

PhD Thesis

THE CRITICAL ROLE OF MONOCYTES IN TRANSPLANT-RELATED ACUTE LUNG INJURY

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Declaration of Originality

I hereby declare that this work is my own, and that by any others is fully acknowledged and referenced herein.

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Dedication

This thesis is dedicated to my parents. Their support and encouragement has given me the strength to juggle clinical training with basic research and numerous other projects, interests and distractions along the way.

I also dedicate this thesis to my sister Amy who stimulated me to go 'back to the bench' and complete a PhD, after she successfully completed hers, and to Paolo, who has provided me with immense support throughout my research and beyond. Thank you.

I would also like to dedicate this work to the lung donors, recipients and their loved ones, who selflessly contributed to the on-going clinical trials in lung transplantation from which this work benefitted. I hope this study contributes in some way to the improving the lives of lung transplant patients.

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Abstract

Lung transplantation remains the final option for patients with end-stage lung disease. A significant proportion will develop primary graft dysfunction acutely post implantation, severely impacting on their morbidity and mortality [1]. A key role for marginated monocytes has been identified in several experimental models of lung injury. Whilst other inflammatory leukocytes have been implicated in the pathogenesis of lung ischaemia-reperfusion injury (and primary graft dysfunction), a specific role for vascular monocytes has not yet been fully investigated.

We hypothesised that marginated lung monocytes played a critical role in the pathogenesis of lung ischaemia-reperfusion injury. Our overall aims were to: (a) develop, optimise and characterise a murine model of ischaemia-reperfusion, utilising the isolated perfused lung system, (b) elucidate the role of monocytes with liposomal-clodronate depletion and adoptive transfer, and (c) to identify and characterise monocytes in human lung transplant samples and correlate them to patient outcomes.

The major findings of this work were that lung marginated monocytes are retained and activated following ischaemia-reperfusion injury, inducing inflammatory mediator release. Clodronate depletion of vascular monocytes resulted in reversal of the injury and abrogated mediator release. Moreover, vascular monocyte repletion (via adoptive transfer), reinstated the injury and inflammation originally observed. Analysis of human lung samples, flushed prior to implantation, also demonstrated significant monocyte retention and activation, which correlated with primary graft dysfunction, in a pilot study of transplant recipients.

In summary, this study was able to confirm a critical role for marginated monocytes in lung ischaemia-reperfusion injury. A significant number were retained, and activated, in both murine and human lung samples despite considerable pulmonary flushing. This indicates a propensity for these monocytes to be transferred as 'passenger' cells to recipients, where they are capable of the propagation of lung inflammation and injury following transplantation.

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Abbreviations

ALI	Acute lung injury
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BB94	Batimastat 94
BSA	Bovine serum albumin
BOS	Bronchiolitis obliterans syndrome
CAM	Cell adhesion molecule
CD	Cluster of differentiation molecule
CO ₂	Carbon dioxide
DLI	Donor lung inflammation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EVLP	<i>Ex vivo</i> lung perfusion
FCM	Flow cytometry
FCS	Fetal calf serum
FiO ₂	Fraction of inspired oxygen
FWB	Flow wash buffer
H ₂ O	Water
HAS	Human serum albumin
ICAM-1	Intercellular cell adhesion molecule-1
IL	Interleukin
IPL	Isolated perfusion lung
IQR	Interquartile range
IR	Ischaemia reperfusion
IT	Intratracheal
IV	Intravenous
K ⁺	Potassium
KC	Keratinocyte-derived chemokine
LPS	Lipopolysaccharide
Ly6C	Leukocyte antigen 6C
MCP-1	Monocyte chemotactic protein 1
MIP-2	Macrophage inflammatory protein 2
Na ⁺	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate H
NFκB	Nuclear Factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffer saline
PECAM-1	Platelet endothelial cell adhesion molecule-1
PEEP	Positive end expiratory pressure
PF ratio	PaO ₂ to FiO ₂ ratio
PGD	Primary graft dysfunction
PaO ₂	Partial pressure of arterial oxygen
PVR	Pulmonary vascular resistance
PWB	Perm wash buffer
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
TNF	Tumour necrosis factor α (alpha)
VCAM-1	Vascular cell adhesion molecule 1
W:D	Wet:dry

Chapter 1

Introduction

Lung Transplantation

Carrel and Lindbergh first described the ability to preserve whole organs *ex vivo* with the invention of their 'perfusion pump' in the 1930s [2]. Based on this work, surgical transplantation techniques were developed in animals in the 1940s-1950s. Although survival was still severely limited by graft rejection, James Hardy went on to transplant a human lung in Mississippi in 1963, in a convicted murder, John Richard Russell [3]. He died 18 days later due to renal failure [4, 5]. On-going problems with the surgical anastomotic techniques and organ rejection hampered subsequent attempts until cardio-pulmonary bypass, and further experience with immunosuppressive agents, improved the procedure some years later. In 1981 the first successful heart-lung transplants were carried out in Stanford, California [6]. Following on from this Joel Cooper in Toronto went on to pioneer the first single, and then double, lung transplants throughout the 1980s [7].

Since this advent, the rates of survival have increased rapidly and currently stand at 80% and 53% at 1 year and 5 years respectively, with over 3700 occurring/year worldwide. The most common primary indication for transplantation remains Chronic Obstructive Pulmonary Disease (COPD) not due to α 1-antitrypsin deficiency (33%), followed closely by Interstitial Lung Diseases (ILD) including Idiopathic Pulmonary Fibrosis (24%), and Cystic Fibrosis (16%) [8]. Median survival is currently 5.7 years worldwide [8].

The prognosis of the chronic lung diseases listed above have vastly improved over recent years with advances in modern medicine. However, transplantation remains the mainstay of treatment for end-stage disease when standard therapies become ineffective. Unfortunately, the lung transplantation process itself is limited by numerous factors, which can be divided into lack of organ availability, poor organ quality and complications following transplantation.

1.1.1 Organ availability

The demand for all organs, including lungs, far outstrips the supply in the UK and availability of organs remains a significant hurdle. Multiple strategies to improve organ donation and consent rates have been initiated, and gradually they are improving. However, the UK still lies far behind other European countries when it comes to organ donation rates. Over 230 people were on the lung transplant list in the UK in 2011-2012 but only 180 lung transplants were carried out that year [9]. Median waiting times are 311 days and 24% of adult registrants have been removed from, or died, whilst on the lung transplant waiting list [10]. In Europe the current leaders in lung transplantation are Norway with 6.5 transplants/million population, with the UK 8 places behind with only 2.6/million population. In 2008 the UK organ donation taskforce published its report 'Organs for Transplant' putting forward a 5 year UK-wide program of recommendations and centralised specialist nurses, clinical leads and a National Organ Retrieval Service, to increase deceased donors [11]. This coincided with a 50% increase in donors and a 30.5% increase in transplants. However over 7000 people still remain on the transplant waiting list in the UK and >1300 people died or became too unwell to receive an organ, in 2013 [9].

The current donor program is seeking to address these issues with strategies in place, and in development, to expedite patient referral and thus optimisation of donors prior to organ harvest.

1.1.2 Organ quality

Grafts made available to organ retrieval teams are frequently of variable quality owing to the fact that the lung is highly susceptible to injury during the last few days and hours of life. For example this may occur following aspiration, sepsis, over ventilation, pneumonia, hypoperfusion states and brainstem death-induced pulmonary oedema. Poor lung quality at the point of assessment resulted in only ~24% of potential grafts being transplanted at this stage in the UK [9].

In addition, accepted lungs may carry with them undetected subclinical infection in up to 50% [12], which could translate a poorer outcome post-transplantation due to resultant immunological activation, dysfunction and bacterial translocation [13, 14].

1.1.3 Maximising organs

As up to 80% of lungs are rejected at the time of harvest and in light of transplant waiting lists greatly exceed organ availability, it is essential to maximise donor lung availability by minimising donor lung injury and developing novel strategies to address it. One such strategy that is now becoming widely adopted is *ex vivo* lung perfusion (EVLP). EVLP is a novel method of assessing, recovering and repairing injured donor lungs deemed unsuitable for transplantation [15-18]. Several groups have now successfully transplanted EVLP-reconditioned lungs [19, 20]. (See below)

1.1.4 Post-transplantation complications

Following transplantation lung recipients are susceptible to several life threatening complications, including the considerable risk from the procedure itself. Post-operative Primary Graft Dysfunction (PGD) is a major concern occurring in 10-25% of this cohort of patients [8]. Of these greater than 60% may die within 30 days [21]. Other common causes of death at up to 30 days include acute rejection, infection and related cardiovascular and surgical complications. At a year, infection becomes the most frequent cause of death (36%). Longer term, survival has a median of 5.7 years, with bronchiolitis obliterans is the leading cause of death at this stage. Other causes include graft failure (17%, lung rejection or bronchiolitis obliterans syndrome) and infection (18%). Malignancy is also common occurring in 14% at 5 years. Overall lung graft survival is substantially less than other solid organ transplants which can last greater than 10 years (renal, liver, heart) [22].

1.1.5 Primary Graft Dysfunction

Of those patients receiving a graft, a significant proportion develop PGD, and many die as a result [1, 21, 23]. Furthermore it increases the risk of developing bronchiolitis obliterans (BO), which limits survival of a recipients to ~5 years [24].

Box 1.1 Primary Graft Dysfunction Grading (International Society of Heart Lung Transplantation) [1]

PaO₂: partial pressure arterial oxygen, FiO₂: fraction of inspired oxygen

Grade 0: PaO₂/FiO₂ >300mmHg and normal chest radiograph (>40kPa)

Grade 1: PaO₂/FiO₂ >300mmHg (>40kPa) and diffuse allograft infiltrates on chest radiograph

Grade 2: PaO₂/FiO₂ between 200 and 300mmHg (26.7-40kPa)

Grade 3: PaO₂/FiO₂ <200mmHg (<26.7kPa)

PGD is a form of acute lung injury occurring within 72 hours of lung transplantation [25]. It presents with acute hypoxia, bilateral pulmonary infiltrates and impaired pulmonary compliance (without alternate cause), accompanied by diffuse alveolar damage microscopically, not dissimilar to the criteria ascribed to ALI and ARDS [25]. Previous titles for PGD allude to its potential mechanisms and include 're-implantation oedema', 'ischaemia-reperfusion injury' and 'early graft dysfunction'. PGD is thought to occur in 10-25% of patients and is the leading cause of death in this group (up to 40%) post transplantation [1, 26]. A variety of factors have been implicated in the pathogenesis in PGD, which can be broadly divided into: pre-transplant (i.e. donor factors) and peri-transplant (i.e. ischaemia-reperfusion (IR)).

Until recently, the majority of research into the pathogenesis of PGD was focussed on the effect of the peri-transplantation exposure of lungs to ischaemia-reperfusion (IR). A combination of mechanisms have been postulated and considerable effort has been undertaken to ameliorate any injury at this stage. Examples include the use of novel preservation solutions such as Perfadex, transportation at 4°C, normothermic perfusion and ventilation during transportation (Organ Care System) and the recent development of EVLP to analyse and optimise borderline organs [18, 27, 28].

However, pre-transplant, or donor factors, are now receiving increasing attention in the study of PGD pathogenesis. Donor lungs are exposed to a variety of insults, inevitable to the circumstances and resulting compromise of becoming a donor. These factors are thought to contribute, at one extreme, to the lungs being deemed unsuitable for transplantation, to, at the other extreme, appearing normal but carrying an underlying subclinical injury ('1st hit'), which may contribute to, and manifest as PGD in the recipient following IR ('2nd hit'). Suggested causes include ventilator associated lung injury, and pneumonia, systemic sepsis, acid aspiration and the autonomic storm, hypertension, neurogenic pulmonary oedema and systemic inflammation, manifested post-

traumatic brain injury [29]. These all may induce, or contribute to, undetected donor lung inflammation, likely to be a previously unrecognised principle determinant in the development of PGD [30].

1.2 Modelling lung ischaemia-reperfusion

1.2.1 *Hypoxia versus ischaemia*

Lung ischaemia is a major feature in the lung transplantation paradigm, and is also relevant during other low perfusion states including cardiopulmonary bypass, severe hypotension, circulatory arrest and pulmonary embolism [31]. Following reperfusion, injury is manifested by microvascular and alveolar damage, hypoxaemia and pulmonary oedema [31]. Ischaemia, taken from the Greek 'to restrict blood', is a unique process in the lungs. In other organs after ischaemia, hypoxia inevitably follows, with reoxygenation likewise occurring at reperfusion. However the lung parenchymal oxygen supply is derived from a dual blood supply (bronchial and pulmonary) as well as oxygen derived from the alveolar space directly, which is maintained during the transplantation process [31]. Currently lungs harvested from donors for transplantation are flushed with large volumes of preservation solution through the pulmonary artery. They are inflated with 50% oxygen enabling aerobic respiration (in contrast to other solid organs) [32] and then stored and transported at 4°C. However, the occurrence of alveolar hypoxia should not be disregarded, occurring e.g. following lung consolidation, and has been shown to directly activate the pulmonary endothelium [33].

Thus owing to the varied manifestations of lung IR due to its three sources of oxygen (pulmonary, bronchial and alveolar), several different models of lung IR have been investigated [31].

To achieve hypoxia as a cause of lung IR the largely inert gas nitrogen has been widely used. This can be supplied in varied concentrations to ventilated lungs or *in vitro* preparations, although the precise effects of nitrogen *per se* on lung cells remains unclear. In contrast, it has also been shown that oxygen can worsen IR injury and could be improved with the application of nitrogen ventilation [34].

1.2.2 *Warm versus cold ischaemia*

Much work has been done to directly compare warm versus cold ischemia. Studies focussing on warm ischaemia have been criticised for not being fully representative of the clinical situation. However a period of warm ischaemia is inevitable immediately prior to organ harvest in human transplantation. Moreover, normothermia occurs with other forms of lung IR, including one-lung ventilation and pulmonary embolism. One study compared an *in situ* warm ischaemia versus 24 hour cold storage of porcine lungs. They showed that shorter periods of warm ischaemia result in pathological changes similar to longer periods of cold ischaemia, and conclude the former may provide a useful accelerated model of lung IR injury [35]. However the perceived protective effects of cold ischaemia

continue to be debated. In fact, cold ischaemia can result in increased intracellular calcium and limited intracellular buffering, inducing cell damage in liver grafts [36].

1.2.3 *In vitro* IR

Basic modelling of IR *in vitro* is popular for its simplicity, low cost and ability to observe single cell line responses. This technique has been widely used and ranges from application of hypoxia/reoxygenation to cell substrate deprivation, to pH and temperature variation.

Exposure of human umbilical vein endothelial cell (HUVEC) line, thought to resemble pulmonary microvascular endothelial, to nutrient deplete Perfadex ('ischaemia') at 4°C and returning them to normal media at 37°C ('reperfusion'), resulted in MAP kinase and NF-κB upregulation and reversible cytoskeletal changes and gap formation in the endothelial monolayer [37]. However the relevance of a non-pulmonary cell line is unclear. A potentially more representative model of lung IR *in vitro* has been performed by one of the major lung transplantation research teams in Toronto. They utilised the lung epithelial cell line A549 and maintained oxygenation, as per the storage of human lungs. They instead induced 'ischaemia' through application of low potassium dextran preservative (with and without calcium and glucose supplementation) at 4°C and 'reperfusion' through reintroduction of warm complete media. This resulted in a time-dependent loss of cells, no protection from the addition of glucose, but benefit from calcium supplementation and use of 5% CO₂/95% O₂ over 100% O₂ [32]. Criticisms of this model may include the lack of modelling changes in endothelial shear stress (i.e. a 'flow'/'stop-flow' phenomenon) and the use of epithelial, rather than endothelial cell line, which one would expect to primarily experience pulmonary ischaemia. Others have developed an oxygenation 'interrupted flow' model of lung IR with bovine pulmonary artery endothelial cells. They showed interruption of media flow over the endothelial cells induced significant upregulation of reactive oxygen species (ROS), nuclear factor kappa B (NF-κB) and nuclear activator protein-1 (AP-1), suggesting a role for mechanotransduction in normoxic lung ischemia cell signalling [38].

1.2.4 *Hilar and pulmonary artery clamping*

The hilar clamping *in vivo* model is frequently used owing to its simplicity. It may either entail pulmonary artery clamping alone, which leaves the bronchial circulation and pulmonary venous drainage intact, or complete *in situ* clamping of the lung hilum. The latter results in complete ischaemia and hypoxia. However it does not exactly resemble the clinical manifestations of IR as all three (bronchial, pulmonary and alveolar) supplies of oxygen are interrupted and remain in a warm (37°C) environment prior to 'reperfusion' (unclamping). Despite this it provides a useful method of comparing IR lungs to matched non-clamped lungs of the same animal.

1.2.5 Isolated Perfused Lung

The Isolated Perfused Lung (IPL) system is ideally placed to study lung ischaemia with its ability to induce 'stop-flow' ischaemia and reperfusion as desired. Moreover it provides an intact organ preparation, without the systemic influence of the other organ system and the ability to modify gas content and ventilator parameters, pulmonary flow, perfusate constituents and ambient temperature. IPL was first used in dog lungs in 1949 [39] and has been widely used since to assess various lung pathologies including IR in several species including rabbits, rats, murine and canine models [40-42]. For example, cessation and reperfusion of pulmonary flow via the IPL has been directly compared to hypoxia/reoxygenation induced injury with 95% N₂/5% CO₂ gas ventilation, in canine lungs [42]. In this study both groups exhibited a significant increase in lung permeability, which were ameliorated by allopurinol, indicating a potential role for xanthine oxidase in both these pathways [42]. However a similar study using a rat IPL model, indicated that allopurinol was only able to inhibit oxidant generation following anoxia-reoxygenation and not IR [43]. The use of IPL for this study is discussed in more detail in Chapter 3.

1.2.6 Experimental Orthotopic Transplantation

Evidently the optimal method to model the IR experienced during transplantation is to perform entire orthotopic transplantation in animals. Until recently this technique was limited to large animals such as pigs, sheep and dogs, where the surgery was technically less demanding. However several groups have more recently described orthotopic transplants in rodents as small as mice, initially in 2007 and improved upon more recently [44-46]. This has expanded the potential of this technique considerably, with the ability to incorporate transgenic species into the experimental protocols, thus shedding more light on the mechanisms of lung IR.

1.3 Mechanisms of lung IR injury

1.3.1 *Ischaemia versus hypoxia*

Owing to its three sources of oxygen, and numerous experimental models, multiple features of the pathogenesis of lung IR have been investigated.

Classically tissue damage to organs subjected to ischaemia is intensified following re-oxygenation occurring at the point of reperfusion. Ischaemia in solid organs typically follows the course of interruption of an arterial blood supply resulting in the abrupt loss of tissue oxygen and nutrient supply, and the build of metabolites. Lack of oxygen results in enforced anaerobic glycolysis and a deficit of the main cellular energy source adenosine triphosphate (ATP). Lack of ATP impairs sodium transport mechanisms resulting in intracellular build up and subsequent oedema within the cells and the organelles, leading to rupture of mitochondria and lysosomes, and enzyme release contributing to cell death [47].

However, if hypoxia is thought not to be a feature, for example during lung transplantation, then it follows that oxidative stress will not occur. In respect of this it has been shown that ATP levels remain normal and that 'stop-flow' ischaemia may be considered therefore less injurious in ventilated lungs [48]. Moreover in a ventilated lung, rendered ischaemic, oxygen tensions will in fact rise as a result. Fisher *et al.*, investigated the effects of varied ventilator gas FiO₂ during a IPL 'stop-flow' rat model of IR; seeking to compare lung ischaemia to anoxia [49]. They found that increasing the inspired oxygen concentration and 'stop-flow' ischaemia time lead to an increased in lipid peroxidation, which was abolished with nitrogen ventilation. In addition they found that a subsequent reperfusion period did not increase lipid peroxidation and that blockade of cyclooxygenase and lipoxygenase pathways accounted for 40-50% of lung lipid peroxide production [49]. In a study designed to separate ischaemia from reperfusion injury, Becker *et al.*, assessed lung permeability (filtration and osmotic reflection coefficients) in ferret lungs. They found that injury occurred primarily during the ischaemic period, as opposed to the reperfusion period [50].

1.3.2 *Loss of shear stress and mechanotransduction injury mechanisms*

Normoxic lung ischaemia, i.e. 'stop-flow', has been reported to induce injury via the loss of shear stress on the endothelium. This activates flow-sensitive channels inducing membrane depolarisation and results in the production of ROS via xanthine oxidase, and raised intracellular calcium [38, 51]. This has been supported by the inhibition of permeability changes following IR with the application of xanthine oxidase inhibitor allopurinol [42]. In addition NADPH and eNOS activation occurs and signalling via MAP kinases is induced by endothelial depolarisation following cessation of flow [52, 53]. Lack of vascular mechanotransduction has also been reported to induce the generation of ROS and nitric oxide (NO) from macrophages and other immune cells [31, 54].

IR has also been shown to induce nuclear factor kappa B (NF κ B), MAP kinase and cell-adhesion molecule (CAM) upregulation in hilar clamping and *in vitro* models (Figure 1.1) [30, 37, 41, 54, 55]. In addition, blockade of TNF/IL1 production has been demonstrated to attenuate IR in a rat model associated with a significant reduction phosphorylated-p38 MAP kinase activation [56]. Upregulated CAMs have been shown to directly contribute to microvascular injury and lung leukocyte extravasation [57]. Studies investigating CAM (Platelet-activating factor, ICAM1, CD18, P-selectin) blockade have also been shown to confer protection to IR [57-60].

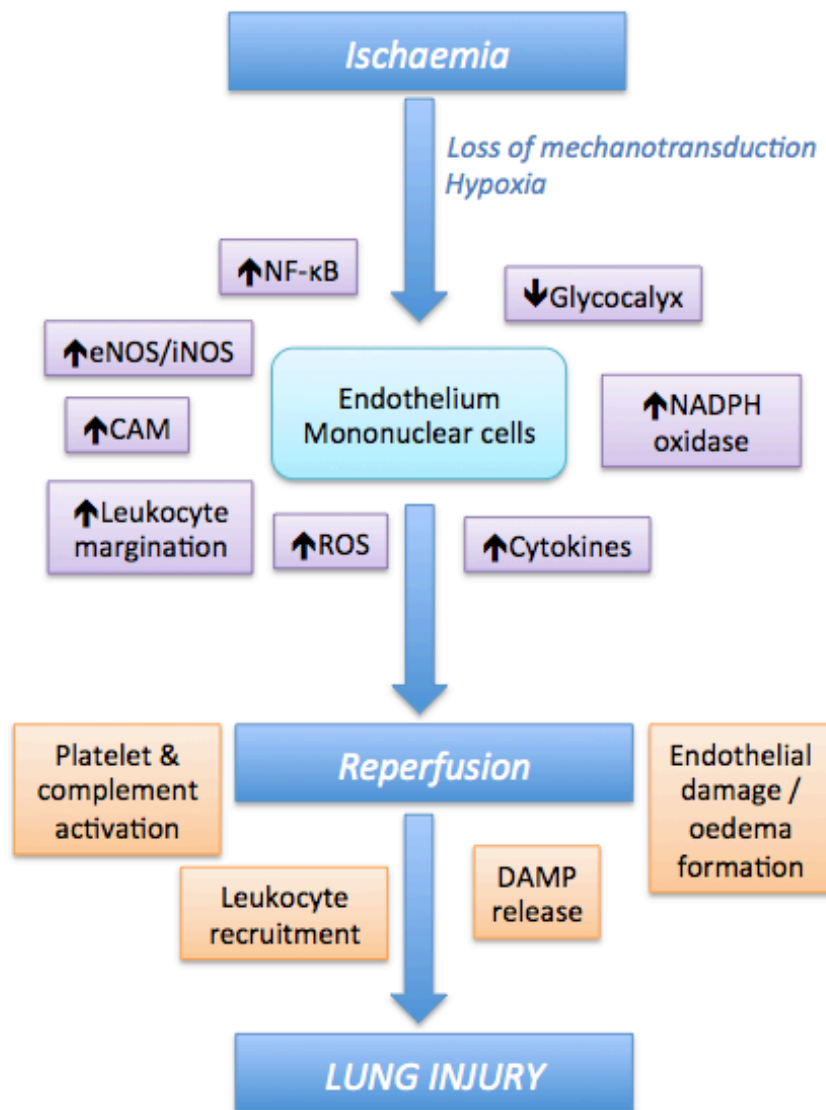


Figure 1.1 Schematic of the mechanisms of IR on the lung

1.3.3 Reperfusion

Reperfusion, as with reoxygenation, is commonly when pathophysiological changes become evident and the injured graft tissue releases DAMPS and comes into contact with recipient blood [61]. At this point endothelial injury and permeability, leukocyte, platelet and complement activation occurs along with further cytokine and ROS release within the lungs and at extra-pulmonary sites [62, 63].

1.3.4 Reactive oxygen species

As outlined above ROS play a key role in the pathogenesis of IR. ROS have been found in models of both ventilated and hypoxic models of lung ischaemia, occurring on reintroduction of oxygen in the latter [64]. As indicated above (cell membrane) lipids are a common target for ROS damage DNA and inactivate proteins. Endothelial ROS production has been linked to NADPH oxidase activity in a rodent IPL lung model comparing 'stop-flow' ischaemia to anoxia/reoxygenation. ROS production was abolished through use of a NADPH oxidase selective inhibitor or gp91^{phox} knockout mice [41]. Furthermore fluorescence imaging in this study showed ROS generation to be localised to the pulmonary endothelium. Other groups have also shown ROS derived from endothelial NADPH oxidase plays a key role in vascular oxidative stress [65]. Moreover, in a IR model of hilar clamping, mice lacking the NADPH oxidase (Nox2^{-/-}), or the transient receptor potential channel 6 (TRPC6^{-/-}) were protected from IR-induced oedema formation, with ROS production being completely abolished in Nox2^{-/-} endothelial cells [66]. In addition activated neutrophils have also been reported to produce injurious ROS in lung injury [67]. The pulmonary microvasculature has also been shown to have the ability to scavenge significant amounts of H₂O₂ produced from exogenous xanthine oxidase addition, but this does not necessarily protect the endothelium from injury [68].

1.3.5 Nitric oxide

In response to cessation of pulmonary blood flow, endothelial nitric oxide synthase (eNOS) is upregulated, elevating levels of nitric oxide (NO) [69]. In a hilar clamp model, upregulation of eNOS mRNA and protein was observed in lung tissue [54]. However, this appeared to be a protective response as pulmonary oedema, leukocyte infiltration and VCAM upregulation were significantly greater in eNOS knockout mice [54]. Of note, no increase in inducible NOS (iNOS) or neural NOS (nNOS) were observed. In addition hypothermic protection against lung IR injury has been shown to be dependent on eNOS [70].

1.3.6 Endothelial glycocalyx

The vascular endothelium is coated with a surface layer referred to as the glycocalyx, consisting of a thick hydrated lattice of negatively charged glycoproteins and glycosaminoglycans. It provides several functions including maintenance of endothelial homeostasis, vascular tone and permeability, and shelters the surface cell adhesion

molecules from circulating leukocytes [71, 72]. However, following IR, surgery, cardiopulmonary bypass and inflammatory insults, the glycocalyx layer has been shown to shed, enhancing leukocyte interaction with endothelial surface CAMs and increasing permeability [73-79]. Shedding of the glycosaminoglycans occurs in response to enzymatic activity of endoglycosidases, heparanase and hyaluronidase in the lung [79-81]. In addition shedding of glycoproteins has been shown by metalloproteinases and serine proteases, released during inflammation [82, 83]. Loss of glycosaminoglycans has also been shown to occur in the lungs in response to inflammation via tumour necrosis factor alpha (TNF) mediated mechanisms [84] and are thought likely to play a key role maintaining barrier function in lung injury [79]. Moreover direct heparanase infusion has been shown to increase lung oedema formation in murine lungs [84, 85] and ROS has also been shown to directly contribute to degradation following IR [73, 86].

1.4 Acute Lung Injury and Acute Respiratory Distress Syndrome

Acute Respiratory Distress Syndrome (ARDS) was initially described in the 1960s by Ashbaugh who authored a case series of 12 patients suffering from refractory hypoxaemia, decreased compliance and diffuse infiltrates on chest radiography requiring positive end expiratory pressure (PEEP) ventilation [87]. Since then diagnostic criteria have been defined and redefined to diagnose ARDS, most recently in Berlin in 2012 (Table 1.1) [88]. Previous definitions have included the term 'Acute Lung Injury' as a less severe form of ARDS, a term which remains in use in the scientific literature and within this thesis. However, the most recent definition now refers to mild, moderate and severe ARDS. The syndrome can occur in response to a wide variety of pulmonary (e.g. pneumonia, ventilator-induced lung injury, aspiration, inhalational injury) and non-pulmonary insults (e.g. sepsis, pancreatitis, head injury, trauma, surgery). Estimates of current incidence are reported to be ~190,000 patients/year in the US with an estimated in hospital mortality of 38.5% [89].

Box 1.2 The Berlin Definition of Acute Respiratory Distress Syndrome [88]

Timing	Within 1 week of known clinical insult or new or worsening respiratory symptoms
Chest imaging	Bilateral opacities – not fully explained by effusion, lobar/lung collapse, or nodules
Origin of oedema	Respiratory failure – not fully explained by cardiac failure or fluid overload Need objective assessment (e.g. echocardiography) to exclude hydrostatic oedema if no risk factor present
Oxygenation:	
<i>Mild:</i>	$200\text{mmHg} < \text{PaO}_2/\text{FiO}_2 \leq 300\text{mmHg}$ with PEEP or CPAP $\geq 5\text{cmH}_2\text{O}$
<i>Moderate:</i>	$100\text{mmHg} < \text{PaO}_2/\text{FiO}_2 \leq 200\text{mmHg}$ with PEEP $\geq 5\text{cmH}_2\text{O}$
<i>Severe:</i>	$\text{PaO}_2/\text{FiO}_2 \leq 100\text{mmHg}$ with PEEP $\geq 5\text{cmH}_2\text{O}$

The acute phase of ALI/ARDS is characterised by pulmonary endothelial injury and subsequent capillary barrier leak and influx of protein-rich oedema into the alveolar space (diffuse alveolar damage). This is compounded by epithelial injury and reduced fluid clearance and surfactant production. Impaired alveolar gas exchange and airway collapse ensues, resulting in shunt and significant hypoxaemia. This is accompanied by neutrophil influx and hyaline membrane deposition [90] and followed by a fibro-proliferative phase and in some, resolution.

The role of inflammatory leukocytes in ALI have focussed until recently on neutrophils and alveolar macrophages, likely related to the ease of their recovery and identification from broncho-alveolar lavage (BAL) samples in patients and animal models. However evidence is mounting for the roles of several other lung leukocytes, including the other mononuclear phagocytic cell populations. Moreover, the precise location of these cells is key as leukocytes within each distinct lung parenchymal compartment (intravascular versus interstitial versus alveolar) are likely to have distinct roles. These may range from sensing perturbations of the endothelium in response to ischaemia to effecting increased permeability, neutrophil recruitment and resultant injury.

1.5 Neutrophils and ALI

The recruitment and activation of leukocytes within the pulmonary vasculature has long been described in the pathogenesis of ALI. Of those, neutrophils have been widely identified as central players in this process with numerous studies in animals and humans labelling them as such [67]. They are recruited to the small capillaries of the lungs where they are activated and sequestered from the blood, migrating to the interstitial and then alveolar space. Their size is such that lung transit time of activated neutrophils is prolonged and the required shape change to allow access through the narrow lung capillaries is impeded by inflammatory stimuli, which reduce deformability through changes in the cytoskeleton [91-93]. Once lodged or marginated to the microvasculature they contribute to endothelial damage, increasing permeability within minutes to hours of induction of experimental ALI [94]. Neutrophils are attracted to the tissues by other cells (e.g. monocytes), endothelial injury, and microvesicle, chemokine, and cytokine release in response to pathogen-associated and damage-associated molecule patterns (PAMPS and DAMPS) [95, 96]. Subsequently selectins and cell adhesion molecules mediate endothelial binding and specific recognition of tight junctions allows passage of neutrophils through the endothelium into the interstitium and alveolar space [92].

Neutrophils have been implicated in several different models of ALI, namely caecal ligation and puncture, shock, intratracheal LPS, lung ischaemia-reperfusion, transfusion-related ALI, ventilator-induced lung injury and pneumonia [40, 97-102]. The mechanisms by which neutrophils are thought to induce ALI are varied. The neutrophil-complement pathway has attracted much attention. Neutrophil mediated damage has been reported to occur following release of their granule proteins seen specifically in a Streptococcal model of ALI, whereby injury was reversed following neutrophil depletion and reinstated with application of activated neutrophil supernatant [103]. Similarly neutrophil-mediated shedding of the pulmonary vascular endothelial glycocalyx surface layer, which can increase permeability, has been attributed specifically to neutrophil supernatant application and abrogated by serine protease inhibition [104]. Several groups have found that the serine protease neutrophil elastase is key to the pathogenesis of ALI, with plasma and BAL levels correlating with severity of injury in patients, inducing damage when exogenously applied in mouse models and abrogating injury when directly inhibited [105-110]. This is likely to occur through destruction of the basement membrane and other protein structures. The neutrophil role has been

further confirmed with reduced ALI severity following their depletion and through blockade of the neutrophil chemoattractant IL-8 [111, 112].

However, ARDS has been reported to occur in patients with neutropaenia, indicating that neutrophil-independent mechanisms are also of relevance and are not necessary essential in the pathogenesis of this disease [113-116]. This has also been supported by animal data where neutrophil depletion had no effect on lung injury following ischaemia-reperfusion [117].

1.6 Mononuclear Phagocytes and ALI

1.6.1 Monocytes

Monocytes are bone-marrow derived multipotent cells of the mononuclear-phagocytic innate immune system [118]. They originate from a progenitor cell common to neutrophils in the bone marrow and after being released into the blood for a few days, move into the tissues where they differentiate to macrophages and dendritic cells [119-121]. Circulating monocytes have been shown to give rise to several tissue macrophages including lung and alveolar macrophages, dendritic cells and osteoclasts (see Figure 1.1). Multiple signals have been shown to facilitate the trafficking of monocytes to the tissues, including pro-inflammatory, immune and metabolic stimuli [122]. Once within the tissues they modulate the host defence and remodelling.

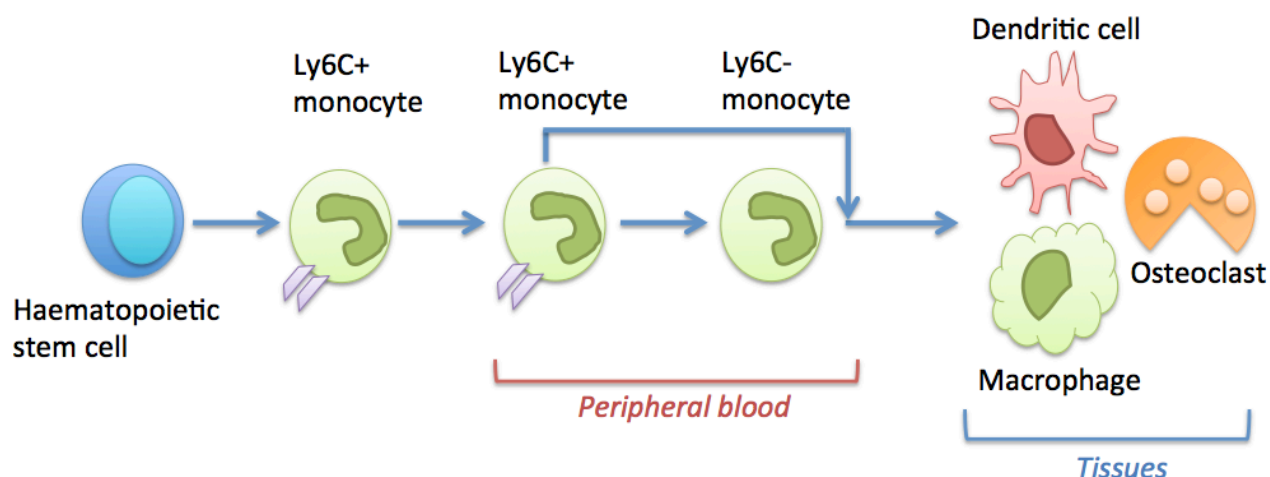


Figure 1.2 The murine mononuclear-phagocytic system (abbreviated).

Several heterogeneous circulating monocyte groups exist in both humans and animals, identifiable by differing cell surface receptor expression, likely to reflect the ultimate fate of these subsets. Work, initially in humans, identified two separate circulating monocyte phenotypes, distinguished by their differential expression of CD14, a co-

receptor for bacterial lipopolysaccharide (LPS) (with Toll-like receptor 4 and MD-2), and CD16, comprising of Fc receptors FcγRIIIa and FcγRIIIb. CD14^{High}/CD16⁻ monocytes are referred to as classical monocytes and are regarded as the 'inflammatory' subset. They typically exhibit higher levels of the chemokine receptors CCR1, 2, 4, 7 and CXCR1, 2 and 4. CD14⁺/CD16⁺ monocytes as referred to as non-classical or 'resident' subset as they are thought to resemble tissue macrophages, and express greater amounts of FcγRII (CD32), Major Histocompatibility Complex II (MHC II) and CCR5. Despite this, both pools exhibit the ability to migrate across endothelial beds and differentiate into dendritic cells or macrophages depending on the chemokine signals applied to induce such movement. More recently a third subset has been identified in humans referred to as the 'intermediate' subset, with, as the name suggests, an intermediate CD14⁺/CD16⁺ phenotype. They express FcγRI (CD64) along with high levels of CD86 and HLA-DR and are co-stimulatory to T cells thus greatly resembling dendritic cells, and have been proposed to have an immune regulatory function owing to their intermediate phenotype.

In mice, distinct subsets of CD11b⁺/ F4/80⁺ monocytes were more recently identified on the basis of their expression of CCR2, L-selectin (CD62L) and the fractalkine receptor CX₃CR1. Geissman *et al.*, investigated the fate of the monocyte subsets with the use of adoptive transfer of Green Fluorescent Protein (GFP) expressing monocytes from CX₃CR1 knock in mice. They found that the short-lived 'inflammatory' subset was designated as such owing to its propensity to travel to inflamed tissue and its expression of CCR2 and migration to the chemokine CCL2 (also known as Monocyte Chemoattractant Protein 1 (MCP1)) [123]. This subset also expressed higher levels of L-selectin, shed to its soluble form by activation, but lower levels of CX₃CR1. In contrast, the other murine subset does not express either L-selectin or CCR2, but does express higher levels of CX₃CR1. In 2003, Geissmann *et al.*, characterised the peripheral mouse monocyte populations further with the use of the cell surface marker Ly6C present on the CCR2⁺ subset [123]. In addition they showed that the CCR2⁺CD62L⁺CX₃CR1^{low}Ly6C⁺ inflammatory subset corresponded to the 'classical' human CD14^{High}/CD16⁻ monocytes, with corresponding levels of CCR2 and CX₃CR1. Moreover the non-classical human CD14⁺/CD16⁺ (CD64⁻) monocytes express low CCR2 and high CX₃CR1, corresponding to the Ly6C^{Low} murine monocyte population. This discovery validated the use of mouse models for studies investigating monocyte subsets.

They also found that after migration to areas of inflammation the 'inflammatory' subset upregulated cell surface CD11c and MHC II and trafficked to nearby lymph nodes, indicating differentiation of these monocytes to dendritic cells [123]. This was further complimented by the ability of these same monocytes to prime T cells. The CX₃CR1-subset however was shown to persist for longer and trafficked to the lung, liver, spleen and brain, and also upregulated CD11c and MHC II. To elucidate the differences in murine monocyte subsets, liposomal clodronate depletion experiments were performed. These indicated that the Ly6C^{High} subset were first to be released from the bone marrow and therefore may precede the maturation of their Ly6C^{Low} counterparts. In addition a potential intermediate inflammatory Ly6C population has also been identified [124]. Both of these have the potential to migrate to the tissues at sites of inflammation and differentiate into dendritic cells and macrophages. In the

absence of inflammatory cues the 'resident' population are thought to replenish tissue macrophages, likely responsible for remodelling and repair following injury.

1.6.2 Monocytes and ALI

Whilst several studies have highlighted a specific role for alveolar macrophages in ALI; from the initiation of the inflammatory response to causing direct tissue injury [125-129], recent attention has shifted to the role of monocytes. Numbers of lung-margined monocytes under resting conditions are substantial, in the $0.5-2.0 \times 10^6$ range for mice, which is far excess of total numbers of their circulating counterparts [130]. This abundance of lung-margined monocytes suggests a significant contribution to lung physiology and immunology, yet neither of these roles have been addressed to any great extent. Early work in the 1990s observed significant CD18 dependent and independent monocyte margination in the lungs of endotoxaemic rabbits [131].

In other species of animals (predominantly pigs and sheep) pulmonary intravascular macrophages (PIM) reside on the pulmonary endothelium, producing potent inflammatory mediators, thought to be responsible for their unique susceptibility to experimental ALI [132-134]. It has therefore been proposed that lung-margined monocytes may resemble at PIM-like phenotype during sustained inflammation, in the species that lack them [135, 136].

Inflammatory (Ly6C^{High}) monocytes have been implicated in the pathogenesis of acute lung injury as a result of a variety of insults including ventilator-induced lung injury (VILI), atelectatrauma and endotoxaemia [137-140]. Monocytes, like neutrophils, are rapidly sequestered to the lungs in response to inflammation [131, 141, 142] but their role under resting or inflamed conditions is not well characterised. We have previously shown that monocytes margined to the pulmonary microcirculation induce TNF-mediated endothelial dysfunction during acute endotoxaemia [137]. Moreover low-grade sub-clinical endotoxaemia has been shown to induce latent priming in the lungs, predisposing them to subsequent ALI [140, 143]. This process is dependent on margination and activation of monocytes, in particular the Ly6C^{High} subset, and induces a more significant injury to the pulmonary microcirculation on exposure to a secondary microbial challenge or injurious mechanical ventilation [140, 143]. This suggests that lung-margined inflammatory monocytes play a previously unappreciated, critical role in the evolution of ALI of various aetiologies.

A recent study sought to investigate the role the monocytes in intra-alveolar LPS-induced ALI with three depletion techniques: systemic liposomal clodronate, CD11b diphtheria toxin transgenic mice and anti-CCR2 antibody administration. They found that both Gr1^{high} and ^{low} populations contributed to lung injury and that depletion significantly reduced injury parameters. Moreover they showed that injury prevention was reversed with reintroduction of peripheral monocytes but crucially not mature macrophages [95].

1.6.3 Alveolar Macrophages

Alveolar macrophages have been shown to be derived from both peripheral (Gr1^{Low}) and local fetal precursors, under the control of nuclear receptor PPAR- γ [144-146]. Radiation and clodronate-induced peripheral monocyte depletion has been shown to not significantly alter their numbers in the lung [147] with evidence for repopulation within the lung occurring for up to a year [148]. Moreover depletion of alveolar macrophages has indicated repletion to occur from peripheral circulating precursors, although as yet this has not been definitively shown.

1.7 The role of leukocytes in lung IR

As outlined above leukocyte recruitment and activation within the pulmonary vasculature is likely to be key to the pathogenesis of acute lung injury. Their role however in IR-induced lung injury is not so clearly defined. However, pro-inflammatory cytokines and chemokines, found in both animal models and human lung samples as discussed below, have pointed to a significant role of inflammatory leukocytes in this process. However, many of these studies rely on samples taken following lung reperfusion in recipients, and may therefore only represent a *recipient* leukocyte response to implantation.

1.7.1 Neutrophils and IR

As in other forms of ALI, neutrophils are likely to have a significant role in the pathophysiology of IR-associated ALI. This has been evidenced by their increased margination, activation and migration into the alveoli of lung-donor brain injury models in animals [149]. Furthermore the neutrophil chemoattractant IL-8 has been found in high levels in BAL fluid samples from human donor lungs, and shown to correlate with subsequent incidence of PGD and survival [150]. IL-8 has also been shown to be increased following reperfusion correlating with increased PF ratios, mean airway pressures and APACHE scores immediately post transplantation [151]. In addition, blockade of platelet-activating factor (PAF), produced by epithelial cells in response to hypoxia and known to mediate increased lung permeability, has been shown to improve oxygen delivery in human recipients, when lungs are flushed with PAF inhibitor prior to transplantation [152]. However, as with other forms of ALI, recent animal data has suggested that neutrophil depletion had no effect on lung oedema in a rabbit hilar clamp model of IR, pointing to other cellular mediators [117]. Another study using the hilar clamping model, this time in rats, found increased myeloperoxidase activity and increased permeability at 30 minutes in the group exposed to IR. Rats rendered neutropaenic however, did not affect this early stage but did display a significant reduction in permeability at 4 hours [19].

1.7.2 Mononuclear phagocytes and IR

Much work had until recently focused on the neutrophils as the cellular mediators on inflammation and injury in lung IR. However, whilst the role of mononuclear phagocytic cells in this paradigm has also attracted interest, very little work to investigate the role of vascular monocytes in IR. Instead initial work has surrounded the role of resident alveolar macrophages as key initiators in lung injury following IR, with intratracheal liposomal clodronate depletion significantly attenuating lung injury in an murine IPL model of IR [153]. However others found that intratracheal depletion of alveolar macrophages aggravated lung IR injury in a rat IPL model, although a direct inflammatory effect of liposomal clodronate on the lung epithelium cannot be excluded in this particular case [154]. Moreover, a recent study using a murine pulmonary artery occlusion model of ischaemia found that both systemic liposomal clodronate and CD11b reporter-diphtheria toxin transgenic mice methods of lung macrophage depletion, significantly attenuated the IR injury [155]. However these approaches are likely to have also depleted the marginated monocyte pool, thus making it difficult to isolated this response to a single mononuclear cell subset.

A further study utilising a rat hilar clamp model suggested that lung macrophage induced injury was TNF mediated, with release occurring with 15minutes of reperfusion. Moreover they found that the TNF staining by immunohistochemistry was localised to the mononuclear phagocytic cells situated in the alveoli, interstitium and around the blood vessels, co-staining positive to the alveolar macrophage marker HAM 56 [156]. In this study the macrophage inhibitor gadolinium chloride abrogated this affect and the IR-induced vascular permeability [156], although again one must bear in mind this method also has the ability to deplete vascular monocytes [157, 158]. In a rabbit IPL model also using gadolinium depletion pre-treatment or leukocyte-depleted (filtered) 'recipient' whole blood reperfusion, a biphasic response to IR was observed. They found that early IR injury was mediated by the resident donor macrophages, and the later stages driven by recipient circulating leukocytes [159].

Additionally, as the key antigen presenting cells (APCs) of the innate immune system, host recipient monocytes will respond to DAMPS released by necrotic or dying cells from transplanted organs, inducing maturation and subsequent activation of host T-cells. To this end, monocyte depletion in a model on allogenic heart transplantation, has been shown to delay graft rejection and demonstrate reduced T cell infiltration [160]. Following pulmonary artery ligation *in situ*, the role of lung monocytes/macrophages had been further investigated with the use of liposomal clodronate depletion. Moldobaeva *et al.*, showed that MHC II^{int} CD11c⁺ CD11b⁺ lung macrophage numbers increased following this form of IR injury and were the main producers of the pro-inflammatory chemokine MIP-2 α [161].

