

Differential tumour necrosis factor signalling in human models of acute lung injury

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by
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Declaration of Originality

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. This thesis was composed on the basis of work carried out under the supervision of Dr Mark Griffiths and Dr Matthew Hind (Department of Leukocyte Biology, Imperial College, London) and Dr Charlotte Summers (Department of Medicine, Cambridge University).

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Abstract

Numerous clinical and experimental studies have implicated tumour necrosis factor (TNF) as a central mediator of acute lung injury (ALI). TNF signals through two cell surface receptors, TNFR1 and TNFR2, which initiate distinct signalling pathways and cellular responses. Using a novel, highly selective TNFR1 domain antibody GSK1995057 allied with TNF-receptor (TNFR) specific muteins, the work in this thesis investigated the roles of differential TNFR signalling in a range of human *in vitro* models of ALI using human lung microvascular endothelial cells (HMVEC-L), human alveolar type 2 cells (hAT2) and human neutrophils. In particular we focused on the cascade of events, which lead to neutrophil migration into the alveolar space during ALI. The TNFR1 signalling pathway mediated HMVEC-L activation (neutrophil adhesion molecule expression and chemokine / cytokine release) and injury (trans-endothelial resistance). These effects were associated with an amelioration of neutrophil migration through HMVEC-L monolayers. TNFR1 signalling also played key roles in neutrophil priming, activation, and cell fate. TNFR1 signalling mediated TNF-induced alterations in neutrophil cell surface molecule expression and reactive oxygen species production. TNFR1 antagonism also reversed TNF induced late survival (20 hours) of human neutrophils. In an attempt to generate translational data investigating the effects of differential TNFR1 signalling, we established a human *ex vivo* model of ALI. Using an LPS method of injury we demonstrated release of the pro-inflammatory cytokines TNF, interleukin (IL)-8, IL-6 and IL-1 β in the alveolar space. This was associated with a significant influx of healthy donor neutrophils and pulmonary oedema in LPS injured lung compared to control treated contralateral lung. These data suggest a central role for TNFR1 signalling in ALI. Translation of these *in vitro* data in our human *ex vivo* lung model may provide the basis for future clinical trials of selective TNFR1 antagonism in ALI. (Words 295)

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Abbreviations

Akt	Protein kinase B
ALI	Acute lung injury
ANOVA	Analysis of variance
AP-1	Activator protein -1
ARDS	Acute respiratory distress syndrome
BALF	Broncho-alveolar lavage fluid
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
cFLIP	FLICE-inhibitory protein
CLP	Caecal ligation and puncture
CMS	Cyclic mechanical strain
CPB	Cardiopulmonary bypass
CXCR2	Chemokine receptor 2
dAb	Domain antibody
DD	Death domain
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid, cDNA (Complementary) dsDNA (Double stranded DNA)
DNA-SB	DNA strand breaks
dNTP	Deoxyribonucleotide triphosphate
DPI	Diphenylene iodonium
DTT	Dithiothreitol
EC	Epithelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ENA-78	Epithelial neutrophil activating peptide-78
ERK	Extracellular signal related kinase
EVL	Ex vivo lung perfusion
FACS	Fluorescence activated cell sorting
FADD	Fas associated death domain
FCS	Fetal calf serum
FGF-7/KGF	Keratinocyte growth factor
FITC	Fluorescein isothiocyanate

fMLP	Formyl-methionyl-leucyl-phenylalanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-monocyte colony stimulating factor
GPCR	G-protein coupled receptor
GRO	Growth-related oncogenes
GSK	GlaxoSmithKline
GRO α	Growth related oncogene-alpha
H ₂ O ₂	Hydrogen peroxide
hAT1	Human alveolar type 1 cells
hAT2	Human alveolar type 2 cells
HBSS	Hanks balanced salt solution
HDAC	Histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-Ethanesulphonic acid
HGF	Hepatocyte growth factor
HIF1 α	Hypoxia-inducible factor-1-alpha
HRP	Horse radish peroxidase
HMVEC-L	Human lung microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
IC ₅₀	Concentration producing 50% inhibition
ICAM-1	Intercellular adhesion molecule 1
ICU	Intensive Care Unit
IgG1	Immunoglobulin-G1
IFN	Interferon
IKK	Inhibitor of nuclear factor kappa B kinase
IKK2	Inhibitor of nuclear factor kappa B kinase beta subunit
IL	Interleukin
IL1 β	Interleukin-1 beta
IL-1R	Interleukin-1 receptor
IL-1RA	Interleukin-1 receptor antagonist
IL-6	Interleukin 6
IL-8	Interleukin 8
IP3	Inositol-triphosphate
JNK	c-Jun N-terminal kinase
KC/CXCL1	Keratinocyte-derived chemokine
KGF7	Keratinocyte growth factor-7
LDH	Lactate dehydrogenase
LFA	Lymphocyte function-associated antigen-1
LOX	Lipoxygenases
LPS	Lipopolysaccharide
LT	Leukotriene

JAM	Junctional adhesion molecules
MAP3Ks	Mitogen activated protein kinase kinase kinases
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic peptide-1
MEKK	MAP kinase kinase kinase
MEKK3	Mitogen activated protein kinase kinase kinase
MIP-1 α	Macrophage chemotactic peptide-1
MIP-2	Macrophage inflammatory protein-2
MKK	Mitogen activated protein kinase kinase
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger RNA
MTT	Methylthiazolitetrazolium
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaN ₃	Sodium azide
NCS	Newborn calf serum
NEMO	NF- κ B essential modulator
NF κ B	Nuclear factor-kappa B
NGFR	Nerve growth factor receptor
OD	Optical density
OLV	One lung ventilation
p38MAPK	p38 mitogen activated protein kinases
PAF	Platelet activating factor
PaO ₂	Partial pressure of oxygen in arterial blood
PBM _s	Peripheral blood monocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PEEP	Positive end-expiratory pressure
PI	Propidium iodide
PI3K	Phosphatidyl inositol 3 kinase
PKC	Protein kinase C
PTK	Protein tyrosine kinase
RANTES	Regulated upon activation normal T-cell expressed and secreted
RIPK1	Receptor interacting kinase protein-1
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction

sE-selectin	Soluble E-selectin
SEM	Standard error of the mean
sICAM-1	Soluble ICAM-1
SODD	Silencer of death domain
SP-A	Surfactant-associated protein A
SP-B	Surfactant-associated protein B
SP-C	Surfactant-associated protein C
SP-D	Surfactant-associated protein D
STAT	Signal transduced and activator of transcription
sVCAM-1	Soluble VCAM-1
TACE	TNF alpha converting enzyme
TGF	Transforming growth factor
TGF β	Transforming growth factor beta-1
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TRADD	TNFR-associated death domain
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
VALI	Ventilator associated lung injury
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
V _H	Heavy chain
V _L	Light chain
V _t	Tidal volume

1 Chapter 1: Introduction

1.1 Acute lung injury

1.1.1 Clinical perspective

Despite almost 40 years of scientific research, outcomes in acute respiratory distress syndrome (ARDS) are comparable to breast cancer or HIV (Camporota, 2006; Rubenfeld et al., 2005) and no specific pharmacotherapy exists. ARDS, which was first described in by Ashbaugh and Petty in 1967 (Ashbaugh et al., 1967), is characterised by acute inflammation that affects the function of the gas exchange surface of the lung, presenting clinically with acute onset of breathlessness and hypoxaemia in the presence of diffuse pulmonary oedema on the chest radiograph. A working definition of ARDS was established in 1994 by the American European Consensus Conference (AECC) (Bernard et al., 1994). This definition identified patients with bilateral pulmonary infiltrates and hypoxaemia ($\text{PaO}_2:\text{FiO}_2$ ratio of less than 300mmHg) as having acute lung injury (ALI). Those with more severe derangements of hypoxaemia ($\text{PaO}_2:\text{FiO}_2$ ratio of less than 200mmHg) were given a diagnosis of ARDS. A recent report - called the Berlin definition (Ranieri et al., 2012) - recommends the use of three categories of ARDS, mild, moderate and severe based on the degree of hypoxaemia (table 1.1). Additionally, the exclusion of pulmonary oedema secondary to cardiac failure is now not mandated, positive end expiratory pressure (PEEP) is accounted for as an indicator of severity and a known risk factor for ARDS has to be present at onset. Whether stratification of patients based on these revised descriptions will enhance clinical detection of ARDS remains to be determined. For the purposes of this thesis, the umbrella term acute lung injury will be used to encompass clinical and experimental definitions of ARDS.

Table 1.1 Berlin Definition of ARDS

	Mild ARDS	Moderate ARDS	Severe ARDS
Gas exchange	PaO ₂ :FiO ₂ 201-300 mmHg	PaO ₂ :FiO ₂ ≤200 mmHg	PaO ₂ :FiO ₂ ≤100 mmHg
PEEP	PEEP ≥ 5cm H ₂ O	PEEP ≥ 5cm H ₂ O	PEEP ≥ 10 cm H ₂ O
Chest x-ray	Bilateral opacities	Bilateral opacities	Bilateral opacities

PaO₂/FiO₂: arterial partial pressure of oxygen/ inspired oxygen fraction PEEP: positive end expiratory pressure

Many conditions are associated with the onset of ALI. The risk factors for ALI can be divided into two groups depending on whether injury to the lung is direct such as pneumonia, or indirect blood-borne insults – for example, severe sepsis (Ware, 2006). Indirect injury, via blood-borne inflammatory mediators, is more common. Sepsis (including pneumonia) is the commonest risk factor (Hudson et al., 1995), as well as being the risk factor most likely to cause ALI. Risk of progression to ALI varies according to the type, number and severity of predisposing conditions, as well as the genetics and other characteristics of the patient (Marshall et al., 2002). Diagnosing and treating conditions that mimic or are associated with ALI is the first principal of successful management of these patients.

A prospective, population-based, cohort study in 21 hospitals in the USA over 14 months in the late 1990s identified 1113 adults who met AECC criteria for ALI (Rubenfeld et al., 2005). The age-adjusted incidence of ALI was 86.2 per 100,000 person-years. The in-hospital mortality rate was 38.5%; mortality was 24% for teenagers and 60% for patients 85 years of age or older. More recent data collected between 2001 and 2008 demonstrated that the incidence of ALI in hospitalized adults declined from 82.4 to 38.9 per 100,000 person-years during the study period (Li et al., 2011). A fall in hospital-acquired ALI was responsible for the observed decline as there was no change in

incidence of ALI on hospital admission during the study period. Improvements in healthcare delivery and process such as adherence to low-tidal volume ventilation and conservative use of blood products were sighted as possible reasons for the observed decline. Based on the most recent ARDS Network trials, a 60-day mortality of 22% has been reported despite higher severity of illness scores and a higher incidence of shock at enrollment compared with previous trial data (National Heart et al., 2011).

Once treatment of the precipitating condition and patient support has begun, prognostic factors relate to the patient's response to therapy. Severe arterial hypoxemia ($\text{PaO}_2/\text{FiO}_2 < 100\text{mmHg}$) and an increase in the pulmonary dead space fraction (>0.60) are associated with higher mortality (Nuckton et al., 2002). Mortality rates correlate with the number of organ system failures, increasing to 83% when three or more are present. Complications appearing during the course of ARDS, including circulatory shock, acute renal failure and liver dysfunction, allied with age over 60 years are associated with a higher mortality (Liu et al., 2007; Cooke et al., 2008; Brown et al., 2011). The majority of late deaths are related to sepsis syndrome, present in 73% of ALI patients, in whom the predominant site of infection is the lung (Bachofen and Weibel, 1982). Several investigators have indicated that sepsis rather than respiratory failure is the leading cause of death in patients with ARDS (Montgomery et al., 1985; Luhr et al., 2000; Fein et al., 1983; Doyle et al., 1995).

Most ALI patients either die or are weaned from supportive treatment within 1-2 weeks, although up to 10% of patients require prolonged (30 days or more) mechanical ventilation. Lung function in most survivors returns to normal over 6-12 months although the majority have persistent, abnormal exercise endurance; the six minute walk distance at one, three and five years was 66, 67 and 76 percent of predicted respectively in a prospective study of 109 ARDS survivors (Herridge et al., 2011). However,

neuromuscular and neurocognitive morbidity can significantly impair longer- term health-related quality of life (Herridge et al., 2003; Hopkins et al., 1999; Wilcox and Herridge, 2010).

1.1.2 Pathogenesis of acute lung injury

1.1.2.1 Epithelial activation and injury

The normal alveolar epithelium is composed of approximately equal numbers of flat type I cells (hAT1) which account for 95% of the alveolar surface area but only 8% of the total cells in the adult human lung and cuboidal type II cells (hAT2) which comprise 15% of the total cells of the lung, but only 5% of the alveolar surface area (Crapo et al., 1982). In contrast to hAT2, hAT1 are susceptible to injury and cell death. hAT2 have several critical functions, including surfactant production and ion transport. They also function as progenitor cells for regeneration of hAT1 cells after injury. Hence, alveolar epithelial injury has critical implications for the pathogenesis of ALI. Indeed, the extent of alveolar epithelial damage is an important predictor of outcome (Matthay and Wiener-Kronish, 1990). Typical histological appearances include extensive necrosis of alveolar type I cells and the formation of protein-rich hyaline membranes on a denuded basement membrane.

Alveolar fluid clearance by hAT2 is primarily driven through sodium uptake on the apical membrane, followed by extrusion of sodium on the baso-lateral surface by Na⁺K⁺- ATPase (Matthay et al., 2000). Loss of alveolar epithelial integrity through apoptosis and necrosis of hAT2 results in the accumulation of protein-rich and highly cellular oedema fluid in the interstitium and alveoli. Furthermore, *in vitro* data suggest that inflammation further impairs fluid clearance through down-regulation of sodium and chloride transporters on epithelial cells (Lee et al., 2007).

Loss of surfactant producing hAT2, allied with the effect of plasma proteins in the airspace, impairs surfactant turnover and production contributing to surfactant deficiency and dysfunction (Lewis and Jobe, 1993; Gregory et al., 1991). Surfactant is a complex mixture of phospholipids and proteins, which reduces alveolar surface tension preventing collapse of the airspace. Thus, abnormalities in surfactant production and function may contribute to the pathogenesis of ALI through exacerbation of atelectasis and impairment of gas exchange, increased oedema formation, and impairment of local host defense. This presents clinically with collapse of lung units and reduced lung compliance.

Epithelial cells also play a key role in regulating the inflammatory response in the alveolar space through:

1. Production of injury-driven pro-inflammatory cytokines, including tumour necrosis factor (TNF), interleukins (IL) -1, -4, -6, -13, substance P, and platelet-activating factor (PAF)
2. Release of chemokines including IL-8, monocyte chemotactic protein-1 (MCP-1), growth related oncogene-alpha (GRO- α), and epithelial-derived neutrophil-activating peptide 78 (ENA-78)
3. Cell-cell interactions with resident lung cells, in particular alveolar macrophages.
4. Expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), to facilitate leukocyte trafficking into the alveolar space

These processes are central to both the onset and progression of ALI (Bermudez and Goodman, 1996; Steinberg et al., 1994; Tate and Repine, 1983). Neutrophil migration into the alveolar space, in response to a chemokine gradient, further disrupts epithelial cell barrier function, fluid clearance and surfactant production. This occurs through the release of oxidants, proteases, leukotrienes, and other pro-inflammatory mediators (discussed in chapter 1.4.1).

1.1.2.2 Endothelial activation and injury

The pulmonary endothelium forms a continuous, tight barrier of squamous endothelial cells, which regulate fluid permeability as well as modulating host inflammation, vascular tone, angiogenesis and interactions with blood-borne cells. Loss of barrier integrity, characterized by the formation of reversible intercellular gaps between endothelial cells, is accepted as the ultra-structural basis for the increased permeability pulmonary oedema observed in ALI (Hurley, 1982). Hyper-filtration of plasma across the lung vascular bed results in the accumulation of protein-rich oedema in the interstitium and the alveoli, even in the context of normal lung vascular pressure (Calfee and Matthay, 2007). Gap formation is induced by the binding of mediators, including thrombin, thromboxane A₂ and transforming growth factor beta (TGFβ) to their cognate receptors on the endothelial cell surface, which induces cytoskeletal rearrangement and endothelial barrier disruption. Sources of these mediators include platelets, leukocytes and epithelial cells. Gap formation may also occur as a result of endothelial cell apoptosis in response to specific stimuli, such as endotoxin.

Similar to the alveolar epithelium, the lung endothelium orchestrates and propagates the inflammatory response. After stimulation by a range of mediators, endothelial cells release cytokines and chemokines, and up-regulate the expression of neutrophil (and platelet) adhesion molecules, such as ICAM-1, vascular cell adhesion molecule 1 (VCAM- 1) and E-Selectin. Leukocytes, attracted via a chemokine gradient accumulate in the microcirculation of the lung, where cytokine-induced cytoskeletal alterations reduce their deformability, promoting sequestration in the lung microvasculature (reviewed in chapter 1.4). Neutrophils, activated by local mediators and cytokines subsequently degranulate releasing toxic mediators, which can further increase

lung vascular permeability through alterations in focal adhesions, transmembrane integrins and the cytoskeleton of endothelial cells.

In response to a range of stimuli including hypoxia, cytokines, chemokines, and activated platelets and neutrophils, the pulmonary endothelium shifts from an anti-thrombotic and anti-inflammatory phenotype to an activated state, characterized by pro-thrombotic and pro-adhesive properties. Central to this transformation is the up-regulation and activation of tissue factor and loss of the ability to activate proteins C and S, resulting in capillary thrombosis and extra-vascular fibrin deposition, potentiating pulmonary dysfunction and the acute inflammatory response (Bastarache et al., 2006; Idell, 2003; Wang et al., 2008). It may also contribute to the increased dead-space fraction seen in ALI patients (Nuckton et al., 2002).

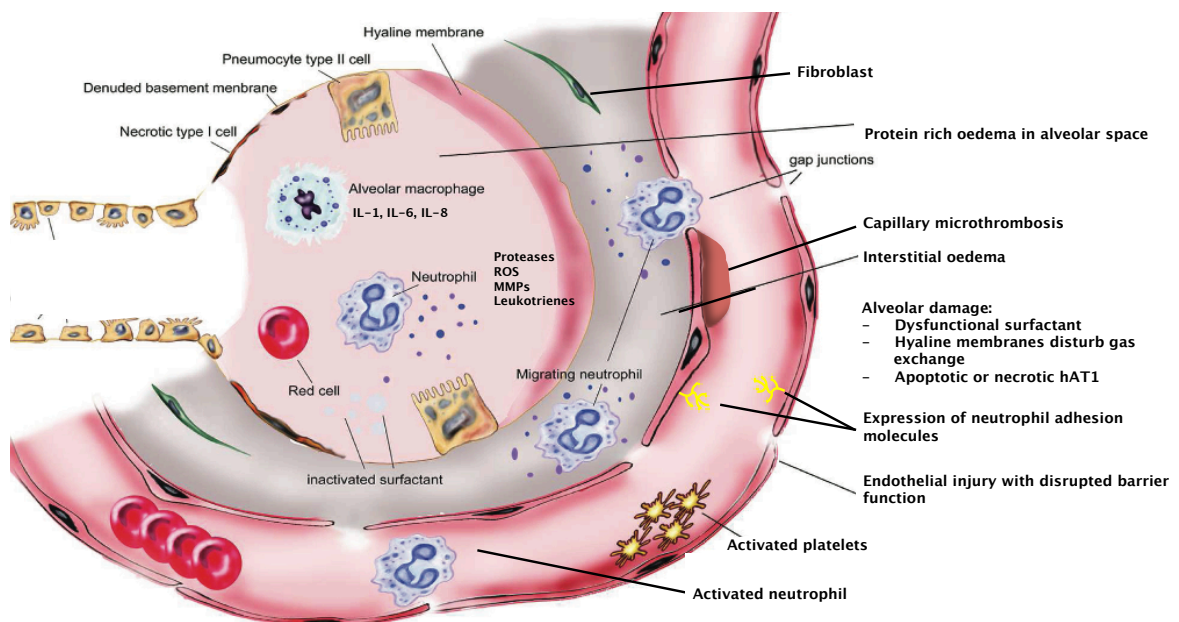


Figure 1.1 Pathogenesis of acute lung injury

Adapted from (Grommes and Soehnlein, 2011), ROS: reactive oxygen species, MMP: matrix metalloproteinases

1.1.2.3 Ventilator associated lung injury

The application of mechanical ventilation, required in the majority of patients, exacerbates the syndrome further in a process called ventilator associated lung injury

(VALI). Mechanical forces applied during ventilation cause physical disruption of the alveolar-capillary membrane leading to pulmonary oedema, whilst cyclical stretch that is insufficient to mechanically disrupt tissues, induces activation of cell-signalling pathways in epithelial, endothelial and inflammatory cells. These adverse effects, in the context of the injured lung, may result in cell death or the expression of pro-inflammatory and/or pro-fibrotic mediators (e.g. TGF β , TNF, IL-1 β , IL-6, IL-8) (Stuber et al., 2002; Ranieri et al., 1999; Belperio et al., 2006). These pro-inflammatory mediators further propagate injury and phagocyte influx into the alveolar space, resulting in a pathophysiology that is indistinguishable from ALI of other aetiologies. The precise source, and indeed role of individual mediators remains unresolved.

The observation that injurious mechanical ventilation could also lead to the systemic release of inflammatory mediators into the circulation (Slutsky, 2005; Tremblay and Slutsky, 1998) as well as translocation of bacteria (Nahum et al., 1997) or endotoxin (Murphy et al., 2000) from the airspace into the bloodstream has led to the biotrauma hypothesis of VALI. Thus, in addition to local injury, VALI can drive systemic inflammation and extra-pulmonary organ damage. This is reflected in the fact that the majority of ARDS patients die from multi-system organ failure, as opposed to hypoxaemic respiratory failure (Stapleton et al., 2005; Vincent and Zambon, 2006).

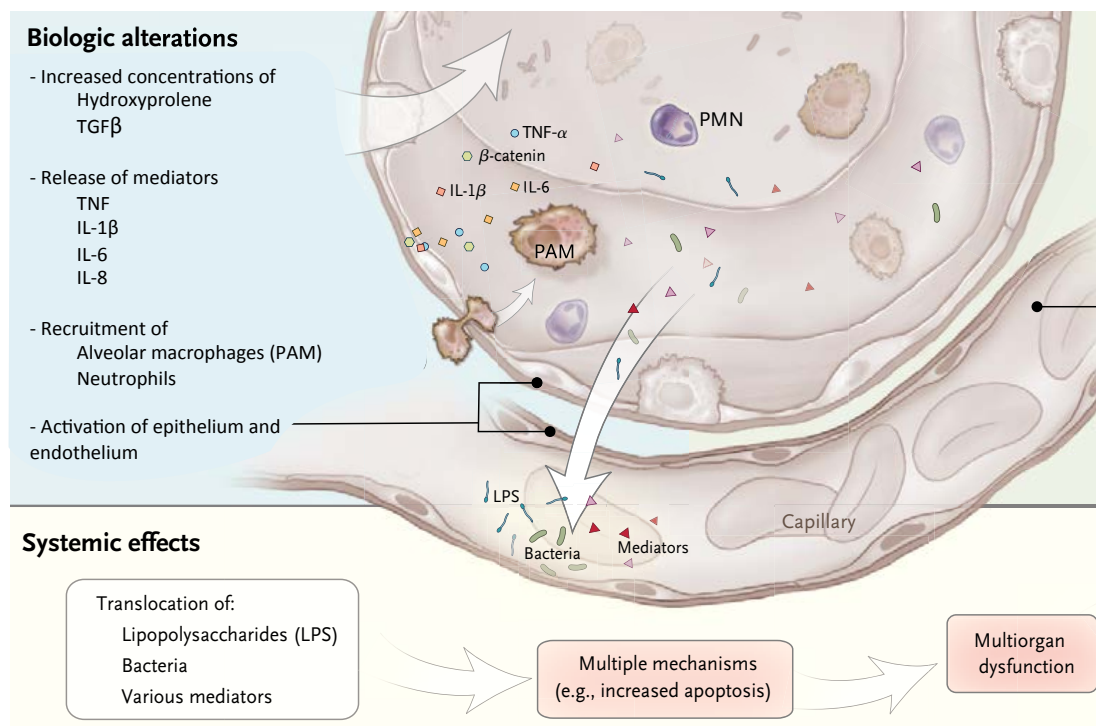


Figure 1.2 Pathobiology of ventilator associated lung injury

Adapted from (Slutsky and Ranieri, 2013), TGF β: transforming growth factor-β, TNF: tumour necrosis factor

The magnitude of the clinical burden of VALI was demonstrated by the ARDS Network study, in which 861 patients with ARDS were randomized to receive either a high tidal volume (12ml/kg predicted body weight), or a low tidal volume ventilation strategy (6ml/kg predicted body weight) (Network, 2000). The low tidal volume cohort demonstrated a 9% absolute reduction in mortality from 39.8 percent in the conventional group to 31 percent in the low volume group. There were also more ventilator- and organ failure-free days in survivors in the low volume group.

1.1.2.4 Resolution of inflammation and repair of the injured lung

Following the acute inflammatory phase, most patients gradually recover normal physiology and lung function. In the alveolar epithelium, which is normally lined by approximately equal numbers of type I and type II cells, type II cells proliferate in response to stimulation by epithelial growth factors, including keratinocyte (KGF) and hepatocyte growth factors. Type II cells are also thought to act as progenitor cells for both

daughter type II cells and type I cells (Sannes, 1991), differentiating into type I cells in response to specific developmental cues. Local and bone marrow-derived stem cells, recruited to the injured lung by soluble factors (Rojas et al., 2005), may also contribute to repair (Lee et al., 2009; Kumar et al., 2011; Burnham et al., 2005). Collectively, these mechanisms combine to reconstitute the epithelial barrier and restore lung function.

However, if epithelial injury is severe, or epithelial repair is impaired, a fibroproliferative phase of ALI can ensue either following or in parallel with epithelial injury possibly through activation of proteinase-activated receptors (Jose et al., 2014; Howell et al., 2005). During this phase, mesenchymal cells proliferate, neovascularisation occurs and the alveolar space becomes filled with activated fibroblasts and myofibroblasts that synthesise excessive collagenous extracellular matrix. A small proportion of patients progress to a chronic phase of respiratory insufficiency characterized by widespread pulmonary fibrosis with disordered lung architecture and evidence of sub-pleural and intrapulmonary cysts (Gattinoni et al., 1994).

Resolution of inflammation in ALI occurs through clearance of alveolar neutrophils, monocytes, and necrotic debris from the distal airspaces of the lung. Macrophages remove apoptotic neutrophils and monocytes via a process called efferocytosis, involving phagocyte migration towards apoptotic cells, receptor adhesion, and ingestion (Bratton and Henson, 2011; McCubrey and Curtis, 2013). Regulatory T cells may play an essential role in this process by enhancing neutrophil apoptosis and suppressing cytokine secretion, in part by release of TGF- β (D'Alessio et al., 2009).

1.1.3 Treatment of acute lung injury

Despite over 40 years of research, none of the pharmacological interventions developed and investigated in ALI have translated into measurable clinical benefit. Current best practice focuses on treatment of the precipitating insult, supportive therapy and the avoidance of complications, which add further insult to injury (Slutsky and Ranieri, 2013).

Owing to the heterogeneity of causes and biology of ALI, efforts to develop adjunctive treatments have largely focused on targeting specific aspects of the pathogenesis of ALI (reviewed in (Sweeney et al., 2013; Roch et al., 2013), for example, anti-inflammatory therapies. Despite promising experimental data supporting their potential efficacy, clinical trials of granulocyte-macrophage colony stimulating factor (GM-CSF), corticosteroids, antioxidants, and anti-cytokine therapies have failed to demonstrate a mortality benefit. Alternatively, agents have been aimed at improving physiological abnormalities in ALI; for example, diuretics and β_2 agonists to minimise pulmonary oedema, vasodilators and anticoagulants to optimise alveolar perfusion, and surfactant and neuromuscular blocking agents to limit VALI. With the exception of one recent trial of neuromuscular blockade (Papazian et al., 2010), none of these agents have demonstrated clinical benefit. More recently, investigators have focused on cell-based therapy to enhance alveolar repair (Matthay et al., 2010; Lee et al., 2009) and modulation of signalling pathways to reduce lung vascular permeability (London et al., 2010).

Numerous factors explain the failure of pharmacotherapy trials in ALI. Many of these issues relate to the logistics of researching and treating a heterogeneous population with a range of underlying pathologies and comorbidities, suffering from a syndrome with no definitive diagnostic test or biomarker. Nonetheless, there are unquestionably deficiencies in the translation of promising experimental data into clinically effective

therapies, in part, is due to limitations of the currently available pre-clinical (animal) models of ALI (Matute-Bello et al., 2008; Bastarache and Blackwell, 2009).

1.2 Current experimental modelling of acute lung injury

1.2.1 Animal models

Animal models are invaluable in the study of complex syndromes such as ALI, allowing the testing of hypotheses in complex biological systems. Rodent ALI models, in particular, have revealed important biologic mechanisms underlying the temporal sequence of injury, inflammation and dysregulated repair.

In parallel with the aetiologies of human ALI, animal models are broadly divided into those inducing direct and indirect injury. Modes of direct injury include noxious stimuli (e.g. intra-tracheal or intranasal administration of bacteria, or bacterial products such as lipopolysaccharide (LPS)) to model pneumonia, the administration of intra-tracheal acid to reproduce aspiration pneumonitis, and simulation of VALI using high tidal volume ventilation. Indirect injury models include sepsis models such as caecal ligation and puncture (CLP) and the administration of intravenous bacteria or LPS or intravenous oleic acid model, which simulates the release of bone marrow fats in ALI patients following long bone fracture.

The injury in these models broadly targets either the capillary endothelium or alveolar epithelium (Matute-Bello et al., 2008). Although each can reproduce components of the key pathological changes that occur in ALI, none is capable of fully recapitulating the clinical features. For example, although LPS administration elicits a neutrophilic inflammatory response with an increase in intrapulmonary cytokines, changes in alveolar-capillary permeability are mild (Wiener-Kronish et al., 1991). In fact, of the currently available animal models, only acid aspiration, transfusion associated lung injury (TRALI)

and VALI models mimic human aetiologies of ALI to a reasonable extent. Combination models using two mechanisms of injury, such as surfactant depletion and high tidal volume ventilation, might overcome some of these failings. Recapitulating the coagulation and fibrinolytic abnormalities of ALI, in particular, continues to pose a scientific challenge.

Despite extensive study in current animal models, none of the pharmacological interventions developed has translated into measurable clinical benefit. Additional limitations might therefore exist – for example, there are fundamental differences in anatomy and physiology between humans and both small and large animals. Indeed, comparative proteomic analysis between bronchoalveolar lavage fluid (BALF) from an ALI patient and a mouse model of ALI identified only 21 homologous proteins (Gharib et al., 2010). In addition, airway architecture and pleural anatomy show marked differences (Albertine et al., 1984; Shapiro, 2006), and normal rates of alveolar epithelial fluid transport differ markedly across species (Matthay et al., 1985; Sakuma et al., 1997; Smedira et al., 1991). Primate studies are more closely representative of human disease but are logistically complicated, financially prohibitive and fewer genetic and genomic tools are available. A further drawback of animal models is interspecies difference in host immune response to epithelial injury and the coagulopathy of ALI (Bastarache and Blackwell, 2009). Animal size places limitations on sampling volume of, for example, blood and BALF, as well as obtaining accurate physiological measurements. Modelling of established, severe ALI presents logistical problems in the provision of prolonged intensive care to critically ill rodents; most animals die from shock or hypoxemia. Finally, there are no robust biomarkers in animal models of ALI that translate into the human syndrome, and *vice versa*. Despite these limitations, animal models will continue to have a considerable role in the study of ALI for the foreseeable future, principally because few

alternatives allow investigation (including imaging modalities) and therapeutic testing in a complex biological system.

1.2.2 Human models

1.2.2.1 *In vivo* models

One lung ventilation (OLV) is a technique used in thoracic surgery describing ventilation of a single lung, allowing the other one to collapse. The method optimises surgical access during lung resection and oesophagectomy procedures. OLV is associated with pulmonary inflammation, reflecting what is defined as subclinical ALI in most patients (Moloney et al., 2004). The majority of the pathophysiological features of OLV-induced ALI, including endothelial and epithelial dysfunction, neutrophil infiltration and vascular congestion, are common to ALI of other aetiologies (Carden and Granger, 2000; Her and Mandy, 2004; Jordan et al., 2000; Yin et al., 2007). The incidence of ALI after lung resection is 2-4% after lobectomy and up to 8% after pneumonectomy, with a mortality of 40-60% (Alam et al., 2007; Dulu et al., 2006; Kutlu et al., 2000; Ruffini et al., 2001). The incidence of ALI after oesophagectomy in a single centre study was 23.8% (Tandon et al., 2001).

Pulmonary dysfunction following cardiopulmonary bypass (CPB) is common, with ALI occurring in 2% of patients. Causative mechanisms include ischaemia-reperfusion, endothelial dysfunction, systemic inflammation and atelectasis (Dodd-o et al., 2004; Fan et al., 2006; Syed et al., 2004). Analysis of intra-operative lung specimens showed evidence of alveolar oedema, extravasation of erythrocytes and neutrophils, and congested alveolar capillaries (Wasowicz et al., 1999). Electron microscopy demonstrated swollen and necrotic pneumocytes and endothelial cells (Wasowicz et al., 1999).

The elective nature of surgery affords an opportunity to identify a cohort of potentially predictable ALI patients for future study. Indeed, the efficacy of hydroxymethylglutaryl-CoA (HMGCoA) reductase inhibitors to modulate physiological and biological parameters and beta agonists to improve clinical outcomes in patients undergoing oesophagectomy has recently been assessed by investigators in the UK (Shyamsundar et al., 2014; Perkins et al., 2014). Whilst, beta agonists had no impact on ALI prevention, the study recruited over 350 patients suggesting that the post-oesophagectomy model is viable and feasible human model for future study of novel interventions in ALI.

Inhalation of LPS, a component of the outer membrane of gram-negative bacteria, results in a local subclinical alveolar inflammatory response with neutrophil recruitment and an increase in cytokine and chemokine production (Jagiello et al., 1996; Nick et al., 2004; O'Grady et al., 2001; Sandstrom et al., 1994), analogous to that which occurs in ALI. Notably, the coagulopathic and fibrinolytic abnormalities associated with ALI are also replicated in the inhaled LPS model (Maris et al., 2005). Although a mild systemic inflammatory response is also observed, neither the pulmonary or systemic effects have been associated with significant adverse clinical events.

Models of nebulized or bronchoscopically instilled LPS have been applied to test a range of putative therapies in ALI, including recombinant human activated protein C, β -agonists, corticosteroids and phosphodiesterase-4 inhibitors (Chen et al., 2009; Michel et al., 2007; Nick et al., 2004; Shyamsundar et al., 2009). More recently the inhaled LPS model was used as part of a randomized control trial to investigate monocyte depletion as a means of modulating early neutrophil recruitment to inflamed lung (Barr et al., 2013). Following LPS inhalation, 30 volunteers were randomised to actual or sham leukapheresis to reduce peripheral blood monocytes. Despite failing to detect any clinical or biological

effect, this translational study demonstrated the potential of human models to inform later-phase clinical trials.

There are several pathological, ethical and logistical challenges that limit the use of *in vivo* surgical models in humans. First, these are short-term models of low-level inflammation and although many of the pathological hallmarks of ALI are re-created, the physiological decrements in gas exchange and the levels of pro-inflammatory cytokines induced are modest. Second, *in vivo* studies are inevitably constrained by clinical priorities and ethical considerations. Third, it is important to recognise that the pathogenesis of ALI after operations involving both OLV and CPB is multi-factorial, and patient factors combine with the direct effects of OLV and CPB to compromise pulmonary function in the early post-operative phase.

1.2.2.2 *Ex vivo* models

Ex vivo lung research has been widely used to study lung function in small animals and other species (Broccard et al., 2002; Wang et al., 1989; Wisser et al., 1993). Human *ex vivo* research uses consented donor organs that are unsuitable for transplantation based on International Society for Heart and Lung Transplant (ISHLT) criteria (Orens et al., 2006). Organs are maintained in a viable state with established ventilation and perfusion systems.

Human *ex vivo* models of ALI involve similar noxious stimuli to those used in animals, including live bacteria, which can be either infused into lung tissue via the perfused vasculature, or delivered to the airways and distal lung by nebulisation, or delivered under direct vision to a targeted area using a bronchoscope. Such models may permit testing of disease-modifying therapies in ALI to generate relevant, reliable and predictable human pharmacodynamic, pharmacokinetic and toxicology data through analysis at the organ, cell, genomic and molecular level, before early phase clinical

studies. *Ex vivo* modelling is superior to tissue and cell-based assays, as the architectural integrity of the lung is preserved. For example, type I pneumocytes that cover over 90% of the gas exchange surface of the lung and are difficult to culture *in vitro*; therefore, little is known about the response of this cell type to injury and subsequent mechanisms of repair. Moreover, *ex vivo* ALI models might allow recapitulation of severe ALI, experimental manipulation and temporal analysis of distal lung tissue, which are not achievable using *in vivo* human models.

Currently, there are two main techniques for maintaining lungs *ex vivo*: The isolated perfused lung model and the *ex vivo* lung perfusion (EVLP) model. In the isolated perfused lung model, the lungs, together with the heart, are surgically removed and suspended by the trachea in a humidified, jacketed chamber maintained at 36-37°C. The lungs are perfused with a buffer solution that enters the lung by the pulmonary artery and drains via the pulmonary vein. The buffer solution is then collected or re-circulated. The lung is either left unventilated, or continuous positive airway pressure is applied.

Ex vivo lung perfusion was developed by Steen (Steen et al., 2003) as a method to assess the quality of donor lungs from non-heart beating donors. It is used clinically to facilitate reconditioning and subsequent transplantation of unacceptable donor lungs (Egan et al., 2006; Steen et al., 2007; Wierup et al., 2006). An elaborate and expensive extracorporeal circuit and ventilator system replicate *in vivo* physiology; lungs are ventilated with a low tidal volume strategy, while an extracorporeal circuit with a centrifugal or roller pump generates perfusate flow which can be as high as 40% of the donor's cardiac output. An albumin-based perfusate, with or without matched red cells is used to optimise oncotic pressure and reduce endogenous lung oedema. Whole lungs have been maintained on EVLP for up to 12 hours with preservation of lung function and avoidance of additional lung injury (Cypel et al., 2009; Cypel et al., 2008). Clinically,

EVLP has been used as a platform for administration of antimicrobial therapy to reduce the burden of bacterial load in lungs that otherwise would be rejected for transplantation. Moreover, gene therapy with an adenoviral vector encoding human interleukin-10, attenuated inflammation in injured human donor lungs resulting in improved lung function after 12 hours of *ex vivo* perfusion (Cypel et al., 2009).

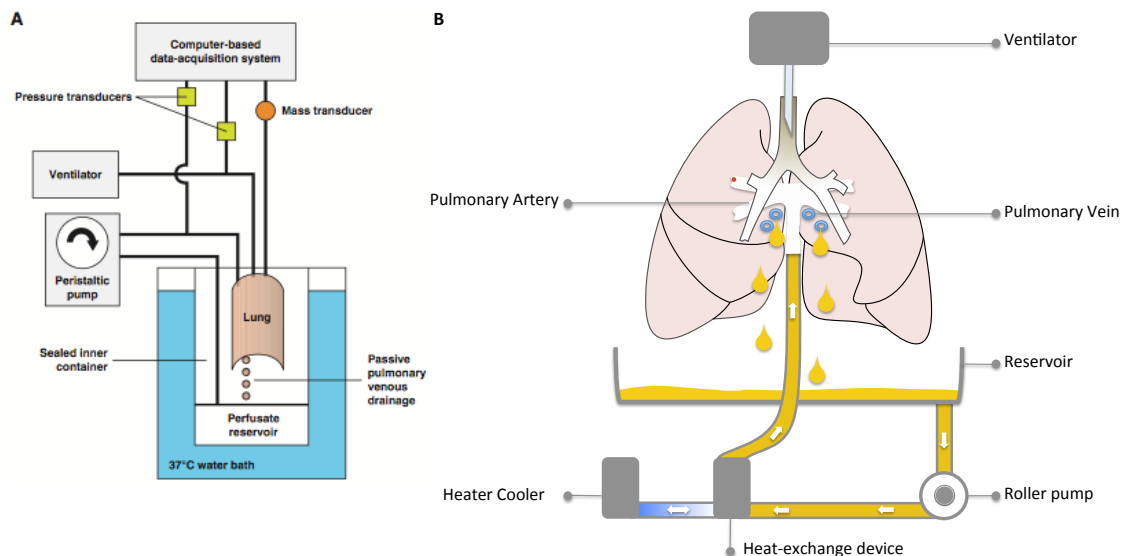


Figure 1.3 Ex vivo human models of ALI

The isolated perfused lung model (panel A) is a simplified version of the *ex vivo* lung perfusion model (EVLP, panel B). In the isolated perfused lung model whole human lungs are suspended by the trachea in a humidified chamber. The lungs are perfused with a buffer solution, at a low flow rate, that drains passively via the pulmonary vein. The lungs are either left unventilated or continuous positive airway pressure (CPAP) is applied. The EVLP uses a modified cardiopulmonary bypass circuit to perfuse a high oncotic pressure solution with red blood cells. A ventilator is connected to the trachea via an endotracheal tube and a protective ventilatory strategy (6 ml/kg tidal volume) used. Adapted from (Lee et al., 2009)

Although *ex vivo* modelling has many potential benefits, there are several limitations. It is important to recognise that most lungs donated for research have been declined for transplantation, baseline physiology is often abnormal and there will inevitably be a degree of background ALI present prior to use of the tissue for experimental modelling. Moreover, donated lungs are unlikely to be homogenous, with a wide variation in ischaemic times and donor co-morbidities, including smoking. The

direct impact of cold preservation of the lungs for transport on the development of ALI remains poorly understood. Available donor lungs are, by necessity, at a premium. Organ procurement is further limited by legal, ethical and cultural considerations, which vary between states and countries. This process is facilitated by collaboration with donor networks and organ procurement organisations. The fact that renal and hepatic metabolism are absent in *ex vivo* models also needs to be taken into consideration when interpreting pharmacokinetic data.

1.3 Tumour Necrosis Factor

1.3.1 TNF as a pro-inflammatory cytokine

TNF is a pleiotropic cytokine regulating many biological processes including immune function, inflammation, energy metabolism, cellular fate, proliferation and differentiation. In general however, it may be considered to represent a major pro-inflammatory and immuno-regulatory mediator, with the capacity to induce necrosis and apoptosis (MacEwan, 2002). TNF is mainly produced by a range of cells including macrophages, dendritic cells, T-lymphocytes, mast cells, endothelial cells, fibroblasts and neuronal tissue (MacEwan, 2002; Wajant et al., 2003) and most cells show at least some TNF responsiveness.

The central role of TNF as a regulator of inflammation was highlighted by the side effect profile of recombinant TNF administered to patients as an anti-cancer therapy in the late 1980's. Patients developed a symptom profile that paralleled clinical sepsis including headache, nausea, vomiting, fever, rigors, anorexia, hypotension diarrhoea and coagulopathy (Bauer et al., 1989; Jakubowski et al., 1989). These symptoms and the associated metabolic responses closely mimicked those following systemic LPS administration to healthy volunteers (Michie et al., 1988). Furthermore, lethal septic shock

does not occur in TNF deficient mice (Ito et al., 2006). Accordingly, TNF is implicated in the pathogenesis of many diseases including atherosclerosis, Alzheimer's disease, and multiple sclerosis. TNF has a central role in auto-immune diseases including rheumatoid arthritis, psoriasis and inflammatory bowel disease, where anti-TNF therapy has demonstrated clinical benefit (Klareskog et al., 2004; Breedveld et al., 2006). Concomitantly, inhibition of TNF increases susceptibility to infections with bacteria or mycobacteria, further emphasizing the essential role of TNF in immune responses and inflammation (Feldmann and Steinman, 2005; Galli et al., 2008).

1.3.2 TNF biology

TNF is synthesized as a 34kDa type II transmembrane protein, meaning that it crosses the membrane once and is orientated such that its amino terminus is intracellular and its carboxy terminus is extracellular. TNF can either function in its membrane-bound form, or it can be released as a soluble circulating (cleaved) form of a 17kDa polypeptide by a metalloproteinase called TNF alpha converting enzyme (TACE/ADAM17) (Black et al., 1997; Moss et al., 1997) and other sheddases (Krunkosky et al., 2005; Wajant et al., 2003; Aggarwal, 2003).

TNF signals through one of two type I transmembrane receptors designated TNF receptor-1 (TNFR1, p55) and TNF receptor-2 (TNFR2, p75) (Locksley et al., 2001) to initiate distinct signalling pathways and cellular responses (Aggarwal, 2003). The extracellular domains of both TNFR are similar and contain four cysteine rich structural motifs that form TNF-binding domains. By contrast, intracellular domains are quite different. Both receptors are devoid of intrinsic enzymatic activity, and each signal by recruiting cytosolic proteins to their intracellular domains as a result of the conformational changes induced by ligand binding. Subsequent events are best understood for TNFR1 and are illustrated in figure 1.4. Cell surface receptor expression is regulated by protein

synthesis, the action of sheddases and by receptor internalisation (Higuchi and Aggarwal, 1994). TNFR1 is predominantly sequestered in the Golgi apparatus and mobilized to the cell surface, whereas TNFR2 is typically localized to the plasma membrane. TNFR1 expression is thought to be ubiquitous, whereas expression of TNFR2 is mainly restricted to immune and endothelial cells (Gomez et al., 2006; Ermert et al., 2003).

The actions of TNF depend on the relative abundance of these receptors and are antagonised by circulating inhibitors (TNF binding proteins) and soluble receptors. Whilst both receptors bind soluble TNF with similar affinity, membrane TNF may preferentially interact with TNFR2 (Grell et al., 1998; Grell et al., 1995). In addition to responding to TNF, both TNFR may be activated by a structurally homologous ligand called lymphotoxin. It has also been suggested that TNFR2, which can be more abundantly expressed, may engage in a phenomenon called 'ligand passing'; TNFR2 binds and then immediately dissociates from TNF in close proximity to TNFR1, resulting in transfer of TNF to TNFR1, and promotion of TNFR1 activation (Tartaglia et al., 1993). This phenomenon appears to occur when TNFR2 and soluble TNF levels are low.

1.3.3 TNF signalling pathways

1.3.3.1 The TNFR1 signalling pathway

The cytoplasmic region of TNFR1 contains a death domain (DD) that couples TNFR1 to either of two distinct signalling pathways via binding of the adapter protein TNFR-associated death domain (TRADD). In the absence of ligand, TNFR1 is thought to associate with silencer of death domain (SODD) to prevent signalling, although genetic deletion does not induce constitutive signalling. TNF binding releases SODD and promotes the binding of TRADD. In the main signalling pathway, TRADD recruits two additional proteins, namely receptor interacting kinase protein-1 (RIPK1) and TNFR-associated factor 2 (TRAF2). This complex is internalised and released from TNFR1;

inhibition of internalisation interferes with endothelial cell signalling (Schutze et al., 1999). The internalised complex along with other incorporated proteins (called a signalosome), then functions through recruitment and activation of cytosolic proteins including; mitogen activated protein kinase kinase kinases (MAP3Ks), c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinases (p38MAPK), apoptosis signalling kinase (ASK)-1 and the inhibitor of nuclear factor kappa B kinase (IKK) complex. Hence, the transcription factors, nuclear factor kappa-B (NF- κ B) and activator protein-1 are activated culminating in the induction of expression in genes important for cell growth and death, development, oncogenesis, immune, inflammatory, and stress responses (Grell et al., 1998). Thus, TNFR1 modulates recruitment of inflammatory leukocytes, mediates production of pro-inflammatory chemokines and promotes trans-endothelial migration of leukocytes of cells (Paleolog et al., 1994; Slowik et al., 1993) and oxidative burst in neutrophils (Dri et al., 2000).

After several hours, the signalosomes assembled on TRAD may be inactivated by a de-ubiquinating enzyme called A20 (induced by activation of NF- κ B), or further modified by recruitment of Fas associated death domain (FADD). FADD converts the signalosome to a death inducing signalling complex (DISC), which can activate caspase-8, and subsequently caspase-3 to induce apoptosis (Higuchi and Aggarwal, 1994; Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004). However, cells lacking FADD are also susceptible to cell death via a poorly understood pathway involving receptor interacting kinase protein-3 (RIPK3) in a process called necroptosis (Osborn et al., 2010).

In addition to activating cell death pathways, TNF can inhibit cell death via the caspase and JNK pathways. Activated NF- κ B induces transcription of FLICE-inhibitory protein (cFLIP), which competes with procaspase-8 for binding to FADD, preventing activation of caspase-8 by the TRADD/RIPK1/TRAF2/FADD complex (Hehlhans and

Pfeffer, 2005; Naude et al., 2011; Ware, 2005). It has also been suggested that TNF can activate mitochondria to sequentially release reactive oxygen species, cytochrome C, and Bax, leading to activation of caspase-9 and caspase-3 and thereby causing apoptosis (Morgan and Liu, 2010).

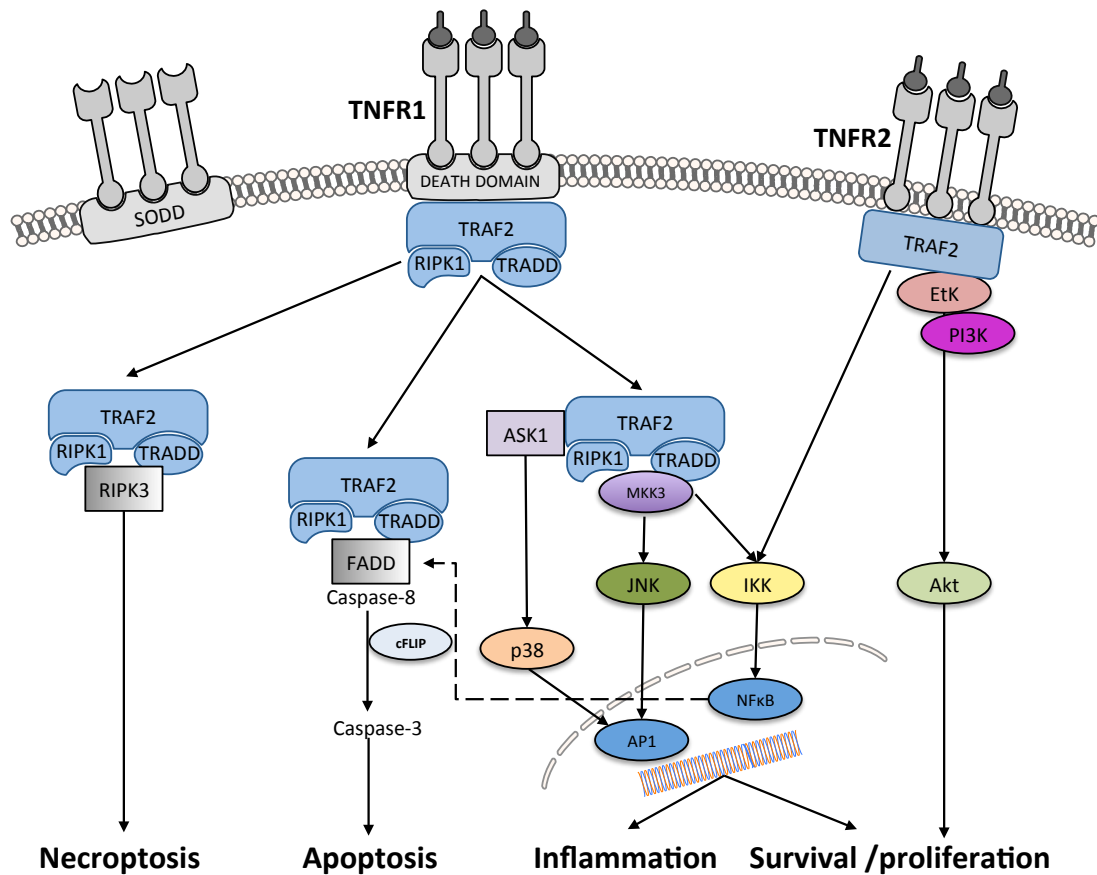


Figure 1.4 TNF signalling pathways

Adapted from (Waters et al., 2013) Akt – protein kinase B ; ASK 1 - apoptosis signalling kinase; AP1 – Activator protein-1 ; cFLIP - FLICE-inhibitory protein; Etk - epithelial/endothelial tyrosine kinase; FADD - Fas associated death domain ; IKK - IκB kinase; JNK - c-Jun N-terminal kinase; MKK3 – mitogen activated protein kinases kinase 3; NFκB – nuclear factor kappa B; PI3K - phosphatidylinositol 3 kinase ; RIPK1 - receptor interacting kinase protein-1; RIPK3 - receptor interacting kinase protein-3; SODD - silencer of death domain; TRADD - TNFR-associated death domain; TRAF - TNFR-associated factor 2

1.3.3.2 The TNFR2 signalling pathway

Significantly less is known about TNFR2 signalling pathways. TNFR2 lacks an intracellular DD to recruit TRADD, but can directly interact with TRAF2 via a TRAF binding site. Following ligand binding TNFR2 recruits TRAF2, leading to NF- κ B activation independent of the TNFR1 signalling pathway (Thommesen and Laegreid, 2005); over-expression of TNFR2 can lead to NF- κ B activation via this mechanism, although the intracellular signalling mechanisms are unclear (Rothe et al., 1995). Moreover, TNFR2 induced NF- κ B activation appears to be active in only some cell types (Naude et al., 2011). A further downstream signalling pathway of TNFR2 involves activation of a cytosolic protein called epithelial/endothelial tyrosine kinase (Etk). Etk activation via TNFR2 can partly activate vascular endothelial growth factor receptor 2, leading to activation of PI3K and its effector protein called Akt (Zhang et al., 2003). This pathway was cytoprotective and promoted cell growth, although it may not be sufficient to do so on its own. In some cell types (including T-cells), TNFR2 can also induce cell death although the signalling pathways responsible remain elusive. Some studies suggest it may be mediated indirectly by up-regulation of transmembrane TNF, which then secondarily stimulates TNFR1 (Grell et al., 1999; Vercammen et al., 1995).

1.3.4 The roles of differential TNFR signalling in host immunity and repair

TNF and its differential signalling pathways, play key roles in host immunity and tissue repair mechanisms. Upon release, TNF proceeds throughout the lifecycle to exert regulatory roles over immune cells by triggering transcription of genes responsible for inflammation, proliferation, differentiation, and apoptosis. To counter invading micro-organisms, TNF facilitates proliferation of immune cell clones and subsequent

differentiation and recruitment of naïve immune cells. Subsequently, TNF orchestrates destruction of superfluous immune cell clones to reduce inflammation and tissue damage.

The relative contribution of TNFR signalling in these processes is not well understood and often contradictory. Studies using transgenic mice deficient in TNFR1, demonstrate that some signalling via TNFR1 is important for controlling infection by intracellular pathogens such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Schluter and Deckert, 2000). Interestingly, TNFR1 deficient animals are resistant to TNF and IL-1 β -mediated mortality, and to endotoxic shock induced by LPS or D-galactosamine. However, there is evidence to support divergence in the role of TNF receptors in host immunity. For example, TNFR2 facilitated generation of Fas ligand-dependent cytotoxic T-cell effector pathways play an important role in antiviral-immune responses in the liver and lungs (Kafrouni et al., 2003; Richter and Topham, 2007), and TNFR2 may also be involved in the pathogenesis of cerebral malaria (Piguet et al., 2002). Finally, TNFR2 may also be important in the control of inflammation, since TNFR2 is expressed on a highly suppressive subset of regulatory T cells (Tregs) (Chen et al., 2008). Adoptive transfer of TNFR2 deficient CD4⁺ T cells resulted in more severe inflammation and tissue destruction in the CD4⁺ T-cell transfer murine model of colitis (Dayer Schneider et al., 2009). Moreover, an agonist for TNFR2 selectively destroyed autoreactive T-cells in blood samples from patients with type I diabetes (Ban et al., 2008), whilst TNFR1 could deliver pro-survival signals to these autoreactive T lymphocytes (Twu et al., 2011). TNFR2 is an important receptor expressed on regulatory T cells necessary for their activation, proliferation and proper functioning (Chen et al., 2007). Furthermore, TNFR2 signalling plays a non-redundant role in natural killer cell-dendritic cell activation (Xu et al., 2007).

The central role of TNFR2 signalling in tissue repair has been highlighted by experimental studies using transgenic mice deficient in TNFR2 and TNFR selective muteins. Murine models have demonstrated that TNFR2 is protective in the pathogenesis of heart failure (Hamid et al., 2009; Higuchi et al., 2004), renal allograft failure due to ischaemia-reperfusion injury (Al-Lamki et al., 2005), neuronal injury (Fontaine et al., 2002), and ischaemic neovascularisation (Goukassian et al., 2007). TNFR2 is also crucial for TNF-induced regeneration of oligodendrocyte precursors that make up myelin (Arnett et al., 2001), and TNFR2 signalling has a role in angiogenesis; endothelial-specific transgenesis of TNFR2 promoted adaptive arteriogenesis and angiogenesis in a femoral artery ligation model (Luo et al., 2010). In contrast to the suggested essential role of TNFR2 signalling in tissue repair, TNFR1 is required for liver regeneration (Yamada et al., 1998), but it nevertheless plays a non-redundant role in hepatitis (Shibata et al., 2008).

1.3.5 The role of TNF in acute lung injury

Although TNFR have been shown to be differentially expressed on both alveolar epithelial cells and the pulmonary endothelium (Ermer et al., 2003; Grau et al., 1996), many of the pro-inflammatory effects of TNF can be explained based on the effects of TNF on vascular endothelium and leukocyte-endothelial interactions. Endothelial cells up-regulate the expression of leukocyte adhesion molecules including ICAM-1, VCAM-1 (vascular cell adhesion molecule-1) and E-selectin (Mackay et al., 1993; Slowik et al., 1993; Neumann et al., 1996; Eriksson et al., 2005), in a distinct temporal, spatial and anatomical pattern (Petzelbauer et al., 1994; Bradley and Pober, 1996) in response to TNF exposure. Similarly, TNF induces release of chemokines including IL-8, MCP-1, GM-CSF and interferon-gamma induced protein-10 (Rollins et al., 1990; Paleolog et al., 1994), leading to leukocyte recruitment, independent of antigen recognition. Finally, TNF disrupts adherens junctions in endothelial cells (Angelini et al., 2006; Petrache et al.,

2001) promoting fluid leak and leukocyte influx. Given that the pulmonary vascular bed (including the lung capillaries, the site of pulmonary neutrophil recruitment) is unique in receiving the body's entire cardiac output, and that gap formation in lung endothelium is the primary cause of fluid leak in ALI, the lungs would seem to be exquisitely sensitive to TNF-induced inflammation and lung injury.

TNF has been strongly implicated in the pathogenesis of ALI, initiating the inflammatory cytokine cascade by inducing up-regulation of pro-inflammatory neutrophil chemokines including IL-8 (Matsushima et al., 1989). Levels of TNF and its soluble receptors are elevated in BALF (Armstrong and Millar, 1997; Pugin et al., 1999) and blood of patients with ALI (Hyers et al., 1991), and levels of downstream TNFR1 signalling products (e.g. IL-6, IL-8, TNFR1 and TNFR2) in blood have been associated with a poor outcome (Calfee et al., 2009; Parsons et al., 2005) and sequential organ failure in ALI patients (Liu et al., 2007). Alveolar macrophages from ALI patients express higher levels of bioactive cell-surface TNF, with a trend for increased soluble TNF production (Armstrong et al., 2000; Armstrong and Millar, 1997).

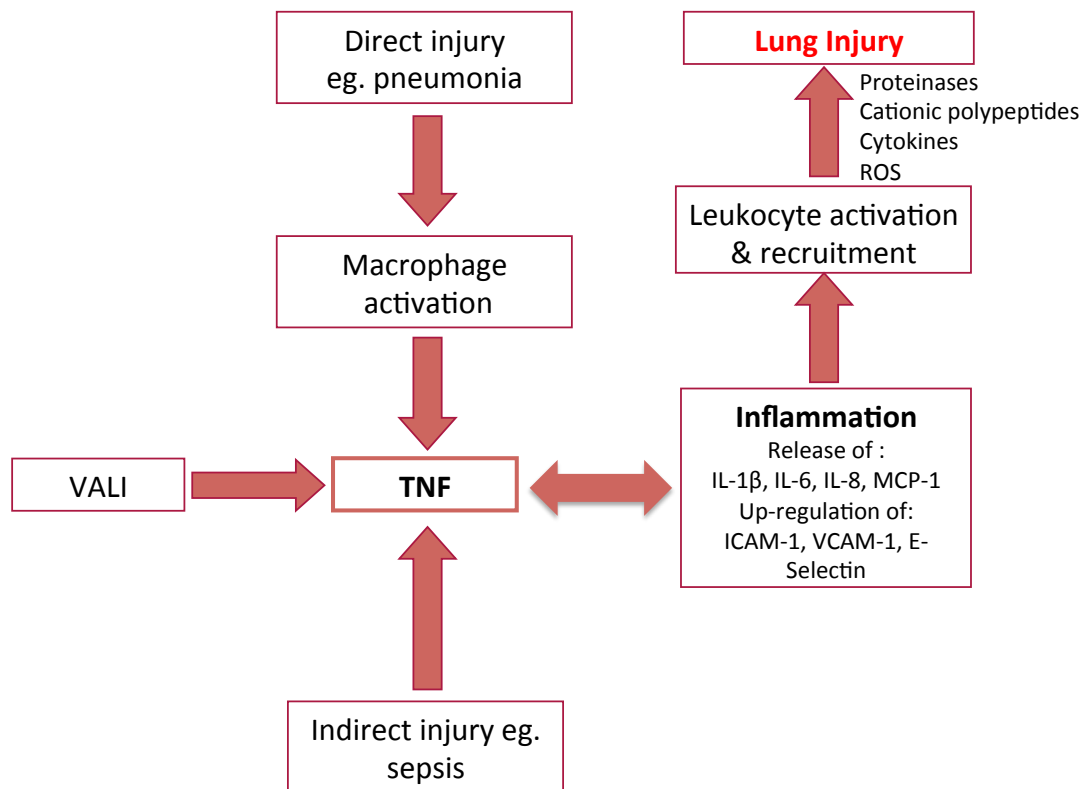


Figure 1.5 The central role of TNF in the pathogenesis of ALI

ROS: Reactive oxygen species, VALI: Ventilator associated lung injury, ICAM-1: Intracellular adhesion molecule-1, VCAM-1: vascular cell adhesion molecule -1, MCP-1 Monocyte chemotactic protein-1

TNF has similarly been implicated in the pathogenesis of both experimental and clinical VALI. Indeed, in a clinical study, BALF and plasma levels of TNF and its soluble receptors fell after 36 to 40 hours in patients with ALI receiving protective ventilation, compared to those ventilated using a conventional strategy (Ranieri et al., 1999). Early experimental studies yielded conflicting results (Imai et al., 1999; Murphy et al., 2000; Ricard et al., 2001; Tremblay et al., 2002), however recent work has demonstrated that the complexities of differential TNF signalling may underlie some of the confusion. In genetically modified mice, pulmonary oedema formation in the early phases of VALI was mediated through the TNFR1 receptor, whilst TNFR2 was protective (Wilson et al., 2007). The mechanism of this divergent effect is unclear, although TNFR1 may increase alveolar-capillary membrane permeability via p38 mediated cytoskeletal rearrangement in endothelial cells (Ferrero et al., 2001), whereas TNFR2 activation may maintain epithelial

barrier function through the endothelial/epithelial tyrosine kinase (Etk/Bmx) (Hamm-Alvarez et al., 2001).

1.3.6 TNF antagonism

1.3.6.1 Currently available therapies

Over the past decade, the use of TNF antagonists, has led to substantial improvements in the quality of life of patients with several autoimmune diseases including rheumatoid arthritis and Crohn's disease. In these diseases and others, anti-TNF therapy interferes with disease pathogenesis at multiple levels, inhibiting inflammatory cell recruitment, inducing inflammatory cell death and lowering cytokine production by multiple cell types, thus limiting aberrant inflammatory damage and promoting functional recovery of the affected tissues. Over two million patients have been treated with anti-TNF therapy to date, and three of the commercially available TNF-blockers are among the top ten best-selling drugs in the world.

Different compounds targeting TNF are available, the mouse-human chimeric antibody Infliximab, the fully human antibodies Adalimumab and Golimumab, a fusion protein composed of the extracellular part of two TNFR2 molecules coupled to the fragment crystallisable (Fc) region of an Immunoglobulin-G1 (IgG1) molecule, Etanercept and a PEGylated fragment antigen-binding (Fab) fragment lacking an Fc region, Certolizumab. These compounds differ in composition, mechanism of inhibition, dosing, and route of application. For example, whilst they all inhibit soluble TNF with comparable efficiency (Kaymakcalan et al., 2009), they have different binding kinetics to membrane bound TNF (Scallan et al., 2002) which may account for their varying efficacies in different auto-immune diseases.

Despite the promising clinical outcomes, anti-TNF therapy has been associated with important complications, most notably an increased risk of infection from intracellular bacteria and certain viruses (Gardam et al., 2003). Moreover, anti-TNF therapy has been associated with the development of *de-novo* auto-immune diseases such as systemic lupus erythematosus and multiple sclerosis, and a propensity towards a higher incidence of malignancies and lymphoma. These effects may relate to unwanted effects on TNFR2 signalling in host immunity, particularly T-regulatory cells (chapter 1.3.4).

Based on these data and the data suggesting a role for TNFR2 signalling in tissue repair and regeneration (chapter 1.3.3.2), a variety of compounds that selectively block TNFR1 have been developed including monoclonal antibodies and antagonistic TNF mutants with specificity for TNFR1. Ideally these would reduce the pro-inflammatory effects of TNFR1 induced NF- κ B signalling in the relevant tissues and cells whilst maintaining the putative benefits of TNFR2 signalling.

1.3.6.2 TNF blockade in acute lung injury

Despite anti-TNF therapy or TNF receptor blockade ameliorating the severity of lung injury in animal models (Windsor et al., 1993; Van Zee et al., 1996; Tracey et al., 1987; Imai et al., 1999; Goldman et al., 1990) clinical studies of a recombinant TNF neutralizing fusion protein in sepsis, which included subgroup analysis of patients with ALI, yielded largely disappointing results (Abraham et al., 1998; Abraham et al., 1995; Fisher et al., 1996), culminating in a large (1340 patients) phase III study demonstrating no 28-day mortality benefit (Abraham et al., 2001). The failure of this trial may be explained by the kinetics of TNF release in sepsis. Despite targeting early sepsis in the trial (<4 hours of vasopressor dependent shock), in animals serum TNF peaked 90 minutes after endotoxin injection having a circulating half-life of just 14-18 minutes (DeForge et al., 1990). Indeed, in experimental sepsis models, neutralizing antibody to

TNF was protective only when it was administered during a window beginning before, and ending very soon after, injection of endotoxin (Beutler et al., 1985) or *E. coli* (Tracey et al., 1987). Moreover, in patients with severe meningococcal sepsis, TNF release occurred early but was absent in the mid- and later- phases of fatal infections (Waage et al., 1987). Alternatively, accumulating data in a range of experimental models (chapter 1.3.4) suggest that the complexity of differential TNFR signalling in host inflammation and immunity, may account for the lack of clinical efficacy of TNF blockade. For example, patients with Gram-positive infection tended to have an increased mortality whereas those with Gram-negative infection were unaffected (Fisher et al., 1996).

