidence of hyperacute rejection in modern transplantation has been dramatically reduced due to the introduction of pre-transplant screenings and tissue typing.

Acute rejection:

AR is the result of both cell-mediated and humoral mediated immunity to MHC mismatched antigens present on donor cells, as described above. This commonly occurs over a period of 7 to 10 days and is likely to occur to some degree in all transplants. Acute cellular rejection is primarily mediated by lymphocytes that have been activated owing to their antigen-specific receptors and infiltrate the graft. Typically this is a result of donor APCs that enter the recipient circulation and present alloantigen to naïve T cells (25). Ultimately T cell activation leads to the induction of cell death either via apoptosis or necrotic pathways. CD8+ T cells can induce lytic cell death via perforin and granzyme release, or apoptotic cell death via Fas-Fas ligand interactions (26). In contrast, humoral rejection primarily involves antibodies that can either be pre-formed or occur as a result of alloantigen recognition. Complement activation can also occur, with C4d now becoming the hallmark of humoral rejection. Indeed, C4d is now detectable in transplanted organs before its histological presentation (27). Ultimately, this leads to increased graft infiltration, necrosis of endothelial cells and vasculitis (28). This is particularly evident earlier in highly vascularised tissues, such as the kidney and liver.

In most cases, a single episode of AR can be treated with immunosuppression and monitoring and organ failure can be prevented. However, recurrent AR episodes are a major risk factor for the development of chronic rejection (29).
Chronic rejection:

In contrast to AR, chronic rejection develops slowly following transplantation, occurring in the months to years’ post-transplantation. The mechanisms behind this process are still poorly understood but it has been well documented that this is a multifactorial process, with a range of immune processes contributing. However unlike AR, donor APCs contribute minimally to chronic rejection, with allografts depleted of donor-derived cells still eventually rejecting the graft (21). It should however be noted that donor DCs have been detected in hand transplant recipients 10 years post-transplant and therefore clearly still contribute to the rejection mechanism (30). Despite this, it is postulated that recipient APCs gradually replace donor APCs over-time as the primary initiator in antigen presentation, leading to a switch from direct to indirect presentation (31, 32). The specific histological picture varies between organs, yet the common characteristic is fibrosis of the graft and vasculature. Indeed, many of the features of chronic rejection resemble those of wound healing including the proliferation of endothelial or epithelial cells along with collagen deposition. For this reason, chronic rejection is postulated to be primarily driven by a type 2 T cell response. Eloquent studies have demonstrated this, with the injection of type 2 related cytokines into immunodeficient animals inducing chronic rejection (33). Conversely, blocking IL-4 with antibodies blocks the progression of chronic rejection (34). IL-4 induces fibroblasts to produce extracellular matrix proteins, and together with IL-10 inhibits mφ from producing metallopeoteinases (35, 36). This may explain the fibrotic histological picture of chronic rejection. Ultimately, the resultant interstitial fibrosis can cause ischaemic regions within the graft leading to a loss of graft function (37, 38).

Once the mechanisms of rejection were established, the failures in the early experience of organ transplantation can be mainly contributed to inadequate immunosuppression. Improvements in mortality following transplant did occur and were correlated with advances in surgical techniques, post-operative care and a greater understanding of the anatomy and physiology of individual organs. However, a major advance in transplant success occurred in the early 1980’s with the introduction of the first specific immunosuppressive agents. Patients now routinely take permanent immunosuppression.
1.2 Immunosuppression

1.2.1 Cell cycle inhibitors

Initial attempts at immunosuppressive therapy began with irradiation techniques but this proved to be ineffective, and in some cases lethal. This subsequently led to patients being treated with steroids alone although this also proved to be unsuccessful. It was not until the early 1960’s that immunosuppression with either Purinethol or the cell cycle inhibitor azathioprine became the standard of care following transplantation. Azathioprine is a purine analogue that disrupts RNA synthesis and metabolism via inhibition of the purine nucleotide synthesis within the cellular DNA. However, the long-term outcomes in patients remained poor until an additive and synergistic effect of combining corticosteroids with these therapies was demonstrated in renal transplants. Such treatment strategies were rapidly adopted by several transplant centres and in 1994 this became the standard treatment regime worldwide (39). In combination with this, a major breakthrough in controlling rejection occurred following the discovery of the HLA, which then allowed donor and recipient tissue to be matched as closely as possible (40, 41). This was in part due to a greater understanding of the immune system, which subsequently provided the insight for targeted immunotherapies to be developed.

1.2.2 Calcineurin inhibitors

Cyclosporine is a calcineurin inhibitor (CNI) that was originally isolated from the fungus *Tolypocladium inflatum* by Jean Borel in 1976, but was not approved for clinical use until 1983 (42). This was the first therapeutic compound available that inhibited lymphocytes in a specific and reversible manner by inhibiting a critical step in T cell proliferation. Whilst other pharmacological effects have been reported, cyclosporine primarily exerts its method of action by binding to an intracellular immunophilin known as cyclophilin, to form a complex that inhibits the phosphatase activity of calcineurin (43). Calcineurin is activated following a rise in intracellular calcium upon binding and activation of T cells. Once active, calcineurin cleaves the terminal phosphate group on the cytoplasmic subunit of the family of transcription factors known as the nuclear factor of activated T cell. This reveals a nuclear localisation signal allowing its migration to the nucleus and ultimately induces the transcription of genes including those for interleukin (IL) 2, CD25 and CD40 ligand (44). Cyclosporine therefore inhibits the activation of T cells via a blockade of IL-2 secretion, a cytokine necessary for T
cell activity. This now represents the first line of CNI treatment for transplant recipients. Since the development of cyclosporine, a new generation of CNIs have been formed. This includes Tacrolimus, a chemically distinct molecule that binds to the intracellular immunophilin FKBP-12 leading to an interaction and inhibition of calcineurin in a similar fashion to cyclosporine (45).

1.2.3 Mammalian target of Rapamycin Inhibitors (mTOR inhibitors)

Sirolimus, which was isolated from the soil of Easter Island in 1975, was the first of the mTOR inhibitors and was originally developed as an antifungal medication. In 1999 it was approved for use in preventing renal allograft rejection. Despite being structurally similar to Tacrolimus, also binding to FKBP-12, the mode of action of Sirolimus is quite different. Rather than inhibiting calcineurin, Sirolimus inhibits the activation of mTOR, a key regulatory kinase of the cell cycle. In this way it prevents T cell proliferation via an inhibition of cell cycle progression from the G1 (cell growth) phase to the S (DNA synthesis) phase. This effect is also seen in B cells, vascular smooth muscle cells, endothelial cells and fibroblasts (46). This drug is principally used in renal transplantation owing to its reduced toxicity towards kidneys compared to calcineurin inhibitors.

Although Sirolimus can be used by itself as an immunosuppressive agent, it is not unusual to combine this with CNI to negate the need for corticosteroids.

1.2.4 Post-transplant Complications

Whilst the introduction of immunosuppression into clinical practice has reduced patient mortality rates following transplantation via a reduction in incidence of rejection, there are several adverse effects associated with their use. Patients receiving a transplant are typically put on a cocktail of immunosuppressive drugs containing a CNI, a cell-cycle inhibitor and corticosteroids. Although the main therapeutic target of these drugs is to inhibit T cell proliferation, their non-specific nature means that both cellular and humoral immunity are affected. Suppression of the recipient’s immune system severely inhibits normal immune surveillance, leaving patients susceptible to infection and malignancies (47). The incidence of infections can vary depending on the level of immunosuppression required but includes viral, bacterial and fungal pathogens. These can range from commonly encountered organisms such as influenza, to more invasive pathogens such as *Aspergillus* and *Tuberculosis* (48, 49).
However, one of the most common infections is Cytomegalovirus (CMV), a viral infection that often occurs in the first month post-transplant. Infection with CMV is a major cause of morbidity and mortality post-transplantation (50). In some cases, viral infections post-transplant have been association with an increase in viral-related malignancies, although the aetiology of the malignancies if multifactorial (51). This includes a direct carcinogenic effect of immunosuppressive drugs themselves, with a cancer promoting effect of CNI previously being demonstrated (52). One of the most common cancers reported is post-transplant lymphoproliferative disorder (PTLD); occurring in up to 10% of transplant recipients. This is most frequently associated with Epstein-Barr virus (EBV) infection of B cells, which can result from a primary infection or from the reactivation of the virus from a previous infection post-transplantation. EBV becomes incorporated into B cells and becomes established as a lifelong, asymptomatic, latent disease (53). When occurring as a primary infection, it is often a result of B lymphocytes of donor origin that have the capacity to transform into a lymphoproliferative disorder due to the utilisation of immunosuppression (54).

However, there is evidence in the literature that immunosuppressive drugs, such as the mTOR family of drugs, exert anti-neoplastic effects. Whilst clinical data surrounding this remains poor, these drugs could simultaneously reduce the incidence of rejection and minimise the risk of post-transplant malignancies (51). Indeed, a switch from Cyclosporine to Sirolimus in kidney transplant patients induced a regression of Kaposi’s sarcoma (55-57). This is proposed to be due, at least in part, due to the antiangiogenic properties of mTOR inhibitors (58). Despite this, there still remains a distinct lack of therapeutics targeting the donor immune compartment, with all current immunosuppression targeted at recipient lymphocytes. Given the importance of passenger leukocytes in the initiation of AR, this is an untapped area that clearly warrants further research.

Aside from the complications associated, the introduction and refinement of immunosuppression regimes have dramatically improved post-transplant outcomes. This has led to the field of transplantation becoming a victim of its own success, with increasing numbers of patients being listed for transplant. As a consequence, one of the major caveats to organ transplantation has become a lack of donor organs.
1.3 Donor Organ Shortage

With an increasing global population and a growing number of patients with end stage disease, the number of patients listed for transplant is continually rising. As a result of this, the demand for transplantation continually outweighs the supply of organs through donation. In the 2013-2014 financial year there were 3505 organ transplant procedures performed from a total of 1320 organ donors in the UK. During the same period there were 7026 patients registered on the transplant waiting list, of which almost 20% either died whilst waiting or became too sick to receive a transplant. This leads to accessibility to the transplant waiting list being limited and many patients who would benefit from organ transplantation are not considered. In combination with this, current preservation practice can lead to organ damage prior to and following transplantation. This alone can lead to delayed graft function (DGF) and shorter graft survival rates, resulting in the need for re-transplantation. In turn, this has the potential to increase patient morbidity.

1.3.1 Static Cold Storage

Following the decision that an organ is suitable for transplant, the organ is exposed to a period of reduced or complete lack of blood supply. This is referred to as the warm ischaemic (WI) phase and may occur following the withdrawal of life support or at the time of cardiac arrest. During this time there is substantial cellular stress and tissue damage which subsequently impairs organ function. It is therefore imperative to reduce the exposure of organs to WI by cooling as quickly as possible, with the current gold standard for organ preservation remaining static cold storage (SCS). This relies on hypothermic temperatures to lower metabolic activity and as such, reduce the requirement of oxygen and nutrients. The vasculature is flushed through with a cold preservation solution to cool the organ and remove microthrombi before being submerged in an ice-cold solution and placed in an icebox until implantation. Whilst this is essential for organ storage, hypothermic temperatures have been associated with oxidative stress and sodium pump (Na⁺/K⁺ATPase) inactivation. Furthermore, long periods of cold ischaemia result in a decrease in pH (resulting in acidosis) and ATP levels as a result of anaerobic metabolism and lactate accumulation. This results in dysfunctional ATP-ase dependent ion transport mechanisms, leading to increasing intracellular and mitochondrial calcium levels. Ultimately, this causes cell swelling and rupture leading to cell death, leaving organs sensitive to damage upon reperfusion (59). Prolonged SCS, which is measured by the cold ischaemic time (CIT) from the start of
preservation flush to implantation, is therefore one of the key limiting factors in transplantation and long CIT is associated with reduced long-term graft survival (60).

1.3.2 Ischaemia Reperfusion Injury

In all organ transplants, following a period of ischaemia the organ is connected to the recipients’ vasculature and normal blood flow is resumed. It is well documented that this reperfusion process exacerbates the cellular injury that was endured during the ischaemic period. This is commonly known as ischaemia-reperfusion injury (IRI) and encompasses several complex pathophysiological processes. The mechanisms contributing to this are multifactorial, but include the generation of reactive oxygen species (ROS), ionic imbalance-induced oedema, oxidative stress, endothelial activation and dysfunction, complement activation and a pronounced pro-inflammatory response (61). The relative importance of these contributing factors remains unclear in the literature, although ROS production has been suggested as a major contributor to the resulting injury (62).

The Effect of IRI on cells:

**Ischaemia:** Organs exposed to prolonged ischaemia exhibit several cellular metabolic and ultrastructural changes. The magnitude of cellular dysfunction is intrinsically linked to the duration of ischaemic period. The switch from aerobic to anaerobic metabolism during ischaemia results in a decrease in cellular pH and an accumulation of hydrogen ions within the cell. To counteract this build up, the sodium-hydrogen exchange pump excretes excess hydrogen ions (63). At the same time, cellular oxidative phosphorylation declines, meaning that energy rich phosphates including adenosine 5′-triphosphate (ATP) cannot be re-synthesised. The end result of this is an alteration in the function of membrane ATP-dependent ionic pumps causing the entry of calcium, sodium and water into cells leading to cell swelling and the induction of cell death (both necrotic and apoptotic). In addition, the expression of leukocyte adhesion molecules, cytokines and bioactive agents such as endothelin are up-regulated on endothelial cells whilst gene products such as constitutive nitric oxide synthase are down-regulated. The reduced bioavailability of these agents leads to vasoconstriction and can facilitate the formation of microvascular thrombi, reducing blood flow to the tissue thereby inducing ischaemic injury to the organ (64). Furthermore, this creates a pro-inflammatory environment that leaves the organ susceptible for enhanced injury following reperfusion (65).
Reperfusion: The re-introduction of blood flow to ischaemic tissue restores the oxygen supply, allowing aerobic metabolism to be restored and cellular pH to be normalised. However, Jennings et al established over 50 years ago that this process has adverse consequences associated with it (66). The reperfusion of blood to coronary arteries that had been ligated accelerated the development of necrosis. Whilst the mechanisms underlying this injury are multifactorial and include the opening of the mitochondrial pore coupled with inflammatory responses, it is largely considered that the generation of ROS is the main contributing factor.

The role of Reactive Oxygen Species:

The generation of ROS increases during both the cold ischaemic phase and following reperfusion when molecular oxygen is reintroduced to the tissue. This includes a variety of molecules and free radicals derived from molecular oxygen such as superoxide anions, hydrogen peroxide and hydroxyl radicals. Indeed, superoxide becomes hydrogen peroxide in a process involving superoxide dismutase. These are potent oxidising and reducing agents that can damage cells via a number of mechanisms that includes peroxidation of cell membrane lipids and oxidising DNA. Additionally, ROS generation can lead to the activation of matrix metalloproteinases and calpains, proteases that can cleave proteins and receptors on the cell surface. Ultimately this results in the opening of the mitochondrial permeability transition pore, contributing to cellular swelling and lysis (62).

The generation of ROS post-ischaemia can result in leukocyte activation (of both donor and recipient origin) as well as inducing the up-regulation of adhesion molecules, cytokines and chemokines that lead to cellular influx. Complement activation can also occur, generating several key inflammatory mediators that have the potential to alter vascular homeostasis. This includes the anaphylatoxins C3a and C5a that induce the production and secretion of pro-inflammatory cytokines (67). Together, these processes also contribute to swelling and lysis of cells including vascular endothelial cells which can causes plugging in capillaries. This reduces blood flow in these areas, promoting a second phase of ischaemia and propagates ROS generation and cellular injury.

The extent of IRI and cell dysfunction is influenced by the duration of the ischaemic period (68). The initial cellular injury and dysfunction is reported to be reversible, yet this can
become irreversible if the ischaemic insult is prolonged (62). It is therefore imperative to limit the time organs are preserved in an ischaemic state.

1.3.3 Expanded Criteria Donors

In an attempt to overcome the donor organ shortage there has been an increase in the use of organs transplanted using expanded criteria donors (ECD), organs not previously considered suitable for transplantation. This includes the use of older donors and donors with characteristics or even illnesses once thought to preclude donation. Whilst this has offered survival benefit to patients compared to remaining on the transplant waiting list, ECD are more susceptible to damage during transplantation, leading to high rates of primary graft dysfunction and shorter graft survival. In addition, ECD are more sensitive to SCS compared with that of standard criteria donors, leading to an increased risk of IRI injury post-transplant.

In combination with the use of ECD there has been a change in the pathway of donation. Traditionally, organs are retrieved from donors following brain-stem death, known as donation after brain death (DBD) donors. These are patients whose circulations are working right up to the point of organ retrieval. However, there has been increasing interest in utilising donation after circulatory death (DCD) donors where organ retrieval takes place a short time after cardiac arrest, usually following the planned withdrawal of life-supporting treatments. Organs retrieved from such donors are subjected to a prolonged WI time compared to heart beating donors making them more susceptible to the subsequent damaging effects of SCS. In the UK, this was evident in kidney transplantation alone with outcome data demonstrating substantially worse outcomes in DCD kidneys with a CIT of over 12 hours (69).

1.3.4 Donation after Brain-stem Death

Organ function is retained within DBD donors due to continued cardiac function and intensive care respiratory support. However, the irreversible damage to the brain stem typically results in several broad hemodynamic changes, along with hormonal and metabolic imbalance that can impair organ function (70). In the early period following injury, ischaemia disseminates down from the cerebrum to the spinal cord. Pontine ischaemia leads to a both vagal and sympathetic stimulation, ultimately leading to bradycardia and hypertension. As the ischaemia progresses towards the distal medulla oblongata, the vagal motor nucleus becomes ischaemic which results in a marked catecholamine release (commonly termed a ‘catecholamine storm’).
The initial result of increased circulating catecholamines is a dramatic hypertensive crisis with extensive peripheral vasoconstriction, arterial hypertension, tachycardia and cardiac arrhythmias, which leads to organ ischaemia (72). The initial vasoconstriction only lasts for a short period, on average minutes, and is followed by systemic vasodilation owing to a loss of vascular tone when ischaemia reaches the spinal cord. There is also a gradual decline in catecholamine release, although this occurs at a slower rate. These processes cause haemodynamic collapse within the donor, generating impaired blood flow and perfusion pressure to the donor organs (70, 71). Significant damage ensues, which activates several inflammatory cells, including DCs, within the organ itself prior to transplantation. Additionally, the catecholamine storm can cause activation of immune cells by prompting an aerobic to anaerobic shift in metabolism. This switch induces ROS generation, the release of lipases, proteases and endonucleases as well as the activation of nuclear factor kappa-B, the protein complex that regulates the immune response. An increase in the transcription of several pro-inflammatory cytokines and mediators ensues, with animal studies identifying the systemic release of several inflammatory cytokines, including IL-1, IL-6, tumour necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) (70, 71, 73, 74). The extent of this pro-inflammatory and damaging environment created within the donor determines the probability of AR occurring, by instigating the events promoting graft rejection within the recipient. However, the process of brain death does not elicit a uniform pro-inflammatory environment across all organs of the donor, which results in varying degrees of damage to different organs (70, 71, 74). Delineating these pre-transplant complications associated with brain death could provide therapeutic targets, with the aim of alleviating its damaging effects and improving post-transplant success rates.

1.3.5 Donation after Circulatory Death

Despite DBD being the conventional donors for transplantation, the damage that ensues following brain death can render the organs unsuitable for transplantation. It is estimated in the UK that only 25% of lungs offered for donation are utilised due to safety concerns. This has led to resurgence in the interest of using DCD organs for transplantation, not only for kidneys but for liver, pancreas and lungs that have a lower tolerance for WI (75). The UK alone has seen a 10-fold increase in their utilisation with DCD donors representing 40% of all deceased organ donations from 2014 to 2015. More recently this has included the use of a
DCD heart, something that was previously thought to be unusable due to the detrimental effects of WI on the myocardium.

Identifying patients as suitable DCD donors remains a challenge in clinical practice. Principally two defined categories have been outlined, typically referred to as controlled or uncontrolled DCD. Uncontrolled DCD is organ retrieval after an unexpected cardiac arrest, whilst controlled DCD takes place after the declaration of death, which follows the planned withdrawal of life-sustaining treatments. Additional sub-categories exist based on the Maastricht classification regarding the clinical circumstances surrounding the process of cardiac death (Table 1.1). Categories I, II and V describe organs that are retrieved from uncontrolled DCD donors, with categories III and IV referring to controlled DCD donors (76).

<table>
<thead>
<tr>
<th>Category</th>
<th>Definition</th>
<th>Type of DCD</th>
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<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>Cardiac arrest following planned withdrawal of life sustaining treatment</td>
<td>Controlled</td>
</tr>
<tr>
<td>IV</td>
<td>Cardiac arrest in a brain dead patient</td>
<td>Controlled</td>
</tr>
<tr>
<td>V</td>
<td>Circulatory arrest in hospital</td>
<td>Uncontrolled</td>
</tr>
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Table 1.1 - Maastricht classification of DCD donors

Historically, the major reason for the lower utilisation of DCD organs is due to the increased ischaemic injury experienced between the withdrawal of treatment and the start of cold preservation. In contrast to DBD, the circulation in DCD donors ceases following cardiac arrest and death is confirmed following a ‘hands off period’ of typically 5-10 minutes. During this time the patient cannot be touched and the organs are not being perfused, leading to the increased WI and cellular injury previously mentioned (77). For controlled DCD, withdrawal of treatment is planned and the WI is typically 15 minutes. However, uncontrolled DCD donors experience an unexpected cardiac arrest following brain stem death causing the WI time to be considerably longer in most cases. The increase in WI exacerbates the risk of primary graft failure, DGF and other ischaemic complications such as biliary strictures (77). This leads the retrieval team to be cautious of accepting organs from older DCD donors or donors with comorbidities including diabetes mellitus, hypertension and peripheral vascular
disease that have the potential to add to the ischaemic insult (76). However, DCD donors do not encounter the deleterious effects encountered following brain death. In combination with this, the repeated demonstration that transplantation of kidneys from DCD donors has similar long-term outcomes to those from DBD donors has instigated several countries to reintroduce DCD schemes. Despite this, the number and quality of transplants performed from DBD donors is unlikely to be matched by those from DCD donors (76).

1.4 Ex Vivo Organ Perfusion

Aside from the increasing necessity to use ECD and DCD donors to meet the waiting list demand, potentially viable organs are wrongly declined for use in transplantation based on ambiguous functional assessments within the donor body, which is inherently a suboptimal environment. In an attempt to address these issues, a novel technique known as ex vivo organ perfusion (EVOP) was established to enable detailed physiological assessments of organ function prior to transplantation. This approach aims to increase donor organ utilisation by simulating the in vivo environment on an ex vivo circuit. As current preservation techniques do not allow this, EVOP is now considered essential to maximise organ utilisation. Organs are retrieved from the donor following current standard transplant protocols where they subsequently undergo SCS as described previously. Prior to transplantation, the organ is then removed from ice and attached to a modified cardiopulmonary bypass machine that circulates an oxygenated blood-based perfusate through the vasculature of the organ at normothermia. Many of the clinical protocols also include a leukocyte depletion filter to remove donor-derived cells before transplantation (78). This process provides metabolic support and restores functionality to organs allowing detailed assessments of the organ during perfusion. This theme of research is now considered a priority by the Department of Health’s special authority for transplantation (NHSBT) in delivering a world-class transplant service in the UK.

1.4.1 Ex Vivo Lung Perfusion

The concept of EVOP was originally proposed as early as 1938 by Carrel and was subsequently refined for the evaluation and preservation of lungs in 1970 by Jirsch et al. These early attempts were unsuccessful due to the development of oedema caused by the preservation solutions failing to hold fluid within the vasculature (79, 80). Stig Steen and colleagues in Lund, Sweden developed a major breakthrough in organ preservation. Their primary interest was to develop a technique to allow lungs previously deemed unsuitable for
transplant (DCD lungs) to undergo an extended period of assessment and evaluation prior to transplantation (termed ex vivo lung perfusion or EVLP). One of the successes of this team was the development of a preservation solution, known as Steen solution, which could be combined with cell-saved, packed red blood cells to a haematocrit of 15-20% to prevent lung oedema during perfusion. This is a buffered, extracellular solution with optimal colloid osmotic pressure, the composition of which cannot be disclosed due to patent protection. The aim of this solution is to retain fluid within the intravascular space whilst simultaneously providing nutrients to the lung, helping to maintain tissue viability. To achieve this, Steen and colleagues modified a current organ preservation solution, low potassium dextran-glucose (LPD-glucose) with the addition of human serum albumin. This increases the plasma colloid osmotic pressure to 38mmHg, compared to the 23mmHg in human plasma. This is now the solution routinely used for clinical EVLP.

In combination with the development of his unique perfusate, Steen identified that controlled reperfusion and controlled ventilation were key to maintaining the integrity of the lung tissue and to minimise the IRI damage that occurs following revascularisation. This helps to reduce oedema formation in the early reperfusion period when endothelial permeability is increased and limits ventilator-induced lung injury (81, 82). The lungs are placed in an organ chamber and are gradually re-warmed to normothermia (37°C) with a maximum difference of delta T less than 8°C between the water in the heater-cooler and the perfusate. At 32°C ventilation is commenced and is gradually increased to achieve maximal ventilation at 37°C with a tidal volume of 5-7 ml/kg. At this point, the gas mixture supplied to the circuit is switched to represent that of venous blood. The oxygen supply is removed, thereby allowing the oxygenation of the blood from the lung itself to be determined via arterial and venous blood gases.

Following the development of Steen’s protocol, the so called Lund protocol, a large animal model of EVLP was utilised to demonstrate the feasibility and safety of preserving, evaluating and transplanting DCD donor lungs using this procedure. To simulate a DCD donor, porcine lungs from a non-heart beating donor were assessed in situ, exposed to 65 minutes of WI followed by 6 hours of topical cooling. Lungs were subsequently excised and placed onto the EVLP circuit where they were assessed for 60 minutes followed by a single lung transplant.Recipient pigs then received a contralateral pneumonectomy to make the animal completely dependent on the donor lung. All lungs were successfully evaluated without oedema.
formation and retained normal function during a 24 hour observation period post-transplant (83).

Steen et al successfully used this procedure to evaluate human DCD lungs in 2001, which were subsequently transplanted with excellent function confirmed post-transplant (84). This demonstrated that DCD lungs can be successfully evaluated and safely transplanted using EVLP. However, Steen went on to use the EVLP circuit for donor lungs from a brain dead patient that had been rejected by all Swedish transplant centres. The left lung was oedematous with bleeding spots but was successfully reconditioned during EVLP and was transplanted with good post-operative function (85). This was the first reported case of EVLP reconditioning rejected donor lungs. Despite this, the mechanisms underlying the reconditioning process remain unclear.

The aim of the Lund protocol was to allow evaluation of donor lungs in the short term and therefore perfusion times typically did not exceed 3 hours (86, 87). To utilise EVLP as a method of extended evaluation and preservation and to allow therapeutic delivery to donor lungs prior to transplant, a prolonged EVLP protocol is required. Rakhorst et al were the first group to attempt 6 hours of EVLP but whilst the lung function remained stable, the pulmonary artery pressure and ventilator pressure increased significantly (88). The first successful attempt at prolonged EVLP was achieved by Cypel et al who maintained lungs in a viable state for 12 hours (89). To establish 12 hours of EVLP with no decline in lung function, the team utilised a lung maintenance strategy with several protective aspects included in the protocol to avoid any stress to the lung during perfusion (89). Whilst this protocol was largely based on the Lund protocol outlined by Steen and colleagues, several changes were incorporated to develop the Toronto protocol. One of these changes was the use of an acellular perfusate containing no packed red blood cells. The rationale for this was that there was no need for the red blood cells to supply oxygen to lung cells as this could come directly from the ventilator. This concept was outlined by Date et al who demonstrated that lung cells can maintain aerobic metabolism from the oxygen within the alveoli during preservation (90). It was also proposed that red blood cells become damaged over-time due to the mechanical nature of the circuit, which may cause complications during prolonged perfusion. Furthermore, in contrast to the Lund protocol where the target flow is 100% of the estimated cardiac output with a maximum PA pressure of 20mmHg, the Toronto protocol maintains flow at 40% of cardiac output with PA pressure of 10-15mmHg. This makes this a low flow,
low pressure circuit causing non-dependent areas of the lung to receive sub-optimal perfusion. Despite this the histology post-transplant remains equivalent to dependent areas that received greater perfusion. This protocol has been used successfully to evaluate and recondition high-risk donor lungs, with equivalent clinical outcomes compared to standard transplants (91).