One of the most significant studies implicated circulating monocytes, as opposed to their tissue counterparts, in the pathophysiology of IR has been performed in a model of renal IR injury. Ferenbach *et al.*, compared clodronate depletion of circulating monocytes to the CD11b reported diphtheria-toxin mediated monocyte and tissue macrophage depletion technique. Intriguingly the former method produced superior protection against IR injury

than the more effective depletion of the latter, implicating circulating monocytes as key protagonists and tissue macrophages as protective in this model [162].

Moreover scanning electron microscopy has been used to visualise the effects of IR on an orthotopic mouse model of transplantation [163]. They found several patho-morphological changes following IR, including the significantly increased presence of alveolar leukocyte and macrophages.

1.7.3 'Passenger' donor lung leukocytes

Traditionally 'passenger' leukocytes refer to the substantial extravascular donor leukocyte pool inevitably transferred to the recipient following transplantation. The transfer of intravascular leukocytes has until recently [164] not been considered relevant, likely owing to the flushing procedure of the pulmonary vasculature pre-implantation. However, any retention of intra-vascular lung-marginated leukocytes within donor lungs is likely to be crucial following transplantation. These cells are prone to pre-activation in the donor following the events preceding death (e.g. aspiration, sepsis, VILI), and in response to insults such as the surgical harvest and ischaemic storage [165, 166]. They would then be carried over as 'passengers' into the recipient where they could interact with and be exposed to recipient blood cells immediately. To date flushing of human lungs pre-implantation is regarded as an appropriate technique to remove of intravascular donor leukocytes, including through more extended protocols of *ex vivo* lung perfusion [164]. Attempts to remove alveolar macrophages through lavage have also been attempted. However we, and other groups, have shown that neither of these techniques can reliably remove either of these compartment leukocyte populations, with marginated leukocytes remaining intravascular [167] and a large proportion of alveolar macrophage resistant to removal via BAL [167-169]. All of the residual donor marginated, interstitial and alveolar leukocyte populations are therefore capable of subsequent propagation of injury and inflammation in the recipient.

1.8 Prevention and treatment of Lung IR Injury

1.8.1 Expanding the donor pool

As outline above both in the UK and worldwide numerous initiatives are in place to increase awareness and therefore donation rates. For example the Spanish have employed active management of cadaveric donors with use of extracorporeal membrane oxygenation to preserve the organs prior to harvest. The UK currently has one of the highest levels of family refusal with 43% of families refusing permission at the time of donation [170]. The recent centralization of UK services has resulted in a significant increase in donation rates, but this is mostly around an increase in donation after cardiac death (DCD). How 58% of UK donation occurs following donation after brain-stem death (DBD), which represent an ideal source of lungs and hearts. However, non-heart beating donors are now also being increasingly utilised as a further source of potential lungs grafts [171].

1.8.2 Maximising Organs: Ex Vivo Lung Perfusion (EVLP)

As up to 80% of lungs might be rejected at the time of harvest and in light of transplant waiting lists greatly exceed organ availability, efforts have been made to maximise donor lung quality by improving recognition of lung injury and developing novel strategies to treat it prior to transplantation. An increasingly adopted strategy for this is *ex vivo* lung perfusion (EVLP). EVLP is a novel method of assessing, recovering and repairing injured donor lungs considered unsuitable for transplantation [15-18]. It has also permitted further investigation of the pathophysiology and mechanisms resulting in PGD post transplantation or donor lungs.

Despite the early work by Carrel and Lindbergh in the 1930s, *ex vivo* organ perfused remained a research tool in animal models for decades following this. Not until the 1990s did Professor Stig Steen of Lund University in Sweden, pioneer the use of EVLP for the purpose of preservation and reconditioning borderline or rejected lungs, from any type of donor, with protective perfusion and ventilation. The time on EVLP allowed for more precise and thorough functional assessment and the opportunity for therapeutic interventions, especially of use in lungs from non-heart beating donors [172]. Studies subsequent to this suggested EVLP to be of potential use to allow treatment of pulmonary oedema and emboli, infection and aspiration and allow removal of donor alveolar and vascular inflammatory leukocytes [173-176]. Moreover, other groups have shown encouraging results with the application of stem cell and anti-inflammatory therapy directly to the isolated lungs [177, 178].

In 2007 Steen's group reported the successful transplantation of a human reconditioned lung through the use of EVLP [179]. Since then, several other groups worldwide have also successfully transplanted EVLP-reconditioned lungs [19, 20]. Enthusiasm for the adoption of the technique has lead to technical improvement including evidence that acellular perfusates might be superior to blood-based solution for organ assessment and protection [16]. Also a recent study has suggested that maintenance of the organs at 37°C with EVLP might be superior to cold ischaemic storage [180].

In the time since Steen's initial publication several clinical trials have taken place. The Toronto group, who pioneered lung transplantation in the 1960s, have gone on to develop a very successful EVLP program, enabling improvement to the waiting list [181]. They recently published a high-profile trial prospectively comparing standard lung transplants to EVLP reconditioned lung transplants. They were able to show a significant increase in EVLP lung PF ratio from 335mmHg to 443mmHg at 4hours of perfusion. 20 EVLP reconditioned lungs were transplanted and compared to 116 standard lungs. They were able to show no significant difference in the incidence of PGD at 72hours post transplantation, with 15% in the EVLP group versus 30% in the control group. There was also no significant difference in any of the secondary end points such as airway complications, ICU or hospital stay and 30-day mortality [182]. Since then this group have transplanted over 40 EVLP reconditioned lungs successfully and their protocol has been adopted as part of a large multi-centre trial [183].

1.8.3 Preventing and treating PGD

To date no clear strategy exists for the prevention or treatment of PGD. One such strategy is the Organ Care System (OCS). This has been reported to provide successful preservation of grafts with normothermic perfusion and ventilation of donor lungs during transportation [28]. Comparison of this to standard cold ischaemic storage is currently under investigation in the INSPIRE trial. Lung protective ventilation in donors is also recommended, as evidenced by a multicentre trial by Mascia *et al.*, in Turin. They found that significantly more donor lungs were suitable for transplantation in the protective ventilation groups (54%) compared to conventional ventilation group (27%) [184].

In addition there are several experimental strategies evidenced to potentially prevent PGD in transplant recipients. Evidence for ischaemic preconditioning in other organs (e.g. hearts, livers) already exists. Similarly 15 minutes of ischaemic preconditioning has been shown to enhance donor lung preservation in a rabbit model of lung IR [185]. Other specific therapies include exogenous surfactant therapy has been shown to improve oxygenation in a model of porcine lung transplantation [186, 187] and is now being trialled in human PGD [188, 189]. It has also been shown to be of benefit when applied to donor lungs before retrieval [190]. Other promising pathways under investigation include complement activation inhibition [191] and PAF antagonism [152]. Gene therapy has also had promising results with IL-10, Ik8 and soluble type 1 interleukin receptor suppressor genes reducing experimental IR injury and may well represent the basis of future therapies in this field [55, 192].

1.9 Aims of Thesis

The overall aims of this thesis were as follows:

The murine IPL

- ⇒ Characterise the IPL system
- ⇒ Identify areas of optimisation and modification
- ⇒ Develop a relevant model of lung IR injury

Murine model of lung IR Injury

- ⇒ Quantify IR-induced monocyte activation and soluble mediator expression
- ⇒ Determine the effect of monocyte depletion on IR
- ⇒ Determine the mechanisms of monocyte-mediated IR lung injury
- ⇒ Assess the role of 'donor inflammation'
- ⇒ Quantify and evaluate the role of the endothelial glycocalyx response lung IR
- ⇒ Identify and validate the precise compartmental location of lung mononuclear phagocytes using a dual staining technique
- ⇒ Characterise the expression of the co-stimulatory inflammatory molecule CD86 on mononuclear cells in response to IR
- ⇒ Restore IR-induced lung injury in depleted lungs with monocyte adoptive transfer

Human lung IR injury

- ⇒ Identify and quantify mononuclear cells in human lung transplant samples with flow cytometry
- ⇒ Quantify mononuclear cell activation
- ⇒ Correlate mononuclear cell numbers and activation to clinical outcomes in recipients following lung transplantation

Chapter 2

Materials and Methods

2.1 Animals

All protocols were reviewed and approved by the U.K. Home Office in accordance with the Animals (Scientific Procedures) Act 1986, U.K.. All experiments were performed using male C57BL/6 mice (Charles River Laboratories, Margate UK) aged between 8–12 weeks, weighing between 22–28g. The mice were able to access food and water freely at all times.

2.2 Equipment, Software and Reagents

Table 2.1 Equipment

Equipment	Application	Supplier
Nylon cell strainer (40µm)	Lung homogenate filtration	<i>BD Falcon, New Jersey, USA</i>
Custom-made ventilator	Murine ventilation	<i>Harvard Apparatus, Holliston, MA, USA</i>
CyAn ADP Analyzer	Flow cytometry	<i>Beckman-Coulter, High Wycombe, UK</i>
Eppendorf microcentrifuge 5417R	Microcentrifugation	<i>Eppendorf, Hamburg, Germany</i>
FLx 800 microplate reader	Fluorescence plate reader	<i>Biotek Instruments Inc</i>
Heraeus Multifuge 3 S-R	Centrifugation	<i>Thermo Scientific, Waltham, MA, USA</i>
IPL-1 Type 839	Isolated lung perfusion	<i>Hugo-Sachs Elektronik, March, Germany</i>
Leucosep Tube 163 290	Whole blood separation	<i>Greiner Bio-One, Stonehouse, UK</i>
MRW Plate washer AM68	96 well plate washer	<i>Dynex Technologies Inc, Chantilly, VA, USA</i>
MRX II microplate reader	96 well plate reader	<i>Dynex Technologies Inc, Chantilly, VA, USA</i>
Nuaire DH autoflow incubator	Cell culture	<i>Nuaire Group, Caerphilly, Wales</i>
Purple 'C' and orange 'M' dissociation tubes	Mechanical lung dissociation	<i>gentleMACS, Miltenyi Biotec, Bisley, UK</i>
Precision Stereo Zoom Trinocular Microscope (III)	10x microscope for tissue dissection/microsurgery	<i>World Precision Instruments, Sarasota, FL, USA</i>
Stuart SB 2 rotators	Sample mixing	<i>Bibby Scientific, Stone, UK</i>
Tissue dissociator	Mechanical lung dissociation	<i>gentleMACS, Miltenyi Biotec, Bisley, UK</i>

Table 2.2 Software

Software	Application	Supplier
Chart 5 v5.5.6	Physiology data acquisition	AD Instruments, Oxford, UK
FlowJo 10.0.8r1 (for MAC OS X)	Flow cytometry analysis	Flowjo, LLC, Ashland, OR, USA
GraphPad Prism version 6 (MAC)	Figure/graph plotting	GraphPad, La Jolla, CA, USA
KC4	Fluorescence plate reader analysis	BioTek, Winooski, VT, USA
PowerLab	Physiology data acquisition	AD Instruments, Oxford, UK
Revelation v4.22 200	Plate reader analysis	Dynex Technologies Inc, Chantilly, VA, USA
SPSS version 20 (MAC)	Statistical analysis	IBM, Portsmouth, UK
Summit v4.3.04	Flow cytometry sample acquisition	Beckman-Coulter, High Wycombe, UK

Table 2.3 Reagents and pharmacological agents

Reagent	Application	Supplier
Alexa Fluor 594 conjugated albumin	Permeability assay	Sigma Aldrich, St Louis MO, USA
Batimastat (BB94)	Prevention of TACE-mediated TNF (RAW assay) and L-selectin shedding	Vernalis, Winnersh, UK
BD Cytofix/Cytoperm	Cell fixation/permeabilisation	BD Biosciences, Oxford, UK
BD Lyse/Fix	Red cell lysis	BD Biosciences, Oxford, UK
Bovine serum albumin (BSA)	Buffer/media constituent	Sigma Aldrich, St Louis MO, USA
BrdU Flow Kit	<i>In vivo</i> cell labelling	BD Biosciences, Oxford, UK
CFSE (CellTrace)	Monocyte labelling dye	Thermo Fisher Scientific, Waltham, MA, USA
Coomassie brilliant blue dye G-250	BAL protein quantification	Bio-Rad Laboratories, Hemel Hempstead, UK
DMEM F12 media	RAW cell culture media	Invitrogen, Paisley, UK
DNase 2mg/ml	Lung tissue digestion	Sigma Aldrich, St Louis, MO, USA
Duoset ELISAs (KC, MIP-2, IL-6, MCP-1, RAGE, TNF)	Soluble mediator detection	RnD Systems, Abingdon, UK
EasySep Mouse Monocyte Enrichment Kit and Purple Magnet	Blood monocyte isolation	StemCell Technologies, Cambridge, UK
Ethylenediaminetetraacetic acid (EDTA)	Flow wash buffer constituent	Sigma Aldrich, St Louis, MO, USA
Fetal calf serum (FCS)	Buffer constituent	Sigma Aldrich, St Louis, MO, USA
Hanks Balanced Salt Solution (HBSS)	Buffer	Sigma Aldrich, St Louis, MO, USA

Histopaque 1083	Blood monocyte isolation	<i>Sigma Aldrich, St Louis MO, USA</i>
Human serum albumin (HAS)	Perfusate constituent	<i>Baxter, Newbury, UK and Biotest, Brimingham UK</i>
IC Fixation buffer	Cell fixation	<i>eBioscience, Inc, San Diego, CA, USA</i>
Liberase 2.5mg/ml	Lung tissue digestion	<i>Roche, Burgess Hill, UK</i>
Lipopolysaccharide	Ultra Pure Escherichia coli O111:B4;	<i>Autogen Bioclear, Calne, UK</i>
Liposomal clodronate	Monocyte depletion	<i>FormuMax Scientific Inc., Palo Alto CA, USA</i>
Penicillin/Streptomycin/Glutamine (PSG)	IPL perfusate supplement	<i>Invitrogen, Paisley, UK</i>
Phosphate buffered saline (PBS)	Buffer	<i>Invitrogen, Paisley, UK</i>
RPMI-1640 (no glutamine, no phenol red)	IPL perfusate base	<i>Invitrogen, Paisley, UK</i>
Saponin	Perm wash buffer constituent	<i>Sigma Aldrich, St Louis MO, USA</i>
Sodium azide	Flow wash buffer constituent	<i>Sigma Aldrich, St Louis MO, USA</i>
Sodium bicarbonate 8.4%	IPL perfusate supplement	<i>Sigma Aldrich, St Louis MO, USA</i>
Sodium chloride powder	IPL perfusate supplement	<i>Sigma Aldrich, St Louis MO, USA</i>
Tween	ELISA detergent	<i>Sigma Aldrich, St Louis MO, USA</i>

Table 2.4 'In-house' buffers

Name	Application	Constituents
Flow wash buffer (FWB)	Flow cytometry	PBS, 2% FCS, 0.1% sodium azide, 5mM EDTA
Perm wash buffer (PWB)	Flow cytometry	PBS, 2% FCS, 0.2% saponin, 0.1% sodium azide
Lung perfusate	IPL	RPMI-1640, 4% HAS, 0.1% Sodium chloride, 0.08% Sodium Bicarbonate, PSG

Table 2.5 Murine antibodies

Epitope	Fluorophore	Clone	Final concentration	Supplier
BrdU	APC	3D4	1/200	BD Biosciences, Oxford, UK
CD11b	PE	M1/70	1/400	Biolegend, San Diego, USA
	PE Texas Red (PE-CF594)	M1/70	1/400	BD Horizon, Oxford, UK
	APC-Cy7	M1/70	1/400	Biolegend, San Diego, USA
CD11c	PeCy7	N418	1/400	Biolegend, San Diego, USA
	APC eFluor 780	N418	1/200	eBioscience, Inc, San Diego, CA, USA
CD31	FITC	MEC 13.3	1/200	BD Biosciences, Oxford, UK
	APC	MEC 13.3	1/2000	Biolegend, San Diego, USA
CD45	PE-Texas Red (PE-CF594)	30-F11	1/400	BD Horizon, Oxford, UK
	PerCP	30-F11	1/400	Biolegend, San Diego, USA
CD45.2	APC	104	1/400	Biolegend, San Diego, USA
CD86	PE	GL1	1/200	eBioscience, Inc, San Diego, CA, USA
F4/80	FITC	BM8	1/400	eBioscience, Inc, San Diego, CA, USA
Heparan sulfate	Unconjugated*	F58-10E4	1/200	Amsbio, Abingdon, UK
IgM	PE	RMM-1	1/200	Biolegend, San Diego, USA
L-selectin	PE	MEL-14	1/200	BD Biosciences, Oxford, UK
Ly6C	PeCy7	HK1.4	1/400	Biolegend, San Diego, USA
Ly6C/Ly6G (Gr1)	PerCP	RB6-8C5	1/400	Biolegend, San Diego, USA
Ly6G	PE	1A8	1/400	BD Biosciences, Oxford, UK
	APC	1A8	1/2000	Biolegend, San Diego, USA
MHC II (I-A/I-E)	PerCP	M5/114.15.2	1/400	Biolegend, San Diego, USA
NK1.1	PeCy7	PK 136	1/400	Biolegend, San Diego, USA
Phospho-NF-κB	APC	93H1	1/50	Cell Signalling, Danvers MA, USA
Phospho-p38	APC	28B10	1/100	Cell Signalling, Danvers MA, USA
Phospho-MK2	APC	27B7	1/800	Cell Signalling, Danvers MA, USA
Phospho-ERK1/2	APC	D13.14.4E	1/100	Cell Signalling, Danvers MA, USA
TNF	PE	MP6-XT22	1/200	eBioscience, Inc, San Diego, CA, USA

*PE-IgM (clone RMM-1) conjugated secondary required

Table 2.6 Human Antibodies

Epitope	Fluorophore	Clone	Final concentration	Supplier
Calgranulin A/B	FITC	27E10	1/40	<i>Santa Cruz, Dallas, TX, USA</i>
CD11b	PE-Texas Red (CF 594)	M1/70	1/400	<i>BD Horizon, Oxford, UK</i>
CD14	PerCP	M5E2	1/80	<i>Biolegend, San Diego, USA</i>
CD16	PeCy7	3G8	1/400	<i>Biolegend, San Diego, USA</i>
CD45	APC-Cy7	HI30	1/200	<i>Biolegend, San Diego, USA</i>
CD56	PE	HCD56	1/80	<i>Biolegend, San Diego, USA</i>
CD66b	PE	G10F5	1/40	<i>Biolegend, San Diego, USA</i>
CD86	APC	IT2.2	1/10	<i>Biolegend, San Diego, USA</i>
CCR2	APC	48607	1/20	<i>BD Biosciences, Oxford, UK</i>
HLA-DR	APC	L243	1/10	<i>Biolegend, San Diego, USA</i>
TREM-1	APC	TREM-26	1/10	<i>Biolegend, San Diego, USA</i>

Conjugate dye abbreviations:

- FITC: fluorescein, isothiocyanate
- PE: phycoerythrin
- PerCP: perinidin chlorophyll protein
- APC: allophycocyanin
- Cy: cyanine

2.3 The Isolated Perfused Lung

Whilst the final isolated perfused lung (IPL) methods are described here, Chapter 3 describes in full the development of this protocol.

The IL-1 IPL system (Hugo Sachs, March, Germany) was used to model IR in this project. This system comprises three principal components; the perfusion circuit, the thermostatic circuit and the ventilator/respiratory circuit.

2.3.1 The perfusion circuit

As shown in Figure 2.1 below the perfusion circuit consists of several parts accessed through 4 roller pump lanes. This allows it to infuse fresh perfusate (1) to pass via lane 2 (2) to the lungs via a heat exchanger (3) to the lungs through the pulmonary artery (PA). On exiting the pulmonary circulation the perfusate passes into the left atrial (LA) cannula and through the reservoir (4) and onto the waste container (5) via lane 3 of the pump (2). This constitutes 'open' or non-recirculating lung perfusion of fresh perfusate. Alternatively, the 'closed' recirculating perfusion circuit (6) can be selected by opening lane 4 of the pump and clamping off the lane 3 inlet/lane 2 outlet. Fresh perfusate ± additives can also be infused via lane 1 or directly through an auxiliary port point B (8) into the PA, although this increases the chances of bubble entrainment as it bypasses the bubble trap/heat exchanger and thus delivers perfusate at room temperature. Perfusate that has passed through the pulmonary circulation was sampled at point A (9). PA and LA pressures are monitored by pressure transducers located immediately prior to, and immediately after, the respective cannulae.

2.3.2 The thermostatic circuit

The entire plexiglass chamber in which the IPL sits was warmed to 37°C. This includes a heat exchanger, which warms the afferent perfusates (Figure 2.1 (3)). Water flowing through the chamber lid also conserves humidity within the chamber. A detailed diagram overlying a photograph of the IPL is discussed further in Chapter 3.

2.3.3 The ventilation circuit

Ventilation of the lungs is performed by a custom-made mouse ventilator (Harvard Apparatus, Holliston, MA, USA) consisting of a solenoid valve alternating inspiratory/expiratory flow. Flow is measured by a pneumotachometer and airway pressure by a pressure transducer. The inspired gas is also humidified

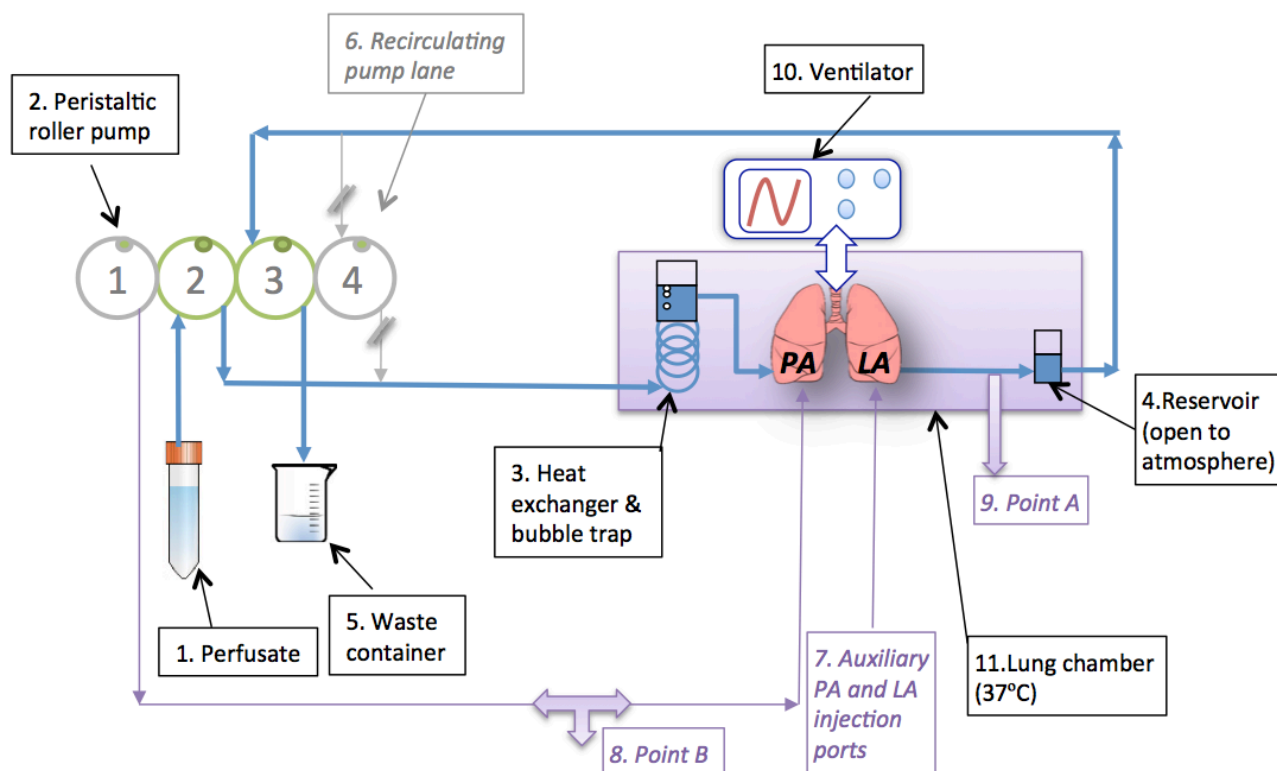


Figure 2.1 Schematic of IPL

2.4 Lipopolysaccharide administration *in vivo*

Intravenous

Our group had previously induced significant monocyte margination in mouse lungs, priming them to subsequent injury, with low dose lipopolysaccharide (LPS) administration, without producing systemic upset [143]. To model underlying subclinical inflammation prior to our IPL protocol 20ng (in 200µl PBS) of LPS (Ultra Pure Escherichia coli O111:B4; Autogen Bioclear) was injected via the tail vein.

Intratracheal

To validate our dual-staining compartment method, neutrophil dynamics were assessed following intratracheal LPS administration. Mice were anaesthetized and the vocal cords visualised with use of an external light source and microscope as previously described by our group [193-195]. A fine catheter was inserted through the vocal cords and 20µg of LPS in 50µl of 0.9% normal saline was instilled. Mice continued to spontaneously breathe throughout and were recovered in a warm environment.

2.5 Ischaemia-Reperfusion protocol

2.5.1 Preparation for IPL

2.1.1.1 Perfusate

We chose to use an acellular nutrient replete complete media perfusate for all our IPL protocols to allow the measurement of the lungs intrinsic response to 'stop-flow' ischaemia, rather than alteration of physiological parameters i.e. pH, osmolality and glucose concentration. To this end Roswell Park Memorial Institute Media-1640 without phenol red (RPMI-1640, Invitrogen, Paisley, UK) was used as described by others [196, 197]. To maintain appropriate oncotic pressure, and thus prevent oedema formation, the RPMI-1640 was supplemented with human albumin solution (HAS, Octopharma, Switzerland) to a final concentration of 4%. HAS was used in preference to bovine albumin to minimise contamination, which is discussed in more detail in Chapter 3. Furthermore sterile sodium chloride was added to adjust the osmolality that that of a mouse to approximately 330mosmol/kg.

2.1.1.2 Anaesthetic, tracheostomy and ventilation

To prevent coagulation within the vasculature all mice received 50iu of heparin intravenously 5minutes prior to anaesthetic overdose with intraperitoneal ketamine (130g/kg) and xylazine (13mg/kg). After loss of the foot pinch reflex, mice were weighed and a surgical tracheostomy was performed via a horizontal incision and the trachea was intubated, secured with a 3/0 silk suture and connected to a custom-made ventilator. Ventilation was commenced at a rate of 80/minute, positive end expiratory pressure (PEEP) of 5cmH₂O, with 21% FiO₂ and the flow was adjusted to maintain tidal volumes between 6-7ml/kg.

2.5.2 Mounting on the IPL

Following tracheostomy a midline laparotomy was performed and the inferior vena cava identified and blood sampled. The mouse was then exsanguinated through complete transection of the inferior vena cava and abdominal aorta. A proportion of blood was kept on ice and the remaining volume spun at 1700g, 4°C for 5minutes to allow removal of the plasma for subsequent flow cytometry and ELISA analysis. Following exsanguination the lungs were switched to a continuous positive airway pressure (CPAP) of 5cmH₂O (to prevent accidentally injury during subsequent thoracotomy) and the inhaled gas was exchanged for 5% CO₂/21% O₂ to ensure a normocapnic environment for the lung. The lower abdomen was removed to prevent gut contamination of the preparation.

A small incision was made in the anterior diaphragm to eliminate negative intra-thoracic pressure and allow the lungs to move away from the chest wall. A midline thoracotomy incision was then made along the left sternal edge and then the right, removing the sternum, taking care not to injury underlying lung or large vessels. The diaphragm was then incised bilaterally to open up the thoracic cavity. A 2/0 silk suture was passed through the apex of the

heart and was held in gentle tension to facilitate cannulation. A 3/0 suture was then gently placed behind the root of the pulmonary artery (PA) and aorta and tied in a loose single throw knot. A 2mm incision was then made in the base of the right ventricle and after extensive priming and confirmation of bubble exclusion of the PA cannula and tubing, the cannula was inserted into the root of the PA and secured by tightening of the pre-prepared knot. The apex of the left ventricle was then incised and the effluent cannula was passed into the left atrium. The left atrial cannula was then secured with a 2/0 silk above the apex, around the whole heart. Pulmonary vascular perfusion was then commenced at 0.2ml/min for 1 minute, increasing to 0.4ml/min for another minute in an 'open' non-recirculating fashion. At this point 2 inflation breaths to 25cmH₂O were performed and ventilation was recommenced as before. The perfusion rate was then increased to 25ml/kg/min for a further 3 minutes. After discarding the initial 250µl of dead space perfusate, 1.5ml of perfusate (which had passed through the lungs) was collected at 'point A'. To simulate ischaemia, following the initial 5 minute 'washout' period, perfusion was ceased and ventilation was switched back to CPAP of 5cmH₂O (5% CO₂ /21% O₂) for a 2 hour period. 'Normoxic' ischaemia was chosen over hypoxia to represent clinical scenario as discussed further in Chapter 3. The left atrial pressure was maintained throughout at 2.5mmHg by adjusting the height of the reservoir. A 10x Precision Stereo Zoom Trinocular microscope was utilised for all the surgical procedures described above (World Precision Instruments, Sarasota, FL, USA).

2.5.3 Ischaemia-reperfusion protocol

Following the 2 hour ischaemic period described above, perfused and ventilation was recommenced in an identical fashion to the 5 minute initial washout period with fresh perfusate, again collecting 1.5ml of effluent. Following this the IPL circuit was switched to the 'closed' non-recirculating circuit and the flow increased to 40ml/kg/min for a 2 hour 'reperfusion' and ventilation period (see Figure 2.2). At 15 minute intervals the ventilation was held to complete 8 'end-inspiratory pause' breaths and a 'sigh' recruitment manoeuvre was applied to 25cmH₂O for 5 seconds to allow calculation of respiratory mechanics. At the end of the 2hour reperfusion period a final 5 minute 'open' (non-recirculating) washout was completed and the first 1.5ml of perfusate was sampled via point A.

Throughout the protocols used in this study the respiratory mechanics remained within normal ranges. The plexiglass chamber surrounding the lungs was maintained at 37°C throughout the protocols as discussed further in Chapter 3.

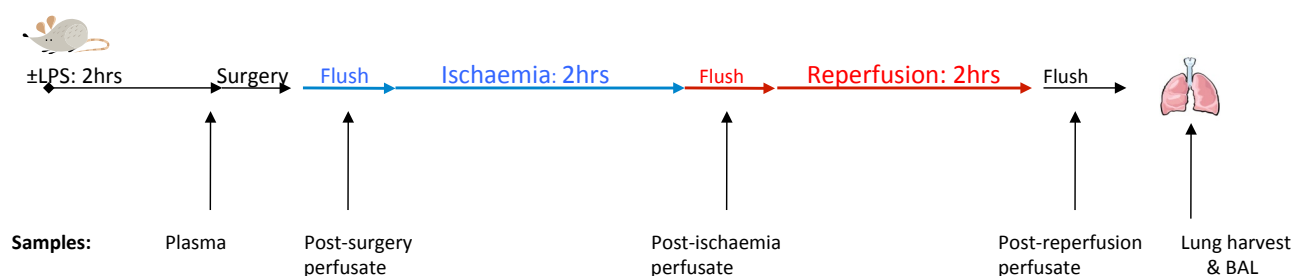


Figure 2.2 IPL ischaemia-reperfusion model protocol

2.5.4 Bronchoalveolar lavage (BAL) fluid sampling

After collection of the final perfusate samples at the end of the reperfusion period, perfusion and ventilation were ceased and a silk 2/0 suture was placed around the left lung, gently occluding it at the hilum to prevent BAL fluid entering. 500µl of normal saline (0.9%) was then slowly injected into the remaining lung lobes via the tracheal tube and lavaged gently a total of three times and before the residual fluid was removed and centrifuged at 200g for 5 minutes at 4°C. The supernatant was then used for immediate protein quantification (Bradford protein assay) and the remainder was stored at -80°C for subsequent soluble mediator analysis via ELISA.

2.5.5 Lung harvest and analysis

Following right lung BAL, the left lung lobe was blotted twice and weighed after which it was placed in a 60°C oven for wet:dry weight analysis. The remaining lung lobes were also blotted and weighed for lung cell count calculation and then subdivided for subsequent homogenisation.

2.6 Flow cytometric analysis

2.6.1 Lung homogenisation and staining

After weighing, lung tissue was homogenised in one of two ways. We have previously shown that the simultaneous fixation/homogenisation procedure is ideal for optimal cell recovery for when accurate cell counts are required [198]. However use of a fixative can result in loss of the Gr1 antibody dual specificity (Ly6C and Ly6G antigens) reactivity with monocytes (Ly6C) but not neutrophils (Ly6G). Therefore a Ly6C specific antibody was used to improve monocyte subset discrimination in fixed samples. For instances where antigen epitopes are completely denatured by fixation (e.g. anti-L-selectin clone MEL-14), homogenisation is performed in non-fixing medium. The Gr1 antibody was used in the unfixed lung samples to full effect.

‘Fixed’ lung samples were placed in an orange gentleMACs ‘M’ dissociation tube with 2ml of IC Fixation Buffer at room temperature. ‘Unfixed’ samples were placed in a purple gentleMACs ‘C’ tube with 2ml of ‘flow wash buffer’ (FWB) at 4°C containing phosphate buffered saline (PBS), 2% fetal calf serum (FCS), 0.1% sodium azide, 5mM EDTA and 10µM batimastat (BB94) to prevent L-selectin cleavage. They were then run on the ‘Spleen_01_01’ setting of a gentleMACs dissociator for 1 minute. Immediately following homogenisation both samples were diluted with 8mls of FWB at kept at 4°C thereafter. Fixed lung homogenates were then pushed through a 40µm nylon strainer (BD Falcon, New Jersey, USA) with a 2ml syringe plunger and 10ml of 4°C FWB to maximise cell yield for subsequent counts. Unfixed homogenates were flushed through strainers with 4°C FWB, to limit damage and/or activation of

unfixed cells. Both sets of samples were then centrifuged at 600g (Heraeus Multifuge 3 S-R) for 5 minutes at 4°C. The unfixed cells pellet were resuspended in FWB/BB94 10µM at 4°C. The fixed cell pellet were resuspended in a further 4ml of FWB and re-centrifuged at 600g/4°C/5 minutes. Supernatants were discarded and resuspended and stored at 4°C prior to cells staining.

Lung cell suspensions were then stained with a variety of antibody panels to allow identification of constituent cells and their activation status, as listed in Table 2.7 below.

2.6.2 Flow cytometry data acquisition

Cell counts were determined by flow cytometry with the addition of microsphere counting beads (Caltag, Medsystems). Samples were acquired using a CyAn ADP Analyzer flow cytometer and Summit software (both supplied by Beckman Coulter, High Wycombe, UK). Data were subsequently analysed with FlowJo software (V.10.0.8, Ashland, OR, USA).

Channel	Leukocyte counts (fixed)	Leukocyte activation (unfixed)	Endothelium (fixed)
1. FITC	F4/80 (1/400)	F480 (1/400)	CD31 (1/200)
2. PE	±CD86 (1/200)	L-selectin/isotype (1/200)	IgM (Heparn Sulfate secondary 1/200)
3. PE TxRed	CD11b (1/400)	-	
4. PerCP	MHC II (1/400)	Gr1 (1/400)	CD45 (1/400)
5. Pe-Cy7	Ly6C (1/400)	NK1.1 (1/400)	
8. APC	Ly6G (1/2000)	-	
9. APC-Cy7 (Ax780)	CD11b (1/400)	CD11b (1/400)	

Table 2.7 Fluorophore conjugated anti-mouse antibody combinations

2.6.3 Intracellular phosphorylated p38/MAP Kinase quantification

To analyse intracellular p38/MAP kinase activation lungs were dissociated in 2ml BD cytofix/cytoperm at 37°C in an orange 'M' MACs tube and gentleMACs dissociator on the spleen_01.01 setting for 1 minute. A 10 minute further incubation was then completed at 37°C. Samples were then diluted up to 10ml with 'perm wash buffer' (PWB) containing PBS, 2% FCS, 0.2% saponin, 0.1% sodium azide, and filtered through a 40µm cell strainer and kept on ice. Cells were washed twice in PWB, centrifuging at 600g at 4°C for 5 minutes. Phospho-p38/MAPK antibodies phospho-p38, phospho-ERK1/2, phospho-MK2 and phospho-NF-κB (Cell Signalling, Danvers, MA, USA) were analysed in APC channel 8 (see Table 2.5).

2.7 Assays

2.7.1 RAW 264.7 cell assay

To provide an accurate, efficient and reproducible method of assessment contamination and intrinsic activation in our IPL system we developed a novel *in vitro* assay. RAW 264.7 murine macrophage blood cell line cells (ATCC, Virginia, USA), routinely cultured in DMEM/2mM glutamine/10% FCS in 5% CO₂ at 37°C, were seeded in 24 well plates (Nunc, Thermo Fisher Scientific, MA, USA) with DMEM complete media 24 hours prior to experimentation at a density of 2×10^5 /well. Perfusate samples taken from a recirculating test IPL circuit without lungs *in situ*, were diluted (1/2) in DMEM/10μM BB94 (metalloproteinase inhibitor) to minimise RAW cell TNF surface cleavage during the assay, to a final volume of 400μl and applied to the RAW cells for 90minutes at 37°C. RPMI-1640 perfusate served as a negative control and 100ng/ml lipopolysaccharide as a positive control. Following this samples were removed, cells were washed with PBS and incubated with cell dissociation buffer for 5minutes at room temperature before gentle removal with a cell scraper and place in flow cytometry tubes. Cells were then incubated in the dark at 4°C for 30minutes with PE-conjugated rat anti-mouse TNF or isotype control. They were then diluted with 'flow wash buffer' (FWB) and centrifuged at 600g at 4°C for 5minutes. Cells were resuspended and TNF expression quantified via flow cytometry.

2.7.2 Bradford protein assay

Bronchoalveolar lavage fluid protein was quantified with the Bradford method [199] utilising Coomassie Brilliant Blue G-250 dye. BAL samples were diluted to 1/10, 1/20, 1/40, 1/80 and 1/160 and 10μl of each was added in triplicate to a clear flat-bottomed 96 well polystyrene plate (Nunc, Thermo Fisher Scientific, Dublin, Ireland). Standards were also applied in triplicate at 10μl/well, diluted from stock human albumin solution. 200μl of Coomassie Brilliant Blue G-250 dye was added to each well and samples were gently agitated for 5minutes. They were then analysed using the MRX II absorbance plate reader and Revelation software (see Tables 2.3 and 2.4).

2.7.3 Soluble mediator quantification (ELISAs)

Several soluble mediators were quantified in both the perfusate and BAL samples, which were stored at -80°C in the interim. These included macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), soluble Receptor for Advanced Glycation Endproducts (sRAGE), monocyte chemotactic protein 1 (MCP-1), interleukin 6 (IL-6) and tumour necrosis factor α (TNF). Quantification was performed via sandwich DuoSet (R&D systems, Abingdon, UK) enzyme linked immunosorbent assays (ELISA). Capture antibody diluted in sterile PBS was applied to a 96 well MaxiSorp flat-bottomed polystyrene plate (Nunc, Thermo Fisher Scientific, Dublin, Ireland) at the concentration specified by the individual kits) and left at room temperature overnight. Unbound capture antibody washed in 0.05% Tween/PBS and removed from the plate with a MRW plate washer (Table 2.1), blotted and blocked with diluent (1% BSA/PBS) for 1 hour. Washing and blotting was repeated and standards and samples

were added in duplicate to the plate and left covered at room temperature for 2 hours. Plates were then washed/blotted again and biotinylated detection antibody (at the recommended concentration) was placed in each well for a further 2 hours. Plates were rewashed/blotted and streptavidin-HRP 1/200 was applied to each well for 20 minutes. Plates were washed/blotted one final time and 50µl of substrate solution (3, 3',5',5'-Tetramethylbenzidine, Sigma-Aldrich, St Louis MO, USA) was added to each well before protecting the plate from the light for 30 minutes at room temperature. Finally 'stop' solution (2N H₂SO₄) was added to each well and the plate was read at 450nm (with wavelength correction at 540nm) on an MRX II absorbance plate reader (Table 2.1) and data analysed using Revelation software (Table 2.2).

2.7.4 Permeability Dye Assessment

To assess lung permeability 100µl of Alexa Fluor 594 conjugated bovine albumin was added to the recirculating perfusate in the IPL reservoir, 30 minutes prior to the end of the protocol. Lungs underwent the final 5 minute non-recirculating washout period, as per the IR protocol, and lung was harvested as normal. Lungs were placed in an orange GentleMACS 'M' tube with 2ml of normal saline and dissociated with tissue destructive the RNA_01.01 setting on the gentleMACS machine (Table 2.1) for 60 seconds. 250µg of liberase (Table 2.3) was added and vortexed and the sample was incubated light-protected at 37°C for 30 minutes to ensure full degradation of connective tissue matrix. The 'M' tube was then dissociated a second time on the RNA_01.01 setting before the contents were centrifuged at 4000g for 5 minutes, room temperature. Lung supernatant samples were then applied in triplicate to a black 96 well flat-bottomed plate (Nunc Microwell Polypropylene) and constituent fluorescence quantified with the FLx 800 fluorescence microplate reader using KC4 software (Tables 2.1, 2.2). BAL and final perfusate sample supernatants were also analysed, alongside fresh perfusate, sodium chloride and neat dye for comparison.

2.8 Monocyte harvest and labelling

2.8.1 *Fresh blood harvest*

For adoptive transfer of murine monocytes into the IPL system, C57BL/6 mice received 50iu intravenous heparin and were terminally anaesthetised with an overdose of isoflurane anaesthesia. Following confirmation of death ~1ml of blood was taken via cardiac puncture.

2.8.2 *Histopaque separation and fluorescent labelling*

Mononuclear cells were isolated from the heparinised blood via density gradient separation whereby 3ml of murine Histopaque 1083 (Sigma Aldrich, St Louis MO, USA) was added to a 15ml Leucosep tube (Greiner Bio-One, Stonehouse, UK) and centrifuged for 30 second at 1000g at room temperature to relocate the medium below the porous barrier. Fresh whole blood (3-8ml) was then gently layered onto the membrane and centrifuged for 15 minutes at 800g at room temperature in a swinging bucket rotor (no brake). The enriched cell fraction was gently removed with a Pasteur pipette and diluted in 10ml of PBS and centrifuged again for 10 minutes at 250g. The resultant mononuclear cell pellet was then incubated with either CFSE (2 μ M 5mins at room temperature) or CD45.2-APC-Cy7 (4 μ g/ml, 5mins at room temperature). These cells were then injected into the isolated perfused lung, directly into the flushed pulmonary circulation.

2.8.3 *Magnetic bead separation*

Mouse monocytes were isolated using the negative selection magnetic bead EasySep Mouse Monocyte Enrichment Kit (StemCell Technologies, Grenoble, France). Whole blood was initially lysed in ammonium chloride 1:9 on ice for 15 minutes. This was then centrifuged at 300g at room temperature for 6 minutes and the supernatant discarded and pellet washed with EasySep buffer at 300g for 10 minutes. Supernatant was again discarded and the cells were resuspended in EasySep buffer supplemented with 5% rat serum at a concentration of $\leq 1 \times 10^8$ /ml and placed in 5ml round-bottomed polystyrene tube. Cells were then incubated with EasySep Mouse Monocyte Enrichment Cocktail 50 μ g/ml for 15 minutes at 4°C. The cells were then washed again with EasySep buffer at 300g for 10 minutes and resuspended at $\leq 1 \times 10^8$ /ml and incubated with EasySep Biotin Selection Cocktail at 60 μ l/ml at 4°C for 15 minutes. Following this EasySep magnetic particles were added to the solution at 150 μ l/ml and incubated for a further 10 minutes at 4°C. Finally the volume was increased to 2.5ml with EasySep buffer and the tube was placed within the magnet for 5 minutes at room temperature. The tube was then swiftly inverted leaving the unwanted labelled cells within the tube. Isolated monocytes were then quantified via the flow cytometry using the 'unfixed' antibody panel in Table 2.7 and counting beads, before being resuspended in 1ml final volume of our supplement RPMI-1640 perfusate and injected into the pulmonary artery cannula of the IPL adoptive transfer experiments.

2.8.4 BrdU Staining

We piloted *in vivo* labelling of recently divided bone marrow cells with the thymidine analogue bromodeoxyuridine (BrdU) as previously described, to allow assessment of monocyte trafficking [143]. Mice received 200µl (2mg) of intraperitoneal BrdU 24hours prior to the IR protocol. Following lung harvest a portion of lung was weighed and dissociated in an orange Miltenyi MACs 'M' tube with 1ml of BD CytoFix CytoPerm at room temperature on the spleen setting of the gentleMACs dissociator for 1 minute and then left to stand for a further 29 minutes. Cells were then washed through a 40µm cell strainer assisted by a 2ml syringe plunger and washed in 9mls of BD Perm/Wash buffer, centrifuging at 200g for 5 minutes at room temperature. After discarding the supernatant, the cells were resuspended in 100µl of BD CytoPerm Plus Buffer for 10 minutes on ice, washing them in the same fashion again thereafter. The cells were then re-fixed again by resuspending them in 100µl of BD Perm-Wash buffer for 5 minutes at room temperature and washed again. The supernatant was then discarded and the cells resuspended in 100µl of DNase (300µg/ml) to expose the incorporated BrdU for 1 hour at 37°C before washing again as above. Supernatant was discarded and cells were then resuspended in staining buffer and incubated with identifying antibodies including APC-antiBrdU clone 3D4 (Table 2.5).

2.9 Human Sample Collection

2.9.1 Protocols and Ethics

Human lung, blood and BAL samples were collected and stored in accordance with the protocol and ethics of the POPSTAR and EXTRALUNG lung transplant studies on-going at Royal Brompton & Harefield NHS Foundation Trust by Dr Rosalba Romano, in conjunction with the Harefield Hospital transplantation team

2.9.2 Lung Transportation and Storage

Lung samples were taken from standard and organ care system (OCS) transplants from the above studies. Following harvest standard transplants were flushed anterogradely with 4 litres Perfadex (Vitrolife, Sweden), and 1 litre retrogradely for donation after brain-stem death (DBD) donors. Donation after circulatory death donor lungs underwent 3 litres antegrade/2 litres retrograde flush. In both cases the perfusion pressures were maintained between 10-15mmHg. They were transported half-inflated (FiO₂ 0.5) on ice.

Organ Care System lungs were perfused with between 1.7-2l/min 'OCS' solution (including 2 units of O negative blood) and ventilated at 6ml/kg ideal body weight, PEEP 5-7cmH₂O, respiratory rate of 12 bpm, FiO₂ 0.21. These parameters varied slightly depending on whether they were in the 'preservation' or 'monitoring' mode.