1.3.6.3 GSK1995057

1.3.6.3.1 Domain antibodies

Domain antibodies (dAb) are an emerging class of drug comprising the variable domains of either the heavy (V_H) or light chain (V_L), and are thus the smallest functional antigen-binding portions of the immunoglobulin-G (IgG) molecule. Each dAb therefore contains three of the six naturally occurring complementary determining regions from an antibody, which are the diversified loop regions that bind to target antigen. They were discovered in the late 1980s by a group from Cambridge when a repertoire of isolated murine V_H domains was screened for binding to lysozyme (Ward et al., 1989). Given that they only comprise the V_H domain of an antibody, they are one quarter the size of a fragment antigen-binding region and half the size of single chain fragments. Variable domains bind a wide range of antigens from small molecules to large proteins and inhibit enzymatic activity by penetrating into enzymatic active sites. dAbs are readily expressed in bacteria and are therefore suitable for isolation by *in vitro* selection via phage display (Holt et al., 2003). Thus, the potential exists to generate fully human dAbs with high

target affinity and specificity with the advantages of conventional antibodies but considerably greater ease of manufacturing.

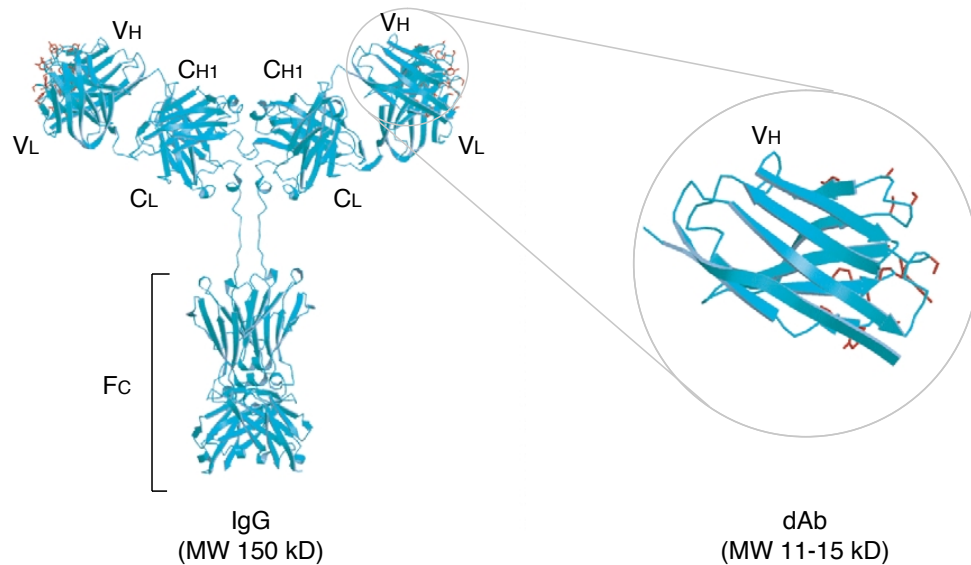


Figure 1.6 Variable domain antibodies

A human Immunoglobulin-G (IgG) molecule has both variable and constant regions. An IgG contains two variable regions (each composed of a V_H and V_L domain) that confer antigen-binding specificity on the antibody, and an Fc fragment in the constant region that recruits the effector functions of the immune system. A domain antibody (dAb) is an isolated antibody V_H (shown here) or V_L domain. Taken from (Holt et al., 2003). MW: molecular weight (kilodaltons), C_H : heavy chain, C_L : light chain, Fc: fragment crystallisable portion.

Domain antibodies offer multiple advantages over conventional antibody technology: they bind monovalently to their targets, avoiding the potential for receptor cross-linking; they lack the Fc portions and do not induce Fc effector responses such as complement- or antibody-dependent cytotoxicity; their small size (~12 kD) enables better tissue penetration and delivery at much higher concentrations per unit mass and potentially fewer long-term adverse effects (e.g. immunosuppression) due to more rapid excretion; and they are thermodynamically and potentially proteolytically stable.

1.3.6.4 Selective TNFR1 antagonism using GSK1995057

A number of antibodies have been described that bind specifically to TNFR1, however, several of these cross-link the receptor resulting in activation of downstream

signalling cascades (Menegazzi et al., 1994; Weingartner et al., 2002). Therefore, investigation of the role of TNFR1 is largely limited to studies in knockout mice or over-expression systems.

GSK1995057 is a variable domain antibody developed by GlaxoSmithKline (GSK) that comprises a single heavy chain variable domain of ~13kDa. It was selected from a phage display dAb library comprising the framework sequences of the human germline gene 3-23; gene 3-23 is the most commonly used in human antibodies. GSK1995057 is expressed as a recombinant protein from *E. coli* and is purified by a multi-step sequential chromatography process. Degradation testing confirmed that it remains monomeric and stable during storage and during nebulisation (personal communication from GSK). The biological consequences of GSK1995057 pharmacology appear similar to monoclonal antibody TNF antagonists with subtle differences predicted based on differential signalling through TNFR1.

GSK1995057 specifically binds TNFR1 with high affinity (assessed by both Biacore™ assay and ELISA, table 1.2) and inhibits the binding of TNF and subsequent signal transduction via TNFR1. Surface plasmon resonance studies and immunoassay studies demonstrated that GSK1995057 does not show evidence of binding to other TNFR superfamily receptors including TNFR2, nerve growth factor receptor (NGFR), recombinant lymphotoxin beta-receptor, TNF-related apoptosis inducing ligand (TRAIL) receptor 2, TRAIL receptor 3, TRAIL receptor 4 and CD27 (a TNFR superfamily receptor.) Hence GSK1995057 is highly selective for human TNFR1. When bound to TNFR1, GSK1995057 (0.001 to 1000nM) prevented TNF (10ng/mL) from binding to TNFR1 with a mean half maximal inhibitory concentration (IC₅₀) of 0.07nM. Thus GSK1995057 can be considered as a competitive antagonist.

Table 1.2 Binding Characteristics of GSK1995057 to Human TNFR1

Assay	GSK1995057 (nM)
Binding to human TNFR1 (Biacore) (K_D) using recombinant mammalian expressed cynomolgus Fc-TNFR1	0.017
Binding to human TNFR1 (Biacore) (K_D) using recombinant mammalian expressed human Fc-TNFR1	0.015
Binding to human TNFR1 (Biacore) (K_D) using <i>E. coli</i> expressed biotinylated human TNFR1	0.47
Binding to human TNFR1 (ELISA) (EC_{50})	0.113

To improve its efficacy in distal lung tissue, GSK1995057 was developed to be protease resistant via selection in the presence of bovine trypsin. Although susceptible to proteolytic degradation, it demonstrated >50% residual binding activity to TNFR1 after incubation with trypsin, elastase or leucozyme. These data compare favourably with other domain antibodies (personal communication GSK).

Inhibition of TNFR1 induced downstream signalling by GSK1995057 was evidenced in a range of human cell types using a variety of signalling events. Accordingly, in the human lung epithelial cell-line, A549, TNF (3ng/mL) induced activation of NF- κ B transactivation was inhibited in the presence of GSK1995057. Similarly, in human lung fibroblasts, lung epithelial cells, monocytes and in human whole blood, IL-8 release was completely abrogated by GSK1995057 whilst TNF induced apoptosis was inhibited in human lung fibroblasts. Finally, TNF-induced activation of human umbilical vein endothelial cells (HUVEC), measured by VCAM-1 up-regulation was abrogated by pre-treatment with GSK1995057 (personal communication with GSK).

Having confirmed *in vitro* efficacy, single doses of nebulised GSK1995057 were evaluated in a well-characterised Cynomolgus monkey model of acute lung inflammation, elicited by aerosol challenge with bacterial LPS. This model simulates a number of features of direct ALI, including recruitment of inflammatory cell types into

the lung lumen and up-regulation cytokines, chemokines, acute phase proteins and other inflammatory mediators. Nebulised GSK1995057 was well tolerated and inhibited both neutrophil infiltration and the expression of a broad range of inflammatory mediators in the BALF (interleukin-1 receptor alpha, IL-6, macrophage inflammatory protein -1 beta, myeloperoxidase, TNFR2 and von Willebrand factor) of animals in a dose-dependent manner. Serum mediators were largely unaffected with the exception of transient elevations in IL-6, sTNFR1 and inhibition of IL-1 receptor alpha. Thus GSK1995057 effectively engages and selectively inhibits TNFR1 function in the lungs of non-human primates leading to protection from the pro-inflammatory effects of LPS challenge (personal communication with GSK).

Most recently, data demonstrating the effects of GSK1995057 in a murine model of 'pure' VALI and an LPS plus VALI model were published (Bertok et al., 2012). Treatment of mice with a specific TNFR1 antagonist attenuated deteriorations in respiratory physiology compared to a dummy version of the dAb and a dual TNFR antagonist in the 'pure' VALI model. This was associated with attenuation of alveolar capillary permeability. In the LPS plus VALI model, GSK1995057 decreased levels of pro-inflammatory cytokines in the BALF (IL-6 and MCP-1) with a trend towards reductions in leukocyte chemokines. Moreover, surface alveolar macrophage expression of ICAM-1 was attenuated. Both the dAb and the monoclonal antibody were administered intra-tracheally. Of note, the beneficial effect of TNFR antagonism in the VALI model was not observed with a monoclonal anti-TNF antibody lending support to the concept of a potentially protective effect of TNFR2 signalling and improved efficacy owing to the small size of the dAb compared to the monoclonal antibody (both were delivered intra-tracheally).

Cumulatively, these data suggest that GSK1995057 potentially inhibits multiple signalling pathways downstream of human TNFR1. Allied with its small size and proteolytic stability, this dAb may offer selective disruption of TNFR1 signalling, whilst preserving TNFR2 signalling, in a compound capable of penetrating and retaining stability in distal lung tissue.

1.3.7 TNF agonism using TNFR specific muteins

Whilst analysis of receptor specific effects *in vivo* is facilitated by receptor knockout mice, the investigation of comparative signalling between 2 different receptors *in vitro* is more challenging. Muteins describe a general term for mutated recombinant proteins, which carry single or multiple amino acid substitutions. These proteins are derived frequently from cloned genes that have been subjected to site-directed or random mutagenesis, or from completely synthetic genes.

Hence, when arginine at position 32 was replaced by tryptophan in recombinant TNF, the TNFR1 selective mutein R32W was generated (Van Ostade et al., 1993). This demonstrated a 3300 fold decreased affinity for TNFR2 with nominal (1.4 fold) reduction in binding to TNFR1. Despite mutation, R32W maintains full cytotoxic potential *in vitro* and *in vivo* (Van Ostade et al., 1993). Similarly D143N (aspartic acid at position 143 replaced by phenyl-alanine) (Van Ostade et al., 1994) is a TNFR2 selective mutant (Van Ostade et al., 1994), which has no demonstrable binding to TNFR1 but also exhibits a 30-fold decrease in binding to TNFR2. These muteins therefore represent unique tools to define the roles of differential TNFR signalling in human cells and potentially corroborate findings using the selective TNFR1 antagonist GSK1995057.

1.4 The role of neutrophil endothelial interactions in acute lung injury

1.4.1 Neutrophils – a key regulator of inflammation

Neutrophils are the most abundant circulating leukocyte (50-70% of total leukocytes) in man and play a fundamental role in the innate immune response. The importance of neutrophils in preserving health is exemplified by patients suffering from neutropenia, who are prone to life-threatening bacterial and fungal infections. Neutrophils are required to be highly motile, respond to the finest of chemotactic trails and have the ability to kill bacteria even under the most adverse (e.g. hypoxic) conditions.

Neutrophils are multi-lobar cells that contain a plethora of granules formed sequentially during myeloid differentiation. Granule formation commences in early promyelocytes, when immature transport vesicles bud off from the Golgi, fuse and form azurophilic (primary) granules, which contain large amounts of myeloperoxidase (MPO). Production of MPO halts at the promyelocyte-myelocyte transition therefore granules formed in the later stages of myelopoiesis are peroxidase negative, and can be divided into specific (secondary) containing lactoferrin and gelatinase (tertiary) granules containing matrix metalloproteinase 9 (MMP9). Neutrophils also contain secretory vesicles that can rapidly transport their contents, for example $\beta 2$ integrins required for cell adhesion, to the cell surface upon activation. Tertiary granules then deliver proteases that can digest basement membrane and extracellular matrix, perhaps allowing for neutrophil transmigration (Hager et al., 2010).

Neutrophils are continuously generated in the bone marrow from myeloid precursors. Their daily production can reach up to 2×10^{11} cells, a process controlled by G-CSF (Lieschke et al., 1994), which is produced in response to IL-17A synthesized by regulatory T cells (Ley et al., 2006). Release of IL-17A is in turn under the control of IL-

23 originating from tissue-resident macrophages and dendritic cells. In response to inflammation, neutrophil numbers increase, and they are recruited rapidly to inflamed sites, where their primary role is to kill invading bacteria and certain fungal species through phagocytosis, release of preformed granular enzymes and proteins, and by the production of a range of oxygen species through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Borregaard, 2010; Galli et al., 2011; Hager et al., 2010).

1.4.2 Neutrophil priming, activation and degranulation

Circulating neutrophils may be viewed as quiescent cells that require effective processes to remain functionally dormant thus preventing inappropriate accumulation and activation with release of potentially damaging reactive oxygen intermediates and granular contents. The key mechanism for this is ‘priming’. The ability of neutrophils to produce superoxide anions in response to a particular secretagogue agonist is significantly enriched when cells are exposed to mediators including TNF, GM-CSF or PAF; this has been described as the gold standard priming response (Condliffe et al., 1998). Priming agents themselves do not generate effector functions, unless applied at very high concentrations (Condliffe et al., 1998) and neutrophil degranulation and activation of the NADPH oxidase occur only in cells that have been primed prior to activation (Guthrie et al. 1984b). Critically, priming has been shown to be important for the induction of endothelial injury both *in vitro* (Smedly et al., 1986) and *in vivo* (Worthen et al., 1987) and affects neutrophil cytoskeletal organisation to induce a highly irregular polarized cell contour and reduced deformability (Worthen et al. 1987). Furthermore, priming agents, including TNF, modulate neutrophil adhesion molecule expression and function, inducing β 2-integrin expression (Lauterbach et al., 2008) and L-selectin shedding thus regulating the adhesive and migratory capacity (Montecuccio et al., 2008).

1.4.3 Neutrophil dependency of acute lung injury

Neutrophils are central to the initiation and propagation of inflammation and injury in ALI (Abraham, 2003). Neutrophil accumulation within the pulmonary vasculature occurs early in the evolution of clinical ALI (Bachofen and Weibel, 1977; Bachofen and Weibel, 1982), and neutrophilic alveolitis is a histological hallmark (Lee and Downey, 2001). Neutrophilia is common in the BALF of patients with ALI (Parsons et al., 1985) and the extent of this correlates with clinical outcome (Steinberg et al., 1994). Whilst ALI has been described in neutropenic patients (Ognibene et al., 1986), recovery from neutropenia in patients with ALI was associated with a deterioration in pulmonary function (Azoulay et al., 2002). Animal models further support the importance of neutrophils in ALI; depletion of neutrophils, or their products, attenuated ALI (Abraham et al., 2000; Folkesson et al., 1995; Folz et al., 1999; Kawabata et al., 2000; Looney et al., 2006; Shimizu et al., 2009), and delayed neutrophil apoptosis or failure of apoptotic cell clearance, was associated with worsened inflammation and increased mortality (Matute-Bello and Martin, 2003). Moreover, activated neutrophils directly induced vascular leak by free radical production (Gao et al., 2007).

However, the presence of neutrophils *per se* is not necessarily damaging, and the priming/activation status of these cells is a major determinant of their subsequent injury limiting/inducing behavior (Chollet-Martin et al., 1992; Zimmerman et al., 1983). Hence, whilst neutrophil products are abundant in the BALF of patients with ARDS (Cochrane et al., 1983; Ricou et al., 1996; Weiland et al., 1986), when neutrophils were recruited into the normal human lung by instillation of the chemoattractant leukotriene-B₄, neutrophils migrated into the airspace without causing injury to the endothelial or epithelial barriers of the lung (Martin et al., 1989).

1.4.4 Mediators of neutrophil migration

1.4.4.1 Chemokines

Chemokines are a family of small (6 to 15 kD), structurally related cytokines with chemotactic properties for leukocytes. Chemokines provide directional cues for the movement of leukocytes in development, homeostasis and inflammation. Hence, in response to inflammation, the local secretion of chemokines, results in the recruitment of leukocytes into activated or injured tissues. Chemokine production was originally thought to be limited to activated macrophages and monocytes. However, under appropriate conditions, their production and release has been elicited from neutrophils, endothelial and epithelial cells, platelets, and a variety of parenchymal cells *in vitro* (Crippen et al., 1995; Furie and Randolph, 1995; Huang et al., 1992).

Over 50 chemokines have been identified with between 20 and 70% homology in amino acid sequences (Luster, 1998; D'Ambrosio et al., 2003), and they have been subdivided into families on the basis of the relative position of cysteine residues. Structurally, the majority of chemokines possess four cysteine residues in their amino-terminal end that form disulfide bridges and are divided into the CXC (α) and CC (β) families dependent upon whether the two most NH₂-proximal cysteines are separated by one amino acid (CXC) or lie adjacent to each other (CC). CXC chemokines are further subdivided based on the presence or absence of a Glu-Leu-Arg (ELR) motif preceding the first cysteine residue. In general, ELR-positive chemokines regulate neutrophil migration via an interaction with the CXCR2 chemokine receptor, and the ELR-negative chemokines modulate mononuclear leukocyte migration (Strieter et al., 1999). Whilst over 50 human chemokines exist only 20 G-protein coupled chemokine receptors have been identified (Zlotnik and Yoshie, 2000). This disparity is balanced by the promiscuity of

ligand-receptor interactions, with multiple chemokines binding to the same receptor and several chemokines binding to more than one receptor.

The most well studied neutrophil chemokine is IL-8, which is the primary stimulus for neutrophil migration in humans (and rabbits). In addition to IL-8, at least six other CXC chemokines mediate neutrophil responses in humans: neutrophil-activating peptide-2, three isoforms (α , β and γ) of growth-related oncogenes (GRO), and epithelial cell-derived neutrophil-activating peptide-78 (ENA-78). There is no evidence that different chemokines are used to attract neutrophils to various organs; it was empirically shown that the differences in specific chemoattractants and the order of their action might be more stimulus-specific rather than tissue-specific (reviewed in (Sadik et al., 2011)).

As a result of the increasing number of chemokines described, a standardised nomenclature was adopted in 2002; chemokines were named according to their family (CXC, CC, CX3C or XC) then L to denote ligand prior to a number. Thus IL-8, and MCP-1 are named CXCL8 and CCL2 respectively; the traditional nomenclature is still used in this thesis.

In ALI, endothelial cells, alveolar epithelial cells and macrophages release chemokines in response to bacterial products and early response inflammatory mediators. These chemokines are retained by matrix heparin sulphate glycoproteins at the site of inflammation, thus establishing a chemokine gradient toward the inflammatory focus (Puneet et al., 2005). In the lung microvasculature, neutrophils, are first attracted via a gradient of ELR-positive chemokines, and subsequently retained via chemokine (and other mediator) induced alterations in neutrophil cytoskeletal arrangements and activation of neutrophil integrins (see 1.4.4.3.2). For example, IL-8, ENA-78, and GRO- α were present in biologically significant concentrations in BALF from patients with ALI (Goodman et al., 2003; Villard et al., 1995) and their concentrations correlated with

neutrophil numbers (Goodman et al., 1996; Miller et al., 1996; Villard et al., 1995) and patient outcome (Michel et al., 1997). IL-8 and ENA-78 mediated neutrophil adhesion to HUVEC *in vitro* (Zimmerman et al., 1999) as well as directing neutrophil trafficking during LPS-induced lung inflammation (Jeyaseelan et al., 2004). Moreover, in animal models, neutralisation of chemokines (Colletti et al., 1995; Folkesson et al., 1995; Miller et al., 1992) or their receptors (Sue et al., 2004) attenuated lung injury.

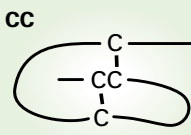
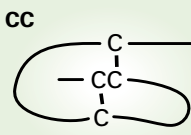
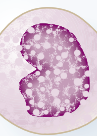
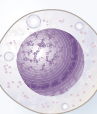
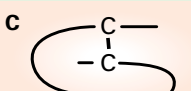
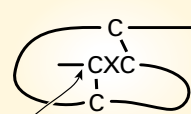
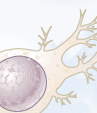
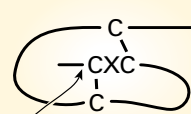
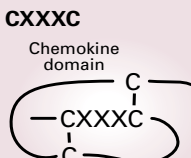
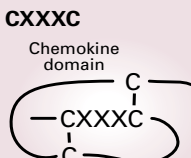
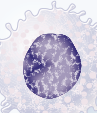
Chemokine	Receptor	Cell Type
 MCP-3, -4; MIP-1α; RANTES MCP-3, -4; eotaxin-1, -2; RANTES	 CCR1 CCR3	Eosinophil 
MCP-1, -2, -3, -4, -5 MCP-3, -4; eotaxin-1, -2; RANTES	CCR2 CCR3	Basophil 
 MCP-3, -4; MIP-1α; RANTES MCP-1, -2, -3, -4, -5 MIP-1α, MIP-1β, RANTES I-309 MDC, HCC-1, TECK	CCR1 CCR2 CCR5 CCR8 ?	Monocyte 
Fractalkine	CX ₃ CR1	
SDF-1	CXCR4	
MCP-3, -4; MIP-1 α ; RANTES MCP-1, -2, -3, -4, -5 TARC MIP-1 α , MIP-1 β , RANTES MIP-3 β (ELC) PARC, SLC, 6CKine (Exodus-2)	CCR1 CCR2 CCR4 CCR5 CCR7 ?	Activated T cell 
Fractalkine	CX ₃ CR1	
IP-10, MIG, I-TAC	CXCR3	
 PARC, DC-CK1 Lymphotactin	? ?	Resting T cell 
SDF-1	CXCR4	
 MCP-3, -4; MIP-1α; RANTES MCP-1, -2, -3, -4, -5 MCP-3, -4; eotaxin-1, -2; RANTES TARC MIP-1α, MIP-1β, RANTES MIP-3α (LARC, Exodus-1) MDC, TECK	CCR1 CCR2 CCR3 CCR4 CCR5 CCR6 ?	Dendritic cell 
SDF-1	CXCR4	
 Glutamic acid-leucine-arginine Interleukin-8, GCP-2 Interleukin-8, GCP-2; GRO-α, -β, -γ; ENA-78; NAP-2; LIX	CXCR1 CXCR2	Neutrophil 
 MCP-1, -2, -3, -4, -5 MIP-1α, MIP-1β, RANTES	CCR2 CCR5	
 Chemokine domain Mucin-like domain Cytoplasmic domain Fractalkine	CX ₃ CR1	Natural killer cell 
IP-10, MIG, I-TAC	CXCR3	

Figure 1.7 Chemokines and their receptors

Taken from Luster (Luster, 1998) MCP- monocyte chemoattractant protein; MIP- macrophage inflammatory protein; RANTES- regulated upon activation normal T-cell expressed and secreted; MDC- macrophage derived chemokine; HCC-1- hemofiltrate CC chemokine; TECK- thymus-expressed chemokine; SDF-1- stromal- cell derived factor-1; TARC- thymus and activation-regulated chemokine; ELC EBL-1- (Epstein Barr virus- induced gene-1)-ligand chemokine; PARC- pulmonary and activation regulated chemokine; SLC- secondary lymphoid-tissue chemokine; 6CKine- 6-cysteine chemokine; IP-10- interferon-inducible protein- 10; MIG- monokine induced by interferon γ ; I-TAC- interferon inducible T-cell alpha chemoattractant; DC-CK1- dendritic-cell chemokine 1; LARC- liver and activation regulated chemokine; GCP- granulocyte chemotactic protein; GRO- growth regulated oncogene; ENA-78- epithelial cell derived neutrophil activating peptide 78; NAP-2- neutrophil activating peptide 2, LIX- lipopolysaccharide induced CXC chemokine

1.4.4.2 Cytokines

Whilst many of the inflammatory processes in ALI, including neutrophil migration, are accomplished through direct cell-to-cell adhesive interaction via adhesion molecule expression, cells also transmit signals between neighbouring cells through low molecular weight (mostly <40kDa) protein mediators called cytokines. Cytokines are involved in almost all biological processes, from cell proliferation to inflammation, immunity, migration, fibrosis, repair, and angiogenesis (Vilcek and Feldmann, 2004), displaying concentration dependent effects. Individual subpopulations of immune cells in the lung, in particular alveolar macrophages, possess different capacities to elaborate and secrete different cytokines in response to particular stimuli. In addition to immune cells, endothelial cells, fibroblasts and epithelial cells also release cytokines in response to specific stimuli.

Evidence from several clinical studies indicates that pro-inflammatory cytokine networks (as well as chemokines) mediate, amplify, and perpetuate lung injury. Whilst the precise roles of individual cytokines in ALI remains elusive, several cytokines including IL-1 β , IL-6 and TNF have been identified as key mediators of ALI (Nathan, 1987) stimulating local cells to release chemokines, and activating leukocytes and endothelial cells in preparation for neutrophil-endothelial interactions (Springer, 1994). For example, neutrophil activation directly correlates with severity of ALI and with levels of TNF and IL-6 (Chollet-Martin et al., 1996). Intra-tracheal instillation of IL-1 β and TNF induced an intra-alveolar inflammatory response composed of predominantly neutrophils (Ulich et al., 1991). Moreover, TNF and IL-1 stimulated expression of adhesion molecules on activated vascular endothelial cells (Springer, 1994) thus mediating firm adhesion of neutrophils to the endothelium. TNF and IL-1 also amplified neutrophil infiltration in an

autocrine and paracrine manner by activating macrophages to secrete chemokines including IL-8 (Fong et al., 1989).

1.4.4.3 Adhesion Molecules

Neutrophil emigration from the vascular compartment is dependent upon the coordinated and regulated expression and function of a number of cell surface molecules, including the selectins CD62L (L selectin) and CD62E (E selectin), neutrophil integrins including CD11b and lung microvascular endothelial neutrophil ligands including ICAM-1 and VCAM-1. Stimulation of endothelial cells or neutrophils with a range of agonists (including TNF) leads to altered expression of these molecules (Condliffe et al., 1996; Yang et al., 2005; Zhou et al., 2007) promoting neutrophil transendothelial migration.

1.4.4.3.1 CD62L

CD62L is a member of the selectin family of molecules with a molecular weight of 95kDa. CD62L is constitutively expressed on tips of micro-processes of neutrophils (Picker et al., 1991) as well as monocytes and lymphocytes. It is rapidly shed from the cell surface following cellular activation by chemoattractants and activating factors, including CD18 cross-linking (Walzog et al., 1994), via endoproteolytic cleavage from the cell surface at the membrane proximal extracellular site. In addition to a role in cellular adhesion, CD62L may signal cell responses directly; CD62L cross-linking stimulated intracellular release of Ca^{2+} , O_2^- release and induction of mRNA for cytokine production in neutrophils (Crockett-Torabi et al., 1995; Laudanna et al., 1994; Simon et al., 1995; Waddell et al., 1994).

Studies to further elucidate the role CD62L in knockout mice have produced varying results. CD62L may play a role in the margination and sequestration of neutrophils within the pulmonary circulation in certain circumstances, for example following formyl-methionyl-leucyl-phenylalanine (fMLP) and LPS installation (Olson et

al., 2002; Wong et al., 2000) but not in others, such as on stimulation with complement 5a (Doyle et al., 1997). In patients at-risk for ALI, plasma soluble CD62L (thought to be shed from leukocytes) was significantly lower in those who subsequently progressed to ALI than in those who did not or in normal controls (Donnelly et al., 1994). Significant correlations were also found between CD62L levels and the requirement for ventilation, the degree of respiratory failure, and patient mortality.

1.4.4.3.2 Integrins

Integrins are transmembrane glycoproteins expressed on neutrophils comprised of α and β -chains (Rose et al., 2007). The $\beta 2$ integrins are composed of a common β chain (CD18) that is non-covalently associated with any of four types of α subunits: CD11a (lymphocyte function-associated antigen-1 (LFA-1)), CD11b (Mac-1), CD11c or CD11d80 (Carlos and Harlan, 1994). Detection of molecules such as IL-8, fMLP and TNF by neutrophils results in activation by conformational change in $\beta 2$ integrin receptors on their cell surface, which interact with endothelial ligands to mediate adhesion.

Integrins play a crucial role in many neutrophil functions, particularly cell adhesion, migration and complement-mediated phagocytosis as evidenced by defects in these processes in patients with inherited deficiencies of the CD18 common $\beta 2$ integrin subunit (Arnaout, 1990). CD11a/CD18 and CD11b/CD18 are the predominant receptors mediating neutrophil adhesion to the endothelium, interacting with the Ig gene superfamily (IgSF) counter-ligands on the endothelial surface, including ICAM-1 and ICAM-2 (Etzioni et al., 1999). ICAM-1, the major ligand for CD11b, is present constitutively on endothelial cells but its expression is increased by pro-inflammatory cytokines. For example, pre-treatment of human umbilical vein endothelial cells (HUVEC) with TNF increased transcellular neutrophil emigration attributable to an increase in ICAM-1 expression (Yang et al., 2005). Elevated neutrophil CD11b levels and

activation status positively correlated with indicators of disease severity in septic shock and adverse outcomes following cardio-pulmonary bypass (Rinder et al., 2003), both of which are common precipitants of ALI.

Of note, shape change, CD62L, and CD11b expression are affected during neutrophil isolation, with primed cells having less CD62L expression, greater CD11b expression, and a blunted response to neutrophil agonists (Haslett et al., 1985). These markers are therefore useful *in vitro* indices of neutrophil priming, either through isolation alone or following exposure to a stimulus.

1.4.4.3.3 Endothelial neutrophil adhesion molecules

P-selectin, the largest selectin, is stored in α -granules of platelets and in Weibel–Palade bodies of endothelial cells, and is translocated to the endothelial cell surface by stimuli including bacterial components and hypoxia. E-selectin is not expressed (or expressed at extremely low levels) under baseline conditions, except in skin microvessels, but is rapidly induced by inflammatory cytokines, including TNF. Whilst both E- and P-selectin are critical for initiating neutrophil rolling and tethering neutrophils to inflamed endothelium in the systemic circulation (Patel et al., 2002), their role in mediating neutrophil sequestration in the pulmonary circulation is presumed to be less important and possibly even redundant owing to spatial constraints (discussed in 1.4.5) and data suggesting that, at least in animals P-selectin is not expressed on pulmonary capillary endothelium (Mulligan et al., 1992). Moreover, any role of selectins in experimental models appears to be stimulus dependent. Hence, neutrophil migration associated with Streptococcal pneumonia was unaffected in mice deficient in E- and P-selectins (Mizgerd et al., 1996) and blocking of E-, P- and L-selectin did not attenuate LPS-induced alveolar migration of neutrophils (Burns et al., 2001). In contrast, all 3 selectins were involved in the progression of complement induced ALI (Mulligan et al., 1996).

The role of other adhesion molecules in mediating neutrophil-endothelial interactions in ALI remains similarly elusive. Nonetheless, in post-mortem lung biopsy specimens of patients with ALI, ICAM-1, VCAM-1 (and E-selectin) were strongly up-regulated; only ICAM-1 was up-regulated in lung capillaries, suggesting that these adhesion molecules may have a role in human ALI (Muller et al., 2002). Additionally, lung microvascular endothelial cells purified from ALI patients post-mortem revealed a significantly higher constitutive expression of ICAM-1 than control cells (Grau et al., 1996). When treated with TNF, both ALI-patient derived and control patient derived cells showed a dose-dependent increase in ICAM-1 expression that was significantly higher in the ALI-derived endothelial cells.

1.4.4.4 Selected other mediators

Platelets may promote the extravasation of neutrophils from the vasculature through a range of mechanisms; first, platelet adherence to activated vascular endothelium may further propagate endothelial activation (Gawaz et al., 2005) and second, activated platelets release growth factors, cytokines and chemokines to activate leukocytes (von Hundelshausen and Weber, 2007). Platelets, aggregating with neutrophils via P-selectin, facilitate neutrophil recruitment during sterile ALI (Grommes and Soehnlein, 2011; Zarbock et al., 2006) and LPS-induced lung inflammation (Kornerup et al., 2010). Moreover, platelet-derived neutrophil-attracting chemokine heterodimers of RANTES (regulated on activation, normal T cell expressed and secreted) and platelet factor 4 are important for neutrophil recruitment during ALI (Grommes et al., 2012) and endothelial presentation of RANTES is platelet-dependent (Drechsler et al., 2010).

Experimental models of LPS induced lung injury suggest that peripheral blood monocytes (PBMs), along with resident alveolar macrophages and recruited alveolar

monocytes, play a pivotal role in the neutrophilic alveolitis pathognomonic of ALI (Auffray et al., 2007; Henderson et al., 2003; Li et al., 1998). Indeed, depletion of monocytes in mice significantly reduced LPS-induced blood neutrophilia, alveolar neutrophilia, and markers of lung injury (Dhaliwal et al., 2012; Maus et al., 2002). Furthermore, a study employing two-photon imaging demonstrated that monocyte depletion by liposomal clodronate reduced pulmonary neutrophil transendothelial migration (Kreisel et al., 2010). A recent trial of sham versus actual leukapheresis to deplete PBMs (although lymphocytes and platelets were also reduced) in human model of subclinical LPS-induced lung inflammation (n=30) detected no clinical or biological effect (Barr et al., 2013). Whilst the lack of treatment effect may (in part) relate to methodological nuances of the study (for example monocyte depletion was incomplete despite four blood volume leukapheresis), the role of monocytes in the recruitment of neutrophils in human ALI remains to be fully established.

1.4.5 Neutrophil migration from the pulmonary vasculature to the alveolus

The mechanisms by which neutrophils are sequestered at a site of inflammation within the alveolar space appear to be quite different in the pulmonary microcirculation compared to the systemic microcirculation. In the systemic microcirculation, extravasation of neutrophils takes place in post-capillary venules in a cascade-like sequence of capture, rolling, slow rolling, arrest and transmigration. Rolling is mediated by selectins, a family of calcium-dependent lectins that includes L-selectin expressed on neutrophils, P-selectin expressed on platelets and endothelial cells, and E-selectin expressed on endothelial cells (Kansas, 1996). Rolling is followed by chemokine receptor-mediated signalling via G-protein-coupled receptors which, trigger activation of neutrophil $\beta 2$ integrins. Activated integrins (LFA-1 and Mac-1) subsequently interact with

their endothelium-expressed ligands, including ICAM-1 and VCAM-1, leading to slow rolling and eventually to firm adhesion and leukocyte arrest on the endothelium. Subsequently, neutrophils transmigrate into tissues using a paracellular or transcellular route (Ley et al., 2007).

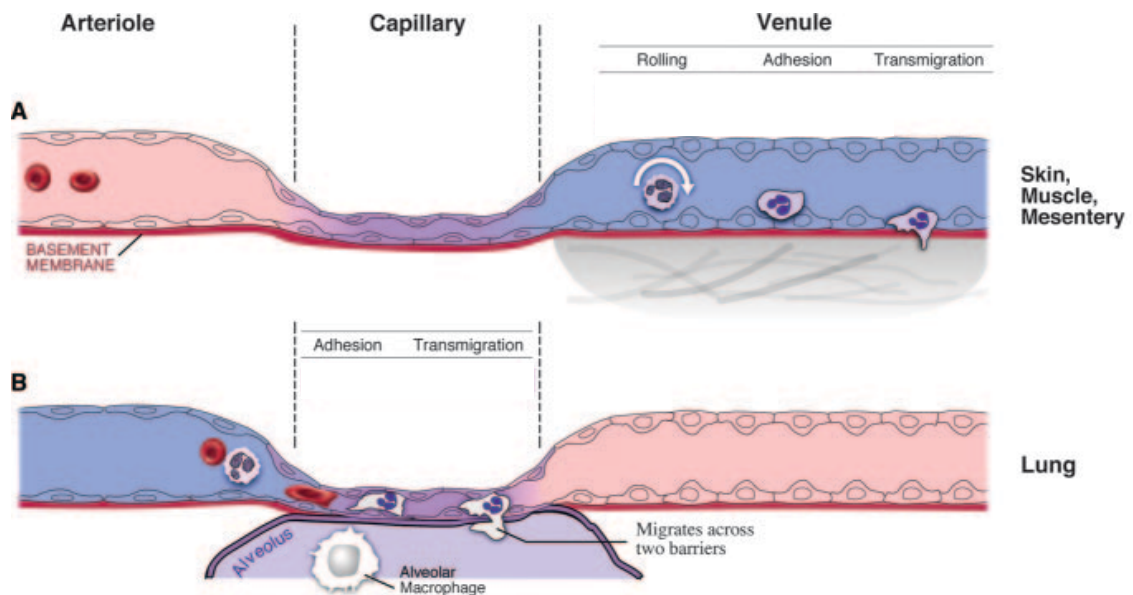


Figure 1.8 Neutrophil migration in the systemic circulation compared to the pulmonary circulation

Adapted from (Aird, 2007)

By contrast, the major site of neutrophil sequestration in the lung appears to be the alveolar capillary bed, where rolling does not occur (Doerschuk, 2000; Burns et al., 2003) as a consequence of the unique pulmonary capillary microanatomy; the diameters of spherical neutrophils (6-8 μm) are larger than the diameters of many capillary segments (2-15 μm). Approximately half of the capillary segments require neutrophils to change their shape to pass through (Doerschuk et al., 1993; Gebb et al., 1995). In addition to spatial limitations, the binding of neutrophil G-protein coupled receptors by chemokines or bacterial products induces an f-actin rim to form beneath the cell membrane rendering neutrophils stiff (Downey et al., 1991; Inano et al., 1992), further promoting neutrophil

retention in pulmonary capillaries. Indeed, during bacterial pneumonia in rodents, neutrophils with f-actin rims were preferentially retained in the lungs over those without f-actin rims at a time when sequestration was actively ongoing (Yoshida et al., 2006). Hence, in the pulmonary capillaries, spatial limitations allied with mediator-induced changes in the biomechanical properties of neutrophils promote neutrophil sequestration in the pulmonary capillaries. This is highlighted by animal studies demonstrating that the neutrophil adhesion molecules L-selectin and CD11/CD18 were not required for neutrophil sequestration in the pulmonary capillaries (Doerschuk, 1992; Doyle et al., 1997; Kubo et al., 1999). However, once sequestered, both were critical for prolonged (>5 minutes) pulmonary neutrophil retention (Doerschuk, 1992; Kubo et al., 1999).

As the inflammatory response progresses, cytokines including TNF, are produced which activate neutrophils and lung parenchymal cells including the lung microvascular endothelium; up-regulation of adhesion molecules on neutrophils and their cognate endothelial receptors thus ensues. In addition, chemokines are induced, which activate integrins on neutrophils to further promote pulmonary neutrophil sequestration. Neutrophils then spread and crawl on the endothelial surface until they reach the site for transmigration (Figure 1.9).

Intravascular crawling is mediated by CD11b/CD18 on neutrophils and ICAM-1 on endothelial cells. This spreading and crawling implies that the adhesive bonds between integrins and their ligands must be displaced while new bonds are formed. This requires coordinated signalling from chemokines, integrins, ICAM-1 and possibly VCAM-1 (Burns et al., 2003; Carlos and Harlan, 1994). Besides regulating neutrophil adhesion and locomotion on endothelial cells, signalling during adhesion also modulates endothelial cell junctions, inducing expression of inflammatory genes. These responses probably play important roles in the progression of an inflammatory response by modulating endothelial

cell permeability, neutrophil transmigration and production of inflammatory mediators. Neutrophil transmigration through the endothelium can occur at endothelial cell borders (paracellular route) or through endothelial cells (transcellular route) *in vitro* and *in vivo*. During paracellular migration another set of adhesion molecules are engaged that include platelet endothelial cell adhesion molecule (PECAM)-1, VE-cadherin, junctional adhesion molecules (JAMs) and CD99 (Muller, 2009; Woodfin et al., 2010).

In other organs and *in vitro*, neutrophil endothelial transmigration via the paracellular or transcellular route is contingent upon the nature of the inflammatory stimulus, the tissue microenvironment, the level of ICAM-1 expression, and the localization of ICAM-1 (Millan et al., 2006; Woodfin et al., 2010; Yang et al., 2005). However, in the pulmonary microvasculature, the majority of neutrophils (and perhaps nearly all) appear to take the paracellular route between endothelial cells (Burns et al., 2003; Walker et al., 1995; Behzad et al., 1996).

Migration of neutrophils across the pulmonary interstitium is thought to be mediated by fibroblasts, which establish defined conduits assisting neutrophils to gain access to the basal surface of the epithelium (Walker et al., 1995), whilst maintaining alveolar-capillary integrity (Sirianni et al., 2003). Owing to the discontinuous nature of tight junctions at these sites, neutrophils then migrate into the alveolar space through tricellular corners at the junctions of type I and type II epithelial cells (Burns et al., 2003; Walker et al., 1995). Roles for both CD11/CD18 and the β 1 integrin CD29 in neutrophil migration through the pulmonary interstitium toward the alveolar space have been established (Ong et al., 2003; Ridger et al., 2001). Once in the alveolar space, neutrophils adhere to the apical surface of epithelial cells, possibly through ICAM-1 interactions.

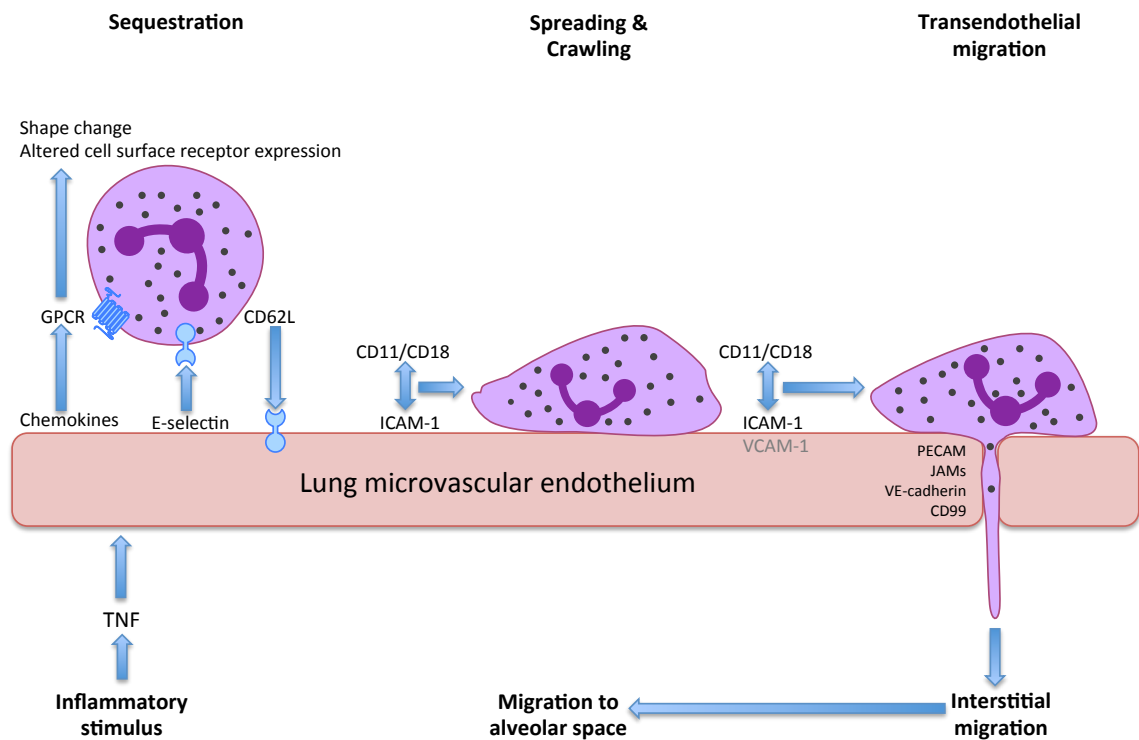


Figure 1.9 Neutrophil transendothelial migration

CD62L – neutrophil L –selectin; GPCR – G protein coupled receptor; ICAM-1 – intracellular adhesion molecule 1; JAMs – junctional adhesion molecules; PECAM - Platelet endothelial cell adhesion molecule; TNF – tumour necrosis factor; VCAM-1 - vascular cell adhesion molecule 1; VE-cadherin – vascular endothelial cadherin (CD144).

1.4.6 Resolution of neutrophilic inflammation

1.4.6.1 Neutrophil Apoptosis

Once neutrophils have migrated to the alveolar space, their lifespan can be modified by the local inflammatory environment (Geering and Simon, 2011). For example, macrophages secrete death receptor ligands, such as Fas-ligand and TNF, to promote neutrophil survival at low concentrations and neutrophil death at higher concentrations (Murray et al., 1997; Salamone et al., 2001; van den Berg et al., 2001). In this regulatory pathway, phosphoinositide 3-kinase (PI3K)-mediated reactive oxygen species (ROS) production represents a key event in limiting cell survival and triggering apoptosis (Geering et al., 2011), whilst Btk, a negative regulator of NADPH activation, is

associated with neutrophil survival (Honda et al., 2012). Hence, Btk-deficient neutrophils demonstrate high levels of ROS production, driven by excessive NADPH and PI3K activation (Honda et al., 2012). Other environmental signals such as hypoxia prolong neutrophil lifespan via hypoxia-inducible factor-1 α (HIF1 α) induced mechanisms (Walmsley et al., 2011). Finally, GM-CSF, a survival factor for neutrophils, triggers extracellular signal-regulated kinases (ERK) activation; interference with ERK or other lifespan prolonging signalling pathways may promote neutrophil apoptosis.

Neutrophil apoptosis is thought to be central to the restoration phase of ALI; it leads to functional down-regulation, including shape change, degranulation and respiratory burst (Whyte et al., 1993) and to recognition and clearance of the apoptotic neutrophils by resident macrophages (Savill et al., 1989). Dysregulated neutrophil apoptosis has been proposed as an important mechanism contributing to the propagation of lung injury (Cox et al., 1995). Indeed, several human studies have demonstrated decreased neutrophil apoptosis in ALI and modulation of neutrophil apoptosis when treated with BALF from patients in the early phase of ALI (Lesur et al., 2000; Matute-Bello et al., 2000; Matute-Bello et al., 1997). This inhibitory effect of BALF on neutrophil apoptosis is thought to be mediated by the pro-inflammatory cytokines granulocyte colony-stimulating factor (G-CSF), and GM-CSF (Matute-Bello et al., 1997; Matute-Bello and Martin, 2003) possibly acting through PI3K. Concentrations of G-CSF and GM-CSF in BALF from ARDS patients correlated with the anti-apoptotic effect of ARDS BAL fluid on neutrophils (Matute-Bello et al., 2000). Additionally, pre-B cell colony-enhancing factor has been identified as a novel cytokine that plays a requisite role in delayed neutrophil apoptosis in clinical and experimental sepsis (Jia et al., 2004).

1.4.6.2 Neutrophil de-priming

Neutrophil priming, including shape change, has been shown to be reversible *in vitro* (Kitchen et al., 1996). Two hours after challenge with PAF, cells demonstrated baseline values of superoxide anion generation, cell polarisation and CD11b activity. Interestingly, once recovered, these cells were capable of subsequent re-priming following stimulation with PAF or TNF. Recently the concept that the pulmonary vasculature provides a protective mechanism in the role of neutrophil priming has been proposed (Summers et al., 2014). Hence, the healthy lung microvasculature retains primed cells and facilitates their de-priming and release (into the systemic circulation) in an un-primed quiescent state. Failure of these mechanisms, for example in endothelial injury in ALI, results in high circulating levels of primed neutrophils, which correlates with the severity of lung injury (Chollet-Martin et al., 1992).

1.5 Experimental Strategy

Numerous clinical and experimental studies have implicated TNF as a central mediator of ALI. Previous clinical trials of anti-TNF therapy have, however, failed to demonstrate clinical benefit; this, in part, may relate to the complex biology of differential TNF signalling through its two cell surface receptors, TNFR1 and TNFR2, which initiate distinct signalling pathways and cellular responses. Using a novel, highly selective TNFR1 domain antibody GSK1995057 allied with TNFR specific muteins, we sought to investigate the roles of differential TNFR signalling in a range of human *in vitro* models of ALI. In particular, we focused on the role of differential TNFR signalling in components of the cascade of events, which lead to neutrophil migration into the alveolar space during ALI including; chemokine and cytokine release; lung microvascular

permeability, neutrophil priming and activation, neutrophil chemotaxis and adhesion molecule expression on both neutrophils and lung microvascular endothelial cells.

Experiments were performed using human neutrophils, human lung microvascular cells and human alveolar type II epithelial cells to represent the major cell types involved in modulating neutrophil migration in human lung.

Finally, we aimed to establish a robust and reproducible human *ex vivo* model of LPS induced ALI. The purpose of this work was to overcome the failings of current animal models of ALI to translate promising preclinical data into clinically beneficial therapies and to confirm our *in vitro* data in a highly relevant and tractable whole human lung model.

2 Chapter 2: Materials & methods

2.1 Materials

2.1.1 Cells and cell culture reagents and materials

Human alveolar type 2AT2 cells and human alveolar macrophages

40µm cell strainer	BD Biosciences (352340)
Collagen I Coated Bioflex plates	DunnLab (BF30010/40)
DCCM-1 Medium	React Scientific (05-010-1A)
DNase I	Sigma-Aldrich (DN-25)
Hanks balanced salt solution (HBSS)	Invitrogen (14025-050)
Newborn calf Serum (NCS)	Invitrogen (26010-074)
Purecol, type I collagen	Advanced BioMatrix (5005-B)
Penicillin/Streptomycin/Glutamine	Invitrogen (10378-016)
Trypsin (from bovine pancreas)	Sigma-Aldrich (T8003)

HUVEC

Endothelial Growth Media-2 Bullet	Lonza (CC3162)
Human Umbilical Vein Endothelial Cells (Pooled)	Lonza (C2519A)
Reagent Pack - subculture kit	Lonza (CC5034)

HMVEC-L

3µm polycarbonate membrane inserts 12mm membrane	VWR (734-1570)
Cryo SFM	Promocell (C29910)
Detach Kit 30	Promocell (C41200)
ENDOHEM for 6mm culture cup	World Precision Instruments (ENDOHEM-6)
Endothelial Growth Media MV2 Kit	Promocell (22121)
Human Pulmonary Microvascular Endothelial Cells (Proliferating)	Promocell (C12282)
Growth factor reduced matrigel	BD Biosciences (734-0269)
µ-slide angiogenesis slide	IBIDI (81506)

Neutrophils

Citrate Concentrated Solution 4%	Sigma-Aldrich (S5770)
Dextran 500 from Leuconostoc sp	Sigma-Aldrich (31392-10G)
Dulbecco's phosphate buffered saline (with CaCl ₂ & MgCl ₂)	Sigma-Aldrich (D8662)
Dulbecco's phosphate buffered saline (without CaCl ₂ & MgCl ₂)	Sigma-Aldrich (D8537)
Iscove's Modified Dulbecco's medium (IMDM) IMDM media	Invitrogen (12440-046)
Iscove's Modified Dulbecco's medium IMDM media (no without phenol red)	Life technologies (21056-023)
Percoll	VWR (17-0891-0L)

2.1.2 Cytokines, inhibitors and other drugs

Evans Blue	Sigma Aldrich (E2129)
Human Interleukin-8 (IL-8)	R&D Systems (208-IL-100)
Human GM-CSF	R&D Systems (285-IF-100)
Human Tumour Necrosis Factor	R&D Systems (210-TA-10)
Human Vascular Endothelial Growth factor	R&D Systems (293-VE-10)
Lipopolysaccharide from Escherichia coli 011:B4	Sigma Aldrich (L2630)
Luminol	Sigma-Aldrich (123072)
N-formyl-Met-Leu-Phe	Sigma-Aldrich (F3506)
TNFR1-Fc	R&D Systems (372-R1)
TNFR2-Fc	R&D Systems (726-R2)
U0126	Calbiochem (662005)

2.1.3 Flow Cytometry and Immunofluorescence antibodies and reagents

Alexa Fluor 594 Donkey anti-goat IgG Antibody	Life Technologies (A-11058)
CellFIX	BD Biosciences (340181)
Cell dissociation solution Non-enzymatic x1	Sigma Aldrich (C5789)
Compensation beads	BD Biosciences (552843)
Goat Polyclonal IgG VE-Cadherin Antibody	Santa Cruz Biotech (sc-6458)
Mouse IgG1 κ Isotype APC Isotype Control Antibody	Cambridge Biosciences (400120)
Mouse IgG1 κ Isotype APC Isotype Control Antibody	Biolegend (400119)
Mouse IgG1 κ Brilliant Violet 421 Isotype Control Antibody	Cambridge Biosciences (400158)
Mouse IgG1 Phycoerythrin Isotype Control Antibody	R&D Systems (10002P)
Mouse IgG1 Pacific Blue Isotype Control Antibody	Biolegend (400131)
Mouse IgG1 Fluorescein Isotype Control Antibody	R&D Systems (10002F)
Mouse IgG2a FITC κ Isotype Control Antibody	Biolegend (400209)
Mouse IgG2 Phycoerythrin Isotype Control Antibody	Biolegend (400213)
Cell dissociation solution Non-enzymatic x1	Sigma Aldrich (C5789)
ProLong Antifade Reagent with DAPI	Life Technologies (P-36931)

2.1.4 Western blotting antibodies and reagents

5x Loading buffer	Invitrogen
10% Tris-HCl ready gel	Bio-Rad (161-1155)
10X Cell Lysis Buffer	New England Biolabs (9803)
10x Tris/Glycine/SDS (TGS) buffer	Bio-Rad (161-0732)
Amersham Rainbow Markers (full range)	GE Healthcare (RPN 800E)
Anti BMX (phosphor 456) Antibody	Abcam (ab59409)
Bmx Antibody (C-17)	Insight Biotech (sc-8874)
Hyperfilm ECL (X ray film)	Amersham Biosciences (RPN203D)

Laemmli Sample Buffer	Bio-Rad (161-0737)
LumiGLO™ Reagent and Peroxide	New England Biolabs (7003)
Milk powder	Waitrose
Phototope®-HRP Western Blot Detection	New England Biolabs
Phospho-p44/42 MAPK Antibody	Cell Signalling Tech (9101)
Phospho-Etk (Tyr40) Antibody	Cell Signalling Tech (3211)
Phospho-STAT-1 Antibody	Cell Signalling Tech (9167)
Phospho-STAT-3 Antibody	Cell Signalling Tech (9131)
p44/42 MAPK Antibody	Cell Signalling Tech (9102)
STAT-1 Antibody	Cell Signalling Tech (9172)
STAT-3 Antibody	Cell Signalling Tech (9132)

2.1.5 Molecular Biology Reagents

dNTP (set)	Sigma-Aldrich (DNTP100A)
GAPDH primer	Qiagen (QT01192646)
Moloney murine leukaemia virus reverse transcriptase	Invitrogen (28025-13)
Oligo (dT) 12-18 primer	Invitrogen (18418-012)
PCR oligonucleotide primers	Invitrogen Life Sciences
PCR tubes and caps	Qiagen
QuantiTect primer pairs	Qiagen
RNase	Sigma-Aldrich (R4875)
RNeasy Mini Kit	Qiagen (74104)
RNase free DNase set	Qiagen (79254)
Surfactant Protein C Primer	Qiagen (QT00000714)
Trizol	Sigma-Aldrich (1559-026)

2.1.5.1 Primers

Both were purchased from Qiagen and selected with the aid of Primer3 software.

Primer	Sequence
Surfactant Protein C	Forward GCAGGGAGAGACAGGTGAAG Reverse GGTGGCCTGGATTCTAACAA
GAPDH	Forward GAAGGTGAAGGTCGGAGT Reverse GAAGATGGTGTGATGGGATTTC

2.1.6 Kits

Annexin V FITC Apoptosis Detection Kit	BD Biosciences (556547)
Cytoselect Leukocyte Transmigration Assay	Cambridge Bioscience (CBA 212)
Human CXCL8/IL-8 DuoSet ELISA Kit	R&D Systems (DY208)
Human sTNFR-1 duoset	R&D Systems (DY225)
Human sTNFR-2 duoset	R&D Systems (DY726)
Mini Trans-Blot Electrophoretic Transfer Cell	Bio-Rad (170-3930)
MPO Human ELISA kit	Hycult biotech (HK324-02)
pP38 MAPK alpha (pT180/Y182) PhosphoTracer ELISA kit	Abcam (ab119638)
QuantiTect Reverse Transcription kit	Qiagen (205310)
QuantiTect SYBR Green PCR kit	Qiagen (74104)

2.1.7 MSD plates

Custom V-plex	Meso Scale Discovery (K15047D)
Prototype duplex	Meso Scale Discovery (N45ZA)
Human cytokine 9-plex	Meso Scale Discovery (K15007B)
Human chemokine 7-plex	Meso Scale Discovery (K15031C)
Custom 5-plex	Meso Scale Discovery (N751A)
Vascular Injury Panel II	Meso Scale Discovery (K15136C)

2.1.8 *Ex vivo* lung perfusion hardware

Biomedicus BPx80 Pump head	Medtronic (95204)
Bovine serum albumin (BSA) Low Endotoxin	Fisher Scientific (BPE9705)
DMEM high glucose	Sigma-Aldrich (D5671)
ECMOTHERM II Heat Exchanger	Medtronic (ECMOTHERM II)
Heater Cooler 3T	Sorin Biomedical (16-02-80)
Lung Catheter Pulmonary Artery (unwashed)	Vivoline Medical (410018-01)
Lung Research Pack	Maquet (H19702)
Organ Box Insert (unwashed)	Vivoline Medical (410008-01)
Organ Box Lid (unwashed)	Vivoline Medical (410007-01)
Organ Box Reservoir (unwashed)	Vivoline Medical (410006-01)
Perfadex	XVIVO perfusion (19017)
Red cell lysis buffer	Sigma-Aldrich (R7757)
Steen Solution	XVIVO Perfusion (19004)
XVIVO Organ Chamber	XVIVO Perfusion (19020)
XVIVO Lung cannula set	XVIVO Perfusion (19022)

2.1.9 General reagents

2-mercaptoethanol	Sigma-Aldrich (M-7522)
Agarose, low gelling temperature	Sigma-Aldrich (A9414)
Albumin FITC-conjugated	Sigma-Aldrich (A9771)
Benzamide	Sigma-Aldrich (125828)
Bovine serum albumin (BSA)	Sigma-Aldrich (A7030)
Bradford protein assay dye reagent concentrate	Bio-Rad (500-0203)
Dextran Blue (from Leuconostoc sp)	Sigma-Aldrich (31393)
Dimethyl sulphoxide (5×5mlmL DMSO)	Sigma-Aldrich (D2650)
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich (D5879)
Dithiothreitol (DTT)	Sigma-Aldrich (D5545)
Reastain DiffQuick solution	Gentauro Molecular Products (102035)
ELISA substrate reagents A+B	BD Biosciences (555214)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (E6511)
Evans Blue	Sigma Aldrich (E21299)
Ethanol	BDH (L227007)
Formalin solution, neutral buffered, 10%	Sigma-Aldrich (HT501128)
HEPES	Sigma-Aldrich (H7006)
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich (55021C)
Human albumin solution (20%)	Bioproducts Laboratory
Hydrogen Peroxide (H ₂ O ₂)	Sigma-Aldrich (H6520)
Glutamine	Invitrogen (25030-024)
Growth factor reduced Matrigel	BD Biosciences (734-0269)
Methanol	BDH (10158BD)
Methylthiazolitetrazolium (MTT)	Sigma-Aldrich (M2128)
Penicillin / Streptomycin	Invitrogen (15140-122)
Phenylmethanesulphonyl fluoride (PMSF)	Sigma-Aldrich (D7626)
Phosphate buffered saline tablets	Sigma-Aldrich (P4417)
Propidium Iodide	Sigma-Aldrich (P4864)
Protein standard	Sigma-Aldrich (P5619)
Red Blood Cell Lysing Buffer Hybri-Max	Sigma-Aldrich (R7757)
Sodium Azide (NaN ₃)	Sigma-Aldrich (S2002)
Sodium chloride 0.9%	Baxter (TRF7124)
Sodium fluoride	Sigma-Aldrich (S7920)
Sodium pyrophosphate	Sigma-Aldrich (P8135)
Streptavidin Peroxidase (for ELISA)	Sigma-Aldrich (S5512)
Sucrose	BDH (102745C)
Triton X-100	BDH (30632)
Trypsin (0.25%) + EDTA (1mM)	Invitrogen (15400-054)
TRIZMA base	Sigma-Aldrich (T6791)
TRIZMA hydrochloride	Sigma-Aldrich (T3253)
Vectashield mounting medium	Vector Labs (H1000)

2.1.10 Company Locations

Abcam	Cambridge, UK
Advanced BioMatrix Inc	San Diego, CA, USA
Amersham Biosciences	Little Chalfont, UK
Baxter	Deerfield, IL, USA
BD Bioscience	Palo Alto, CA, USA
BDH	Poole, UK
Biolegend	San Diego, CA, USA
Bio-Rad Laboratories	Hercules, CA, USA
Calbiochem	San Diego, CA, USA
Cambridge Bioscience	Cambridge, UK
Cell Signaling Technologies	Beverly, MA, USA
DunnLab	Asbach, Germany
Fisher Scientific	Loughborough, UK
GE Healthcare	Little Chalfont, UK
Gentaur	Kampenhout, Belgium
Hycult biotech	Uden, The Netherlands
Invitrogen	Carlsbad, CA, USA
Insight Biotech	Wembley, UK
Life Technologies	Carlsbad, CA, USA
Lonza	Basel, Switzerland
Maquet	Rastatt, Germany
Meso Scale Discovery	Rockville, MD, USA
Medtronic	Minneapolis, MN, USA
New England Biolabs	Hitchin, UK
Promega	Madison, WI, USA
Promocell	Heidelberg, Germany
Qiagen	Venlo, Netherlands
R&D Systems	Abingdon, UK
React scientific	Troon, UK
Santa Cruz Biotechnology	Santa Cruz, CA, USA
Sigma-Aldrich	Poole, UK
Vector Labs	Burlingame, CA, USA
XVIVO Perfusion	Gothenburg, Sweden
Vivoline Medical	Lund, Sweden
VWR	Radnor, PA, USA
World Precision Instruments	Sarasota, FL, USA

2.2 General Methods

2.2.1 Centrifugation

Centrifugations were carried out for volumes less than 2mL in one of two fixed angle machines Hawk 15/05 (Sanyo Gallenkemp, Etten Leur, Netherlands) or Boeco M-240 R (Hettich, Germany). For larger volumes a Hettich Rotina 46R swing bucket centrifuge was used (Hettich AG). Centrifugation speeds are quoted as the relative centrifugal force (rcf) or centrifuge rotor speed (revolutions per minute: rpm) followed by time, and temperature. For neutrophil isolation, the acceleration and brake parameters are also quoted.

2.2.2 Cell counting

Cells were counted by using a haemocytometer (improved Neubauer model) under a phase contrast microscope (CK40, Olympus UK, Southend, UK). The total cell number in the four corner squares was counted including cells that overlapped the bottom and right border of squares but not those overlapped top and left border. Appropriate dilutions were made such that the total number of cells counted was between 100 and 400. The volume of each square's area was 10^{-4} mL, and the number of cells per mL was calculated using the following formula:

$$\text{Cells/mL} = \frac{\text{Total cell number of four corner squares}}{4} \times 10000$$

2.2.3 MTT determination of cell viability and proliferation

Yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is cleaved into purple formazan by metabolically active cells, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilised and quantified spectrophotometrically. In a homogeneous cell

population, the amount of formazan generated is directly proportional to the number of viable cells over a wide range. After cell treatments, cell supernatants were removed, the cells washed with buffer and incubated at 37°C with MTT (1mg/mL) in medium for a period of time dependent on the cell type. Following incubation, MTT solution was removed completely and the plates dried. Formazan dye was dissolved in DMSO for 15 minutes and excitation measured at 550nm.

2.2.4 Trypan blue determination of cell viability

Non-viable cells with compromised membranes absorb and fail to exclude trypan blue, whilst healthy cells are refractile (unstained). An equal volume of cell suspension and Trypan Blue (0.4% w/v) were mixed and observed after 2-3 minutes. Viability was expressed as the percentage of unstained cells relative to the total number of cells and determined according to the following equation:

$$\text{Viability (\%)} = \frac{\text{Viable cell number}}{\text{Total cell number}} \times 100\%$$

2.2.5 Cell culture Protocols

2.2.5.1 Isolation of human alveolar type 2 cells

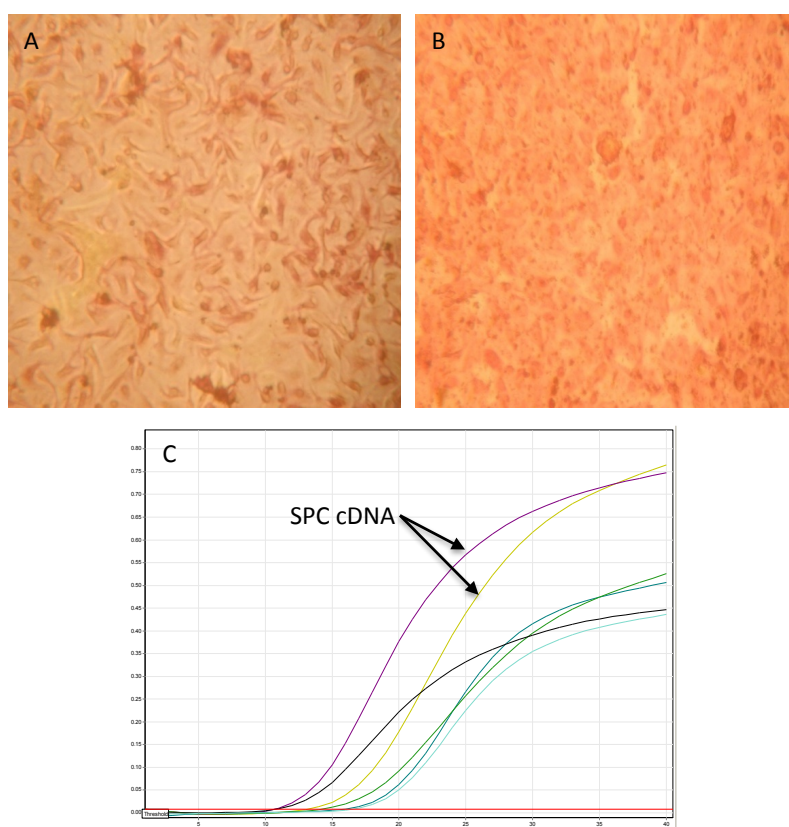
Human alveolar type 2 (hAT2) cells were isolated, purified and cultured from disease-free lung parenchyma removed as part of a tumour resection (Pinhu et al., 2008; Witherden and Tetley, 2001). This practice was approved by the Local Ethics Committee (REC reference 10/H0504/9). Lung sections were perfused by injection of 0.9% sterile 0.9% sodium chloride (saline) until the draining lavage ran clear and the cell count was less than 1×10^4 cells/mL. The tissue was then perfused and inflated with trypsin (0.25% in HBSS; Sigma, Poole, UK) and incubated at 37°C for 45 min; trypsin was replaced twice during this time. The tissue was finely chopped in the presence of newborn calf serum

(NCS; Invitrogen). The chopped tissue was then incubated with DNase (250 µg/mL; Sigma) and the mixture shaken by hand for 5 minutes before being passed through a 300µm filter, followed by a 40µm filter to remove large tissue debris. The cell suspension was then centrifuged at 1300 rpm for 10 min and the cell pellet re-suspended in DCCM-1 media (React Scientific) containing DNase (50µg/mL). These cells were incubated in tissue culture flasks for 2 hours at 37°C, 5% CO₂ to allow differential adherence of mononuclear cells.

