Preserving myocardium and regulating inflammation by *ex vivo* perfusion

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ATP – Adenosine triphosphate

ANOVA – Analysis of variance

Bcl-x – B cell lymphoma-x

CCHP – Cold cardioplegic heart perfusion

CCL – Chemokine C-C motif ligand

CD – Cluster of differentiation

CREB – Cyclic adenosine monophosphate response element binding protein

CXCL - CXC motif chemokine ligand

DBD – Donation after brain death

DCD – Donation after circulatory death

EDTA - Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FAK – Focal adhesion kinase

GADPH – Glyceraldehyde 3-phosphate dehydrogenase

HBSS – Hank's buffered salt solution

HSP – Heat shock protein

IFN-γ – Interferon gamma

IL - Interleukin

MFI – Mean fluorescent intensity

MHC – Major histocompatibility complex

NK – Natural killer

NYHA – New York Heart Association

PBS – Phosphate buffered saline

PD-L1 – Programmed death ligand 1

qPCR – Quantitative polymerase chain reaction

SLA – Swine leucocyte antigen

SMAC/Diablo – Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI

SPSS – Statistical package for the social sciences

STAT – Signal transducer and activator of transcription

TNF – Tumour necrosis factor

WNK1 – With no K (lysine) protein kinase 1

XIAP – X-linked inhibitor of apoptosis protein

Abstract:

Heart transplantation is a lifesaving therapy required by patients with end stage heart failure, yet there are a number of caveats to long term success. Primary amongst these is the immunological rejection of the donor graft by recipient leucocytes. The transfer of passenger leucocytes from the donor organ is important in stimulating this response, although the cardiac immune content is currently unknown. Despite the increase in demand for transplantation there is a shortfall in the number of suitable donor organs. This programme of work aimed to profile the cardiac immune reservoir and to establish whether novel *ex vivo* perfusion devices could be used for the preservation and evaluation of donor hearts. The effect of preservation and evaluation on the donor cardiac immune reservoir was then established. The findings presented herein provide novel evidence that the heart contains a significant immune reservoir capable of mobilisation upon reperfusion. Moreover, traditional static cold storage is associated with endothelial and myocardial injury, as well as broad inflammatory cytokine release. Such injury can be avoided for at least 8 hours by preserving the heart using hypothermic cardioplegic perfusion. Such preservation diminishes ischaemia-reperfusion related protein signalling, demonstrating a protective effect in the acute period. This method provides auxiliary benefits by mediating the significant immunodepletion of the donor organ, diminishing the population able to be transferred into the recipient. Importantly, hypothermic perfusion is also associated with an interferon gamma driven inflammatory storm, which promotes the upregulation of a protective immune checkpoint molecule. This may confer the ability to delete or anergise recipient T cells. Perfused organs that were subsequently transplanted initially displayed improved graft infiltration although this could not be confirmed in a larger study. Finally, this thesis demonstrates that donor hearts can undergo a thorough and sustained *ex vivo* functional evaluation over a four hour period without imparting myocardial injury. Surprisingly, this method of perfusion was not associated with large immune mobilisation from the heart, although it did stimulate a significant inflammatory response on the circuit. Overall, this work demonstrates that *ex vivo* perfusion can play a dual role in enhancing access to and improving outcomes of transplantation. This can be achieved by extended preservation, impaired direct allorecognition as a result of immunodepletion and improved evaluation of marginal organs.

Declaration:

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

Copyright statement:

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Chapter 1: Introduction

1.1 The historical context of heart transplantation

Heart transplantation currently represents the only viable treatment option for patients with end stage heart failure refractive to maximal pharmacological therapy. This complex procedure was the result of many decades of research, requiring significant advances in surgical technique to enable its utilisation. Of particular importance was the development by Alexis Carrel of techniques for vascular anastomosis in the early 1900s, allowing the successful connection of donor and recipient blood vessels, which was an essential step in paving the way for organ transplantation[1]. Despite this pivotal achievement (for which Carrel received the Nobel prize in Physiology or Medicine in 1912) occurring before 1910, heart transplantation would not become a clinical reality for decades. Pioneering research by Norman Shumway in the United States between the late 1950s and early 1960s in canines proved that the donor vasculature could be successfully connected to that of the recipient, and that the recipient circulation was sufficient to maintain donor organ viability over the subsequent days[2]. This research demonstrated that the transplant procedure was technically achievable, although recipient mortality was high. Whilst Shumway was central to the development of the technical expertise required for heart transplantation, and is often recognised as the father of heart transplantation for his contributions, he did not perform the first procedure in humans. In 1967, Christiaan Barnard performed the first successful human heart transplant in a patient in Cape Town using many of the techniques developed by Shumway but with modifications of his own devising[3]. Whilst the patient survived for only a couple of weeks after developing pneumonia as a complication of immunosuppression, the operation generated a media storm and over 100 transplants were performed worldwide in 1968[4]. However, mortality rates were very high due to immunological rejection of the donor organ, which could not be adequately controlled at this time and as a result the number of transplants performed annually dropped dramatically for a short period. In the UK, the first transplant was performed a matter of months after Barnard's pioneering operation, yet only six more would be performed over the next decade.

Another breakthrough would be required before the heart transplantation procedure could become the viable option that it is today. Between 1967 and 1980, immunosuppression was essentially restricted to the use of azathioprine, corticosteroids and antithymocyte globulin[5]. Although some patients survived for

extended periods of over 20 years, most did not and these drugs were either insufficient to control rejection or were associated with the development of lethal infections. The discovery of the immunosuppressive properties of cyclosporine A, a calcineurin inhibitor, in 1976 by Borel[6] revolutionised post-transplant care. Following trials in kidney and liver transplantation, cyclosporine was introduced for use in heart transplantation in the early 1980s. This significantly improved regulation of the recipient immune system, albeit with unavoidable side effects including renal toxicity. Further advances in immunosuppression have followed with the development of Tacrolimus, mycophenolate mofetil and monoclonal antibodies to cytokine receptors and immune checkpoint molecules, extending life expectancy of transplant recipients.

1.2 Modern heart transplantation

The prevalence of heart failure is high with approximately 900,000 sufferers in the UK alone[7] and up to 23 million people estimated to suffer worldwide[8]. As a result of this high disease burden, around 5000 heart transplant procedures are performed globally each year, including nearly 200 per year in the UK across six centres[9]. Heart failure severity is quantified using a number of scales, the most commonly used of which is the New York Heart Association (NYHA) classification, which ranges from I to IV[10]. Patients with NYHA class I have cardiac disease but display no limitation of physical activity whereas mild limitation in activity is observed with class II and marked limitation in activity is displayed with class III. Both class II and class III patients are comfortable at rest. The most severe patients (class IV) display heart failure symptoms at rest and are unable to perform any physical activity without discomfort. Patients receive transplants as a result of heart failure from a variety of disease aetiologies, although the most common indications are coronary artery disease and dilated or hypertrophic cardiomyopathies[11]. The variation in aetiology and severity as well as individual recipient circumstances means that there are differences in urgency level for transplantation. Indeed, this is reflected in UK centres having urgent and non-urgent transplant waiting lists, with prioritised organ allocation from around the UK rather than their local zone for the most critical patients. Whilst the characteristics of the recipient and their particular requirements are important in determining their eligibility, suitability and urgency for a transplant, many other factors must also be considered, particularly donor-specific variables.

Traditional guidelines for donor organ acceptability vary from country to country and between centres, yet most agree on a number of fundamental factors that may influence post-transplant outcomes, particularly mode of death and donor age restrictions[12]. Historically, almost all organs have been retrieved and transplanted from patients declared dead by neurological criteria (donation after brain death, DBD), as the donor remains on ventilator support and cardiopulmonary function is maintained. This ensures that the perfusion of the heart (and thus delivery of oxygen and nutrients to the muscle) does not cease and the heart can be retrieved in a controlled manner without warm ischaemia. As a result, excess trauma to the heart can be avoided and the functional parameters observed in the donor should theoretically be achievable in the recipient.

Although original guidance suggested that hearts should not be used from donors aged over 35 years[13], this was considered to be a rather conservative estimate and most centres now have an upper donor age limit of around 50-55. Transplants are less often performed with organs older than this owing to the fact that donor age is an established risk factor for poorer outcomes[13]. Further traditional criteria for donor organ acceptability include: close matching of organ size to that of the recipient[14], negative serology (such as HIV), absence of cardiac disease or trauma and appropriate donor cardiac function/haemodynamics.

Despite many improvements over the past few years, several limitations remain to successful transplantation, including a lack of suitable donor organs meaning that there are far more patients on the waiting list than there are organs available for use. Indeed, according to the latest figures published in 2016, only 16% of patients registered in the UK for a non-urgent transplant between April 2012 and March 2013 received an organ within 12 months of listing and only 20% had received a transplant by 3 years[9]. This has led to a high incidence of mortality on the transplant waiting list (10% and 14% at 1 and 3 years respectively for patients in the same cohort)[9]. As such, novel methods to increase the available pool of donor organs are required. One approach that has been utilised recently is the use of extended criteria donor organs, which encompasses organs from donors normally deemed to be unsuitable for transplantation. This may involve accepting hearts from donors with history of drug or long term alcohol abuse, donors aged >55 years[15, 16] or with diabetes mellitus[17].

Importantly, this may also include hearts donated after circulatory death (DCD), which are associated with an inevitable amount of cardiac injury. Whilst extended criteria donor organ use has improved access to transplantation, these marginal donor organs push the boundaries of organ acceptability and inevitably represent an increase in risk to the recipient. As such the utilisation of such organs must be balanced against the increasing risk of mortality on the waiting list. Further improvements in organ preservation are required to increase the donor pool and minimise waiting times, thereby diminishing waiting list mortality without exposing the recipient to increased risk.

1.3 Current organ preservation practice and cold ischaemia

Following declaration of brain death and consent to retrieve organs for transplantation, the retrieval team will perform a number of procedures to protect the donor heart prior to removal, storage and transport. Initially, a cold cardioplegic solution is perfused through the aorta into the coronary vessels, and in this manner is delivered to the entire organ[18]. A number of solutions are available for this purpose, which vary significantly in their composition and mechanism of action. However, all aim to cease cardiac contractility such that the metabolic requirement of the organ is reduced and provide some protection by maintaining appropriate electrolyte balance so that cell membrane integrity is not compromised. Once the heart has been fully flushed and contractions have ceased, the organ can be removed from the donor. From this point until revascularisation in the recipient, the organ receives no further oxygenation or active nutritional support. The isolated heart is then simply submerged in cardioplegia and insulated with the aim of maintaining the organ at approximately 4°C. This low temperature storage (known as static cold storage) further ensures that cell metabolism is diminished, with a 1.5-2 fold reduction for every 10°C drop in temperature[19]. This is essential to minimise the impact of ischaemia, an inevitable consequence of discontinuing oxygenation. Unfortunately, the isolated heart can only be maintained for a matter of hours with static cold storage due to the damage to the organ that accrues over time and cold ischaemic time has a well-established impact upon post-transplant outcomes[20]. Indeed, the shorter the cold ischaemia time, the lower the risk of immediate graft dysfunction. The median cold ischaemic time for donor hearts transplanted in 2015-2016 was 3.2 hours, highlighting the very limited time available for transplant surgeons to prepare the recipient and perform the procedure[9].

During storage, the myocardium utilises adenosine triphosphate (ATP) as usual (albeit at a slower rate at 4°C than at 37°C) to perform vital cellular processes such as ion transport through the cell membrane. This ATP pool would ordinarily be replenished through oxidative phosphorylation[21]. However, as the oxygen supply is limited during storage and is quickly depleted in the myocardium, aerobic metabolism is no longer possible and ATP stores are depleted. In order to ensure cell survival, the myocardium induces a metabolic switch to anaerobic metabolism, converting its glycogen stores to ATP, and releasing lactate as a by-product[22]. However, cardiac glycogen stores are also very limited and ATP production by this pathway is much less efficient. Furthermore, anaerobic metabolism drives a significant drop in intracellular pH due to the accumulation of protons that are not subsequently utilised in the synthesis of more ATP[23]. As a result, the Na^+/H^+ ion exchanger removes protons from the cell in order to buffer this acidification, although this leads to an increase in intracellular sodium ion concentration[24]. Importantly, due to ATP depletion there is a loss of ATPase activity, including that of the Na^+/K^+ ATPase pump meaning that this excess Na^+ is not removed from the cell. The accumulation of intracellular sodium ions therefore reduces Na^+/Ca^{2+} exchange, inhibiting Ca^{2+} efflux and thereby inducing significant calcium overload[25, 26]. Calcium overload has a well-documented role in the stimulation of reactive oxygen species release[27], as well as opening of the mitochondrial permeability transition pore[28], which ultimately induces mitochondrial swelling and cell death via necrosis. However, if mitochondrial permeability transition pore opening occurs only transiently, then there may be sufficient restoration of ATP production for the cell to be salvaged or for the more regulated apoptotic cell death pathway to be initiated.

Throughout the period from harvesting to implantation, the heart is exposed to a number of toxic metabolites, including reactive oxygen species, which are injurious to the organ via lipid peroxidation and protein oxidation[29]. Furthermore, there is likely to be significant cytokine release locally into the vasculature of the isolated organ, although this has not been studied in depth. If this is the case, then upon reperfusion these signals would initiate significant activation of recipient immune cells, which then induce severe inflammation and enhance graft infiltration. Avoidance of ischaemia is impossible with static cold storage, yet there is clear benefit to minimising the exposure time during which cytokines, chemokines and other inflammatory factors

could accumulate. It is noteworthy that the duration of ischaemia correlates with the extent of damage upon reperfusion and risk of primary graft dysfunction[30]. Consequently, minimising the time between harvest and implantation is an important step in improving the chance of a successful transplant outcome.

1.4 Perfusion as an alternative method of storage

Static cold storage is inherently limited by the inability to provide nutritional support or oxygenation to the organ, limiting the period for which the donor heart can be safely preserved. The development of devices to allow clinical *ex vivo* perfusion of donor organs has recently become a reality, particularly for kidneys[31], livers[32] and lungs[33, 34]. However, establishing a device to specifically maintain the heart *ex vivo* for clinical purposes has not reached the same point, despite the fact that *ex vivo* perfusion of hearts is not new.

1.5 The development of *ex vivo* heart perfusion

The first description of perfusion of an isolated heart was provided by Langendorff in 1895[35]. In this method, the heart is perfused in 'retrograde' fashion via the aorta, which is cannulated upon isolation. The delivery of perfusate in this manner induces the closure of the aortic valve due to the pressure of fluid movement. As such, all of the perfusate medium, which can be oxygenated and provided with an appropriate balance of electrolytes and nutrients, is thus directed to enter the coronary ostia at the aortic root, and flows down the left and right coronary arteries. The perfusate then flows through the entire vasculature of the heart, allowing all of the tissue to be oxygenated. Ultimately, the perfusate will then pass through the coronary veins and enter the right atrium via the coronary sinus and be pumped into the right ventricle, from which it is expelled out of the pulmonary artery. In the Langendorff perfusion model, assuming that the buffer being used as perfusate is appropriate and not cardioplegic, then the heart is able to maintain contractile function and should be able to attain sinus rhythm. However, the left ventricle should not technically receive any fluid unless there is an aortic valve insufficiency. It is therefore only performing minimal activity due to the lack of loading. As a result of this, the Langendorff perfusion model cannot strictly be considered a 'working heart' model, as only the right side of the heart is required to pump to eject any perfusate. Indeed, even the right side of the heart is only required to eject the coronary flow volume and does not receive the full volume that would

physiologically be returned via the vena cavae. A representative overview of the setup required for Langendorff perfusion is provided in figure 1.1. It is important to understand that there is significant variation depending upon the nature of the study and figure 1.1 only describes the essential components required for maintaining the heart *ex vivo* and not for performing functional evaluation.

Most Langendorff preparations utilise pressure restricted perfusion, which mimics the physiological coronary perfusion pressure and forces the aortic valve to remain closed throughout perfusion. *In vivo*, coronary perfusion occurs in response to the pressure gradient between the diastolic aortic pressure and the left ventricular end diastolic pressure. This can vary widely depending upon energetic requirements at any particular time but is generally set in Langendorff perfusion at 60mmHg to 100mmHg[36-38]. In some cases, constant pressure is not the optimal setup and rather it is more important to maintain constant coronary flow rates. In these experiments, it is possible to then monitor the perfusion pressure that is required to achieve the target coronary flow rate, and from this discern the vascular resistance[39].

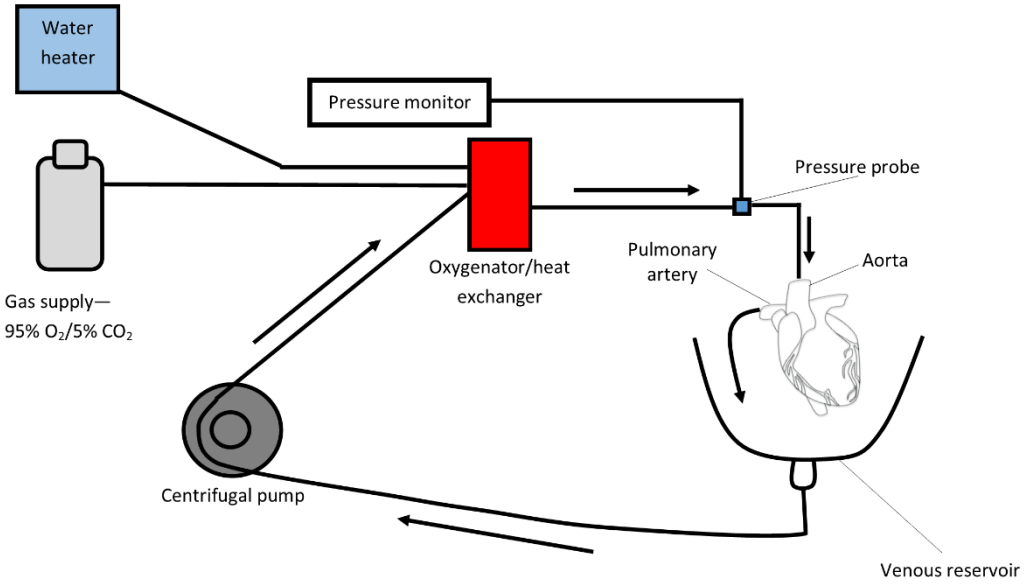

Figure 1.1 – An overview of the Langendorff model. Perfusate is drawn from the venous reservoir by the centrifugal pump and directed through the oxygenator/heat exchanger. The oxygenator receives 95% oxygen and 5% carbon dioxide from the gas cylinder and the perfusate is heated to 37°C by the water heater. The perfusate is then directed into the aortic cannula, immediately prior to which the pressure is assessed and adjusted to an appropriate level (60-100mmHg). The aortic valve is forced shut and the perfusate is directed through the coronary arteries, emerging into the right atrium, from where it is passed into the right ventricle and expelled back into the venous reservoir. Arrows represent the direction of flow of perfusate.

Using the original preparation, functional assessment was limited. Coronary flow rate could be estimated by measuring the output from the pulmonary artery, from where all perfusate that has passed through the vasculature is expelled. However, this provides little definitive information about the function of the heart and can simply demonstrate whether or not the heart is receiving sufficient perfusate delivery. In addition to this, Langendorff included a simple method of evaluating contractile force, which involved connecting thread from the apex of the heart to a mechanical recorder to establish the extent of movement along that axis. Over time, the model has been modified to incorporate further elements for evaluation of cardiac function. Commonly, contractile function can be assessed with the insertion of a fluid-filled balloon into the left ventricle, connected to a pressure transducer, which allows a measurement of isovolumic pressures[40].

Building upon the success of the Langendorff model, Neely *et al.* pioneered the development of a more physiological isolated heart model, which was capable of contractile work to eject the perfusate delivered to the left atrium[41]. In this model, the heart is initially perfused in a retrograde manner via the aorta as per the Langendorff method. However, this early period is required for only a matter of minutes and allows the heart to stabilise outside of the body whilst additional cannulation is being performed. When all preparation is completed, the delivery of perfusate is directed to fill the left atrium. The perfusate will then pass into and fill the left ventricle until it is ejected out of the aortic cannula (reversing the initial direction of flow) into the aortic pressure chamber. The pressure from the contraction increases the pressure in the chamber, driving perfusate into the aortic bubble trap. In this manner, the coronary vessels will be perfused as they would *in vivo* when the pressure gradient between aortic pressure and left ventricular end diastolic pressure is appropriate to close the aortic valve. An integral component of the setup that allows coronary flow is the aortic bubble trap, which acts as an afterload reservoir and must be placed at a height sufficient to induce backflow during diastole. In the Neely *et al.* setup[41], the bubble trap is 70cm above the heart, which should equate to approximately 70mmHg pressure exerted by gravity. This was demonstrated to induce coronary flow sufficient to maintain the viability of the heart. In this elegant model, the amount of work required to be performed by the heart is determined by the height of the preload reservoir, the 'atrial bubble trap'. By increasing the height of this reservoir, the left atrial filling

pressure is increased and the heart must work harder to eject this volume. In their initial experiments, Neely *et al* demonstrated that with an increase in left atrial filling pressure, there were associated increases in heart rate, coronary flow rate, cardiac output and oxygen consumption[41]. This was an important finding clarifying the mechanisms of normal cardiac physiology and introduced a crucial element in the development of *ex vivo* perfusion with functional assessment under a range of variable conditions. A representative overview of the modern setup for *ex vivo* 'working mode' perfusion is provided in figure 1.2, and illustrates those components required for maintaining the heart and allowing ejection of perfusate from the left ventricle.

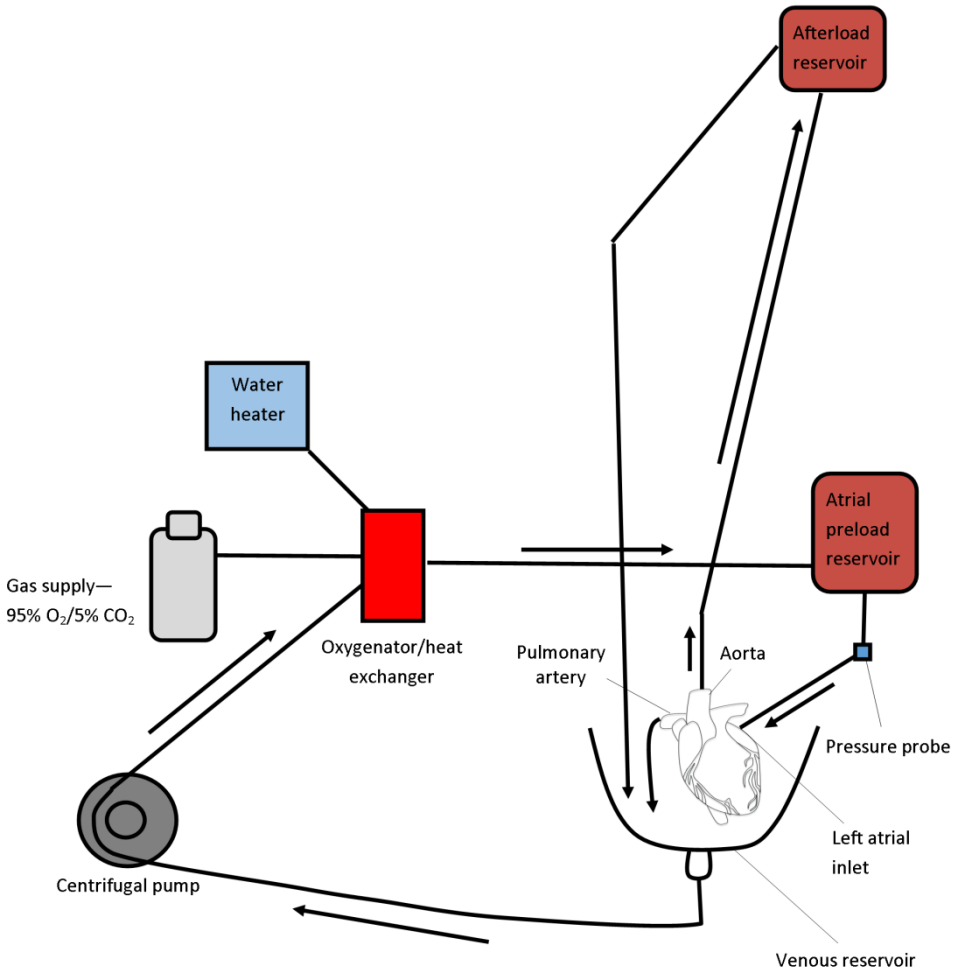

Figure 1.2 – An overview of the 'working mode' model. Perfusate is drawn from the venous reservoir by the centrifugal pump and directed through the oxygenator/heat exchanger. The oxygenator receives 95% O_2/5% CO_2 from the gas cylinder and the perfusate is heated to 37°C by the water heater. The perfusate is then directed into the left atrial preload reservoir, which is placed at a height to allow flow into the left atrium by gravity at a pressure of approximately 5mmHg. This pressure can be adjusted to increase the workload placed upon the heart in order for evaluation to take place. The perfusate will exit the left atrium into the left ventricle, from which it is ejected into the afterload reservoir. Perfusion of the coronary arteries occurs in a physiological manner due to this afterload pressure. The perfusate is returned back from the afterload reservoir to the venous reservoir via gravity. Afterload reservoir height can be adjusted to increase the pressure against which the left ventricle must eject to fully evaluate left ventricular function. Arrows represent the direction of flow of perfusate.

1.6 Hypothermic *ex vivo* heart perfusion for donor organ preservation

Whilst both the Langendorff and working mode models are useful for laboratory evaluation of cardiac physiology, they have mostly remained simply research tools rather than clinically relevant methods for organ preservation. However, early incorporation of a modified, hypothermic version of Langendorff's isolated perfusion procedure as a method of donor organ storage demonstrated significant benefit, with storage times of 24 and 48 hours achieved with strong functional performance post-transplant[42-44]. These encouraging results did not however translate into widespread incorporation of the technique into clinical practice although it did stimulate interest in the area, leading to greater evaluation of different perfusion methods and perfusate compositions.

Further research into extending donor heart preservation times evaluated the use of short term *ex vivo* perfusion following extended cold storage prior to transplantation. Findings by Ohtaki *et al.* demonstrated that coronary perfusion for 1 hour with University of Wisconsin solution at 4°C significantly improved high energy phosphate stores compared to organs cold stored for 12 hours without subsequent perfusion[45]. Moreover, following transplantation, those organs which had undergone 1 hour of hypothermic perfusion were able to produce significantly higher left ventricular pressures, indicating a greater functional capacity compared to those receiving only cold storage.

In agreement with these findings, Nickless *et al.* demonstrated that organs receiving hypothermic perfusion during storage were associated with improved recovery of function compared with those receiving static preservation[46]. Furthermore, these authors suggested that the implantation period is a critical phase, during which warming could account for a significant proportion of the ischaemic damage to the organ, although this could be minimised by hypothermic perfusion.

At this stage, it was becoming clear that there were benefits to hypothermic perfusion as a method of donor heart preservation, however the apparatus was considered too complicated for use in the clinical arena. As such, there was a drive to simplify the perfusion procedure in order to allow greater incorporation of the technique. Oshima *et al* demonstrated that coronary perfusion could be achieved without the requirement for sophisticated equipment, and that adequate delivery of perfusate was possible

simply by gravity[47]. This simple and elegant solution significantly simplified the perfusion procedure, maintained perfusion pressures within physiological range and was associated with significant improvements in post-transplant function compared with the standard control group.

More recently, hypothermic perfusion of rat hearts demonstrated important improvements in cardiac metabolic status during storage, associated with higher myocardial oxygen usage and enhanced graft function compared with static storage[48]. Importantly, ATP levels remained higher in the perfusion groups due to their capacity to continue to metabolise throughout preservation to replenish ATP stores as necessary. These findings are in keeping with an additional study that perfused canine hearts with a commercially available asanguineous perfusate solution[49]. These hearts were subsequently evaluated using a normothermic Langendorff perfusion system and it was demonstrated that continuous perfusion maintained significantly higher myocardial ATP stores, which were not depleted as with the static cold storage control hearts. Furthermore, intramyocardial pH was maintained without acidosis, which was present in control hearts, indicating a significant metabolic switch likely responsible for lactic acid production. Upon reperfusion with blood in Langendorff mode, all perfused hearts demonstrated greater functional recovery with less indication of cellular injury or apoptosis, albeit with some limited oedema after preservation, although this was rectified during normothermic perfusion.

1.7 Normothermic *ex vivo* heart perfusion for donor organ preservation

Rao *et al.* demonstrated in 1997 that the use of donor blood as perfusate was associated with significantly improved recovery of function post-transplant[50]. This study utilised only standard transfusion apparatus in order to minimise the equipment requirements that had been discouraging groups from using the method. Furthermore, this method represented a marked deviation from other *ex vivo* perfusion procedures as it did not control temperature, but instead involved the continuous perfusion of the organ with donor blood at room temperature. Despite the significant functional benefits observed with this technique, the storage period was only 4 hours and as such did not represent an improvement in graft preservation duration beyond that achievable with static cold storage.

Shortly after this study, however, Hassanein *et al.* published their findings with continuous machine perfusion using donor blood at normothermia, allowing the donor heart to be stored in a beating state during storage and transport[51]. This resulted in a reduction in myocardial oedema development and significantly improved left ventricular pressures compared to the static cold stored control group when both were assessed *ex vivo* in working mode. The authors suggested that these benefits were associated with the ability to continually provide nutrition and oxygenation to the organ, and as such remove the period of ischaemia normally associated with organ storage for transplantation.

More recent evaluation of beating heart perfusion for donor organ preservation in experimental animal models by a group in China has equally demonstrated success for 8 hours of storage at both mild hypothermia and at normothermia[52, 53]. Lin *et al.* utilised an *ex vivo* setup based around the Langendorff model comprising a roller pump that delivers blood as perfusate from a reservoir through a membrane oxygenator/heat exchanger, which ensured a constant perfusate temperature of 32-34°C[52]. The blood is then filtered and subsequently directed into the aorta at a rate of 60-80ml per minute. This preservation method was sufficient to maintain significantly greater ATP levels post-storage compared to static hypothermic storage and was associated with less myocardial injury assessed by electron microscopy. When transplanted, hearts stored in this manner displayed significantly higher left and right ventricular systolic pressures, as well as increased cardiac output, higher cardiac index and lower requirement for dobutamine in comparison to organs preserved by static cold storage. In their follow-up study of 8 hours of normothermic perfusion, Yang *et al.* utilised normothermic perfusion with autologous blood as the perfusate[53]. The perfusion setup was similar to that utilised for hypothermic perfusion, although the aortic cannula was modified to allow continuous perfusion throughout cardioplegia, preservation and during implantation, avoiding the requirement for topical cooling prior to transplantation. As with the previous study, the authors demonstrate that cardiac function is significantly improved at both 2 and 3.5 hours post-transplantation, with lower incidence of arrhythmias and higher cardiac output.

The aim of *ex vivo* perfusion of donor hearts is to increase access to transplantation by safely extending preservation times, yet it may also be possible to increase the donor

heart pool by utilising marginal donors, including DCD hearts. Recent pioneering work by Large *et al*[54] using normothermic regional perfusion has demonstrated the feasibility of utilising DCD hearts to significantly increase the number of transplants that can be performed. Their work utilises perfusion technology for the purposes of clinical evaluation, yet performs this *in cadavero* rather than *ex vivo*. This innovative approach allows the DCD heart to be reanimated and fully tested to determine its suitability for transplantation at the donor site. If deemed suitable, the heart is then transported using *ex vivo* normothermic perfusion to the recipient site. In many ways it is the work by this group that has fuelled the interest in *ex vivo* heart perfusion through their provision of clear evidence that DCD hearts can be utilised as a means to significantly increase the donor pool.

Previous studies have utilised *ex vivo* perfusion for the purpose of evaluation in experimental models with success. Suehiro *et al.* assessed the function of donor hearts after 60 minutes of warm ischaemia to determine whether they were capable of ejecting against 80mmHg afterload pressure with a left atrial preload pressure of 10mmHg[55]. Four of the 13 hearts in the study met this criteria whereas the remaining nine were unable to eject. All hearts were subsequently transplanted and the authors demonstrate that those predicted to be able to support circulation (4/13) were indeed capable. Hearts that did not meet the *ex vivo* assessment criteria all required dobutamine post-transplant and were otherwise unable to support the recipient circulation. This method could potentially be of significant value in predicting post-transplant function[56]. Similar conclusions about the utility of *ex vivo* perfusion for this purpose have been made in work by Colah *et al.,* who also demonstrated significant *ex vivo* functional capacity in hearts obtained following circulatory determination of death[57]. This work was advanced further by the same group, who suggested that the current strategy for *ex vivo* transport of hearts may actually impact function. This strategy involves delivering cold cardioplegia in the donor, followed by normothermic perfusion and then a second cold cardioplegic arrest prior to transplantation. To combat this, they developed a 'cardioprotective' *ex vivo* heart perfusion protocol, in which the heart receives 22°C cardioplegia and is then continually perfused throughout storage and transplantation[58]. This method led to reduced oedema, lower oxidative stress and provided significant improvements in immediate post-transplant graft function.

Clearly, the accumulated evidence from animal models of transplantation suggests that there are a number of benefits to storage with perfusion compared with static cold storage. However, the optimal method of perfusion remains uncertain, with some approaches still having unresolved problems, such as the development of oedema, albeit after a significantly greater period of preservation than achievable with traditional storage methods[59]. It remains unclear from current evidence whether normothermic perfusion provides any additional benefits over those observed with hypothermic perfusion outside of those cases with marginal donor organs requiring evaluation. Both techniques minimise ischaemia through the active delivery of oxygen and nutritional support. Hypothermic perfusion provides an additional level of protection for the heart by significantly reducing metabolic demand through cardioplegia and temperature reduction, both of which are avoided with normothermic perfusion. However, normothermic perfusion in a beating state does allow some (albeit crude) evaluation of cardiac function. This could aid in the decision of whether or not to utilise the organ, although it provides much less information than normothermic regional perfusion. Furthermore, it is unclear whether there is significant benefit to the use of an asanguineous perfusate compared to donor blood or a perfusate-blood mixture. Indeed, there remains no consensus regarding the best perfusate solution to use and a wide range have been used thus far.

1.8 Perfusate solutions for *ex vivo* heart perfusion

A number of different perfusate solutions have been utilised by groups investigating and developing *ex vivo* protocols for clinical use. University of Wisconsin solution has been utilised for the static cold preservation of a number of different organs for many years and has a composition that can be considered ‘intracellular-like’ due to its high potassium concentration. This makes it a useful solution for cardiac perfusion as the potassium ensures that cardioplegia is maintained. Whilst it was originally designed for static storage, it is by far the most utilised perfusate in the current literature for *ex vivo* heart preservation[44-48]. An alternative perfusate that has been readily utilised is Celsior[48, 60]. Celsior represents an alternative preservation strategy in that its composition is an ‘extracellular-like’ solution, with a much lower potassium content, albeit still cardioplegic. Direct comparison between these perfusates is scarce, however one study did evaluate their use[48]. Whilst perfusion with either solution was preferable to static preservation, the authors described improved performance with

Celsior over University of Wisconsin solution. Further evaluation of these solutions is required to definitively determine their suitability for use as perfusate, and potentially the development of other solutions specifically for the purpose of *ex vivo* heart perfusion may be warranted.

Of course, the cardioplegic nature of the solutions described above makes them unsuitable for *ex vivo* perfusion at normothermia if the heart is required to be beating in order to perform some form of functional evaluation of the organ. In each case to date where beating heart perfusion has been performed, autologous blood has been utilised as the perfusate[50-53]. The use of blood has both advantages and disadvantages. Firstly, erythrocytes are the ideal delivery method for oxygenation and although oxygenation of acellular perfusates will provide oxygen to the heart, the efficiency of this process is reduced in the absence of erythrocytes. Secondly, donor blood contains all the constituents of normal plasma, many of which are not accounted for in perfusate solutions, and thus can provide a wide range of beneficial metabolites and signals to the heart. However, this also means that the signals provided are not controllable by those performing the perfusion. Additionally, as blood is the only physiological solution that ordinarily perfuses through the heart, it has all the essential components required to maintain appropriate oncotic pressure and thus avoid oedema formation. However, whole blood will also contain leucocytes, which must be depleted prior to perfusion otherwise they will become activated within the circuit and release inflammatory factors. Whilst the potential impact of donor-derived leucocytes on the isolated organ is worthy of consideration, there are further roles for both donor and recipient leucocytes that are crucial in determining clinical outcomes post-transplant.

1.9 Allorecognition

Despite significant advances in donor-recipient matching and organ management, graft rejection remains a significant problem post-transplant and necessitates significant immunosuppressive therapy for the lifetime of the recipient. Graft rejection represents the process whereby recipient immune cells recognise the donor organ as ‘foreign’ and mount a specific response toward it. This response may take one of three forms: hyperacute, acute and chronic rejection. Hyperacute rejection occurs within hours or days of reperfusion and is the result of preformed antibodies to donor antigen, although this is a rare problem outside of xenotransplantation due to the use of pre-

operative panel-reactive antibody screening[61]. Acute rejection generally represents the development of an allospecific T cell-mediated response toward the graft and is most likely to occur early in the post-transplant period (weeks to months, but can be observed after years)[62]. Chronic rejection occurs late following transplantation, generally requiring years before being observed and culminates in intimal hyperplasia within the graft vasculature[63].

Graft rejection requires recognition of the donor organ by recipient leucocytes and this process can occur by 3 main pathways: direct, semi-direct and indirect allorecognition[64]. Direct allorecognition occurs when passenger leucocytes (donor leucocytes transplanted within the donor organ) migrate out of the organ. Immediately upon reperfusion, donor leucocytes will diapedese into the recipient circulation and enter the lymphatic organs[65]. Within the lymphatic sites, the donor leucocytes will directly self-present intact major histocompatibility complex (MHC) on their cell surface to recipient T cells. As this does not require any form of antigenic processing by the antigen presenting cell, this is a rapid pathway through which recipient alloimmunity is stimulated. This process will continue for as long as there remain donor leucocytes able to migrate to the recipient lymphoid organs.

The indirect pathway of allorecognition represents the more conventional route to immune activation, involving surveillance by the antigen presenting cells of the recipient. These recipient cells infiltrate into the donor organ upon revascularisation and acquire donor antigen. Following internalisation of donor antigen, it is processed and cleaved to small peptides, which are transported to the cell surface and presented in association with self MHC to naïve recipient T cells[66]. This indirect pathway directs the recipient T cell response to a smaller number of dominant determinants than that observed for direct presentation[67]. Semi-direct allorecognition remains the least studied of the 3 pathways and has much less direct evidence of a role in graft rejection following transplantation, but could have a significant impact. Semi-direct presentation requires the acquisition of donor peptide-associated MHC from donor leucocytes by recipient antigen presenting cells[68]. It has been postulated that this could occur by direct cell-cell transfer or by fusion of extracellular vesicles to recipient antigen presenting cells[69].

It is important to note that in the direct pathway of allorecognition, the magnitude of the recipient response is significant greater than that of the indirect pathway as there is a high frequency of recipient T cells able to respond to allo-MHC[70]. A much lower proportion of recipient T cells are activated in response to alloantigen in the context of self-MHC as presentation is more restricted to dominant antigenic epitopes. Importantly, this initial pathway of antigen delivery is largely ignored from a clinical perspective, as no current therapies target the donor immune repertoire, despite the important role it plays at this stage. The concept of an important role for passenger leucocytes in stimulating the alloresponse is not new, and was in fact proposed a decade prior to the first human heart transplant[71]. Indeed, more recently it has been demonstrated that antigen presentation in the context of donor MHC is sufficient to induce acute rejection of the transplanted heart without the requirement for T cell priming with recipient MHC and thus the direct pathway has a major role in initiating the alloresponse[72]. Furthermore, in other transplanted organs, the depletion of passenger dendritic cells is able to significantly reduce rejection, confirming that this role for tissue resident leucocytes within the transplanted organ has a clinically-relevant impact[73]. This effect is further verified by pioneering work by Lechler[74], in which the reintroduction of donor-derived dendritic cells into long-survived recipients restimulates the alloresponse, highlighting the potency of donor-derived compared to recipient-derived antigen presenting cells. More recently, a study of *ex vivo* lung perfusion demonstrated the importance of passenger leucocyte populations and the potential benefits of their removal[65].

Even though the major response to the graft is induced by the interaction between antigen presenting cells and recipient T cells, the inflammatory status of the tissue plays a major role and can significantly influence innate immune cells. The donor organ undergoes a number of sequential injurious events during transplantation as a result of donor death, cold ischaemia, surgical manipulation/dissection leading to trauma, and reperfusion injury. This induces the release of a number of damage associated factors, which can influence the local environment through the activation of innate immunity, the production of pro-inflammatory cytokines and chemokines, and the upregulation of adhesion molecules on endothelium stimulating further infiltration[75]. Indeed, following injury, neutrophils (of either donor or recipient origin) will become activated rapidly in response to cellular stress signals[76]. These cells are

subsequently able to degranulate to release a host of pro-inflammatory factors, and thereby mediate infiltration of the graft by recipient antigen presenting cells triggering the indirect allorecognition response. The direct allorecognition response, in which donor antigen presenting cells migrate into the recipient circulation, also requires the activation of these leucocytes. This process may similarly occur as a result of their detection of tissue injury. Alongside neutrophils, other innate cells are important in the early response to transplantation. Natural killer (NK) cells are a primary producer of both interferon(IFN)-γ and tumour necrosis factor (TNF)-α, which promote macrophage and dendritic cell activation[77]. Furthermore, stimulated NK cells release a number chemokines, such as chemokine C-C motif ligand (CCL)5, a known chemoattractant for T cells[78]. As such, this leucocyte is integrally involved in both the stimulation of antigen presentation and the induction of the adaptive alloimmune response through promotion of T cell infiltration.

1.10 Allospecific T cell priming

Alloantigen presentation to the recipient T cell via the T cell receptor represents the first signal in the process leading to graft rejection and provides the T cell with the specific target that it must attack. However, there are further signals that are required in order for rejection to occur and which represent important regulatory checkpoints to ensure that the response is tightly controlled and only those antigenic determinants that were presented appropriately are targeted. The second signal, known as costimulation, requires the interaction of cluster of differentiation (CD)80/CD86 from an antigen presenting cell with CD28 expressed on the surface of the T cell receiving the antigenic stimulus[79]. Importantly, in order for this costimulatory signal to be delivered, the antigen presenting cell must first be activated to induce expression of CD80 and CD86[80], which does not occur simply in response to the acquisition of antigen. Antigen presenting cells may be activated by a wide variety of stimuli, particularly those associated with tissue damage, known as damage associated molecular patterns, which include intracellular material not normally exposed to the extracellular environment, including ATP[81]. Further signals associated with stress or damage to the tissue can provide this activation signal to antigen presenting cells via their expression of toll-like receptors, which are innate pattern recognition receptors able to bind a variety of conserved determinants[82]. Once activated and matured to express CD80 and CD86, costimulation can be provided by antigen presenting cells to

recipient T cells, promoting their activation, proliferation and in some cells the development of immunological memory toward the alloantigen.

1.11 T cells as the target for current immunosuppression

Graft rejection remains a significant clinical problem, and a number of immunosuppressive drugs are utilised in an attempt to diminish graft infiltration and damage. Typically, patients receive a combination of a calcineurin inhibitor, cell-cycle inhibitor and steroids. Each drug has a different mechanism of action, but as a general rule, all tend to target recipient T cells to inhibit either their activation or their proliferation and clonal expansion.

Azathioprine is a cell-cycle inhibitor that has been utilised successfully for decades. However, it is now being phased out as more targeted therapies are introduced. Indeed, as of 2012, only 1.1% of new heart transplant recipients in the USA were treated with azathioprine[83]. Its replacement, mycophenolate mofetil, was used in 92% of new heart transplant recipients in the same period[83]. This drug also inhibits the proliferation of immune cells and has demonstrated greater efficacy than azathioprine in cardiac transplant recipients[84].

Cyclosporine represented the first of the class of calcineurin inhibitor drugs. Cyclosporine inhibits T cell clonal expansion by disrupting the activity of calcineurin, an enzyme essential for the production of pro-proliferative cytokines. Similar to the trends observed with azathioprine, cyclosporine use in the USA has significantly diminished over the last decade with 57.2% of new heart transplant recipients receiving cyclosporine in 2002, compared with only 6.0% of recipients in 2012[83]. Tacrolimus, a newer calcineurin inhibitor acting with a similar mechanism, now represents the dominant therapy, with 88.6% of new heart transplant recipients receiving Tacrolimus in 2012[83].

In addition to the calcineurin inhibitors and antimetabolite treatments, most heart transplant recipients will receive corticosteroids such as prednisolone as induction and/or maintenance therapy and will receive addition doses if rejection is suspected. Generally, corticosteroids affect the nuclear factor kappa-light-chain-enhancer of activated B cells[85] and activator protein 1[86] transcription factors, causing significant disruption to the production and secretion of a number of inflammatory cytokines

(including interleukin(IL)-2) from a wide range of immunological cells and is thus a broad acting therapy.

It is clear from the immunosuppressive therapies described above that the main focus of current treatments is the recipient immune system. Whilst this is logical given that these are the cells that ultimately are responsible for effecting the destruction of the donor organ, it is not an ideal solution. Firstly, the non-specific nature of these treatments (in that they alter the entire immune population, not simply those cells that are allospecific) ensures that the recipient is at an increased risk of complications such as infection and malignancy, which cannot be controlled as readily by the compromised immune system. Secondly, most therapies target the leucocyte following antigenic stimulation and the delivery of costimulation and aim to halt the release of cytokines and growth factors that promote proliferation. Whilst this is effective, it does also mean that tolerance to the graft cannot be induced. T cells become tolerant or anergic when they receive an antigenic stimulus in the absence of costimulation. Anergy can also be induced when co-inhibitory signals are provided by the antigen presenting cell.

Clearly, alternative strategies to modulate the immune response of the recipient are warranted and perhaps broadening the approach to include altering the donor passenger leucocyte pool could be appropriate. However, until this point, modulation of the donor immune reservoir has been impossible for ethical reasons as only the minimum manipulation of the donor required for retrieval is allowed *in situ*. The development of *ex vivo* perfusion devices that allow the isolated organ to be maintained and transported in optimal conditions with nutritional support and oxygenation may also allow manipulation of the organ prior to transplantation. Donor organs contain a significant repertoire of passenger leucocytes that are likely to migrate to recipient lymphoid organs upon revascularisation and stimulate the alloresponse via direct allorecognition. However, the immune system of each organ varies significantly, and it remains unclear whether the heart contains such a population of leucocytes able to rapidly migrate.

1.12 The cardiac immune repertoire

All organs have resident immune cells present with the purpose of surveillance for infection, malignant cells and tissue injury requiring repair. There has been significant

interest in the role of immune cells following injury within the heart, however this has mostly related to the impact of circulating cells, including monocytes, which traffic into the organ upon sensing damage[87, 88].

Whilst there remains very little clarity regarding the map of immune cells present in the heart under normal conditions, there are indications that these cells exist, albeit perhaps not to the extent of some other organs such as the lungs or kidney that are more readily exposed to pathogens. Early findings by Dvorak *et al*. identified a population of mast cells within the human heart[89], later confirmed to contain significant amounts of TNF-α and histamine, which could be readily degranulated upon ischaemia[90]. Whilst this work related to ischaemia reperfusion injury in the context of infarction rather than transplantation, it is likely that a similar process of mast cell degranulation would occur following donor organ retrieval, introducing a highly inflammatory state within the organ.

Further evidence of a large antigen presenting population was provided by Pinto *et al.*, who describe a sizable proportion of macrophages within the murine heart during homeostasis[91]. These cells have a number of important roles within the tissue including the release of inflammatory cytokines, causing widespread activation and are capable of migrating and thus self-presenting antigen to T cells in the context of donor MHC. Additionally, populations of dendritic cells, thought to be the most efficient cell type for antigen presentation, have been identified within the aorta and valve regions, from which they can sample antigen[92]. Characterisation of these cells demonstrated their ability to present antigen as capably as splenic dendritic cells and could thus be considered highly immunogenic. Transfer of these cells would undoubtedly result in direct allorecognition. Recent evidence suggests that donor-derived dendritic cells can initiate the alloresponse without specifically self-presenting antigen, but instead by transferring the MHC-antigen complex to a recipient dendritic cell, using the semi-direct pathway of allorecognition[93]. This highlights an additional mode of action and reason for which targeting these cells could be clinically relevant in the prevention of acute rejection.

Most recently, a study of murine cardiac ischaemic injury was performed to assess changes in enzyme expression in resident immune cells. Under normal conditions, the murine heart was suggested to contain approximately $2.3x10^3$ leucocytes per mg of

tissue[94], of which the majority were considered to be antigen presenting in nature. Indeed, the authors demonstrate that antigen presenting cells comprise approximately 73% of the total leucocyte population present in the heart under resting conditions. Additional large populations of B cells and monocytes were also observed but other populations such as granulocytes, NK cells and T cells were relatively scarce. This study highlights the importance therefore of the donor heart as an immunogenic stimulus carrying a significant number leucocytes capable of self-presenting antigen to recipient T cells.

A direct evaluation of passenger leucocyte trafficking from the donor heart has been performed recently using bioluminescence imaging[95]. These authors demonstrate small numbers of CD5+ passenger leucocytes that cannot be visualised by bioluminescence within the donor organ. However, immediately upon revascularisation these cells migrate into the recipient and are easily detectable within as little as 30 minutes. Interestingly, these cells proliferate upon diapedesis and their numbers increase significantly up until approximately 8 hours. This important finding suggests that despite the low initial numbers of passenger leucocytes likely to be resident within the heart, they are able to multiply and potentiate their effect within the recipient.

Whilst the majority of this evidence relates to murine heart biology, this reflects the difficulty in obtaining appropriate human samples, which generally will have some pathology altering the natural resident populations and thus affecting interpretation of the findings. Furthermore, it is important to be aware that the donor heart prior to transplantation will have an altered immune content, particularly following brain death. Brain death is associated with significant inflammation and mobilisation of leucocytes into the circulation and peripheral organs, through a number of mechanisms including complement activation and upregulation of adhesion molecules on the graft[96]. This results in significantly increased levels of myocardial leucocyte infiltration prior to transplant, and their transfer into the recipient could be of clinical relevance.

There are only a limited number of studies that have attempted to profile the resting leucocyte repertoire of the heart. However, there is sufficient evidence to suggest that there resides a significant enough population to contribute to recipient T cell priming.

Further work is clearly necessary to fully evaluate the leucocyte content of cardiac tissue and to phenotype their behaviour in the context of ischaemia-reperfusion and downstream impact following transplantation.