2.9.3 Sample Collection

Lung

A pre-implantation lung biopsy was taken from the right lower lobe by a Covidien GIA stapler 80 mm (Medtronic, Watford, UK). This was followed by a second biopsy from the same site, after reperfusion, before the closure of the chest. Each biopsy measured approximately 3cm x 1cm x 1cm. Biopsies were stored in a humidified sterile container on ice during transport and on arrival subdivided and weighed. The bulk of the sample was then manually minced and subjected to enzymatic disaggregation with DNase (1/80) and Liberase (1/25) at 37°C for 20 minutes, agitated in a vertically rotating device. Samples were then diluted up to 6ml in FWB and passed through a 40µm cell strainer with use of a 2ml syringe plunger. They were washed for 7 minutes at 4°C at 300g, supernatant removed and the wash repeated. Cells were resuspended and stained with the antibody combinations listed in Table 2.8 for 30 minutes in the dark at 4°C. Supernatants were again discarded and cell pellets vortexed whilst 500µl of BD cytofix/cytoperm was added and then left to incubate for 5 minutes at room temperature. Cells were then washed twice with PWB to assist membrane permeabilisation and cells were resuspended in PWB and incubated with FITC-27E10 for 15 minutes at 4°C in the dark. They cells were then washed and resuspended one final time before counting beads were added and flow cytometry analysis commenced.

BAL

Bronchoalveolar lavage was performed via fibre-optic bronchoscopy after reperfusion and chest closure and/or on reaching intensive care with ~30mls of normal saline (0.9%) and passed through a gauze filter and centrifuged at

300g for 10minutes at 4°C. The supernatant was stored at -80°C for subsequent analysis and the cell pellet was stored on ice until being stained for flow cytometry analysis as per the lung samples.

Blood

Blood was collected in sodium heparin bottles and samples were stained as per the lung samples above.

All the human sample processing and analysis was done jointly with Dr Rosalba Romano.

2.9.4 Human Antibody Panel

Based on previous work by our group and others we developed an antibody panel that would enable optimal identification of the monocyte subsets in question with maximal data output from limited tissue biopsies.

Various cell markers were quantified in the APC channel including TREM-1, CD86, CCR2 and HLA-DR.

Table 2.8 Antibody panel for identification of lung constituent leukocytes

	FITC	PE	PE Texas Red	PerCP	PeCy7	APC	Ax780
Antibody:	27E10*	CD66b	CD11B	CD14	CD16	<i>Functional markers</i>	CD45

* applied after permeabilisation

2.10 Statistical Analysis

2.10.1 Statistical tests

All data underwent assessment of normality with Shapiro Wilk and Levene's homogeneity of variance tests. Parametric data was expressed as mean± standard deviation and analysed for significance using T-test or ANOVA with Bonferroni correction. Non-parametric data were expressed as median (interquartile range) and analysed via Mann-Whitney U or Kruskal-Wallis with Dunns correction. Clinical data in Chapter 6 was assessed using linear correlations. For all tests a p value of less than 0.05 was considered statistically significant.

2.10.2 Statistical software

Data were analysed and graphed using SPSS (IBM, version 20) and GraphPad Prism (version 6, La Jolla, CA, USA) software.

Chapter 3

Development and Optimisation
of the Isolated Perfused Lung Model of
Ischaemia-Reperfusion Injury

3.1 Abstract

Introduction: The murine isolated perfused lung is a well-established tool for investigating multiple lung pathologies. It especially lends itself to the analysis of lung ischaemia-reperfusion as multiple physiological parameters can be altered to reproduce the disease model of choice. After successful use of murine IPL in our group for studies of ventilator-induced lung injury, atelectatrauma and intratracheal inflammation models, we developed a protocol to investigate the pathophysiology of lung ischaemia-reperfusion in more detail. This included characterisation of the perfusate volumes, optimal sampling modifications, perfusate content optimisation and stringent system decontamination development.

Methods: To develop a reproducible model we piloted several combinations of ischaemia-reperfusion with varied time scales, gas constituents, flow rates and lipopolysaccharide priming. A combination of BAL protein and lung dye permeability were utilised to guide the degree of lung 'injury'.

To characterise the perfusate sampling and thus maximise yield, we undertook washout kinetics of the IPL system using red blood cell washout (post surgery and post ischaemia) and dye washout (post perfusion) techniques.

To identify microbial contamination in our system we quantified membrane TNF expression via flow cytometry in the RAW 264.7 mouse monocyte/macrophage cell line following exposure to IPL system perfusate samples.

Results: Pilot experiments altering the nitrogen concentration in the inhaled gas and perfusate flow rate appeared to significantly influence the lung injury seen. In addition long ischaemia and perfusion times also increased injury parameters. To combine maximal exposure to IR, without inducing excessive lung injury, a 2 hour ischaemia – 2 hour perfusion protocol was settled upon which produced reproducible injury, susceptible to LPS priming augmentation.

Use of a RAW 264.7 cell membrane TNF assay provided a sensitive tool to assess system contamination, including that derived from commonly used buffer constituent 'low-endotoxin' bovine serum albumin. Moreover, adoption of an improved cleaning regimen and addition of antimicrobials to the RPMI-1640 based perfusate contributed to maintaining a contamination-free system.

Conclusion: We successfully modified the IPL system to create a sensitive and reproducible sterile IR injury model. We were able to eliminate the confounding effect of background inflammation derived from exogenous sources and thus allowed detection of the effects of *in vivo* LPS inflammatory priming'

3.2 Background

3.2.1 Modelling ischaemia-reperfusion

Models of lung ischaemia-reperfusion (IR) have been under investigation for many years, following the increasing success of lung transplantation in the latter half of the last century. Several models of lung IR have since been developed which address various aspects of lung ischaemia-reperfusion. As outlined in the Chapter 1, ischaemia, or restriction of blood flow in solid organs, results in lack of oxygen supply. However the multiple oxygen sources of the lung, (pulmonary, bronchial circulations and direct alveolar diffusion) requires more than just cessation of pulmonary blood flow to induce hypoxia. 'Stop-flow' ischaemia in this case will only induce shear stress cessation, reduction in nutrient provision and subsequent build up of metabolites secondary to interrupted blood flow

Clinically, lung-ischaemia reperfusion injury occurs in response to several distinct insults. Iatrogenic IR occurs secondary to cardiopulmonary bypass, inducing cessation of pulmonary flow and halting of ventilation during cardiac surgery. In this case the bronchial circulation is maintained. In contrast, one-lung ventilation, commonly used during intra-thoracic and oesophageal surgery, entails preferential deflation of one lung by the anaesthetist, whilst pulmonary and bronchial perfusion of the collapsed lung is on-going. This frequently results in a significant shunt fraction from the large proportion of deoxygenated pulmonary blood passing through the lungs without being oxygenated. In contrast, global reductions in blood flow, with or without interruption in alveolar ventilation can occur secondary to low cardiac, hypoperfusion and peri-arrest states. The most severe form of ischaemia and reperfusion a lung undergoes is during lung transplantation where lungs are subjected to extensive periods of both normothermic hypoperfusion (in peri-arrest or donors undergoing cardiopulmonary resuscitation) and to cold ischaemic storage during transportation. Of note, harvested lungs are transported inflated with oxygen and thus the alveolar supply remains intact and are therefore only subjected to 'stop-flow' ischaemia.

Current models of IR vary. *In vitro* models are favoured for the ability to easily alter atmospheric gas constituents and substrate provision, however single cell cultures provide limited insight into whole organ pathophysiology. One frequently used animal model is the substitution of alveolar oxygen supply with nitrogen. Whilst this clearly eliminates the alveolar oxygen competent, inducing a subsequent reduction in systemic and pulmonary partial pressure, it only provides a model alveolar hypoxia/reoxygenation and not necessarily lung IR *per se*. However, despite the generally accepted inertness of inhaled nitrogen, it is unclear what the physiological impact of it might have in this model. Hilar clamping *in vivo* represents a more clinically relevant model although renders the entire lung without an oxygen supply, which is likely to be only of relevance to hypoperfusion and hypoventilation (peri-arrest) states. Orthotopic transplants have been undertaken for some time in large mammals and are likely to be the most representative model of lung transplantation, but are expensive. More recently however technical advances have allowed orthotopic transplantation to be performed in small mammals including mice, which when combined transgenic strains allows for greater investigative power.

3.2.2 The isolated perfused lung (IPL)

The isolated perfusion of organs was first described in 1903 [200] but isolated perfusion of (canine) lungs was not described until 1949 [39]. The combined IPL benefits of assessing a whole organ system without systemic influence are clearly advantageous [201]. Furthermore the ability to individually manipulate the pulmonary circulation and alveolar ventilation, lends itself to a broad range of experimental questions. The murine IPL is now widely reported for all aspects of investigating acute and chronic lung pathophysiologies. Examples include investigation of ventilator-induced lung injury, atelectrauma, pneumonia, sepsis, aspiration and pulmonary hypertension [139, 143, 202]. It has also been integral to the recent advances in intravital lung fluorescent microscopy [203]. The ability to temporarily halt ventilation in a lung, maintained in homeostasis with perfusion and continuous positive airway pressure, has allowed this imaging modality to advance considerably the knowledge of cell-cell interactions in the small capillaries and alveoli of the lung.

IPL is of particular use when investigating IR. It allows manipulation of ventilator gases and well as the ability to start and stop pulmonary blood flow, depending on the specific 'ischaemic' clinical scenario you intend to model. Several groups have utilised it for this purpose, ranging from investigating the role of neutrophils in rabbit lung IR, the role of ROS and NADPH in rat lung IR, alveolar macrophages depletion in murine IR and the effects of IR on pulmonary vascular resistance and permeability in a canine IPL [40-42]. It is for that reason that we selected this system to investigate the role of monocytes in ischaemia-reperfusion injury.

3.2.3 Modelling donor lung inflammation

To model the influence of pre-existing donor lung inflammation on IR-induced lung injury, and specifically the role of monocytes, we incorporated a 'priming' low dose of intravenous lipopolysaccharide 2hours prior to our IR protocol. Previous work in our group had shown that endotoxaemia induced by an intravenous low 20ng dose of lipopolysaccharide (LPS) achieved maximal Gr1^{High} monocytes recruitment (20x increase) to the lungs without producing symptoms or signs of sepsis, whilst priming the lungs to subsequent injury [143]. This will be discussed in more detail in Chapter 4 but was also utilised as part of the development process.

3.3 Aims

Our aims were to:

- ⇒ Characterise the IPL system
- ⇒ To reduced activation and contamination
- ⇒ Develop protocols to produce a reproducible model of transplanted-associated acute lung injury, whereby the roles of ischaemia-reperfusion and subclinical inflammation can be separated

3.4 Methodology

3.4.1 *The isolated perfused lung*

The murine *ex vivo* isolated perfused lung (IPL) system consists of three principal components: the perfusion, respiratory and thermostatic circuits. The perfusion circuit (blue lines) consists of a 4-lane peristaltic roller pump, heat exchanger, bubble trap, pulmonary artery cannula, left atrial/ventricular drainage cannula, reservoir and connecting tubing (Figure 3.1). Two transducers monitor the perfusion pressure, one immediately prior to the PA cannula and one immediately after the LA cannula. The respiratory circuit (green arrows) consists of a custom-made mouse ventilator and a humidifier, with a pneumotachometer and pressure transducer to measure airflow and airway pressure, respectively. The thermostatic circuit (red box) circulates water at 37°C through a plexiglass chamber and through a heat exchanger warming the perfusate entering the lung. Warmed water flowing through the chamber lid conserves humidity in the central lung chamber. A detailed diagram of the circuits overlaid on a photograph of the IPL can be found in figure 3.2 below.

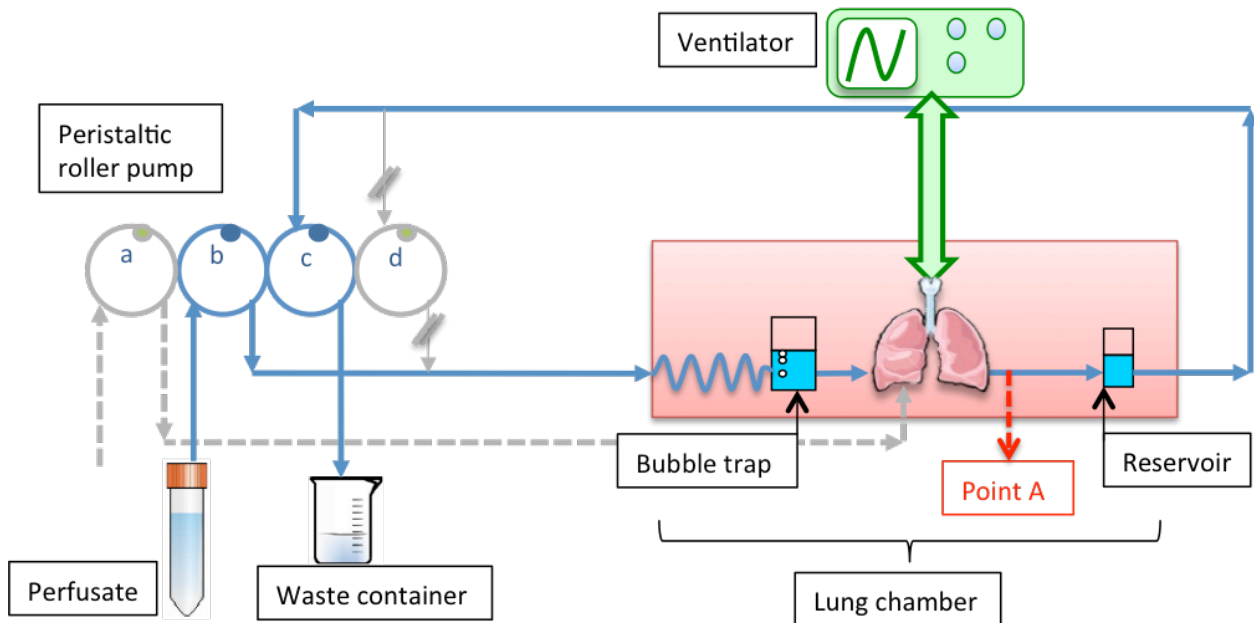


Figure 3.1 Schematic illustration of the IPL system, in the 'open' non-recirculating setting.

3.4.2 Placing murine lungs on the IPL

Lungs were mounted on the IPL as described in detail in Chapter 2. In brief mice were heparinised intravenously and then anaesthetised with intraperitoneal ketamine (130g/kg) and xylaxine (13mg/kg). Tracheostomy was performed once under general anaesthesia and ventilation was commenced (80bpm, tidal volume 6-7ml/kg, PEEP of 5cmH₂O, FiO₂ 0.21%). The mouse was then exsanguinated and ventilation switched to 5cmH₂O CPAP (5% CO₂, 21% O₂). A thoracotomy was performed and the pulmonary artery (PA) and left atrial (LA) were both cannulated. Lung perfusion was commenced in a non-recirculating fashion at 0.2ml/min for 1 min, increasing to 0.4 (regardless of body weight) and then increased to 25-50ml/kg/min at 3minutes and thereafter depending on the protocol requirements.

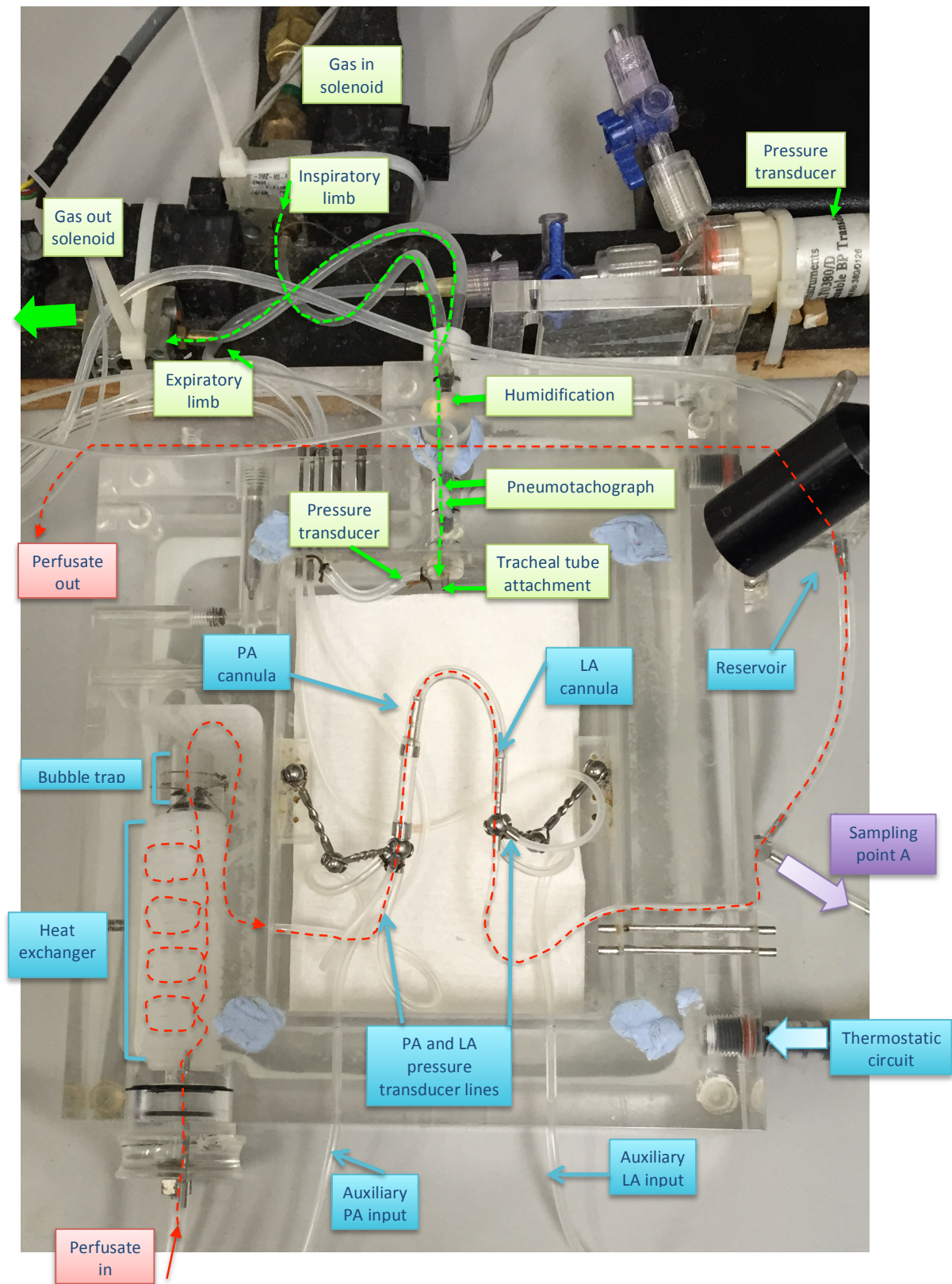


Figure 3.2 Detailed diagram overlaid on a photograph of the IPL

3.4.3 Inducing ischaemia and reperfusion

Following the initial flush period, perfused was halted and ischaemia induced. During this period CPAP of 5cmH₂O was applied to the lungs and LA pressure was maintained at 2.5mmHg by altering the height of the reservoir. After ischaemia, reperfusion was commenced, initially in a non-recirculating fashion to washout and collection of the post-ischaemic cells and mediators. Reperfusion was commenced at the same incremental rate as above for a total flush period of 5 minutes, with ventilation recommencing at 3 minutes with 2 initial inflation breaths to 25cmH₂O. The circuit was then switched to the recirculating pump for the subsequent reperfusion and ventilation period (see Figure 3.3, reproduced from Chapter 2). At 15minute intervals the mechanics of the respiratory system were measured using an end-inspiratory pause technique and 25cmH₂O deep inflation was applied for 5 seconds to recruit the lung. These readings remained within the normal range in all the IR protocols in this study.

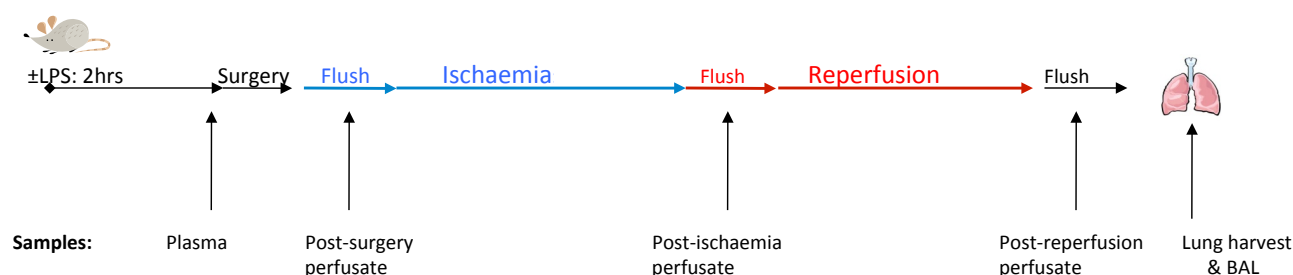


Figure 3.3 IPL ischaemia-reperfusion model protocol

3.4.4 Perfusate

The non-blood physiological buffer RPMI-1640 without phenol red was used as previously described [196, 197]. Clinical grade human albumin solution was added to a final concentration of 4% to maintain oncotic pressure and sterile sodium chloride was added to increase the osmolality to ~330mosmol/kg which is the normal value for mice. HAS was used in place of the more conventional bovine serum albumin (BSA) for reasons explained in the results below. We opted to use a complete culture media for the ischaemia period as it was (a) a generic representation of the perfusates used to flush and preserve human lungs and (b) because we wanted to measure the direct response to 'stop-flow' IR, as opposed to manipulation of physiological parameters (e.g. glucose, pH, osmolality). This also prevented the exhaustion of metabolites during ischaemia at 37°C, which is unlikely to occur during organ preparation with current perfusate formulations.

3.4.5 Perfusate, BAL fluid and lung collection

At the end of the reperfusion period, 1.5ml of perfusate was sampled immediately from point A. Then perfusion and ventilation were ceased and the left lung tied off (3/0 silk) for subsequent wet:dry weight analysis, preventing entry of BAL fluid. The tracheal tube was then disconnected from the ventilator and 0.5ml of sterile NaCl (0.9%) was gently lavaged into the remaining lung lobes 3 times, and removed. The supernatant then underwent BAL protein analysis using the Bradford method (Chapter 2) and the remainder was stored at -20°C for ELISA analysis. All the lung lobes were then carefully excised. The left lobe was immediately weighed and placed in a 60°C oven for future dry weight analysis. The remaining lobes were split into two, homogenised (with or without paraformaldehyde fixation) and used for flow cytometry analysis of the cellular constituents (see below).

3.4.6 Flow cytometric analysis of lung homogenate

On harvest lung tissue was weighed and homogenised with a gentleMACs dissociator in either in paraformaldehyde IC fixation buffer or flow wash buffer (+ 10µM BB94) at 4°C. Homogenised lungs were then flushed through a 40µm nylon sieve with FWB and were centrifuged at 600g, 4°C for 5 minutes. The supernatant was discarded and this last step repeated. Fixed cells (to assess leukocyte numbers and endothelial activation) were resuspended in FWB. Unfixed samples (to assess leukocyte activation, as described in Chapter 2) were resuspended in FWB 10µM BB94 to prevent L-selectin cleavage, and both sets were kept on ice at all times. Lungs cell suspensions were then stained in the dark at 4°C for 30 minutes with a variety of fluorophore conjugated anti-mouse antibodies and isotype-matched controls to enable constituent leukocyte identification (as described in Chapter 2). Cell counts were calculated via flow cytometry with the assistance of microsphere beads and data were acquired using a Beckman Coulter Cyan flow cytometer and Summit software. The data were analysed with FlowJo software.

3.4.7 RAW 264.7 assay

To assess microbial contamination within the isolated perfused lung system tubing we developed a sensitive *in vitro* assay to assess the pro-inflammatory activity of perfusate samples from our IPL system. Complete media or perfusate that had passed through the system were sampled at point A (Figure 3.1) and incubated for 90 minutes at 37°C with murine macrophage RAW 264.7 cells. BB94, a metalloprotease inhibitor, was added to the perfusate to maximise membrane TNF signal (memTNF) through inhibition of its proteolytic cleavage from the cell surface [137]. Cells were then stained with PE-conjugated rat anti-mouse TNF or isotype control, and TNF expression was quantified via flow cytometry.

3.4.8 Endotoxaemia

20ng (in 200µl PBS) of LPS (Ultra Pure *Escherichia coli* 0111:B4) was injected via the tail vein 2 hours prior to pilot IR IPL protocols.

3.5 Results

3.5.1 Optimisation of the IPL protocol

Previously, the IPL model had been developed in our laboratory to investigate different aspects of mechanical lung injury. To develop a new IR-based injury model, we decided to carefully review the IPL methodology and optimise or redesign where appropriate. A number of IR pilot experiments had been performed already in the group based largely on pre-existing methods described in the literature, and these served as a reference point for the protocol design and optimisation. The protocol based on these pilots consisted of surgical setup (~20min) followed by the flushing of the pulmonary circulation (5min) with perfusate buffer to remove blood components (including non-adhered leukocytes), a no-flow ischaemia step and then reperfusion by recirculating 'closed circuit' perfusion.

To develop a suitable protocol, which displayed a baseline mild-moderate IR injury, and could be augmented by priming with low dose lipopolysaccharide, we trialled a number of protocol variations, as shown in Figure 3.4 below. We selected BAL protein as the key injury endpoint to help determine the response of the lungs to varied IR insults.

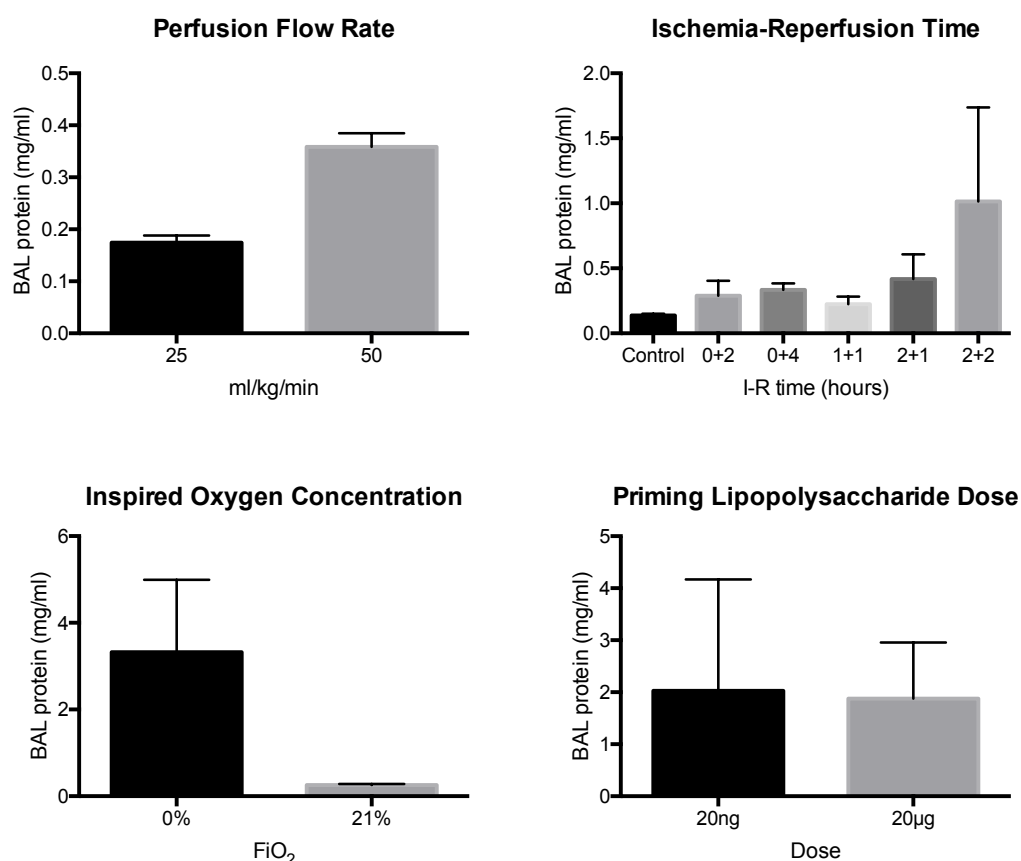


Figure 3.4 Assessment of IPL variables

A series of pilot experiments were carried out to assess (A) the optimal flow rates, (B) ischaemia-reperfusion times, (C) oxygen levels and (D) LPS priming dose; n=2-5

As a result of these pilot experiments, we opted for the following parameters: 2 hours of ischaemia + 2 hours of reperfusion, washout perfusion rate of 25ml/kg/min, reperfusion rate of 40ml/kg/min, gas content of 21% O₂/ 5% CO₂, $\pm$ 20ng of LPS priming and a temperature of 37°C for the reasons listed that follow. Crucially this combination produced a reproducible low-grade lung injury, which is studied in greater detail in Chapter 4.

Ischaemia/reperfusion times and flow rates

Ischaemia time of 2 hours enabled a moderate injury to occur at 37°C (see '*Temperature*' below). A perfusion time of 2 hours was selected to study lung injury in the acute perfusion period, while avoiding the risks of long-term perfusion artefacts and noise intrinsic to the IPL system. In addition, owing to the increase in permeability seen with higher perfusion rates, but the desire to avoid the complications of extended perfusion periods at slower rates, we opted for an initial gentler flush-out rate of 25ml/kg/min for the 5 minute washout periods immediately following the post-surgical and ischaemic periods. A final rate of 40ml/kg/min was ultimately utilised for the 2 hour 'reperfusion' rate, to minimise any direct injury from the higher 50ml/kg/min perfusion rate.

Gas content

Owing to the significant effects of 95% nitrogen in our system, and the fact that human lung grafts are transported inflated with oxygen during ischaemia, we opted for 21% O₂/74% N₂/5% CO₂ gas mixture. The use of 95% O₂ /5% CO₂ was avoided due to concerns over the potential effects of oxygen toxicity as previously reported [204, 205].

LPS priming

To induce adequate lung 'priming' (and monocyte margination), without evidence of systemic side effects [143], we opted for the lowest possible dose of iv LPS (20ng), 2hours prior to the IPL protocol, to augment IR injury.

Temperature

IR models previously reported in the literature had suggested that excessive ischaemic times, exceeding those seen clinically at 4°C, were required to model the IR injury observed in human lungs. In fact they demonstrated that a short warm ischaemia can accurately replicate an extended cold ischaemia in a porcine model of IR [35]. Moreover, warm ischaemia occurs clinically during the hypoperfusion states, which many donors are prone to prior to lung harvest. Thus, based on pilot work, a warm ischaemia period of 2 hours was selected, representing the normothermic ischemia during lung graft preparation and an optimal period to ensure a reproducible injury within the constraints of an *ex vivo* organ preparation system.

3.5.2 Perfusate Sampling

For reliable assessment of soluble mediators (e.g. chemokine and cytokine) production within the pulmonary vasculature, the sample volume and collection point in the IPL circuit are crucial variables. Standardised sampling protocols are not evident in the literature, restricting cross-study comparisons. There are two distinct sampling options: 1) washout and collect the total perfusate volume in the circuit and lung, enabling the estimation of the total amount of soluble mediator production or, 2) sample a relatively small, fixed volume to obtain an estimate of mediator concentration in the lung vasculature only. We considered the latter a more pertinent and sensitive indicator of the pulmonary vascular content, particularly following the surgical setup and ischaemia steps. To achieve this aim, it was first necessary to modify the IPL apparatus to perform sampling as near to the left atrial exit as possible to collect minimally diluted pulmonary vasculature effluent. Then, to define appropriate sampling volumes, our approach was based on the use of tracer (cells or dye) with reference to internal tubing dimensions and volumes.

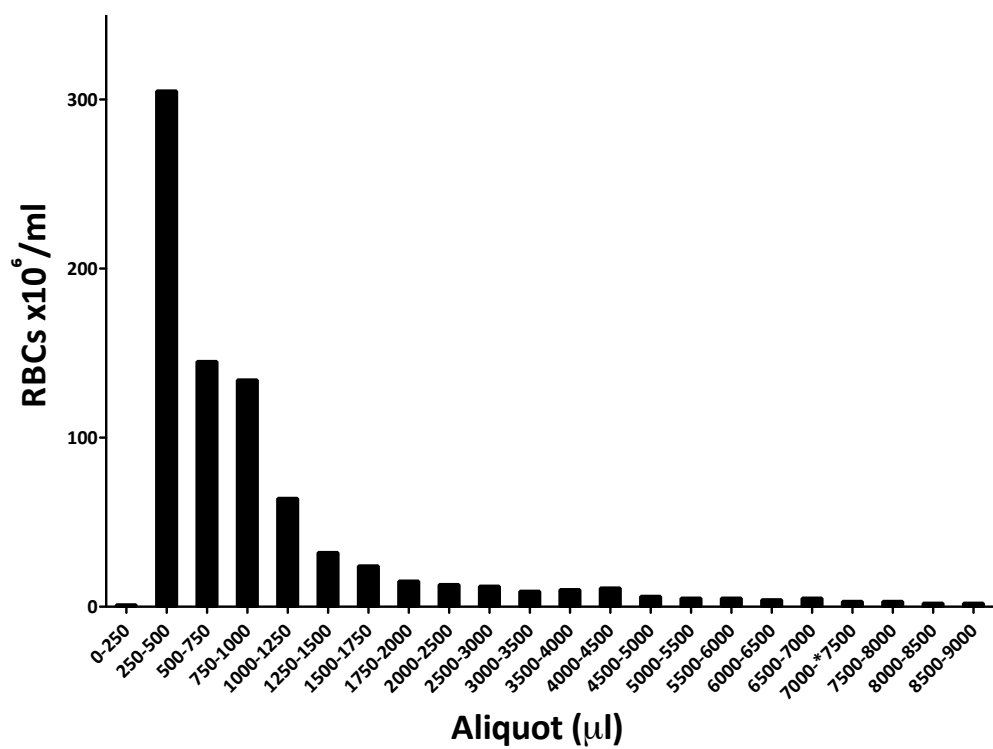
Sampling port

The first step was to therefore modify the sampling port, to be as close to the left atrium as possible, from the previously used reservoir to a newly inserted point A (see Figure 3.1). The minimal dead space volume (between the LA and the port) was calculated from the tubing dimensions and verified from washout results below.

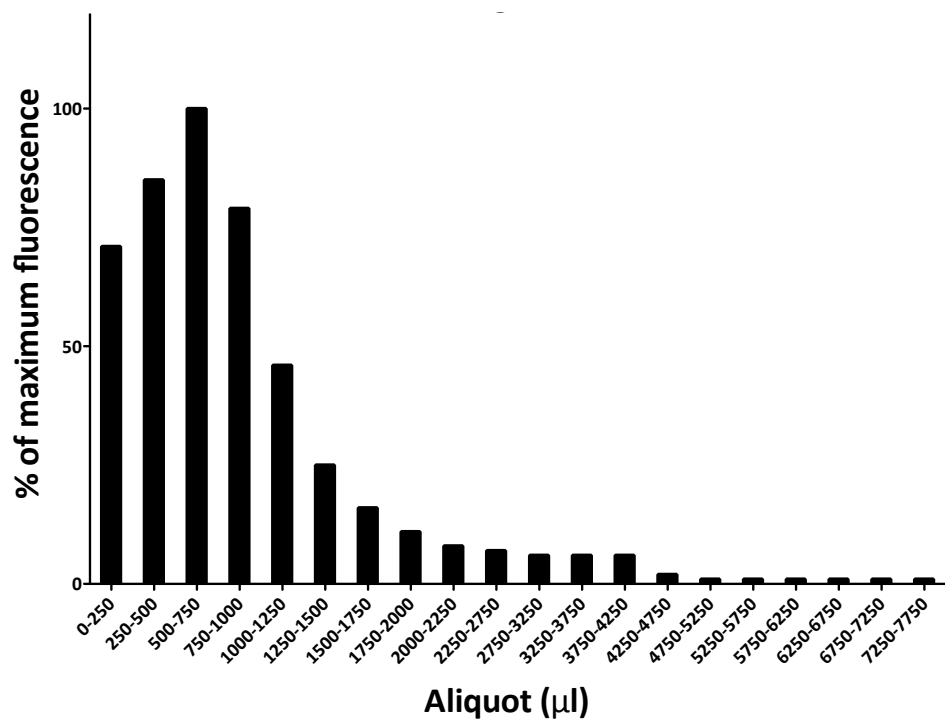
Post-surgical sampling measuring erythrocyte outflow and post-ischaemia dye sampling

The method for evaluation of perfusate sampling volumes was tailored to the relevant step in the IR IPL protocol, based on sampling from the lungs only (post-surgery and post-ischaemia) or from the lungs *and* perfusion circuit after perfusate recirculation (post-reperfusion). Sampling post-surgery and post-ischaemia involves collecting the small volume within the lungs, while discarding the initial tubing dead space perfusate (from the LA to point A) and minimising dilution from the fresh perfusate in the proximal tubing.

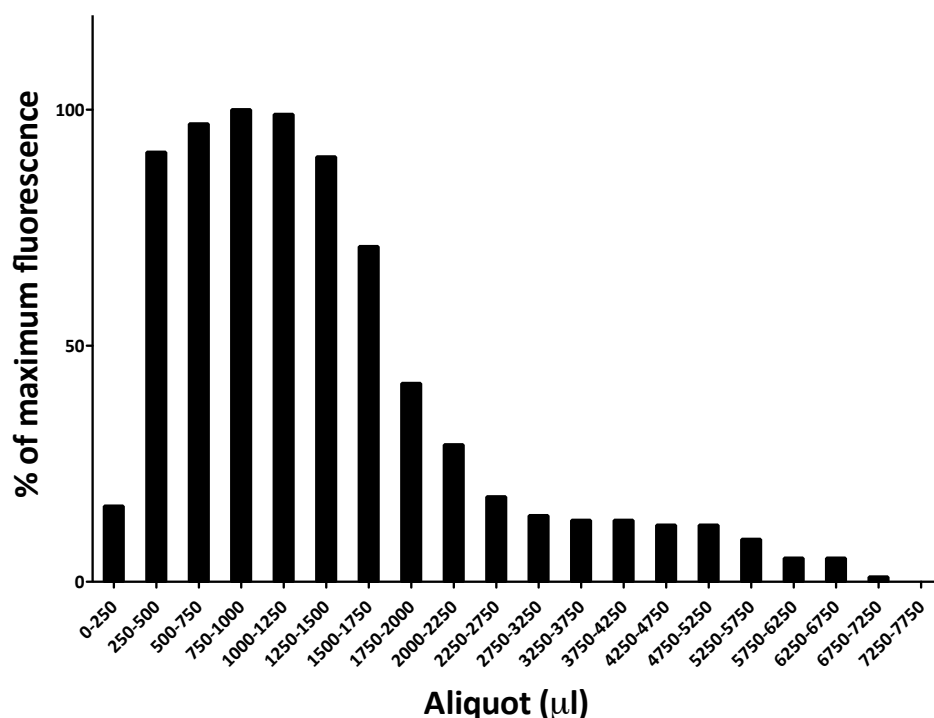
For evaluation of post-surgery sampling, red blood cells already present in the pulmonary vasculature were used as a convenient cellular tracer. As an estimate of dead space, the initial volume of clear, blood-free perfusate was found to be 250µl, consistent with a calculated dead space volume based on tubing diameter and length. On this basis sequential 250µl volumes, and then 500µl, aliquots of perfusate were collected from port A, as shown in Figure 3.5. Flow cytometry was used to quantify red blood cells and construct a blood washout curve. This confirmed that the first 250µl represented 'clean' perfusate with virtually no cells present, but subsequently ~90% of cells collected were washed out in the first 2mls of sampling, half of these being collected in the 2nd 250µl aliquot. It also indicated that after washout of ~5mls volume, <5% of total red cells remained.



A: Post surgery red blood cell washout



B: Post ischaemia dye washout



C: Post reperfusion dye washout

Figure 3.5 Red cell and dye washout dynamics of the IPL system

A series of IPL washout experiments were performed to characterise the features of (A) the post surgery washout period of red blood cells, (B) the post ischaemia washout of soluble mediators/cells from the lung with dye and (C) the washout of soluble mediators/cells from the lungs and circuit post reperfusion, also with dye.

For the post-ischaemia sampling it was necessary to directly infuse a bolus (200μl) of a fluorescent tracer, albumin-Alexa-fluor 594nm, directly into the pulmonary artery (PA) side of the circulation. Sequential 250μl aliquots were again taken from point A and fluorescence measured immediately afterwards (Figure 3.5B). This produced a similar curve to the red cell washout (Figure 3.5A), with an initial dead space volume of ~250μl and ~90% of fluorescent dye collected within the first 2ml (Figure 3.5B). On the basis of these results we standardised post-surgery and post-ischaemia sampling by discarding the initial 250μl and collecting the next 1.5ml volume containing the majority of the lung vascular fluid contents. This was verified by performing this method on two subsequent occasions with minimal variation.

Post-reperfusion sampling

To assess the optimal sampling technique post reperfusion (i.e. 2 hours of perfusate recirculation) we again employed the fluorescent dye technique. This stage differs as any cells or soluble factors are distributed throughout the IPL circuit. Therefore on this occasion the dye was introduced to the reservoir and allowed to circulate and equilibrate for 5 minutes. The circuit was then opened to wash through with fresh perfusate, which did not contain

dye. Perfusate aliquots (250µl) were then collected from point A as before. As expected, dye was present in first aliquot, but as before ~90% was removed from the system within the first 2mls. Therefore, we were able to perform the same sampling procedure as used for the post-surgery and post-ischaemia, bar discarding the initial 250µl fraction, and instead simply collecting the first 1.5ml.

3.5.3 Minimisation of baseline IPL inflammation

The IPL may be affected by inflammation due to the procedure itself and exogenous sources of (i.e. microbial contamination), which can only be defined by elimination of the latter. To measure and reduce this potential system 'noise', we established an assay system to detect and minimise external sources of inflammation, allowing us to properly assess IR-induced inflammation and the impact that pre-existing sub-acute endotoxaemia may have on this response.

We developed a rapid and sensitive *in vitro* bioassay to perform same-day monitoring of inflammation-inducing activity in the IPL fluidics. Confluent RAW 264.7 monocyte/macrophage cells were incubated for 90minutes with samples from different stages of the IPL protocol. Expression of cell-associated membrane TNF was determined after a short antibody staining step by flow cytometry. The metalloprotease inhibitor, BB94 (10µM), was used throughout to prevent TNF release from the cell membrane and thereby amplify its signal, as described previously [137].

Despite a daily apparatus cleaning procedure using a high quality detergent (Mucosol®) and polymyxin B (endotoxin neutralising antibiotic) after each run, we detected a relatively potent activity in the RAW-TNF bioassay as illustrated in Figure 3.6A. This activity was produced even when the system was run for several hours without lungs in situ (instead with simple 'bypass' tubing their place). As this activity was present in the absence of lungs it seemed likely that this represented contamination of the system. Prolonged overnight soaking of the circuit with polymyxin B (PMB) reduced but did not eliminate TNF inducing activity. Only by replacing all the system tubing could the background signal be reduced substantially (Figure 3.6B).

We considered the most likely source of contamination arose during the experimental period, where the IPL system is maintained at 37°C throughout and for a large part in a nutrient rich medium (RPMI-1640). We therefore introduced the antibiotics penicillin and streptomycin into the perfusate buffer, to minimise microbial build up during and between runs. Routine monitoring of samples (pre- and post-surgical) using the RAW-TNF bioassay indicated that inclusion of antibiotics following tube replacement prevented circuit contamination. However, periodic full replacement of system tubing was performed as a precautionary measure (Figure 3.6B).

Even with the use of fresh circuit tubing, we still observed RAW cell stimulatory effects in system perfusates, albeit at very low levels, when comparing TNF expression to control RPMI-1640 incubations. Closer examination of the stock perfusate buffer (RPMI-1640 + low endotoxin bovine serum albumin BSA) indicated that this also contained

TNF inducing activity. Despite the widespread use of this buffer in the IPL model of IR, we found that TNF-inducing activity emanated from the low-endotoxin BSA (Figure 3.6C). The importance of using low endotoxin BSA, instead of normal grade BSA, has been recognised to minimise constitutive inflammatory cytokine from the system [197] (although its use has not been adopted by all groups). However, our data indicate that commercially-available low endotoxin BSA is likely to not be completely free from LPS or other TNF-inducing substances. We therefore changed our source of albumin to clinical grade human albumin solution (HAS), which appeared to ameliorate this response.

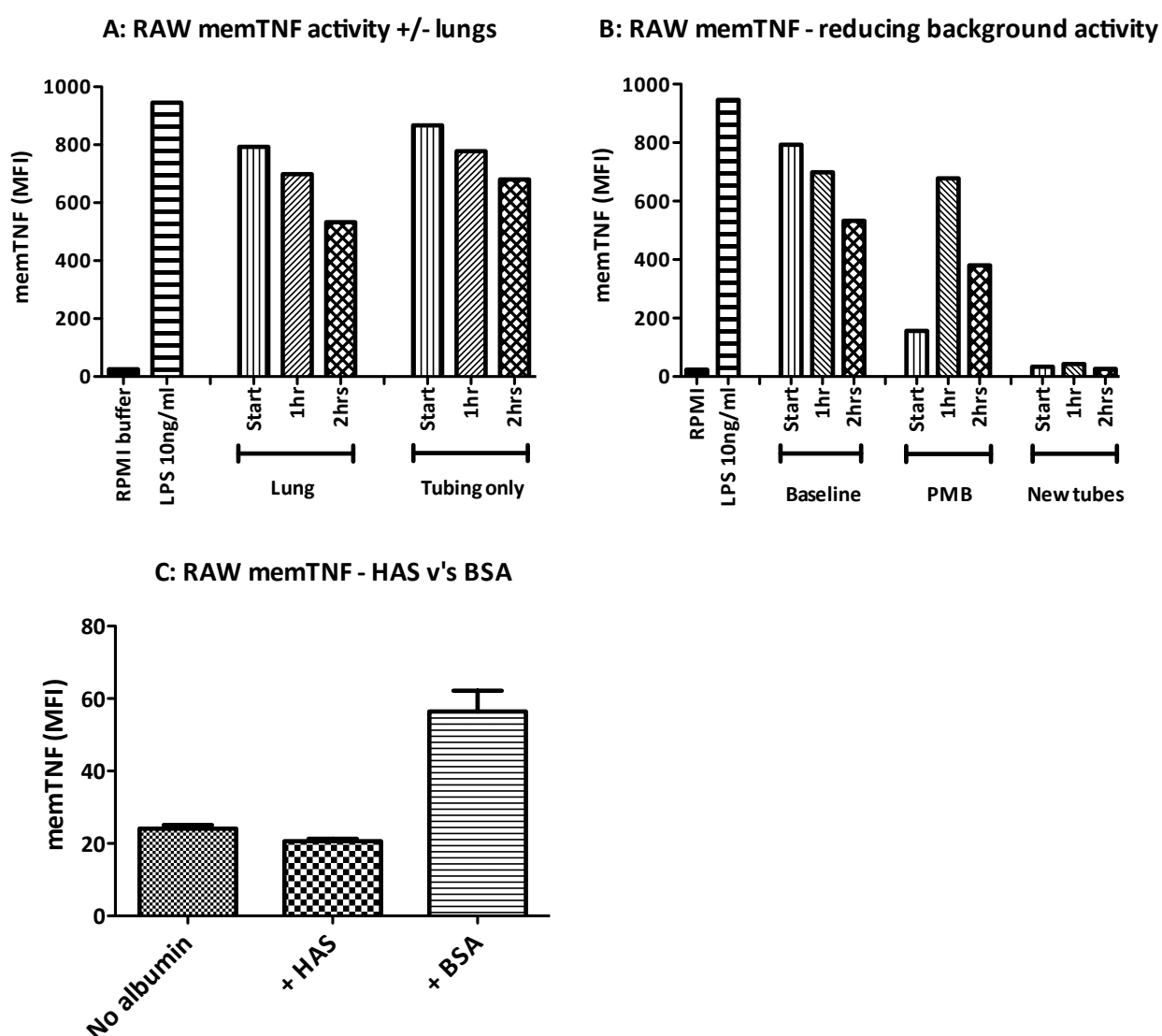


Figure 3.6 'RAW' Assay assessment of IPL contamination

memTNF expression by RAW cells was quantified after 90minute co-incubation with perfusate samples from the start and at 1 and 2hrs time points of baseline perfusion experiments. (A) High levels of memTNF were noted with or without mouse lung present. (B) Prolonged treatment with PMB reduced but did not eliminate this activity but was substantially reduced by replacement of the tubing. (C) RPMI-1640 buffer supplemented with BSA induced background memTNF expression which was reduced by replacement with HAS. Mean±SD, n= 6-8

3.6 The final IR protocol

Based on the pilot work and redesign undertaken above a final protocol was established.