After 2 h the non-adherent ATII cells were removed and the cell suspension centrifuged at 1300 rpm for 10 min. The cell pellet was then re-suspended in DCCM-1 containing 10% NCS and 1% PSG at a concentration of 1×10^6 cells/mL. Following isolation, cells were counted by using a haemocytometer under a phase contrast microscope and seeded onto type 1 collagen-coated well (1% Purecol 3mg/mL) at the optimum density (Table 2.1). hAT2 cells were stained with alkaline phosphatase, which has been used to characterise isolated cells (Edelson et al., 1988). The intensity of pink stain reflects the amount of alkaline phosphatase present (Figure 2.1). The cells used for experimentation were over 95% “alkaline phosphatase-positive” representing hAT2 purity, and expressed surfactant protein C (figure 2.1). After 48h the confluent monolayer was washed 3 times with DCCM-1 medium containing 10% NCS to remove debris and blood. New medium was added and cells were ready for experimentation 72 hours after seeding.

Table 2.1 Optimum seeding density of hAT2 cells in various well sizes

	Growth Surface (cm ²)	Culture medium (mL)	Recommend density
6 well plate	9.6	2.0	2.5-5.0×10 ⁶ per well
12 well plate	3.8	1.0	1.0-2.0×10 ⁶ per well
24 well plate	2.0	0.5	0.5-1.0×10 ⁶ per well
48 well plate	0.75	0.3	2.0-3.75×10 ⁵ per well
96 well plate	0.32	0.1	1.0-1.5×10 ⁵ per well

**Figure 2.1 Characterisation of human alveolar type 2 cells**

Human alveolar type II (hAT2) cells were stained with alkaline phosphatase. Image A shows cells at x10 power, 72h hours following seeding while Image B shows cells at x5 power 96 hours after seeding. The cells are virtually 100% alkaline phosphatase-positive, representing the hAT2 cell purity. To further confirm purity, RNA was extracted from monolayers of hAT2. Polymerase chain reaction of RNA demonstrated surfactant protein C cDNA (arrowed lines, Image C) with co-expression of reference genes GAPDH and HMBS. These are representative images of n=4.

2.2.5.2 Isolation of human alveolar macrophages

The draining lavage from perfused lung sections outlined above was collected into 50mL Falcon tubes. The first two Falcons, which contained large numbers of red blood cells were discarded and the remaining falcons centrifuged at 300rcf for 10 minutes. The cell pellets was re-suspended in pre-warmed serum-free DCCM-1 containing 1% penicillin/streptomycin/ glutamine and plated in 24-well tissue culture plates (0.5×10^6 macrophages/well). After 3 hours, macrophages had adhered and the medium was removed and the wells were washed with pre-warmed DCCM-1 four times, to remove non-adherent cells and ensure minimal red cell contamination. The macrophages were maintained in serum-free DCCM-1 and treated within 15 hours of plating.

2.2.5.3 Human lung microvascular endothelial cell line

Primary human lung microvascular endothelial cells (HMVEC-L) were purchased from Promocell (Germany). These cells are isolated from post mortem normal human tissue and stain positive for CD31 and von Willebrand factor, and negative for smooth muscle alpha-actin. They are shipped as proliferating cells at passage 2. The cells were maintained in microvascular endothelial cell growth medium-2 (EGM-MV2, Promocell) supplemented with 2% FBS and growth factors, antibiotics and other additions according to the manufacturer's guidelines. They were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were passaged when 70-90% confluent (figure 2.2) using Trypsin/EDTA for between 2 and 3 minutes, until 90% of cells were detached from the growth flask. Trypsin activity was stopped using trypsin neutralising solution (TNS, Promocell). The cells were centrifuged at 220rcf for 3 min at room temperature and re-suspended in culture medium. The morphology of confluent HLMVEC under phase contrast microscopy is shown in figure 2.2. After a variable number of passages, cell growth pattern or morphology altered, at which point cells were abandoned and a new vial

purchased. For all experiments cells were used at passage 3-6. Cells were routinely seeded at a density of 1×10^4 cells/cm² unless otherwise stated.

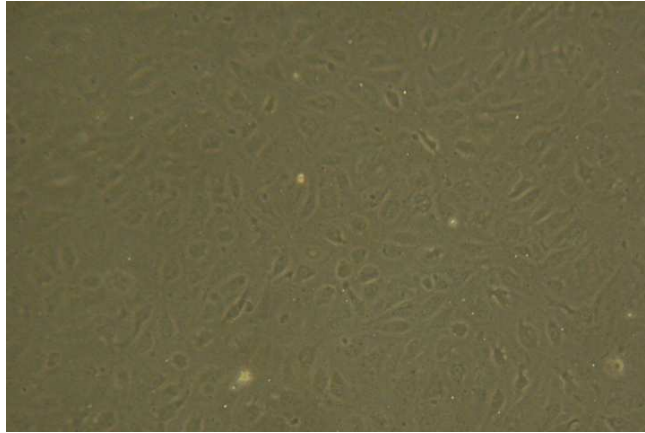


Figure 2.2 Morphology of confluent HMVEC-L

2.2.5.4 Human umbilical vein endothelial cells cell line

Pooled human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (UK). These cells are isolated from up to 4 different umbilical cords and stain positive for CD31 and von Willebrand factor and negative for smooth muscle alpha-actin. They are shipped as cryopreserved cells at passage 2. The cells were maintained in microvascular endothelial basal medium-2 (EGM-2) supplemented with 5% FBS and growth factors, antibiotics, and additives according to the manufacturer's guidance. HUVEC were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and passaged when 80% confluent (figure 2.3) using Trypsin/EDTA for between 2 and 3 minutes, until 90% of cells were detached from the growth flask. Trypsin activity was stopped using trypsin neutralising solution (TNS). The cells were centrifuged at 220rcf for 3 min at room temperature and re-suspended in culture medium. The morphology of confluent HUVEC under phase contrast microscopy is shown in figure 2.3. For all

experiments cells were used at passage 3-7. Cells were routinely seeded at a density of 5×10^3 cells/cm² unless otherwise stated.

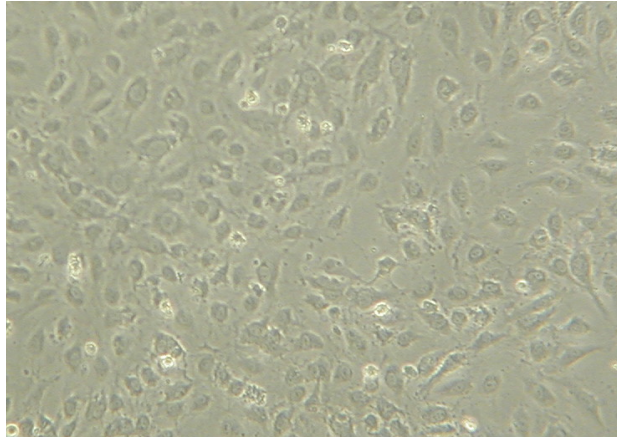


Figure 2.3 Morphology of confluent HUVEC

2.2.5.5 Purification of human neutrophils for *in vitro* studies

Ethical permission was granted by the local ethics committee (REC reference 11/LO/1092), for obtaining peripheral venous blood from healthy non-medicated volunteers. Neutrophil isolation was undertaken at room temperature, using endotoxin-free reagents and plastic-ware, under sterile conditions in a laminar flow cell culture hood (Microflow Class II cabinet) as previously described by Haslett (Haslett et al., 1985).

Venupuncture was undertaken using a sterile 19 gauge Butterfly needle (Hospira Venisystems) and 50mL Plastipak disposable syringes. The blood was immediately transferred into 50mL Falcon tubes (BD) containing 4mL (3.8%) sodium citrate to make a total volume of 40mL. Cells were centrifuged at 320 rcf for 20 min at room temperature. The platelet-rich plasma (PRP) supernatant layer was removed and used to make platelet poor plasma (PPP) and autologous serum. To prepare PPP, the PRP was centrifuged at 1400 rcf for 20 min and the pellet discarded. Simultaneously, the pelleted cells from the initial blood centrifugation were subjected to dextran sedimentation using 6% dextran 500

(2.5mL/10mL of cell pellet). The volume was made up to 50mL with 0.9% pre-warmed (37°C) sterile 0.9% saline and the tubes then mixed gently and allowed to stand for 30 min to allow the erythrocytes to sediment.

The leukocyte-rich plasma was aspirated and centrifuged at 320 g for 6 minutes. The pellet was gently re-suspended in 2mL PPP and transferred to a 15mL Falcon tube, where it was under-layered with 2mL freshly prepared 42% Percoll in PPP, which was in turn under layered with 2mL of freshly prepared 51% Percoll in PPP. Under-layering was performed using a Pasteur pipette (2mL, Sigma-Aldrich). The gradients were centrifuged for 14 min at 150 rcf, with the brake and acceleration speed set to zero. Mononuclear cells and some platelets remained at the top interface between the plasma and 42% Percoll layer, with neutrophils in a wider band at the interface of the 42% and 51% layer Percoll layers and extending into the 51% Percoll layer to a few millimeters above the erythrocyte pellet (figure 2.4). Each band was aspirated with a Pasteur pipette. The neutrophils were sequentially washed in PPP, phosphate buffered saline without $\text{Ca}^{2+}/\text{Mg}^{2+}$ ($\text{PBS}^{-/-}$) and phosphate buffered saline with $\text{Ca}^{2+}/\text{Mg}^{2+}$ ($\text{PBS}^{+/+}$) (centrifuged at 256 rcf, 5 min) prior to re-suspension at 5×10^6 cells/mL in $\text{PBS}^{+/+}$ or Iscove's Modified Dulbecco's medium (IMDM) containing 1% autologous serum.

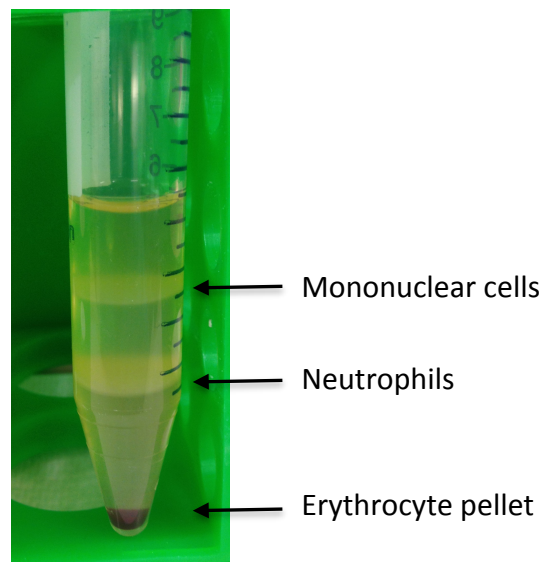


Figure 2.4 Plasma-Percoll gradation of freshly isolated leukocytes

Cell purity was confirmed by examination of cytocentrifuged neutrophils. Labelled Polycine microscope slides were placed in funnel clips in a cytospin (Cytospin 2, Thermo Scientific). The cell suspension (100 μ L of 2×10^6 cells /mL) was placed into the funnel and centrifuged at 300 rpm for 3 minutes, at high acceleration. Slides were removed and allowed to air dry before being placed in methanol for 4 minutes. Slides were then sequentially placed in DiffQuick stain; orange/red for 1 minute, then blue for 2 minutes, with excess stain being rinsed off in between with distilled water. Stained slides were allowed to air dry for 30 minutes before being coverslip mounted using DPX (Sigma Aldrich). Cells were examined by light microscopy, magnification x20, 300 cells/ per slide in triplicate. Analysis of freshly isolated neutrophils confirmed that neutrophil preparations were routinely >99% viable (trypan blue negative) and analysis of cytospun neutrophils were $95 \pm 1.8\%$ pure (eosinophils being the dominant non-neutrophil contaminant), with less than 0.1% monocytes. In addition, basal median forward scatter (shape change) (Qu et al., 1995) did not significantly differ between donors ($p=0.0443$, figure 2.5).

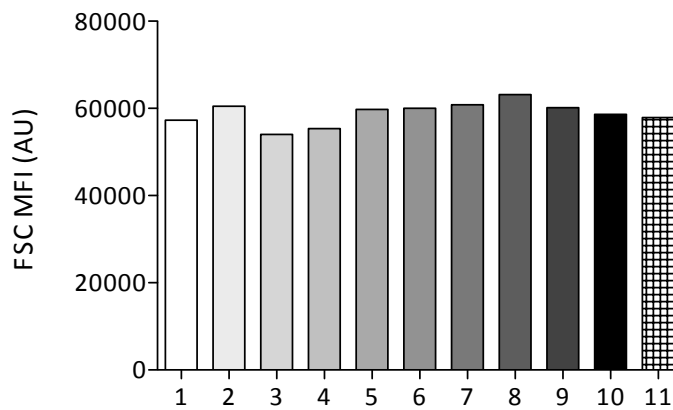


Figure 2.5 Mean forward scatter in freshly isolated human neutrophils

Freshly isolated human granulocytes ($5 \times 10^6/\text{mL}$) were analysed by flow cytometry. Shape change was assessed by mean forward scatter (FSC, $n=11$).

2.2.6 *In vitro* ALI modelling

2.2.6.1 Applying cyclic mechanical strain to hAT2

The FX-4000TM system was used to apply cyclic mechanical strain (CMS, stretch) to cell monolayers according to computer-controlled protocols. Amplitudes of 0, 5, 20 and 30% elongation were used at a frequency of 20 cycles per minute. The system consists of a FlexCentral® computer, BioFlex® Baseplate & Gaskets, BioFlex® Loading stations and BioFlex® plates (figure 2.6). The system applies strain using a vacuum to deform the flexible membrane on which cells are cultured (Gilbert et al., 1994; Schaffer et al., 1994).

The BioFlex culture plate has 6-wells, which can be deformed by up to 30% elongation. The BioFlex loading stations are designed to provide uniform radial and circumferential strain across the membrane surface up to 20% elongation. The membrane is pulled over the post by the vacuum; each radius is stretched by same amount at any point in the circle, providing uniform “equibiaxial” strain for all cells on the membrane when applied elongation to the cell is ranged between 0 to 20%. Loading posts are

removed when greater than 20% elongation is used. The inability to achieve 30% strain whilst using the loading posts is a limitation of the Flexercell™ system. Without loading posts a radially non-uniform strain field is produced, such that only a fraction of cells in the well's periphery are exposed to the desired amplitude of strain (i.e. 30%) (Gilbert et al. 1994; Schaffer et al. 1994). Furthermore, mechanical strain is not confined to the basolateral cell surface. The radial acceleration of cells relative to the stationary medium produces shear forces over the apical cell surface that are of uncertain significance.

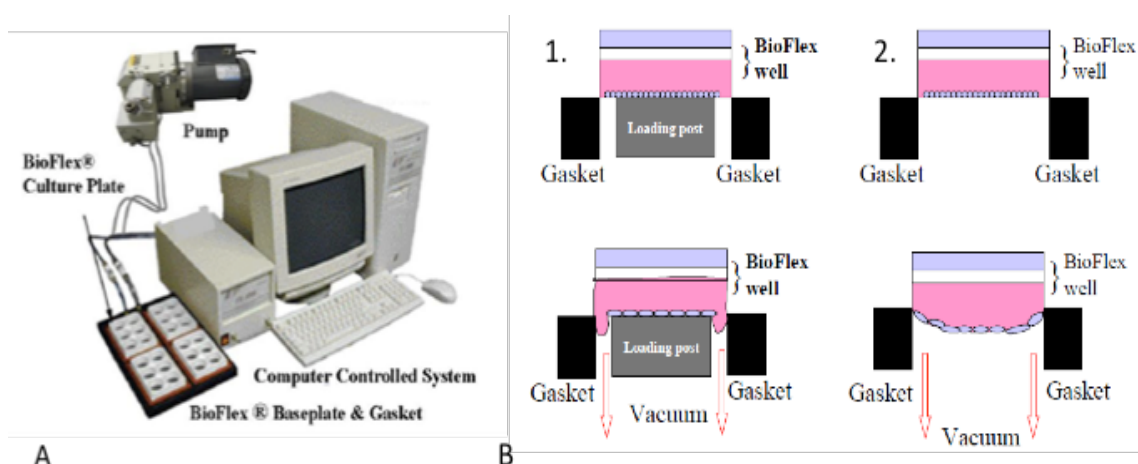


Figure 2.6 Flexcell FX-4000 system

Image A – shows the Flexcell® FX-4000™ computational system. Image B - diagram to illustrate the application of the vacuum to the flexible membrane with (B1) and without (B2) loading posts.

2.2.6.2 Endothelial monolayer permeability assay

Electrical resistance of endothelial cell monolayers was measured using an electrical cell impedance sensor (ECIS) technique (Applied Biophysics, Inc., NY, USA). HMVEC-L (2.5×10^5 cells/mL, 400μL) were cultured in proprietary chamber slides with 8 wells per slide. Each well contains ten circular 250μm diameter active electrodes connected in parallel on a common gold pad, measuring approximately 500 to 1000 cells. The chamber slide was connected to the 16 well array station and placed in an incubator at

37°C, 5% CO₂. Following growth to confluence (normally over 72 hours), the endothelial monolayer acts as insulating particles and the total resistance across the monolayer is composed of the resistance between the ventral cell surface and the electrode and the resistance between cells. A 4,000-Hz AC signal with 1-volt amplitude through a 1mΩ resistor created an approximate constant current source (1μA). The lock-in amplifier attached to the electrodes detects changes in both magnitude and phase of the voltage appearing across the endothelial cell and was controlled by an IBM-compatible personal computer, which was used both for data accumulation and processing. Transendothelial resistance (TER, generated by a proprietary algorithm) increased immediately after cell attachment and achieved a steady state when endothelial cells became confluent. Thus, experiments were conducted after the electrical resistance achieved a steady state of 3000 ohms. Resistance data were normalised to the initial voltage and plotted as a normalised TER over 20 hours.

2.2.6.3 Neutrophil Apoptosis Assay

Neutrophil apoptosis was assessed using a commercial kit purchased from Becton Dickinson (Annexin V FITC Apoptosis Detection Kit). Double labelling of neutrophils with Annexin-V-7AAD (AnV), which binds to phosphatidylserine on the surface of apoptotic cells, and the nuclear stain propidium iodide (PI), which allows assessment of membrane integrity, enables differentiation between viable non-apoptotic cells (AnV⁻, PI⁻), viable early apoptotic cells (AnV⁺, PI⁻), and late apoptotic/necrotic cells (AnV⁺, PI⁺).

Purified neutrophils were re-suspended at 5x10⁶ (200μL) in IMDM supplemented with autologous serum (10%) and streptomycin and penicillin (50U/mL) and cultured in Falcon flexiwell 96-well plate inserts. Following treatment for 6 or 20 hours, neutrophils were pulse-centrifuged, then re-suspended in binding buffer (100μL: 0.1M HEPES pH 7.4, 1.4M NaCl, 25mM CaCl₂), containing annexin-V-fluorescein isothiocyanate (FITC:

2.5µL) and propidium iodide (PI: 5µL). Samples were incubated for 20 minutes at 4°C in the dark, and the volume increased to 500µL with buffer immediately before analysis using a fluorescence-activated cell sorter with FACS Diva software. Ten thousand events were collected for each sample, and data analysed using FlowJo software (v10.0, Treestar, USA). Viable, non-apoptotic neutrophils were defined as being negative for both annexin V-FITC and PI, and apoptotic neutrophils were defined as being positive for annexin V-FITC. Apoptosis was expressed as a percentage in relation to the total number of counted neutrophils.

These data were confirmed by assessing microscope slides (×100 objective) of cytocentrifuged neutrophils fixed in methanol and stained with DiffQuick™. Apoptotic neutrophils were identified by cell shrinkage, blebbing of the cell membrane and the presence of darkly stained, condensed and fragmented nuclei. For each condition, duplicate slides were prepared and 300 neutrophils counted per slide in a blinded fashion.

2.2.6.4 Oxidative burst in human neutrophils

The kinetics of TNFR dependency on oxidative burst in TNF primed, fMLP stimulated neutrophils was obtained assessed using luminol-dependent chemiluminescence. Human neutrophils (5×10^6 /mL, 400µL) were harvested as outlined previously. Neutrophils were exposed to treatments and controls for 45 minutes at 37°C at 300rpm in a thermomixer (Thermomixer Comfort, EppendorfEppendorf, USA). Luminol (1µM) and horseradish peroxidase (HRP: 62.5U/mL) in PBS were added to the treated cells. After 3 minutes, 150µL of this the cell suspension was transferred to a pre-warmed 96-well luminometer plate. Neutrophils were stimulated with fMLP (100nM); fMLP was added through the injection port and light emission was recorded by a Berthold MicroLumat Plus luminometer (Berthold Technologies, UK). Data output was measured

in relative light units per second and data analysed as fold change in peak height of luminescence intensity over a period of 10 minutes.

2.2.6.5 Leukocyte transendothelial migration assay

2.2.6.5.1 Confirmation of HMVEC-L monolayer integrity on Transwell filters

Prior to assessing neutrophil migration, monolayer integrity in HMVEC-L grown on Transwell inserts was confirmed. At a seeding density of 4×10^4 cells/well, by 72 hours fluorescent confocal microscopy of Transwell filters stained strongly positive for VE-cadherin suggesting intact adherens junctions in monolayers (Figure 2.7A & B). Transendothelial resistance increased over 72 hours, suggestive of monolayer integrity (Figure 2.7C); this effect was associated with impermeability to FITC-labelled albumin (Figure 2.7D).

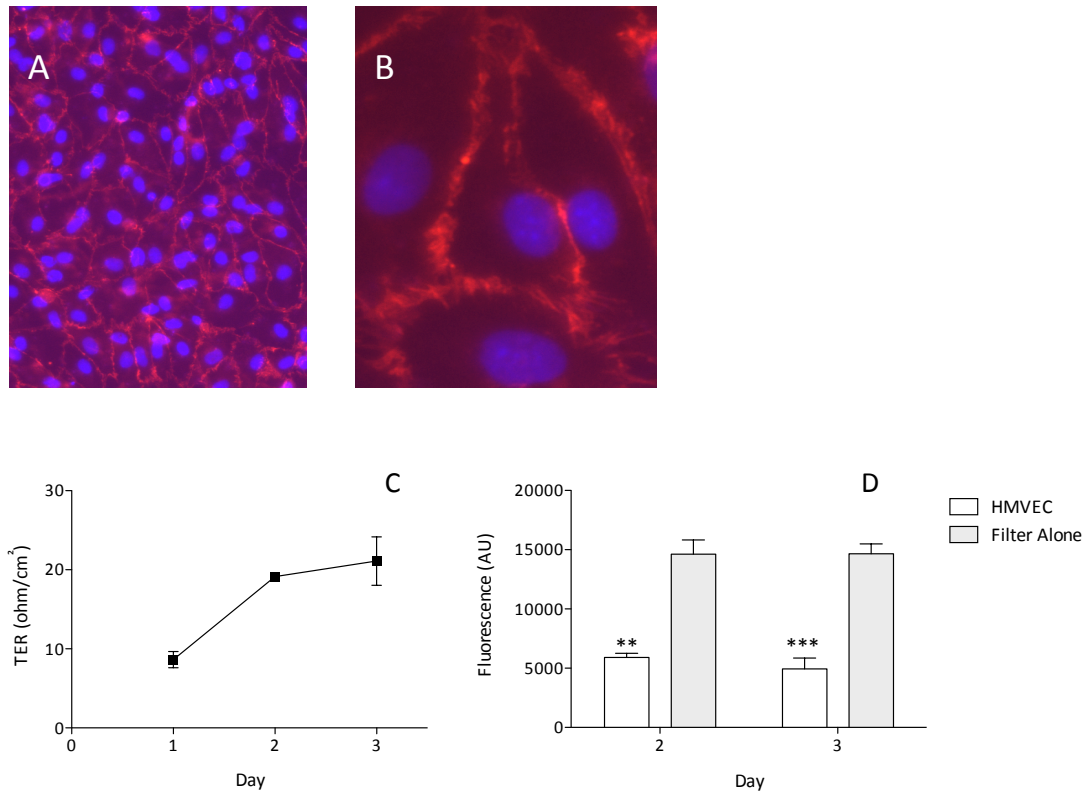


Figure 2.7 Confirmation of HMVEC-L monolayer integrity on Transwell filters

HMVEC-L (5×10^4) on Transwell filters were stained with antibody to VE-cadherin (anti-goat Alexafluor 594) for imaging using fluorescent confocal microscopy. The red stain was indicative of intact VE cadherin staining across the field of view (Image A, 10x, Image B, 40x magnification). Transendothelial resistance was measured at days 1-3 (Image C) (n=3). Permeability of FITC labelled albumin (1mg/mL, 200 μ L) across monolayers was measured on days 2 and 3 of HMVEC-L culture. After 3 hours, 100 μ L was removed and measured in triplicate on a fluorescent plate reader at 480nm (n=3). Data are presented as mean $\pm$ SEM and analysed by Mann Whitney U test **p<0.01, *** p<0.001

The possibility that fluorescent staining with Leukotracker™ solution adversely affected baseline neutrophil priming status, was also excluded prior to migration studies. Ideally, thorough interrogation of the combination of shape change, CD11b and CD62L expression would have been undertaken. However, the green fluorescence of Leukotracker™ excluded analysis of CD11b or CD62L expression by flow cytometry. Nonetheless, fluorescently labelled neutrophils did not show any evidence of shape change suggestive of neutrophil priming (Figure 2.8)

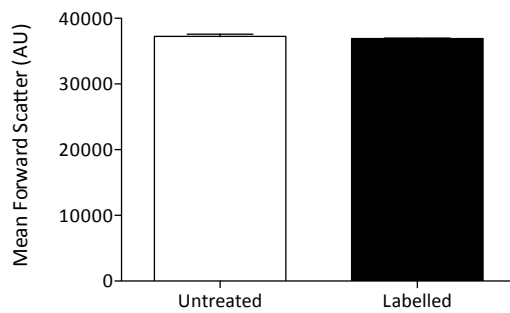


Figure 2.8 Leukotracker™ labelled neutrophils did not undergo shape-change

Neutrophils were exposed to Leukotracker™ or PBS. Following washing according to the manufacturer's guidelines, cells were analysed for shape change. Data are presented as mean $\pm$ SEM (n=3)

IL-8 was chosen as the most relevant neutrophil chemokine in migration experiments. The dose response curve of neutrophil migration through HMVEC-L monolayers is shown in figure 2.9. Based on these data a dose of 100ng/mL was used for further experiments.

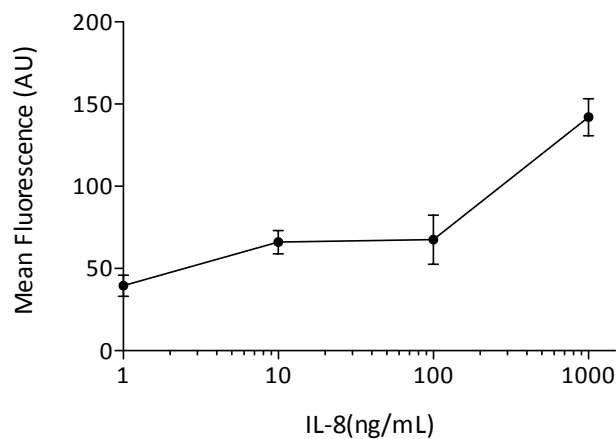


Figure 2.9 Dose response curve of neutrophil migration through HMVEC-L to IL-8

Leukotracker™ labelled neutrophils (1x10⁶/mL, 200 μ L) were added to the upper chamber of a Transwell covered with confluent HMVEC-L. Migration was triggered by the addition of interleukin (IL)-8 1, 10, 100 or 1000 ng/mL in the lower chamber. Neutrophil migration was assessed by measurement of fluorescence accumulated in the lower chamber after 3 hours, read at 480nm on a fluorescent plate reader. Each condition was measured in triplicate and data presented as mean $\pm$ SEM (n=3)

2.2.6.6 Neutrophil migration through HMVEC-L monolayers

To assess the functional consequences of selective TNFR antagonism on neutrophil migration through TNF stimulated HMVEC-L, a static proprietary migration assay (CytoSelect™ Leukocyte Transmigration assay, Cambridge Biosciences, UK) was used. The principal of this assay is outlined in figure 2.10. HMVEC-L (1.6×10^5 /mL, 200 μ L) were seeded onto the upper chamber of Transwell filters (24-well size, 3 μ m pore size) and left for 72 hours. Medium (700 μ L) was placed in the lower chamber. Growth to confluence was confirmed by 3 distinct methods outlined above; light microscopy, TER measurement and confocal microscopy to assess adherens junction formation. Cells were then washed twice with PBS and exposed to treatments for 4 hours at 37°C, 5% CO₂. All conditions were performed in triplicate. Following a further 2 washes in PBS (to remove basal chemokine release from stimulated HMVEC-L), the media in the lower chambers was changed to IMDM containing IL-8 (50ng/mL, 700 μ L).

During HMVEC-L monolayer stimulation, healthy volunteer neutrophils were harvested as outlined previously. Neutrophils were suspended at 1×10^6 cell/mL in serum free IMDM and Leukotracker™ was added at the manufacturer's recommended dilution. Cells were incubated at 37°C, 5% CO₂ for 1 hour then washed twice and re-suspended at 1×10^6 cells/mL in serum free media. 200 μ L of this solution was added to the upper chamber and neutrophils left to migrate over 3 hours. After 3 hours, the medium in the upper chamber was discarded and non-migratory cells were removed with a cotton swab. The Transwell filter was then placed in a well containing proprietary lysis buffer (150 μ L) and 400 μ L of media from the lower chamber containing migrated neutrophils. The plate was gently shaken for 5 minutes at room temperature then 150 μ L of the mixture was transferred to black 96 well plates suitable for fluorescence measurement. Fluorescence was read in triplicate at 480nm/520nm.

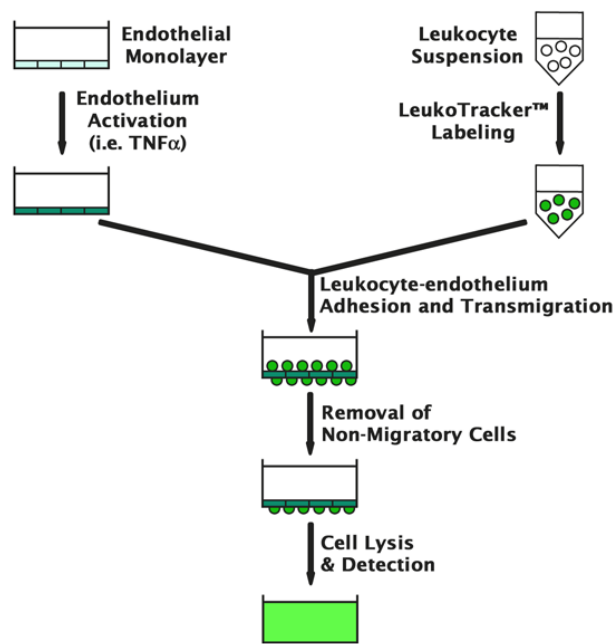


Figure 2.10 Principle of the leukocyte transmigration assay

2.2.6.7 Scratch assay in human alveolar type 2 cells

The bottoms of collagen coated, 48 well plates were marked with a scalpel or fine pen across the centre of the well, and hAT2 seeded at 3×10^5 cells per well. Following growth to confluence, the medium was changed to serum free DCCM-1 overnight prior to scratch assay. The cell monolayer was then disrupted in a straight line to create a “scratch” with a p200 pipette tip; this was performed through the marker on the well base outlined below (figure 2.11). Cell debris was removed washing the cells twice with DCCM-1 media and then replaced with medium (500 μ L) containing relevant inhibitors and controls. It is important to create scratches of approximately similar size to minimize any possible variation in healing rates caused by the difference in the width of the scratches. The wounded monolayer was then imaged by phase-contrast microscopy with reference mark outside the capture image field but within the eyepiece field of view. Wounded cells are then incubated for 20 hours at 37°C and cells re-imaged aligning the reference point with the baseline image. The images acquired for each sample were

analysed quantitatively using Image J software (v1.45, National institutes of Health, USA). Repair was expressed as the percentage of the original wound area covered by cells.

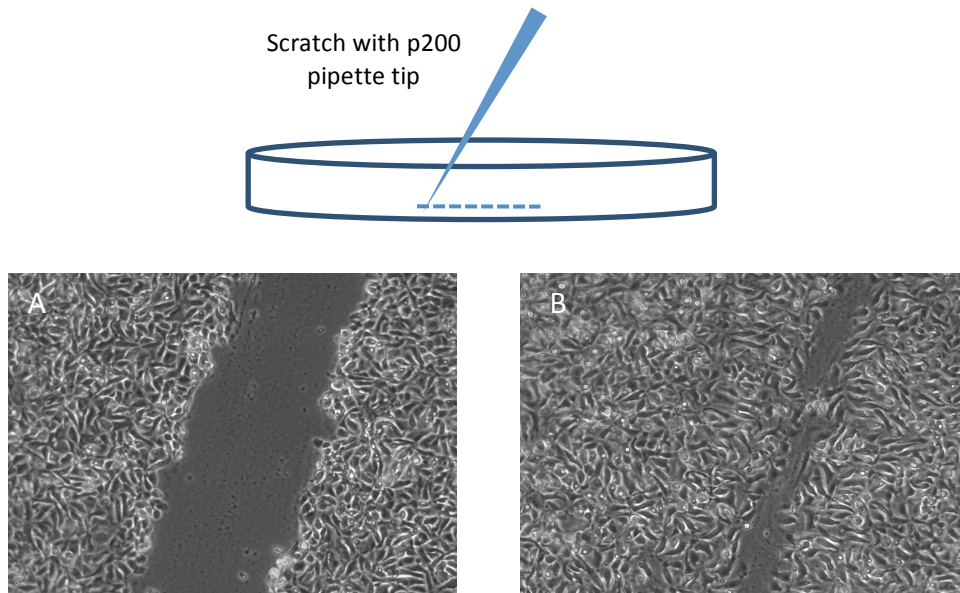


Figure 2.11 Mechanical wounding of hAT2 cells

Following scratch with a pipette tip a homogenous wound is created (Image A). Depending on ambient conditions and treatment, the wound heals over 18-24 hours (Image B)

2.2.6.8 *In vitro* angiogenesis assay

The *in vitro* formation of capillary-like tubes by endothelial cells on a basement membrane matrix is a powerful *in vitro* method to screen for various factors that promote or inhibit angiogenesis. The basement membrane extract used for this assay, sold commercially as Matrigel (BD Biosciences), is obtained from a murine tumour that produces an abundant extracellular matrix of basement membrane (Kleinman and Martin, 2005). The extract is a liquid at 4°C and it forms a gel at 16–37°C.

The angiogenesis assay was based on published methodology (Arnaoutova and Kleinman, 2010). 10µL Matrigel was added to wells in a pre-warmed ibiTreat Angiogenesis µ-slide (Ibidi, Germany) and incubated at 37°C for ≥60 minutes. HUVEC

and HMVEC were then admixed with the relevant treatments and controls. Cells at passages 4 and below were seeded in triplicate wells at a density of 1×10^4 HUVEC and 6×10^3 HMVEC-L per well. EGM-2 and EGM-MV2 media containing 2% FCS and without VEGF (for HUVEC and HMVEC-L respectively) were used for cell seeding and the additions of treatments. A total volume of 50 μ L (cells plus treatment) was added to each well.

Cells were incubated for 4 hours at 37°C, 5% CO₂; preliminary experiments (data not shown) determined that this was the optimal time-course for tube formation in both cell types. After 4 hours, the medium was aspirated from the wells and replaced with 75 μ L of warmed PBS. Wells were imaged using a digital camera attached to an inverted phase contrast microscope (CK40, Olympus, UK) UK with x4 objective (figure 2.12). Total tube length per well was measured using Image J software. The observer was blinded to the treatment conditions to avoid measurement bias and the mean total tube length of triplicate wells analysed.

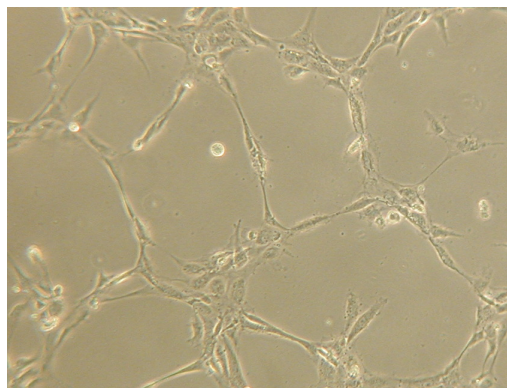


Figure 2.12 A representative image of tube formation in HMVEC-L after 4 hours

2.2.7 Antibody based assays

2.2.7.1 Protein Assay

The Bradford assay is a colorimetric assay based on the principle of binding brilliant blue to protein (Bradford, 1976). Protein standards (BSA at 400 μ g/mL) were prepared between 5 and 400 μ g/mL in distilled water. Standard or diluted samples (100 μ L) were added to 96-well micro-titre plate in triplicate. Bio-Rad Bradford Reagent (200 μ L) diluted reagent was added to each well. Absorbance was measured at 620nm using an Anthos Ht2plate reader (Anthos, Italy). The absorbance of cell lysis buffer alone was measured to exclude the possibility of interference with the assay. Unknown protein concentrations were calculated using a standard curve.

2.2.7.2 Enzyme Linked Immunosorbent Assay (ELISA)

2.2.7.2.1 Basic Principles

Enzyme linked immunosorbent assay (ELISA) is an immunologic assay that uses specific antibodies to detect and quantify solid phase bound proteins on a membrane, and proteins in solution. There are two common types of ELISA (figure 2.13). Sandwich ELISAs detect proteins in solution using an immobilized capture antibody. A washing step removes non-specifically bound material and an enzyme-marked secondary antibody

with a second epitope to target protein is added. An enzyme-substrate reaction is used to amplify the signal (2.13A). Alternatively, the target protein is immobilized and captured by the antibody in solution (2.13B). The basic methodology of a sandwich ELISA is described in Figure 2.14.

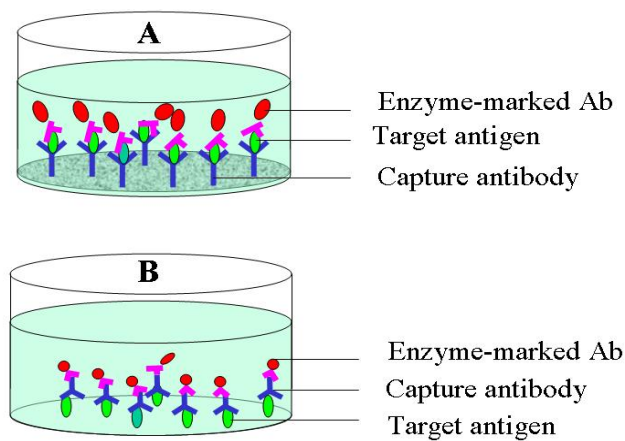


Figure 2.13 Two common types of Enzyme Linked Immunosorbent Assay (ELISA)

A: Sandwich ELISA, capture antibody is immobilized on a solid support and captures target antigen in solution. B: Target protein is immobilized (such as expressed on the cell surface) and captures antibody in solution.

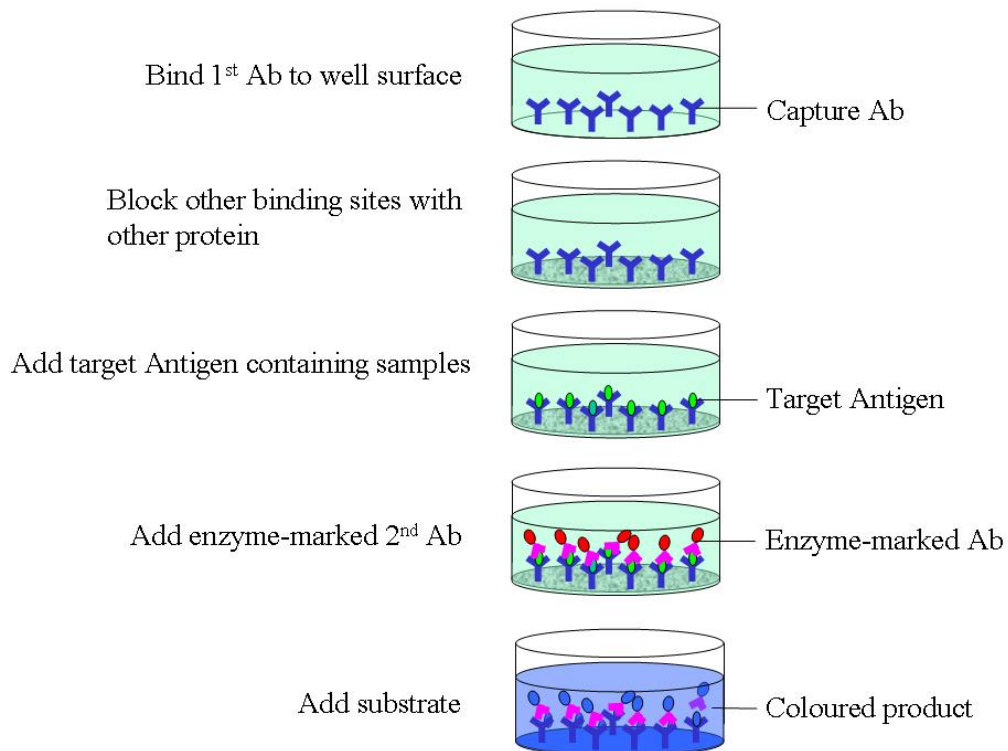


Figure 2.14 Basic principle of a sandwich ELISA

2.2.7.2.2 Duoset ELISA

Sandwich ELISAs for detection of a variety of proteins were performed using a generic ELISA protocol according to the guidelines provided by the supplier of the paired

antibodies. Supernatants were examined immediately after experiments or frozen at -80°C and used when necessary. The constituents of the reagents for the ELISA and ELISA protocol are shown in table 2.2 and figure 2.15. The DuoSet ELISAs were used to analyse for IL-8, human sTNFR1, human sTNFR2 (R&D Systems, UK).

Table 2.2 DuoSet ELISA reagents

	Wash buffer	Blocking buffer	Assay Diluent	Substrate solution	Stop solution
IL-8	0.05% Tween 20 in PBS	1% BSA in PBS (pH 7.2-7.4)	Tris buffered saline (20mM Trizma base, 150mM NaCl), 0.1% BSA, 0.05% Tween-20	Reagents A & B	2N H ₂ SO ₄
sTNFR1	0.05% Tween 20 in PBS		1% BSA in PBS (pH 7.2-7.4)	Reagents A & B	2N H ₂ SO ₄
sTNFR2	0.05% Tween 20 in PBS	1% BSA in PBS (pH 7.2-7.4)	1% BSA in PBS (pH 7.2-7.4)	Reagents A & B	2N H ₂ SO ₄

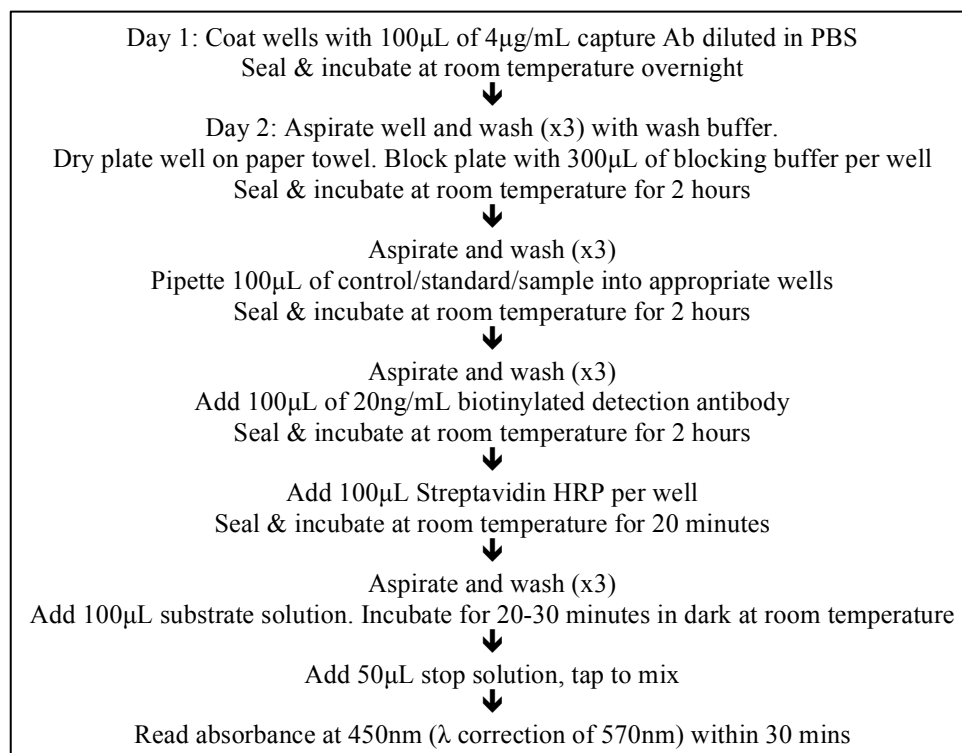


Figure 2.15 Duoset ELISA protocol and reagents

2.2.7.2.3 Human MPO ELISA

Myeloperoxidase (MPO) is a glycoprotein present in azurophilic granules of neutrophils, which is rapidly released upon neutrophil activation. MPO activity can therefore be used as a surrogate marker of neutrophil influx and activation into tissues. Classical MPO assays are often hampered by the presence of tissue inhibitors of MPO (Ormrod et al., 1987). The human MPO ELISA (Hycult biotech, The Netherlands) is not influenced by these inhibitors and was therefore chosen for this assay.

Protein was harvested from *ex vivo* lung tissue according to the protocol outlined in 2.2.15.3 and stored at -80°C until analysis. Proprietary wash buffer, dilution buffers A and B, standard, biotinylated tracer, streptavidin-peroxidase, TMB substrate and stop solutions were reconstituted and used at the manufacturer's recommended concentration. Control/sample/standard (100µl) was added in duplicate to microtitre wells pre-coated with antibodies recognizing human MPO and stored at room temperature for 60 minutes.

Following washing (x4), 100µL of biotinylated tracer antibody was added to each well and left for 60 minutes at room temperature. Wells were washed (x4) and 100µL of streptavidin-peroxidase conjugate added and left for 60 minutes at room temperature. After a final wash (x4) 100µL of TMB solution was added, incubated for 30 minutes at room temperature before 100µL of stop solution was added. Plates were immediately read at 450 nm using a spectrophotometer. MPO activity was corrected for the total protein content of the samples as measured by Bradford assay and calculated from the standard curve.

2.2.7.2.4 pP38 MAPK alpha PhosphoTracer ELISA

PhosphoTracer assays use a traditional immuno-sandwich format, but with a major difference; both the analyte and the assay reagents are added to the PhosphoTracer assay microplate (optimised to form an active surface for the capture of immunocomplexes formed as part of the assay procedure) at the same time. After a short incubation period, unbound assay reagents and analytes are washed away, and immuno-complexes containing both antibodies are detected. Protein extracts from control and LPS injured *ex vivo* human lung tissue were analysed according to the protocol outlined in figure 2.16 and read on a fluorescent plate reader with excitation band width of 530nm and an emission filter of 590nm. Assay control lysates are provided for use as an assay positive controls, and the Hardie lysis buffer (chapter 2.2.15.3) was used as the negative control.

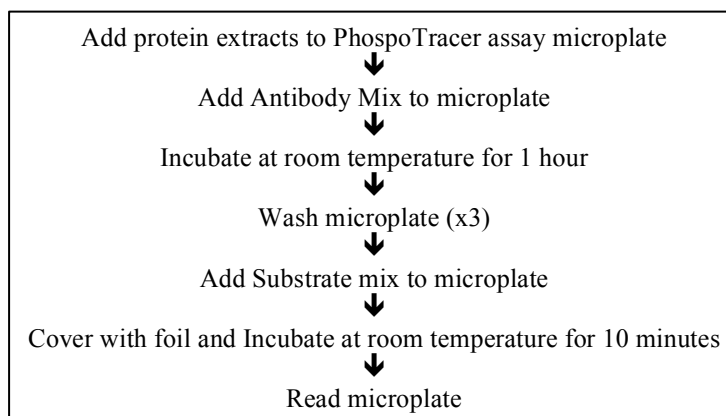


Figure 2.16 p38 MAPK alpha PhosphoTracer ELISA protocol

The antibody mix is comprised of a 1:1 mixture of proprietary Capture Antibody Reagent and the Detection Antibody Reagent and contains all the antibodies required for the assay. The substrate mix is a combination of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) and a stabilised H₂O₂ solution.

2.2.7.3 Electrochemiluminescence (MSD)

2.2.7.3.1 General principles of MSD multiplex plates

Meso-Scale Discovery (MSD) multiplex plates allows the detection of multiple proteins from a single biological sample, across a wide range of concentrations. Each well of a microplate features several micro-spots, each corresponding to a specific analyte (figure 2.17); plates are available in 3 geometries with 4, 7 or 10 spots. The samples are added to the wells and processed similarly to a standard ELISA. The detection antibody is conjugated to a Sulpho-Tag™ group. When the protocol has been completed the plate is placed within a plate-reader (MSD SECTOR® imager) and a small electric current run across each micro-spot within each well. By electrochemiluminescence, a luminous intensity is generated in proportion to the extent the Sulpho-tagged antibody present and this varies in proportion to the quantity of analyte present within each well (figure 2.15). A series of dilutions of known concentrations of analytes provided a reference and the on-computer software (MSD Discovery workbench) calculates the levels of each analyte in each well and exports the data to a spreadsheet.

In addition to measuring multiple analytes simultaneously, the advantage of the MSD system is that wide range of concentrations that can be measured without sample dilution – a traditional ELISA can measure over 2 to 3 log ranges, the MSD system can measure over 4 to 5. The main disadvantage of the system is expense.

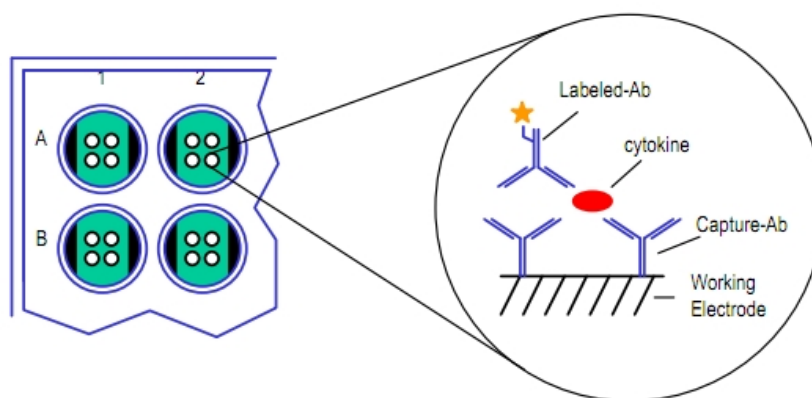


Figure 2.17 Cytokine capture antibody is pre-coated on specific spots of a 4-Spot MSD MULTI-SPOT plate.

Calibrator solutions or samples are incubated in the MULTI-SPOT plate, and each cytokine binds to its corresponding capture antibody spot. Cytokine levels are quantitated using a cytokine-specific detection antibody labelled with MSD SULFO-TAG reagent.

2.2.7.3.2 MSD protocol

Samples were collected and reagents prepared as per the manufacturers' instructions. Undiluted supernatants were used for analysis. Calibrators were diluted in Calibrator Diluent generating a standard curve from 10,000 to 2.4pg/mL with minor adjustments to ensure that analytes for all samples were within the standard curve. Diluent 2 (25µL) was pipetted into each well, ensuring coverage of the entire bottom of the well; a slight tap may be necessary to allow the fluid to settle to the bottom. The plate was then sealed with an adhesive plate seal and incubated for 30 minutes with vigorous shaking (700 rpm) at room temperature. 25µL of each calibrator or sample solution was then added into a separate wells of the MSD plate. Calibrators were measured in duplicate, whilst samples were measured in triplicate. The plate was sealed and incubated

for 2 hours with vigorous shaking (700 rpm) at room temperature. Following washing (x3) with PBS + 0.05% Tween-20, 25 μ L of the 1X Detection Antibody Solution was added to each well of the MSD plate. The plate was again sealed and incubated for 2 hours with vigorous shaking (700 rpm) at room temperature. Following a final wash (x3) with PBS + 0.05% Tween-20, 150 μ L of 2x Read Buffer T was added to each well. The plate was read immediately on the SECTOR[®] reader. Standard curves were fitted using MSD Workbench software (version 4.0) and the samples back-calculated to the curve. The mean and standard deviation for sample replicates were calculated in MSD Workbench software and exported into Microsoft Excel for plotting.

2.2.7.4 Confocal laser scanning microscopy

2.2.7.4.1 Basic principle

The principle of immunocytochemistry is the same as that of ELISA and immunoblotting but this technique uses antibodies to visualize target antigens in cultured cells. The following protocol uses fluorescent antibodies and confocal microscopy. The cultured cells are incubated with antibody to identify specific antigens. After washing, the cells are incubated with secondary antibody labelled with fluorescence. The fluorescent antibody can be visualized directly observed by a laser or UV microscope.

2.2.7.4.2 Immunocytochemistry and image acquisition

HMVEC-L were seeded on Transwell filters (3 μ m pore size, 12mm polycarbonate filter) and grown to confluence over 72 hours in an incubator at 37°C in a humidified atmosphere of 5% CO₂ in air. Growth to confluence was confirmed by light microscopy. The medium was removed from both upper and lower wells of the Transwell filter and replaced with 3.5% formaldehyde (700 μ L bottom well, 200 μ L top well) and left for 5 minutes at 37°C. Cells were then permeabilised by treatment with 0.5% Triton X-100 for

10 minutes at room temperature before incubation with 0.5% bovine serum albumin in PBS for 30 minutes at room temperature.

The filter with the confluent monolayer on the apical surface was then cut out using a scalpel and placed onto a labelled polycine slide with the apical surface of the filter to air. The filter was then incubated with 125µL goat polyclonal IgG VE-cadherin antibody (1:250 dilution in PBS/0.1% BSA) for 60 minutes at room temperature. Following incubation, the filter was gently washed three times with PBS. 125µL Alexa Fluor 594 donkey anti-goat IgG antibody (1:250 dilution in PBS/0.1% BSA) was then added and filters left at room temperature for 60 minutes in the dark. A negative control, with the primary antibody omitted, was used to quantify non-specific background fluorescence. Filters were washed 3 times before mounting with ProLong Gold anti-fade reagent containing 4',6-diamidino-2-phenylindole (DAPI) to stain endothelial cell nuclei. Confocal imaging was carried out using a Leica SP5 inverted confocal microscope (Leica, Switzerland), with a ×40 1.25 oil objective and 405 diode, argon, and HeNe 543 lasers. Leica Application Systems AF software (v2.7, Leica Microsystems, Switzerland) was used for quantification.

2.2.7.5 Western Blotting

2.2.7.5.1 Basic Principle

Western analysis or immunoblotting is a direct method for identifying and quantifying specific proteins in complex mixtures such as cell lysates. The complex protein mixture is fractionated according to molecular weight by gel electrophoresis. Separated proteins are transferred and blotted onto a membrane made of nitrocellulose, most commonly polyvinylidene fluoride (PVDF) or nylon. The membrane is incubated with a blocking reagent to shield non-specific binding sites. The primary antibody captures the target protein on the membrane. After washing steps to remove the unbound

primary antibody, horseradish peroxidase conjugated secondary antibody binds to the primary antibody immobilized on the membrane with the specific protein. Horseradish peroxidase secondary antibodies are utilized in conjunction with specific chemiluminescent substrates to generate a light signal. The amount of peroxidase activity present is related to the amount of the target protein fixed to the membrane. The general principle for western blotting is summarised in Figure 2.18.

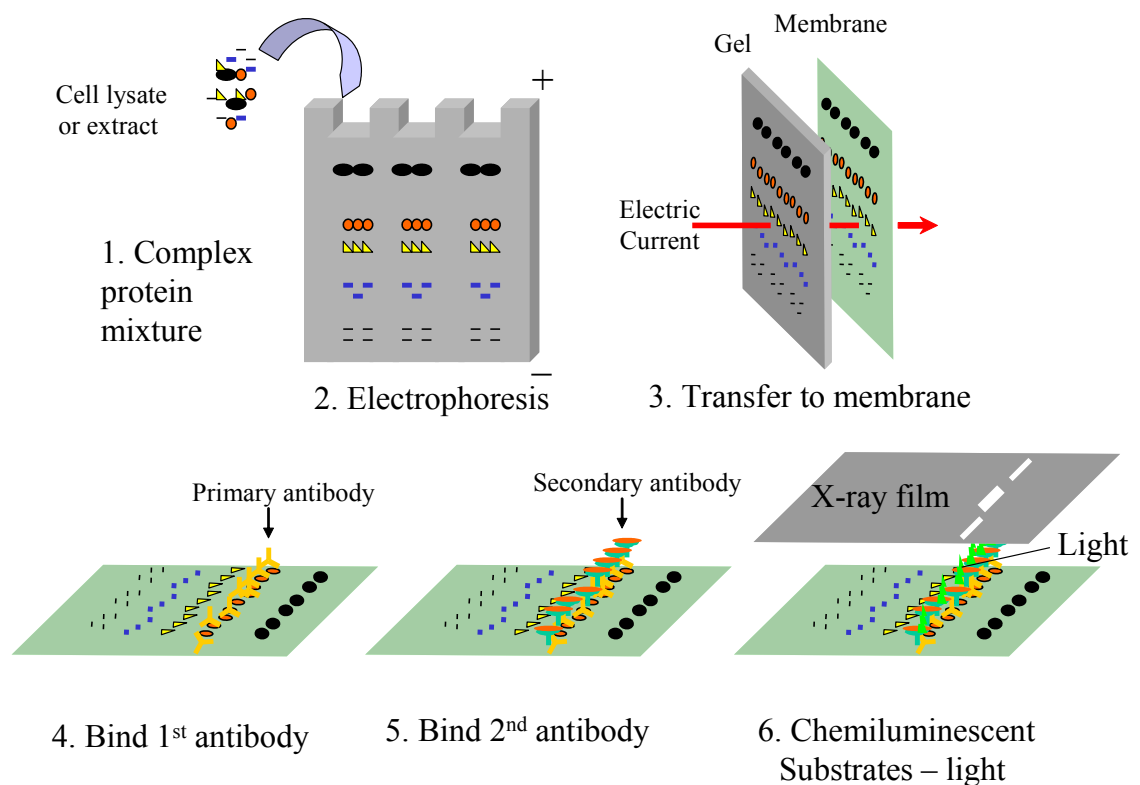


Figure 2.18 The general principle of western blotting

Image courtesy of L Pinhu

2.2.7.5.2 Preparation of whole cell protein extracts

Whole cell extracts were prepared by harvesting cells in $1\times$ cell lysis buffer (CLB) including 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na_2EDTA , 1mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5mM sodium pyrophosphate, 1mM β -glycerophosphate, 1mM Na_3VO_4 , $1\mu\text{g/mL}$ leupeptin and 1mM phenylmethylsulfonyl

fluoride (PMSF). The whole cell extract was kept at -20°C until required. Samples were then centrifuged and supernatants collected as described in figure 2.19.

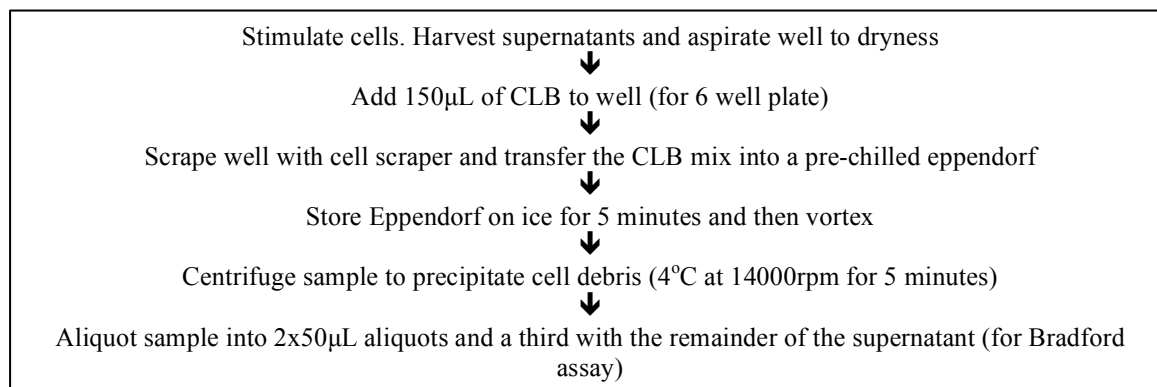


Figure 2.19 Protocol for intracellular whole cell protein harvest

2.2.7.5.3 Gel electrophoresis, blotting and immunodetection of proteins

The preparation of cell lysates is described in figure 2.19. The protocol for electrophoresis and immunodetection of proteins is shown in Tables 2.3 and 2.4 and figure 2.20. Samples were boiled with dithiothreitol (DTT) to reduce oxidised disulphide bonds and loaded onto sodium dodecyl sulphate (SDS) gels for electrophoresis. Molecular weight markers were also added to one well on each gel to monitor the progress of the electrophoresis. The membrane was incubated with LumiGLO (0.5mL 20x LumiGLO, 0.5mL 20x peroxide and 9mL Milli-Q water) with gentle agitation for 1-5 minutes at room temperature following incubation of secondary antibody. Duration of exposure to x-ray film varied between antibodies and the amount of protein in the sample loaded.

Table 2.3 Western blot buffer and solutions

Solution	Preparation of working solution	Use
x SDS sample buffer	62.5mM 5mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50mM DTT, 0.01% w/v bromophenol blue or phenol red	Sample loading
Running buffer	For 2L – Trizma base 21.11g, SDS 2g, Glycine 57.65g, H ₂ O 2L	
Transfer buffer	25mM Tris base, 0.2M Glycine, 20% methanol (pH 8.5). For 1.5L – Trizma base 4.5g, Glycine 21.525, dH ₂ O 1200mL, methanol 300mL	Transfer
Blocking buffer	1xTBS, 0.1% Tween-20, 5% w/v non-fat dry milk (100mL TBS and 5g milk powder)	Blocking
Wash buffer TBS	20mM Tris base, 137mM NaCl, 0.1% Tween	Washing solutions

Table 2.4 Gels for western blotting (for 2x 10% gels)

	Separation gel	Stacking gel
Protogel	8.3mL	1.3mL
Protogel buffer	6.5mL	2.5mL (stacking buffer)
dH ₂ O	10.2mL	6.1mL
Aluminummmmonium persulphate (10% 0.1g in 1mL H ₂ O)	300μL	150μL
TEMED (add last)	30μL	30μL

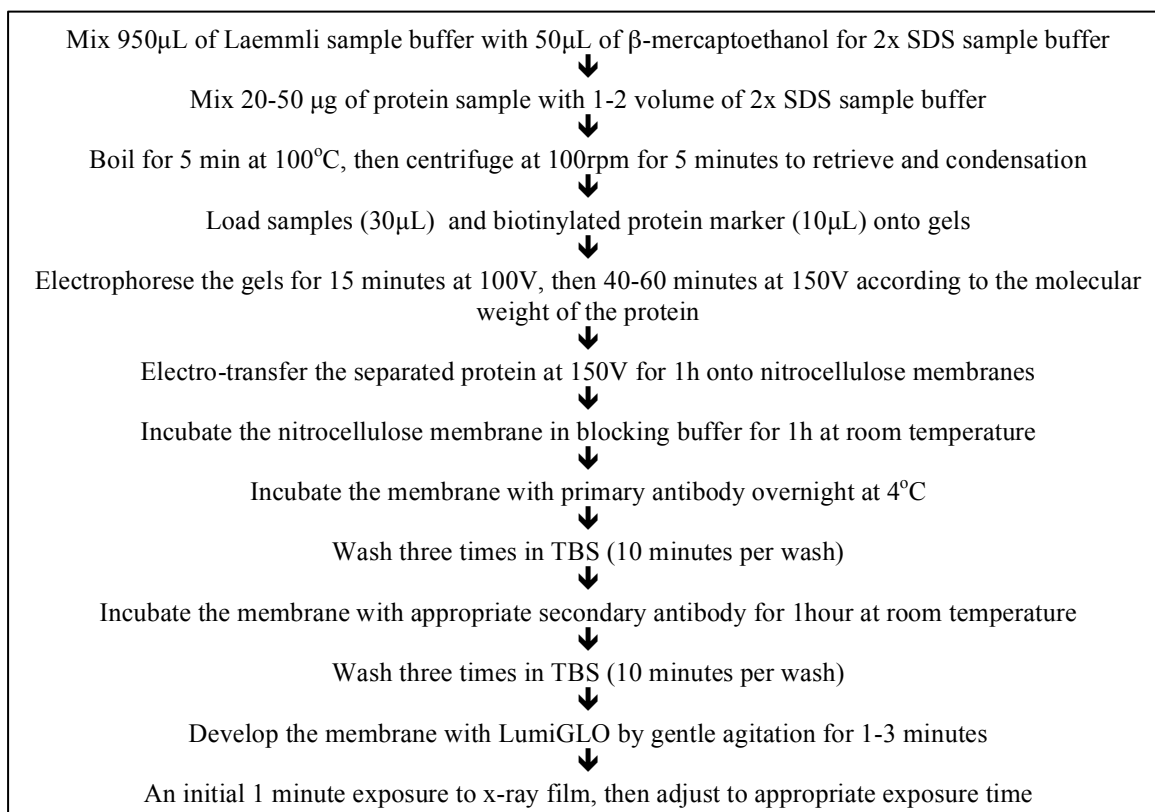


Figure 2.20 Protocol for western blotting

2.2.7.6 Flow Cytometry

2.2.7.6.1 Basic principles of Flow Cytometry

Flow cytometry is a technique that can be used for counting, characterising, and sorting cells suspended in a stream of fluid, either by their physical characteristics, or by the extent of presence of bound, labelled antibodies or dyes. Individual cells are scanned as they flow past an excitation light source and the scattered light is picked up by detectors. Cells are processed to allow binding of fluorescently-labelled monoclonal antibodies to the cells. Following removal of excess antibodies by washing, cell fluid dynamics are used to generate a stream of single cells from a cell suspension which pass through a series of lasers and paired detectors. Light scattered in the forward direction of a laser beam is focused by a confocal lens and detected by a light detector which converts it into an electrical signal that is digitalised to generate a parameter known as Forward

Scatter (FSC). The FSC signal will give information about the size and shape of the cell, and information can also be gathered by a side confocal lens and detected by a detector reading side scattered light. The Side Scatter (SSC) signal gives information about the granularity of the cell and surface topography. As FSC and SSC are unique for each type of particle, the combination of the two can help identify distinguish different types of cell types.