1.4.2 Ex-Vivo Normothermic Perfusion

The success of EVLP and the demand for utilising marginal donor organs in the last decade has led to a renewed interest in developing normothermic perfusion as a means to optimise preservation of other solid-organs.

Liver

Whilst livers from DBD donors tolerate prolonged periods of SCS much better than hearts or lungs, DCD donor livers are much more sensitive to damage. Normothermic preservation may therefore offer an alternative method of preserving DCD livers, helping to increase donor organ utilisation. Indeed, following Steen and colleagues’ successful transplant of EVLP lungs, Schön et al performed a study to assess normothermic preservation of donor livers, known as ex vivo normothermic preservation (EVNP) or normothermic machine perfusion. Livers from DBD donors were subjected to either 4 hours of SCS or 4 hours of EVNP, followed by transplantation. No significant difference was noted between the groups demonstrating the feasibility of preserving livers using EVNP. Perhaps more importantly, Schöns group have subsequently demonstrated a substantial survival benefit in recipients receiving DCD donor livers preserved using EVNP compared to those receiving a standard SCS transplant (92). In this study, livers were subjected to 1 hour of WI followed by either 4 hours of EVNP or 4 hours of SCS. All six animals that received EVNP preserved livers survived, whilst none survived following SCS. Successful EVNP of porcine livers for up to 72 hours was also achieved at this time and demonstrated conserved hepatic metabolic and synthetic activity. This confirmed the superiority of EVNP over SCS of donor livers (93-95). Since these early experiments, several refinements have been made to the experimental set up which includes replacing bile salts within the perfusate. This has subsequently improved liver function and viability allowing more detailed evaluation of organs prior to transplantation (96, 97).

Much of the evidence surrounding EVNP of isolated livers with autologous blood has come from large animal models, particularly using pigs. These studies provided sufficient
preclinical data to warrant proceeding clinical trials. Friend et al secured a European Commission Seventh Framework Programme (FP7) Award to perform a multinational randomised trial comparing the efficacy of EVNP with SCS in human liver transplantation. The pioneering work by this group has demonstrated the non-inferiority of EVNP, whilst also providing evidence of the safety and feasibility of this technique (98).

**Kidney**

Although EVNP has been successfully utilised to repair damaged kidneys, the interest in the clinical application of ex vivo perfusion of kidneys has remained lower than that for other organs (99). This is perhaps due to the tolerant nature of DBD kidneys to SCS, although the IRI that ensues following transplantation of DCD kidneys after SCS results in higher rates of DGF. In addition, hypothermic machine perfusion is increasingly used over SCS in kidney transplantation with growing evidence of its benefits at reducing DGF post-transplantation (100). Despite this, restoring renal metabolism and function provides the opportunity for a more comprehensive assessment of kidney function, which facilitates in transplant decision making. Furthermore, a large percentage of donor kidneys are rejected due to inadequate perfusion in situ yet this may not reflect the true function of the organ. Indeed, Hosgood et al demonstrate that kidneys declined for transplantation based on inadequate in situ perfusion can be assessed and restored using normothermic perfusion (101).

Brasile et al was one of the first to demonstrate that a short period of normothermic preservation using an acellular perfusate following SCS reduced reperfusion injury in a canine autologous transplant model (102). Since then Nicholson et al have dominated the field of EVNP, providing the majority of the evidence for EVNP of donor kidneys. Much of their early work was performed in large animal models where they demonstrated that the addition of EVNP towards the end of SCS preservation was a feasible method and offered benefit over SCS alone (103). Additionally, this period of restored metabolism allows reparative processes to occur prior to transplantation (104). This group then led the first clinical study to assess the effects of EVNP in kidneys from marginal donors, demonstrating it is both a feasible and safe method of preservation (105). Despite this, the use of EVNP for donor kidneys remains largely underutilised both clinically and for research purposes.

**Heart**
Whilst other organs have successfully utilised DCD and ECD organs to increase the donor pool, DCD donor hearts have previously been considered too high risk by clinicians for use in transplantation due to the prolonged warm ischaemia encountered (106). However, with current transplant demand increasing, enabling the use of these organs is essential. One of the major constraints of using DCD donors is the inability to evaluate the function or structural integrity of the heart whilst asystole. An effective means of assessing pre-transplant function and viability would allow a greater utilisation of donor hearts. In addition, hearts do not tolerate long periods of cold ischaemia, with SCS times limited to 4-6 hours before irreversible damage occurs. Normothermic preservation or ex vivo heart perfusion (EVHP) provides the opportunity to alleviate both of these constraints, allowing donor hearts to be rapidly transferred from the donor onto the ex vivo device to be immediately re-warmed. This significantly reduces the cold ischaemic time and allows the donor heart to be re-animated so its function can be determined. Recent evidence from an international trial involving 10 cardiac transplant centres compared SCS and EVHP, demonstrating that EVHP can be safely used to evaluate and transport donor hearts. Furthermore, long-term benefits in patients receiving EVHP compared to SCS hearts have been reported with a lower incidence of severe AR and reduced primary graft failure (107). A commercially available device for EVHP, known as the organ care system, has recently been developed. A global clinical trial, PROCEED II, has recently demonstrated the non-inferiority of EVHP preserved hearts compared to those preserved using SCS (108).

1.5 Rationale, Aims and Objectives

As previously discussed, one of the major caveats to long-term transplant success is the high incidence of AR. This occurs as a recipient dependent allospecific response to donor antigens present on donor-derived cells. Despite this knowledge there remains a limited understanding of the natural history of passenger leukocyte transfer from the donor organ to the recipient, or the contribution of donor organs to the inflammatory cascade that ensues post-transplant. This is partly due to it previously being impossible to evaluate the temporal kinetics of leukocyte migration. However, the development of EVOP has provided a platform to assess immune cell extravasation following revascularisation. As donor organs are attached to a circuit that mimics the circulatory system, it is likely that passenger leukocytes will mobilise as they would in the post-transplant setting. In addition, mobilisation of donor cells into the circuit
may allow their removal prior to transplantation. This is particularly true in the case of EVLP where a leukocyte filter is attached to the circuit to capture circulating immune cells. As these cells play a major role in AR, their removal prior to transplantation may impart significant clinical benefit.

1.5.1 Hypothesis:

Ex vivo perfusion induces donor leukocyte mobilisation and removal prior to transplantation, thereby reducing graft immunogenicity and the incidence of AR.

1.5.2 Aims:

This PhD studentship was designed to address the following aims;

1) Characterise the temporal kinetics of passenger leukocytes from donor lungs following transplantation

2) Determine if a removal of passenger leukocytes during EVLP reduces the donor burden post-transplant and the effect of this on clinical outcomes

3) Establish a clinically relevant model of EVNP using porcine kidneys

4) Assess if the effects of EVLP are organ specific or can be translated into EVNP

5) Determine if a second preservation flush of donor kidneys impacts on graft immunogenicity prior to transplantation

The research described herein is divided into different experimental approaches. To characterise the extravasation of donor-derived cells following lung transplantation a large animal model of EVLP was utilised. Lung transplantation was chosen as the primary organ of interest owing to the sensitive nature of the lungs, the significant population of leukocytes they contain and because EVLP is the most developed of the ex vivo systems. In addition, lung transplant patients typically have the poorest post-transplant outcomes with an estimated 50% survival rate at 5 years post-transplant. Understanding the contribution of passenger leukocytes following transplantation may provide better immunosuppressive targets. This study proposes that a removal of passenger leukocytes during perfusion would reduce
leukocyte transfer into recipients, and thereby improve clinical outcomes manifesting as a reduction in AR. Kakishita et al provide evidence that EVLP is capable of removing inflammatory cytokines prior to transplantation (109, 110). This is a clinically important finding as Sekido et al demonstrate that using monoclonal antibodies to inflammatory cytokines can reduce the incidence of lung injury (111). It is therefore possible that removal of the cells responsible for the secretion of these cytokines during the EVLP procedure may reduce lung injury, and thereby decrease the incidence of graft rejection following transplantation. These results suggest that EVOP can alter post-transplant outcomes, making it plausible that this technique provides other clinical benefits that have yet to be explored. To demonstrate any improvement in post-transplant outcomes, a porcine transplant model was utilised, comparing standard transplantation to transplanting lungs following EVLP treatment. Using this model the primary aim was to determine the early effect of reducing the passenger leukocyte burden on the clinical outcome post-transplantation.

To determine if the processes identified during EVLP translate into other organs or if this is an organ specific phenomenon, a clinically relevant model of EVNP in porcine kidneys was established. Kidneys were chosen as the model of choice due to the increasing number of kidney transplants performed annually. In combination with this, EVNP remains an underutilised technique in kidney transplantation. Providing evidence of benefit using this technique may renew clinical interest in this preservation method, thereby increasing the number of donor kidneys available for transplantation. Once a clinically relevant EVNP model has been established it will be utilised to describe the inflammatory contribution of the donor kidney and determine the effects of perfusion on the kidney immune profile. In addition, a secondary preservation flush of the donor kidney following cold storage will be performed to determine if this also impacts on the immune burden of donor kidneys immediately prior to transplantation.

Insights into the immune content of donor organs and their migratory pattern following transplantation may greatly impact on treatment strategies and has the potential to change current clinical practice.
Section 2 – Cellular Diadepesis during EVLP

A version of this paper is published in The American Journal of Transplantation:

Title of paper – Altered Immunogenicity of Donor Lungs via Removal of Passenger Leukocytes Using Ex Vivo Lung Perfusion


Within this chapter, the EVLP procedure was performed by Professor Stig Steen and his team in Lund, Sweden. I collected all of the samples during the EVLP procedure, performed all of the experiments (flow cytometry and luminex) and analysed all of the data. All of the statistical analysis was checked by our statistician prior to publication.

Sections of this chapter have also been presented as a poster:

Title of Poster - Characterising passenger leukocyte migration from the donor lung using ex vivo lung

2.1 Abstract

**Introduction:** Passenger leukocyte transfer from the donor lung to the recipient is intrinsically involved in acute allograft rejection. Direct presentation of complete alloantigen expressed on donor leukocytes is recognised by recipient T cells, inducing allospecificity, which is a prerequisite for acute cellular rejection. Using EVLP the natural history of the inflammatory response and passenger leukocyte migration from the donor lung has been characterised.

**Methods:** Explanted porcine lungs (n=6) underwent 3 hours of EVLP. Perfusate samples were collected at 30 minute intervals for a total of 180 minutes, and upon completion the leukocyte filter was removed from the circuit. A range of immune cells were characterised via flow cytometry. An inflammatory profile was generated via quantification of cytokines and chemokines within the perfusate.

**Results:** Throughout perfusion there were continuous increases in IFN-γ (p<0.001), IL-1α (p=0.014), IL-1β (p<0.001), IL-1RA (p<0.001), C-X-C Motif Chemokine Ligand 8 (CXCL-8) (p<0.001), IL-10 (p<0.001), IL-12 (p<0.001), IL-6 (p<0.001), TNF-α (p=0.003) and IL-18 (p<0.001). Significant populations of mature donor basophils, eosinophils and T cells (comprising of equal ratios of CD4+ and CD8+ cells) were identified within the first 30 minutes of perfusion. Of the monocyte repertoire, classical monocytes represented only a minor fraction, with non-classical monocytes found in much greater abundance. Minor populations of neutrophils, B cells and NK cells were also detected at all time points. On completion of perfusion the cell content within the leukocyte filter was assessed, with cell populations comparable to above.

**Discussion:** The donor lung possesses a significant immune compartment capable of direct presentation of self (donor) antigens, provision of co-stimulation via APC, T cell activation, immunologic help via B cells, innate immunity via a significant granulocyte population, and the induction of non-specific systemic inflammation via cytokine secretion. These findings also indicate that EVLP may be of benefit in removing this inflammatory content prior to transplantation.
2.2 Introduction

Respiratory diseases represent a leading cause of death worldwide, with many patients developing end stage lung disease. Whilst current therapeutics can provide symptomatic relief, the only treatment option remains a lung transplant. However, one of the major caveats to long-term transplant success is the high incidence of AR. It is well established in the literature that this occurs as a recipient dependent allospecific response to donor antigens present on donor-derived cells (112). Despite this, to date we have a limited understanding of the natural history of passenger leukocyte transfer from the donor lung to the recipient. Given that the lung represents a primary barrier to the external environment and is equipped with a sophisticated localised immune system, such data are important.

During homeostasis, the lung remains in a regulatory state and despite noticeable environmental fluctuations, maintains a regulated immune system. Several key mechanisms have been identified that prevent inflammatory responses from occurring in healthy individuals. One of the major regulators of immune function in the lung is mediated by cell-cell interactions of alveolar mφ and respiratory epithelial cells. A large population of alveolar mφ reside within the airspace in conjunction with type I and II alveolar epithelial cells, as well as in the interstitial space between the alveoli and blood vessels where populations of DCs, T cells and B cells are found. In humans, these mφ are poor antigen presenters and lack the expression of co-stimulatory molecules such as CD86, which induces T cell antigen-specific unresponsiveness and promotes tolerance (113). In addition, alveolar mφ have reduced phagocytic activity but produce immunosuppressive prostaglandins and transforming growth factor-β, which also suppresses T cell activity (114). However, following an insult, a significant immune response can occur. In severe cases of injury and inflammation, such as brain or cardiac death, airway epithelium can become damaged and endogenous ligands exposed leading to a robust respiratory immune response (115). This includes pulmonary and systemic inflammation manifesting in a local cytokine storm and mass cellular infiltrate. Using current strategies for transplantation, it is at this point that inflamed donor lungs with a globally activated immune system are implanted into the recipient.

The understanding of the immediate events following transplantation remains limited, but a significant mobilisation of the donor leukocyte repertoire from the organ to the recipient is believed to occur (116). Although the importance of donor-derived immune cells in the
rejection process has been demonstrated previously, a detailed evaluation of the temporal kinetics of leukocyte migration from the lung has not been performed. In addition, the contribution of different immune cell populations following transplantation remains unclear as it has previously been impossible to assess this in vivo.

The advent of EVLP provides a unique opportunity to study immune extravasation immediately following revascularisation in a physiologically controlled environment outside of the human body. EVLP simulates the in vivo transplant setting, with the introduction of oxygenated blood following a period of ischaemia. For these purposes, this study was designed to provide an in-depth characterisation of the migratory behaviour of major immune populations following transplantation. In addition, the inflammatory profile will be described as well as the influence of EVLP on the donor lung immune cell repertoire.
2.3 Material and Methods

2.3.1 Ethical Approval:

The Ethical Committee of the University of Lund approved the study. Animals were treated in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication 85-23, revised 1985).

2.3.2 Donor and Recipient Preparation:

Free-ranging domestic pigs (n=6) with a mean weight of 63±2kg were used in this experiment.

Prior to any surgical procedure, all animals received 30mg/kg body weight of intramuscular ketamine medication (Ketalar, Parke-Davis, Morris Plains, NJ), and 4 mg/kg of xylasin medication (Rompun, Bayer, Gothenburg, Sweden). Animals were then anaesthetised intravenously using 5-8 mg/kg body weight of sodium thiopental (Pentothal; Abbot, North Chicago, IL) and 0.015 mg/kg of atropine (Atropine; Kabi Pharmacia, Uppsala, Sweden). Prior to tracheotomy and the introduction of the tracheal tube, pancuronium (Pavulon, Organon Teknika, Boxel, The Netherlands) was given intravenously. An anaesthetic mix was given throughout the experiment consisting of 8g ketamine, 30mg midazolam and 300mg pancuronium bromide dissolved in 5% glucose to 500ml at a continuous infusion rate of 20ml/hour. Fentanyl (Leptanal, Lilly, France) injections (2mg/kg/h) were also given intermittently throughout the experiment. Animals were ventilated using a Siemens Servo ventilator 300 (Siemens-Elema AB, Solna, Sweden). Ventilation was volume controlled and pressure regulated at 10L/min (20 breaths/min; positive end-expiratory pressure [PEEP], 8cm H2O).

2.3.3 Procurement and Preservation of the Donor Lungs:

A medial sternotomy was performed and a Swan-Ganz catheter was introduced into the pulmonary artery (PA). Catheters were placed into the arch of the aorta and the PA to allow the continuous monitoring of pressures. The hemodynamic values of the donor animal were
obtained and blood gases were recorded using a data acquisition system (Testpoint; Capital Equipment Corp, Billerica, MA).

Prior to the initiation of the preservation flush of the donor lungs, ventricular fibrillation was induced by electrical stimulation delivered directly to the epicardial surface. The PA was then perfused with approximately 2L of Perfadex solution (XIVO Perfusion AB, Gothenburg, Sweden), the composition of which cannot be disclosed in this thesis due to patent protection. Lungs were fully ventilated during this procedure. Following this, the tracheal tube was disconnected from the ventilator and the lungs were allowed to collapse. Lungs were then procured, submerged in cold Perfadex in a semi-inflated state with 100% oxygen and placed in a refrigerator (4°C) for 2 hours.

2.3.4 Ex Vivo Lung Perfusion:
EVLP was performed for 3 hours on 6 donor lungs as previously described (83). The procedure was performed using the Vivoline LS1 system (Vivoline Medical AB, Lund, Sweden). The circuit was primed with 2 litres of pneumoprotective STEEN Solution (VT029, Vitrolife AB) and donor blood type specific leukocyte depleted packed red blood cells to achieve a target haematocrit of 15% ± 3%. Additional supplements of 20iU insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) and 10,000iU heparin (Leo Pharma, Malmö, Sweden) were also added and the perfusate was buffered to a physiological level of pH 7.4 with trometamol (Addex-THAM, Kabi, Sweden). Finally, 100mg imipenem was added to the perfusate. A blend of oxygen, nitrogen and carbon dioxide was supplied to the membrane oxygenator through two gas mixes (one for oxygen/nitrogen and one for oxygen/carbon dioxide) and the flow was adjusted to achieve a physiological venous blood gas.

After a period of cold storage, the PA cannula was connected to the perfusion circuit with careful de-airing through the open bypass shunt. Reperfusion was initiated at a low flow rate of 0.5 L/minute. The first 200ml of blood exiting the cannula in the left atrium was discarded. The lungs were slowly re-warmed, maintaining a delta T between lung temperature and perfusate temperature less than 8°C. The flow rate was increased whilst ensuring that PA pressure was initially maintained at 15mmHg and then if stable, at 20mmHg. At 25°C the bypass shunt was closed, diverting all of the perfusate through the lung vasculature. When the temperature of the lungs reached 32°C, protective mechanical ventilation was initiated (less than 1.5 times the circulation in minute volume) maintaining peak airway pressures of less
than 30mmHg. Ventilation was gradually increased as the lungs continued to warm, with full ventilation achieved at 37°C. During this time, if there were areas of persistent atelectasis, recruitment manoeuvres were performed through transient increases in PEEP. When a steady state was achieved, with stable perfusate flow and ventilator settings, blood gases and haemodynamics were registered. At this point the oxygenator was disconnected from the circuit, so that the perfusate was representative of mixed venous blood and the functionality of the lungs could be assessed via the oxygenation of the blood. An arterial and venous blood gas was run on a fraction of inspired oxygen (FiO$_2$) of 50%, 100% and 21%. Lungs were deemed acceptable if pO$_2$ was greater than 13kPa and pCO$_2$ was less than 6kPa with an FiO$_2$ of 21%, or if pO$_2$ was greater than 40kPa with a pCO$_2$ less than 6kPa with an FiO$_2$ of 100%. If lungs were suitable then they were cooled, oxygen was reconnected, ventilation was stopped and the trachea was clamped at half inspiration.

2.3.5 Sample Collection during EVLP

**Perfusate:**

Prior to initiation of EVLP, 5ml of perfusate was collected into Ethylenediaminetetraacetic acid (EDTA) vacutainers. Additional samples were then collected at 30-minute intervals. At each time point, 100µl of perfusate was analysed by flow cytometry. To obtain plasma, EDTA tubes were centrifuged at 500g for 10 minutes. Plasma was aliquoted into 1ml vials and stored at -80°C. When EVLP assessment was completed, the leukocyte filter (Vivoline Medical AB, Lund, Sweden) was clamped to prevent the release of cells into the circuit, and removed for analysis. Trypsin was added in a retrograde direction, incubated at 37°C for 5 minutes, washed with phosphate-buffered saline (PBS) and centrifuged. Cells were re-suspended in PBS and assessed using flow cytometry.

**Bronchoalveolar Lavage (BAL):**

BAL samples were collected via standard protocols both pre- and post EVLP. Briefly, approximately 100mls normal saline was passed into the lungs via a bronchoscope and collected for analysis. The collected BAL was passed through a 100µm pore, nylon mesh cell strainer (BD Bioscience, Oxford, UK), washed at 450g for 10mins at 4°C and re-suspended in 1ml PBS. Cells were analysed via flow cytometry.
2.3.6 Flow Cytometry:

Immunophenotyping of the samples was performed on an EPICS XL flow cytometer (Beckman Coulter, UK). A panel of antibodies was utilised to characterise T helper cells (CD3ε+ CD4α+), cytotoxic T cells (CD3ε+CD8β+), B cells (CD21+CD79+), classical monocytes (CD14+CD163-), non-classical monocytes (CD14+CD163+), immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+) and mature eosinophils/basophils (6D10-2B2+) and NK cells (CD3ε-CD8α+CD16+). Cells were treated with red blood cell lysing solution (BD Biosciences, UK), washed and re-suspended in 0.5ml PBS. Samples were analysed for 3 minutes. All gating strategies were performed using FlowJo version 10.0.6 (Figure 2.1).

Flow cytometry materials:

Spectral red (SPRD)-conjugated anti-CD3ε and PE-conjugated mouse anti-CD8α were purchased from Southern Biotech (Birmingham, USA). PE-Cy5-conjugated mouse anti-CD21 and purified mouse anti-CD8β were obtained from BD Bioscience (Oxford, UK). FITC-conjugated mouse anti-CD4, FITC-conjugated mouse anti-CD16, FITC-conjugated mouse anti-CD79, PE-Cy5-conjugated mouse anti-CD14, FITC-conjugated mouse anti-CD163, FITC-conjugated mouse anti-2B2, PE-conjugated mouse anti-6D10 and Lynx Rapid R-PE antibody conjugation kit were all purchased from AbD Serotec (Kidlington, UK).
Tube 1

Tube 2

Tube 3
Figure 2.1 – Gating strategy used to identify leukocyte populations in the EVLP perfusate

Representative image of the gating strategy used to identify leukocyte populations in the EVLP perfusate using flow cytometry. In tubes 1-3, all leukocyte populations were selected from the forward and side scatter and leukocyte populations identified as follows. In tube 1, granulocytes were gated on their positive or negative stain of 2B2 and 6D10. In tube 2, B cells were identified as CD79+ and CD21+. In tube 3, monocytes were gated depending on their positive or negative staining for CD14 and CD163. In tubes 4-6, a lymphocyte population was initially selected from the forward and side scatter. NK cells were then identified in tubes 4 and 5 by first selecting a population of CD3- cells to remove T cells. Tube 4 then selected CD8+ cells and from this a population of CD16+ cells were defined as NK cells. To confirm this population in tube 5, a population of CD4- followed by CD8+ cells were selected from the CD3- population. Finally in tube 6, pan T cells were gated as CD3+ and then CD4+ and CD8+ populations identified from this.


2.3.7 Luminex Analysis:

The level of cytokines and chemokines within the perfusate was assessed using a porcine 13-plex magnetic bead panel (Merck Millipore, Missouri, USA). All reagents used were supplied within the kit. Plasma samples were thawed and a 96-well plate was prepared by adding 200µl assay buffer before being shaken for 10 minutes at room temperature. Following this, the buffer was decanted and 25µl of standards and controls were added to the appropriate wells, along with 25µl of matrix solution. To the sample wells, 25µl of assay buffer was added with 25µl of neat plasma. The pre-mixed antibody solution was thoroughly vortexed to ensure appropriate separation of the beads and 25µl was added to all wells. The plate was sealed, wrapped in foil and incubated with agitation on a plate shaker overnight at 4°C. After incubation, the plate was washed three times and 50µl of detection antibodies were added. The plate was re-sealed, wrapped in foil and incubated for a further 2 hours at room temperature. An additional 50µl of Streptavidin-Phycoerythrin was added to each well and the plate was re-sealed and incubated for 30 minutes at room temperature. The plate was then washed three times and 100µl of sheath fluid was added to all wells and the plate was agitated for 5 minutes on a plate shaker. Finally, the plate was read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, UK).

2.3.8 Statistical Analysis:

Data normality was determined by assessing the descriptive statistics, mean, standard deviation, skewness and kurtosis. The analysis of the change in cell numbers over the time course of the experiment was performed using the General Linear Model. Where data were not normally distributed, log-values were derived prior to analysis. For the comparison of BAL-derived cells and cells within the perfusate at baseline and 30 minutes, the paired Student’s t-test was used. For those parameters with non-parametric data distribution, the Mann-Whitney U test was utilised. Data were considered significantly different if a p value of <0.05 was observed.
2.4 Results

2.4.1 Perfusate Characterisation:

Major populations of mature basophils and eosinophils migrated into the circuit within the first hour of perfusion, although this was significantly reduced thereafter (p<0.001). Donor T cells comprising of an approximately equal ratio of CD4+ and CD8+ cells were identified within the first 30 minutes of perfusion, and remained stable over time (p=0.060 and p=0.068 for CD4+ and CD8+ cells respectively). Both mature and immature neutrophils diapedesed into the perfusate within the first hour, and were significantly reduced over time (p=0.045 and p=0.024 respectively). Classical monocytes represented a minor fraction, with non-classical monocytes found in greater abundance despite this cell type being considered rare. However, the size of both populations remained consistent during perfusion (p=0.345 and p=0.164 for classical and non-classical repertoires respectively). Minor populations of B cells and NK cells were also detected, diminishing over time (p=0.001 and p=0.039 respectively; Figure 2.2).