1.13 Rationale, aims and objectives:

The incidence of graft rejection is a major impediment to long term success following heart transplantation. This process can be stimulated through the presentation of antigen by donor leucocytes transferred within the organ. However, there remains a poor understanding of the immune content of the donor heart and their capacity for migration upon revascularisation. Until recently, this has been difficult to establish due to the inability to study the isolated heart in a physiologically relevant context without altering viability or function. *Ex vivo* perfusion technology has now provided unprecedented access to study the organ in detail without confounders from the recipient and over a period that is relevant to transplantation. Importantly, both normothermic evaluation models and hypothermic preservation models have become available as pre-clinical research platforms and could differentially alter the donor organ. By providing continuous flow through the vasculature, these devices provide useful *ex vivo* models to recreate the early period post-transplant and as such it is possible to characterise the donor immune pool and their response to reperfusion in detail. Finally, the use of an *ex vivo* perfusion model in combination with in-line leucocyte filters may facilitate the depletion of the organ prior to transplant and as such ameliorate the impact of their transfer on the induction of acute rejection through the direct allorecognition pathway.

1.14 Hypothesis:

The donor heart will contain a significant immunological reservoir, which could be transferred into the recipient upon transplantation. *Ex vivo* perfusion of the organ in both normothermic and hypothermic settings will promote leucocyte migration into the circuit, and thus diminish the donor immune pool at the point of transplantation. The immunodepleted heart will induce less stimulation of the recipient through the direct allorecognition pathway and lower graft infiltration.

1.15 Aims:

This programme of work was designed to investigate the following objectives:

1) Characterise the immune populations resident within the healthy porcine heart
2) Characterise the exudate from a secondary cardioplegic flush following static cold storage
3) Establish the migratory characteristics of resident cardiac leucocytes upon reperfusion
4) Determine the effect of hypothermic perfusion on both the viability and the immune content of the donor organ
5) Determine whether donor heart immunomodulation alters the level of graft infiltration following heterotopic transplantation
6) Investigate whether an *ex vivo* normothermic four-chamber beating heart model could be used to perform an in-depth evaluation of cardiac function

Chapter 2: General methodology:

2.0 Methods statement:

Methods performed across multiple experiments and therefore required in more than one chapter are described here to avoid unnecessary repetition. Where modifications to these general protocols were required, further details are provided in the specific chapter describing that experiment.

2.1 Porcine experimental models:

2.1.1 Ethical approval:

All porcine experiments performed as part of this programme of work were approved by the local Ethics Committee for Experimental Research. The animals were treated in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (Eight Edition, revised 2011) and the directive "On the protection of animals used for scientific purposes" of The European Parliament and of the Council (Directive 2010/63/EU).

2.1.2 Donor organ retrieval:

For all porcine models, anaesthesia was induced in each pig via the intramuscular delivery of ketamine hydrochloride (25mg/kg; Pfizer, Sweden) and xylasin (4mg/kg; Bayer, Sweden) and ventilation was performed with a Servo ventilator 300 (Siemens, Stockholm) throughout the donor heart retrieval process. Following appropriate anaesthesia, each pig received systemic heparin, and a median sternotomy was performed. The ascending aorta was exposed and cannulated with a cardioplegic cannula. The superior and inferior vena cavae were clamped until the heart was emptied, at which point the distal ascending aorta was cross-clamped. A total volume of 600ml cardioplegia (the type of which differed between experiments) was flushed through the heart via antegrade coronary perfusion at a fixed 60mmHg perfusion pressure until cardioplegia was accomplished. The left and right atrial appendages were cut and a the left ventricle vented to ensure that the heart remained decompressed throughout. Once the flush was completed, the donor heart was excised and preserved as required for the study.

2.1.3 Cold cardioplegic heart preservation device:

All experiments involving cold cardioplegic heart perfusion utilised a single preservation device. The heart preservation system consists of an insulated box with a large capacity perfusate reservoir. Perfusate is drawn from the reservoir by a pressure

and flow-regulated roller pump, which directs the fluid through a membrane gas-exchanger, heater-cooler unit and leucocyte filter. The device is fully automated and can be run at fixed pressure or flow at a range of temperatures and is programmable to run continuously or intermittently depending on the nature of the experiment.

2.1.4 Preparation of the heart preservation system and donor heart perfusion:

The perfusate reservoir was filled with 2.5L of high albumin, hyperoncotic cardioplegic perfusate medium produced in-house. The perfusate composition has been published previously[97] and is displayed in table 2.1. Autologous whole blood was washed using a Medtronic Autolog cellsaver (Medtronic Inc, Minneapolis, USA) and leucocyte filtered using a Pall Leucocyte filter (Pall Corp., Port Washington, USA) to acquire packed erythrocytes, which were added to the circuit to a haematocrit of 15%. The perfusate was circulated at a temperature of 8°C. Once all components of the perfusate were combined in the system and the temperature reached 8°C, the donor heart was connected to the device. After careful de-airing, perfusion was commenced with the pressure set to 20mmHg. The heart was perfused continuously for 8 hours at 8°C. The vena cavae and pulmonary artery were left open to allow the perfusate to be returned to the reservoir from the coronary sinus. Throughout perfusion, the donor heart remained submerged within the perfusate reservoir and received a minimum of 100ml/min perfusate.

Na^+	136 mmol/L
K^+	23 mmol/L
Ca^{2+}	1.3 mmol/L
Mg^{2+}	8.0 mmol/L
Cl^-	142 mmol/L
HCO_3^-	25 mmol/L
PO_4^{2-}	1.3 mmol/L
d-Glucose	6.3 mmol/L
Albumin	7.5%
Cocaine	6 pmol/L
Noradrenaline	6 pmol/L
Adrenaline	6 pmol/L
T3	3 pmol/L
T4	2 pmol/L
Cortisol	420 pmol/L
Insulin	8 U/L
Imipenem	20 mg/L

Table 2.1 – Composition of cardioplegic perfusate.

The table displays the composition of the high albumin cardioplegic solution used for all hypothermic perfusion experiments. Adapted with permission from previous publications by Steen et al.[97, 98].

2.1.5 Heterotopic transplantation:

All recipient pigs received anaesthesia as above via intramuscular delivery of ketamine and xylasin. Recipient pigs were ventilated with a Servo ventilator 300 throughout the procedure. Once anaesthetised, a longitudinal incision was made to the left of the linea alba, and the viscera were displaced to allow visualisation of the infrarenal aorta and caval vein. At implantation, the aorta of the preserved heart was sutured end-to-side to the infrarenal aorta and the pulmonary artery was connected end-to-side to the vena cava. Reperfusion of the donor heart was commenced at the earliest opportunity and the flow was allowed to stabilise for approximately 20 minutes before the hearts were defibrillated if sinus rhythm was not spontaneously established. Once the donor heart had achieved sinus rhythm, the incision was sutured closed and the pig was awakened. Fluid support was provided to all recipient pigs by infusion of Ringer's solution, saline, 20% albumin, 5% glucose and 10% dextran as necessary over the early period post-transplant. All recipient pigs were maintained in their pens for 48 hours without immunosuppression until sacrifice.

2.2 Analytical methods:

2.2.1 Flow cytometry:

Flow cytometry was performed as a core component of each study. The protocol significantly differed only between the porcine and human experiments, although there was some variation between studies with regard to the specific antibodies utilised. Specific modifications are detailed in the relevant chapters.

For porcine experiments, the cells to be analysed were stained with LIVE/DEAD fixable violet dye (Thermo Fisher, Massachusetts, USA) and incubated in the dark for 30 minutes. Each sample was then washed with the addition of 1ml staining buffer (consisting of phosphate buffered saline (PBS) supplemented with 2% foetal bovine serum) followed by centrifugation at 500g for 5 minutes. This was then repeated before primary antibodies were added. The stained cells were then incubated for 15 minutes in the dark at room temperature. The cells were washed a further two times with PBS and secondary antibodies added where necessary before incubation for a further 20 minutes at 4°C. A final two wash steps were performed with PBS before the cells were resuspended in 1.2ml staining buffer. A total of 1ml cells were acquired at a rate of 1ml/minute by an Attune 1st generation flow cytometer (Thermo Fisher,

Massachusetts, USA). All gating strategies and absolute cell counts were determined using Attune Cytometric software.

For human experiments, cells were stained with Zombie UV fixable viability dye (BioLegend, London, UK) and incubated for 30 minutes in the dark. The cells were subsequently washed with 2ml of staining buffer and centrifuged twice at 500g for 5 minutes. The cell pellet was resuspended in 100µl of staining buffer and primary antibodies added to detect cell surface antigens. The cells were incubated with these antibodies for 10 minutes in the dark before being washed and centrifuged twice at 500g for 5 minutes. The cells were finally resuspended in 500µl staining buffer and analysis performed using a BD LSRII flow cytometer (Becton Dickinson, Oxford, UK). Gating strategies and mean fluorescence intensities were obtained using FlowJo software version 10 (FlowJo, Oregon, USA).

2.2.2 Quantitative polymerase chain reaction (qPCR):

For all porcine experiments, it was important to quantify the level of cell-free DNA released into either perfusate or plasma. The same protocol was utilised for all experiments. All primers were designed using Primer Express® Software v3.0.1 (LifeTech, Paisley, UK) and their homology assessed using BLAST. Primers designed for the detection of mitochondrial DNA (cytochrome b) and genomic DNA (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) were obtained from Sigma Aldrich (Sigma Aldrich, Dorset, UK). The following primers were used:

GAPDH forward: 5’ TGCTCCTCCCCGTTCGA 3’

GAPDH reverse: 5’ GGCTTTACCTGGCAATGCA 3’

Cytochrome b forward: 5’ ACACATCAGACACAACAACA 3’

Cytochrome b reverse: 5’ GTAGCGAATAACTCATCCGTAA 3’

GAPDH and cytochrome b primers were resuspended and adjusted to 150nM and 50nM respectively using nuclease-free water (Ambion, USA). All qPCR analysis was performed using a QuantStudio 12K Flex system using a Power SYBR green PCR master mix (LifeTech, Paisley, UK).

2.2.3 Multiplex cytokine evaluation:

Inflammatory profiling of perfusate or plasma was performed using the same protocol and the same assay across a number of experiments in this programme of work. A total of 13 cytokines were quantified in the required samples using a porcine 13-plex Luminex assay (Merck Millipore, Billerica, MA, USA). The concentrations of 12 cytokines (granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-10, IL-12, IL-18 and TNF-α) and 1 chemokine (CXC motif chemokine ligand (CXCL)8) could be detected. To perform the assay, the Luminex plate was blocked with assay buffer provided as a component of the kit prior to the addition of sample. Once blocked, undiluted sample was added to each well. Subsequently, premixed beads attached to antibodies specific for the 13 cytokines under analysis were added to the plate before it was sealed and incubated for 16 hours at 4°C. The plate was washed three times followed by addition of detection antibodies and the plate was incubated for two hours at room temperature. Streptavidin-phycoerythrin was next added to each well and the plate was again incubated for 30 minutes at room temperature. A final three washes were performed before the beads were resuspended in sheath fluid to allow analysis by a Bio-Plex 200 system (Bio-Rad, Herts, UK).

2.2.4 Troponin I detection:

Cardiac troponin I was quantified using a commercially available porcine enzyme-linked immunosorbent assay (ELISA) kit (Abbexa, Cambridge, UK). The ELISA plate was twice rinsed with wash buffer supplied with the kit to ensure specificity of the binding. Undiluted samples were then added to the plate, which was incubated at 37°C for 90 minutes before the contents were discarded. Biotin-conjugated detection antibody was then added and the plate was sealed and incubated at 37°C for a further 60 minutes. A series of three washes was performed before horseradish peroxidase streptavidin conjugate solution was added. The plate was then incubated for 30 minutes at 37°C. The plate was washed five times before tetramethylbenzidine substrate reagent was added and the plate incubated at 37°C for 15 minutes. The reaction was halted with the addition of stop solution and absorbance was read at 450nm using a Tecan infinite 200 PRO system (Tecan Group, Männedorf).

2.2.5 Proteomic evaluation of phosphokinase and apoptosis signalling:

Homology between porcine and human proteins was assessed by protein BLAST alignment to ensure that the human assays would cross-react with porcine antigens. Proteomic changes between biopsy samples were compared using human phosphokinase and apoptosis antibody proteome profiler arrays (R&D systems, Abingdon, UK). For apoptosis detection, 30mg snap frozen tissue was homogenised in lysis buffer supplemented with 1% HALT protease inhibitor cocktail (ThermoFisher, Waltham, Massachusetts, US). For phosphokinase assessment, 40mg snap frozen left ventricular tissue was homogenised in lysis buffer supplied with the kit. For both assays, the cells were homogenised using a TissueLyser II instrument (Qiagen, Germantown, MD, USA). Protein concentration was quantitated by bicinchoninic acid assay (Sigma Aldrich, Dorset, UK) and 400µg added to each apoptosis membrane and 600µg added to each phosphokinase membrane. A separate membrane was utilised for each sample to provide the greatest power for analysis and a more in-depth appreciation of the events occurring in each individual organ. The membranes containing the samples were incubated overnight at 4°C before a series of three 10 minute washes was performed. Detection antibody cocktails were then added to each membrane before incubation for either 1 hour (apoptosis array) or 2 hours (phosphokinase array). The series of three washes was repeated before streptavidin-horseradish peroxidase was added to each membrane. Each membrane was incubated at room temperature for 30 minutes to allow binding of the streptavidin to the biotinylated detection antibody. A final three washes were performed before chemiluminescence reagent was added and incubated for 1 minute. A ChemiDoc MP imaging system was utilised for chemiluminescence detection (Bio-Rad, Herts, UK). Pixel density analysis was performed using ImageJ (NIH, USA). Further processing was required for some experiments and modifications are described in detail in their respective chapters.

2.2.6 Histological processing:

Samples for histological evaluation were immediately stored in formalin and later embedded in paraffin. Sections were cut at 4µm, de-paraffinised and stained with haematoxylin and eosin. All samples were prepared and assessed in a blinded manner by a consultant histopathologist. The nature of the assessment differed between studies.

2.2.7 Statistical analysis:

A combination of the statistical package for the social sciences (SPSS) version 22.0 (IBM, NY, USA) and Prism 7 (GraphPad, CA, USA) were used for statistical analysis. Normality of the data was always determined formally with the used of the Shapiro-Wilk statistic. Descriptive data are expressed as mean ± standard deviation if normally distributed or as median [interquartile range] if non-normally distributed. The specific tests used for analyses varied between experiments and are described in greater detail in each subsequent chapter. For all experiments, statistical significance was accepted when $p \leq 0.05$. Graphs were always produced using Prism 7.

Chapter 3: Donor hearts preserved by static cold storage are associated with endothelial denudation, inflammation and rapid leucocyte mobilisation upon revascularisation

3.1 Introduction:

Transplantation is associated with a number of adverse immunological sequelae within the recipient. Recipient immune activation occurs in response to recognition of the donor organ as 'non-self', due to mismatches in proteins expressed on the surface of tissues[62, 96]. Once activated, recipient allospecific T cells infiltrate the donor graft causing tissue damage[99], which can lead to graft dysfunction and rejection. Recipient immunosuppression aims to prevent graft loss but the side effect profile of commonly administered medication imparts a degree of toxicity to the recipient[100].

It is broadly recognised that recipient T cell activation occurs via both indirect and direct presentation. Recipient antigen presenting cells can traffic into the donor organ and acquire donor-peptides for later 'indirect' presentation to recipient T cells[101]. Alternatively, antigen presenting cells from the donor graft can present donor peptide antigens via 'direct' presentation to activate recipient T cells. The importance of direct allorecognition in the rejection of cardiac allografts has been described previously[72]. This elegant study utilised an adoptive transfer method, which confirmed a primary role for donor MHC II expression in acute rejection and demonstrated that graft infiltration could occur in the absence of recipient MHC II bearing antigen presenting cells. Therefore, graft-expressed MHC II molecules were sufficient to activate recipient T cells, independent of indirect presentation. However, these findings only considered the presentation of donor antigen in the context of MHC II by the graft but did not account for or establish the isolate for the presence and contribution of passenger leucocytes.

Passenger leucocytes are ordinarily resident in the donor organ and are transferred within the graft at the time of transplantation[102]. By their very nature, these immune cells continually express MHC class II and other proteins recognised by the recipient as 'non-self'. Once transferred into the recipient, it is believed that they migrate to recipient lymph nodes, where they are able to directly stimulate activation and clonal expansion of alloreactive recipient T cells toward the antigenic determinants on their surface[65]. In this manner, it is believed that these cells are able to cause acute rejection of the donor organ. For this reason, a greater degree of understanding is required of the impact and outcomes on the donor organ from the transfer of donor leucocytes following revascularisation. Furthermore, little is known about the activation status of

these passenger leucocytes following transfer and collectively this requires further investigation.

It is widely understood that surgical trauma induces leucocyte activation[103] and it is therefore plausible that donor immune cells could be transferred in an inflammatory, activated state. As such, the transfer of passenger leucocytes into the recipient could have further important implications, contributing to significant acute phase inflammation and potentially promoting early graft dysfunction. Previous studies exploring the role of passenger leucocytes are scarce, and as yet no evaluation of their early migration from the donor heart into the recipient circulation has been performed. Indeed, little is known about the cardiac immune capacity and whether or not these cells are strictly resident or possess significant migratory capacity.

Importantly, it is not only passenger leucocytes that are transferred upon revascularisation of the donor organ. Indeed, cold storage is associated with altered cellular metabolism[22], contributing to the accumulation of toxic by-products within the graft. This is inherently damaging to the tissue, and could induce the release of damage-associated molecular patterns into the vasculature. Additionally, it is likely that passenger leucocytes are sufficiently active during storage to be able to secrete a variety of inflammatory mediators, including cytokines, which could potentiate non-specific inflammation in the early post-transplant period. It is therefore important to evaluate the soluble contents within the vasculature after static cold preservation to provide insight into the transferred inflammatory burden following revascularisation.

To evaluate whether passenger leucocytes have a strong influence on outcomes post-transplantation, a greater understanding of their migration and activation within the donor heart is needed. This study aimed to provide novel insight into leucocyte extravasation out of the donor heart using a post-preservation cardioplegic flush. This flush replicates the immediate period of revascularisation and allows, for the first time, the ability to quantify donor immune load and the associated signals that would be transferred into the recipient.

3.2 Methods:

3.2.1 Donor organ retrieval:

Ten healthy Swedish pigs of native breed were sacrificed, with donor hearts retrieved as previously described in section 2.1.2. Briefly, each pig was anaesthetised and a median sternotomy was performed. The ascending aorta was cannulated and the superior and inferior vena cavae were clamped until blood was drained from all chambers of the heart. The distal ascending aorta was cross-clamped and 600ml of St Thomas cardioplegic solution (8°C) delivered by antegrade coronary perfusion. The donor heart was then excised and preserved by static cold storage by submerging in St Thomas cardioplegia solution at 8°C for approximately 2 hours, to replicate clinical cold ischaemic intervals prior to transplantation.

3.2.2 Post-preservation flush of the coronary vasculature:

All veins and arteries were sutured closed or clamped with the exception of the aorta and pulmonary artery. After 2 hours of static cold preservation, a second flush of the donor heart was performed using a similar method to the initial preservation flush. A total volume of 200ml of St Thomas cardioplegia solution was flushed through the heart via antegrade coronary perfusion at a fixed 60mmHg pressure, allowing controlled reperfusion. The effluent from the pulmonary artery was collected into a flask and the total volume recorded to allow standardisation between subjects.

3.2.3 Cardiac effluent collection:

The effluent from the post-preservation flush was collected after passive outflow from the pulmonary artery. To evaluate leucocyte content, 100µl of the flush effluent underwent processing for flow cytometry. The remaining effluent was split equally across four 50ml falcon tubes and centrifuged for 10 minutes at 2000g to separate plasma from the cellular fraction. The plasma was aliquoted into 1ml volumes and stored at -80°C for later assessment of cytokine content and markers of tissue injury.

3.2.4 Flow cytometry:

Leucocyte phenotyping was performed as previously described in section 2.2.1, with minor modifications. Cell viability was determined with LIVE/DEAD fixable violet dye, which diffuses across the compromised cell membrane in dying or dead leucocytes. Primary antibodies specific for porcine CD45 and porcine CD31 were used for cell surface staining. Leucocytes were identified by their expression of CD45, a

pan-leucocyte marker and endothelial cells were identified by cell surface CD31 expression in the absence of CD45. Following surface staining, red blood cell lysis buffer (BD Biosciences, UK) was added and the cells incubated for 10 minutes in the dark at room temperature. Cells were washed twice and finally resuspended in 1.2ml staining buffer prior to data acquisition by flow cytometry. Cell counts were adjusted to the total volume of effluent retrieved to quantify the number of cells that would have been transferred into the recipient.

3.2.5 Cytokine profiling:

A porcine 13-plex Luminex assay was performed as described in section 2.2.3 to quantify cytokine release during static cold storage. Plasma from the post-preservation flush effluent was analysed without prior dilution to determine the concentration of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α. Concentrations were adjusted to pg/ml for standardisation.

3.2.6 Troponin I quantification:

Cardiac troponin I was quantified in the flush effluent using a porcine specific ELISA kit as previously described in section 2.2.4 to determine the extent of myocardial injury as a result of cold ischaemia. All samples were analysed without prior dilution.

3.2.7 Statistical analysis:

Prism 7 (GraphPad, La Jolla, CA, USA) was used to perform all statistical analyses. Data are expressed either as mean ± standard deviation if normally distributed or as median [interquartile range] if non-normally distributed. An exception to this is the troponin I data, which was displayed as median [minimum-maximum] as values were highly skewed. Only descriptive data was provided as this study was designed to give insight into the contents of the vasculature upon revascularisation and no statistical comparisons could be made.

3.3 Results:

3.3.1 Post-preservation flush effluent volume:

A total of 200ml cardioplegic solution was infused into the coronary vessels to mimic reperfusion in a controlled manner. The effluent volume freely returned from the pulmonary artery was much lower (mean 92.94±11.18ml). Total egress upon reperfusion for each variable was adjusted for the volume returned by that individual heart. Unfortunately, errors in sample preparation meant that 2/10 effluents could not be evaluated for cellular migration into the flush effluent (by leucocytes and endothelium).

3.3.2 Leucocytes migrate out of the donor heart upon reperfusion:

The number of leucocytes that were present in 8/10 effluents was quantified following a post-preservation flush in order to evaluate the donor cardiac leucocyte load transferred immediately into the recipient at the point of revascularisation. These findings demonstrate that leucocytes are indeed able to rapidly move out of the donor vasculature in response to restoration of flow through the arteries. The post-preservation flush yielded a large number of leucocytes ($1.83x10^8 \pm 7.29x10^7$ cells, figure 3.1) despite the low flush volume used.

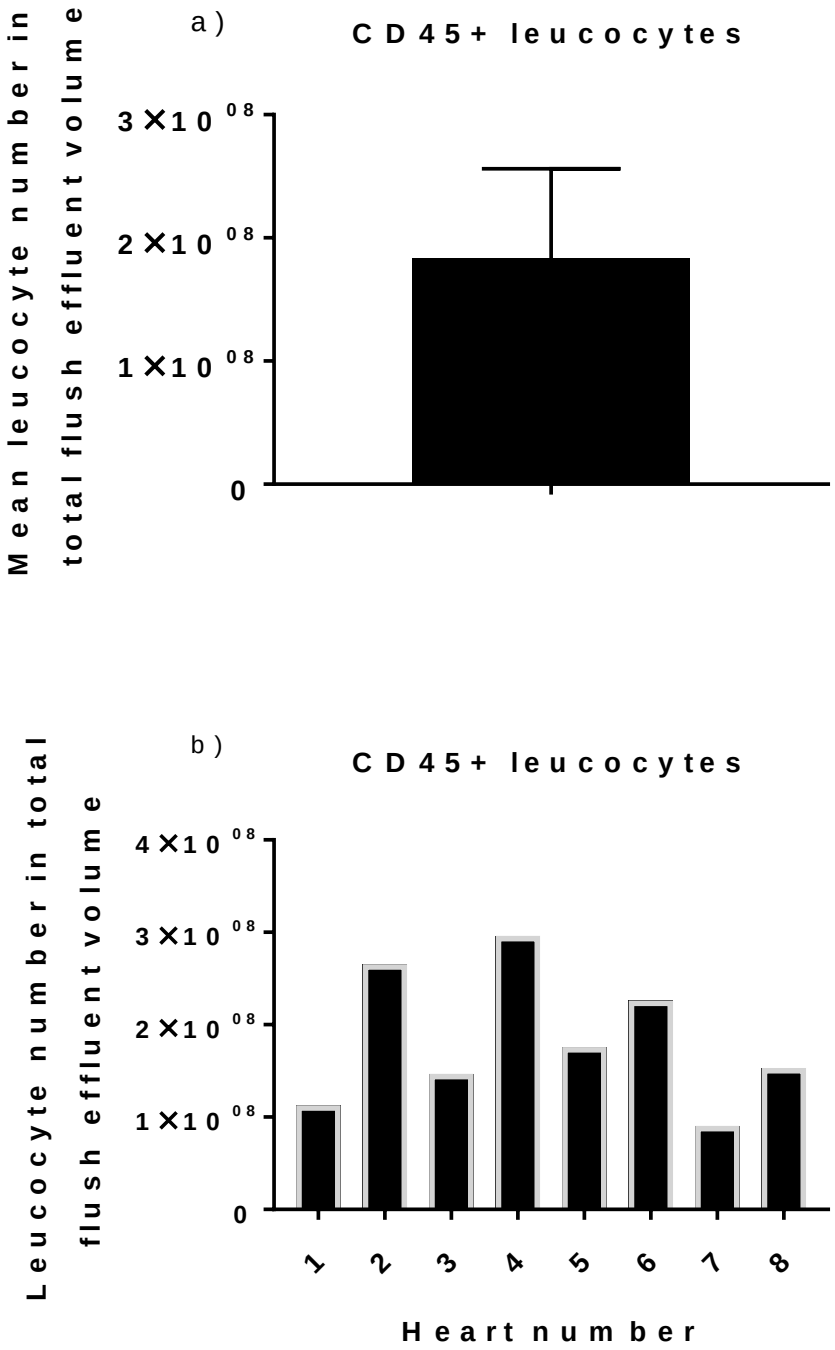

Figure 3.1 – Leucocyte mobilisation upon revascularisation. Large numbers of donor leucocytes are rapidly flushed from the vasculature upon restoration of flow through the graft. This replicates the events that would occur after revascularisation and highlights the donor immune load that would be transferred into the recipient after reperfusion with a small volume flush (200ml). Both mean leucocyte count (a) and individual counts for each donor heart (b) are shown. The bar represents mean value, and the whisker illustrates the standard deviation.

3.3.3 Endothelial denudation of the graft vasculature occurs in response to ischaemia-reperfusion:

Endothelial viability is an important factor in determining coronary arterial function. The number of free endothelial cells released into the flush was evaluated as a marker of disruption to the tissue during ischaemia-reperfusion. This study demonstrates that controlled revascularisation by way of a post-preservation flush induces significant endothelial loss from the vessels. In total, a mean of $3.47x10^6 \pm 3.16x10^6$ endothelial cells were detected in the total effluent volume (figure 3.2).

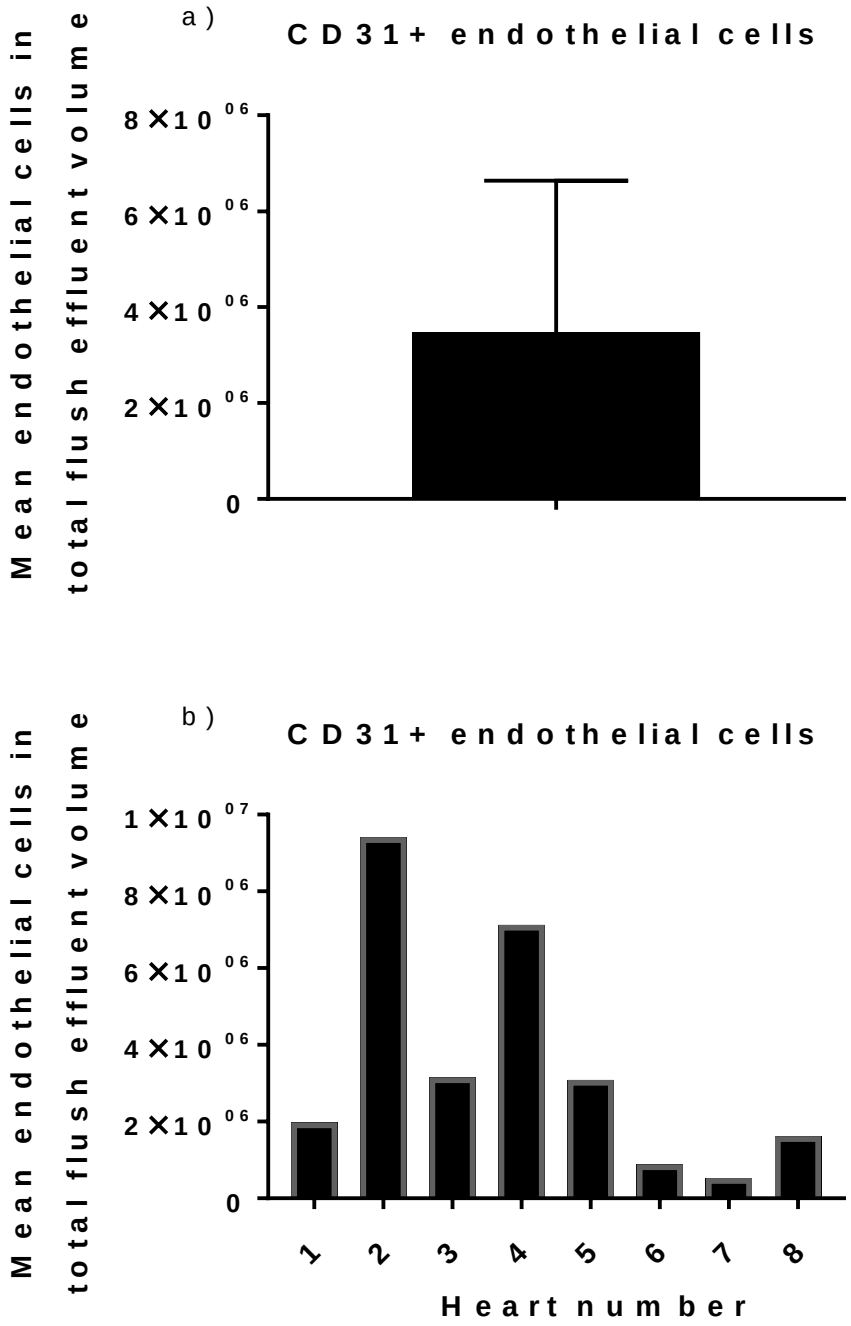

Figure 3.2 – Endothelial cell loss from the coronary vessels is detected upon reperfusion. Flush effluents from all grafts displayed significant endothelial cell populations, which is suggestive of damage to the vasculature during procurement and graft storage. These endothelial cells would be transferred into the recipient upon reperfusion. Both mean endothelial cell count (a) and individual counts for each donor heart (b) are shown.

3.3.4 Inflammatory cytokines are released during ischaemia-reperfusion:

To determine the inflammatory profile of the donor heart and the immune environment during static cold storage, a panel of cytokines were quantified in the flush effluent. The presence of cytokines was taken as an indication of activation of the donor graft and the leucocyte compartment in response to cold ischaemia and reperfusion. Of the 13 cytokines assessed, only 5 were detectable in at least half of the n=10 organ flushes. The absence of GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-6, IL-10, and IL-12 was noted in most donor hearts. IFN-γ was only detected in three flush effluents, but for each of these it was detected at high concentrations (>1000pg/ml), demonstrating high variability between subjects. Of those consistently detected across the majority of the hearts, IL-1α had the lowest titre (5.0 [0.0-42.5] pg/ml, figure 3.3). Relatively low concentrations of IL-4 (10.0 [0.0-12.5] pg/ml), IL-8 (20.0 [7.5-40.0] pg/ml) and TNF-α (20.0 [10.0-30.0] pg/ml) were observed, with IL-18 the dominant cytokine detected (60.0 [0.0-265.0] pg/ml, figure 3.3). Despite the relatively low concentrations observed in the effluent, the volume flushed through the vasculature was 200ml. As such, when scaled up to the volume of effluent retrieved, the amount of cytokine potentially being transferred rises to more significant levels (IL-1α: 465.0 [0.0-3953.0] pg; IL-4: 930.0 [0.0-1163] pg; IL-8: 1860.0 [697.5-3720.0] pg; TNF-α: 1860.0 [930.0-2790.0] pg; and IL-18: 5580.0 [0.0-24645.0] pg, figure 3.3).

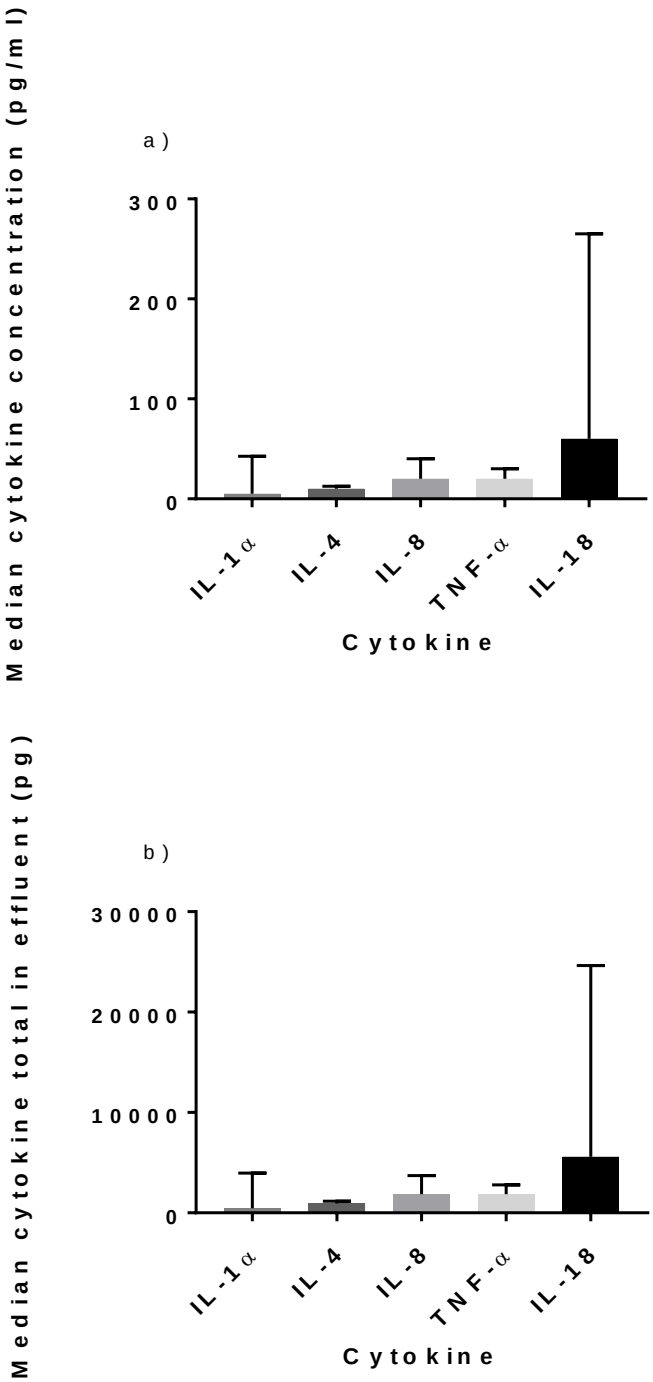

Figure 3.3 – Inflammatory cytokines are released from the donor heart and venous effluent during static cold storage. Only five of the 13 cytokines assessed were detected in the effluent, highlighting a selective pattern of release. All cytokines were detected at relatively low concentrations (a) but when scaled up to the total volume (b), this adds up to a significant bolus of signal that would be transferred to the recipient. Graphs present data from n=10 post-preservation flush experiments. Bars represent median values and whiskers illustrate the interquartile range.

3.3.5 Static cold storage is associated with myocardial injury:

Cold ischaemia during donor organ storage is associated with significant alterations to intracellular pH and metabolic activity. The concentration of cardiac troponin I was quantified in the post-preservation flush as a sensitive marker of myocardial injury to determine the impact of storage on the tissue on a cellular level. Of the 10 effluents analysed, eight demonstrated troponin I levels greater than the limit of detection of the assay (2000pg/ml). In order to provide some descriptive evaluation of troponin I release, 2000pg/ml was therefore assigned as a conservative value for each subject without a concentration in range. This data demonstrates that even a relatively short period of cold ischaemia is sufficient to induce significant myocardial injury as represented by cardiac troponin I release (median [minimum-maximum]: 2000.0 [410.6-2000.0] pg/ml).

3.4 Discussion:

Passenger leucocyte transfer into the recipient upon transplantation is well documented to play a role in the induction of the alloresponse and may therefore significantly alter clinical outcomes, particularly the incidence of primary graft dysfunction and acute rejection episodes. Previous work has provided strong and clear evidence of the donor immune contribution in recipient leucocyte priming in other organs[65, 104, 105], yet the direct transfer of such cells from the heart has not been described in detail. Indeed, the heart has long been considered to have a limited resident immune repertoire that could be transferred during transplantation. In part, this is due to the lack of established lymphatic tissue such as lymph nodes within or associated with the organ. Although there is the potential for some leucocytes from the circulation to remain within the organ at the time of transplantation, it is likely that the vast majority of marginal leucocytes are expelled from the vasculature during the initial protective cardioplegic flush. This study attempted to address whether a residual donor leucocyte load would be transferred from within the donor organ by utilising a secondary *ex vivo* post-preservation flush to mimic the moment of revascularisation.

This study demonstrates that large populations of viable donor leucocytes can be removed using this simple method at the time of graft retrieval. Furthermore, cells that are removed are in an activated state as demonstrated by increased concentrations of pro-inflammatory cytokines such as IL-18. It can therefore be deduced that the donor heart contains donor leucocytes that rapidly migrate from the tissue upon reperfusion. This population was relatively large considering the small volume of flush infused to restore flow and indicates that the leucocytes are likely marginated close to the vascular bed. This finding has significance due to the fact that these cells would ordinarily be directly transferred into the recipient circulation, where they would have the potential to migrate to secondary lymph nodes and activate recipient T cells to mount a response against the donor heart. It remains unclear how the number of cells migrating would be affected by a larger flush volume as a longer period of exposure to the “circulation” could induce further diapedesis into the vasculature from the surrounding tissue. Despite this, the main aim of this study was determine the migratory capacity of donor leucocytes, and was therefore observational in nature. However, it does provide evidence to suggest that the use of a simple secondary post-preservation flush could alter the donor immune content immediately prior to

transplantation, and this could confer benefit through a reduction in stimulation of direct allorecognition.

Importantly, the findings of this study highlight that the static cold stored heart is associated with detachment or release of vascular endothelium in the acute phase of reperfusion. The presence of endothelial cells in the flush effluent suggests a certain level of damage to the vessels, which has resulted in their loss of adherence to the cellular substratum and subsequent release into the vessel lumen. It is unclear whether these denuded cells were released prior to the restoration of flow as a result of ischaemic injury or the adhesion was weakened during the cold ischaemic period and the rapid increase in pressure associated with reperfusion was responsible for the detachment. Regardless, this same process would occur during clinical implantation, and as such these findings demonstrate the sensitivity of the cardiac vascular endothelial lining. In this study, a conservative cold ischaemic interval of two hours was utilised, representing a relatively short preservation window. If this time was increased beyond four hours, a more common occurrence in transplantation, then the extent of endothelial disruption would potentially be further amplified.

Endothelial damage is associated with a number of detrimental effects due to the importance of the endothelial lining to vessel function. Whilst the level of damage detected here is difficult to contextualise, even a small amount of loss could be relevant. The endothelium has numerous roles, particularly as a regulator of vascular tone through the activation of endothelial nitric oxide synthase, leading to the release of nitric oxide[106]. Disruption of this intimal layer could therefore significantly impact upon vessel integrity, and consequently affect organ function within the recipient. Perhaps of greater relevance within the remit of this study, the endothelium has a major role as a barrier regulating cellular movement across from the vessel lumen into the surrounding tissue (and vice versa)[107]. The loss of endothelial cells, as observed in this study, is associated with impaired barrier function and greater vascular permeability[108], which may have contributed to the rapid mobilisation of leucocytes from the surrounding tissue into the vasculature and flush effluent. Furthermore, it is known that upon endothelial damage, platelets are rapidly recruited to the site and are able to promote greater adhesion of leucocytes[109], mediating more efficient extravasation. Although this is generally demonstrated in the context of inflammation

and leucocyte migration *into* the tissue, this may also be the case for movement into the vasculature. Clearly, endothelial loss has a significant impact, as it may enhance both passenger leucocyte egress from the tissue into the recipient circulation as well as promoting enhanced recipient leucocyte adhesion and graft infiltration.

The endothelium is a key source of immunological signals, including cytokines and cell surface receptors associated with antigen presentation and stimulation of leucocytes[110]. This is particularly so when the cells are activated, as is likely to be the case following denudation from the vasculature. The transfer of these cells into the recipient (as this data suggests would occur upon revascularisation) represents a significant immunological stimulus. These cells express a wide variety of alloantigens that could be utilised to prime recipient T cells via the direct allorecognition pathway. Whilst the endothelium is not as efficient at priming T cells as dendritic cells, they can certainly potentiate the response.

The role of the endothelium following transplantation has been investigated in some depth, as endothelial cells represent a major target for recipient leucocytes. Damage to the endothelial lining by recipient leucocytes is a major factor responsible for the development of cardiac allograft vasculopathy, a primary manifestation of which is intimal thickening[111]. The data from this study suggests that acute damage to the endothelium is induced independent of recipient leucocytes, which could contribute to the development of cardiac allograft vasculopathy as early as revascularisation. Strategies to prevent damage to the endothelium may be required prior to transplantation and may necessitate alteration of the cardioplegic flush pressure or cardioplegia composition to include protective components.

Donor leucocyte extravasation upon reperfusion may be enhanced by the release of cytokines during the storage period. These data support the notion that cold storage does not fully impair the ability of the donor heart and its associated immune contents to function during the preservation period. Indeed, a relatively high concentration of a number of cytokines was detected in the flush effluent despite the diminished metabolism promoted by the preservation strategy. The cytokines detected in this study are primarily pro-inflammatory in nature, which provides a strong indication that cardiac resident donor leucocytes are activated in response to cold ischaemia and the associated accumulation of toxic metabolites and free radicals. Transplantation of

the donor heart at this point would therefore also result in the transfer of leucocytes in an activated, pro-inflammatory state, augmenting their impact on both the local tissue microenvironment and direct stimulation of recipient allospecific immune cells. Furthermore, the cytokines themselves have direct detrimental effects upon the donor tissue. TNF-α has an established role in enhancing vascular permeability[112, 113], which promotes greater extravasation of leucocytes in a bi-directional manner into the circulation (donor cells) and into the tissue (recipient cells).

The high concentration of IL-18 is indicative of cardiac injury and has a role in the loss of myocardial function[114], induced through ultrastructural alterations[115]. Although this downstream effect likely requires exposure over a much longer period than the cold storage duration described herein, it still demonstrates that such cytokine exposure is detrimental to the donor organ. The use of a secondary flush prior to transplantation clears the accumulated cytokines from the heart such that they are not transferred alongside the organ and cannot further affect the tissue or direct the recipient alloresponse.

The data from this study indicates that in addition to endothelial disruption, there is also significant myocardial injury induced over the two-hour storage period. This was detected by the presence of acellular troponin I in the flush effluent. Troponin I is a major intracellular component of the myocardium responsible for contractility[116], and is released upon loss of integrity of the myocardial cell membrane[117]. The concentration observed here is difficult to contextualise due to the low flush volume used for revascularisation. Despite this complexity, the fact that troponin I is detected at all is a strong indicator of damage at a cellular level. Interestingly, free troponin I is not simply an inert biomarker of cellular injury and has been linked to the development of exacerbated inflammation in the heart after ischaemia-reperfusion injury in murine experimental myocardial infarction models[118]. Transferring a concentrated bolus of free troponin I into the recipient may thus increase the magnitude of inflammation post-transplantation. Removing this stimulus with a simple pre-transplant flush minimises the burden of this potential complication, although further delayed release of troponin is still possible.

Collectively, this study has established the presence of a large leucocyte compartment within the donor heart capable of rapid mobilisation and pro-inflammatory cytokine

release upon reperfusion. Transfer of this population may be detrimental to clinical outcomes following transplantation, although further research with orthotopic or heterotopic transplantation and a no-flush control group would be required to validate this. These findings support the concept of the donor immune compartment as a target for therapeutic intervention. The loss of endothelial integrity in response to static cold storage is a further important and novel finding of this study, which could significantly impair vascular function post-transplant. The addition of endothelial protective agents, such as dextran 40[119], to the initial cardioplegic flush may diminish the effect of cold ischaemia on intimal integrity. Further exploration of this concept is warranted but was outside the remit of the current study.

3.5 Limitations:

The donor pigs utilised in this study did not undergo brain death prior to retrieval. Furthermore, the donor heart was completely functional and remained injury free until the point of cardioplegia delivery. As such, the donor heart can be considered healthy and is therefore not a true representation of the status of donor hearts retrieved in the clinical setting following brain death or circulatory arrest. Consequently, the leucocyte content of the organ is reflective of the size of the resident population in the healthy heart. This may be altered in the clinical setting of transplantation. No transplantations were performed to evaluate the consequences of the transfer of donor immune cells or soluble inflammatory mediators, limiting the conclusions that can be drawn although the study was specifically designed with an observational focus. The study is further limited by the fact that the flush was performed at a single time point with consistent volume and the total effluent evaluated as a single sample, and would benefit from evaluating the effects of ischaemia by flushing at more prolonged ischaemic intervals. As such, no temporal kinetics of leucocyte mobilisation can be elucidated.

3.6 Conclusion:

The donor heart contains a resident population that is able to migrate into the recipient circulation after being transferred out of the donor heart. A pro-inflammatory profile was observed in the effluent, highlighting the impact of cold ischaemia on the tissue and resident leucocytes. Taken together, these findings suggest that static cold storage promotes significant donor immune activation, with potential detrimental impact post-

transplantation. Endothelial denudation may also potentiate graft infiltration by recipient leucocytes as a result of increased vascular permeability.

Chapter 4: Cold cardioplegic perfusion induces immunodepletion of donor hearts associated with activation of the IFN-γ signalling axis

4.1 Introduction:

Transplant waiting list mortality remains high due to the lack of suitable donor organs. In an effort to increase the donor pool, novel preservation methods have been developed that aim to extend the time between organ retrieval and transplantation without a deleterious impact on graft viability. Standard donor heart retrieval requires a cardioplegic flush followed by static storage on ice enabling preservation via reduced metabolism[120]. During this cold ischaemic period, the organ is deprived of oxygen and nutrients, inevitably imparting increasing damage to the tissue, limiting the duration for which the heart can be stored before transplantation. In order to address this problem, a method of cold cardioplegic *ex vivo* heart perfusion (CCHP) has now been developed, which can safely extend preservation times to 24 hours with stable function upon transplantation in pigs[97]. This method combines the protective effect of minimised metabolic demand with optimal nutritional support and oxygenation. Whilst this has clear implications for improved donor organ preservation and management, the potential for auxiliary benefits following transplantation have not been fully explored, particularly with regard to acute graft rejection.

Acute graft rejection represents a major barrier to successful transplantation and involves broad immune activation targeted towards the graft. This requires permanent immunosuppression which is associated with severe adverse side effects that contribute significantly to morbidity and mortality. Novel methods to manipulate the immune response during transplantation are therefore essential to improve clinical outcome. Current therapies predominantly target recipient T cells to diminish their activation and proliferation. However, little attention is paid to the donor immune content transplanted with the organ, despite the knowledge that passenger leucocytes induce acute rejection of the transplanted heart[72]. Depletion of donor dendritic cells is sufficient to prevent rejection[73] and their reintroduction restores the immune response[74]. It has been demonstrated previously that *ex vivo* perfusion is sufficient to alter the immunogenicity of the donor lung via the removal of passenger leucocytes[65]. This significantly reduced the magnitude of graft infiltration by recipient allospecific T cells at 24 hours post-transplantation.

This study aimed to determine whether *ex vivo* perfusion of the donor heart alters the passenger leucocyte repertoire and tissue inflammatory status prior to transplantation.

4.2 Methods:

4.2.1 Donor organ retrieval:

Six healthy Swedish pigs of native breed were used in the study. Donor organ retrieval was performed as previously described in section 2.1.2. Briefly, the ascending aorta was cannulated after median sternotomy. The superior and inferior vena cavae were clamped until the heart was emptied. The distal ascending aorta was cross-clamped and 600ml of 8°C cardioplegic perfusate solution was flushed through the heart via antegrade coronary perfusion at a fixed 60mmHg perfusion pressure. The donor heart was excised and maintained at approximately 8°C submerged in cardioplegic perfusate whilst the heart preservation system was being prepared. The mitral valve was made insufficient by inserting tubing through the valve and into the left ventricle in order that any perfusate leaking through the aortic valve during perfusate delivery would be able to flow freely out of the open left atrium and not cause left ventricular stretching.

4.2.2 Device preparation and cold cardioplegic perfusion:

The heart preservation system was prepared as previously described in section 2.1.4. The reservoir was filled with 2.5L of cardioplegic perfusate medium containing packed leucocyte-depleted erythrocytes at a haematocrit of 15%. The perfusate was circulated at 8°C before the donor heart was connected and submerged for the duration of the experiment. The heart was perfused continuously for 8 hours at 8°C at a constant pressure of 20mmHg receiving a minimum 100ml/minute perfusate. The vena cavae and pulmonary artery were left open to allow the perfusate to be returned to the reservoir from the coronary sinus.

4.2.3 Sample collection during CCHP:

4.2.3.1 Biopsy:

Left ventricle tissue was obtained from porcine hearts before and after 8 hours of CCHP. The tissue sample was split into 3 sections. Biopsy tissue weighing 30-100mg was dissected into small pieces (<2mm^3, placed in 25ml calcium and magnesium-free Hank's buffered salt solution (HBSS) and then homogenised at the lowest speed for 2 minutes on ice. The homogenate was filtered through 500µm then 250µm filters then centrifuged at 2000g for 2 minutes at 4°C. The cells were resuspended in 5ml HBSS before a final filtration through a 40µm cell strainer. The filtered solution was washed at 2000g for 2 minutes at 4°C and the cells resuspended in 0.5ml staining buffer, which

was split into 5 tubes for flow cytometry. The second section was snap frozen in liquid nitrogen and stored immediately at -80°C for later analysis of cardiac protein expression. The final section was fixed in 10% buffered formalin and paraffin embedded for later histological evaluation.