Several optical detectors called photomultiplier tubes (PMT) are used in a flow cytometer to read measure fluorescence. These read detect the light emitted from the particle crossing the laser beams that excite the attached fluorochromes at one range of wavelengths. Different fluorochromes will emit light at a second range of wavelengths and these are split into specific colours by optical filters and sent to PMT's (figure 2.21). PMT's convert light into an electrical pulse that is digitalised by other converters into signals readable by a computer as events, which are then used to generate histograms and dot plots.

Identification of specific populations of cells is possible through comparison of the fluorescence in several channels and the use of 'gating' strategies. Extent of fluorescence data is usually represented as a histogram with number of events on the y- axis and the fluorescence along the x-axis as a logarithmic scale. The distribution of events may be presented as the median fluorescence intensity (MFI) to represent the degree of fluorescence emitted by certain populations (assuming non-parametric distribution).

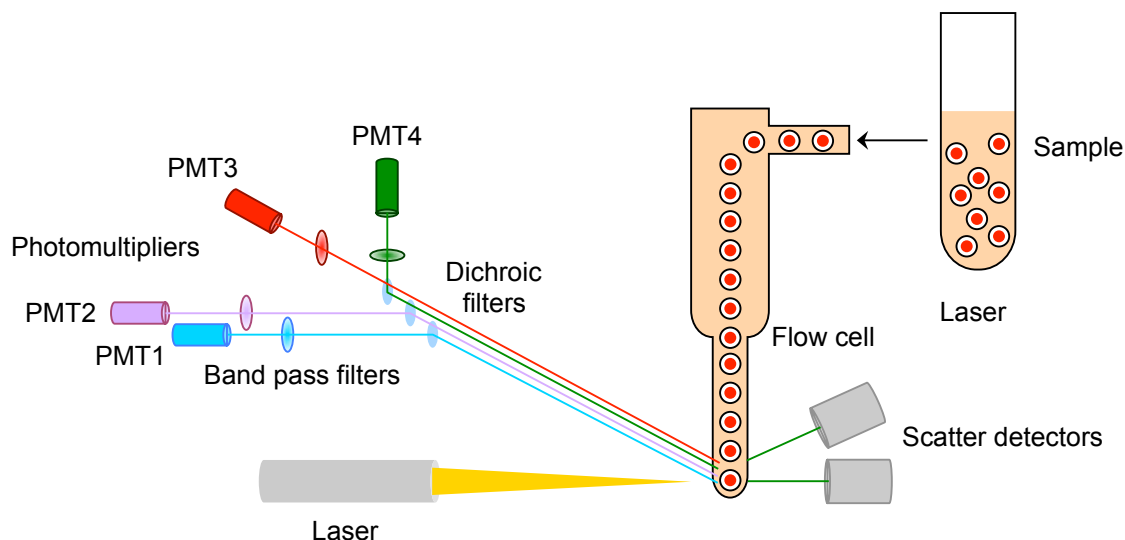


Figure 2.21 Schematic of FACS CantoII flow cytometer

Table 2.5 Antibodies used for flow cytometric analyses

Antibody	Flouorochrome conjugate	Host species	Clone	Supplier	Code
CD11b	Brilliant Violet	Mouse	ICRF44	Cambridge Bioscience	301324
CD54	Pacific Blue	Mouse	HA58	Biolegend	353109
CD62E	Phycoerytherin	Mouse	HCD62E	Biolegend	322605
CD62L	APC	Mouse	DREG-56	Cambridge Bioscience	304810
CD62P	FITC	Mouse	AK4	Biolegend	304903
CD102	APC	Mouse	CBR-IC2/2	Biolegend	328507
CD106	APC	Mouse	STA	Biolegend	305809
CD321	Phycoerytherin	Mouse	M.Ab.F11	BD Bioscience	552556
TNFR1	Phycoerytherin	Mouse	16803	R&D Systems	FAB225P
TNFR2	Fluorescein	Mouse	22235	R&D Systems	FAB226F

2.2.7.6.2 Flow cytometer set-up and sample acquisition

Compensation beads (BD Biosciences) were used to set up single colour compensation. Compensation was calculated automatically using FACSDiva™ software (v6.0, BD Biosciences, USA). Unstimulated, stained controls were used to detect the level of basal receptor expression and singlet discrimination was performed for each experiment. Titration experiments were performed to determine the optimum volume of antibody per test, to optimise signal-to-noise ratio and to minimise background.

Cells stained with isotype control antibodies were used as the negative control population; a gated marker was placed around the negative peak of the isotype control ($\geq 98\%$ events) stained cells (figure 2.22). Expression of the ligand of interest was determined by gating of fluorescence outside the negative control population and was expressed as either median fluorescence, or percentage of cells positively expressing the ligand compared to control cells. A minimum of 10,000 cells were analysed, in triplicate, per condition and the mean value of triplicate experiments used for analysis. A FACS Canto II Flow cytometer was used for all experiments. Data collected using FACSDiva™ software (BD Biosciences, USA) and analysed using FlowJo™ software (v10.0, TreeStar Inc, USA)

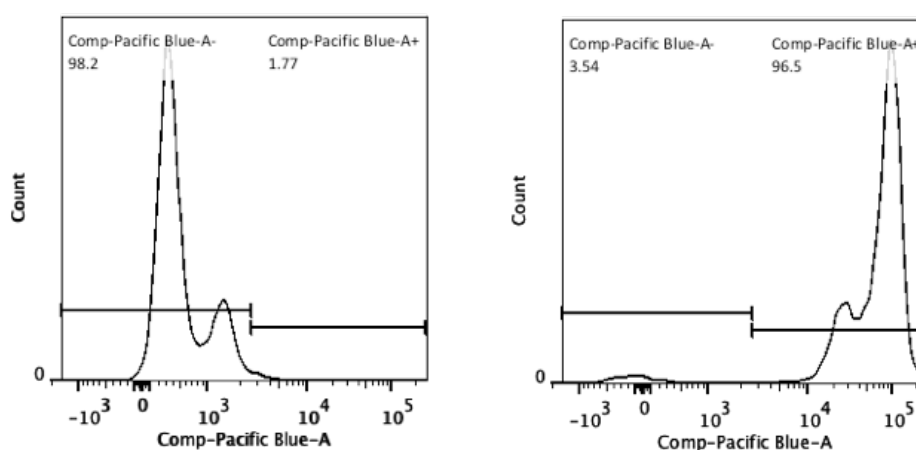


Figure 2.22 Gating of negative and positive populations by flow cytometry

The negative isotype control population was gated to include $\geq 98\%$ of events (left hand image). The positive population was defined the median fluorescence outside this gate or percentage of cells outside this gate (right hand image).

2.2.7.6.3 Cell surface staining of neutrophils

Healthy volunteer peripheral blood neutrophils ($5 \times 10^6/\text{mL}$) were harvested from healthy volunteers by dextran/percoll gradation from whole blood, as outlined in chapter 2.2.5.4 and re-suspended in PBS. Cell solution (200 μL) was left to stand at room

temperature for 5 minutes then treated and placed on a thermomixer (300rpm 21°C). After 45 minutes the cells were placed on ice then centrifuged at 12000rpm for 7 seconds. Supernatants were removed and stored at -80°C for ELISA analysis and the cell pellet was re-suspended in PBS containing both anti-human CD11b Pacific Blue conjugate, anti-human CD62L APC conjugate, anti-human TNFR1 Phycoerytherin conjugate, anti-human TNFR2 Fluorescein conjugate, or the relevant isotype control antibody. Samples were incubated at 4°C in the dark then washed twice in PBS before being re-suspended in 250µL of optimised CellFIX solution (1mL CellFIX, 9mL distilled water, 30mL PBS) in FACS tubes. Samples were kept at 4°C in the dark until analysis using a FACSCanto™ II flow cytometer (BD Biosciences). Neutrophils granulocytes were gated based on their characteristic side scatter and forward scatter criteria pattern (Dorward et al., 2013).

2.2.7.6.4 Cell surface staining of HMVEC-L

HMVEC-L cells were seeded into 6-well plates at a density of 1.2×10^5 cells/well and grown to confluence over 3 days in an incubator at 37°C in a humidified atmosphere of 5% CO₂ in air. Owing to limited cell numbers, treatments were performed in single wells only. After 4 hours treatment, cells were first washed 3 times using HBSS (400µL), then harvested using non-enzymatic cell dissociation buffer (400µL). The cell suspension was then placed in an eppendorf on ice and centrifuged at 400rcf for 5 minutes at 4°C. The supernatant was removed and the cell pellet re-suspended in 50µL of blocking buffer (5% FBS in 0.5% BSA) for 10 minutes at 4°C; 5µL was taken from each eppendorf and added to a labelled tube for unstained cells and a further 20µL was taken from each eppendorf and combined to generate the isotype control samples; 20µL of this mixed sample was then removed to generate compensation and isotype controls. The blocked cells were then incubated with the relevant antibodies (CD54, CD62E, CD62P, CD102, CD106 and CD321) and isotype controls for 30 minutes at 4°C in the dark. Cells were

washed twice with the addition of 800µL of PBS and centrifugated at 400g for 5 minutes at 4°C. The washed, stained cell pellet was re-suspended in 200µL of stain buffer (PBS +1% FBS + 0.09% sodium azide) and transferred to a FACS tube containing 200µL of stain buffer. Cells were stored for a maximum of 1 hour at 4°C in the dark prior to analysis on a FACSCanto™ II flow cytometer (BD Biosciences).

2.2.7.6.5 Cell surface staining of TNFR expression in hAT2

hAT2 cells were seeded into 24-well plates at a density of 1×10^6 cells/well and grown to confluence over 4 days in an incubator at 37°C in a humidified atmosphere of 5% CO₂. Cells were first washed 3 times using HBSS (100µL) then harvested using non-enzymatic cell dissociation buffer (100µL). The cell suspensions were then placed in an Eppendorf on ice and centrifuged at 400rcf for 5 minutes at 4°C. The supernatant was removed and the cell pellet re-suspended in 50µL of blocking buffer (5% FBS in 0.5% BSA) for 10 minutes at 4°C. 5µL was taken from each Eppendorf and added to a labelled tube for unstained cells and a further 20µL was taken from each Eppendorf and combined to generate the isotype control samples; 20µL of this mixed sample was then removed to generate compensation and isotype controls. The blocked cells were then incubated with the relevant antibodies (TNFR1 and TNFR2) and isotype controls for 30 minutes at 4°C in the dark. Cells were washed twice with the addition of 400µL PBS-/- and centrifugation at 400g for 5 minutes at 4°C. The washed, stained cell pellet was re-suspended in 200µL of stain buffer (PBS-/- +1% FBS + 0.09% sodium azide) and transferred to a FACS tube containing 200µL of stain buffer. Cells were stored for a maximum of 1 hour at 4°C in the dark prior to analysis on a FACSCanto™ II flow cytometer (BD Biosciences). Cells were analysed in triplicate.

2.2.8 Generation of surfactant protein C cDNA

2.2.8.1 Ribose nucleic acid extraction from hAT2

Total ribose nucleic acid (RNA) was extracted from cultured cells using RNeasy mini Kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Cells were scraped from the surface of the well in buffer supplied (RLT buffer) and dissolved in equal volumes of 70% ethanol. The mixture was then added to an RNeasy mini spin column and centrifuged to fix total RNA to the column. The RNA in the column was then washed with further buffers and eluted into an Eppendorf using RNase free water and used or stored at -20°C (figure 2.23).

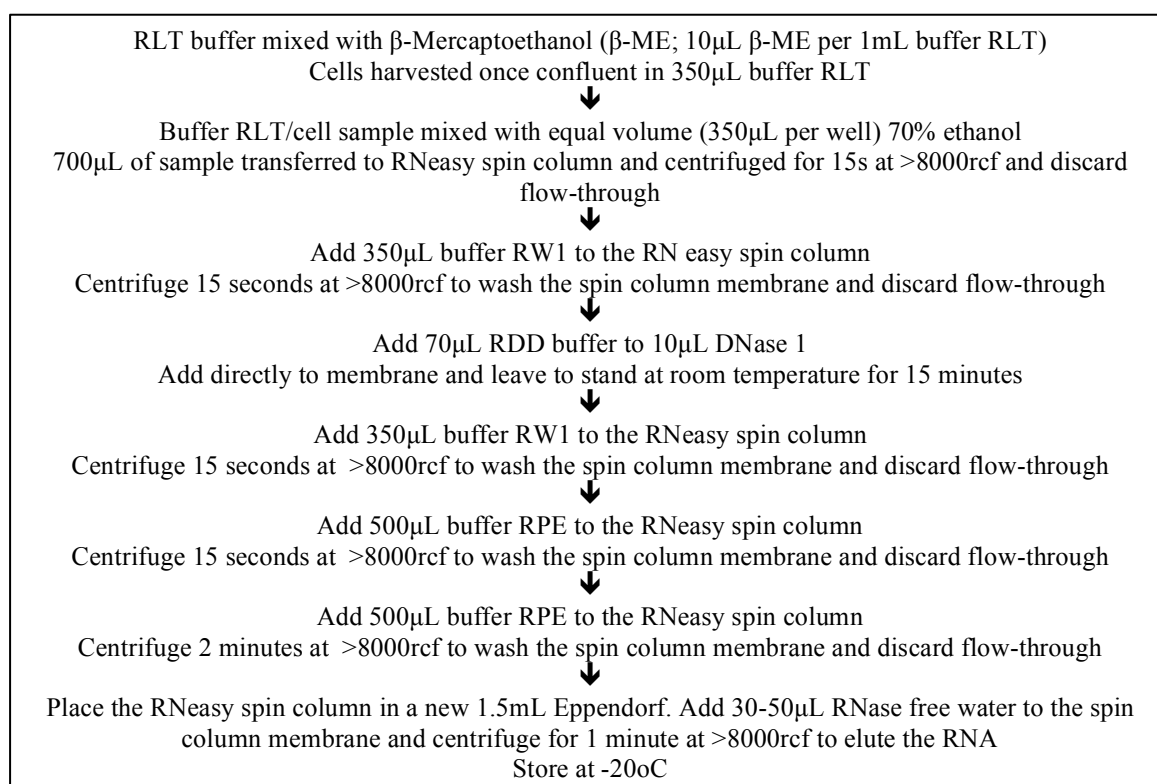


Figure 2.23 Protocol for RNA extraction using RNeasy spin columns

2.2.8.2 Determining the concentration of RNA

The RNA concentration was determined using the nano-drop ND1000 spectrophotometer (supplied by Lab Tech International, East Sussex). RNA can be

quantified using the absorption of light at 260 and 280nm. For high quality nucleic acid the ratio of Abs 260/280 should be close to 2. However ratios above 1.6 still result in useful real -time PCR results.

2.2.8.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

mRNA was reverse transcribed into complementary DNA (cDNA) using the QuantiTect Reverse Transcription kit (Qiagen). The kit components and samples were thawed on ice. Total mRNA (0.6µg) diluted in RNase free water (7µL) was used for reverse transcription and incubated with oligo(dT)12-18 primer (1µL) at 65°C for 10min. The reverse transcriptase mixture containing first strand buffer (5×, 2.8µL), DTT (0.1M, 2µL), deoxyribonucleotide triphosphate (dNTP, 10mM, 2µL), RNase free H₂O (1.7µL), RNasine (0.5U/µL, 0.5µL) and Moloney Murine Leukemia Virus Reverse Transcriptase (MLV-RT, 200U/µL, 1µL) was added (10µL for each sample) and incubated for 1 hour at 37°C, then at 65°C for 5min. The synthesized cDNA could be used immediately or stored at -20°C for several months until required.

2.2.8.4 Real Time Polymerase Chain Reaction

Surfactant protein C gene expression in hAT2 was assessed by real time polymerase chain reaction (RT-PCR) using the Rotor-Gene[®] SYBR[®] Green PCR Kit (Qiagen) and Rotor-Gene 6000 thermal cycler (Corbett, Australia) according to the manufacturer's instructions. The RT-PCR reaction was performed in a total volume of 20µL reaction solution containing QuantiTect SYBR Green PCR Master Mix (2×, 10µL), forward primer (10µM, 1µL), reverse primer (10µM, 1µL), template cDNA (1µM, 1µL) and RNase-free water (7µL). SYBR Green fluoresces when it binds to the minor groove of the double stranded DNA. SYBR Green emits a low signal when in solution, but a much higher signal when bound to DNA. Thus, as the reaction proceeds there is a corresponding increase in signal intensity (Figure 2.24). Target gene amplification was

performed under the following cycling conditions: initial denaturing at 95°C for 15 minutes and then 65 cycles of sample denaturation at 95°C for 10 seconds and annealing at 55°C for 20 seconds, extension at 72°C for 20 seconds and a final extension at 72°C for 1 minute. This was followed by a final hold at 25°C for 1 minute. The house-keeping gene GAPDH (Qiagen) was used as the reference molecule for PCR amplifications. The surfactant protein C primer (Qiagen) was selected with the aid of Primer3 software.

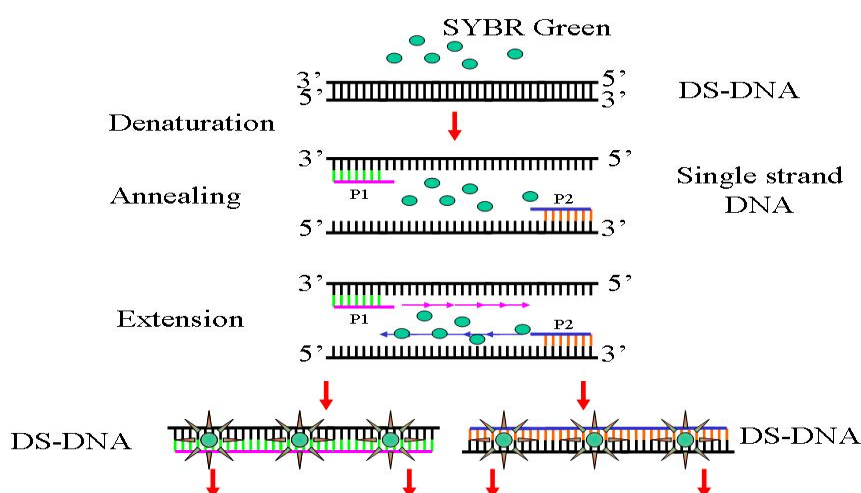


Figure 2.24 The basic principle of real-time PCR using SYBR green

2.2.9 Generation of TNFR specific muteins

The TNFR1 and TNFR2 specific muteins, R32W and D143N respectively, were gifts from GSK. Muteins were generated using site-directed mutagenesis using the pUC19 cloning vector as a template. The primer sequences for each mutein are outlined in table 2.6. The gene product was confirmed as low endotoxin (<0.16EU/mL) and the predicted molecular weight of each product was confirmed by mass spectrometry.

Table 2.6 Primer sequences for TNFR specific muteins

(forward strand below/reverse is complementary)

R32W	5'-CAG CTG CAG TGG CTG AAC CGT TGG GCT AAC GCC CTG CTG GCA AAC- 3'
D143N	5'- CGT CCT GAC TAT CTA AAT TTC CGT GAA TCT GGC CAG GTG-3'

To confirm receptor specificity, Surface Plasmon Resonance (SPR) measurements were carried out on a Biacore T100 instrument (GE Healthcare Life Sciences) under standard conditions (25°C and 1 atmosphere). Anti-human IgG specifically directed towards the Fc-gamma fragment was immobilized covalently on all channels of a CM4 chip by amine derivatisation. TNF (50µg/mL, R&D Systems) diluted in HBS-EP buffer (GE Healthcare Life Sciences) was passed over the sensor in three flow channels in a range of concentrations. A 0nM TNF curve was used for double referencing. The association (ka) and dissociation (kd) rate constants for binding of R32W (11mg/mL) and D143N (4.7mg/mL) were measured during each set of SPR runs.

As expected, TNF bound to both receptors. The TNFR1 mutein and TNFR2 mutein bound specifically to TNFR1 and TNFR2 respectively, with no evidence of non-specific binding. The biphasic curves for both TNF and the TNFR2 mutein suggested that both TNF and D143N have a low affinity for TNFR2, with D143N being a weaker binder than TNF (figure 2.25).

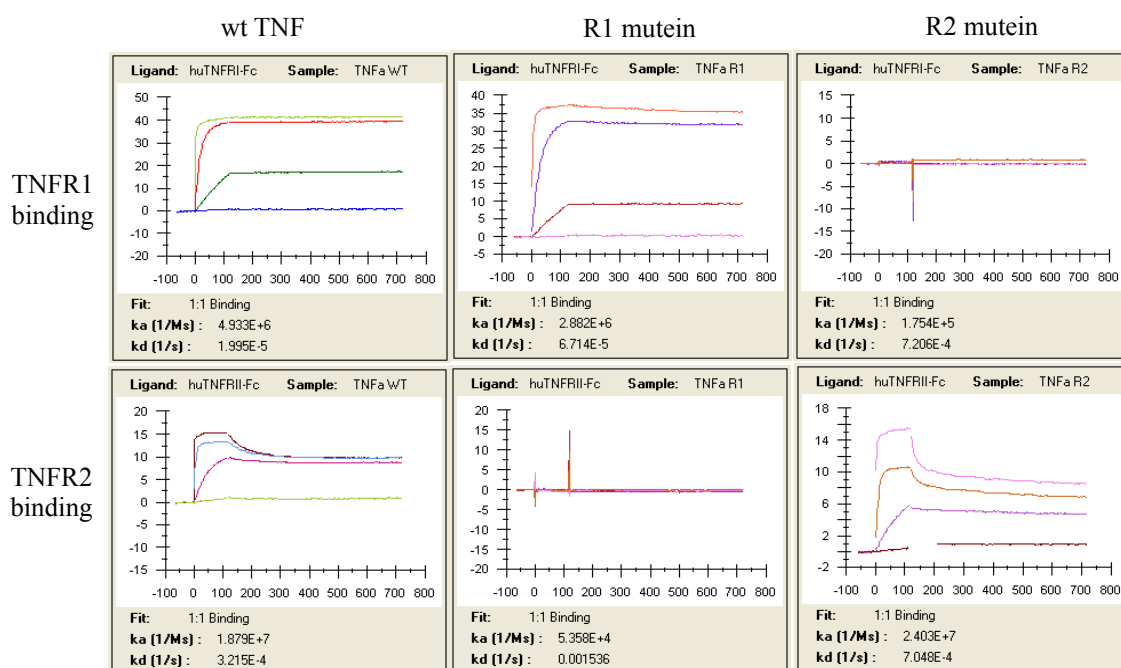


Figure 2.25 TNF muteins display receptor specificity

Anti-human IgG specifically directed towards the Fc-gamma fragment was immobilized covalently on all channels of a CM4 chip by amine derivatisation. TNF (50µg/mL) diluted in HBS-EP buffer was passed over the sensor in three flow channels in a range of concentrations. A 0nM TNF curve was used for double referencing. The association (k_a) and dissociation (k_d) rate constants for binding of R32W (11mg/mL) and D143N (4.7mg/mL) were measured during each set of SPR runs.

2.2.10 Preparation of precision cut human lung slices

Precision cut human lung slices (PCLS) were generated from disease-free lung parenchyma removed as part of a tumour resection (~6g). The tissue was washed twice with ice-cold HBSS containing penicillin (100µg/mL), streptomycin (100µg/mL). Tissue was immersed in ice-cold HBSS containing penicillin and streptomycin and inflated by injecting 3% (w/v) low melt agarose (Sigma-Aldrich, UK) using a total volume of ~3-5mL/g wet weight of tissue. The tissue was cooled and cut using a microtome blade, into slices approximately 0.5cm thick. Using an 8mm coring tool, cylindrical cores of the inflated tissue were prepared and cut into ~400µm thick slices by a Krumdieck Tissue Slicer (TSE Systems, Germany). Lung slices were transferred to a 12-well tissue culture plate containing 1mL of RPMI medium (Life Technologies, UK) supplemented with 1%

penicillin, streptomycin, L-glutamine and gentamicin and incubated at 37 °C in 5% CO₂. Slices were washed six times in RPMI media with a 30min incubation period between each wash to remove endogenous factors released from the slices. Slices were then incubated overnight and medium removed and replaced with 1mL of fresh medium approximately 30 min prior to treatment protocols.

2.2.11 *Ex vivo* lung perfusion

2.2.11.1 Harvesting of porcine lungs for model development experiments

Heart-lung blocks from domestic pigs were obtained from a local abattoir where pigs are treated according to European Union legislation governing such facilities. The trachea was cannulated with an endotracheal tube and the lungs were gently inflated several times with room air. The trachea was then clamped in inflation. The main pulmonary artery (PA) was cannulated and flushed with preservation solution (Perfadex; Vitrolife, Sweden: 2000mL at 4°C) supplemented with heparin (20,000iu; LEO Laboratories, Denmark: 20,000iu), THAM (3mL; Northwick Park Hospital, UK), Calcium Chloride (10%, 0.6mL, Martindale Pharmaceuticals, UK) and Glyceryltrinitrate (8mL, Hameln pharma, Germany; 8mL). The lungs were transported soaked in preservation solution (1000mL) surrounded by crushed ice.

2.2.11.2 Establishment of a contract for shipment of whole human lungs

While current UK legislation permits organ donation for primary research purposes, available numbers are small as the vast majority of potential donors consent for organ donation for the purposes of transplantation. Thus, study in of whole human lungs from the UK donor pool is logistically problematic. Moreover, with the

development of clinical EVLP, increasing numbers of borderline donor lungs should be used for clinical EVLP assessment to increase the donor pool in the UK.

The International Institution for the Advancement of Medicine (IIAM) in the USA is a non-profit organisation that works with almost all of the 58 organ procurement organizations (OPOs) in the United States to provide non-transplantable, human biomaterials for medical research. Consent for donation for research purposes is obtained in accordance with hospital, state and federal regulations in the USA. In conjunction with the Tissue Governance Manager at our institution, we established a contract with IIAM ensuring compliance with The Human Tissue Act (HTA 2004) code of practice relating to the import and export of human bodies, body parts and tissue. Information is anonymised but demographic data, organ procurement times, donor medical history and pre-explant biochemical and physiological parameters are provided with the tissue for baseline data collection. To improve the quality of donor lungs we stipulated a cold ischaemic time of less than 36 hours. Approval by the National Research Ethics Service was not deemed necessary as our proposed study does not involve NHS patients or use tissue from NHS patients (assuming an HTA license was in place).

2.2.11.3 Selection criteria for donor human lungs

All cases were discussed with a coordinator at IIAM. A copy of the lung harvesting protocol and shipping contract are contained in Appendix B. Lungs were cooled *in situ* with preservation solution (University of Wisconsin solution, 50mL/kg) and transferred in 1L of preservation solution on ice. Donor criteria are outlined in table 2.7.

Table 2.7 Donor criteria for *ex vivo* human lungs

Clinical variable	Criteria
Age	16 - 65 years
Smoking history	Smokers or non-smokers
Cold Ischaemic Time	<36 hours
Warm Ischaemic Time (if donation after cardiac death)	< 1 hour from cross-clamp time
Serology	HIV, HBsAb, EBV, CMV negative
Bacteriology	Sputum culture –ve, MRSA -ve
Time on ventilator	<10 days
Radiology	Absence of dense consolidation Diffuse shadowing consistent with pulmonary oedema or ALI
Oxygenation	PaO ₂ :FiO ₂ ratio >200mmHg
Anatomical	Adequate PA cuff or trachea Absence of lung contusions or haemorrhage or persistent pneumothorax

HBsAb: Hepatitis B surface antibody, EBV: Epstein Barr virus, CMV: cytomegalovirus, PA: pulmonary artery

2.2.11.4 EVLP circuit

Following receipt of the lungs at our institution, they were inspected for contusions and anatomical suitability for connection to the *ex vivo* circuit, namely; at least 2cm of main PA, a left atrial cuff (closed system) or intact pulmonary venous drainage and at least 5cm of trachea. As soon as the lungs were deemed anatomically acceptable, the main pulmonary artery (PA) was cannulated with a pre-fashioned cannula (Vivoline, Sweden) and the left atrium (LA) was cannulated with a conical cannula (Vitrolife, Sweden: closed system), or the pulmonary veins were left open to air (open system). The trachea was cannulated with an endotracheal tube and secured with 2.0 silk. The lungs were then transferred to the perfusion container (Vivoline, Sweden) and connected to the perfusion circuit and ventilator. The perfusion circuit (Maquet, Germany), outlined in appendix 7.2, was connected to an ECMO[®]therm II heat exchanger (Medtronic, USA). A

paediatric nasogastric tube was inserted via a side-port into the pulmonary artery (PA) cannula and connected to a pressure transducer to allow continuous PA pressure monitoring.

The circuit was de-aired with perfusion solution (high glucose DMEM containing 5% human albumin solution, 1350mL). Perfusion was started at the highest flow rate that maintained PA pressure at less than 15mmHg, to avoid hydrostatic damage. Following re-warming to 37°C, ventilation was commenced (Evita XL, Drager, Germany) with settings as follows: tidal volume 300mL, respiratory rate 10/min, positive end-expiratory pressure 8cm water and fraction of inspired oxygen 0.21. Perfusate flow rate was increased incrementally to a target of 0.8L/minute without exceeding a target PA pressure of 15mmHg. In experiments using a closed system, the left atrial pressure was maintained at less than 5mmHg by adjusting the height of the reservoir. In experiments, using an open system, the pulmonary veins were left open to air and assumed to have a pressure of 0mmHg.

2.2.11.5 ALI modelling in *ex vivo* human lung

Following rewarming, 150mL of group matched healthy volunteer whole blood was added to the perfusate. Baseline tissue and BALF sampling was undertaken and the lungs were maintained with a protective ventilator strategy perfusate flow as outlined in figure 2.25. When logistically possible (a clinical scanning facility was used), baseline computed tomography was undertaken to exclude significant radiological infiltrates or consolidation. Where infiltrates or consolidation were present, the respective lobe(s) were excluded from experimental intervention.

Immediately after baseline sampling, *E.coli* endotoxin (LPS, 0.6mg/mL, 10mL) and vehicle control (10mL, 5% human albumin solution) were instilled via a bronchoscope or blindly placed catheter (7 French size, coronary guide catheter,

Medtronic, USA). The periphery of the lung was viewed to identify ‘blebbing’ from the injectate, firstly to confirm that the LPS or vehicle control reached distal lung, and secondly to allow demarcation of the injured area for subsequent tissue sampling (figure 2.29D). The right lower lobe was used preferentially for LPS injury and the left lower lobe for vehicle control. Following injury, BALF and tissue were harvested at 4 and 8 hours post-LPS injury (figure 2.26). In experiments investigating soluble TNF receptor release in the alveolar space, an additional BALF was performed 2 hours after LPS injury.

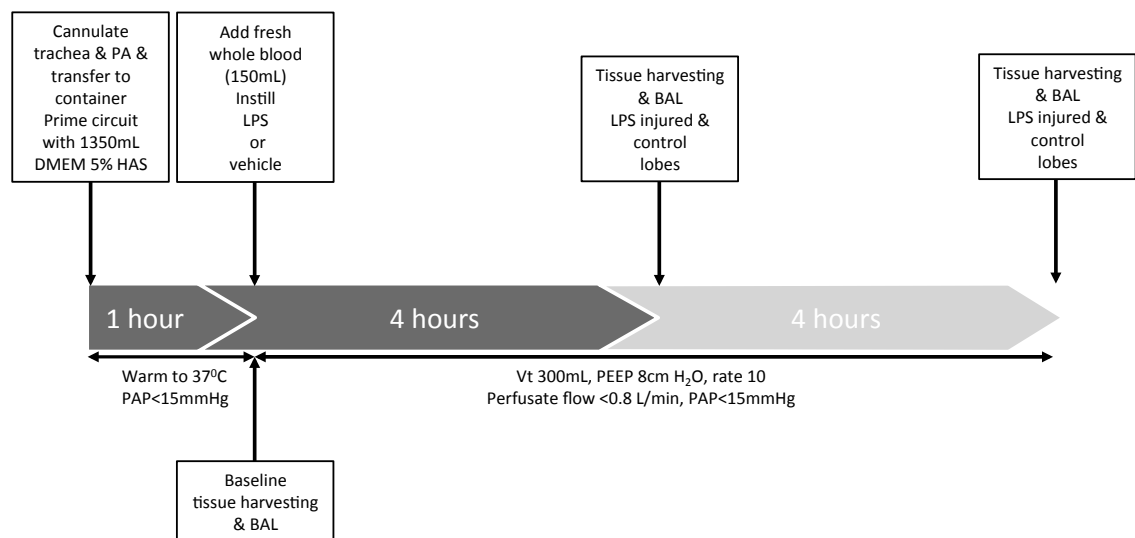


Figure 2.26 Protocol for LPS induced human *ex vivo* ALI model

BAL: broncho-alveolar lavage, DMEM: Dulbecco’s modified eagle medium, HAS: human albumin solution, LPS: lipopolysaccharide, PA: pulmonary artery, PAP: pulmonary artery pressure, PEEP: positive end expiratory pressure, Vt: tidal volume

2.2.11.6 Development of a robust and reproducible model of human *ex vivo* ALI

Having established a technique that maintained human lungs for up to 8 hours *ex vivo*, it was necessary to produce a robust method to deliver LPS / vehicle control and subsequently sample the respective lobes of *ex vivo* human lung.

A fibre-optic bronchoscope with a catheter in the working channel was used to firstly allow passage to distal lung and secondly to avoid contamination of injured and control BALF samples. Preliminary data did not demonstrate robust and reproducible

injury as measured by BALF cytokine IL-8 (figure 2.27A) release and neutrophil infiltration. We therefore moved to a model where the LPS (2.4mg/mL, 2.5mL), was injected locally to discrete segments of the right middle lobe and vehicle control in similar fashion to the left lower lobe (figure 2.28A). Tissue samples from the relevant infiltrated area were then collected and analysed. Whilst this provided a mechanism to sample tissue that we knew had been infiltrated with LPS or vehicle control, the corresponding BALF IL-8 data again were not suggestive of localised injury (figure 2.27B). This was probably due to an inability to direct the bronchoscope to the extreme periphery of the lung where LPS was at its highest concentration. Indeed, LPS concentrations in the BALF were markedly lower in these samples compared to samples derived from bronchoscopy–delivered LPS (figure 2.28).

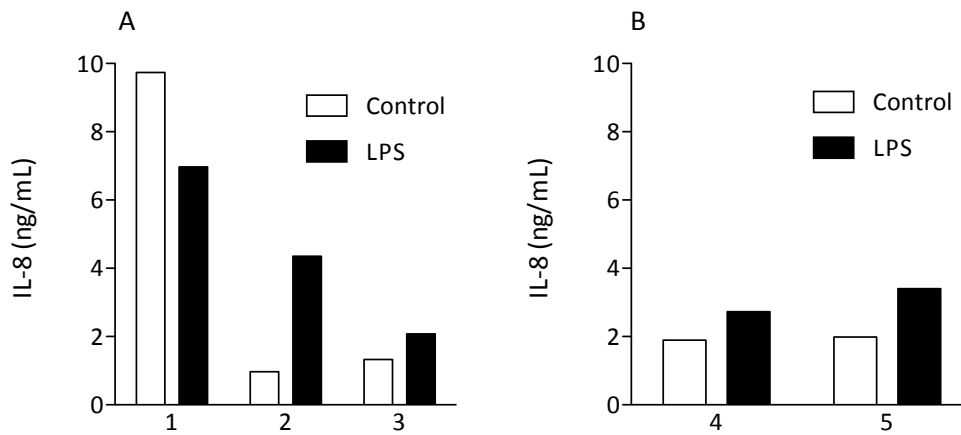


Figure 2.27 BALF IL-8 concentrations in endobronchially delivered vs locally delivered LPS in human *ex vivo* lung

LPS (0.1mg/kg, 10mL) or vehicle control (10mL) were instilled blindly via a bronchoscope into the right middle lobe or left lower lobe respectively of human lungs on the EVLP circuit (panel A). In separate experiments LPS (0.4mg/kg, 2.5mL) or vehicle control (2.5mL) were injected via an orange needle into discrete areas of peripheral lung tissue. After 4 hours, the injury and control lobes were lavaged with 5% human albumin solution (50mL). Interleukin-8 was measured by sandwich enzyme linked immunosorbent assay (ELISA). Each number represents a separate experiment.

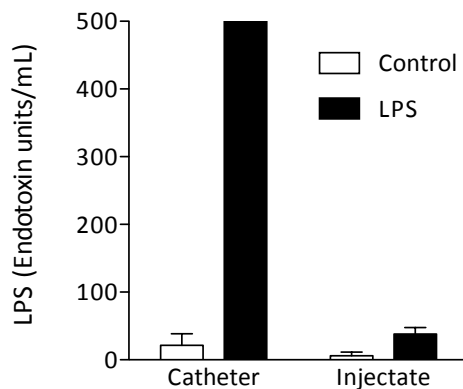


Figure 2.28 Comparison of LPS concentrations in human lung tissue using 2 different methods of LPS administration

LPS (0.1mg/kg, 10mL) or vehicle control (10mL) were instilled via a catheter into the right middle lobe or left lower lobe respectively of human lungs on the EVLP circuit (Catheter, n=3). In separate experiments LPS (0.4mg/kg, 2.5mL) or vehicle control (2.5mL) were injected via an orange needle into discrete areas of peripheral lung tissue (injectate, n=2). After 4 hours, the injury and control lobes were lavaged with 5% human albumin solution (50mL). LPS concentration in the corresponding BALF was measured by Limulus Amebocyte Lysate (LAL) assay using the PTS Endosafe®PTS™ system (0.05 EU/mL sensitivity),

Finally, we developed a model whereby angiography catheters (Medtronic, USA) were fed either directly into the trachea, or via a port in the endotracheal tube mount. The catheters were gently passed into distal lung until resistance was felt, ensuring that the pleural surface was not compromised. Three catheters were placed in discrete areas of the right lower lobe and 3 catheters were placed in the left lower lobe (figures 2.29 panels B & C). LPS (0.6mg/mL, 10mL) or vehicle control (10mL) was instilled via each catheter into distal lung and then flushed with air (10mL). The area of infiltrated lung, evidenced by 'blebbing' of the peripheral tissue was delineated with a marker pen to guide subsequent tissue harvesting (figure 2.29D). After 4 and 8 hours perfusion, BALF (0.9% saline with 5% HAS, 50mL) was undertaken in duplicate catheters for both LPS and vehicle control, and tissue was harvested in the demarcated area. The 3rd catheter was used to guide tissue harvesting for wet:dry ratio. This method provided robust, reproducible data, at least for BALF cytokine release and neutrophil infiltration into the alveolar space.

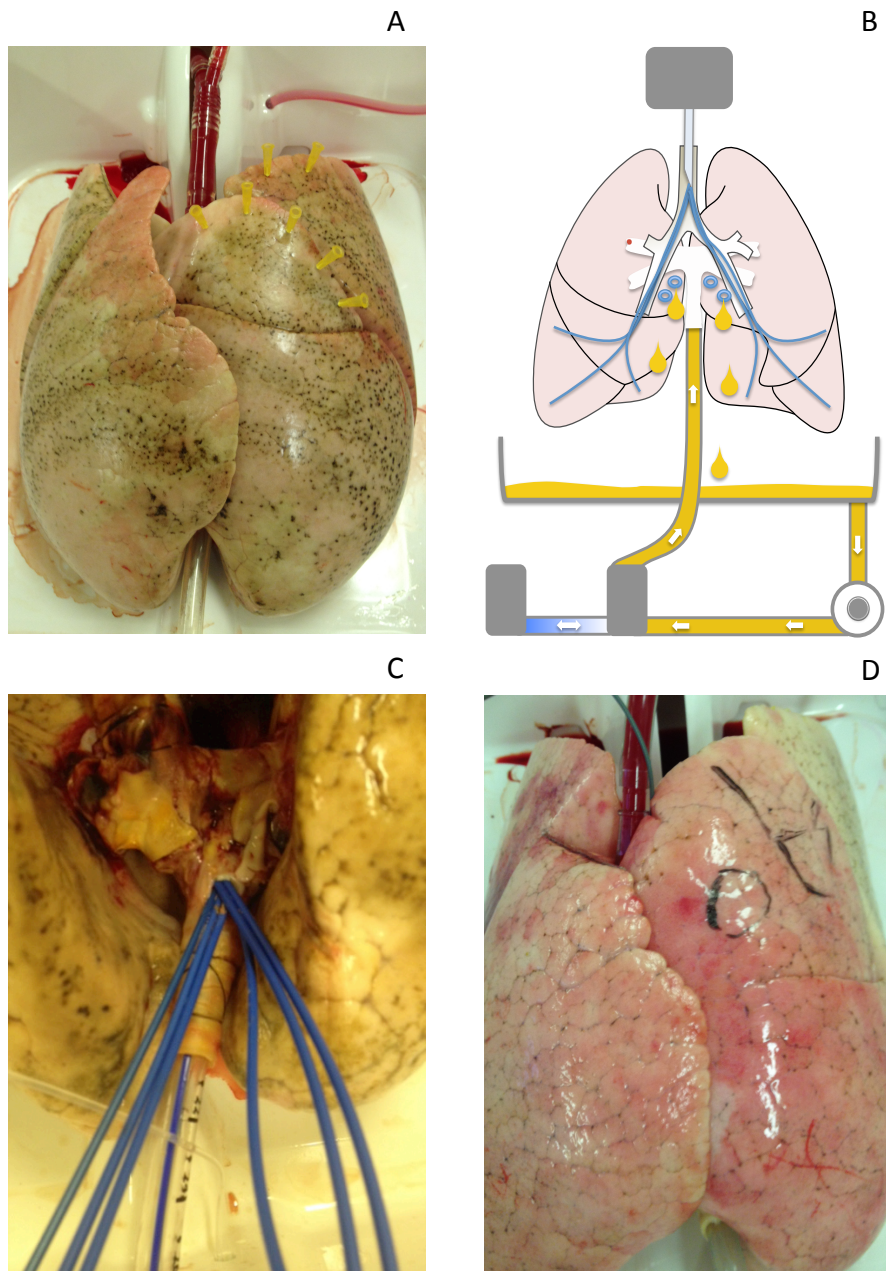


Figure 2.29 Administration of localised injury to the distal lung *ex vivo* perfused lung

LPS (0.6mg/mL, 2mL) or vehicle control was instilled via an orange (22G) needle into discrete areas of peripheral lung (panel A). Localised tissue was then resected after 4 hours perfusion and analysed. Alternatively, endotoxin (0.6mg/mL, 10mL) or vehicle control (10mL) was administered via cardiac catheters inserted endobronchially to distal lung (panels B, C). During infusion of endotoxin, the area perfused was marked externally with a marker pen to guide biopsy samples at 4 hours following injury (panel D).

2.2.11.7 Methods of drug delivery to *ex vivo* lung

Having established a human *ex vivo* model of LPS induced ALI with robust and reproducible data output, we next investigated means of administering therapeutic intervention in *ex vivo* models to harness their translational potential. These experiments were undertaken to establish the optimum method of administration of GSK1995057 in our human ALI model. Given the paucity of available human lung tissue, this component of model development was undertaken in porcine lung.

Nebulisation of Evans blue dye via the endotracheal tube demonstrated homogeneous distribution of dye throughout the lower lobe of porcine *ex vivo* lung (figure 2.30). Significant deposition also occurred in the upper airways, however.

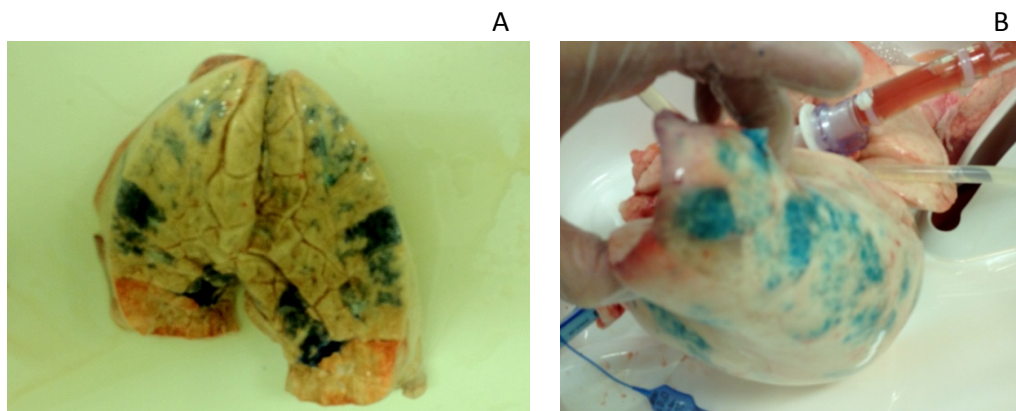


Figure 2.30 Distribution of nebulised Evans blue dye in porcine *ex vivo* lung

Evans blue (1mg/mL, 5mL) was nebulised endotracheally into porcine tissue lungs ventilated and perfused *ex vivo*. After 2 hours, the lung demonstrated homogenous distribution of nebulised Evans blue in coronal section (panel A) and peripheral distribution of the dye (panel B)

2.2.12 Statistical analysis

2.2.12.1 Data processing

Data were entered in Excel spreadsheets prior to statistical analysis using PRISM v5.0 (GraphPad software, CA, USA).

2.2.12.2 Data presentation and analysis

All data (unless where stated) were analysed using non-parametric methods (Kruskal Wallis, Mann Whitney), as the sample sizes were not sufficient to ensure that the data were normally distributed. All p values were adjusted for multiple comparisons and a value of <0.05 was considered significant. Post hoc testing of groups to determine the underlying source of statistical significance was only undertaken if there was an overall statistically significant difference.

Table 2.8 Statistical tests used according to dataset distribution and number of groups

Distribution	Groups	Paired	Test
Non-parametric	2	N	Mann Whitney U
Parametric	>2	N	Dunnet's
Non-parametric	>2	N	Kruskal-Wallis test with Dunn's multiple comparisons test correction

Data in this thesis are almost exclusively presented as bar charts with errors bars delineating standard error of the mean. This allows any treatment effect to be readily observed, and is in keeping with the method employed by the majority of scientific journals. Boxplots may be more informative about the spread of the data for large datasets and facilitate the identification of outliers. However, the majority of *in vitro* experiments undertaken in this thesis have sample sizes of 5 or less, with a small intrinsic spread despite the use of primary cells. Therefore boxplots were only employed for *ex vivo* experimental data where the spread of values was more apparent.

3 Chapter 3: Effect of selective TNFR1 inhibition on *in -vitro* models of ALI

3.1 Introduction

The cells lining both the pulmonary capillaries and alveolar compartments are the primary barrier to inflammatory cell migration into the alveolar space and as such, are ideally situated to regulate the activation and recruitment of inflammatory cells into the alveolar space. Although alveolar macrophages are a major source of chemokines in the air spaces (Goodman et al., 1998), the pulmonary epithelium and endothelium release a complex network of cytokines and chemokines, which, in unison with alterations in the expression of cell adhesion molecules (chapter 1.1.2), direct leukocyte trafficking and orchestrate the inflammatory response in ALI. For example, hAT2 release a range of neutrophil chemokines after stimulation with LPS, including IL-8, monocyte chemotactic protein-1 (MCP-1) and growth related oncogene-alpha (GRO- α) (Witherden et al., 2004). Although neutrophils provide a first line of defence against invading pathogens, excessive recruitment and activation of neutrophils (and other inflammatory cells including monocytes) can initiate the loss of alveolar capillary membrane function and permeability pathognomonic of ALI (chapter 1.4.3). Therefore, the release of neutrophil chemotactic factors is thought to contribute significantly to the progression of ALI.

Chemokine release from lung epithelium and endothelium occurs predominantly in response to early phase cytokines, including TNF, released from alveolar macrophages and circulating monocytes triggered by pathogen recognition. Levels of TNF and its soluble receptors are consistently elevated in bronchoalveolar lavage (BAL) and blood of patients with ALI (Hyers et al., 1991), and levels of downstream TNFR1 signalling products (e.g. interleukin- 6, soluble TNFR1, soluble TNFR2 and IL-8) in blood have

been associated with a poor outcome (Parsons et al., 2005) and sequential organ failure in ALI patients (Liu et al., 2007).

Alveolar capillary membrane inflammation is further propagated by ventilator associated lung injury (VALI) whereby mechanical forces induce signal transduction in epithelial (and endothelial) cells, further promoting local chemokine release, amplifying local cellular injury and neutrophil influx into the alveolar space. TNF has similarly been implicated in the pathogenesis of both experimental and clinical VALI. Indeed, in a clinical study, BALF and plasma levels of TNF and its soluble receptors fell after 36 to 40 hours in patients with ALI subjected protective ventilation compared to those ventilated using a conventional strategy (Ranieri et al., 1999). Early experimental studies yielded conflicting results (Imai et al., 1999; Murphy et al., 2000; Ricard et al., 2001; Tremblay et al., 2002), however recent work has demonstrated that the complexities of differential TNF signalling may underlie some of the confusion. Hence TNFR2^{-/-} mice exposed to high tidal volume ventilation developed pulmonary oedema whereas TNFR1^{-/-} mice were protected (Wilson et al., 2007).

Disruption of the alveolar capillary membrane and the associated influx of protein-rich and highly cellular oedema fluid into the interstitium and alveoli is pathognomic of ALI. Recent results have indicated a dichotomous role for TNF in this process, perhaps suggestive of differential TNFR signalling effects. TNF has been proposed to mediate the induction of apoptosis of lung microvascular endothelial cells, thus contributing to the disruption of the endothelial barrier (Hamacher et al., 2002; Lucas et al., 1998) while also indirectly promoting oedema formation by decreasing the expression of ENaC and the Na⁺/K⁺ ATPase (Dada and Sznajder, 2007). TNF directly induced barrier dysfunction via disruption of adherens junctions in endothelial cells (Angelini et al., 2006; Petrache et al., 2001). In rat alveolar epithelial cells, TNF inhibited sodium uptake, which is crucial for

alveolar fluid clearance, by reducing the expression of at least 3 subunits (alpha, beta, gamma) of the epithelial sodium channel ENaC (Dagenais et al., 2004). By contrast, TNF opposed oedema formation by enhancing alveolar fluid reabsorption in a rat pneumonia model (Rezaiguia et al., 1997) and in a rat intestinal ischemia reperfusion model (Borjesson et al., 2000).

Experimental studies have suggested a beneficial role of TNFR2 signalling in tissue repair, regeneration, and the resolution phase of tissue injury following trauma, ischaemia/reperfusion (IR) injury, or acute inflammatory insults. For example, in mice TNFR2 was protective in the pathogenesis of heart failure (Hamid et al., 2009; Higuchi et al., 2004), renal allograft failure due IR injury (Al-Lamki et al., 2005), neuronal injury (Fontaine et al., 2002), and ischaemic neovascularisation (Goukassian et al., 2007; Zhang et al., 2013). Moreover, TNF or TNFR2 agonism was associated with pancreatic regeneration (Kodama et al., 2003; Ban et al., 2008), remyelination (McCoy and Tansey, 2008; Arnett et al., 2001) and stem cell proliferation (Al-Lamki et al., 2013; Bocker et al., 2008).

Resolution of ALI requires reabsorption of alveolar oedema, repair of the epithelial and endothelial barriers, and removal of inflammatory cells from the distal airspaces. Hence, early epithelial repair with re-epithelialisation of the denuded basement membrane and regeneration of the lung endothelial barrier is important for the restoration of the normal architecture and alveolar fluid clearance of the lung. Indeed, repair of the lung epithelium correlated with prognosis in ALI (Ware and Matthay, 2001). hAT2 are thought to act as progenitor cells for both daughter type II cells and type I cells (Gonzalez et al., 2005; Sannes, 1991), differentiating into type I cells in response to specific developmental cues; these de-differentiated hAT2 cells *in vivo* may have a similar phenotype to hAT2 *in vitro* as both are attempting to re-epithelialise a denuded membrane.

Owing to the ethical and logistical difficulties of procuring lung tissue for hAT2 cell harvest, most research in the role of epithelial cell activation and injury in ALI has used the human A549 adenocarcinoma cell line. However, the A549 cell line has little in common with hAT2 cells as they do not form tight junctions, contain no surfactant apoproteins (including surfactant protein C) and possess no contact inhibition of growth. In addition chemokine release, including IL-8, was higher in hAT2 cells (Masubuchi et al., 1998; Witherden et al., 2004). Human alveolar type I cells are extremely fragile and to date their culture *in vitro* has proved challenging; some researchers rely upon hAT2 cell differentiation into type I cells *in vitro* over several days, however, these terminally differentiated cells cannot be passaged. Highly sophisticated three-dimensional models may arguably be more representative of *in vivo* pathobiology, but these require the use of immortalised cell lines (Carterson et al., 2005; Lehmann et al., 2011). We have developed the facility and experience in our laboratory to isolate hAT2 and consider these cells most representative of the *in vivo* alveolar epithelium.

The experiments in this chapter were designed to establish clinically relevant human *in vitro* models of alveolar-capillary cell activation and injury to allow characterization of the role of differential TNFR signalling in these processes. In view of data suggesting beneficial effects of differential TNFR signalling in the resolution phase of tissue injury, we also investigated the role of TNF and TNFR signalling in lung microvascular endothelial regeneration and alveolar epithelial repair.

3.2 Specific methods

hAT2 were seeded on BioFlex™ 6 well plates (Dunn Laboratories, Germany) at a density of 2.5×10^6 cells/well and exposed to 2 hours of 30% CMS (0.33Hz). This regimen was chosen because it demonstrated robust production of IL-8 from hAT2 cells in

previous experiments by our group (Pinhu et al., 2008). Following overnight serum starvation, cells were exposed to CMS for 2 hours at 0.33Hz and then incubated for 24 hours prior to the removal of supernatants for the assessment of cytokine levels by Meso Scale Discovery (MSD[®]) analysis. These experiments were repeated following 1-hour pre-treatment of the cells with GSK1995057 (10nM), Adalimumab (1nM) or dummy domain antibody (10nM) followed by 2 hours of 30% CMS.

3.2.1 Treatment of hAT2 with BALF from ALI patients

Following consent, BALF was harvested from patients with moderate to severe ARDS (table 1.1). Lavage fluid was filtered through gauze to remove debris then irradiated with ultraviolet light (458nm) for 1 hour to kill contaminating bacteria. Following overnight serum starvation, cells were treated with BALF diluted 5-fold with serum free DCCM-1 media. Supernatants were harvested over 24 hours and IL-8 concentrations determined by ELISA.

3.2.2 Wound healing assay

hAT2 were grown to confluence in 24 well plates. Following overnight serum starvation cells were exposed to GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) or vehicle control for 1-hour, scratched with a p200 pipette tip, washed prior to the addition of new 2% medium containing TNF (10ng/mL). In experiments using selective TNFR muteins, cells were serum starved, scratched then washed, followed by the addition of serum free DCCM-1 media, R32W (10,100ng/mL), D143N (10,100ng/mL) or 10% serum. A time course of 24 hours was chosen as preliminary experiments suggested sub-maximal (75-80%) wound closure in positive control (10% serum) treated cells. Wound closure was measured by subtracting the 24-hour wound area from the initial wound area (measured in pixels) and calculating this as a percentage of initial wound area using Image J software.

In experiments using supernatants from treated alveolar macrophages, isolated from disease free lung resection specimens (chapter 2.2.5.2) macrophages were exposed to GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) or vehicle control for 1-hour, then TNF (10ng/mL) added overnight (16 hours). Harvested supernatants were then added to scratched cells and imaged as above.

3.2.3 Tube formation assay

HUVEC and HMVEC-L were treated in suspension with vehicle control (media alone), TNF (10ng/mL), R32W (10-100ng/mL), D143N (10-100ng/mL) and VEGF (100ng/mL). Cells were added to an ibiTreat angiogenesis μ -slide (Ibidi, Germany) pre-coated with reduced growth factor Matrigel™ matrix (chapter 2.2.6.8). Tube formation was analysed after 4 hours and recorded as total tube length analysed by image analysis software (Image J, USA).

3.2.4 Assessment of permeability in HMVEC-L monolayers

HMVEC-L (1×10^6 /mL, 400 μ L) were grown in complete EGM-MV2 media (chapter 2.2.5.3) on proprietary 8 well Electrical Cell-substrate Impedance Sending (ECIS®) cultureware slides (Applied Biophysics, USA) containing gold film electrodes to allow time-course measurement of electrical resistance, capacitance and impedance (chapter 2.2.6.2). Growth to confluence was confirmed by light microscopy and steady state resistance of $>3000\Omega$. Cells were pre-treated with GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) for 1 hour then TNF (10ng/mL) or vehicle control was added. Transendothelial resistance was measured at 4000 Hz over 20 hours using the ECIS® ZTheta array station (Applied Biophysics, USA) and time-course resistance data normalised to the initial voltage.

3.2.5 Analysis of cytokine release from stimulated hAT2 and HMVEC-L

hAT2 and HMVEC-L were grown to confluence (chapter 2.2.5) then pre-exposed to GSK1995057 (10nM), dummy domain antibody (10nM), Adalimumab (1nM) or vehicle control (media alone) for 1 hour prior to the addition of TNF (10ng/mL) or vehicle control. Cells were incubated at 37°C, 5% CO₂ for 24 hours then supernatants were collected and immediately frozen at -80°C. MTT assay (chapter 2.2.3) was performed on cells immediately after supernatant harvesting. Analysis of cytokine release was undertaken by MSD analysis according to the manufacturer's instructions (chapter 2.2.7.3.2). The cytokine panel was partially bespoke; specific analytes of interest were added to a proprietary standard human 9 plex kit that contained the majority of clinically relevant cytokines and chemokines.

3.3 Results

3.3.1 *In vitro* models of acute lung injury induced a dose dependent increase in IL-8 release from human alveolar type 2 cells

Initial experiments were designed to develop *in vitro* models of ALI, which could be used to analyse the effects of differential TNFR signalling. The *in vitro* injury models consisted of TNF a clinically relevant pro-inflammatory cytokine, hydrogen peroxide (H₂O₂) as a model of oxidative stress, cyclic mechanical strain (CMS) as a model of VALI, and finally BALF from ARDS patients as a model of the inflammatory milieu in ALI. TNF and CMS elicited a dose dependent increase in IL-8 release from stimulated hAT2 monolayers (figure 3.1 A & D). Bronchoalveolar lavage fluid (BALF) induced a time-dependent increase in IL-8 release compared to treatment with vehicle control (figure 3.1 C). Reassuringly, the IL-8 release could not be attributed to cell death as the MTT assay for each condition did not demonstrate a significant loss of cell viability, despite increasing doses of each respective injury (figure 3.2).

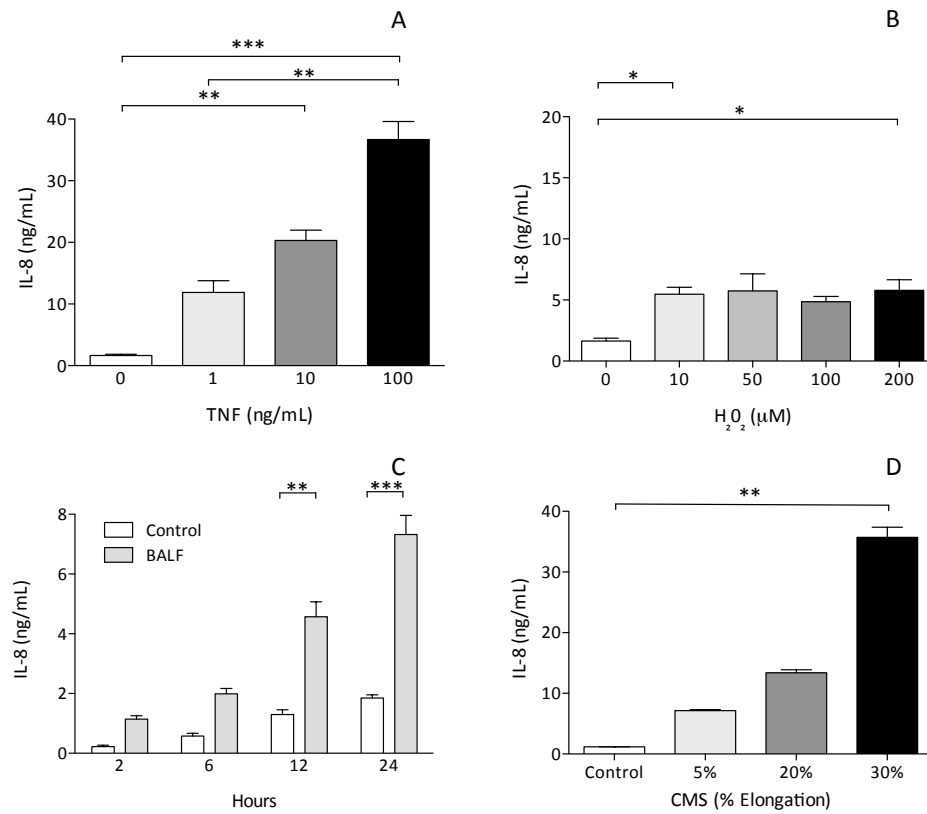


Figure 3.1 *In vitro* ALI models demonstrated a dose dependent increase in IL-8 production

hAT2 cells were grown to confluence and treated with tumour necrosis factor (TNF 0, 1, 10, 100 pg/ml) $n=8-10$ panel A or Hydrogen Peroxide (H₂O₂ 10, 50, 100, 200nM) $n=5-8$ panel B for twenty-four hours in the absence of serum. Alternatively, they were exposed to bronchoalveolar lavage fluid (BALF) from ALI ARDS patients for 2, 12, 24, 48 hours (panel C, $n=6$) or 0,5,20, 30% CMS for two hours (panel D, $n=4$). In all experiments, the medium was collected and concentrations of Interleukin-8 (IL-8) were measured by sandwich ELISA in supernatant. Data are presented as mean $\pm$ SEM and analysed by Kruskal-Wallis (Dunns) A, B & D or two way ANOVA, C * $p<0.05$ ** $p<0.01$ *** $p<0.001$

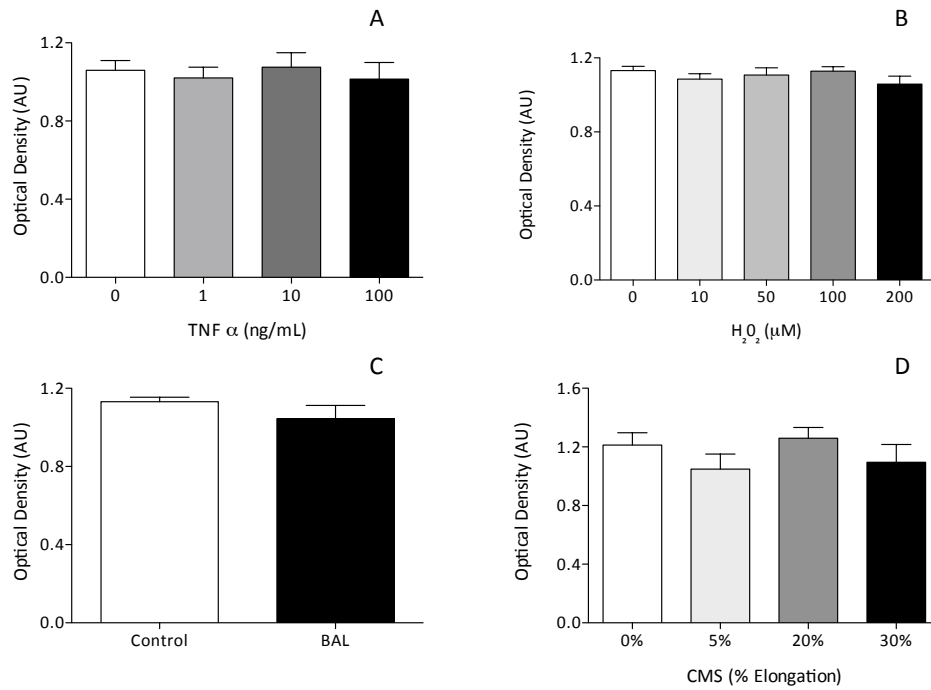


Figure 3.2 *In vitro* injury ALI models had no effect on hAT2 viability

Confluent hAT2 were subjected to TNF (Image A, 0, 1, 10, 100ng/mL), H₂O₂ (Image B, 0, 10, 50, 100, 200uM), BALF diluted 5 fold (Image C) and cyclic mechanical strain (image D, 0, 5, 20, 30%, 2 hrs.). Cell viability was measured 24 hours after injury by MTT assay. Data are presented as mean optical density at 550nm ± SEM from 4 independent experiments.

3.3.2 hAT2 and HMVEC-L expressed both TNFR

Prior to undertaking experiments investigating selective TNFR antagonism and agonism we confirmed that hAT2 (figure 3.3) and HMVEC-L (figure 3.4) expressed both TNF receptors. In preliminary experiments, the human alveolar epithelial cell line A549 expressed TNFR1 only (personal communication from GSK).