All populations increased in the circuit within the first 30 minutes of perfusion (Table 2.1).
Figure 2.2 - Donor leukocytes migrate out of the lung into the perfusate during EVLP

Serial perfusate samples were analysed by flow cytometry to identify migrating leukocytes and their change over time assessed using the general linear model. A similar pattern of migration occurred for immature neutrophils, mature neutrophils, mature basophils and eosinophils, CD21+CD79+ B cells, CD21-CD79+ B cells, CD4+ T cells, CD8+ T cells, non-classical monocytes, classical monocytes and NK cells. The largest populations of extravasating cells were mature eosinophils and basophils.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Baseline Mean ± SD/median [IQR]</th>
<th>30 minutes Mean ± SD/median [IQR]</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells</td>
<td>1444.17 ± 1567.78</td>
<td>6051.83 ± 4609.34</td>
<td>0.023</td>
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<tr>
<td>CD8+ T cells</td>
<td>2013.00 ± 2351.31</td>
<td>7201.33 ± 4842.38</td>
<td>0.026</td>
</tr>
<tr>
<td>CD21+ CD79+ B cells</td>
<td>4.50 [11.50]</td>
<td>41.50 [78.25]</td>
<td>0.028</td>
</tr>
<tr>
<td>CD21- CD79+ B cells</td>
<td>11.50 [54.50]</td>
<td>75.00 [91.00]</td>
<td>0.046</td>
</tr>
<tr>
<td>NK Cells</td>
<td>3.00 [372.25]</td>
<td>301.00 [926.75]</td>
<td>0.043</td>
</tr>
<tr>
<td>Immature neutrophils</td>
<td>21.00 [18.75]</td>
<td>1125.50 [2885.50]</td>
<td>0.028</td>
</tr>
<tr>
<td>Mature neutrophils</td>
<td>151.50 [1478.75]</td>
<td>498.00 [3686.50]</td>
<td>0.028</td>
</tr>
<tr>
<td>Mature eosinophils and basophils</td>
<td>982.83 ± 1069.91</td>
<td>9704.00 ± 6660.10</td>
<td>0.016</td>
</tr>
<tr>
<td>Classical monocytes</td>
<td>78.017 ± 89.46</td>
<td>581.50 ± 480.29</td>
<td>0.040</td>
</tr>
<tr>
<td>Non-classical monocytes</td>
<td>203.00 [357.25]</td>
<td>806.00 ± [3580.50]</td>
<td>0.028</td>
</tr>
</tbody>
</table>

**Table 2.1 - Number of donor leukocytes at baseline and 30 minutes post perfusion**

The number of donor leukocytes within n=6 EVLPs significantly increased in the circuit between baseline and 30 minutes, demonstrating rapid extravasation from the donor lung.
2.4.2 Leukocyte Filter:

Eosinophils, basophils and neutrophils were abundant in the leukocyte filter at the end of perfusion. Both CD4+ and CD8+ T cells were found in large numbers. In a similar fashion to the perfusate, non-classical monocytes were found in higher numbers than their classical counterparts. NK cells and B cells represented only minor populations of the immune cell reservoir. The total number and populations of cells identified within the filter is comparable to that seen in the perfusate samples (Figure 2.3).
Figure 2.3 - Donor leukocytes are removed in the leukocyte filter

At the end of perfusion the leukocyte filter was removed and leukocyte populations identified by flow cytometry (n=6). Leukocyte populations mirrored that observed within the perfusate during perfusion, with granulocytes being most abundant. For graphical purposes, an outlier has been omitted from the mature neutrophil population.
2.4.3 BAL Characterisation:

No significant difference was observed between immune cells in BAL taken both pre and post EVLP, with the exception of CD21⁺ CD79⁺ B cells (Table 2.2). Neutrophils were the most abundant cells both pre and post EVLP, whilst NK cells were scarcely observed. The non-classical phenotype predominated within the observed monocyte repertoire.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mean (Pre-EVLP) ± SD</th>
<th>Mean (Post-EVLP) ± SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells</td>
<td>2472.57 ± 3207.15</td>
<td>1891.71 ± 2031.78</td>
<td>1.000</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>1427.71 ± 955.66</td>
<td>1680.57 ± 1589.32</td>
<td>0.612</td>
</tr>
<tr>
<td>CD21+ CD79+ B cells</td>
<td>292.71 ± 350.54</td>
<td>743.43 ± 716.90</td>
<td>0.043</td>
</tr>
<tr>
<td>CD21- CD79+ B cells</td>
<td>2082.14 ± 3532.90</td>
<td>714.57 ± 748.71</td>
<td>0.398</td>
</tr>
<tr>
<td>NK cells</td>
<td>14.83 ± 18.65</td>
<td>17.83 ± 23.48</td>
<td>0.685</td>
</tr>
<tr>
<td>Immature neutrophils</td>
<td>67264.14 ± 173767.25</td>
<td>9520.43 ± 19713.50</td>
<td>1.000</td>
</tr>
<tr>
<td>Mature neutrophils</td>
<td>62911.29 ± 136688.64</td>
<td>23516.29 ± 27696.26</td>
<td>0.735</td>
</tr>
<tr>
<td>Mature eosinophils and basophils</td>
<td>4590.00 ± 4859.55</td>
<td>6063.14 ± 8018.13</td>
<td>0.398</td>
</tr>
<tr>
<td>Classical monocytes</td>
<td>832.54 ± 1394.23</td>
<td>565.86 ± 1272.70</td>
<td>0.345</td>
</tr>
<tr>
<td>Non-classical monocytes</td>
<td>3920.67 ± 1958.26</td>
<td>5798.57 ± 3233.22</td>
<td>0.122</td>
</tr>
</tbody>
</table>

Table 2.2 - Number of donor leukocytes in BAL samples pre and post EVLP

Donor leukocyte numbers do not change in the airway of the lungs (n=6) during EVLP. Leukocytes were assessed in pre and post BAL samples by flow cytometry. No significant differences were noted between the pre and post BAL samples, with the exception of CD21+ CD79+ B cells (p=0.043).
2.4.4 Inflammatory Profile:

Throughout perfusion there were continuous increases in IFN-γ (p<0.001), IL-1α (p=0.014), IL-1β (p<0.001), IL-1RA (p<0.001), CXCL-8 (p<0.001), IL-10 (p<0.001), IL-12 (p<0.001), IL-6 (p<0.001), TNF-α (p=0.003) and IL-18 (p<0.001). No significant alterations in the concentration of circulating IL-4 were detected during perfusion (p=0.321), and both IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were undetectable (Figure 2.4).
Figure 2.4 - Cytokine secretion increases over time during ex vivo perfusion
Serial perfusate samples from *n* = 6 EVLPs were analysed by Luminex to detect a range of cytokines and chemokines and the change in concentration over time was assessed using the general linear model. After approximately 90 minutes on the circuit, EVLP is associated with a rapid increase in the secretion of IFN-γ, IL-1α, IL-1β, IL-1Ra, IL-6, CXCL-8, IL-10, IL-12, IL-18 and TNF-α, although IL-4 remains unaffected.
2.5 Discussion

The lungs are continually exposed to a milieu of inhaled environmental and microbiological agents, and as such contain a large reservoir of immune cells. It is estimated that each healthy lung contains approximately $40 \times 10^9$ immune cells, playing an essential role in tissue homeostasis and surveillance. Following inflammation, there is down-regulation of regulatory mechanisms and immune cells become exposed to endogenous ligands leading to activation. Indeed, the phenotype of alveolar mφ is changed upon inflammatory stimulation and can lead to pro-inflammatory, rather than regulatory responses (117). This is particularly true in the case of lung transplantation when the donor has been through the inflammatory process of brain or cardiac death, followed by trauma caused by surgical removal of the organ. Lungs obtained for transplantation therefore contain a significant population of primed donor leukocytes, representing a major adjuvant for clinical rejection.

Despite this, the natural history of donor cell diapedesis following transplantation remains unclear, although mobilisation of donor leukocytes from the lung into the recipient circulation is believed to occur. The introduction of donor immune cells to the recipient has long been known to contribute to allograft rejection. Lechler et al demonstrated that injection of donor-derived DC into rats receiving donor devoid, re-transplanted kidneys induced AR, highlighting the essential role of these cells in determining the success of a transplant. It is now considered that donor APCs migrate to recipient lymph nodes following transplantation. Here they present alloantigen via intact donor MHC directly to naïve recipient T cells, inducing clonal expansion, activation and migration to the graft (118, 119). This is an area that warrants exploration, as current immunosuppression regimes only target the recipient immune repertoire, ignoring the contribution of donor leukocytes. Immunomodulation of the donor immune system prior to transplantation could offer therapeutic potential.

The development of EVLP has not only revolutionised the way lung transplantation is conducted, but has provided the ideal platform to study the immune mechanisms that occur within the donor lung. The advantage of such a system is that normothermic perfusion mimics the circulatory environment encountered immediately following revascularisation. As such, the perfusate simulates the continuous flow of blood into the donor organ, and provides the opportunity to study the fate of passenger leukocytes following engraftment in an ex vivo setting. In combination with the leukocyte filter, this method may be therapeutically utilised
for the removal of pro-inflammatory donor-derived cells, which would typically be transferred directly into the recipient following lung transplantation. The findings of this study indicate that during EVLP there is significant mobilisation of donor-derived immune cells into the perfusate. These cells were detected as early as 30 minutes post EVLP initiation, and therefore based on the temporal kinetics of donor leukocyte trafficking into the perfusate; these cells are likely to be predominantly marginated to the vasculature of the lung. In the transplant setting, these cells will encounter recipient immune cells and induce AR. The early migration of these cells is likely to occur in response to various danger signals, including the catecholamine secretion following brain death and DAMP release, as well as to organ stress following donor preservation and retrieval.

A number of these cells were removed via the leukocyte filter attached to the EVLP circuit, preventing these cells from re-entering the lung prior to transplantation. However, the number of cells in the leukocyte filter remains comparable to that observed in the perfusate in the early period. This suggests that the filter may become saturated over time. The use of multiple filters may therefore be of benefit to continuously remove donor-derived cells throughout the EVLP procedure.

In this study, there was a significant population of monocytes within the perfusate and filter. Monocytes have the capacity to differentiate into DCs and mφ that can orchestrate AR via rapid migration from the donor lung to recipient lymph nodes. Monocytes, DCs and mφ perform direct presentation of self (donor) antigens to naïve T cells, and secrete a plethora of inflammatory cytokines following exposure to DAMPs. A removal of these donor-derived cells during EVLP may therefore impart therapeutic benefit in the lung transplant setting. Within this study, a surprising reversal of the expected monocyte population dynamics was noted. A markedly greater number of the minor, non-classical monocyte population was observed compared to the number of classical monocytes present. Non-classical monocytes typically represent 10% of the monocyte population in blood, and an estimated 1% of the immune cell repertoire. However, the data suggest that the donor lung contains a large reservoir of non-classical monocytes, which has not previously been reported. This is in corroboration with work described by our group in human EVLP (120). These cells are inflammatory in nature, expressing high levels of MHC Class II on their surface and secrete inflammatory cytokines during inflammation. In addition, non-classical monocytes exhibit high endothelial affinity with the capacity for rapid mobilisation and extravasation, which
may explain the large population found in the perfusate samples. Whilst the fate of the extravasating non-classical monocytes cannot be determined in this model, evidence in the literature suggests that lymphoid homing and DC differentiation is likely to occur. Monocyte derived DCs that are intravenously injected into mouse models home to various lymph nodes, including splenic, pancreatic, mesenteric and renal nodes (121). In addition, the long-term effects of non-classical monocyte transfer is unknown, yet it has been reported that monocytes and DCs are identifiable 12 months post-transplantation, suggesting that donor-recipient interactions continue for long periods after transplant (122). This is a novel finding that in combination with a low population of classical monocytes in both the BAL and perfusate samples clearly warrants further research.

Populations of CD4+ and CD8+ T cells, along with a small population of B cells, were also present within the EVLP perfusate and leukocyte filter. Donor-derived B and T cells possess the ability to both clonally expand and secrete a plethora of cytokines that can drive an inflammatory response. In addition, a significant efflux of neutrophils was observed following revascularisation. It is likely that these cells will be primed and activated prior to transplantation, particularly given the inflammatory profile observed. Active neutrophils have the ability to produce ROS, contributing to both oxidative stress and DAMP release which can potentiate the inflammatory response. Furthermore, neutrophils are responsible for IL-17 production, driving a Th17 response and reducing Treg cell expansion (123). Indeed, neutrophilia in the transplant setting has been attributed to chronic rejection. A removal of these cells prior to transplantation is therefore likely to confer significant clinical benefit.

The direct comparison of immune cell frequencies in both pre and post EVLP BAL samples indicates that the major immune populations remain static in the airway throughout EVLP. This would suggest that airway immunity is maintained prior to transplantation and remains unchanged in the post-transplant setting. Whether this increases allore cognition and immune responsiveness within the recipient, or provides a degree of airway resistance to community infection cannot be confirmed until long term clinical data become available.

Over the course of the EVLP experiment, increasing concentrations of a range of cytokines were detected, although levels of IL-4 remained unchanged throughout. Despite this, lung function remained stable suggesting that the cytokine release did not impact upon the integrity of the lung tissue. Whilst the source of these cytokines is unknown, it is likely that this non-specific inflammatory response is unexpected due to the exposure of the lung and its contents
to the plastic nature of the circuit. This is commonly reported in patients following cardiopulmonary bypass (CPB), where blood cells coming into contact with the artificial environment of the extracorporeal circuit become activated, yet this does not appear to contribute to clinical outcome (124). One of the largest cytokines present during perfusion was IFN-γ, with levels exceeding 60,000 pg/ml at the end of EVLP. To put this into context, pigs with severe swine influenza infection have IFN-γ concentrations of less than 1,000 pg/ml (125). The role of IFN-γ on immune cell function during inflammation is well characterised and involves the up-regulation of PRRs, antigen processing and presentation, leukocyte trafficking and inhibition of cellular proliferation (126). Together, these processes would increase graft immunogenicity and induce both donor and recipient cellular mobilisation, contributing to AR. However, it has recently been demonstrated that IFN-γ can induce the expression of the programmed death ligand 1 (PD-L1) (127, 128). PD-L1 is a type I transmembrane protein that is expressed on mφ, myeloid DCs, mφ, and activated T and B cells as well as non-haematopoietic stem cells. Under certain conditions, such as in the presence of IFN-γ, PD-L1 is up-regulated. Upon ligation of its receptor, programmed cell death protein 1 (PD-1), which is expressed on several immune cells including T cells, it drives a signal that down-regulates immune responses via an inhibition in IL-2 secretion. This occurs primarily via an inhibition of ZAP70 phosphorylation (129). At the same time, apoptosis is induced leading to T cell death. A blockade of this pathway can restore T cell function (130). Whilst the expression of PD-L1 was not determined in this study, it is plausible that the high level of IFN-γ within the EVLP circuit induces the expression of PD-L1 on donor cells prior to transplantation, thereby inhibiting T cell responsiveness. This would not only prevent tissue injury, explaining the stability of lung function during EVLP, but would induce a tolerogenic environment prior to transplant. Evidence in the literature demonstrates that CD4+ T cells expressing PD-1 induce IL-10 secretion from monocytes when ligating PD-L1 on the cell surface (131).

This study provides novel data surrounding the migration of immune cells from healthy donor tissue into the vasculature. In the transplant setting, lungs are obtained from patients that have undergone brain death or circulatory death, which induces an inflammatory cascade ultimately leading to tissue injury. Importantly, this is accompanied by a recruitment and influx of activated inflammatory cells into the donor organ. It is important to note therefore, that the data presented are representative of the ‘normal’ lung environment, as the lungs used in this study were sourced from healthy animals, rather than following brain or cardiac death.
Despite this the results of this study demonstrate that donor-derived immune cells rapidly migrate from the lung, where following standard lung transplant protocols, they would enter the recipient circulation and induce AR.

This finding is inherently important for the design of novel therapeutics that target donor-derived cells as current therapeutic regimes only target the recipient’s immune cells. In addition, EVLP is currently only considered as a tool for the reassessment of donor organs at the recipient hospital prior to transplantation, and as a potential reconditioning strategy for marginal organs. However, this study suggests that EVLP may impart benefit following transplantation via the mechanical removal of inflammatory cells directly involved in immune mediated processes that lead to AR. Clearly, characterising these cells and assessing the effect of their removal prior to transplantation warrants further research.
Section 3 – Reduced Passenger Leukocyte Transfer following EVLP Impairs Direct Allorecognition Post-Transplantation

A version of this paper is published in The American Journal of Transplantation:

Title of paper – Altered Immunogenicity of Donor Lungs via Removal of Passenger Leukocytes Using Ex Vivo Lung Perfusion


Within this chapter, the EVLP procedure and the transplants were performed by Professor Stig Steen and his team in Lund, Sweden. I collected all of the samples prior to the transplants being performed and in the post-transplant period. I also performed all of the experiments with the assistance of student’s within our laboratory, with the exception of the immunohistochemistry and histological grading of the tissue. This was performed by a collaborator in Norway. I analysed all of the data. I performed all of the statistical analysis which was checked by our statistician prior to publication.

Sections of this chapter have also been presented as an oral presentation. This presentation was shortlisted for the Medawar award:

Title of Presentation – Ex vivo lung perfusion impairs direct allorecognition reducing recipient T cell infiltration

3.1 Abstract

Introduction: Depletion of immune cells from the donor organ prior to transplantation prevents rejection, whereas restoration of the depleted immune compartment restores the alloresponse. Strategies to remove donor immune cells prior to transplantation may therefore represent a potential therapeutic intervention. For the purpose of this study, a porcine EVLP transplant model was used to determine if donor immune cell depletion impacts on recipient T cell responses.

Methods: 12 female recipient pigs were randomised to receive either i) a left male lung following 3 hours of EVLP or ii) a left male lung retrieved using standard protocols, followed by right pneumonectomy. Recipients were monitored for 24 hours and samples were collected at 0, 6, 12 and 24 hours. Donor or recipient cells were identified via Y chromosomal selection. T cell infiltration was assessed and graded using standard transplant guidelines. In all cases EVLP transplantation was compared against standard lung transplantation.

Results: At all time points EVLP reduced donor leukocyte transfer following transplantation, determined by total donor DNA quantification and Y+ cell counts. Donor leukocyte migration to the recipient splenic and liver lymph nodes was also reduced (spleen – 0.16ng/ul vs. 0.09ng/ul, p=0.006, liver 0.17ng/ul vs. 0.12ng/ul, p=0.038). Recipient T cell infiltration of the donor lung was significantly lower in EVLP recipients when compared to the standard transplant group (p=0.039).

Conclusions: These data suggests that passenger leukocyte removal during EVLP reduces direct allorecognition and T cell priming within the recipient, leading to a loss of recipient T cell infiltration of the donor lung. Immunomodulation of donor lungs prior to transplantation clearly warrants further investigation.
3.2 Introduction

Allorecognition, the process of donor antigen presentation to recipient T cells precedes allograft rejection. This occurs by two primary routes, firstly the direct recognition of MHC Class II restricted alloantigens presented by donor APCs to recipient T cells, and secondly via indirect recognition of donor antigens which have been processed by recipient APCs (118). Direct allorecognition alone can result in AR, even without indirect mechanisms (132). Furthermore, depletion of donor immune cells from an organ prior to transplantation prevents rejection (133), and restoration re-establishes the alloresponse (134). It therefore appears that clinical outcome following transplantation is dependent on the early processes of recipient allorecognition of donor antigens. This is especially true in the context of lung transplantation, where the lung represents a primary barrier to the external environment and is equipped with a sophisticated immune compartment. In severe cases of injury and inflammation such as brain or cardiac death, a robust systemic immune response ensues (135), which includes pulmonary inflammation manifesting in a local cytokine storm (136) and mass cellular infiltrate (135). Using current strategies for transplantation, it is here that inflamed donor lungs with a globally activated immune system are transplanted into the recipient.

Although the importance of direct allorecognition is well documented, the donor leukocyte compartment is not targeted by current immunosuppressive strategies prior to transplantation. This is partly the result of a limited understanding of the natural history of passenger leukocyte transfer from the donor organ to the recipient, although a significant mobilisation of donor leukocytes is believed to occur (137, 138). A detailed evaluation of the temporal kinetics of leukocyte migration from the lung has not previously been possible. However, the advent of EVLP provides a unique opportunity to study immune extravasation immediately following revascularisation.

EVLP allows donor lungs to be removed from the inflammatory donor environment and placed onto an ex vivo circuit where a more detailed evaluation of donor lungs can be performed. A reconditioning effect has also been reported during EVLP, providing the opportunity to utilise lungs that would otherwise be clinically rejected for transplantation (78, 139). A protective strategy of organ reperfusion is employed that replicates the in vivo environment, providing a physiological representation of the events occurring immediately post-transplantation. In section 2 the immune cell extravasation from the donor lung into the
recipient in the early post-operative period was characterised using EVLP. Here it was demonstrated that a significant proportion of donor immune cells rapidly migrate out of the donor lung following revascularisation where they will interact with the recipient immune repertoire. However, as EVLP is a closed system the donor immune cells that would typically enter a recipient’s circulation enter the circuit instead and are therefore removed prior to transplantation. Furthermore, the EVLP circuit contains a leukocyte filter, which prevents a proportion of donor cells from re-entering the lung during perfusion. Whilst the fate of these cells following transplantation is unknown, it is likely that lymphoid homing and alloantigen presentation will occur. Creusot et al demonstrate the homing of monocyte derived DC to the splenic, pancreatic, paratracheal, mesenteric, lumbar, renal and inguinal lymph nodes when intravenously delivered (121). The homing of donor-derived DCs to T cell rich recipient lymph nodes was also analysed by Pietra et al, where it was established that this process is vital for the induction of direct allorecognition and is considered to be the primary cause of AR (140). A removal of donor-derived cells prior to transplantation may therefore impart clinical benefit in the post-transplant setting via a reduction in AR.

To determine if removal of passenger leukocytes during EVLP reduces the incidence of AR following transplantation, a large animal model of EVLP was utilised. Pigs were randomised to either receive a standard lung transplant or a lung transplant following EVLP. Male donors to female recipient lung transplants were performed, allowing donor leukocyte migration to be determined via the presence of the Y chromosome. Clinical outcome was determined by a histological grading of AR at 24 hours post-transplant.
3.3 Materials and Methods

3.3.1 Ethical Approval:

The Ethical Committee of the University of Lund approved the study. Animals were treated in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication 85-23, revised 1985).

3.3.2 Procurement of Donor Organ:

Free-ranging domestic pigs (n=24) with a mean weight of 63±2kg were used in this experiment (12 donors and 12 recipients). All transplantations were male to female, allowing the detection of male cells via the Y chromosome. Preparation of the donor and procurement of organs was performed as previously described in section 2.

3.3.3 Experimental Procedure:

The donor lung was preserved with Perfadex (XVIVO Perfusion AB, Gothenburg, Sweden). After harvesting, lungs were stored at 4°C for 2 hours. Following cooling, the lungs were randomly assigned to standard or EVLP groups. In the standard group, the left lung was prepared for transplantation and implanted into a recipient pig in which left pneumonectomy had been performed. In the EVLP group, lungs were perfused for 3 hours with core cooling to 8°C in the last 30 minutes. The left lung was then transplanted as in the control group.

3.3.4 Ex Vivo Lung Perfusion (EVLP):

Male donor lungs (n=6) underwent 3 hours of EVLP prior to transplantation as previously described in section 2. The procedure was performed with the Vivoline system (Vivoline Medical AB, Lund, Sweden).

3.3.5 Lung Transplantation:

A female recipient of similar size and blood group as the male donor animal was anaesthetised
and prepared as described in section 2 and as previously described in the literature (141). A tracheostomy was performed and a number 7 endotracheal tube was inserted. Animals were ventilated as described for donor pigs. Two central venous catheters were introduced via the internal jugular vein, with two further catheters being placed within the aorta through the carotid artery. A Foley catheter was inserted into the urinary bladder through a suprapubic cystotomy.

A right thoracotomy was performed through the seventh intercostal space. The pulmonary ligaments were cut up to the hilus while the pericardium was opened posterior to the phrenic nerve. A left thoracotomy was then performed through the sixth intercostal space, followed by a left pneumonectomy, leaving the long ends of the pulmonary veins. The left main bronchus was occluded near the carina with a Satinski vascular clamp. A gracile Satinski vascular clamp was placed intrapericardially across the lateral wall of the left atrium, incorporating the stumps of the left pulmonary veins. An arterial cuff was formed by an incision joining the inferior and superior pulmonary veins. The Satinski vascular clamp on the bronchus was removed and ventilation was started. A catheter was inserted into the left atrium to measure pressure. Once the blood flow through the transplanted was stable and consistent, the left thoracotomy was closed using towel clips. Pigs were then placed right side up and a right pneumonectomy was performed within 3 hours after transplantation using a TA-90 surgical stapler (United States Surgical Corp, Norwalk, CT).

Recipient pigs remained anaesthetised throughout the 24-hour period. At 24 hours post-transplantation, a final assessment of lung function was made before pigs were sacrificed via an intravenous administration of a high dose of potassium to stop the heart. Ventilation was then stopped and the trachea was disconnected from the ventilator.

3.3.6 Serological Post-transplant Peripheral Blood Sample Collection:

Peripheral blood (20ml) was collected from donor and recipient pigs pre-transplantation and sequentially from recipients at 0, 6, 12 and 24 hours post-transplantation. Samples were centrifuged at 500g for 10 minutes. Aliquots of plasma and whole blood were stored at -80°C.


3.3.7 Cytospin Preparation:

**Peripheral Blood:**

To isolate leukocytes, 100µl of peripheral blood was taken and mixed with 500µl of red blood cell lysis solution (BD Biosciences, Oxford, UK) to lyse erythrocytes, before being placed in the dark for 10 minutes. After 10 minutes, 1ml of Phosphate Buffered Saline (PBS) solution was added to the sample before placing it back in the dark for 5 minutes. The cells were than washed by centrifugation at 300g for 10 minutes. Supernatant was decanted and the cells were re-suspended in 1ml PBS. A cell count was performed by mixing 20µl of 0.4% trypan blue (Sigma-Aldrich, Dorset, UK) with 20µl of the sample in an Eppendorf. A haemocytometer was prepared by placing a cover slip over the grids and 20µl was added. Cells were adjusted to a concentration of 1 x 10⁶/ml using PBS and 100µl of the cell suspension was added to a cytofunnel (ThermoFisher Scientific, Waltham, MA, USA) slide chamber with a pre-labelled slide (ThermoFisher Scientific, Waltham, MA, USA) and added to a cytocentrifuge (Shandon Cytospin 3). Centrifugation then began at 40g for 3 minutes. Superforst plus slides (ThermoFisher Scientific, Waltham, MA, USA) containing the isolated leukocytes were removed, checked under a microscope to ensure sufficient cell coverage with no overlapping cells and allowed to air dry. Slides were then placed into methanol for 10 minutes to fix the cells for later processing.

**Bronchoalveolar Lavage:**

BAL samples collected 24 hours post-transplantation were passed through a 100µm pore, nylon mesh cell strainer and centrifuged at 300g for 10 minutes at 4°C. Supernatant was removed and stored in aliquots at -80°C. The remaining cells were re-suspended in 1ml PBS and enumerated using trypan blue differentiation as described above. Cells were adjusted to a concentration of 1 x 10⁶/ml using PBS. 100µl of the cell suspension was added to a slide chamber with a pre-labelled slide and added to a cytocentrifuge. Centrifugation was carried out at 40g for 3 minutes. Slides were removed and allowed to air dry before being fixed in methanol for 10 minutes.
3.3.8 Lymph Node and Biopsy Collection:

Following sacrifice at 24 hours, biopsies were collected from the spleen, liver and lung. Liver and splenic lymph nodes were also collected. Pulmonary lymph nodes were not studied as they were of donor origin. Samples were either stored at -80°C, or fixed in 10% neutral buffered formalin.

3.3.9 DNA Extraction:

DNA was extracted from peripheral blood and lymph node lysate samples using the QIAamp DNA Mini and Blood Mini kit, according to the manufacturer’s protocol (Qiagen, Manchester, UK). For peripheral blood, 200µl was added to a sterile, DNase free microcentrifuge tube, along with 20µl proteinase K and 20µl RNase A. Following 2 minute incubation at room temperature, 200µl of lysis buffer was added and samples were incubated in a water bath at 55°C. After 10 minutes, samples were removed from the water bath and 200µl of 100% ethanol was added to the lysate. Prepared cell suspensions were added to purelink spin columns and centrifuged at 10,000g for 1 minute. Spin columns were removed and placed into clean purelink collection tubes and the sample was washed with 500µl wash buffer 1 before centrifugation at 10,000g for 1 minute. This process was repeated using 500µl wash buffer 2, with centrifugation at maximum speed (approximately 21,000g) for 3 minutes. To elute the DNA, spin columns were placed into clean, sterile 1.5ml microcentrifuge tubes and 50µl of purelink genomic elution buffer was added. After 1-minute incubation at room temperature, columns were centrifuged at maximum speed for 1 minute. DNA concentration was determined spectrophotometrically using a nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and adjusted to 50ng/ml using DNase/RNase-free distilled water (Life Technologies, Paisley, UK). Samples were stored at -20°C.

For lymph nodes, samples were prepared using a mortar and pestle. The powder was weighed and 25mg was added to a sterile, DNase free microcentrifuge tube with 180µl of purelink digestion buffer and 20µl proteinase K. Samples were incubated in a water bath at 55°C until lysis was complete (1-4 hours). Cells were centrifuged at maximum speed for 3 minutes to remove any particulate material. Supernatant was transferred to a new 1.5ml sterile microcentrifuge tube before the addition of 20µl RNase. Following 2 minutes incubation, 200µl purelink genomic lysis/binding buffer and 200µl 100% ethanol was added. Samples
were then transferred to purelink spin columns and treated as per the protocol for peripheral blood samples.