Following perfusion, a transverse section of the heart was retrieved and weighed (wet weight) before being dried at 60C before being reweighed (dry weight). No pre-perfusion ratio was determined as no biopsy was retrieved in order to minimise the damage to the heart prior to perfusion. The percentage water content of the heart was calculated as follows:

((Wet weight – dry weight)/wet weight) x 100

4.2.3.2 Perfusate:

Serial perfusate samples measuring 20ml were collected prior to organ connection and every 2 hours throughout perfusion. Each sample was centrifuged at 2000g for 10 minutes to separate plasma, which was stored at -80°C for later inflammatory profiling and quantification of cellular injury biomarkers

4.2.3.3 Leucocyte Filter:

Following perfusion, the ingress and egress tubes were clamped either side of the leucocyte filter, which was then removed from the circuit. The filter contents were then poured into a sample collection flask in a retrograde direction. Trypsin-Ethylenediaminetetraacetic acid (EDTA) was then added to the filter in a retrograde fashion and incubated at 37°C for 15 minutes in order to detach any adherent leucocytes from the filter. The filter contents were combined into the sample collection flask and the total volume retrieved was recorded. The enzyme was subsequently quenched with foetal bovine serum to a final 10% concentration (vol:vol). The perfusate was washed by centrifugation at 2000g for 10 minutes before the supernatant was discarded. The entire filter volume was assessed using flow cytometry.

4.2.4 Inflammatory profiling:

Perfusate samples from serial time points were analysed without prior dilution according to the protocol described in section 2.2.3. The concentrations of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α

were determined to provide a broad overview of the inflammatory signalling occurring in response to hypothermic perfusion.

4.2.5 Chemokine quantification:

ELISA kits were utilised to quantify chemokines CCL2, CCL4, CCL5, CXCL9, CXCL10 (Insight Biotechnology, Wembley, UK) and CXCL11 (2BScientific, Oxfordshire, UK) at serial time points in perfusate. Undiluted perfusate samples were added to wells of each ELISA plate in duplicate and incubated for 2.5 hours at room temperature. Biotinylated antibody was then added to each well and further incubated for one hour at room temperature. Streptavidin solution was subsequently added and incubated for 45 minutes before tetramethylbenzidine substrate reagent was added. The plate was incubated for a final 30 minutes at room temperature before the reaction was halted with stop solution. Absorbance was read at 450nm using a Tecan infinite 200 PRO system (Tecan Group, Männedorf).

4.2.6 Flow cytometry:

Flow cytometry analysis was performed as per the method described in section 2.2.1. A panel of antibodies was utilised to phenotype immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10-2B2+), helper T cells (CD3ε+CD4α+), cytotoxic T cells (CD3ε+CD8β+), γδ T cells (γδ+), NK cells (CD335+), B cells (CD21+), classical monocytes (CD14+CD163-), non-classical monocytes (CD14+CD163+), intermediate monocytes ($CD14^{dim}$ $CD163^{bright}$) and macrophages (CD203a+). Swine leucocyte antigen (SLA)-DR expression was also assessed as a marker of antigen presentation. Toll-like receptor 4 (TLR4) expression was assessed on each population.

Following cell surface staining with the above antibodies, cells were treated with red blood cell lysis buffer (BD Biosciences, UK), washed with PBS and resuspended in a final 1.2ml volume of PBS. Propidium iodide (Sigma Aldrich, Dorset, UK) was added to each sample and incubated for 5 minutes prior to acquisition on the flow cytometer as a marker of cellular viability. Cell counts were normalised per milligram of starting tissue obtained for analysis or adjusted to the total volume present in the filter.

4.2.7 Cell-free DNA quantification:

The presence of cell-free mitochondrial and genomic DNA in the perfusate was evaluated using qPCR as previously described in section 2.2.2. Briefly, each sample was assessed without dilution using primers specific for porcine cytochrome b (mitochondrial DNA) and porcine GAPDH (genomic DNA) and the concentration was standardised per millilitre of perfusate. Samples were analysed using a QuantStudio 12K Flex system using a Power SYBR green PCR master mix.

4.2.8 Phosphokinase and apoptosis signalling:

A pre and post perfusion tissue biopsy from each pig was obtained. Human phosphokinase and apoptosis antibody proteome profiler arrays were utilised to evaluate proteomic changes between biopsies (R&D systems, Abingdon, UK). Proteomic evaluation was performed as described in section 2.2.5. A separate membrane was utilised for each sample to provide the greatest power for analysis and to allow a readout for each individual organ. Pixel density analysis was performed using ImageJ (NIH, USA) and data is expressed as the percentage change in protein expression from the pre-perfusion baseline following 8 hours of perfusion (i.e. ([post/pre] x 100)-100). A paired analysis of pre and post perfusion pixel density values was performed.

4.2.9 Cardiac tissue viability:

Cardiac troponin I concentration in the perfusate was quantified as a sensitive marker to detect cardiac injury. The presence of troponin I was detected using a commercially available porcine-specific ELISA kit, which was performed as described in section 2.2.4. All samples were assessed without prior dilution and comparisons made across serial time points during perfusion.

4.2.10 Histological evaluation:

To confirm the viability of the donor heart, a histological assessment was performed using formalin-fixed tissue obtained pre and post perfusion from the left ventricle as described in section 2.2.6. Sections were cut at 4µm, de-paraffinised and stained with haematoxylin and eosin. All samples were prepared and assessed in a blinded manner by a consultant histopathologist, who reported on the structural integrity of the tissue, oedema formation and endothelial disruption.

4.2.11 Immunohistochemistry:

An assessment of apoptosis induction in the tissue was performed on formalin-fixed tissue obtained before and after perfusion. Sections were de-paraffinised and antigen retrieval (Tris-EDTA pH 9.0 buffer for 3 minutes at 750W and 20 minutes at 900W in a Whirlpool Talent Microwave Oven) was performed prior to 30 minute incubation at 37°C with the rabbit polyclonal anti-porcine caspase 3 antibody (Abcam, Cambridge, UK), diluted 1:100 in Ventana Antibody Diluent (Ventana Medical System, Tucson, USA). Binding of primary antibody to the tissue samples 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. All assessments of tissue caspase-3 expression were performed in a blinded manner by a consultant histopathologist.

4.2.12 Pilot heterotopic transplant study design:

In order to determine whether immunodepletion by cold cardioplegic perfusion resulted in downstream clinically-relevant effects on graft rejection warranting further investigation, an initial series of heterotopic transplants was performed. Donor organs were harvested as above. Five organs were preserved by 2 hours of static cold storage and six organs preserved by 8 hours of cold cardioplegic perfusion. No perfusate or biopsy samples were obtained throughout the organ preservation process and no post-transplant blood samples were withdrawn.

4.2.13 Heterotopic transplant procedure:

Heterotopic transplants were performed as previously described in section 2.1.5. Briefly, all recipient pigs received anaesthesia before a longitudinal incision was made to the left of the linea alba. At implantation, the aorta of the preserved heart was connected to the infrarenal aorta and the pulmonary artery was connected to the vena cava. Reperfusion was commenced and the hearts were defibrillated where necessary to achieve sinus rhythm. The incision was sutured closed and the pig was awakened. All recipient pigs were awakened and maintained without immunosuppression. Pigs were sacrificed across a number of different time points to allow an assessment of the most appropriate time for comparison in a subsequent larger study. Pigs were sacrificed at 48 hours (n=2 perfused and n=2 static cold stored), 72 hours (n=2 perfused and n=1 static cold stored) and on days 5 (n=1 each for perfused and static

cold stored) and 10 (n=1 each for perfused and static cold stored). Biopsies were collected from across the heart for histological analysis of leucocyte infiltration.

4.2.14 Histological evaluation of graft infiltration:

Histological assessment of leucocyte presence in the donor heart was performed using formalin-fixed tissue sections obtained at sacrifice from across the regions of the heart. Sections were cut at 4μm, de-paraffinised and stained with haematoxylin and eosin. All samples were prepared and assessed in a blinded manner by a consultant histopathologist, who reported on the intensity of leucocyte infiltration (on an ordinal scale of severity from 0-3 (0=no infiltration, 1=mild infiltration, 2=moderate infiltration and 3=severe infiltration). The distribution of infiltration across the section was also analysed and presented as a percentage of the field of view affected. Only descriptive data were produced and no formal statistical comparisons were made between groups.

4.2.15 Statistical analysis:

SPSS version 22.0 (IBM, Armonk, NY, USA) was used to perform all statistical analyses. Data are expressed either as mean ± standard deviation if normally distributed or as median [interquartile range] if non-normally distributed. Paired samples T tests were utilised to assess the difference in leucocyte content of the heart between pre and post perfusion tissue samples. The related samples Wilcoxon signed rank test was utilised to determine changes in tissue protein expression as a result of perfusion. The related samples Friedman's two-way analysis of variance (ANOVA) by ranks was utilised to assess changes in markers from the perfusate over time. Only descriptive data was produced for the pilot heterotopic transplant experiments due to the low number performed. Statistical significance was accepted when $p \leq 0.05$. All graphs were produced using Prism 7 software (GraphPad, La Jolla, CA, USA).

4.3 Results:

4.3.1 Cardiac immune characterisation:

The donor heart immune repertoire was first profiled to generate a baseline reference of the healthy donor heart. These findings demonstrate that the heart contains a significant immune repertoire including large populations of both innate and adaptive cells (figure 4.1). NK cells represent the largest single immune phenotype resident in the tissue ($1.13x10^6 \pm 5.49x10^5$ cells/g). Mature basophils and eosinophils ($3.7x10^5 \pm 2.3x10^5$ cells/g) and B cells ($3.0x10^5 \pm 2.1x10^5$ cells/g) were also abundant in the left ventricle following standard donor organ retrieval.

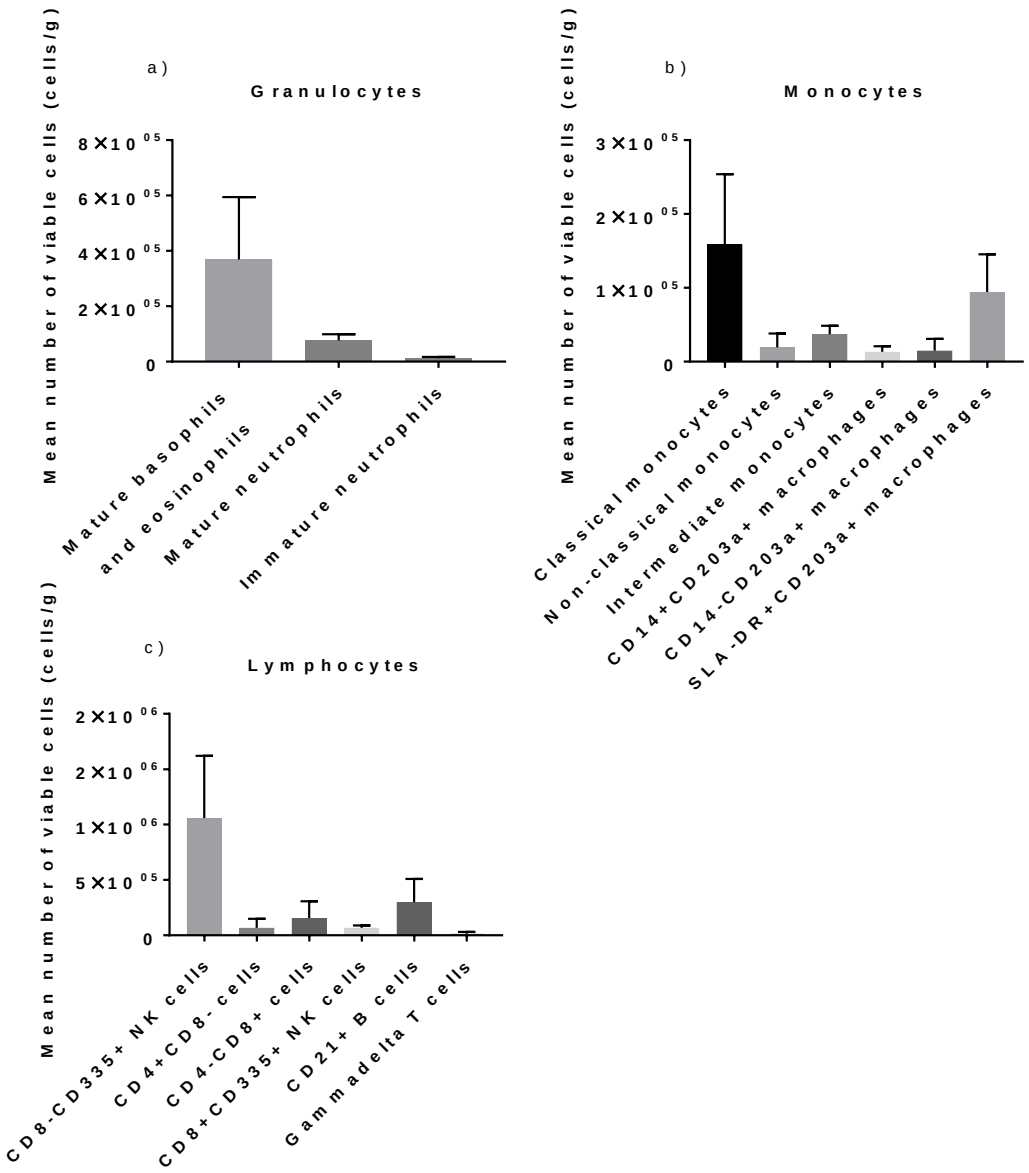

Figure 4.1 – A baseline reference of the leucocyte repertoire resident within the healthy donor heart. Granulocytes are observed in the tissue, which are predominantly basophils and eosinophils (a). Low numbers of monocytes and macrophages are present, dominated by the classical monocytes phenotype (b). Lymphocytes are the most highly abundant leucocyte population with significant numbers of NK cells and B cells present (c). These cells are likely to be tissue-resident rather than intravascular as the cardioplegic flush performed as part of the standard retrieval process removes the vast majority of blood cells from the vasculature.

4.3.2 Perfusion variables:

Clinically relevant parameters associated with either organ retrieval and CCHP were recorded for each pig. For this study, a mean cold ischaemic time of 18.5±7.66 minutes between organ retrieval and initiation of CCHP was recorded. The coronary flow was constantly maintained in the range of 100 to 200 mL/min depending on the weight of the heart.

4.3.3 CCHP induces significant immunodepletion of the donor heart:

It was next determined whether perfusion alters the immune profile of the donor heart. Indeed, these data demonstrate the significant loss of viable leucocytes from the tissue following perfusion (figure 4.2). This is particularly the case for mature neutrophils (85% reduction, p=0.003), mature basophils/eosinophils (84% reduction, p=0.023), classical monocytes (72% reduction, p=0.024) and B cells (60% reduction, p=0.042). Depletion of immature neutrophils (p=0.011), CD14+CD203a+ and CD14-CD203a+ macrophages (both p=0.043) and CD8+ NK cells (p=0.003) was also observed. However, no overall effect of perfusion was observed on T cell populations, which remained consistent despite CCHP (p=0.409 and p=0.140 for helper and cytotoxic T cells respectively). The number of non-classical monocytes (p=0.117) and γδ T cells (p=0.119) was reduced for each sample pair but this did not reach significance. CD8- NK cells were markedly reduced in all but one heart following perfusion although this was not statistically significant (p=0.129). Intermediate monocytes (p=0.225) and SLA-DR+CD203a+ macrophages (p=0.500) were not significantly altered by perfusion. Toll-like receptor 4 expression on leucocytes within the tissue was also assessed before and after perfusion but did not change significantly on any population except for mature neutrophils, which displayed lower expression (see figure 4.3).

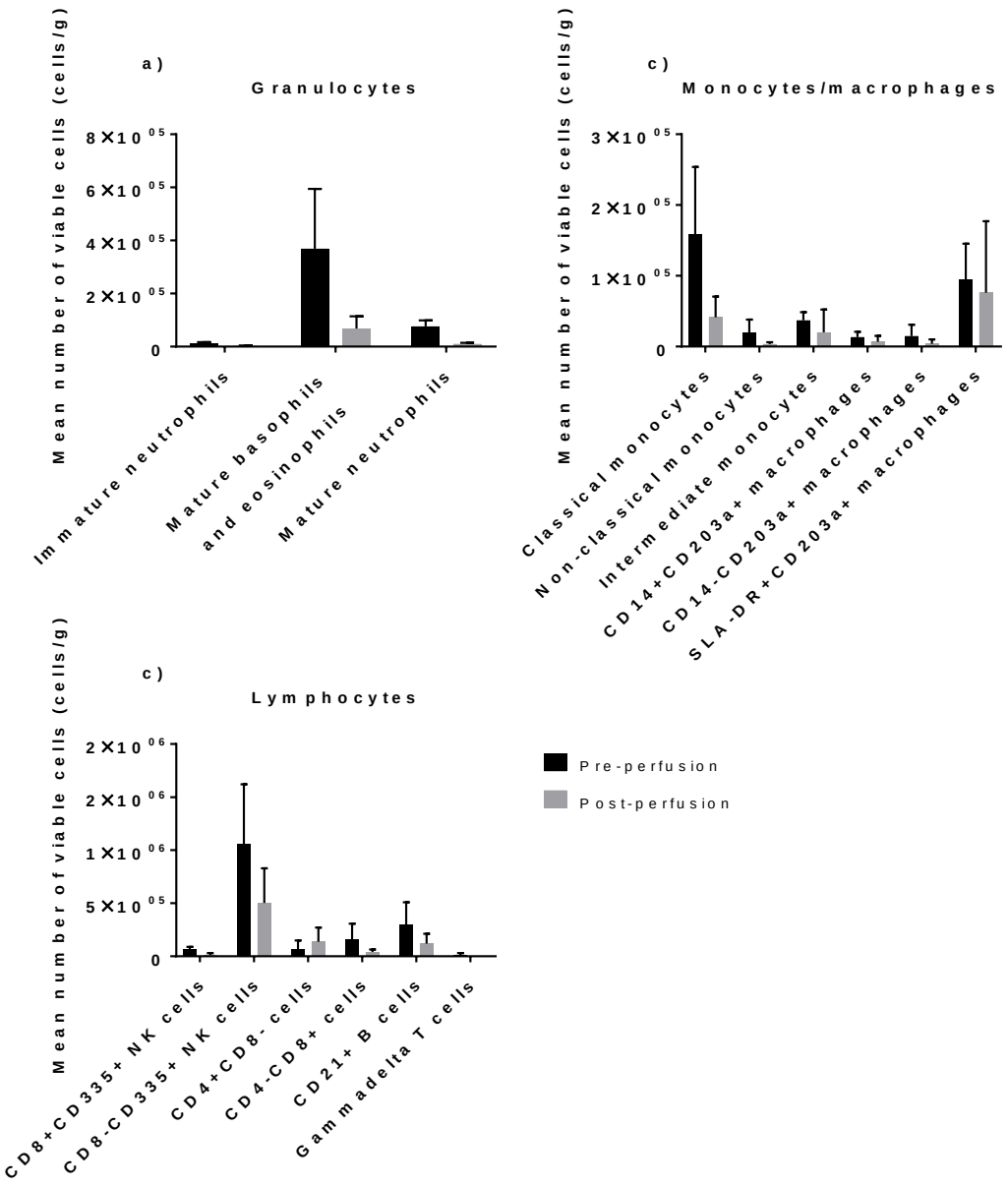

Figure 4.2 – Immunodepletion of the donor heart via CCHP. Significant leucocyte loss was observed from the tissue across a range of phenotypes, including granulocytes (a), monocytes/macrophages (b) and lymphocytes (c). All granulocyte populations were markedly reduced, in particular mature neutrophils and mature basophils/eosinophils (86% and 84% reductions respectively).

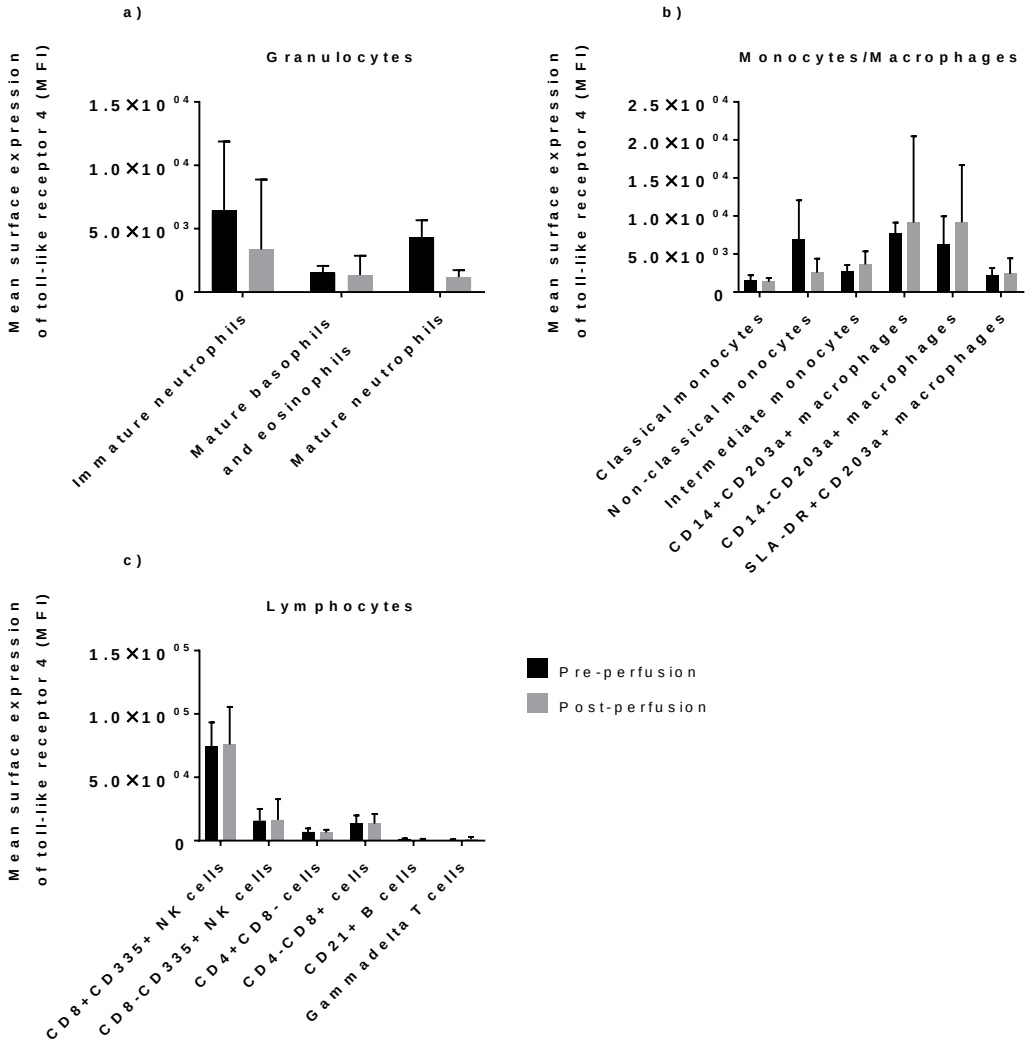

Figure 4.3 – TLR4 expression on leucocytes during perfusion. TLR4 expression is displayed for granulocytes (a), monocytes and macrophages (b), and lymphocytes (c). TLR4 is significantly reduced only on mature neutrophils following CCHP. MFI = mean fluorescent intensity.

4.3.4 Leucocyte filtration plays a role in immunodepletion during perfusion:

At the end of perfusion, the content of the in-line leucocyte filter was profiled to determine the phenotypes of cells that had become trapped. A significant immune repertoire was demonstrated to be sequestered by the filter (figure 4.4). The leucocyte filter population comprised varying numbers of each population, with NK cells again dominant ($7.2x10^6 \pm 12.1x10^6$ cells/filter) although all phenotypes were detected. Immature neutrophils were detected in greater numbers within the filter than either mature basophils/eosinophils or mature neutrophils ($5.1x10^5 \pm 5.4x10^5$, $4.5x10^5 \pm 2.9x10^5$ and $2.1x10^5 \pm 2.8x10^5$ cells/filter respectively). Classical monocytes ($5.7x10^5 \pm 6.4\ x10^5$ cells/filter) were much more abundant than either non-classical ($5.6x10^4 \pm 5.8x10^4$ cells/filter) or intermediate monocyte ($6.5x10^4 \pm 3.3x10^4$ cells/filter) phenotypes. The total macrophage number in the filter was $5.0x10^5 \pm 1.8x10^5$ cells. T cells and B cells were found in relative abundance in the filter, despite not having significant changes in the tissue itself ($6.7x10^5 \pm 5.9x10^5$ cells/filter and $2.7x10^5 \pm 1.2x10^5$ cells/filter respectively). Gammadelta T cells were only scarcely retained by the filter ($2.7x10^4 \pm 8.3x10^3$ cells/filter).

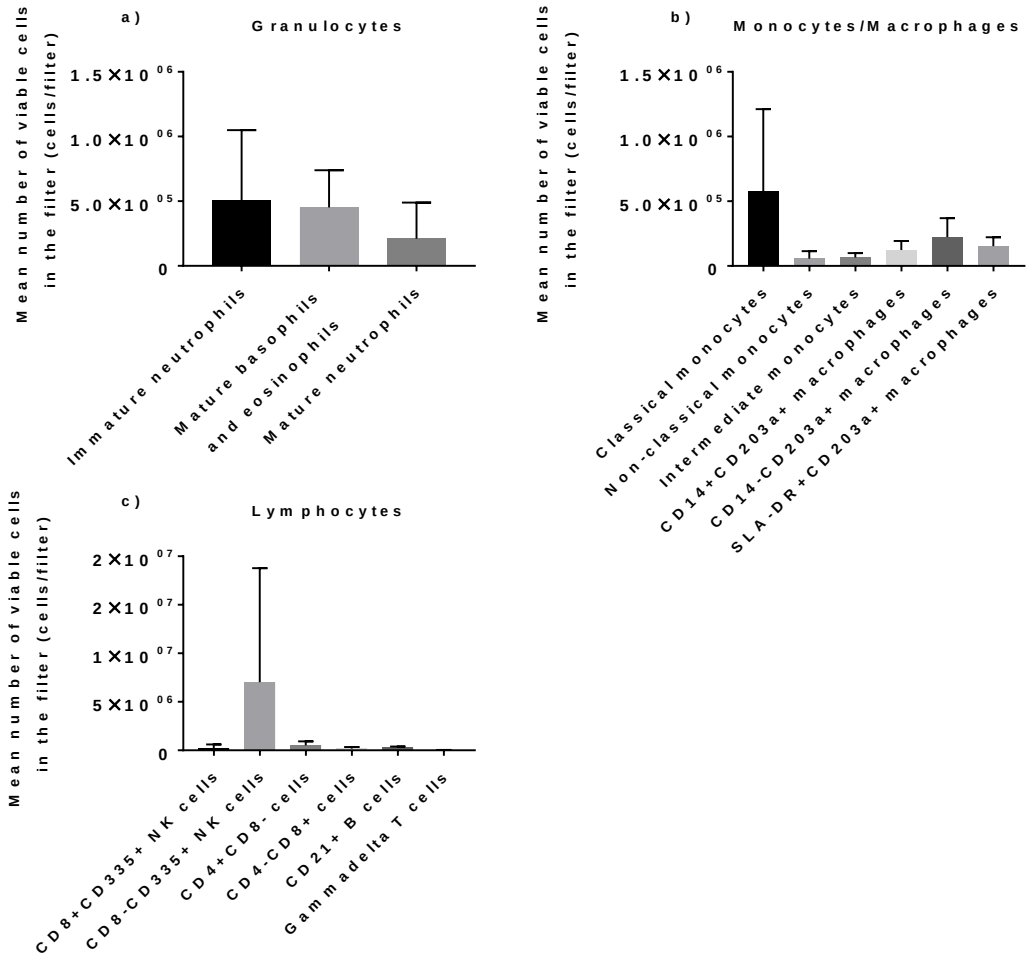

Figure 4.4 – Immune populations are sequestered by the leucocyte filter. Cells are grouped as granulocytes (a), monocytes and macrophages (b), and lymphocytes (c). The leucocyte pattern detected in the filter reflects that observed in the baseline reference of the donor heart, with NK cells the major population. However, immature neutrophils represent the largest granulocyte population rather than basophils and eosinophils. Only low numbers of B cells are detected in the filter.

4.3.5 CCHP mediates a specific inflammatory storm dominated by interferon-γ release:

A profile of the cytokine content of the perfusate was produced to determine the signals released from the donor heart. Of the 12 cytokines analysed, only four were detected in the perfusate (IFN-γ, TNF-α, IL-18 and GM-CSF, figure 4.5). The concentration of IFN-γ increased significantly over the perfusion period (p=0.003), starting off low at baseline (883.3±1379pg/ml), but rose rapidly to 4448±980pg/ml and 6223±2194pg/ml at 2 and 4 hours respectively, reaching a peak at 6 hours (7518±1348pg/ml) and remaining elevated at 8 hours (6883±1830pg/ml). TNF-α also increased significantly in the perfusate over time, peaking at 4 hours and remaining elevated thereafter (baseline: undetectable; 2 hours: 38.33±11.69pg/ml; 4 hours: 55.00±13.78pg/ml; 6 hours: 51.67±7.53pg/ml; 8 hours: 50.00±8.94pg/ml, p=0.001). IL-18 increases significantly during perfusion, although the concentration remains relatively stable from 4 hours on (baseline: 40±80pg/ml; 2 hours: 81.67±85.19pg/ml; 4 hours: 123.3±77.37pg/ml; 6 hours: 125±65.65pg/ml; 8 hours: 128.3±69.98pg/ml, p=0.001). GM-CSF also increases significantly over time but remains at a relatively low concentration throughout (baseline: 15±16.43pg/ml; 2 hours: 40±8.94pg/ml; 4 hours: 45±12.25pg/ml; 6 hours: 50±12.65pg/ml; 8 hours: 46.67±35.59pg/ml, p=0.021). The concentration of IFN-γ released by the isolated donor heart and its contents was by far the most markedly increased by CCHP.

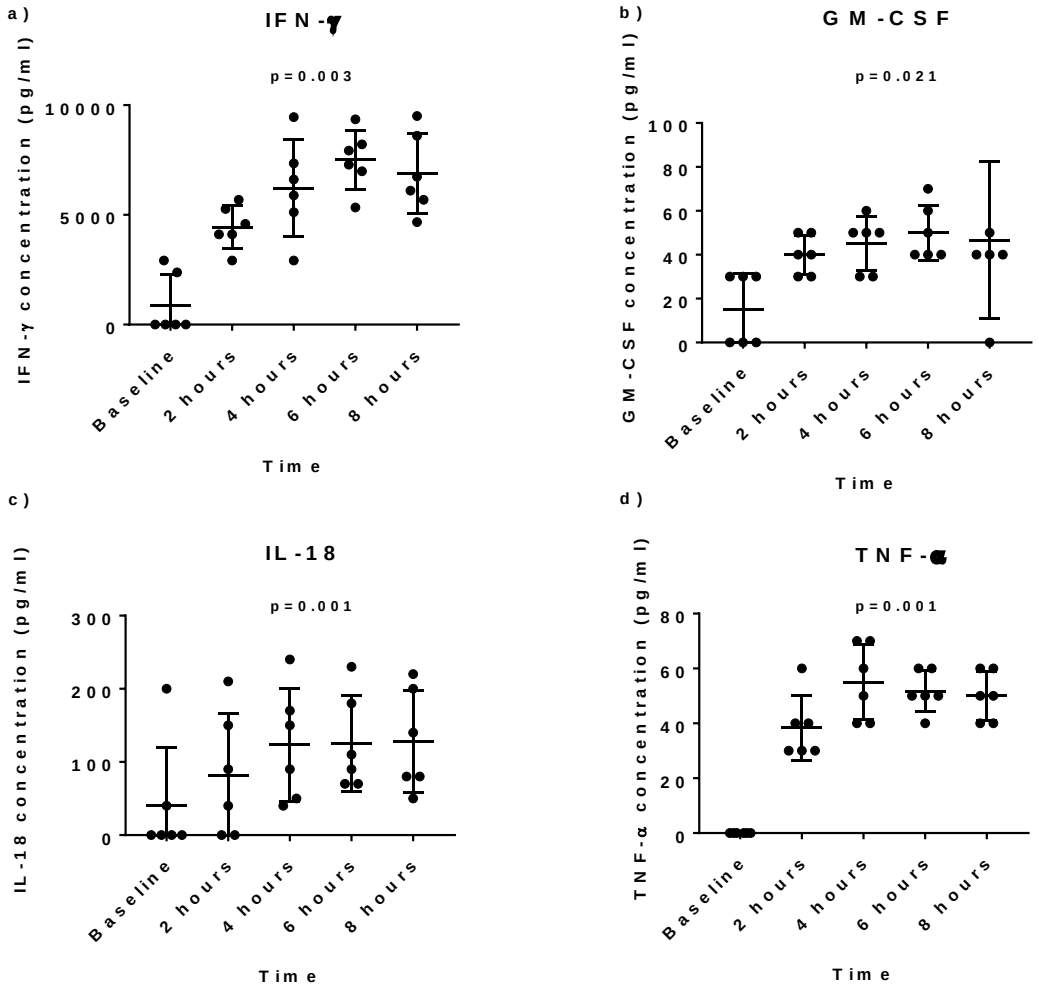

Figure 4.5 – Cytokine secretion increases over time during perfusion. All 4 cytokines detected are increased significantly as perfusion progresses, although IFN-γ (a) is released at markedly greater concentrations than GM-CSF (b), IL-18 (c) and TNF-α (d). The isolated heart and its contents demonstrate the capacity to generate and secrete a significant pro-inflammatory response, driven mostly by IFN-γ.

4.3.6 Chemokine release is induced by CCHP:

In order to determine whether leucocyte migration from the tissue occurred in response to specific chemotactic signals, the perfusate was assessed to detect 7 chemokines (figure 4.6). Due to the high level of IFN-γ previously detected, a predominant focus was placed on chemokines induced by or responsive to IFN-γ stimulation. CCL5 and CXCL11 were not detectable at any point throughout perfusion. However, all other chemokines were released into the circuit. CXCL8 concentration increased significantly over time, peaking at 4 hours but remaining elevated up to 8 hours (baseline: 16.67±13.66pg/ml; 2 hours: 28.33±7.53pg/ml; 4 hours: 38.33±7.53pg/ml; 6 hours: 35.00±5.48pg/ml; 8 hours: 35.00±8.37pg/ml, p=0.001). A stepwise but small increase in CCL2 was observed in the perfusate over time, peaking at 8 hours (baseline: 31.93±4.19pg/ml; 2 hours: 33.45±5.56pg/ml; 4 hours: 33.70±6.23pg/ml; 6 hours: 36.16±9.45pg/ml; 8 hours: 37.47±12.35, p=0.021). A large increase in CXCL9 occurred in response to perfusion up to 8 hours (baseline: 9.67±15.48pg/ml; 2 hours: 39.82±41.72pg/ml; 4 hours: 45.68±44.60pg/ml; 6 hours: 59.90±54.72pg/ml; 8 hours: 69.40±58.99pg/ml, p<0.001). CCL4 and CXCL10 were detected in the perfusate from 4/6 and 2/6 pigs respectively, and thus demonstrated no statistically significant changes over time (p=0.184 and p=0.255 respectively).

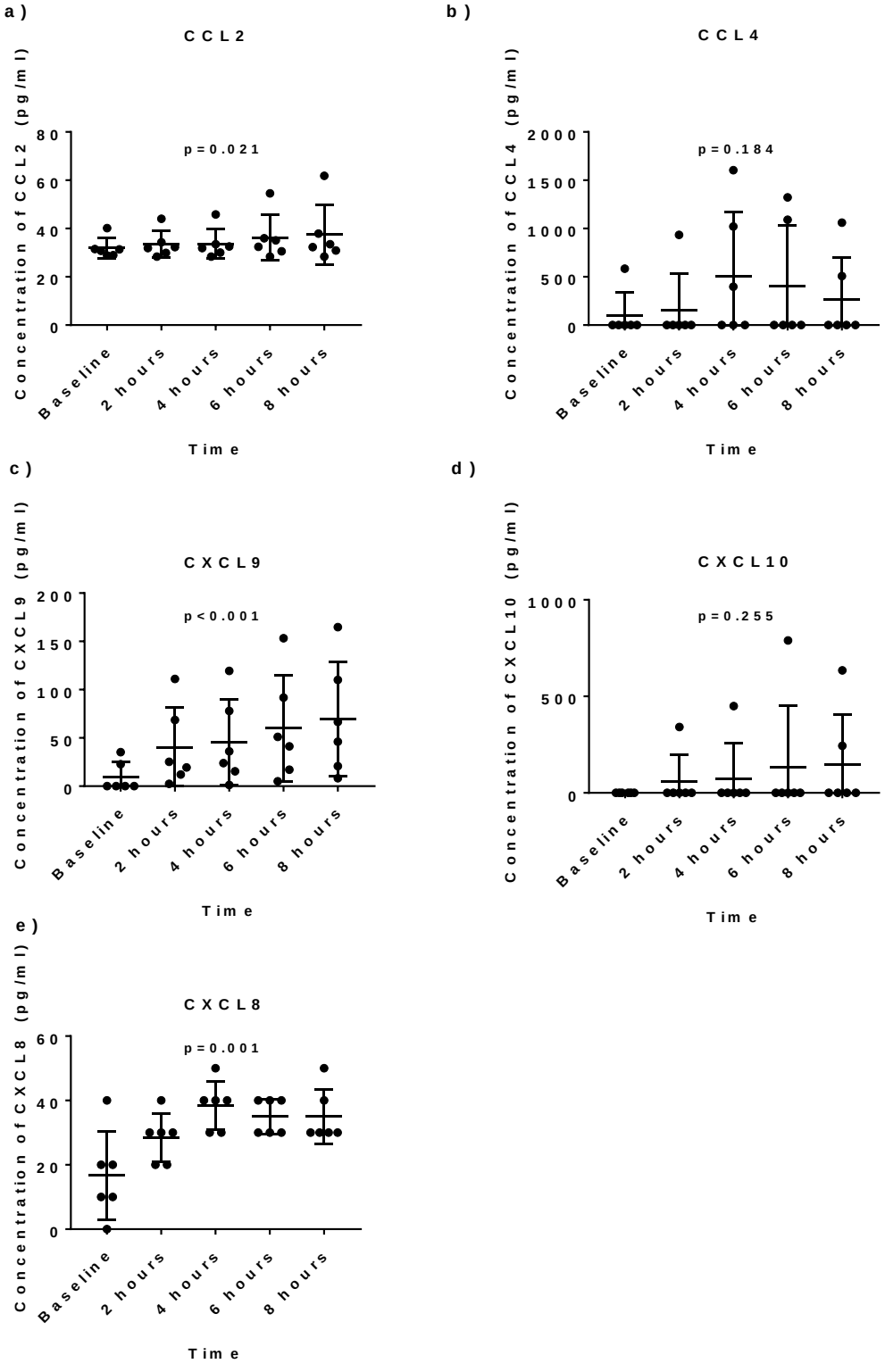

Figure 4.6 – Chemokine release during perfusion is dominated by CXCL9, CXCL8 and CCL2. The release of each of these chemokines is significantly increased in response to perfusion. These chemokines may contribute to the active migration of leucocytes out of the heart and into the circuit.

4.3.7 Impact of ischaemia reperfusion injury following CCHP:

A profile of the immunodepleted tissue was performed to assess whether the phosphorylation status of a broad range of protein kinases was altered following CCHP. Biopsies taken prior to and following CCHP were analysed from n=6 organs. Of the phosphokinases analysed, these findings demonstrate that 6 that are intrinsically linked to ischaemia reperfusion injury were significantly diminished in the left ventricle following CCHP. Significantly reduced tissue expression was observed for Tyrosine^{689}-phosphorylated signal transducer and activator of transcription (STAT)2 (14.62 [0.76-19.55]% reduction, p=0.044), Tyrosine^{694}/Tyrosine^{699}-phosphorylated STAT5a/b (18.26 [13.35-22.85]% reduction, p=0.011), Tyrosine^{694}-phosphorylated STAT5a (19.71 [14.23-35.82]% reduction, p=0.028), Tyrosine^{641}-phosphorylated STAT6 (13.00 [6.29-20.61]% reduction, p=0.009), Serine^{133}-phosphorylated cyclic adenosine monophosphate response element binding protein (CREB) (28.81 [6.40-61.51]% reduction, p=0.045) and Threonine^{60}-phosphorylated with no K (lysine) protein kinase 1 (WNK1) (50.79 [12.52-55.34]% reduction, p=0.022).

4.3.8 CCHP is associated with improved apoptotic status:

Next, a broad proteomic screen of the donor heart tissue was performed to determine whether perfusion alters the expression of apoptosis/survival proteins. Biopsies taken prior to and following CCHP were analysed from n=6 organs. Of the 35 proteins analysed, 9 demonstrated diminished expression in the immunodepleted tissue compared to baseline. Significantly reduced relative expression of Serine^{46}-phosphorylated p53 (17.06 [0.49-25.3]% reduction, p=0.046), TNF receptor 1 (12.58 [7.20-19.95]% reduction, p=0.009), death receptor 5 (10.27 [8.96-18.52]% reduction, p=0.001), heme oxygenase 1 (12.83 [4.48-19.01]% reduction, p=0.015), Bad (12.03 [6.74-20.19]% reduction, p=0.034), B cell lymphoma-x (Bcl-x) (35.56 [0.74-43.62]% reduction, p=0.041), pro-caspase-3 (14.45 [5.58-30.46]% reduction, p=0.019), claspin (13.97 [3.49-27.14]% reduction, p=0.045) and clusterin (22.23 [9.30-32.17]% reduction, p=0.018) was observed following CCHP (figure 4.7).

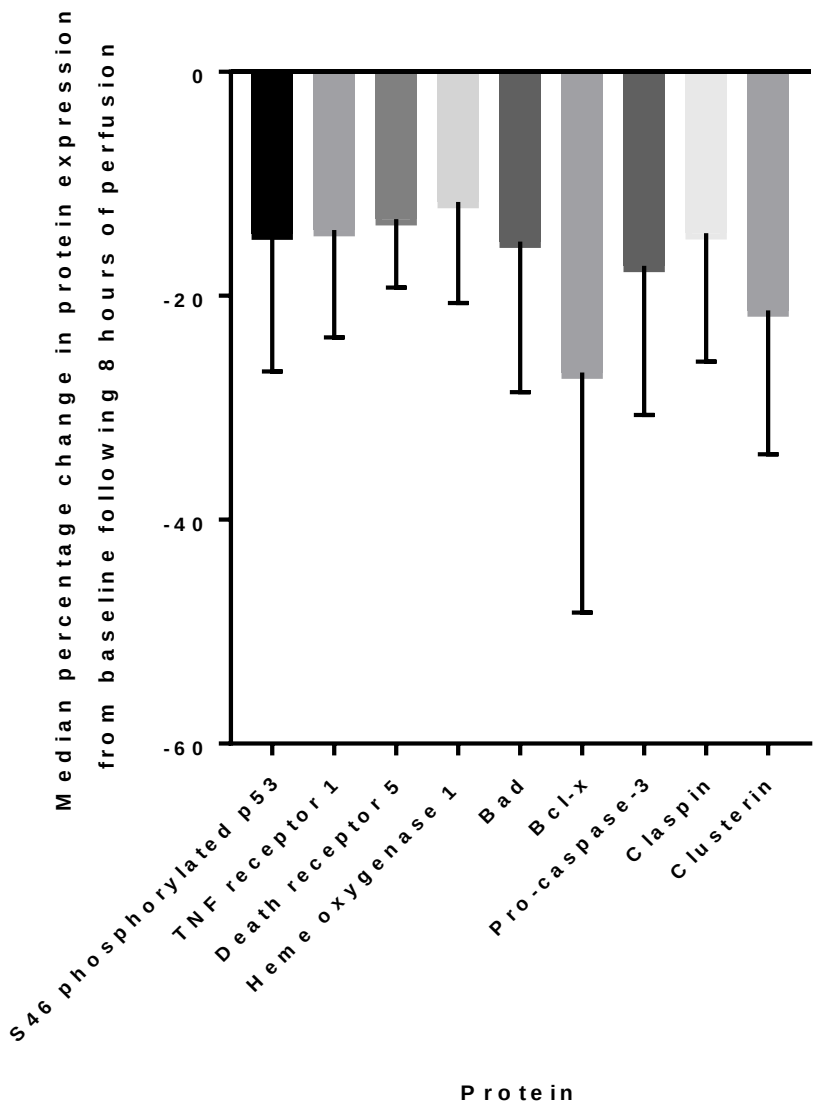

Figure 4.7 – Apoptosis-related protein expression is diminished compared to baseline tissue following 8 hours of CCHP. This may be related to active downregulation of apoptosis pathways, promoting tissue survival after perfusion and thus inducing protective pathways prior to reperfusion. It may also be related to the loss of apoptosis proteins from leucocytes as a result of perfusion. TNF = Tumour necrosis factor, S46 = serine 46, Bcl = B cell lymphoma.

4.3.9 Cell-free DNA is released during perfusion:

In order to further characterise the inflammatory properties of the perfusate, qPCR was utilised to detect cell-free DNA. Cell-free DNA represents a marker of leucocyte activation, inflammation and cell injury. These data demonstrate that CCHP is associated with steadily increasing release of both mitochondrial (baseline: 0.00±0.00ng/µl; 2 hours: 0.012±0.015ng/µl; 4 hours: 0.017±0.010ng/µl; 6 hours: 0.014±0.011ng/µl; 8 hours: 0.012±0.008ng/µl, p=0.063) and genomic DNA (baseline: 0.006±0.013ng/µl; 2 hours: 0.038±0.034ng/µl; 4 hours: 0.063±0.042ng/µl; 6 hours: 0.072±0.050ng/µl; 8 hours: 0.077±0.055ng/µl, p=0.037) within the perfusate (figure 4.8). Genomic DNA was released at a significantly higher concentration than mitochondrial DNA at all time points (p=0.009).

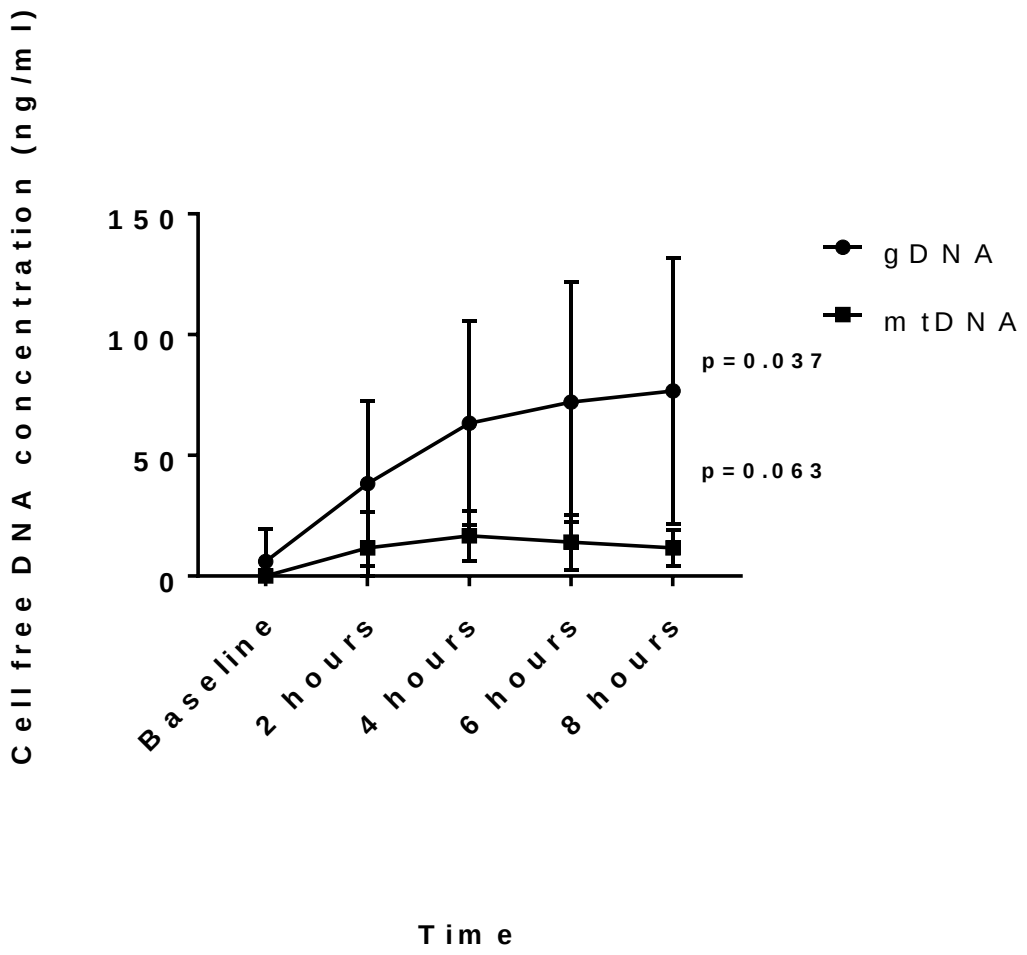

Figure 4.8 – Perfusion is associated with cell-free DNA release. Cell-free DNA is released into the perfusate at increasing concentrations over time. Mitochondrial DNA peaks at approximately 4 hours, whereas genomic DNA peaks at 8 hours. Genomic DNA is detected at higher concentrations than mitochondrial DNA.

4.3.10 Tissue viability is maintained throughout the course of perfusion:

Any novel strategy to immunodeplete a donor organ must also ensure that tissue integrity is not impaired. The development of oedema is a problem that can lead to poor cardiac function. Post-perfusion water content of the heart was demonstrated to be 80.58±1.46%, which is similar to previously reported data for control hearts[121, 122], suggesting a lack of oedema formation.

As a clinically relevant end-point, a blinded histological analysis of pre and post perfusion tissue architecture and apoptotic signalling was performed by a consultant histopathologist. These findings indicate that CCHP preserves the myocardium with no significant ischaemia or endothelial disruption after 8 hours (figure 4.9). Furthermore, caspase-3 expression remained undetectable in the muscle, endothelium and fibroblasts, although apoptotic leucocytes were observed (figure 4.9). Interestingly, 3 of the 6 hearts displayed signs of low level injury prior to CCHP, including oedema and endothelial swelling, which was normalised by 8 hours of hypothermic perfusion.