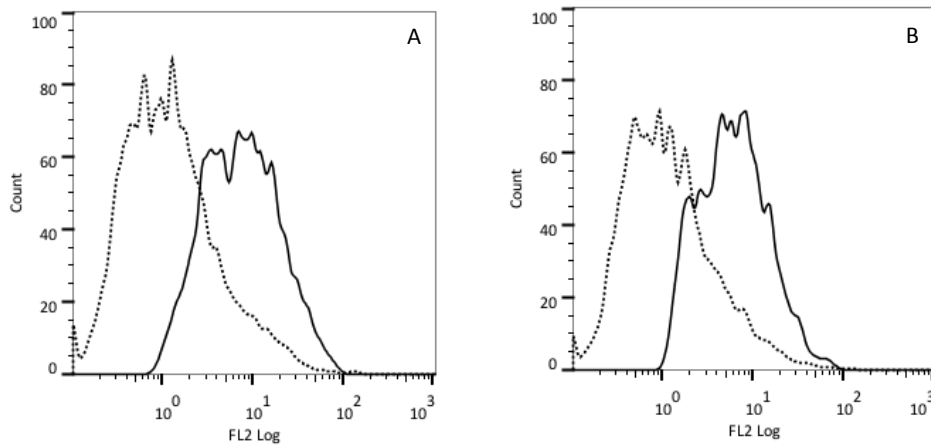


Figure 3.3 hAT2 expressed both TNFR

hAT2 were grown to confluence over 5 days then re-suspended using non-enzymatic dissociation buffer, transferred on ice and stained with anti-human TNFR1 PE and anti-human TNFR2 PE or isotype controls. Receptor expression was analysed by flow cytometry. Panels A and B are representative of 2 separate experiments and demonstrate TNFR1 expression (Panel A) and TNFR2 expression (Panel B). IgG isotype controls are shown with the dotted line

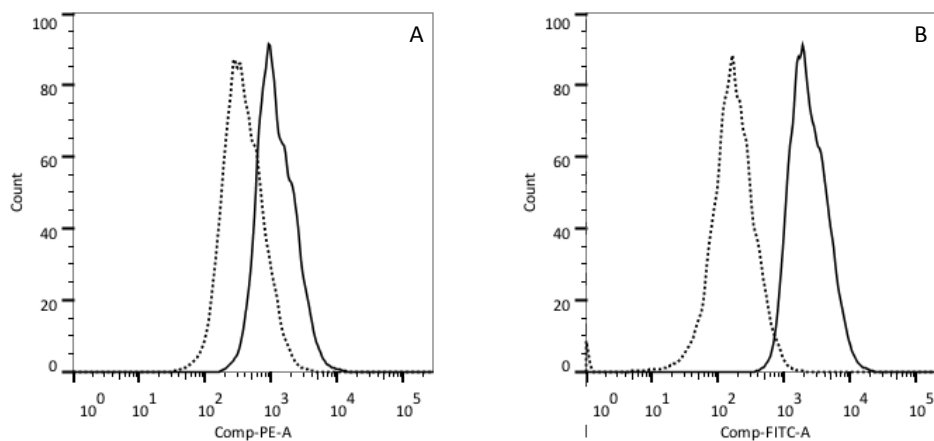


Figure 3.4 HMVEC-L expressed both TNFR

HMVEC-L were grown to confluence over 3 days then re-suspended and stained with anti-human TNFR1 PE and anti-human TNFR2 FITC or isotype controls. Receptor expression was analysed by flow cytometry. Panels A and B are representative of 2 separate experiments and demonstrate TNFR1 expression (Panel A) and TNFR2 expression (Panel B). IgG isotype controls are shown with the dotted line

3.3.3 Selective TNFR1 antagonism abrogated IL-8 release from human alveolar type 2 cells in a dose responsive manner

The effect of TNFR antagonism was investigated in TNF stimulated hAT2, pre-incubated with GSK1995057, Adalimumab and a dummy domain antibody. The dose of TNF used (10ng/mL) was the approximate half maximal effective concentration (EC_{50}) from the dose response experiments shown in figure 3.1 A. GSK1995057 (10nM) and Adalimumab (1nM) abrogated IL-8 release from TNF stimulated hAT2 (Figure 3.5A). The dummy domain antibody had no effect on IL-8 release and none of the treatments influenced cell viability, measured by MTT assay (figure 3.6). Based on these data, all future experiments investigating selective TNFR antagonism used a dose of 1nM Adalimumab and 10nM GSK1995057 and dummy domain antibody.

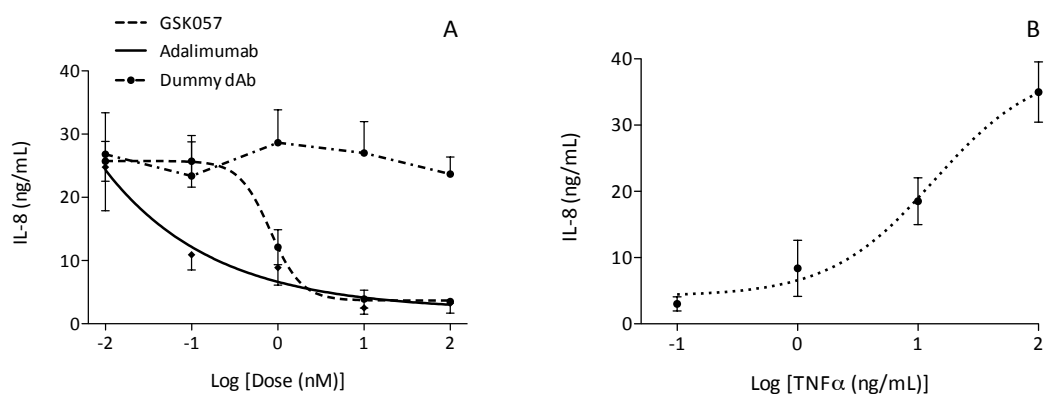


Figure 3.5 Selective TNFR antagonism abrogated IL-8 release from TNF stimulated human alveolar type 2 cells

hAT2 were exposed to GSK1995057, Adalimumab or dummy domain antibody for 1 hour. Cells were then treated with TNF 10ng/mL for 24 hours. The medium was collected after a further 24 hours and concentrations of Interleukin-8 (IL-8) measured by ELISA (Panel A, n=4-5). Panel B shows the TNF dose response curve (n=4). Data are presented as mean $\pm$ SEM.

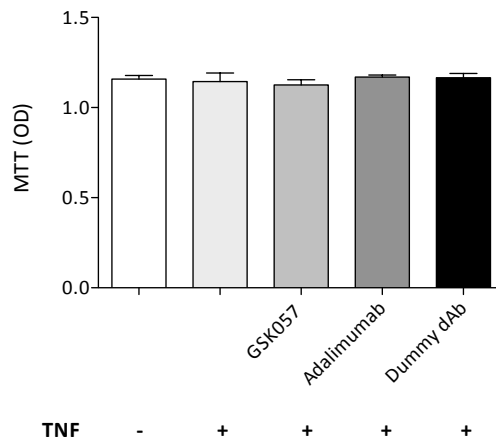


Figure 3.6 Effect of selective TNFR antagonism on hAT2 viability

hAT2 were pre-incubated with the GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) for 1 hour then exposed to exogenous TNF (10ng/mL) or vehicle control. After 24 hours cell viability was measured by MTT assay. Data are presented as median optical density at 550nm $\pm$ interquartile range (n=2).

3.3.4 Effect of selective TNFR1 antagonism on cytokine and chemokine release in TNF treated hAT2

Exposure of hAT2 to TNF (10ng/mL) induced release of IL-8, IL-6 and GM-CSF. Levels of IL-1 β , CXCL-1 and ENA-78 were increased at least 2 fold but failed to reach statistical significance owing to small sample numbers (n=3, figure 3.7). GSK1995057 reduced release of IL-8, IL-6, GM-CSF and ENA-78 towards control levels, but again this failed to reach statistical significance owing to the small sample size (n=3-6).

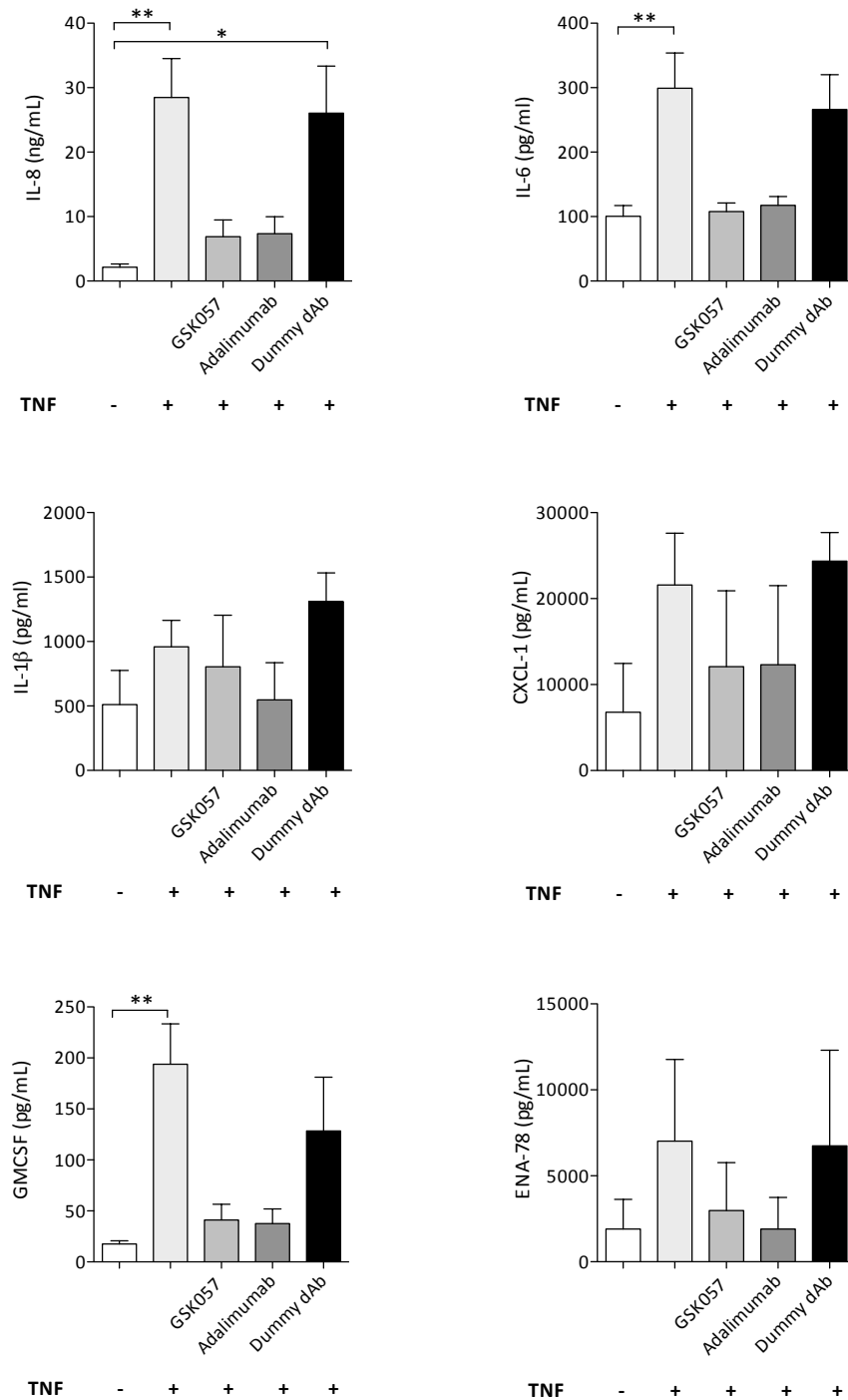


Figure 3.7 Effect of selective TNFR1 antagonism on cytokine and chemokine release from TNF treated hAT2

Monolayers of hAT2 were pre-incubated with GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) for 1 hour then exposed to exogenous TNF (10ng/mL) or vehicle control. Supernatants were collected after 24 hours and analysed for cytokines and chemokines using MSD® multiplex panels (n=4). Data are presented as mean ± SEM and analysed using Kruskal-Wallis (Dunns) test *p<0.05, **p<0.01.

3.3.5 Effect of selective TNFR1 antagonism on cytokine and chemokine release in hAT2 exposed to cyclic mechanical strain

30% CMS for 2 hours significantly increased IL-8, IL-6 and IL-1 β protein release from hAT2 cells (figure 3.8). Selective TNFR1 antagonism demonstrated no effect on release of IL-8, IL-6 or IL-1 β . There was no effect of CMS on release of other cytokines or chemokines, in particular TNF, hence it was impossible to establish any effect of selective TNFR1 antagonism.

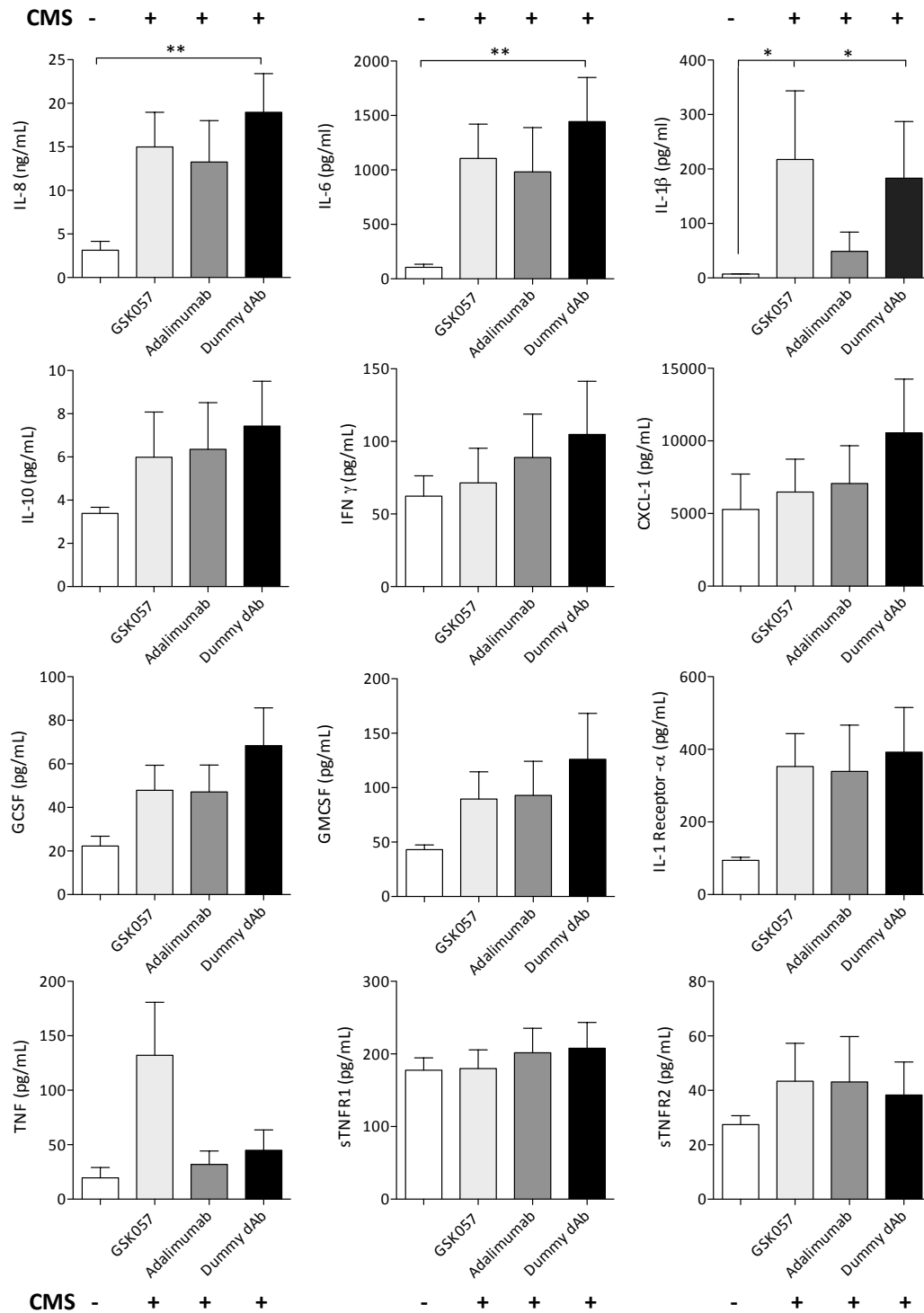


Figure 3.8 Effect of selective TNFR1 antagonism on cytokine and chemokine release from hAT2 exposed to 30% cyclic mechanical strain

Monolayers of hAT2 were pre-incubated with GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) for 1 hour then exposed to cyclic mechanical stretch (30%, 2 hours) or no stretch. Supernatants were collected 24 hours after cessation of stretch and analysed for cytokines and chemokines using MSD® multiplex panels or duoset ELISA kits (sTNFR1 & sTNFR2) n=3-5. Data are presented as mean ± SEM and analysed using Kruskal-Wallis (Dunns) test *p<0.05, **p<0.01

3.3.6 Effect of selective TNFR1 antagonism on cytokine and chemokine release from TNF stimulated HMVEC-L

Treatment of HMVEC-L monolayers with TNF (10ng/mL) induced release of the cytokines IL-8, IL-6, IL-1 β and IL-10 and the chemokines ENA-78, CXCL-1 and GM-CSF (figure 3.9). Pre-treatment of cells with GSK1995057 significantly abrogated IL-8 release whilst TNF induced release of IL-6, IL-1 β , ENA-78 and GM-CSF was abrogated by GSK1995057 and Adalimumab as evidenced by the loss of significance compared to TNF (or dummy dAb) treatment. Dual TNFR blockade with Adalimumab paralleled the effects observed with GSK1995057. Pre-treatment with GSK1995057 had no effect on IL-10 release from TNF stimulated HMVEC-L whilst dual receptor blockade with Adalimumab returned IL-10 release towards control levels suggesting that IL-10 release may be regulated predominantly via TNFR2 signalling.

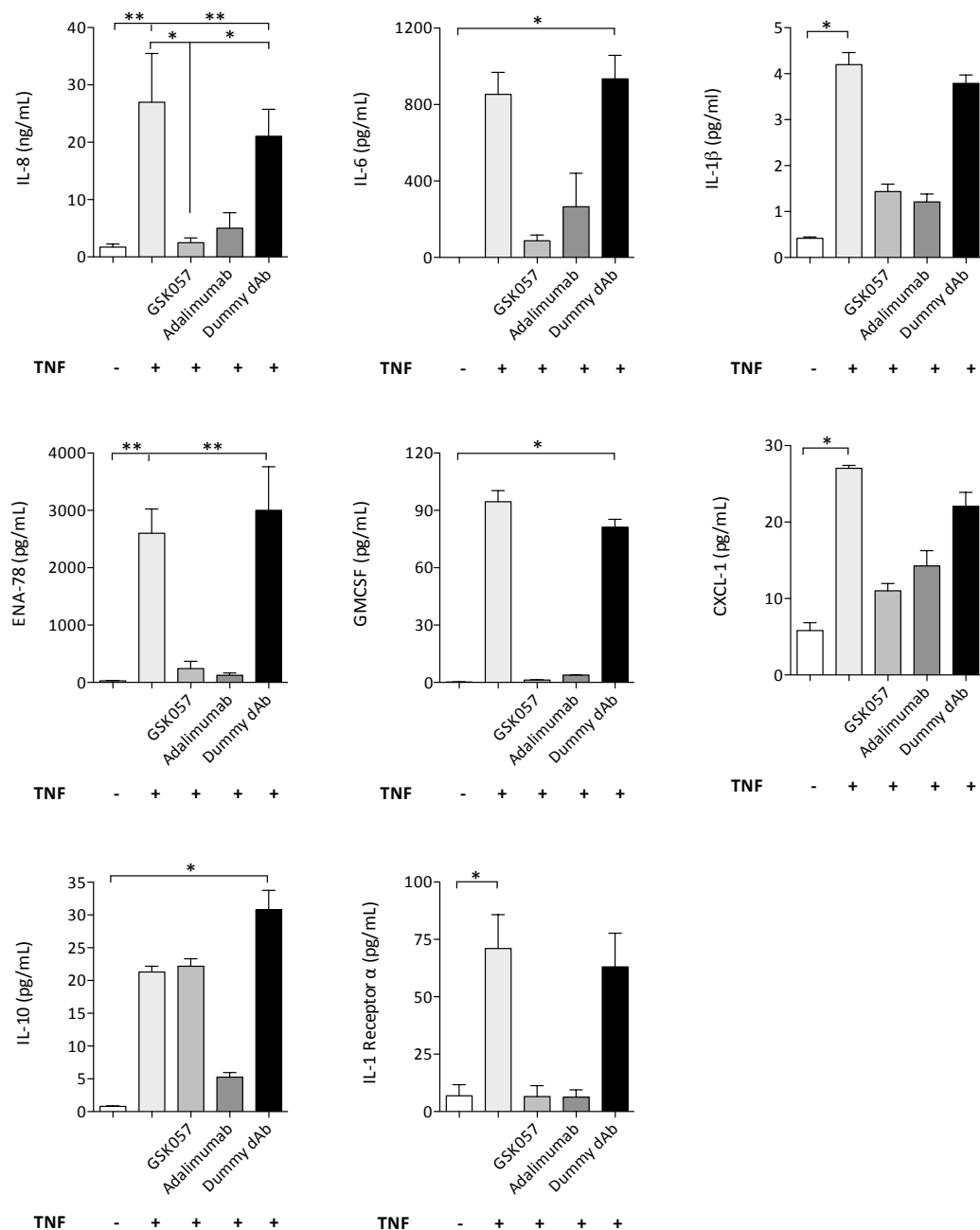


Figure 3.9 Effect of selective TNFR1 antagonism on cytokine and chemokine release from TNF treated HMVEC-L

Monolayers of HMVEC-L were pre-incubated with GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) for 1 hour then exposed to exogenous TNF (10ng/mL) or vehicle control. Supernatants were collected after 24 hours and analysed for cytokines and chemokines using MSD® multiplex panels (n=4). Data are presented as mean ± SEM and analysed using Kruskal-Wallis (Dunns) test *p<0.05, **p<0.01

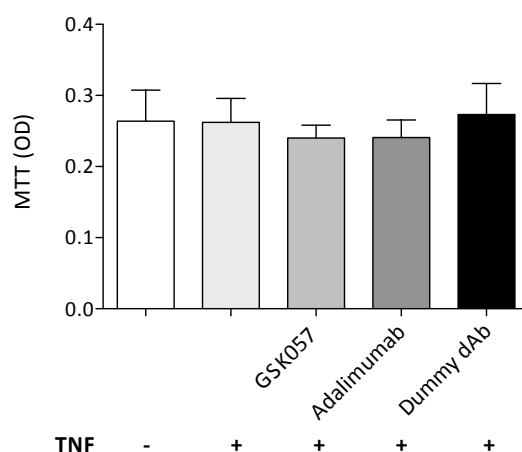


Figure 3.10 Effect of selective TNFR antagonism on HMVEC-L viability

HMVEC-L monolayers were pre-incubated with the GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) for 1 hour then exposed to exogenous TNF (10ng/mL) or vehicle control. After 24 hours, cell viability was measured by MTT assay. Data are presented as median optical density at 550nm $\pm$ interquartile range (n=2).

3.3.7 Differential TNFR signalling did not promote wound healing in hAT2

Across a range of TNF concentrations (1-100ng/mL respectively), wound healing of scratched hAT2 did not increase beyond basal levels (figure 3.11). The effect of TNF was further investigated using selective TNFR antagonism and agonism. Neither selective TNFR1 antagonism, in principal 'forcing' TNFR2 signalling in the presence of TNF (figure 3.12), nor TNFR2 agonism with D143N (figure 3.13) promoted wound healing. TNF stimulation alone had no effect on wound healing, however pre-incubation with the dummy domain antibody stimulated wound healing similarly to experiments in figure 3.12. Of note, treatment with media containing 10% serum did not induce a significant increase in wound healing compared to media without serum.

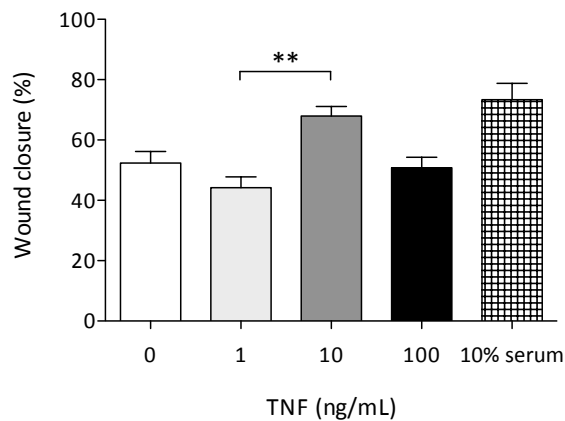


Figure 3.11 TNF dose response of wound closure in hAT2

Wounded hAT2 monolayers were incubated with TNF (1, 10 or 100ng/mL), vehicle control, or media containing 10% serum. After 24 hours the wound was re-imaged and percentage wound closure calculated using image analysis software (n=12). Data are presented as mean $\pm$ SEM analysed using Kruskal-Wallis (Dunns) test. ** $p < 0.01$

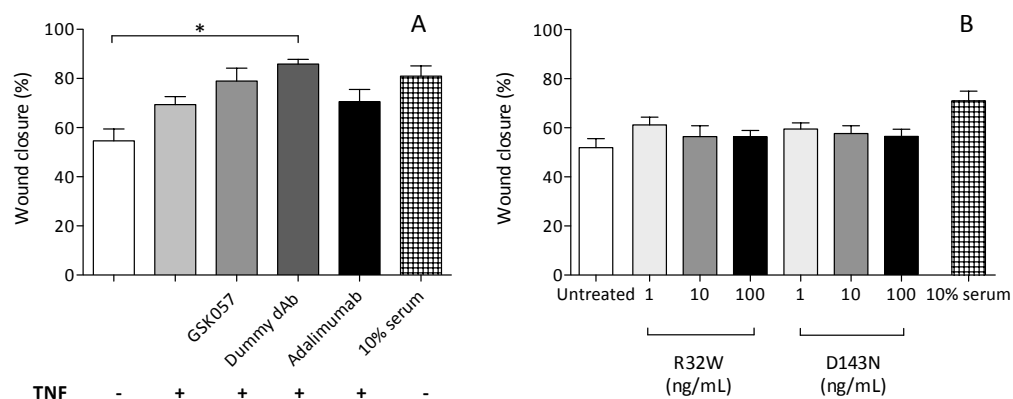


Figure 3.12 Effect of differential TNFR signalling on wound healing in hAT2

Wounded hAT2 monolayers were incubated with GSK1995057 (10nM), Adalimumab (1nM) and a dummy domain antibody (10nM) for 1 hour then exposed to exogenous TNF (10ng/mL), vehicle control, or media containing 10% serum (Figure A, n=3-5) or treated with R32W (1, 10 or 100ng/mL), D143N (1, 10 or 100ng/mL), vehicle control or 10% serum (Figure B n=4-6). After 24 hours the wound was re-imaged and wound closure calculated using image analysis software. Data are presented as mean $\pm$ SEM and analysed using Kruskal-Wallis (Dunns) test. * $p < 0.05$

To assess the effect of cytokine release from TNF stimulated cells on wound healing of hAT2 monolayers and the effects of differential TNF signalling, we stimulated alveolar macrophages with TNF in the presence of GSK1995057 and controls. The harvested supernatant did not significantly promote wound healing (figure 3.14).

Similarly, selective TNFR antagonism had no effect. In separate experiments, IL-8 levels in the harvested supernatant were measured and this demonstrated nominal IL-8 release and no difference between treatments.

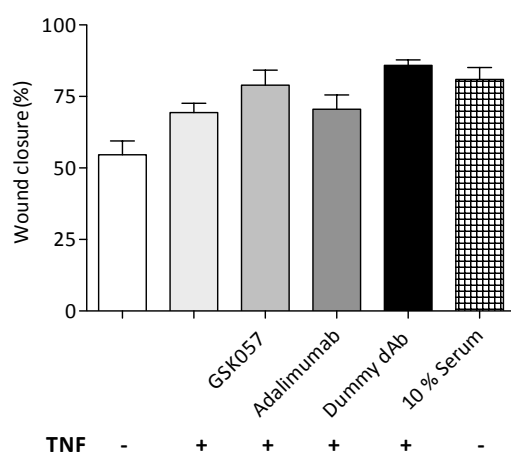


Figure 3.13 Effect of supernatants from alveolar macrophages stimulated with TNF in the presence of GSK1995057 and controls on hAT2 wound healing

Human alveolar macrophages were incubated with GSK1995057 (10nM), Adalimumab (1nM) or dummy domain antibody (10nM) then exposed to exogenous TNF (10ng/mL) or vehicle control. After 20 hours, the supernatants were collected and frozen at -80°C. Supernatants were later added to wounded hAT2 and wound closure measured over 24 hours was calculated using image analysis software (n=3-5). Data are presented as mean $\pm$ SEM

3.3.8 Effect of selective TNFR agonism on tube formation in HUVEC and HMVEC-L

In an angiogenesis or vascular wound healing assay, selective TNFR2 agonism significantly promoted tube formation in HUVEC compared to unstimulated cells (figure 3.14A). The magnitude of this effect was similar to that of the positive control, vascular endothelial growth factor (VEGF), a known angiogenic factor *in vitro*. A similar trend was observed in HMVEC-L but this effect was non-significant (figure 3.14B). Both cell types expressed TNFR1 and TNFR2 although relative expression between cell types was not demonstrated.

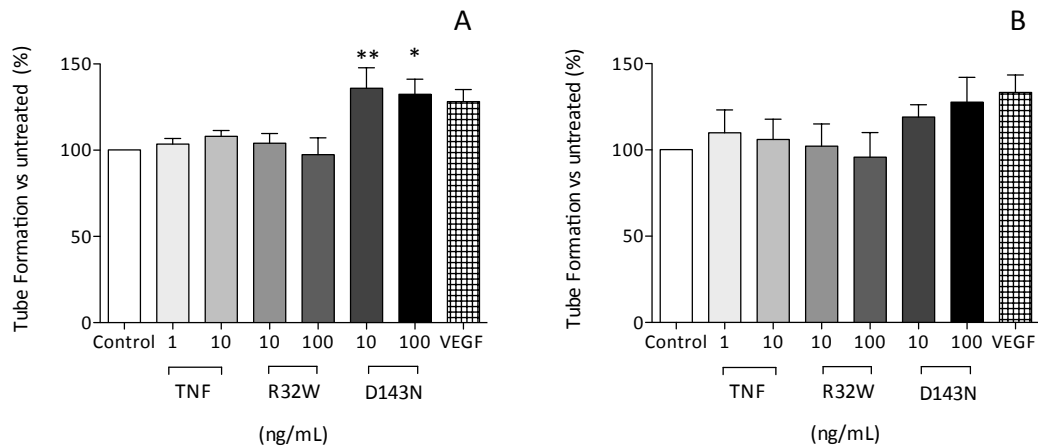


Figure 3.14 Effect of selective TNFR agonism on tube formation in HUVEC and HMVEC-L

HUVEC and HMVEC-L were diluted in TNF (1 or 10ng/mL), R32W (10 or 100ng/mL), D143N (10 or 100ng/mL), VEGF (10ng/mL) or vehicle control and seeded onto angiogenesis slides coated with Matrigel. After 4 hours, the well was imaged and total tube length measured using image analysis software. Figure A represents tube formation in HUVEC (n=7) and figure B tube formation in HMVEC-L (n=4). Data are presented as mean $\pm$ SEM analysed using one way ANOVA (Dunnett's) test *p<0.05, **p<0.01

3.3.9 Selective TNFR1 antagonism abrogated TNF induced permeability in HMVEC-L

Both selective TNFR1 antagonism and dual blockade of both TNFR abrogated TNF induced changes in endothelial permeability as measured by trans-endothelial resistance (figure 3.15B). The effects of TNF were maximal at around 8 hours following stimulation and plateaued for the duration of the experiment (figure 3.15A). Electrical impedance and capacitance across the cells remained unchanged suggesting that the observed changes in electrical resistance were not due to alterations in cell morphology or cell death.

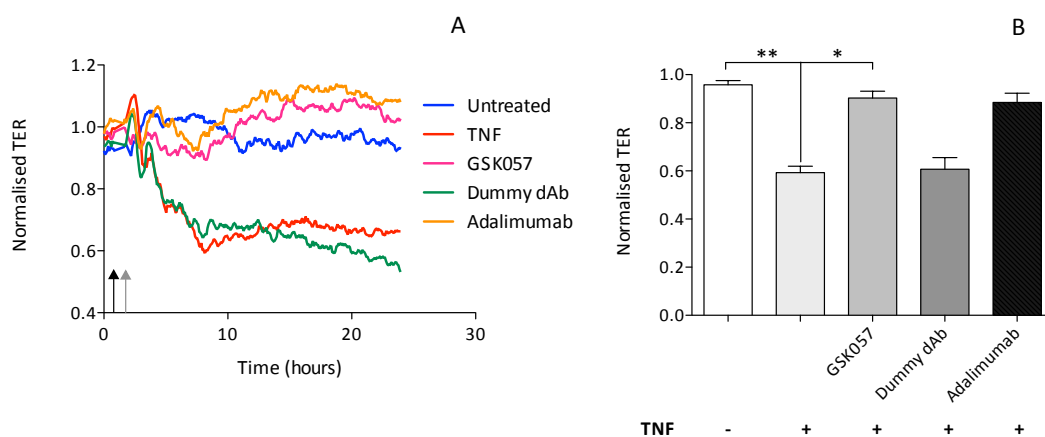


Figure 3.15 Effect of selective TNFR antagonist on TNF induced endothelial permeability, as measured by transendothelial resistance

HMVEC-L plated on an ECIS 8-well electrode array plate were incubated with GSK1995057 (10nM), Adalimumab (1nM) or a dummy domain antibody (10nM) for 1 hour then exposed to exogenous TNF (10ng/mL) or vehicle control. Transendothelial resistance (TER) was measured continuously over 20 hours. Figure A shows a representative tracing of normalised resistance where the black arrow indicates timing of pre-incubation and the grey arrow TNF administration. Figure B shows the trough-normalised resistance over the 20 hour period for each condition (n=3-5). Data are presented as mean $\pm$ SEM analysed by Kruskal Wallis (Dunns) *p<0.05, **p<0.01

3.3.10 Effect of selective TNFR1 antagonism on release of vascular injury markers from TNF stimulated HMVEC-L

Treatment of HMVEC-L monolayers with TNF (10ng/ml) resulted in elevation of the vascular injury markers, soluble ICAM-1, soluble VCAM-1 and soluble E-selectin. There was a 3-fold increase in C-reactive protein (CRP) and serum amyloid protein A (SAA) release but this did not reach statistical significance (figure 3.16). Pre-treatment of cell monolayers with GSK1995057 resulted in a reduction of these vascular injury markers to basal (unstimulated) levels although again none of these data reached significance owing to small n numbers (n=4). MTT data confirmed that release of these markers was not due to cell death (figure 3.10)

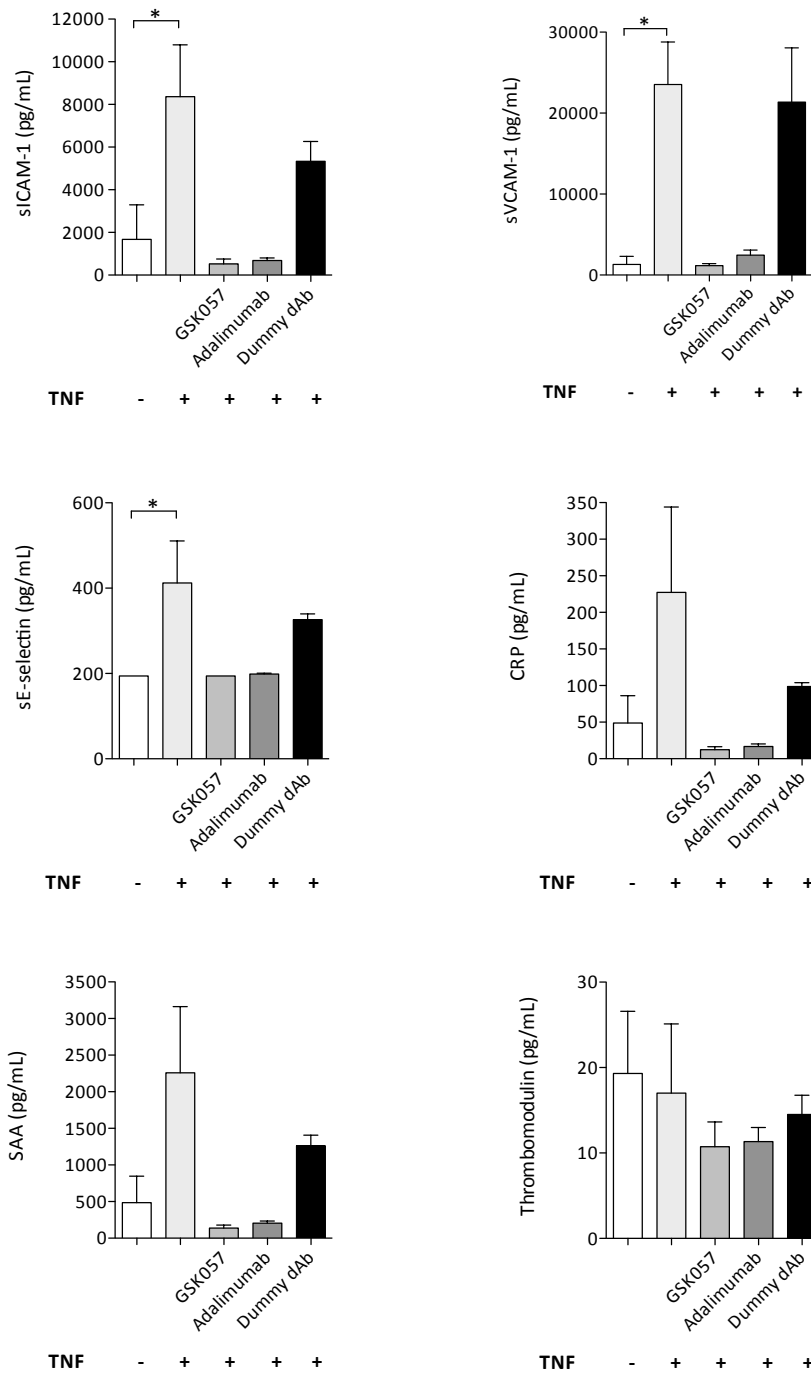


Figure 3.16 Effect of selective TNFR1 antagonism on release of vascular injury markers release from TNF treated HMVEC-L

Monolayers of HMVEC-L were incubated with GSK1995057 (10nM), Adalimumab (1nM) or a dummy domain antibody (10nM) for 1 hour then exposed to exogenous TNF (10ng/mL) or vehicle control. Supernatants were collected after 24 hours and analysed for cytokines and chemokines using MSD® multiplex panels (n=4). Data are presented as mean $\pm$ SEM and analysed using Kruskal-Wallis (Dunns) test *p<0.05 SAA; serum amyloid protein A, CRP; C-reactive protein

3.3.11 Summary of important findings in this chapter

TNF induced release of IL-8, IL-6 and GM-CSF from hAT2. TNF stimulation of hAT2 increased release of IL-1 β , CXCL-1 and ENA-78 at least 2- fold but these data failed to reach statistical significance. GSK1995057 pre-treatment reduced release of IL-8, IL-6, GM-CSF and ENA-78 towards control levels but again this failed to reach statistical significance.

Similarly, chemokine and cytokine release from TNF stimulated HMVEC-L was markedly attenuated by GSK1995057 suggesting that pro-inflammatory signalling in these cells is significantly influenced by TNFR1 signalling. Pre-treatment of HMVEC-L with GSK1995057 had no effect on IL-10 release from TNF stimulated cells, whereas dual TNFR blockade reduced IL-10 release to basal levels, suggesting that IL-10 release may be under the dominant control of TNFR2 signalling

hAT2 monolayers exposed to 30% CMS released IL-8, IL-6, and IL-1 β but the release of the majority of cytokines and chemokines assayed was unaffected. TNFR1 antagonism had no significant effect IL-8, IL-6, and IL-1 β release. Interestingly, there was no effect of CMS on either sTNFR1 or sTNFR2 release.

TNF had no effect on wound healing in hAT2. In addition, neither selective TNFR antagonism nor TNFR agonism promoted wound healing. TNFR2 signalling appeared to promote tube formation in HUVEC. Owing to lower experimental numbers (n=4), whilst there was a trend towards a similar effect in HMVEC-L, this was not statistically significant.

TNF reduced transendothelial resistance in HMVEC-L. This was mediated by TNFR1 signalling as GSK1995057 pre-treatment abrogated the effect. Similarly TNF activated HMVEC-L as evidenced by release of sICAM-1, sVCAM-1 and soluble E-

selectin. Whilst there was a reduction to basal (untreated) levels in cells pre-exposed to GSK1995057, these data failed to reach statistical significance.

3.3.12 Development of *in vitro* models of ALI and selective TNFR antagonism thereof

Stimulation of hAT2 cells with TNF, hydrogen peroxide, CMS and bronchoalveolar lavage fluid (BALF) from ARDS patients demonstrated a dose-dependent increase in IL-8 production (figure 3.1). These *in vitro* models were designed to reflect several key pathomechanisms of *in vivo* ALI, including direct ALI, oxidative stress and VALI. IL-8 is a potent neutrophil chemokine produced by respiratory epithelium (Standiford et al., 1990) in response to pro-inflammatory cytokines and is the predominant chemokine in BALF in human ALI (Goodman et al., 1996); concentrations of IL-8 in BALF from patients with ALI were elevated compared to those with cardiogenic pulmonary oedema (Miller et al., 1996; Pugin et al., 1999). Moreover, increased BALF IL-8 levels predicted both the development of ALI in an at-risk population and mortality (Donnelly et al., 1993; Miller et al., 1996; Rodriguez et al., 1992). As a primary read-out of epithelial injury with relevance to *in vivo* injury, IL-8 therefore seemed an appropriately robust and relevant marker.

TNF induced significant release of IL-8 from hAT2 (figure 3.1). Under inflammatory conditions, TNF concentrations between 1 and 10 ng/mL have been measured in the BALF of ARDS patients (Meduri et al., 1995). A concentration of 10nM GSK1995057 and 1nM Adalimumab abrogated IL-8 release from TNF stimulated hAT2 (Figure 3.5). These effects and concentrations paralleled preliminary experiments performed in fibroblasts and A549 cells (personal communication from GSK). Accordingly, we chose a concentration of 10ng/mL TNF and 10nM GSK1995057 (and

dummy domain antibody) with 1nM Adalimumab for all future experiments in all cell types.

Whilst BALF from ARDS patients induced IL-8 release (figure 3.1), in subsequent experiments investigating the effect of selective TNFR1 antagonism on IL-8 release from BALF stimulated hAT2, IL-8 release failed to reach statistical significance so further analysis was abandoned. Similarly, hydrogen peroxide (200 μ M) induced IL-8 release from hAT2 (figure 3.1), but the treatment effect was small and further analysis was not pursued.

3.3.13 The effect of differential TNFR signalling on cytokine and chemokine release from TNF stimulated hAT2 and HMVEC-L

Selective TNFR antagonism in both hAT2 and HMVEC-L reduced cytokine (IL-8 and IL-6) and neutrophil chemokine (ENA-78, CXCL-1 and GMCSF) release to basal (unstimulated levels) although these data were not statistically significant (figures 3.7 and 3.9). Although the precise role of each cytokine and chemokine in the pathogenesis of ALI is not fully understood, the putative benefits of blockade of pro-inflammatory cytokine and neutrophil chemokine release have been highlighted by a number of both clinical and experimental studies. Hence, passive immunisation of rabbits with neutralising antibodies to IL-8 abrogated neutrophil recruitment and tissue injury in a rabbit model of acid aspiration lung injury (Folkesson et al., 1995) and ischemia–reperfusion injury (Strieter et al., 1999). Furthermore, antibody neutralization of ENA-78 attenuated lung injury in a rodent model of hepatic ischemia/reperfusion injury (Colletti et al., 1995) whilst pretreatment of rats with a neutralising antibody to CINC, cytokine-induced neutrophil chemoattractant (CINC), the homolog of human CXCL-1, protected rats against pancreatitis-associated lung injury (Bhatia et al., 2000).

Moreover, human data suggest that early enhanced neutrophil migration due to elevated alveolar IL-8 concentrations may be critical to neutrophilic alveolitis in ALI or those at risk (Pallister et al., 2002) and neutrophil activation directly correlates with disease severity and with levels of IL-8, TNF and IL-6 (Chollet-Martin et al., 1996). Furthermore, whilst other chemokines are elevated in BALF from ARDS patients, only IL-8 consistently correlates with the number of neutrophils and disease severity (Goodman et al., 2003; Villard et al., 1995).

Plasma IL-6 was associated with oxygenation index, fewer ventilator free days and 28-day mortality in patients enrolled in the ALVEOLI (high PEEP vs low PEEP) trial (Brower et al., 2004). Other investigators have reported elevated plasma IL-6 in patients who developed ALI in comparison to those who did not (Takala et al., 2002) and high baseline IL-6 levels were seen in patients with ARDS who died (Parsons et al., 2005; Meduri et al., 1995) and a persistently elevated circulating level of IL-6 predicted mortality (Meduri et al., 1995). Whilst IL-1 β is the major inflammatory mediator in BALF of ALI patients (Pugin et al., 1996), large multi-centre studies investigating the role of IL-1 β are lacking, but small studies showed persistent elevation of plasma IL-1 β (Meduri et al., 1995) and elevated BALF levels of IL-1 β (Pugin et al., 1996) were associated with worse outcomes

Despite IL-8 being considered the main neutrophil chemoattractant in ALI, neutralisation of IL-8 in the BALF of patients with mild ALI did not completely abolish its neutrophil chemotactic activity (Miller et al., 1992), suggesting that other chemotactic factors are present that contribute to neutrophil migration. Hence, plasma and BALF levels of chemokines including CXCL-1 and ENA-78 have been elevated in patients at risk for, and with established, ALI (Goodman et al., 1996; Villard et al., 1995; Donnelly et al., 1993) and levels correlated with outcome (Miller et al., 1992). GM-CSF is recognised

to have effects on neutrophil recruitment, macrophage biology, surfactant homeostasis, and host defence in the lungs (Trapnell et al., 2009). Acute blockade of GM-CSF decreases pulmonary neutrophil recruitment elicited by LPS or cytokines (Bozinovski et al., 2004; Bozinovski et al., 2002; Laan et al., 2003). Furthermore, in 31 patients with ALI, whilst GM-CSF did not correlate with BALF neutrophil levels, G-CSF but not GMCSF negatively correlated with survival (Aggarwal et al., 2000).

TNFR1 inhibition of pro-inflammatory cytokine and chemokine release from both hAT2 and HMVEC-L is probably mediated via inhibition of NF- κ B signalling. Fifty-five genes known to be regulated by NF- κ B were analysed in TNF stimulated human microvascular endothelial cells (HMVEC) and HUVEC (Perrot-Applanat et al., 2011). The chemokines IL-8, GRO- α , MCP-1 and GMCSF cytokines IL-6 and IL-1 β and neutrophil adhesion molecules ICAM-1 and VCAM-1 were up-regulated more than 5 fold in HMVEC and HUVEC. The TNFR1 dependency of this effect was investigated using small inhibitory RNA (siRNA) to TNFR1. The expression of IL-8, IL-6, IL1- α , IL-1 β , GMCSF, ICAM-1 and VCAM-1 were inhibited more than 30% compared to TNF stimulated siRNA control-transfected cells. Interestingly, transfection of HMEC with TNFR2 siRNA also inhibited up-regulation of IL-1 β and GMCSF suggesting that TNFR2 signalling is additionally involved in the control of the NF- κ B gene signature of TNF stimulated HMVEC. In agreement with these data TNF-stimulated ICAM-1 and VCAM-1 expression in HUVEC was mediated exclusively NF- κ B signalling, but not the ERK, p38MAPK or JNK kinase signalling; this effect was under the exclusive control of TNFR1 signalling (Zhou et al., 2007). Similarly, TNF-induction of inflammatory genes was under the dominant control of TNFR1 in airway smooth muscle (Amrani et al., 2001).

Interestingly, TNFR1 antagonism had no effect on IL-10 levels in TNF stimulated HMVEC-L whilst dual TNFR blockade reduced IL-10 release to basal levels (figure 3.9)

suggesting that TNFR2 signalling has anti-inflammatory and reparative roles in certain tissues (chapter 1.3.4). IL-10 therapy abrogated LPS induced ALI via its anti-inflammatory actions including inhibition of TH₁ differentiation, suppression of neutrophil activation, and neutrophil chemokine down regulation (Inoue, 2000). IL-10 has also been shown to counteract LPS and TNF induced inhibition of neutrophil apoptosis (Keel et al., 1997) and trans-epithelial migration of neutrophils (Ajuebor et al., 1999). Moreover, IL-10 may be central to the resolution of inflammation; intravenous administration of mesenchymal stem cells in a murine sepsis model reduced mortality and organ injury, an effect mediated primarily by increased IL-10 levels. Analysis of plasma samples from ARDS patients enrolled in the 2000 ARMA study (Network, 2000), demonstrated a negative correlation of plasma IL-10 with a poor outcome (Parsons et al., 2005).

Whether IL-10 release represents a TNFR2 mediated anti-inflammatory pathway in HMVEC-L requires further investigation, but it was notable that TNFR1 antagonism reduced the putative anti-inflammatory cytokine IL-1 receptor alpha (an antagonist to the pro-inflammatory cytokine IL-1 β) to near basal levels.

3.3.14 TNFR1 antagonism had no effect on chemokine or cytokine release from mechanically stretched hAT2 or hAT2 treated with BALF from patients with ALI

Numerous cytokines have been detected in BALF and plasma from ALI patients subjected to mechanical ventilation. Similar variation in results has been observed in animal models of VALI (Held et al., 2001; Imai et al., 1999; Narimanbekov and Rozycki, 1995; Tremblay et al., 1997) and from *in vitro* models where mechanical strain was applied to tissues or cells. These results indicate that the release of a variety of pro-inflammatory mediators and several cytokines are dependent upon species, cell line and ventilation or CMS conditions. Using a protocol of 30% elongation, 20 cycles per minute

for 2 hours, we observed significant increases in IL-8, IL-6, and IL-1 β from hAT2 (figure 3.8) whilst release of the majority of cytokines and chemokines was unaffected. Our regimen was chosen because data from our group has demonstrated robust production of IL-8 from hAT2 cells (Pinhu et al., 2008). In animal models, however, a 2-hour high stretch regimen up-regulated TNF in the lavage fluid but was insufficient to up-regulate neutrophil chemokines (Tremblay et al., 1997; Tremblay et al., 2002; Wilson et al., 2005). It is therefore plausible that our protocol did not allow for analysis of the kinetics of cytokine and chemokine release thus accounting for the observed lack of stretch response for the majority of analytes. MTT data (figure 3.2D) suggest that release of cytokines is unlikely to be due to cell death; previous unpublished data from our group has confirmed that, at least in A549 cells, our regimen did not induce cell death although cell membrane damage was evident, as previously described (Tschumperlin and Margulies, 1998; Vlahakis et al., 1999).

IL-8, IL-6, and IL-1 β are clinically relevant pro-inflammatory cytokines in ALI; BALF levels of these cytokines decreased over 36 hours in patients exposed to low tidal volume ventilation compared to conventional ventilation (Ranieri et al., 1999), whilst in the ARDS Network low tidal volume study (Network, 2000), plasma levels of IL-6 decreased by 26% and IL-8 by 12% in the low tidal volume group. Similarly, plasma levels of IL-6- and IL-8 increased in patients exposed to high tidal volume ventilation (Stuber et al., 2002); this effect was also seen in the plasma with levels returning to baseline with tidal volume reduction.

Our group (unpublished data) and others (Vlahakis et al., 1999) have failed to detect TNF release from A549 cells exposed to 30% CMS. Similarly, we detected no response of hAT2 to CMS induced TNF protein release (figure 3.8). As outlined previously (chapter 1.3.5) experimental data regarding the role of TNF in VALI has been

inconsistent. One explanation for these conflicting data relates to the kinetics of TNF release. Despite BALF TNF levels in Ranieri's clinical study increasing at both 24 and 36 hours after conventional (12ml/kg) ventilation (Ranieri et al., 1999), data in a murine VALI model demonstrated that TNF expression was transient; animals terminated at the onset of VALI when physiological signs of injury were starting to become apparent expressed significantly higher levels of TNF than animals with advanced lung injury (Wilson et al., 2003). An interrogation of the time course of TNF release, as opposed to measurement at a single time point, may therefore have yielded more information about TNF release from hAT2 exposed to CMS.

More definitive data regarding the role of TNF and in particular differential TNFR signalling was provided by data from the Takata group (Wilson et al., 2007). Using selective TNFR knockout mice, signalling through TNFR1 promoted the development of high stretch-induced pulmonary oedema, while signalling through TNFR2 was protective. These opposing effects were independent of TNF-mediated leucocyte recruitment. Interestingly, mice devoid of both TNF receptors showed no protection against oedema suggesting that TNFR1 signalling is injurious whilst TNFR2 signalling is protective, at least in murine VALI. These observations were further confirmed by the same group, using the selective TNFR1 antagonist GSK1995057 (Bertok et al., 2012). GSK1995057 attenuated deteriorations in respiratory mechanics and oedema formation in a murine model of VALI whilst dual TNFR blockade had no effect. The mechanisms of TNFR1 mediated pulmonary oedema have yet to be established although recent results from a murine acid induced ALI model suggested that TNFR1 mediated caspase-8 signalling induced lung epithelial injury and dysfunction (Patel et al., 2013).

In our *in vitro* study, we observed no effect of selective TNFR1 antagonism on IL-8, IL-6 or IL-1 β release from hAT2 exposed to CMS (figure 3.8). The absence of

effect may again relate to the kinetics of TNF release in VALI models; a time course experiment, including early time points where TNF release may have yielded positive data. Alternatively, TNF production in VALI may be predominantly macrophage derived as opposed to released from alveolar epithelium. Indeed, alveolar macrophages (co-stimulated with LPS) respond to mechanical stress by releasing TNF (Pugin et al., 1998) and depletion of alveolar macrophages in a rat VALI model decreased lung endothelial and epithelial permeability and lowered BALF neutrophil counts (Frank et al., 2006). Moreover, in an LPS plus VALI (20mL/kg tidal volume) model, up-regulation of cytokines and chemokines in the BALF, and accordingly neutrophil recruitment, was abrogated by selective TNFR1 antagonism (Bertok et al., 2012). This effect was associated with reduced activation of alveolar macrophages further suggesting that alveolar macrophages are a major source of cytokines in the alveolar compartment and that this process may be partly mediated by TNFR1 signalling.

Levels of sTNFR in the plasma and BALF fell after 36 to 40 hours in patients with ALI subjected protective ventilation compared to those ventilated using a conventional strategy, whilst BALF sTNFR1 levels, but not sTNFR2, increased in patients exposed to injurious ventilation (12mL/kg) compared to protective ventilation (6mL/kg) (Parsons et al., 2005). Despite hAT2 expressing both TNFR (figure 3.3), CMS did not induce sTNFR release from hAT2 in our study. Our human *in vitro* data therefore support murine *in vivo* data suggesting that elevated levels of sTNFR in the airspace associated with high stretch ventilation reflects increased alveolar epithelial permeability as opposed to pulmonary epithelial cell sTNFR release (Dorr et al., 2011). Injurious ventilation induced alveolar protein influx, which strongly correlated with BALF levels of both sTNFR but this effect was abolished in isolated buffer-perfused lungs devoid of circulating sTNFRs.

3.3.15 Differential TNFR signalling did not promote wound healing in hAT2

Neither TNF nor TNFR specific muteins promoted wound healing in hAT2 (figure 3.11 and 3.12). TNF induced urokinase-type plasminogen activator (u-PA), a key component of fibrinolysis that accompanies tissue repair promoting tissue remodeling and cell migration in A549 cells (Marshall et al., 1992). Further studies confirmed that recombinant TNF stimulated bovine bronchial epithelial cell migration, an effect mediated by activation of PKC (Wyatt et al., 1997), whilst TNF induced airway epithelial cell wound healing required matrix metalloproteinase-9 release, epidermal growth factor (EGF) shedding and subsequent EGF receptor transactivation (Maille et al., 2011).

Why was no effect observed in wounded hAT2? The concentration of TNF used was similar to other studies investigating cell proliferation and migration. In endothelial cells, however, wound repair was promoted at low concentrations (1ng/mL) and inhibited at higher concentrations (5 and 10ng/mL) (Pan et al., 2002). Both TNFR were present on the cell surface of hAT2 (figure 3.3) and previous data suggest that in response to injury, TNF responses are diminished by down-regulating TNFR1 mRNA and protein (Al-Lamki et al., 2003) or shedding of TNFR1 (Wang et al., 2003) whilst in tissues, TNFR2 is up-regulated at sites of injury (Al-Lamki et al., 2009; Al-Lamki et al., 2010; Al-Lamki et al., 2005). Indeed, in microvascular endothelial cells harvested from patients with established ALI, TNFR2 expression was up-regulated compared to both TNFR1 in the same cells and TNFR2 expression in control cells from cancer-free lobectomy specimens (Lucas et al., 1997). Although we did not measure TNFR expression in cells at the wound edge, it would seem unlikely that the lack of response was therefore due to a lack or indeed absence of TNFR2. It remains unclear whether the selective TNFR mutein D143N has functional activity. Whilst a small but significant increase in tube formation in HUVEC was demonstrated with D143N (figure 3.14A), suggestive of functional activity, this is the

only positive ‘read-out’ across a range of cell types. Receptor binding studies demonstrate selective binding to TNFR2 with no cross-reactivity with TNFR1 (chapter 1.3.6.3) but we have been unable to demonstrate downstream signalling specific to TNFR2 (Appendix A). In organ cultures, TNFR2 promoted cell adhesion, migration, proliferation, survival and angiogenesis through activation of endothelial/epithelial tyrosine kinase (Etk) (Al-Lamki et al., 2009; Pan et al., 2002). Indeed, in epithelial cells, Etk may regulate cell junctions by promoting a redistribution of actin filaments (Hamm-Alvarez et al., 2001). Despite attempts to measure Etk phosphorylation and upstream AKT phosphorylation in both cell lysates and organ culture (precision cut lung slices), we have been unable to demonstrate any robust effect of either selective TNFR1 antagonism or TNFR2 agonism (Appendix A).

Consistent with observations in other organ injury (chapter 1.3.4), we speculated that preferential TNFR2 signalling would promote wound healing in injured hAT2. Pre-exposure of cells to the selective TNFR1 antagonist GSK1995057, in parallel with TNFR2 agonism demonstrated no effect on hAT2 wound healing (figure 3.12A). This regimen should, in principal ‘force’ TNFR2 signalling. One explanation for this lack of treatment effect could be a report suggesting TNFR2 was not efficiently activated *in vitro*; the membrane bound 26-kDa form of TNF has been shown to be vastly superior to recombinant 17kDa soluble TNF in activating TNFR2 (Grell et al., 1995; Grell et al., 1998). Alternatively, TNFR2 signalling may contribute at lower concentrations of TNF (Slowik et al., 1993); hence the doses of the TNFR2 specific mutein D143N used in our studies (10 and 100ng/mL) may have been too high to observe a measurable effect. Nonetheless, it is difficult to draw firm conclusions from this assay as we did not observe a significant effect with the addition of 10% serum to cells compared to serum free treatment (figure 3.11), suggesting sub-optimal baseline experimental conditions.

As a final line of investigation, we speculated that conditioned medium from TNF treated macrophages in the presence of GSK1995057 and controls may promote hAT2 wound healing. Mononuclear cell conditioned medium enhanced bovine bronchial epithelial cell migration in a TNF dependent manner (Ito et al., 1996), whilst in a rodent model, TNF released from LPS activated alveolar macrophages induced epithelial GM-CSF expression, which in turn initiated alveolar epithelial cell proliferation, contributing to alveolar repair (Cakarova et al., 2009). We did not observe any differences in wound healing between TNF and vehicle treated macrophages, with no further effect from selective TNFR1 antagonism (figure 3.13). These data are consistent with those generated by the Matthay group in San Francisco (Geiser et al., 2001). Pulmonary oedema fluid from patients with ALI increased alveolar epithelial repair by 33% compared to pooled plasma from healthy donors. Consistent with their own animal data (Geiser et al., 2000) this effect appeared to be mediated by IL-1 β . Of note, TNF did not promote wound healing in their models and inhibition of TNF in the BALF by recombinant sTNFR1 had no effect on wound healing *in vitro*.

The *in vitro* scratch assay has a number of limitations. Since the scratch is introduced manually with a pipette tip, the wound sizes are variable and hard to reproduce. Moreover, the biologic relevance of scratch wounding for lung disease is unclear. Electron microscopic images of type I alveolar epithelial cells of severely stressed rat lungs show large cell breaks and fenestrations of denuded basement membrane (Dreyfuss et al., 1985; Tsukimoto et al., 1991; West et al., 1991), which resemble some of the lesions produced by *in vitro* scratch. Nevertheless, the mechanisms responsible for repair of these very large cell wounds could be very different from those observed in other *in vitro* models (e.g. CMS), and presumably in patients. Despite these

limitations, the *in vitro* scratch assay offered a simple method to screen for effects of differential TNFR signalling on hAT2 wound healing.

3.3.16 Effect of differential TNFR signalling on tube formation in HUVEC and HMVEC-L

We observed a modest but significant increase in tube formation in HUVEC stimulated with the selective TNFR2 mutein D143N (figure 3.14). A trend towards this effect was observed in HMVEC-L but this was non-significant (figure 3.3.8) probably due to lower sample numbers in the HMVEC-L group. Further experiments to increase the size of this dataset (currently on-going) may delineate any pro-angiogenic effect of selective TNFR2 agonism in HMVEC-L. Of note, TNF at 1ng/mL and 10ng/mL had no significant effect.

The role of TNF in angiogenesis is controversial with numerous studies showing that it is either pro- or anti-angiogenic, with the preponderance of published studies suggesting that TNF has inconsistent effects *in vitro*, but enhances angiogenesis *in vivo*. Hence, TNF (2.5ng/ml) failed to induce dermal HMVEC tube formation in a fibrin matrix (Koolwijk et al., 1996), while TNF (1ng/ml) induced HUVEC tube formation in a proprietary angiogenesis system (Zhu et al., 2007). Using growth factor reduced-Matrigel, similar to that used in our study, TNF induced an angiogenic effect in dermal HMVEC at 0.1ng/ml (Shu et al., 2012). These data were in agreement with previous studies, in which TNF induced tube formation at low concentrations of TNF (Pan et al., 2002) but the effect was lost at higher concentrations of TNF (Leibovich et al., 1987). Thus, the lowest dose of TNF used in our studies may have been a log fold too high to demonstrate an effect.

Alternatively, these differing reports suggest that *in vitro* tube formation may rely on cell type, and the different matrixes and/or methods employed.

TNFR2 activates Etk, a member of the Bruton's tyrosine kinase non-receptor tyrosine kinase family involved in TNF-induced angiogenic events (Pan et al., 2002; Zhang et al., 2003) in vascular endothelial cells. Etk signalling involves tyrosine phosphorylation of the intracellular regions of vascular endothelial growth factor receptor 2 (VEGFR2), resulting in a VEGF-independent pro-angiogenic response mediated through a signalling cascade including phosphatidylinositol 3-kinase and Akt (Zhang et al., 2003). *In vivo*, loss of TNFR1 (p55 receptor) leads to enhanced angiogenesis in both retina (Sainson et al., 2008) and wounded skin (Mori et al., 2002). Furthermore, TNFR1 knockout mice demonstrated enhanced hind limb angiogenesis after temporary ischaemia compared to TNFR2 knockout animals (Luo et al., 2006). The same authors went on to demonstrate that, in a femoral artery ligation model, TNFR2 transgenic mice had enhanced ischemia-mediated arteriogenesis and angiogenesis (Luo et al., 2010). Endothelial cell specific expression of TNFR2 in TNFR2-deficient mice was sufficient to augment ischemia-induced Etk induced VEGFR2 signalling and functional flow recovery, thus further substantiating the concept that TNFR2 in endothelial cells uses Etk to trans-activate VEGFR2 and promote angiogenesis.

In our *in vitro* assay, we failed to observe a reproducible effect with respect to tube formation across different endothelial cell types with TNFR2 specific agonism (figure 3.14). This may relate to a combination of factors. First, differences in cell type, and possibly quantitative TNFR expression, may influence tube formation *in vitro*. Second, we have failed to demonstrate conclusive evidence of downstream signalling by Etk following TNFR2 agonism in HMVEC-L (Appendix A). Third, whilst a modest effect was observed in HUVEC, the use of TNFR2 agonistic antibody to enhance effects of

D143N may have amplified any potential effect; hence in KYM-1 and HeLa cells the agonistic TNFR2-specific anti-serum MR2-1 had an additive effect on TNFR2 stimulation of JNK activity when combined with a TNFR2 specific mutein. The mechanism of this effect remains elusive (Jupp et al., 2001). Finally, owing to limited cell numbers, we were unable to replicate the experimental numbers in HMVEC-L compared to HUVEC. The trend towards an effect of TNFR2 signalling was observed in HMVEC-L but this failed to reach significance owing to a small treatment effect and low experimental numbers (n=4). Efforts to increase the dataset are underway. Furthermore, in neither tube formation assay were we able to confirm any treatment effect of selective TNFR agonism with TNFR1 antagonism owing to technical difficulties of the assay protocol.

3.3.17 TNF induced permeability in HMVEC-L was TNFR1 dependent

TNF increased HMVEC-L permeability as measured by changes in TER (figure 3.15). TNF increases endothelial permeability by a variety of mechanisms including microtubule rearrangement and adherens junction disassembly via p38 MAPK activation (Kierner et al., 2002; Nwariaku et al., 2002; Petrache et al., 2003), increasing tyrosine phosphorylation of inter-endothelial junction proteins, actin stress fiber formation, and Ca^{2+} influx by a Src-dependent mechanism (Tiruppathi et al., 2001), by promoting Ca^{2+} influx via transient receptor potential channel 1 (Paria et al., 2003) and via protein kinase C activation of RhoA (Wojciak-Stothard et al., 1998). All of these data, however, were generated in HUVEC, representative of post-capillary venules in the systemic circulation, which are known not to be representative of pulmonary microvascular endothelium. Indeed, myosin light-chain kinase (MLCK) phosphorylation causing actinomyosin-based cell contraction and the formation of micrometer-scale gaps is proposed as one of the main effector pathways in systemic vascular bed hyperpermeability (Dudek and Garcia,

2001). The role of this mechanism in pulmonary oedema formation however, was questioned by a study demonstrating that LPS induced lung injury was not attenuated in mice with MLCK selectively deleted from endothelial cells (Yu et al., 2012).

TNF induced changes in trans-endothelial resistance appeared to be mediated by TNFR1 signalling as GSK1995057 abrogated the effect (figure 3.15). These data are concordant with a study that measured the TNFR dependency of transendothelial ¹²⁵I albumin flux across HUVEC monolayers (Ferrero et al., 2001). TNF induced increases in albumin flux were abrogated by a monoclonal anti-TNFR1 antibody and replicated by the anti-TNFR1 monoclonal antibody htr-9 and the TNFR1 selective mutein R32W-S86T. Moreover, TNF induced hyper-permeability was unaffected by anti-TNFR2, rabbit polyclonal anti-TNFR2 antiserum or the TNFR2 mutein D143N-A154R. These effects were mediated by p38 MAP kinase dependent TNFR1 induced cytoskeletal reorganization.

One potential mechanism of TNFR1 mediated pulmonary hyper-permeability, independent of the action of MLCK, is disruption of vascular endothelial cadherin (VE-cadherin). VE-cadherin, an endothelium-specific member of the cadherin family of adhesion proteins that binds to several cytosolic protein partners, including α , β - and p120-catenins, is a major component of adherens junctions. Endothelial permeability is regulated, in part, by the dynamic opening and closure of cell-cell adherens junctions. Thus, disruption in function and organisation of VE-cadherin increases vascular permeability. In HMVEC-L, TNF induced junctional instability by Src-family tyrosine kinase-mediated phosphorylation of adherens junction proteins including VE-cadherin (Angelini et al., 2006). These changes were associated with intercellular gap formation in HMVEC-L monolayers and increased trans-endothelial flux of [¹⁴C] bovine serum albumin in the absence of endothelial cell injury or apoptosis. Of note, further data have

demonstrated a reduction in expression of VE-cadherin in post-mortem biopsies of patients with ALI compared to control tissue from cancer-free lobectomy specimens (Herwig et al., 2013); these changes were most marked in the pulmonary capillaries.

A further explanation for these data would be TNFR2 induced death signalling. Whilst we did not specifically investigate HMVEC-L viability or apoptosis in this assay, both TNF and GSK1995057 treated cells demonstrated no evidence of loss of viability as measured by MTT assay in previous experiments (figure 3.4).

3.3.18 The effect of selective TNFR1 antagonism on markers of injury in TNF stimulated HMVEC-L

Injury and activation of the pulmonary endothelium are recognized as inciting events in the pathogenesis of ALI (chapter 1.1.2.2). TNF stimulation of HMVEC-L increased release of soluble forms of ICAM-1 (sICAM-1), VCAM-1 (sVCAM-1) and E-selectin (sE-selectin), markers of endothelial injury, into cell supernatants (figure 3.16). TNF induced HMVEC-L injury was mediated predominantly by TNFR1 as selective TNFR1 antagonism reduced adhesion molecule release to basal levels, although these data failed to reach statistical significance owing to small numbers (figure 3.16).