3.3.10 Quantitative PCR (qPCR):

All primers used for qPCR were designed using the Primer Express® Software v3.0.1 (Life Technologies, Paisley, UK) and their homology assessed using BLAST. The forward primer designed for the SRY gene was (5’ CAAGTGGCTGGGATGCAAGT 3’) and the reverse was (5’ TCGAAGAATGGGCGCTTTT 3’). For glyceraldehyde 3-phosphate dehydrogenase (GAPDH) the forward primer (5’ TGCTCCTCCCCGTTCCA 3’) and reverse primer (5’ GGCTTTACCTGGCAATGCA 3’) were used. Prior to use, primers (Sigma Aldrich, Dorset, UK) were de-salted and adjusted to 150nM using nuclease-free water (ThermoFisher Scientific, Waltham, MA, USA).

3.3.11 PCR Plate Set-up:

qPCR was performed using a QuantStudio™ 12K Flex system (Life Technologies, Paisley, UK) using a Power SYBR green PCR master mix (Life Technologies, Paisley, UK). The following reactions were made up for each well of a MicroAmp Optical 384-Well Reaction Plate (Life Technologies, Paisley, UK); 5µl Power SYBR green PCR master mix, 3.9µl H₂O, 0.3µl Forward Primer (at 0.15µM), 0.3µl Reverse Primer (at 0.15µM) and 0.5µl DNA. A sheet of optical adhesive film was used to seal the plate and the following protocol was used for the qPCR procedure; 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence of each well was then read and a melt curve from 60°C to 95°C was included at the end of the program to analyse the products. Ct values (the number of cycles needed to produce a threshold amount of fluorescence) of more than 40 were discarded, along with any samples that produced multiple peaks in the melt curve as this may have been due to primer dimers. In all cases, each PCR was performed with quadruplet samples. For quantification, standard curves were constructed using serial dilutions of DNA from a male donor pig lymph node sample. Gene expression was assessed as a ratio of SRY expression to the housekeeping gene, GAPDH.
3.3.12 Fluorescent in situ Hybridisation (FISH):

Preparation of Solutions:

All solutions required for FISH analysis were prepared fresh on the day before starting the experiment using the ZytoLight FISH-Cytology Implementation Kit (Zytovision, Germany)

A 1x TBS (Tris-buffered saline) wash solution was prepared by diluting the 20x wash buffer TBS in 1:20 with distilled water. A 1% formaldehyde solution was prepared by diluting 10mls 10% neutrally buffered formaldehyde with 10mls of 10x magnesium chloride and 10mls 10x PBS. The volume was adjusted to 100mls with distilled water. A series of ethanol dilutions were prepared with distilled water to achieve 70%, 90% and 100% solutions.

Slide Pre-treatment:

Cytology pepsin solution was added dropwise to prepared cytospin slides and samples were incubated for 10 minutes at 37°C in a water bath. Slides were then incubated in a coplin jar containing the 1x TBS solution for 5 minutes at room temperature, followed by a 5 minute incubation in the 1% formaldehyde solution. Cells were then washed again in the 1x TBS solution for 5 minutes. Finally, slides were dehydrated by immersing them sequentially in 70%, 90% and 100% ethanol for 1 minute at room temperature.

Denaturation and Hybridisation:

Slides were allowed to air dry and then 10µl of ZytoLight porcine X/Y dual color probe (Zytovision, Germany) was placed onto the pretreated cytospin slides and a cover slip was added and sealed using rubber cement (MP Biomedicals Europe). Slides were placed on a preheated hot plate at 72°C ± 1°C for 2 minutes to allow the probe to co-denature with the chromosomal DNA. Slides were placed into a coplin jar and left overnight at 37°C in a water bath (ensuring the slides remained dry) to allow hybridisation to occur.

Post-hybridisation Washes:

Following overnight hybridisation, rubber cement was carefully removed from the slides, before placing them in a coplin jar containing cytology stringency wash buffer SSC for 2 minutes at 72°C ± 1°C. The jar was periodically shaken to remove the cover slips from the slides. Slides were placed in a coplin jar containing cytology wash buffer SSC solution and
incubated for 1 minute at room temperature. The slides were then air dried before being mounted with 30µl DAPI/Antifade solution.

**Slide Visualisation:**

To detect the X and Y chromosome a fluorescent microscope was used. Images were collected on an *Olympus BX51* upright microscope using a 60x/0.65-1.25 Plan Fln objective and captured using a Coolsnap ES camera (Photometrics, Tucson, USA)) through MetaVue Software (Molecular Devices, Sunnyvale, USA). Specific band pass filter sets for DAPI, ZyOrange and ZyGreen (Zytovision, Germany) were used. Images were processed and analysed using ImageJ.

**3.3.13 Luminex Analysis:**

The concentration of cytokines and chemokines within the circulation post-transplant was assessed using a commercially available porcine 13-plex magnetic bead panel (Merck Millipore, Billerica, MA, USA). Briefly, plasma samples were thawed and a 96-well plate was prepared by adding 200µl assay buffer before being shaken for 10 minutes at room temperature. Buffer was decanted and 25µl of standards and controls were added to the appropriate wells, along with 25µl of matrix solution. To the remaining wells, 25µl of assay buffer was added with 25µl of neat plasma. Pre-mixed antibodies were mixed and 25µl was added to all wells. The plate was incubated overnight at 4°C with gentle agitation. After incubation, the plate was washed three times and 50µl of detection antibodies were added for 30 minutes at room temperature. Following this, 50µl of Streptavidin-Phycoerythrin was added to each well and incubated for 30 minutes at room temperature. The plate was washed three times, re-suspended in 100µl sheath fluid and read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, UK).

**3.3.14 Enzyme-linked Immunosorbent Assay (ELISA):**

The plasma concentration of high sensitivity C-reactive protein (hs-CRP) was assessed using a commercially available porcine hs-CRP ELISA kit (2b Scientific, Oxfordshire, UK). Standards were prepared and 50µl were added to a 96 well plate, along with 50µl streptomycin-HRP. To sample wells, 40µl of neat plasma, 10µl hs-CRP antibodies and 50µl streptomycin-HRP were added. The plate was incubated at 37°C for 60 minutes followed by 5
washes. To all wells, 50µl chromogen solution A and 50µl chromogen solution B were added and incubated at 37°C for 10 minutes. Finally, 50µl of stop solution was added to all wells and the ELISA plate was read using a Tecan infinite 200 PRO system (Tecan Group, Männedorf). Plasma samples from anaesthetised pigs were used as a baseline, and compared to recipient post-transplant samples.

3.3.15 Histological Preparation of Lung Tissue:

Lung tissue biopsies, one central and two peripheral from each lobe, were fixed in 10% buffered formalin solution and paraffin-embedded (142). Four-micron sections were cut, de-paraffinized and stained with haematoxylin, eosin and saffron. One specimen from the periphery of the upper and one from the lower lobe from each pig, showing least atelectasis and granulocyte infiltration, were selected for immunohistochemistry.

3.3.16 Immunohistochemistry:

Sections were positioned on Superfrost Plus slides (Menzei-Glaser, Braunschweig, Germany) and de-paraffinized. Antigen retrieval (Tris-EDTA pH 9.0 buffer for 3 minutes at 750W and 20 min at 900W in a Whirlpool Talent Microwave Oven) was performed prior to 30 minute incubation at 37°C with the primary polyclonal rabbit anti-CD3 antibody (Neo Markers), diluted 1:200 in Ventana Antibody Diluent (Ventana Medical System, Tucson, USA). Binding of the primary antibody was detected using the Ultra View Universal DAB Detection Kit (Ventana Medical System, Tucson, USA). The slides were washed in Ventana APK detergent between incubations.

3.3.17 T cell Quantification:

Perivascular lymphocyte infiltrates were utilised as an indication of T cell recruitment to the grafts. Vessels with the largest accumulations surrounding them were marked. T cells were counted in the 10 largest infiltrates in each section, and the size of the corresponding infiltrate was measured. T cell infiltrates around vessels close to bronchi and bronchioles were avoided. T cells were also counted in alveolar septa in five randomly selected areas in each immunostained section, each 0.5 x 0.5 mm. The histopathologist remained independent to the study and was blinded to all sample identification.
3.3.18 Statistical Analysis:

All statistical analyses were performed using SPSS version 20.0. Data normality was determined by assessing mean, standard deviation, skewness and kurtosis. Formal evaluation was performed using the Shapiro-Wilk test. For direct comparisons between EVLP and standard samples obtained at a single time point, either the Independent samples t-test or the Mann-Whitney U test were utilised depending on the distribution of the data. For the comparison of BAL-derived cells, the paired Student’s t-test was used. Changes in cell number and cytokine concentration over time in the perfusate were analysed using the Related-Samples Friedman’s 2-Way ANOVA by Ranks. Comparisons of hs-CRP and cytokine concentration alterations between groups post-transplant was performed using the General Linear Model. Data were considered significantly different if a p value of <0.05 was observed.
3.4 Results

3.4.1 Transplantation of EVLP vs. standard lungs:

**Clinical variables:**

Before donor lung harvesting there was no difference in partial pressure arterial oxygen/fraction of inspired oxygen (PaO$_2$/FiO$_2$) between the groups (64.1 ± 3.1 and 62.3 ± 2.5 for EVLP and standard, respectively). After reperfusion there were no differences in lung function determined via serial measurements of PO$_2$/FiO$_2$ and partial pressure of carbon dioxide (PCO$_2$) except at 12 hours after reperfusion, when the PO$_2$/FiO$_2$ was significantly higher in the EVLP group (73.5 ± 1.9 compared to 56.1 ± 4.9 in the standard group (Table 3.1)). Mean arterial pressure (MAP) and mean pulmonary artery pressure (MPAP) did not differ significantly throughout the study. This enabled the study to proceed as any differences between groups could be causally related to perfusion rather than the function of the lung.
<table>
<thead>
<tr>
<th>Time</th>
<th>MAP (mmHg)</th>
<th>MPAP (mmHg)</th>
<th>PO2/FiO2 (kPa)</th>
<th>PCO2 (kPa)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Standard</td>
<td>EVLP</td>
<td>Standard</td>
<td>EVLP</td>
</tr>
<tr>
<td>6 hours</td>
<td>78.2±2.6</td>
<td>76.5±3.5</td>
<td>28.7±2.3</td>
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<td>18 hours</td>
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<td>69.0±1.8</td>
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<td>28.5±3.8</td>
</tr>
<tr>
<td>24 hours</td>
<td>70.2±4.1</td>
<td>73.7±4.3</td>
<td>31.3±4.0</td>
<td>31.2±2.4</td>
</tr>
</tbody>
</table>

Table 3.1 – Physiological parameters post-transplantation

Table demonstrating the physiological parameters in pigs following lung transplantation. All parameters remained similar in the two groups at all time points, with the exception of 12 hours when the PO2/FiO2 was significantly higher in the EVLP cohort. Standard values are given as mean and the standard error of the mean (±SEM), (n=6). ** p<0.01.
3.4.2 EVLP induces short-term non-specific inflammation post-transplantation:

A systemic inflammatory response was observed in pigs receiving EVLP treated lungs, which was similar to that described during perfusion (Table 3.2). Immediately post-transplant, a greater concentration of IL-1β (p=0.051), IL-2 (p=0.032), IL-4 (p=0.009), CXCL-8 (p=0.042) and IL-10 (p=0.028) was observed in the EVLP cohort compared to standard. The inflammatory response generally dissipated by 6 hours post-transplantation, although the levels of IFN-γ (p=0.041), IL-6 (p=0.027) and TNF-α (p=0.053) remained elevated until 12 hours in the EVLP group. No differences in cytokine concentrations were observed within BAL samples obtained from the donor airway post-transplantation (Figure 3.1).

Surprisingly, there were no differences in hs-CRP levels between the groups at any point (p=1.000, p=1.000, p=0.818 and p=0.937 for 0, 6, 12 and 24 hours respectively, Figure 3.2). Immediately following removal of cross-clamp there was a small increase in hs-CRP in both groups (both p=0.002), most likely driven by mild IRI, which remained consistently low without returning to baseline.
### Table 3.2 – Cytokine and chemokine concentrations post-transplantation

Comparison of the cytokine/chemokine secretion in the post-operative period in standard (n=6) vs. EVLP (n=6) transplant recipients.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>EVLP</th>
<th>Standard</th>
<th>p-value</th>
<th>EVLP</th>
<th>Standard</th>
<th>p-value</th>
<th>EVLP</th>
<th>Standard</th>
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<th>Standard</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Median</td>
<td>Mean</td>
<td>SD</td>
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<td>SD</td>
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<tr>
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<td>TNF-α</td>
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</table>

Time point (hours)

0 | 6 | 12 | 24

Cytokine Concentration (pg/ml) - mean±SD/median[IQR]
Figure 3.1 - EVLP does not alter cytokine concentrations in the donor airway following transplantation

Bronchoalveolar lavage samples were taken at 24 hours from n=12 pigs (n=6 standard and n=6 EVLP) and analysed by Luminex to detect a number of cytokines and chemokines. The following cytokines all remained statistically similar to those observed in standard lungs: GM-CSF (a), IFN-γ (b), IL-1α (c), IL-1β (d), IL-1Ra (e), IL-4 (f), IL-6 (g), IL-10 (h), IL-12 (i), IL-18 (j) and TNF-α (k).
Figure 3.2 - EVLP is not associated with clinical inflammation following transplantation

Blood samples were taken from healthy pigs (n=6) to provide a baseline value, and at 0, 6, 12 and 24 hours following transplantation (n=6 standard and n=6 EVLP recipients). hs-CRP concentration was then analysed by ELISA. The circulating concentration of hs-CRP remained similar between EVLP and standard groups.
3.4.3 EVLP reduces donor leukocyte transfer post-transplantation:

Donor-derived diapedesis into the recipient circulation was assessed over the 24-hour period via the detection of $Y^+$ chromosomal DNA using qPCR (Figure 3.3A). Immediately post-transplantation there was significantly less circulating $Y^+$ chromosomal DNA in the EVLP group compared to the standard ($p=0.002$). This pattern was also conserved at 6, 12 and 24 hours ($p=0.041$, $p=0.038$ and $p=0.040$ respectively). To confirm that the reduction was not cell-free artefact, FISH was performed on cytospins to quantify the number of $Y^+$ donor leukocytes within the recipient circulation. In corroboration with the DNA findings, significantly less donor-derived cells were identified in the EVLP cohort compared to standard (0 hours $p=0.010$, 6 hours $p=0.002$, 12 hours $p=0.030$ and 24 hours $p=0.026$ respectively, Figure 3.3B and Figure 3.4).
Figure 3.3 A and B - Donor leukocyte migration is impaired from EVLP treated lungs compared to standard lungs

Blood samples taken post-transplant were either analysed by qPCR to detect Y+ DNA (A) or used to prepare cytopsins for assessment by FISH (B). The reduction in donor leukocyte migration by EVLP was indicated by the loss of Y+ DNA in the circulation at all time points following transplantation (A). This translated into a loss of passenger leukocyte transfer into the recipient circulation as determined by Y+ cell quantification (B).
Figure 3.4 – The number of male (donor) cells in recipient peripheral circulation is reduced following EVLP

A representative image of FISH-stained cytospins from (a) standard and (b) EVLP transplanted lungs. Cytospins were prepared from whole blood taken at multiple time points and stained using X (red) and Y (green) chromosome specific FISH probes.
3.4.4 EVLP reduces donor leukocyte migration to recipient lymph nodes:
We quantified Y⁺ chromosomal DNA in lymph nodes. There was significantly less Y⁺ chromosomal DNA in both spleen (p=0.006) and liver (p=0.038) lymph nodes from pigs receiving EVLP reconditioned lungs compared to standard transplants (Figure 3.5).
Figure 3.5 - EVLP ameliorates passenger leukocyte trafficking to recipient lymph nodes

Splenic and liver lymph nodes were homogenized and Y-chromosome DNA was detected by qPCR. The concentration of donor DNA within the recipient splenic (A) and liver (B) lymph nodes at 24 hours post-transplantation was significantly reduced by EVLP.
3.4.5 EVLP reduces T cell infiltration post-transplantation:

The impact of reduced passenger leukocyte transfer was assessed to determine if this diminished graft infiltration of recipient T cells where reduced direct allore cognition would impair T cell priming. There were cuffs of lymphocytes around some of the small vessels, which were comparable to cuffs used diagnostically for clinical AR (Figure 3.6). Recipient T cell infiltration was significantly lower in the EVLP cohort when compared to standard (p=0.039, Figure 3.7). Furthermore, there was a greater T cell infiltrate within the upper compared to the lower lobe in 5 out of 6 donor lungs of each group (Table 3.3). T cells were also counted in alveolar septae and no significant difference in alveolar T cells could be found between the groups (p=0.484, Figure 3.8 and 3.9 respectively).
Figure 3.6 – Histological representation of lymphocyte cuffing around vessels
Lymphocytes were counted in the 10 largest infiltrates in each tissue section, and the size of the corresponding infiltrate was measured.
Figure 3.7 - T cell infiltration into the graft was impaired following EVLP
T cells were identified within lung tissue sections by immunohistochemistry. The number of T cells quantified surrounding the donor organ vasculature was significantly greater in standard compared with EVLP treated lungs.
<table>
<thead>
<tr>
<th></th>
<th>Upper Lobe</th>
<th>Lower Lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVLP 1</td>
<td>2854</td>
<td>967</td>
</tr>
<tr>
<td>EVLP 2</td>
<td>1509</td>
<td>1077</td>
</tr>
<tr>
<td>EVLP 3</td>
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<tr>
<td>EVLP 4</td>
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<thead>
<tr>
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</tr>
</thead>
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<tr>
<td>EVLP: upper vs. lower</td>
<td>0.041</td>
</tr>
<tr>
<td>Standard: upper vs. lower</td>
<td>0.108</td>
</tr>
<tr>
<td>Upper lobe: EVLP vs. standard</td>
<td>0.009</td>
</tr>
<tr>
<td>Lower lobe: EVLP vs. standard</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Table 3.3 – Number of T cells in upper and lower lobes of both standard and EVLP treated lungs

T cell infiltrates in the upper and lower lobes of donor lungs 24 hours post-transplantation. T cell infiltrate within the upper compared to the lower lobe was greater in 5 out of 6 donor lungs of each group.
Figure 3.8 - Histological representation of lymphocyte staining in the alveolar septa

Histological sections of the alveolar septae were prepared and lymphocytes were stained and quantified within lung tissue sections using immunohistochemistry.
No difference was detected in airway T cell infiltration between EVLP and standard lungs. The number of T cells quantified per square mm of alveolar lung tissue was similar in standard compared with EVLP treated lungs. T cells were quantified within lung tissue sections using immunohistochemistry.
3.5 Discussion

A significant barrier to long-term success following lung transplantation is the immune response following recognition of alloantigen by recipient leukocytes. Standard immunosuppressive regimens target recipient lymphocyte proliferation and activation without considering the impact of passenger leukocytes. Donor lungs contain a significant population of leukocytes, representing a major adjuvant for clinical rejection. However, the natural history of donor cell diapedesis following transplantation remains unclear. The introduction of donor immune cells to the recipient is known to contribute to AR. Injection of donor-derived dendritic cells into rats receiving donor-devoid, re-transplanted kidneys induces AR, highlighting the role of donor-derived APCs in determining the success of transplantation (134). Donor APCs migrate to recipient lymphoid organs following transplant and present alloantigen to naïve recipient T cells, inducing clonal expansion, activation and migration to the graft (118, 119). This area warrants exploration as immunomodulation of donor immunity prior to transplantation may offer therapeutic potential.

Normothermic EVLP mimics the circulatory conditions encountered following revascularisation, providing unique insight regarding donor leukocyte movement into the recipient. In combination with leukocyte filtration, this method may be used to remove pro-inflammatory donor-derived cells, typically transferred into the recipient post-transplantation. The early extravasation of cells from the donor lung into the perfusate may reflect a stress response following death, ischaemia and surgical trauma. In accordance with this, a plethora of soluble mediators were detected as part of a generalised inflammatory environment within the perfusate, similar to that described recently (143). Previous studies have demonstrated a systemic inflammatory response comparable to the systemic inflammatory response syndrome in patients during CPB, whereby interactions between blood constituents and artificial circuit components lead to cellular activation (144). Whilst this may have contributed to the inflammatory environment observed, it is expected that the detrimental processes following death and organ retrieval would also represent a major initiator of inflammation. Therefore it is likely that standard lungs would also exhibit a significant inflammatory response to revascularisation, although this could not be investigated in this study. Passenger leukocytes exposed to this environment may display a pro-inflammatory phenotype and are thus primed prior to transplantation (120). These data demonstrates that EVLP induces mobilization of donor leukocytes into the perfusate and their removal via the leukocyte filter. However, the
number of cells in the filter remained comparable to that observed in the perfusate in the early period, suggesting saturation. Multiple filters may therefore be beneficial to continuously remove donor-derived cells throughout EVLP. Further depletion of the donor immune repertoire before transplantation may reduce the post-operative inflammatory cascade, leading to a more tolerogenic environment.

At all times, there was a significant reduction in donor-derived DNA within the recipient circulation. This study indicates that EVLP ameliorates donor-derived leukocyte transfer into the recipient circulation following revascularisation, potentially reducing the potency of the direct allore cognition pathway. Additionally, passenger cell migration to recipient lymph nodes was significantly diminished at 24-hours post-transplant following EVLP, as confirmed by quantifying Y+ DNA at these sites. According to the principles of direct allore cognition, the loss of donor leukocyte trafficking to recipient lymph nodes would result in perturbed naïve recipient allospecific T cell priming and subsequent infiltration into the graft. Indeed, a significantly greater T cell infiltrate into the donor lung, seen as cuffs around small vessels as in cellular rejection, was reported following standard versus EVLP transplantation. Considering that immunosuppression was not administered throughout this study, the marked decrease in T cell infiltration is clinically significant, highlighting the importance of the donor leukocyte compartment as a therapeutic target. Whether the addition of immunosuppression would amplify the beneficial effects of EVLP remains unclear, however targeting both the donor (via EVLP) and the recipient (via current immunosuppressive strategies) will likely improve outcomes.

Following EVLP lung transplantation, an early increase in a broad spectrum of cytokines was observed compared to standard transplant. However, this did not influence lung function or immune priming over the experimental period. Furthermore, hs-CRP concentration, a clinical indicator of inflammation remained comparable between the groups. Additionally, the inflammatory profile of the airway demonstrated equivalent cytokine concentrations, indicating the alveolar septae remained unaffected by EVLP. This was corroborated by the similarity in recipient T cell recruitment into the alveolar septa 24-hours post-transplantation. The elevated cytokine profile observed following EVLP transplantation might be unexpected considering the loss of infiltrating T cells. However, this may be a non-specific inflammatory response following exposure to the circuit, as described in patients following cardiopulmonary bypass, which does not appear to contribute to clinical outcome.
Previous studies have demonstrated the importance of direct allorecognition, yet limited detail of immune transfer and donor-recipient interaction has been described. EVLP provides a novel method for evaluating early leukocyte trafficking from donor to recipient, although not all immune cell populations could be evaluated in this study due to the limited availability of robust and reliable porcine antibodies. The short-term follow-up is a limitation that precludes interpretation of long term outcomes following EVLP; however T cell recruitment into the graft is a hallmark of AR, which was significantly reduced without immunosuppression. This suggests that EVLP may represent a clinical method for the removal of donor leukocytes, thus allowing insight into their importance following transplantation. Moreover, the early interactions between donor and recipient leukocytes are thought to play a critical role in long-term outcome following transplantation. The demonstration that EVLP-mediated donor leukocyte depletion impairs early recipient T cell priming without immunosuppression represents a novel and exciting approach to tackling AR. However, it remains unclear from these data whether the reduction in passenger leukocyte trafficking via EVLP subsequently alters indirect presentation by recipient APCs. It is plausible that the reduction in graft immunogenicity observed at 24 hours would at least delay T cell infiltration following indirect allorecognition due to greater preservation of graft cells.

Early immune activation remains a significant problem following transplantation. This study highlights that removal of passenger leukocytes during EVLP reduces donor leukocyte transfer into the recipient culminating in reduced recipient T cell infiltration into the graft. These findings can be evaluated in the clinical setting to provide an additional level of validation as EVLP has become an established procedure (145). Adaptation of this technique as a platform for pharmacological delivery of immunomodulatory agents may allow novel intervention to target passenger leukocytes. Indeed, the use of adenoviral vectors encoding IL-10 within the circuit has proven successful in a previous study (146). This could have significant implications in terms of improved treatment strategies that regulate both donor and recipient immunity. It has already been demonstrated that removal of donor leukocytes, particularly non-classical monocytes, is possible via EVLP in humans (120). As a significant non-classical monocyte population was discovered in this study, it is likely that the benefits observed post-transplant will be conferred in human EVLP. Early clinical trial data has indicated equivalence in clinical outcome between standard transplant and EVLP reconditioned lungs, allowing an expanded donor organ pool (147-150). Whilst significantly
beneficial in itself, there is scope for further improvements in clinical outcome with EVLP via modulation of the donor leukocyte repertoire.
Section 4 – Development of an Ex Vivo Normothermic Perfusion Circuit

Part of this chapter is taken from a paper published in Kidney International Reports:

Title of paper – Ex Vivo Normothermic Perfusion Induces Donor-derived Leukocyte Mobilisation and Removal Prior to Renal Transplantation


DOI: http://dx.doi.org/10.1016/j.ekir.2016.07.009

For this section, I retrieved all of the organs from the abattoir and performed all of the normothermic perfusions. My colleagues primed the normothermic perfusion circuits to allow the 2 hour cold ischaemic times to be adhered to while the organs were being retrieved. I collected all of the samples and analysed all of the data. All of the statistical analysis was checked by our statistician prior to publication.
4.1 Abstract

**Introduction:** EVNP offers an alternative method of organ preservation allowing donor kidneys to be reanimated and evaluated prior to transplantation. Beyond preservation, it can be used to characterise the immunological contribution of the donor kidney in isolation. Furthermore, it has the potential to be used as an immunomodulatory strategy to manipulate donor kidneys prior to transplantation. For this purpose, the aim of this study was to develop a clinically relevant model of EVNP using porcine kidneys.

**Methods:** Explanted porcine kidneys were retrieved from a local abattoir following standard transplant retrieval guidelines and placed on ice for a standardised cold ischaemic time of 2 hours. Following this kidneys were placed onto an EVNP circuit and perfused for 6 hours. Renal haemodynamic observations were continually recorded and regular blood gases performed to determine the biochemistry of the circuit.

**Results:** All kidneys reperfused immediately and tubular function was restored. Renal blood flow remained stable throughout the experiment; although remained below the 50mls/min/100g threshold defined for adequate perfusion. In addition, urine production declined following 60 minutes of perfusion. Renal biochemistry remained stable, with the exception of glucose and lactate which had an increasing trend suggesting hypoperfusion and cellular injury. At the end of perfusion kidneys did not meet the criteria for transplantation, with evidence of ischaemic injury.

**Discussion:** Kidneys perfused during this study deteriorated during EVNP, with a loss of cellular viability and integrity. Clearly the current clinical protocol for EVNP does not provide adequate perfusion for kidneys retrieved from abattoir pigs and therefore a modified protocol is required. Until this is achieved and the model is clinically relevant, it cannot be used to establish the cellular and inflammatory contribution of donor kidneys.
4.2 Introduction

Kidney transplantation is severely limited by a shortage of suitable donor organs. This has led to prolonged waiting list times and a subsequently higher waiting list mortality rate. In an attempt to overcome this problem there has been a drive to develop EVNP as a tool to evaluate and recondition ECD kidneys with marginal function, and DCD kidneys. Aside from the survival benefit EVNP has offered to patients by enabling the safe evaluation and transplantation of such organs, this technology may also represent a novel therapy to manipulate the donor leukocyte repertoire prior to transplantation (103, 104, 151).