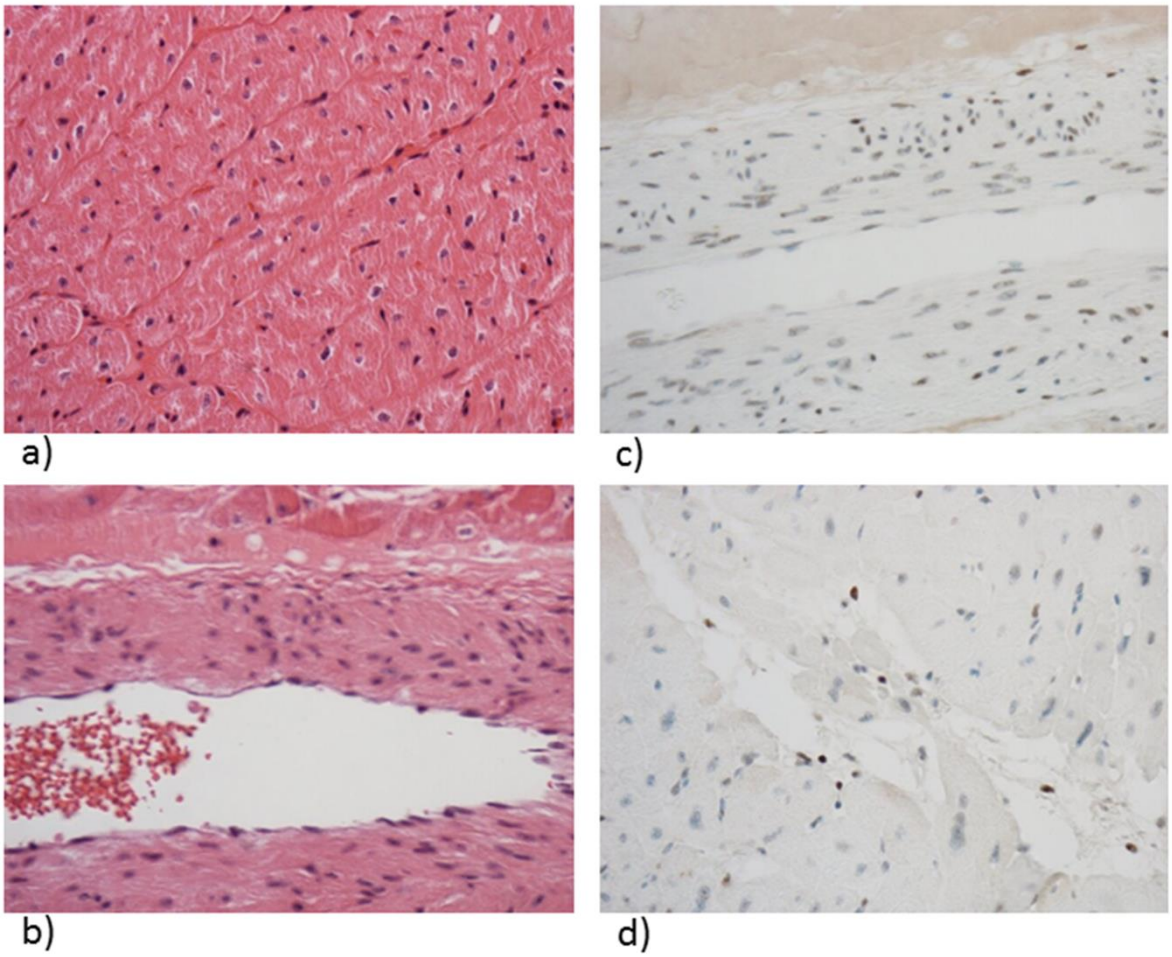

Figure 4.9 – Tissue architecture and structural integrity are maintained throughout perfusion. No oedema or damage to muscle (a) or endothelial cells (b) were observed after perfusion. No caspase-3 induction was observed in the muscle, endothelium or fibroblasts (c), but was detected in leucocytes (d).

4.3.11 CCHP is not associated with myocardial injury:

These experiments have demonstrated that perfusion immunodepletes the heart whilst preserving organ viability at a tissue level. As a final validation that tissue viability is maintained, extracellular cardiac troponin I was quantified in perfusate from all n=6 hearts as a sensitive marker of myocardial damage at a cellular level. These findings demonstrate that CCHP does not mediate significant injury to the myocardium, with cardiac troponin I undetectable at 8 hours in 4/6 hearts. Overall, troponin concentration remained stable during perfusion (median [IQR]; baseline: 0.00 [71.16]; 2 hours: 0.00 [53.83]; 4 hours: 27.31 [61.56]; 6 hours: 0.00 [78.01]; 8 hours: 0.00 [86.75], p=0.930).

4.3.12 CCHP significantly diminishes post-transplant graft infiltration at 48 hours in the absence of immunosuppression:

A pilot series of heterotopic transplants were performed to determine the downstream effects of perfusion upon recipient immune recruitment into the graft post-transplantation. CCHP was associated with diminished graft infiltration compared to static cold storage as determined by percentage of the total cardiac tissue affected at 48 hours (cold stored vs. perfused: 23.0±2.8 vs. 11±2.8, figure 4.10). This was true for distribution of leucocytes within the coronary arteries (cold stored vs. perfused: 50.0±28.3 vs. 20.0±0.0, figure 4.11), left ventricle (cold stored vs. perfused: 12.5±3.5 vs. 7.5±3.5, figure 4.11), right ventricle (cold stored vs. perfused: 15.0±14.1 vs. 5.0±0.0, figure 4.11) and septum (cold stored vs. perfused: 15.0±0.0 vs. 12.5±10.6, figure 4.11). It was further observed that alongside the effect on tissue distribution, the intensity of the infiltration was also diminished by CCHP at 48 hours. Overall intensity of infiltration for perfused donor hearts was considered to be mild, whereas overall intensity in the cold stored hearts was determined as moderate infiltration at 48 hours (figure 4.12).

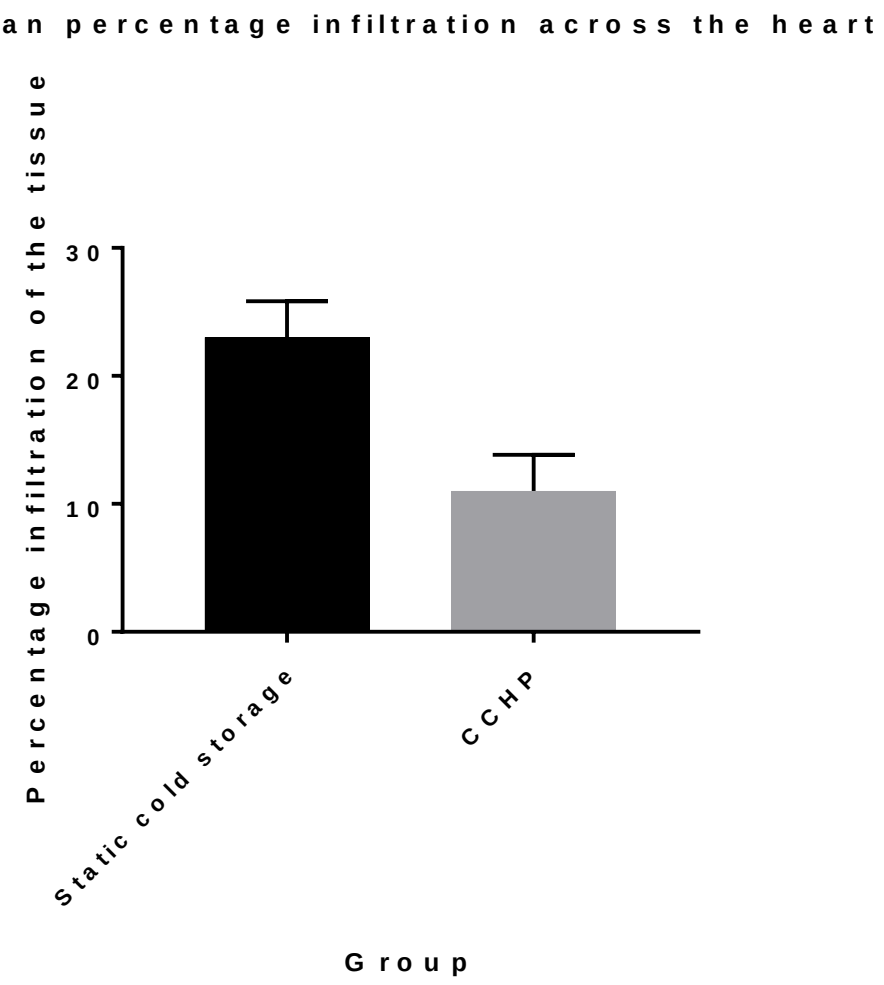

Figure 4.10 – Graft infiltration after heterotopic transplantation. Cold cardioplegic perfusion is associated with a reduction in mean leucocyte infiltration at 48 hours. Data is displayed for n=2 transplants in each group. CCHP = cold cardioplegic heart perfusion.

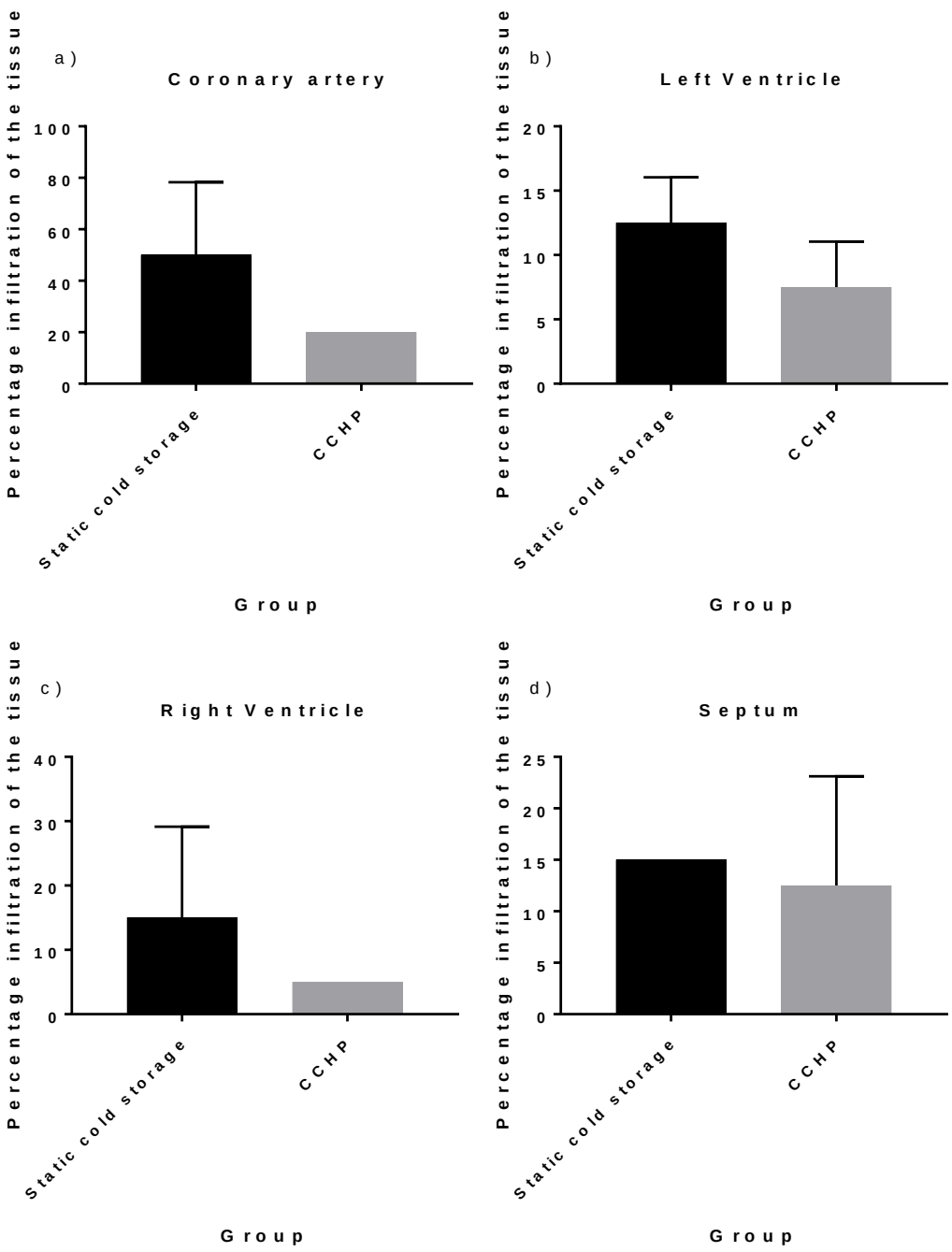

Figure 4.11 – The distribution of leucocytes across each region of the heart in cold stored and perfused donor hearts. Lower infiltration by recipient leucocytes was observed in the CCHP group compared to static cold storage in the coronary arteries (a), left ventricle (b), right ventricle (c) and septum (d). Data is displayed for n=2 transplants in each group. CCHP = cold cardioplegic heart perfusion.

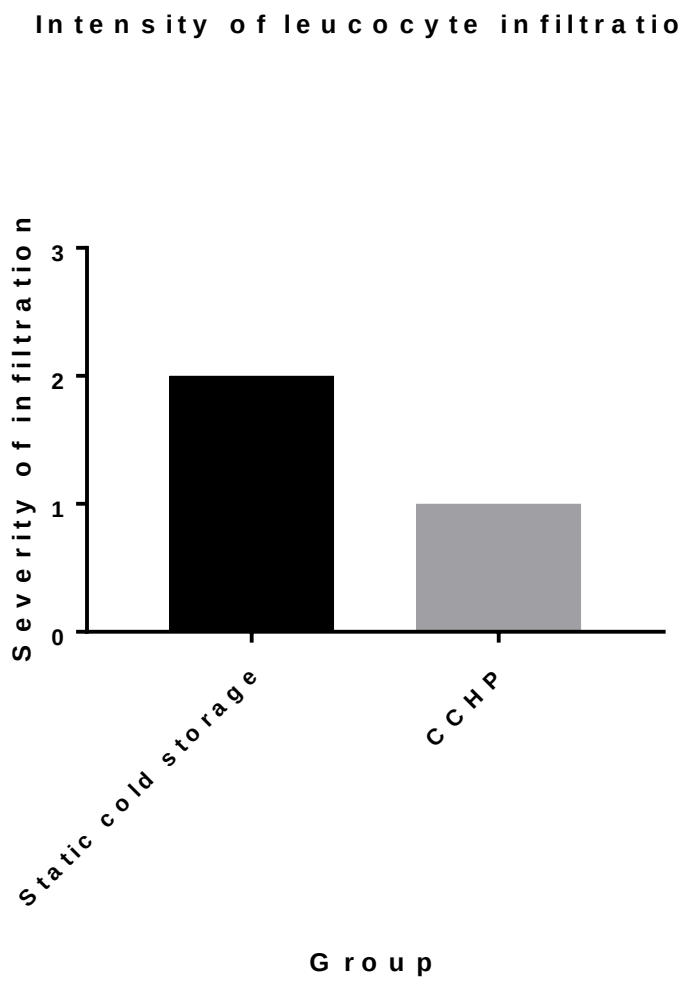

Figure 4.12 – Intensity of leucocyte infiltration is reduced by CCHP. Both donor hearts preserved by cold cardioplegic perfusion were deemed to have only mild infiltration across the heart, whereas those preserved by static cold storage were considered to have moderate infiltration. Data displayed is representative of n=2 transplants in each group. CCHP = cold cardioplegic heart perfusion.

4.3.13 The benefit of CCHP on graft infiltration is not sustained beyond 48 hours:

In order to determine the optimal time point for further evaluation of the effects of perfusion-mediated immunodepletion on graft infiltration, further heterotopic transplants were performed. By day 3, the level of graft infiltration was increased in both groups compared to that observed at 48 hours. There was a much greater level of variability in the perfused group at day 3, with one organ still displaying very low infiltration whilst the other displayed far greater lymphocytic presence. Taken together, the perfused group still demonstrated lower mean infiltration compared with the static cold stored organ (mean 46.0±0.0% vs. 23.5±19.1%, for cold stored and perfused respectively, figure 4.13). The severity of infiltration was similar between groups, with the cold stored heart considered severe and the perfused hearts each scored as moderate and severe (figure 4.13). By day 5 and day 10, all hearts from both groups had ceased to contract and displayed macroscopically apparent infiltrates along all major vessels. The loss of contractility as a result of the infiltration highlighted the extent of the effect and no further analysis was performed on these organs.

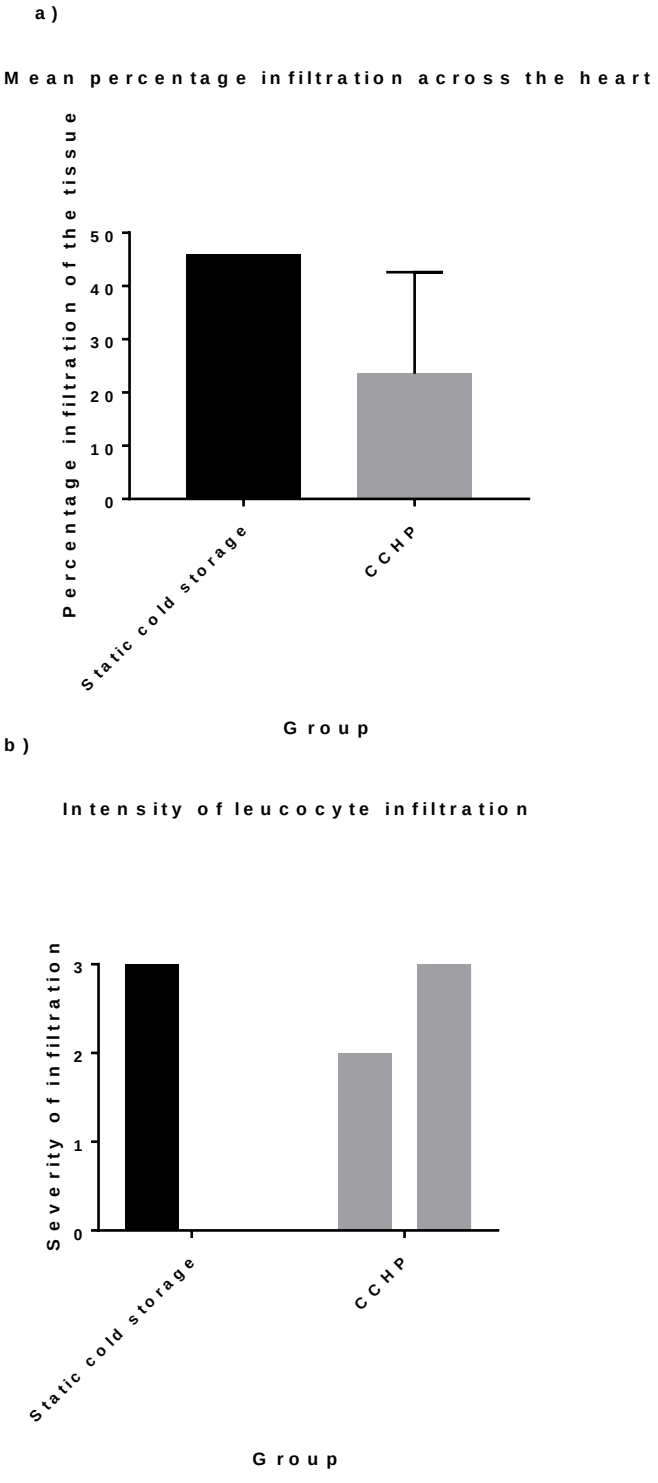

Figure 4.13 – Graft infiltration at 72 hours post-transplant. Infiltration of the graft is lower in the perfused group at 72 hours (a), although there was high variation in the level of lymphocytic presence observed. The intensity of leucocyte infiltration into the graft was similar at 72 hours between the groups (b), with an improved score only in 1/2 CCHP hearts.

4.4 Discussion:

Allograft rejection occurs via priming of recipient allospecific T cells which subsequently infiltrate and damage the heart. Whilst current therapies predominantly target recipient T cells, immunomodulation at an earlier stage would be advantageous. A significant role has been described previously for passenger leucocytes in the induction of T cell alloreactivity following lung transplantation[65]. *Ex vivo* lung perfusion significantly diminished donor leucocyte transfer into recipient lymph nodes, where T cell priming occurs. This led to a marked reduction in T cell graft infiltration, a hallmark of acute rejection. However, it remained unclear whether similar benefits would be observed in other organs with less well defined resident immune repertoires.

This series of experiments utilised a recently developed heart preservation device to assess the impact of perfusion on the donor heart immune repertoire. This study describes for the first time that the heart possesses a significant immune population which could contribute significantly to the activation of recipient immunity. The large proportion of NK cells in the donor heart is novel and surprising considering the low ratio of the lymphocyte population that NK cells normally comprise in other organs[123]. However, important roles have previously been ascribed to NK cells in a range of cardiac-related conditions[124], including transplantation[125]. The potential role of donor derived NK cells in allorecognition following transplantation warrants further investigation.

Antigen presenting cells represent other significant cardiac-resident leucocyte populations which mobilise to recipient lymph nodes upon revascularisation. Once there, these donor leucocytes prime recipient allospecific T cells to promote graft infiltration. Importantly, this study demonstrates that perfusion induces a broad mobilisation and immunodepletion of the donor heart without the use of targeted therapeutics or chemoattractants within the circuit. This mobilisation is likely to occur immediately following revascularisation of the donor heart, and highlights the potential importance of the donor as a therapeutic target for immunomodulation. The cause of this mobilisation is unclear but may occur in response to high cytokine and chemokine levels in the perfusate, which was dominated by IFN-γ. The high concentration of IFN-γ (reaching >7000pg/ml) was unexpected from a donor heart in

isolation, and again brings further evidence to the role of the donor immune response in the immediate events following transplantation. The source of IFN-γ remains unknown, but NK cells are a prominent source of this cytokine and given the large numbers observed leaving the donor myocardium, may represent a target cell to immunomodulate the donor[126]. Hypothetically, CCHP may exhaust the IFN-γ response from the donor heart during perfusion, reducing this cytokine being secreted following transplantation. Again, this warrants investigation.

IFN-γ is not known to have direct chemotactic properties but has a well-documented role in inducing the release of chemokines[127], many of which were detected in this study. The release of such a milieu of IFN-γ associated proteins suggests a prominent role for this signalling network in mediating leucocyte migration from the heart. This is particularly true for CXCL9 which if neutralised, prevents IFN-γ secretion and is essential for donor specific T cell reactivity[128]. CXCL9 increases rapidly following revascularisation and plays a key role in recruitment of a broad range of leucocytes to the graft[129]. As such it has been proposed as a potential therapeutic target as well as a biomarker of acute rejection, where peripheral blood concentrations above 160pg/ml have been suggested as a 'rejection positive' result. Given that this study reports CXCL9 levels ranging between 70-160pg/ml, the donor heart may represent a primary source immediately following transplantation. Aside from CXCL9 significant increases were also observed in CCL2, which is a potent inducer of monocyte migration[130]. The loss of granulocytes, in particular neutrophils, may occur in response to the secretion of CXCL8, which is an established neutrophil chemoattractant.

Interestingly, significant T cell depletion from donor hearts was not observed despite the presence of CXCL9. This suggests that these T cells represent either a static non-migratory resident population, require further signals such as CCL5 or CXCL11 (which were not detected), or that these cells are able to home back to the tissue after migration. It remains unclear whether additional benefit would be derived from actively encouraging the removal of this and other populations not depleted by CCHP. Further characterisation of the behavioural phenotypes of these cells and the differences between resident and migrated leucocytes is warranted.

The loss of donor leucocytes could have profound effects once transplanted with the organ. The in-line leucocyte filter may be responsible for sequestering a proportion of

these cells, although it is likely to have become saturated relatively quickly. The interaction with the plastic consumables within the circuit may have induced leucocyte death, thus accounting for the additional population lost. This is supported by the detection of increasing levels of cell-free DNA which is released upon apoptosis and necrosis[131].

Whilst immunodepletion of the organ has potentially vast clinical implications due to reduced donor-recipient interactions, it is critical that this occurs without stress to the donor heart. This study demonstrates that 8 hours of CCHP maintains the viability of the tissue, with no observable oedema or endothelial damage. This was accompanied by low/undetectable levels of cardiac troponin I in the perfusate, suggesting myocardial integrity was maintained at a sub-cellular level. Moreover, tissue obtained at the end of perfusion displayed a molecular signature indicative of reduced ischaemia reperfusion injury compared with matched tissue taken freshly at retrieval following cardioplegia. From this study it appears that CCHP alone reduces STAT5 and STAT6 pathway activation which contributes to myocardial injury following ischaemia and reperfusion[132, 133]. This was further confirmed by histological analysis with no detectable caspase-3 induction in endothelium, cardiomyocytes, smooth muscle or fibroblasts. However, some apoptosis was observed in the remaining tissue resident leucocytes, supporting the suggestion of immune cell death as the source of cell-free DNA.

The clinical implications of 8 hour preservation without loss of tissue integrity and with diminished donor immune load are clear and substantial. This method represents a significant improvement over current strategies, with minimal ischaemic time and the induction of protective survival pathways prior to the ischaemic period required during implantation. This may reduce the initial injurious impact of reperfusion and minimise the transfer of passenger leucocytes, which are important in determining post-transplant outcomes.

Most importantly, these initial results post-transplantation indicate that the use of CCHP is associated with a clinically relevant reduction in leucocyte recruitment into the graft until at least 48 hours in the absence of immunosuppression when compared to standard storage on ice. It is possible that this effect may be sustained until 72 hours, as there still appeared to be lower levels of lymphocytes present at this point. However,

by day 5 any benefit is undetectable as all hearts are grossly dysfunctional and highly oedematous, potentially as a result of the activation of the indirect allorecognition pathway. Despite the loss of effect after the early period, this study provides novel evidence that immunodepletion of the donor heart can significantly alter the early clinical course post-transplant without the requirement for further therapeutic intervention. Incorporating this method into clinical practice could potentially allow the use of more immunosuppression-sparing regimens. These exciting findings require translation and validation with discarded human tissue prior to incorporation into clinical practice, although the technique clearly holds great promise for revolutionising donor heart storage. Further work is necessary to establish why this effect is not sustained beyond 48-72 hours.

4.5 Limitations:

The donor pigs utilised in this study did not undergo brain death prior to heart retrieval. Furthermore, no injury was induced to the heart to replicate a DCD scenario, meaning that the heart was healthy and functional upon retrieval. The leucocyte content of the donor hearts in this study therefore represents the repertoire of the healthy heart, which may be altered upon brain death or circulatory arrest as would be experienced prior to retrieval in the clinical setting. Furthermore, this data did not allow the source of the cell-free DNA observed within the perfusate to be determined definitively. However, leucocyte apoptosis/necrosis may be a major contributor as no damage was observed to the myocardium, smooth muscle, endothelium or fibroblasts, yet there was notable leucocyte loss from the tissue combined with observable caspase-3 expression in immune cells. Only the left ventricle was studied in terms of both leucocyte numbers and molecular alterations. It is assumed that this would be observed globally across the heart, although it cannot be confirmed by the data in this study. Furthermore, the number of leucocytes present at the end of perfusion is complicated by the fact that the heart was not flushed prior to obtaining the biopsy. As such, the vessels will still retain some perfusate, and any leucocytes present in the perfusate will thus be included in the left ventricular count. Whilst this is a limitation of the study, it does mean that the level of immunodepletion presented here is a conservative estimate. The true immunodepletion effect is actually likely to be greater than that detailed here. No biopsy was taken prior to perfusion for the purposes of calculating water content of the tissue, which limits the strength of the weight:dry weight data presented post-

perfusion as no paired comparison with the same tissue could be performed. However, it was necessary to minimise the damage induced to the heart prior to perfusion to ensure the clearest possible effect. The values obtained are instead compared with data from the published literature, which suggest that the post-perfusion tissue was within the expected range for control hearts. The number of heterotopic transplant experiments performed was low, precluding any direct comparison between the groups although these were included to provide pilot data to guide subsequent investigation.

4.6 Conclusion:

This study demonstrates for the first time that the donor myocardium contains a significant immune population capable of mediating a strong recipient alloresponse and reinforces the importance of the donor as a therapeutic target for immunomodulation. It also provides evidence that *ex vivo* perfusion alters the immune content and molecular signature of the donor heart, which in turn reduces recipient T cell recruitment to the graft up to 48 hours following transplantation in the absence of immunosuppression. Incorporating cold cardioplegic perfusion into clinical practice could potentially allow the use of more immunosuppression-sparing regimens. The preservation of donor hearts via hypothermic, cardioplegic perfusion may provide significant benefit through both improved organ viability for an extended period and diminished immunogenicity.

Chapter 5: Programmed death ligand 1 is upregulated on graft endothelium in response to IFN-γ release during cold cardioplegic heart perfusion

5.1 Introduction:

Current immunosuppressive protocols are non-selective and inhibit the proliferation or activation of all recipient T cells, irrespective of the antigenic determinant to which they have specificity. This means that the protective action of T cells with specificity towards, for example, infectious agents are also inhibited. The development of alternative strategies to promote acceptance of the donor graft without the requirement for such potent drugs is necessary.

Graft rejection occurs as a result of recipient allospecific T cell activation and recruitment into the donor organ, where it can induce significant damage to the tissue[134]. This is a highly co-ordinated series of events, requiring the interplay of many different cell types. The role of donor endothelial cells is incompletely defined but they are known to be a significant target for the recipient alloresponse and are involved in graft rejection through a number of mechanisms[110]. This includes modulation of leucocyte function through direct cell-cell communication as well as enabling the diapedesis of leucocytes from the vasculature into the donor tissue itself. Leucocyte extravasation across the endothelial barrier initially takes place in the form of rolling adhesion, whereby adhesion molecules on the endothelium bind to corresponding ligands on the leucocyte. Whilst this bond is only transitory due to the velocity of leucocyte movement through the blood, it continues along the length of the vessel and the affinity of binding is increased as the leucocyte gets closer to the site of interest[135]. This causes the number of binding events to increase and their duration is extended, effectively slowing the movement of the cell. Once the cell is stopped and adhered to the endothelium at the required site, the leucocyte is then able to either communicate directly with the endothelial cell or transmigrate across this barrier. Activation of the endothelium, which occurs in response to damage associated molecular patterns and surgical trauma, inevitable during transplantation, leads to significantly augmented expression of adhesion molecules[136], promoting the infiltration of recipient leucocytes.

Endothelial cells are further able to modulate T cell function during the period in which the cells are in direct contact. T cell responsiveness requires more than simply antigenic challenge; secondary signals are also necessary. Co-stimulation is required for activation of the T cell, which is generally provided by dendritic cells but can also be provided to memory T cells by the activated endothelium[137]. Endothelial cell

activation is well-documented in response to the trauma of transplantation, leading to significant upregulation of adhesion molecules and MHC class II[138], amplifying antigen presentation capability.

Improving endothelial cell status during organ preservation could significantly alter the signalling provided by these cells to the recipient leucocytes. Endothelial quiescence rather than activation could minimise the expression of adhesion molecules, diminishing the infiltration of the graft following transplantation. Importantly, whilst endothelial cells are able to provide antigenic delivery and T cell stimulation, they are also able to induce expression of co-inhibitory molecules, which in turn induces T cell tolerance rather than activation[139, 140]. Enhancing the expression of co-inhibitory molecules could significantly alter the post-transplant tissue environment and subsequent alloresponse. Programmed death ligand 1 (PD-L1) is one such inhibitory immune checkpoint molecule, which has demonstrated the capacity to prevent T cell activation[141], and blockade of this pathway has been suggested as a therapeutic strategy against cancer[142]. PD-L1 could thus play an important role if elevated prior to transplantation.

Findings from chapter 4 have demonstrated that *ex vivo* perfusion of the donor heart maintains viable endothelium for at least 8 hours. However, the graft is exposed to significantly elevated concentrations of IFN-γ, a pleiotropic cytokine with both pro- and anti-inflammatory functions under distinct conditions. As such, it is unclear what effect this cytokine would have on the endothelium, particularly with regard to the expression of immunologically-important markers. Furthermore, *ex vivo* perfusion appears to exert a protective effect in terms of reduced graft infiltration over the first 48 hours (as demonstrated in chapter 4). It is possible that this is related to an immune inhibitory mechanism that is upregulated during perfusion. However, infiltration is restored by day 5, indicating that this mechanism of protection is lost at this point. It was hypothesised that immune checkpoint molecules could be responsible for mediating the protective effect of perfusion but that there expression may be only transient. This study aimed to determine the effect of IFN-γ at a range of concentrations on the expression of MHC class II and the co-inhibitory immune checkpoint molecule PD-L1 on human endothelial cells. The duration of any expressional changes were subsequently established.

5.2 Methods:

5.2.1 Cell culture:

Human aortic endothelial cells from more than two individual donors (Promocell, Heidelberg, Germany) were utilised for all *in vitro* experiments. Cells were cultured in a humidified incubator at 37°C in commercially available endothelial growth medium MV2 (Promocoll, Heidelberg, Germany). The growth medium was supplemented with 5% foetal bovine serum, 100mg/ml streptomycin and 100U/ml penicillin (all from Sigma Aldrich, Dorset, UK).

5.2.2 The effect of IFN-γ on the endothelium:

In order to determine the effect of different concentrations of IFN-γ on the endothelium, human aortic endothelial cells at passage 6 were plated onto cell culture wells at $1x10^6$ cells/ml. The cells were grown to 60% confluency (after approximately 24 hours) before stimulation with IFN-γ at varying levels: 1000pg/ml, 2000pg/ml, 4000pg/ml, 8000pg/ml or 80000pg/ml or diluent as an unstimulated control. After 24 hours of incubation with the cytokine, the cell culture medium was removed and discarded. The cells were subsequently obtained from the plate by manual scraping. This method was preferable to enzymatic harvesting using trypsin, which is associated with protein expression changes that could have altered the findings[143]. Cells retrieved from the plate were resuspended in 1ml phosphate buffered saline for subsequent analysis by flow cytometry. All cultures were performed in triplicate.

5.2.3 Temporal effect of IFN-γ on the endothelium:

To delineate the duration of effect of IFN-γ, a series of cultures were performed with different cytokine exposure periods. Human aortic endothelial cells ($1x10^6$ cells/ml) at passage 8 were plated onto cell culture wells and grown to 60% confluency. At this point, the cells were stimulated differentially with 1) diluent for 48 hours without washing; 2) diluent for 24 hours followed by medium replacement and a further 24 hour incubation; 3) 4000pg/ml IFN-γ for 24 hours followed by removal of IFN-γ and a further 24 hour incubation with unstimulated medium; 4) 4000pg/ml IFN-γ for 48 hours without washing. After a total of 48 hours, the cell culture medium was discarded. The cells were then retrieved from the plate by manual scraping. Cells were resuspended in 1ml phosphate buffered saline for subsequent analysis by flow cytometry. All cultures were performed in triplicate.

5.2.4 Donor heart retrieval and cold cardioplegic perfusion:

Organ retrieval and perfusion was performed as described previously in sections 2.1.2 and 2.1.4. Briefly, five healthy Swedish pigs were anaesthetised and median sternotomy performed to allow cannulation of the ascending aorta. Both caval veins were clamped until the heart was empty. The distal ascending aorta was cross-clamped and the heart was stopped with the delivery of 600ml of cardioplegic perfusate. The heart was then perfused continuously for 8 hours at 8°C with a high albumin cardioplegic solution supplemented with autologous leucocyte depleted packed erythrocytes using the automated device described previously in section 2.1.3. The donor heart remained submerged in the perfusate reservoir and received at least 100ml/min perfusate at a fixed pressure of 20mmHg. The left ventricle was decompressed by venting.

5.2.5 Biopsy processing:

Left ventricular biopsies weighing 16-41mg were obtained immediately after donor heart retrieval and following 8 hours of cold cardioplegic perfusion. The tissue was immediately submerged in 25ml calcium and magnesium-free HBSS and dissected into small ($<2mm^3$) pieces. The biopsy was then homogenised at the lowest speed on ice for 2 minutes to achieve a single cell suspension. This suspension was then serially filtered through 500µm and100µm cell strainers before being centrifuged at 2000g for 2 minutes. The supernatant was discarded and the pellet resuspended in 5ml HBSS before being filtered through a final 40µm filter. The filtered cells were then washed and centrifuged at 2000g for 2 minutes and resuspended in 1ml PBS for flow cytometry staining.

5.2.6 Flow cytometry:

For analysis of cultured human aortic endothelial cells, flow cytometry was performed as described in section 2.2.1. Briefly, the cells were stained with mouse anti-human antibodies directed toward MHC class II (AlexaFluor700-conjugated) and PD-L1 (brilliant violet 510-conjugated) antigens. The cells underwent a series of washes and were then resuspended in 500µl staining buffer prior to analysis. The expression of MHC class II and PD-L1 was then determined using a BD LSRII flow cytometer (Becton Dickinson, Oxford, UK).

For analysis of MHC class II and PD-L1 expression on the porcine endothelium of the left ventricle, flow cytometry was performed as previously described in section 2.2.1. Cell surface staining was performed using a combination of mouse anti-porcine CD45 as a negative selector for leucocytes, mouse anti-SLA-DR (porcine MHC II) and unconjugated anti-porcine PD-L1 antibodies. The brilliant violet 510 secondary antibody was added to the cells to enable detection of the PD-L1 primary antibody and incubated for 20 minutes at 4°C. The cells were washed twice and then resuspended in 1.2ml staining buffer. A total of 1ml cell suspension was acquired and analysed by an Attune 1st generation flow cytometer (Thermo Fisher, Massachusetts, USA) at a flow rate of 1ml/min.

For both human and porcine samples, gating strategies and mean fluorescence intensities were obtained using FlowJo software version 10 (FlowJo, Oregon, USA).

MHC class II was chosen as a marker due to its primary role in antigen presentation. PD-L1 was chosen as a marker of interest due to its previously described role in the inhibition of T cell activity, which is of significant interest in the area of transplantation. Other checkpoint inhibitor molecules may also be of interest but they have not been profiled to the same extent in the existing literature and thus may have additional effects.

5.2.7 Statistical analysis:

Prism 7 (GraphPad, La Jolla, CA, USA) was used to perform all statistical analyses. Data are expressed either as mean ± standard deviation if normally distributed or as median [interquartile range] if non-normally distributed. One-way ANOVA was used to determine the change in PD-L1 and MHC II expression on endothelial cells exposed to IFN-γ. Post-hoc multiple comparisons were performed using Tukey's test. Paired T tests were used to compare PD-L1 and MHC II expression between pre and post perfusion samples. Statistical significance was accepted when $p \leq 0.05$.

5.3 Results:

5.3.1 IFN-γ significantly increases MHC class II expression:

The expression of class II MHC molecules was quantified on purified endothelial cells following IFN-γ stimulation. These findings demonstrate that 24 hour exposure markedly elevates the expression of MHC class II on the surface of the endothelium, which increases steadily in relation to the concentration of IFN-γ utilised (mean fluorescent intensity, diluent: 93.8±8.9; 1000pg/ml: 154±12.12; 2000pg/ml: 176±8.89; 4000pg/ml: 194.7±1.53; 8000pg/ml: 209±13.23; 80000pg/ml: 232±17.44, $p=0.004$, figure 5.1). Post-hoc multiple comparisons indicates that all concentrations used for stimulation induce significantly greater MHC class II expression compared to the diluent control with the exception of 1000pg/ml (all versus diluent: 1000pg/ml, $p=0.06$; 2000pg/ml, $p<0.0001$; 4000pg/ml, $p=0.01$; 8000pg/ml, $p=0.03$; 80000pg/ml, $p=0.01$). No differences were observed between stimulation with 80000pg/ml and 4000pg/ml or 8000pg/ml (versus 80000pg/ml: 4000pg/ml, $p=0.21$; 8000pg/ml, $p=0.77$), suggesting stimulation above 4000pg/ml induces little further change.

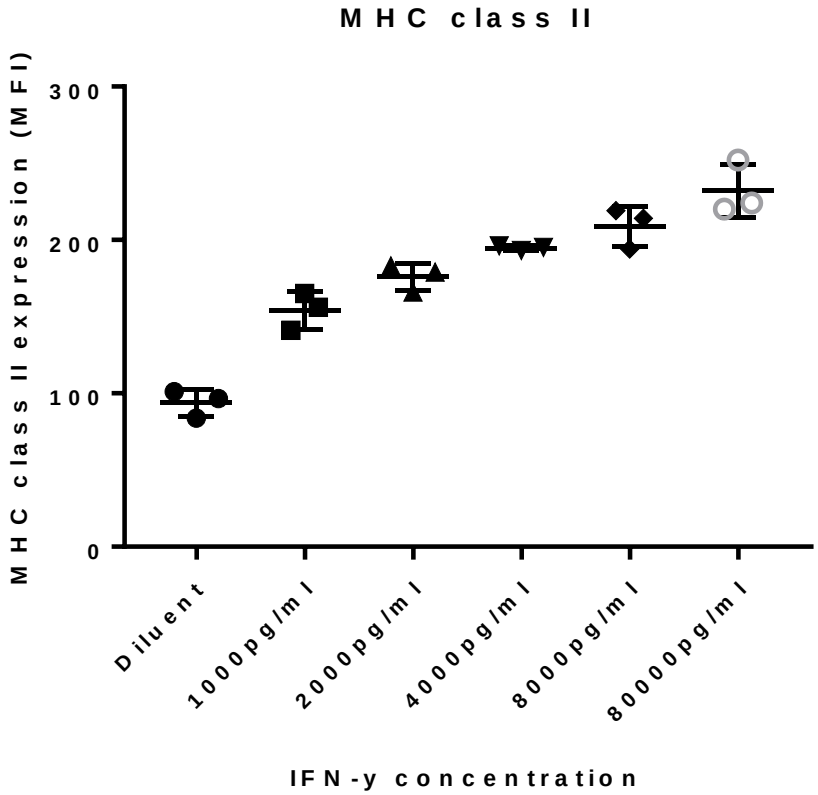

Figure 5.1 – IFN-γ significantly increases the expression of MHC class II on endothelial cells. MHC II expression continues to increase up to 80000pg/ml stimulation, although all cells stimulated with more than 1000pg/ml display greater expression than unstimulated cells (all $p<0.05$). Graph displays data from n=3 replicates. MFI = mean fluorescent intensity.

5.3.2 Programmed-death ligand 1 expression is significantly upregulated by IFN-γ stimulation:

The expression of PD-L1 was evaluated on single endothelial cells exposed to increasing concentrations of IFN-γ. This data indicates that 24 hours of exposure to IFN-γ is sufficient to mediate a marked elevation in co-inhibitory PD-L1 molecule expression on the endothelial cell surface. PD-L1 expression was increased in relation to the concentration of IFN-γ present (mean fluorescent intensity, diluent: 2973±198.3; 1000pg/ml: 4521±179.1; 2000pg/ml: 5011±73.93; 4000pg/ml: 5687±291; 8000pg/ml: 5932±119.6; 80000pg/ml: 6396±178.5, p=0.003, figure 5.2). Post-hoc multiple comparisons between concentrations demonstrates that all stimulated cells display statistically greater PD-L1 expression than the cells exposed only to diluent control (all versus diluent: 1000pg/ml, p=0.006; 2000pg/ml, p=0.01; 4000pg/ml, p=0.04; 8000pg/ml, p=0.01; 80000pg/ml, p=0.0002). Similar to MHC class II, no significant difference between 80000pg/ml and either 4000pg/ml or 8000pg/ml stimulation could be observed (versus 80000pg/ml: 4000pg/ml, p=0.37; 8000pg/ml, p=0.20), indicating stimulation above 4000pg/ml does not induce additional PD-L1 expression. All further experimentation will thus involve stimulation with only 4000pg/ml as this was determined to stimulate maximal change.

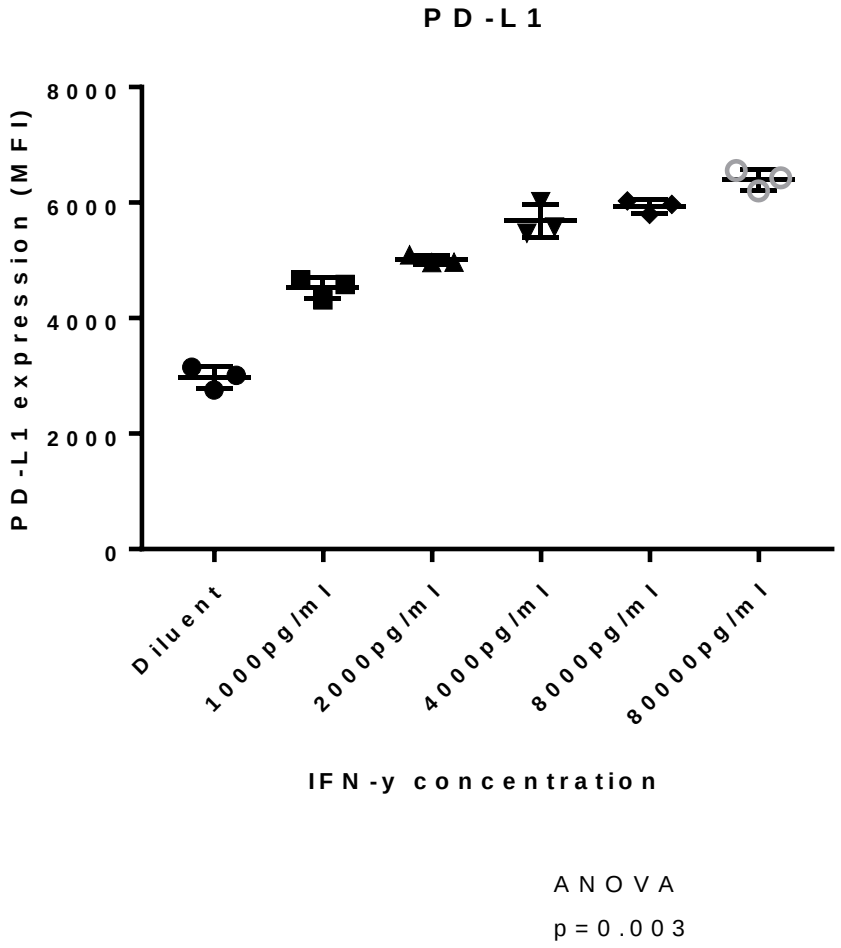

Figure 5.2 – PD-L1 expression is significantly increased upon endothelial cells exposed for 24 hours to IFN-γ. The expression of PD-L1 continues to increase until 80000pg/ml stimulation but all stimulated cells display greater expression than unstimulated cells (all $p<0.05$). Graph displays data from n=3 replicates. MFI = mean fluorescent intensity.

5.3.3 Class II MHC upregulation requires continual exposure to IFN-γ:

To evaluate the temporal kinetics of MHC class II expressional changes with IFN-γ, a series of cultures were performed with either constant exposure to the cytokine or 24 hours exposure followed by removal of the IFN-γ and replacement with diluent. These findings demonstrate that the effect of 24 hours IFN-γ exposure is not maintained in the absence of stimulation for the subsequent 24 hours. By 48 hours, the expression of MHC class II is similar to that observed with unstimulated control cells. By contrast, 48 hours of continual exposure to IFN-γ increased MHC class II expression above and beyond that previously observed with 24 hours exposure. Only in cultures with IFN-γ present at the time of evaluation was significant MHC class II expression observed (mean fluorescent intensity, 48 hour diluent control: 122±4; 48 hour diluent control with wash at 24 hours: 98.5±2.6; 24 hours IFN-γ followed by 24 hours diluent: 166.3±26.5; 48 hours IFN-γ: 8620±425, p=0.0009, figure 5.3). Post-hoc multiple comparisons demonstrates that constant exposure to IFN-γ for 48 hours led to significantly increased MHC class II expression compared to all other conditions (all versus 48 hours IFN-γ: 48 hours diluent, p=0.0007; 48 hours diluent with wash at 24 hours, p=0.0007; 24 hours IFN-γ with 24 hours diluent, p=0.001).

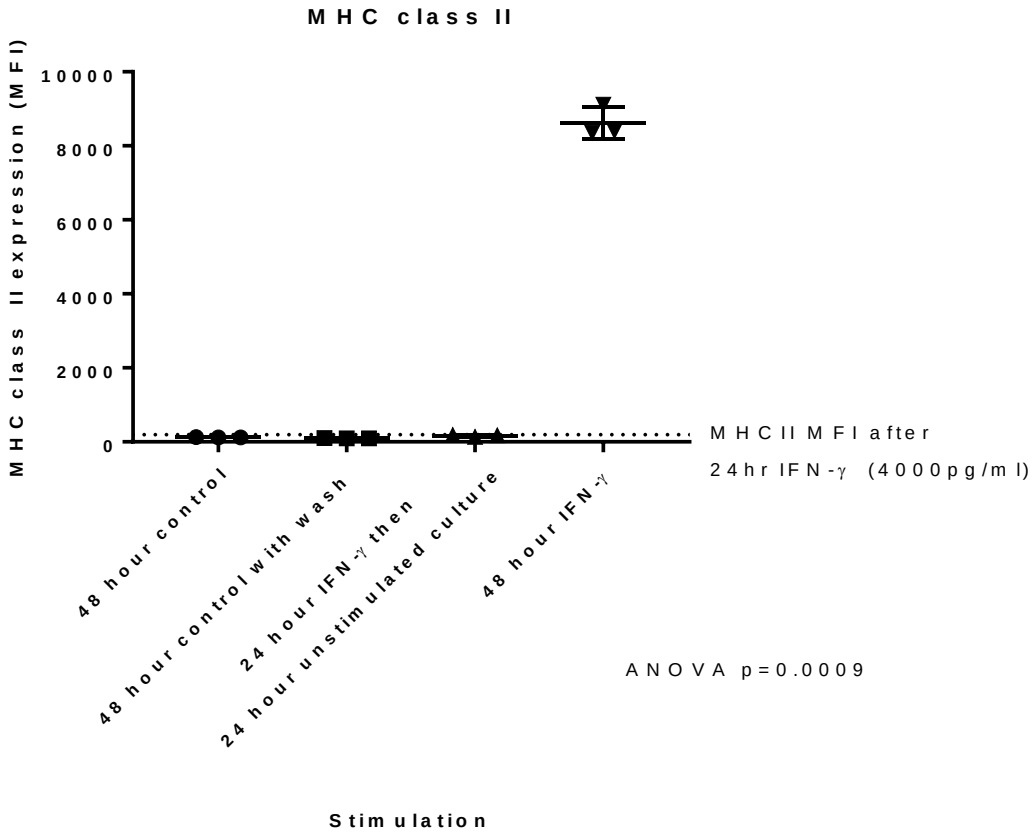

Figure 5.3 – Temporal kinetics of IFN-γ induced MHC II expression. Stimulation with IFN-γ increases MHC class II expression, although expression returns to unstimulated levels when IFN-γ is removed. Stimulation for 48 hours increases expression beyond that observed in the previous experiment at 24 hours. All data is representative of n=3 replicates. MFI = mean fluorescent intensity.

5.3.4 Removal of IFN-γ stimulation reverses IFN-γ mediated upregulation of PD-L1:

Next, a series of cultures was performed to assess whether continual exposure of endothelial cells to IFN-γ was required for PD-L1 upregulation. Indeed, similar to MHC class II, constant stimulation with IFN-γ is required for its maintenance of elevated PD-L1 expression. Whilst IFN-γ induces markedly increased PD-L1 expression, when the cytokine is removed at 24 hours, PD-L1 expression returns to a similar level observed with unstimulated cells. Only cells stimulated continuously for 48 hours displayed elevated PD-L1 expression in this experiment (mean fluorescent intensity, 48 hour diluent control: 2242±44.8; 48 hour diluent control with wash at 24 hours: 2108±55.2; 24 hours IFN-γ followed by 24 hours diluent: 2165±54.5; 48 hours IFN-γ: 9008±247.6, $p=0.0004$, figure 5.4). Post-hoc multiple comparisons demonstrates constant exposure to IFN-γ increased PD-L1 expression beyond that observed in any other condition (all versus 48 hours IFN-γ: 48 hours diluent, $p<0.0001$; 48 hours diluent with wash at 24 hours, $p<0.0001$; 24 hours IFN-γ with 24 hours diluent, $p=0.0003$).