The key features of this included:

1. A 'clean' system incorporating antibiotics in the perfusate, regular tubing changes and stringent cleaning protocol
2. 5 minute 'open' (non-recirculating) pulmonary washout period immediately post thoracotomy, post ischaemia and post perfusion periods, i.e. at time 0hrs, 2hrs and 4hrs
3. 2 hour ischaemia ('stop-flow') at 37°C, CPAP of 5cm H₂O Air/5% CO₂ applied via tracheostomy
4. 2 hour recirculating pulmonary perfusion ('reperfusion') period with 6ml/kg ventilation at 80 breaths/minute, PEEP 5cmH₂O

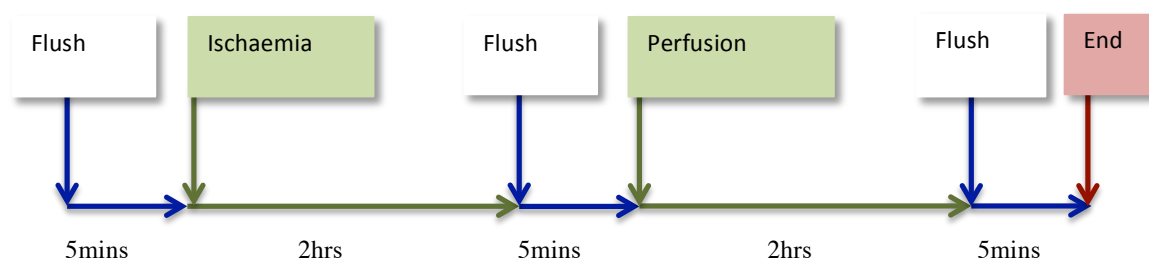


Figure 3.7 The final ischaemia-reperfusion protocol

3.7 Discussion

3.7.1 *The 'pros and cons' of IPL*

Isolated organ systems are a unique and powerful method of experimentation. They especially lend themselves to study of the primary function of the lungs with the ability to modulate and monitor ventilatory parameters and inhaled gas composition [206]. In addition, modulation of the pulmonary vasculature is also permitted and with it the ability to alter flow dynamics and apply cellular and chemical mediators directly to the pulmonary circulation via the perfusate. Other key attributes of the system include its low cost, simplicity and reproducibility [207].

As with many experimental models, the IPL includes some inherent limitations. Whilst an intact organ system is beneficial, a completely intact mouse, for example undergoing orthotopic lung transplantation, may be considered superior. The surgical procedure required to place a mouse on IPL is complex and time consuming and requires training to perfect. Contamination, as shown above, can persist despite efforts to reduce it, and awareness of this is key when interpreting inflammation-related readouts. Other disadvantages include the lack of humoral or neural inputs. The absence of a beating heart alters pulmonary circulation dynamics and the peristaltic roller pump does not resemble true pulsatile flow. Moreover the intermittent occlusive nature of a peristaltic pump has the potential to injure circulating cells that remain and induce a subsequent inflammatory response [208]. This system is also limited by organ viability, only lasting several hours at a time. With these limitations in mind and careful evaluation and optimisation of the system, we were able to create an optimal reproducible model of IR lung injury.

3.7.2 *Modelling IR*

We sought to model IR related to lung transplantation and to this end the IPL was considered most versatile and manageable and therefore ideally suited. However, as with any animal model, some compromise has to be made regarding exact replication of the clinical scenario. We chose to use IPL over hilar clamping, for example, as it better represents the IR experienced during lung donor harvest and implantation period. This also influenced the choice of ischaemia temperature at 37°C, over 4°C, which is traditionally used clinically. However, to ensure the model was practical in terms of execution and time scale, a warm ischaemia was opted for. The use of warm ischaemia experimentally has been validated in other animal models to provide a suitable alternative condensed protocol akin to longer-term cold ischaemia [35]. Reperfusion was performed in a recirculating manner. In view of our new minimally contaminated system, we opted for this option to prevent washout thus allowing positive feedback of released cytokines and chemokines to occur, and to be readily detectable.

The choice of gas composition was assessed in our pilot studies. As clinically ~50% oxygen is supplied to the clamped and inflated human ischaemic lungs in transit, and the potentially injurious effects of high concentrations of nitrogen seen here, a normoxic gas mixture was opted for [209, 210]. Furthermore a hyperoxic (>70%) gas mix

was avoided owing to the widely reported incidence of lung injury following prolonged exposure to a high FiO_2 [205].

We opted for a model that only induced mild/moderate lung injury to ensure reproducibility. For example, lavage protein can be highly variable in excessively injurious models, and instead we sought to create a model that would allow amelioration and exacerbation of the injury, with minimal noise-signal ratio from a range experimental readouts.

3.7.3 Optimising the system

The choice of perfusate IPL varies considerably between different research groups. Frequently, supplemented HBSS, RPMI-1640, DMEM or Krebs-Henseleit buffers are utilised [211]. In addition, some use autologous heparinised whole blood or diluted blood solutions [201]. We chose RPMI-1640 as a replete media, ensuring that any response was one purely of 'stop-flow' ischaemia and not nutrient deprivation *per se*. In addition we found that supplementing the media with antimicrobials, as commonly performed in *in vitro* techniques, also helped maintain a 'clean' system and minimal intrinsic activation.

Maintenance of oncotic pressure is one of the key components to prevent lung oedema formation, often achieved by the addition of albumin. Several sources utilised BSA for this purpose, however we found that even 'low endotoxin' BSA, maintained in a sterile environment, had the ability to induce system activation as shown by our RAW cell assay. Moving to clinical grade human albumin eliminated this problem.

3.7.4 Investigating the role of monocytes in lung IR

Previous work by our group has highlighted the key role of marginated monocytes in other models of lung injury. This study sought to elucidate their role in IR-induced lung injury, and to this end modulate their numbers, for example augmenting them through the use of a priming low dose of LPS. When piloting such experiments, a key observation was made in that LPS-treated mice not only behaved identically to their untreated cage-mates, but their lungs were also visually and functionally indistinguishable throughout the majority of the protocol, (despite having greater injury at the conclusion). If extrapolated to the clinical scenario, it becomes clear how primed or pre-activated lungs might go unnoticed at the point of selection, carrying an undetected propensity to injury following subsequent IR.

Chapter 4

The Role of Lung-Marginated Monocytes in a Murine Model of Ischaemia-Reperfusion Induced Lung Injury

4.1 Abstract

Introduction: In mice, inflammatory (Ly6C^{High}) monocyte margination and subsequent activation within the pulmonary vasculature has been shown to contribute to the development acute lung injury (microbial challenge, ventilator-induced lung injury)[137, 140]. We hypothesised that during lung transplantation, lung-marginated monocytes in donor lungs could enhance the response to ischaemia-reperfusion, contributing to development of acute lung injury in lung transplant recipients.

Methods: We utilised our mouse isolated perfused lung (IPL) model of 2 hours stop-flow followed by 2 hours of recirculating perfusion as a model of IR-induced lung injury. Monocytes were depleted with intravenous clodronate-liposomes 24hrs prior to IR. Enhanced monocyte margination was induced with low dose lipopolysaccharide pre-treatment. Lung injury was quantified via wet:dry ratio and broncho-alveolar lavage fluid protein. Lung-marginated leukocyte numbers and activation were analysed by flow cytometry of lung cell suspensions. Perfusate and broncho-alveolar lavage samples were analysed by ELISA for soluble inflammatory mediators.

Results: Substantial numbers of monocytes and neutrophils were retained by the lungs following the IR protocol. Furthermore these inflammatory leukocytes demonstrated activation evidenced by L-selectin shedding and CD11b upregulation in response to IR. Murine lungs exposed to IR exhibited higher levels of broncho-alveolar lavage protein and wet:dry ratios, compared to 2 hour perfusion-only controls. Depletion of monocytes resulted in a significant attenuation of lung injury parameters and neutrophil activation. Levels of inflammatory chemokines MIP2, KC, RAGE released into the perfusate and broncho-alveolar lavage following ischaemia-reperfusion were also attenuated by monocyte depletion. Lipopolysaccharide augmentation of monocyte lung margination was associated with enhanced lung injury.

Conclusion: In this model, monocytes are activated by IR and are retained within the lungs. Depletion of monocytes attenuates IR-induced injury and activation, and inflammatory chemokine release, whereas monocyte augmentation through sub-clinical endotoxaemia may enhance IR lung injury. These results suggest that donor monocytes could be an important determinant in IR injury and represent an important new target for prevention of post-transplantation lung injury.

4.2 Introduction

4.2.1 Lung Transplantation and Primary Graft Dysfunction

Lung transplantation remains the gold standard therapy for end-stage lung disease from a variety of aetiologies. However graft quality is very variable as the lung is highly susceptible to injury in the last days of life. This includes exposure to events such as acid aspiration, ventilator-induced lung injury and brainstem death-related pulmonary oedema. As a result up to 80% of potential lungs are deemed unsuitable at the point of donation. Of those patients receiving a graft, significant proportions develop primary graft dysfunction and many die as a result [23, 212]. Primary graft dysfunction (PGD) is a form of acute lung injury (ALI) occurring within 72 hours of lung transplantation [25]. It presents with acute hypoxia, bilateral pulmonary infiltrates and impaired pulmonary compliance (without alternate cause), accompanied by diffuse alveolar damage microscopically, not dissimilar to acute respiratory distress syndrome (ARDS) [25].

The majority of research into the pathogenesis of PGD had until now focussed on the effects of the peri-transplantation exposure to IR. However, increasing attention is now being paid to pre-transplant donor factors rendering lungs, at one extreme, unsuitable for transplantation, and at the other appearing normal but carrying undetectable underlying inflammation, which may determine the development of PGD post implantation [30]. Suggested causes include exposure to aspiration, sepsis, over ventilation and neurogenic pulmonary oedema, manifesting post-traumatic brain injury [29].

4.2.2 The role of monocytes in acute lung injury

Inflammatory leukocytes have long been implicated in the pathogenesis of acute lung injury with considerable evidence mounting for the roles of neutrophils, monocytes, alveolar macrophages, dendritic cells and eosinophils [67, 95, 129, 140, 143, 213-215]. In addition to this, numerous pro-inflammatory cytokines and chemokines have been proposed as causative and have attracted significant attention in the search for elucidating ALI mechanisms and therapies to treat and prevent its occurrence.

The phenomenon of vascular margination of inflammatory leukocytes in the narrow capillaries of the lung has been reported since the 1970's [216, 217] and is thought to be central to the development of acute lung injury [218]. Much work has focussed the role of neutrophils owing to their capacity for inducing injury, however more recently focus has shifted to the role of marginated monocytes owing to their wider repertoire of functions including inflammation-enhanced trafficking and differentiation in extravascular compartments [137, 140, 143, 198, 213, 219]. Under resting conditions the number of lung-marginated monocytes is substantially greater than the already significant circulating pool ($3-6 \times 10^5/\text{ml}$), ranging from $0.5-2.0 \times 10^6$ range/lung for adult mice [130], and a further expansion of this pool during inflammation [131, 141]. On activation monocytes lose their deformability and

become lodged in the small diameter lung capillaries; a process termed margination, inducing sequestration. The precise significance of this abundance of cells remains unclear, but almost certainly implies a significant role in lung innate immunity, with the lungs representing one of the body's first lines of defence to exogenous pathogens.

The mechanisms by which marginated monocytes contribute to lung injury have been linked to direct inflammatory mediator release (TNF, KC, MIP-1), inducing further inflammatory cells sequestration (monocytes, neutrophils) and local cell activation (alveolar macrophages, dendritic cells, T cells) [95, 137, 140, 143, 167, 198, 214]. These can then induce direct activation and injury to the pulmonary endothelium and epithelium, resulting in lung injury [137, 220]. In support of this, our group has shown that during acute endotoxaemia, large numbers of monocytes marginate to the lungs inducing TNF-mediated endothelial dysfunction [137]. We have also shown that in 'two-hit' models, sub-clinical endotoxaemia produces latent priming in the lungs, predisposing them to subsequent ALI [140, 143]. This process is dependent on margination and activation of monocytes, in particular the Ly6C^{High} 'inflammatory subset', and induces a more significant injury to the pulmonary microcirculation on exposure to non-sterile stimuli (zymosan, endotoxaemia) [137, 143, 221] and sterile (VILI) insults [140]. In these studies the importance of margination and priming during subclinical endotoxaemia was demonstrated, which may have relevance to donor lung status, specifically underlying undetected lung inflammation prior to transplantation.

4.2.3 The Critical Role of Leukocytes and their Cellular Mediators in Ischaemia-Reperfusion

Leukocytes play a prominent role in IR, enhancing inflammation and injury. The tissue resident inflammatory cell populations of the donor lungs (e.g. interstitial and alveolar macrophages, dendritic cells) are activated in response to ischaemia [153, 161]. Intravascular leukocytes have been shown to be rapidly recruited following reperfusion in recipients, along with a concurrent expansion of interstitial dendritic cells populations [222, 223]. However, the role of the donor 'resident' marginated (vascular) monocytes has not been studied in either the ischaemic or reperfusion phases.

Until now neutrophils have been regarded as the mediators of acute transplant-associated lung injury as shown in animal models of brain death (a common source of donor lungs clinically) and lung IR injury. These studies revealed enhanced neutrophil margination, activation and alveolar migration [19, 149]. Moreover BAL fluid neutrophil chemoattractant IL-8, from human transplants, has been shown to correlate with incidence of PGD and survival [150]. Additionally pulmonary endothelial cell adhesion molecule expression, which facilitates leukocyte extravasation, is upregulated during IR, contributing to microvascular injury [224]. Thus it is logical that selective blockade of these molecules (Platelet-activating factor, ICAM1, CD18, P-selectin) confers some protection to IR [224-227]. However, the mechanism by which monocytes and neutrophils induce pulmonary vascular injury is broad, likely to involve paracrine mediators, reactive oxygen species, cytokines, eicosanoids, and proteases, and is not well defined.

In IR, various mediators have been identified, yet their source or precise compartment of action remains unclear. Monocyte/macrophage-derived pro-inflammatory cytokines have been widely reported in BAL fluid in animal models after lung transplantation (TNF, IL2, IF γ) [228] and in human subjects (TNF, IL8, 10, 12 and 18, IF γ) during ischaemia and reperfusion. Similarly donor lung tissue profiling via real time PCR to assist identification of sub-clinically injured lungs (susceptible to PGD) prior to transplantation, produced similar results with likely monocyte-derived TNF, IL6, 8 and IL1 β associated with greater risk of mortality post transplantation [229]. IL8 levels in BAL and lung tissue, have also been negatively correlated by others to graft function and outcome post transplantation [150, 151]. In addition plasma MCP-1 and IFN inducible protein 10 are significantly raised in patients with PGD, also implicating a key role of monocytes [230].

In view of the previous implication of lung-marginated monocytes in one and two-hit ALI models and the presence of monocyte-derived mediators in IR studies outlined above, we hypothesise that monocyte recruitment and activation within the pulmonary vasculature is likely to be key to the pathogenesis of transplant-related ALI (PGD). Moreover, the potential for lung-marginated donor monocytes to be carried over as pre-activated 'passenger' leukocytes to the lung recipient is likely to be a significant factor in the orchestration and propagation of PGD, during and after transplantation, and is therefore the focus of this study.

4.2.4 Leukocyte-mediated endothelial injury and the glycocalyx in ischaemia-reperfusion

Pulmonary vascular inflammation and increased permeability, occurring in response to leukocyte margination and activation, are central to the pathogenesis of ALI [198]. In recent years more attention had been drawn to the role of the endothelial surface layer now known as the glycocalyx. In microvessels it consists of a 0.4-0.5 μ m thick zone separating the blood from the capillary wall, sometimes exceeding the width of the endothelium itself [231]. It serves numerous functions and has been implicated in the regulation of endothelial permeability through prevention of water and protein extravasation, mechanotransduction of shear stress and modulation of red cell and leukocyte interactions and thus inflammation [232, 233]. Consequently damage to the glycocalyx causes disruption to these processes, increasing adhesion of leukocytes and impairing endothelial barrier function [76]. Specifically IR has been shown by several groups to induce shedding and impair glycocalyx function in human and mouse models, increasing permeability [76, 234-237] and impacting on inflammatory processes [233, 238, 239]. Specifically increased exposure of endothelial cell adhesion molecules, due to glycocalyx shedding, promotes adhesion of circulating leukocytes and subsequent endothelial disruption [240]. Based on this we hypothesised that ischaemia-induced glycocalyx disruption and altered leukocyte margination are likely to be heavily implicated in the pathophysiology of transplant-related acute lung injury.

4.3 Hypothesis and Aims

Utilising our newly adapted *ex vivo* IPL mouse model of 2 hours ischaemia and 2 hours reperfusion, we investigated monocyte margination in the lungs and their contribution to IR-induced acute lung injury.

Based on previous findings we hypothesised that donor lung-marginated leukocytes, specifically monocytes, would be activated on exposure to ischaemia-reperfusion.

The aims were to:

- ⇒ Quantify monocyte activation and soluble mediator expression
- ⇒ Determine the effect of monocyte depletion on injury, inflammation parameters and mediator expression
- ⇒ Determine the potential mechanisms of monocyte-mediated lung injury in response to ischaemia-reperfusion
- ⇒ Assess the role of 'donor inflammation' utilising a model of LPS priming
- ⇒ Evaluate the endothelial glycocalyx response to IR

4.4 Methodology

4.4.1 *An isolated perfused lung model of ischaemia – reperfusion*

Utilising our new ‘clean’ IPL system described in Chapter 3 we opted for a combination of 2 hours ischaemia plus 2 hours perfusion, owing to the reproducible mild-moderate injury produced with this protocol. This incorporated a initial 5 minute non-recirculating ‘flush’ washout period (at 25ml/kg/min) immediately after PA and LA cannulation, following the 2 hours of ischaemia and at following the 2 hours of perfusion, (i.e. at 0, 2 and 4 hours). During the ischaemia (‘stop-flow’) period the lungs were maintained at 37°C with a CPAP of 5cm H₂O Air/5% CO₂ applied via the tracheal tube (tracheostomy). The 2 hour recirculating perfusion (‘reperfusion’) at 40ml/kg/min incorporated 6ml/kg tidal volume ventilation at 80 breaths/minute with a PEEP of 5cmH₂O Air/5% CO₂. (See Chapters 2 and 3 for more details.)

4.4.2 *Sample collection, processing and measurements*

BAL samples were obtained and analysed for protein content and soluble mediators via sandwich ELISA as described in detail in Chapter 2 and 3, along with perfusate samples. Similarly the left lung was used for wet:dry weight ratio analysis and the right lung was subdivided and dissociated to create single cell suspensions for flow cytometry as per Chapter 2 and 3.

4.4.3 *Monocyte depletion and augmentation*

Circulating monocytes were depleted with an intravenous dose of 200µl liposomal clodronate, given 24 hours prior to the IR protocol. Monocyte augmentation was achieved through intravenous low dose lipopolysaccharide (20ng) administration 2 hours prior to the IR protocol. We have previously found this dose optimal for maximal monocyte margination to the pulmonary circulation without inducing systemic upset [143].

4.5 Results

4.5.1 Flow cytometric identification of monocytes in lung cell suspension

Lung monocytes were identified, counted and activation quantified via flow cytometry (Figure 4.1).

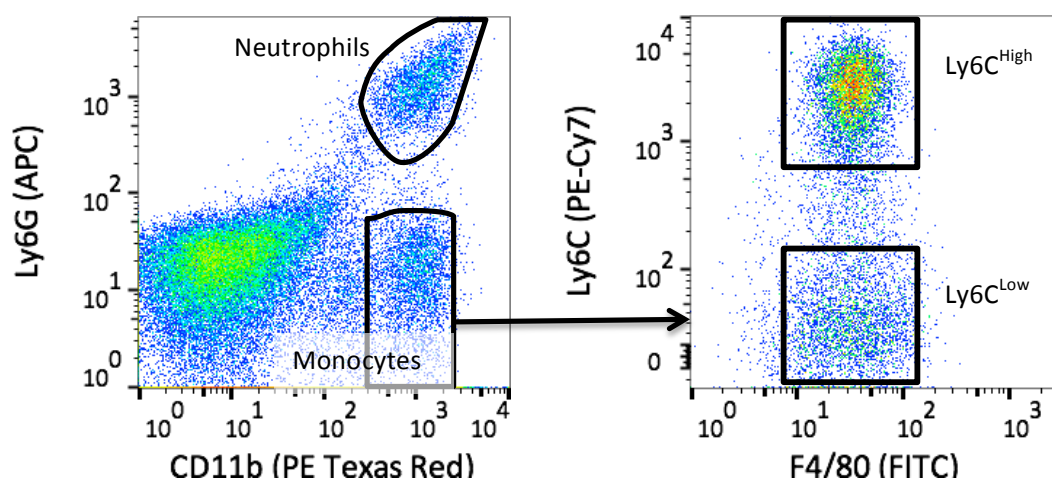


Figure 4.1 Representative flow plots depicting phenotype of monocytes

Lung monocytes were initially identified as CD11b⁺/Ly6G⁻, then as F4/80⁺ and either Ly6C^{High} or Ly6C^{Low} cells. (Neither monocyte population expressed the NK cell marker NK1.1; data not shown).

Neutrophils were identified as CD11b⁺, F4/80⁻, NK1.1⁻ and Ly6G⁺.

4.5.2 Inflammatory lung leukocytes are retained following ischaemia-reperfusion

Following the IR protocol and despite the three 'flush-out' periods this incorporated, significant numbers of monocytes and neutrophils still remained in the lungs as compared to the non-surgical controls (Figure 4.2). Trapping of these cells within the pulmonary microvasculature is potentially due to a reduction in deformability as a result of activation within the capillaries, increased cell adhesion molecule activity, or that they are residing outside the vascular space. Further assessment of precise monocyte compartment location was carried out in Chapter 5.

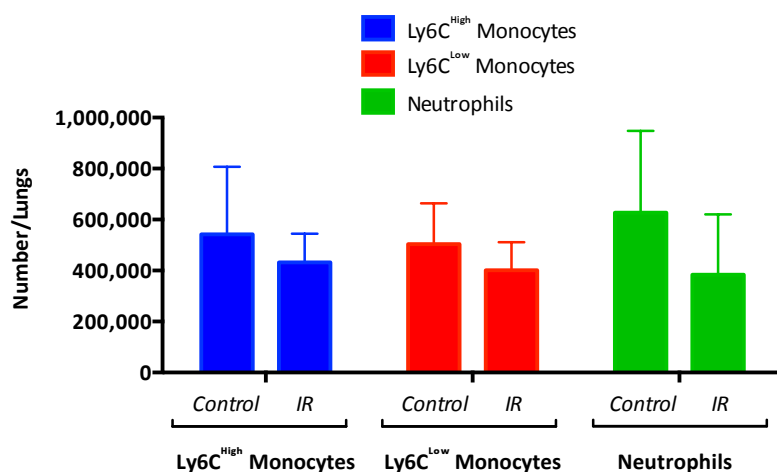


Figure 4.2 Substantial numbers of leukocytes remain in the lungs despite exposure to 2hrs ischaemia-2hrs reperfusion (IR), as compared to non-surgical controls.

Median ± IQR, n = 6-9. Lung leukocytes proportions were quantified from a single cell suspension via flow cytometry

4.5.3 Inflammatory lung leukocytes are activated following ischaemia-reperfusion

Ly6C^{High} monocytes were activated by the ischaemia-reperfusion protocol as evidenced by L-selectin shedding (Figure 4.3A) in response to IR. Neutrophils also exhibited activation shown by L-selectin shedding and upregulation of CD11b as previously shown [241-243] (Figure 4.3B&C). (NB: Ly6C^{Low} monocytes do not express significant levels of L-selectin.)

2hr perfusion alone also resulted in evidence of activation, but this was limited to a relatively small effect in Ly6C^{High} monocyte L-selectin shedding.

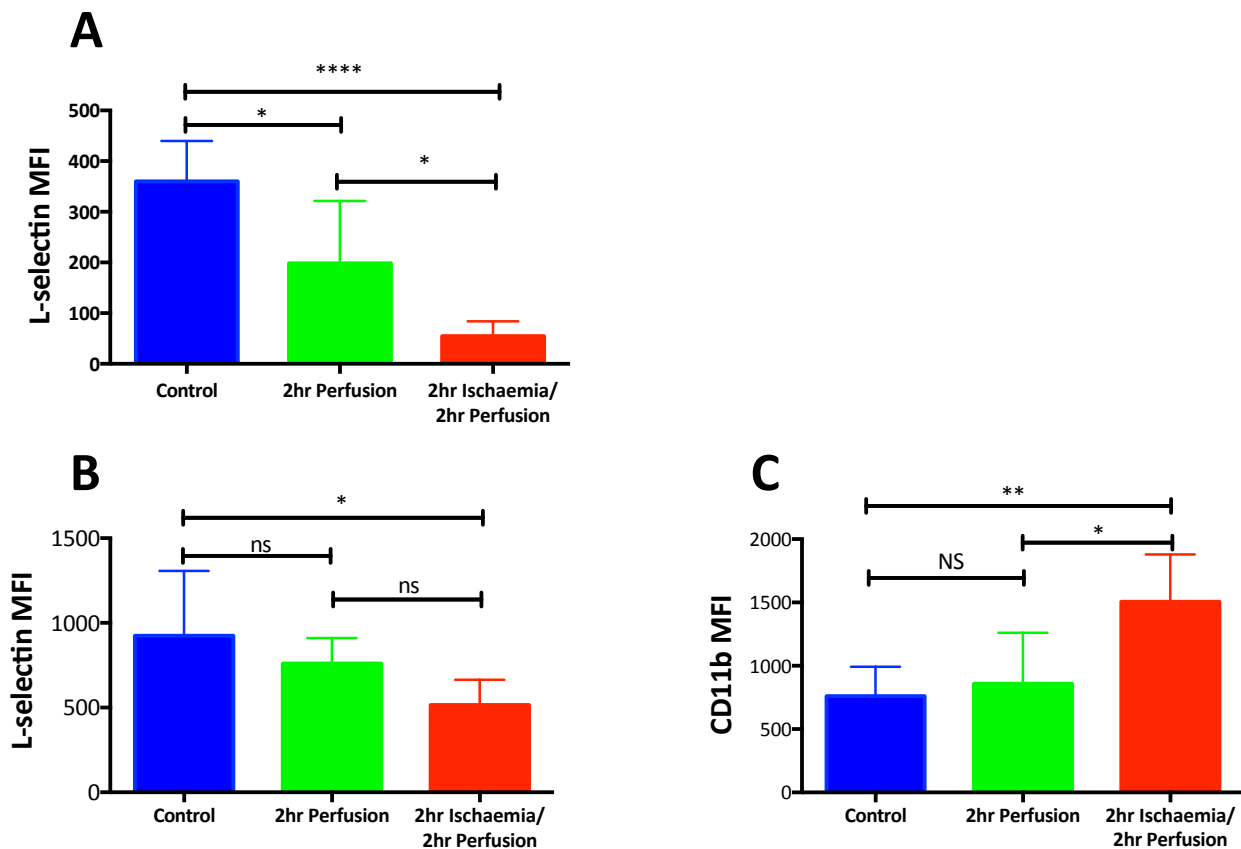


Figure 4.3 Inflammatory leukocyte markers of activation in response to ischaemia-reperfusion

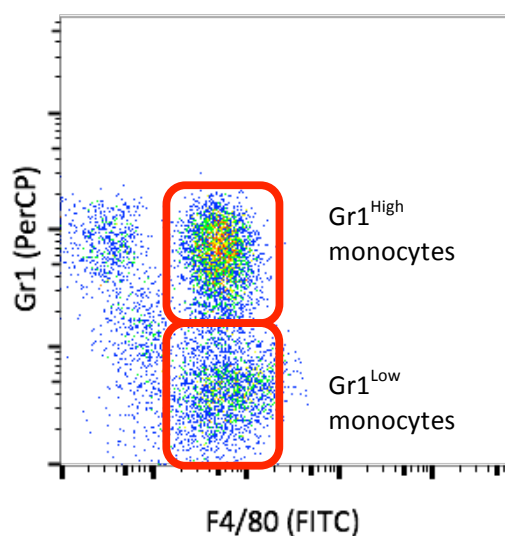
Activation was observed with L-selectin shedding on both Ly6C^{High} monocytes (A) and neutrophils (B), along with CD11b upregulation on neutrophils (C).

(Mean±SD, n= 7-9, ANOVA+Bonferroni * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$)

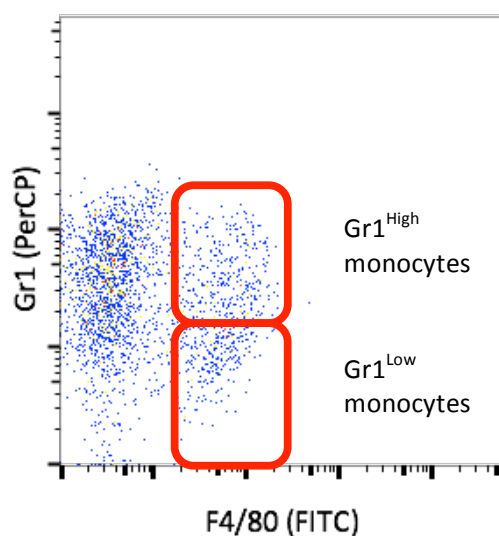
4.5.4 Clodronate Depletion of Lung-Marginated Monocytes

To further assess the role of monocytes, mice were treated with intravenous clodronate liposomes 24 hours prior to the 2hr ischaemia/2hr reperfusion protocol. Monocyte numbers in the lungs of clodronate treated mice exhibited substantial drops in Ly6C^{High} (~60%) and Ly6C^{Low} (~65%) monocytes (Figure 4.4 and Table 4.1). The total number of neutrophils did not significantly change although displayed an upward trend, in keeping with reports from others [244].

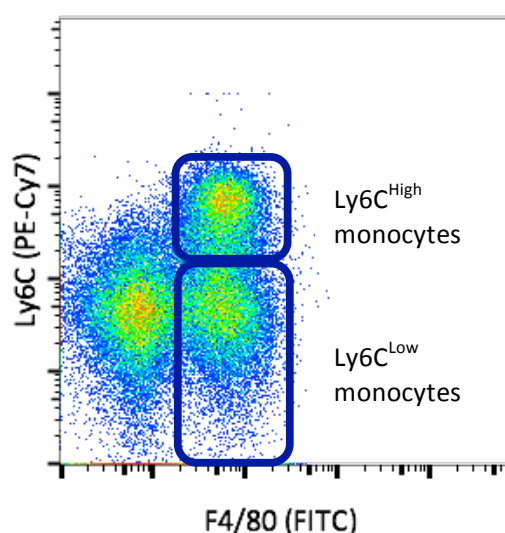
A. Blood: Control



B. Blood: Clodronate pre-treated



C. Lungs: Control



D. Lungs: Clodronate pre-treated

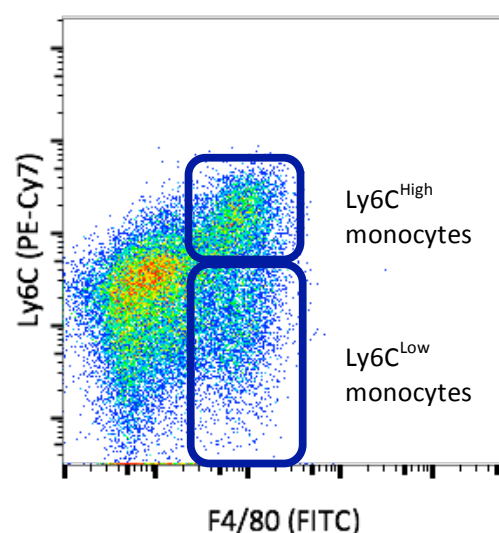


Figure 4.4 Leukocytes remaining in the blood and lung following liposomal clodronate depletion in control mice

Representative flow cytometry plots of monocytes populations following depletion in the blood (A control, B clodronate) and lungs (C control, D clodronate). All plots were initially gated as CD11B positive (not shown). Monocytes were identified as F4/80+ and either Ly6C^{High} or ^{Low} in lungs or Gr1^{High} or Gr1^{Low} in blood.

Table 4.1 Total number of leukocytes in the blood and lungs with or without clodronate pre-treatment

Median(IQR), n= 6-10, Mann Whitney U, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs' control.
All values $\times 10^5$ /ml for blood or per lungs

		Neutrophils $\times 10^5$	Gr1 ^{High} /Ly6C ^{High} Monocytes $\times 10^5$	Gr1 ^{Low} /Ly6C ^{Low} Monocytes $\times 10^5$	Alveolar Macrophages $\times 10^6$
Blood	Control	4.81(3.8-7.7)	2.40(1.9-3.9)	2.85(2.2-4.6)	N/A
	Monocyte-depleted	9.07(7.4-16.0)*	0.39(0.2-0.5)****	0.19(0.2-0.4)****	N/A
Lung	Control	5.41(4.1-7.8)	4.51 (3.3-7.3)	4.57(4.2-5.7)	18.8(17-24)
	Monocyte-depleted	10.01(6.3-18.7) NS	1.97(0.16-2.3)***	1.60(0.8-2.4)**	19.0(18-21) NS

The apparent reduced efficacy of clodronate on the lung monocytes, as opposed to blood, may be due to several factors. Clodronate remains vascular, being unable to penetrate the endothelium, therefore at least some of the residual lung monocytes seen here could represent an inaccessible extravascular pool. Lung monocyte compartmentalisation is investigated in more detail in Chapter 5. In addition, the propensity for monocytes to marginate under even very low levels of inflammation is significant and may result in accumulation of the residual monocytes in the lungs following liposomal-clodronate depletion. Moreover, even if the monocytes have been rendered apoptotic, margination may delay their clearance by trapping in the lung microvasculature.

4.5.5 Monocyte depletion attenuates ischaemia-reperfusion lung injury

Depletion of vascular monocytes significantly reduced the oedema induced by IR. (Figure 4.5A, purple bar). Protein in the broncho-alveolar lavage fluid, which is likely to represent both epithelial *and* endothelial leak, was increased by exposure to IR and reduced to the levels approaching the non-surgical controls following monocyte depletion. Clodronate treatment also significantly reduced the elevated wet:dry ratios observed after ischaemia-reperfusion, however only to the levels observed with the 2 hour perfusion controls (Figure 4.5B).

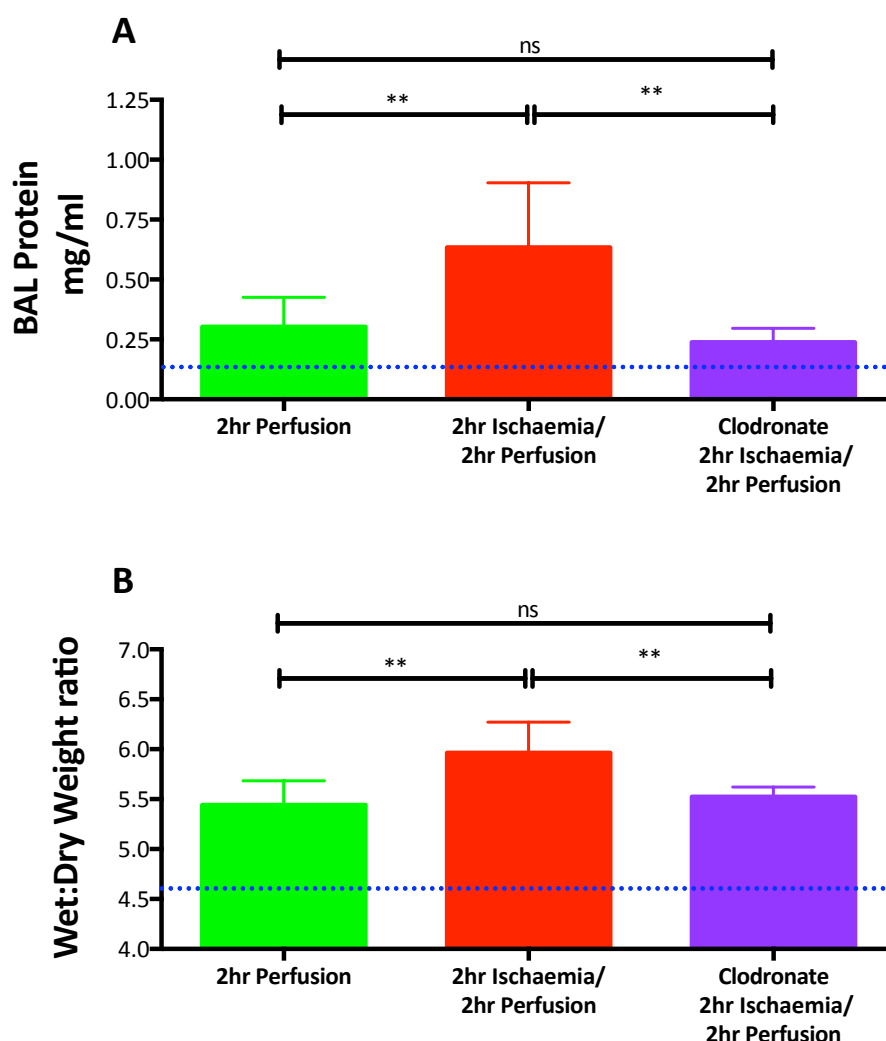


Figure 4.5 Lung injury quantified by BAL protein content (A) and lung Wet:Dry weight ratio

Mean±SD, n= 7-8, ANOVA+Bonferroni, **p<0.01. The blue dotted line represents non-surgical control values.

To examine the relationship between IR and BAL versus wet:dry ratio we infused BSA-conjugated Alexa Fluor 594 dye into the reservoir of the IPL, allowing it to recirculate for 30 minutes prior to the end of the protocol. The lungs were then flushed, lavaged and then chopped and digested with Liberase and DNase to promote dye release.

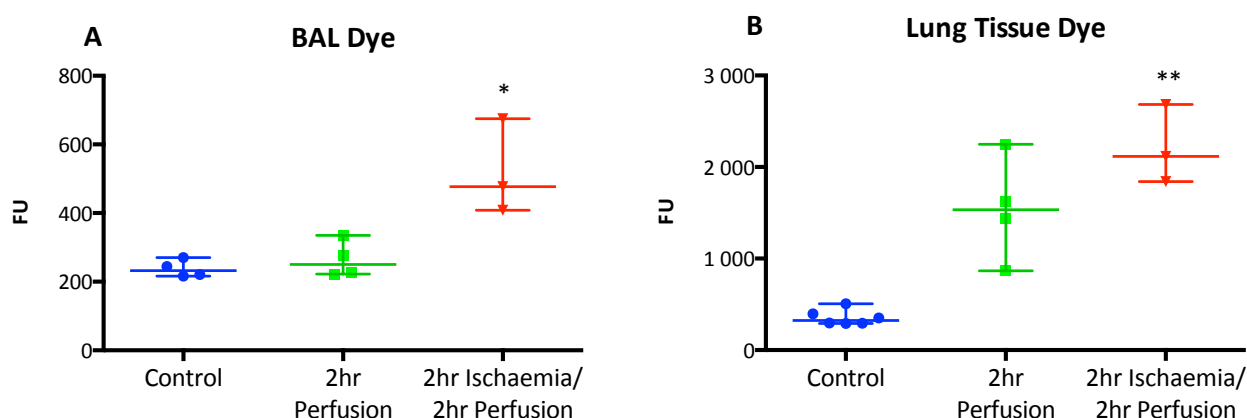


Figure 4.6 Fluorescent dye in BAL (A) and lungs (B) following IR.

Alexa Fluor 594 dye was circulated 30minutes prior to 5minute washout and cessation of IR protocol and lung lavage and harvest. Residual dye in fluorescent units FU are shown. Control values (blue dots) represent baseline lung/BAL fluorescence. Median±Range, n=3-6, Kruskal-Wallis+Dunns, **p<0.01, *p<0.05 vs' control.

These data mirrored those observed with BAL protein and wet:dry ratios following IR above (Figure 4.5); 2 hour perfusion exhibited increased 'interstitial' fluid leak (enhanced lung tissue fluorescence), without concomitant 'epithelial' leak (BAL fluorescence remains at control levels) (Figure 4.6). This reinforces the concept that *ex vivo* acellular perfusion may permit interstitial influx of fluid seen by relative increases in both wet:dry ratio and lung tissue fluorescent dye penetration with 2 hours perfusion. Similarly alveolar leak of oedema fluid, signified by increased BAL protein in Figure 4.5 is replicated by significantly increased BAL fluorescence following IR in these experiments (Figure 4.6). Of note no significant difference in terms of physiological and ultrastructural changes has been found in lungs perfused with acellular compared to cellular perfusate solutions [245].

4.5.6 Monocyte depletion attenuates IR-induced vascular chemokine release

Lung perfusate samples were collected at 0 (post thoracotomy), 2 (post ischaemia) and 4 hours (post perfusion). MIP-2, KC, RAGE and MCP-1, released into the perfusate from the lungs, increased over the IR period. Clodronate-liposome depletion of monocytes, however, induced a significant reduction in MIP-2, KC and RAGE after reperfusion. In the case of RAGE, a significant increase was seen at the end of ischaemia in addition to perfusion, both of which were attenuated by monocyte depletion. In contrast, clodronate treatment actually increased soluble perfusate MCP-1 levels prior to, as well as during, the protocol, which is likely to be due a lack of monocytes which might normally be expected to take up this cytokine [246, 247] (Figure 4.7). Perfusate IL-6 levels increased, particularly during the reperfusion phase, but were not affected by clodronate treatment. IL-1 β and TNF levels were below the lower limit of detection (data not shown).

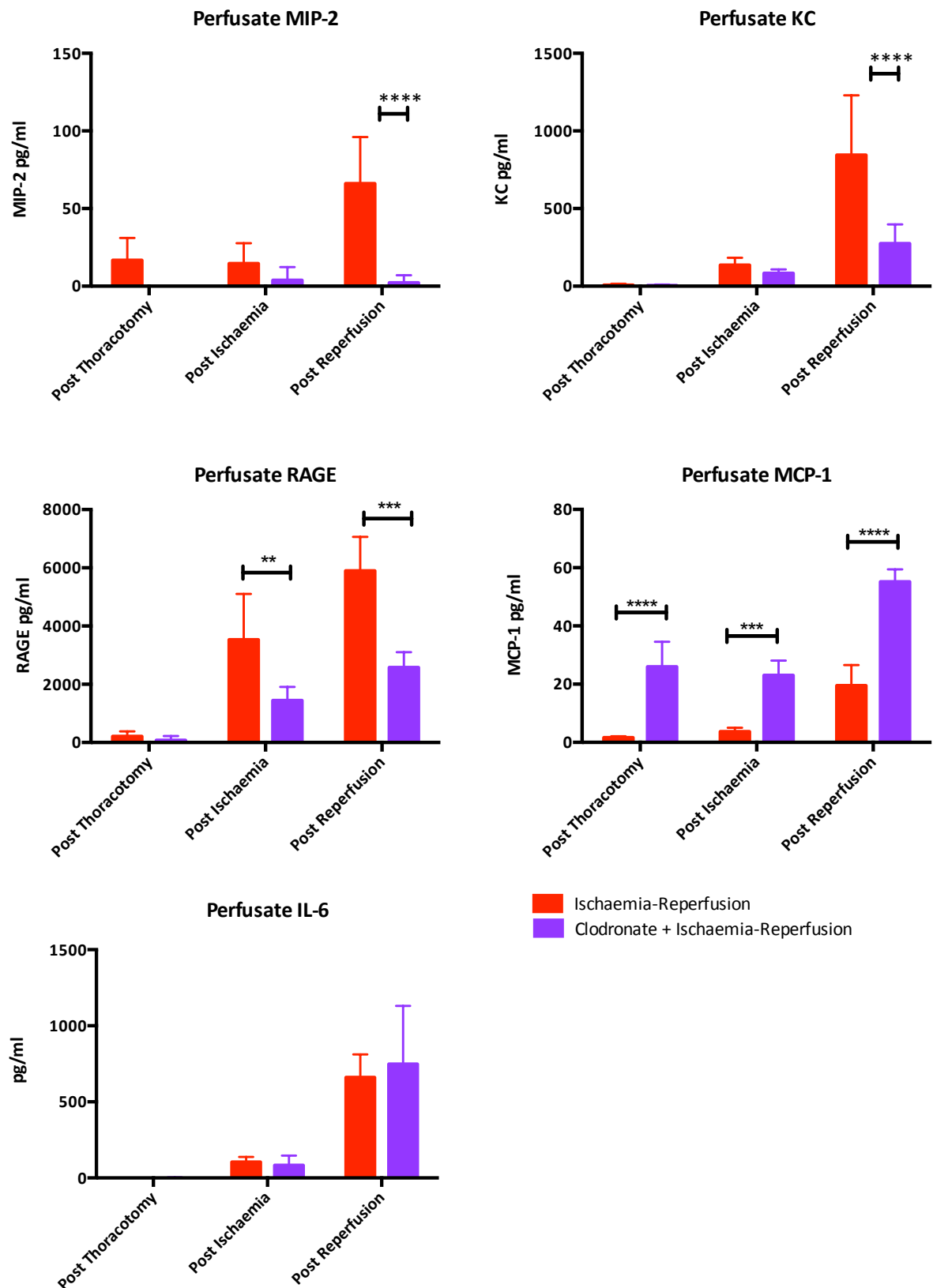


Figure 4.7 Perfusate chemokines quantified by ELISA.

(Median(Range), n= 4-6, Mann-Whitney **p<0.01, ***p<0.001, ****p<0.0001)

4.5.7 Ischaemia-reperfusion induces chemokine and cytokine release into the broncho-alveolar lavage fluid

Chemokines were also detectable in the broncho-alveolar lavage fluid. KC and RAGE were significantly attenuated by monocyte depletion. MIP-2 and TNF however remain unchanged by depletion, with the latter trending upwards.