Although the importance of donor-derived leukocytes in the allore cognition and rejection process has been demonstrated previously, a detailed evaluation of the temporal kinetics of leukocyte migration from the kidney has not been possible prior to EVNP. To date there is limited understanding of the natural history of passenger leukocyte transfer from the donor kidney to the recipient. Given that the kidney is equipped with a sophisticated localised immune system, such data are important. During homeostasis, the kidney remains in a regulatory state. However, following insult a significant immune response can occur. In severe cases of injury and inflammation such as brain death, a robust immune response ensues. This includes local and eventually, systemic inflammation manifesting in a local cytokine storm and mass cellular infiltrate in the donor (136, 152, 153). Using current strategies for transplantation, it is at this point that the inflamed donor kidney with global immune activation is implanted into the recipient. Direct presentation of alloantigens by donor APCs to recipient T cells occurs rapidly following transplantation (118). The latter cells infiltrate the graft and the rejection process is initiated. These early interactions are key to the long-term clinical outcome of the patient, and as such warrant detailed investigation.

This study was designed to determine the immune and inflammatory contribution of the donor kidney following reperfusion in isolation. For this purpose, a porcine model of EVNP was utilised to replicate the response of the donor kidney to revascularisation in the absence of any contribution from a recipient immune system. Additionally, it is likely that mobilisation and removal of a proportion of passenger leukocytes using EVNP will reduce graft immunogenicity prior to transplantation, thereby reducing AR and improving clinical outcome (154).
For the first part of this study the focus was on the creation of a functional and clinically relevant model of EVNP that was reproducible. Samples were therefore not collected until the model was optimal and the kidney parameters are relevant for the transplant setting. To achieve this, a working EVNP model was utilised using the established protocol described by Nicholson et al in the first instance (155). The circuit components and perfusion solutions were largely based on this model, however porcine kidneys retrieved from a local abattoir were used. Porcine kidneys were chosen owing to their anatomical and physiological similarity to humans, providing improved translation from animal models to humans. In addition, obtaining organs from a slaughterhouse negates the need for further ethical applications, adheres to the National Centre for the Replacement, Refinement and Reduction of Animals in Research guidelines (NC3Rs) and increases output by using organs that would otherwise be disposed of (156).

Once a reproducibly viable circuit is successfully established, the system will be utilised to assess the inflammatory contribution of the donor kidney and determine the effects of perfusion on the kidney immune profile.
4.3 Materials and Methods

4.3.1 Study Design:

Porcine kidneys were utilised, given the similarity in their anatomy and physiology to humans. A total of n=5 porcine kidneys were retrieved from a local abattoir from Landrace pigs with a mean weight of 80kg.

4.3.2 Kidney Retrieval:

As the kidneys were retrieved from a local abattoir, all pigs were culled in line with EU Council Regulation (EC) 1099/2009 on the protection of animals at the time of killing. This required pigs to be rendered unconscious via electrical stunning, followed by exsanguination. At the point of exsanguination, the blood (approximately 4L) was collected into a sterile container containing 100ml normal saline (0.9%) supplemented with 40,000iU unfractionated heparin to prevent the blood from clotting. The blood was then stored on ice for transport back to the university. Following exsanguination, an abattoir worker excised the kidneys from the abdominal cavity, which remain within the renal fascia and adipose tissue. The kidneys were inspected for any lacerations or cysts and if the kidneys were deemed suitable they were placed on ice and underwent immediate standard donor kidney procurement. The renal artery was isolated from the renal vein and cannulated. Owing to the high vascular resistance experienced in pigs, 20mls of glyceryl trinitrate (GTN; Hameln, Gloucester, UK; 1mg/ml) was initially flushed down the renal artery to promote vasodilation of the vasculature. This was followed by an anterograde flush with hyperosmolar citrate solution (Soltran; Baxter Healthcare, Thetford, UK, osmolality 486 mOsm/kg) that is maintained at 4°C to cool the kidneys (Figure 4.1). In addition, Soltran was supplemented with 20,000iU unfractionated heparin to ensure dissemination and removal of microthrombi. During the preservation flush the ureter was also isolated and cannulated so that urine production could be determined. Once the kidneys had fully blanched, an indicator of adequate perfusion, the preservation flush was stopped and the kidneys were submerged in ice cold Soltran and placed on ice for transport.
Figure 4.1 - Comparison of a kidney being flushed with a preservation solution with a freshly isolated kidney

A representative image comparing a freshly isolated kidney (left) with a kidney blanching during the preservation flush with Soltran (right). Kidneys are excised from the pig immediately following exsanguination, placed on ice and cannulated. A 1L bag of Soltran is flushed through the renal artery cannula, following which the kidney is submerged in Soltran and placed on ice for transport.
4.3.3 EVNP Circuit:

EVNP was carried out using an isolated organ system based on models previously described (155). This consists of an organ chamber containing a platform that the kidney is placed on, with a reservoir to collect the venous outflow. A centrifugal pump was used to re-circulate the perfusate, passing through a membranous oxygenator with a built in heat exchanger to provide oxygen to the blood before it re-enters the kidney via the renal artery (Figure 4.2 and 4.3). Temperature and pressure probes were also incorporated to allow continual monitoring of these parameters.

4.3.4 EVNP Procedure:

**Priming**

Prior to placing the kidneys onto the EVNP circuit, the system was primed with a perfusate to ensure there was no air within the circuit. This consisted of 350mls Ringer’s solution supplemented with approximately 900ml of autologous, leukocyte depleted (Haemonetics, UK), packed red blood cells to achieve a target haematocrit of 25-30%. Following re-circulation, the Ringer’s solution was further supplemented with 8mg Dexamethasone, 25mls 10% mannitol, 20mls 8.4% sodium bicarbonate and 10,000iU-unfractionated heparin. Using a syringe driver a continuous infusion of 50mls nutriflex (B. Braun, Sheffield, UK), 10mls epoprostenol (Sandoz, Surrey, UK; 0.5mg) and 40iU insulin (Actrapid; Novo Nordisk, Denmark; 100iU/ml) was supplemented into the circuit at a flow rate of 20ml/hr. Finally, a gas mixture of 95% O2 and 5% CO2 was supplied to the membrane oxygenator at a flow rate of approximately 0.5L/min. A blood gas analyser was utilised to assess the pO2, pCO2 and electrolyte concentrations within the circuit. The pH of the circuit was also determined and adjusted using sodium bicarbonate if the pH was low, or the CO2 was increased if the pH was high.

**Preparation for Perfusion**

Once the perfusion circuit had been fully primed and de-aired, and the blood gas variables were within physiological reference ranges, the kidneys were removed from ice. A baseline picture was taken to provide a reference during perfusion so that any structural changes in the kidney could be monitored. An additional 10mls of GTN (1mg/ml) was flushed down the renal artery, followed by 200mls of Ringer’s solution to remove any residual Soltran residue.
All kidneys were weighed to provide the baseline parameters and allow renal blood flow (RBF) per 100g to be calculated.

**Perfusion**

Following preparation, the kidneys were connected to the perfusion circuit via the renal artery cannula. A pressure probe was attached to the arterial arm of the circuit to allow the pressure of perfusate entering the artery to be measured and controlled. The initial flow rate was reduced to approximately 0.05L/min to allow adequate de-airing of the kidney prior to attachment, and to prevent endothelial damage due to haemodynamic shear stress. The flow rate was then gradually increased until the arterial pressure reached 75mmHg. Haemodynamic observations were continually recorded and a blood gas analyser utilised to determine the biochemistry of the perfusate. At the same time, the ureter cannula was placed into a 50ml falcon tube to collect the urine. Urine output (UO) was regularly monitored and replaced with Ringer’s solution to maintain normal physiology, specifically sodium levels, and to maintain circuit volume to provide hydration. The circulating perfusate temperature was set to 38°C ± 2°C and the organ chamber was covered to maintain this temperature and to retain the humidity throughout the perfusion period. All kidneys were perfused for 6 hours.
Figure 4.2 - Schematic diagram demonstrating the setup of the EVNP circuit

A schematic diagram depicting the EVNP circuit. Kidneys are placed onto a membranous platform within the venous reservoir and attached to the circuit via a renal artery cannula. Perfusate passes into the renal artery and drains out of the renal vein into the venous reservoir. It is re-circulated via a centrifugal pump, passing through an oxygenator that supplies oxygen and carbon dioxide to the perfusate and warms the perfusate to normothermia via a heater-cooler that is attached to it. The perfusate then re-enters the kidney via the renal artery to complete the circuit. Urine is collected into a receptacle via the ureter cannula.
Figure 4.3 – Image demonstrating the setup of the EVNP circuit

A representative image of the EVNP circuit demonstrating the complete circuit. The kidney is placed onto the porous membrane where the venous efflux drains into the venous reservoir. From here the centrifugal pump recirculates the perfusate, where it passes through the oxygenator to be supplied with 95% O₂ and 5% CO₂. At the same time the perfusate is heated to normothermia here, before entering the kidney via the renal artery cannula.
4.3.5 Sample Collection during EVNP:

**Perfusate:** Prior to initiation of EVNP, 15ml of perfusate was collected to provide a baseline sample. Additional samples were then collected at 15 and 30 minutes, followed by 30 minute intervals up to 3 hours of perfusion when samples were collected hourly. Samples were centrifuged at 2000g for 5 minutes at 4°C and 1ml aliquots of plasma were prepared and stored at -80°C.

**Urine:** Following 15 minutes of perfusion, 5ml of urine was collected. Additional samples were collected at 30 minute intervals up to 3 hours of perfusion when samples were collected hourly. Samples were separated into 1ml aliquots and stored at -80°C.

4.3.6 Statistical Analysis:

All statistical analysis was carried out using IBM SPSS software version 22. Data normality was determined by assessing mean, standard deviation, skewness and kurtosis. Formal evaluation was performed using the Shapiro-Wilk test. Normally distributed data are expressed as mean ± standard deviation, or as median [interquartile range] if non-normally distributed. Depending on data distribution the changes in renal haemodynamics and renal biochemistry over time were either analysed using the General Linear Model or the Friedman's 2 way ANOVA by ranks. The correlation between RBF and intra-renal resistance (IRR) was assessed using Spearman’s rho correlation coefficient. For comparisons between RBF at different time points either a paired t-test or the Wilcoxon Signed Rank test was utilised depending on the distribution of the data. Data were considered significantly different if a p value of <0.05 was observed.
4.4 Results

4.4.1 Functional Assessment of the Kidney:

To assess the validity of the model and the viability of the kidneys, the functional parameters of the kidney were determined. UO, MAP and RBF were continually recorded. IRR was also determined by calculating the MAP/RBF.

All kidneys were reperfused without any complications and began producing urine upon connection to the circuit. The average urine production per hour was 54.74 ± 44.52mls, with a mean total UO of 328.44 ± 185.57mls. The pattern of UO changed during the perfusion period (p=0.011), with a decline in UO after 60 minutes of perfusion. All kidneys appeared evenly perfused, with a significant increase in RBF between 0 and 30 minutes of perfusion (p=0.011). Following reperfusion RBF and IRR remained stable throughout the 6 hour experiment (p=0.272 and p=0.433 respectively, Figure 4.4). A significant correlation was observed between RBF and IRR, with RBF increasing as IRR decreased (r_s=-0.888, p<0.001; Figure 4.5).
Figure 4.4 - Renal haemodynamics during perfusion

Kidney haemodynamic data over time representing A) RBF, B) IRR and C) UO per hour per 100g of tissue. RBF and IRR remained stable during perfusion. Conversely, UO declined over time.
A significant correlation was observed between RBF and IRR. RBF increased as IRR decreased ($p<0.001$).

Figure 4.5 - RBF and IRR were inversely proportional during perfusion
4.4.2 Renal Biochemistry:

In combination with the functional data, assessing the renal biochemistry provides a detailed analysis of the kidney homeostasis. The pH of the circuit was continually monitored using a blood gas analyser, along with the concentration of pO₂, pCO₂, and circulating electrolytes. The concentration of lactate was also determined and provided an indicator of kidney function, level of perfusion and the degree of renal injury (Figure 4.6).

During perfusion, the pH, pO₂, pCO₂ and concentration of electrolytes (HCO₃⁻, Na⁺ and K⁺) remained stable (p=0.365, p=0.223, p=0.291, p=0.169, p=0.412 and p=0.066 respectively). However, a general increase in the concentration of both glucose and lactate was observed over time (p=0.016 and p=0.040 respectively), suggesting a degree of cellular injury has been incurred.
Figure 4.6 - Renal biochemistry during perfusion

Kidney biochemical concentrations over time representing A) sodium bicarbonate and potassium, B) PCO₂ and PO₂, C) Sodium, D) pH, E) Lactate and F) Glucose. All variables remained stable (p>0.05), with the exception of glucose and lactate where a general increase during perfusion was observed (p=0.016 and p=0.040 respectively).
4.5 Discussion

The purpose of this study was to establish a clinically relevant EVNP model based on established protocols (105). The components of the EVNP circuit and the perfusate solution used were largely based upon those described by Nicholson et al, with the only major difference being the use of abattoir pigs as the source of organs. This negates the need for ethical approval and follows the NC3Rs guidelines that aim to reduce the number of animals used for research purposes only. For this reason, the first aim of the study was to determine if the protocol used clinically could be utilised in animals that have been sourced with an unknown medical history. Once a clinically relevant model has been established it will be utilised to determine the immediate events that occur following kidney revascularisation.

All of the kidneys retrieved were placed onto the EVNP circuit and immediately reperfused, with renal function being restored and an immediate production of urine observed. Following the initial reperfusion phase, RBF remained stable but low for the course of the experiment, with macroscopic evidence of tissue injury presenting as black speckles on the kidney surface (Figure 4.7). Nicholson et al have created an EVNP assessment tool to provide an index of organ quality, which includes a threshold rate for RBF. Here they suggest that a flow rate less than 50mls/min/100g is inadequate and is likely to lead to ischaemic injury (101). Throughout this experiment the mean RBF was 48mls/min/100g and therefore following the guidelines set the kidneys were being under-perfused. It is essential to provide an adequate blood supply to the kidney to ensure optimal delivery of oxygen to the tissue, thereby preventing ischaemic insults. According to Ohm’s Law, RBF is dictated by the haemodynamic relationship between the perfusion pressures, defined as the difference between arterial pressure and venous pressure, and the IRR. This creates a reciprocal relationship between RBF and IRR, with an increase in IRR resulting in a subsequent decrease in RBF. Within this circuit the vein was not cannulated and instead was left open to provide no resistance and as such keep the pressure at 0mmHg. Rather, the circuit was set to a defined perfusion pressure in the arterial arm of 75mmHg meaning that in this setting the RBF was dictated by the IRR. Furthermore, the aim was to minimise the IRR to maximise RBF through the organ. As expected, the defined relationship between IRR and RBF was observed in this study with increasing IRR coupling with a decrease in RBF. The poor RBF and high IRR may be a result of the combined effects of vascular injury, tissue injury and oedema. Vascular injury accompanies IRI as a result of both the local and systemic inflammatory response that ensues and manifests as microvascular
dysfunction. Additionally, IRI alters the tissue barrier function leading to an increase in permeability and a reduction in the oncotic pressure gradient, ultimately causing tissue oedema.
Macroscopic evidence of ischaemic regions manifesting as black speckles on the kidney surface

Figure 4.7 – Macroscopic evidence of tissue damage post perfusion

Representative images of kidneys being perfused on the EVNP circuit. Macroscopic evidence of tissue damage can be seen as black speckles on the kidney surface due to hypoperfusion.
The production of urine following transplantation is often used as a parameter of kidney function and therefore was used as an indicator of glomerular filtration rate (GFR) and tubular function. A cessation can indicate acute tubular injury, although it should be noted that this can be recoverable and therefore urine production alone cannot be used as a reliable marker of functionality. During this study, urine production began immediately following reperfusion and continued for the duration of the study. Despite this, a general decline was observed overtime. This may be reflective of acute kidney injury as a result of the IRI that ensues. In addition, the low RBF can result in tubular injury and a reduction in GFR. Within the kidneys the majority of the blood flow supplies the cortex containing the glomeruli and convoluted tubules. These areas require good perfusion in order to achieve filtration and reabsorption, processes that are metabolically demanding. Comparatively the outer medulla is starved of oxygen as its blood supply transverses the glomerular capillary bed, where hydrostatic pressure is also lost, prior to entering the medulla. Oxygen is then counter currently exchanged with the venous vasa recta (157). Whilst these features are essential to establish and maintain the osmotic gradients within the medulla, and therefore urine production, it leaves the medulla susceptible to changes in RBF (158). Indeed, a decline in RBF often results in impaired tubular sodium reabsorption causing the afferent arterioles to constrict and GFR to decline. This is a compensatory mechanism of the kidney known as tubuloglomerular feedback which aims to protect the downstream nephrons during periods of reduced oxygen supply (157). However, sustained hypoperfusion of the kidney tissue results in severe depletion of ATP and ischaemic injury to vascular endothelial cells. Cell swelling occurs in combination with leukocyte activation and a further reduction of RBF ensues. Renal tubular epithelial cells also swell, lose their brush borders and cellular polarity is lost. A higher oncotic pressure in the glomerulus follows, which contributes to the loss of GFR and diminished UO (159). Increasing RBF through the kidneys is therefore likely to overcome the rapid decline in UO seen in this study and will be a major focus for the next phase of this study.

All kidneys remained biochemically stable, though a general increase in glucose and lactate was noted. The interpretation of glucose and lactate levels has been discussed in other organ perfusions but the significance remains unclear for the prediction of graft function. It has been proposed that an increase in lactate indicates a switch from aerobic to anaerobic metabolism, which can occur due to poor oxygen supply or a decrease in its clearance by the kidney. During aerobic metabolism, pyruvate is produced by glycolysis and then enters the
Krebs cycle. Conversely, lactate is the end product of glycolysis under anaerobic conditions and for this reason it has been proposed that increasing lactate can be used as an indicator of regional ischaemia and hypoperfusion. However, in a study of lung perfusion it has been suggested that the increase in lactate is reflective of normal lung metabolism but with reduced clearance owing to the closed nature of the perfusion circuit (160). In a clinical setting, it is widely considered that a lactate concentration above 4mmol/L is associated with poorer patient outcomes (161, 162). Additionally, patients admitted with a blood lactate level of 2.5mmol/L or above are closely monitored (163). Despite this, it has been proposed that a lactate of less than 5mmol/L would be acceptable criterion for transplantation for organs perfused on a closed system. Throughout this study the mean lactate concentration reached 4mmol/L. The increase during perfusion matched the decline in kidney function and therefore it is likely that this is indicative of ischaemic injury in this model. Indeed, the gradual increase in glucose during perfusion may also support this. It has previously been suggested that active metabolism during perfusion is indicated by a decline in lactate and glucose levels (164). The increase observed during this study may therefore be indicative of a loss of cellular viability, and as such a decline in metabolism. However, it is entirely possible that the increase in glucose is not reflective of a reduction in cellular metabolism but merely a result of the increased lactate within the circuit. The kidney, like the liver, has the ability to produce and release glucose into the circulation via both gluconeogenesis and glycogenolysis, with lactate being the primary precursor (165). Conversely, glucose utilisation within the renal medulla results in several metabolic by-products being produced, including lactate. Due to the closed nature of the circuit, this may provide a feedback loop. Lactate produced via the glycolysis of glucose within the medulla can be absorbed by the kidney and converted to produce glucose (166). This would also explain the increase of both glucose and lactate over-time. Despite this, when taking into account the decline in other parameters, including RBF, and macroscopic evidence of tissue injury, it is more likely that the lactate and glucose are a result of poor perfusion.

The functionality of the kidneys in this study does not comply with those reported in the literature using this protocol. This may be a reflection of the source of the organs in this study, as well as the mode of death that the animals are exposed to. The organs utilised were obtained from abattoir pigs that have been bred outdoors in close proximity to other pigs, leaving them susceptible to disease. In a bid to overcome this, animals are treated with several antibiotics, which can lead to antibiotic resistance within the population. This is particularly
problematic when attempting to perfuse these organs at normothermia as this can lead to a rapid increase in bacterial load, which itself can impact upon organ functionality. These animals are also subjected to hormone injections to encourage growth and to aid in the production of leaner meat. The impact of this on the organs is unknown and baseline functional parameters cannot be obtained from the pigs used in this study prior to culling. It is therefore unclear what the target parameters for these animals should be. The vascular resistance within pigs has also been reported to be higher than that seen in humans, which can lead to poor RBF. This is likely to be enhanced in abattoir animals due to the catecholamine storm that can occur as a result of the stress when animals are prepared for slaughter. To overcome this, the renal artery was flushed with GTN prior to the preservation flush to dilate the vasculature and promote thorough clearing of any micro thrombi. Prostacyclin was also included in the circuit to promote good RBF. Despite this the flows remained low and therefore the infusion rate of the prostacyclin needs to be addressed. In addition to this, the mechanism of death may contribute to the poor organ function seen during this study. Animals were subjected to electrical stunning to render them unconscious prior to exsanguination. It can often take several minutes for the blood to drain out of the pig before they are considered dead. These pigs are therefore representative of DCD donors, rather than DBD. Organs from DCD are subjected to prolonged warm ischaemia compared to organs from brain-dead donors. This is known to negatively impact on graft function and is associated with an increased risk of primary graft failure. These organs are also more susceptible to IRI following revascularisation. Indeed, in this study kidneys were subjected to approximately 20 minutes of warm ischaemia, defined as the time from exsanguination to the start of the cold preservation flush. This may account for the poor RBF and tubular injury observed. Clearly the protocol used needs to be tailored to account for the issues experienced.

The current clinical protocol for EVNP does not provide adequate perfusion for organs retrieved from abattoir pigs. It should also be noted that current clinical practice is only to provide EVNP for a short period, typically 1-3 hours. Prolonged perfusion using this protocol has not been attempted but it is likely that the results demonstrated here over 6 hours of perfusion would also be seen in human kidneys following this protocol. Due to the poor perfusion of the organs and the evidence of ischaemic injury the immune cell efflux from these kidneys was not assessed as this does not represent the clinical transplant setting. The protocol will now be modified to establish a usable and reproducible protocol for the preservation of kidneys retrieved from abattoir pigs. The main aim of this protocol will be to
achieve increase RBF, which should impact on the UO. Once this has been established and validated, the inflammatory contribution of the donor kidney following revascularisation will be determined.
Section 5 - Optimisation and Validation of an Ex Vivo Normothermic Perfusion Model

A version of this paper is published in Kidney International Reports:

Title of paper – Ex Vivo Normothermic Perfusion Induces Donor-derived Leukocyte Mobilisation and Removal Prior to Renal Transplantation


DOI: http://dx.doi.org/10.1016/j.ekir.2016.07.009

As per section 4, I retrieved all of the organs from the abattoir and performed all of the normothermic perfusions. My colleagues assisted with priming the normothermic perfusion circuits and in the running of the perfusions. I collected all of the samples and analysed all of the data. All of the statistical analysis was checked by our statistician prior to publication.

Sections of this chapter have also been presented as an oral presentation.

Title of Presentation – Ex-vivo normothermic perfusion reduces kidney immunogenicity prior to transplantation via removal of passenger leukocytes

5.1 Abstract

**Introduction:** To determine the immune and inflammatory contribution of donor kidneys using EVNP, clinical parameters need to be maintained during perfusion. Through a series of optimisation experiments, a protocol has been established that allows porcine kidneys obtained from an abattoir to be perfused for 6 hours with good function retained throughout. Following validation of its reproducibility, it will be used to determine the cellular content and inflammatory contributions of the donor kidney upon revascularisation.

**Methods:** Explanted porcine kidneys underwent 6 hours of perfusion (n=5 for validation and n=5 for the study). Renal haemodynamics were recorded and the biochemistry determined by serial blood gas analysis. Following validation, sequential perfusate samples were collected and leukocytes characterised via flow cytometry. An inflammatory profile was generated via cytokine quantification. Cell-free DNA was also determined as markers of cell death.

**Results:** All kidneys functioned within normal parameters and met the criteria for transplantation at the end of perfusion. Throughout perfusion there were continuous increases in pro-inflammatory cytokines, including large concentrations of IFN-γ, suggesting perfusion drives a significant inflammatory response. Increasing concentrations of cell-free DNA were also observed, suggesting cell death occurs. During perfusion there was a marked cellular diapedesis of T cells, B cells, NK cells and monocytes from the kidney into the circuit. Minor populations of granulocytes and mφ were also detected.

**Discussion:** This study demonstrates that ex vivo normothermic perfusion initiates an inflammatory cytokine storm and release of mitochondrial and genomic DNA. This is likely to be responsible for immune cell activation and mobilisation into the circuit prior to transplantation. Interestingly this did not impact upon renal function. These data therefore suggests that normothermic perfusion can be used to immunodeplete and saturate the pro-inflammatory capacity of donor kidneys prior to transplantation.
5.2 Introduction

In the previous chapter the aim was to establish a clinically viable model of EVNP in order to track donor leukocyte migration upon revascularisation. In the first instance the currently established clinical protocol was utilised, however this did not provide adequate perfusion when using kidneys obtained from abattoir pigs. This resulted in tissue damage and a decline in UO and therefore these kidneys did not represent the post-transplant setting and samples could not be collected. For this reason a different protocol needed to be explored that focused on improving RBF to maintain both tissue viability and UO.

The poor RBF and subsequent decline in UO described in the previous chapter was likely to be as a result of a low oncotic pressure within the circuit. The blood used within the circuit goes through a process of washing to remove the plasma with only the red blood cells being utilised in the perfusate solution. This means that the albumin within the whole blood is removed during this process and does not enter the circuit. Albumin is a protein that plays an important role in maintaining homeostasis within the body via maintenance of intravascular oncotic pressure. A reduction in the level of albumin within the plasma results in both oedema of the tissue and a decrease in UO owing to the loss of pressure in the glomerular capillary bed. For this reason, the perfusate used in the previous section was supplemented with 7% bovine serum albumin (BSA) to determine if this would improve RBF. This concentration of BSA was chosen owing to its success during EVLP in both preventing tissue oedema and encouraging good perfusion of the organs. In combination with this the sodium concentration within the Ringer’s solution was reduced in a bid to maintain the concentration of sodium within physiological parameters. A high concentration of sodium results in an alteration to the fluid reabsorption, with an increase in fluid retention which in turn leads to poor UO. To overcome the problem of infection that is often observed within pigs obtained from an abattoir, antibiotics were incorporated into the perfusion circuit. The concentration of dexamethasone was also increased to reduce inflammation following reperfusion. Finally, the epoprostenol concentration within the infusion, as well as the rate of infusion, was adjusted to follow that used clinically. Together, these changes resulted in significant improvements in RBF, with an average flow of over 100mls/min/100g. Sodium was maintained within physiological parameters and the lactate concentration did not exceed 2mmol/L. However, RBF was not maintained for the duration of the experiment with a trend towards increasing IRR in combination with a very poor UO throughout.
To determine if the loss of RBF was reflective of the changes made to the Ringer’s solution the experiment was repeated using 7% BSA but with standard Ringer’s solution. This was the only change made to this experiment to allow us to establish what was causing the loss of RBF during perfusion. During the 6 hour experiment good RBF was observed and maintained with little change in arterial pressure. However, UO remained very poor throughout the experiment, with an average UO per hour of 9mls. This may be due to the BSA concentration being too high, resulting in the oncotic pressure exceeding the threshold for glomerular-tubular balance. When the oncotic pressure within the peritubular fraction of the nephron is high a subsequent increase in sodium and water reabsorption occurs causing a decrease in UO. It is essential to maintain UO to sustain tubular integrity and ensure that waste products are removed from the perfusion circuit. The development of a new protocol therefore focused on optimising the circuit to improve UO whilst maintaining good perfusion.