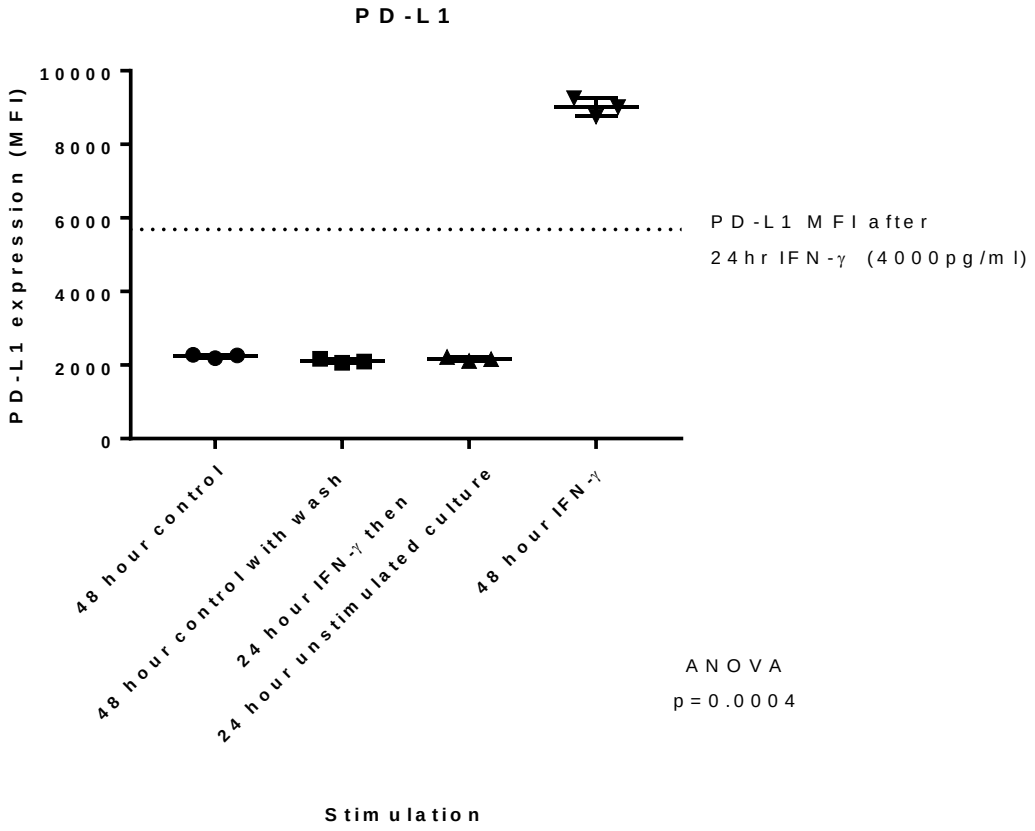

Figure 5.4 – Loss of IFN-γ stimulation diminishes PD-L1 expression. PD-L1 is increased at 24 hours but expression returns to unstimulated levels when IFN-γ is removed at 24 hours. Stimulation for 48 hours increases expression beyond that observed in the previous experiment at 24 hours. All data is representative of n=3 replicates. MFI = mean fluorescent intensity.

5.3.5 Cold cardioplegic heart perfusion increases PD-L1 but does not alter MHC class II expression on endothelial cells:

To provide a clinically relevant outcome measure, a series of *ex vivo* heart perfusions (associated with high IFN-γ exposure) were performed to establish whether the expression of MHC class II and PD-L1 was altered on porcine endothelium. No significant difference in MHC class II expression was observed between pre-perfusion and post-perfusion on endothelial cells obtained from the porcine left ventricle (mean fluorescent intensity: 6431±7152 vs. 11281±19703 for pre and post-perfusion respectively, p=0.81, figure 5.5). Significant variation in the response to perfusion was observed, leading to no overall statistical pattern. However, PD-L1 expression was significantly elevated in response to *ex vivo* perfusion in these same cells (mean fluorescent intensity: 1989±617.6 vs. 2764±507 for pre and post-perfusion respectively, p=0.03, figure 5.6).

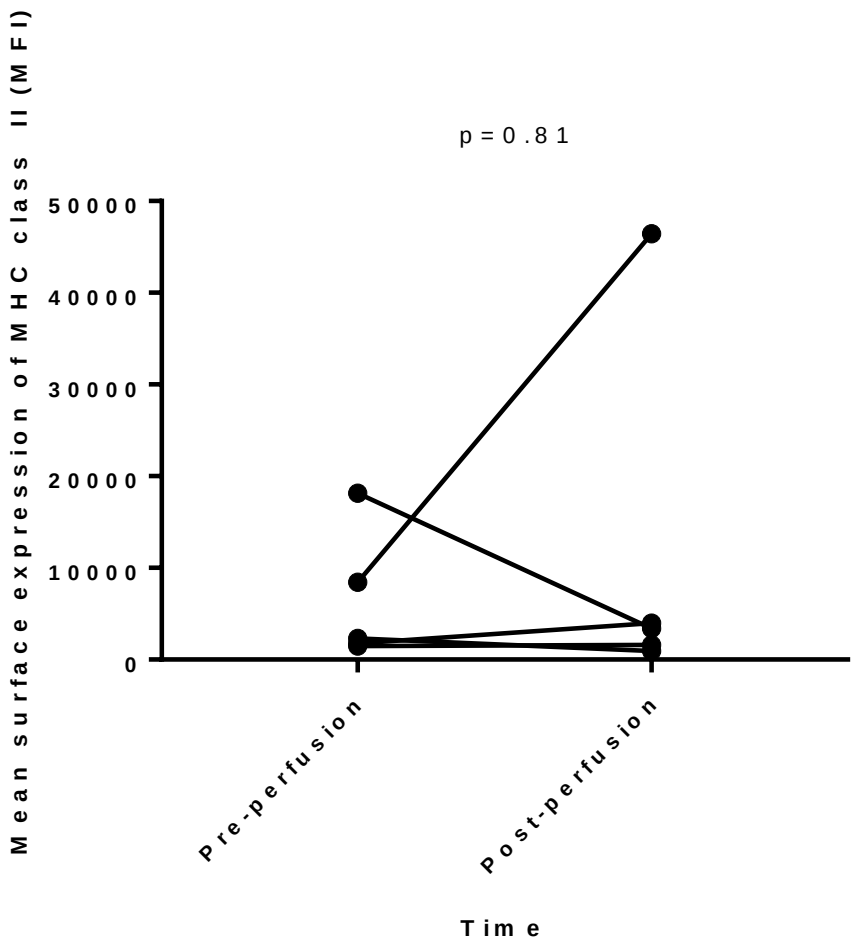

Figure 5.5 – *Ex vivo* perfusion of the donor heart does not alter MHC class II expression. There was no discernible pattern of response by the endothelium to *ex vivo* perfusion with regard to the expression of MHC class II. Graph displays data from n=5 porcine perfusion experiments. MFI = mean fluorescent intensity.

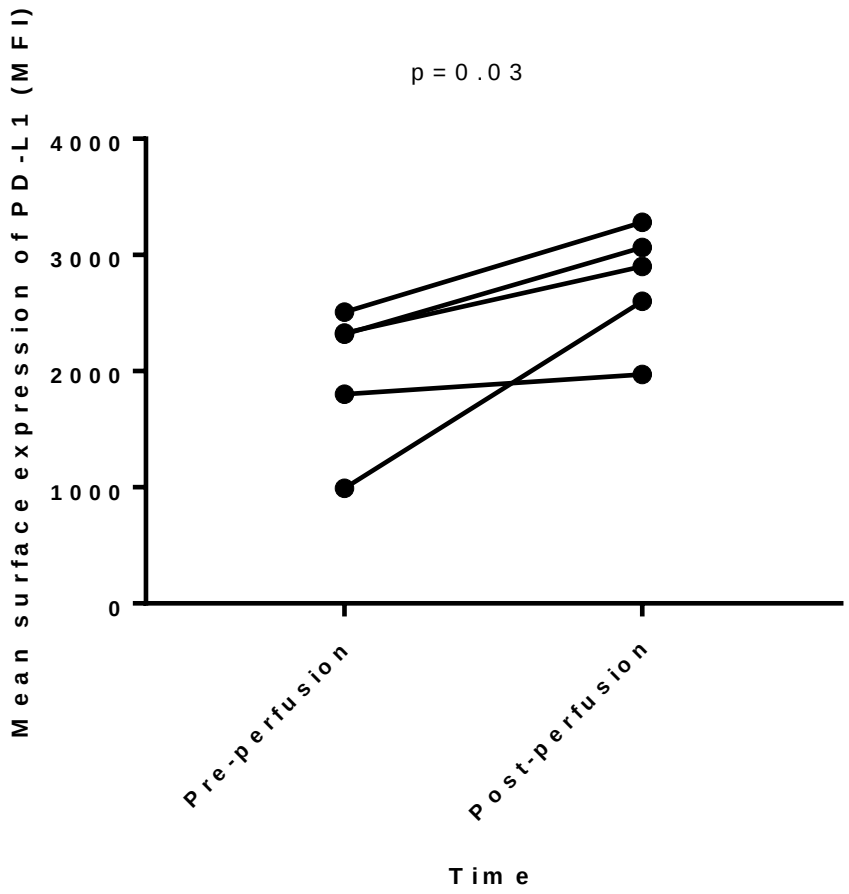

Figure 5.6 – PD-L1 expression is significantly upregulated following *ex vivo* perfusion of the donor heart. Endothelium cells from all hearts displayed increases in PD-L1 on the surface of the cell after 8 hours of cold perfusion. Graph displays data from n=5 porcine perfusion experiments. MFI = mean fluorescent intensity.

5.4 Discussion:

Graft endothelial cells are an important component of the tissue required for adequate function and regulation of vascular compliance. However, their physiological role in regulating vascular tone is only one function of these cells. In the context of transplantation, these cells often represent a prominent target for the recipient alloresponse and are affected by both acute[144] and chronic rejection[145] responses. Endothelial cells are able to sustain the alloresponse through their combined expression and presentation of MHC and antigenic peptides to recipient T cells. Importantly, endothelial cells lack CD80 and CD86 expression[146], and as such cannot co-stimulate naïve T cells via this traditional interaction but can maintain the anti-graft response through repeat stimulation of memory T cells. Given that the delivery of antigen to recipient T cells in the absence of co-stimulation leads to anergy or deletion of that specific T cell clone, the endothelium could have a more important role in the promotion of graft tolerance[147]. Furthermore, antigen presenting cells can also deliver inhibitory checkpoint signals, which perform the opposing function to co-stimulation and drives the loss of T cell reactivity. PD-L1 represents one such molecule, which has attracted significant interest in the field of cancer research[148], yet little research has been performed to determine its potential as a therapeutic target in transplantation. Previous data (presented in chapter 4) demonstrates that hypothermic *ex vivo* perfusion of the donor heart exposes the endothelium to a significant level of cytokines, particularly IFN-γ, which could alter the balance of markers expressed on the endothelial surface. In this study, endothelial expression of important markers involved in antigen presentation and co-inhibition was assessed *in vitro* in response to IFN-γ stimulation. This was followed by an assessment of protein expression in response to *ex vivo* perfusion of the isolated heart.

These findings provide strong evidence that IFN-γ signalling is sufficient to significantly upregulate the expression of MHC class II molecules. Whilst these cells display constitutive MHC expression under basal conditions, its upregulation indicates an increased capacity to interact with and deliver antigenic determinants to T cells. In the post-transplant setting, high MHC expression on the graft endothelium is traditionally considered detrimental to clinical outcome as this could contribute to stimulation of the alloresponse. Indeed, the absence of graft MHC class II has previously been associated with diminished acute rejection in experimental rodent

models[149], although this involved secondary stimulation of effector memory T cells by the endothelium and did not strengthen primary responses. However, it is possible that the increased MHC class II expression on graft endothelium in response to IFN-γ, if translated *in vivo*, could increase endothelial-T cell interactions as part of the primary response. This would provide the opportunity for delivery of antigen in the absence of co-stimulation (fundamentally absent in endothelial cells), therefore promoting graft tolerance through T cell deletion or anergy.

The promotion of allospecific T cell anergy is the ultimate goal following transplantation, as this would represent the acceptance of the graft by the recipient. However, it is likely that this could not be achieved simply through the lack of co-stimulation delivery by graft endothelium as there are sufficient alternative pathways to allow T cells to be rescued from deletion. This includes both direct presentation by donor antigen presenting cells and indirect presentation by the recipient, which can both provide costimulation. It is therefore critical that methods to promote graft acceptance involve active mechanisms and are not simply reliant upon the lack of a signal. Endothelial delivery of antigen in combination with co-inhibitory signals may provide the additional stimulus to prevent allospecific T cell activation. Notably, this study demonstrates that IFN-γ stimulated endothelial cells also significantly upregulate their expression of PD-L1, a key inhibitory immune checkpoint molecule. In this manner, it may be possible for graft endothelium to override stimulatory signals and thereby preclude immune activation. Furthermore, the co-expression of both PD-L1 and MHC class II would promote a selective, antigen-specific deletion of T cells. Not only can PD-L1 inhibit T cell reactivity but high PD-L1 expression may also provide additional benefits. Previous studies have demonstrated that PD-L1 promotes the development and sustained function of regulatory T cells, with a concomitant decrease in helper T cell function[150]. As such, it is clear that IFN-γ is able to induce a pro-tolerogenic effect on endothelial cells *in vitro*.

The temporal kinetics of IFN-γ induced MHC and PD-L1 upregulation indicate that incorporating this pathway as a viable graft protection strategy would be complex. Indeed, the sustained presence of IFN-γ is required for the maintenance of this effect and the removal of this cytokine rapidly restores both proteins to basal expression levels. The effect of high IFN-γ post-transplant is unclear, and both beneficial[151] and

detrimental[152, 153] findings have been presented previously. Developing methods to prevent the loss of PD-L1 following the removal of IFN-γ would be advantageous. Importantly, whilst the use of IFN-γ to stimulate beneficial protein expression on the endothelium is a viable method *in vitro*, it is not likely to represent a feasible approach *in vivo*.

As the concentration of IFN-γ utilised to stimulate the endothelium in this study was determined on the basis of previous findings with *ex vivo* perfusion, it was important to next evaluate how isolated perfusion itself affected MHC and PD-L1 expression. It was expected that the effects observed *in vitro* should be relatively well translated *ex vivo* due to the similarities in cytokine concentration. Whilst the effect on PD-L1 was retained, MHC class II expression remained stable in response to perfusion. It is possible that MHC upregulation requires longer exposure to IFN-γ, although it cannot be discounted that alternative mechanisms have prevented this increase. The dominance of the IFN-γ signal in the previous perfusion experiments directed the *in vitro* strategy, but other cytokines were certainly present and how they contribute to the overall protein expression profile is unclear. Nevertheless, MHC is constitutively expressed and as such endothelial-T cell interactions will occur, allowing the PD-L1 signal to be delivered. These exciting results suggest that cold cardioplegic perfusion of the donor heart could significantly diminish graft immunogenicity, a major auxiliary benefit of this preservation method.

Importantly, the impact of hypothermic perfusion on graft infiltration appears to be protective up to 48 hours. It is possible that infiltration is inhibited by the increased PD-L1 expression on the graft endothelium, which could be lost post-transplant after 48 hours when the local graft environment is different to that experienced *ex vivo*. Further work is required to validate these findings and confirm their downstream effect on T cell function, yet these data provide strong proof of concept with regard to the induction of protective protein expression on the donor organ without the requirement for additional pharmacological intervention. It may yet be possible to develop agonists that could be incorporated into the perfusate to further establish PD-L1 expression over a longer time course in order to ameliorate the alloresponse.

5.5 Limitations:

Only IFN-γ was evaluated as a mediator of PD-L1 upregulation, and it may be that other cytokines could potentiate or ameliorate this effect. Whilst the upregulation of PD-L1 is suggestive of major benefit as its only role is as a regulatory checkpoint for T cells, it was not possible to determine the effect on T cells in the current study. The upregulation of PD-L1 was observed *in vitro* and *ex vivo* but no transplant was performed, so the duration of this effect on the tissue was not determined and so may only be transient. Similarly, without a transplant it was impossible to assess the downstream effect of PD-L1 upregulation on graft infiltration.

5.6 Conclusion:

This study provides strong evidence of a basic immunological mechanism that can be harnessed for potential benefit post-transplantation via the regulation of recipient immunity. Indeed, endothelial expression of PD-L1 and MHC class II molecules can be efficiently induced by the presence of IFN-γ *in vitro*. Cold cardioplegic perfusion is sufficient to mediate this effect *ex vivo* for PD-L1, demonstrating further auxiliary benefits to the use of this preservation method over static cold storage. Whilst MHC class II was not affected by perfusion, recipient T cell activation and graft infiltration may still be diminished as a result of stimulation of this protective pathway.

Chapter 6: Donor heart immunodepletion by cold cardioplegic perfusion does not diminish graft infiltration following heterotopic transplantation

6.1 Introduction:

Transplantation is associated with severe recipient immune activation and infiltration of the donor organ, necessitating the lifelong use of potent immunosuppressants. This graft rejection process is stimulated by the presentation of donor antigenic peptides to recipient allospecific T cells, and can occur by two main pathways: direct and indirect allorecognition. Indirect allorecognition occurs through the acquisition and processing of antigen by recipient antigen presenting cells, which subsequently present antigenic peptides on the cell surface in association with recipient MHC[154]. In this manner, recipient antigen presenting cells are able to deliver antigenic stimulation to recipient T cells in lymphoid tissue, driving their activation and proliferation. As such, this process reflects that required for stimulation of T cells with nominal antigen. Conversely, direct presentation represents a pathway unique to transplantation and involves the presentation of donor antigen in the context of donor MHC[155]. Direct presentation occurs through the transfer of donor antigen presenting cells present within and transplanted alongside the donor organ. These passenger leucocytes display intact antigenic peptides on their cell surface in association with donor MHC and as such are able to directly present to recipient T cells without antigenic processing[74, 156]. This leads to significant and rapid activation of a large proportion of the recipient T cell repertoire, which can subsequently infiltrate the graft.

Current immunosuppression protocols predominantly target the recipient T cell on the basis that these cells are the effectors responsible for damage to the graft. However, these therapies are non-specific and systemically diminish T cell function regardless of their antigen specificity, leading to high risk of infection and malignancy. Furthermore, no targeted therapies are currently available to alter the presentation of alloantigen to recipient T cells. Indeed, it would be extremely challenging to develop a method of inhibiting alloantigen presentation by recipient cells without concomitantly disrupting presentation of nominal antigen. However, it may be possible to immunomodulate the donor organ and its passenger leucocytes following the development of *ex vivo* heart perfusion technology and in this manner significantly impede direct allorecognition and recipient T cell infiltration of the graft.

In other organs, including the lung, *ex vivo* perfusion is sufficient to induce the loss of significant numbers of passenger leucocytes, which migrate into the circuit and are

thus not transferred into the recipient[65]. This significantly diminishes the population of antigen presenting cells able to directly stimulate recipient T cells and a similar finding could be achieved with donor hearts.

Previous data presented in chapter 4 illustrates that cold cardioplegic heart perfusion is able to successfully immunodeplete the donor organ to a significant degree leading to a clinically important reduction in recipient recruitment to the graft up to 48 hours. This was associated with a significant IFN-γ dominated inflammatory storm, which may have enhanced the migration of donor leucocytes out of the organ through the release of IFN-γ associated chemokines. Importantly, this preservation technique maintained myocardial and endothelial integrity, demonstrating a selective alteration of the donor immune compartment without impacting upon cardiac tissue viability. Indeed, in a subset of organs that were subsequently transplanted, all recipients survived with intact contractility of the heterotopic heart, suggesting that the perfusion process maintained adequate function. Furthermore, hypothermic perfusion mediated an improvement in myocardial status via the selective downregulation of ischaemia-reperfusion related proteins and shutdown of cell death pathways, intimating tissue protection.

This study aimed to strengthen the earlier post-transplant findings and fully evaluate in a larger group whether donor organ immunodepletion by *ex vivo* perfusion translates into diminished graft infiltration. On the basis of the pilot heterotopic transplant study from chapter 4, 48 hours was chosen as the optimal point for sacrifice to evaluate the impact of perfusion following heterotopic transplantation.

6.2 Methods:

6.2.1 Power Calculation:

Using data obtained from chapter 4, a power calculation was performed to determine the number of animals required for the next stage of the study. To detect an overall 48.8% reduction in leucocyte numbers by perfusion with 29.5% standard deviation, when α=0.05 and power =0.8, a sample size of n=5 animals was required per experimental group. In order to ensure that no effects were missed, it was decided that a total of n=6 cold cardioplegic perfusion and n=6 static cold storage heart transplantations were required.

6.2.2 Study design:

Forty healthy Swedish pigs of native breed weighing from 35kg-70kg were used in the study. Twenty male pigs were utilised as organ donors and were split into two preservation groups: standard static cold storage following St. Thomas cardioplegia (n=14) and cold cardioplegic perfusion (n=6). Following organ preservation, donor hearts were transplanted into n=20 female blood matched recipient pigs. All recipients were maintained without immunosuppression until sacrifice at 48 hours.

6.2.3 Donor organ retrieval:

Donor heart retrieval was performed as previously described in section 2.1.2. Briefly, a median sternotomy was performed following anaesthesia and the ascending aorta was cannulated. The superior and inferior caval veins were clamped until the heart was emptied, at which point the distal ascending aorta was cross-clamped. A total of 600ml of 8°C St. Thomas cardioplegia (static cold storage group) or cardioplegic perfusate solution (cold cardioplegic perfusion group) was flushed through the heart via antegrade coronary perfusion. Left and right atrial appendages were cut to ensure decompression throughout the procedure. The left ventricle was vented to ensure decompression throughout. Once the flush was completed, the donor heart was excised.

For the static cold storage group, the donor heart was maintained at approximately 8°C submerged in St. Thomas solution whilst the recipient pig was being prepared. The left and right atria were closed by sutures prior to implantation.

For the perfusion group, the donor heart was submerged in 8°C cardioplegic perfusate whilst the heart preservation system was being prepared.

6.2.4 Cold cardioplegic perfusion:

The heart preservation system was prepared as previously described in chapter 2.1.4. Briefly, 2.5L of high albumin, hyperoncotic cardioplegic cellular (15% haematocrit) perfusate medium was used to fill the reservoir. The donor heart was perfused continuously for 8 hours at 8°C with a constant perfusion pressure of 20mmHg. The vena cavae and pulmonary artery were left open to allow the perfusate to be returned to the reservoir from the coronary sinus. Throughout perfusion, the donor heart remained submerged within the perfusate reservoir and received a minimum of 100ml/min perfusate. After 8 hours and just before implantation, the left and right atria were closed by sutures.

6.2.5 Recipient preparation and heterotopic transplantation:

All 20 female recipient pigs were prepared as described in section 2.1.5. Heterotopic transplantations were performed for both static cold stored and hypothermic perfusion groups as described previously in section 2.1.5. Briefly, a longitudinal incision was made to the left of the linea alba and the infrarenal aorta and caval vein visualised. The aorta of the donor heart was sutured end-to-side to the infrarenal aorta and the pulmonary artery was attached end-to-side to the vena cava. The donor heart was reperfused by the recipient circulation as soon as possible. Donor hearts were defibrillated where necessary before the incision was closed. The pig was awakened and fluid support provided over the next 48 hours. All recipient pigs were allowed to move freely in their pens for 48 hours without immunosuppression until sacrifice.

6.2.6 Sample collection:

6.2.6.1 Biopsy:

Left ventricle tissue was obtained from porcine hearts before and after organ preservation (8 hours duration for the perfusion group and approximately 2 hours for the static cold storage group). The tissue sample was weighed before being dissected into $<2mm^3$ pieces and placed into 25ml calcium and magnesium-free HBSS. Each sample was subsequently homogenised for 2 minutes on ice. The homogenate was serially filtered through 500µm then 100µm filters then centrifuged at 2000g for 2 minutes at 4°C. The cells were resuspended in 5ml HBSS before a final filtration

through a 40µm cell strainer. The filtered solution was washed at 2000g for 2 minutes at 4°C and the cells resuspended in 1ml PBS ready for downstream flow cytometry processing.

6.2.6.2 Perfusate:

A 20ml sample of perfusate was withdrawn immediately prior to organ attachment and after 4 and 8 hours of continuous perfusion. Each sample was centrifuged at 2000g for 10 minutes to separate plasma, which was stored at -80°C for the purposes of cytokine quantification and injury biomarker assessment.

6.2.6.3 Blood collection post-transplantation:

Blood samples measuring 30ml were obtained from recipient pigs within 30 minutes of reperfusion and at 48 hours post-transplantation. Each sample was centrifuged at 2000g for 10 minutes to separate out the plasma from the cellular component. The plasma was stored at -80°C for later quantification of cytokines and injury biomarkers.

6.2.6.4 Tissue collection at sacrifice:

Left ventricular tissue was obtained upon sacrifice at 48 hours. Each sample was split into 2 pieces and either stored in neutral buffered formalin and for later histological assessment or immediately snap frozen in liquid nitrogen. Snap frozen samples were rapidly transferred to a -80°C freezer for longer term storage until downstream analysis could be performed. Formalin-fixed samples were later paraffin embedded in tissue blocks for longer term storage.

6.2.7 Flow cytometry:

Flow cytometry assessment was performed as previously described in section 2.2.1. Briefly, cells were stained with viability dye before primary antibody was added to detect the cell surface expression of CD45 as a pan-leucocyte marker. Once stained, the cells underwent a series of washes before resuspension in 1.2ml staining buffer. A total of 1ml of these cells was assessed by the cytometer and absolute leucocyte count was determined using Attune Cytometric software. Cell counts were adjusted to the weight of the left ventricular biopsy obtained to provide a standardised value for comparison between pre and post perfusion tissue.

6.2.8 Troponin I quantification:

Cardiac troponin I concentration was measured in both the perfusate and in the recipient plasma post-transplantation as a marker of cardiac injury using a commercially available porcine ELISA kit. Quantification was performed as previously described in section 2.2.4. All samples were analysed without prior dilution and comparisons were made over time (for perfusate) and between groups post-transplantation.

6.2.9 Cytokine quantification:

A porcine 13-plex Luminex assay was performed to quantify cytokine release in recipient plasma obtained post-transplantation as previously described in section 2.2.3. The concentrations of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α were measured to provide a broad profile of the extent of inflammation within the transplant recipient at each time point. All plasma samples were assessed without prior dilution.

6.2.10 Cell-free DNA detection:

Quantitative PCR was utilised as previously described in section 2.2.2 to determine the concentration of cell-free DNA of mitochondrial and genomic origin in the recipient plasma obtained within 30 minutes of reperfusion and at 48 hours post-transplant. All samples were analysed undiluted and were standardised per millilitre of plasma analysed.

6.2.11 Left ventricular phosphokinase and apoptosis signalling:

Left ventricular tissue biopsies were obtained from each pig upon sacrifice at 48 hours post-transplantation. Human phosphokinase and apoptosis antibody proteome profiler arrays (R&D systems, Abingdon, UK) were utilised to compare the expression of a broad range of proteins between groups. For determination of apoptosis, snap frozen tissue weighing 37-85mg was homogenised and assessed as described in section 2.2.5. For phosphokinase assessment, 41-95mg snap frozen left ventricular tissue was homogenised and analysed as described in section 2.2.5. For analysis, 400µg protein was added to each apoptosis membrane and 600µg added to each phosphokinase membrane. A separate membrane was utilised for each sample. Pixel density analysis was performed using ImageJ (NIH, USA) and data is expressed as integrated pixel density scores.

6.2.12 Histological evaluation:

To assess the viability and magnitude of lymphocytic infiltration of the transplanted heart, a histological assessment was performed using formalin-fixed paraffin embedded tissue obtained at 48 hours from the anterior, lateral and posterior region of the left ventricle as well as the septum and right ventricle. Sections were cut at 4μm, de-paraffinised and stained with haematoxylin and eosin. All samples were prepared and assessed in a blinded manner by a consultant histopathologist, who reported on the extent of myocardial damage to the tissue (reported as the percentage of the myocardium displaying damage). The same histopathologist also provided a clinically-relevant score of the overall level of tissue infiltration (reported as a percentage of the tissue displaying lymphocytic infiltrates). A composite score of the overall donor heart was produced by the histopathologist by averaging the scores for each region. The same field of view was utilised each time to produce the score for lymphocytic infiltration and myocardial damage. Comparison between preservation groups was performed following unblinding of the data.

6.2.13 Statistical analysis:

Prism 7 (GraphPad, La Jolla, CA, USA) was used to perform all statistical analyses. Data are expressed either as mean ± standard deviation if normally distributed or as median [interquartile range] if non-normally distributed. Paired T tests or Wilcoxon signed rank tests were utilised to determine the difference in cardiac leucocyte content between pre and post perfusion tissue. One-way ANOVA was used to determine the change in troponin I concentration in the perfusate. Multiple comparisons were performed using the Sidak method to detect changes in troponin I concentration between the two groups over time post-transplantation. The Holm-Sidak multiple comparisons method was utilised to determine differences between the groups for apoptosis and phosphokinase expression on the left ventricle post-transplant. The Holm-Sidak multiple comparisons test was utilised to determine the difference in concentration of cytokines and cell-free DNA between groups at each time point post-transplantation. Statistical significance was accepted when $p \leq 0.05$.

6.3 Results:

6.3.1 High mortality rate following transplantation:

In order to determine whether cold cardioplegic perfusion altered the early clinical course post-transplant, it was decided that n=12 heterotopic transplants would be performed with n=6 organs preserved by static cold storage and n=6 organs preserved by hypothermic perfusion. Data from n=6 vs. n=6 remained the target through the study. However, there were significant technical issues apparent with the surgical procedure, which led to the loss of a large number of recipient pigs. In total, n=6 transplants were performed for the hypothermic perfusion group, without any mortality. However, n=14 transplants had to be performed for the static cold storage group in order to obtain data at 48 hours from n=6. All assessments were subsequently performed for n=6 vs. n=6 transplants as originally intended.

The loss of eight pigs post-transplant in the static cold storage group did not relate to acute rejection. An autopsy was performed for one of the pigs that died within 12 hours post-transplant. There were no detectable problems with the donor heart, nor was there significant infiltration. Of all those pigs that were lost post-transplant, only one survived beyond 24 hours. One pig died whilst on the operating table, although most were lost between 6 and 12 hours. Whole blood samples were obtained within 30 minutes post-transplant and again at 6 and 12 hours according to the original design. However, it was decided that the blood samples retrieved could have promoted haemoconcentration and potentially increased the likelihood of clots. As such, the 6 hour and 12 hours blood samples were not taken after the first two deaths, although this did not diminish the mortality rate.

6.3.2 Cold cardioplegic perfusion but not static cold storage induces immunodepletion of the donor heart:

To further validate the earlier findings from chapter 4, the total leucocyte content of the left ventricle was quantified prior to and following cold perfusion or static cold storage. These findings demonstrate that perfusion was associated with a large reduction in total leucocyte content (median 50.31% decrease, figure 6.1), similar to the mean 48.4% reduction described previously. This was not the case for static cold storage (median 11.32% increase, figure 6.1).

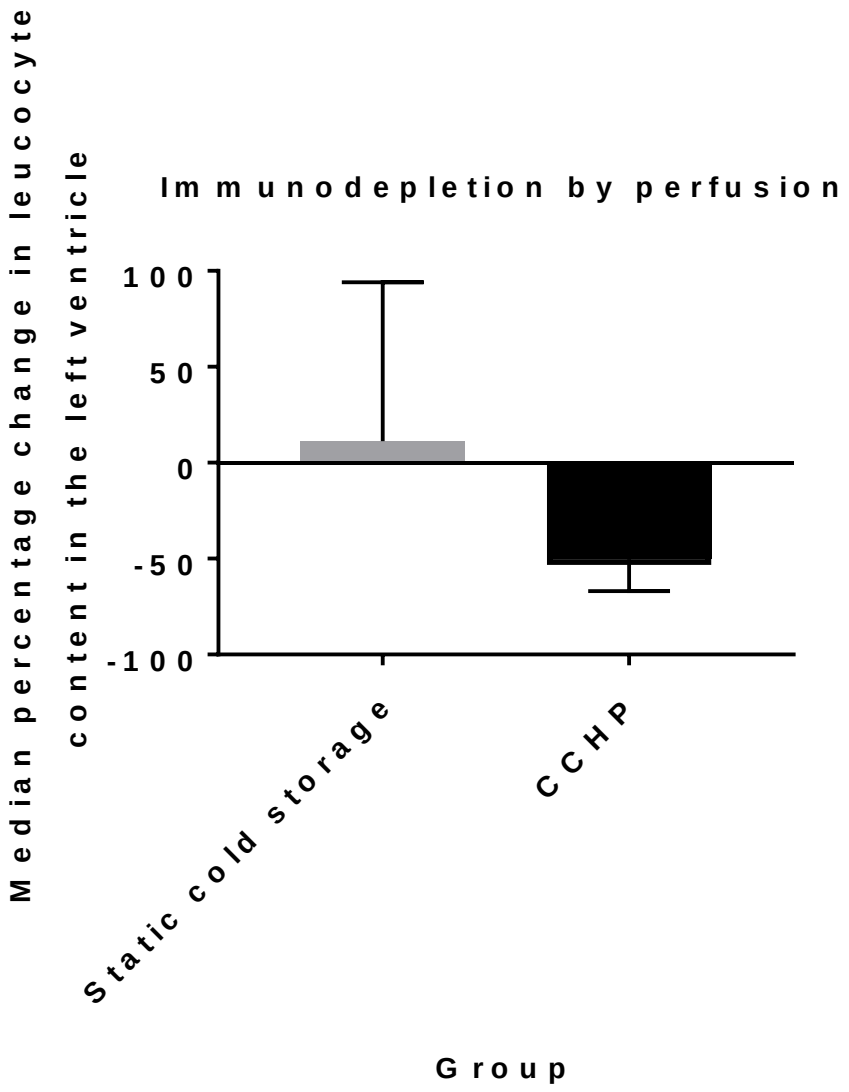

Figure 6.1 – Cold cardioplegic perfusion induces a reduction in total leucocyte content. However, this does not occur with static cold storage. This validates earlier findings of significant immunodepletion with perfusion and is strengthened by the addition of the static cold storage control group. Graph depicts data from n=5 porcine perfusions (due to a missed post-perfusion sample) and n=6 static cold storage experiments. Bars represent median values and whiskers represent interquartile range. CCHP = cold cardioplegic heart perfusion.

6.3.3 Perfusion induces significant cardiac troponin I release into the *ex vivo* circuit:

To validate the previous finding that cold cardioplegic perfusion was not associated with myocardial damage, the concentration of cardiac troponin I was again measured in the perfusate. Interestingly, greater release of troponin I was detected throughout perfusion compared to previous data (presented in chapter 4). Indeed, baseline values (prior to organ attachment) were elevated beyond that detected at any point during the previous set of perfusion experiments. Further significant increases in troponin I were detected after 4 and 8 hours of perfusion, peaking at >2000pg/ml (the detection limit of the assay) by 4 hours and remaining above this at 8 hours ($p<0.0001$, figure 6.2). For the purposes of analysis, values above the detection limit were conservatively taken as 2000pg/ml.

6.3.4 Post-transplant cardiac troponin I release is not altered by cold cardioplegic perfusion:

The level of myocardial damage was assessed on a cellular level by quantification of cardiac troponin I. The type of organ preservation prior to transplantation did not alter downstream myocardial damage immediately post-transplant (233.2±431.8pg/ml vs. 123.9±200.2pg/ml for perfused vs. static cold storage respectively, p=0.59, figure 6.2) or at 48 hours (1078.2±814.7 vs. 655.9±658.8 for perfusion vs. static cold storage respectively, p=0.57, figure 6.2). In both groups, the level of troponin I was increased from 0 to 48 hours but this did not reach statistical significance (p=0.09 for both groups).

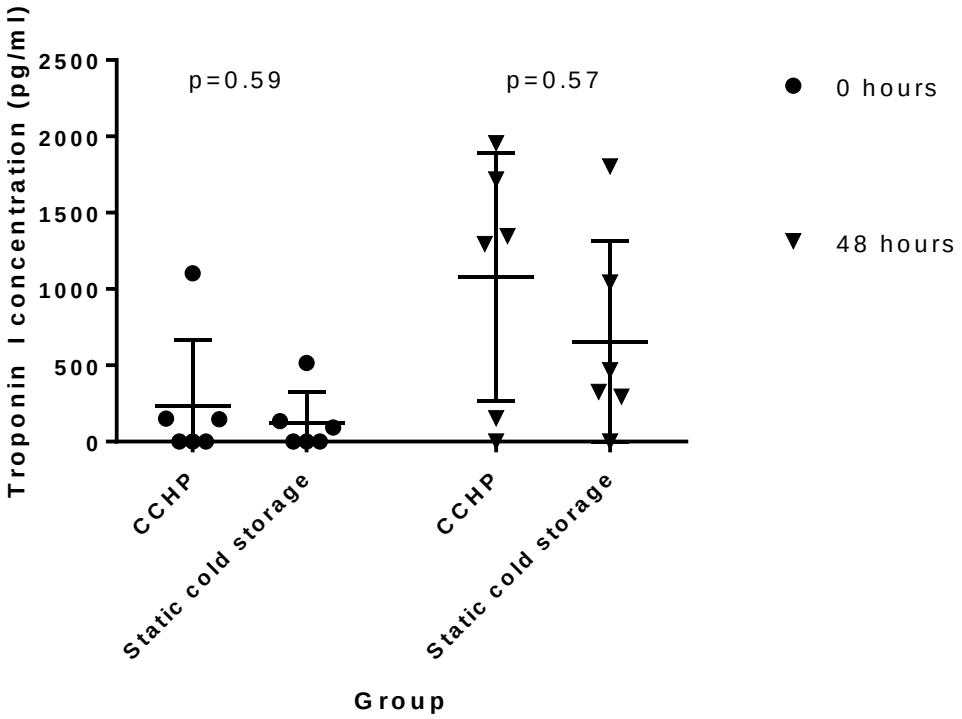

Figure 6.2 – The method of organ preservation does not alter cardiac troponin I release post-transplantation either immediately or at 48 hours. The level of cardiac troponin I in both groups is increased by 48 hours but not to a statistically significant level. Graphs illustrate data from 12 transplants (n=6 perfusion versus n=6 static cold storage). CCHP = cold cardioplegic heart perfusion.

6.3.5 Cold cardioplegic perfusion reduces post-transplant cytokine release:

We next determined the effect of *ex vivo* perfusion on post-transplant inflammatory cytokine secretion. The mean concentration of all cytokines was consistently lower in the cold cardioplegic perfusion group than the static cold storage group at both 0 and 48 hours post-transplant, although most did not reach statistical significance. GM-CSF did not significantly differ between groups at 0 hours (perfused vs. cold storage: 6.7±12.1pg/ml vs. 16.7±24.2pg/ml, p=0.47) or at 48 hours (perfused vs. cold storage: 0.0±0.0pg/ml vs. 26.7±43.7pg/ml, p=0.17, figure 6.3). Donor hearts in the perfused group were completely unable to secrete IFN-γ at any time post-transplant, whereas large amounts of IFN-γ could be secreted in 3/6 hearts from the static cold storage group. However, no statistically significant difference was detected at either 0 hours (perfused vs. cold storage: 0.0±0.0pg/ml vs. 1755.0±2333.9pg/ml, p=0.17) or at 48 hours post-transplant (perfused vs. cold storage: 0.0±0.0pg/ml vs. 2106.7±3370.2pg/ml, p=0.17, figure 6.3). No significant difference in IL-1α was detected post-transplant at 0 hours (perfused vs. cold storage: 13.3±5.2pg/ml vs. 22.2±49.3pg/ml, p=0.40) or at 48 hours (perfused vs. cold storage: 11.7±7.5pg/ml vs. 58.3±62.1pg/ml, p=0.11, figure 6.3). IL-1β was secreted at statistically equivalent levels early following transplant (perfused vs. cold storage: 136.7±79.4pg/ml vs. 723.3±1037.6pg/ml, p=0.25), although significantly greater IL-1β was observed in the cold stored group at 48 hours (perfused vs. cold storage: 305.0±168.3pg/ml vs. 1866.7±1342.9pg/ml, p=0.009, figure 6.3).

IL-1 receptor antagonist was detected at relatively high concentrations in both groups and high intra-group variability was observed following cold storage at 48 hours. No significant difference was observed at either 0 hours (perfused vs. cold storage: 471.7±232.7 vs. 2181.7±2198.1, p=0.90) or 48 hours post-transplant (perfused vs. cold storage: 1400.0±809.0pg/ml vs. 24381.7±44863.7pg/ml, p=0.17, figure 6.5). No significant difference was observed in IL-2 concentration at 0 hours (perfused vs. cold storage: 85.0±47.2pg/ml vs. 211.7±227.3pg/ml, p=0.37), although there was a trend towards higher secretion in the cold stored group at 48 hours (perfused vs. cold storage: 83.33±74.7pg/ml vs. 406.7±414.8pg/ml, p=0.06, figure 6.4). IL-4 secretion was consistent regardless of group at both 0 hours (perfused vs. cold storage: 75.0±44.2pg/ml vs. 493.3±819.5pg/ml, p=0.32) and at 48 hours (perfused vs. cold storage: 75.0±49.3pg/ml vs. 875.0±1167.1pg/ml, p=0.13, figure 6.4). IL-6

concentration was also not statistically different between groups at either 0 hours (perfused vs. cold storage: 60.0±37.4pg/ml vs. 128.33±133.6pg/ml, p=0.27) or 48 hours (perfused vs. cold storage: 108.3±109.1pg/ml vs. 1481.7±2123.0pg/ml, p=0.27, figure 6.4).

IL-8 was not significantly different between groups at 0 hours (perfused vs. cold storage: 21.7±19.4pg/ml vs. 36.7pg/ml, p=0.80) although there was a trend towards higher secretion in the cold stored group at 48 hours (perfused vs. cold storage: 6.7±10.3pg/ml vs. 138.3±194.0pg/ml, p=0.06, figure 6.5). There were no detectable differences in the secretion of IL-10 between groups at 0 hours (perfused vs. cold storage: 70.0±63.9pg/ml vs. 421.7±539.4pg/ml, p=0.24) or at 48 hours (perfused vs. cold storage: 118.3±88.2pg/ml vs. 396.7±535.8pg/ml, p=0.24, figure 6.5). Similarly, IL-12 was not statistically different between preservation groups at 0 hours (perfused vs. cold storage: 491.7±190.1pg/ml vs. 708.33±395.0pg/ml, p=0.26) or at 48 hours post-transplant (perfused vs. cold storage: 328.3±97.0pg/ml vs. 681.7±456.7pg/ml, p=0.14, figure 6.5). IL-18 again displayed no significant difference in concentration between the groups at 0 hours (perfused vs. cold storage: 395.0±228.2pg/ml vs. 1448.3±1785.4pg/ml, p=0.21), although there was a trend toward a difference at 48 hours post-transplant (perfused vs. cold storage: 408.3±394.4pg/ml vs. 2180.0±2143.1pg/ml, p=0.08, figure 6.5). Finally, there were no significant differences detected between groups in the concentration of TNF-α at either 0 hours (perfused vs. cold storage: 10.0±12.6pg/ml vs. 26.7±19.7pg/ml, p=0.44) or at 48 hours post-transplant (perfused vs. cold storage: 8.3±13.3pg/ml vs. 46.7±68.6pg/ml, p=0.17, figure 6.5).

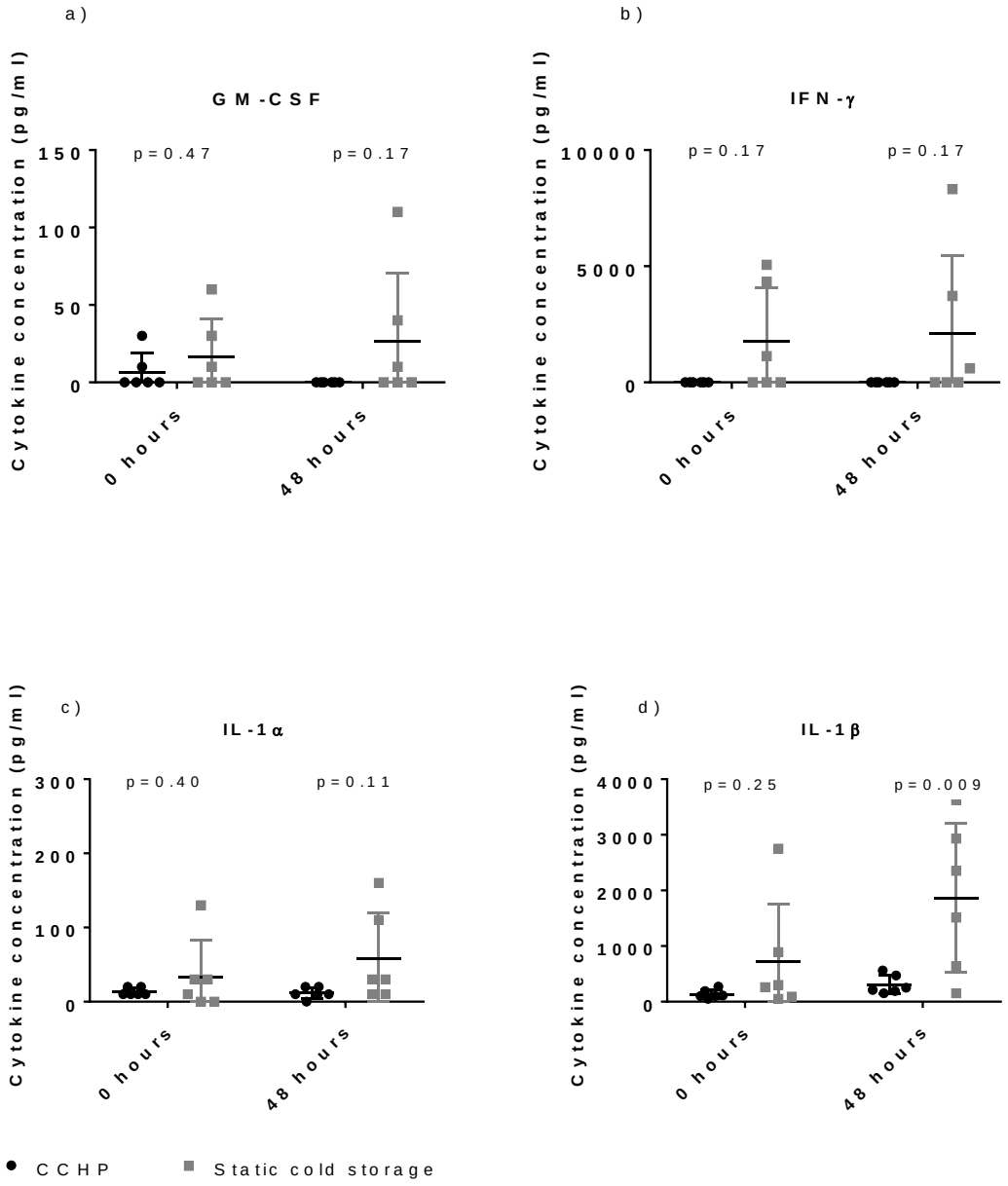

Figure 6.3 – Release of GM-CSF, IFN-γ, IL-1α and IL-1β post-transplant. Post-transplant cytokine release is diminished by cold cardioplegic perfusion of the donor heart. Post-transplant cytokine concentrations are displayed for GM-CSF (a), IFN-γ (b), IL-1α (c), and IL-1β (d). All graphs display data from n=6 transplants per group. CCHP = cold cardioplegic heart perfusion.

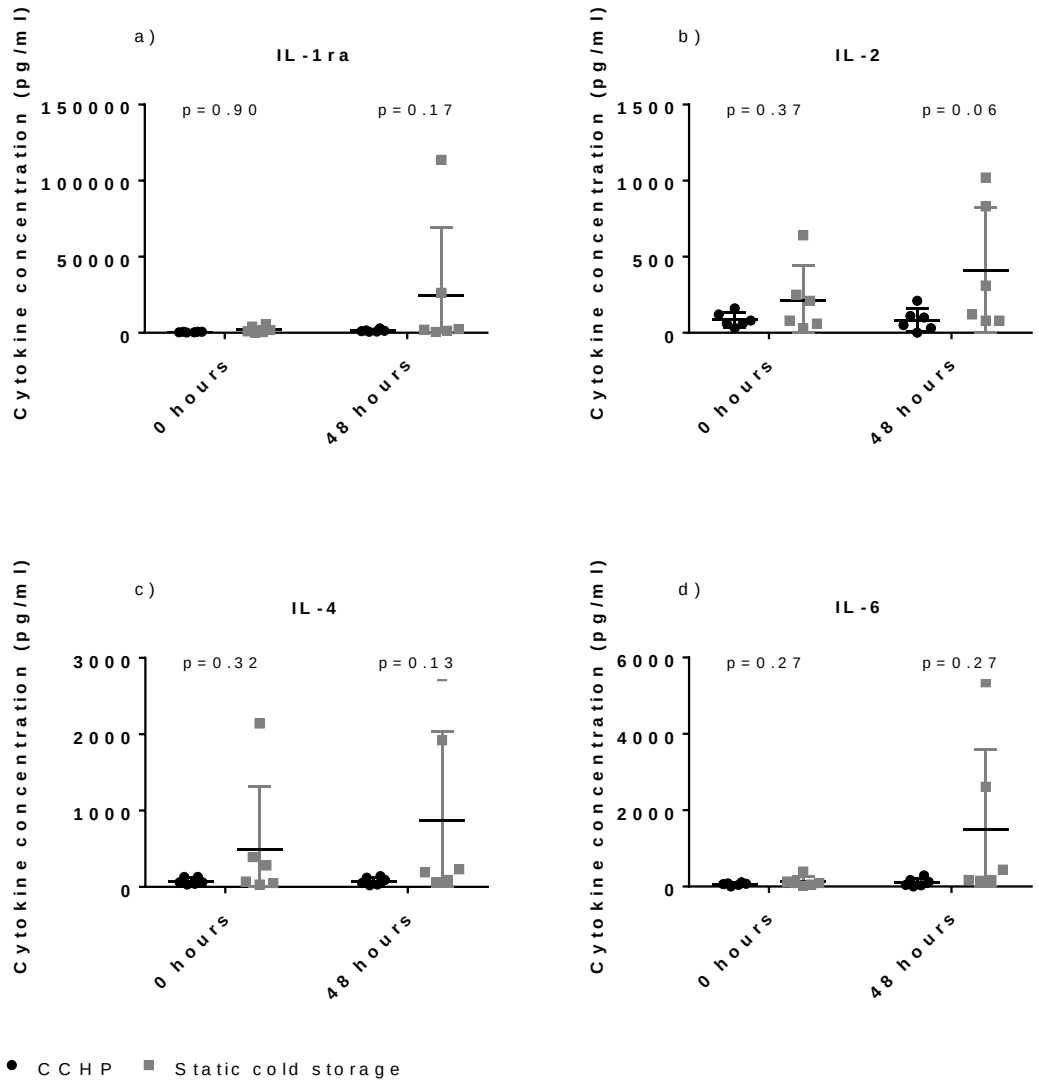

Figure 6.4 – Release of IL-1ra, IL-2, IL-4 and IL-6 cytokines post-transplant. Post-transplant cytokine concentrations were compared at 0 and 48 hours for donor hearts preserved by static cold storage and cold cardioplegic perfusion. No differences between groups were observed for IL-1ra (a), IL-2 (b), IL-4 (c), and IL-6 (d). All graphs display data from n=6 transplants per group. CCHP = cold cardioplegic heart perfusion.