Elevated levels of circulating adhesion molecules are associated with sepsis (the commonest cause of ALI). Hence both sICAM-1 and sVCAM-1 were elevated in septic patients, their levels correlating with multi-organ failure and death (Gearing and Newman, 1993; Tsokos and Fehlaue, 2001), whilst circulating levels of sE-selectin were elevated in patients with sepsis and shock (Kayal et al., 1998; Reinhart et al., 2002)

Moreover, elevated levels of sICAM-1 and sE-selectin have been associated with poor outcomes in clinical ALI. In 67 patients with hydrostatic oedema or ALI, both

plasma and BALF ICAM-1 levels were significantly higher in those with ALI and increasing levels of ICAM-1 over time were associated with subsequent death after controlling for clinical variables (Calfee et al., 2009). Similarly, a threefold increase in BALF levels of ICAM-1 was observed in ALI patients compared to those with hydrostatic pulmonary oedema and levels of sICAM-1 correlated with the duration of mechanical ventilation (Conner et al., 1999). Finally, in a study of 83 paediatric patients with ALI, plasma ICAM-1 levels greater than 1,000ng/mL were associated with an 82% specificity for predicting non-survivors (Flori et al., 2003). In a study of 50 patients admitted with the systemic inflammatory response syndrome, elevated plasma E-selectin on admission predicted the development of ALI (and other organ failures). Mortality was also higher in patients with elevated plasma E-selectin (27.3%vs 0%) (Okajima et al., 2006). Furthermore, sE-selectin (and ICAM-1) levels were greater in the plasma of patients with sepsis than those who had sustained trauma not only when the patients were at risk for ALI but also at the time they had established ALI (Moss et al., 1996). In 27 patients with pneumonia, sE-selectin demonstrated utility in predicting those patients who would develop ALI (Osaka et al., 2011).

The biological function of soluble adhesion molecules remains unclear. In the alveolar compartment sICAM-1 has been reported to bind alveolar macrophages and enhance inflammatory cytokine production by alveolar macrophages in response to injurious stimuli (Schmal et al., 1998). Similarly, sVCAM-1 has been shown to either limit leukocyte integrin binding to endothelial VCAM-1 by binding to leukocytes or stimulate leukocyte (eosinophil) chemotaxis (Kitani et al., 1998; Ueki et al., 2009). Nonetheless, our *in vitro* data support the observation in a human *in vivo* inhaled LPS model that selective TNFR1 antagonism reduced endothelial cell injury/activation (O'Kane et al., 2013); reduced levels of endothelial injury may have contributed to the

31% reduction in BALF neutrophils with selective TNFR1 antagonism observed in this study.

3.3.19 Future directions

The respective roles of TNFR1 and TNFR2 signalling in promoting / preventing pulmonary oedema require further investigation. We have demonstrated that TNFR1 signalling mediates lung microvascular permeability and study of VE-cadherin expression in TNF treated HMVEC-L pre-incubated with GSK1995057 and controls is currently ongoing in our laboratory.

A further understanding of the cues for epithelial repair is undoubtedly one of the major challenges in the field. Whilst the scratch assay yielded equivocal results regarding the role of differential TNFR signalling in hAT2 repair, further investigation in tissue precision cut lung slice models (under development in our laboratory) may offer a more robust and longer-term (up to 5 days) model to define these mechanisms. Moreover, identification of specific downstream signalling events in both hAT2 and HMVEC requires further investigation. These experiments may be optimized by the use of MR2-1 to enhance TNFR2 signalling.

4 Chapter 4: The role of TNFR signalling in neutrophil priming and neutrophil migration into the alveolar space

4.1 Introduction

Numerous experimental and clinical studies suggest that neutrophils are key cells in the pathogenesis of ALI (reviewed in chapter 1.3.4). Indeed, neutrophilic alveolitis is a histological hallmark of ALI (Lee and Downey, 2001). It is also evident that the presence of neutrophils *per se* is not necessarily damaging, and that the activation status of these cells was a major determinant of their role in modulating injury (Chollet-Martin et al., 1992).

The pleiotropism of TNF is central to the processes by which neutrophils interact with the endothelium. First, TNF primes neutrophils in readiness for firm adhesion to pulmonary endothelium and subsequent neutrophil-mediated tissue injury through the enhancement of the expression of CD11b/CD18, triggering adherent neutrophils to release reactive oxygen species (ROS) and by promoting neutrophil degranulation and phagocytosis (Condliffe et al., 1996; Drost and MacNee, 2002; Dri et al., 1991; Nathan, 1987; Gamble et al., 1985; Richter et al., 1989; Shalaby et al., 1987). Second, TNF regulates the expression of key neutrophil adhesion molecules on activated endothelial cells, including ICAM-1, VCAM- 1 and E-selectin. (Mackay et al., 1993; Slowik et al., 1993; Neumann et al., 1996; Eriksson et al., 2005). Finally, TNF modulates neutrophil lifespan, and thus the potential duration of neutrophil-mediated tissue injury (Murray et al., 1997; Salamone et al., 2001).

The complexity of TNF directed neutrophil-endothelial interactions was highlighted in experiments using TNFR^{-/-} mice injected with either wild-type neutrophils or TNFR deficient neutrophils (Woodfin et al., 2009). TNF mediated endothelial activation was

essential for *both* neutrophil adhesion and trans-endothelial migration, whilst neutrophil priming / activation was required for adhesion but not full neutrophil migration. Furthermore, dissecting the distinct roles of TNFR1 and TNFR2 in each phase of neutrophil priming / activation, adhesion and trans-endothelial migration has to date been complicated by the use of a range of monoclonal and polyclonal blocking antibodies, or specific agonist mutant proteins (chapter 1.3.7). Monoclonal antibodies in particular have a tendency to induce receptor cross-linking, thereby inducing partial agonism rather than inhibiting signalling, whilst many receptor specific muteins lack specificity.

Much data establishing potential mechanisms of TNF directed neutrophil-endothelial interactions occurring within the systemic circulation has been reported. Murine *in vivo* models of systemic post-capillary venules, or *in vitro* studies using endothelial cells derived from large veins eg. HUVEC are often used. However, as outlined in chapter 1.4.4, neutrophil trafficking to the lung entails several structural, functional and mechanistic differences to that observed in the systemic circulation. The development of live lung imaging microscopy techniques in animals has unquestionably informed, and will continue to progress, the field of neutrophil-endothelial interactions in the lung (Looney and Bhattacharya, 2013). Nonetheless, validation and translation of such data in relevant human models remains central to the progression of our understanding of human ALI and the development of therapies and therapeutic targets.

The work in the preceding chapter demonstrated that TNFR1 antagonism abrogated selected cytokine and neutrophil chemokine release from TNF-stimulated hAT2 and HMVEC-L *in vitro*. The aim of the work in this chapter was to investigate the hypothesis that human pulmonary neutrophil trafficking is TNFR1 dependent. Therefore, human neutrophils and HMVEC-L were used, along with a highly selective TNFR1 antagonist and TNFR specific muteins. Specifically, the aims were:

- a. To establish whether TNFR1 signalling directs TNF induced neutrophil priming and activation**
- b. To determine the TNFR1 dependency of TNF induced pro-apoptotic and pro-survival effects in human neutrophils, and**
- c. To investigate the functional effects of selective TNFR1 antagonism on expression of neutrophil adhesion molecules by HMVEC-L and neutrophil migration through HMVEC monolayers in response to IL-8**

4.2 Specific methods

4.2.1 Granulocyte experiments

Human granulocytes were isolated according to the protocol outlined in chapter 2.2.5.5 and re-suspended at a concentration of $5 \times 10^6/\text{mL}$. In experiments using inhibitors, granulocytes were pre-incubated with GSK1995057 (10nM), dummy domain antibody (10nM) and Adalimumab (1nM) for 60 minutes then treated with TNF (10ng/mL) or vehicle control for time periods specific to the experimental conditions. These concentrations were established from experiments in chapter 3.3.3; it was decided that a single dose of GSK1995057 and controls would be used throughout all *in vitro* experiments. In experiments using the TNFR specific muteins granulocytes were treated with R32W (TNFR1 specific mutein, 100ng/mL) and D143N (TNFR2 specific mutein, 100ng/mL), TNF (10ng/mL) or vehicle control. The concentration of 100ng/mL for mutein experiments was based on published data in organ culture where a dose range of 10-100ng/mL was used (Al-Lamki et al., 2010; Al-Lamki et al., 2005; Tchorzewski et al., 1993).

4.2.2 HMVEC-L experiments

HMVEC-L were used between passages 3 and 6. Following growth to confluence, cells were exposed to GSK1995057 (10nM), dummy domain antibody (10nM) and Adalimumab (1nM) for 60 minutes then treated with TNF (10ng/mL) or vehicle control for 4 hours. For analysis of neutrophil adhesion molecule expression, cells were washed and resuspended following gentle trypsinisation (2 minutes, trypsin 0.04% / EDTA 0.03%, Promocell, Germany) prior to staining.

4.3 Results

4.3.1 Effect of TNF on granulocyte priming status

Shape change, CD11b up-regulation and cell surface CD62L loss could be induced by TNF, suggesting that cells were minimally primed at baseline, retained normal granulocyte function and that TNF induced priming *in vitro* (figures 4.1 & 4.2).

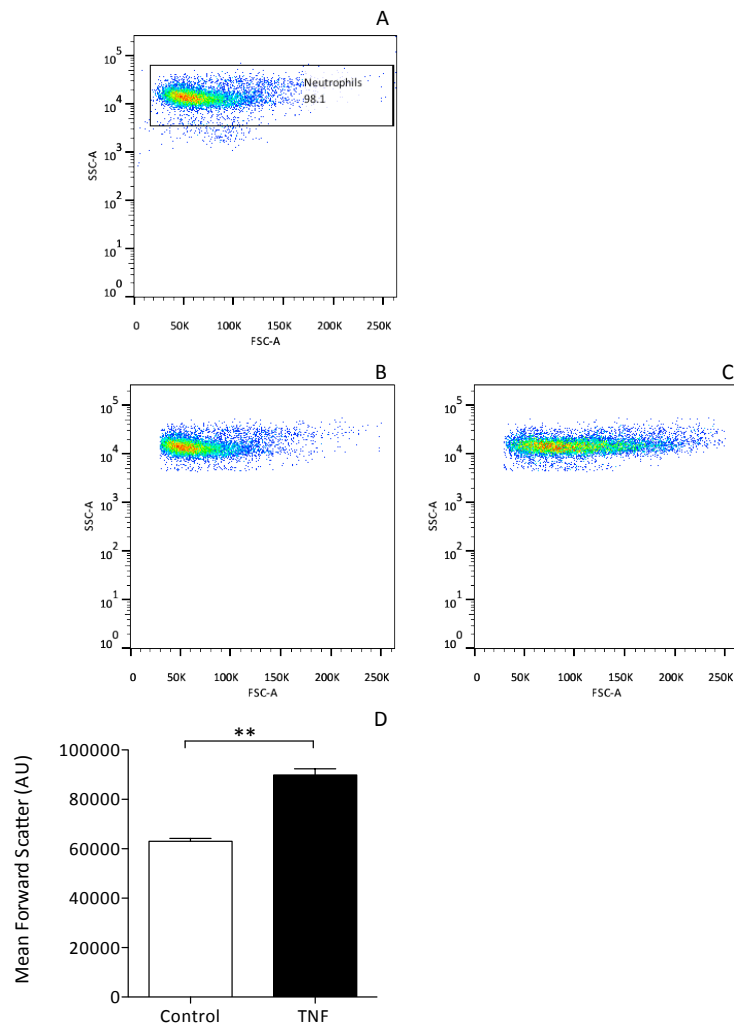


Figure 4.1 TNF induced shape change in granulocytes *in vitro*

Human granulocytes ($5 \times 10^6/\text{mL}$) treated with TNF (10ng/mL) or vehicle control were analysed by flow cytometry. Shape change was assessed by mean forward scatter. Panel A shows a representative density plot of the entire population; the boxed area contains the granulocyte population used for subsequent analysis of forward scatter. Panels B & C show representative density plots of Control and TNF treated granulocytes respectively. Panel D demonstrates data obtained from 5 separate experiments. Data are presented as mean $\pm$ SEM analysed by Mann Whitney U test **p<0.01

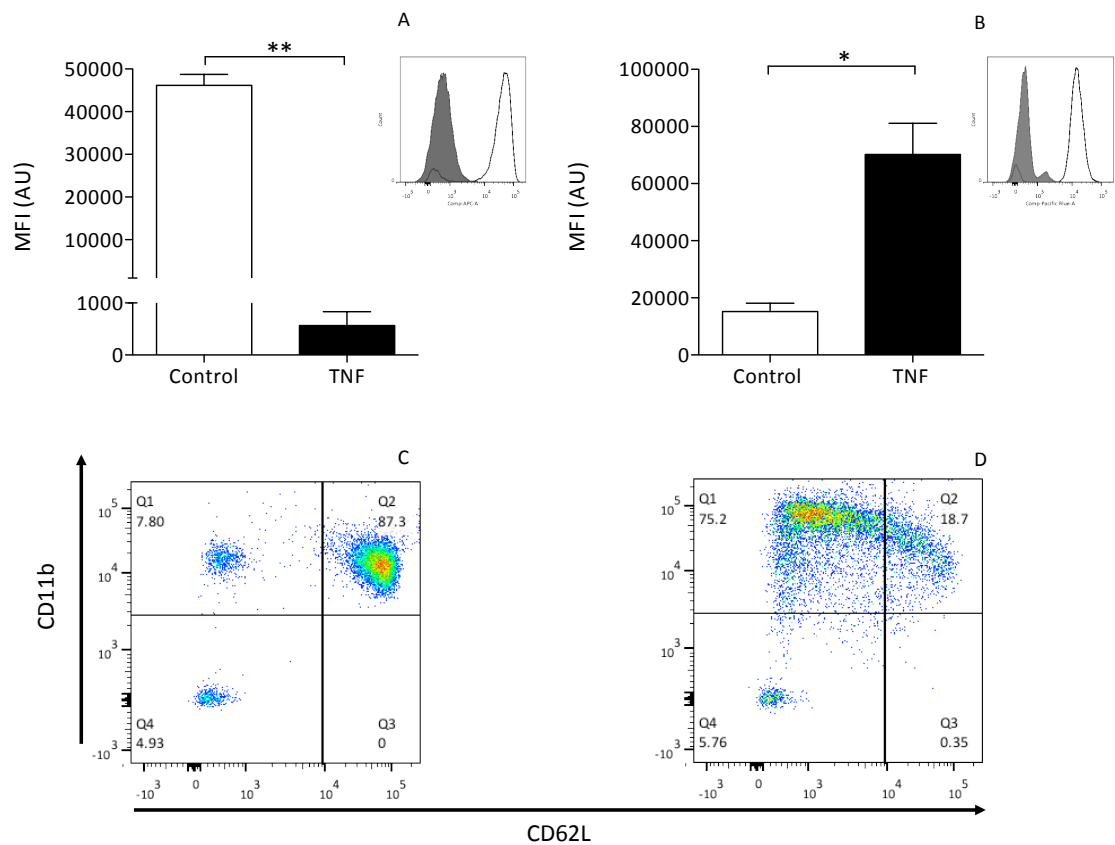


Figure 4.2 TNF induced CD11b up-regulation and CD62L shedding from granulocytes

Human granulocytes ($5 \times 10^6/\text{mL}$) were treated with TNF (10ng/mL) or vehicle control for 45 minutes then stained with anti-human CD11b Pacific Blue and anti-human CD62L APC and before being analysed by flow cytometry. Panel A shows CD62L expression and panel B CD11b expression in control and TNF treated cells ($n=3$). Staining with the respective antibody (solid line) and isotype control (shaded fill) is shown in the inserted histograms. Panel C is a representative scatter plot of control cells (CD62 high, CD11b low), while panel D shows TNF treated cells (CD62 low, CD11b high). Data are presented as mean $\pm$ SEM and analysed by Mann Whitney U test * $p < 0.05$, ** $p < 0.01$ MFI; median fluorescence intensity

4.3.2 Differential TNFR signalling and granulocyte priming

Having demonstrated that TNF primes granulocytes *in vitro*, the aim of subsequent experiments was to determine which TNFR regulates mediates this effect. The TNFR1 mutein R32W induced shape change, CD62L loss and CD11b upregulation in a similar manner to TNF, whilst D143N, a TNFR2 specific mutein had no effect on these parameters compared to vehicle treated cells (figure 4.3).

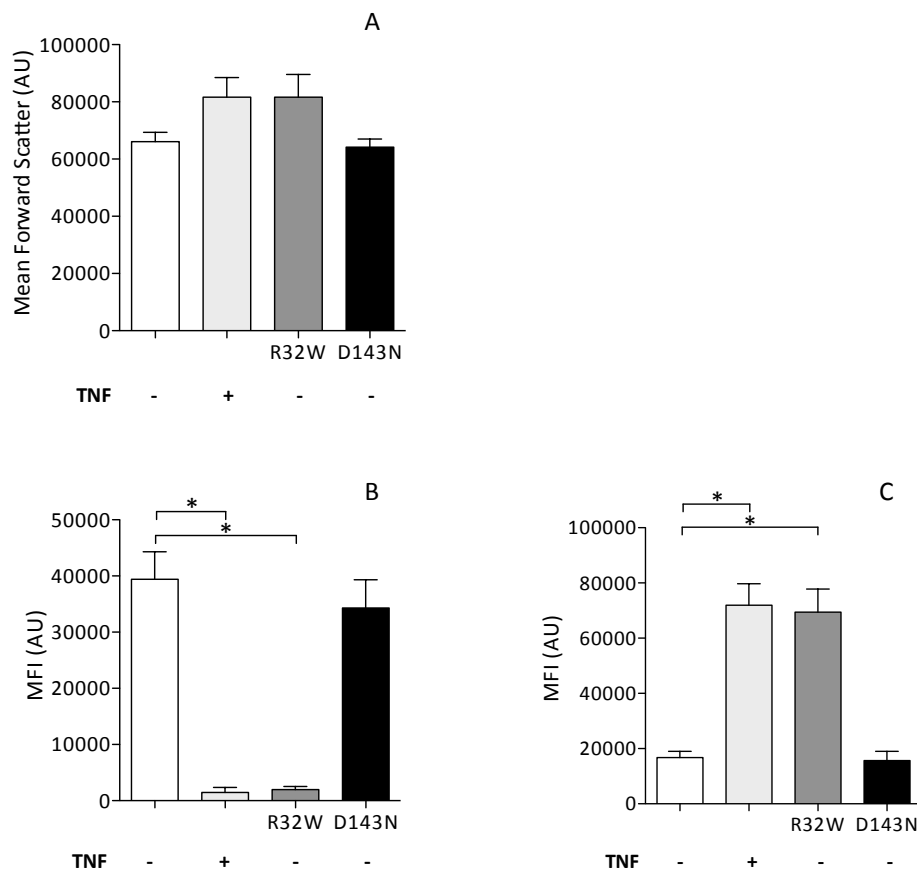


Figure 4.3 TNFR1 signalling induced granulocyte priming

Human granulocytes ($5 \times 10^6/\text{mL}$), treated with TNF (10ng/mL), the TNFR1 specific mutein R32W (100ng/mL), the TNFR2 specific mutein D143N (100ng/mL) or vehicle control for 45 minutes were stained with anti-human CD11b Pacific Blue and anti-human CD62L APC then analysed by flow cytometry. Panel A shows mean forward scatter, Panel B CD62L expression and Panel C CD11b expression in treated granulocytes ($n=5$). Data are presented as mean $\pm$ SEM and analysed by Kruskal-Wallis (Dunns) $*p<0.05$. MFI; median fluorescence intensity

Having shown TNFR1 was likely be the dominant effector with respect to granulocyte priming, we examined whether TNF-induced priming could be abrogated by selective TNFR1 antagonism. Pre-incubation with a TNFR1 specific antagonist (GSK1995057, 10nM) abrogated TNF-induced granulocyte priming to control levels (figure 4.4 A-C). A TNFR1/2 monoclonal antibody (Adalimumab, 1nM) only partially inhibited this effect (figure 4.4 A-C) as demonstrated by 40% of cells remaining CD11b high, CD62 low (figure 4.4 H). A dummy domain antibody (10nM) had no effect on CD62L and CD11B expression paralleling that of TNF treated granulocytes (figure 4.4 E & G).

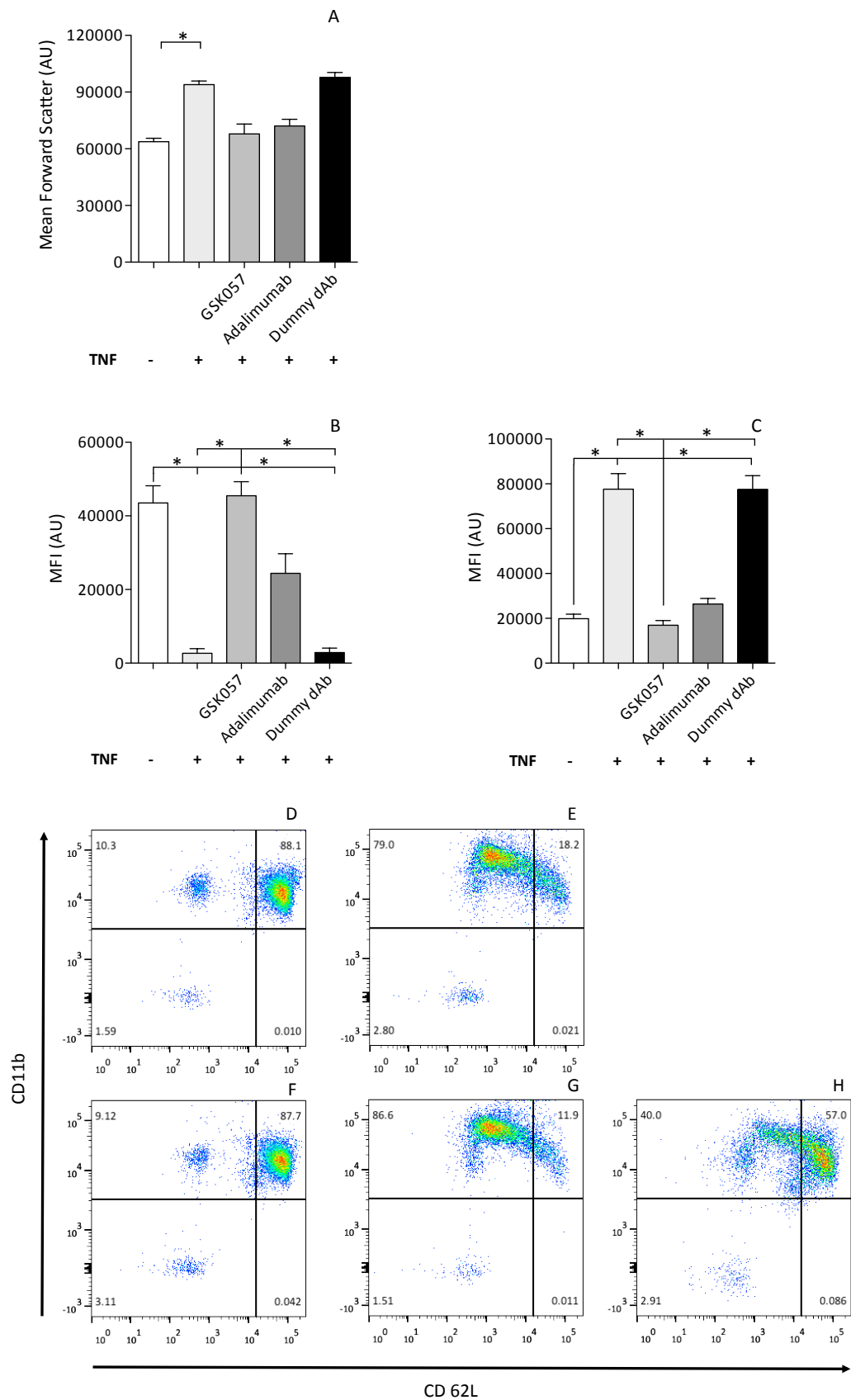


Figure 4.4 TNFR1 antagonism abrogated TNF induced priming of granulocytes

Human granulocytes ($5 \times 10^6/\text{mL}$) were pre-incubated with GSK1995057 (10nM), Adalimumab (1nM), Dummy domain antibody (10nM) or vehicle for 60 minutes then treated with TNF (10ng/mL) or vehicle control for 45 minutes. Cells were stained with anti-human CD11b Pacific

Blue, anti-human CD62L APC or isotype controls then analysed by flow cytometry. Panel A shows mean forward scatter, Panel B CD62L expression and Panel C CD11b expression in treated granulocytes (n=3). Panels D-H are representative scatter plots of CD62L and CD11b in control (D), TNF (E), GSK1995057 (F), dummy dAb (H) and Adalimumab (G) treated granulocytes. The numbers in each corner of the dot plot represent the percentage of cells in each quadrant. Data are presented as mean $\pm$ SEM and analysed by MFI; median fluorescence intensity

4.3.3 The role of TNFR signalling in superoxide burst from TNF primed granulocytes

Having demonstrated that TNFR1 antagonism maintained granulocyte priming at (non TNF primed) basal levels, the question of whether TNFR1 antagonism could inhibit effector functions in TNF primed granulocytes was addressed. Using luminol-dependent chemiluminescence, we examined superoxide burst from TNF and TNFR mutein primed, fMLP-stimulated granulocytes pre-incubated with GSK1995057 and controls. Consistent with the priming data, R32W significantly enhanced respiratory burst in parallel with TNF whereas TNFR1 antagonism by both GSK1995057 and Adalimumab reduced superoxide release towards basal levels (figure 4.5). Priming with D143N induced peak luminescence values that paralleled GSK1995057 suggesting that TNFR1 may be fully competent to induce superoxide generation, although further study is required.

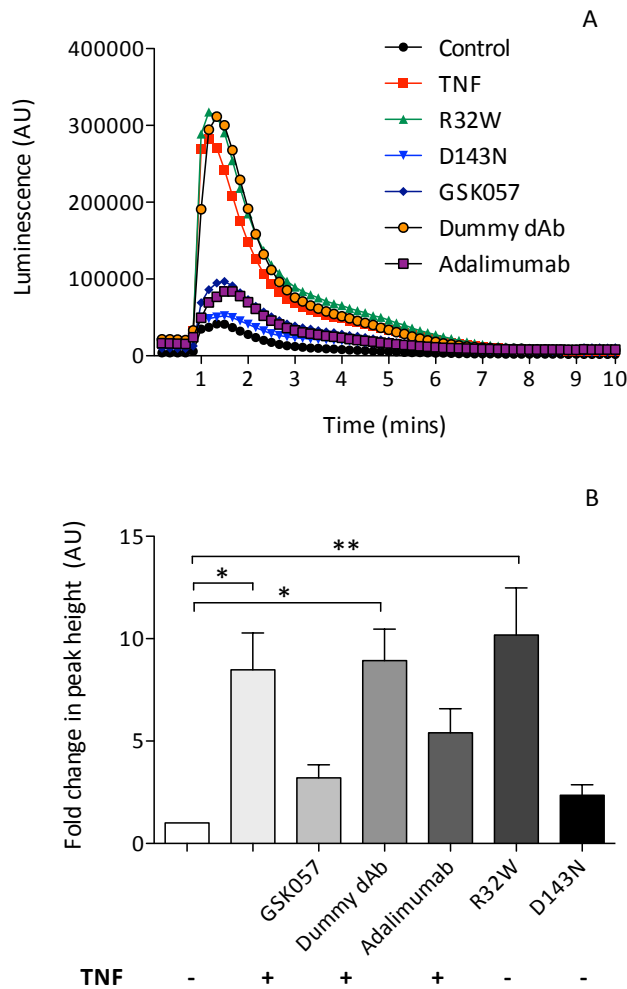


Figure 4.5 Selective TNFR1 antagonism inhibited superoxide release from TNF primed granulocytes

Human granulocytes ($5 \times 10^6/\text{mL}$) were pre-incubated with GSK1995057 or controls before priming with TNF (10ng/mL) or TNFR selective muteins (100ng/mL). Following fMLP stimulation superoxide release was measured by luminol-dependent chemiluminescence as assessed by the change in optical density of the supernatants at 550 nm over 10 minutes. Panel A shows a representative luminescence time course. Panel B demonstrates the cumulative fold change in peak height of luminescence compared to unprimed cells ($n=4$). The dotted line in panel B represents the peak luminescence value of vehicle treated cells. Data are presented as mean $\pm$ SEM and analysed by Kruskal-Wallis (Dunns) * $p < 0.05$, ** $P < 0.01$

4.3.4 TNFR expression and shedding from TNF stimulated granulocytes – the role of differential TNFR signalling

In view of the data suggesting that TNFR1 may modulate granulocyte priming, it was important to establish that the observed effect was not a consequence of alterations in expression of TNFR, in particular loss of cell surface TNFR2. Using flow cytometry, expression of both TNFR was confirmed in granulocytes (figure 4.6). TNFR expression, particularly TNFR 2 expression varied between donors; median fluorescence intensity (MFI) of TNFR1 expression of 5 donors was 31 (interquartile range 21.5-46, figure 4.6A) and the MFI of TNFR2 expression was 384 (IQR 213-3413 figure 4.6B).

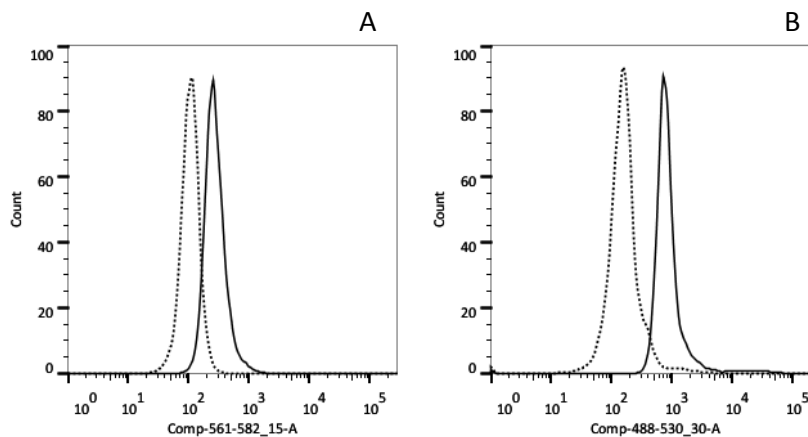


Figure 4.6 Human granulocytes expressed both TNFR1 and TNFR2

Purified human granulocytes ($5 \times 10^6/\text{mL}$) were stained with anti-human TNFR1 PE and antihuman TNFR2 FITC or isotype controls and analysed by flow cytometry. Panels A and B are representative of 3 separate experiments and demonstrate TNFR1 expression (Panel A) and TNFR2 expression (Panel B). IgG isotype controls are shown with the dotted line

TNF stimulation (10ng/mL) of granulocytes induced a significant reduction in TNFR1 cell surface expression (figure 4.7A). ELISA analysis of harvested supernatants suggested concentration-dependent shedding of TNFR1 maximal at 60 minutes and at lower TNF concentrations (figure 4.7B - 15 minute data not shown as sTNFR1 levels were below the lower detection limit of the ELISA). Both the expression and shedding of TNFR1

appeared to be induced via TNFR1 signalling as evidenced by parallel experiments stimulating granulocytes with TNFR selective muteins (figure 4.7 C&D). R32W induced down-modulation of TNFR1 expression and receptor shedding comparable to TNF whereas D143N had no effect on expression or shedding of TNFR1.

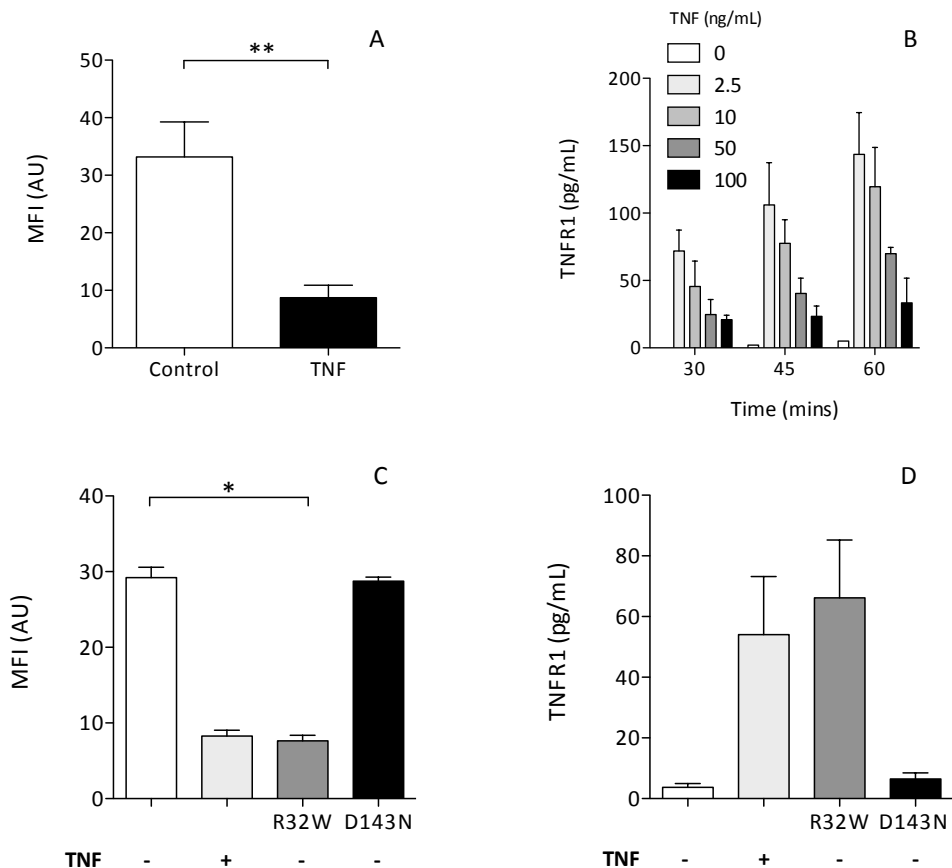


Figure 4.7 TNF receptor dependency on TNFR1 expression and shedding from human granulocytes was TNFR1 dependent

Human granulocytes ($5 \times 10^6/\text{mL}$) were exposed to TNF (2.5, 10, 50 or 100 ng/mL) or vehicle control for 1 hour. The cell suspension from triplicate wells were collected every 15 minutes over 1 hour, spun at 4°C and the supernatant frozen at -80°C for sTNFR1 ELISA. The cell pellet at the 45 minute time point was stained with anti-human TNFR1 PE for 30 minutes, washed twice then analysed by flow cytometry. Panel A shows TNFR1 expression in control and TNF treated granulocytes ($n=5$). Panel B shows the time course and concentration response of sTNFR1 in harvested supernatants ($n=3$). In parallel experiments, TNFR1 expression (panel C) and sTNFR1 release (panel D) was measured in TNF (10 ng/mL), R32W, D143N (both 100 ng/mL) and vehicle treated granulocytes over 45 minutes ($n=2-3$). Data are presented as mean $\pm$ SEM, analysed by Mann Whitney U test $**p < 0.01$ MFI; median fluorescence intensity

Conversely, TNFR2 expression was increased with TNF stimulation; the observed lack of priming response to D143N observed in figure 4.3 cannot, therefore, be ascribed to low expression of TNFR2. TNFR2 shedding was time but not concentration dependent in response to TNF (figure 4.8 A&B). TNFR2 expression increased approximately 3-fold in

response to TNFR1 signalling (figure 4.8C) but there was no clear response to either TNFR mutetin with regards to TNFR2 shedding.

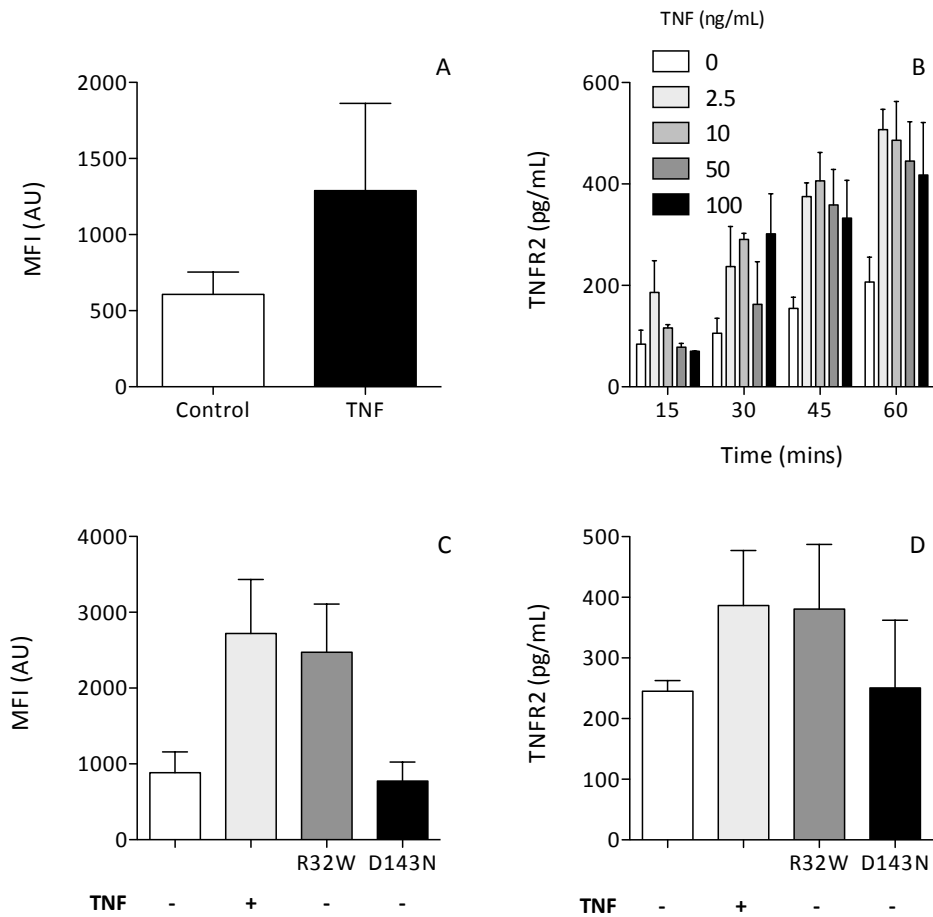


Figure 4.8 Effect of TNF and TNFR specific muteins on TNFR2 expression and shedding from human granulocytes

Human granulocytes ($5 \times 10^6/\text{mL}$) were exposed to TNF (2.5, 10, 50 or 100ng/mL) or vehicle control for 1 hour. The cell suspension from triplicate wells was collected every 15 minutes over 1 hour, spun at 4°C and the supernatant frozen at -80°C for sTNFR2 ELISA. The cell pellet at the 45 minute time point was stained with anti-human TNFR2 FITC for 30 minutes, washed twice then analysed by flow cytometry. Panel A shows TNFR2 expression in control and TNF treated granulocytes ($n=5$). Panel B shows the time course and concentration response of sTNFR2 in harvested supernatants ($n=3$). In parallel experiments, TNFR2 expression (panel C) and sTNFR2 release (panel D) was measured in TNF (10ng/mL), R32W, D143N (both 100ng/mL) and vehicle treated granulocytes over 45 minutes ($n=2-3$). Data are presented as mean $\pm$ SEM. MFI; median fluorescence intensity

4.3.5 TNFR2 agonism does not “de-prime” TNF primed granulocytes *in vitro*

Further to determining the role of TNFR signalling in granulocyte priming, the possibility that TNFR2 signalling could “de-prime” granulocytes was investigated. As outlined in chapter 1.3.4, emerging data suggest that TNFR2 signalling may be anti-inflammatory and contribute to organ repair. We speculated that de-priming of neutrophils, as a mechanism to dampen the inflammatory response, may be a component of these putative TNFR2 mediated processes. Granulocytes primed with TNF over 45 minutes were incubated with GSK1995057 (10nM) to ‘force’ TNFR2 signalling, the selective TNFR2 mutein D143N (100ng/mL) or Adalimumab (1nM) as a negative control for 1 hour before analysis by flow cytometry. Neither GSK1995057 nor D143N had any discernable effect on granulocyte shape change, CD11b or CD62L expression (figure 4.9).

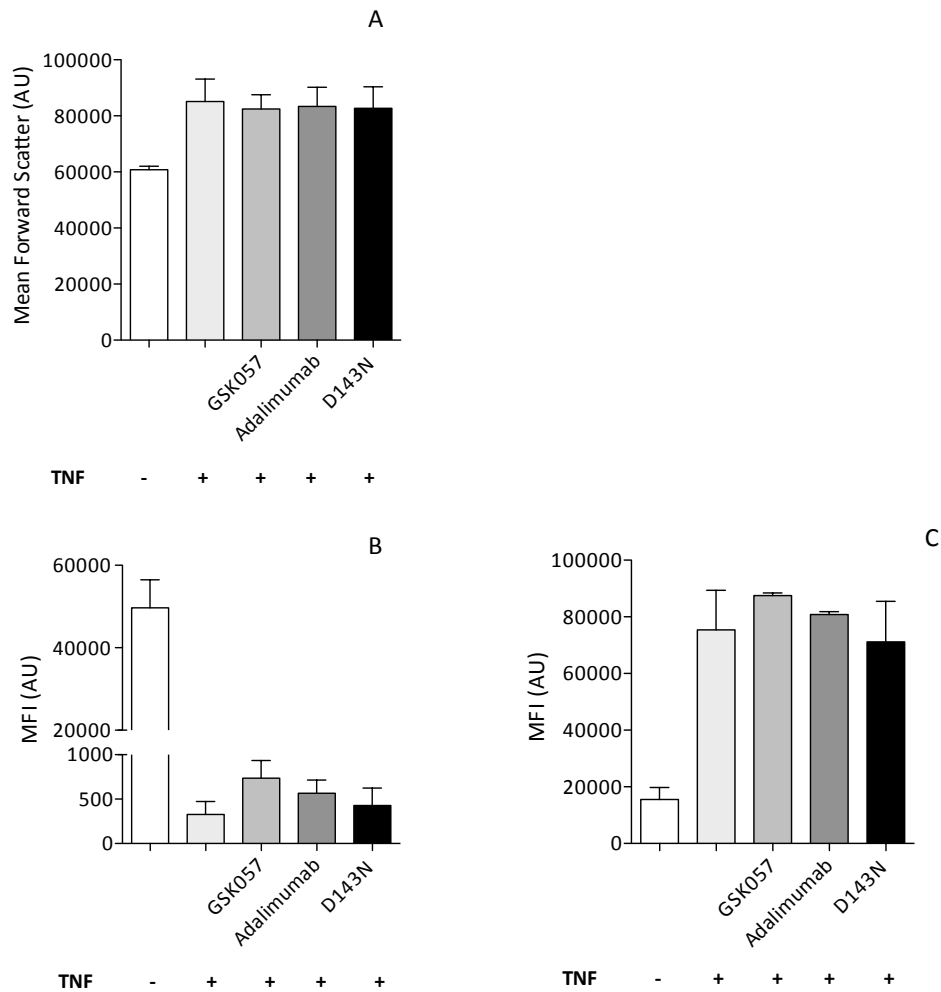


Figure 4.9 Selective TNFR2 signalling did not de-prime TNF primed granulocytes

Human granulocytes ($5 \times 10^6/\text{mL}$) treated with TNF (10ng/mL) then exposed to GSK1995057 (10nM), Adalimumab (1nM) or D143N (100ng/mL) were stained with anti-human CD11b Pacific Blue and anti-human CD62L APC and analysed by flow cytometry. Panel A shows mean forward scatter, Panel B CD62L expression and Panel C CD11b expression in treated granulocytes (n=3). Data are presented as mean $\pm$ SEM MFI; median fluorescence intensity

4.3.1 Differential TNFR signalling and modulation of neutrophil lifespan

TNF significantly induced granulocyte apoptosis at 6 hours whilst TNF induced a modest 10% pro-survival effect at 20 hours (vehicle treated granulocyte 20 hour survival 64.4% $\pm$ 8.8%, TNF treated 54.1% $\pm$ 10.2%) (figures 4.10A and 4.11A respectively). The potential role of TNFR1 signalling in determining these effects was therefore investigated.

Granulocyte-macrophage colony stimulating factor (GM-CSF, 10ng/mL), a potent inhibitor of apoptosis was used as a positive control.

Neither the TNFR1 mutein (R32W) nor the TNFR1 antagonist (GSK1995057) affected basal, or in the latter case, TNF stimulated apoptosis at 6 hours (figure 4.10A). The lack of discernable effect on apoptosis by R32W and GSK1995057 could not be explained by a lack of functional activity as previous experiments had demonstrated their ability to regulate TNF induced priming. These data therefore imply that activation of both TNFRs may be required to mediate the early TNF mediated death signal in granulocytes. To confirm this, both R32W and D143N were added in combination. However, the combination of both TNFR1 and TNFR2 muteins failed to replicate the level of apoptosis seen with TNF (figure 4.10A). Of note, pre-exposure with the non-functional dummy domain antibody, unexpectedly inhibited TNF induced apoptosis.

The involvement of TNFR1 in mediating the 20 hour pro-survival effect of TNF was evaluated in similar experiments. R32W (100ng/mL) replicated the modest pro-survival effect of TNF at 20 hours (R32W apoptosis 50.1% $\pm$ 12.3% vs TNF apoptosis 54.1 $\pm$ 10.2%, figure 4.11A), suggesting a possible role for TNFR1 signalling although these data did not reach statistical significance. This was further investigated by TNFR1 antagonism with GSK1995057 (10nM); cells pre-incubated with GSK1995057 demonstrated levels of apoptosis similar to control treated cells.

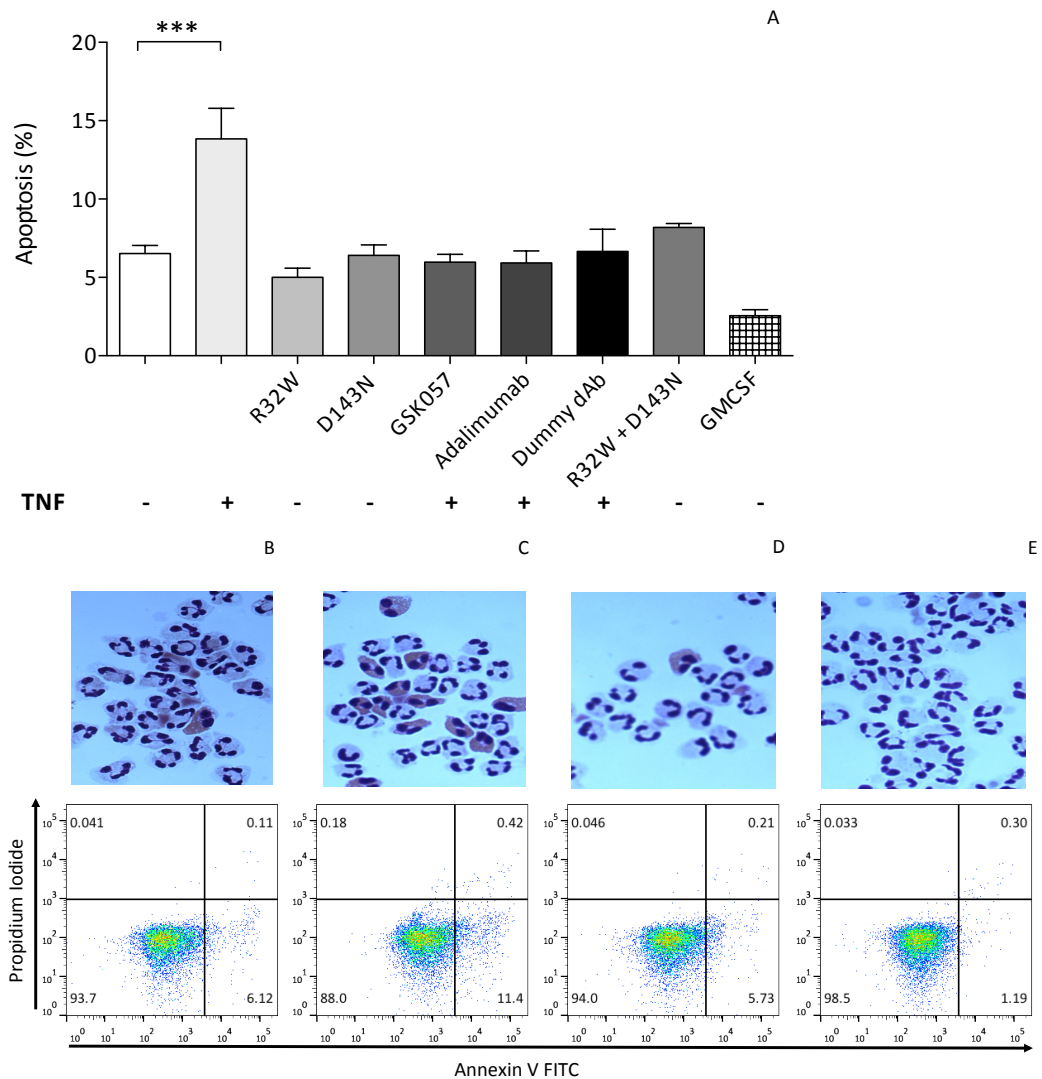


Figure 4.10 Early granulocyte apoptosis required activation of both TNFR

Granulocyte apoptosis was assessed by annexin-V-FITC / propidium iodide (PI) staining analysed by flow cytometry following 6 hour incubation with GSK1995057 (10nM, n=5), Adalimumab (1nM, n=5), dummy dAb (10nM, n=6), TNF (10ng/mL n=6), vehicle control (n=6) or the TNFR specific muteins R32W and D143N alone (100ng/mL, n=5) or in combination (100ng/mL each, n=2). Panel A shows cumulative apoptosis represented as % annexin V positive cells (PI negative plus PI positive). Panels B-E show representative morphological images and dot plots of vehicle (B), TNF (C), GSK1995057 (D) and GMCSF (E) treated cells. Values shown in each corner of the dot plots represent the percentage of cell within each quadrant. Data are presented as mean $\pm$ SEM analysed by one way ANOVA (Dunnett's) *** $p < 0.001$

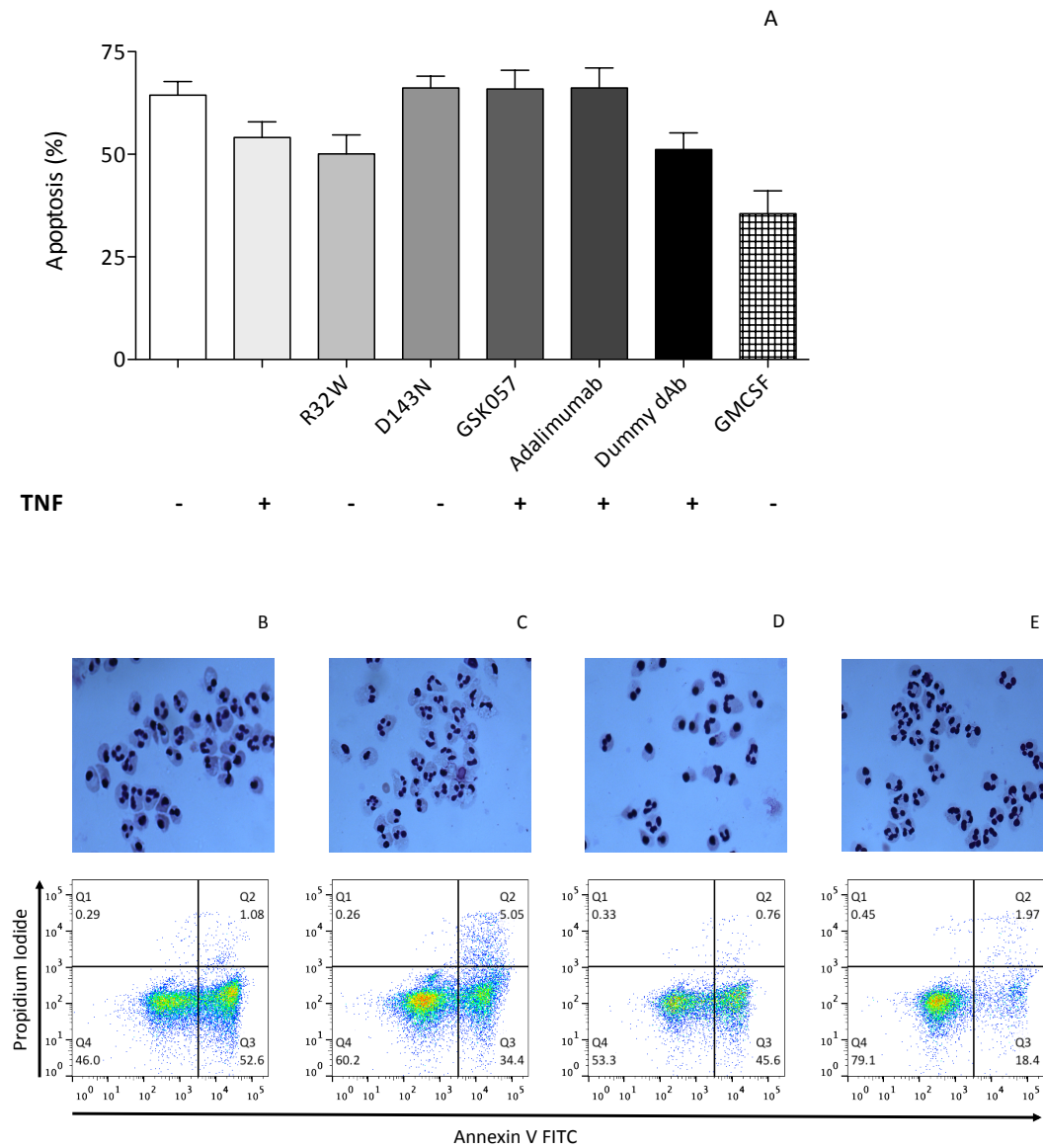


Figure 4.11 The effect of differential TNFR1 signalling directed on late granulocyte survival

Granulocyte apoptosis was assessed by annexin-V-FITC / propidium iodide (PI) staining analyzed by flow cytometry following 20 hour incubation with GSK1995057 (10nM), Adalimumab (1nM), dummy dAb (10nM), TNF (10ng/mL), vehicle control or the TNFR specific muteins R32W and D143N (100ng/mL) Panel A shows cumulative apoptosis; data are represented as % annexin V positive cells (PI negative plus PI positive) (n=6). Panels B-E show representative morphological images and dot plots of vehicle (B), TNF (C), GSK1995057 (D) and GMCSF (E) treated cells. Values shown in each corner of the dot plots represent the percentage of cell within each quadrant. Data are presented as mean $\pm$ SEM

4.3.2 Granulocyte migration through HMVEC and effect TNFR antagonism

The work in this chapter has so far focused on TNFR signalling and granulocyte activation and lifespan. Subsequent experiments were designed to interrogate the role of differential TNFR signalling on endothelial activation and the functional consequences thereof on granulocyte migration. Having optimised baseline conditions (chapter 2.2.6.5.1), granulocyte migration through TNF-stimulated HMVEC-L monolayers in the presence of GSK1995057 or controls was assessed. TNF (10ng/mL) increased granulocyte migration (figure 4.12) whilst pre-exposure of cells to GSK1995057 reduced migration to near basal levels ($p=0.17$), suggesting that TNFR1 may play a role in neutrophil-endothelial interactions.

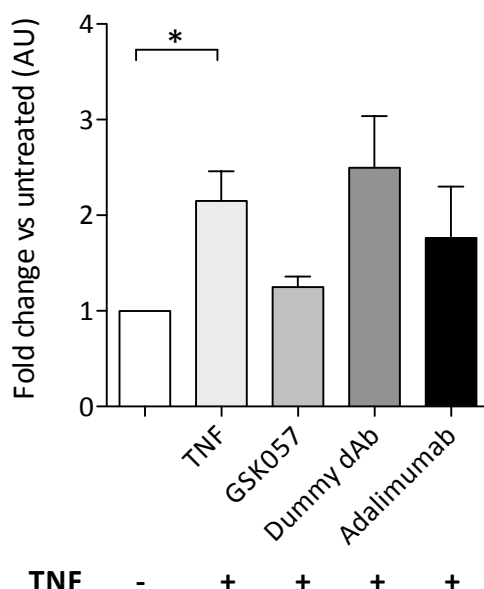


Figure 4.12 TNFR1 antagonism ameliorated granulocyte migration through TNF stimulated HMVEC-L

Leukotracker™ labelled granulocytes ($1 \times 10^6/\text{mL}$, $200 \mu\text{L}$) were added to the upper chamber of a Transwell filter containing confluent HMVEC-L (5×10^4) treated with TNF (10 ng/mL) in the presence of GSK1995057 (10 nM , $n=5$), Adalimumab (1 nM , $n=4$), a dummy dAb (10 nM , $n=5$) and vehicle control ($n=5$) for 4 hours. IL-8 (100 ng/mL , $700 \mu\text{L}$) was added to the lower chamber. Migration was assessed by measurement of fluorescence accumulated in the lower chamber after 3 hours read at 480 nm on a fluorescent plate reader. The dotted line represents granulocyte migration in vehicle treated HMVEC-L. Data are presented as mean $\pm$ SEM, $*p < 0.05$.

4.3.3 TNFR signalling and granulocyte receptor expression on HMVEC

To investigate a possible mechanism to explain the effects of differential TNFR activation on granulocyte migration through HMVEC-L monolayers, we analysed the expression of adhesion molecules known to direct neutrophil migration in TNF activated HMVEC-L pre-incubated with GSK1995057 and controls. Time course analysis of expression of adhesion molecules known to direct neutrophil migration was undertaken in TNF (10 ng/mL) stimulated HMVEC-L monolayers over 24 hours. Neither JAM1, nor ICAM-2 were induced by TNF (Figure 4.13) whilst, P-selectin could neither be detected at baseline nor after TNF stimulation (data not shown). By contrast, ICAM-1 and VCAM-1 were constitutively expressed (54.4% ICAM-1 positive $\pm 10.2\%$, 20.7% VCAM-1

positive $\pm 6\%$) and up-regulated in a time dependent fashion (Figures 4.137 & 4.148). Low-level expression of E-selectin (6.54% cells E-selectin positive ± 1.94) was detected at baseline with expression peaking at 4 hours (Figures 4.13 & 4.14). ICAM-1, VCAM-1 and E-selectin were therefore chosen to investigate the effects of differential TNFR signalling in modulating neutrophil adhesion molecule expression further experiments.

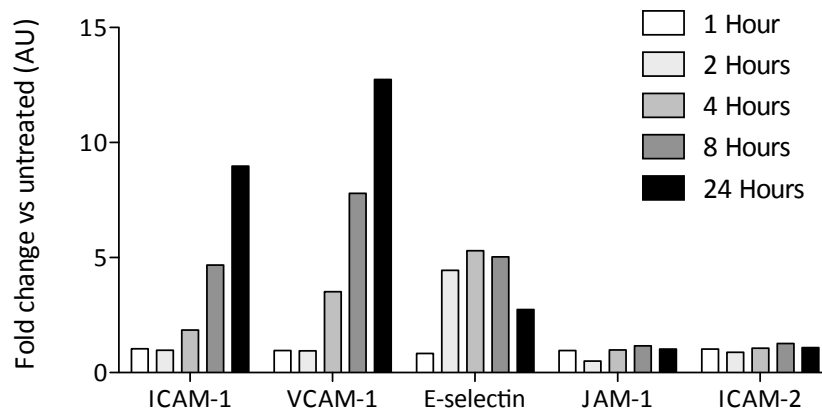


Figure 4.13 Time course of adhesion molecule expression in TNF stimulated HMVEC-L

HMVEC-L treated with TNF (10ng/mL) for 1,2,4,8 and 24 hours were exposed to anti-human ICAM-1 Pacific Blue, anti-human VCAM-1 APC, anti-human E-Selectin APC, anti-human JAM-1 PE, and anti-human ICAM-2 FITC and analysed in duplicate by flow cytometry. (n=1)

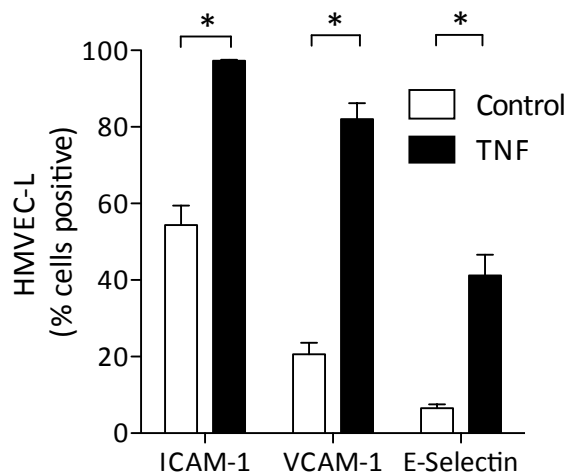


Figure 4.14 Induction of neutrophil adhesion molecule expression in TNF stimulated HMVEC-L

HMVEC-L (1×10^5) treated with TNF (10ng/mL) or vehicle control, were resuspended and exposed to anti-human ICAM-1 Pacific Blue, anti human VCAM-1 APC, anti-human E-Selectin APC, anti-human JAM-1 PE, and anti-human ICAM-2 FITC and analysed in duplicate by flow cytometry (n=4). Data are expressed as mean $\pm$ SEM analysed by Mann Whitney U test *p<0.05

The effect of selective TNFR1 antagonism on neutrophil adhesion molecule expression by HMVEC-L was analysed following 1 hour pre-incubation with GSK1995057 followed by TNF (10ng/mL) stimulation over 4 hours. Selective TNFR1 antagonism abrogated TNF induced expression of ICAM-1, 1 and VCAM-1 and E-selectin to constitutive levels (Figure 4.15). Treatment of HMVEC-L with the TNFR1 mutein R32W induced expression of all 3 neutrophil ligands in parallel with TNF but these preliminary data failed to reach significance owing to low experimental numbers. Dual TNFR receptor antagonism (Adalimumab) only partially blocked VCAM-1 expression (figure 4.15B) implying additional regulatory mechanisms for regulation of TNF induced VCAM-1 expression. Cumulatively, these data suggest a role for TNFR1 in the expression of neutrophil adhesion molecules by HMVEC-L.

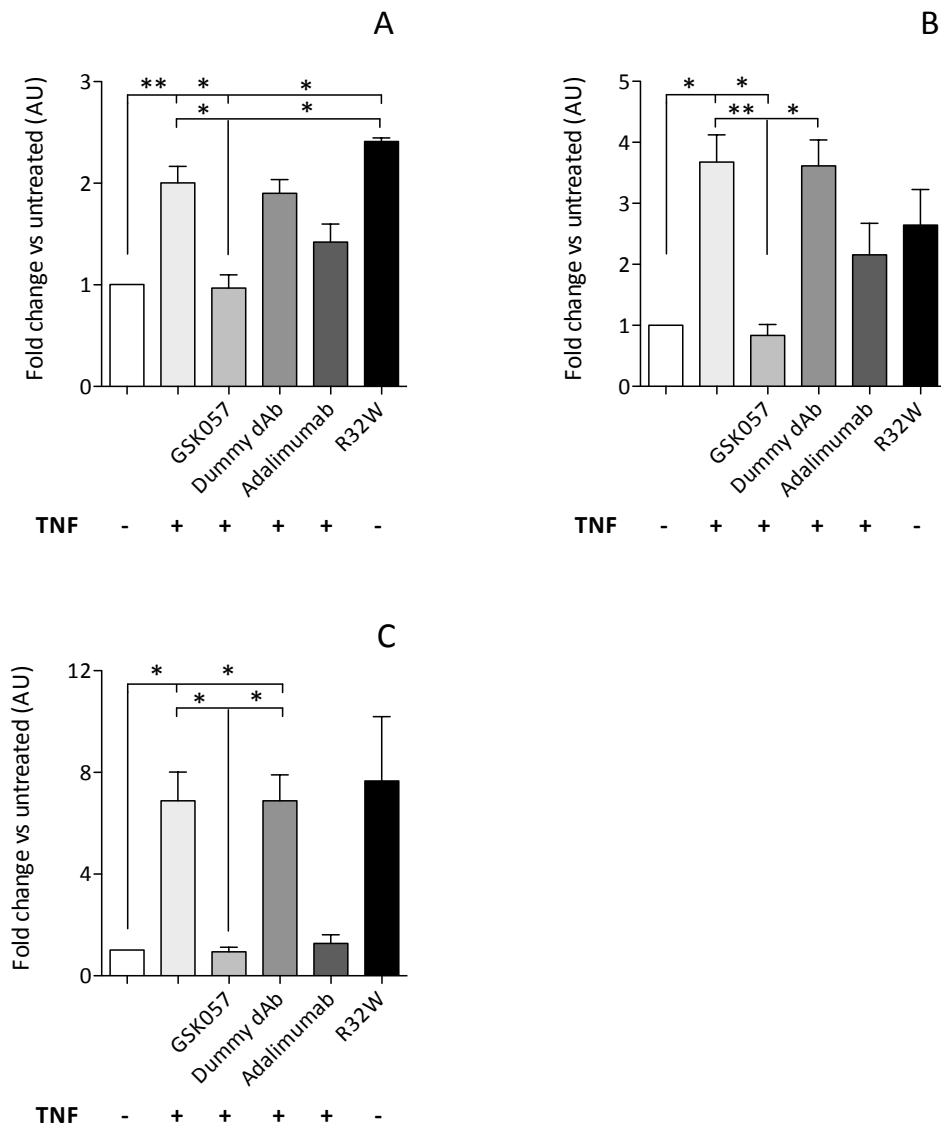


Figure 4.15 Selective TNFR1 antagonism abrogated ICAM-1 and VCAM-1 expression in TNF stimulated HMVEC-L

HMVEC-L (1×10^5) were pre-exposed to GSK1995057 (10nM), Adalimumab (1nM), a dummy dAb (10nM) or vehicle for 1 hour then treated with TNF (10ng/mL), R32W (TNFR1 mutein, 100ng/mL) or vehicle control for 4 hours. Resuspended cells were exposed to anti-human ICAM-1 Pacific Blue, anti human VCAM-1 APC, anti-human E-selectin PE and analysed in duplicate by flow cytometry (n=2-53). Panels show fold-change in proportion of cells expressing ICAM-1 (A), VCAM-1 (B) and E-selectin (C) for each condition. The dotted line represents expression levels in vehicle treated cells. Data are presented as mean $\pm$ SEM analysed by Kruskal Wallis (Dunns) *p<0.05, **p<0.01

4.4 Discussion

4.4.1 Summary of important findings

TNFR1 signalling has a pivotal role in TNF induced priming and respiratory burst activity in human neutrophils. Selective TNFR1 agonism induced alterations in classical priming markers (shape change, CD11b up-regulation and CD62L shedding) and augmented respiratory burst in fMLP-stimulated neutrophils that paralleled the effect of TNF. Selective TNFR2 agonism had no effect compared to PBS control treated cells. These data were further confirmed by selective TNFR1 antagonism, which abrogated neutrophil priming and reduced respiratory burst 2-fold in TNF stimulated cells. Analysis of TNFR expression and shedding suggested that TNFR1 expression was under the dominant control of TNFR1 signalling.