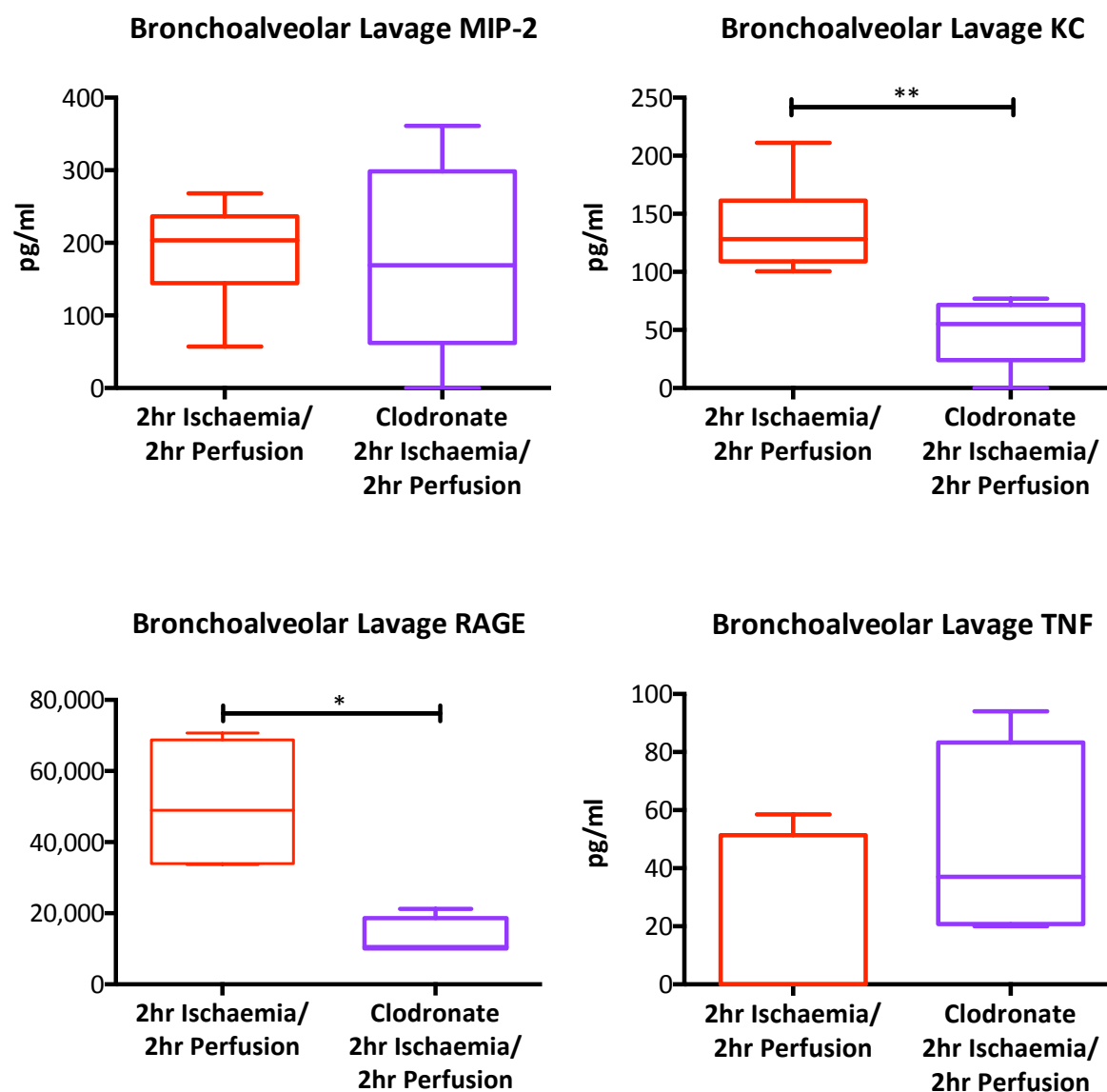


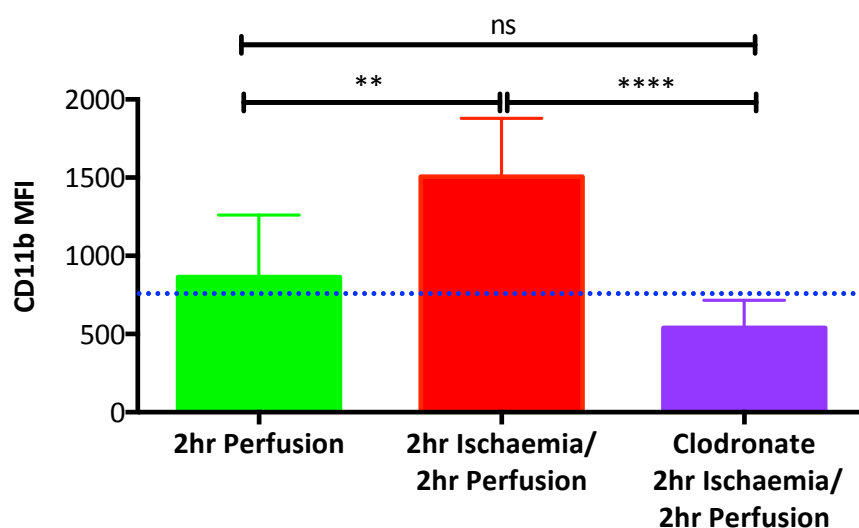
Figure 4.8 Broncho-alveolar lavage soluble chemokines and cytokines quantified by ELISA.

Median(IQR), $n = 4-6$, Mann Whitney, * $p < 0.05$, ** $p < 0.01$

4.5.8 Monocyte depletion prevents ischaemia-reperfusion induced neutrophil activation

As seen in figure 4.3C above, neutrophil activation with ischaemia-reperfusion was evident based on upregulation of surface CD11b expression. A reduction in CD11b expression on lung neutrophils in response to monocyte depletion was observed, suggesting a possible interplay between these cell populations in relation to the development of lung injury in this model (Figure 4.9). However, reduction of neutrophil L-selectin in response to IR was unaffected by monocyte depletion, presumably due to direct effects of ischemia on the L-selectin shedding mechanism, which is distinct from that of CD11b upregulation [248-250].

A



B

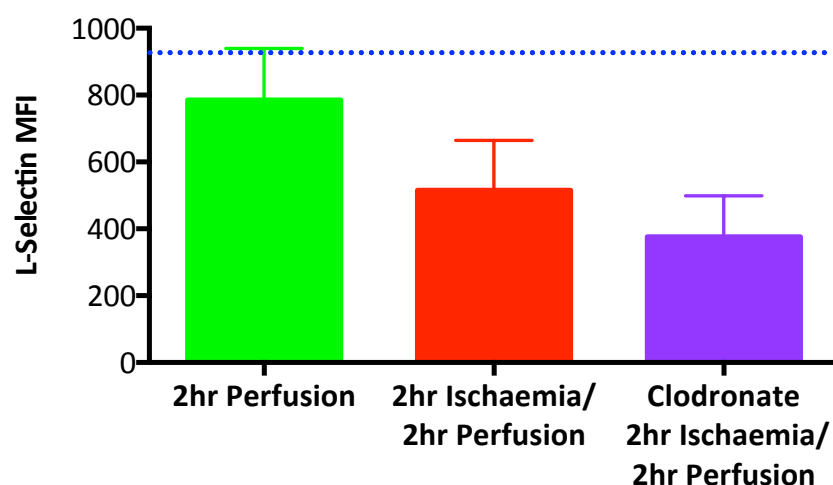


Figure 4.9. Lung neutrophil surface (A) CD11b and (B) L-selectin expression

(Mean±SD, n= 7-8, ANOVA+Bonferroni, ** $p < 0.01$, **** $p < 0.0001$. The blue dotted line represents non-surgical control values)

4.5.9 Subclinical Endotoxaemia Enhances IR Injury

Previous work in our group had shown that endotoxaemia induced by intravenous low dose (20ng) lipopolysaccharide (LPS) was sufficient to induce significant margination of leukocytes to the pulmonary vasculature without producing any signs of clinical symptoms or sepsis at 2hrs, and in doing so, primes the lungs to subsequent injury [143]. The reasoning behind LPS administration was therefore two-fold; to enhance the number of margined monocyte numbers within the lung and to enhance IR-induced lung injury, further developing our model of primary graft dysfunction in lung recipients. In a series of pilot experiments, 20ng of LPS was injected via the tail vein and as expected resulted in substantial increase in lung-marginated leukocytes, producing a significant increase in both neutrophil and Ly6C^{High} monocyte numbers (Figure 4.10).

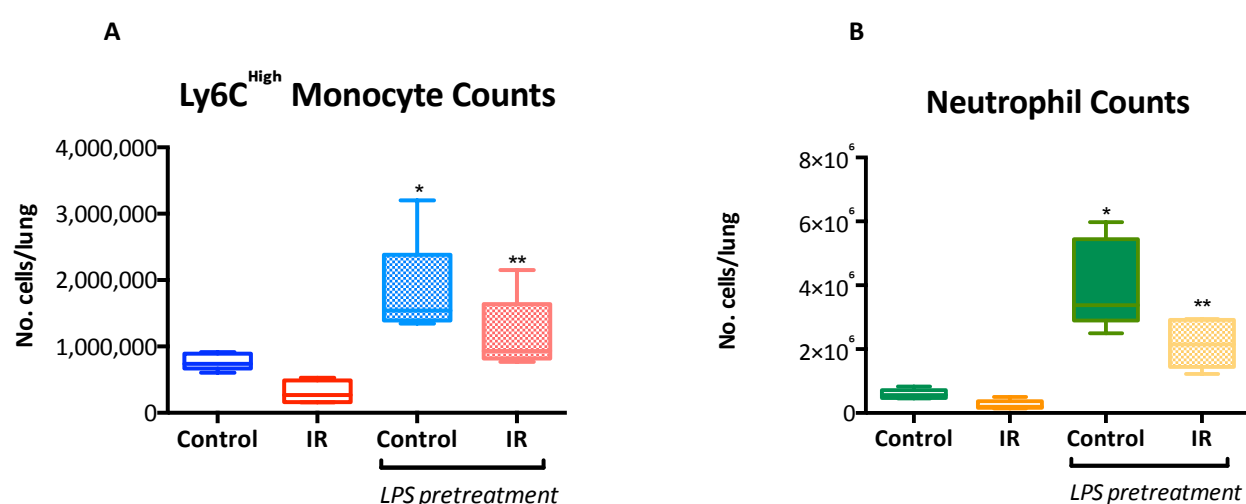


Figure 4.10 Leukocytes numbers are enhanced by prior LPS injection and are retained in the lungs post IR

LPS pre-treatment induced a substantial increase in the numbers of both Ly6C^{High} monocytes (A) and neutrophils (B) as compared to non-surgical controls

*Median(IQR), n=3-4, Kruskal-Wallis+Dunns * p<0.05, ** p<0.01 LPS control v's non-LPS control, or LPS- IR v's non-LPS IR=.*

As shown in Figure 4.5, IR alone induced significant lung injury as evidenced by an increase in both wet:dry ratio and BAL protein concentration attenuated with monocyte depletion. Mice subjected to subclinical endotoxaemia exhibited increased pulmonary vascular permeability in response to IR exposure, with augmented BAL protein and wet:dry ratios (Figure 4.11A and B).

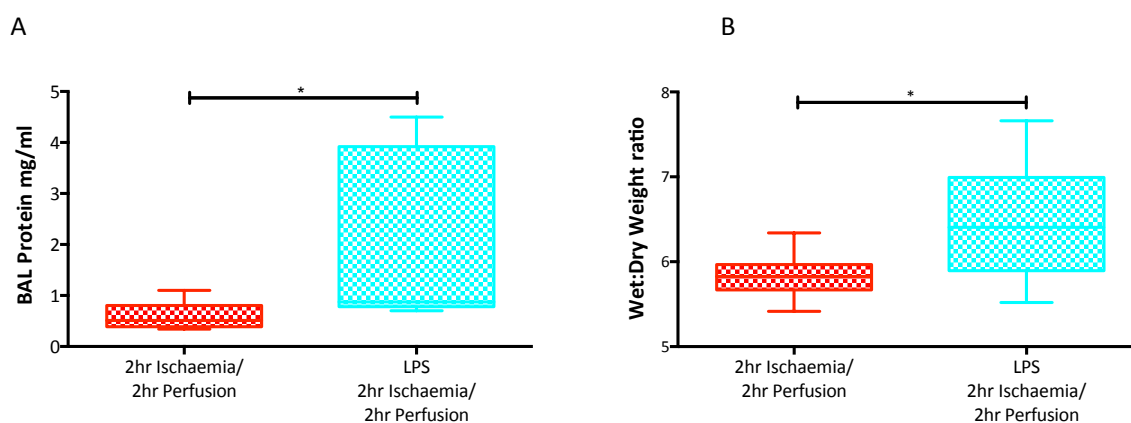


Figure 4.11 Pulmonary vascular permeability is impaired by IR +/-prior endotoxaemia (LPS).

Lung BAL protein concentrations (**A**) and Wet:Dry weight ratios (**B**) were quantified as an indication of pulmonary vascular permeability and lung injury. Median(IQR), $n=6-8$, Mann Whitney, * $p<0.05$.

Perfusate cytokine analysis from LPS-IR lungs showed no difference between the values and 0, 2 and 4 hours for KC, MIP-2 and RAGE concentrations (data not shown). Perfusate TNF and IL-6 potentially showed a trend towards increase following LPS pre-treatment (Figure 4.12 A and B). MCP-1 levels however showed a significant increase after prior pre-treatment with LPS (Figure 4.12 C), which could be attributable to the increased margination of monocytes expressing MCP-1 in response to LPS stimulation in this model [251]. Interpretation of these results is not straightforward as LPS pre-treatment will produce various effects including expansion of the margined cell populations, the direct induction cytokine expression in the constituent and recruited cell populations, as well as their priming to the IR-related insults.

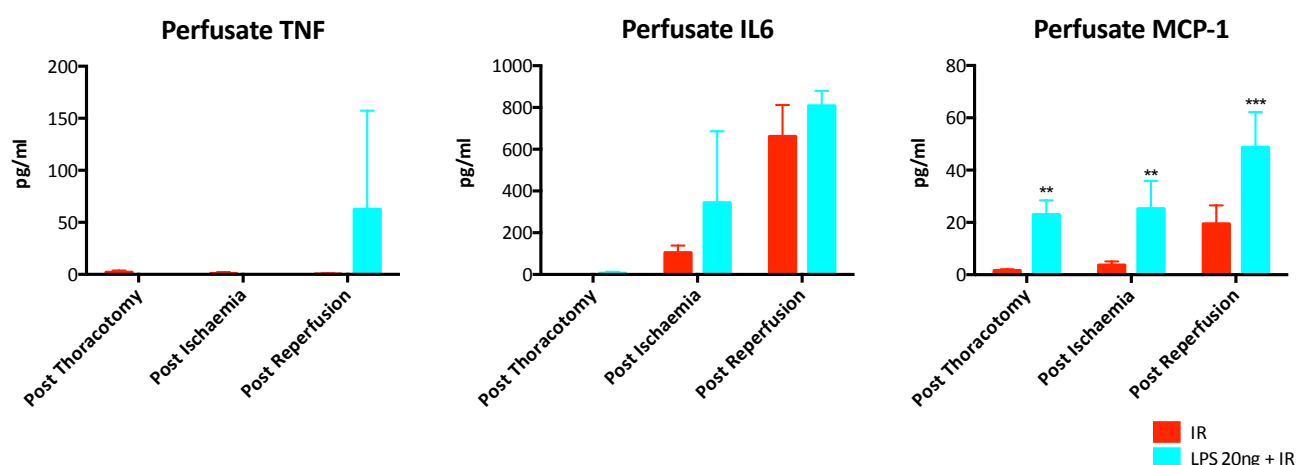


Figure 4.12 Perfusate cytokine levels following LPS injection prior to IR protocol. (A) TNF, (B) IL-6, (C) MCP-1.

Median(IQR) Mann Whitney vs' IR, ** $p<0.01$, *** $p<0.001$

The cytokines and chemokines quantified in the BAL also showed no significant difference when mice were pre-treated with LPS prior to IR, of MIP-2, KC, TNF or RAGE.

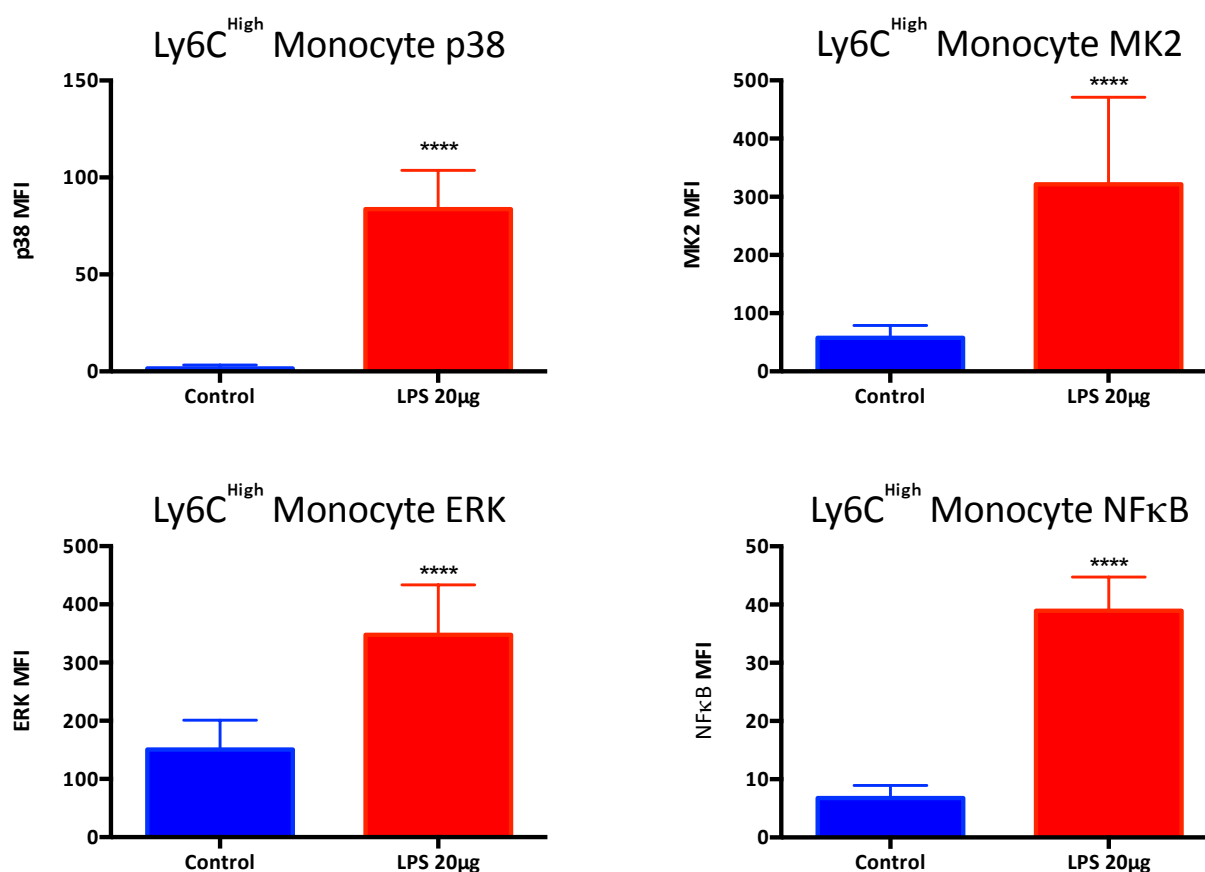
4.5.10 Elucidating the mechanisms on monocyte-mediated IR lung injury

Monocyte activation

Previous work by our group and others has highlighted upregulation of p38 MAPK/MK2 pathway in lung monocyte subsets on exposure to endotoxaemia, intratracheal LPS and injurious ventilation [195, 198]. To elicit the dynamics of the onset of leukocyte activation within our model we sought to quantify upregulation in monocytes in our IR model to shed light on potential mechanisms of lung injury.

We performed positive control treatments to assess levels of kinase activation in response to a strong inflammation stimulus to validate the intracellular detection of phospho-p38, MK2, ERK1/3, NFκB (p65) signals of the monocytes. To this end 20μg of intravenous LPS was given to control mice, and the lungs harvested 10 minutes later. This produced significant upregulation of all the intracellular markers as compared to untreated controls in both lung monocyte populations (Figure 4.13).

A



B

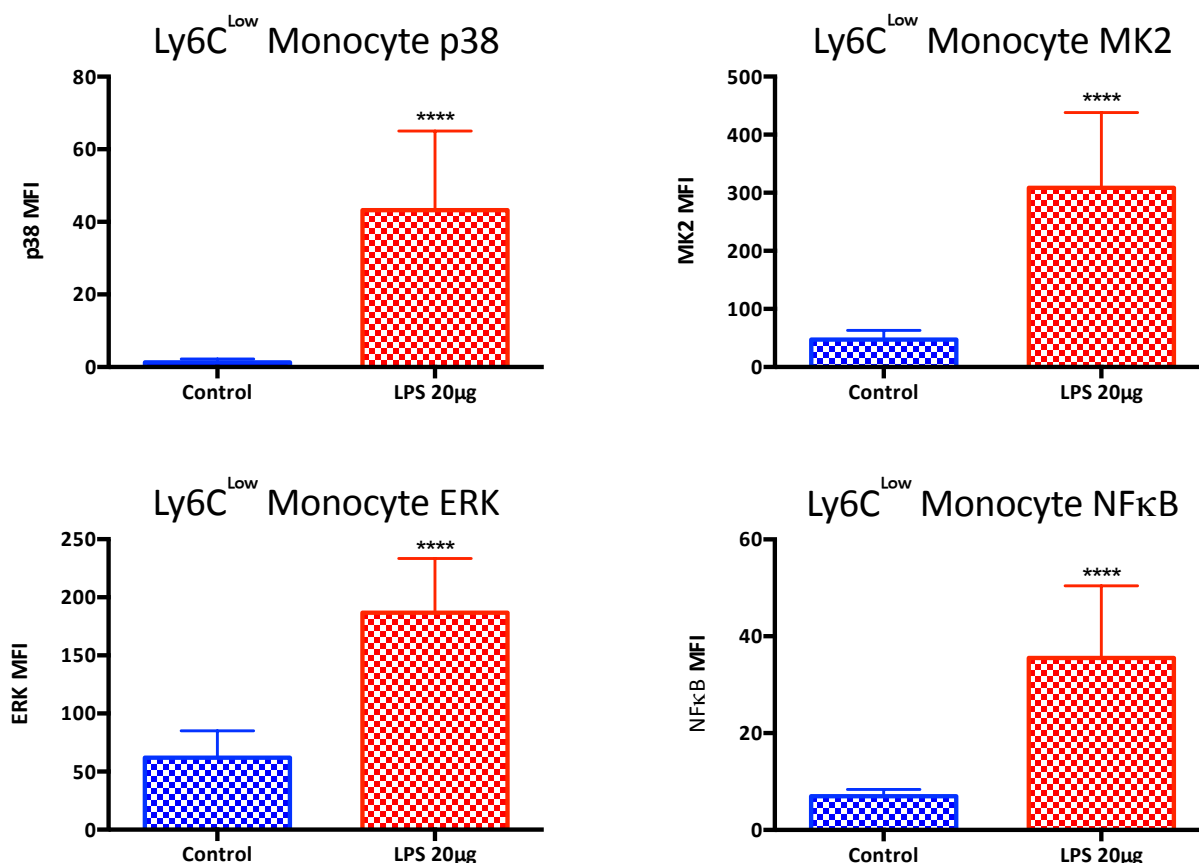


Figure 4.13 Validation of p38, MK2, ERK, NFκB signals in lung $Ly6C^{High}$ (A) and $Ly6C^{Low}$ (B) monocytes.

Low dose intravenous LPS 20 μ g 10minutes prior to lung harvest produced significant upregulation of all markers compared to untreated controls. Mean $\pm$ SD, n=8-10, t-test, ****p<0.001

We then assessed the same markers in lung monocytes following either 2 hours of ischaemia only versus our standard IR protocol. $Ly6C^{High}$ and $Ly6C^{Low}$ monocytes overall showed limited upregulation of the phospho-p38 MK2/MAPK pathway markers in response to IR (Figure 4.14 A and B). Of noted phosph-p38 showed an increase in both monocyte subtypes following IR. $Ly6C^{High}$ ERK also exhibited a trend to increase following IR, although this was overshadowed by a significant increase following only 2 hours ischaemia. Overall a limited response was observed following IR, mostly restricted to the $Ly6C^{High}$ population. However, this did not provide convincing evidence that monocytes necessarily represent the key sensors of ischaemic stress, as well as upstream cellular effectors of injury. These data indicate that ischemia and reperfusion insults do not produce an immediate response detectable with this method, and that the responses detectable in the late stages of the protocol may indicate on-going monocyte activation or secondary responses to mediator release

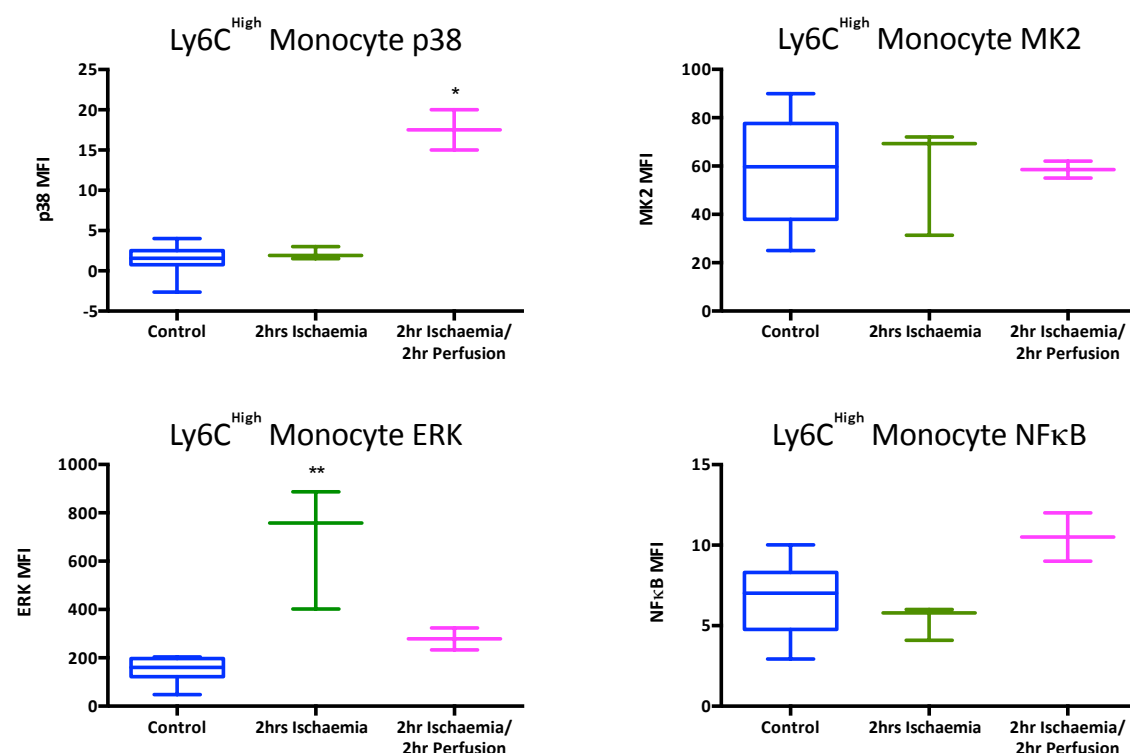
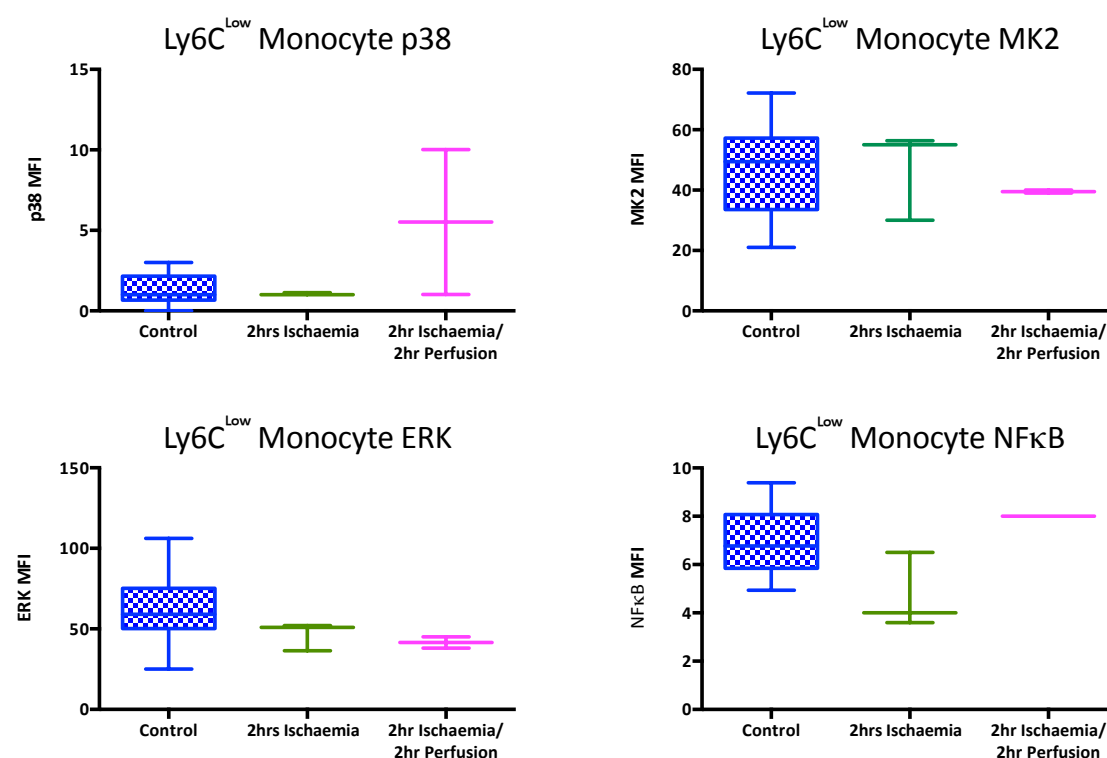
A**B**

Figure 4.14 p38, MK2, ERK, NFκB signals in $Ly6C^{High}$ (A) and $Ly6C^{Low}$ (B) lung monocytes following IR.

$Ly6C^{High}$ (A) and $Ly6C^{Low}$ (B) monocytes exposed to IR overall showed limited upregulation of the p38 MK2/MAPK pathway markers in response to IR. p38 showed an increase in both monocyte subtypes. $Ly6C^{High}$ ERK exhibited a significant increase at 2hrs ischaemia, and trend to increase following the IR. Median(IQR) $n=3-8$, Kruskal-Wallis + Dunns, * $p<0.05$ vs's control

Enzyme upregulation

Cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) are pro-inflammatory intracellular enzymes regulated post transcription by p38 MAPK/MK2 pathway. These enzymes were quantified in lung leukocytes and endothelia via flow cytometry at the end of the protocol, as previously described [195, 198]. Unfortunately no clear changes in enzyme activity were seen following IR compared to controls.

Following this, a group of pilot experiments were undertaken using a range of enzyme inhibitors against potential candidate pathways, shown previously to reverse pulmonary endothelial leak [198]. They included WEB (platelet activating factor antagonist), D609 (phosphatidylcholine-phospholipase C inhibitor), indomethacin (cyclooxygenase 1/2 inhibitor), DPI (NADPH oxidase inhibitor) and the antioxidants catalase and N-acetylcysteine. They were administered prior to onset of IR. However, as potentially predicted by the lack of increase in iNOS and COX activity previously, these enzyme inhibitors failed to protect against oedema formation and lung injury.

It is therefore likely that events occurring earlier during IR exposure, inducing endothelial activation and stress, determine barrier dysfunction and oedema formation in this model of acute lung injury.

4.5.11 The role of the endothelium and the glycocalyx in IR: pilot studies

Our data demonstrate that intravascular leukocytes contribute to barrier dysfunction and thus lung injury in response to IR, augmented by endotoxaemia. It is clear that the endothelium plays a key role in this process and has previously been shown to be activated by IR in the literature [37, 252]. We attempted to quantify activation of pulmonary endothelium in response to IR in the IPL model.

Cell adhesion molecule expression

In an attempt to assess endothelial activation in our model we measured surface VCAM, ICAM-1 and E-selectin expression on endothelial cells as described by our group [253]. This failed to show a clear pattern of expression between the IR groups (data not shown), which may represent too early a time point to assess pulmonary activation.

The endothelial glycocalyx

The ability of the endothelial glycocalyx to regulate permeability and leukocyte adhesion is well known, with degradation of this surface layer implicated in the pathophysiology of acute organ dysfunction. However little is known about the behaviour of the pulmonary microvascular glycocalyx under ischaemic conditions. Glycocalyx degradation in response to inflammation and ischaemic treatments have been shown to occur rapidly in several vascular beds [58, 234, 254, 255] and most recently in the lungs [84, 237, 238, 256]. We therefore quantified the main glycosaminoglycan heparan sulfate (HS) via flow cytometry as a potentially acute marker of endothelial activation and injury. Endothelial cells were identified from lung cell suspensions as CD45⁻/CD31⁺/High SSC population as shown in Figure 4.15. Endothelial surface HS was identified and validated as such by the use of heparinase III degradation as previously described [84, 257]. HS was then quantified following our IR protocols. Whilst a trend towards HS shedding was observed following IR, and seemingly protected against following monocyte depletion, no significant change was observed (Figure 4.16). This may be in part due to the highly variable expression of HS [258].

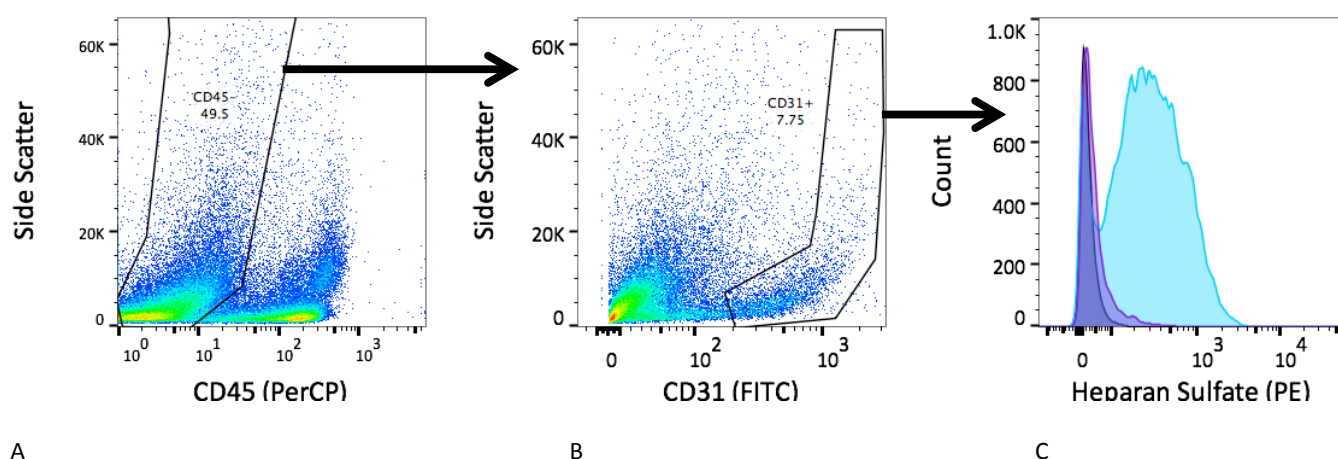


Figure 4.15 Identification and validation of endothelial cell surface heparan sulphate

Representative plots of lung cell suspensions were gated as CD45⁻ (A), CD31/SSC⁺ (B). Heparan sulfate expression is shown by the blue histogram, isotype control by the grey histogram and levels seen after in vitro heparanase treatment in the purple histogram (C).

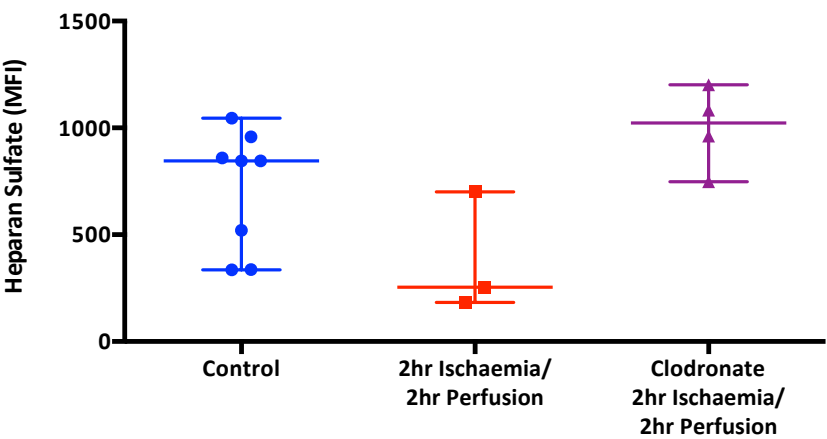


Figure 4.16 The effect of IR ±monocyte depletion on endothelial cell surface heparan sulfate

A trend towards increased HS shedding in response to IR was observed, which was potentially prevented with clodronate monocyte depletion. However the trend observed did not reach significance. Median(IQR), Kruskal-Wallis, n=3-9.

4.6 Discussion

This chapter demonstrates the key role of lung-marginated monocytes, retained in a murine model of lung ischaemia-reperfusion. Moreover it demonstrates monocyte activation and soluble mediator release in response to this form of sterile IR injury.

4.6.1 Monocyte retention and activation

Lung-marginated monocytes have been shown to play a key in the development of acute lung injury [137, 140, 143, 167, 198], accompanied with their likely complex interplay with neutrophil recruitment and activation [95, 142, 259, 260]. The aims of these experiments were elicit a precise role for monocytes in ischaemia-reperfusion, for which use of an isolated perfused lung system is ideally suited, allowing a whole-organ system to be analysed, without systemic interaction or influence. Additionally it enabled us to simplify and apply a pure 'stop flow' ischaemic injury, mirroring the clinical scenario more closely. We were able to demonstrate, for the first time that, in a mouse model of IR, significant numbers of both Ly6C^{High} and Ly6C^{Low} monocytes are retained by the lungs and this occurred despite a prolonged perfusion protocol.

Furthermore, we observed inflammatory monocyte and neutrophil activation in response to the ischaemia and reperfusion. Both Ly6C^{High} monocytes and neutrophils exhibited a drop in the constitutively expressed cell surface molecule L-selectin. In contrast, Ly6C^{Low} monocytes express limited L-selectin. This molecule is rapidly shed in response to cellular activation by the action of TNF alpha converting enzyme (TACE) via ROS pathways [138]. Activation was also exhibited by neutrophil upregulation of CD11b (MAC 1 α), part of the CD11b/CD18 heterodimer, which is strongly expressed on myeloid cells and functions as a C3 complement receptor. In contrast to L-selectin, CD11b is pre-stored in secretory vesicles, which are released on contact with an activated endothelium and rapidly incorporated into the neutrophil plasma membrane [261], playing a key role in leukocyte activation, phagocytosis, adhesion and migration.

4.6.2 Monocyte-dependent lung injury

As initially described in Chapter 3 and shown above, our mouse model of 2 hours ischaemia/2 hours perfusion induces a mild/moderate lung injury. This was evidenced through increased BAL fluid protein levels and increased lung wet:dry weight ratios. Depletion of monocytes however reversed these injury parameters, implying a key role of monocytes in this process. Of note the BAL fluid protein levels were returned to levels approaching baseline, signifying maintenance of barrier integrity at the alveolar epithelial level, by monocyte depletion. This effect could not be attributed to alveolar macrophage depletion, as their numbers remained the same. However, wet:dry weight ratios, a measure of overall lung water, were only returned to the 2 hour perfusion baseline values with

monocyte depletion, suggesting that perfusion alone is somewhat 'injurious', either compromising the endothelial barrier, or due to modified perfusion and fluid clearance dynamics, with the IPL setup producing a degree of unavoidable interstitial oedema. These findings were also supported by similar results using our fluorescent dye perfusion technique. Previous data from IPL preparations in dogs have shown that endothelial permeability to water and protein can change independently to each other [262]. IPL studies in guinea pigs has also shown that at baseline wet:dry ratios can increase as a result of perfusion time, but did not necessarily correlate to overall lung function [263]. However this phenomenon does suggest that ischaemia is integral to injury process, contributing to an alveolar protein leakage, ameliorated by monocyte depletion.

4.6.3 Monocyte depletion

To further delineate the role of intravascular monocytes in this model, we sought to distinguish their role from neutrophils and other inflammatory leukocytes. Several methods of monocyte depletion have been described with clodronate liposomes and an anti-CCR2 antibody use being widely reported. Several other groups have successfully depleted lung monocytes through the use of CD11b diphtheria toxin (DT) reporter gene targeting in the lung and other organs. Although this method claims to be more 'effective', it is likely targeting more than just the vascular population with greater penetration to interstitial and alveolar spaces and therefore is not appropriate for the study of monocytes in a single compartment e.g. vascular or alveolar. Apart from unwanted depletion of all lung mononuclear phagocyte populations, CD11b DT mediated destruction of numerous tissue cells is also likely to produce a degree of local inflammation within the lung parenchyma that may influence the IR response. An anti-CCR2 (Gr1^{high} specific) siRNA method of monocyte ablation has also been successful [264] and could be useful for subset specific studies.

We selected intravenous clodronate as an established method for depletion of vascular monocytes. This method has been widely utilised by many groups, including our own, and therefore considerable data on its physiological effects in murine models exists. It is also superior, to e.g. anti-CCR2 methods, as it is more widely available and selectively depletes all vascular monocytes populations, and crucially does not cross the endothelial barrier. Moreover, the combined use of clodronate with an isolated perfused lung system represents an optimal approach as it eliminates any of the confounding effects of clodronate-mediated depletion of splenic and liver macrophages.

Within 24hrs the liposomal clodronate induced a substantial >87% reduction in circulating blood Ly6C^{High} monocytes and >90% reduction in Ly6C^{Low} monocytes. Likewise lung monocyte numbers also dropped albeit to a slightly lesser extent. This suggests that either the resident lung monocytes are extravascular in origin (described in more detail in Chapter 5) or alternatively that the surviving monocytes are likely to accumulate in the lungs as previously described [143]. These surviving monocytes may be apoptotic but avoid clearance by sequestering in the lungs. It is also possible that clodronate access was not fully effective (to lung capillaries) or ineffective on pre-marginated cells. The desired lack of clodronate penetration through the endothelium, is supported by the number

of alveolar macrophages remaining unchanged. Of note, in support of this, the reverse is true when clodronate liposomes are delivered to the alveolar space, alveolar macrophages are depleted and splenic and liver macrophage numbers remain unchanged [265].

4.6.4 Soluble mediators: Perfusate

To evaluate the inflammation-related mechanisms of monocyte-dependent IR lung injury we quantified pro-inflammatory soluble chemokines and cytokines released in the perfusate samples taken before, during and after the IR protocol. Of these MIP-2, KC, MCP-1 and IL6 (all frequently attributed to monocytes [158, 266, 267]) showed a stepwise increase over the course of the protocol. Initial levels of MIP-2, KC, RAGE and MCP-1 were low and peaked post-reperfusion, supporting the hypothesis of an inflammatory basis for this model of 'sterile' vascular injury. Soluble RAGE showed a stepwise increase following ischaemia and also following reperfusion. This classical DAMP receptor and soluble biomarker typically attributed to epithelial injury (specifically type 1 epithelial cells [268] has also been shown to be highly expressed on endothelium [269] and reportedly involved in endothelial cell membrane repair [270] and shown to predict alveolar fluid clearance in ARDS [271]). Detection at this earlier time point indicates an initial vascular source of RAGE, as this is occurring in advance of any alveolar protein leak, and supported by the relatively large size of this protein (49kDa). Monocyte depletion exhibited a significant reduction in MIP-2, KC and perfusate soluble RAGE concentrations, indicating that they may be responsible for the propagation of ALI in this model. Alternatively these mediators are released as a result of the developing injury, which is abrogated by monocyte depletion. There was however no change in IL6, which is an indicator of injury in other organ ischemia models[272-274]. Of note MCP-1 was increased in the perfusate of mice pre-treated with clodronate, indicating that a lack of monocytes in these animals may induce accumulation of MCP and point to a physiological role in binding, internalisation and effective removal of this molecule by monocytes. It may also, however may be associated with the neutrophil influx (induced by clodronate administration) and activation in response to IR. IL-10 and other monocyte derived mediators, which would normally limit neutrophil influx, have been shown to be reduced following depletion [275].

TNF and IL1 β levels fell below the lower level of detection in our assays for our IR perfusate samples. This may be due to the (successful) significant efforts to maintain a contamination-free system (including the exclusion of commonly used 'low endotoxin' bovine serum albumin), insensitivity of the ELISAs used to low levels of TNF or the relative dynamics of cytokine release in response to this injury. Work in other models of organ ischaemia have highlighted differential release dynamics with TNF being released as earlier, but only transiently, and IL1 β and IL-6 not being released to 3-6hrs later following brain ischemia [276, 277]. The perfusate TNF production was only detectable post-reperfusion in LPS pre-treated mice. This would therefore imply that priming may be a key event in order to produce a demonstrable TNF response during lung IR. This also provides evidence that the TNF seen is not a continuation of the initial response to endotoxaemia but is produced actively in response to the IR insult. However an early rise may have been missed with TNF reported in the literature at as early as 15-30mins in

response to IR [156, 278]. TNF expression has been identified by several groups, including our own, as a marker of pulmonary inflammation in ALI [253]. Moreover TNF has been implicated in the early inflammatory response of IR, is thought to be produced by alveolar macrophages, and to mediate lung injury in this paradigm [153, 156].

4.6.5 Soluble mediators: BAL

Similar patterns to perfusate were observed in the BAL fluid with the levels of KC and RAGE again being significantly attenuated by monocyte depletion. Higher concentrations of MIP-2 (~2x) and RAGE (>10x) were detected in the BAL as compared to the final perfusate samples. In the case of MIP-2 however, the overall absolute amount was approximately the same when accounting for relative volumes of BAL (500µl) versus perfusate sampled (1500µl). When taking into account that the perfusate samples were from open (fresh perfusate non-recirculating perfusion) which would have diluted any MIP-2 effluent from the pulmonary circuit, the overall perfusate amount is in fact likely to exceed that of the BAL, indicating a vascular source. In contrast despite early detection of RAGE in the perfusate following ischaemia, it was subsequently found in large amounts in the BAL at the end of the protocol, indicating that it may well originate from in or near the alveolar space at this later time point, implying indirect alveolar epithelial injury from a vascular insult. The presence of soluble RAGE in ALI is widely reported, being highly associated with injury severity and APACHE 3 scores in human studies [269, 279]. It is regarded as marker of alveolar type 1 cell damage has also been implicated in the mechanism of IR, with RAGE blockade attenuating lung IR injury [280]. TNF was also detected in low levels the BAL but not in the perfusate, but the dilution of the perfusate samples may have limited the detection. MIP-2 and TNF levels in the BAL were not changed by clodronate depletion.

4.6.6 Lipopolysaccharide priming

To further assess the role of monocytes we sought to model the clinical scenario of pre-existing subclinical donor inflammation. This was achieved with prior low dose LPS administration, which we have previously shown to cause minimal physiological upset whilst significantly augmenting inflammatory monocyte numbers and priming the lung to subsequent injury [143]. Of note following IR, only ~25-30% of augmented monocytes and neutrophils were washed out, indicated that the newly marginated cells are likely to be activated and primed to injury, resulting in a significant proportion being retained. In keeping with this augmentation of BAL fluid protein and wet:dry ratio was seen indicating that LPS pre-treatment induces leukocyte recruitment to the lungs, priming them to pulmonary leak on exposure to IR.