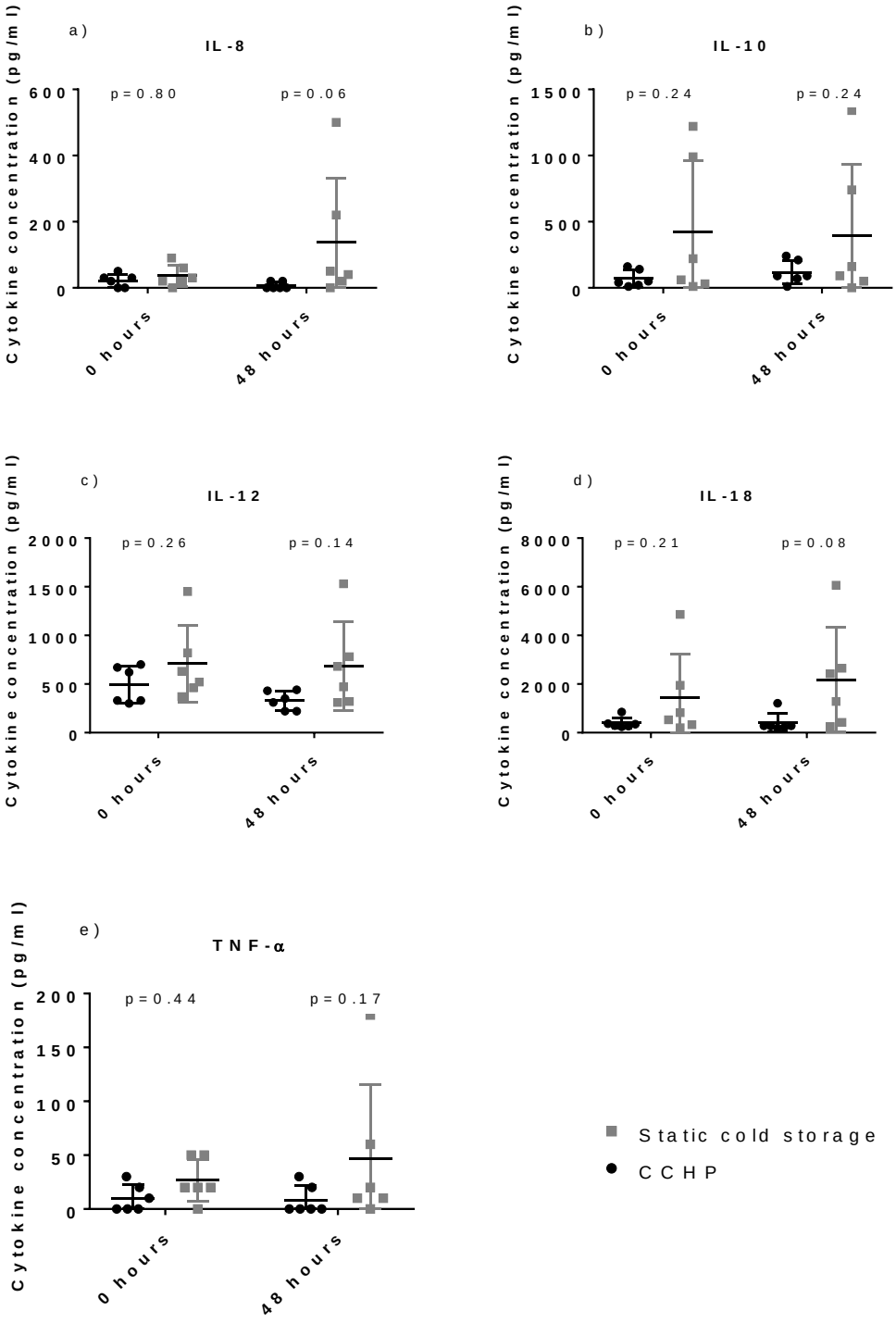

Figure 6.5 – Post-transplant release of IL-8, IL-10, IL-12, IL-18 and TNF-α after static cold storage or cold cardioplegic perfusion. No differences between groups were observed for IL-8 (a), IL-10 (b), IL-12 (c), IL-18 (d), or TNF-α (e). All graphs display data from n=6 transplants per group. CCHP = cold cardioplegic heart perfusion.

6.3.6 Method of donor heart preservation does not alter the concentration of cell-free DNA observed in plasma post-transplantation:

Cell-free DNA is a useful marker of non-specific cell injury and inflammatory activation and has been suggested as having utility as a predictor of acute rejection. As such, cell-free DNA of mitochondrial and genomic origin was next quantified in plasma following transplantation. These findings indicate that following transplantation, cell-free DNA is detectable in the plasma, albeit in low concentrations. There was no observable difference in the concentration of mitochondrial DNA between groups at either 0 hours (perfused vs. cold storage: 0.23±0.2ng/µl vs.0.18±0.1ng/µl, p=0.73) or at 48 hours post-transplantation (perfused vs. cold storage: 0.12±0.2ng/µl vs. 0.19±0.2ng/µl, p=0.73, figure 6.6). Similarly, genomic DNA did not differ between groups at 0 hours (perfused vs. cold storage: 0.06±0.0ng/µl vs.0.09±0.0ng/µl, p=0.34) or at 48 hours (perfused vs. cold storage: 0.03±0.0ng/µl vs.0.05±0.0ng/µl, p=0.54, figure 6.6). At 0 hours, cell-free mitochondrial DNA was present at a higher concentration than genomic DNA for the perfused group (p=0.03) but levels were statistically similar in the static cold storage group (p=0.13). However, no differences were observed at 48 hours in the perfused group (p=0.32) or the static cold storage group (p=0.12).

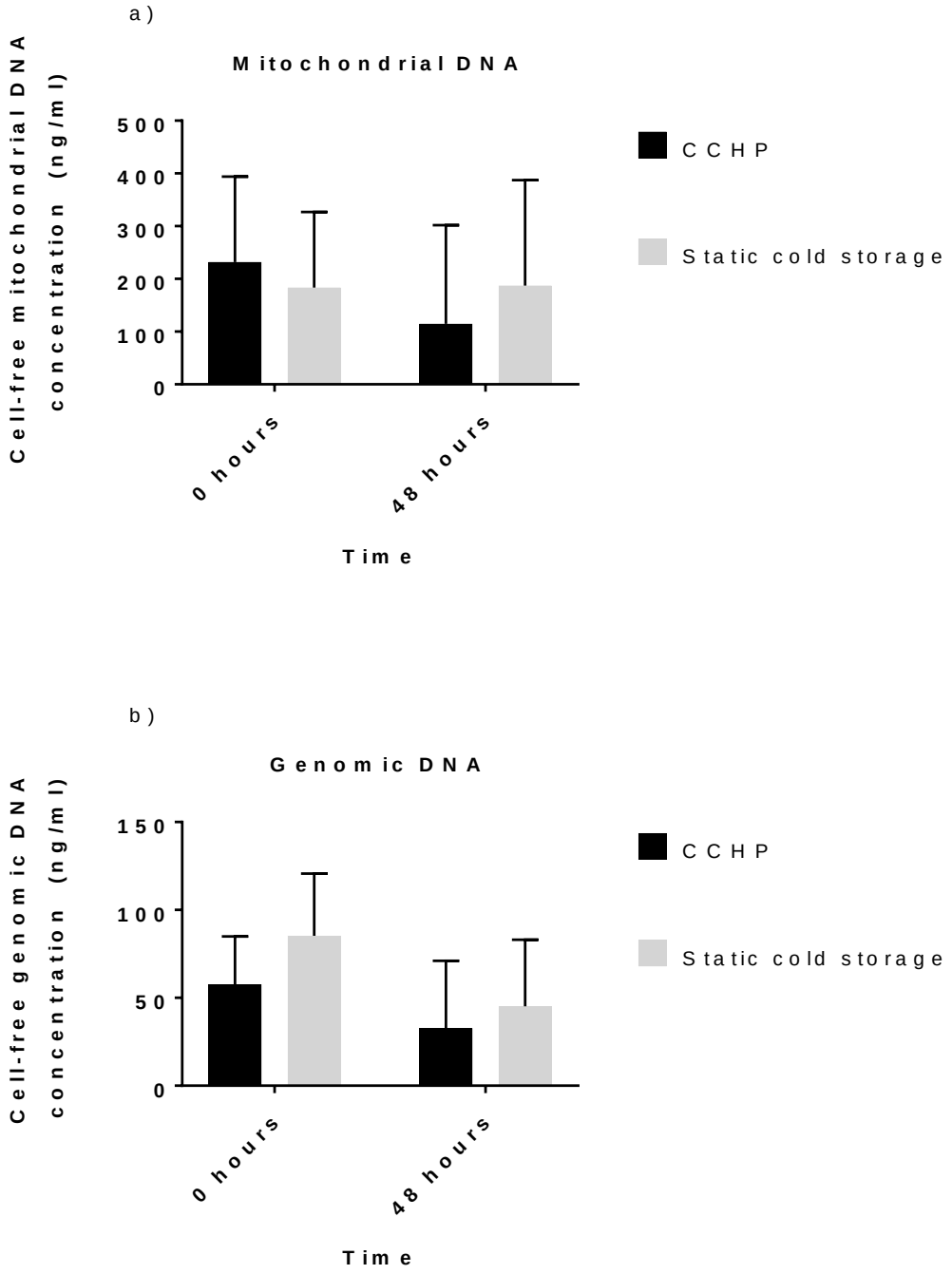

Figure 6.6 – The release of cell-free DNA post-transplant is not altered by donor heart preservation method. Extracellular DNA of both mitochondrial (a) and genomic (b) origin was detected in plasma post-transplant in both groups. Data displayed is from n=6 transplants per group. CCHP = cold cardioplegic heart perfusion.

6.3.7 Cold cardioplegic perfusion does not alter the expression of left ventricular apoptosis and cell survival associated protein expression:

To determine the effect of method of preservation on post-transplant left ventricular cell viability, a proteomic analysis was performed using an array specific for the detection of 35 proteins associated with cell death and survival pathways. Multiple comparisons analysis demonstrated that there were no significant changes in the expression of any proteins associated with cell death or survival pathways upon sacrifice at 48 hours (all $p>0.05$, figure 6.7).

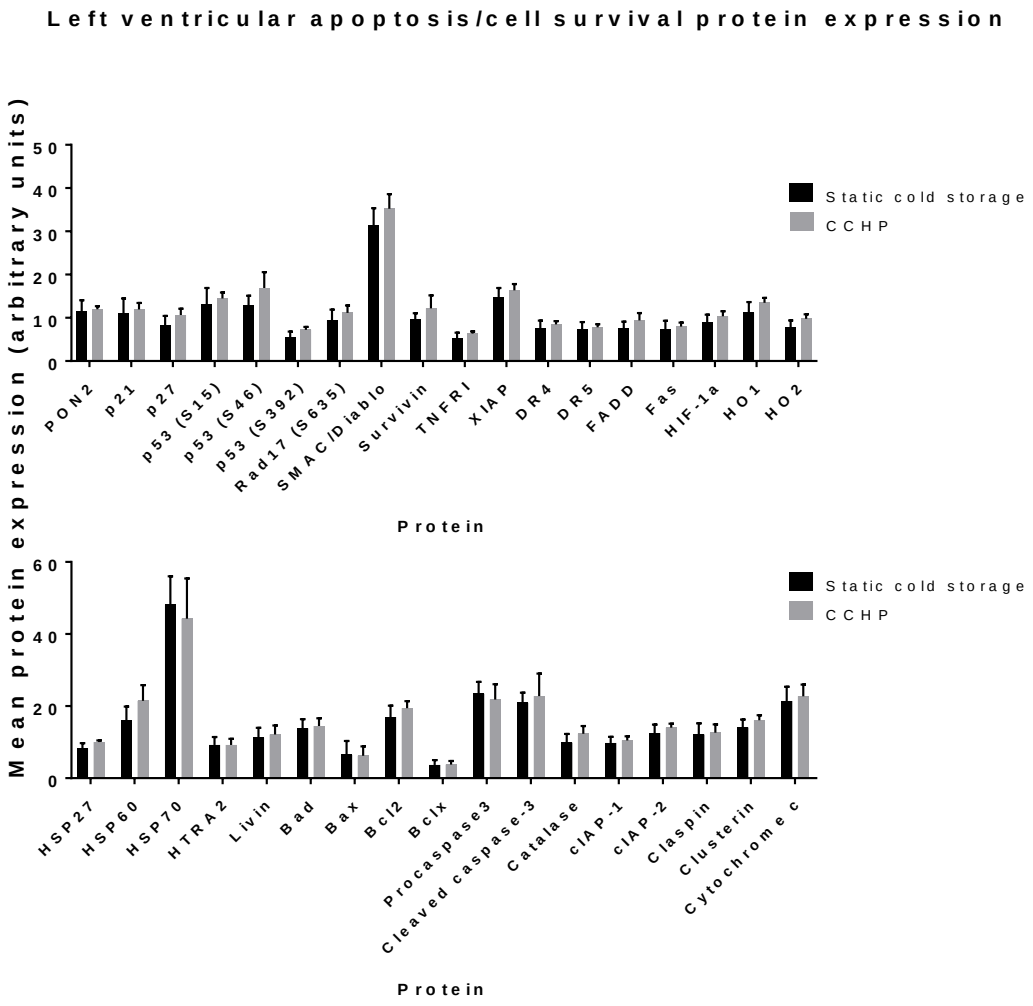

Figure 6.7 – Left ventricular apoptosis and cell-survival related protein expression is not altered by method of donor heart preservation. Data displayed is from n=6 transplants per group. CCHP = cold cardioplegic heart perfusion.

6.3.8 Method of donor heart preservation does not induce alterations in phosphokinase signalling protein expression:

To determine the effect of donor heart preservation method on post-transplant left ventricular signalling pathways, a targeted proteomic assay was utilised to evaluate the expression of 45 phosphokinases. Multiple comparisons analysis demonstrated that focal adhesion kinase (FAK) was expressed to a greater degree in the perfused group than the standard group (mean integrated density: 20.9±7.5 vs. 27.7±7.7, $p=0.005$). All other proteins displayed statistically similar expression (all $p>0.05$, figure 6.8).

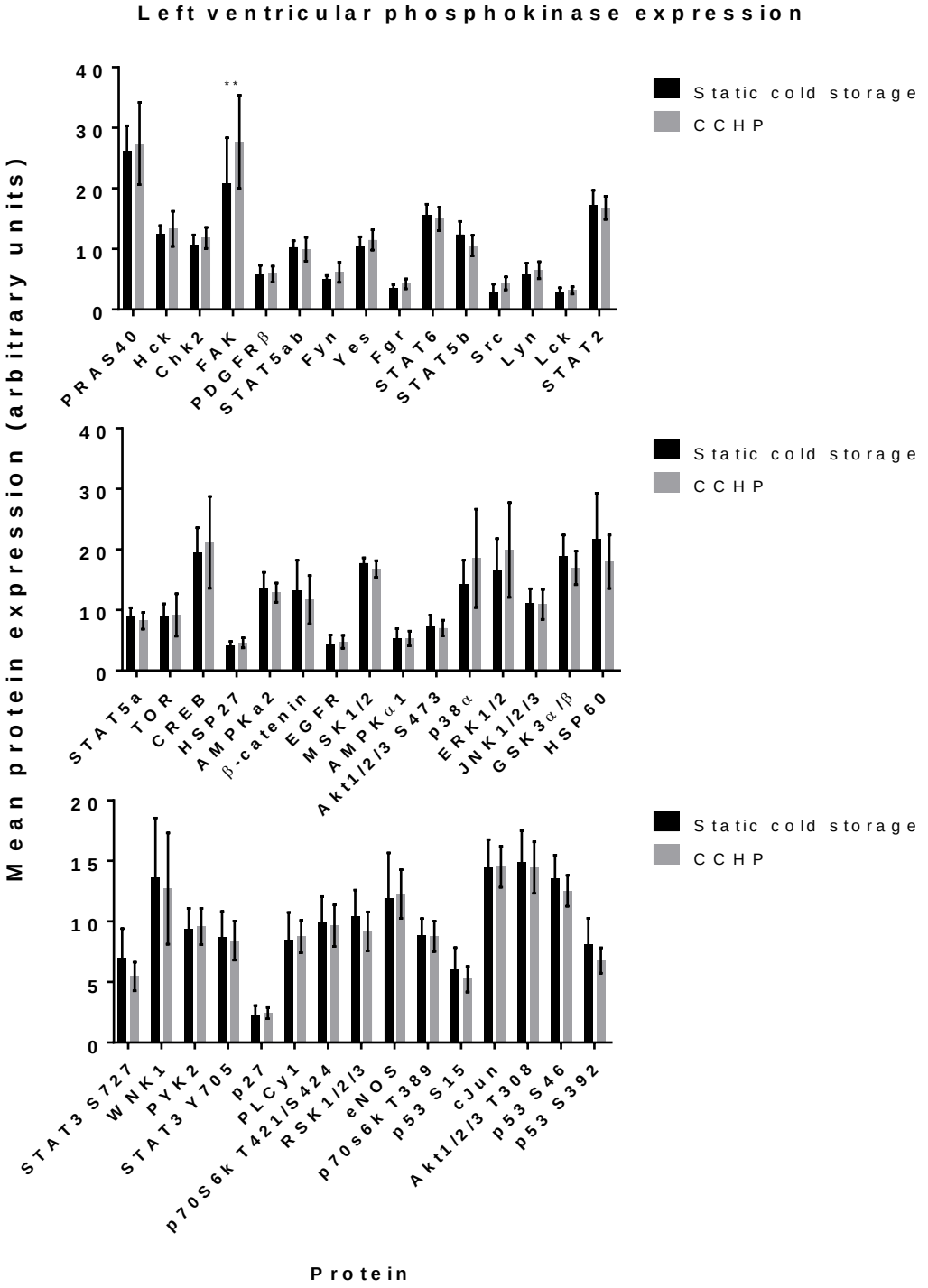

Figure 6.8 – FAK expression is increased at 48 hours post-transplant in response to perfusion. Left ventricular phosphokinase signalling protein expression is not altered by method of donor heart preservation, with the exception of FAK. ** = $p<0.01$. Data displayed is from n=6 transplants per group. CCHP = cold cardioplegic heart perfusion.

6.3.9 Method of donor heart preservation does not alter the extent of post-transplant graft infiltration or tissue damage:

A blinded histological assessment of inflammatory damage and leucocyte recruitment to the graft was performed at 48 hours. The extent of myocardial damage was not significantly different between the groups, and was considered to be relatively high at 48 hours for all pigs (perfused vs. static storage: 1.93±1.4% vs. 7.1±11.1%, p=0.45, figure 6.9). The damage was markedly greater in one pig from the static cold stored group. Where damage was detected, this manifested as a loss of cross-striations and nuclei and was taken to represent ischaemic injury, which was also present in areas without lymphocytic infiltration and thus may have occurred independent of rejection. Representative images from the assessed slides are provided in figure 6.10 and demonstrate the presence of lymphocytes, necrosis and macrophages in the damaged areas from both static cold storage and perfused groups. Importantly, myocyte injury impaired the assessment of graft infiltration as it was unclear whether infiltrating leucocytes were responding to ischaemia or the presence of donor antigen. However, an attempt to score the level of infiltration was made in areas without significant myocyte injury.

These findings demonstrate that the intensity of lymphocytic infiltration in the tissue was similar between the two groups (perfused vs. static storage: 7.3±2.8% vs. 12.9±14.1% infiltrated, p=0.36, figure 6.9), although there was much greater variability in the standard group. The static cold stored group had two recipients with much greater infiltration compared to any other subject, with high lymphocytic foci. Some macrophages can also be detected in all hearts but granulocytes are absent. Some minor lymphocytic infiltration was observed beneath the surface epithelium of some arteries, indicating a limited level of vascular rejection in each heart.

Photographs of n=1 static cold stored heart (with greatest infiltration [37.2%]) and n=1 representative photograph from a perfused heart post-transplant are provided in figure 6.11. Photographs display a macroscopic view of the hearts immediately following retrieval at 48 hours.

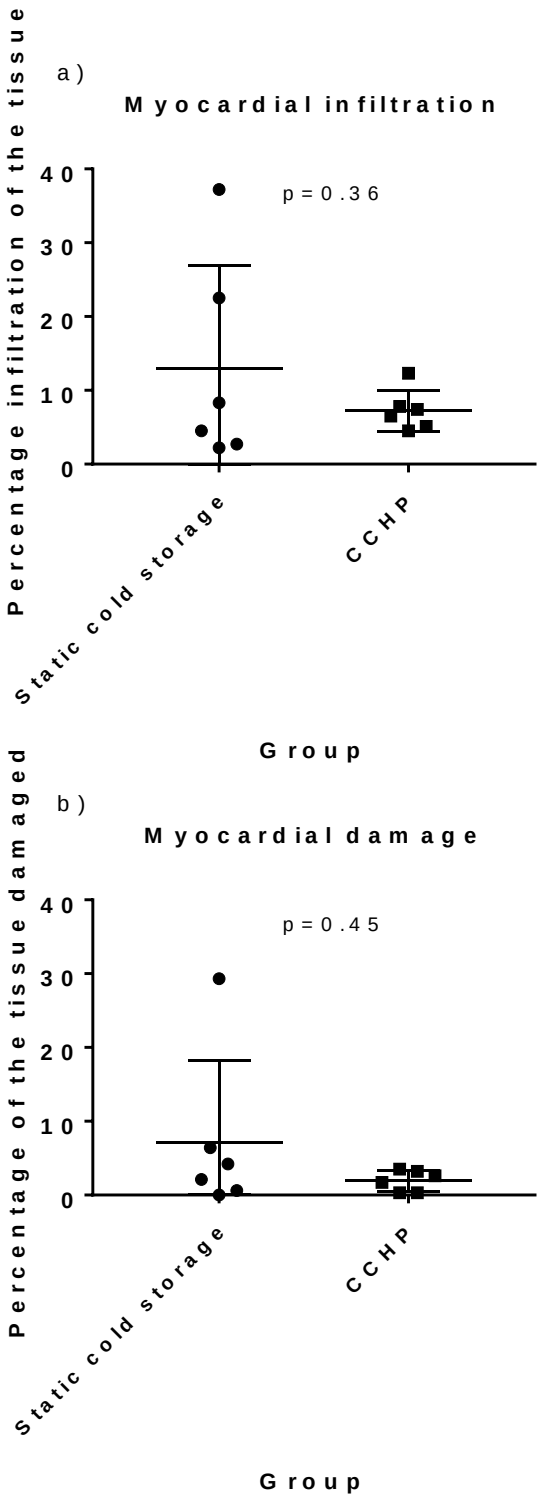

Figure 6.9 – Cold cardioplegic perfusion does not alter graft infiltration post-transplantation compared with static cold storage. Similar levels of graft infiltration were observed regardless of preservation method (a). Myocardial damage was also similar between static cold storage and perfused groups (b). Data displayed is from n=6 transplants per group. CCHP = cold cardioplegic heart perfusion.

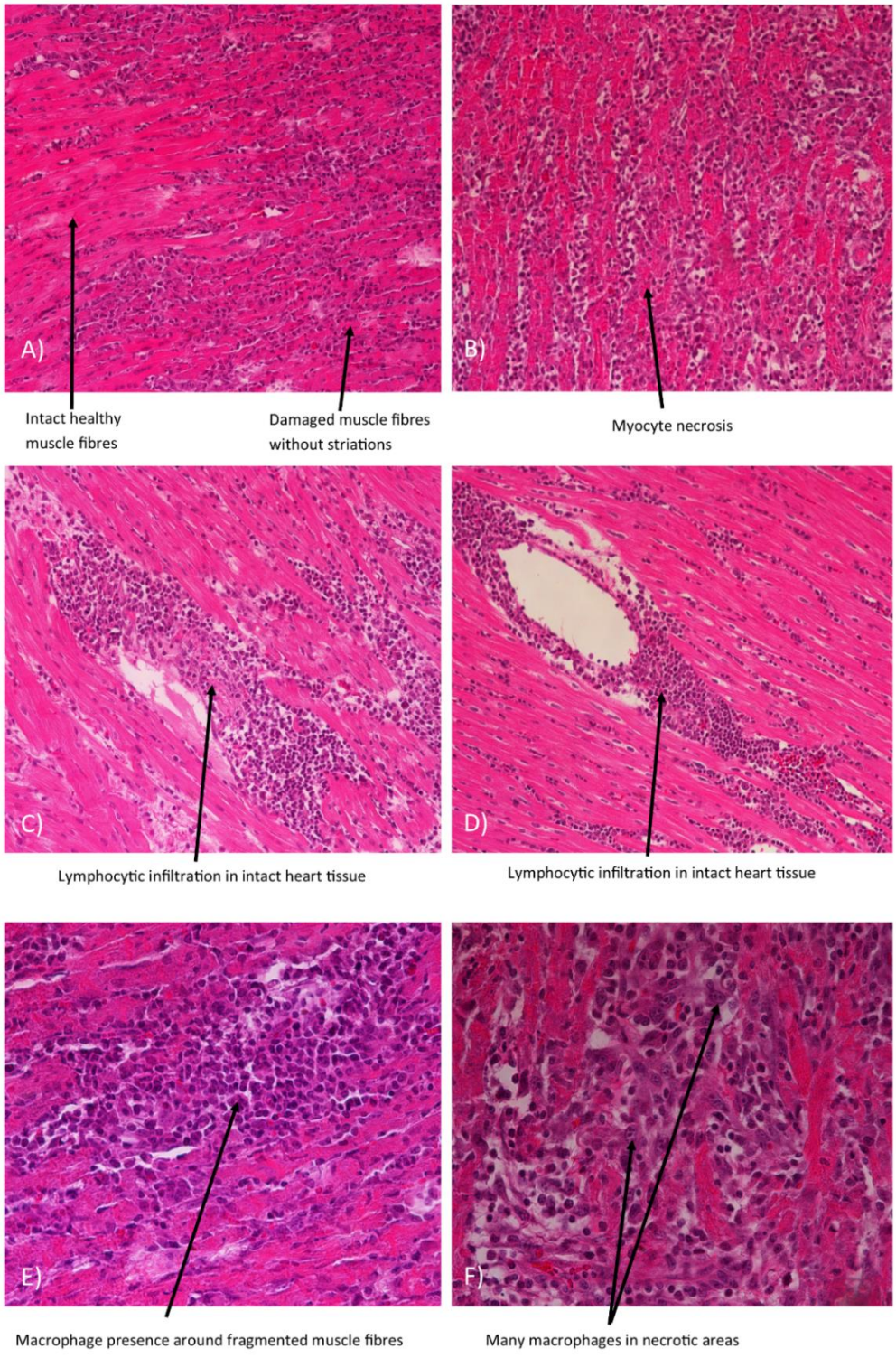

Figure 6.10 – Representative histological images from hearts at 48 hours. Images are presented displaying myocyte damage (A&B, static cold stored and perfused respectively), lymphocytic infiltration (C&D, static cold stored and perfused respectively) and macrophage presence around damaged muscle (E&F, static cold stored and perfused respectively).

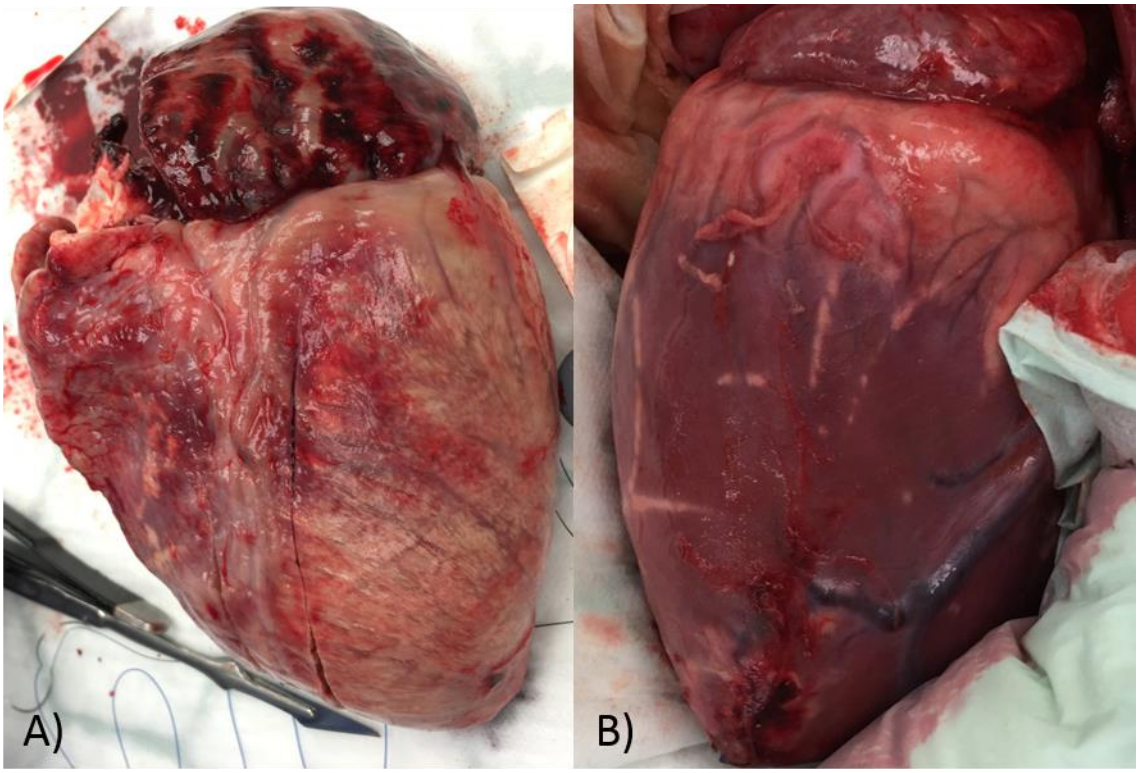

Figure 6.11 – Photographs illustrating the macroscopic appearance of the hearts at 48 hours. Photograph A was preserved by static cold storage and demonstrated a major diffuse infiltration of the ventricle. This heart demonstrated the greatest infiltration of all hearts transplanted. Photograph B was preserved by cold cardioplegic perfusion for 8 hours and demonstrated well preserved ventricle with white infiltrated areas along some vessels. Several hearts preserved by static cold storage were more similar to B than A.

6.4 Discussion:

Donor leucocyte transfer into the recipient upon revascularisation has an established role in directing rejection of the allograft[74]. This occurs via the self-presentation of

peptides on the surface of passenger cells in the context of donor MHC, which induces the activation of recipient T cells resulting in graft infiltration. Previous findings have demonstrated that removal of passenger leucocytes prior to lung transplantation significantly diminishes lymphocytic infiltration of the donor organ at 24 hours post-transplant. Removal of donor leucocytes was achieved by normothermic *ex vivo* lung perfusion, which caused significant diapedesis from the organ into the perfusion circuit and thus precluded their transfer into the recipient[65]. The earlier findings described in chapter 4 suggested that a similar effect could be induced with cold cardioplegic perfusion of the donor heart. Whilst the healthy heart contains far fewer leucocytes than the lung, the extent of immunodepletion associated with hypothermic perfusion was much greater than hypothesised and as such it was proposed that this would translate into significant clinical impact post-transplantation.

Unfortunately, this study was associated with significant technical problems, many of which remain unresolved. This was mostly related to the heterotopic transplant procedure, following which there was a high level of mortality in the static storage group. It is unclear precisely why the number of deaths was so high in this group but not in the perfusion group, although it is not thought to be related to a graft rejection response. Indeed, the reason for performing a heterotopic rather than orthotopic transplant was to account for the possibility of a high level of graft infiltration that could alter the function of the graft. It was believed that by retaining the recipient heart this would alleviate the risk of recipient mortality for immunological reasons. Following autopsy there was no indication of graft infiltration, nor was there any sign of thrombus formation in either the donor or recipient hearts and as such the death remained unexplained.

Despite the success achieved with the pilot heterotopic transplants, this was not translated in this larger study. The differences between the two sets of experiments in terms of protocol are generally minor. In the current study, left ventricular biopsies were obtained from the donor heart prior to and following storage, although these were relatively small and are unlikely to have been able to cause large scale problems for the recipient. Furthermore, blood samples were obtained from the recipient within 30 minutes of transplant and again after 6 and 12 hours. This necessitated the continued sedation of the pigs over this time period, which was achieved with ketamine infusion.

This was not the case with the previous pilot transplants, where the pigs were awakened as early as possible. Whilst the blood samples obtained were relatively large, the total cumulative volume removed across all time points never exceeded 100ml and a larger volume of either Ringer's solution or albumin was infused to support the animal. Due to the high mortality during the early phase of the study, the 6 and 12 hours samples were stopped to ensure that this did not contribute to haemoconcentration and clot formation. This did not alter the clinical course of latter transplants and as such neither the blood retrieval nor the continued sedations were likely to be contributors to the deaths. More likely to be important however was the large volume of blood lost during the transplant procedure in some of the pigs (varying from approximately 300ml up to 2L), which represents a significant proportion of the total blood volume and is likely to have been detrimental. This is particularly the case in smaller recipients weighing only around 35kg, where the effect of blood loss would be amplified.

Further problems may have resulted from the size mismatch between the male donor and female recipient pigs. Although effort was made to try to pair the pigs appropriately, many of the recipients were smaller than the donors. As such, there was little room within the abdomen for placement of the donor heart. During the implantation procedure the bowels were removed from the cavity and held back to allow visualisation of the implant site. This meant that they were constrained for upwards of an hour and some areas were visually noted to turn purple/black during this period suggesting ischaemic injury. This may be responsible for promoting bacterial translocation leading to the development of sepsis[157]. The level of distension and ischaemia of the bowels may therefore have contributed to the deaths of a number of recipient animals. Future improvements could be made to address this by either altering the site of implantation, shortening the period of bowel retraction or establishing a more suitable method for repositioning the bowels to diminish the risk of injury.

As a further modification of the protocol from the pilot heterotopic procedures to try to address the lack of space in the abdomen, a longer section of aorta was retrieved from the donor, and was used to attach the heart. This may have increased the possibility that the vessel became twisted and partially occluded, reducing the amount

of perfusion. The fact that the pigs were awakened and placed back in their pen for the entire follow-up period may also have increased the risk of occlusion. The pigs were freely able to move, which meant that in certain positions they may have been able to increase the pressure on the aorta and cause some constriction.

Although there were numerous problems associated with the surgical procedure in this study, it was eventually possible to gain data from n=6 vs. n=6 transplants. As such, a direct comparison of outcomes post-transplantation after standard static cold storage or cold cardioplegic perfusion was performed as originally intended. Whilst *ex vivo* perfusion but not static cold storage was associated with a major immunodepletion effect as expected, this did not translate into a loss of leucocyte infiltration at 48 hours post-transplantation. The level of ischaemic injury to the hearts in the early post-transplant period confounded the histological assessment of graft infiltration, leading to a lack of clarity in the conclusion. The combination of high mortality due to technical issues and the high post-transplant injury unrelated to graft infiltration means that it is prudent to therefore remain conservative with regard to the inferences that can be drawn on clinical effect. It is certainly surprising that these new findings contradict the earlier substantial differences observed in n=4 pilot transplants at this time point when surgical issues were not present. The lack of effect observed in this study may to a certain extent be related to a lack of statistical power and it may be the case that n=6 vs. n=6 is simply too small a population to observe an effect. The study was powered on the basis of the level of immunodepletion that was observed with cold cardioplegic perfusion. Whilst this immunodepletion was indeed replicated in this study, it may be that the effect size for myocardial injury or infiltration is smaller and thus a larger population is required. The nature of the surgical procedure also increases the level of heterogeneity and so a higher number of subjects may be necessary to improve reliability of the results.

On the assumption that the data obtained here are reliable, this larger study would suggest that immunodepletion by cold cardioplegic perfusion does not promote clinical benefit with regard to acute rejection. However, there are a number of differences between the previous findings and those displayed here, which remain unexplained. Indeed, whilst the level of infiltration in the perfused group is approximately in agreement with that observed with the earlier transplants at day 2

(11.0% vs. 7.3%), the mean infiltration in the standard control group has improved markedly (23.0% previously vs. 12.9%). It is unclear why this is the case when there were such technical difficulties experienced, however it may relate to a lack of perfusion in the donor heart post-transplant, which has impeded the infiltration of the graft. Furthermore, there was significant variation in the level of infiltration in the static cold storage group, with two pigs displaying much higher lymphocytic presence than the rest of the animals. Unsurprisingly, these same two pigs also displayed the highest myocardial damage scores. Whether this variation is reflective of increased disparity between donor and recipient in terms of leucocyte antigen mismatch or is due to some other phenomenon remains unclear. However, it is important to note that the extent of infiltration in the cold cardioplegic group displayed little variability, with low infiltration despite the significantly longer storage time. As such, 8 hours storage using this method of preservation does not display inferiority to 2 hours of static cold storage, which in itself indicates a significant benefit to hypothermic perfusion.

It is intriguing to note that with this set of perfusions the level of cardiac troponin I was significantly elevated by 4 hours and remained so until the end of the preservation period. This is a further marked deviation from the earlier findings presented in chapter 4, in which this biomarker was scarcely detectable and certainly below clinically indicative levels. In this case, concentrations of >2ng/ml were observed, significantly greater than the threshold reported as indicative of acute coronary syndromes in patients (0.04ng/ml)[158]. The levels observed here are further contextualised by those observed following experimental myocardial infarction in porcine subjects, which range from 41-109ng/ml dependent upon the site and severity of infarction[159]. Whilst there are caveats to the relevance of both of these figures in this setting, particularly due to the lower total volume in the circuit compared to that in the adult circulation, it does provide a strong indication that there was damage induced during the preservation period. Whether the greater level of myocardial injury observed in this series of perfusions altered the post-transplant course compared to previous experiments is unclear. However, it is important to note that static cold storage was also associated with significant troponin I release and was thus presumably also subjected to myocardial damage prior to transplantation. Although damage during static cold storage is expected, the presence of troponin I in the perfusate was certainly surprising. One explanation for the possible presence of the biomarker in this

experiment is the fact that the perfusate reservoir was reused despite it being designed as a single-use disposable consumable. It was thought that for research purposes the consumables could be washed and this would be sufficient to allow multiple uses. As it is plastic rather than metallic it could not be autoclaved between uses and it is therefore possible that this has promoted bacterial growth. This may have promoted an overstimulation of donor immunity leading to the release of non-specific cytotoxic products that have damaged the graft.

Importantly, post-transplant troponin I levels were not different between groups, although they were still elevated by at least two-fold compared to the 0.04ng/ml threshold. Clearly, the implantation process requires a certain amount of surgical manipulation of the tissue, which may be responsible for a proportion of this release and makes interpretation of these values more difficult. The histological assessment was in agreement in that there was no observable difference in level of injury between the groups, but was suggested to be predominantly ischaemic in nature rather than inflammatory. It is not possible to discount the potential that the native heart also contributed to the circulating troponin I pool in the post-transplant period, although at sacrifice there was no obvious damage to the organ.

The lack of alteration in graft infiltration post-transplant with perfusion suggests a lack of downstream clinical effect of donor heart immunodepletion. However, this removal of passenger leucocytes was associated with lower inflammatory cytokine release, suggesting that there may still be a beneficial impact upon the local tissue environment. Indeed, this effect was most prominently displayed by the elevated IL-1β titre at 48 hours in the static cold storage group, which remained low with perfusion. This cytokine represents a major pro-inflammatory mediator normally released in the acute phase of inflammation and is a key activator of innate immune cells, and promotes adhesion molecule expression to increase leucocyte recruitment[160]. The diminished release of IL-1β post-transplant in the perfused heart group is indicative of a less inflammatory state in the recipient, which should be beneficial. Furthermore, high IL-1β concentrations are associated with impaired contractility[161] and could therefore contribute to early graft dysfunction. As such, the diminished release of this cytokine by perfusion should prevent this effect, although it was not assessed in this study. Importantly, IFN-γ was not detected at any time point in any recipient of a

hypothermic perfused heart, whereas high levels were observed in the early post-transplant period in a number of those pigs receiving a static cold stored heart. Whilst this difference did not reach statistical significance, the inability to produce IFN-γ may be a downstream consequence of such high levels observed during perfusion, leading to "inflammatory exhaustion" of the donor heart. This may be beneficial as IFN-γ is a highly inflammatory cytokine which promotes activation of macrophages and modulates their behaviour towards a pro-inflammatory phenotype[162], which is likely to cause graft damage. This being said, sustained IFN-γ exposure was required *in vitro* for the elevated expression of the inhibitory checkpoint molecule PD-L1. The lack of IFN-γ post-transplant could have prevented the sustained expression of PD-L1 and therefore the protective effect of this molecule is lost.

Although cell-free DNA was detected in the plasma post-transplant, it was released at a consistent level in both groups. This is suggestive that the level of cellular damage was similar with both methods of preservation and further supports the findings from both troponin I and histological assessment. Injury to the cells leads to passive release of cell-free DNA into the circulation[163] and is the most plausible mechanism responsible for the amount detected. The source of the DNA remains unclear in terms of the predominant cell type injured but the evidence of significant myocyte damage implies that the myocardium could be a major contributor. Whilst activated neutrophils and eosinophils are also able to release cell-free DNA in a more formal mechanism as part of the inflammatory response[164, 165], this is less likely to have occurred in the heart as very few granulocytes were observed in the tissue at 48 hours.

A proteomic evaluation of the tissue provided a broadly similar profile between the groups in terms of both cell death pathways and inflammatory signalling. The relative increase in expression of focal adhesion kinase in the perfused group post-transplant could have a number of important roles. In cardiac homeostasis, focal adhesion kinase is involved in the maintenance of anchorage between the endothelium and substratum[166]. It has been suggested previously that this protein has anti-apoptotic properties[167], although this was observed in an *in vitro* culture model using human leukaemic cells. Later findings have also indicated a similar role for this protein in protection of endothelial cells from apoptosis[168]. Increased expression of focal adhesion kinase would likely impart some benefit to the tissue, although downstream

mediators of a number of these protective pathways were also evaluated as part of this analysis and were not observed to differ limiting the significance of this finding.

These latest results do not provide conclusive evidence of a significant benefit to immunodepletion of the donor heart in the early period post-transplantation in terms of cellular infiltration of the graft. The local tissue environment demonstrates a significantly lower inflammatory milieu, which could be due to a loss of resident leucocytes and the 'cytokine exhaustion' of the graft due to perfusion and this is undoubtedly beneficial. Although there is a lack of clarity in the histological findings, if correct, then it is possible that the relatively low number of resident leucocytes in the healthy donor heart means that this immunodepletion has a more limited downstream impact compared with other organs with more established immune compartments, such as the lung. Of course, the greater the number of passenger leucocytes that are present in the tissue, the greater the contribution of the direct allorecognition pathway. It is important to note that the extent of immunodepletion achieved is high, and thus increased benefit may be imparted in hearts from brain dead donors, which are well documented to have increased donor immune load[96]. Graft infiltration in the standard group would be expected to be significantly higher with this additional population contributing to direct presentation to recipient T cells. It may therefore be appropriate to repeat this experiment in a more clinically relevant model with donor brain death prior to retrieval and to determine whether hypothermic perfusion retains its ability to immunodeplete to such an extent and re-evaluate post-transplant outcomes.

Despite the lack of effect of *ex vivo* perfusion induced immunodepletion on post-transplant graft infiltration, there remain numerous benefits to the use of this preservation method. Foremost is the significantly increased safe preservation time of at least 8 hours, which has been further increased with subsequent successful transplantation after 24 hours of intermittent perfusion[97]. This additional preservation period could allow improved donor-recipient matching and lead to more efficient theatre logistics, with a greater window available for the transplant procedure to be scheduled. Further clarification of the effect of perfused-induced immunodepletion in the post-transplant period is urgently required and it is appropriate to suggest that this study should be repeated when the technical difficulties associated with the transplant

procedure are rectified. It may also be sensible to repeat the study with the use of an orthotopic transplant, which would remove much of the uncertainty found in this experiment. This would also significantly increase the clinical relevance of the findings.

Further research is clearly warranted to discern how to utilise the circuit to allow greater immunomodulation of the donor organ, a process that is not feasible without *ex vivo* perfusion technology. It may be possible to incorporate use of the system as a platform for delivery of targeted therapeutics and thereby diminish graft immunogenicity and increase tolerogenic properties on the tissue itself in an attempt to prevent recipient immune activation.

6.5 Limitations:

This study was associated with a number of technical difficulties that make it difficult to infer any reliable conclusions, and as such these surgical issues represent the major limitation to the study. Besides this, the study was also limited by the lack of an evaluation of graft function post-transplantation, and it is therefore assumed that contractility was equivalent between the groups, although a future assessment is warranted. This is particularly true in light of the inflammatory cytokine findings, which can significantly alter functional capacity. This study also only evaluated graft infiltration at a single time point, and it is possible that 48 hours was not optimal to detect a difference based on the temporal kinetics of leucocyte activation and trafficking into the graft. The donor pigs utilised in this study were healthy at the point of cardioplegia and as such the model did not replicate the DBD or DCD scenario prior to retrieval. As such these organs did not undergo relevant events such as a catecholamine storm, hypertension, hypotension or a profound cytokine storm prior to retrieval, which could alter the results from this study. Hearts from the pigs that died prior to 48 hours were not evaluated histologically and thus there is bias in the results. It is unclear whether the inclusion of these hearts would have significantly altered the findings.

6.6 Conclusion:

Ultimately, this study was severely limited by the technical complications experienced throughout. As such, no firm conclusions can be made. It is crucial that this study is repeated once the heterotopic procedure has been validated in order to discern the true

effects of hypothermic perfusion on clinical outcomes. It may be more appropriate to switch to the use of an orthotopic procedure for evaluation of post-transplant outcomes as this would also represent a more clinically-relevant model. Further evaluation of this preservation method and its potential value for therapeutic delivery of immunomodulatory agents is warranted.

Chapter 7: Normothermic perfusion of donor hearts induces a cytokine storm but does not promote passenger leucocyte migration

7.1 Introduction:

There is a severe shortage of suitable donor hearts available for transplantation globally, significantly limiting the number of procedures that can be performed. This shortfall in suitable donor organs is responsible for elevated mortality whilst on the

transplant waiting list and as such there is currently a drive to increase the number of donor hearts available for transplant. Indeed, the median time between listing and implantation of a donor heart in the UK between 2011 and 2014 was 1280 days, which represents a significant waiting time for a patient with advanced heart failure[9]. Of this same population, 10% of those listed had died within 1 year[9], demonstrating the urgent requirement for additional donor organs to make up the shortfall. Incorporating novel methods of organ preservation could increase the number of transplants that can be performed. Previous data presented in chapter 4 highlights the multitude of potential benefits to hypothermic *ex vivo* perfusion of the donor heart, which could revolutionise the storage procedure for DBD donors. However, whilst the extended safe preservation period with hypothermic perfusion is hugely beneficial, it is unlikely to solve the problem of donor heart shortage. Alternative strategies that allow functional assessment to be performed are now required to expand the potential donor pool.

A number of methods have been proposed for increasing the number of transplants performed, including the use of extended criteria donors, such as those with greater smoking history or older donors[169]. However, these donors are by definition associated with greater risk than those deemed to be within standard criteria. Greater utilisation of these donors has been incorporated into clinical practice over recent years, yet centres are still unable to keep up with the number of transplants required annually. As such, it is clear that further expansion of the donor pool is required, and strategies to allow the use of DCD donors has recently received significant attention.

DCD donor utilisation has increased the availability of donor organs in non-cardiac transplant, but these donors have previously been considered too high risk by clinicians for heart donation. This is in spite of the fact that DCD heart donation has been demonstrated to be possible. Indeed, the first heart transplantation performed in 1967 utilised a heart from a DCD donor[3]. Increased utilisation of such donor hearts has now been identified as an essential part of a national strategy on improving transplantation[170].

Until recently, donor hearts were almost exclusively sourced from DBD donors. In such cases the donor remains on ventilatory support and cardiopulmonary function is maintained. This ensures that there is minimal interruption to oxygenation before cooling and preservation of the donor heart. In contrast, with DCD donors, death is

certified after circulatory arrest. This is associated with injury to the donor organ as there is a period between withdrawal of ventilatory support and the declaration of circulatory arrest, which may impact upon cardiac perfusion and subsequent function. The duration of this period is variable but inevitably imparts warm ischaemic injury to the organ once systolic blood pressure has significantly dropped.

Importantly, whilst DBD hearts have known functional capacity (measured *in vivo*) prior to explantation, DCD hearts have unknown function following circulatory arrest. Transplantation of a DCD heart without functional evaluation would be high risk and thus methods to demonstrate appropriate contractile capacity are necessary. Pioneering work led by Large *et al* has utilised a method known as normothermic regional perfusion to restore optimal conditions to the heart in isolation whilst still in the donor. This technique allows the DCD heart to be functionally evaluated *in cadavero* prior to retrieval to ensure that sufficient function can be retained to support circulation in the recipient[54]. Early findings suggest that this method is associated with a significant increase in the number of transplants being performed and positive clinical outcomes[54]. Whilst this undoubtedly represents an inventive and elegant approach to addressing this problem, its widespread implementation is blocked due to ethical restrictions in place in other countries.

Consequently, there remains an urgent requirement for an alternative method for evaluation of marginal donor organs before their widespread use can be safely achieved. This could also be utilised for further evaluation at the recipient site after normothermic regional perfusion prior to implantation. Current evaluation options include the Organ Care System (TransMedics, Andover, MA, USA), which is a normothermic perfusion device delivering perfusate in a manner similar to the Langendorff method[171]. This was developed in an effort to avoid damage from cold ischaemia. This device allows the heart to beat throughout transport but functional evaluation is limited and decisions are based mostly upon macroscopic evaluation of the organ and perfusate lactate content as an indicator of cardiac metabolic activity[172].

Whilst the hypothermic perfusion device discussed previously is not conducive to cardiac functional evaluation, *ex vivo* perfusion may still hold the key to expanding donor heart availability. A comprehensive functional analysis of the donor heart can only be undertaken effectively at normothermia. As such, the development of *ex vivo*

normothermic perfusion systems has become increasingly important. This has the additional benefit that the organ is stored in a much more physiological manner, which could ensure improved preservation. Furthermore, the continual delivery of perfusate through the vasculature of the heart could have auxiliary benefits similar to those presented earlier in chapter 4 and those described for normothermic *ex vivo* perfusion of the lung[65].

This study aimed to determine whether *ex vivo* normothermic perfusion could be utilised to maintain a donor heart in a viable and active state for four hours. Secondarily, the donor organ was assessed to establish whether *ex vivo* normothermic perfusion altered donor immunity similar to that observed with cold cardioplegic perfusion.