To promote UO the concentration of BSA within the perfusate was reduced from 7% to 5% with the aim of maintaining RBF whilst reducing the reabsorption of sodium and water in the peritubules. In addition, the amount of mannitol within the circuit was increased. Mannitol is an osmotic diuretic that elevates blood plasma osmolality and thus enhances water loss from tissues and inhibits water and sodium reabsorption in the kidney. In addition, it has been reported to prevent endothelial cell swelling, thereby maintaining RBF. Indeed, mannitol infusions are often utilised clinically to treat poor UO during renal failure (167). The changes described provided good renal perfusion combined with adequate UO that maintained for the duration of the perfusion. Renal biochemistry was stable with minimal increases in lactate concentrations. This suggests that the components of this perfusion circuit provided sufficient blood flow to maintain tissue viability whilst allowing tubular function to be retained. To determine if this protocol is reproducible, a series of n=5 porcine kidney perfusions will be performed following the changes described. Success at this stage will be a RBF above 50mls/min/100g, a UO of more than 30mls/hour/100g and a lactate concentration less than 2.5mmol/L at the end of the perfusion. If this is achieved, an additional n=5 perfusions will be performed and the immune cell efflux and inflammatory profile following revascularisation will be determined.
5.3 Materials and Methods

5.3.1 Procurement of Donor Organ:

Kidneys from n=10 Landrace pigs (n=5 for validation of the circuit and n=5 for the study) with a mean weight of 80kg were collected from a local abattoir. All pigs were culled under EU Council Regulation (EC) 1099/2009 on the protection of animals at the time of killing. In brief, pigs were rendered unconscious via electrical stunning followed by exsanguination. Approximately 3L of blood was collected into a sterile receptacle containing 100mls of normal saline supplemented with 40,000iU unfractionated heparin. The abdomen was opened using a midline incision and the kidneys were exposed and excised. Kidneys were immediately placed on ice and the visceral membrane was removed, allowing inspection for lacerations or cysts. If deemed acceptable, the renal artery and ureter were isolated and cannulated and 20mls of GTN (1mg/ml) was flushed through the renal artery. This was immediately followed by 1L 4°C Soltran (Soltran; Baxter Healthcare, Thetford, UK) infused with 10,000iU unfractionated heparin at a hydrostatic pressure of 100mmHg. Kidneys were then submerged in Soltran and placed on ice for a standardised SCS time of 2 hours.
**Figure 5.1 – Porcine kidney following a preservation flush**

Representative image of a porcine kidney that has been flushed with 1L of preservation solution (Soltran). The kidney appears evenly blanched, suggesting all of the erythrocytes have been removed from the vasculature. The renal artery and ureter can also been seen.
5.3.2 Perfusion Circuit:

The EVNP circuit consisted of an organ chamber with a porous platform that the kidney was placed on, allowing the renal outflow to drain into the reservoir. The perfusate was re-circulated using a centrifugal pump, passing through a membranous oxygenator attached to a heater-cooler system to maintain the organ at normothermia. Pressure and temperature probes were incorporated to maintain these parameters.

5.3.3 EVNP Procedure:

EVNP was performed using an adaption of the currently published protocol (168)

**Priming:**

Prior to placing the kidney onto the EVNP circuit, the system was primed with perfusate to ensure the circuit was de-aired. This consisted of 350mls Ringer’s solution supplemented with 5% BSA. Approximately 500ml of autologous, leukocyte depleted (Haemonetics, UK); packed red blood cells were infused to achieve a target haematocrit of 20-25%. Following re-circulation, the perfusate was further supplemented with 13.2mg Dexamethasone (Hameln, Germany-distributed by Gloucester, UK), 30mls 10% mannitol, 20mls 8.4% sodium bicarbonate and 4,000iU unfractionated heparin. A syringe driver enabled the continuous infusion of 20mls nutriflex with 14mls 8.4% sodium bicarbonate; 100iU insulin (Actrapid; Novo Nordisk, Denmark; 100iU/ml) and 25mls 15% glucose to supplement the circuit at a flow rate of 10ml/hr. A second syringe driver containing 18mls epoprostenol (Sandoz, Surrey, UK; 0.5mg) and 42mls normal saline was also infused at an initial rate of 24mls/hr. Finally, a gas mixture of 95% oxygen and 5% carbon dioxide was supplied to the membrane oxygenator at a flow rate of approximately 0.5L/min. A blood gas analyser was utilised to assess the pO$_2$, pCO$_2$ and electrolyte concentrations within the circuit, which were corrected to maintain physiological levels. The oxygen consumption was calculated from venous and arterial blood using the equation (((pO$_2$art – pO$_2$ven) x flow / weight)). Creatinine was added to the perfusate to achieve a circulating concentration of 1,500 µmol/L.
Preparation for Perfusion:

Once the perfusion circuit had been fully primed and de-aired, and the blood gas variables were within physiological reference ranges, the kidneys were removed from ice. A baseline photograph was taken to provide a reference during perfusion so that any structural changes in the kidney could be monitored. Any residual Soltran was removed from the vasculature with 200mls of 4°C Ringer’s solution. Finally, the kidney was weighed to provide the baseline parameters.

Perfusion:

Following preparation, the kidneys were connected to the perfusion circuit via the renal artery cannula (Figure 5.2). A pressure probe was attached to the arterial arm of the circuit to allow the pressure of perfusate entering the artery to be measured and controlled. The initial flow rate was reduced to approximately 0.05L/min to allow adequate de-airing of the kidney prior to attachment and to prevent endothelial damage due to haemodynamic sheer stress. The flow rate was then gradually increased until the arterial pressure reached a mean of 55mmHg and the observations were recorded. At the same time, the ureter cannula was placed into a measuring cylinder to collect and record the UO. The arterial pressure was increased by 5mmHg every 5 minutes until the target pressure of 75mmHg was achieved 20 minutes post-reperfusion. At this time, the infusion of epoprostenol was started. Observations and blood gas analysis were routinely collected and any changes in the physiology were corrected. UO was regularly monitored and replaced with Ringer’s solution to maintain normal physiology and circuit volume. The circulating perfusate temperature was set to 38°C ± 1°C to rapidly re-warm the kidney to physiological temperatures, allowing kidney function to be restored and cellular metabolism to be re-established. The organ chamber was covered to maintain this temperature and to retain the humidity throughout the perfusion period. If there was a drop in functional parameters, therapeutic interventions were made, in line with clinical practice, so as to maintain tissue viability and normal physiology. The rate of epoprostenol infusion was increased in 1.6ml/hr increments for every 10% drop in RBF to a maximum of 34.8ml/hr. If the response was inadequate despite the maximum infusion, or if UO dropped below 10mls/hr, then a bolus of either 10% mannitol or Ringer’s solution supplemented with 5% BSA was added. All interventions did not exceed doses used clinically, and responses were
closely monitored to ensure irreversible renal failure had not occurred. In all cases kidneys were perfused for 6 hours.
Figure 5.2 - Porcine kidneys appear globally pink during perfusion

Representative images of porcine kidneys being perfused on the EVNP circuit. Both kidneys are globally pink in appearance, demonstrating that all of the tissue is being well perfused.
5.3.4 Sample Collection during EVNP:

For the initial n=5 perfusions, samples were not collected as these were validation experiments. For the remaining n=5 kidney perfusions, the following samples were collected and analysed.

**Perfusate:** Prior to initiation of EVNP, 15mls of perfusate was collected to provide a baseline sample. Additional samples were collected at 15 and 30 minutes, followed by 30-minute intervals up to 3 hours of perfusion when samples were collected hourly. At baseline, 1 hour and 6 hours, 100µl of perfusate was analysed by flow cytometry. All perfusate samples were centrifuged at 2000g for 5 minutes at 4°C and 1ml aliquots of plasma and whole blood were stored at -80°C.

**Urine:** Following 15 minutes of perfusion, 5ml of urine was collected. Additional samples were collected at 30 minute intervals up to 3 hours of perfusion when samples were collected hourly. Samples were aliquotted and stored at -80°C.

5.3.5 Mitochondria Isolation:

To obtain mitochondrial DNA (mtDNA) to generate a standard curve for use in qPCR, mitochondria were isolated from porcine lung tissue using a mitochondrial isolation kit (Sciencell Research Laboratories, Carlsbad, CA, USA) and the reagents supplied. Approximately 0.1g of lung tissue was weighed out and washed twice in 5ml ice-cold PBS following which 1ml of 1x mitochondrial isolation buffer A was added. Lung tissue was then homogenised (Polytron pt 1200 E manual disperser, Kinematica, Schweiz, Switzerland) on ice for 20 seconds and the homogenate transferred to a 1.5ml Eppendorf tube and centrifuged at 1000g for 5 minutes at 4°C. The supernatant was transferred to a clean 1.5ml Eppendorf tube and centrifuged at 10,000g for 20 minutes at 4°C. The supernatant was removed and the remaining pellet containing the intact mitochondria was re-suspended in 100µl mitochondrial isolation buffer B and kept on ice. mtDNA was subsequently extracted using the QIAamp DNA Mini and Blood Mini kit (Qiagen, Manchester, UK).
5.3.6 DNA Extraction:

Cell-free DNA was extracted from plasma samples of the perfusate, genomic DNA (gDNA) was extracted from porcine blood and mtDNA from isolated mitochondria using the QIAamp DNA Mini and Blood Mini kit, according to the manufacturer’s protocol (Qiagen, Manchester, UK) using reagents supplied in the kit. Eppendorf tubes were labelled and 20µl proteinase K was added, followed by 200µl of sample and 200µl of Buffer AL. Samples were thoroughly mixed by pulse vortexing for 15s and incubated at 56°C for 10 minutes in a water bath. After 10 minutes, 200µl ethanol (100%) was added to the sample and mixed by pulse vortexing for 15s. The mixture was then removed from the Eppendorf tube and transferred into a pre-labelled QIAamp Mini spin column. Spin columns were centrifuged at 6000g for 1 minute, following which the spin column was placed into a clean 2ml collection tube and 500µl Buffer AW1 was added. Samples were centrifuged again at 6000g for 1 minute and the spin column was placed into a fresh 2ml collection tube. This process was then repeated with 500µl Buffer AW2 and centrifugation at maximum speed (approximately 21,000g) for 3 minutes. To elute the DNA, spin columns were placed into pre-labelled 1.5ml Eppendorf tubes and 200µl Buffer AE was added and incubated at room temperature for 1 minute. A final centrifugation step was performed at 6000g for 1 minute. For gDNA isolated from blood and mtDNA isolated from mitochondria, the concentration of DNA was determined spectrophotometrically using a nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and the samples adjusted to 50, 25, 10, 5, 1 and 0.1ng/µl for use in the PCR standard curve using DNase/RNase-free distilled water (Life Technologies, Paisley, UK). All samples were stored at -20°C.

5.3.7 Quantitative PCR (qPCR):

All primers used for qPCR were designed using the Primer Express® Software v3.0.1 (Life Technologies, Paisley, UK) and their homology assessed using BLAST. Primers (Sigma Aldrich, Dorset, UK) were adjusted to 50nM using nuclease-free water (ThermoFisher Scientific, Waltham, MA, USA).

To identify genomic DNA, primers to the housekeeping gene GAPDH were used, as documented below:
GAPDH forward: 5’ TGCTCCTCCCCGTTCGA 3’
GAPDH reverse: 5’ GGCTTTACCTGGCAATGCA 3’

To identify mtDNA, primers specific to cytochrome B were used, as stated below

Cytochrome b forward: 5’ ACACATCAGACACAACAACA 3’
Cytochrome b reverse: 5’ GTAGCGAATAACTCATCCGTAA 3’

All qPCR was performed using a QuantStudio™ 12K Flex system (Life Technologies, Paisley, UK) with Power SYBR green PCR master mix (Life Technologies, Paisley, UK). The following reactions were made up for each well of a MicroAmp Optical 384-Well Reaction Plate (Life Technologies, Paisley, UK); 10µl Power SYBR green PCR master mix (Life Technologies, Paisley, UK), 4.8µl H₂O, 0.1µl Forward Primer, 0.1µl Reverse Primer and 5µl DNA. A sheet of optical adhesive film was used to seal the plate and the following protocol was used for the qPCR procedure; 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence of each well was then read and a melt curve from 60°C to 95°C was included at the end of the program to analyse the products. Ct values (the number of cycles needed to produce a threshold amount of fluorescence) of more than 40 were discarded, along with any samples that produced multiple peaks in the melt curve as this may have been due to primer dimers. In all cases, each PCR was performed with quadruplet samples. mtDNA extracted from the porcine tissue and gDNA extracted from porcine blood was used to generate the standard curves.

5.3.8 Flow Cytometry:

Immunophenotyping of the perfusate samples was performed on a BD LSR II flow cytometer (Becton Dickinson, Oxford, UK). Leukocytes were identified and gated as CD45+ and their viability assessed using an efluor 506 viability dye (ebioscience, CA, USA). Following this, a panel of antibodies was utilised to characterise T helper cells (CD3ε+ CD4α+), cytotoxic T cells (CD3ε+CD8β+), double positive T Cells (CD3+CD4+CD8+), double negative T cells (CD3+CD4-CD8-), γδ T cells (γδ+), B cells (CD3-CD21+), macrophages (CD203a+), classical monocytes (CD14+CD163-), non-classical monocytes (CD14+CD163+), immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10-2B2+), and natural killer cells (CD335+). Cells were treated with 1x red blood cell
lysing solution (BD Biosciences, UK), washed via centrifugation at 700g for 10 minutes at 4°C and re-suspended in 0.3ml flow staining buffer. The bottle of e123count beads (eBioscience, CA, USA) was thoroughly mixed and 20µl were added to allow an absolute cell count using the equation below:

\[
\text{Absolute cell count} = \frac{\text{cell count} \times \text{eBead volume} \times \text{eBead concentration}}{\text{eBead count} \times \text{cell volume}}
\]

Samples were analysed for 3 minutes. All gating strategies and analysis was performed using FlowJo version 10.0.6 (Figure 5.3).

**Flow cytometry materials:**

PE-Cy7-conjugated anti-CD3ε, PE-conjugated anti-γδ and Alexa Fluor 647-conjugated anti-CD8α were purchased from BD Bioscience (Oxford, UK). PerCP-Cy5.5-conjugated anti-CD45, FITC-conjugated mouse anti-CD21, PE-conjugated mouse anti-CD4, FITC-conjugated anti-CD14, PE-conjugated mouse anti-CD163, Alexa Fluor 488-conjugated mouse anti-CD335, FITC-conjugated mouse anti-2B2, PE-conjugated mouse anti-6D10 and Lynx Rapid PerCP-Cy5.5 antibody conjugation kit were all purchased from AbD Serotec (Kidlington, UK). APC-conjugated mouse anti-CD203a was purchased from Novus Biologicals (Abingdon, UK).
Figure 5.3 – Gating strategy for identifying leukocyte populations in the perfusate

Representative image of the gating strategy used to identify leukocyte populations using flow cytometry. In all 4 tubes, the counting beads added were initially identified and gated out so that they weren’t included in the populations using the ‘make not gate’. Leukocytes were then identified and gated as CD45+ cells. From this population, single cells were gated and from this the viability of cells was determined. In tube 1, granulocytes were gated on their positive or negative stain of 2B2 and 6D10. In tube 2, pan T cells were gated as CD3+ and then CD4+, CD8+ and double negative T cells (CD3+CD4-CD8-) populations gated from this population based on their positive or negative staining for CD4 and CD8 (A). B cells were gated as CD21+ from the CD3- gate (B). In tube 3, NK cells were gated as CD335+ (A) and gamma delta T cells as γδ+ (B). Finally, in tube 4 monocytes and macrophages were gated. Macrophages were gated as CD203a+ (A). For monocytes, a population of CD14- and CD163- were initially gated and any cells not in this gate were then gated on their positivity or negativity for CD14+ and CD163+ (B).
5.3.9 Luminex Analysis:

The concentration of cytokines and chemokines within the perfusate was assessed using plasma samples collected at baseline, 60 minutes and 360 minutes. A commercially available porcine 13-plex magnetic bead panel (Merck Millipore, Billerica, MA, USA) was utilised, following the manufacturer’s protocol as described in section 2. Briefly, plasma samples were thawed and a 96-well plate was blocked by adding 200µl assay buffer before being shaken for 10 minutes at room temperature. Following this, 25µl of standards and controls were added to the appropriate wells, along with 25µl of matrix solution. To the sample wells, 25µl of assay buffer was added with 25µl of neat plasma. The pre-mixed antibody solution was thoroughly vortexed to ensure appropriate separation of the beads and 25µl was added to all wells. The plate was sealed, wrapped in foil and incubated with agitation on a plate shaker overnight at 4°C. After incubation, the plate was washed three times and 50µl of detection antibodies were added. The plate was re-sealed, wrapped in foil and incubated for a further 2 hours at room temperature. An additional 50µl of Streptavidin Phycoerythrin was added to each well and the plate was re-sealed and incubated for 30 minutes at room temperature. The plate was then washed three times and 100µl of sheath fluid was added to all wells and the plate was agitated for 5 minutes on a plate shaker. Finally, the plate was read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, UK).

5.3.10 Statistical Analysis:

All statistical analysis was carried out using IBM SPSS software version 22. Data normality was determined by assessing mean, standard deviation, skewness and kurtosis. Formal evaluation was performed using the Shapiro-Wilk test. Normally distributed data are expressed as mean ± standard deviation, or as median [interquartile range] if non-normally distributed. Changes in renal haemodynamics and biochemistry, cell number and cytokine concentration and DNA concentrations were analysed using the General Linear Model or the Friedman’s 2-way ANOVA by Ranks depending on the distribution of the data. The Spearman’s rho correlation coefficient was used to determine the relationship between RBF and IRR. For comparisons between RBF at different time points a paired t-test or Wilcoxon Signed Rank test was used. Data were considered significantly different if a p value of <0.05 was observed.
5.4 Results

5.4.1 Validation of the Circuit:

To validate the protocol prior to initiating the study, n=5 kidneys were perfused and the functional parameters of the kidney were determined. UO, MAP and RBF were continually recorded. IRR was also determined by calculating the MAP/RBF.

All kidneys were perfused without complications, with IRR decreased following the initial reperfusion period (p=0.011) and remaining low (p=0.149; Figure 5.4). This was met with good RBF that remained stable over the 6 hour perfusion period (p=0.094; Figure 5.4). An inverse relationship was observed between RBF and IRR, with RBF increasing as IRR decreased (r_= -0.894, p<0.001; Figure 5.5). The level of oxygen consumption was high throughout (p=0.070), and the lactate concentration remained stable (p=0.286) with a median value of 1.31 [1.08] mmol/L (Figure 5.6). UO began immediately upon connection to the circuit and remained for the duration of the study, although a decline was observed over-time (p=0.008; Figure 5.7). Finally, serum creatinine concentrations decreased over time suggesting tubular function was maintained (p<0.001; Figure 5.7).
Figure 5.4 – Renal haemodynamics are maintained during perfusion

All kidneys remained functional throughout the perfusion period. The level of RBF (A) remained within clinically acceptable ranges, with IRR (B) reflecting this accordingly.
Figure 5.5 – The relationship between RBF and IRR during EVNP

In accordance with renal physiology, an inverse relationship was observed between IRR and RBF. As IRR decreased there was a subsequent increase in RBF.
Figure 5.6 – Oxygen consumption and lactate concentrations during EVNP

Kidneys continued to consume oxygen throughout the experiment (A). Lactate concentrations decreased in the first hour of perfusion, with an increasing trend thereafter (B).
Figure 5.7 – Renal tubular function is restored and maintained during perfusion

Tubular function was restored and maintained throughout. UO was maintained throughout the perfusion period (A) and creatinine was continually cleared from the circuit (B).
5.4.2 Renal Haemodynamics and Oxygen Consumption:

Following validation of the protocol an additional n=5 kidneys were perfused and samples collected for analysis.

All kidneys were reperfused without any complications and appeared evenly perfused with a significant increase in RBF between 0 and 30 minutes of perfusion (p=0.043). A change in RBF was observed over the 6 hour perfusion (p<0.001, Figure 5.8 A), although the level remained within physiological parameters. Following a short period of decrease, RBF stabilised with an increasing trend following 2-hours of perfusion. IRR also fluctuated during perfusion (p=0.020, Figure 5.8 B) but did not exceed 0.3mmHg/ml/min and was inversely proportionate to the RBF (r=−0.824, p<0.001; Figure 5.9). The level of oxygen consumption observed throughout the perfusion remained high peaking in the first hour. Following this there was a slight decline that plateaued after two hours of perfusion and remained stable for the duration of the procedure (p=0.006, Figure 5.10 A). Lactate concentration also decreased during the first 60 minutes of perfusion with an increasing trend thereafter (p=0.003, Figure 5.10 B)
Figure 5.8 – Renal haemodynamics remained stable during EVNP

Renal haemodynamics remained stable during perfusion. RBF (A) and IRR (B) remained within acceptable ranges during perfusion.
Figure 5.9 – Relationship between RBF and IRR during perfusion

An inverse relationship between RBF and IRR was observed during perfusion. As IRR decreased there was a subsequent increase in RBF.
Figure 5.10 – Changes in oxygen consumption and lactate concentrations during EVNP

Oxygen consumption was maintained throughout the experiment (A). Lactate concentrations decreased in the first hour of perfusion, with an increasing trend thereafter (B).
5.4.3 UO and Creatinine Clearance during EVNP:

All kidneys began producing urine immediately after being connected to the circuit (Figure 5.11). The average urine production per hour was 92.03 ± 78.13mls, with a mean total UO of 530.04 ± 243.78mls. The pattern of UO changed during the perfusion period (p=0.001), with a decline in UO after the first hour of perfusion. The serum creatinine concentration in the circuit significantly decreased during the 6-hour perfusion (p<0.001; Figure 5.11).
Figure 5.11 – Tubular function during 6 hours of perfusion

Tubular function is restored and retained over 6 hours of perfusion. Urine production began immediately following revascularisation, peaking in the first hour of perfusion (A). Creatinine is continually removed by the kidney and excreted in the urine (B).
5.4.4 Chemokine and Cytokine Concentrations within the Perfusate:

To characterise the inflammatory profile during perfusion, 13 cytokines and chemokines were assessed. Increasing concentrations of IFN-γ (p=0.021), IL-1α (p=0.016), IL-1β (p=0.010), IL-1RA (p=0.009), IL-2 (p=0.017), IL-6 (p<0.001), CXCL-8 (p=0.007), IL-10 (p=0.007) and IL-18 (p<0.001) were detected. This occurred immediately following initiation of perfusion and was maintained throughout the procedure. However GM-CSF, IL-4, IL-12 and TNF-α did not change from baseline, suggesting EVNP drives a significant inflammatory response (Figure 5.12).
Figure 5.12 – Cytokine and chemokine profile within the perfusate during EVNP

Cytokine secretion increases over time during ex vivo perfusion. Serial perfusate samples were analysed by Luminex to detect a range of cytokines and chemokines. After approximately 60 minutes on the circuit, EVNP is associated with a rapid increase in the secretion of IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-6, CXCL-8, IL-10 and IL-18. The concentration of GM-CSF, IL-4, IL-12 and TNF-α remains unaffected (A-D).
5.4.5 Concentration of circulating cell-free mitochondrial and genomic DNA:

To determine if EVNP induces cell death during perfusion, circulating cell-free mtDNA and gDNA were quantified. An increase in the concentration of both cell-free gDNA and mtDNA was observed (p<0.001 and p=0.008 respectively), suggesting EVNP causes a degree of cellular injury upon reperfusion (Figure 5.13).
Figure 5.13 – Cell-free genomic and mitochondrial DNA increase in the perfusate over time

EVNP is associated with increasing concentrations of cell-free genomic (A) and mitochondrial (B) DNA. Cell-free mitochondrial and genomic DNA concentrations from plasma samples were quantified using qPCR. Increasing concentrations are detected during perfusion, suggesting cellular injury occurs.
5.4.6 Cellular Efflux from the Donor Kidney into the Perfusate:

The immune cell efflux from the donor kidney into the EVNP circuit was characterised to assess the contribution of passenger leukocytes from the donor kidney following transplantation (Figure 5.14). Major populations of donor T cells including helper, cytotoxic, double negative, and CD4+CD8+ T cells were identified within the first 60 minutes of perfusion and increased throughout the procedure (p=0.015, p=0.012, p=0.022 and p=0.021 respectively). A large population of B cells was also detected, with the greatest number being identified following 6 hours of perfusion (p=0.041).

Of the granulocyte lineage, mature basophils and eosinophils migrated into the circuit with an increase in numbers during perfusion (p=0.015). Equally, the number of mature and immature neutrophils increased (p=0.026 and p=0.038 respectively). Classical monocytes represented the major monocyte population, with non-classical monocytes being less abundant. However, the size of both populations remained consistent during perfusion (p=0.059 and p=0.549 for classical and non-classical repertoires respectively). Minor populations of NK cells and mφ were also detected, with NK cells observed to increase over time (p=0.015 and p=0.449 respectively).
Figure 5.14 – Donor leukocyte mobilisation into the perfusate during EVNP

Donor leukocytes migrate out of the kidney into the perfusate during EVNP. Serial perfusate samples were analysed by flow cytometry to identify migrating leukocytes. Large populations of T cells, B cells and NK cells were detected in increasing concentrations during the perfusion period. Populations of monocytes, macrophages, neutrophils and basophils and eosinophils were also detected (A-D).
5.5 Discussion

The initial focus of this study was to develop and validate an experimental model of EVNP that allowed prolonged perfusion of porcine kidneys. Based on results described in section 4, BSA and was added to the perfusate to create an oncotic pressure within the circuit, thereby improving RBF and promoting UO in the proximal tubules. In addition, mannitol and dexamethasone were increased in the circuit, with antibiotics also being included. Following a series of experiments trialling different concentrations of these within the perfusate a set of validation perfusions (n=5) was performed using the protocol that best maintained kidney function and UO. Here, it was demonstrated that the protocol using 5% BSA Ringer’s solution as the basis of the perfusate provides good tissue perfusion whilst maintaining urine production. The excellent renal function is demonstrated through the high RBF with a concomitant low IRR and high oxygen consumption, suggesting cellular metabolism is restored and remains during perfusion. In addition, the rapid and maintained creatinine clearance within the continued urine production suggests tubular function is retained. In section 4 it was demonstrated that an increasing lactate during EVNP is likely to indicate ischaemic cellular injury and therefore the low concentrations observed in this set of experiments suggest that tissue viability is preserved. Together, this data set demonstrates that this circuit is reproducible and is clinically relevant and therefore can be utilised for research purposes. Indeed, we performed an additional 5 perfusions to collect samples for analysis.

Normothermic EVNP provides a unique insight into the immunological and inflammatory contribution of the donor kidney following revascularisation. In this study EVNP resulted in a significant inflammatory storm with surprisingly high concentrations of IL-6 (80,242pg/ml ± 15950 perfusate), CXCL-8 (49980pg/ml ± 39 perfusate) and IFN-γ (16292pg/ml ± 26917 perfusate) detectable at the end of perfusion. Other pro-inflammatory cytokines also increased throughout the 6 hours perfusion period, including IL-1β, IL-1RA, and IL-12. To a lesser extent, increases in IL-4, IL-10, IL-18 and TNF-α were detected, but did not exceed 500pg/ml throughout perfusion. These data is clearly of importance as they give insight into the potential contribution of the donor kidney to the inflammatory events that occur immediately following transplantation. For example, if the donor kidney drives a similar CXCL-8 response following revascularisation, this will result in both donor and recipient cellular mobilisation and an efflux of cells out of the donor kidney and into the recipient vasculature. Recipient immune cells expressing the CXCL-8 receptors C-X-C chemokine receptor (CXCR) 1 and
CXCR2 (including DCs and T cells) will infiltrate the donor kidney via this CXCL-8 dependent chemokine gradient. Therefore the CXCL-8 response could contribute to the diapedesis of donor leukocytes to recipient lymph nodes, and the recruitment of recipient DC and T cells to the graft. As recipient T cells infiltrate the graft they encounter alloantigen presented directly via donor DC which self-present donor leukocyte antigens or donor peptides. Infiltrating recipient DC also process and present donor peptides to recipient T cells, which collectively represents the hallmark of allore cognition and ultimately leads to permanent T cell alloreactivity (169). IL-6 is an acute phase cytokine that plays a central role in IRI and contributes to graft rejection by orchestrating leukocyte recruitment, activation and proliferation of a range of lymphocytes (170, 171). IL-6 pathway inhibitors have subsequently been used following transplantation and have shown promise in improving early graft survival. Given the profoundly high levels observed in this study, the donor kidney may represent a major source of IL-6 following transplantation and as such may represent a therapeutic target. High levels of IFN-γ are observed, which is a major pro-inflammatory cytokine involved in allore cognition and alloreactivity. IFN-γ induces the up-regulation of Class I MHC on graft epithelial and endothelial cells and drives recipient leukocyte infiltration of the donor kidney (172, 173). However, as EVNP is a closed system, IL-6, CXCL-8 and IFN-γ are released prior to transplantation and had no adverse effect on renal function during perfusion. Instead, the pro-inflammatory cytokine response observed may be responsible for the activation and mobilisation of donor-derived leukocytes out of the donor kidney during perfusion. Furthermore, inducing the secretion of IL-6, CXCL-8 and IFN-γ may exhaust the pro-inflammatory response of the donor kidney prior to transplantation, and may also impede the ability of donor cells to respond in the post-transplant setting. As such, the presence of these cytokines may be reduced following transplantation, thereby inhibiting their deleterious effects and promoting graft survival.