4.6.7 Monocyte effector mechanisms

In addition to the attenuation of perfusate and BAL inflammatory chemokines and cytokines in response to monocyte depletion, a reduction in CD11b expression on lung neutrophils in the absence of monocytes was also observed. This suggests likely interplay between these cell populations in relation to the development of lung injury in this model. Activation observed with L-selectin shedding was also seen in response to IR, however monocyte depletion did not attenuate this, likely due to a fixed 'all or nothing' tendency to L-selectin shedding in response to activation from exposure to ischaemia. In keeping with the literature, these data imply that monocytes and neutrophils are unlikely to act alone and that injury effector mechanisms are potentially neutrophil dependent [95, 259]. It has been well characterised that neutrophils are recruited early to the inflamed lung, with prolonged monocyte influx occurring thereafter [142]. Moreover, CCR2-bearing monocytes have been shown to accelerate and significantly amplify alveolar neutrophil accumulation in a LPS model of lung inflammation [260].

Despite considerable effort to elicit putative mechanisms of monocyte-mediated IR injury in this study, investigation of the possible effector mechanisms on the endothelium, and resultant barrier dysfunction, remained inconclusive and remains a work in progress. Several other putative mechanisms that might contribute to vascular leak in this model may be involved, that were not investigated, including the role of sphingosine 1-phosphate [281]. However preliminary work did suggest that Ly6C^{High} monocyte upregulation of phosph-p38 and ERK may indicate an early cellular response to IR. Moreover monocyte-mediated shedding of the endothelial glycocalyx may also contribute to the changes in alveolar permeability, which is consistent with previous work by us, and others, implicating a leukocyte-dependent pathogenesis [84, 104].

4.6.8 Conclusion

In conclusion, we have shown that IR activates Ly6C^{High} monocytes and neutrophils and that these leukocytes are retained in the lungs after reperfusion. Monocytes-specific depletion reverses lung injury, caused by this sterile insult, and ameliorates activation of neutrophils. Likewise lung injury was attenuated along with pro-inflammatory chemokine release into the perfusate and BAL samples. Moreover we have demonstrated that sub-clinical endotoxaemia dramatically increases leukocyte margination to lungs and that for a pro-inflammatory TNF response to occur, endotoxin priming is required. Finally we have linked this inflammatory response to lung injury, potentiated by endotoxaemia, which is therefore relevant to the clinical setting. As underlying inflammation is common in lung donors, based on our findings it seems likely that primed, donor lung-marginated leukocytes could act as cellular conveyers of inflammation in graft recipients. Clinically the potential for these activated cells to propagate inflammation is likely to induce primary graft dysfunction in recipients. Therefore identification of precise pre- and peri-transplant pro-inflammatory mechanisms are key in assisting in the assessment and optimisation of donor organs. This work has mapped leukocyte margination, activation status and quantity to the production of inflammatory chemokines and TNF, and linked it to direct pulmonary injury. However the precise

location and role of the monocytes retained within the systems remains unclear and in the next chapters we look to clarify these points (Chapter 5) and translate these findings into monocytes identification and quantification of activation in human lung samples (Chapter 6).

Chapter 5

Mononuclear Cell Compartments and Dynamics During Ischaemia-Reperfusion

5.1 Abstract

Introduction: We have previously shown that lung mononuclear cells ('monocytes') play a key role in our IPL model of IR. However the exact location of these cells remains unclear. Several studies have highlighted the role of donor mononuclear cells in lung IR and in the co-stimulation of host T cells in the immune response following transplantation. We sought to identify the phenotype and location of mononuclear phagocytes within the lung and re-establish IR injury through adoptive transfer of blood monocytes.

Methods: To identify cell compartments, the pan-leukocyte marker CD45 was administered intravenously whilst CD45.2 was instilled into the alveolar space immediately prior to lung dissociation. Cell suspensions were subsequently stained with other identifying cell surface markers and for the T cell co-stimulatory mononuclear cell molecule CD86. Several adoptive transfer techniques were trialled, with and without the use of cell stains (CFSE, CD45). BrdU RNA staining was also piloted.

Results: Dual compartment staining allowed identification of 3 distinct lung compartments; 'vascular', 'alveolar' and 'interstitial'. The majority of Ly6C^{High} CD11b⁺ F4/80⁺ monocytes were located in the 'vascular' compartment. In contrast, the Ly6C^{Low} CD11b⁺ F4/80⁺ monocyte population were found in significant proportions in the 'vascular' and 'interstitial' compartments. A significant number of dendritic cells were also identified in the interstitial compartment. CD86 expression in response to IR was greatest on the interstitial Ly6C^{Low} CD11b⁺ F4/80⁺ and dendritic cell populations, but also significantly upregulated on vascular Ly6C^{High} monocytes.

Adoptive transfer of blood monocytes into monocyte-depleted lungs returned their numbers to that of untreated mice. Furthermore, adoptive transfer reinstated the levels of BAL protein, wet:dry weight ratio, and perfusate KC, seen previously in response to IR.

Conclusion: We developed a lung compartment dual staining technique to allow differentiation between 'vascular', alveolar, and by process of elimination, 'interstitial' compartments. This allowed assessment of CD86 upregulation of several lung mononuclear cell populations, specifically highlighting the presence of the interstitial mononuclear cell populations, which are inevitably carried over as 'passengers' on transplantation of a lung.

After trialling several methods, isolated monocytes were successfully adoptively transferred into monocyte-depletion lungs subjected to IR. This also re-established the injury and inflammatory cytokine release seen in control lungs exposed to IR, further verifying the key role for vascular monocytes in this model.

5.2 Introduction

5.2.1 *The role of lung monocytes in graft dysfunction*

The ability to localise mononuclear phagocytes in the different lung compartments is critical to understanding their role in IR injury. Previous work has frequently labelled cells remaining in the lungs following vascular perfusion as 'interstitial'. However very recent data (published during the course of this body of work) has demonstrated a large vascular population remains, following a perfusion protocol designed to wash out non-adherent (non-marginated) cells [130]. This has thrown into question exactly what and where 'interstitial' versus 'marginated' versus 'circulating' populations exist within the lung. In addition to identification of cell phenotypes within these compartments, definition of their role and response to IR is critical to targeting therapy to combat this form of injury in, for example, lung transplantation. With the use of our murine IPL system, it is possible to perform detailed compartment analysis of cell phenotype and activation. This technique supersedes those used with human lung biopsies, which rely on semi- or non-quantitative methods of direct visualisation of constituent cell populations, where the paper-thin alveolar-interstitial-endothelial borders can be difficult to delineate precisely. Although vascular depletion in this study (Chapter 4) strongly suggests a central role for lung-marginated monocytes, the dynamics of their retention and activation, as well as that of other mononuclear phagocytic cells in the lungs, requires further analysis.

Retention of vascular, interstitial and alveolar mononuclear leukocytes is likely to be critical in lung transplantation, where donor mononuclear cells would be carried over as 'passenger' cells, and in the case of intravascular cells will interact directly and immediately with the recipient blood. Moreover the 'passenger' vascular monocytes will be exposed directly to ('stop-flow') ischaemia, which can cause direct activation, inflammation and injury [165]. This may then also modulate recipient blood leukocyte recruitment. Whilst flow cytometry of lung cell suspensions suggests that vascular phenotype monocytes are likely to be margined, trapped in the pulmonary vessels due to conformational changes, there is also a substantial pool of mononuclear cells present in the capillary interstitial space and lymph nodes. Similarly, these interstitial leukocytes may be capable of sensing the response of the pulmonary capillaries to ischaemia. In donor lungs, interstitial cells are also impervious to removal prior to transplantation, and thus represent a further population of leukocytes that will be transferred to and remain in recipients [282]. All of these 'passenger' cells are capable of activation and propagation of short-term injury and long-term rejection in recipients.

5.2.2 *Monocyte dynamics: compartment identification and adoptive transfer*

We developed a novel methodology, and applied it to the IPL, to differentiate between the lung leukocyte compartments and thus monitor their trafficking dynamics in response to ischaemia-reperfusion. This builds on our current flow cytometry based techniques, which only provided representation of the 'whole lung' picture. Classically, histological techniques have been utilised to differentiate between populations, but differentiation

between 'thin-walled' compartments are difficult to distinguish in the lung. In addition, the technique is limited by artefact. For example formaldehyde preservation can damage or mask antibody binding sites. Intra vital fluorescent microscopy provides a novel approach of observing leukocytes *in vivo* [283-286] but is technically time consuming (with low through-put) and complex. It is as yet unable to fully distinguish discreet subpopulations or quantify their activation status. Flow cytometry however, enables the ability to interrogate more complex phenotypes and concomitant functional markers simultaneously.

In the past other groups have relied on IPL or bronchoalveolar lavage techniques to 'washout' the vascular and alveolar compartments respectively. However we have shown that this is ineffective in respect of the vasculature, and colleagues have demonstrated a similar limitation on alveolar washout of macrophages [167, 168]. A variety of techniques to enumerate vascular monocytes and track their locations were undertaken as described below. As shown in Chapter 4, following the IR protocol a significant proportion of cells with the phenotypic characteristics of blood neutrophils and monocyte subsets remained in the lungs (Chapter 4). To investigate this phenomenon further, we modified previous dual-staining flow cytometry-based methods to determine leukocyte locations in different lung compartments (alveolar, interstitial and vascular) [129, 130]. To this end, previous groups had instilled antibodies simultaneously into the vascular and alveolar compartments. They had then lavaged the alveolar space in an effort to prevent contamination of leukocytes with excess antibody during standard enzymatic lung dissociation, thus effectively removing a large quantity of macrophages [130]. In our study and as recently reported by us [287], by omitting this lavage (i.e. leaving the stain *in situ*) and using a rapid 1minute dissociation/fixation and dilution technique, these disadvantages and potential artefacts were largely abrogated, .

To further characterise the dynamics of monocyte margination and its contribution to IR injury, we performed 'adoptive transfer' of labelled monocytes to assess retention of circulating monocytes in perfused lungs, and as a means to restore intravascular monocytes to the lungs after clodronate-liposome depletion, to further define their role in IR-induced injury.

5.3 Hypothesis and Aims

We hypothesised that a significant proportion of lung mononuclear cells resided within distinct compartments of the lung, allowing them to be carried over as ‘passenger’ cells and propagating inflammation in recipients. Furthermore, based on the depletion studies in Chapter 4, we hypothesised that replenishment of vascular monocytes would re-establish lung injury following IR.

Our aims were therefore:

- To identify and validate the precise compartmental location of lung mononuclear phagocytes using a dual staining technique
- To characterise the expression of the co-stimulatory inflammatory molecule CD86 on mononuclear cells in response to IR
- To attempt the restoration of IR-induced lung injury in depleted lungs with monocyte adoptive transfer

5.4 Methods

5.4.1 Compartment staining

The dual-labelling compartment staining method described here was previously developed for *in vivo* use, and evaluated in naïve as well as acid injured mice in our laboratory by Dr Brijesh Patel. Here we describe its application to our *ex vivo* IPL model of IR.

Mice were injected with anti-CD45 antibody (PE Texas Red, 2µg) and euthanised after 5 minutes by terminal anaesthesia. Following exsanguination and tracheostomy, the left lung was tied off for wet:dry analysis and anti-CD45.2 antibody (2µg in 500 µl NaCl 0.9%) instilled into the right lung bronchoalveolar space. The same procedure was applied at the end of two IPL protocols (15 minutes washout or 2hr ischaemia/2hr perfusion). After 5mins the lungs were excised and placed directly into 2ml of fixative in a gentleMACs tube and dissociated for 60 seconds. Flow cytometry buffer (8ml) at 4°C was then added to further dilute labelled antibodies and stop the fixation reaction. Samples were then stained according to the following panels (Table 5.1).

<i>Channel</i>	<i>Monocyte Panel</i>	<i>Neutrophil Panel</i>
1. FITC	F4/80	F4/80
2. PE	CD11b	Ly6G
3. PE Texas Red	Intravenous CD45	Intravenous CD45
4. PerCP	MHC II	MHC II
5. Pe-Cy7	Ly6C	CD11c
8. APC	Intratracheal CD45.2	Intratracheal CD45.2
9. APC-Cy7 (Ax780)	CD11c	CD11b

Table 5.1 Compartment staining antibody panels

5.4.2 CFSE Labelling

To perform adoptive transfer of labelled cells, the intracellular label CFSE (carboxyfluorescein diacetate succinimidyl ester) was utilised as previously described (see Chapter 2) [3]. Leukocytes were isolated from 1ml of heparinised blood and separated via Histopaque density gradient separation for mononuclear leukocytes, before incubation with CFSE (2µM 5mins at room temperature). These cells were then injected into the IPL, directly into the pre-flushed pulmonary circulation.

5.4.3 BRDU Labelling

BrdU labelling of dividing monocytes *in vivo* was used as a strategy to metabolically label cells avoiding the potential artefacts of dye labelling *in vitro*. Recently divided bone marrow cells, including Ly6C^{High} monocytes, were labelled by intraperitoneal injection of 200µl Bromodeoxyuridine (BrdU), 24hrs prior to experimentation. After IPL, lung cell suspensions were prepared as before and then subjected to an extended fixation, permeabilisation and DNase digestion technique, incorporating staining with anti-BrdU antibody (APC, clone 3D4) in addition to the standard leukocyte identification antibodies (see Chapter 2).

5.4.4 Monocyte Isolation and Adoptive Transfer

Bone marrow monocyte isolation was performed from the femurs of control mice using a monocyte MACS magnetic bead separation kit. For freshly harvested whole blood however, a StemCell magnetic bead negative selection column was utilised as per protocol (see Chapter 2).

5.5 Results

5.5.1 Leukocyte dynamics and trafficking

Leukocytes in the intra-alveolar and intra-vascular spaces were labelled separately by administration of two anti-CD45 antibodies with different epitope specificities conjugated to different fluorophores:

- (a) intravenously (anti-CD45 clone 30-F11, PE-Texas Red) *in vivo*, immediately prior to sacrifice
- (b) via broncho-alveolar lavage (anti-CD45.2, clone 104, APC), at the end of the experimental protocol, *ex vivo*.

Single cells suspensions were then prepared using the rapid tissue dissociation and fix method, stained with further identifying anti-leukocyte antibodies and analysed by flow cytometry. Using this technique we could identify the location of three leukocyte compartments: 'vascular', 'alveolar' and an 'interstitial' CD45/CD45.2 double-negative population (Figure 5.1). The distinction between the 'vascular' (Q3) and 'interstitial' compartments however was not entirely clear, likely to be due to the lower CD45 expression on neutrophils compared to mononuclear cells. Importantly, virtually no CD45 double positive events (<1%) were observed in lung cell suspensions indicating that with this method there was no cross-compartment leukocyte staining *in situ* or following tissue harvest and preparation of the single cell suspension (Figure 5.1).

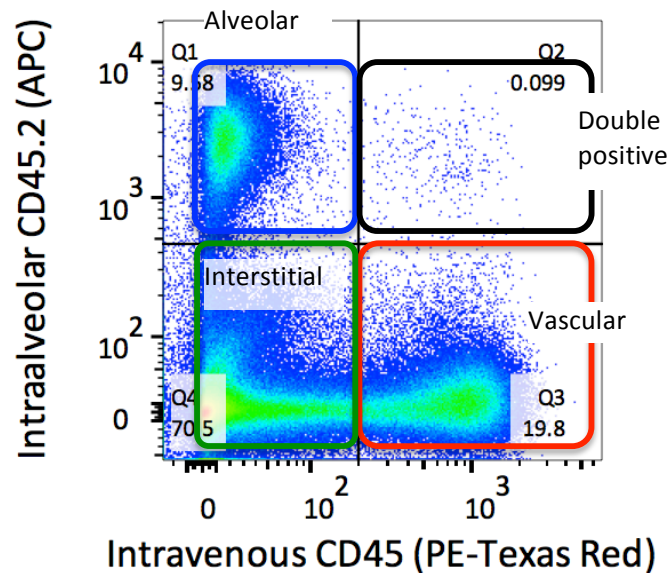


Figure 5.1 Intravenous versus intraalveolar CD45 compartment staining.

Typical plot of stained lung cell suspension gated on intravenous and intraalveolar CD45 antibodies applied *in vivo*. Q1 represents the alveolar CD45⁺ cells, Q3 represents the CD45⁺ intravascular cells, Q4 represents the interstitial compartment. Less than 0.1% of the cells stained double-positive (Q2) indicating minimal compartmental spill-over. Lower expression of CD45 on neutrophils as compared to mononuclear cells limited differentiation between the vascular and interstitial compartments.

To enhance distinction of mononuclear phagocytic cells in the ‘vascular’ and ‘interstitial’ compartments, neutrophils were excluded with initial identification of the CD11b+, F4/80+ populations (Figure 5.2 A&B) and subdivision into Ly6C^{High} and Ly6C^{Low}, before gating on the compartment stains (Figure 5.2 C).

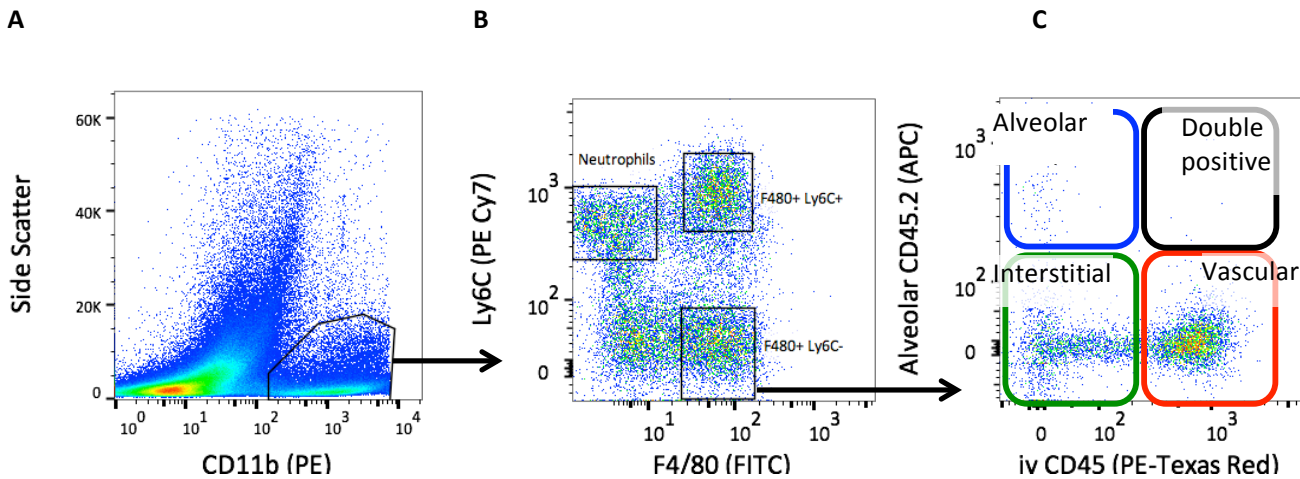


Figure 5.2 Illustrative plots of monocyte/macrophage cells identified in the compartments of the lung.

To aid differentiation between the ‘vascular’ IV CD45+ population from CD45- ‘interstitial’ population, neutrophils were gated out by positive selection of CD11b+ (A), then F4/80+ Ly6C^{High} and Ly6C^{Low} mononuclear cell populations (B) and into the compartments thereafter (C).

5.5.2 Validation of leukocyte trafficking across compartments

To further validate this method, including the identification of leukocytes within the alveolar walls, i.e. the ‘interstitial space’ (as opposed to bronchial or lymphatic tissue), *in vivo* cross-compartment leukocyte trafficking was assessed utilising intra-tracheal application of LPS (20µg). As previously shown neutrophils are rapidly recruited to the alveolar space following intra-tracheal LPS, where they extravasate from the capillaries, and therefore are an ideal population to examine transmigration from the vascular to the interstitial and then alveolar spaces [92, 93, 288-290]. Neutrophils were identified in lung cell suspension as a CD11b⁺ Ly6G⁺ population and assigned to the relevant compartment as per the gating strategy in Figure 5.1. These data confirmed the ability to ‘track’ a leukocyte subpopulation, normally present only in the vascular space, across the three compartments over time using our compartment staining method (Figure 5.3). Moreover, it clearly confirms the presence of an identifiable ‘interstitial’ compartment.

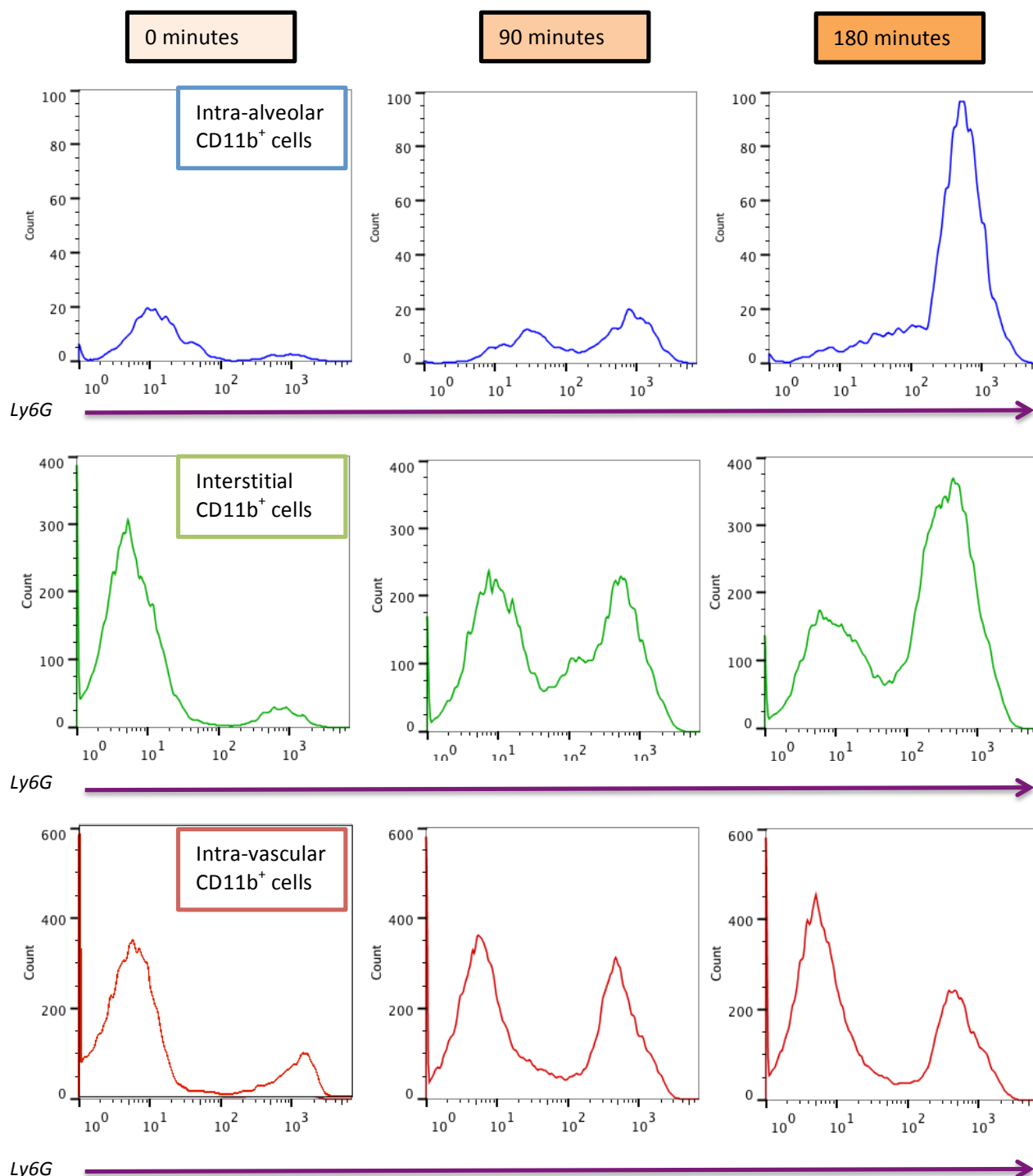


Figure 5.3 Validation of compartment tracking of leukocytes using readily identifiable neutrophils in response to alveolar stimulus (LPS)

Neutrophils (number of CD11b⁺/Ly6G⁺ events) increase initially in the vascular (red), then interstitial (green) and finally the alveolar space (blue) at 90 and 180 minutes post intra-tracheal LPS administration.

5.5.3 Lung leukocyte retention following continuous perfusion

To investigate further the substantial retention of lung monocytes and neutrophils seen after IR in Chapter 4, lungs were subjected to a 15 minute open perfusion ‘washout’ period following CD45 compartment labelling, as described above. Intravascular monocyte numbers were significantly reduced, but a substantial proportion remained: ~50% of Ly6C^{High} and ~65% of Ly6C^{Low} (Figure 5.4), comparable to the values observed at the end of the IR protocols previously. As expected interstitial cells were not significantly altered in number by perfusion, further supporting their extravascular location. Moreover numbers of monocytes recovered in the perfusate samples from the 15 minutes of perfusion approximated to the reduction seen in perfused compared control non-perfused lungs (Table 5.2).

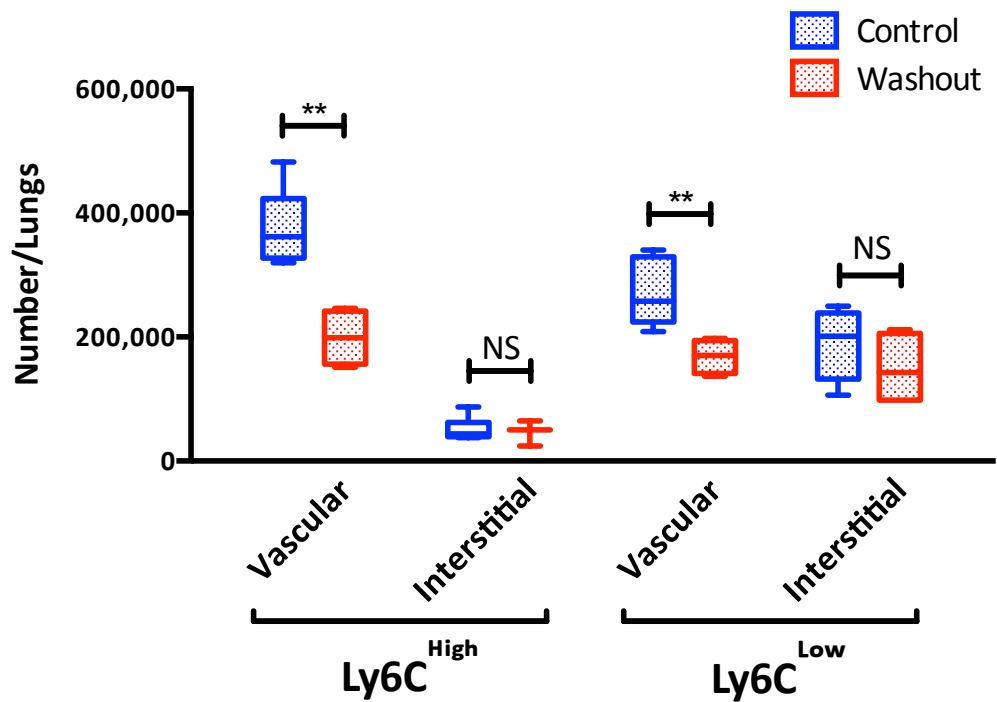


Figure 5.4 Intravascular lung monocytes are partially washed out following perfusion whilst interstitial monocytes are not.

A significant proportion of vascular Ly6C^{High} and Ly6C^{Low} monocytes were washed out during 15 minutes non-recirculating perfusion when compared to unperfused controls. However a substantial number remained within the lungs at the end of this period. Numbers of interstitial Ly6C^{High} and Ly6C^{Low} monocyte/macrophages remained unchanged. Median(range), n= 3 washout, n=6 control, Mann-Whitney, **p<0.01

	Ly6C ^{High} monocytes	Ly6C ^{Low} monocytes
Perfusate monocyte count (x10 ³)	122.5 (100-143)	74.3 (29-115)

Table 5.2 Total number of perfusate monocytes collected following 15minutes lung perfusion

Monocyte numbers were quantified in perfusate samples collected during the entire 15 minute washout periods via flow cytometry. Median (IQR), n=4

5.5.4 Phenotyping of interstitial mononuclear phagocytes and their potential role in IR Injury

Interstitial monocytes can be identified using MHC II

Examination of the interstitial compartment, using the intravascular versus intraalveolar CD45 dual staining technique above, yielded a significant population of interstitial cells. Owing to their location and the ‘washout’ data (Figure 5.4) it follows that these cells remain in the lung post harvest from donors and are transferred over as ‘passengers’ on implantation of the graft in the recipient. Our initial hypothesis explored the possibility that margined monocytes, which can vary dramatically in number [143], are carried over and likely to play a role in determining the severity of subsequent acute lung injury seen in recipients. However, the interstitial mononuclear cell/macrophage population, impervious to washout, may also be critical to the injury process, located strategically between the vascular and alveolar environments with the potential to exert significant effects on barrier function. To define the interstitial macrophage (F4/80⁺) population further, the bulk of which displayed a similar surface marker phenotype to Ly6C^{Low} blood monocytes, we performed additional flow cytometric analysis focussing on MHCII expression, which has been previously described as a marker of lung interstitial macrophages [291, 292]. After initial identification of the Ly6C^{Low} monocytes (CD11b⁺/F4/80⁺/Ly6C^{Low}), the CD45 dual alveolar and intravascular gates are used to identify the ‘interstitial’ and ‘vascular’ population as above. Double negative (intravascular CD45⁻ and alveolar CD45.2⁻) interstitial Ly6C^{Low} monocytes were also positive for MHC II staining (Figure 5.5, green box). Intravascular (intravenous CD45⁺ only) Ly6C^{Low} monocytes were negative for MHCII staining (Figure 5.5, red box). Therefore MHCII was utilised to differentiate intravascular from interstitial Ly6C^{Low} monocytes, obviating the need for the dual staining technique thereafter (Figure 5.5). MHCII was also utilised to positively identify interstitial CD11b⁺/Ly6C^{Low}/F4/80⁻/CD11c⁺ dendritic cells (Figure 5.6). As minimal numbers of Ly6C^{High} monocytes were identified to be in the interstitial compartment they were considered to be all vascular in origin thereafter. Following this validation process a final gating strategy for all lung leukocytes was determined as per Figure 5.6 and a new single antibody panel was utilised thereafter (Table 5.3).

Table 5.3 Compartment antibody final panel

Channel	Antibody
1 FITC	F4/80
2 PE	-
3 PE Texas/CF594	CD11b
4 PerCP/Pe-Cy5	MHC II
5 PeCy7	Ly6C
8 APC/AX 647	Ly6G
9 APC-Cy7 (eFluor 780)	CD11c

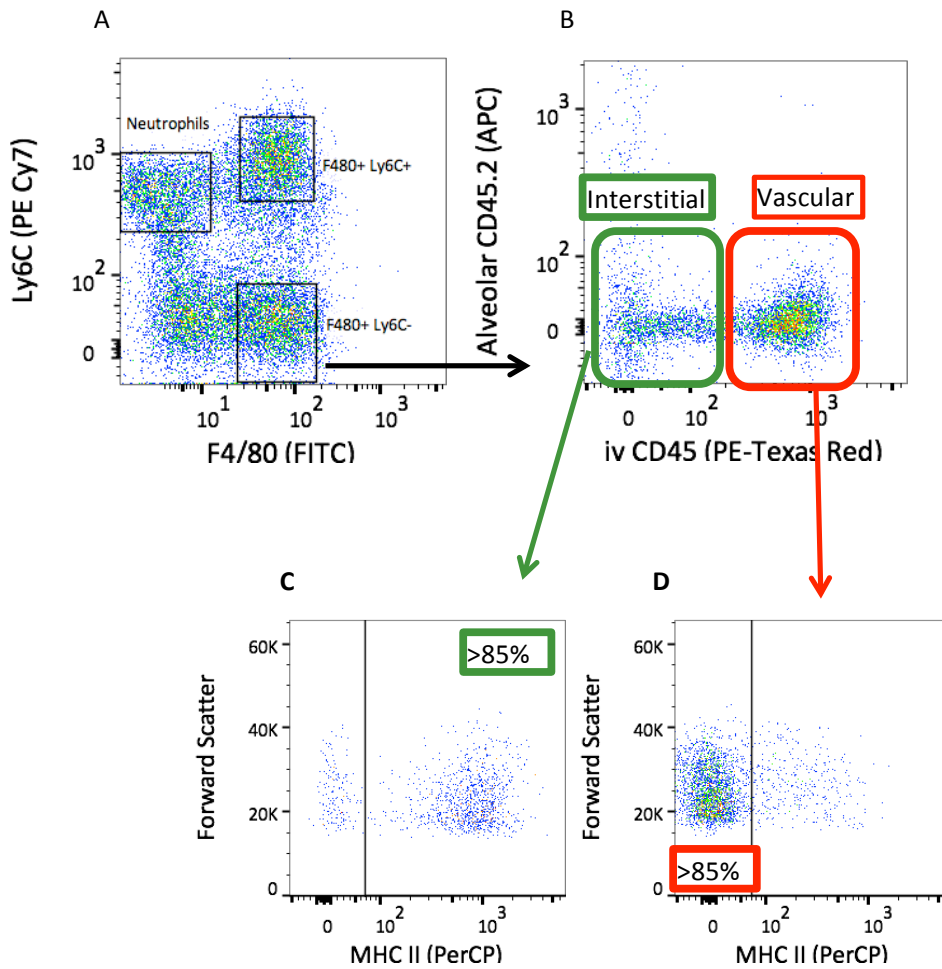


Figure 5.5 Validation of an alternative method of identifying interstitial Ly6C^{Low} monocytes using MHCII.

Mononuclear cells were identified (as per figure 5.1) as $\text{F4/80}^+ \text{Ly6C}^{\text{High}}$ and Ly6C^{Low} (A) and as 'interstitial' and 'vascular' with the compartment staining (B). The majority of the Ly6C^{Low} 'interstitial' population were MHCII+ (C) and likewise the 'vascular' compartment as MHC II negative (D). MHC II was utilised as a surrogate of $\text{CD11b}^+/\text{F4/80}^+/\text{Ly6C}^{\text{Low}}$ mononuclear cell location thereafter.

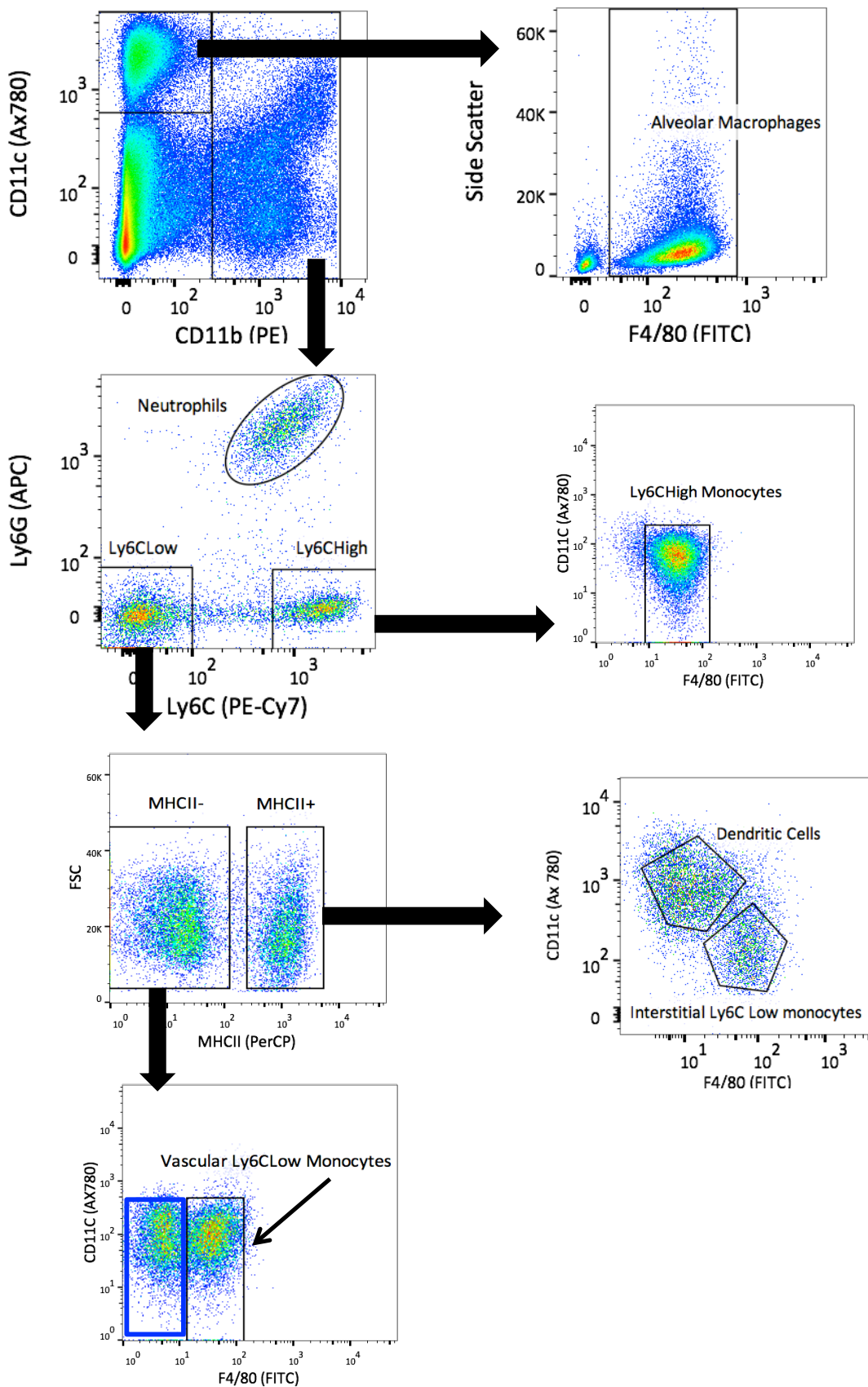


Figure 5.6. Lung leukocyte compartment gating strategy utilising MHCII, without the requirement for compartment labelling

A reproducible gating method was used to identify the constituent lung mononuclear cell population. (The $CD11b^+/Ly6C^-/MHCII^-/F4/80^-$ population (blue box) represent NK cells, verified separately with NK1.1+ staining which is not shown)

5.5.5 Interstitial monocytes are activated in response to vascular inflammation

To investigate the potential for interstitial macrophages to transfer inflammation from lung donors to graft recipients as a ‘passenger’ population, their response to clinical or sub-clinical systemic inflammation was evaluated. In preliminary experiments we determined the response of lung leukocytes 2 hours following systemic LPS injection, quantifying CD86, a T cell co-stimulatory molecule relevant to transplant immunology, and a well described marker of monocyte activation [293-295]. Two hours following intravenous LPS injection, the Ly6C^{Low} interstitial macrophage population exhibited the most marked CD86 upregulation (Figure 5.7) and dose response experiments indicated that maximal activation occurred following 20µg LPS intravenous injection. Even at the sub-clinical dose of 2µg/mouse, and this early time point, a significant upregulation of CD86 was observed on interstitial macrophages. Interestingly, CD86 upregulation was more marked in interstitial populations, including dendritic cells, than in intravascular monocyte subsets exposed directly the LPS challenge. The observations under relatively mild inflammatory conditions suggest the existence of an interstitial sensing mechanism capable of detecting vascular signals in the absence of substantial barrier dysfunction.

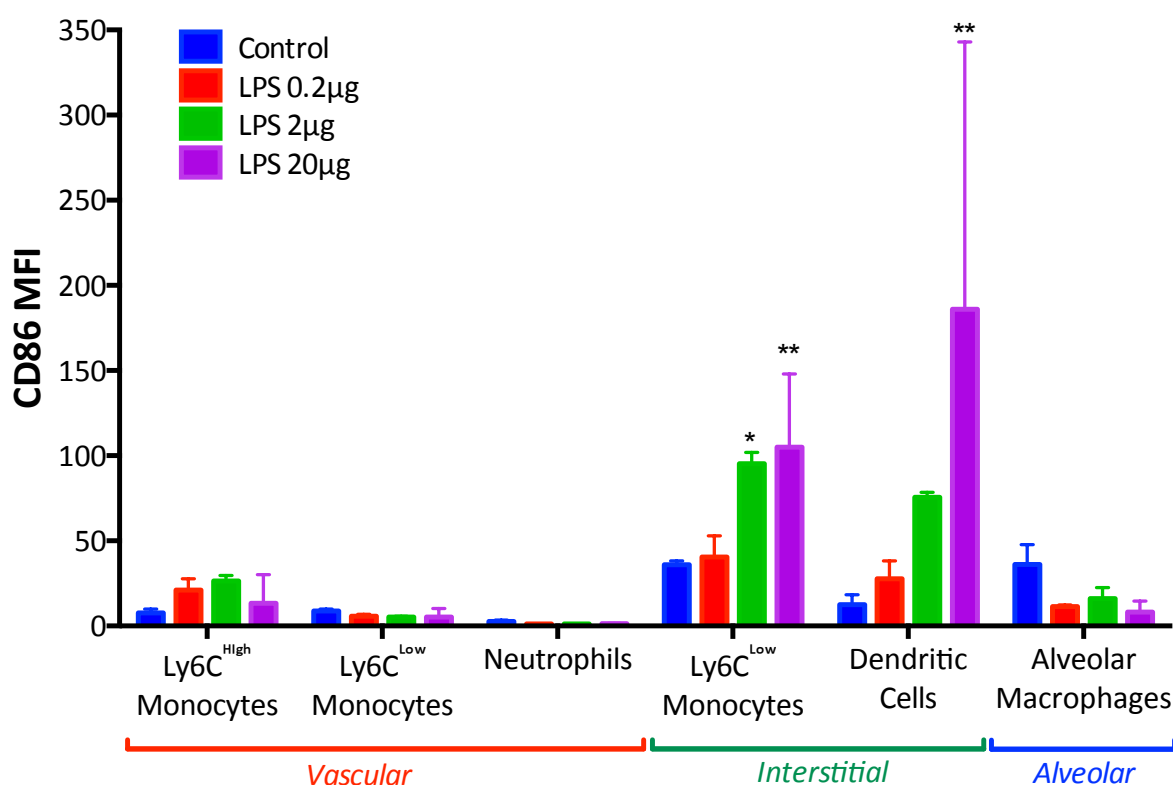


Figure 5.7 CD86 expression on lung leukocytes in response to intravenous LPS

Median(IQR), Kruskal-Wallis, n = 2-6, *p<0.05, **p<0.01, vs' corresponding controls (blue bars)

5.5.6 CD86 upregulation occurs following IR, with and without clodronate-liposome depletion

Following demonstration of this sensitive and early response by interstitial leukocytes to LPS, we investigated upregulation of CD86 expression on mouse lung leukocytes exposed to ischaemia-reperfusion (with or without prior clodronate-liposome depletion). Numbers of interstitial monocytes were not affected by clodronate depletion of vascular monocytes, confirming that they are not directly accessible by the intravenous liposomes (Table 5.4).

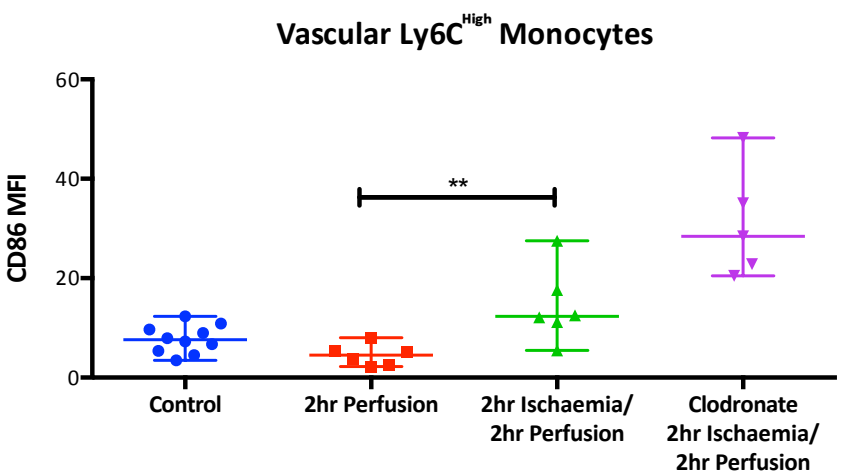
Table 5.4 Number of lung vascular and interstitial leukocytes following clodronate depletion

Median(IQR) $\times 10^5$ /lungs, n=6-10, Mann Whitney ** $p < 0.01$, *** $p < 0.001$ vs' control mice.

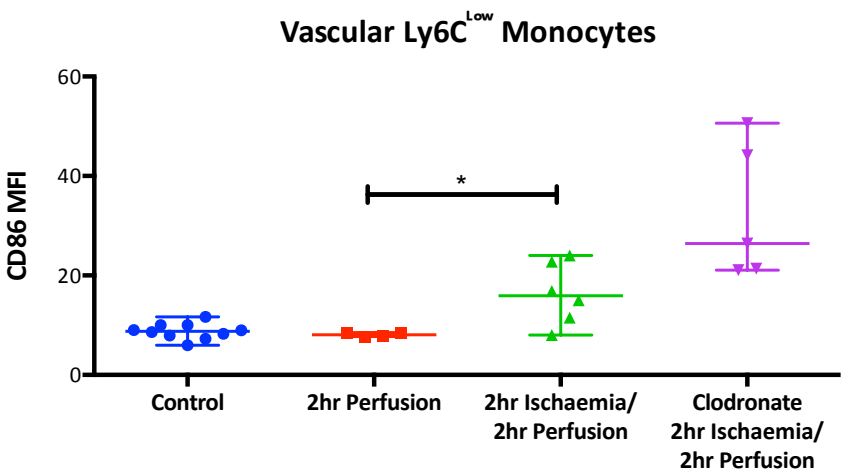
	VASCULAR Ly6C ^{High} Monocytes $\times 10^5$	VASCULAR Ly6C ^{Low} Monocytes $\times 10^5$	INTERSTITIAL Ly6C ^{Low} Monocytes $\times 10^5$	INTERSTITIAL Dendritic Cells $\times 10^5$	ALVEOLAR Macrophages $\times 10^5$
Control mice	4.51(3.3-7.3)	3.15 (3.0-4.8)	0.90(0.7-1.3)	1.93(1.5-2.3)	18.8(17.0-24.4)
Clodronate treated mice	1.98(1.6-2.3)***	0.72(0.3-1.0)**	0.70(0.5-1.6)	1.81(1.6-2.2)	18.98(18.5-21.3)

Vascular Ly6C^{High}, and Ly6C^{Low} leukocytes populations exhibited a significant increase in CD86 expression in response to IR, while 2hrs perfusion alone (no ischaemia) had a negligible effect (Figure 5.8 A & B). Greater CD86 upregulation was noted on Ly6C^{Low} interstitial macrophages exposed to IR (median 89.0; IQR 76-8-99.8) compared to the vascular Ly6C^{Low} (16.0; 10.6-23.1) and Ly6C^{High} (12.3; 9.8-20.1) populations (Figure 5.8 C). Unlike the soluble markers of inflammation in Chapter 4, clodronate-liposome depletion of vascular Ly6C^{High} and ^{Low} monocytes did not impact significantly on interstitial Ly6C^{Low} CD86 upregulation, suggesting that this response is not directly related to the vascular monocyte-dependent IR injury. Although, CD86 expression was upregulated in the small number of residual vascular Ly6C^{High} and ^{Low} monocytes following clodronate depletion and IR (Figure 5.8 A and B purple triangles), this may indicate that this residual population is still capable of driving the interstitial macrophage/DC activation. Dendritic cells exhibited a similar pattern of CD86 expression to the interstitial monocytes. Alveolar macrophages and neutrophil populations showed no difference in CD86 expression following IR.