TNF stimulated early-apoptosis but this effect was not specific to either TNFR suggesting that engagement of both TNFR is required to induce early apoptosis. TNFR1 agonism replicated the modest (10% reduction in apoptosis) late pro-survival effect of TNF, but this effect was not statistically significant. TNFR1 antagonism of TNF stimulated neutrophils, rescued apoptosis to control levels at 20 hours, but again this was not statistically significant.

Selective TNFR1 antagonism ameliorated neutrophil migration through HMVEC-L monolayers. Our data suggest that this may be due to blockade of up-regulation of the neutrophil adhesion molecules ICAM-1, VCAM-1 and E-selectin on HMVEC-L although further functional studies using blocking antibodies to adhesion molecules or adhesion molecule knockout mice are required.

4.4.2 The role of differential TNFR signalling in neutrophil priming and activation

A number of studies have attempted to examine define the role of differential TNFR signalling in neutrophil priming using a range of selective monoclonal and polyclonal blocking antibodies allied with specific agonist mutant proteins. In agreement with our data (figures 4.3 and 4.4), the majority of these suggest a pivotal role for TNFR1 in priming of respiratory burst activity. Using agonistic anti-TNFR antibodies, TNFR1 was shown to be exclusively involved in TNF induced up regulation of CD11b expression and priming of superoxide release (Abe et al., 1995). Similarly, in adherent neutrophils where TNF functions as a full secretagogue (Dri et al., 1991; Nathan, 1987; Nathan, 1989; Richter et al., 1989), TNFR1 was fully competent to induce superoxide generation and, CD11b up-regulation with redundancy of TNFR2 (Menegazzi et al., 1994).

In contrast, it has been suggested that TNFR1 and TNFR2 cooperate to induce the respiratory burst (Barbara et al., 1994; Richter et al., 1989). In both studies, TNFR1 was identified as the major mediator of intracellular signalling but both authors suggested a requirement for TNFR2 to potentiate TNFR1 signalling. Others have suggested that, whilst TNFR1 alone is responsible for the priming effect of TNF induced phagocytosis in human neutrophils, TNFR2 regulates binding of TNF to TNFR1 (Della Bianca et al., 1995). Whilst our data did not support these findings, these data are in keeping with the ligand-passing model proposed by Tartaglia and Goeddel (Tartaglia et al., 1993; Tartaglia et al., 1993) discussed in chapter 1.3.3.

The mechanisms underlying TNF-induced neutrophil priming remain elusive. Both ERK and p38 MAPK mediated serine phosphorylation of p47^{phox}, leading to assembly of the NADPH oxidase and generation of ROS, have been identified as key events in TNF-induced priming (Dang et al., 2006; Kilpatrick et al., 2010). In experiments

using human neutrophils in suspension and neutralizing TNFR antibodies, phosphorylation of serine³⁴⁵ on p47^{phox} was mediated exclusively by TNFR1 via p38MAPK (Dewas et al., 2003). Moreover, in a series of articles by Kilpatrick, binding of TNF to TNFR1, in cooperation with ligation of integrins, has been postulated to activate PI3K, thereby modifying δ -PKC and promoting p47^{phox} phosphorylation (Kilpatrick et al., 2002; Kilpatrick et al., 2004; Kilpatrick et al., 2010; Kilpatrick et al., 2006; Korchak and Kilpatrick, 2001). These conclusions are supported by the observation that inhibition of class IA PI3K and p38MAPK abolished TNFR1 induced ROS generation (Geering et al., 2011).

In this study changes in CD11b and CD62L expression were used as markers of neutrophil priming (figures 4.3 and 4.4). Shape change, CD62L, and CD11b expression, as well as chemotaxis, have been shown previously to be affected during neutrophil isolation, with primed cells being less able to migrate in response to chemotactic stimuli, having less CD62L expression, greater CD11b expression, and a blunted response to neutrophil agonists (Haslett et al., 1985). It is however, intriguing to speculate on the functional implications of alterations in the expression of these neutrophil adhesion molecules. Consistent with the quiescent, non-adherent state of circulating neutrophils, constitutively expressed CD11b/CD18 cannot mediate efficient adhesion or effector functions (Hynes, 1992). Stimulus-induced up-regulation of CD11b, as observed in our *in vitro* data (figures 4.2-4.4), does not augment CD11b/CD18 mediated neutrophil adhesion (Vedder and Harlan, 1988; Schleiffenbaum et al., 1989). Thus, despite clinical data suggesting that CD11b up-regulation correlates with adverse clinical outcomes (Rinder et al., 2003; Orr et al., 2007), qualitative or conformational changes may be more relevant to CD11b function than total cell surface expression (Diamond and Springer, 1993; Oxvig et al., 1999). Indeed, conformational activation of CD11b has been demonstrated in critical

illness (Rosenbloom et al., 1999) and occurred in the absence of CD11b up-regulation (Orr et al., 2007). Moreover, interrogation of the respective roles of the CD11b/CD18 subunits, demonstrated that CD11b mediated cell adhesion and spreading in response to a range of ligands but was unable to support cell migration (Solovjov et al., 2005).

Pharmacological blockade and genetically modified mouse experiments have provided contradictory data regarding the functional implications of CD62L shedding. Initial studies suggested that shedding allowed neutrophils to migrate normally after extravasation (Venturi et al., 2003) and that blockade of shedding did not affect neutrophil rolling, adherence or transmigration in TNF activated HUVEC monolayers under flow (Allport et al., 1997). Conversely, the Doerschuk group demonstrated in knockout mice that initial neutrophil retention in the lung following complement fragment injection was CD62L independent, however, neutrophil sequestration after 5 minutes was CD62L dependent (Doyle et al., 1997) suggesting an essential role in retention of sequestered neutrophils within pulmonary capillaries. Recent murine data has also demonstrated that CD62L is required for PSGL-1–dependent integrin activation, slow rolling, and leukocyte recruitment *in vitro* and *in vivo* (Stadtman et al., 2013). Moreover, pharmacological blockade of CD62L shedding increased CD62L expression on the surface of neutrophils and augmented signal input through CD62L (Hafezi-Moghadam et al., 2001). These changes were associated with reduced rolling velocity (Hafezi-Moghadam and Ley, 1999), increased “smoothness” of rolling, enhanced arrest, and transmigration (Hafezi-Moghadam et al., 2001). Finally, CD62L is cleaved from the cell surface by ADAM17 (and other sheddases) (Smalley and Ley, 2005). Thus, compared to wild-type, ADAM17^{-/-} mice exhibited higher CD62L expression associated with slower neutrophil rolling with improved adherence with or without TNF activation (Tang et al., 2011) and mice

expressing non-cleavable CD62L had enhanced neutrophil infiltration in response to intra-peritoneal *E. coli* . (Long et al., 2012)

Collectively, these data suggest that that blockade of CD11b up-regulation and CD62L shedding, for example by GSK1995057, may augment neutrophil adhesion and arrest at least in the systemic circulation. The implications for pulmonary capillary leukocyte infiltration are, however, unclear as none of these studies investigated functional effects in the lung microvasculature, where, owing to spatial constraints, neutrophil arrest is independent of CD62L mediated rolling and neutrophil migration occurs by both CD11b/CD18 dependent and independent mechanisms, depending on stimulus (Doerschuk, 2001). Recent but highly novel data from Summers *et al* suggest that priming neutrophils with GM-CSF or platelet activating factor (PAF) *ex vivo* prolongs pulmonary transit kinetics compared to control cells (Summers et al 2014). Autologous ^{99m} Technetium labelled cells were injected into healthy volunteers and the peak signal from selected anatomical regions of interest was measured by gamma scintigraphy over 40 minutes. In addition to prolonged transit times, imaging demonstrated a significant retention of GM-CSF primed neutrophils in the lung compared to control cells. These data are in concordance with earlier experiments by Haslett et al in which formyl-norleucyl-leucyl-phenylalanine (FNLP) primed radiolabelled neutrophils were shown to be significantly retained within the lungs of rabbits, compared to control cells (Worthen et al., 1987).

Given that TNFR2 signalling has immunomodulatory functions (Cabal-Hierro and Lazo, 2012) and that our data suggested up-regulation of TNFR2 following priming (figure 4.8), we considered the hypothesis that TNFR2 signalling de-primed neutrophils *in vitro*. Neutrophils challenged with PAF displayed a spontaneous return to baseline values of ROS production, cell polarisation and CD11b activity within 120 min (Kitchen et al.,

1996). The mechanisms underlying neutrophil de-priming are unknown, although it has been postulated that the healthy lung microvasculature may play a role (Singh et al., 2012). TNF, D143N and GSK1995057 were unable to de-prime TNF-primed neutrophils (figure 4.9), suggesting that differential TNFR signalling does not participate in neutrophil de-priming mechanisms, at least *in vitro*.

4.4.3 The role of differential TNFR signalling in receptor expression and shedding in human neutrophils

TNF induced down-modulation of TNFR1 in human neutrophils was associated with shedding of TNFR1 and was mediated by TNFR1 signalling (figure 4.7). Additionally, TNFR2 was shed, but intriguingly expression of TNFR2 increased (figure 4.8). This effect was not clearly mediated by either TNFR.

Previous data regarding the mechanism of surface TNFR loss has been discordant. TNF stimulation of human neutrophils decreased expression of both TNFR (Dri et al., 2000). Using TNFR specific and competing antibodies, these effects were shown to be mediated exclusively via TNFR1 and involved shedding of both receptors. A further study demonstrated loss of TNFR expression in TNF stimulated neutrophils. Owing to low levels of sTNFR1 in harvested supernatants, the authors concluded that the down-modulation of TNFR1 was due to receptor internalisation, while TNFR2 was shed (Porteu and Hieblot, 1994). It has been argued that the observed discrepancy in TNFR1 shedding in this study owed to the low sensitivity of the ELISA employed. Accordingly our data were generated with an identical proprietary ELISA kit used in the study by Dri which demonstrated no cross reactivity with R32W or D143N.

Proteolytic cleavage (shedding) is the principal mechanism of TNF receptor down-modulation that may on the one hand reduce sensitivity to TNF, and on the other hand

limit bioavailability of TNF by providing soluble forms TNFR that could compete for the cytokine with the cellular receptors. The clinical significance of this effect is highlighted by TNFR1-associated periodic syndromes (McDermott et al., 1999); missense mutations of the human TNFR1 gene impair receptor shedding and patients present with increased inflammatory responses and concomitant fever. TNFR shedding appears to be regulated at least in part by the cell surface metalloproteinase TNF converting enzyme (TACE or ADAM17) (Bell et al., 2007; Black et al., 1997). Whilst definitive data to confirm the role of TACE in human neutrophils is lacking, TACE is expressed by human neutrophils at levels comparable to that of TNF receptors (Dri et al., 2000). In irradiated mice reconstituted with blood cells deficient in functional ADAM17 (ADAM 17 gene knockout is lethal), LPS stimulated shedding of TNFR1 and TNFR2 from neutrophils was incompletely abrogated (Bell et al., 2007). These data suggest a primary but not exclusive role for ADAM17 in TNFR shedding in murine neutrophils. Interestingly, resting neutrophils from ADAM17 chimeric mice expressed higher levels of surface TNFR1 and TNFR2 compared with resting neutrophils from wild-type mice.

Interference with TNFR shedding therefore represents an alternative mechanism of modulating TNF induced inflammation. Indeed, a protective role of TNFR1 shedding by ADAM17 was reported in TNF sensitisation of hepatocytes to Fas-mediated death (Murthy et al., 2010). TNF signalling promoted hepatotoxicity, whilst excessive TNFR1 shedding was protective. Interestingly, TNFR1 shedding induced protection via dampening of JNK phosphorylation and NF- κ B activation. This effect was further confirmed in a recent study from the Bhattacharya laboratory applying confocal microscopy to ADAM17 knockout mice and the TNFR1 blocking antibody E20. In lung microvessels, TNF induced TNFR1 shedding was entirely ADAM17 dependent and was regulated by endothelial mitochondrial Ca^{2+} and ROS production (Rowlands et al., 2011).

Blockade of TNFR1 shedding by inhibition of mitochondrial Ca^{2+} and ROS production, propagated inflammation, as evidenced by enhanced (TNF dependent) E-selectin expression and leukocyte adhesion.

Despite contrasting the majority of published data, the finding that TNF up-regulated TNFR2 expression is not without precedent. TNF has been shown to induce increased TNFR2 expression in human malignant epithelial cell lines (Kalthoff et al., 1993) and fibroblasts (Winzen et al., 1993). However, TNF incubations over 20 to 48 hours were used in these experiments. Moreover, in LPS treated mice, whilst TNFR1 expression in lung microvascular cells was down-regulated, TNFR2 expression remained unchanged. Alternatively, the discrepancy may be explained by differences in TNF concentration (2.5ng/mL) and duration of TNF exposure (15 minutes) employed in others' work (Dri et al., 2000).

4.4.4 The role of differential TNFR signalling in modulating neutrophil lifespan

In parallel with TNFR shedding, TNF mediated neutrophil apoptosis contributes to the regulation of duration and intensity during inflammatory responses (Jonsson et al., 2005). In the work presented in this chapter, and consistent with published data (Murray et al., 1997; Salamone et al., 2001), we have demonstrated that TNF has a bimodal effect on apoptosis; inducing apoptosis at 6 hours whilst affecting a modest pro-survival response at 20 hours *in vitro* (figures 4.10 and 4.11). Selective TNFR agonism alone did not replicate this effect suggesting that that dual activation of both TNFR is required for the early apoptotic effect of TNF, whilst our data demonstrated no clear effect of differential TNFR signalling upon late survival.

The finding that engagement of TNFR2 may be required for early apoptosis, was somewhat surprising; TNFR2 lacks a cytoplasmic death domain and is unable to recruit FADD (chapter 1.3.3.2). TNFR2 can specifically enhance TNFR1-mediated apoptosis by depletion of the NF κ B-promoting/caspase-8 inhibitory TRAF2-cIAP1/2 complex from TNFR1 via competitive recruitment to TNFR2 and subsequent proteasomal degradation (Declercq et al., 1998; Weiss et al., 1998). However, our data showing that R32W was unable to induce apoptosis despite maintaining full agonistic activity in priming of fMLP-stimulated ROS release would suggest that this is not the mechanism, at least in neutrophils. A number of studies have demonstrated apoptosis induction after selective stimulation of TNFR2. In most of these reports, apoptosis was not directly triggered by TNFR2, but was mediated indirectly by up-regulation of transmembrane TNF, which then secondarily stimulated TNFR1 (Grell et al., 1999; Vercammen et al., 1995). The absence of effect with D143N in our study would contradict this, although again, we cannot exclude that firstly the mutein is non-functional and secondly that TNFR2 was unresponsive to soluble TNF (Rauert et al., 2010). Data from the rat-mouse cytotoxic T-cell hybridoma PC60 suggest that TNFR2 can induce cell death independently from TNFR1, but the underlying signalling mechanisms are unknown (Vandenabeele et al., 1995).

The failure of the combination of both TNFR muteins to replicate TNF induced apoptosis (figure 4.10) is not unprecedented (Chilvers group, unpublished data) and may reflect either lack of specificity of D143N or an inability to initiate downstream signalling. The ability of R32W to induce neutrophil priming and ROS release would suggest functionality of the TNFR1 mutein used. However, as outlined previously we have been unable to demonstrate specific functional responses to D143N in a range of models. Interestingly, in a study using pre-exposure to anti-TNFR antibodies followed by

TNF (100u/10⁶ neutrophils), neither antibody alone enhanced neutrophil apoptosis at 3 hours, whilst a combination of the two also failed to enhance apoptosis (Gon et al., 1996).

TNF induced a moderate anti-apoptotic effect in neutrophils after 20 hours *in vitro*. Data demonstrating that inhibition of NF- κ B increased constitutive apoptosis (Ward et al., 1999; Choi et al., 2003) would suggest that NF- κ B, probably via transcription of survival proteins is the probable signalling pathway for this pro-survival effect. NF- κ B is regulated via TRAF2 and I κ B kinase (IKK) suggesting that these effects are under dominant control of TNFR1. That R32W mirrored the effects of TNF (10% reduction in apoptosis from baseline), and TNFR1 antagonism returned apoptosis to constitutive levels supports a possible role for TNFR1 signalling.

Kilpatrick and colleagues further added to the field with data proposing an additional regulatory role for ERK1/2 as a downstream regulator of TNFR1 induced anti-apoptosis. Using an indirect measurement of apoptosis, Caspase 3 activity, TNF induced anti-apoptotic signalling was increased six-fold at 20 hours compared to freshly isolated cells (Kilpatrick et al., 2006);. TNF reduced Caspase-3 activity by almost 50%, an effect mediated by phosphorylation of ERK1/2. In parallel with priming mechanisms outlined previously (chapter 5.2.2), δ PKC was required for TNF-triggered activation of ERK1/2. Both of these effects are mediated by TNFR1, further supporting a putative role for TNFR1 signalling in the pro-survival effect.

Other experimental nuances may impact upon constitutive and induced rates of apoptosis. In our experiments, we used a standard dose of TNF throughout (10ng/mL). This dose reflects TNF concentrations in the BALF of ALI patients (Meduri et al., 1995). In the study by Murray et al, a dose of 25ng/mL was anti-apoptotic at 20 hours, however, a concentration-dependent effect has also been observed; low doses (<1ng/mL) were anti-apoptotic, whereas higher doses (10-100ng/mL) were pro-apoptotic (van den Berg et al.,

2001). The authors concluded that at higher doses, the production of respiratory burst may induce apoptosis. This effect was recently investigated by a Swiss group, who reported that TNFR1 stimulation induced p38 MAPK and class IA PI3Ks, which are usually involved in survival pathways, as proximal death players (Geering et al., 2011). These kinases were shown to be essential for the generation of ROS, which furthermore triggered caspase-3 activation resulting in neutrophil apoptosis. We observed a pro-apoptotic effect with GSK1995057 treatment; assuming ROS production modulated apoptosis in our assay, it would be anticipated that TNFR1 antagonism would reduce ROS production, thus promoting survival. We cannot exclude the previously observed effect of contaminating mononuclear cells in propagating this effect (Walmsley et al., 2004); in the data presented here, neutrophils were routinely >95% pure and the conditions were matched for each treatment.

4.4.5 Selective TNFR1 antagonism ameliorates neutrophil migration through HMVEC-L monolayers

The observation that, in murine cremaster vessels, endothelial TNFR were an absolute requirement for neutrophil adherence and migration (Woodfin et al., 2009) suggests a specific role for TNF induced modulation and activation of the endothelium in this process. Thus we sought to investigate these findings in human *in vitro* models. TNF stimulation of HMVEC-L monolayers enhanced neutrophil migration towards IL-8 (figure 4.12). This effect was ameliorated by both TNFR1 antagonism and dual TNFR1/2 blockade suggesting a role for TNFR1 signalling in monolayer activation. The experimental set-up was designed such that treated monolayers were washed extensively prior to the addition of neutrophils, reducing the possibility of priming or activation of neutrophils by cytokines released from stimulated monolayers. Thus, any treatment effect could be attributed to activation of endothelial cells alone. Moreover, we sought extensive

evidence of monolayer integrity to ensure that neutrophils did not preferentially migrate through endothelial gaps.

We used a simple, static, Transwell filter based method to analyse migration. The major criticism of such assays (compared to dynamic flow-based assays) is that they essentially assess neutrophil migration down a chemotactic gradient with endothelial cells “in the way” as opposed to endothelial-driven migration (McGettrick et al., 2007). In addition, the location of non-migrated cells in the filter is ambiguous and the kinetics of static transmigration differ from flow-based migration (Rainger et al., 1999; Rainger et al., 1995). By removing adherent cells in the upper chamber and placing the filter itself in lysis buffer along with migrated cells we attempted to overcome some of these issues (chapter 2.2.6.6) In preliminary experiments we attempted to create a bilayer model of static migration using HMVEC-L on the apical surface and hAT2 on the basolateral surface. This work is ongoing but presents challenges particularly in the seeding of hAT2 on the basolateral surface. Future work should address experiments in both a flow based assay system and LPS injured ex vivo lung to validate our findings.

Having demonstrated a potential role for TNFR1 signalling in neutrophil migration, through endothelial monolayers, we hypothesised that this may be mediated via alterations in the expression of neutrophil ligands by TNF activated HMVEC-L. A range of *in vitro* and *in vivo* data suggest that ICAM-1, VCAM-1, E-selectin, Junctional Adhesion Molecule 1 (JAM1), ICAM-2 and P-selectin are critical adhesion molecules regulating trans-endothelial neutrophil migration in the systemic circulation. Initial experiments determined that only ICAM-1, VCAM-1 and E-selectin were TNF responsive (figure 4.14). Time course data for TNF induced ICAM-1, VCAM-1 and E-selectin expression (figure 4.13) are comparable with *in vitro* data from HUVEC (Mackay et al., 1993) and murine *in vivo* data (Neumann et al., 1996; Bertok et al., 2011). Contrary to

reports in HUVEC and *in vivo*, both VCAM-1 and ICAM-1 were expressed at baseline in HMVEC-L and increased in a time dependent manner in response to TNF; basal expression of ICAM-1 was significantly higher than VCAM-1 (figure 4.14).

ICAM-1, VCAM-1 and E-selectin all contain consensus sites for NF- κ B in their promoter regions, suggesting a central role for TNFR1 acting via this route. Consistent with our data demonstrating that TNFR1 antagonism abrogated adhesion molecule expression, several authors have demonstrated TNFR1 dependency in adhesion molecule expression in both *in vivo* and *in vitro* (Bertok et al., 2011; Chandrasekharan et al., 2007; Neumann et al., 1996; Mackay et al., 1993; Slowik et al., 1993; Kneilling et al., 2009). Perhaps the most compelling data to suggest a definitive role for TNFR1 in adhesion molecule expression comes from experiments in HUVEC using the same clones of muteins employed in this work and a TNFR2 agonistic antibody in combination with small molecule inhibitors of JNK, p38MAPK and MEK1. (Zhou et al., 2007). TNF induced ICAM-1 and VCAM-1 expression and mRNA expression were found to be NF- κ B but not ERK, p38MAPK or JNK dependent. This effect was mediated exclusively by TNFR1, measured by protein, mRNA and cell surface expression levels of ICAM-1 and VCAM-1. Of note, using an NF- κ B inhibitor and NF- κ B reporter construct, this effect was found to be entirely NF- κ B dependent, an effect similarly reported in A549 cells (Holden et al., 2004).

Conversely, both an exclusive and regulatory role for TNFR2 have been proposed. Chandrasekharan et al. suggested that both TNFR were critical for TNF induced E-selectin and VCAM-1 expression while TNFR2 signalling was sufficient for ICAM-1 expression in murine aortic endothelial cells (Chandrasekharan et al., 2007). An additional regulatory role for TNFR2 signalling in murine lung endothelial cells stimulated with TNF was further shown; both TNFR1 knockout mice and lung microvascular cells treated

with a TNFR2 antagonist demonstrated reductions in VCAM and E-selectin (Bertok et al., 2011). Moreover, in a TNFR-knockout mouse model of cerebral malaria, TNFR2 was critical for ICAM-1 induced up-regulation of leukocyte sequestration in brain microvessels (Lucas et al., 1997).

It should be highlighted that none of these studies were undertaken with a focus on the human lung microvasculature. Heterogeneity in both the parent species and vascular bed of endothelial cells employed in these studies and the phenotypic differences thereof (King et al., 2004; Aird, 2007) may account for the conflicting data observed. To our knowledge, this is the first study investigating TNFR dependency on neutrophil migration and adhesion molecule expression in human cells.

Recent data have suggested additional key roles for ICAM-2 and JAM1 in transcellular neutrophil migration. These effects, however, have been shown to be stimulus specific. For example, in receptor knockout mice, IL-1 β induced neutrophil migration via recruitment of pathways involving ICAM-2 and JAM1, whereas TNF induced migration was independent of these adhesion molecules (Huang et al., 2006; Woodfin et al., 2007; Thompson et al., 2001). One possible explanation for these findings is that TNF regulated neutrophil migration via neutrophil activation as opposed to exerting effects on vascular endothelium (TNF was administered systemically to mice in these experiments). Our data showing that JAM and ICAM-2 expression was not inducible in HMVEC-L over 24 hours TNF exposure (figure 4.13) would suggest that in human lung microvasculature, TNF induced neutrophil migration is probably independent of these molecules.

Inducible expression of P-selectin has been proposed as a key regulator of inflammation (McEver and Zhu, 2010), and P-selectin knockout mice exhibit absent leukocyte rolling and impaired recruitment to sites of systemic inflammation (Mayadas et

al., 1993). In HUVEC P-selectin expression is inducible within minutes following treatment with histamine (Hattori et al., 1989). In HMVEC-L P-selectin cell surface expression was neither constitutive nor inducible in response to TNF stimulation (data not shown). These data confirm previous animal studies demonstrating that P-selectin was not expressed on pulmonary capillary endothelium of normal rats, mice, and rabbits (Mulligan et al., 1992). Moreover, whilst TNF induces P-selectin expression in murine endothelium (Hahne et al., 1993; Liu et al., 2010) increasing slow rolling and adhesion (Liu et al., 2010), in HUVEC, TNF has been shown to have no effect, or even decrease P-selectin mRNA (Burns et al., 1995; Yao et al., 1996). Hence, P-selectin expression appears to be stimulus, species and vascular bed specific and its role as a neutrophil ligand in the pulmonary microvasculature, at least in response to TNF, is questionable.

The finding that selective TNFR1 antagonism ameliorated neutrophil migration could alternatively be explained by effects on the endothelial surface layer (ESL) or glycocalyx. Emerging data suggest that ESL loss induces neutrophil adhesion to endothelial cells. Potential mechanisms include the release of pro-adhesion mediators previously sequestered by the glycocalyx, exposure of previously hidden endothelial surface adhesion molecules, and/or the activation via proteoglycans of pro-adhesion endothelial signalling pathways (Schmidt et al., 2011). The TNFR specificity of this loss was investigated by the Downey group, using pulmonary intra-vital microscopy (Schmidt et al., 2012). In the pulmonary microvasculature, LPS induced degradation of the ESL, specifically heparan sulfate (HS), via TNF. This effect was abolished in mice lacking TNFR1 suggesting a central role for TNFR1 signalling in HS homeostasis. LPS induced ESL loss exposed ICAM-1 and was associated with increased adherence of neutrophils *in vivo*.

4.4.6 Future directions

Using the combination of human neutrophils, human lung microvascular cells and a highly selective TNFR1 mutein, reassuringly, we have validated much of the published literature in models relevant to human ALI. Future work should address further the functional effects of differential TNFR induced priming and activation of neutrophils on neutrophil migration in the lung. These experiments could be repeated using *in vivo* imaging techniques in combination with an *ex vivo* ALI model. *Ex vivo* modeling would additionally allow interrogation of the hypothesis that primed neutrophils preferentially migrate to injured lung. Further mechanistic data defining the role of TNFR1 antagonism in modulating neutrophil-endothelial interactions allied with emerging *in vivo* data may encourage early phase trials of selective TNFR1 antagonists, in the prevention of ALI.

Given the persistent failure of animal models of ALI to translate to therapeutic benefit, the parallel development and application of human *in vitro*, *in vivo* and *ex vivo* models (with relevance to the lung microvasculature) to investigate these mechanisms is essential.

5 Chapter 5: Development & characterisation of human ex vivo perfused lung models of ALI

5.1 Introduction

Animal models have been invaluable in the study of complex diseases such as ALI, allowing testing of hypotheses in complex biological systems. Rodent ALI models, in particular, have revealed important biologic mechanisms underlying the temporal sequence of injury, inflammation and dysregulated repair. However, despite over 30 years of promising experimental and preclinical studies, no pharmacological intervention developed thus far (including anti-TNF therapy) has reduced mortality in ALI. This fact alone highlights the limitations of current models of ALI and the urgent need for tractable human models of ALI to develop and test novel therapeutic targets and provide more relevant data to inform subsequent larger clinical trials. In particular, the development of *ex vivo* human lung models of ALI could offer a new paradigm in ALI translational research allowing recapitulation of moderate to severe ALI, experimental manipulation and temporal analysis of distal lung tissue, which are not achievable using current *in vivo* human models (chapter 1.2.2.1).

Several recent studies by the Matthay group at University of California San Francisco (UCSF) have shown the potential of *ex vivo* models for studying the pathophysiology of ALI. In 20 human lungs rejected for transplantation, the investigators demonstrated that firstly changes in alveolar fluid clearance (AFC, as a marker of epithelial barrier function) could be measured in their model and that AFC was preserved in perfused compared to non-perfused *ex vivo* lung tissue (Frank et al., 2007). Secondly, β 2 agonist terbutaline increased rates of AFC compared to basal levels (Frank et al., 2007). Finally, levels of soluble receptor for advanced glycation end products (RAGE, a

marker of alveolar type I epithelial cell injury) correlated inversely with AFC (Briot et al., 2009). Thus, human data regarding a therapeutic intervention for pulmonary oedema and a putative biomarker for alveolar epithelial cell injury were generated highlighting the potential translational impact of such models.

The same investigators then established an injury model in *ex vivo* human lung (Lee et al., 2009). The right middle lobe or left lower lobe was injured with an intra-bronchial instillation of *E. coli* endotoxin. A contra-lateral lobe was used as the control. Instillation of endotoxin into the distal airspaces resulted in acute pulmonary oedema, an increase in lung vascular permeability and an almost complete loss of AFC. These effects were associated with a significant increase in BALF neutrophils and levels pro-inflammatory cytokines implicated in the pathogenesis of ALI (IL-1 β , TNF α and IL-8). The translational potential of the model was realised through studying the effect of mesenchymal stem cells on AFC in the injured lobe over 4 hours: MSCs reduced endotoxin-induced injury and inflammation, and augmented AFC compared to controls. Thus, *ex vivo* ALI modelling allowed assessment of the effect of a therapeutic intervention in abrogating some of the key physiological, immunological and pathological features of clinical ALI.

Most recently, the UCSF group have developed an *ex vivo* live bacteria pneumonia model (Lee et al., 2013). *Escherichia coli* (10^9 to 10^{10} colony forming units) was instilled into the right middle lobe or left lower lobe. In parallel with the LPS model, this induced cytokine release, neutrophil infiltration and impaired AFC in the injured lobe compared to control. Of note, this model was extended to 10 hours perfusion to induce a bacteraemia in the perfusate. Injured lungs remained stable throughout the experimental period with only a modest decline in AFC in the control lobe at 10 hours compared to that measured 6 hours after injury. Thus, this study demonstrated firstly that alternative injury modalities

can induce injury and secondly that longer-term perfusion is feasible without progressive lung injury.

In the previous chapters we have demonstrated the pleiotropic roles of differential TNFR signalling in a range of *in vitro* models of ALI. The aim of this chapter was to translate these data in the UK's first *ex vivo* human lung injury model. The specific aims of experiments in this chapter were:

- a. To establish the *ex vivo* lung perfusion technique to produce stable physiology and long-term viability**
- b. To characterise our own LPS induced human *ex vivo* ALI model**
- c. To establish a method of performing pharmacological study in *ex vivo* lung**
- d. To investigate the effects of selective TNFR1 antagonism on neutrophil infiltration into the alveolar space in an *ex vivo* model**

5.2 Specific Methods

5.2.1 BAL protocol

The control lobe was cannulated under direct vision with a bronchoscope (Olympus, Japan). 25mL of sterile 0.15M saline was injected via the working channel and then aspirated into two sterile collection pots. The collected bronchoalveolar lavage fluid (BALF) was filtered through 4 layers of sterile surgical guaze and then centrifuged at 200rcf for 5 minutes at 4°C to pellet any cells present in the BALF. The supernatant was aliquoted and stored at -80°C for analysis at a later date. The cell pellet was then re-suspended in 500uL of ice cold PBS.

20µL of the original BALF was removed and mixed with 180µl of red cell lysis buffer (Sigma-Aldrich) and left to stand for 5 minutes at room temperature. 10µL of this solution was then mixed and added to 10µL trypan blue stain and left to stand for 2

minutes. 10 μ L of this stained mix was then withdrawn and added to a haemocytometer and a total cell count made.

A further 120 μ L of the original BALF was added to 480 μ L of red cell lysis buffer and incubated for 5 minutes at room temperature. This solution was then centrifuged at 1500rpm for 5 minutes. Following re-suspension of the cell pellet in 600 μ L of PBS 100 and 200 μ L of this solution was added in duplicate to funnel clips in a cytospin (Cytospin 2, Thermo Scientific) and centrifuged at 300rpm for 3 minutes, high acceleration. Slides were removed and allowed to air dry before being placed in methanol for 4 minutes. Slides were then sequentially placed in DiffQuick stain; orange/red for 1 minute then blue for 2 minutes with excess stain being rinsed off in between with distilled water. Stained slides were allowed to air dry for 30 minutes before being coverslip mounted using DPX (Sigma Aldrich). Cells were examined by light microscopy, magnification x20, 300 cells per slide in triplicate and the neutrophil count calculated per condition.

5.2.2 Protein collection

Sections of control and LPS injured lung (weighing 50-100mg) were immediately snap frozen in liquid nitrogen and stored at -80°C for later analysis of protein content. Frozen tissue samples were crushed in liquid nitrogen using a pestle and mortar and scraped into a 2mL Eppendorf. 200-400 μ L of ice-cold Hardie lysis buffer (50 mM Trizma base, 250 mM sucrose, 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.05 mg/mL soybean trypsin inhibitor, 0.1 mM PMSF, 1 mM benzamidine in dH₂O) was added to the Eppendorf and left on ice. The tissue in Hardie lysis buffer was then homogenised using an ultra-turrax (small probe, IKA, Germany) for 20 seconds on ice. The probe was cleaned with clean ice-cold distilled water and a paper towel between homogenisations. Samples were then centrifuged at

15000g for 20 minutes at 4°C and the resulting supernatant removed, placed in a fresh eppendorf and stored at -80°C until analysis by Western Blotting or ELISA.

5.2.3 Wet:dry weight ratio

Following injection of LPS or vehicle control, the injectate area in peripheral lung was marked with a pen (figure 5.7D). After 4 hours, a tissue sample from the demarcated area was harvested and the sample immediately homogenised in a 7mL bijou. After homogenisation the sample was weighed (wet weight) snap frozen in liquid nitrogen and placed in the freeze dryer for 24 hours, after which a dry weight was recorded. A wet dry weight ratio was calculated:

$$\text{Wet:dry weight} = (\text{Wet weight/dry weight}) * 1000$$

5.2.4 Measurement of lung “dynamics”

Lung physiologic parameters including pulmonary artery pressure, perfusate flow rate, dynamic compliance and peak airway pressure were measured throughout EVLP at 1 hourly intervals. Pressures were measured with standard pressure monitoring equipment (Edwards Lifesciences, USA). Flow rate was measured with an ultrasonic flow probe (Maquet, USA). Compliance and airway pressure measurements were recorded continuously from the ventilator (Evita XL, Drager, Germany). Pulmonary vascular resistance ($\text{dyn} \cdot \text{s} \cdot \text{cm}^{-5}$) was calculated:

$$\frac{80 \times (\text{pulmonary artery pressure} - \text{left atrial pressure})}{\text{Perfusate Flow}}$$

5.2.5 Lung injury scoring of human ex vivo lung

Sections of control and LPS injured lung (weighing 50-100mg) were gently inflated with 10% formalin solution (1-2mL, Sigma Aldrich, UK), placed in a bijou

containing 10% formalin (7mL) for 24 hours and then embedded in wax. Sections cut at 5µm were stained with haematoxylin and eosin and then examined by light microscope for pathological changes by a pulmonary pathologist who was unaware of the experimental groups. Semi-quantitative assessment of acute lung injury was performed using interstitial, alveolar and septal oedema, vascular thrombosis, haemorrhage, interstitial and alveolar inflammation and alveolar fibrin accumulation/hyaline membrane. Five regions from each specimen were examined, and each parameter was scored for severity using a 4 point scale; 0 = absent, 1 = mild, 2 = moderate, 3 = severe (Vaschetto et al., 2008). The sum of scores for each parameter gave the Lung Injury Score with a value of 0 to 24.

5.3 Results

5.3.1 Optimisation of the *ex vivo* lung perfusion circuit for future experimentation

Early experiments used a ‘closed’ system whereby both the left atrium and PA were cannulated and the lungs were placed in a specially designed dome which supported both inflow and outflow tubing (figure 5.1 A & B). This system, however, mandated that donor lungs had an intact left atrial cuff.

With the development of alternative clinical equipment, we changed to an ‘open’ system where the PA was cannulated but the left atrium or pulmonary veins were left open to air (figure 5.1 C & D). This system reduced the cold ischaemic time (CIT) as the technical demands of suturing the left atrial cannula often took over 30 minutes and avoided the need for an intact left atrial cuff, thus markedly increasing our potential donor pool.

In both systems, we initially used a centrifugal pump and a membrane oxygenator (without gas) to heat the perfusate and hence the lungs. This was based on equipment applied in the clinical setting (Cypel et al., 2011) (figure 5.2A). However, changing to a roller pump system with a simple heat exchange device (figure 5.2 B) offered physiological advantage by generating pulsatile flow and technical advantage of reducing the hardware costs and the dead-space in the system; hence less perfusate per experiment was required. Both circuits were fitted with ‘quick-release’ connectors to allow the circuit to be clamped, flow temporarily ceased and the lung container taken to the computed tomography (CT) scanner for baseline imaging. Both systems also allowed continuous measurement of PA pressure via a PA transducer in the PA cannula and both were connected to a clinical grade ventilator (figure 5.2 C). The most recent iteration of our model is outlined in figure 5.2D.

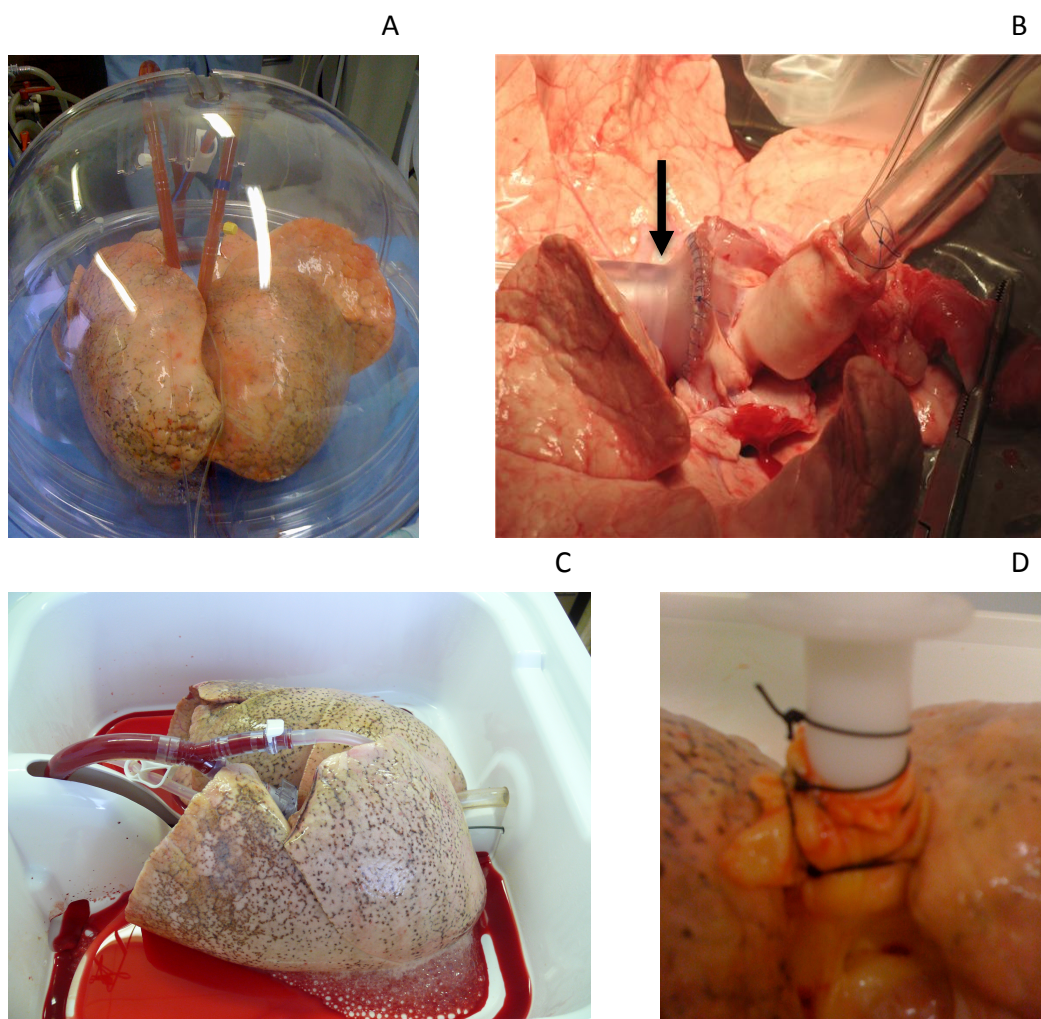


Figure 5.1 Arterial and venous cannulation in the *ex vivo* lung perfusion system

Initial models employed a clinical grade XVIVO™ dome (Image A). A conical XVIVO™ cannula (arrow panel B) was sewn into the left atrium and a straight XVIVO™ cannula was tied into the pulmonary artery thus creating a ‘closed’ perfusion system. Latterly, we have used an open system (panel C) whereby the left atrium is open to air, draining into a specially designed hardshell reservoir. A customised, bevelled cannula was tied into the pulmonary artery (panel D).

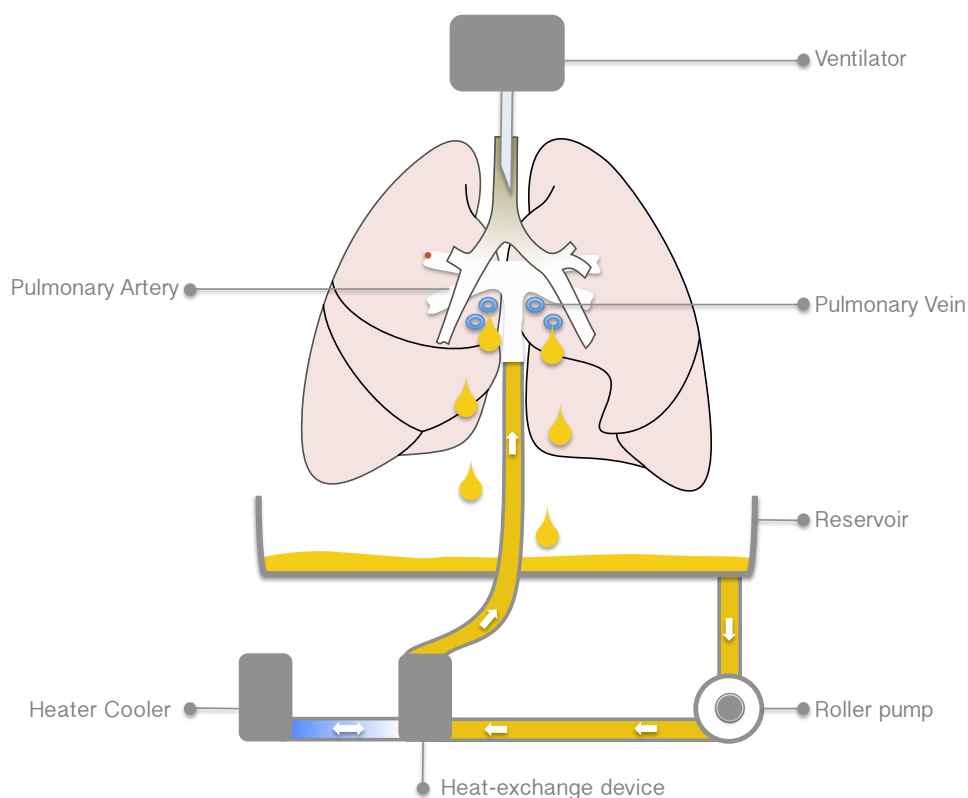
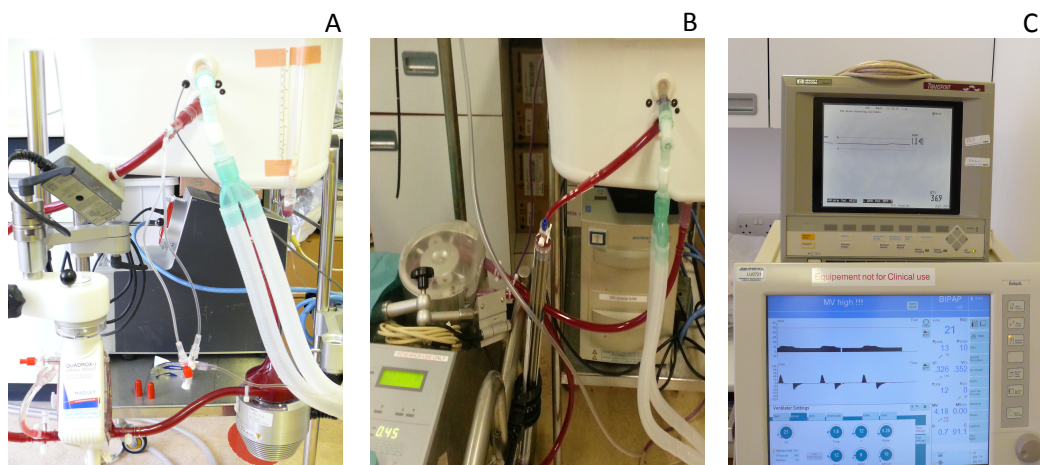


Figure 5.2 *Ex vivo* lung perfusion circuit hardware

Initial models employed a centrifugal pump to generate flow and membrane oxygenator (connected to a heat exchanger) to allow heating of the perfusate and lungs (panel A). Recent models have used a roller pump and a clinical grade heat exchange device (panel B). A standard ICU ventilator was connected via an endotracheal tube secured in the trachea thus providing a breath-by-breath read-out of lung compliance and resistance and a pressure transducer was inserted into the pulmonary artery to allow continuous measurement of pulmonary artery pressures and hence pulmonary vascular resistance (panel C). An overall outline of the current *ex vivo* circuit is demonstrated in the schematic (panel D).

5.3.2 Clinical characteristics of donor human lungs

Lungs were accepted based on the criteria outlined in table 5.1. Referrals were received on average once per fortnight but only about 25% of lung offers were accepted. The majority of lungs were declined on the basis of radiological and gas-exchange criteria. Alternatively acceptable donor lungs could not reach us within the pre-determined CIT limit. The characteristics of the accepted lungs are outlined in table 5.2. The CIT in some cases was as high as 39 hours; despite the projected CIT being within our limit, logistical delays contributed to a prolonged CIT. In 2 cases of young donors with normal radiology, the PaO₂:FiO₂ ratio did not fall strictly within our criteria but the lungs were used.

Table 5.1 Clinical characteristics of donor lungs

Clinical Characteristics	Results (n=10)
Age (years)	54±13
Male/Female (%)	60/40
PaO ₂ :FiO ₂ ratio on acceptance of lungs	370±221
Total cold ischaemic time (hrs)	34±4

5.3.3 Human lungs ventilated and perfused ex vivo for 8 hours demonstrated no signs of injury

Pulmonary vascular resistance and lung compliance remained stable for the duration of prolonged ex vivo lung perfusion in our model set-up (figure 5.3). Biopsies taken before EVLP and after 8 hours EVLP demonstrated no histological evidence of lung injury when examined by a pulmonary pathologist blinded to experimental conditions (figure 5.4).

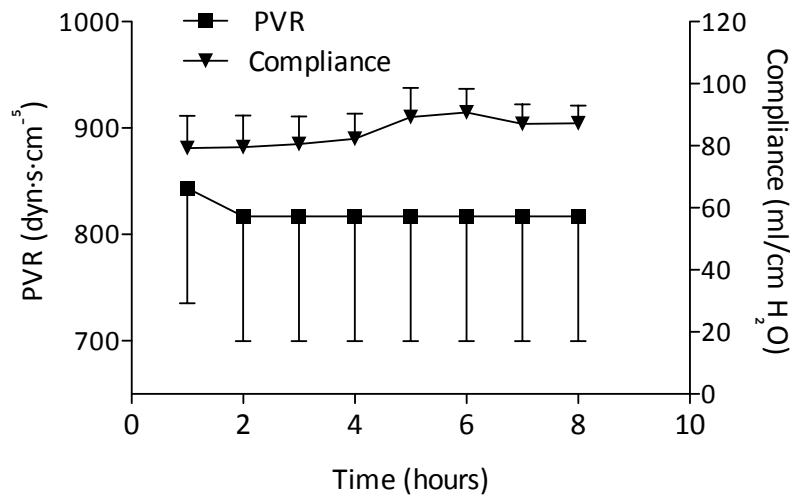


Figure 5.3 Human lungs maintained stable pulmonary vascular resistance (PVR) and compliance throughout *ex vivo* perfusion.

The ventilator gave a breath-by-breath read-out of lung compliance while PVR (dyn·s·cm⁻⁵) was calculated: $(80 \times (\text{pulmonary artery pressure} - \text{left atrial pressure}) / \text{perfusate flow rate})$. Pulmonary artery pressure was measured continuously via a built in pressure transducer n=8. Data are presented as mean $\pm$ SEM

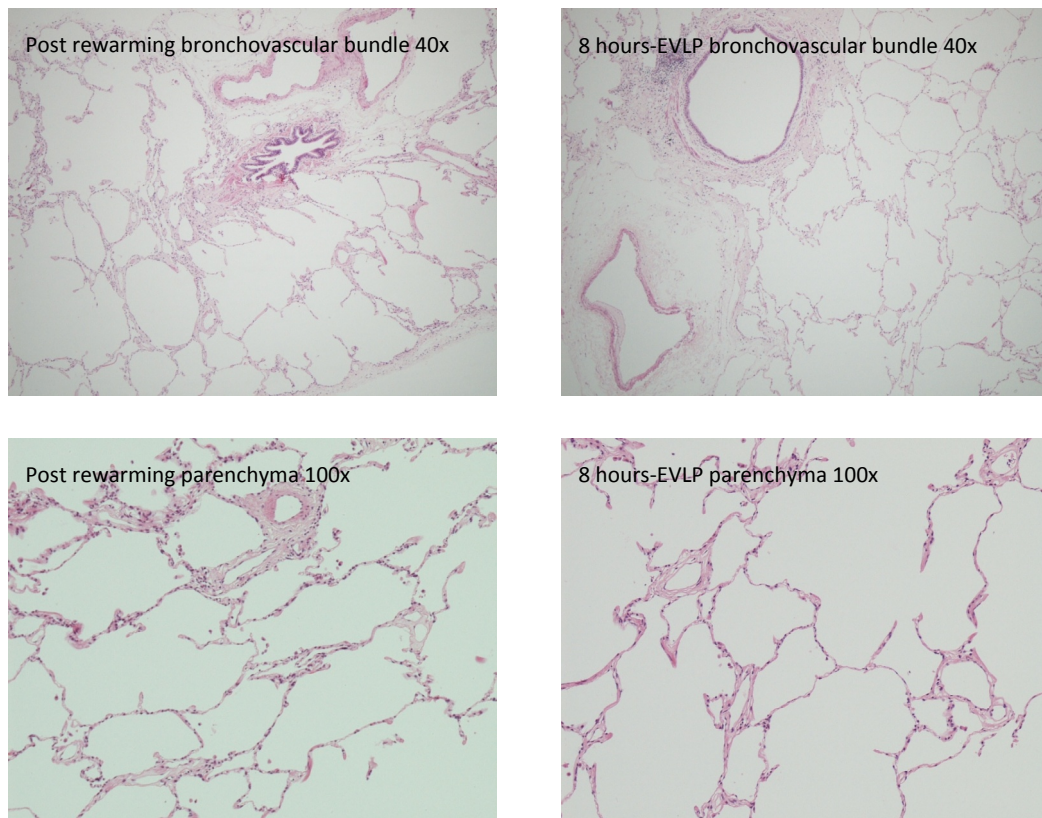


Figure 5.4 Human lungs maintained for 8 hours *ex vivo* demonstrated no histological evidence of injury

Human lungs were rewarmed on the *ex vivo* circuit, perfused and ventilated and maintained for 8 hours. A biopsy from the right lower lobe was taken after rewarming and again after 8 hours

normothermic perfusion and ventilation. Samples stained with haematoxylin and eosin were examined by a pulmonary pathologist blinded to experimental conditions. The above demonstrates representative images at 2 different magnifications before and after 8 hours EVLP of both the bronchovascular bundle and lung parenchyma

5.3.4 Cytokine release into BALF in LPS injured *ex vivo* human lungs

Compared to control, human *ex vivo* lung injured with LPS (0.1mg/kg predicted body weight of donor) demonstrated increased release of the pro-inflammatory cytokines IL-1 β , TNF, IL-6 and IL-8 in BALF (figure 5.5, panels A-D). This effect was evident at 8 hours following injury. After 4 hours, only IL-1 β and TNF showed significant increase compared to vehicle treated lung. Release of soluble TNF receptors type 1 and 2 in the BALF demonstrated no significant difference between vehicle treated lung at both 2 and 4 hours (figure 5.5 panels E & D).

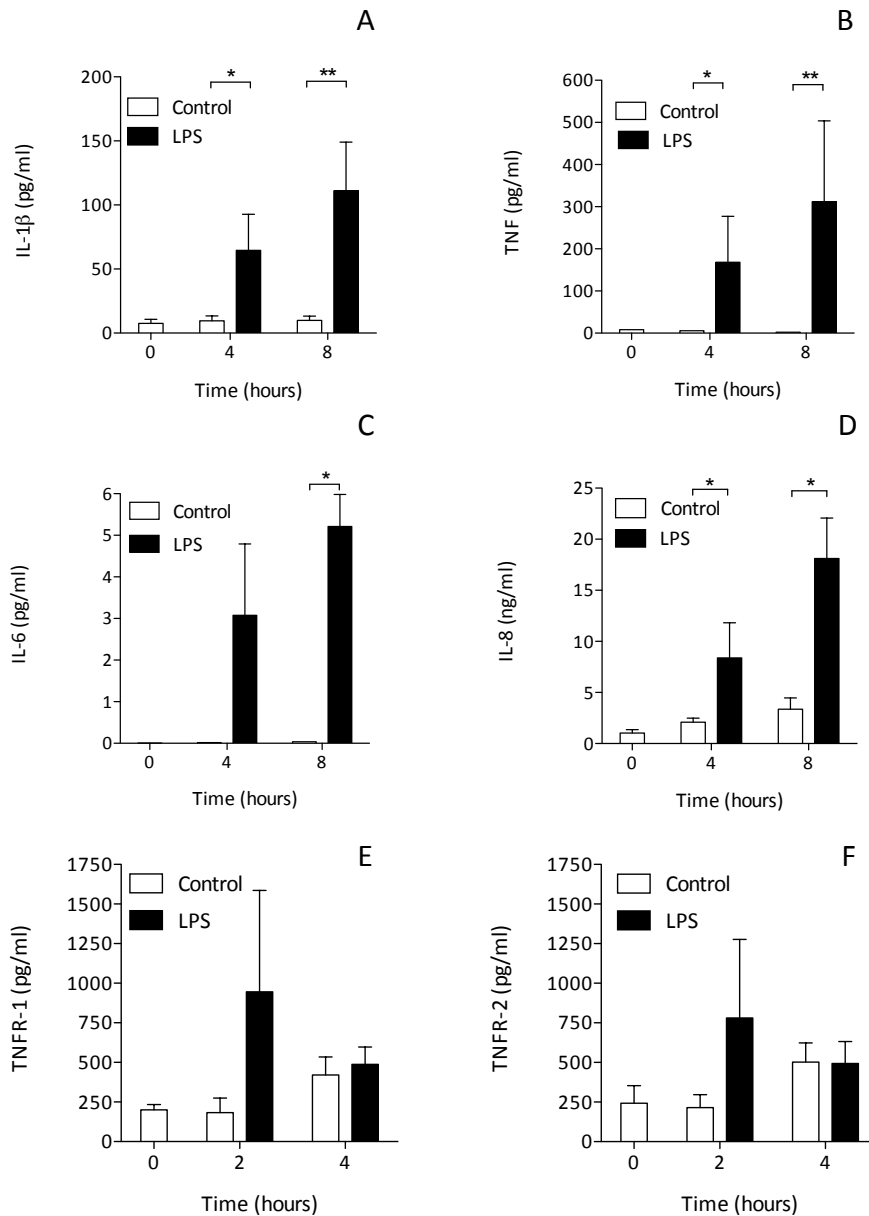


Figure 5.5 LPS injured *ex vivo* human lung demonstrated evidence of alveolar inflammation

LPS (0.1mg/kg, 10mL) or vehicle control (10mL) were instilled into the right middle lobe or left lower lobe respectively of human lungs on the EVLP circuit. After 2, 4 and 8 hours, the injury and control lobes were lavaged with 5% human albumin solution (50mL). Interleukin 1-beta (IL-1 β , panel A), Tumour Necrosis Factor alpha (TNF α , panel B), Interleukin-6 (IL-6, panel C), Interleukin-8 (IL-8, panel D), soluble TNF receptor-1 (TNFR-1, panel E) and soluble TNF receptor-2 (TNFR-2, panel F) were measured by sandwich enzyme linked immunosorbent assay (ELISA) n=3-6. Data are presented as mean $\pm$ SEM analysed using Mann Whitney U test *p<0.05, **p<0.01

5.3.5 The effect of LPS injury on pulmonary oedema, histological lung injury scores and neutrophilic infiltration into the alveolar space

LPS injury induced an elevation in lung tissue wet:dry ratio suggestive of pulmonary oedema in LPS injured segments (figure 5.6A). This effect was not translated into a significant elevation of histological lung injury scores, which included alveolar and septal oedema as part of the scoring system, although there was a trend towards increased injury in LPS treated lung tissue (figure 5.6B)

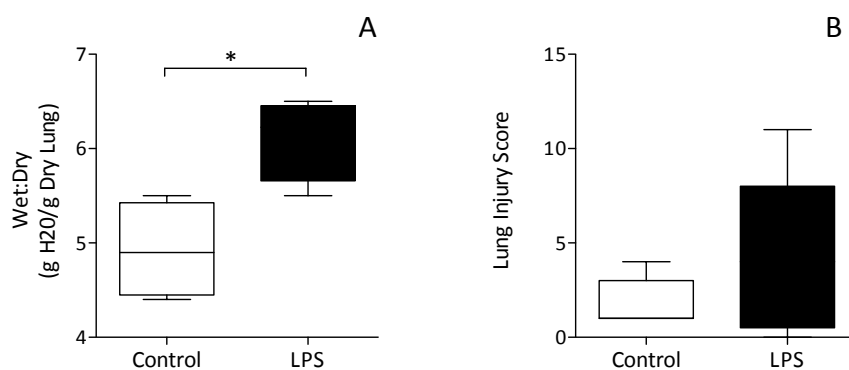


Figure 5.6 The effect of LPS injury on wet:dry ratio and lung injury scores in human *ex vivo* lung

Wet:Dry ratio (panel A) was determined by weighing a homogenised segment of injured or control lung (wet weight) and then re-weighing after 24 hours of freeze-drying (dry weight) and dividing the wet by the dry weight for each sample $n=4$. Lung Injury Scores were assessed by blinded pulmonary pathologist. Based on established scorings systems, a lung injury score between 0 and 24 was given to each sample analysed in duplicate $n=4$. Data are presented as mean $\pm$ SEM analysed by Mann Whitney U test, $*p<0.05$

LPS injured lung demonstrated significant influx of neutrophils into the alveolar space as evidenced by elevated neutrophil counts in the BALF (figure 5.7 A & C) as well as histological evidence of intravascular neutrophils in LPS injured lung (figure 5.7D). The tissue myeloperoxidase level in LPS treated lung tissue increased 2 fold although this failed to reach statistical significance (figure 5.10B).

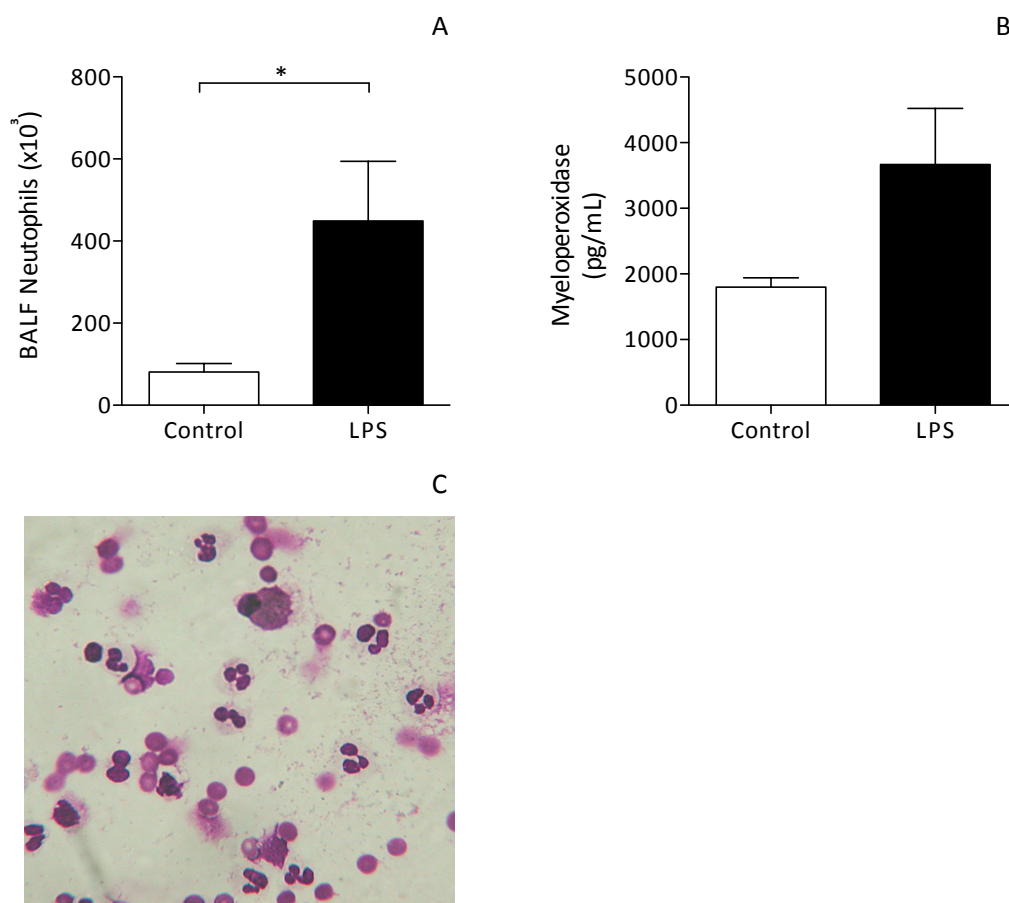


Figure 5.7 BALF of LPS injured *ex vivo* human lung demonstrated neutrophilic infiltration

Human lungs were injured with LPS (0.6mg/mL, 10mL) or vehicle control, After 4 hours the respective lobes were lavaged and representative tissue sections harvested. Panel A represents the neutrophil count in cytopsin of BALF from LPS and control treated lung n=5. Panel B represents the myeloperoxidase levels in protein extracted from harvested tissue n=4-5. Panel C is a representative cytopsin of LPS injured *ex vivo* lung. Data are presented as mean $\pm$ SEM analysed by Mann Whitney U test *p<0.05

5.3.6 LPS injury did not activate the STAT pathway in human *ex vivo* lung

LPS injury had no effect on activation of the signal transducers and activators of transcription (STAT) pathway as evidenced by STAT1/STAT3 phosphorylation measured by western blotting (figure 5.8 panels B & C). There was, however, significant variation in STAT1 phosphorylation; 3 of the samples demonstrated a doubling of STAT1 phosphorylation whilst 3 samples demonstrated no effect beyond basal conditions or vehicle treated tissue. p38 mitogen activated protein (MAP) kinase, an putative upstream

signalling regulator of the STAT pathway (Bode et al., 2012), similarly showed no difference between vehicle and LPS treated tissue (figure 5.8A).

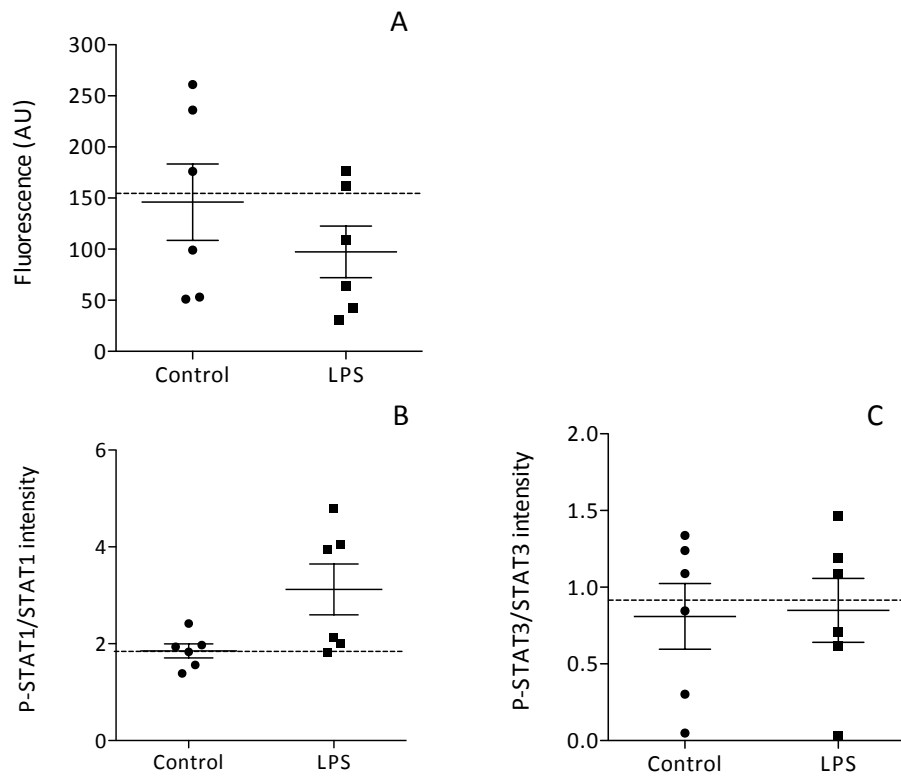
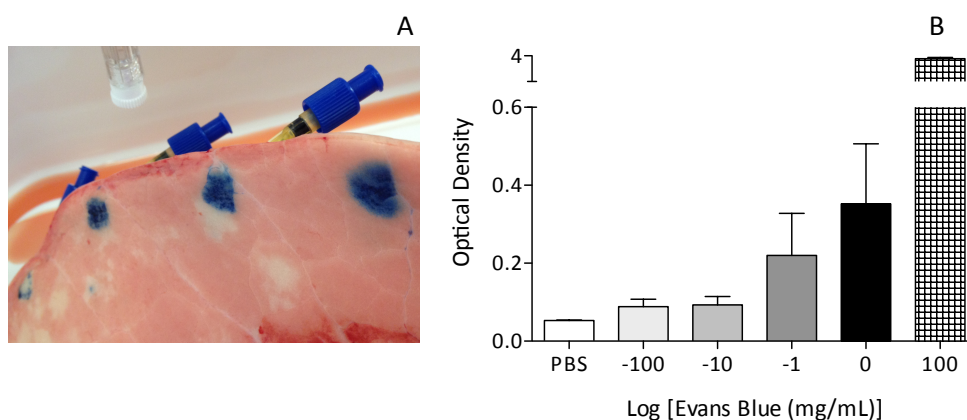


Figure 5.8 LPS did not activate p38MAPK or STAT pathways in *ex vivo* human lung

Human lungs were injured with LPS (0.1mg/kg, 10mL) or vehicle control. After 4 hours representative tissue sections were harvested and snap frozen for later processing. Protein was extracted from the defrosted lung tissue specimens and analysed for p38MAP kinase (ELISA, panel A) or STAT1 and STAT3 phosphorylation (Western blotting, panels B & C) n=5. Data are expressed as mean $\pm$ SEM. The dotted line represents mean fluorescence or intensity at 0 hours.