From the current study it is not possible to decipher the cytokine source; however increasing concentrations of mtDNA and gDNA were detected throughout perfusion. This is likely as a result of a loss of leukocyte viability within the circuit due to the interaction of these cells with the plastic circuit, as well as the in evitable IRI that ensues. The release of endogenous damage-associated molecular patterns such as extracellular DNA are associated with the initiation and maintenance of a pro-inflammatory response and immune activation (174). Whilst this may partly explain the increase in a range of cytokines, the EVNP circuit itself may influence the inflammatory response as it consists of a modified CPB machine. It is well
documented that patients placed on CPB during surgical procedures develop systemic inflammatory response syndrome (175). This is proposed to be due to the interaction of leukocytes with an artificial surface leading to cellular activation, and resultant cytokine secretion (176). Interestingly, this does not appear to influence clinical outcome. Indeed, in this study kidney function was maintained throughout perfusion despite increasing cytokine and extracellular DNA concentrations. All kidneys functioned within normal parameters, remained biochemically stable and met the criteria for transplantation throughout perfusion (101). Urine production began immediately and continued for the duration of the perfusion, although a decline was observed after 1 hour. Following an ischaemic insult and subsequent development of acute tubular necrosis, a polyuric phase is often observed that can then stabilise when tubular function is restored (159). The initial large volume of urine is likely to be reflective of acute tubular injury that stabilises during reperfusion and adequate oxygenation. In corroboration with this, acid-base balance was maintained, suggesting tubular function remained viable. Interestingly, an increase in lactate concentration was observed which can be used as an indicator of regional ischaemia and hypoperfusion. In a clinical setting, it is widely considered that a lactate concentration above 4mmol/L is associated with poorer patient outcomes (161, 162). During this study the mean lactate concentration did not exceed 2.5mmol/L and was therefore unlikely to be representative of impaired kidney function. However, it is important to note that the kidney was only exposed to 2 hours SCS to minimise the ischaemic injury, thereby providing a clear indication of the inflammatory capacity of the donor organ. Prolonged ischaemic times may influence the inflammatory profile described as a result of tissue injury.

Aside from cytokine secretion in the presence of good renal function, this study demonstrates that the kidney contains a large reservoir of leukocytes that rapidly mobilise following revascularisation. Major populations of donor leukocytes were detected in the perfusate as early as 60 minutes post perfusion, suggesting that these cells are marginal and can extravasate from the tissue and enter the recipient circulation following transplantation. This includes large populations of T cells (4,154,908 ± 1,894,451 cells/ml perfusate) and B cells (376,098 ± 275,830 cells/ml perfusate) that have the capacity to drive inflammation; secrete pro-inflammatory cytokines (including IL-6, CXCL-8 and IFN-γ) and orchestrate alloantigen specific immune responses. Ultimately this can lead to AR and graft loss. It should be noted that within this T cell population a proportion may be of regulatory phenotype, although this was not determined in this study. Removing these cells prior to transplantation may
negatively impact on clinical outcomes via a loss of tolerance induction. However, a significant proportion of these cells are unlikely given the predominantly inflammatory environment described. Significant numbers of NK cells were also observed within the perfusate (124,644 ± 109,934 cells/ml perfusate). NK cells have multiple functions and can secrete high levels of IL-6, CXCL-8 and IFN-γ upon stimulation. They are also central in the recruitment of cytotoxic T cells during inflammation, which are recruited into the kidney and contribute to tissue destruction and a loss of graft function. The diapedesis of major populations of cells with antigen presenting capacity also occurred, including monocytes, mφ and B cells. These donor leukocytes are essential for direct presentation and can provide co-stimulatory signals to recipient T cells (177).

A porcine model was utilised in this study owing to the similarity in their physiology and organ development to humans (178). The pigs used were a similar size to humans and all pigs were the same breed and of equal size and the mode of death was standardised to minimise data variation. However, it should be noted that using abattoir animals is not without limitations as these animals are from an uncontrolled environment, although this is more reflective of a heterogeneous population.

Whilst the fate of the immune efflux cannot be determined in this study, the migration of billions of donor leukocytes in conjunction with the secretion of an IFN-γ, IL-6 and CXCL-8 storm is likely to drive direct allorecognition and orchestrate significant recipient immune activation following transplantation. Furthermore, a major proportion of these donor leukocytes are likely to home to lymph nodes and self-present donor antigens to naïve recipient T cells (121). Removing this inflammatory burden prior to transplantation may therefore confer significant clinical benefit (154). Based on these findings, immunomodulatory strategies to alter the donor immune environment prior to transplantation warrant development.
Section 6 – Assessing the impact of a secondary pre-transplantation preservation flush on the donor kidney immune burden

A version of this paper is published in Nephrology Dialysis Transplantation:

Title of paper – Characterising the early inflammatory contribution of the donor kidney following reperfusion


DOI: https://doi.org/10.1093/ndt/gfw464

Within this section I retrieved all of the organs used for this data set and performed the secondary preservation flush with the aid of an undergraduate student to collect the venous effluent. I did all of the experiments with the assistance of a student who was using some of the cells from the preservation flush. I analysed all of the subsequent data. All of the statistical analysis was checked by our statistician prior to publication.
6.1 Abstract:

**Introduction:** Donor kidneys contain a large reservoir of passenger leukocytes that contribute to AR pathway via direct alloantigen presentation and pro-inflammatory cytokine secretion. Current clinical practice depicts an *in situ* preservation flush at the time of organ retrieval, before being placed on ice. In kidney transplantation, re-flushing prior to implantation is rarely performed. Performing a secondary, high volume (2L) preservation flush immediately prior to implantation may remove additional passenger leukocytes and inflammatory mediators, thereby reducing graft immunogenicity.

**Methods:** Explanted porcine kidneys (n=5) were retrieved using a perfusion protocol analogous to clinical practice, stored on ice for 2 hours before being flushed again with 2L of Ringer’s solution. The venous effluent was collected and leukocytes characterised via flow cytometry. Inflammatory mediators, including cytokines and cell-free DNA, were assessed to determine the inflammatory contribution of the donor kidney.

**Results:** Following a secondary flush, a significant number of CD45+ leukocytes are identified. T cells represent the largest population, with inflammatory T cells predominating. Populations of B cells, NK cells, monocytes and granulocytes are also present. A broadly inflammatory cytokine profile was observed, in combination with significant concentrations of cell-free mitochondrial and genomic DNA.

**Discussion:** This study demonstrates that the donor kidney secretes a major inflammatory effluent following revascularisation. Performing a secondary preservation flush prior to implantation is likely to reduce this immune burden via diversion of donor leukocytes and inflammatory mediators from entry into the recipient circulation and secondary lymphoid tissue. This may modulate direct presentation and reduce the inflammatory burden of the donor kidney following transplantation.
6.2 Introduction

Kidney transplantation is the optimal treatment option for patients with end-stage renal disease. However, the limited availability of donor organs available for transplant, coupled with the combined effects of peri-transplant IRI and AR represent major barriers to successful transplantation. Donor-derived leukocytes are integral to the allore cognition of transplanted organs and may stimulate both the innate and adaptive arms of the immune response. In section 3 it was demonstrated that removing donor leukocytes using ex vivo perfusion reduces graft immunogenicity and can impede AR in the absence of immunosuppression (154). However the understanding of the immunological and pro-inflammatory contribution of the donor kidney following transplantation is limited and warrants investigation.

This study has also demonstrated that donor kidneys contain a significant immune compartment consisting of T cells, B cells, NK cells and professional APCs. A major population of these donor leukocytes are marginal and are likely to rapidly mobilise into the recipient circulation following revascularisation (179). Here they directly present alloantigen to recipient naïve T cells via donor MHC and induce antigen specific clonal expansion. This leads to immunological rejection against alloantigens expressed on other cells in the graft. In current clinical practice, an in situ preservation flush of the donor vascular tree is performed at the donor hospital site which washes blood from the vasculature of all organs being retrieved. The organ, flushed and core cooled, is then stored on ice for transport. Once at the recipient hospital, the organ is removed from ice and typically implanted immediately into the recipient. In some instances the donor kidney is reperfused prior to implantation, particularly where residual microsurgical blood traces are suspected, however this is not ubiquitously practiced and the implications post-transplantation remain unclear. The robust donor immune compartment contained within the kidney is therefore usually transplanted into the recipient. In combination with a preservation process, which leaves this resident leukocyte population intact, organ parenchymal cells become damaged during the process of donor death and subsequent cold storage. This results in significant cellular injury and a global inflammatory response in the donor. Together, this increases the immunogenicity of the organ before transplantation, leading to an increased risk of DGF and AR.

Performing a secondary flush of the donor kidney following SCS on ice immediately prior to implantation may remove donor passenger leukocytes and inflammatory mediators that would
typically be transferred into the recipient circulation. For this purpose, kidneys were retrieved using a perfusion protocol analogous to clinical practice, stored on ice and a subsequent secondary preservation flush was performed. The effluent was collected and the leukocyte content along with inflammatory mediators were characterised to determine if an additional pre-transplant flush would be of benefit. Given that direct presentation alone can drive AR, reducing the transfer of donor leukocytes prior to transplantation may impact on rejection and graft survival (132).
6.3 Materials and Methods

6.3.1 Organ Procurement:

Kidneys were obtained from n=8 Landrace pigs (n=5 for the initial study and an additional n=3 for regulatory T cell (Treg) assessment) with a mean dry weight of 80kg were retrieved as described in section 5.2.1. All pigs were culled in accordance with the EU Council Regulation (EC) 1099/2009 on the protection of animals at the time of killing. Briefly, pigs were veterinary inspected and then sacrificed via electrical stunning followed by an incision of the carotid artery, representing a DCD model. A midline incision was performed and kidneys were immediately excised and placed on ice. Following inspection for evidence of disease or injury, the renal artery and ureter were isolated and cannulated. The renal artery was flushed with 20mls glyceryl trinitrate (GTN; Hameln, Germany-distributed by Gloucester, UK) followed by a standardised 1L flush of 4°C Marshall's hypertonic citrate (Soltran; Baxter Healthcare, Thetford, UK) supplemented with 10,000iU-unfractionated heparin (to aid clot dissemination; Fannin, UK). The pressure of the preservation flush did not exceed 100mmHg. Kidneys were then submerged in Soltran and placed on ice for transport.

6.3.2 Secondary Preservation Flush:

Following 2 hours SCS, kidneys were removed from ice and placed onto a porous platform within a venous reservoir. A clinical grade giving set (Baxter Healthcare, Thetford, UK) was then attached to the renal artery. The vasculature of the kidney was flushed with 2L of room temperature Ringer’s solution. This is an isotonic, physiological solution that does not impact on cellular viability and remains the solution of choice for pre-flushing kidneys prior to EVNP (181). The venous outflow drained into the reservoir and the venous effluent was collected. The ureter cannula was also placed into a separate measuring cylinder to prevent contamination of the sample with filtered excretory tract fluid and to determine fluid loss via this route (Figure 6.1).
Figure 6.1 – Images representing the secondary preservation flush

Representative images of the procedure used for the second preservation flush. Kidneys were placed on a porous membrane within a venous reservoir. Ringer’s solution (2L) was flushed through the kidney via the renal artery using a clinical grade giving set that is attached to a funnel. The venous effluent was collected within the venous reservoir and then a collection tube. The ureter was also cannulated and the filtered excretory tract fluid collected into a measuring cylinder to prevent contamination of the venous effluent.
6.3.3 Sample Processing:

At the end of the preservation flush the venous effluent was thoroughly mixed and 10mls was placed into a falcon tube. Samples were centrifuged at 2000g for 2 minutes at 4°C and 200µl aliquots of supernatant were removed and stored at -80°C. The remaining cell pellets were re-suspended in 500µl flow staining buffer, split between 4 tubes and analysed by flow cytometry. For Tregs, cell pellets were re-suspended in 100µl flow staining buffer in 1 flow tube.

6.3.4 Flow Cytometry:

Immunophenotyping of the preservation flush was performed on a BD LSR II flow cytometer (Becton Dickinson, Oxford, UK). Leukocytes were identified and gated as CD45+ and their viability determined using a Zombie UV viability dye (BioLegend, San Diego, USA). From this a panel of antibodies was utilised to characterise T helper cells (CD3ε+ CD4α+), cytotoxic T cells (CD3ε+CD8β+), double positive T Cells (CD3+CD4+CD8+), double negative T cells (CD3+CD4-CD8-), γδ T cells (γδ+), B cells (CD3-CD21+), classical monocytes (CD14+CD163-), non-classical monocytes (CD14+CD163+), immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10-2B2+), and natural killer cells (CD335+).

Tregs (CD3+CD4+CD25+FoxP3+) were also assessed in an additional n=3 cell pellets, in which fixation and permeabilisation steps were included (BD Biosciences, UK).

All cells were treated with 1x red blood cell lysing solution (BD Biosciences, UK), washed via centrifugation at 700g for 10 minutes at 4°C and re-suspended in 0.3ml flow staining buffer. Finally, 20µl e123count beads (ebioscience, CA, USA) were added to allow absolute cell counts to be performed using the equation below:

\[
\text{Absolute cell count} = \frac{(\text{cell count} \times \text{eBead volume}) \times \text{eBead concentration}}{(\text{eBead count} \times \text{cell volume})}
\]

Samples were analysed for 2 minutes. All gating strategies and analysis was performed using FlowJo version 10.1 (Figures 6.2 and 6.3).
**Flow cytometry materials:**

PE-Cy7-conjugated anti-CD3ε, PE-conjugated anti-γδ and Alexa Fluor 647-conjugated anti-CD8α were purchased from BD Bioscience (Oxford, UK). PerCP-Cy5.5-conjugated anti-CD45, FITC-conjugated mouse anti-CD21, PE-conjugated mouse anti-CD4, FITC-conjugated anti-CD14, PE-conjugated mouse anti-CD163, Alexa Fluor 488-conjugated mouse anti-CD335, FITC-conjugated mouse anti-2B2, PE-conjugated mouse anti-6D10, PE-conjugated CD25, FITC-conjugated anti-CD4α, PR-Texas Red-conjugated anti-CD8α and Lynx Rapid RPE-Texas Red antibody conjugation kit were all purchased from AbD Serotec (Kidlington, UK).
Figure 6.2 – The gating strategy used to identify different leukocyte populations within the venous effluent

Representative image of the gating strategy used to identify leukocyte populations using flow cytometry. In all 4 tubes, the counting beads were identified and gated out of the populations using the ‘make not gate’. Leukocytes were then identified and gated as CD45+ cells. From this population, single cells were gated and from this the viability of cells was determined. In tube 1, granulocytes were gated on their positive or negative stain of 2B2 and 6D10. In tube 2, pan T cells were gated as CD3+ and then CD4+, CD8+ and double negative (CD3+CD4-CD8-) populations gated from this population based on their positive or negative staining for CD4 and CD8 (A). B cells were gated as CD21+ from the CD3- gate (B). In tube 3, NK cells were gated as CD335+ (A) and gamma delta T cells as γδ+ (B). Monocytes were gated in tube 4 on their positive or negative staining for CD14+ and CD163+. 
Figure 6.3 - The gating strategy used to identify T cell populations within the venous effluent

Representative image of the gating strategy used to identify T cell populations using flow cytometry. Initially, the counting beads were identified and gated out of the populations using a ‘make not gate’. Leukocytes were then identified and gated as CD45+ cells. From this population, single cells were gated and from this the viability of cells was determined. Pan T cells were gated as CD3+ and then CD4+, CD8+ and double negative (CD3+CD4-CD8-) populations gated from this population based on their positive or negative staining for CD4 and CD8. From the CD3+CD4+ population, regulatory T cells were identified using CD25 and FoxP3.
6.3.5 Luminex Analysis:

The concentration of cytokines and chemokines within the second preservation flush was assessed using supernatant samples collected from the total effluent. A commercially available porcine 13-plex magnetic bead panel (Merck Millipore, Billerica, MA, USA) was utilised, following the manufacturer’s protocol as described in section 2. A commercially available porcine 13-plex magnetic bead panel (Merck Millipore, Billerica, MA, USA) was utilised, following the manufacturer’s protocol as described in section 2. Briefly, plasma samples were thawed and a 96-well plate was blocked by adding 200µl assay buffer before being shaken for 10 minutes at room temperature. Following this, 25µl of standards and controls were added to the appropriate wells, along with 25µl of matrix solution. To the sample wells, 25µl of assay buffer was added with 25µl of neat plasma. The pre-mixed antibody solution was thoroughly vortexed to ensure appropriate separation of the beads and 25µl was added to all wells. The plate was sealed, wrapped in foil and incubated with agitation on a plate shaker overnight at 4°C. After incubation, the plate was washed three times and 50µl of detection antibodies were added. The plate was re-sealed, wrapped in foil and incubated for a further 2 hours at room temperature. An additional 50µl of Streptavidin-Phycoerythrin was added to each well and the plate was re-sealed and incubated for 30 minutes at room temperature. The plate was then washed three times and 100µl of sheath fluid was added to all wells and the plate was agitated for 5 minutes on a plate shaker. Finally, the plate was read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, UK).

6.3.6 Mitochondria Isolation:

Mitochondria were isolated from porcine lung tissue to utilise as qPCR standards. A mitochondrial isolation kit (Sciencell Research Laboratories, Carlsbad, CA, USA) was used under sterile conditions at 4°C as described in section 5. Briefly, lung tissue was homogenised on ice and the resultant lysate centrifuged at 1000g for 5mins at 4°C. The supernatant was removed and centrifuged at 10,000g for 20 minutes at 4°C. The cell pellet was re-suspended in 50µl buffer to retain intact mitochondria for DNA extraction.
6.3.7 DNA Extraction:

QIAamp DNA Mini and Blood Mini kits were used to extract genomic DNA from porcine blood and mtDNA from mitochondria isolated in 6.3.6. In addition, cell-free DNA from supernatant samples of the flush was extracted according to the manufacturer’s protocol (Qiagen, Manchester, UK) as described in section 5. Briefly, 200µl of sample was added to the relevant buffers provided and incubated at 56°C for 10 minutes. Following this 200µl 100% ethanol was added and the samples were washed twice before being eluted in 200µl of buffer AE. DNA concentration was determined spectrophotometrically using a nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and the samples adjusted to 25, 10, 5, 1 and 0.1ng/µl for use in the PCR standard curve using DNase/RNase-free distilled water (Life Technologies, Paisley, UK). All samples were stored at -20°C.

6.3.8 Quantitative PCR (qPCR):

Following DNA extraction, qPCR was used to determine the concentration of mitochondrial and genomic DNA. All primers used were designed using the Primer Express® Software v3.0.1 (Life Technologies, Paisley, UK) and their homology to other genes assessed using BLAST. Primers (Sigma Aldrich, Dorset, UK) for gDNA were adjusted to 50nM and for mtDNA to 25nM using nuclease-free water (ThermoFisher Scientific, Waltham, MA, USA).

To identify gDNA, primers to the housekeeping gene, GAPDH, were used as documented below:

GAPDH forward: 5’ TGCTCCTCCCGTTCGA 3’
GAPDH reverse: 5’ GGCTTTACCTGGCAATGCA 3’

The mtDNA was identified using primers specific to cytochrome B, as stated below

Cytochrome B forward: 5’ ACACATCAGACACACAACAACA 3’
Cytochrome B reverse: 5’ GTAGCGAATAACTCATCGGTA 3’

All qPCR was performed using a QuantStudio™ 12K Flex system (Life Technologies, Paisley, UK) with Power SYBR green PCR master mix (Life Technologies, Paisley, UK). For genomic DNA the following reactions were made up for each well of a MicroAmp Optical
384-Well Reaction Plate (Life Technologies, Paisley, UK); 10µl Power SYBR green PCR master mix, 4.8µl H₂O, 0.1µl Forward Primer, 0.1µl Reverse Primer and 5µl DNA. For mtDNA the following reactions were made up for each well of a MicroAmp Optical 384-Well Reaction Plate (Life Technologies, Paisley, UK); 10µl Power SYBR green PCR master mix, 3µl H₂O, 1µl Forward Primer, 1µl Reverse Primer and 5µl DNA. A sheet of optical adhesive film was used to seal the plate and the following protocol was used for the qPCR procedure; 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence of each well was then read and a melt curve from 60°C to 95°C was included at the end of the program to analyse the products. Ct values (the number of cycles needed to produce a threshold amount of fluorescence) of more than 40 were discarded, along with any samples that produced multiple peaks in the melt curve as this may have been due to primer dimers. In all cases, each PCR was performed with quadruplet samples. mtDNA extracted from the porcine tissue and gDNA extracted from porcine blood was used to generate the standard curves.

6.3.9 Statistical Analysis:

All statistical analysis was carried out using IBM SPSS software version 22. Data normality was determined by assessing mean, standard deviation, skewness and kurtosis. Formal evaluation was performed using the Shapiro-Wilk test. Data are presented as mean ± standard deviation. For the comparison between gDNA and mtDNA, the Independent samples t-test was utilised. Data were considered significantly different if a p value of <0.05 was observed.
6.4 Results

6.4.1 Organ Procurement:

All kidneys utilised were free from any immediate evidence of disease and injury. The time taken for organ retrieval (taken as the time from death to organ explant) was comparable between all pigs, averaging 3 minutes (± 0.71 minutes). The average WI time (taken as the time from death to the start of the cold preservation flush) was 10 minutes (± 1.14 minutes).
6.4.2 Total Number of Leukocytes:

The total number of leukocytes within the secondary flush efflux was assessed to determine the number of cells that immediately enter the recipient circulation upon revascularisation. A significant population of donor CD45+ immune cells were identified within the effluent following the 2L secondary flush, averaging $473,532,589 \pm 135,007,679$ cells (Figure 6.4).
Figure 6.4 – A significant population of leukocytes are present within the venous effluent following the secondary flush

Performing a secondary preservation flush of the donor kidney immediately prior to implantation removed a large number of passenger leukocytes, defined at CD45$^+$ (n=5). The majority of these cells retain their viability, although dead cells are also detected.
6.4.3 Leukocyte Phenotypes:

The leukocyte content within the secondary flush efflux was characterised to determine the populations that mobilised following reperfusion. A major population of donor T cells was identified within the venous outflow, averaging 299,600,000 ± 208,804,454 cells. Populations of B cells, NK cells, monocytes and neutrophils were also detected in abundance; however only a minor population of eosinophils and basophils were identified (Figure 6.5 and 6.6). Tregs represented only a minor proportion (<0.05%) of the T cell repertoire (Figure 6.7).
Leukocyte populations removed during the secondary preservation flush are dominated by T cells. Populations of B cells, NK cells, monocytes and granulocytes are also detected (n=5).

Figure 6.5 – Immune cell populations within the venous effluent following the secondary flush
Figure 6.6 – Individual immune cell populations within the venous effluent

Leukocyte populations removed during the secondary preservation flush are dominated by T cells. Populations of B cells, NK cells, monocytes and granulocytes are also detected. Within these populations a large number of cells remain viable, although cell death does occur. This is particularly evident in the T cell population (n=5).
Figure 6.7 – T cell phenotypes within the venous effluent of the secondary flush

T cell populations were characterised in an additional n=3 flushes. Large populations of T cells, including CD4⁺ and CD8⁺ T cells, are removed from the donor kidney during the secondary preservation flush (A and B). Regulatory T cells represent only a minor proportion of these T cells (C).
6.4.4 Cytokine and Chemokine Profile:

To determine the inflammatory profile within the secondary flush, a range of cytokines and chemokines were assessed via Luminex analysis. A significant concentration of IFN-γ was detected, with an average of 2,074,667 ± 736,927 pg in the total venous effluent (averaging 1,600mls). Minor concentrations of GM-CSF, IL-1β, IL-2, IL-4, CXCL-8, IL-18 and TNF-α were also detectable. The concentration of IL-1α, IL-1Ra, IL-6, IL-10 and IL-12 was undetectable (Figure 6.8)
A significant concentration of cytokines and chemokines are detected within the secondary preservation flush (n=5). IFN-γ predominates, with minor concentrations of GM-CSF, IL-1β, IL-2, IL-4, CXCL-8, IL-18 and TNF-α also detectable.
6.4.5 Cell-free Mitochondrial and Genomic DNA:

To determine the level of inflammatory mediators that enter the recipient circulation upon revascularisation, cell-free mtDNA and gDNA concentrations were quantified. Within the total 1,600ml venous efflux, both mtDNA and gDNA were identified in high concentrations, with significantly more gDNA than mtDNA (p<0.001, Figure 6.9).
Figure 6.9 – Cell-free DNA in the total venous effluent

Significant concentrations of cell-free genomic and mitochondrial DNA are detected within the venous effluent (approximating 1,600mls) following the secondary preservation flush (n=5), suggesting a loss of cellular viability. Genomic DNA is detected at a significantly greater concentration than mitochondrial DNA.
6.5 Discussion

Donor kidneys possess a significant repertoire of leukocytes that are transferred into the recipient during transplantation. These donor-derived leukocytes are intrinsically linked to the rejection process following transplantation, with direct allorerecognition alone capable of initiating AR (181). Current clinical practice requires only an in situ preservation flush of the donor kidney to be carried out as part of the retrieval procedure, usually focused on removing all macroscopic traces of erythrocytes. This process is typically followed by SCS and then immediate transplantation with no routine effort to reduce donor leukocyte transfer. The results of this study suggest that in the conditions of standard renal transplant practice, globally activated donor-derived leukocytes will rapidly infiltrate the recipient circulation upon revascularisation and directly present alloantigen to naïve and memory recipient T cells. Despite this phenomenon being well described, all immunosuppressive regimes are principally designed to target the recipient immune system, not the donor immune compartment. Immunomodulation of the donor kidney prior to transplantation may offer a pathway for therapeutic intervention not currently utilised.

This study aimed to determine if performing an additional flush immediately following SCS (prior to implantation) can be used to further ‘wash out’ donor leukocytes and inflammatory mediators before transplantation. Indeed, the results of this study demonstrate that flushing the donor kidney with 2L of crystalloid solution following a period of cold storage removes a significant population of donor leukocytes. In an average venous return of 1,600mls, the mean number of leukocytes that migrated from the donor kidney was 473 million (±135m). Given that it is estimated a healthy kidney is perfused with 750mls of blood per minute (or 20% of cardiac output), then as a conservative estimate it would take less than 5 minutes following revascularisation for this to occur in the recipient. Exactly what extent diversion of this vast leukocyte population eluted in a 2L flush will reduce recipient exposure to donor leukocytes is unknown and requires further studies in transplanted human subjects.