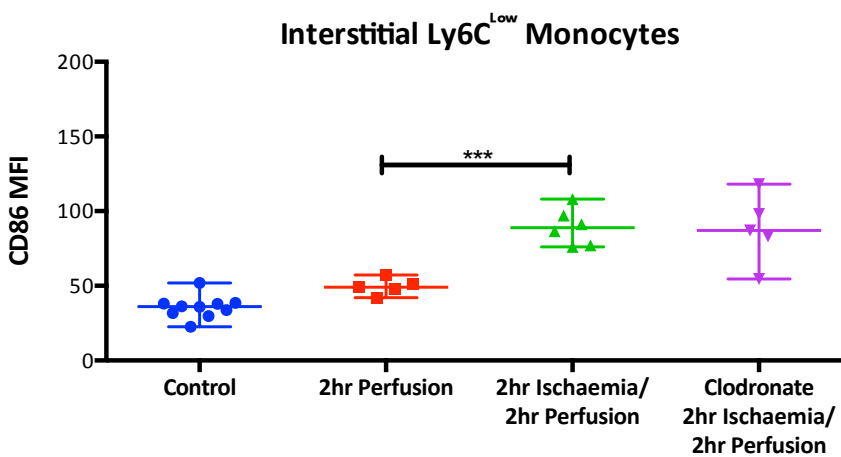
A



B



C



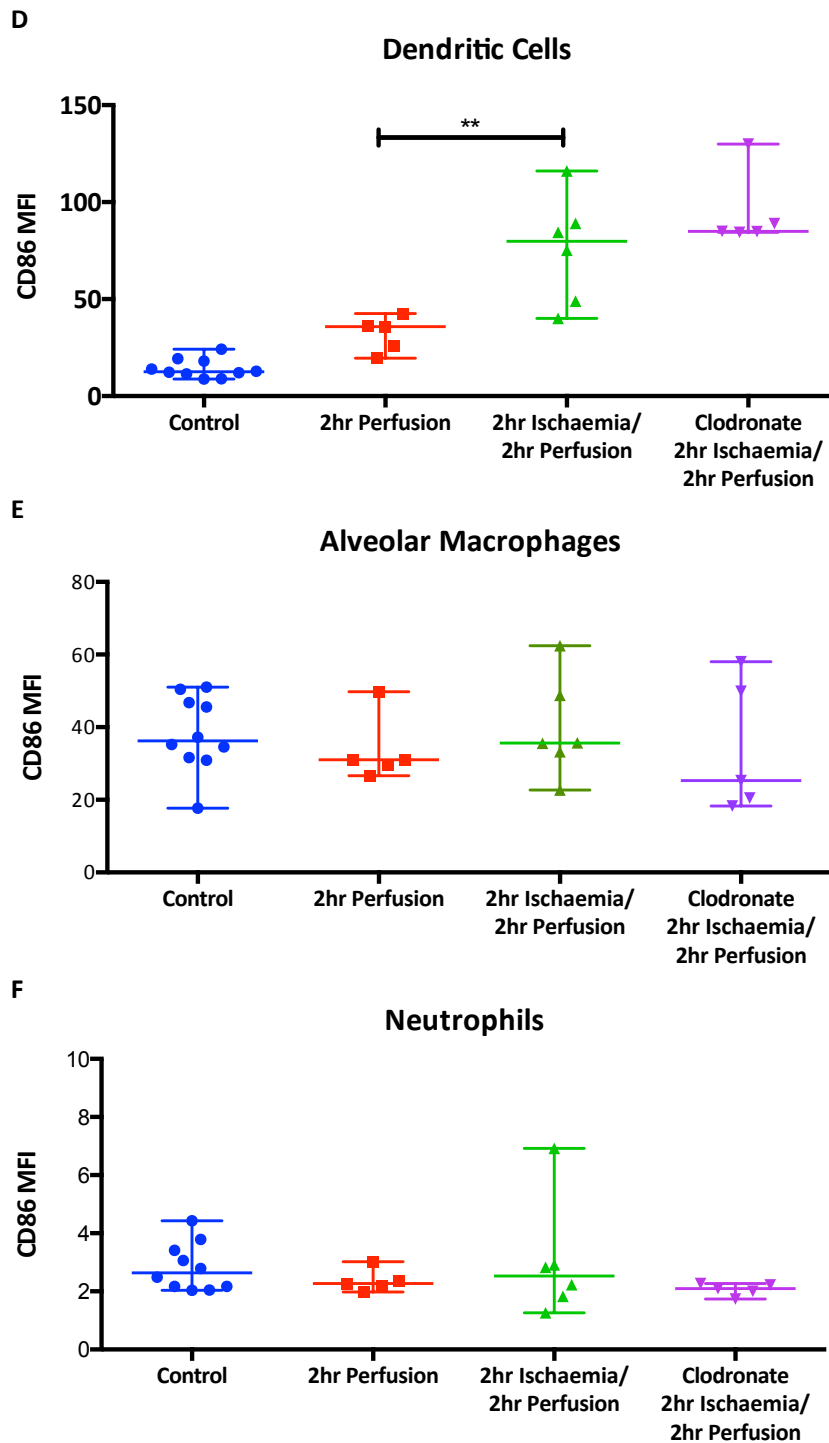


Figure 5.8 Lung leukocyte CD86 activation following ischaemia-reperfusion ±vascular monocyte depletion CD86 expression

Vascular $Ly6C^{High}$ monocytes exhibited increased CD86 expression in response to IR (A, green triangles). This appeared to be further augmented in the few remaining $Ly6C^{High}$ monocytes following clodronate depletion, although this may represent the phenotype of the residual firmly adhered/marginated and activated, cells (A, purple triangles). A similar pattern of activation was observed for the vascular $Ly6C^{Low}$ monocytes (B). The interstitial $Ly6C^{Low}$ monocytes exhibited a significantly higher level of CD86 expression following IR, and remained at this level despite vascular monocyte depletion (C). This pattern was replicated by the dendritic cells (D). No change in CD86 expression was observed on the alveolar macrophage (E) and neutrophil (F) populations. (Median(IQR), $n=5-10$, Kruskal-Wallis/Dunns * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

5.5.7 Investigation of monocyte margination by cell adoptive transfer

CFSE Labelling

Two alternative explanations for the substantial retention of vascular monocytes during perfusion were proposed:

- (a) They are a resident non-circulating sub-population firmly attached to the endothelial luminal surface
- (b) They are normally circulating cells, but have become adhered or sequestered due to the IPL surgical procedure, prior to perfusion

To address these, or other possible explanations, we performed adoptive transfer with circulating monocytes isolated by Histopaque density gradient separation from donor mouse blood, and labelled with the intracellular fluorescent dye CFSE. These cells were then adoptively transferred via the IPL into the pre-flushed lungs (5 minutes) of a recipient mouse. This protocol entailed a 2 minute CFSE-labelled cell infusion and 15 minute washout period.

Figure 5.9 indicates that a significant proportion of CFSE-labelled $\text{Ly6C}^{\text{High}}$ monocytes (median 38%, IQR 31-66) remained in the lungs after the 15min washout period (red bar). The total number of CFSE-labelled cells recovered in the perfusate was however minimal (green bar), with a significant proportion manually aspirated from the input, bubble trap and output/efferent tubing after the protocol (purple bar) suggesting monocyte adherence. Thus, although substantial numbers of transferred monocytes were retained in perfused lungs suggesting a surgery-related effect, the *in vitro* labelling procedure itself (pre-IPL) may also have contributed through increased adhesiveness or reduced deformability. In addition to tubing retention (reducing cell recovery from lung), cell loss during their harvest from lung tissue and subsequent processing, is also quite likely and points to potentially a general underestimation of margined cells based on flow cytometric analysis.

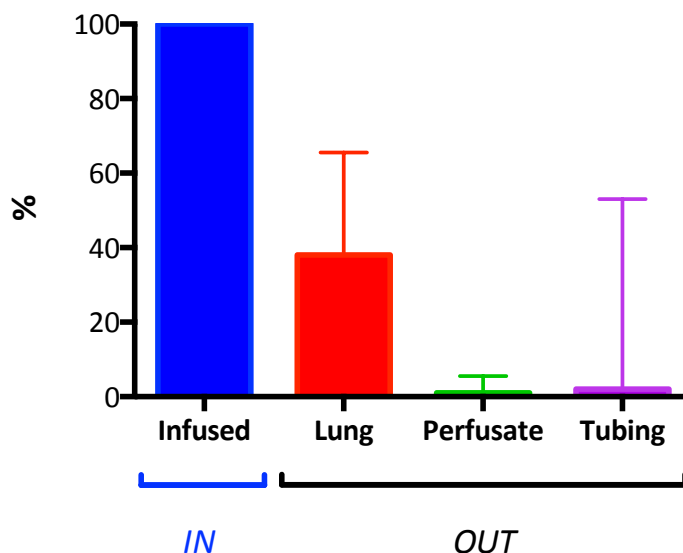


Figure 5.9 Number of CFSE-labelled $\text{Ly6C}^{\text{High}}$ monocytes infused in ('IN') and collected ('OUT') following a 15 minute open washout period.

On average less than half of infused CFSE-labelled cells remained in the lungs at the end of the protocol, with very few being recovered from the perfusate and tubing. Median±IQR, n= 3-5, 'OUT' monocytes expressed as percentage of total 'IN'.

CD45 Labelling

To attempt a simplified alternative method of labelled cell transfer, leukocytes were isolated in a similar fashion and pre-stained briefly with CD45.2 *in vitro* and infused into the lungs via the isolated perfused lung. Again a large proportion of CD45.2-labelled cells remained unaccountable, likely to be due to similar reasons to the CFSE labelled cells outlined above. (Data not shown)

Investigation of BRDU as an *in vivo* labelling method

Owing to the uncertainties produced by fluorescence labelling of cells *in vitro*, we investigated an alternative strategy of identifying donor cells, with BrdU *in vivo*. BrdU is a nucleoside analogue of thymidine and is substituted for it during DNA replication, allowing identification of recently divided bone marrow derived cells after their entry into the blood circulation. BrdU was injected intra-peritoneally 24hrs prior to experimentation to label Ly6C^{High} monocytes. As a preliminary experiment to assess the behaviour of these newly emergent cells in the lungs, we prepared and stained fixed lung cell suspensions with a fluorophore-conjugated anti-BrdU antibody, detected by flow cytometry. It should be noted that due to the relatively short half-life of the injected BrdU, only a proportion ($\leq 50\%$) of recently divided cells were labelled by this method, as previously reported [296]. Using the IPL 2hr ischaemia/2hr perfusion protocol, 41% of Ly6C^{High} monocytes retained in the lung were BRDU-positive, compared to a control BRDU-stained mouse (52% BRDU positive Ly6C^{High}). These similar proportions confirmed that this *in vivo* labelling technique does not cause a significant alteration in washout or retention characteristics of monocytes, and that marginated recently bone marrow derived Ly6C^{High} monocytes are unlikely to be a 'resident' vascular population. This may therefore provide a suitable alternative for labelling in future *ex vivo* adoptive transfer techniques.

5.5.8 Augmenting lung vascular monocyte numbers: adoptive transfer

Data from Chapter 4 highlighted the key role of vascular marginated monocytes in IR injury through the use of clodronate depletion, and lipopolysaccharide augmentation, of monocytes. This was also confirmed by compartmental washout data above (Figure 5.4). To reinforce these findings and rule out any non-specific effects of clodronate liposome treatment we investigated the effects of monocyte adoptive transfer in the IPL model of IR injury.

Bone marrow derived monocytes and blood derived monocytes were both isolated by magnetic bead negative-selection techniques. Following 5 minute washout of the pulmonary circulation, the isolated monocytes were then slowly injected via the IPL pulmonary artery cannula at 1ml/minute into clodronate-depleted lungs. The infused monocytes were then recirculated through the lungs for 10 minutes and the standard IR protocol completed as above.

5.5.9 Transfer of Bone Marrow Derived Monocytes

Bone marrow derived monocytes were isolated (using a negative magnetic bead selection technique) from the femurs of control mice. This method allowed us to harvest up to 7×10^6 monocytes, which were infused into lungs of monocyte-depleted mice. An increase in vascular Ly6C^{High} monocytes retained within the lung was found following the IR protocol. However, BAL protein and soluble KC (measured in perfusate at the end of the protocols), did not suggest replication of the previous injury or inflammatory changes. However there was a return to pre-depletion levels of wet:dry ratios, implying the additional monocytes may have been sufficient to induce an interstitial, but not epithelial/alveolar leak (data not shown). Owing to the observed limited ability of these cells to reproduce injury following IR, isolation and adoptive transfer of blood-derived monocytes was subsequently undertaken.

5.5.10 Transfer of Blood Derived Monocytes

Despite the requirement for more donor mice, we considered the use of blood monocytes as alternative to the bone marrow source because of the presence of both subsets and the greater maturity of the Ly6C^{High} subset. (To enable harvest of sufficient numbers of cells up to 7mls of murine blood was required.) On average $\sim 7.8 \times 10^5 \pm \text{SD} 3.1$ vascular monocytes were adoptively transferred into recipient lungs using the same methodology as above. Although the numbers transferred were less than with bone marrow derived cells, a larger increase in monocytes remaining in the lung post IR was observed (Table 5.5). Moreover, wet:dry ratios and perfusate KC were increased in the monocyte-replete IR experiments, indicating that blood-derived vascular monocytes play a key role in this injury model (Table 5.5).

Table 5.5 Injury and inflammation parameters following blood monocyte adoptive transfer.

*Adoptive transfer increased lung monocyte numbers to levels seen in original IR experiments. Injury (BAL protein, Wet:Dry ratios) and inflammation (perfusate KC) levels were also reinstated to levels akin to those seen in original IR experiments. (Median(IQR), n=4-6, Kruskal-Wallis +Dunns **p<0.01. *p<0.05 vs' corresponding Depletion+IR value)*

	Ly6C ^{High} Monocytes/ lungs ($\times 10^5$)	BAL protein (mg/ml)	Wet:Dry ratio	Perfusate KC post-reperfusion (pg/ml)
IR	3.86(3.5-5.2)*	0.57(0.41-0.83)*	5.88(5.7-6.2)*	748(572-1149)**
Depletion + IR	1.67(1.4-2.3)	0.24(0.18-0.29)	5.52(5.4-5.6)	235(196-373)
Depletion + Blood monocyte adoptive transfer +IR	5.84(2.6-8.4)*	0.31(0.17-1.1) NS	6.08(5.7-6.5)*	633(449-2389)*

5.6 Discussion

An essential role for monocytes in inducing injury and inflammation following IR was demonstrated by clodronate depletion experiments in Chapter 4. Because of the ubiquitous presence of mononuclear phagocytic cells in all tissue compartments, we sought to fully characterise the locations of monocyte-macrophage subpopulations and their responses within the lungs. We confirmed, by lung compartment-specific leukocyte labelling, that a substantial proportion of the normal lung-marginated monocyte and neutrophil pools were retained in the lungs during the ischaemia-reperfusion protocol. To further elucidate monocyte margination and its role in the IR-injury process, we developed adoptive transfer system with blood-derived monocytes in which we were able to demonstrate that transferred monocytes could restore the injury process in the lungs of clodronate-liposome pre-treated mice.

5.6.1 Defining lung leukocyte compartments

Numerous leukocyte subpopulations have been isolated in the lungs, all likely to contribute to their immunological function, however their exact role and location remains contentious. Localisation of lung leukocytes has been addressed by several methods with varied results. Classically histological techniques have been utilised but difficulties differentiating between the closely adjoining alveolar, vascular and interstitial compartments and artefacts from antigen damage and masking during processing, can limit interpretation of images from this technique [297]. Flow cytometry is a widely adopted technique for the simultaneous identification and functional assessment of single cells in suspension. However, localising the precise compartment from whence they originate, relies on known cell surface (or intracellular) markers identifying them as such. To address these uncertainties, studies have attempted to ‘remove’ cells from the vascular compartment (through pulmonary vascular flushing) and alveolar compartment (through bronchoalveolar lavage) thus leaving the assumption that those remaining are only interstitial in nature [130, 291, 292, 298]. However, we, and others, have shown this method to be ineffective with up to half of vascular monocytes remaining despite substantial flushing (Chapter 4) and only ~10% of alveolar cells removable with lavage [129, 167, 169]. In addition to this caveat, activated and margined cells are even less likely to be removable, thus skewing the reliability of this method further.

A major advance in the field of lung physiology is the development of intravital fluorescence microscopy. This high fidelity technique allows direct visualisation of leukocyte dynamics in real time in *in vivo* preparations. Early work with this technique focused on cremaster muscle vasculature, with its thin structure lending itself to direct fluorescent microscopy with minimal physiological impact on the animal [299]. More recently several groups worldwide have developed this technique to image the pulmonary vasculature in real time through thoracic windows [283, 285]. Whilst this may be regarded as the current ‘gold standard’, it is limited by the artefacts of application of suction and heat source (especially with 2-photon lasers) to a lung surface, the ability to observe only 2-3 epitopes at anyone time and the prolonged observation periods.

To optimise the ability to identify and quantify the contents of the lung compartments more reliably, incorporating the benefits of flow cytometry, we developed a compartmental staining technique. Using concomitant vascular and alveolar staining with the pan-leukocyte marker CD45 (and the allele specific antibody CD45.2 to prevent crosslinking) we were able to reliably identify the 3 compartments: vascular, alveolar and, by process of elimination, interstitial. A similar technique was previously described [130], however we opted for the BAL antibody (and therefore the alveolar population) to remain *in situ* [287]. The rapid subsequent 1 minute dissociation/fixation and dilution method meant that crucially no double positive population was seen, indicating that in the intact lung there was no spill-over between compartments of antibody.

5.6.2 Neutrophils traffic from the vascular to interstitial to alveolar space in response to lipopolysaccharide

To further validate the compartment technique we tracked blood leukocyte migration into the lungs in a well-established neutrophil model of lung inflammation and injury; intra-tracheal LPS. This enabled us to track neutrophils from their enhanced vascular margination in the pulmonary vasculature to their appearance within the interstitial and alveolar compartments over a 180 minute period. This implies that over this time course, neutrophils are sequestered to the lungs, migrate into the interstitium and later into the alveolar space. The novel ability to differentiate between the three compartments using this methodology allows greatly improved differentiation between cell location and therefore phenotype, number and activation. This lies beyond the capabilities of histology and even emerging imaging techniques, where the very thin borders between these compartments are not readily discernable. In addition imaging techniques do not provide as accurate 'whole lung' assessment of cell numbers, provided by flow cytometry. However, whilst great care was taken to remove the lungs *without* the bronchial lymphatics, it is not possible to completely rule out the inclusion of bronchial-associated or lymph node derived cells in the unstained 'interstitial' compartment analysis [130]. Loss of alveolar barrier integrity may also limit this technique, with cross contamination of antibody in model of severe lung injury. However confirmation of minimal double-positive populations assists in determining this not to be the case.

5.6.3 Leukocytes are retained following IR

Following this validation we applied the dual staining technique to the IPL system. To replicate the three 5 minute washout periods performed in the IR protocol, we subjected murine lungs to a 15 minute non-recirculating washout period. This showed that of the ~90% of Ly6C^{High} monocytes that were identified as vascular, only ~50% of these were removed by the perfusion protocol. Retention of half of the monocytes was similar to the proportion observed following the IR protocol (Chapter 4). As expected there was minimal change in the small number of interstitial Ly6C^{High} population. The Ly6C^{Low} (CD11b+, F4/80+) monocytes however showed a different pattern, with an approximate 3:2 vascular:interstitial number ratio. Approximately 35% of Ly6C^{Low} vascular monocytes were washed out, with the interstitial numbers, as expected, being left unchanged. These data suggest that not only are

vascular (marginated) monocytes retained following perfusion, but a substantial interstitial monocyte/macrophage population is also identifiable, both of which would be transferred to the recipient and are likely to contribute to the subsequent activation and lung injury seen in recipients. As a significant proportion of these F4/80⁺ interstitial cells shared the same marker phenotypes as Ly6C^{Low} monocytes, we went on to develop a further flow cytometric identification strategy for these cells. Using the dual staining technique we were able to identify and validate that the interstitial Ly6C^{Low} population were MHCII positive, whilst the Ly6C^{Low} vascular population were negative as previously described [168, 291, 300]. This marker was therefore utilised thereafter (in addition to F4/80 and CD11c to differentiate them from dendritic cells), omitting the requirement for the dual staining technique.

5.6.4 The key role of CD86 on lung mononuclear leukocytes

To assess the activation of these interstitial macrophage populations relative to vascular monocytes, we used the stable surface marker, CD86, an indicator of antigen presenting cell maturation and development of T cell cytotoxicity. Firstly we characterised the expression of CD86 on our lung leukocyte populations in response to intravenous LPS. As previously suggested, LPS induced CD86 was highest on interstitial Ly6C^{Low} monocytes and dendritic cells, with LPS-induced upregulation also seen on vascular Ly6C^{High} and ^{Low} populations [291, 294]. In contrast the CD86 expression on alveolar macrophage appeared to be reduced by LPS pre-treatment. CD86 expression was highest after administration of 20µg of LPS on the interstitial populations.

Following this demonstration of the vascular and interstitial responses to a systemic inflammatory challenge, we sought to quantify CD86 on the same leukocytes in response to ischaemia-reperfusion. This followed similar patterns to the LPS dosing, with CD86 expression maximal in the interstitial Ly6C^{Low} and dendritic cell populations following IR. However, in contrast to the effect on soluble mediator release (Chapter 4), vascular monocyte depletion (via clodronate) did not affect CD86 expression on the interstitial populations.

Work by others has similarly shown that host T cells are activated early on by donor-derived CD11c⁺ cells [301]. In addition blockade of this T cell co-stimulation can reverse acute allograft rejection in mice, further implicating CD11c⁺ cells in immune rejection of lung grafts. The upregulation of CD86 on mononuclear cells may therefore play a role in the lung injury seen in our model. Additionally, others have shown CD86 blockade has ameliorated lung injury following caecal ligation and puncture [302]. Reduction of marginated monocytes expressing high levels of CD86 may have also played a role in the reduction of injury seen in our model, and likewise is likely to be of benefit by limiting upregulation of a T cell priming molecule on the interstitial cell populations also, within the transplant paradigm. Upregulation of CD86 on monocyte-derived human dendritic cells has also been documented following liver transplantation, further implying a role in this paradigm [303].

5.6.5 Monocyte adoptive transfer re-establishes IR injury

Optimising adoptive transfer techniques

To further evaluate the dynamics of the margined monocytes in the IPL system, we developed an adoptive transfer technique whereby cells were labelled with CFSE *ex vivo* before being perfused into the IPL system. This produced intriguing results whereby a significant proportion of labelled cells were not locatable at the end of the procedure. This suggests that cells may have either lost their CFSE label during cell recovery and preparation or that they were retained elsewhere within the IPL systems. Adherence to the tubing may have been enhanced by *ex vivo* isolation, manipulation and CFSE staining of the cells. To develop this method further we substituted the CFSE dye for an APC fluorophore conjugated CD45.2 antibody. This, however, produced similar results and again *ex vivo* handling of the adoptively transferred cells is likely to have artificially enhanced the cell retention within the system.

In an attempt to limit *ex vivo* manipulation and activation, we instead labelled cells *in vivo*. To this end the nucleoside BrdU was injected 24hrs prior to IPL experiments, labelling recently divided bone marrow derived cells. This revealed that Ly6C^{High} monocytes were preferentially labelled at this time point, with neutrophils remaining unlabelled (owing to their prolonged maturation time). Similar proportions of BrdU monocytes were retained with or without IR exposure, indicating that these cells are unaltered by the surgical and washout procedures the lungs are exposed to in this protocol. BrdU genetic labelling of leukocytes *in vivo* therefore potentially provides a useful way to selectively track inflammatory monocytes, newly released from the bone marrow and for future monocyte adoptive transfer work as shown to be successful by others in pneumonia models [304].

Owing to the difficulties encountered with labelled cell adoptive transfer, we sought to reintroduce monocytes without the systemic effects of exogenous dye administration. Thus we went on to refine our adoptive transfer method further, also attempting to limit *ex vivo* cell activation affecting the retention dynamics. To this end we performed a negative magnetic bead separation technique to extract both fresh bone marrow and whole blood, to minimise activation. This process produced purified monocytes, which were then infused into the IPL system, prior to the IR protocol, in an effort 'add back' the monocytes following clodronate depletion. However, as found with our previous adoptive transfer attempts, only a small proportion of the infused bone marrow-derived monocytes were retained. In contrast, a much larger proportion of the infused blood-derived monocytes were retained by the lungs. This may indicate that non-margined monocytes have likely functional or structural differences that limit entrapment on passing through the pulmonary vasculature, or that such entrapment is only short-lived.

Further pilot experiments were also undertaken (data not shown) whereby isolated monocytes were exposed briefly to LPS *in vitro* (100ng/ml, 5 minutes) prior to adoptive transfer, to induce monocyte activation, increased stiffness and thus retention [131, 305]. However no significant increase in monocyte retention was seen. Others have performed similar studies in a human model of adoptive transfer of neutrophils, whereby unprimed cells were shown to pass straight through the lungs (<5% retention) with a transit time of 14.2s (only 2.3s slower than

erythrocytes). However they found that priming neutrophils with PAF enhanced retention to 14% at 40mins and with GCSF to 48% at 40mins [242]. Crucially, we found that restoration of the lung-marginated monocyte pool with blood-derived cells reinstated the injury (BAL protein, wet:dry ratio increases) and perfusate KC levels described in our original IR experiments, strongly implicating their role in this model.

5.6.6 Retention of donor leukocytes

Retention of donor-marginated cells (along with interstitial mononuclear cells) is likely to be highly relevant in human lung transplantation, and investigated further in Chapter 6. The advent of perfusion/ventilation technologies during human lung transportation, and for optimisation of borderline organs, provides a unique opportunity for monocyte/macrophage populations to be removed. Current work addressing this is discussed in more detail in the next chapter, but as of yet has only focussed on perfusion as method of washing out margined cells that these data show is likely to be incomplete and ineffective with regards to interstitial populations [164]. Other human work, where peripheral circulating monocyte depletion has been achieved with leukapheresis, has shown a lack of efficacy in an LPS model of acute lung inflammation, indirectly implying that margined/retained, not circulating monocytes, may be playing the key role [306]. However pan-vascular monocyte depletion techniques, such as liposomal clodronate as used here (shown previously not to affect CD11c⁺ cells), diphtheria toxin-CD11b (able to deplete *all* mononuclear phagocytic cells more effectively) and gadolinium chloride, along with more specific therapies to impede monocyte-endothelial adherence, (anti-CCR2 (MCP-1) receptor and anti CD18 antibodies), have proved efficacious in models of LPS induced inflammation [95, 131, 162, 259] and lung IR [307]. In addition CCR2 has been shown to control bone marrow egress of monocytes to, and dendritic cell populations in, lung allografts [308].

5.6.7 Conclusion

In summary, analysis of leukocyte trafficking, using *in vivo* compartment labelling techniques, indicate that many leukocytes are retained within the vascular compartment of the lungs, despite washout protocols. In addition to the retention of margined vascular monocytes, it follows that interstitial mononuclear cells are also retained and carried over to graft recipients as passenger cells. Identification of this sub-population was validated with a dual-staining technique and simplified with the use of MHCII. Of note, IR induced a significant increase in vascular and interstitial mononuclear cell CD86, which may represent a likely candidate for propagation of inflammation and primary graft dysfunction in recipients. However, confirmation that only the intravascular monocyte population was depleted with clodronate, and that adoptive transfer of vascular monocytes re-established IR injury, indicates that this population are the key determinant of lung injury in response to IR in this model.

Chapter 6

Donor Monocytes Play a Critical Role
In Primary Graft Dysfunction
Following Human Lung Transplantation

6.1 Abstract

Introduction: Primary Graft Dysfunction is a major limiting factor in the success of lung transplantation. This form of acute lung injury is thought to occur primarily due to lung ischaemia-reperfusion, but evidence is mounting to support the role of activated immune cells, specifically monocytes in its pathogenesis. We sought to analyse leukocyte numbers and activation in human lung transplant samples and looked to link these to incidence of PGD in graft recipients.

Methods: Samples were collected as part of the on-going POPSTAR and EXTRALUNG lung transplantation studies at Harefield Hospital. Small sections of tissue were harvested from flushed lungs prior to transplantation and again following reperfusion, on implantation. All samples were analysed for leukocyte content, specifically monocyte subsets, and activation with the markers CD86, TREM-1 and HLA-DR. These data were compared to patient outcomes; specifically P:F ratios, PGD score and length of stay.

Results: Samples were collected from standard and organ care system (OCS) transplant lungs. Phenotypes of lung mononuclear cells were validated with comparison to blood and BAL constituent populations and the use of the monocyte intracellular marker 27E10. Substantial numbers of mononuclear cells and neutrophils were retained by the pre-implantation lungs, despite flushing, and were also present in abundance in post-transplantation samples. Numbers and activation of classical and non-classical 'monocytes' correlated with donor and recipient P:F ratios, ischaemia time, and incidence of PGD.

Conclusion: These data suggest that pre-existing lung 'monocytes' may be key players in the pathogenesis of PGD following transplantation. Contrary to current literature, lungs flushed with preservation solution pre-implantation, or following prolonged *ex vivo* machine perfusion of the lungs (OCS), contained significant numbers of mononuclear cells, implying firm margination or an extravascular location of these cells.

6.2 Introduction

6.2.1 Primary Graft Dysfunction (PGD)

PGD remains a major limitation to the success of lung transplantation. It is the leading cause of morbidity, graft rejection and mortality, short and long term [309]. It occurs within 72 hours transplantation and is defined as the presence of severe hypoxaemia and pulmonary oedema, diffuse pulmonary infiltrates on chest x-ray, in the absence of any other cause (See Box 1.1, Chapter 1).

6.2.2 Monocytes and lung IR

Several groups have alluded to the key role of monocytes in PGD. Bronchoalveolar lavage fluid IL-8, a chemoattractant produced predominantly by mononuclear cells, has been linked to poorer oxygenation and early death of patients following transplantation [150]. Similarly IL-8 released from lung tissue homogenates has been shown to positively correlate to early graft failure [151] and patients suffering from PGD have been shown to exhibit increased plasma MCP-1 and CXCL10 [230, 310]; all classically monocyte-derived chemokines. However these data are by no means conclusive and may simply represent a host (recipient) monocyte inflammatory response. Recent examination of pre-implantation lung samples however, has confirmed the presence of a large pool of monocytes within the pulmonary vasculature [164]. Additionally donor leukocytes have been identified in graft recipients many months following lung transplantation, of which monocytes accounted for the greatest proportion (>1.7% of all cells microchimerism), confirming the potential for carry over and persistence of such cells [282]. Work as part of this study with a murine model of IR strongly suggests that monocytes are key players in the pathogenesis of transplant-related ALI. Furthermore we have found that marginated vascular monocytes and interstitial monocytes are activated in response to IR and are likely to persist in the lungs following harvest, being transferred as 'passengers' to the recipient, propagating lung injury post transplantation.

The diverse role of monocyte subsets in human lungs to date remains uncertain. Their circulatory role in other forms of sterile inflammation has been demonstrated in humans following trauma [311] and burns [312], surgery [313] and also following cardiopulmonary bypass (CPB) [314]. CPB is of specific relevance to this work, with lung ischaemia being an integral component of its use in cardiac surgery. In addition to human studies, work with cynomolgus monkeys has shown that CPB alone induces a rapid release of monocytes from the bone marrow, a subsequent increase in their presence in the alveoli and associated production of IL-6 and IL-8 in the BAL 24 hours later, and impaired partial oxygen tension [315].

6.2.3 Human monocyte subsets

Analogous to murine leukocyte biology, human monocytes have been subdivided into classical (CD14^{High}, CD16^{Low}) and non-classical (CD14^{Low}, CD16^{Very High}) subsets, with the classical phenotype accounting for >80% of circulating monocytes [316]. The non-classical phenotype are thought to have a patrolling function and are readily recruitable

to sites of inflammation where they will extravasate and promote classical monocyte and neutrophil recruitment [317]. Both monocyte subsets may therefore be retained within the lungs, margined in the pulmonary circulation and in the interstitium, following activation. They also have the ability to differentiate into macrophages and dendritic cells, capable of donor antigen presentation to host T cells and subsequent graft cell loss [318]. In addition to these two distinct groups of monocytes, we, and others, have confirmed the presence of a third ‘intermediate’ ($CD14^{\text{High}}$, $CD16^{\text{High}}$) circulating population as shown in Figure 6.1 [166, 319].

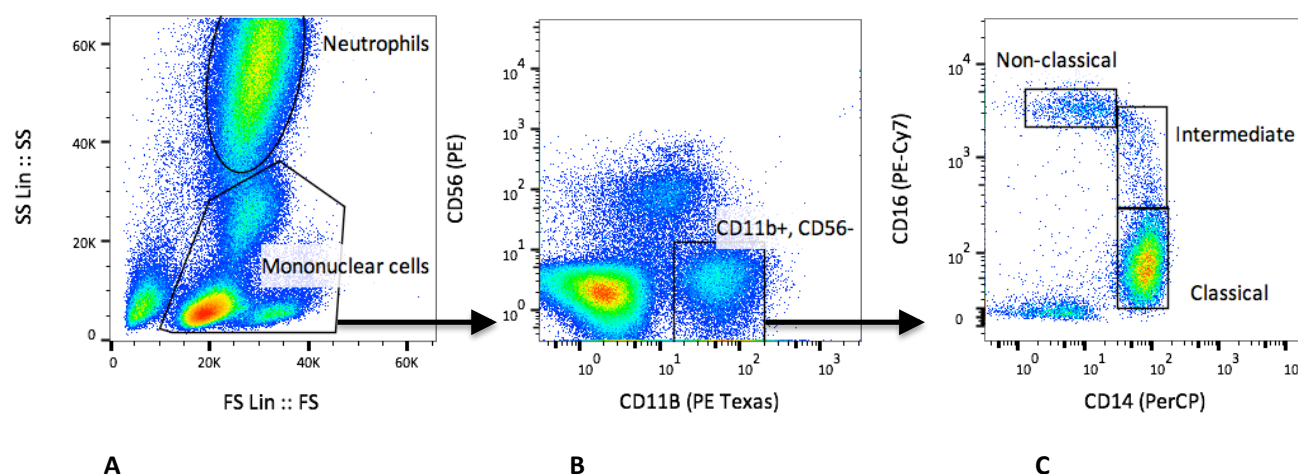


Figure 6.1 Typical gating of classical ($CD14^{\text{High}}/CD16^{\text{Low}}$), intermediate ($CD14^{\text{High}}/CD16^{\text{High}}$) and non-classical ($CD14^{\text{dim}}/CD16^{\text{Very High}}$) monocyte subsets in human blood.

Mononuclear cells from a healthy volunteer were initially separated from neutrophils as low side scatter events (A) and then gated as $CD11b+$ and $CD56-$ to eliminate lymphocytes and NK cells respectively (B). The three monocyte populations were then defined according to their $CD14/CD16$ expression (C).

The intermediate population has been shown to have distinct phenotypic and functional differences, including significantly higher HLA-DR surface expression and a lack of L-selectin [319]. In addition to this, monocytes (especially the non-classical subset) are likely to represent the haematopoietic cell precursors of lung dendritic cells, which are known to populate the lungs in immature and mature forms. Intermediate monocytes however, exhibit high intracellular and then later surface MHCII (mature) along with co-stimulatory molecules (e.g. CD86, CD80) required for (host) T cell activation, and have the greatest capacity to become migratory dendritic cells [320, 321].

However, definitive differentiation between alveolar macrophages, dendritic cells and the monocyte populations is inherently difficult owing to the maturation/differentiation continuum from which they originate, and common precursor ancestry these cells share. Moreover, whilst analysis of the cellular constituents of BAL fluid has been widely performed, a final consensus on the precise phenotypic identification of its constituent cells remains lacking. In addition advanced flow cytometric analysis of human lung mononuclear cells lies even further behind, with only traditional histological techniques providing current insights, notwithstanding its considerable inherent artefacts.

Recently published studies on BAL constituent leukocytes have indicated several distinct populations of monocyte/macrophage-like cells, which can vary greatly in cell surface marker expression depending on the clinical scenario [322, 323].

6.3 Hypothesis and Aims

Based on the outcome of our mouse work, our hypotheses were as follows:

- Analogous to murine lungs, human donor lungs would contain significant numbers of pro-inflammatory mononuclear phagocytic cells, which could be transferred to transplant recipients as ‘passengers’.
- Mononuclear cells would be retained despite lung washout or perfusion (via the Organ Care System)
- The number and activation of the mononuclear cells would correlate to the development of PGD

We therefore sought to:

- ⇒ Identify and quantify mononuclear cell subtypes human lung transplant samples undergoing standard transplantation and *ex vivo* lung perfusion techniques
- ⇒ Quantify activation status in these leukocyte subsets
- ⇒ Relate all of the above to clinical outcomes in recipients following transplantation

6.4 Methodology

6.4.1 *Protocols and Ethics*

All human samples and patient data were obtained and stored in accordance with the ethical approval granted to the POPSTAR and EXTRALUNG studies (Harefield hospital, RB&H NHS Foundation Trust).

6.4.2 *Lung Transportation and Storage*

As detailed in Chapter 2, samples were harvested from standard and OCS pre- and post-implantation lung grafts. Lungs from either protocol were extensively flushed or perfused prior to implantation. Standard lungs were transported on ice, partially inflated with 50% oxygen prior to transplantation. OCS lung samples (some of which were donated for research purposes only) underwent varying periods of perfusion and ventilation prior to implantation.

6.4.3 *Sample Collection*

Lung samples were stored on ice in a humidified container before being subdivided and weighted for wet:dry and flow cytometric analysis. For the latter the lungs were manually minced and disaggregated at 37°C for 20 minutes with a DNase/Liberase enzyme combination. They were then pushed through a 40µm cell strainer and washed twice in FWB at 300g for 7 minutes at 4°C. Cells were then stained with the antibody combination below protected from the light for 30 minutes at 4°C before being washed again.

Bronchoalveolar lavage was performed following implantation and arrival in Intensive Care with 40-60mls normal saline via a fibre-optic bronchoscope. Fluid was strained through a gauze filter, cells pelleted and stored on ice, until antibody staining with the same panel as below

For monocyte phenotype comparisons blood was collected in sodium heparin vacutainers and stained with the same antibody combination listed below.

All samples were harvested by Dr Rosalba Romano. Lung processing and flow cytometry data acquisition was performed equally Dr Romano and myself.

6.4.4 *Antibody Panel*

We utilised a single antibody panel, including various functional markers (TREM-1, CD86, CCR2 and HLA-DR), which enabled optimal identification of monocyte/mononuclear cells phenotypes within our samples.

Following the initial stain with this panel, cell suspensions were washed, fixed and permeabilised with BD cytofix/cytoperm for 5 minutes at room temperature. They were then washed twice in PWB before being stained with the intracellular label 27E10 for a further 15minutes (protected from light, 4°C) and being washed one additional time prior to sample acquisition with the additional of counting beads.

Table 6.1 Antibody panel for identification of lung constituent leukocytes

	FITC	PE	PE Texas Red	PerCP	PeCy7	APC	Ax780
Antibody	27E10*	CD66b	CD11B	CD14	CD16	Functional markers	CD45

** applied after permeabilisation*

6.5 Results

6.5.1 Demographic data

Samples were donated from a total of 21 lungs; 13 from standard transplants and 8 from lungs exposed to the Organ Care System (OCS). Some of the OCS lungs did not continue to transplantation and were examined only for research purposes.

Table 6.2 Demographic and outcome data for standard transplants

¶: Mean $\pm$ SD, T-test. #: Number n (% of total)

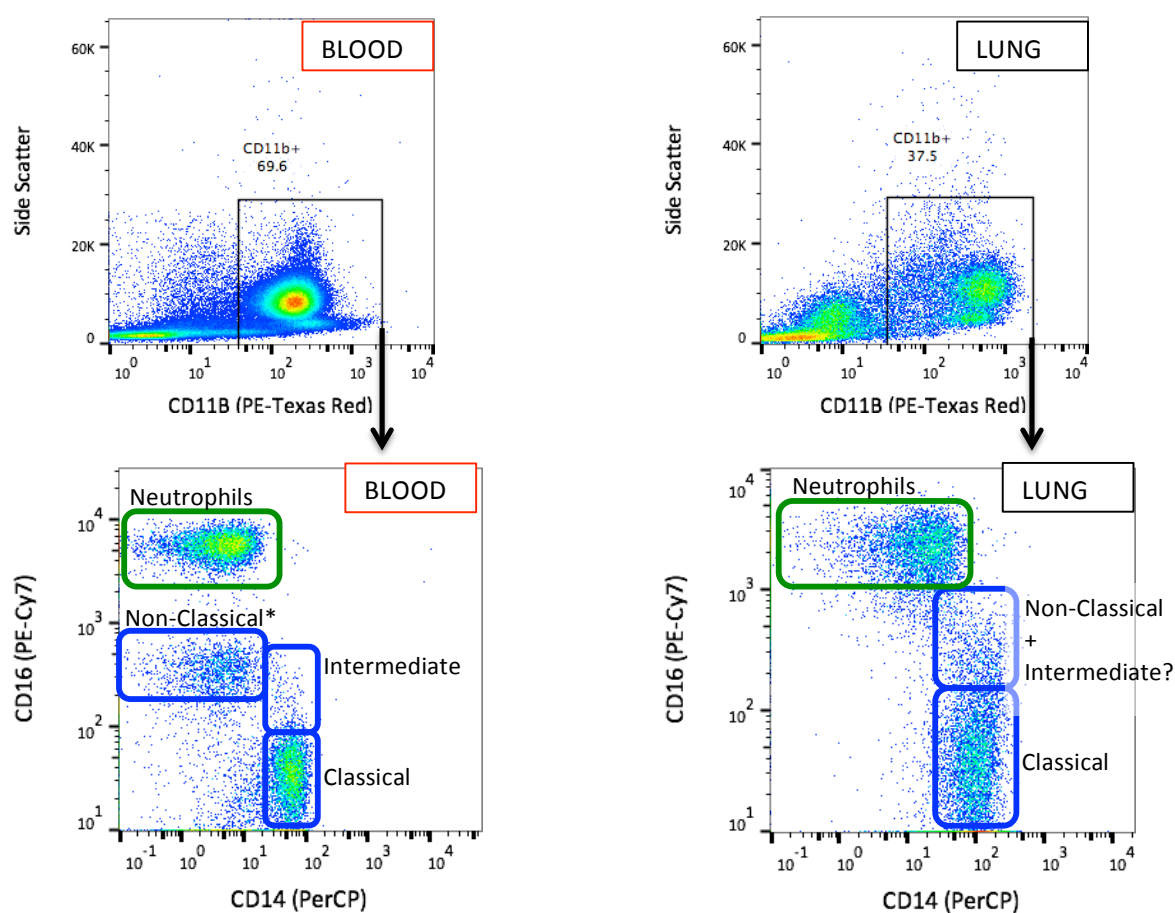
Standard lung transplant characteristics (n= 13)	
Donor age (yr), mean $\pm$ SD ¶	46.6 $\pm$ 14.6
Male donor sex, n (%)#	6 (46.2)
Intracranial haemorrhage, n (%)#	7 (53.8)
Donor PF ratio (kPa), mean $\pm$ SD¶	56.7 $\pm$ 15.1
Donor score, mean $\pm$ SD¶	4.5 $\pm$ 3.2
Ischaemic time (mins), mean $\pm$ SD¶	461.6 $\pm$ 141.1
Recipient age (yr), mean $\pm$ SD¶	51.3 $\pm$ 11.1
Male recipient sex, n (%)#	5 (38.5)
Recipient PF ratio at 24hrs (kPa), mean $\pm$ SD¶	33.0 $\pm$ 13.4
Recipient PF ratio at 48hrs (kPa), mean $\pm$ SD¶	41.8 $\pm$ 14.2
Recipient PF ratio at 72hrs (kPa), mean $\pm$ SD¶	46.1 $\pm$ 12.0
PGD 2-3 at 24hrs, n (% of transplanted)#	8 (61.5)
PGD 2-3 at 48hrs, n (% of transplanted)#	4 (30.8)
PGD 2-3 at 72hrs, n (% of transplanted)#	3 (23.1)
Ventilator hours, mean $\pm$ SD¶	177.8 $\pm$ 249.2
ICU length of stay (d), mean $\pm$ SD¶	12.9 $\pm$ 11.3
Hospital mortality, n (% of transplanted)#	2 (15.4)

The donor demographics show a typical distribution of age, sex and presentation. Rates of PGD at 48 and 72hrs were similar to those reported in the literature [324], as was the mortality rate [8].

6.5.2 Identification of mononuclear cells from human lung samples

Samples of human lung transplant tissue were harvested prior to implantation and post implantation before closure of the chest of graft recipients, and stored, processed and analysed via flow cytometry as outlined above.

Various fluorophore-conjugated surface and intracellular (27E10) anti-human mononuclear cell antibodies were utilised, as previously described, to identify and differentiate the constituent cell phenotypes [313, 322, 323, 325, 326]. These included CD45, CD11B, CD14, CD16, 27E10, HLA-DR and CCR2.



A

B

Figure 6.2 Typical blood monocyte population phenotype compared to lung homogenate phenotypes.

Lung mononuclear cell CD14/CD16 expression did not completely replicate the typical blood monocyte CD14/CD16 patterns. (Neutrophils were identified as CD16^{very high}, CD14⁺.)

**The blood non-classical gate also contains NK cells. These were subsequently gated out with the use of NK marker CD56.*

To enable identification and validation of the phenotypes of the various lung monocyte/macrophage populations, comparison was made to human blood sample phenotypes. However, the human lung sample mononuclear cell phenotypes did not fully resemble the 3 typical blood monocyte phenotypes commonly observed (Figure 6.2). This is perhaps unsurprising as lung cells suspensions contain multiple cells types (including tissue macrophages) from various compartments, which will express varying degrees of the monocyte/macrophage surface markers CD14 and CD16. This is likely to therefore confound identification with the lung tissue, exemplified by the loss of the non-classical/intermediate distinction shown in Figure 6.2B.

To address the issue of loss of population delineation, we utilised the 'vascular' monocyte/neutrophil marker 27E10 to aid subset identification, along with HLA-DR and CCR2, to confirm mononuclear cell subtypes. This identification technique is consistent with recently published lung mononuclear cell migration studies [322, 323, 325, 326].

27E10 displayed a similar pattern of expression to CD14 in blood samples, whereby the three typical monocyte populations were visible with use of either antibody plotted against CD16 (Figure 6.3 A and B). In addition the NK cells were now clearly distinguishable as the CD16^{High} 27E10⁻ (CD56⁺) population, negating the requirement for CD56 in the final antibody panel (purple box, Figure 6.3 A).