7.2 Methods:

7.2.1 Donor organ retrieval:

Six healthy Swedish pigs of native breed were used in the study. Donor organ retrieval was performed as previously described in section 2.1.2. Briefly, anaesthesia was induced and a median sternotomy performed. The ascending aorta was cannulated and the superior and inferior caval veins were clamped until the heart was empty. The distal ascending aorta was cross-clamped and the heart received 600ml of 8°C St

Thomas cardioplegia. The heart was then explanted and maintained at approximately 8°C submerged in St Thomas solution for approximately 30 minutes whilst the heart evaluation system was being prepared.

7.2.2 Heart evaluation device:

The heart evaluation system consists of an organ chamber, which sits above a large capacity perfusate reservoir. The system has separate circuits for independent perfusion of the left and right sides of the heart, although both are fed by the same perfusate reservoir and flow is directed through a shared membrane gas-exchanger, heater-cooler unit and leucocyte filter. By separating the delivery circuits, both left and right sided function can be evaluated in isolation and adjustments can be made as required depending upon the nature of the evaluation. Preload and afterload are controlled by pressure-regulated pumps and are adjusted by software produced in-house, which allows the perfusion to be continued in a fully automated manner.

7.2.3 Evaluation system preparation and donor heart perfusion:

The perfusate reservoir was filled with 3L of high albumin, hyperoncotic perfusate medium produced in-house. Autologous whole blood was washed using a Medtronic Autolog cellsaver (Medtronic Inc, Minneapolis, USA) and leucocyte filtered using a Pall Leucocyte filter (Pall Corp., Port Washington, USA) to acquire packed erythrocytes, which were added to the circuit to achieve a target haematocrit of 20%. The perfusate was circulated at a temperature of 37°C before the donor heart was attached. After careful de-airing, perfusion was commenced in a Langendorff manner, with delivery to the coronary arteries via retrograde aortic flow. Once stabilised and all other perfusate inlets were carefully de-aired, the flow was switched such that perfusate was delivered to the left atrium via the cannulated pulmonary veins. Perfusate was simultaneously delivered to the right atrium via the cannulated caval veins. The appropriate pressure at which the perfusate was delivered to the left and right side of the heart was determined for each individual pig and was set to a maximum of that measured *in vivo* prior to explantation. Afterload pressures and flow rates were adjusted to ensure that left ventricular output was maintained at 3L/minute throughout the experiment and was not increased beyond this. The heart was perfused continuously at normothermia for four hours.

7.2.4 Sample collection:

7.2.4.1 Perfusate:

A perfusate sample measuring 20ml was obtained prior to attachment of the organ and at hourly intervals until the perfusion was ceased at four hours. From each sample, 1ml was withdrawn and reserved for analysis of leucocyte content by flow cytometry. The remaining sample was centrifuged at 2000g for 10 minutes at 4°C to separate plasma, which was stored at -80°C for later analysis.

7.2.4.2 Biopsy:

Biopsies were not obtained prior to perfusion due to concern that this would induce sufficient damage to alter function whilst on the circuit. However, left ventricular tissue was obtained once the perfusion was ceased. Each biopsy was split into two sections, the first of which was snap frozen in liquid nitrogen and transferred to a -80°C freezer for longer term storage. The second sample was fixed in 10% neutral buffered formalin and paraffin embedded for later histological evaluation.

7.2.4.3 Leucocyte Filter:

Following perfusion, entry and exit tubes were clamped either side of the leucocyte filter, which was then removed from the circuit. The contents of the filter were poured into a sample collection flask in a retrograde direction and retained. Adherent leucocytes within the filter were detached by incubating at 37°C for 15 minutes in the presence of trypsin-EDTA. This mixture was decanted from the filter and combined into the sample collection flask. The total volume retrieved was recorded and the trypsin was quenched with foetal bovine serum to a final 10% concentration (vol:vol). The perfusate was washed by centrifugation at 2000g for 10 minutes at 4°C before the supernatant was discarded. The entire filter volume was assessed using flow cytometry.

7.2.5 Flow cytometry:

A panel of antibodies was utilised to characterise a broad range of leucocytes within the circulating perfusate. Expression of key cell surface markers was used to phenotype and quantify immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10-2B2+), helper T cells (CD3ε+CD4α+), cytotoxic T cells (CD3ε+CD8β+), γδ T cells (γδ+), NK cells (CD335+), B cells (CD21+), classical monocytes (CD14+CD163-), non-classical

monocytes (CD14+CD163+), intermediate monocytes ($CD14^{dim}$ $CD163^{bright}$) and macrophages (CD203a+). SLA-DR expression was also assessed as a marker of antigen presentation. TLR4 expression was also assessed on each population to determine whether its ability to detect damage associated molecular patterns was altered during perfusion.

Leucocyte phenotyping was performed using flow cytometry as described in section 2.2.1. Briefly, leucocytes were stained with antibodies specific for the above cell surface markers before contaminating erythrocytes were lysed with red blood cell lysis buffer (BD Biosciences, UK). Cells received a series of washes and were finally resuspended in 1.2ml staining buffer. From each sample, 1ml was evaluated by the cytometer at a flow rate of 1ml/min. Cell numbers were analysed as standardised absolute counts per millilitre of perfusate or were extrapolated to the total filter volume to calculate the total number of cells trapped in the leucocyte filter.

7.2.6 Cytokine quantification:

In order to determine whether *ex vivo* normothermic perfusion altered inflammatory mediator production and release, a porcine 13-plex magnetic Luminex assay was performed as described in section 2.2.3. The cytokine content in the perfusate was determined using undiluted samples at serial time points. The presence of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α was determined and compared between time points.

7.2.7 Quantitative PCR:

The detection of mitochondrial and genomic DNA in perfusate was performed using qPCR as previously described in section 2.2.2. Briefly, each sample was assessed without dilution using primers for cytochrome b (mitochondrial DNA) and GAPDH (genomic DNA) and the concentration was standardised per millilitre of perfusate. All qPCR analysis was performed using a QuantStudio 12K Flex system using a Power SYBR green PCR master mix.

7.2.8 Apoptosis signalling:

A post perfusion left ventricle biopsy was obtained from n=4 pigs. Left ventricle biopsy tissue was also obtained from n=4 healthy pigs (from a separate study) immediately following standard cardioplegic retrieval (as detailed in section 2.1.2) for

comparison. A human apoptosis antibody proteome profiler array was utilised to evaluate the change in 35 cell death and survival signalling proteins in response to four hours of normothermic perfusion and evaluation. The assay was performed as described in section 2.2.5. A total of 30mg snap frozen left ventricle was used for each sample and a standardised 400µg of extracted protein was added to each membrane. Eight individual membranes were used to allow each sample to be analysed in isolation, and thus improve the sensitivity of the assay. Pixel density analysis was performed using ImageJ (NIH, USA) and data is expressed as integrated pixel densities for each membrane spot. Post-perfusion values were compared with pre-perfusion healthy reference values.

7.2.9 Histological evaluation:

To confirm the viability of the donor heart following four hours of continuous normothermic evaluation, a histological assessment was performed using formalin-fixed tissue obtained from the left ventricle as described in section 2.2.6. Briefly, sections were cut at 4µm, de-paraffinised and stained with haematoxylin and eosin. All samples were prepared and assessed in a blinded manner by a consultant histopathologist, who reported on the structural integrity of the tissue and oedema formation.

7.2.10 Statistical analysis:

SPSS version 22.0 (IBM, Armonk, NY, USA) was used to perform all statistical analyses. Data are expressed either as mean ± standard deviation if normally distributed or as median [interquartile range] if non-normally distributed. The related samples Friedman's two-way ANOVA by ranks was utilised to assess changes in leucocyte number and cytokine concentrations in the perfusate over time. *Post hoc* multiple comparisons were performed for perfusate leucocyte and cytokine assessment with corrections using Dunn's method. Only descriptive data was produced to highlight the content of the leucocyte filter. Repeated measured one-way ANOVA was used to determine the change in cell-free genomic DNA over time in the perfusate. The Friedman one-way ANOVA was used to assess the change in perfusate cell-free mitochondrial DNA over time. Sidak's multiple comparisons method was utilised to determine whether apoptosis signalling was altered by four hours of normothermic perfusion and evaluation. Statistical significance was accepted when

$p \leq 0.05$. All graphs were produced using Prism 7 software (GraphPad, La Jolla, CA, USA).

7.3 Results:

7.3.1 Isolated normothermic perfusion of the donor heart does not induce leucocyte migration into the circuit:

To determine whether delivery of perfusate through the coronary vasculature induces the diapedesis of cardiac leucocytes into the circuit, serial samples were obtained at hourly intervals. No significant difference in granulocyte counts was observed in the perfusate between any of the time points sampled, with stable numbers of immature neutrophils ($p=0.52$) and mature basophils/eosinophils ($p=0.15$) throughout and a trend towards increased mature neutrophils ($p=0.07$) detected (figure 7.1). However, leucocytes from the mononuclear phagocyte system had variable responses to

perfusion (figure 7.2). Whilst classical monocytes appeared to diminish in number, this did not reach statistical significant (p=0.08). Intermediate monocytes were not affected at all by perfusion (p=0.44), although non-classical monocytes were significantly diminished within the perfusate following connection of the donor heart (p=0.008). Furthermore, although CD14- macrophages (p=0.98) and antigen-presenting SLA-DR+ macrophages (p=0.07) did not change with perfusion, a substantial drop in the number of CD14+ macrophages was observed after initiation of perfusion of the heart (p=0.003). Post-hoc multiple comparisons demonstrated that the change in non-classical monocytes was driven by a marked loss of cells at 2 hours (baseline vs. 2 hours: p=0.02). This time point was also where the biggest change was observed in CD14+ macrophages (baseline vs. 2 hours: p=0.001). No difference in lymphocyte populations were observed in the perfusate over time (figure 7.3). The connection of the donor heart did not lead to substantial changes in the number of CD8+ NK cells (p=0.81), CD8- NK cells (p=0.15), helper T cells (p=0.12), cytotoxic T cells (p=0.69), B cells (p=0.17) or γδ T cells (p=0.78).

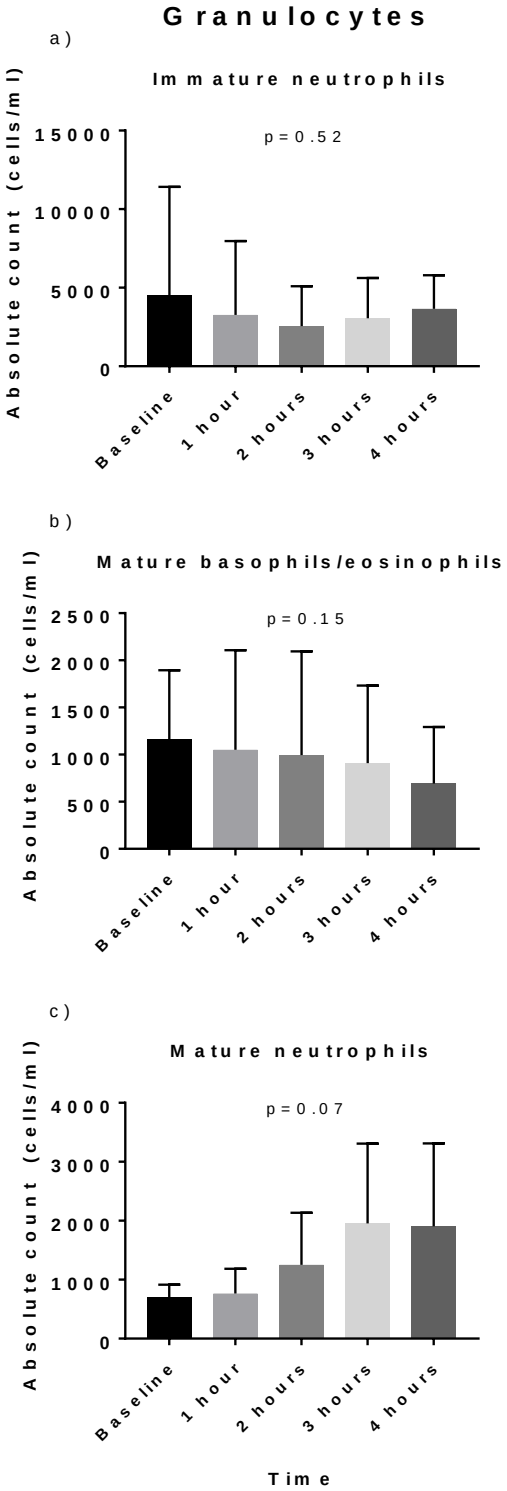

Figure 7.1 – *Ex vivo* normothermic perfusion does not induce migration of granulocytes into the circuit from the donor heart. Stable numbers of immature neutrophils (a) and mature basophils/eosinophils (b) were detected throughout the experiment, although mature neutrophils appeared to be slightly increased by 3 hours (c). Graphs depict data from n=6 porcine perfusions.

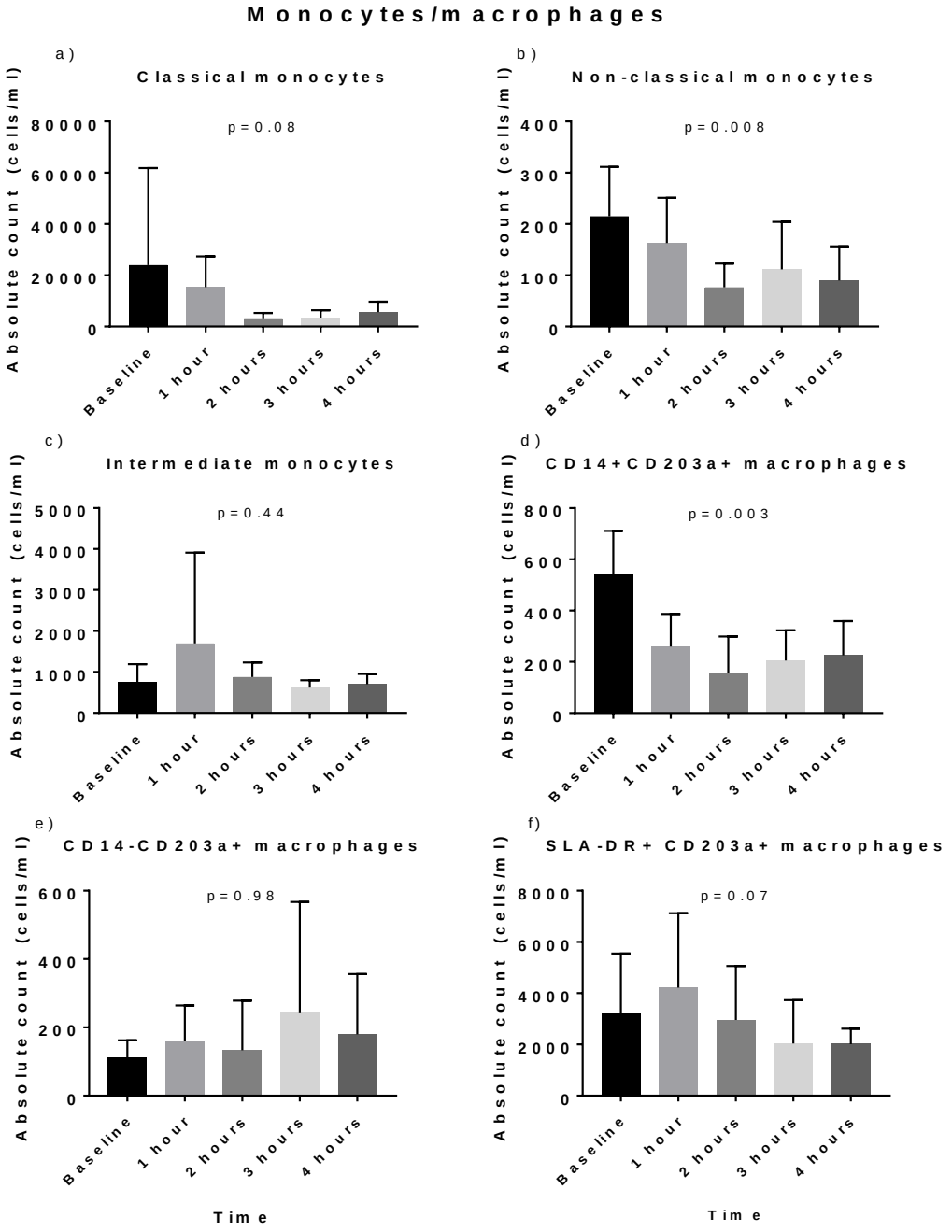

Figure 7.2 – Monocyte and macrophage response to perfusion. Classical monocytes (a) were reduced over time but this was not statistically significant. Non-classical monocytes were markedly reduced at 2 hours following perfusion (b), but no effect on intermediate monocytes (c) was observed. CD14+ macrophages were associated with significantly reduced numbers at 2 hours compared to pre-perfusion baseline (d). Neither CD14- macrophage (e) nor SLA-DR+ macrophage (f) counts were altered by perfusion. Graphs depict data from n=6 porcine perfusions.

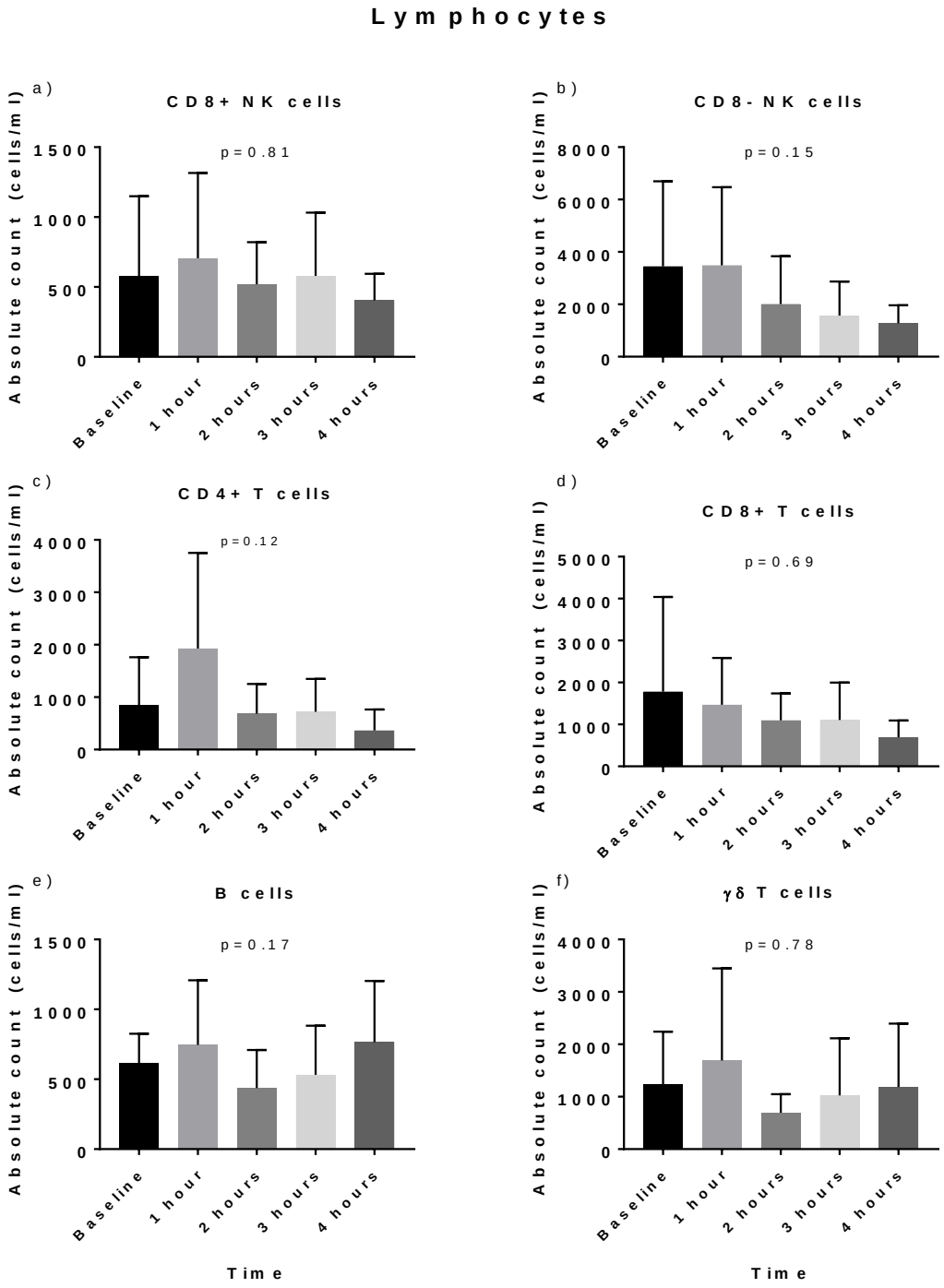

Figure 7.3 – *Ex vivo* perfusion did not alter the number of lymphocytes detectable in the perfusate. Stable absolute counts were observed for CD8+ NK cells (a), CD8- NK cells (b), CD4+ T cells (c), CD8+ T cells (d), B cells (e), and γδ T cells (f). High inter-subject variability was observed. Graphs depict data from n=6 porcine perfusions.

7.3.2 Toll-like receptor 4 expression is altered by perfusion on several but not all leucocyte populations:

TLR4 is one of a number of cell surface surveillance proteins involved in the detection of local tissue damage by leucocytes. The change in expression of this protein over time was quantified in leucocytes within the perfusate to determine whether there were any alterations in response to *ex vivo* donor heart evaluation. No consistent effect was observed on granulocytes, with stable expression detected on immature neutrophils (p=0.74), mature basophils/eosinophils (p=0.09) and mature neutrophils (p=0.23, figure 7.4).

Populations belonging to the mononuclear phagocyte system displayed variable responses to perfusion in terms of TLR4 expression. Intermediate monocytes demonstrated significantly elevated expression over time, peaking at 4 hours (mean fluorescent intensity – arbitrary units, baseline: 47785±9216; 1 hour: 50536±17578; 2 hours: 61064±22402; 3 hours: 70614±22804; 4 hours: 73007±15699; p=0.03, figure 7.5). Additionally, CD14+ macrophages also displayed a significant response to perfusion, with expression increasing over time until its peak at 2 hours (mean fluorescent intensity – arbitrary units, baseline: 97332±49584; 1 hour: 186638±84445; 2 hours: 297968±136575; 3 hours: 281620±161951; 4 hours: 226704±82270; p=0.008, figure 7.5). Stable expression of TLR4 was observed on classical monocytes (p=0.57), non-classical monocytes (p=0.29), CD14- macrophages (p=0.50) and SLA-DR+ macrophages (p=0.22, figure 7.5).

There was no statistically significant change in TLR4 expression on lymphocyte populations. However, there was a trend towards increased expression over time on helper CD4+ T cells (mean fluorescent intensity – arbitrary units, baseline: 63767±41591; 1 hour: 41841±25299; 2 hours: 76612±40630; 3 hours: 104104±111139; 4 hours: 148925±69758; p=0.06, figure 7.6). This was also true for CD8+ T cells (mean fluorescent intensity – arbitrary units, baseline: 44743±15512; 1 hour: 48330±28274; 2 hours: 62026±15820; 3 hours: 84806±54720; 4 hours: 85464±55337; p=0.06, figure 7.6). No changes in TLR4 were observed for CD8+ NK cells (p=0.86), CD8- NK cells (p=0.25), B cells (p=0.34) or γδ T cells (p=0.22).

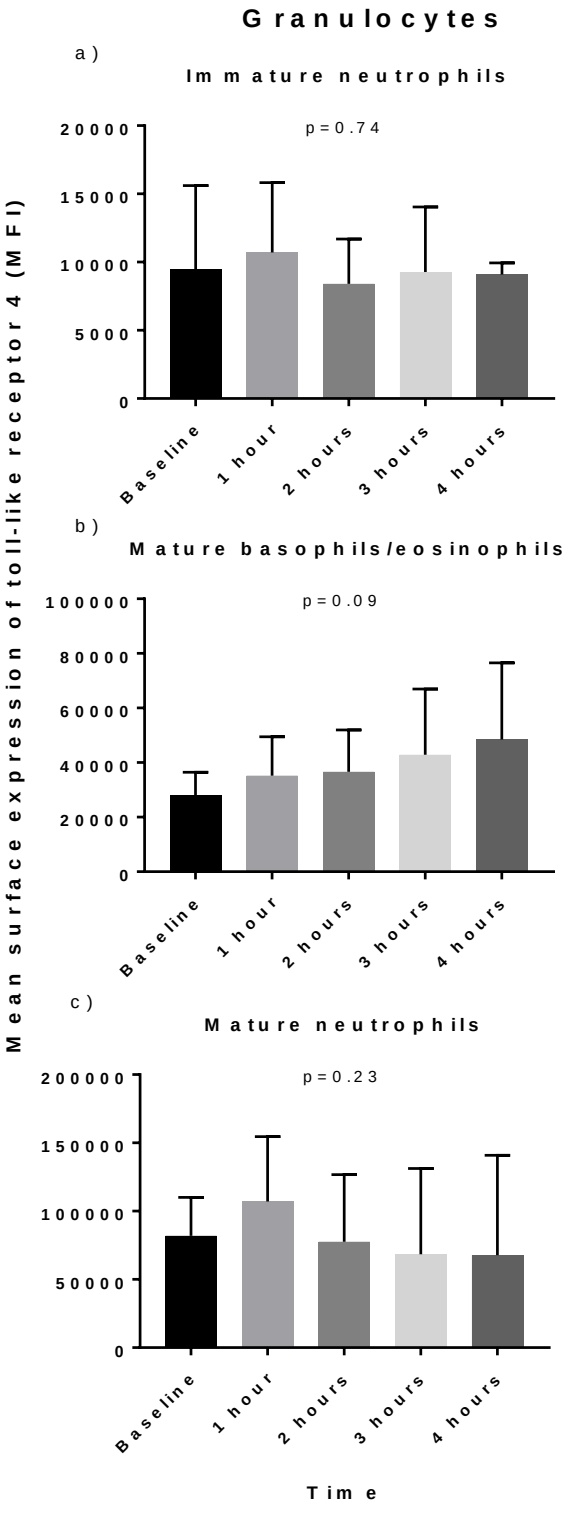

Figure 7.4 – The mean expression of TLR4 on granulocytes was consistent throughout perfusion. This was true for immature neutrophils (a), mature basophils/eosinophils (b) and mature neutrophils (c). Graphs depict data from n=6 porcine perfusions.

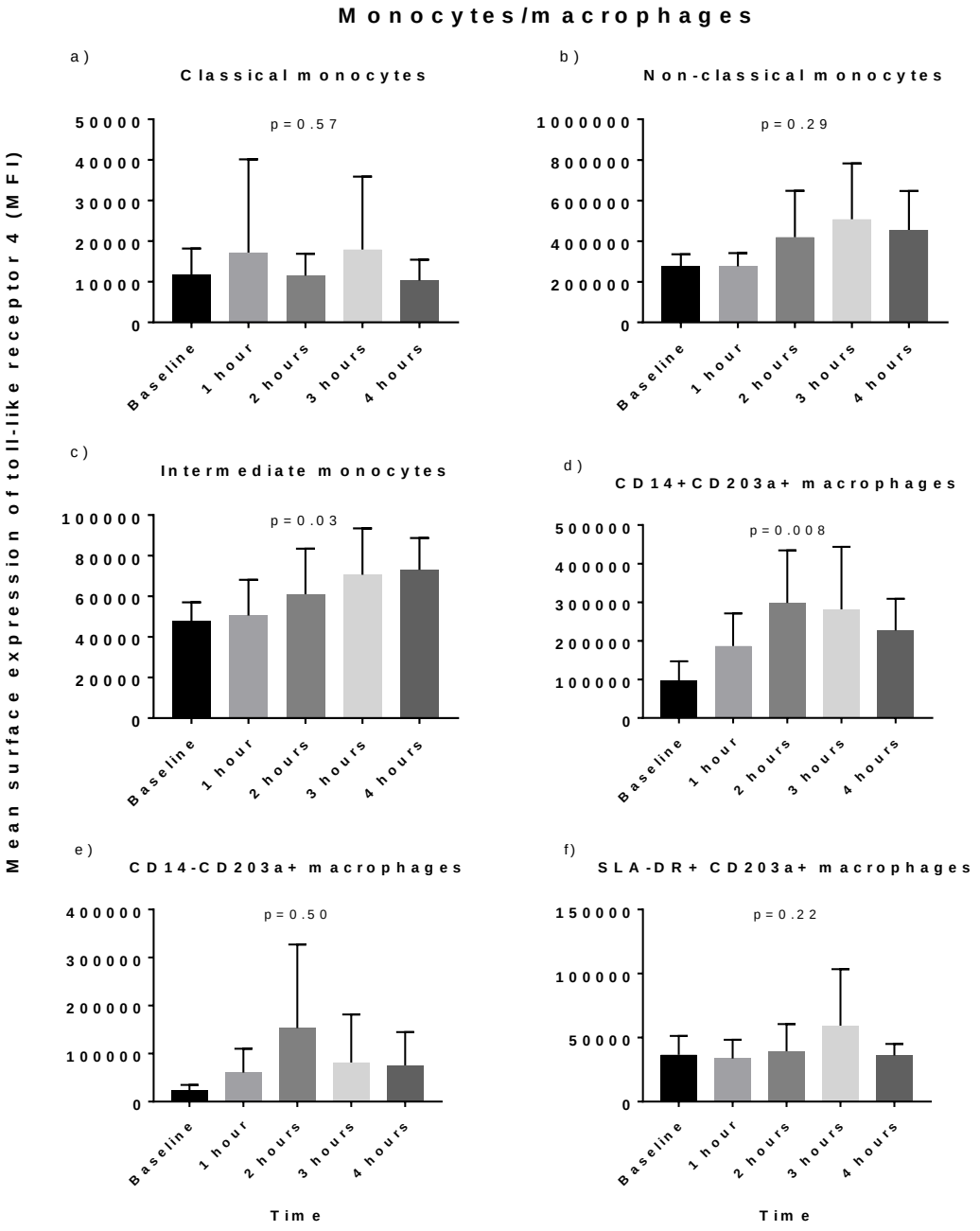

Figure 7.5 – TLR4 expression is altered by perfusion on some phenotypes within the mononuclear phagocyte system. Whilst there is no change for classical monocytes (a) or non-classical monocytes (b), intermediate monocytes (c) display elevated TLR4 expression with perfusion. CD14+ macrophages (d) also demonstrate increased expression upon perfusion, yet this is not the case for CD14- macrophages (e) or SLA-DR+ macrophages (f). Graphs depict data from n=6 porcine perfusions.

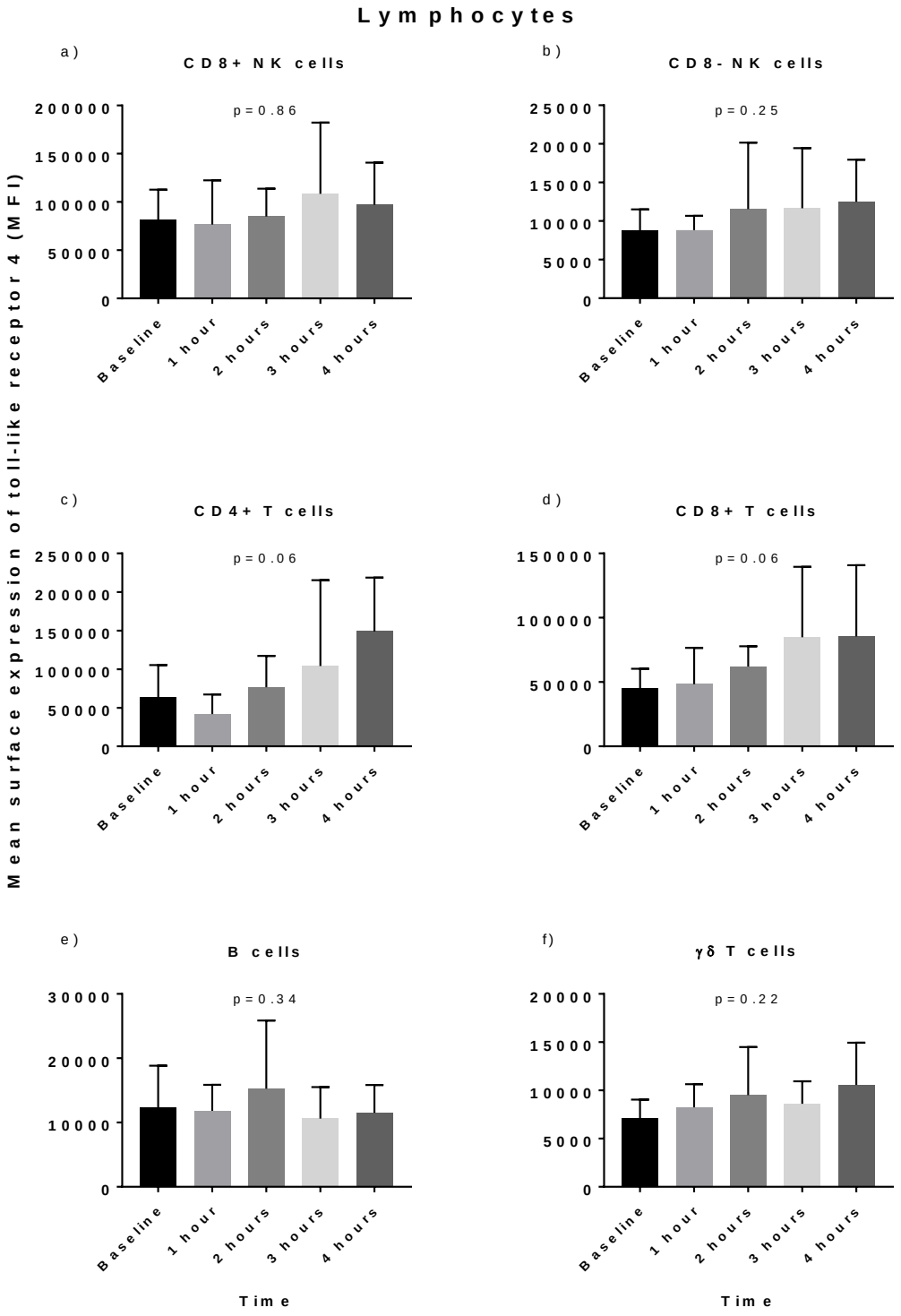

Figure 7.6 – TLR4 expression on the surface of lymphocytes was not significantly affected by perfusion. Both CD8+ NK cells (a) and CD8- NK cells (b) displayed stable expression with perfusion, although there was a trend towards increased TLR4 on the surface of CD4+ helper T cells (c) and CD8+ cytotoxic T cells (d). Again, no changes were observed over time for B cells (e) or γδ T cells (f). Graphs depict data from n=6 porcine perfusions.

7.3.3 Sequestration of leucocytes by the in-line leucocyte filter:

Although active perfusion of the donor heart at normothermia did not induce significant migration of leucocytes out of the organ, the filter was next assessed to determine the presence of sequestered immune cells. All leucocyte populations were found to be sequestered by the leucocyte filter, particularly classical monocytes (mean cell count: $6.1x10^6 \pm 9.9x10^6$ cells/filter), which was four times more abundant than any other population (figure 7.7). A total of $1.8x10^7 \pm 1.2\ x10^7$ leucocytes were observed in each filter.

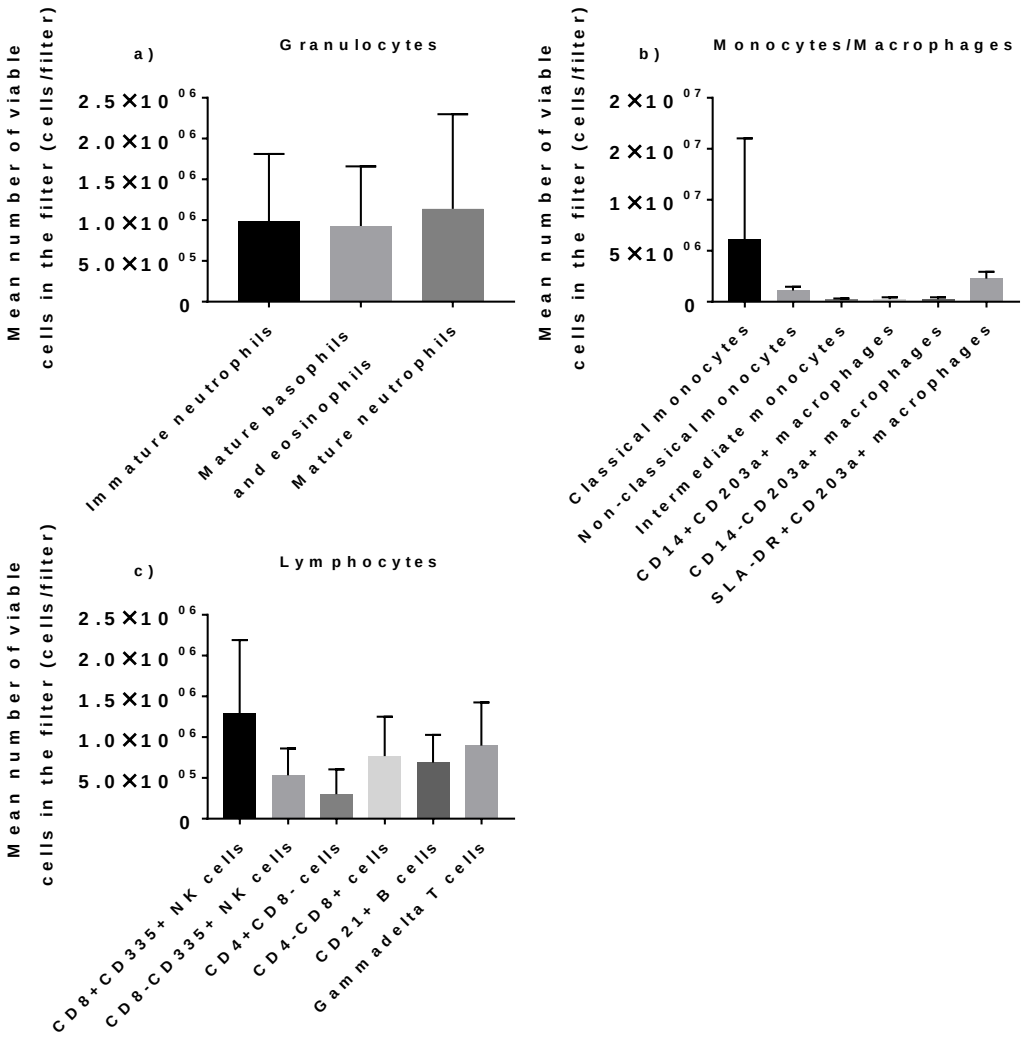

Figure 7.7 – The leucocyte filter is sufficient to sequester a large number of immune cells. These cells could otherwise have been transferred into the recipient. All phenotypes were detectable, with granulocytes (a), monocytes/macrophages (b) and lymphocytes (c) observed at high numbers. The most abundant cell population detected in the filter was classical monocytes. Graphs depict data from n=6 porcine perfusions.

7.3.4 Inflammatory cytokine release is induced by normothermic perfusion:

Next, the cytokine profile of the perfusate was evaluated in order to determine the nature of the signals released from the donor heart. Of the 13 cytokines and chemokines analysed, nine were detected in the perfusate. IFN-γ, IL-2, IL-4 and IL-12 were not detected as a result of perfusion.

A consistent pattern was observed for IL-1 cytokine family members, with low or undetectable concentrations early during perfusion followed by significantly elevated titre from 3 hours (figure 7.8). IL-1α increased significantly over the time course (p=0.0004), with none detectable in any subject until 3 hours (5.0 [0.0-10.0] pg/ml), and this remained elevated at 4 hours (10.0 [10.0-22.5] pg/ml). Similarly, IL-1β was significantly increased at later time points with perfusion (baseline, 1 and 2 hours: 0.00 [0.00-0.00] pg/ml; 3 hours: 0.0 [0.0-210.0] pg/ml; 4 hours: 375.0 [252.5-755.0] pg/ml; p=0.0003). IL-1ra rose steadily throughout perfusion, and was actually detectable in some subjects as early as 1 hour (baseline: 0.0 [0.0-0.0] pg/ml; 1 hour: 0.0 [0.0-5.0] pg/ml; 2 hours: 0.0 [0.0-10.0] pg/ml; 3 hours: 60.0 [40.0-77.5] pg/ml; 4 hours: 155.0 [117.5-255.0] pg/ml; p=0.0001).

GM-CSF was more acutely released during perfusion, and peaked at 1 hour before falling slightly for the remainder of the evaluation (baseline: 0.0 [0.0-42.5] pg/ml; 1 hour: 100.0 [60.0-135.0] pg/ml; 2 hours: 95.0 [22.5-115.0] pg/ml; 3 hours: 45.0 [15.0-115.0] pg/ml; 4 hours: 70.0 [22.5-125.0] pg/ml; p=0.0001, figure 7.9). IL-6 was low prior to attachment of the donor heart but was rapidly increased by perfusion (baseline: 0.0 [0.0-12.5] pg/ml; 1 hour: 35.0 [15.0-112.5] pg/ml; 2 hours: 915.0 [597.5-1463.0] pg/ml; 3 hours: 4610.0 [2605.0-6660.0] pg/ml; 4 hours: 10830.0 [6560.0-14625.0] pg/ml; p<0.0001, figure 7.9). IL-8 was the only chemokine measured in this experiment, and whilst it was low initially, its concentration rapidly increased and was detected at concentrations above the sensitivity of the assay (50,000pg/ml) at 4 hours in a number of subjects. For each of these subjects, the IL-8 titre was assigned as 50,000pg/ml for analysis. IL-8 was one of the most markedly increased signals detected in the experiment (baseline: 35.0 [27.5-40.0] pg/ml; 1 hour: 40.0 [37.5-60.0] pg/ml; 2 hours: 305.0 [187.5-562.5] pg/ml; 3 hours: 3865.0 [1130.0-18575.0] pg/ml; 4 hours: 33975.0 [7388.0-50000.0] pg/ml; p<0.0001, figure 7.9).

IL-10 was not detected at high levels at any point during perfusion but did increase over time (baseline and 1 hour: 0.0 [0.0-0.0] pg/ml; 2 hours: 0.0 [0.0-7.5] pg/ml; 3 hours: 0.0 [0.0-35.0] pg/ml; 4 hours: 40.0 [30.0-60.0] pg/ml; p=0.002, figure 7.10). IL-18 was detectable throughout perfusion and increased in a stepwise manner over time (baseline: 10.0 [0.0-42.5] pg/ml; 1 hour: 80.0 [50.0-107.5] pg/ml; 2 hours: 110.0 [77.5-197.5] pg/ml; 3 hours: 155.0 [105.0-257.5] pg/ml; 4 hours: 200.0 [142.5-322.5] pg/ml; p=0.001, figure 7.10). Finally, TNF-α was also observed to increase significantly in a stepwise manner over the course of perfusion (baseline: 0.0 [0.0-0.0] pg/ml; 1 hour: 40.0 [30.0-42.5] pg/ml; 2 hours: 270.0 [195.0-530.0] pg/ml; 3 hours: 605.0 [455.0-1308.0] pg/ml; 4 hours: 790.0 [487.5-1775.0] pg/ml; p<0.0001, figure 7.10).

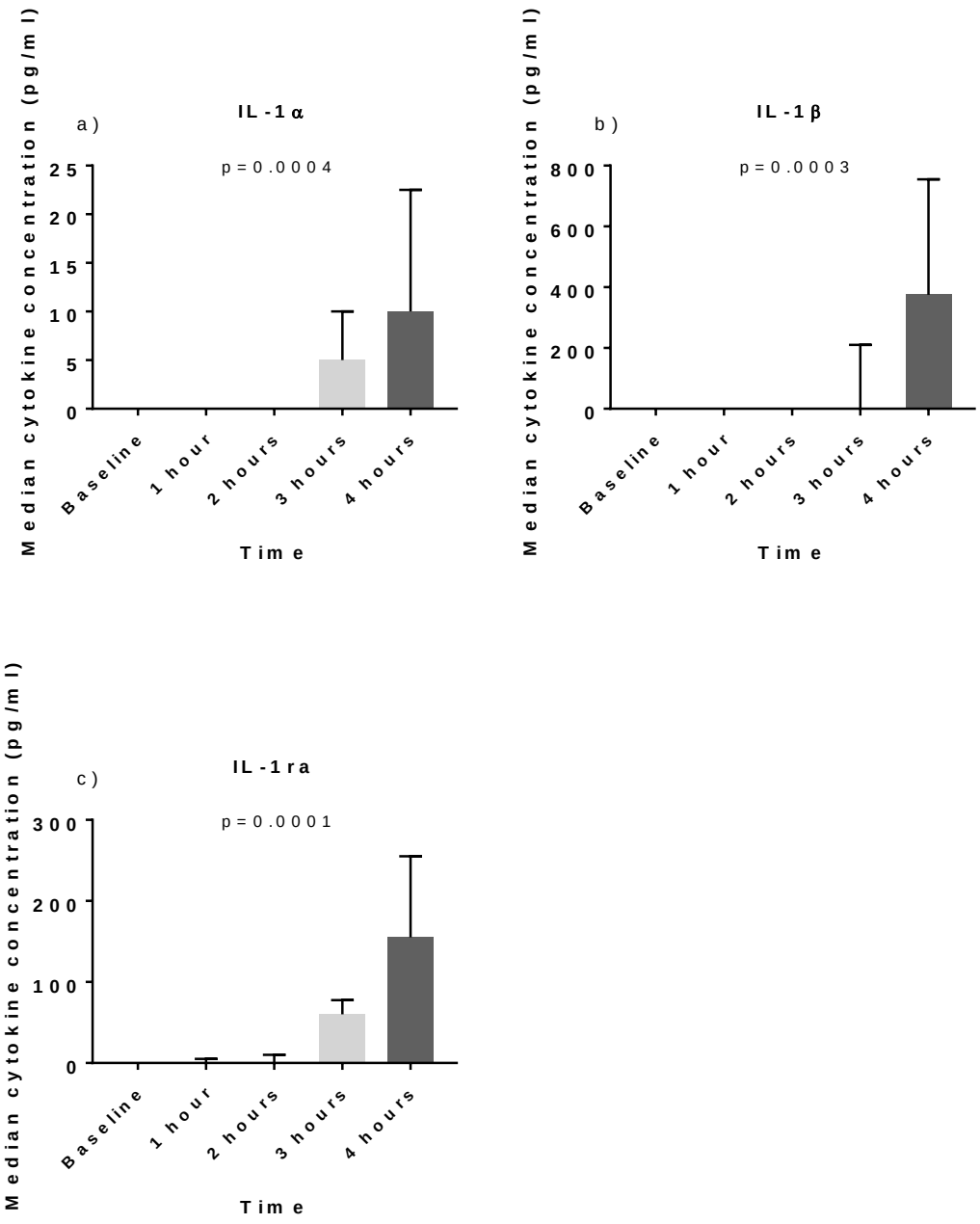

Figure 7.8 – IL-1 family cytokines are significantly increased as a result of *ex vivo* normothermic heart perfusion. The concentrations of IL-1α (a), IL-1β (b) and IL-1ra (c) are all highest at 4 hours. Graphs depict data from n=6 porcine perfusions. Bars represent median values and whiskers represent the interquartile range.

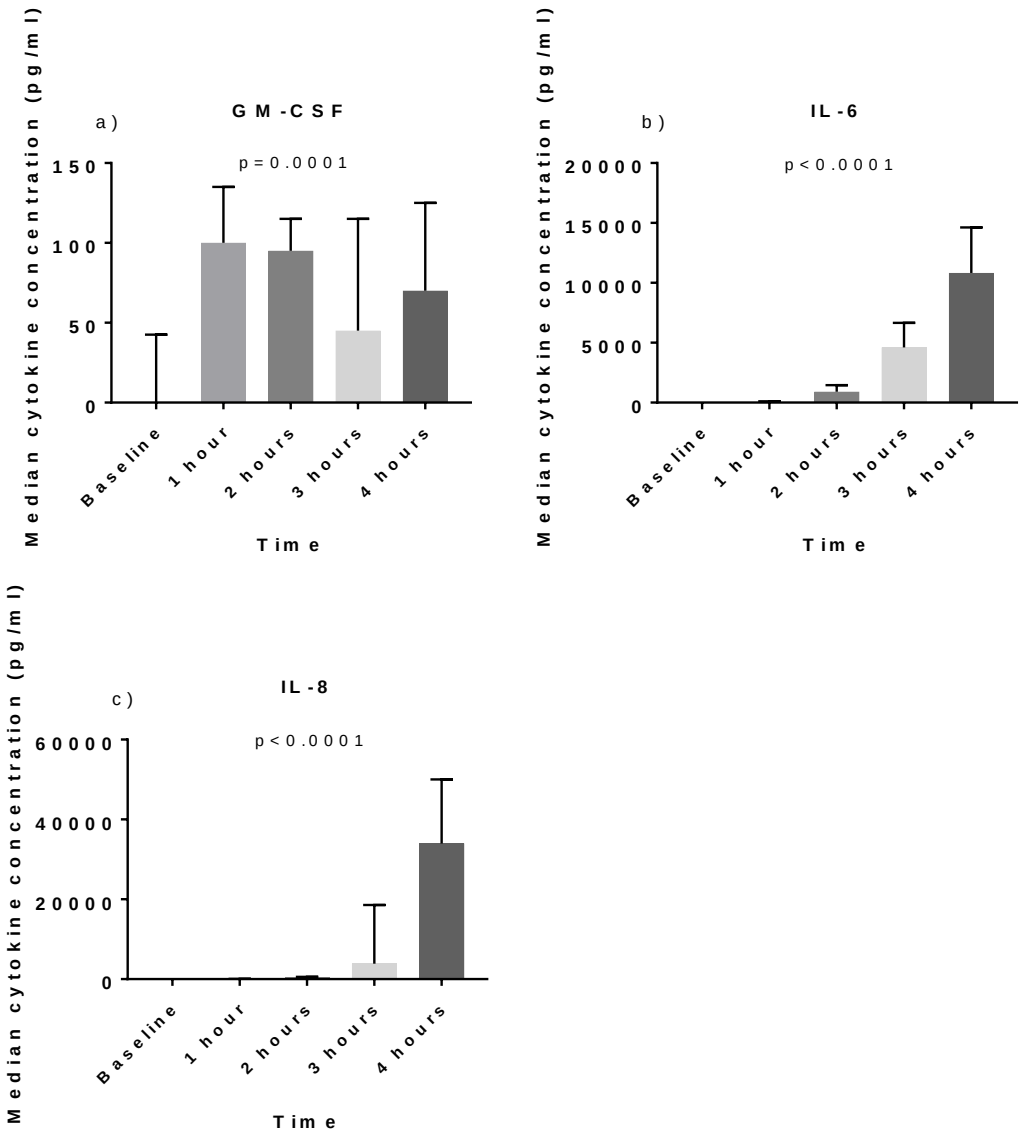

Figure 7.9 – *Ex vivo* normothermic perfusion of the donor heart induces the release of inflammatory cytokines in increasing concentrations over time. The concentration of GM-CSF (a) peaks by 1 hour and but remains elevated over baseline values throughout. IL-6 (b) and IL-8 (c) both demonstrate staggered increases in release and are at their highest concentration at 4 hours. Graphs depict data from n=6 porcine perfusions. Bars represent median values and whiskers represent the interquartile range.