Detailed analysis of the cellular effluent revealed that T cells represented the primary population of donor leukocytes that diapedesed from the kidney following SCS. The majority of these T cells were inflammatory in nature, with only a minor population of cells from a Treg origin (representing less than 0.05% of the T cell repertoire). In addition, IL-10 was not detectable in the flush exudate. This is important as removing donor Tregs and IL-10 prior to
transplantation could drive alloreactivity via loss of tolerance induction and would therefore be undesirable. Instead, the loss of donor FOXP3- T cells in the presence of high IFN-γ concentrations is likely to be of benefit, as T cells in a pro-inflammatory environment orchestrate alloreactivity via direct presentation of donor MHC and secretion of pro-inflammatory cytokines that drive inflammation. More recent evidence suggests that donor CD4+ T cells drive a major alloreactive response and are essential for early AR (182). It is clear that donor FOXP3+ T cells contribute to the rejection process, so their removal via a secondary flush of the kidney immediately prior to transplantation is likely to be of benefit. Interestingly, there is evidence in the literature that mixed chimerism, the co-existence of haematopoietic cells from both donor and recipient origin, occurs following transplantation and induces long-term tolerance to the donor organ (183-185). Several attempts have been made to induce chimerism in patients, typically involving irradiation, cytotoxic drugs or anti-T cell depleting antibodies to remove both recipient and donor T cells (186-188). However, the effect of removing this early, pro-inflammatory population on the degree of mixed chimerism is worthy of further specific study.

Additional to the T cell population, a significant population of APCs including large numbers of monocytes and B cells were also removed. Following revascularisation, donor monocytes rapidly mobilise to recipient lymph nodes and self-present donor antigens to naïve T cells, resulting in alloantigen specific clonal expansion and infiltration of the graft. A recipient T cell response is initiated which manifests as AR. The donor B cell component is also likely to contribute further via recognition and MHC Class II presentation of recipient alloantigens to both donor and recipient T cells. Finally, NK cells were detected in the flush exudate. In keeping with donor B cells, to date there is no definitive understanding of the role of donor NK cells following transplantation. Recipient NK cells can contribute to graft rejection via direct recognition of alloantigen, resulting in cytolytic activation and pro-inflammatory cytokine secretion. This includes IFN-γ, IL-6 and CXCL-8 that have central roles in the up-regulation of MHC Class I on APCs, recruiting cytotoxic T cells to the graft and promoting antigen presentation by DCs (189). In addition, recipient NK cells have been reported to reduce inflammatory burden immediately following transplantation by killing donor leukocytes (182). Indeed, there is evidence of tolerance and graft acceptance induced by recipient NK cell killing of donor DCs (190, 191). It is also possible that donor NK cells may kill recipient leukocytes; however the impact of this remains unknown. Again, the potential
roles of donor NK cells, and the impact of their removal prior to transplantation needs detailed investigation.

Aside from leukocyte removal, a secondary flush also sequestered a significant amount of IFN-γ from the donor kidney prior to transplantation. IFN-γ is a major pro-inflammatory cytokine that contributes to graft immunogenicity via a host of different mechanisms affecting the graft, the donor immune compartment and the recipient immune system. For example, IFN-γ up-regulates donor MHC Class I which accelerates allore cognition. IFN-γ also contributes to chemotaxis via the IFN-γ-chemokine axis, enabling both donor leukocyte mobilisation from the donor kidney and recipient infiltration of the kidney (172, 173). Indeed, IFN-γ is capable of inducing CXCL-10 secretion, a chemokine with potent T cell chemotactic properties (192). This may explain the significant population of T cells present within the secondary preservation flush. CXCL-8 was also detected, which further contributes to donor and recipient cell mobilisation. Evidence of the role of CXCL-8 in reperfusion injury has previously been demonstrated, with anti-CXCL-8 antibodies preventing injury (193). Therefore reducing CXCL-8 transfer from the donor immediately following reperfusion may be of therapeutic benefit. Minor concentrations of IL-1β and IL-18 were detected within the exudate. These inflammasome related cytokines are associated with inflammation and apoptosis post-transplantation, and have a key role in inducing adaptive immune responses (172, 194). Removing this inflammatory milieu prior to transplantation is therefore likely to reduce graft immunogenicity.

Whilst the source of the cytokine and chemokines - and hence the capacity for further synthesis and secretion after implantation - cannot be determined from this study, it is likely that cellular injury causes intracellular components to be present in the extracellular spaces. This will activate donor leukocytes during cold storage, and induce cytokine secretion. Significant quantities of cell-free gDNA and mtDNA were identified within the flush exudate, which have well reported inflammatory effects. Both gDNA and mtDNA initiate alloreactivity via ligation of TLRs on both donor and recipient DCs and mφ. TLR ligation induces the up-regulation of co-stimulatory molecules on DC and mφ which is essential for the allospecific clonal expansion of recipient T cells. The source of extracellular DNA is unclear but the impact of cold storage on graft function is well established, with increasing cold ischaemic times enhancing the risk of graft failure due to a loss of tissue viability (195).
Whilst cold storage remains the standard for kidney preservation, cellular metabolism is not completely abolished and mitochondrial function is retained, albeit at a slower turnover rate. Due to the lack of oxygen for ATP production, metabolism changes to anaerobic respiration leading to succinate accumulation, lactic acid production and a resultant intracellular acidosis (196). Ultimately, these changes lead to both necrotic and apoptotic cell death and the release of cell contents. Indeed, a loss of leukocyte viability is observed in the venous effluent; with subsequent significant concentrations of cell-free gDNA and mtDNA. This suggests that following a brief period (2 hours) of cold storage there is a significant loss of cellular integrity resulting in endogenous ligands immediately entering the recipient circulation upon revascularisation. In the context of transplantation, this will lead to the initiation of rejection via enhanced donor-recipient interactions. Removing this inflammatory burden prior to transplantation is therefore likely to reduce recipient leukocyte activation following implantation and revascularisation, thereby diminishing the incidence of AR.

In summary, the donor kidney contains an intrinsic leukocyte population, the characteristics of which strongly suggest it will drive a major inflammatory response following revascularisation. Performing a secondary flush immediately prior to transplantation reduces the inflammatory burden of the donor kidney and may represent a cost effective and clinically viable therapeutic intervention. This method clearly warrants further investigation to provide translational evidence of its clinical implications.
Section 7 – Discussion
7.1 Discussion

The success of modern transplantation is severely limited by a lack of donor organs available, coupled with the significant risk of rejection post-transplant. Whilst this is true for all solid-organ transplants, it is particularly problematic in the case of lung transplantation owing to their susceptibility to damage. It is estimated that only 25% of all lungs offered for transplantation are utilised due to concerns about their suitability and risk of primary graft dysfunction. To combat this, EVLP has been developed as an alternative method of preservation that enables an extended evaluation of lung function on an isolated circuit. Providing adequate oxygen and nutrition to the organ in isolation facilitates tissue regeneration as well as a more accurate portrayal of functionality. Early evidence suggests that this technique can be used to safely evaluate and recondition donor organs prior to transplantation, with patient outcomes comparable to standard transplantation. This has led to an increased interest in the use of normothermic preservation for the extended evaluation and potential reconditioning of other donor organs including hearts, kidneys and livers, prior to transplantation. In addition, as organs are revascularised in isolation with no confounding factors, such as the presence of the recipient’s immune system, ex vivo perfusion can be utilised as a research tool.

The initial aim of this study was to characterise the immune efflux from donor lungs following reperfusion using EVLP. Understanding the temporal migratory behaviour of donor immune cells is inherently important, as current therapeutics do not target these cells, despite their importance in the rejection cascade. This study demonstrates that donor lungs contain a large reservoir of immune cells that rapidly diapedese into the recipient circulation following revascularisation. Large numbers of these donor cells were detected within the EVLP circuit as early as 30 minutes post-reperfusion, suggesting that these cells are marginal and can rapidly migrate out of the tissue and into the vasculature. Owing to the well-defined role of passenger leukocytes in the initiation of rejection, the demonstration that these cells rapidly enter the recipient circulation is significant as this can impact the clinical course of the organ. For this reason, it was proposed that their removal prior to transplantation may impart benefit in the post-transplant setting. Indeed, when EVLP treated lungs were transplanted and the tissue assessed histologically, a significant reduction in T cell infiltrates around the small vessels compared to a standard lung transplant was observed. Given that all pigs in this study received no immunosuppression, it demonstrates that EVLP alone has the potential to alter
clinical outcome via a removal of passenger leukocytes. This not only highlights the importance of donor-derived cells but also provides a novel therapeutic target as current therapeutics only target the recipient immune system. Whilst limited by the short-term follow up in this study, the findings can be rapidly translated into humans given the establishment of clinical EVLP in many centres worldwide.

Interestingly, despite the large immune efflux during perfusion, no changes were observed in the immune cell content within the airways when comparing the pre and post EVLP BAL samples. This suggests that EVLP only impacts upon marginal immune cells with airway immunity remaining unaffected. Whilst this may reflect the clinical scenario, it could be reflective of the short perfusion period in this study, which was restricted to 3 hours. A longer perfusion may provide sufficient time for the airway immune compartment to be altered.

To determine if the effect of normothermic perfusion on organ cellular content occurred in other systems, a porcine model of kidney perfusion was established. Using this model extravasation of donor cells from the kidney into the perfusion circuit was observed. Given the early migration of cells from the donor kidney, it also suggests that these cells are marginal, with the potential to rapidly diapedese from the tissue and into the recipient circulation upon revascularisation. However, whilst the pattern of migration is similar to that observed during EVLP, the population demographics were vastly different. The donor kidney contains a large reservoir of T cells, particularly CD8+, as well as B cells and NK cells. T cells were also predominant in the donor lung, although in contrast to the kidney a major population of monocytes and granulocytes were also present. Other work by our group and collaborators demonstrates that the heart, liver and pancreas also have different immune profiles. This suggests that the immune compartment of an organ is unique and highlights that treatment strategies need to be tailored accordingly. Rather than solely targeting the recipient immune system, targeted immunotherapies should perhaps be organ specific. In this way both the donor and the recipient immune system can be modulated, thereby reducing inflammation post-transplantation and hence the incidence of AR.

The reason why each organ contains a different immune profile cannot be deduced from this study and this was not the primary aim, yet this is an interesting finding. This is likely to be reflective of the difference in organ functionality and environmental exposure, although this is only speculative at this stage. For example, the lung is constantly exposed to a milieu of
external antigens, yet only generates an immune response to potentially harmful agents, thereby maintaining pulmonary homeostasis to innocuous agents. For this reason they contain a large reservoir of immune cells that constantly patrol the tissue. It is well documented that the lungs are comprised of a large population of APCs, yet the relative contribution of the monocyte populations remains unclear (197). This study demonstrates that donor lungs contain a significant population of non-classical phenotype. Non-classical monocytes are directly involved in immune surveillance and inducing inflammatory responses (198). This may explain the large number of these cells present within the donor lung that rapidly migrate upon reperfusion. However, what is perhaps more surprising is the large population of T cells that reside within the kidney and rapidly mobilise from the tissue into the circulation. The early detection of these cells in the perfusate during EVNP, as well as during the secondary preservation flush, indicates that these cells are marginal. As memory T cells constantly scan host cell surfaces it is likely that the T cells observed in this study are of this phenotype (199). Although the importance of tissue-resident and memory T cells is emerging, it is not clear why the kidney contains a large reservoir of these cells (200). However, Ascon et al have reported a similar finding in mice kidneys (201). Further characterisation of these cells is clearly needed to determine their function and decipher the reason for such a large proportion within the kidney.

Whilst the post-transplant impact of removing a large number of passenger leukocytes during EVNP cannot be determined without performing a transplant, it is possible that post-transplant clinical outcomes will be improved. This will likely manifest as a reduction in DGF and incidence of rejection in the early post-operative period. Given the results described following transplantation with EVLP compared to standard transplant lungs, this is probable as the diapedesis of cells from the donor kidney was more pronounced than that observed from the lung. This is a surprising result owing to the fact that the lung contains a large immune compartment, although it should be noted that EVNP was performed for 6 hours compared to the 3 hours of EVLP. The inclusion of a leukocyte filter during EVLP aided in the removal of cells prior to transplantation, although the number of cells within the filter remained comparable to those identified within the perfusate in the early period. This suggests that the filters become saturated and therefore multiple filters may be needed for enhanced benefit, enabling the continual removal of passenger leukocytes rather than allowing them to re-enter the organ following activation. Filters were not included in the EVNP model described in this thesis to allow the temporal kinetics of cellular mobilisation to be determined.
in isolation. Additionally, they are not currently utilised in the clinical protocol, which this study aimed to replicate as closely as possible. Despite this, the addition of a leukocyte filter to the EVNP circuit is likely to impart additional benefit, particularly given the vast number of immune cells identified in the circuit as early as 60 minutes following reperfusion.

Interestingly, major populations of T cells were identified within the EVLP and EVNP perfusion circuits, suggesting that both the lung and kidney possess a large T cell repertoire that rapidly mobilise. Whilst these cells have capacity to drive inflammation and orchestrate alloantigen specific immune responses that lead to a loss of graft function, it is entirely possible that a proportion of these cells will be of a regulatory phenotype. Several mechanisms of tolerance induction post-transplantation have been described, which includes anergy and deletion of allospecific cells. However, the capacity of Tregs to induce and maintain immune unresponsiveness to alloantigen has recently been implicated as the major component of transplant tolerance (202, 203). Indeed, Tregs can prevent the incidence of graft versus host disease following allogenic bone-marrow transplantation (204, 205). Although this subset of T cells was not quantified within the perfusate, the proportion of Tregs within the secondary kidney flushes was very low. This suggests that the major population of T cells identified are inflammatory in nature and removing these cells prior to transplantation would be beneficial. Furthermore, given the predominant inflammatory environment described, Tregs are not likely to be prominent. Instead, the significant concentration of IFN-γ within both circuits is likely to preferentially polarise the T cell phenotype to an inflammatory Th1 response. Finally, the demonstration that removing the immune burden during perfusion confers benefit post-transplantation makes it unlikely that a large removal of Tregs is occurring.

The finding that both EVLP and EVNP induce leukocyte mobilisation out of donor organs suggests that ex vivo perfusion models can be utilised to manipulate the donor immune compartment prior to transplantation. Importantly, this process occurred in the absence of any drug or therapeutic agent and was therefore solely due to the perfusion technique itself. Furthermore, the impaired recipient T cell priming following EVLP transplantation in the absence of immunosuppression demonstrates that this is a novel and viable approach to tackling AR. Extrapolating this result to other organs, the need for immunosuppression post-transplantation could be significantly reduced for all solid-organ transplant recipients. Immunosuppression is a major contributor to many of the post-transplant complications,
including infection, nephrotoxicity and PTLD, therefore reducing their need is of critical importance. Attempts have already been made to use ex vivo perfusion to manipulate donor organs prior to transplantation and improve clinical outcomes via the delivery of immunomodulatory agents. In a model of EVLP, an adenoviral vector encoding IL-10 was incorporated into the circuit to accelerate cellular repair, with results demonstrating an improvement in lung function and a reduction in inflammation (146). However, the results of this study indicate that perfusion induces the secretion of a range of cytokines and chemokines, driving an inflammatory response. Due to the isolated nature of the circuits this is likely to be beneficial as this may be the driving force behind the donor mobilisation out of the tissue and into the perfusate. Importantly, this inflammatory environment had no adverse effect on tissue integrity or organ function in this study. In corroborating with this, removing inflammatory cytokines during EVLP via an absorbent membrane had no effect on lung function (206). As such, switching the circulating environment from inflammatory to anti-inflammatory may diminish cellular efflux out of donor organs, and therefore impact on the early benefit of a lower incidence of AR previously demonstrated. Rather, methods to enhance cell mobilisation out of the donor organ during perfusion are likely to improve clinical outcomes. This could be via chemical induction of cellular movement by chemotaxis or selectively inducing inflammation within the circuit to induce cellular efflux whilst retaining graft function. Combining this with other protective mechanisms during perfusion has the potential to change treatment strategies in clinical transplantation. Indeed, Brasile et al have demonstrated the feasibility of reducing AR in the absence of immunosuppression using a bioengineered interface consisting of a nano-barrier membrane to coat the vasculature during EVNP (207).

Whilst the inflammatory profile observed during perfusion may be responsible for the induction and maintenance of cellular diapedesis, it is possible that this may also saturate the inflammatory capacity of the donor cells. Immune exhaustion describes the reduced capacity of immune cells to respond in the continued presence of antigen. This has most extensively been described in patients with chronic infections and cancer patients where T cells progressively lose effector functions and up-regulate inhibitory receptors. In this instance immune cell exhaustion is associated with inefficient control of persisting infections and a failure to eradicate tumours. More recently this phenomenon has also been identified in B cells and NK cells in an analogous manner (208). In the context of transplantation, promoting immune cell exhaustion and hyporesponsiveness is likely to favour graft survival. In a
mismatched model of heart transplantation, CD4+ T cell exhaustion was achieved with a consequential prevention of chronic rejection. The closed nature of the perfusion circuits means that passenger leukocytes are continually exposed to both the plastic component of the model and any DAMPs released following reperfusion injury. This exaggerated immune activation is reflected in the high concentrations of cytokines and chemokines observed during both EVLP and EVNP. Indeed, levels of IFN-γ during EVLP significantly exceeded those typically detected in pigs with severe swine flu (209). In addition, significant levels of IL-6 were detected within the EVNP model, a cytokine that amplifies alloreactive CD4+ and CD8+ T cells. In the absence of IL-6, the incidence of post-transplant vascular injury is reduced, likely due to the significant reduction in T cell accumulation (210). Inducing cytokine release during perfusion therefore has the potential to reduce the capacity of these cells to respond following transplantation. This would not only reduce the inflammatory burden of the donor organ prior to transplantation, but would diminish recipient immune cell activation and recruitment into the graft. To confirm this further work would be needed to determine if the donor cells exhibit an exhaustion phenotype with functional alterations and a longer-term transplant performed. Despite this, these results provide novel pathways that can be explored for donor manipulation and tolerance induction prior to transplantation.

The long-term impact of removing passenger leukocytes remains unclear. Evidence in the literature suggests that mixed chimerism, the co-existence of haematopoietic cells from both donor and recipient origin, occurs in the post-transplant setting. Cells of donor origin have been reported in renal transplant recipients up to 30 years post-transplantation (211). The clinical outcome of patients with mixed chimerism remains unclear, although surprisingly the presence of chimerism has been reported to be successful at inducing long-term tolerance to the donor organ (183-185). Recent research has focused on methods of chimerism induction post-transplantation with the aim of modulating recipient immune responsiveness to transplanted organs. To achieve such tolerance, T cells of both recipient and donor origin need to be eliminated or inactivated which is typically achieved by irradiation, cytotoxic drugs or anti-T cell depleting antibodies (186-188). Whilst success has been described, rejection of donor organs has also been reported in chimeric animals suggesting that this method alone cannot prevent allore cognition (212). Combination techniques are clearly warranted to enhance the benefits of this therapeutic approach. Throughout this study a reduction of donor leukocytes is described during perfusion, including large populations of lymphocytes prior to transplantation, yet complete depletion does not occur. Removing donor-
reactive T cells prior to transplantation whilst still transferring smaller populations of donor leukocytes may have the same effect as T cell irradiation prior to transplantation. It is highly plausible that diminishing the early interactions between donor and recipient immune cells and reducing the inflammatory response that ensues will allow donor cell migration and repopulation post-transplantation. If true, removing a proportion of donor cells prior to transplantation may not only reduce AR but may confer longer-term benefit via chimera induction.

One of the major complications following transplantation remains the detrimental effect of the inevitable IRI that occurs. Major efforts have been made to reduce the deleterious events that occur following revascularisation but with little success. It is one of the leading causes of DGF and rejection episodes and is intrinsically linked to the length of cold storage. This is especially true for ECD and DCD donor organs as these organs are more susceptible to damage during cold storage. During this time, ROS production occurs in combination with potassium efflux leading to cytokine production and inflammasome activation. Following reperfusion these inflammatory mediators immediately enter the recipient’s circulation and prime recipient immune cells, orchestrating an immune response. Revascularisation of a donor organ on an isolated ex vivo circuit can initiate these events prior to transplantation. This is evident through the immediate detection of cytokines that increase over time, in combination with the increasing concentrations of cell-free mtDNA and gDNA. Due to the isolated nature of the ex vivo circuits, these mediators cannot induce systemic effects, as they would within the recipient following transplantation. Termed ‘pre-conditioning’ this may, in combination with the reduction in the passenger leukocyte burden, be responsible for the reduced incidence of rejection observed following transplantation with EVLP treated lungs. In a rat model of EVLP, oxygen free radical scavengers within the circuit improved lung function and reduced weight gain during the perfusion (213). Whilst this demonstrates the detrimental effects IRI can have on organ function, it also highlights further avenues for therapeutic intervention when organ perfusion is utilised. Given that the inflammatory responses described during perfusion do not impact on tissue integrity, likely due to the absence of the recipient immune system, it can be proposed that exposing organs to a period of EVNP should be routine practice prior to transplantation.

Whilst several reports have described the clinical benefit of EVNP compared to the current standard of cold storage, its utilisation in clinical practice remains low. This is particularly
true in the case of EVNP of donor kidneys. This is possibly due to the increased financial cost associated with the technique, given the need for hardware, consumables and technical training. That said, reducing the incidence of DGF and improving clinical outcome is likely to reduce the economic burden post-transplantation. Furthermore, given that organ perfusion has the potential to reduce the requirement for immunosuppression following transplantation, it may provide long-term benefit, as well as improve patient quality of life. Despite the perceived benefits, restoring cellular metabolism in an ex vivo environment carries additional risks including the potential introduction of infection or damage to the organ during cannulation and organ manipulation. This perhaps explains the reluctance to introduce this into routine clinical practice. However, with more time and further research, confidence in the technique is likely to grow and its use will increase.

This study demonstrates the importance of passenger leukocytes and describes for the first time the impact of removing these cells during ex vivo perfusion. Whilst this reduces the incidence of AR following transplantation, there are significant financial implications associated with this technique. The final aim of the study sought to determine if a secondary preservation flush of donor kidneys would also impact on the immune burden prior to transplantation. Indeed, flushing the vasculature, following storage on ice, demonstrated the significant inflammatory profile of donor organs at the point of implantation. Major populations of donor leukocytes were identified in the venous effluent, in combination with high concentrations of inflammatory mediators. Removing this inflammatory load prior to transplantation is likely to confer benefit via diminished rejection episodes and increased graft longevity. Given that current clinical practice is to immediately transplant an organ following storage on ice, this finding is significant. In addition, this method is cheap, with minimal impact on organ ischaemic times, and therefore has the potential to be rapidly introduced into clinical practice. Whilst the effect of a secondary preservation flush was only assessed in donor kidneys, given the rapid diapedesis of cells from donor lungs during EVLP, it is likely a similar effect would be observed in this organ. The results therefore further demonstrate the vast immunological burden of solid organs prior to transplantation. As such, combining a secondary preservation flush with normothermic perfusion has the potential to enhance immunodepletion of donor organs prior to transplantation and further improve the clinical benefits described.
If the results of this thesis confer long-term benefit, EVOP may become the gold standard for clinical transplantation, with all organs undergoing perfusion prior to transplantation. This thesis therefore not only highlights new pathways for therapeutic target but also has the potential to influence the way clinical transplantation is performed.

7.2 Limitations

Whilst the first part of this study offers insight into donor immune cell trafficking following transplantation, one of the major limitations is the short-term follow up of pigs post-transplant. Due to ethical constraints pigs were sacrificed at 24 hours meaning that the long-term outcomes following EVLP can only be predicted based on the current findings. However, as T cell recruitment into the donor lung is the hallmark of rejection, the reduction following EVLP in the absence of immunosuppression is still of clinical importance. It should also be noted that the inflammatory response observed during perfusion may in part be due to the artificial nature of the circuits leading to cellular activation. It is therefore impossible to determine if this would also occur in a standard transplant setting. However, this is likely to occur following revascularisation due to the inflammatory process of death, combined with the effects of organ retrieval that lead to cellular priming prior to transplantation. Aside from this, the data highlights the importance of passenger leukocytes following transplantation and provides a novel approach to tackling rejection.

The use of EVNP provides a platform to investigate the migration of passenger leukocytes from the donor kidney following revascularisation. The demonstration of donor trafficking from the kidney into the perfusate, combined with the large immune repertoire in the secondary preservation flush, highlights the significant immune burden of the donor organ. Despite this, the outcome of removing these cells cannot be determined without performing a transplant. For this reason, the results are limited only to a description of the events that occur during ex vivo perfusion of donor kidneys. Nevertheless, extrapolation of the results from the EVLP transplant model indicates that removing passenger leukocytes is likely to confer clinical benefit post-transplantation.
7.3 Future Work

1) EVNP Transplant Model

The reduced passenger leukocyte burden following EVNP has the potential to translate into improved clinical outcomes of transplant recipients, as demonstrated with EVLP. Given the profound efflux of donor cells during EVNP, which is more pronounced that that from lungs during EVLP, this is probable. However, the effect of removing these cells on the incidence of AR and post-transplant clinical outcomes is currently unknown. To determine if removing cells during EVNP improves kidney outcome, a transplant needs to be performed. To confirm clinical benefit, a control transplant following normal standard transplant procedures will also need to be performed. In a similar manner to the EVLP study, male donors and female recipients could be utilised to allow donor-derived cell transfer to be determined via the presence of the Y chromosome. Solid clinical endpoints would be determined, including the incidence of AR, the inflammatory profile, composite morbidity and overall mortality. Success would be clinically relevant improvements in these parameters.

2) Hypothermic perfusion

The large population of leukocytes identified within the secondary preservation flush described in section 6, in combination with the early detection of leukocytes during perfusion, indicates that these cells are marginal and highly mobile. Additionally, it is possible that these immune cells are simply mechanically removed during perfusion of an organ, meaning that this effect may also occur during hypothermic machine perfusion. It is currently unknown what effect storage at hypothermia has on immune cell mobility, although it is believed that a reduction of temperature reduces immune cell activation and movement. However, perfusion at hypothermia offers benefit over traditional SCS as the organ has a constant supply of oxygen which facilitates the reduced metabolism that occurs at these temperatures. This minimises the ischaemic damage to the organ, retaining tissue viability and reducing the IRI that ensues post-transplant. This is reflected in the reduced risk of DGF post-transplantation following hypothermic perfusion of kidneys compared to cold static preservation (214). If hypothermic perfusion is also capable of inducing leukocyte mobilisation prior to transplantation it may in fact be superior to normothermic perfusion. Perfusing an organ at normothermia creates a significant metabolic demand which can leave the organ susceptible to injury. Whilst removing passenger leukocytes during EVNP may reduce graft
immunogenicity, any tissue damage incurred will negate the potential benefit. The reduced metabolism that occurs at hypothermia offers the advantage of retaining tissue integrity, which if combined with a similar reduction in passenger leukocytes, has the potential to significantly improve clinical outcomes. This is an area that clearly warrants investigation.

3) Chemokine induction

Despite EVLP conferring post-transplant benefit, a significant number of passenger leukocytes will still be present within the donor tissue. This study has demonstrated that the perfusion did not impact on the airway of the lungs. This is perhaps not surprising given that perfusion to date is limited by the number of hours the organs can be kept viable on the circuits. Instead, methods to enhance diapedesis during this time frame should be investigated. In the first instance, chemokine induction should be trialed given their ability to induce the migration of cells down a chemotactic gradient. Chemotaxis to target particular cellular subsets, such as CCL2 to induce monocyte movement, could be achieved via the introduction of specific chemokines to the perfusate prior to organ attachment. Combining a range of chemokines has the potential to deplete the organ of a large number of leukocytes before transplantation. Following this treatment, the post-transplant benefits described in this thesis have the potential to be more pronounced and may provide longer-term benefit.

4) Portable perfusion

The current gold standard for organ preservation remains SCS, yet this limits the distance an organ can be transported prior to transplantation as increasing storage time is associated with an increased severity of IRI post-transplantation. Whilst ex vivo perfusion offers the potential to recondition organs prior to transplantation and ameliorate some of this damage, current practice only permits this to occur at the recipient hospital. For this reason, the organ is still exposed to a prolonged period of cold storage and the IRI that ensues can damage the tissue and increases the graft immunogenicity. Establishing a portable version of the current ex vivo circuits will allow the machine to be taken to the donor hospital where the organs can be immediately connected following the standard preservation flush. This provides the opportunity to dramatically reduce the cold ischaemic time of organs from several hours to minutes, thereby minimising the deleterious effects of IRI. Reducing tissue injury prior to transplantation not only impacts on graft function but will also reduce graft immunogenicity.
Combined with the perceived effects of organ perfusion described in this thesis, this has further potential to significantly change the clinical outcome of transplant recipients.
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