When comparing 27E10/CD16 in our lung samples to CD14/CD16 gating, a similar pattern to the blood was observed. This also allowed easy identification of CD16^{High} NK cells (purple box) and produced clearer separation between the likely classical (blue box) and non-classical (black box) populations (Figure 6.3, plots C & D). Neutrophils (CD11B⁺ CD16^{Very High} CD14⁻) were gated out of these plots beforehand with the additional marker CD66b.

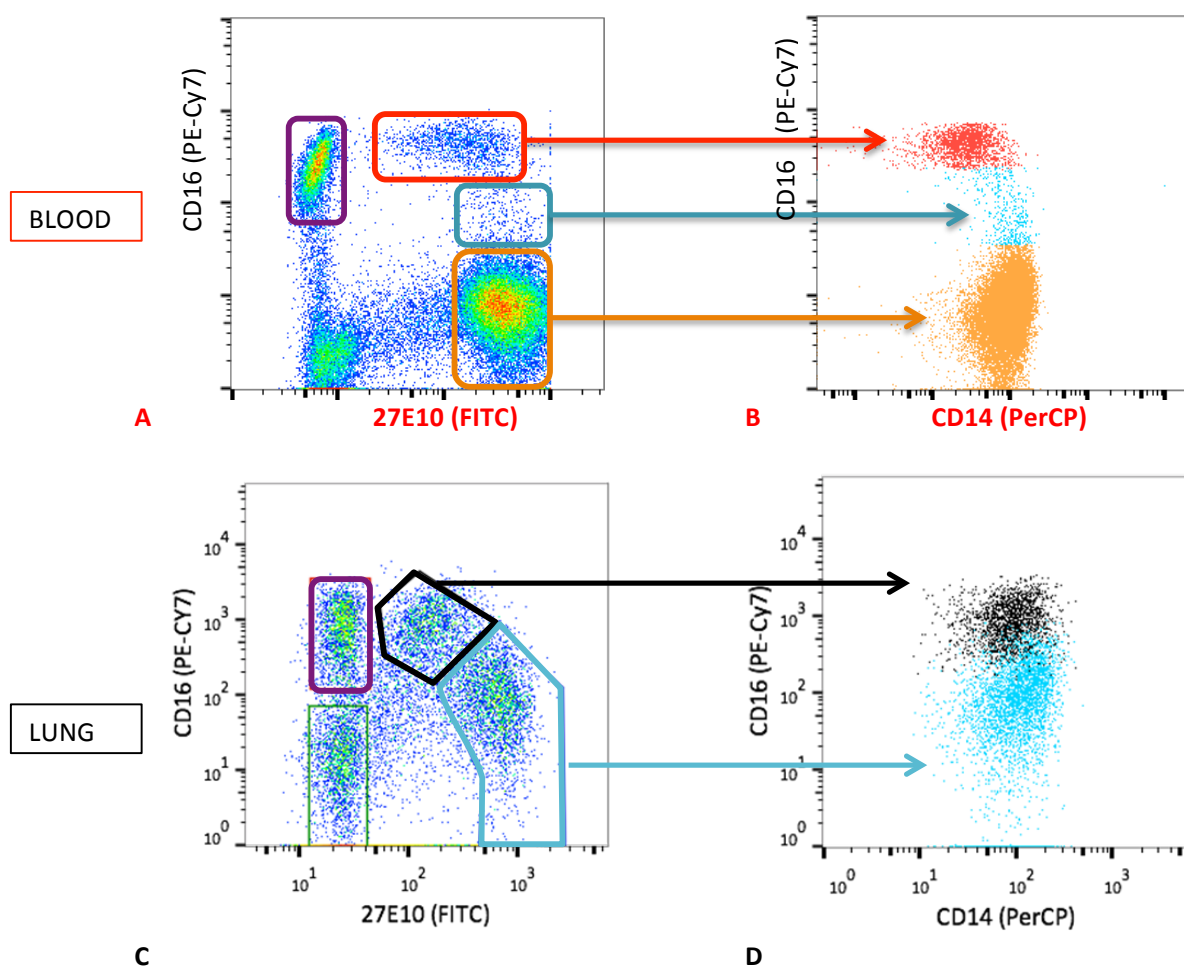


Figure 6.3 Validation of the use of 27E10 in blood and lung monocyte subset gating. 27E10/CD16 (A) compared to CD14/CD16 (B) in blood and in lung (C/D)

All samples were initially gated as CD11b⁺ (as shown in figure 6.2). Reproducible blood monocyte subset identification was achieved with the use of 27E10/CD16 combination gating. This was verified with corresponding CD14 staining in blood samples (A&B).

A similar loss of a distinct intermediate mononuclear cell population in the lung samples was seen with 27E10/CD16 combination (C), as well as with the CD14/16 combination (D). However, 27E10/CD16 combination provided superior distinction between likely classical (blue box) and non-classical subsets (black box).

NB CD16^{very high}, CD14⁻ neutrophils were gated out of the above plots with additional use of the marker CD66b – not shown.

Following this phenotype validation we went on to compare blood and lung to BAL samples, using a similar combination of makers. This firstly identified alveolar macrophages as CD14⁺ and high side scatter, as previously described [323]. Low side scatter events were then subdivided firstly using CD66b (neutrophil marker) and CD45 (pan-leukocyte marker) (Figure 6.4). CD45⁺, CD66b⁻ cells were then subdivided into four separate populations on the basis of their 27E10 and CD16 expression (Figure 6.4 A-C), to differentiate between the mononuclear cell subsets.

The subsequent populations identified then underwent CD14, CD11b, HLA-DR and CCR2 expression quantification as shown in Figure 6.5. This validation defined the constituent populations as (1) 'non-classical' mononuclear cells, (2) 'classical' mononuclear cells, (3) NK cells and lung/BAL alveolar macrophages, summarised in Table 6.3. (For brevity the mononuclear cells will be hereafter be referred to as 'monocytes', whilst we recognise this may not be fully substantiated as this stage.)

Unfortunately BAL samples were not always available and were frequently heavily blood-stained from the surgical retrieval and anastomotic procedure. This therefore compromised and precluded subsequent quantification of constituent monocyte/macrophage populations found within the majority of samples gained from this compartment.

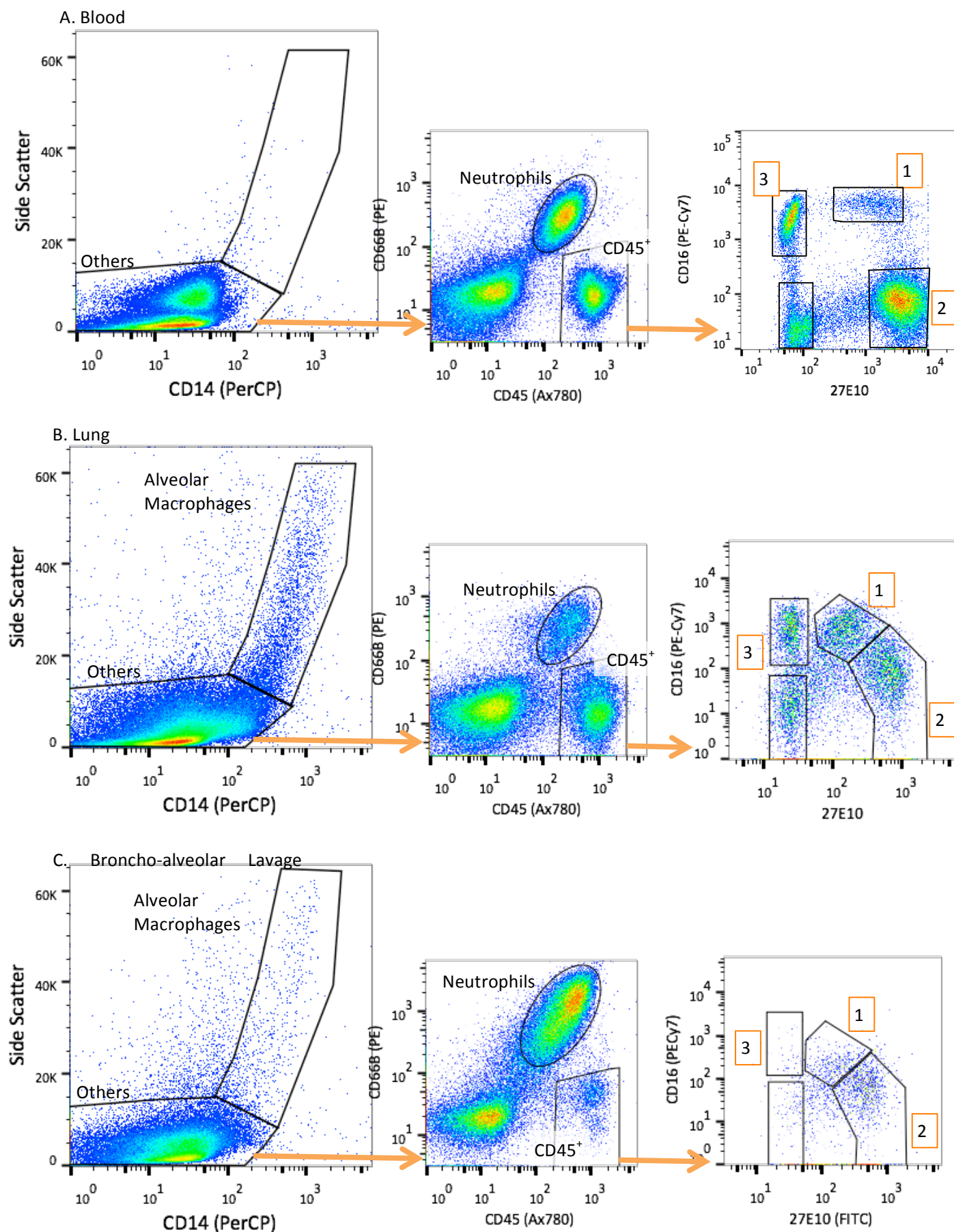


Figure 6.4 Monocyte and macrophage gating via flow cytometry.

Validation of lung flow cytometry plots was performed in comparison to concomitant blood and BAL samples.

Representative plots of (A) blood gating, (B) lung and (C) broncho-alveolar lavage samples of: 1. Non-classical, 2. Classical, 3. NK cells and 4. Lymphocytes/others

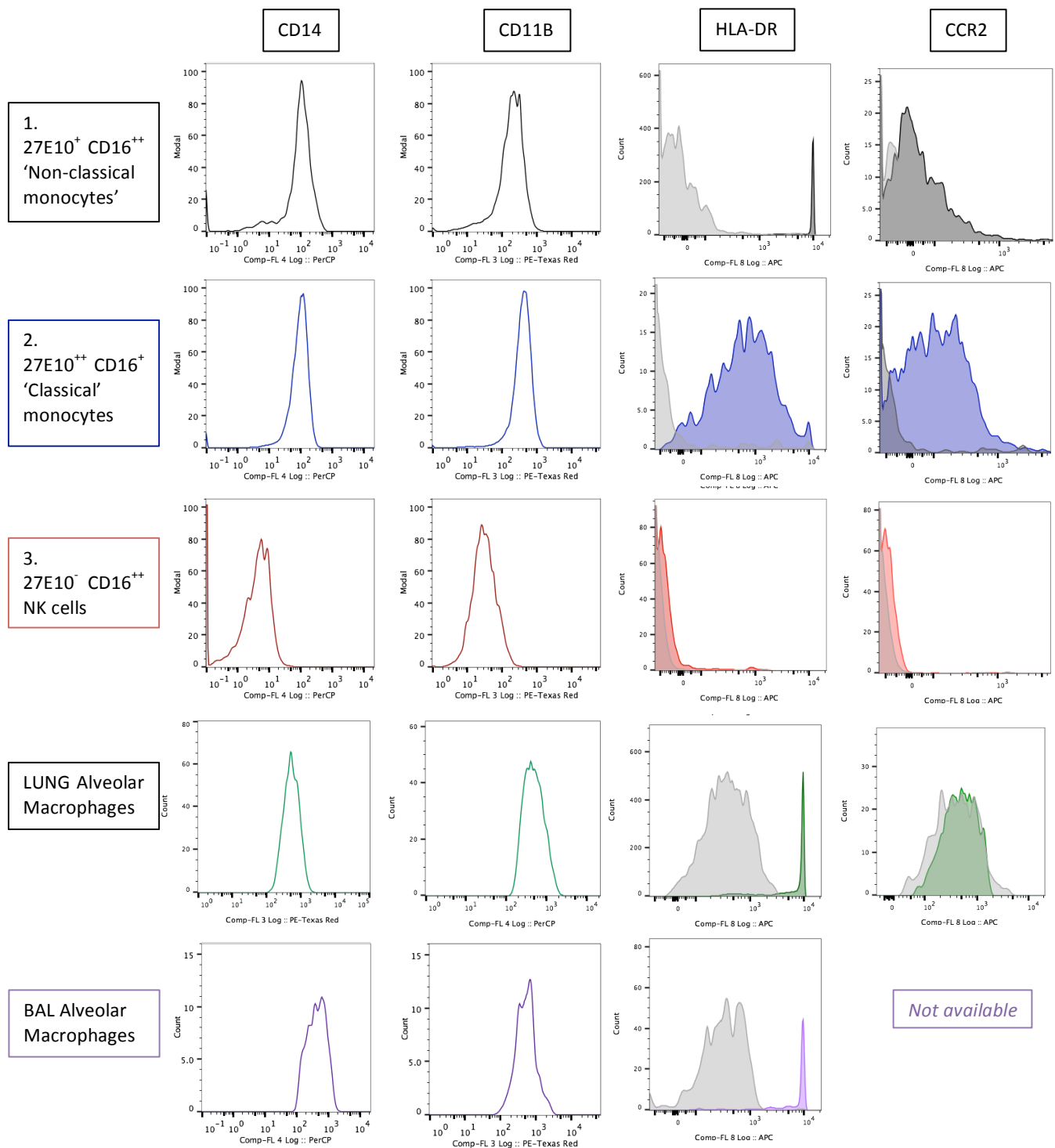


Figure 6.5 Monocyte and macrophage marker expression of lung leukocytes

To further identify phenotypes of the monocytes identified in figure 6.4, plots were initially gated as CD45⁺/CD66b⁻ and then according to their 27E10/CD16 staining in (1) 'non-classical', (2) 'classical', (3) NK cells and lung/BAL alveolar macrophages. These subsets were then gated for CD14, CD11B, HLA-DR and CCR2 expression. (Grey histograms represent blank values; blanks were not performed for CD14 and CD11b)

Table 6.3 Summary of lung mononuclear cell phenotypes

	SSC	CD45	CD66b	27E10	CD16	CD14	HLA-DR	CCR2
'Non-classical' monocytes	Low	+	-	+	+	+	++	+
'Classical' monocytes	Low	+	-	+	-	+	+	++
NK cells	Low	+	-	-	+	-	-	-
Lung alveolar macrophages	High	+	-	+	+	+	++	-
BAL alveolar macrophages	High	+	-	+	+	+	++	N/A

6.5.3 Monocytes are retained in donor lungs and carried over to recipients

After identifying cells with monocyte-subset like phenotypes in our lung samples, we quantified them per gram of lung dry weight, with the addition of microsphere counting beads to the flow cytometry samples. As shown in table 6.4, a substantial number of both monocytes subsets (and neutrophils) were found in the flushed and lavaged standard lung samples immediately prior to implantation ('pre-implantation' samples) and post-implantation. For comparison, lungs perfused on the Organ Care System exhibited similar numbers of leukocytes.

		<i>Classical</i> <i>x10⁶/g</i>	<i>Non-Classical</i> <i>x10⁶/g</i>	<i>Neutrophils</i> <i>x10⁶/g</i>
Standard transplant	Pre-implantation	3.23 ±2.2	2.89 ±10.0	8.05±6.7
	Post-implantation	3.74 ±2.8	1.61 ±2.3	15.48±9.5**
OCS	Pre-implantation	2.22 ±1.6	2.71 ±3.6	5.13±3.7
	Post-implantation	2.78 ±2.7	0.89 ±1.2	11.28±2.2*

Table 6.4 Numbers of monocytes and neutrophils/g dry weight of lung tissue

Numbers of monocyte subsets and neutrophils were calculated via flow cytometry with the use of microsphere counting beads per gram dry weight of lung tissue, taken from paired samples utilised for wet:dry analysis. There was no significant difference between the pre and post-implantation monocyte subset numbers, however neutrophil numbers were greater post-implantation in both groups of lungs.

There was no significant difference when comparing any pre-implantation or post-implantation numbers of standard versus OCS lung samples.

*Mean±SD, n=5-13, x10⁶/g lung dry weight. *p<0.05 vs' OCS pre-implantation, **p<0.01 vs' standard pre-implantation sample*

The number calculated with this methodology is therefore estimated to be greater than $\sim 1.2 \times 10^9$ monocytes in total/lungs (classical + non-classical), based on adult human reference lung weights [327]. These numbers (following flushing) are far in excess of monocytes that may have been derived from, for example, estimated residual pulmonary blood (without flushing) – see box 6.1.

Box 6.1 Estimated and calculated total monocytes (classical + non-classical) in standard pre-implantation lungs.

➤ **Estimated pulmonary blood monocytes:**

Adult monocyte count $\sim 500\,000/\text{ml}$ x pulmonary blood volume $\sim 500\text{ml}$ = **250×10^6 monocytes**

➤ **Calculated total monocytes from flushed pre-implantation lung samples:**

Flow cytometry monocyte count $\sim 6 \times 10^6/\text{g}$ x $\sim 200\text{g}$ dry weight = **1200×10^6 monocytes**

These data therefore strongly suggest that significant numbers of monocytes are retained by the lung and are carried over as passengers to the recipient. Interestingly the numbers of post-implantation monocytes was similar to those found pre-implantation. The post-implantation group of (non-flushed) lung samples were taken once the lung had undergone reperfusion within the recipient. This therefore suggests that either the residual monocytes remain *in situ*, with minimal adhesion of circulating recipient monocytes, or that donor monocytes have been released and replaced by adhered recipient circulating monocytes, or a combination of the two. Moreover, lungs undergoing OCS underwent an average of 484 ± 356 minutes of *ex vivo* perfusion after retrieval (compared to none with the standard lungs). However the final numbers of monocytes of pre-implantation samples are very similar despite prolonged pulmonary perfusion, again providing evidence for substantial monocyte retention in these samples (Table 6.4).

6.5.4 Monocyte activation in donor lungs predicts Primary Graft Dysfunction

Using the gating strategies above we quantified key cell surface activation markers. CD86 was selected as an inducible activation marker based in part on its role in monocyte/T cell co-stimulation, therefore highly relevant to the transplantation paradigm, complementing our mouse work. Previous work by others has highlighted enhanced CD86 expression on macrophages during lung transplant rejection [328]. In addition TREM-1 and HLA-DR have also been used as biomarkers of immune function in blood and BAL monocytes. We also sought to correlate our clinical parameters with pre-implantation lung monocyte numbers and activation status (Table 6.5). Of note the number of lung ‘classical’ monocytes negatively correlated with PF ratio at 72hrs. Furthermore HLA-DR and CD86 expression correlated negatively with PF ratio at 24 and 48hrs, implying a potential link between ‘classical’ monocyte activation and outcome in these patients. Of note ‘non-classical’ monocyte number correlated with PF ratio in the donors, although the significance of this is unclear. There were no demonstrated correlations between neutrophil numbers and any outcome parameter.

Table 6.5 Correlation matrix of standard pre-implantation lung parameters and monocyte numbers and activation, and neutrophil numbers. r^2 (p value), * $p < 0.05$

		Donor PF ratio	PF ratio 24hrs	PF ratio 48hrs	PF ratio 72hrs
		<i>r</i> (p value)	<i>r</i> (p value)	<i>r</i> (p value)	<i>r</i> (p value)
Classical monocyte	Number	0.25 (0.506)	0.34 (0.201)	-0.41 (0.208)	-0.72 (0.012)*
	CD86	-1.20 (0.709)	-0.26 (0.402)	-0.67 (0.016)*	-0.18 (0.574)
	HLA-DR	-0.22 (0.470)	-0.63 (0.021)*	-0.06 (0.871)	0.09 (0.767)
	TREM 1	-0.16 (0.602)	-0.32 (0.291)	-0.39 (0.184)	0.04 (0.896)
Non-classical monocyte	Number	-0.77 (0.003)*	-0.01 (0.981)	0.33 (0.294)	0.22 (0.486)
	CD86	-0.16 (0.605)	-0.35 (0.238)	-0.04 (0.900)	-0.06 (0.835)
	HLA-DR	-0.08 (0.800)	-0.38 (0.199)	0.06 (0.837)	0.07 (0.815)
	TREM 1	0.22 (0.477)	-0.15 (0.630)	-0.39 (0.611)	0.23 (0.451)
Neutrophils	Number	0.13 (0.218)	0.30 (0.053)	0.00 (0.96)	0.00 (0.97)

When stratified against primary graft dysfunction, the markers of inflammation correlated with PGD severity (Figure 6.6), specifically monocyte CD86 and TREM-1, in both monocyte subsets at 48hrs.

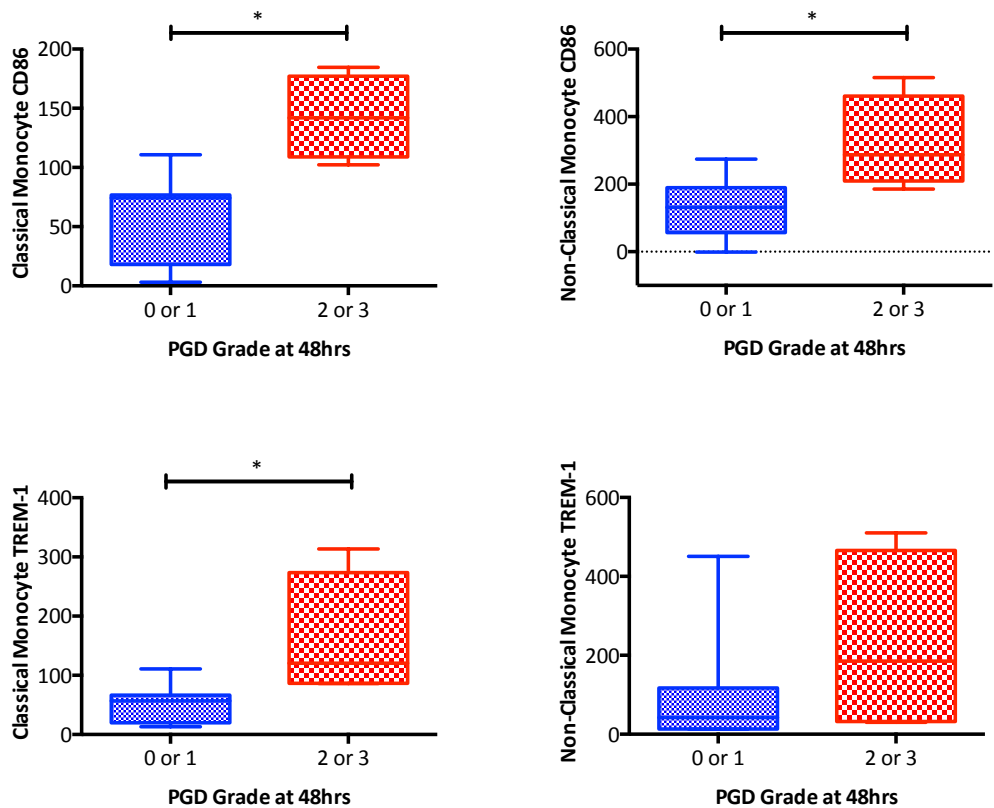


Figure 6.6 Pre-implantation standard transplant lung monocyte CD86 and TREM-1 and severity of PGD

Classical and non-classical monocyte CD86 was significantly increased in pre-implantation lungs which later went on to develop grade 2 or 3 PGD following transplantation. A similar pattern was observed with classical and non-classical monocyte TREM-1, only reaching significance however in the former subset. Median(range); Mann-Whitney, * $p < 0.05$.

In addition CD86 was on average greater with PGD levels 2-3 at 48 and 72 hours post transplantation (figure 6.7).

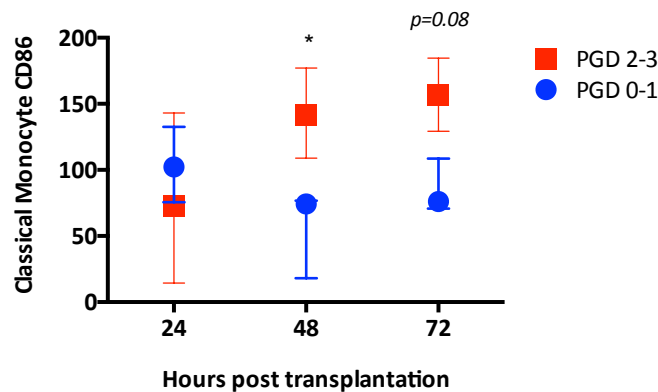


Figure 6.7 Pre-implantation standard transplant lung monocyte CD86 and severity of PGD over time.

Patients with PGD grade 2-3 at 48 or 72 hours exhibited a greater classical monocyte CD86. Median(IQR), $n = 2-8$, Mann Whitney, PGD 0-1 vs' PGD 2-3 at each time point, * $p < 0.05$

6.5.5 Endothelial permeability correlates with PGD and monocyte activation

	<i>Wet:Dry weight ratio</i>	
	Pre-implantation	Post-implantation
Standard transplant	Pre-implantation	6.58 ±2.50
	Post-implantation	6.25 ±1.85

Table 6.6 Overall wet:dry weight ratio of lungs pre- and post-implantation in standard lung transplants.

*There was no significant difference found between pre and post-implantation Wet:Dry ratios.
Mean±SD, n=13, T-test.*

As with our animal model, we also assessed the wet:dry weight ratios of all lung samples as a marker of injury. There was no significant difference between pre and post implantation wet:dry values (Table 6.6). In addition the extensively perfused OCS lungs wet:dry ratios were not significantly different to the standard transplant pre-implantation values (data not shown).

Lung samples were initially transported in saline-moistened tubes, but subsequently we introduced a moistened piece of gauze to control humidity without risking tissue wetting. For consistency only the more recent ‘gauze method’ data were compared to clinical outcomes below. Of note PF ratios at 48hrs correlated with pre-implantation lung wet:dry ratios, and with severity of PGD, indicating a potential link between wet:dry ratios in pre-implantation lungs and severity of PGD, as one might expect with this surrogate of endothelial leak.

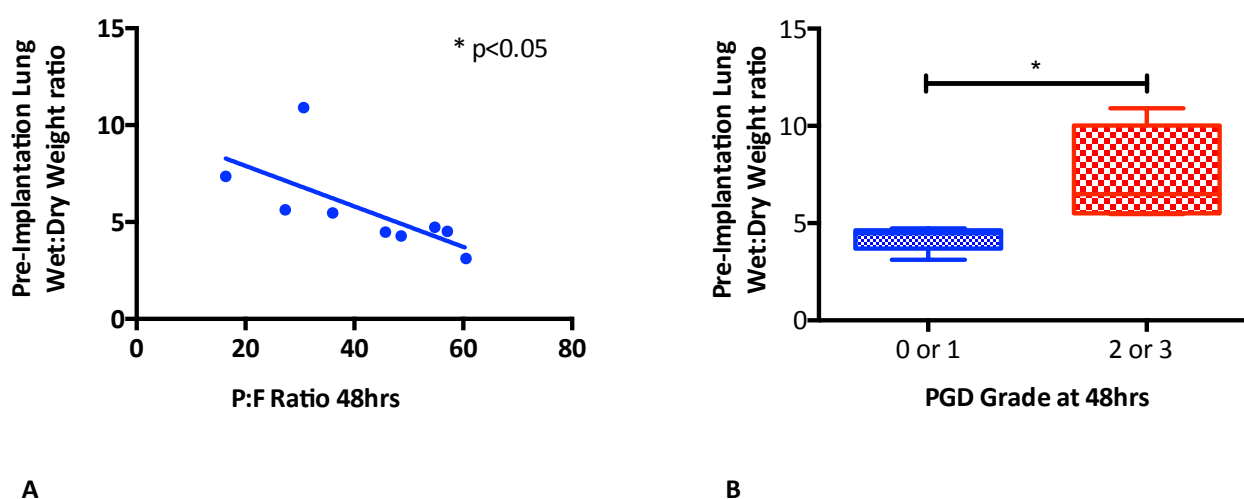


Figure 6.8 Standard transplant pre-implantation lung wet:dry weight ratio to PF ratio and PGD grade at 48hrs

*A: Linear correlation, B: Median(IQR), n= 3-6, Mann Whitney. *p<0.05, **p<0.01*

Wet Dry ratios of pre-implantation lungs also correlated with non-classical monocyte CD86 levels ($p<0.01$) and ICU length of stay ($p<0.05$).

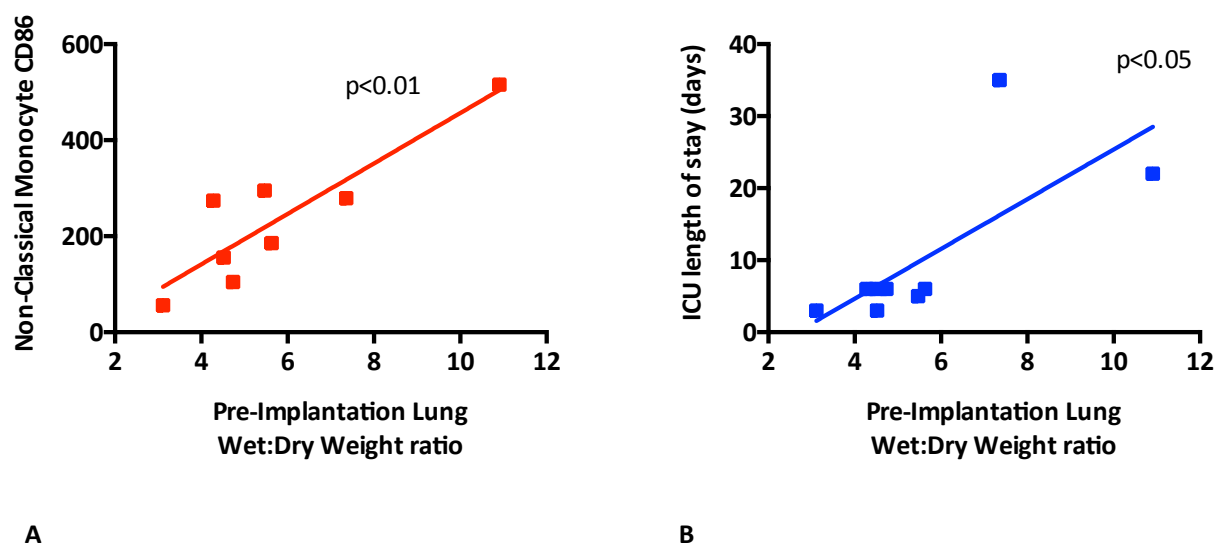


Figure 6.9 Pre-implantation lung wet:dry weight ratios correlated to non-classical monocyte CD86 and ICU length of stay in standard transplants.

Lung wet:dry weight ratios positively correlated with the activation (CD86 expression) of non-classical monocytes and with the length of ICU stay of transplant recipients.
Linear correlation, $n = 8$, linear correlation.

6.6 Discussion

6.6.1 *The role of monocytes and macrophages in ALI*

Lung monocytes have been shown to be key in the pathophysiology of lung injury with several groups highlighting the role of lung monocytes/macrophages in ARDS, IR and lung transplantation [129, 214, 283, 326].

Until now the majority of human work around mononuclear cell population in human lung injury has focussed on BAL leukocyte analysis. For example Rosseau *et al.*, found that a predominance of monocyte-like macrophages correlated with severity of respiratory failure [322]. Moreover Brittan *et al.*, analysed BAL samples from healthy volunteers compared to those from volunteers subjected to inhaled LPS. The latter group exhibited a significant expansion of pro-inflammatory 'inducible pulmonary monocyte-like' cells (CD14⁺⁺/CD16⁻) in contrast to 'resident pulmonary monocyte-like' (CD14⁺⁺/CD16⁺) cells, both identified within the BAL [323, 326]. Moreover they stressed that the inducible population strongly resembles the classical circulating monocyte phenotype and thus suggest a vascular source, supporting previous similar assertions [329].

A recent animal study, performed in Indian Rhesus macaques, sought to determine the dynamics of lung interstitial versus alveolar mononuclear cells [330]. They found that interstitial monocytes have a high turnover, similar to their circulating counterparts, and stained positive for intravenous BrdU, indicating their likely origin being vascular. In addition they were able to demonstrate a potential functional role for interstitial macrophages, exhibiting an increased TNF response to IFN- γ and LPS when compared to the alveolar counterparts [330].

6.6.2 *Identification of lung monocytes and macrophages*

Currently phenotyping of lung monocyte/macrophage populations varies, with several methods of identification being proposed, predominantly based on analysis of BAL samples. We aimed to differentiate blood from lung tissue and BAL with contemporaneous samples, so as to validate their identity in our flow cytometric technique. The ability to completely separate subtypes of mononuclear leukocytes is challenging owing to their shared markers and phenotypes, ostensibly as they originate from a common precursor. In addition to alteration of cell surface expression during maturation and migration of circulating to interstitial and alveolar cells, these same cell markers are also differentially upregulated in response to various stressors. Flow cytometric analysis of BAL samples from healthy volunteers versus samples of patients suffering from cardiogenic lung oedema, ARDS and pneumonia patients, showed distinct variation of CD14, HLA-DR, 27E10 and 25F9 expression between these four patient groups [322]. This is particularly pertinent to our study as obtaining a 'control' tissue sample was not possible and is difficult to strictly define in these circumstances. Lung samples from otherwise healthy donors may in fact represent one of the best 'controls'.

In this pilot work we were able to reliably identify mononuclear cell subtypes for the first time in human lung samples, using flow cytometry. Of note the number of monocytes retained in flushed pre-transplantation lungs, far exceeds the number possibly present from residual pulmonary blood. In addition the classical and non-classical 'monocytes' we identified both stained positive for intracellular 27E10. This monoclonal antibody recognises the heterodimer of MRP8 and MRP14, cytosolic calcium binding proteins present in monocytes of circulating origin. Crucially MRP8 and MRP14 are reportedly absent in the cytosol of tissue macrophages, but are upregulated in monocytes by inflammation whereby they translocate to the cell surface [325, 331]. 27E10 staining of lung monocytes in this study thereby supports the hypothesis that they too are of vascular origin and are therefore likely to be either retained through vascular margination or transmigration into the interstitial space, prior to transplantation.

6.6.3 Lung monocyte retention

Large numbers of potentially marginated monocytes in this study suggest inflammation and activation-induced changes in their deformability, and thus retention, in line with our mouse data. This is in accordance with work by others where transit of neutrophils through human lungs has been shown to be rapid with minimal retention unless subjected to prior activation (again inducing deformability and thus margination) [242, 305]. The large numbers of retained neutrophils found in our pre-implantation, flushed lungs, therefore indicates significant activation of these leukocytes.

Sources of leukocyte activation are likely to be numerous in donors and include systemic sepsis, pneumonia, acid aspiration and ventilator-induced lung injury. Activation-induced monocyte retention is further supported by Stone *et al.*, who showed a significant reservoir of non-classical monocytes in perfusate samples collected from rejected human lungs selected for optimisation with an Ex Vivo Lung Perfusion (EVLP) protocol [164]. In addition they demonstrate that non-classical monocytes recovered from the pulmonary perfusate readily differentiate to dendritic cells, and secreted pro-inflammatory cytokines, exhibiting potential for T cell co-stimulation. The 'non-classical' phenotype they describe strongly resemble the non-classical monocytes (27E10+, CD14+, CD16+) seen in our study. They conclude that the prolonged perfusion during EVLP may represent a mechanism for (pro-inflammatory) monocyte 'washout' prior to transplantation. The murine and human data from this study would suggest that whilst perfusion or flushing of the lungs does remove some leukocytes, it is however, not completely effective at removing the majority of marginated cells.

6.6.4 Lung perfusion and leukocyte removal

Utilising a similar technique to EVLP, the Organ Care System (OCS) utilises normothermic perfusion of donor lungs to eliminate static cold storage during the transplantation process and potentially attenuate ischaemic damage. A

recent pilot study indicated that OCS is safe for lung preservation, with a 100% success rate in a 12 patient cohort [28]. The results of the INSPIRE trial comparing OCS with standard storage, however, will shed more light on the viability of this technique. Pulmonary perfusion, integral to this technique, may also represent a unique opportunity to remove retained vascular donor monocytes. Although the samples we processed did not support this as an effective method for monocyte removal, with similar residual numbers being found compared to our standard transplants, despite prolonged perfusion periods. It does however provide an opportunity to apply therapies to the lungs prior to transplant with potential monocyte depletion techniques similar to those used in animal models (e.g. clodronate liposomes, anti CCR2, anti CD18). However, even with theoretical total removal of marginated cells, in addition to the use of similar methodologies to target alveolar macrophages, significant interstitial mononuclear cells populations will likely continue to remain, impervious to removal. It remains unknown if the method of red cell addition to the OCS perfusate may have a confounding effect depending on the number of leukocytes being introduced from stored blood used for this purpose [28].

The concept of ‘passenger’ lung leukocytes is not new with the inevitable carry-over of alveolar macrophages in donor lungs being investigated in rat model of orthotopic transplantation in the late 1990s. Selective depletion of >95% of alveolar macrophages with intratracheal clodronate liposomes significantly reduced TNF production by approximately two thirds. However, depletion did not prevent development of severe acute rejection [332]. This may indicate that whilst depletion was very effective, some TNF was still being produced, potentially from other cellular sources (dendritic cells, mononuclear vascular or interstitial cells), contributing the acute rejection, which still occurred in this group. Of note the number of B and T cells isolated in the BAL of the transplanted rats was also unchanged indicating that AM are not the antigen presenting cells solely responsible for their presence [332].

6.6.5 The role of mononuclear cell CD86

The increased prevalence of acute and chronic lung allograft rejection, in addition to the already limited life span of transplanted lungs (likely to be related to their constant exposure to airborne pathogens), compared to other solid organs, suggests a greater immunological burden in this organ system [333]. The significance of the correlation between expression of the co-stimulatory molecule CD86 and PGD in our lung mononuclear cell population is therefore of particular interest. The full capacity for mononuclear cells to differentiate to dendritic cells remains unclear, however the latter’s ability to present allograft antigens to T cells, (along with MHC II) is well described [222, 294]. This process occurs via the co-stimulatory molecules CD80 and CD86, which regulate T cell proliferation and cytokine production. Of note several studies have shown that CD86 blockade may prolong allograft survival, which supports our findings correlating monocyte CD86 and PGD following transplantation[334-336].

6.6.6 Mononuclear cells and lung injury

In parallel with the previous murine data in this study, we were able to demonstrate a link between lung 'monocyte' number and activation, with injury parameters and graft function post implantation. Whilst this only represents pilot data, it does indicate that assessment of lung graft samples pre-transplantation may reveal previously undetected underlying inflammation, increasing susceptibility to subsequent PGD. Early identification at this stage could therefore enable therapeutic intervention prior to implantation, limiting graft dysfunction in recipients in the future.

6.6.7 Conclusion

In summary, the evidence for monocytes in the propagation of human lung injury following ischaemia-reperfusion is accumulating. This pilot study is the first of its kind to identify and validate the presence of large numbers of monocytes in donor lungs which are carried over as 'passengers' to graft recipients. Crucially, the number and activation of these monocytes correlates with lung injury and primary graft dysfunction, confirming their potential role in the paradigm.

Chapter 7

Conclusion and Future Directions

7.1 The role of vascular monocytes in lung ischaemia-reperfusion injury

The specific role of vascular monocytes in lung ischaemia has, until now, not been fully appreciated. Whilst our group and others have demonstrated a key role for marginated monocytes in other experimental models of lung injury, their role in IR injury has not been investigated [130, 137, 140, 167]. Persistence of these inflammatory cells within the pulmonary circulation is of particular relevance to transplantation whereby marginated monocytes are carried over as ‘passenger’ cells to the recipient, with the propensity to mediate inflammation.

7.1.1 Modelling IR

The first aim of this study was to develop a clinically relevant model of lung IR, which would allow detailed investigation and manipulation of vascular monocytes. To this end we adapted a well-established IPL model to simulate a reproducible sterile lung injury. Whilst no animal model can be regarded as ‘perfect’ the IPL system lends itself well to the initiation of ‘stop-flow’ ischaemia, similar to that seen clinically with lung transplantation. It also allows manipulation of perfusion parameters and content, permitting the omission or addition of any substance or cells to the perfusate itself. This is not possible in *in vivo* based models of lung IR and transplantation.

7.1.2 Investigating the role of vascular monocytes in lung IR injury

Flow cytometry analysis of lung homogenates was a key methodology in this study. It enabled us to identify and enumerate monocytes in lung samples, which persisted in the pulmonary circulation despite considerable vascular perfusion.

We were also able to go on to identify monocyte/macrophage subtypes and their precise location, achieved using a modified dual-compartment staining technique. This confirmed that liposomal-clodronate specifically depleted vascular monocytes and reversed lung injury. We were also able to identify monocyte and neutrophil activation in response to IR, as well as soluble mediator release. Neutrophil activation and mediator release were reversed in the absence of vascular monocytes.

Of note, the interstitial mononuclear cell populations (Ly6C^{Low} ‘monocytes’, dendritic cells) also demonstrated activation in response to our vascular ‘stop-flow’ ischaemic insult. However intravenous clodronate only depleted the vascular monocytes thus identifying them as the key drivers of IR injury in our model. In support of this interpretation, reintroduction of vascular monocytes by adoptive transfer re-established the lung injury prevented by clodronate depletion.

7.1.3 Identifying mononuclear phagocytic cells in human lungs

Formal assessment of mononuclear cells in human lungs following IR has rarely been performed. We therefore set out to develop a robust technique for lung digestion and constituent leukocyte identification using flow cytometry. Based on well-characterised blood leukocyte phenotypes we validated a novel method of lung mononuclear cell identification. This was achieved through the use of 27E10, a monoclonal antibody reportedly present in only circulating or inflammatory monocytes/macrophages [322, 331, 337, 338]. This, and the fact that cells with the classical monocyte phenotype were identified in extensively flushed human lung samples, strongly implies that vascular/marginated monocytes also persist in human lungs and are transferred as ‘passenger’ cells into recipients. Our pilot data also suggested an on-going injurious role for these monocytes with their number and activation correlating with lung injury in the recipients.

7.2 Limitations

As with all studies, this work incorporates a number of limitations.

The limitations of mimicking human pathophysiology with animal models are not insignificant, and whilst they have undoubtedly contributed considerably to our understanding of disease pathophysiology, the species difference cannot be ignored. Moreover, as discussed in length in the introduction, modelling lung ischaemia-reperfusion injury is challenging owing to the multiple oxygen sources and manifestations of ‘ischaemia’, depending on the causes, and the variability in temperature and nutrient availability. The IPL system, despite being a powerful investigative tool, is also prone to considerable variability and susceptibility to issues such as contamination, as outlined in the Chapter 3.

Flow cytometry is also a powerful analytical tool. However, it resembles an indirect method of cell enumeration and activation quantification, influenced by issues such as compensation, autofluorescence (especially of certain cell types e.g. alveolar macrophages) and co-expression of similar markers by many myeloid cells, that represent a spectrum of expression depending on their migration and differentiation states. Combination with other techniques, such as immunohistochemistry and intravital microscopy, would be a desirable adjunct to future work.

Aside from the same flow cytometry based constraints listed above, analysis of the human lung is also limited by several factors. The variable times lungs were exposed to ischaemia in transit, along with all the inherent differences between both donors and recipients, are inevitable and difficult to control in such a select patient population. There is also likely unavoidable regional heterogeneity in transplanted lungs, despite controlling for attempting to control for site of biopsy.

7.3 Future work

While this study has provided important insights into the role of monocytes in both murine and human lung IR injury, our findings also give rise to further important questions that merit investigation.

7.3.1 Monocyte dynamics

The dynamic behaviour and interactions of lung mononuclear cells, as well as the origin and fate of vascular monocytes, require further examination. In the short term using autologous blood at the reperfusion phase of the murine IPL experiments would provide insight into the interaction between the donor passenger monocytes and recipient leukocytes during reperfusion. In addition expanding the use of BrdU labelled cells would enable tracking of monocyte dynamics over time in our mouse model. Alternatively use of a 'chimaeric' system with transgenic e.g. Green Fluorescent Protein mice would also be of benefit, especially to differentiate between donor and recipient monocytes origin following adoptive transfer experiments. It would also allow use of knock-out strains to help elucidate mechanisms of injury further.

Longer term, this system would also benefit from the use of intravital microscopy. This would allow labelled monocytes to be tracked in the pulmonary circulation before, during and after exposure to IR in real time, shedding more light on chemotaxis and trafficking dynamics.

7.3.2 Mechanisms

The mechanisms by which vascular monocytes exert their effect on lung injury in response to IR were not fully elucidated despite considerable efforts. Initial work quantifying phospho-MAP kinases indicated that they may play a proximal role in the process, but preliminary investigation of potential effector mechanisms with inhibitor experiments were inconclusive.

As lung injury *per se* is mediated by loss of endothelial integrity, the interaction of monocytes with the endothelium is likely to be key to this process. Preliminary work investigating glycocalyx integrity as a marker of endothelial injury following IR was encouraging but further refinement of this approach may provide more robust evidence for its role in lung IR.

Other potential mechanisms worth pursuing include the role of signalling lipid sphingosine-1-phosphate (S1P). This plasma molecule has been reported to regulate inflammation-induced vascular leakage in mice through inter-endothelial cell gap integrity and monocyte-endothelial chemotaxis [281, 339]. S1P administration has in fact been shown to ameliorate lung injury in an orthotopic model of lung transplantation in mice [340].

Further investigation into the contribution of caspase and death-signalling pathways are also likely to be of benefit. Evidence in a piglet IR model suggests pro-apoptotic activity peaks with caspase activity suggesting that caspase blockade may reduce lung injury following IR [31, 341].

Investigation of other methods of vascular monocyte depletion may also be warranted including cell adhesion molecule/integrin/CD18 inhibition to limit margination and effectively remove them from the pulmonary vasculature, as shown to be of benefit in a canine transplantation model [225]. The currently popular *ex vivo* perfusion of lung transplants, used for transportation and assessment, allows therapies such as these to be applied prior to implantation and therefore heralds an exciting new era in transplantation.

7.3.3 Human monocyte dynamics

To expand on our pilot work with human lung samples, the ability to adapt compartment staining to whole lungs (or lobes) would elucidate further the precise location and role of the substantial numbers of passenger monocytes found in our samples. Further analysis is underway of the BAL, perfusate and plasma samples obtained from the human lung recipients in the trials mentioned in this work. These will provide key information about the soluble mediator release into these compartments and the presence of other key mediators released in response to IR, such as microvesicles and glycocalyx degradation products.

7.4 Conclusion

This work has clearly emphasised the role of passenger vascular monocytes in lung IR injury both in a murine model and human samples. While further work is required to elucidate this role completely, it is clear that therapies targeting monocyte margination, activation and subsequent barrier dysfunction may be highly beneficial to the transplant population.

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