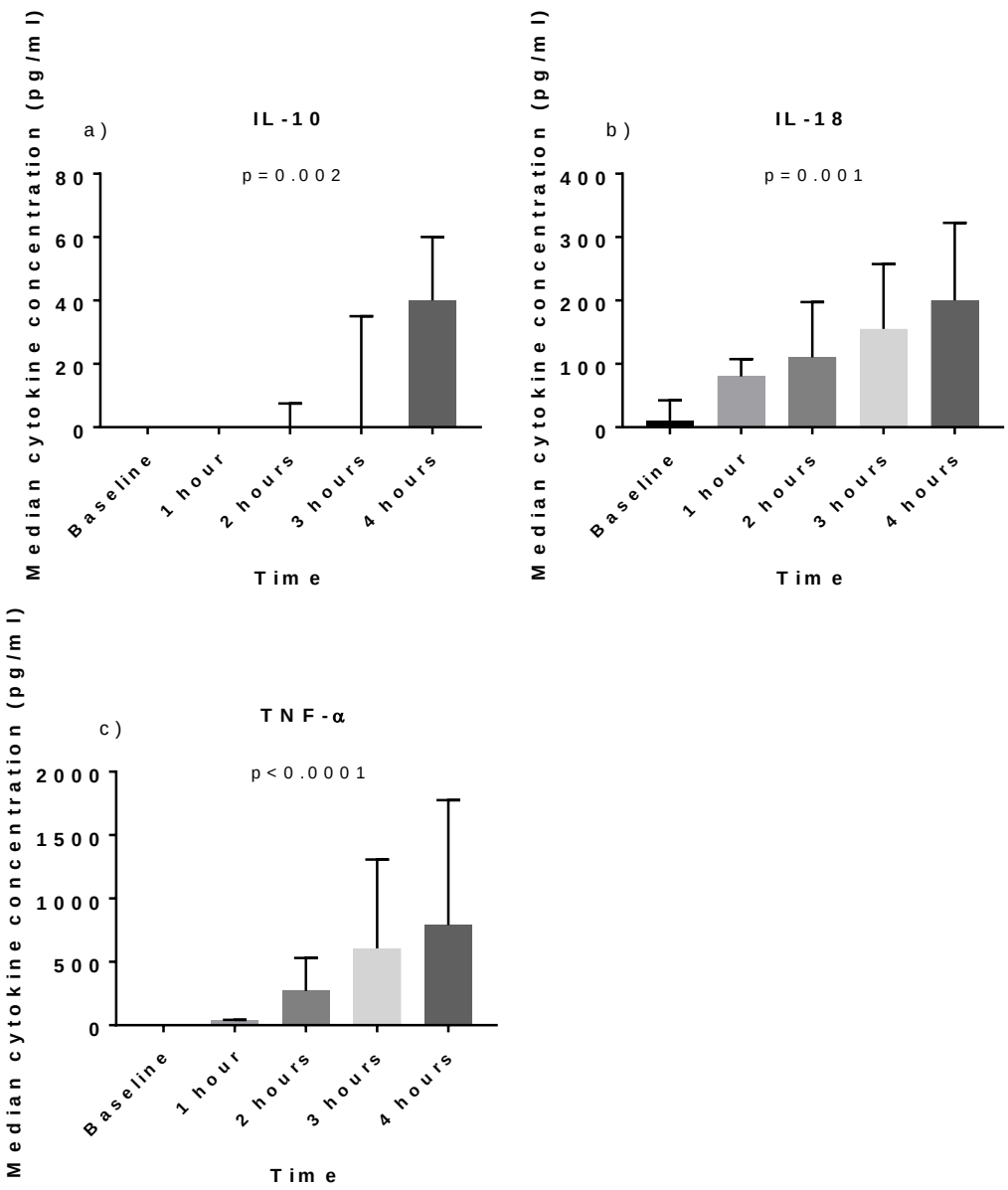

Figure 7.10 – *Ex vivo* normothermic perfusion of the donor heart promotes cytokine release in relation to the duration of perfusion. Whilst IL-10 (a) is only really detected at 4 hours, IL-18 (b) and TNF-α (c) are each associated with a stepwise increase in secretion throughout perfusion, again peaking at 4 hours. Graphs depict data from n=6 porcine perfusions. Bars represent median values and whiskers represent the interquartile range.

7.3.5 Cell-free DNA is released into the circuit following initiation of perfusion:

To further evaluate the inflammatory capacity of the isolated donor heart and its immunological contents, the release of cell-free DNA was evaluated in the perfusate as a marker of non-specific inflammation and cellular injury. These findings illustrate that *ex vivo* normothermic perfusion induces release of cell-free DNA of both genomic and mitochondrial origin (figure 7.11). Cell-free genomic DNA was undetectable at baseline but was then observed at low levels from 1 hour onwards, peaking at 3 hours and remaining elevated at 4 hours, although the change over time did not reach statistical significance (baseline: 0.00±0.00ng/µl; 1 hour: 0.07±0.04ng/µl; 2 hours: 0.04±0.05ng/µl; 3 hours: 0.10±0.12ng/µl; 4 hours: 0.10±0.13ng/µl, p=0.30). Cell-free mitochondrial DNA was undetectable at baseline and remained low throughout perfusion, and was detected in only two subjects at any time. The concentration of cell-free mitochondrial DNA was not significantly altered across the perfusion period (baseline, 1 and 2 hours: 0.00 [0.00-0.00] ng/µl; 3 hours: 0.00 [0.00-0.02] ng/µl; 4 hours: 0.00 [0.00-0.01] ng/µl, p=0.30). Genomic DNA was always detected at greater concentrations than mitochondrial DNA throughout perfusion.

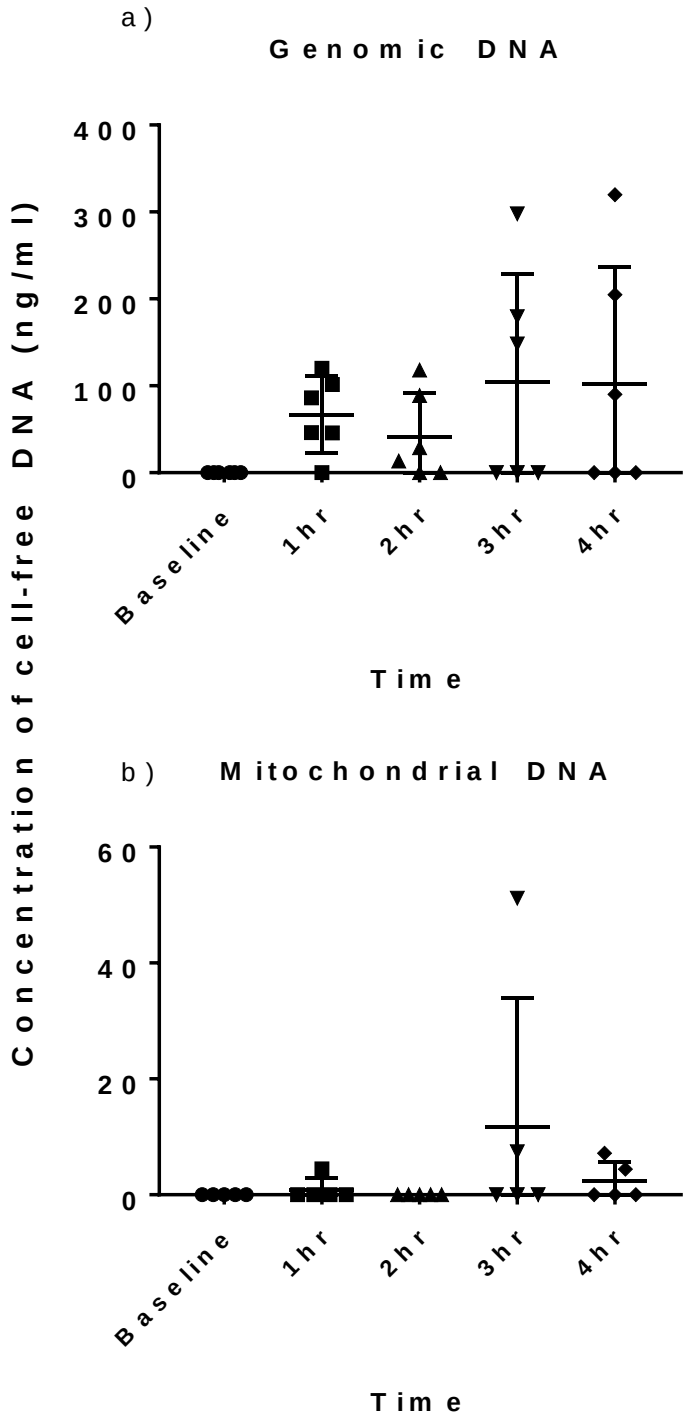

Figure 7.11 – Cell-free DNA is released as a result of *ex vivo* normothermic heart perfusion. Much greater release of genomic DNA (a) is observed compared to mitochondrial DNA (b). No statistical changes in concentration are observed for either DNA source. Graph depicts data from n=6 porcine perfusions. For genomic DNA, lines represent mean and standard deviation. For mitochondrial DNA, lines represent median and interquartile range.

7.3.6 Apoptosis and cell survival signalling is perturbed by *ex vivo* normothermic donor heart perfusion and evaluation:

In order to determine the effect of isolated perfusion upon the tissue itself, a broad proteomic analysis of the left ventricle was performed and the expression of proteins associated with cell death or survival were compared between pre and post-perfusion tissue biopsies. These findings demonstrate that four hours of isolated normothermic perfusion is sufficient to promote significant alterations in both pro and anti-apoptotic signalling pathways in the left ventricle. Heat shock protein (HSP) 70 demonstrated the greatest change in expression, being significantly elevated after perfusion (arbitrary pixel density units, pre vs. post-perfusion: 37.6±8.7 vs. 63.2±15.0, $p<0.0001$, figure 7.12). In addition, p53 phosphorylation at serine-46 was significantly decreased following perfusion (pre vs. post-perfusion: 17.0±1.8 vs. 9.8±1.1, $p=0.02$, figure 7.12).

Significant increases in left ventricular expression of both pro-caspase-3 (pre vs. post-perfusion: 18.94±5.5 vs. 28.6±4.3, $p=0.0002$, figure 7.12) and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/Diablo) (pre vs. post-perfusion: 26.3±1.5 vs. 34.4±2.7, $p=0.005$, figure 7.12) were observed following *ex vivo* normothermic perfusion of the donor heart. Moreover, a significant decrease in the expression of X-linked inhibitor of apoptosis protein (XIAP) was also observed as a result of perfusion (pre vs. post-perfusion: 21.4±6.1 vs. 13.0±1.1, $p=0.003$, figure 7.12). The remaining 30 proteins analysed did not change as a result of perfusion (figure 7.13).

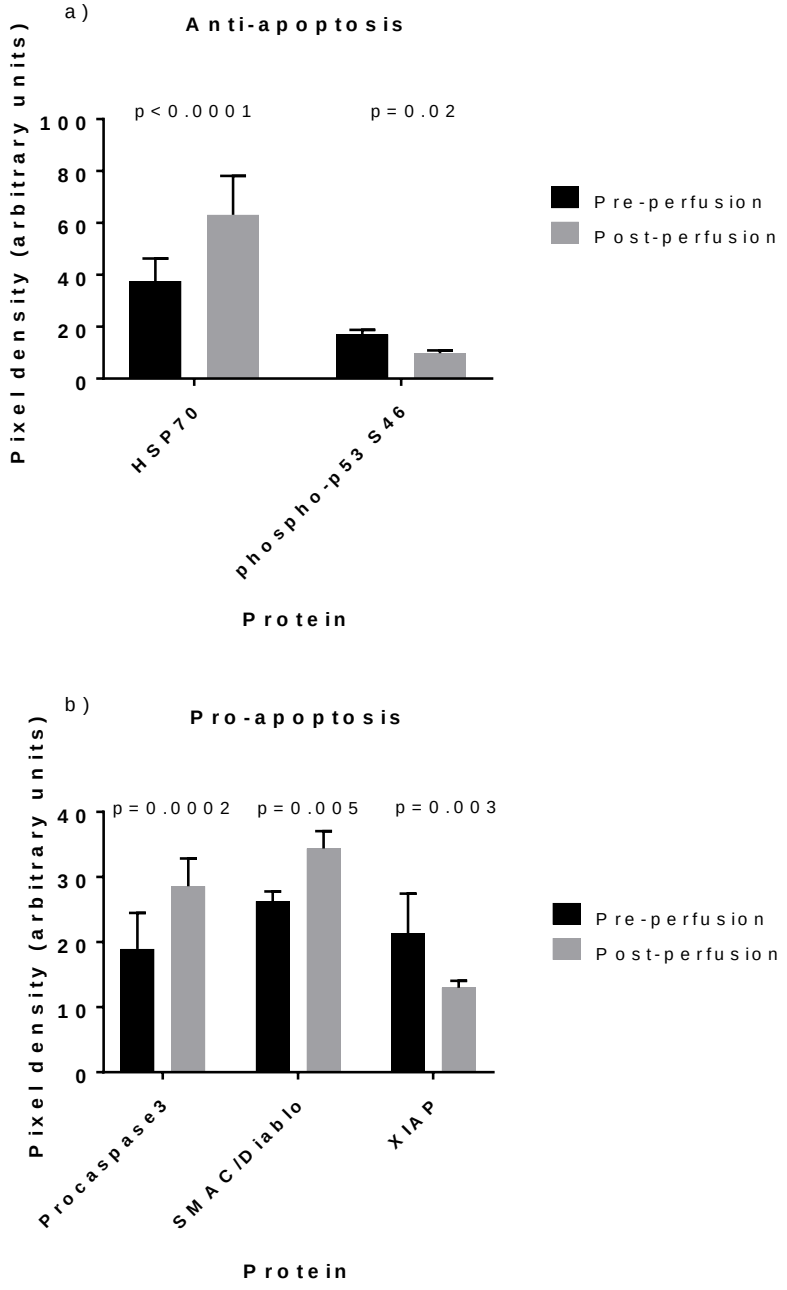

Figure 7.12 – *Ex vivo* perfusion of the donor heart alters both anti and pro-apoptotic protein signalling. Loss of apoptosis is promoted by the increase in protective heat shock protein 70 and loss of the pro-apoptotic p53 phosphorylated at serine 46 (a). Conversely, apoptosis is promoted by the increases in pro-caspase-3 and SMAC/Diablo as well as the loss of the protective XIAP (b). Graph depicts data from n=4 porcine perfusions. HSP70 = Heat shock protein 70. SMAC/Diablo = Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI. S46 = serine 46. XIAP = X-linked inhibitor of apoptosis protein.

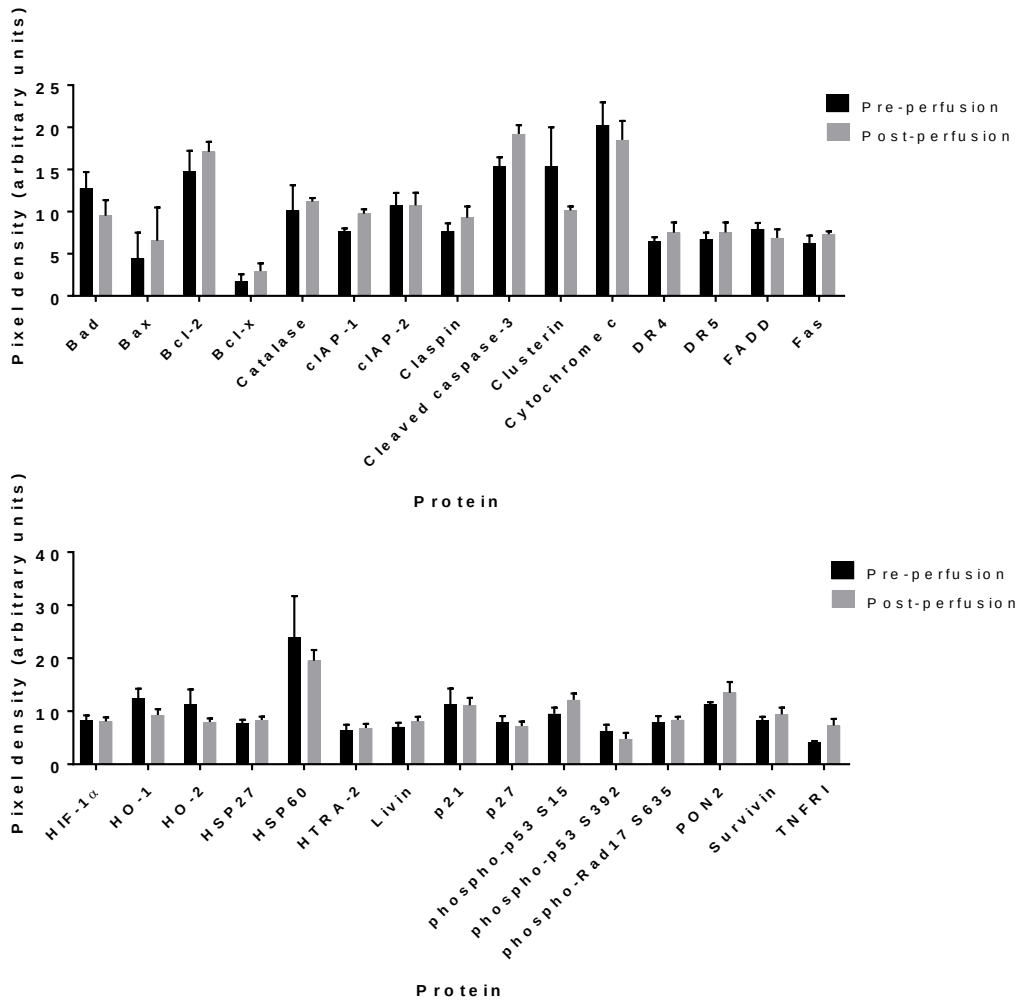

Figure 7.13 – The majority of proteins associated with cell death and survival were not affected by *ex vivo* perfusion. Graph depicts data from n=4 porcine perfusions. Bars represent the mean pixel density values, with whiskers representing standard deviation.

7.3.7 *Ex vivo* normothermic heart perfusion for four hours does not affect left ventricular tissue integrity:

In order to determine the effect of isolated normothermic perfusion on the viability of the donor heart tissue, a histological analysis was performed. Normothermic *ex vivo* perfusion of the heart was not associated with any adverse histological findings. All of the left ventricle samples assessed after cessation of perfusion were considered to display high structural integrity and no observable loss of morphology. Furthermore, no indication of oedema formation was detected after four hours of perfusion, and all muscle fibres were intact (figure 7.14). All hearts were functionally capable of ejecting perfusate at 3L/minute from the left ventricle for the entirety of the perfusion duration.

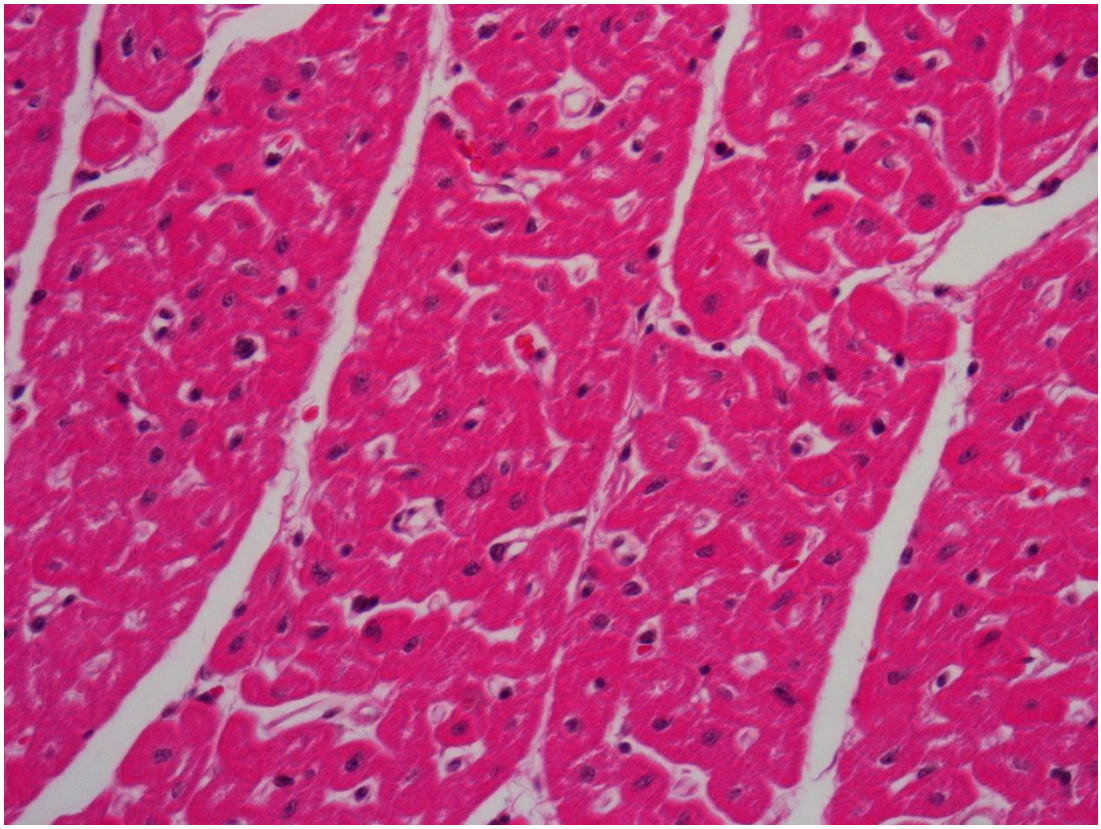

Figure 7.14 – Left ventricle structural integrity is maintained during *ex vivo* normothermic perfusion. No damage to the muscle fibres was detectable, suggesting that the isolated perfusion process is sufficient to meet the oxygen and nutritional demands of the myocytes. Image displayed is a representative image from the left ventricle of a single pig.

7.4 Discussion:

The shortfall in suitable donor hearts for transplantation is a major problem, significantly limiting the number of procedures that can be performed. A number of strategies have been proposed to address the disparity between donor numbers and recipients on the waiting list, particularly the use of extended criteria donor organs. However, there remain concerns over the safety of expanding the acceptance criteria for donor hearts for transplantation. This is particularly the case for the use of donor hearts retrieved from DCD donors that are associated with a greater level of myocardial damage, which could impair function following transplantation.

Improvements in donor organ preservation that provide a more physiological environment could extend the safe period of storage prior to transplantation, allowing a greater number of procedures to be performed. The Organ Care System utilises a Langendorff-style perfusion device to provide nutrient and oxygen delivery at normothermia, with no observed effect on short-term or intermediate term outcomes compared with standard storage[173, 174]. Whilst this device may have advantages over current storage protocols, it does not facilitate in-depth functional assessment of the organ, which would reduce the risk associated with extended criteria organs. Moreover, there have been no studies performed to assess whether active perfusion of the donor heart is associated with additional auxiliary benefits beyond improved storage times. Continuous perfusate delivery through the vasculature has been associated with immunodepletion in the lung[65], and similar beneficial effects may be observed with the donor heart. This study evaluated whether a novel *ex vivo* normothermic perfusion system was able to maintain the viability of the beating heart over a four hour preservation period. The study was further designed to assess whether this method could additionally modulate the cardiac immune repertoire.

These findings demonstrate that a period of four hours of *ex vivo* normothermic perfusion does not impair the viability of the myocardium. This is comparable to perfusion times currently performed in clinical practice with the Organ Care System (mean 227 minutes)[174], despite the significantly increased workload required for full four-chamber function in this model. Of course, the setup described in this chapter does not represent a true functional assessment of the organ and is rather a proof of concept that the system can maintain the viability of the heart for this period. Further

modifications to the system are required to allow a more comprehensive functional evaluation to be performed. Importantly, this model is indeed sufficient to ensure that the tissue is well-preserved without significant tissue injury as determined by histological assessment. Furthermore, there was limited release of cell-free DNA of either genomic or mitochondrial origin, indicative of a healthy tissue environment.

The broad proteomic profile produced in this study provides insight into the molecular properties of the preserved tissue. The majority of cell death and survival-associated proteins evaluated were not significantly altered by perfusion, intimating that the post-perfusion tissue is not inferior at a molecular level to the healthy tissue obtained immediately at retrieval. Changes that were observed provided a further indication that the balance between pro and anti-apoptotic pathways is unlikely to be significantly perturbed by perfusion. Indeed, the tissue displayed a rather mixed picture, with both pro and anti-apoptosis signals altered, which may cancel each other out somewhat.

Increased HSP70 expression following perfusion is anti-apoptotic, suggesting a beneficial effect. The protective effect of this protein on cardiomyocytes has been demonstrated previously *in vitro* and in experimental rat models[175, 176]. However, it is possible that this increase is actually caused by stress within the tissue, which has prompted the induction of protective pathways. This finding was accompanied by a loss of the pro-apoptotic p53[177-179], further contributing to a beneficial environment within the tissue. Conversely, there was a significantly increased expression of pro-caspase-3, which although not active itself, represents the precursor to a primary apoptosis effector[180] and suggests the induction of signalling to prime the cells for apoptosis. Significant pro-caspase-3 accumulation could be highly detrimental, although the source of pro-caspase-3 remains unclear due to the heterogeneity of the tissue assessed. Interestingly, normothermic perfusion induced a significant upregulation of SMAC/Diablo, a known promoter of apoptosis, which acts through the sequestration of inhibitor of apoptosis proteins[181]. This was accompanied by a loss of XIAP protein expression, to which SMAC/Diablo could ligate, supporting the notion of activation of this pro-apoptotic pathway following perfusion. Taken together, there are numerous factors activated by perfusion related to cell death and survival pathways, although there is no clear overarching pro or anti-apoptotic effect.

This suggests that this preservation method does not significantly impact upon tissue viability and is thus safe for use over this period.

Ex vivo perfusion of the donor heart may be associated with significant auxiliary benefits beyond improved preservation or evaluation of marginal organs. However, unlike previous findings in the lung[65], there was no marked mobilisation of cardiac resident leucocytes into the perfusion circuit. Whether this reflects that normothermic perfusion is an insufficient stimulus to induce migration is unclear. However, there was a large leucocyte population in the perfusate at baseline, reflecting an inefficiency of the leucocyte filter used in the acquisition of packed erythrocytes. It is therefore possible that cardiac immune mobilisation was masked somewhat by the large population already present in the perfusate. This is further complicated by the ability of numerous populations to adhere to the plastic of the circuit, which may mean that some leucocytes were missed by this analysis. Importantly, this trait was highlighted by the profile of the leucocytes trapped within the in-line leucocyte filter, which demonstrated a prominent monocyte population. As monocytes are both highly mobile and adherent, these cells are most likely to be sequestered after extravasation out of the donor organ and the incorporation of a leucocyte filter is therefore important to maximise the immunodepletion of the donor organ. Clearer findings may have been achieved if the leucocyte populations were examined within the tissue itself rather than in the perfusate, although it was not possible to take biopsies prior to perfusion in this experiment due to the downstream effect this may have had on the status of the organ during the preservation.

In this study, the impact on tissue leucocytes appears relatively modest, although increased expression of TLR4 was observed on some immune populations. This is a key receptor with a role in surveillance and detection of soluble markers of tissue damage[182]. Upregulation of this receptor is indicative of enhanced activation of these cells, which would ordinarily be considered detrimental as the transfer of activated cells into the recipient would be efficient at priming the alloresponse. However, this upregulation was observed on cells within the perfusate and as such they are removed from the tissue and would therefore not be transferred into the recipient.

These findings demonstrate that *ex vivo* normothermic perfusion induces a significant pro-inflammatory cytokine storm, with major secretion occurring by four hours. This

is consistent with previous studies with cardiopulmonary bypass, in which leucocyte contact with the plastic consumables in the circuit induces major immune activation and cytokine release[183]. This large inflammatory response establishes the donor heart as a major contributor to the cytokine pool post-transplant and demonstrates the impact of ischaemia-reperfusion on early inflammation. Such inflammatory secretion occurring on the circuit is likely to be of significant benefit compared to standard transplantation as the impact of reperfusion is contained in isolation and this inflammatory burden is not transferred into the recipient. Should this reperfusion-induced cytokine storm occur following transplantation, this would induce significant recipient leucocyte activation, driving mass infiltration of the graft. By ensuring that this effect is mediated *ex vivo*, it may diminish the ability of the donor heart to rapidly produce these cytokines, particularly within the early post-transplant period.

Taken together, it is clear that this model has a number of advantages, allowing the heart to be preserved in a more physiological manner than either cold storage or hypothermic perfusion for at least four hours without significant injury. This is a crucial step as it provides the foundations for a full evaluation to be performed. This is important considering that as recently as 2010, only 32% of potential donor hearts were utilised for transplantation in the USA[184]. This means that 68% of those hearts offered for transplantation were declined for various reasons. Of course, many of those would likely remain unsuitable without some form of reconditioning to improve their viability and function. The restoration of an optimal physiological environment by *ex vivo* normothermic perfusion may be able to aid in promoting rehabilitation of the donor organ and in this sense increase the donor pool. Furthermore, there are certainly many marginal donor hearts that are not transplanted that would be suitable but cannot be properly evaluated. Such organs are therefore considered too high risk to allow transplantation to occur. The development of this *ex vivo* protocol to incorporate a full evaluation of the organ may be able to alleviate the burden of donor heart shortage by allowing the identification of marginal organs that are suitable for transplantation. Importantly, in this study the model was utilised to determine whether the left ventricle was able to eject 3L/minute within physiological pressure ranges measured in the donor *in vivo*. The right side was not flow limited and was set to match the pressures of the donor. However, whilst this endpoint was sufficient for the purposes of this experiment, the functional evaluation could be much more tailored to ensure that the

heart was capable of coping with the conditions present in the recipient. For this purpose, the preload and afterload pressures could be matched to the recipient rather than the donor and only if appropriate output could be achieved to sustain the circulation would the organ be deemed suitable for transplant. Moreover, as the model utilises separate circuits for delivery of perfusate to the left and right atria, both sides can be fully evaluated in isolation. Indeed, the systemic and pulmonary vascular resistance of the recipient can be incorporated into the model and therefore functional capacity can be tested against clinically relevant criteria.

All organs utilised in this study were able to meet the functional targets originally set. However, these same levels may not be achievable in the clinical setting. This is particularly the case for marginal donor hearts, which are realistically the only organs that would require a full evaluation using this setup. Despite this caveat, the primary aim of this study was to determine the feasibility of the device as a method of preservation, and to ensure that the protocol itself does not promote adverse effects on the organ, which has been achieved. Further work is necessary to ensure the translatability of this procedure for use in clinical protocols and to discern the level of function required for successful transplantation.

7.5 Limitations:

The hearts utilised in this study were obtained from healthy donor pigs that did not experience brain death prior to retrieval. No circulatory arrest was induced prior to cardioplegia infusion and as such further work is needed to ensure that the same effects would translate in a clinically relevant DBD or DCD scenario. Furthermore, they were cold stored for only a short period prior to attachment and evaluation on the circuit, significantly limiting the extent of injury to which they were exposed. This means that the level of functional restoration observed here may not be achievable in the clinical scenario. Further work is required to establish the threshold level of function that could be considered acceptable, although this may vary from donor to donor. Additionally, no transplants were performed following the evaluation protocol as the study aims were limited to monitoring function in real time and establishing the effects of normothermic perfusion on the tissue itself. This would have provided further evidence to confirm that these organs were suitable for transplantation and not sufficiently damaged by the evaluation process to preclude their downstream function.

7.6 Conclusion:

Normothermic *ex vivo* perfusion of the donor heart is a valid method of physiological preservation that can be performed prior to transplantation. Incorporation of a full evaluation step is crucial if the use of marginal donor hearts is to be increased to address the growing demand for transplantation. Evaluation should ideally be performed immediately before transplant to maximise the reliability of the assessment and reduce the potential for confounding effects during an additional storage period. As such, *ex vivo* perfusion may significantly aid in clinical decision making and increase the number of suitable donor organs available, thereby allowing more procedures to be performed. In turn, this would improve waiting list mortality. *Ex vivo* perfusion may also have additional benefits, particularly with regard to modulation of the inflammatory response to reperfusion. Restoring blood flow *ex vivo* ensures that toxic metabolites and pro-inflammatory cytokines are not transferred to the recipient, improving the local tissue environment. Modulating the organ response to ischaemia-reperfusion may therefore further enable improvements in the incidence of primary graft dysfunction and acute rejection episodes.

Chapter 8: General Discussion

The number of patients living with end stage cardiac disease is rapidly growing, leading to a markedly increased requirement for heart transplantation as a final treatment option. In contrast, the number of potential donor hearts available for use has not increased in line with demand, leading to a significant shortfall. This has resulted in high mortality on the heart transplant waiting list. Although other options, including mechanical unloading with a ventricular assist device, have more recently become available they are not routinely utilised for purposes other than bridge to transplant. As such, there is an urgent requirement to increase donor organ availability and strategies to achieve this are a major focus of current research in the field of transplantation. Furthermore, despite the use of potent immunosuppression in an attempt to prevent graft loss, rejection remains an important impediment to long term success following transplantation. This programme of work was designed to investigate the potential benefits of *ex vivo* perfusion technologies and whether these strategies could confer significant improvements over current approaches.

The findings presented in this thesis provide novel insight into a number of important processes that affect clinical outcome post-transplantation. Firstly, the transfer of passenger leucocytes from the donor organ into the recipient circulation has been proposed but never evaluated in the heart. This thesis describes for the first time that the healthy heart contains a significant immune repertoire able to mobilise immediately upon revascularisation. The activation of such cells during static cold storage, as demonstrated by the release of pro-inflammatory cytokines, is an important finding. The transfer of activated leucocytes into the recipient would stimulate activation of the direct pathway of allorecognition, contributing significantly to infiltration of the graft by recipient allospecific T cells. The presence of cardiac leucocytes prior to transplant reiterates the importance of the donor heart as a target for intervention, yet until this point there has been little attention paid to donor immunomodulation as a potential therapeutic strategy. Importantly, this study further demonstrates that the static cold stored heart is not only associated with immune activation but that reperfusion induces significant endothelial denudation of the graft. Indeed, this occurred with as little as two hours of cold ischaemia, and is likely to be augmented with longer storage. There are clearly important implications of this graft injury, which could significantly affect downstream function of the organ through a reduced ability to regulate both vascular permeability and vascular tone. As such it is

apparent that the standard method of preservation of donor hearts prior to transplantation is associated with adverse changes to the ultrastructure of graft vessels, which is manifest rapidly during storage. Improved methods of preservation of the graft are therefore required to avoid any potential complications post-transplant.

In this thesis, a novel method of preservation is presented which utilises *ex vivo* hypothermic cardioplegic perfusion of the donor heart to maintain the graft. This strategy combines the protective strategy of cold cardioplegia to minimise the metabolic demands of the heart with the delivery of important nutrients and oxygenation to ensure that oxidative metabolism can continue at a diminished level throughout the preservation period. A major benefit of hypothermic perfusion for preservation purposes is that should there be any technical issues with the device, the heart is still contained within an insulated box and should remain at 8°C even without perfusate delivery. In this manner, a loss of power to the pump would mean that the preservation is reverted to static cold storage. As such, this therefore represents an in-built safeguard to avoid the unnecessary loss of organs once translated and incorporated into the clinical setting. Consequently, translation into clinical practice at this stage would appear to be relatively low risk and potentially highly beneficial.

The ability to extend preservation beyond the four hours of cold ischaemia is that is generally considered acceptable would have enormous benefits. Firstly, this would vastly extend the geographical area to which the donor heart could be sent, thereby allowing the most urgent recipient to be transplanted without restriction on the basis of retrieval site. Alternatively, prolonged preservation could allow a more in-depth assessment of the donor organ with regard to tissue typing, and as such promote improved matching of donor and recipient. Furthermore, better preservation may extend the window of opportunity for transplantation, negating much of the urgency and allowing greater flexibility with the scheduling of the procedure.

In this study, the donor heart could be safely preserved without deleterious effects on the myocardium or endothelium for 8 hours. Whilst this is a major achievement in itself and would undoubtedly lead to improvements for transplant surgeons, further benefits to the tissue were also observed. Remarkably, there was an overall improvement in the balance between cell death and cell survival pathways, with a number of anti-apoptotic proteins upregulated by the end of perfusion. This suggests

that not only was the organ optimally perfused to sustain cellular integrity, but that perfusion actively stimulated the expression of proteins preventing cell loss. This induction of protective mechanisms is a novel benefit of hypothermic perfusion not described previously in the heart.

Interestingly, the hypothermic perfusion of the donor heart conferred an additional protective effect on the tissue through the downregulation of ischaemia-reperfusion associated proteins. This may be considered as a form of pre-conditioning effect as the organ has undergone a very short cold ischaemic period prior to connection to the device and the reperfusion occurs in isolation on the circuit. This means that the donor organ is more prepared for a second ischaemic event as protective signalling pathways have already been stimulated. Moreover, the extent of reperfusion injury is further limited in two major ways. Firstly, the diminished cold ischaemic time ensures that there is much less aberrant metabolism occurring and less opportunity for the build-up of toxic by-products. As the duration of cold ischaemia is inherently linked to the extent of reperfusion injury, this is a crucial improvement. Secondly, as reperfusion of the organ occurs in isolation on the circuit, there are no recipient leucocytes present, significantly limiting the level of inflammatory response. Should the initial reperfusion occur with the recipient circulation then this would likely introduce a much greater inflammatory burden as any toxic metabolites or by-products as well as donor leucocytes and cytokines would be transferred into the recipient. Recipient leucocytes would immediately respond to the inflammatory signals from the donor in a positive feedback mechanism and thereby exacerbating the inflammation.

Further protection of the graft is provided in the form of inhibitory immune checkpoint expression. Hypothermic perfusion promotes the upregulation of PD-L1, a prototypic co-inhibitory molecule. The only described role of this protein is that of precluding T cell activation and directing either the anergy or deletion of any T cells that are contacted. Importantly, this expression was observed on graft endothelial cells, which are key regulators of vascular permeability and control leucocyte extravasation. As such, recipient T cells trafficking to the graft must come into contact with the endothelium in order to move from the vasculature into the tissue and thus are likely to receive the inhibitory signal. However, it is unclear from the data presented in this thesis whether this signal remains upregulated following transplantation. Indeed, the

transient nature of its expression in *in vitro* endothelial cultures suggests that continued stimulation is likely necessary and therefore this protection may only be relatively short lived. The use of *ex vivo* perfusion may provide a platform for therapeutic delivery of agonists to stimulate a more sustained expression of immune-inhibitory molecules that can selectively prevent recipient T cell activation. As a whole, these findings suggest that hypothermic cardioplegic perfusion is able to preserve the donor heart to a far higher degree and for a much longer period than standard storage methods.

Excitingly, the use of *ex vivo* perfusion presented greater advantages beyond the improvement and prolongation of safe preservation. Indeed, the significant leucocyte reservoir initially observed in healthy donor hearts could be markedly depleted by perfusion to the extent that some populations were almost entirely absent from the left ventricle at the end of perfusion. Leucocyte migration from the tissue into the circuit was associated with a selective pro-inflammatory cytokine storm driven primarily by IFN-γ, which may have played a role in exacerbating immune mobilisation through the stimulation of chemokine release. Importantly, the increased PD-L1 expression observed following perfusion may be related to the secretion of IFN-γ, which these experiments demonstrate is a potent inducer of this molecule.

The loss of donor leucocytes observed with hypothermic perfusion ensures that there is a much smaller population available to migrate into the recipient at revascularisation and thus a diminished stimulus for direct allorecognition. Initial pilot data suggested that this immunodepletion translated into significant clinical benefit with a marked decrease in graft infiltration by recipient lymphocytes, the hallmark of acute rejection, up to 48 hours in the absence of immunosuppression. This is a major finding that could have important clinical implications by allowing the use of immunosuppression sparing regimens, thus reducing the adverse effects associated with these drugs. However, it is important to note that this protection from graft infiltration was not sustained beyond this point, and both perfused and static cold stored organs were heavily infiltrated by day 5. This occurred to the extent that contractile activity was precluded. It is unclear whether this restoration of infiltration relates primarily to a loss of some protective mechanism on the graft itself (such as PD-L1) or reflects the activation of the indirect allorecognition pathway.

Surprisingly, subsequent investigation with a larger number of heterotopic transplantations failed to replicate these initial results. Although the lack of corroboration in the larger study limits the strength of conclusions that can be drawn from these data, the heterotopic transplant model was beset with technical problems. As such, the reliability of the post-transplant data from the larger study remains in question as the complications appeared to be surgery-related rather than immunological. Therefore it is unclear to what extent there was graft infiltration as a result of the alloresponse and how much of the leucocyte presence was related to an acute phase inflammation in response to injury. Of note, there was a significantly higher cytokine secretion observed post-transplant in the static cold storage group compared with the perfused group. The extent to which all hearts were injured post-transplant was inconsistent with previous findings suggesting a confounding factor as yet unidentified. It is therefore critical that improvements to the transplant model be made if greater clarity is to be provided on the downstream clinical effect of cold cardioplegic perfusion. This may involve the replacement of the model with an orthotopic procedure, which would be more clinically relevant but would require greater monitoring of the donor heart function to ensure that the level of infiltration did not impair contractility.

Although the heterotopic transplant study was unable to detect differences in the level of graft infiltration, it was clear that there was a reduction in cytokine secretion post-transplant in the perfused group. Importantly, the cytokine profile obtained in the plasma post-transplant was predominantly pro-inflammatory. The immunodepleted heart may therefore represent a lower inflammatory stimulus in comparison to the static cold stored heart, inducing a diminished response from the recipient.

Overall, it is apparent that the use of cold cardioplegic heart perfusion is a successful method for prolonged donor organ preservation, with safety demonstrated in this study for 8 hours. Previous data with this device confirmed that the donor heart could be preserved in this manner for 24 hours without affecting post-transplant function[97]. Although further translation of the method is required with the use of discarded donor organs before it can be utilised in the clinical setting, the technique has clearly demonstrable advantages over static cold storage. The additional safe preservation period alone makes the device an attractive solution to increase the number of suitable

donor hearts for transplantation, and should minimise the unnecessary loss of organs. When combined with the auxiliary benefits of immunodepletion and pre-conditioning against ischaemia-reperfusion injury, this novel method of preservation could revolutionise heart transplantation and there is a strong argument that its widespread use with all potential hearts obtained from brain dead donors could be warranted. However, in its current form it is unlikely to be of such benefit with regard to marginal donors, which require a functional evaluation to determine their suitability for transplantation. It is possible that this device could be utilised as a preservation method if an evaluation were to be performed at the donor site rather than at the recipient hospital. This may take the form of normothermic regional perfusion, which would allow an assessment of the organ *in cadavero* prior to retrieval[54]. Once the suitability of the organ has been determined it may not be necessary to utilise a beating heart transport system, and this system would provide a better alternative to static cold storage.

Whilst the use of normothermic regional perfusion is in itself an elegant solution for evaluation of donor hearts, its use is restricted in many countries due to ethical considerations. Furthermore, it would be useful to be able to perform an evaluation at the recipient site. This would mean that the functional performance of the organ can be monitored immediately prior to transplantation. For this purpose, a four chamber beating heart preservation device has been developed, which could be utilised to perform a thorough examination of the donor organ. The findings presented in this thesis demonstrate that normothermic *ex vivo* perfusion of the donor heart can be utilised as a means to preserve the organ and test its capacity to pump against the pressures of the recipient. Although only a relatively simple evaluation was performed in this study, the results indicate that this model may be sufficient for determining the suitability of an organ. The cardiac output of the donor heart can be determined at a range of physiologically relevant perfusion pressures and afterload pressures to mimic the systemic and pulmonary vascular resistance. This enables the assessor to confirm that the organ has retained sufficient contractile capacity following retrieval to support the recipient circulation. In this study, it was confirmed that this evaluation could be performed for up to four hours without deleterious impact upon the tissue. This is likely to be a longer assessment period than would be required in the clinical setting, although it does imply that the device could be used to preserve the organ in a more

physiological environment with real time monitoring should there be a requirement to do so. The incorporation of such technology into the clinical setting would increase the utilisation of donor organs and avoid the unnecessary loss of hearts that would ordinarily be deemed unsuitable for transplantation simply due to a lack of supporting information. The increasing use of hearts obtained from donors following circulatory arrest is necessary if the donor organ shortfall is to be addressed, and this could safely be achieved by utilising *ex vivo* normothermic perfusion.

The use of normothermic perfusion may not be associated with the same auxiliary benefits as hypothermic perfusion with regard to the extent of immunodepletion. However, the same in-depth immunological profile of the tissue was not achievable as the required biopsy collection may have introduced confounding factors to the functional evaluation. It is unclear then whether a similar immunodepletion effect is observed with normothermia, although the lack of mobilisation into the perfusate suggests this is not the case. Similarly, the upregulation of cell survival factors that was observed with cold cardioplegic perfusion was not replicated in this model, suggesting that the increased workload and metabolic demand required for the functional assessment impairs the initiation of these protective pathways. Some smaller benefit may be conferred for reduced ischaemia-reperfusion injury with the use of this circuit, as the incorporation of a normothermic perfusion period would likely be initiated as early as possible. This would potentially lead to a reduced cold ischaemic time, and therefore restoration of oxygenation and nutritional support would occur earlier than would be the case in the standard clinical setting following static cold storage. Furthermore, the initiation of reperfusion in an isolated circuit should ensure that any metabolic waste or pro-inflammatory factors that are released upon reperfusion are not transferred into the recipient. This would induce significant inflammation and avoidance of this transfer would be beneficial. Of course, the data from this thesis also demonstrates the importance of a simple post-preservation flush in the context of static cold storage, which could be incorporated into standard clinical practice without difficulty. This would achieve a similar metabolic and inflammatory washout, which would be undoubtedly beneficial.

In conclusion, this programme of work highlights the marked potential benefits of *ex vivo* perfusion as a technology to enable the greater utilisation of donor organs with

advantages for both preservation and evaluation. Improved preservation over an extended period can be safely achieved with hypothermic delivery of oxygenated cardioplegic perfusate, whilst normothermic *ex vivo* perfusion allows a functional assessment to be performed. The immunological modulation of the donor heart by perfusion observed in these studies demonstrates the powerful effects of this technology and provides insight into how this method could be further developed to promote even greater benefit. Together, these devices could lead to a stepwise change in how organs are preserved for transplantation, improving the utilisation of hearts following both brain death and circulatory arrest. Further research is required to determine whether an *ex vivo* perfusion platform could be utilised as a method to recondition marginal donor organs in addition to observational functional assessment. The improvements in myocardial status with hypothermic perfusion demonstrate the potential for this, although it is now necessary to assess the response of the organ to preservation in the context of donor injury. Although further translation of these findings is required, this work provides a clear indication that the widespread utilisation of such protocols could revolutionise organ procurement and storage, leading to significant patient benefit.

Chapter 9: Future work

There are a number of important experiments that are required in order to fully translate the findings of this work into clinical benefit. Firstly, it will be important to determine the impact of donor brain death on the immune reservoir contained within the donor heart. Similarly, donor cardiac death may also impact on the cardiac immune system, albeit without the expected influx of cells as a result of the cessation of flow. Should the mode of donor death significantly alter either the leucocyte populations present in the heart or the behavioural phenotype of said cells then this may have a downstream impact upon the extent to which hypothermic perfusion modulates the organ. It is therefore important that future experiments focus on translating these initial exciting findings into a more clinically relevant model incorporating donor brain or circulatory death.

In addition, it is essential that this device is evaluated with human tissue in order to ensure that the same mechanisms presented herein are also stimulated in potential donor organs. This may primarily involve the use of discarded donor hearts not acceptable for transplantation. Perfusing these organs *ex vivo* may not provide conclusive data regarding the status of the tissue at the end of the preservation period if the heart is significantly injured prior to its connection. Regardless, such future experiments must be performed to ensure the reliability of the device in a clinical context and this may actually provide some indication of whether cold cardioplegic perfusion can promote organ reconditioning. It is plausible that the metabolic unloading of the heart could stimulate this process in a similar to that sometimes observed with patients receiving a ventricular assist device. Histological and proteomic evaluation of the tissue prior to and following perfusion would achieve this aim. Moreover, the immunodepletion effect of hypothermic perfusion must be evaluated in human tissue to ensure that this secondary benefit is also translated.

The upregulation of PD-L1 on the graft endothelium is a notable finding that has highlighted an avenue for further investigation. The expression of this protein on tissues is part of a complex signalling pathway that prevents autoimmunity during homeostasis. By enhancing the expression of this protein, it may be possible to replicate this process in order to promote recipient immune tolerance to the graft. Further cell culture experiments are required to verify that enhanced expression of this protein can inhibit the action of T cells towards mismatched tissue. As this protein is

not alone in promoting this effect, it will also be important to identify other molecules that can further consolidate graft tolerance. Additional experiments to identify agonists or methods to prolong the expression of PD-L1 are also warranted. The use of *ex vivo* perfusion as a delivery platform is necessary for such agonists to ensure that the effect is specific to graft. The delivery of soluble PD-L1 to the recipient would promote a much more non-specific immunosuppressive effect, with no benefit beyond that observed with current immunosuppression.

Most importantly with regard to hypothermic perfusion, future work must attempt to provide more conclusive evidence of the events post-transplant in order to determine whether donor immunodepletion diminishes direct allorecognition and acute rejection. In order to do this, a validated transplant model must be utilised. A series of experiments designed to optimise the heterotopic transplant model are warranted to ensure that the high mortality rate observed here is not a continued confounder. For the purposes of assessing graft infiltration, it may be that an orthotopic procedure would be preferable, and indeed this would have the added advantage that it would be a more clinically relevant endpoint. This would be performed once again without immunosuppression during the follow-up period. However, in addition to this, future experiments evaluating the effect of hypothermic perfusion post-transplantation should be designed to incorporate immunosuppression over a longer follow-up period. The use of cold cardioplegic perfusion with a tailored immunosuppression-sparing regimen could then be compared with static cold storage and full immunosuppression.

Future work is also required for the normothermic *ex vivo* perfusion device to determine how long the evaluation can be continued and to incorporate a more thorough means of evaluation of the organ. Indeed, whilst this device has demonstrated enormous potential as a tool for functional assessment, preservation at normothermia may also be a realistic prospect and it would be interesting to perform a comparison of hearts perfused at hypothermia and normothermia. The evaluation of donor hearts that have undergone brain death or circulatory arrest prior to retrieval should also be performed in porcine studies. Indeed, a series of experiments exploring the functional recovery of hearts exposed to varying levels of injury followed by transplantation would be useful to provide insight into what degree of impairment can be sustained without adverse clinical outcome.

Following on from these investigations, the evaluation device may then be used to assess human donor hearts that have been declined for transplant. The feasibility of translation from pig to patient could then be confirmed.

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