5.3.7 Methods to administer therapeutic intervention in *ex vivo* lung models

In preparation for experiments investigating the effect of GSK1995057 on LPS induced human lung injury we investigated whether discrete doses of Evans blue could be injected into areas of lung to facilitate a dose-response experiment. Using log fold dilutions of Evans blue we were able to demonstrate that this resulted in a sigmoidal-type dose response effect when harvested tissue was analysed (figure 5.9)



Figure

5.9 Evans blue dose response in *ex vivo* porcine lung

Porcine lungs were harvested from abattoir culled male pigs, placed on the *ex vivo* circuit and ventilated and perfused. Following rewarming, Evans blue was injected via a 23 gauge cannula into discrete areas of the right lower lobe at log fold dilutions of neat solution (100mg/mL). Representative tissue specimens were resected at 4 hours and placed in formamide for 24 hours. The supernatant was collected at absorbance measured at 610nm via a fluorescent plate reader n=3. Data are presented as mean $\pm$ SEM

We then repeated this experiment using UO126 (Cell Signalling, USA), a selective inhibitor of the extracellular signal-regulated kinase (Erk1/2) pathway (MEK1). At concentrations of 100 μ M there was a trend towards inhibition of Erk phosphorylation, however, this was non-significant (figure 5.10) reflecting both low sample numbers and probable sampling error as in previous LPS experiments.

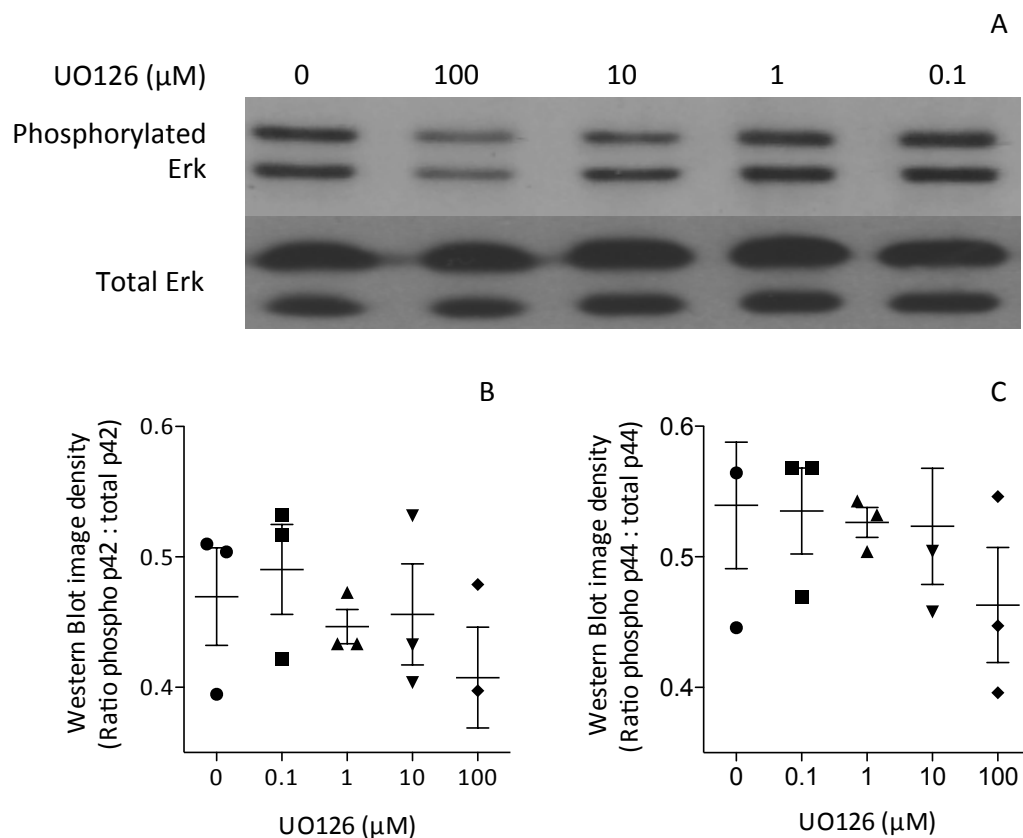


Figure 5.10 Dose response of UO126 a selective Erk1/2 MAP kinase inhibitor in *ex vivo* porcine lung

Porcine lungs were harvested from abattoir culled male pigs, placed on the *ex vivo* circuit and ventilated and perfused. Following rewarming, UO126 was injected into discrete areas of the right lower lobe at log fold dilutions. Representative tissue specimens were resected at 4 hours and snap frozen. Intracellular protein was then extracted and total and phosphorylated Erk measured in 20 μg of protein by Western blotting (panel A). The developed blots were analysed using Image J software to quantitate the density of the bands and the relative values of phosphorylated MAPK to total MAPK (panel B p42 Erk, panel C p44 Erk) $n=3$. Data are presented as mean $\pm$ SEM

5.4 Summary of important findings in this chapter

We have established a method for obtaining human lungs declined for transplant and re-animating them *ex vivo*. Despite prolonged CITs (up to 39 hours in individual cases) these lungs do not demonstrate evidence of physiological deterioration or histological injury after 8 hours of normothermic EVLP.

We have refined our method of LPS administration such that we are able reliably to induce release of pro-inflammatory cytokines in the alveolar space. This is associated with influx of donor neutrophils and pulmonary oedema, as evidence by elevated wet:dry ratios in LPS treated lungs. However, there was no histological evidence of ALI.

Method development designed to facilitate pharmacological study in *ex vivo* lungs demonstrated that dose-response-experiments in single lobes are limited by sampling error

5.5 Discussion

5.5.1 Model Development

We used group-specific healthy volunteer whole blood added at a ratio of 1:10 to the perfusate based on published methodology from the UCSF group. Autologous whole blood was not available due to logistical and ethical considerations. The addition of whole blood was critical to induce impaired AFC (Lee et al., 2009) and it would seem likely, that neutrophil infiltration into the alveolar space is dependent on the presence of circulating neutrophils (and possibly monocytes and platelets, chapter 1.4.4.4) (Abraham et al., 2000; Folkesson et al., 1995; Folz et al., 1999; Kawabata et al., 2000; Looney et al., 2006; Shimizu et al., 2009). In the study by Lee *et al*, the majority of experiments were performed using blood donated by AB, Rh+ volunteers. Despite this, no adverse changes

in lung physiology or endothelial permeability in control lung lobes were observed suggesting that, for short term study at least, non-matched whole blood can be used.

Currently, there exist 2 clinical methods of EVLP; the ‘Toronto’ method using a closed system and an acellular perfusate (Cypel et al., 2011; Cypel et al., 2008) and the ‘Lund’ method applying an open system with the addition of matched red cells to the perfusate (Ingemansson et al., 2009). We prioritised technical simplicity and were discouraged from using the closed system by initial experiences suggesting that the need for an intact left atrial cuff added a premium to the number of available lungs. Maintenance of a positive left atrial (LA) pressure of 3-5mmHg may, however, be vital. This small, but positive LA pressure may prevent collapse of distal veins during inspiration (Petak et al., 2002), whilst stabilizing alveolar geometry and lung compliance (Broccard et al., 2002). In the only published series of prolonged EVLP, application of a closed system with protective ventilation maintained stable lung function over 12 hours in human lungs and demonstrated good lung function and low levels of oedema in porcine lungs perfused for 12 hours subsequently transplanted into recipient pigs (Cypel et al., 2008). Despite employing an open system, we demonstrated stable physiology with no evidence of injury over 8 hours EVLP (figures 5.3 and 5.4). Nonetheless, for future experiments requiring longer durations of EVLP, for example investigation of the kinetics of cytokine release in LPS injured lungs, reverting to a closed system may be optimal.

5.5.2 The impact of prolonged cold ischaemia

Prolonged CITs have variably been associated with poor outcomes. Whilst some studies concluded that graft CIT was not an independent predictor of increased adverse outcomes (Gammie et al., 1999; Fiser et al., 2001) except with donors over 50 years (Meyer et al., 2000) other clinical and experimental studies have suggested that CITs beyond periods as short as 5 hours are detrimental (Snell et al., 1996; Fischer et al.,

2000). Moreover, in patients with reperfusion oedema after lung transplantation, a correlation was found between CIT and capillary-alveolar protein permeability (Ware et al., 1999) and in a human EVLP model, prolonged CIT was associated with alveolar epithelial injury as evidenced by submaximal alveolar fluid clearance and higher levels of RAGE (Briot et al., 2009). We adopted a pragmatic approach to CIT in our model. Our protocol targeted a CIT of less than 36 hours, as this seemed a practical compromise that increased the potential donor pool such that we could select lungs in younger donors and with better baseline gas exchange that might have prolonged CITs.

Our lungs did not demonstrate histological evidence of injury (figure 5.4) nor baseline inflammation (figures 5.5 and 5.6) despite a mean CIT of 34 hours (table 5.1). However, previous data has suggested that many lungs rejected for transplantation based on conventional clinical criteria did not demonstrate significant histological abnormalities nor did they demonstrate abnormal alveolar epithelial fluid transport capacity (Ware et al., 2002). We were also unable to definitively confirm that our lungs, despite the presence of structural integrity, were biologically viable. Previous data suggest that, at least in porcine lung, 12 hours of EVLP allows successful transplantation with good organ function (Cypel et al., 2008). Such categorical proof is clearly not logistically feasible in experimental human lungs. Nonetheless, as surrogate markers of viability, we have been able to culture hAT2 after 8 hours EVLP and have maintained precision cut lung slices (PCLS) from EVLP *in vitro* for a further 3-4 days with no loss of viability as measured by lactate dehydrogenase release and synthesis of surfactant lipids (data not shown).

Previous studies by the group at UCSF have used baseline AFC as both a marker of suitability for experimentation and as an outcome measure in interventional studies (Lee et al., 2009; Briot et al., 2009; Lee et al., 2013). They were able to demonstrate firstly that perfusion *per se* increased AFC 4-fold and that the basal AFC rate (Frank et

al., 2007) was comparable to that measured in other species (Matthay et al., 2002). Moreover, the AFC determined in their model was comparable to estimated AFC rates in patients with pulmonary oedema (Ware and Matthay, 2001; Verghese et al., 1999). It is notable that AFC was preserved, despite the absence of a pulmonary flush with preservation solution prior to cold storage; this has been developed to both cool the lungs (to reduce metabolic activity during transport) and provide endothelial protection in the immediate post-transplantation phase (de Perrot and Keshavjee, 2003). In initial experiments, we attempted to use AFC as a baseline measure of suitability for experimentation and baseline injury. However, we were unable to reliably reproduce the published methodology (Lee et al., 2009; Frank et al., 2007). We therefore used computed tomography assessment of interstitial oedema as a clinically relevant index of baseline injury and 'leak' (data not shown) prior to our interventional studies.

5.5.3 Endotoxin induced acute lung injury

We developed a reproducible *E. coli* endotoxin-induced ALI model in human *ex vivo* lung preparation perfused partially with group matched donor whole blood. Instillation of LPS into the distal airspaces of the right lower lobe increased lung water (figure 5.6A) and induced an acute neutrophilic inflammatory response within the injured alveolus (figure 5.7) associated with elevated levels of IL-1 β , TNF, and IL-8 (figure 5.5), the most biologically active cytokines found in ALI pulmonary edema fluid (Ware and Matthay, 2000).

In the only comparative *ex vivo* model, Lee *et al* (Lee et al., 2009) demonstrated similar trends in cytokine release and an associated neutrophilic alveolitis but at an approximately 10-fold higher magnitude in both control and LPS injured lungs; in their analysis, both the cytokine concentrations and neutrophil counts were multiplied by the volume of BAL injectate (125mL). In parallel with our findings (figure 5.4), they did not

demonstrate significant histological injury other than neutrophil infiltration into the alveolar space, suggesting that whilst sufficient to induce a neutrophilic alveolitis both the concentration of endotoxin and the duration of assessment may be insufficient to induce classical histological changes of ALI such as a denuded basement membrane and hyaline membrane formation (chapter 1.1.2.1). Absence of loss of alveolar type 1 cells or proliferation of alveolar type 2 cells was further confirmed in tissue sections examined by transmission electron microscopy (data not shown). The absence of significant alveolar epithelial damage is similarly demonstrated in currently available animal models of ALI (Bastarache and Blackwell, 2009; Matute-Bello et al., 2008; Ware, 2008) and represents one of the major failings and indeed criticisms of current ALI modeling (Matute-Bello et al., 2008).

In vivo human data using intra-tracheal administration of LPS at doses ranging from 1 to 4 ng/kg similarly demonstrated increases in BALF neutrophils and IL-1 β , TNF, and IL-8 (as well as other neutrophil chemokines) (O'Grady et al., 2001). This effect peaked at 6-8 hours and was associated with increased permeability to protein and albumin. Interestingly, the authors also noticed an increase in both TNFR1 and TNFR2 in the BALF, an effect which was sustained for 24 hours; whilst we noticed a trend towards elevated TNFR release at 2 hours, this was not sustained at 4 hours (figure 5.5). CMS and TNF treated hAT2 *in vitro* did not release significant TNFR (figure 3.8) suggesting that TNFR release into the BALF may originate from the systemic circulation as opposed to direct epithelial injury.

We could not demonstrate activation of the STAT pathway or upstream signalling kinases such as p38 MAP kinase in LPS injured lungs (figure 5.10). The STAT family of transcription factors (as well as NF- κ B, MAPK, and phosphatidylinositol 3-kinase pathways) have been shown to participate in mediating lung inflammatory responses

(Severgnini et al., 2004; Rahman and MacNee, 1998), in particular the host response to LPS (Kovarik et al., 1998). Indeed in 3 different murine models of ALI including intra-peritoneal LPS, STAT activation was demonstrated throughout the lung in resident cells as well as recruited inflammatory cells (Severgnini et al., 2004). Moreover, this effect occurred before evidence of histological lung injury or release of TNF in the BALF. Therefore, in the absence of demonstrable histological injury (figure 5.8B), we used activation of the STAT pathway as an early injury marker in our model.

Potential reasons for the observed lack of STAT activation are multiple. First, this may represent sampling error. These samples in these experiments were collected using blind endobronchial instillation of endotoxin. The wide variation in STAT1 activation in our dataset (figure 5.8B) may indicate that the LPS exposure of the samples was variable owing to sampling error. This represents a current limitation of the model as discussed above. Second, HMVEC-L and murine epithelial cells treated with LPS did not demonstrate evidence of STAT activation (Severgnini et al., 2004) suggesting that STAT activation requires resident phagocytic cells, via LPS driven production of cytokines or ROS; LPS induced STAT activation was ROS dependent in mice and IL-6 but not TNF or IL-1 β induced STAT activation *in vitro* (Severgnini et al., 2004). We did not demonstrate significant release of IL-6 in our model (figure 5.5). Finally, in mice, maximal pulmonary STAT activation was observed after that seen in the liver (Severgnini et al., 2004) suggesting that a hepatic acute phase response may propagate lung inflammation. Whilst the EVLP model affords an opportunity to investigate the lungs in isolation, the absence of other organs does not allow for analysis of the role of the systemic inflammatory response.

5.5.4 The *ex vivo* lung model as a platform for pharmacological study

We undertook dose response experiments (figures 5.9 & 5.10) to establish a method of performing dose-response studies in *ex vivo* lung. By virtue of the scarcity of resource and the current cost of obtaining human lung tissue (£1800 per set of lungs), it would be optimal to investigate multiple doses of a therapeutic compound in a single lung. Our preliminary data (figures 2.26) suggested that nebulised solutions administered to *ex vivo* lung result in homogenous distribution to distal lung. In a pilot gene transfer experiment, nebulisation of the combination of the cationic liposome (GL67A) and plasmid DNA expressing CFTR (pGM169) in porcine *ex vivo* lung demonstrated protein expression in distal lung and perfusate (data not shown) implying that nebulised therapeutics successfully penetrate the alveolar space in *ex vivo* lung models. The use of a double lumen endotracheal tube or bronchial blocker to obstruct specific lobes may facilitate analysis of a nebulised compound compared to control but nebulisation, as a means of drug delivery, does not generally allow for multiple dosing schedules. Nonetheless, the nebulised Evans blue data and these gene transfer data suggest that the model, in principal, holds promise for assessment of nebulised therapies.

Our data (figures 5.9 & 5.10) suggested that peripheral administration of compounds is not sufficiently sensitive to be a viable future methodology; this probably relates to sampling error. Moreover, for ALI models in particular, the most robust markers of injury appear to be BALF inflammatory cytokines and neutrophil infiltration into the alveolar space. Systemic administration of either an injurious agent or novel therapeutic into the perfusate does not allow for an internal control, one of the strong advantages of the current set-up. Hence, endobronchial administration of experimental therapies is the more promising technique for pharmacological experiments.

Our current experimental design for investigation of selective TNFR1 antagonism in a human *ex vivo* ALI model is outlined in figure 5.11. We have established this model on 2 occasions, suggesting that the method is feasible, however, on both occasions we were unable to complete the protocol due to early (2 hours after LPS administration) pulmonary oedema affecting both lungs. Whilst a dose response is not technically possible, the experimental design has an internal control for both the intervention (GSK1995057 vs dummy domain antibody) and the injury (LPS vs vehicle), allowing a large volume of data to be generated from a single experiment. Furthermore, despite the dilution factor of the BALF and elevated levels of soluble TNFR (figure 5.5), TNF bioactivity was detectable during the first 4-8 hours suggesting high local lung concentrations of TNF. Thus, in parallel with our *in vitro* data, we speculate that selective TNFR antagonism may modulate the local inflammatory response in human *ex vivo* LPS induced ALI models.

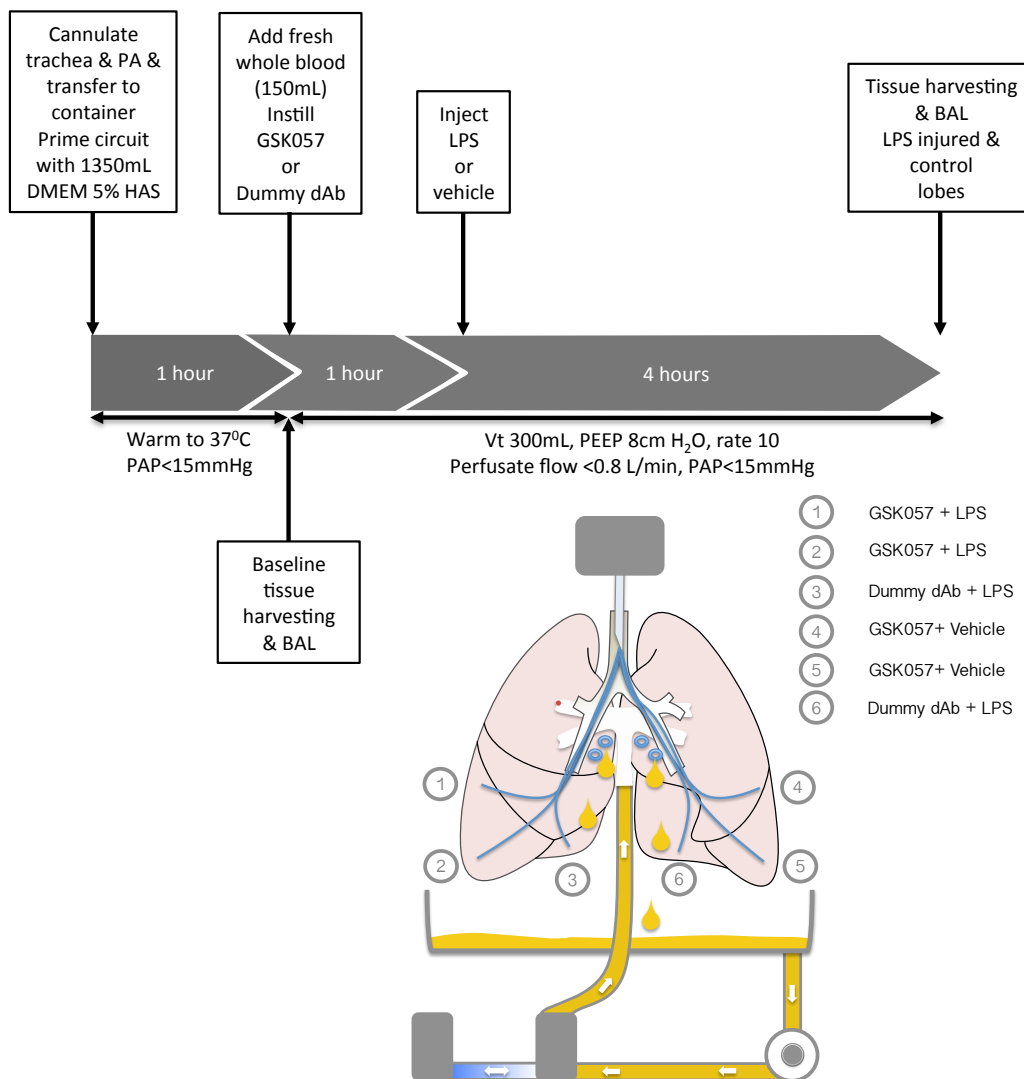


Figure 5.11 Protocol for investigation of effect of selective TNFR1 antagonism in LPS injured *ex vivo* human lung

Human lungs are rewarmed on the EVLP circuit and group-specific whole blood (150mL) added to the perfusate. GSK1995057 (0.2µg/mL, 10mL) or dummy domain antibody (0.2µg/mL, 10mL) is injected via catheters positioned in distal lung segments. After 1 hour, LPS (0.1mg/kg, 10mL) or vehicle control (10mL) is injected via the same catheters. Sampling of BAL and tissue is undertaken at 4 hours post-LPS injury. BAL: broncho-alveolar lavage, DMEM: Dulbecco's modified eagle medium, HAS: human albumin solution, LPS: lipopolysaccharide, PA: pulmonary artery, PAP: pulmonary artery pressure, PEEP: positive end expiratory pressure, Vt: tidal volume

5.5.5 Harnessing the potential of *ex vivo* ALI models

The applications of our model are currently limited by the quality of lung substrate and the availability of lung tissue. We have now completed a total of 16 human *ex vivo* lung perfusion experiments ie. ventilation and perfusion were established, with or without ALI modelling, for at least 4 hours. Around 25% of our experiments were abandoned because the lungs developed pulmonary oedema at an early stage. This probably reflects the prolonged CIT in our lung substrate allied to donor factors. Furthermore, the frequency of lung offers was sporadic. As a result, our cumulative understanding and experience of the model has been hindered; by comparison, the UCSF group gain access to around 1 *ex vivo* lung per week albeit that only 10-20% of their lungs generate meaningful data (personal communication JW Lee, UCSF). Nonetheless, the potential of the model to facilitate investigation of a range of pathological processes in ALI is significant.

Our model and others' undoubtedly recapitulate some of the key features of *in vivo* ALI in a humanized model that is highly tractable and allows temporal sampling of distal lung tissue. Moreover, such models have already generated novel translational data (Lee et al., 2009; Lee et al., 2013), which have formed a considerable component of the preclinical evidence base for a phase 1 trial investigating the role of mesenchymal stem cells in the treatment of ALI (clinical trials number NCT01775774). It is clear that no model, animal or human, can replicate a multi-faceted syndrome. Nonetheless, the refinement of LPS human models allied with the development of alternative injury models (eg. acid induced injury) and data from *in vivo* human models such as post-oesophagectomy or inhaled LPS, has the potential to generate meaningful data to inform future clinical trials in ALI.

Hence a mechanism to gain access to increased numbers of donor lungs with shorter CITs would be desirable. To this end, we have submitted an ethics application with NHS Blood & Transplant to allow us to gain access to lungs from brain-dead donors in UK hospitals who have consented for donation of their organs for research purposes, if rejected for solid organ transplantation. The resulting increase in tissue availability and significantly shorter CITs would enable us to adopt a more rigorous approach to assessing and characterising organs, such that the best lungs will be used for complex functional experiments.

5.6 Future directions

The immediate aim is to complete experiments investigating selective TNFR1 antagonism in LPS injured *ex vivo* human lung to complement much of the *in vitro* data generated from work in this thesis. In particular, the effect of GSK1995057 on neutrophil migration into the alveolar space in an *ex vivo* model merits further study.

Subsequently, we plan to extend our own *in vitro* and others' animal *in vivo* observations regarding cellular trafficking in pulmonary inflammation into an *ex vivo* human model. For example, recruitment of neutrophils to the pulmonary interstitium in animal models is both platelet and monocyte dependent (Pitchford et al., 2003; Kreisel et al., 2010; Kornerup et al., 2010). Combining our ALI model with collaborators' experience with *ex vivo* radiolabelling techniques, nuclear imaging and two-photon intravital microscopy may allow novel mechanistic insights into inflammatory cell trafficking in human lung.

We have based our model almost entirely on published methodology from the UCSF group, using LPS as the injurious stimulus. With increasing experience of the technique, ideally in the context of greater throughput of human lungs, there exists the

potential to develop alternative injury models such as human gastric acid aspiration and live bacteria to broaden the translational potential of the platform.

6 Chapter 6: General Discussions

6.1 Summary of important results

TNF induced release of IL-8, IL-6 and GM-CSF from lung alveolar epithelial cells and lung microvascular endothelial cells, was predominantly controlled by TNFR1 signalling. Pre-treatment of lung microvascular cells with the selective TNFR1 antagonist GSK1995057 had no effect on IL-10 release from TNF stimulated cells, whereas dual TNFR blockade reduced IL-10 release to basal levels, suggesting that IL-10 release may be predominantly controlled by TNFR2 signalling.

Cyclic mechanical strain induced release of IL-8, IL-6, and IL-1 β from hAT2 but this effect did not appear to be mediated by either TNFR. There was no effect of CMS on either sTNFR1 or sTNFR2 release, suggesting that sTNFR release in clinical studies (of high versus low tidal volume ventilation) does not originate directly from the effect of mechanical stimulation of the alveolar epithelium.

Neither TNF alone nor differential TNFR signalling affected wound healing in hAT2. In human umbilical vein endothelial cells, TNFR2 signalling promoted tube formation but this effect was not paralleled in cells from the lung microvascular endothelium.

The TNFR1 signalling pathway mediated lung microvascular cell injury, measured as decreased in trans-endothelial resistance in HMVEC-L and shedding of sICAM-1, sVCAM-1 and sE-selectin. Similarly, up-regulation of the neutrophil adhesion molecules ICAM-1, VCAM-1 and E-selectin (indicative of endothelial cell activation) was TNFR1 signalling-dependent. Accordingly, selective TNFR1 antagonism ameliorated neutrophil migration through HMVEC-L monolayers, an effect that may be due to blockade of up-

regulation of these neutrophil adhesion molecules although further functional studies, (discussed in chapter 6.4), are required.

TNFR1 signalling played key roles in neutrophil priming, activation, and cell fate. Hence, TNFR1 was the main receptor mediating TNF-induced alterations in neutrophil cell surface molecule expression, modulating changes in CD11b, CD62L, TNFR1 and TNFR2. TNFR1 was also the dominant receptor mediating TNF-induced reactive oxygen species production. The role of TNF receptors in modulating neutrophil apoptosis was examined; whilst engagement of both receptors was required to induce early neutrophil apoptosis, TNFR1 antagonism reversed TNF induced late survival to constitutive levels of apoptosis. In contrast to previous animal studies, we found no evidence that TNFR2 signalling modulated neutrophil priming (measured by ROS generation and cell surface molecule expression).

In an attempt to generate translational data investigating the effects of differential TNFR1 signalling, we established a human *ex vivo* model of ALI. Using human lungs declined for transplant, we used an LPS method of injury based on previously published methods. We were able reliably to induce release of the pro-inflammatory cytokines TNF, IL-8, IL-6 and IL-1 β in the alveolar space. This was associated with a significant influx of healthy donor neutrophils and pulmonary oedema in LPS injured lung compared to control treated contralateral lung, despite the absence of histological evidence of ALI. Unfortunately our attempts to investigate the effects of TNFR1 antagonism on neutrophil migration into the alveolar space in this model have been unsuccessful to date but study is on-going.

In summary, these data suggest that TNFR1 has a central role in multiple phases of neutrophil transmigration from the lung microvasculature into the alveolar space. Additionally, TNFR1 signalling may play key roles in TNF mediated neutrophil priming,

activation, and cell fate (summarised in figure 6.1). Hence, neutrophils are attracted to sites of injured microvascular endothelium down a chemotactic gradient in both the vascular and alveolar compartments; chemokine production appears to be regulated via TNFR1 signalling. Owing to priming induced shape-change and TNFR1 induced changes in cell surface adhesion molecules on both neutrophils and lung endothelial cells, neutrophils arrest in the pulmonary circulation before transmigrating to the alveolar space. TNFR1 signalling may promote neutrophil transmigration through the lung microvascular endothelium through endothelial cell activation and TNFR1 induced gap junction formation. Once in the alveolar space, neutrophils further propagate injury via TNFR1 driven respiratory burst. Neutrophilic alveolitis is perpetuated by TNF induced survival signalling, a process, which is also probably TNFR1 mediated.

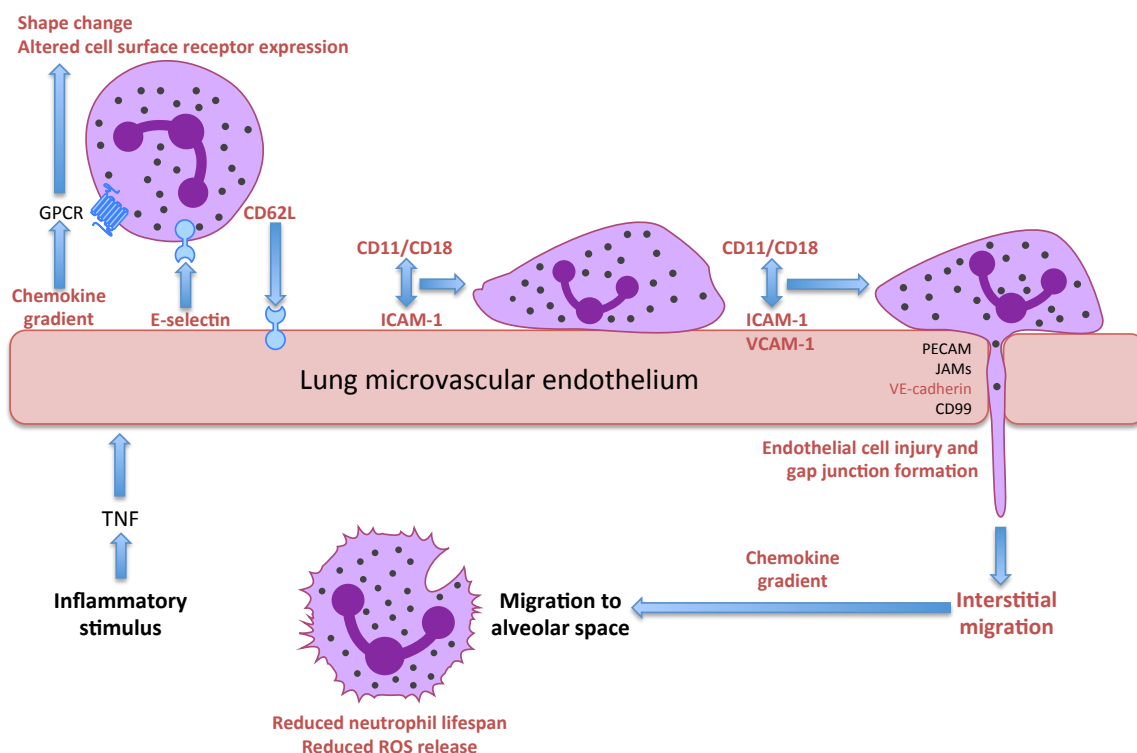


Figure 6.1 Summary of important results of this thesis and potential effects of selective TNFR1 antagonism on neutrophilic alveolitis in ALI

The annotation in red highlight processes that may potentially be modulated by selective TNFR1 antagonism

ICAM-1 – intracellular adhesion molecule 1; JAMs – junctional adhesion molecules; PECAM - Platelet endothelial cell adhesion molecule; VCAM-1 - vascular cell adhesion molecule 1

6.2 Experimental models

6.2.1 Co-culture experiments

For simplicity, we investigated differential TNFR signalling in cell monolayers and suspensions of purified neutrophils. Lung injury, however, is increasingly recognized to result from a complex interplay between cells of the alveolar capillary membrane, alveolar macrophages, neutrophils, monocytes and platelets. For example, numerous studies have demonstrated that activated neutrophils induce inter-endothelial gap formation via secreted neutrophil products, through adhesion dependent mechanisms and by alterations in the balance between ROS and nitric oxide (Wang and Doerschuk, 2002). Hence, total neutrophil depletion and disruption of surface integrins or oxidant production, blunted lung vascular permeability increases (Gao et al., 2005; Gao et al., 2002; Xu et al., 2002). Intriguingly, neutrophil migration has also been shown to trigger repair of alveolar epithelial cells after transmigration induced injury (Zemans et al., 2011). Whilst we have demonstrated that differential TNFR signalling may affect neutrophil migration through TNF stimulated HMVEC-L (figure 4.12), further investigation of neutrophil-endothelial and neutrophil-epithelial interactions in co-culture experiments would further delineate the role of differential TNFR signalling in these processes. Similar co-culture experiments could be undertaken with platelet and neutrophil co-cultures as well as monocyte-neutrophil co-cultures, in particular examining the role of TNF signalling in platelet and monocyte adhesion molecule expression and their subsequent interactions with both neutrophils and the lung microvascular endothelium. Significant *in vitro* findings, in principle, could then be translated in human *ex vivo* lung experiments.

In addition the relative role of alveolar macrophages in inflammation induced by mechanical ventilation could be assessed. Alveolar macrophages from the same lung specimens would be added to the culture medium immediately before CMS is performed

in hAT2 cells. Such experiments could shed light on the relative contribution of alveolar macrophages to cytokine and chemokine production in high stretch ventilation.

Alveolar epithelial permeability is critical in alveolar flooding (Wiener-Kronish et al., 1991) and implicated in intra-alveolar fibrin deposition and hyaline membrane formation (Bastarache et al., 2009), whilst preserved epithelial barrier function is inversely associated with patient mortality (Ware and Matthay, 2001). A dichotomous role for TNF in regulating alveolar permeability has been suggested. Hence, TNF inhibited epithelial sodium uptake, which is crucial for alveolar liquid clearance, by means of reducing the expression of subunits of the epithelial sodium channel ENaC (Dagenais et al., 2004). This effect was probably mediated by a TNFR1 and ceramide-dependent mechanism (Bao et al., 2007). By contrast, TNF increased alveolar fluid clearance in a rat pneumonia model (Rezaiguia et al., 1997). Experiments investigating the role of differential TNFR signalling in mediating epithelial permeability are currently on-going.

6.2.2 siRNA knockdown of TNFR in endothelial and epithelial cells

Despite the large number of studies investigating TNF as a potential therapeutic agent, the complexity of TNF biology in ALI and in general is poorly appreciated. This work has confirmed that many of the potentially injurious events in TNF mediated lung inflammation occur through TNFR1 mediated signalling. As such, TNFR1 antagonism represents a potentially attractive therapeutic target. Nonetheless, we have failed to convincingly answer the hypothesis, based on animal studies of TNFR signalling (Bertok et al., 2012; Wilson et al., 2007), that selective antagonism of TNFR1 may also potentiate the protective effects of TNFR2 signalling. Hence, based on the data presented in this thesis, it is difficult to argue the putative benefits of selective TNFR1 antagonism compared to dual blockade of both receptors. Whilst we endeavoured to interrogate

TNFR2 signalling using a TNFR2 specific mutein, the results from these experiments were unconvincing and we could not demonstrate a TNFR2 specific signalling event.

The use of selective siRNA knockdown of TNFR in alveolar epithelial and lung microvascular endothelial cells may allow interrogation of the hypotheses that TNFR2 signalling plays a central role in arteriogenesis (Luo et al., 2010), endothelial and epithelial cell apoptosis (Fontaine et al., 2002) and maintenance of the alveolar capillary membrane through cytoskeletal rearrangements (Hamm-Alvarez et al., 2001; Pan et al., 2002). Whilst technically challenging, this methodology has been successfully used in non-pulmonary endothelial cells (Perrot-Appianat et al., 2011) and these data may more definitively outline the role of TNFR2 signalling in human models of ALI.

6.3 Clinical Importance

Despite 40 years of investigation no pharmacotherapy can be recommended as standard management of ALI. Deficiencies in the translation of promising experimental data into clinically effective therapies may be partly explained by limitations of the currently available pre-clinical animal models of ALI (Matute-Bello et al., 2008; Bastarache and Blackwell, 2009). Failed clinical trials in ALI also probably result from delayed recognition of patients at risk and the subsequent development of the full-blown syndrome.

Recognising these limitations, the work in this thesis was designed to target both these short-comings. Hence, we have developed a tractable human *ex vivo* ALI model and translational *in vitro* human data underpinned by extensive animal pharmacodynamic and pharmacokinetic data from a novel domain antibody from GSK. Moreover, the data using the selective TNFR1 antagonist GSK1995057 in this thesis were generated exclusively by exposure of cells to GSK1995057, prior to TNF stimulation. This experimental strategy was employed for 3 reasons. First, data regarding the kinetics of TNF release and anti-

TNF strategies in sepsis models (Beutler et al., 1985; DeForge et al., 1990; Tracey et al., 1987) suggest that anti-TNF therapy in ALI (to demonstrate clinical efficacy) would need to be given within hours of onset of lung injury. Second, there is a growing recognition within the ALI scientific community that treatment of established ALI has thus far been unsuccessful (Kor et al., 2012; Levitt and Matthay, 2012). Contemporary clinical trials have largely targeted a patient population within 48 hours of meeting ARDS criteria, who are already requiring mechanical ventilatory support for established gas exchange abnormalities. The administration of preventative ALI therapies may therefore be a more effective treatment strategy. The application of risk stratification scoring, for example the Lung Injury Prevention Study (LIPS) score (Gajic et al., 2011) or informatics technology to identify high-risk patients (Herasevich et al., 2009) may provide an opportunity for the administration of therapies such as selective TNFR1 antagonism prior to the onset of the full-blown ARDS. Those patients undergoing elective cardiothoracic surgery who are at high risk of developing ALI (chapter 1.2.2.1) may represent a further cohort of patients that may benefit, although the safety of this approach would need to be established. Using this paradigm, and similarly targeting neutrophil activation and leukocyte adhesion, a recent multi-centre, placebo controlled, phase II trial of aspirin in patients at high risk of ALI (defined by LIPS scoring) has recently commenced in the USA (Kor et al., 2012). Third, in established ALI owing to loss of surfactant, a large volume of dependent lung becomes collapsed and consolidated (chapter 1.1.2) thus reducing the likelihood of inhaled compounds accessing the most severely injured lung units. This observation was cited as one of the reasons that inhaled albuterol failed to improve outcomes (ventilator-free days) in a recent clinical trial, despite protocol design to ensure optimal alveolar drug delivery (Matthay et al., 2011). Indeed, in this trial, albuterol could be measured systemically, suggesting that the majority of drug was delivered to non-injured alveoli allowing systemic absorption. Our own data (chapter 2.2.12.7) demonstrated that

nebulization of dye to normal lung resulted in homogenous distribution throughout distal lung *ex vivo*, suggesting that administration of prophylactic inhaled GSK1995057 (assuming droplet size and administration method were optimized) would reach distal lung prior to the onset of injury. Not only would this possibly confer protection to the alveolar capillary epithelium, but any compound absorbed systemically would be at maximal concentration in the lung microvasculature where the putative benefits outlined in figure 6.1 could be realised.

Hence, the effect *in vivo* of selective TNFR1 antagonism as a preventative therapy to modulate neutrophil migration in the lung was recently highlighted in an inhaled LPS model of ALI (O'Kane et al., 2013; Proudfoot et al., 2014). Healthy volunteers were randomised to nebulized GSK1995057 or placebo, 1 hour prior to administration of inhaled LPS. Inhalation of GSK1995057 was associated with a trend towards reduced BAL neutrophilia with significant reductions in BAL cytokines and chemokines. Interestingly, and in parallel with our *in vitro* data, inhaled GSK1995057 appeared to reduce endothelial activation as evidenced by reduced BALF levels of the endothelial injury marker von Willebrand factor. This study also demonstrated that single doses of inhaled GSK1995057 in healthy subjects had an acceptable safety profile, was well tolerated, and demonstrated reproducible pharmacokinetic and pharmacodynamics data (Proudfoot et al., 2014; O'Kane et al., 2013).

6.4 Future Directions

Given the absence of available pharmacological interventions, there is a clear and urgent need for novel therapies in ARDS. The work from this thesis, combined with extensive animal data from GSK (unpublished data) and *in vivo* human data from the McAuley group in Belfast (O'Kane et al., 2013) suggest that leukocyte-endothelial interactions and endothelial activation are potential targets for selective TNFR1 inhibition

in clinical trials. Accordingly a multi-centre phase II trial of selective TNFR1 inhibition as prophylaxis against ALI in oesophagectomy patients will commence in October 2014. Further mechanistic insight is nonetheless required.

Our collaborators at Cambridge University have established a neutrophil-endothelial flow chamber system whereby neutrophils are passed over monolayers of HMVEC-L at variable flow rates and video-microscopy used to quantify neutrophil migration. This assay would allow interrogation of the effects of selective TNFR1 antagonism (and TNFR1 agonism) on leukocyte migration under flow conditions, and may be more representative of *in vivo* neutrophil-endothelial interactions. The mechanism of any TNFR1-mediated effect in the flow-based assay could be further explored using selective TNFR knockout mice, or mice treated with GSK1995057 combined with two-photon intra-vital lung microscopy techniques. Expertise with these techniques is currently being developed with our collaborators.

Moreover, whilst the putative benefits of GSK1995057 induced disruption of TNFR1 signalling have been outlined in this thesis, the impact of preservation of TNFR2 signalling, in our own models and others, has been limited. TNF signalling through TNFR2 was recently shown to be indispensably required for the protection of the skin against oxidative stress-induced inflammation in the zebrafish. The absence of TNFR2 signalling in zebrafish triggered the local production of H_2O_2 , which activated NF- κ B to induce up-regulation of genes encoding pro-inflammatory mediators and neutrophil infiltration (Candel et al., 2014). Our own data suggest that IL-10 release and possibly tube formation are TNFR2 specific signalling events. Genomic analysis using DNA microarray technology of HMVEC-L treated with either GSK1995057 or the TNFR specific muteins may help identify candidate genes specific to the TNFR2 signalling

cascade, adding further insight into the potential effects (clinically appealing or indeed harmful) of selective TNFR1 antagonism in human cells.

Appendix A

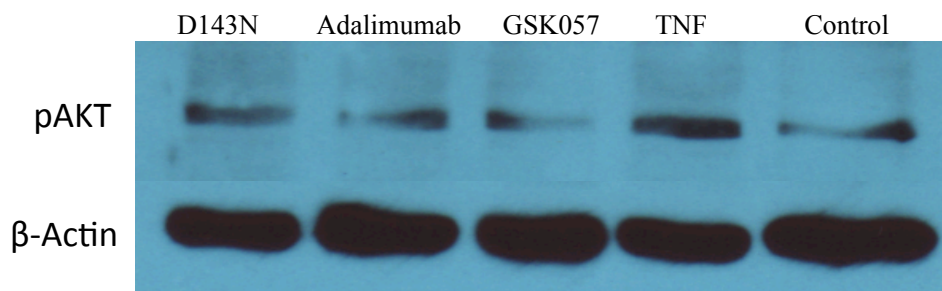


Figure A.1 Representative western blot for phosphorylated AKT in TNF treated human precision cut lung slices

Human precision cut lung slices $\sim 400\mu\text{m}$ in 12-well tissue culture plates containing 1ml of RPMI were treated with TNF (10ng/mL) in the presence of GSK057 (10nM), Adalimumab (1nM), a dummy dAb (10nM) and vehicle control for 30 minutes. Intracellular protein was then extracted and phosphorylated Akt measured in $20\mu\text{g}$ of protein by Western blotting. β Actin was used as a loading control. The above image is representative of $n=2$.

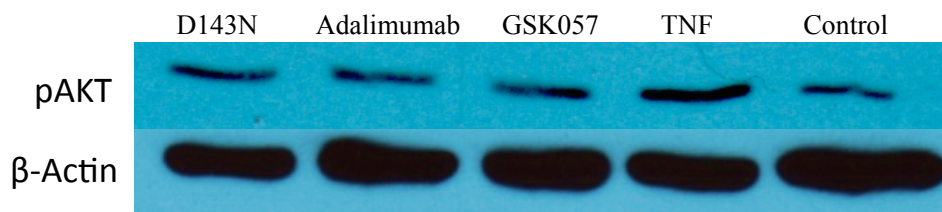


Figure A.2 Representative western blot for phosphorylated AKT in TNF treated HMVEC-L

HMVEC-L were grown to confluence over 72 hours then treated with TNF (10ng/mL) in the presence of GSK057 (10nM), Adalimumab (1nM) and vehicle control or D143N (100ng/mL) alone for 30 minutes. Intracellular protein was then extracted and phosphorylated Akt measured in $20\mu\text{g}$ of protein by Western blotting. β Actin was used as a loading control. The above image is representative of $n=2$.

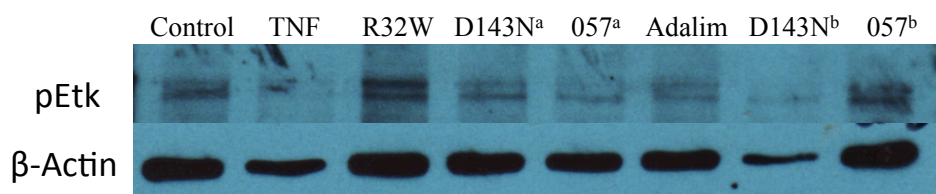


Figure A.3 Representative western blot for phosphorylated Etk in TNF treated HMVEC-L

HMVEC-L were grown to confluence over 72 hours then treated with TNF (10ng/mL) in the presence of GSK057 (10nM, n=2), Adalimumab (1nM) and vehicle control or R32W (100ng/mL) and D143N (100ng/mL, n=2) alone for 30 minutes. Intracellular protein was then extracted and phosphorylated Etk measured in 20μg of protein by Western blotting. The above image is representative of n=3

Appendix B



Lung Recovery Protocol (21)

Background

The purpose of our research is to take whole human lungs and re-animate them ex vivo by ventilating and perfusing both lungs on a specially designed ex-vivo circuit. We therefore require specific anatomy and hence a tight harvesting protocol to enable us insert a large pulmonary artery cannula and endotracheal tube.

Surgical Recovery Instructions

- Administer systemic heparin 25000iu IV
- Flush lungs (via pulmonary trunk) in situ with 1 litre of cold preservation solution (ideal – perfadex but alternatives eg. UW satisfactory)
- Harvest en bloc lungs as per double lung transplant methodology
- It is **absolutely mandatory that we have at least 3-4cm of intact pulmonary trunk** (see images below)
- **Dissect as close to pulmonary valve as possible**
 - This will require dissection of pulmonary trunk from aorta
- The pulmonary venous circulation can be incomplete
- **Minimum 3cm trachea**
- **The lungs must be free of contusions or air leaks**
 - We ventilate the lungs on arrival in the UK
- The en bloc lungs should be placed in a bag on ice

Special Instructions

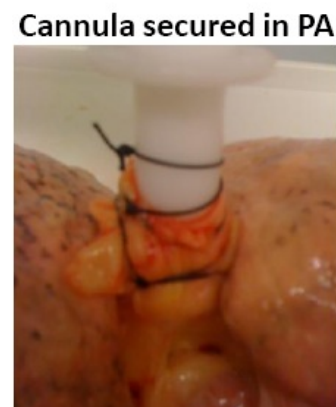
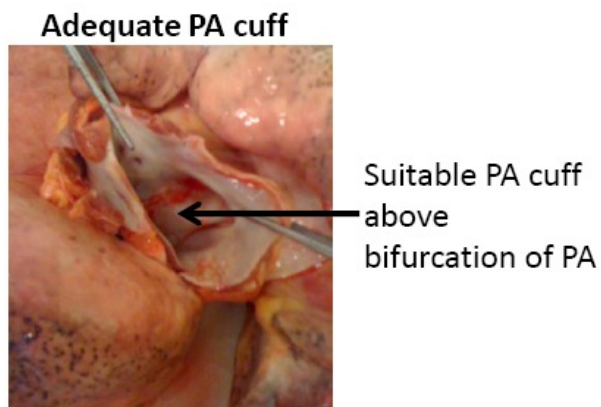
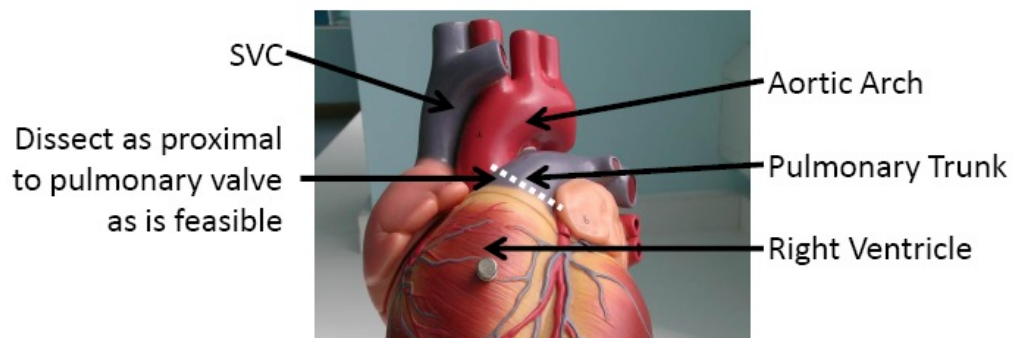
- **Please leave out any type of identifying donor information including, donor chart and labelling stickers.**
- Fax donor chart to IIAM at **(732) 661-2527** within 24 hours of cross clamp time.

Researcher Synopsis

The researcher will utilize the lung to enhance their understanding of the pathogenesis of lung diseases with the hopes of revealing novel therapeutic targets.

Images for Reference

We require the pulmonary trunk not the right or left PA as we need to homogenously perfuse both lungs simultaneously via a single cannula



Appendix C

Edison Corporate

Center

International Institute for the Advancement of Medicine (IIAM)

Research Biomaterials Policy

I. PURPOSE

To provide non-transplantable, human biomaterials for medical research, education and development, and other uses demonstrating scientific merit.

II. AVAILABILITY

All requests for biomaterials will be considered. Availability will be contingent on donor referrals and criteria.

III. PROCEDURE

All applicants must complete and submit the IIAM Application/Biomaterials Transfer Agreement (BMTA), and Exhibits A-C.

IV. BIOMATERIALS USE

Biomaterials shall be used only for the purposes cited in the Application, and may not be transferred to a 3rd party without prior written authorization from IIAM.

V. RESEARCH PRECAUTIONS

IIAM will provide documentation of serological test results, and other donor demographic information. Researcher is responsible for using appropriate precautions in handling human biomaterials.

VI. RECORD KEEPING

Applicant agrees to maintain and provide upon request by IIAM a timely and complete accounting of biomaterials received. This information shall be used in part to ensure adequate tracking of biomaterials, and can be made available for inspection by any regulatory agency. Upon approval of IIAM's Application Review Committee, an executed copy of the BMTA will be returned to the applicant/researcher, and biomaterial placement will commence.

VII. ACKNOWLEDGEMENT

All researchers must acknowledge IIAM in any presentations or publications. A copy of any published material should be forwarded to IIAM.

I. APPLICANT DATA

Name of Researcher: **Dr Mark Griffiths**_____

Phone (day): **0044 (0)2073518523**

Ext 2030

Institution Name or Corporation Name: **Royal Brompton Hospital** (Nights/weekends):
0044(0)7875999126

Is institution a non-profit organization? ☒ Yes ☐ No
n/a _____

Fax:

Name and mailing address: **c/o AICU** _____
m.griffiths@imperial.ac.uk _____

E-mail address:

Royal Brompton Hospital _____
Proudfoot _____

Add'l contact: **Dr Alastair**

Sydney Street _____

Phone

(day): **0044 (0)2073518523 Ext 8528** _____

London SW3 6NP, United Kingdom _____
(0)7779011194 _____

(Nights/weekends): **0044**

II. HUMAN BIOMATERIALS

Description of Biomaterials requested: **Whole human lungs with intact trachea, bronchi, pulmonary arteries and cuff of left atrium sufficient to attach a perfusion cannula (as large as possible). Intact vagus nerve. 1 unit (250mL) of autologous whole blood.**

Specimen size or weight: **Any suitable adult lungs (>16 years of age), donor weight >30kg**

Number of samples, frequency & duration, proposed start date: **As required – we are likely to request around one per week, maximally two per week. During academic holiday periods eg Christmas, Easter etc. Depending on the success of our study, we will use lungs for a minimum of 18 months. We plan to start as soon as possible – ideally mid September (13th) if possible.**

Preferred Source(s) of Specimens and Condition of Biomaterials Requested:

- ☒ Organ donor (**from IIAM's Organ Division only**): non-transplantable organs recovered under sterile conditions within

0-60 minutes of cessation of cardiovascular circulation, and typically delivered to researcher within 12-48 hours from aortic cross-clamp. Please indicate acceptable time from cross-clamp to delivery; up to ____ hours

- ☐ Deceased donor (**from IIAM's Anatomical Division only**): biomaterials are typically recovered under clean, non-sterile

conditions within 24-96 hours post-mortem. Please indicate acceptable period of post-mortem time; up to ____ hours

Disease Screening:

IIAM requires that all biomaterials provided for research are obtained from donors after receiving appropriate consent or authorization for donation, a detailed medical/social history screening and serological testing. Serological testing is included as part of IIAM's service, and is administered for:

Organ Division: Anti-HIV I-II, HBsAg, HBcAb, Anti-HCV, Anti-HTLV I-II, RPR, Anti-CMV and EBV

Anatomical Division: Anti-HIV I-II, HBsAg, Anti-HCV, RPR, and HB core Antibody IgM

These test results can be provided to Applicant if desired but should never be considered conclusive. Biomaterials from a donor testing positive for any of the infectious diseases (e.g. Anti-HCV) in the standard panel will not be shipped unless specific written authorization is obtained from the Applicant at that time. Please check all that apply:

- ☒ Standard infectious disease screening panel (listed above)
- ☐ Please include the following tests in addition to the standard panel (extra charge):
- _____

☐ Biomaterials from donors testing positive for the presence of one or more infectious agents are acceptable because:

III. PRESERVATION METHODS

☒ Fresh; stored in preservation solution and shipped on wet ice. (UW and HTK are considered industry-standard for preservation and are the primary preservation solutions used in IIAM's Organ Division.

☐ Passive freezing at -20° C and shipped on dry ice (the primary mode of preservation used in IIAM's Anatomical Division).

***Fixative, Medium, or Other Requirements** (if other than those listed above. * Researcher may be required to

supply any non-standard preservation media or fixatives):**IV. RESEARCH PROJECT**

Title: Development of a Human *Ex Vivo* Lung Research Model

Use of biomaterials (complete attached Exhibit A for detailed summary): Study of whole human lung *ex vivo*

Funding Source (and identification number, if applicable): **Host Institutional Funding +/- competitive grant (Wellcome Trust)**

Institutional Review Board (IRB) approval: If required by APPLICANT'S institution, please attach letter of approval or exemption from your IRB.

IRB Alternative: If IRB approval is not required by APPLICANT'S institution, it is incumbent on the researcher to obtain approval from his/her supervisor or the authority designated to undertake and review the proposed research, whose signature is required on page 6 of the Biological Materials Transfer Agreement.

IIAM
INTERNATIONAL INSTITUTE FOR THE ADVANCEMENT OF MEDICINE
APPLICATION AND AGREEMENT FOR HUMAN BIOLOGICAL MATERIALS FOR
RESEARCH USE

BIOLOGICAL MATERIALS TRANSFER AGREEMENT

This BIOLOGICAL MATERIALS TRANSFER AGREEMENT (the Agreement) is made this **2nd**

day of September (Year) 2010 by and between **The Royal Brompton Hospital**, hereinafter referred to as "**APPLICANT**", and the Musculoskeletal Transplant Foundation, a District of Columbia non-profit organization ("**MTF**") acting by and through the International Institute for the Advancement of Medicine, an unincorporated division of MTF ("**IIAM**").

WHEREAS MTF, acting by and through IIAM, acquires or procures and distributes non-transplantable human biomaterials (hereinafter "**biomaterials**") for the advancement of medical research, education, and development, and other approved uses;

WHEREAS, APPLICANT desires IIAM's services in obtaining such biomaterials; and

WHEREAS, the parties desire to cooperate for the purposes of providing APPLICANT with such biomaterials,

NOW, THEREFORE, in consideration of the premises and the mutual covenants and agreements hereinafter set forth and other good and valuable consideration, the receipt of which is hereby acknowledged, the parties hereby agree as follows:

1) During the term of this Agreement, IIAM will use reasonable efforts to provide biomaterials suitable, in its sole judgment, and that meet criteria provided by the APPLICANT in the Biomaterials Request Form, which is attached hereto as Exhibit B, for APPLICANT's requirements and in the amounts requested. IIAM acknowledges that APPLICANT's needs are subject to change; such changes will be communicated via an updated Exhibit B. This Agreement and any transfer of biomaterials made pursuant hereto constitutes a limited and conditional license to APPLICANT for use of the biomaterials or biomaterial progeny (cells, cell lines, subcellular fractions, isolates, or recombinant materials, DNA, RNA, or any derivatives thereof), and use thereof shall be limited to the specific use set forth in APPLICANT's Application and Agreement for Human Biological Materials for Research Use to which this Agreement is attached. Uses of immortalized or cloned material are prohibited unless prior written consent is obtained from IIAM. Use of human biomaterials as a therapy is strictly prohibited without prior written authorization from IIAM. In addition, the APPLICANT's use of biomaterials shall not be subject to any licensing, contractual, or consulting obligation to another party without IIAM's prior written consent, which may be withheld in IIAM's sole discretion, except that APPLICANT may share the data and results with third parties.

2) a) APPLICANT agrees to pay service fees ("**Service Fees**") to IIAM for its biomaterials acquisition, processing, preservation, storage and delivery charges as set forth on Exhibit C attached hereto, and which may be amended by IIAM from time to time, provided that any such changes in the Service Fees shall only be effective on 30 day written notice.

b) Payment terms are net thirty (30) days.

c) APPLICANT will receive a 100% credit for the Service Fee of IIAM biomaterials if the biomaterials fall outside of the APPLICANT'S approved protocol, provided that the Applicant notify IIAM within 24 hours of receiving the biomaterials. In the event that IIAM is not notified within 24 hours of biomaterial receipt, the credit may be reduced by at least 50% subject to IIAM's sole judgment. APPLICANT shall either destroy the biomaterials at its facility in accordance with local, state and federal regulations or, if APPLICANT is unable to destroy the biomaterials, APPLICANT may return the biomaterials to IIAM,

with prior permission from IIAM, within five business days of delivery to APPLICANT. IIAM is responsible for the costs of transportation and tissue destruction in such cases

3) IIAM represents and warrants that it has reviewed consent or authorization for research from the donor or next- of-kin which was obtained, to the best of IIAM's knowledge, in accordance with all applicable state and federal laws and regulations prior to recovery and distribution. IIAM will maintain approved protocols, consent or authorization forms for biomaterial acquisition for research.

4) APPLICANT hereby makes the following representations and warranties to IIAM:

a) APPLICANT will not transfer any biomaterials or biomaterial progeny (as defined in Section 1 of the Agreement) received from IIAM to any third party without prior written authorization from IIAM.

b) APPLICANT agrees to maintain and provide upon request by IIAM a timely and complete accounting of biomaterials being used. This information shall be used in part to ensure adequate tracking of biomaterials, and may be made available for inspection by any regulatory agency.

c) APPLICANT or anyone on its behalf will not intentionally contact or acquire human biomaterials from IIAM's sources, which are available upon request, during the term of the Agreement or any extension thereof and for a period of three (3) years thereafter. This restriction does not apply to those IIAM sources with whom the APPLICANT previously had a biomaterials acquisition or procurement agreement prior to the execution date of this Agreement. Furthermore, this restriction shall not apply if during such period IIAM ceases to exist or ceases to engage in the supply of human biomaterials.

d) APPLICANT'S employees, representatives, and agents shall handle human biomaterials obtained under this Agreement using universal precautions, as mandated by the U.S. Occupational Safety and Health Administration Bloodborne Pathogens Final Standard (OSHA). APPLICANT and its employees, representatives and agents shall use, handle, store and dispose of all human biomaterials supplied to APPLICANT pursuant to this Agreement in compliance with all applicable local, state, and federal statutes, laws, rules and regulations including but not limited to OSHA.

e) During the term of this Agreement, APPLICANT will utilize the services of an entity authorized under applicable law to dispose of the biological materials provided by IIAM. APPLICANT agrees to use the entity listed on the attached Biomaterials Request Form to dispose of all biological materials provided by IIAM to APPLICANT pursuant to this Agreement.

f) APPLICANT shall not use or store any biological materials in any facility other than one primarily used as a medical facility or laboratory (a “**Medical Facility**”), unless prior to utilizing such biological materials: (i) APPLICANT requests in writing that IIAM permit the use of such biological materials in a facility other than a Medical Facility (a “**Non-Medical Facility**”), and indicates the proposed use of such biological materials as well as how they will be disposed, and (ii) the owner or operator of said Non-Medical Facility provides to IIAM a written certification that (X) such owner or operator is aware of the proposed use of such biological materials in said Non-Medical Facility, (Y) such owner or operator consents to such use in the Non-Medical Facility, and (Z) the proposed use of such biological materials in said Non-Medical Facility is permitted under applicable law.

g) APPLICANT may request that IIAM and IIAM, at its sole discretion, may agree to dispose of biological material provided by IIAM to APPLICANT pursuant to this Agreement. In the event IIAM agrees to dispose of any such biological materials, APPLICANT shall arrange for the shipment of such biological materials to IIAM or an IIAM designated facility (as directed by IIAM) in accordance with all applicable laws, including proper wrapping and containment of all such biological materials to ensure their safe shipment. APPLICANT agrees to pay all shipping costs and IIAM’s fees related to the disposal of any such biological materials.

h) APPLICANT shall not identify or attempt to identify the donor of any biomaterials provided by IIAM.

i) All statements in the Application are complete and correct.

5) All shipments of biomaterials shall be made on the following terms:

a) Shipments will be made in the best possible manner so as to preserve the quality of the biomaterials. The parties hereto understand that the fragility of human biomaterials is such that

damage may occur during shipment; nevertheless, IIAM shall use its best efforts to comply with the handling and shipping protocols provided by APPLICANT.

b) Except as otherwise expressly stated herein, all biomaterials are provided by IIAM "as is" and without additional warranty. **ALL OTHER WARRANTIES, EXPRESS OR IMPLIED, INCLUDING BUT NOT LIMITED TO WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, ARE DISCLAIMED.**

6) APPLICANT will acknowledge IIAM as a provider of human biomaterials and agrees to provide IIAM with a copy of any non-confidential publication that makes reference to IIAM.

7) a) APPLICANT will indemnify and save harmless IIAM and their respective officers, directors, agents, employees, consultants and/or IIAM's human biomaterials sources from any actual loss or damage resulting from the use by APPLICANT of human biomaterials specimens provided by IIAM or IIAM's human biomaterials sources, except for loss or damage resulting from any breach of warranties or representations made by IIAM in this Agreement or as a result of IIAM's gross negligence or willful misconduct in performing its obligations hereunder.

b) IIAM will indemnify and save harmless APPLICANT and its respective officers, directors, agents, employees, and/or consultants from any actual loss or damage arising from IIAM's performance of this

Agreement except for loss or damage resulting from any breach of warranties or representations made by APPLICANT in this Agreement or as a result of APPLICANT'S gross negligence or willful misconduct in performing its obligations hereunder.

8) a) All medical or other information relating to a donor shall be treated as Confidential Information of each party, and notwithstanding any provision to the contrary continued herein, shall not be disclosed except to the extent otherwise required or permitted by law. Both parties to this Agreement shall act, and shall take reasonable measures to cause their respective agents, contractors, employees, officers and directors to act, in accordance with all applicable laws, rules and regulations

regarding the confidentiality of donor specific medical information. This obligation shall expressly survive termination of this Agreement.

b) Each party understands that the other party and its agents, contractors, employees, officers and directors will or may be provided valuable technical and non-technical information and trade secrets relating to, among other things, the referral, recovery and use of biomaterials and/or medical or other information relating to donors ("**Confidential Information**") that is proprietary and valuable to the disclosing party (the "**Disclosing Party**") and that to protect the Disclosing Party's legitimate interests, the Disclosing Party must protect such Confidential Information. Therefore, the receiving party (the "**Receiving Party**") will hold in confidence and not disclose to third parties or use any such Confidential Information except in connection with the performance of its obligations hereunder, and will take reasonable steps to cause its agents, contractors, employees, officers and directors to hold in confidence and not disclose to third parties or use any Confidential Information except in connection with the performance of the Receiving Party's obligations hereunder. The Receiving Party agrees that the Disclosing Party's failure to mark any such information as "Confidential Information" shall not relieve the Receiving Party of its obligation to treat such information as Confidential Information.

c) The Receiving Party shall cause each of its agents, contractors, employees and officers who Confidential Information to enter into a confidentiality agreement that contain confidentiality provisions comparable to those set forth herein. The provisions of this paragraph shall not apply to any information that is in the public domain, is known to the Receiving Party at the time of its disclosure, is acquired or developed by the Receiving Party following disclosure without violation of this Agreement or is disclosed by the Disclosing Party to any third party without similar non-disclosure restrictions.

d) The Receiving Party shall promptly notify the Disclosing Party if the Receiving Party receives a subpoena seeking any Confidential Information so that the Disclosing Party has an opportunity to take appropriate action with the entity issuing the subpoena before any such Confidential Information is released. The parties agree that the terms and conditions of this Agreement will be considered Confidential Information.

9) The term of this Agreement shall be one (1) year beginning on the date of execution and automatically continuing for further successive terms of one (1) year thereafter, unless either of the parties hereto shall have given to the other thirty (30) days prior written notice (by certified mail at their respective addresses listed at the end hereof, return receipt requested) of its intention to terminate this Agreement, whereupon this Agreement shall terminate thirty (30) days after receipt of such notice. The Agreement may also be terminated by either party before the end of an annual term by giving the other thirty (30) days prior written notice of its intention to terminate this Agreement, whereupon this Agreement shall terminate thirty (30) days after receipt of such notice. Termination shall not relieve the parties hereto of any of their obligations by the nature thereof extending past such termination.

10) a) This Agreement may not be amended without the prior written consent of both parties hereto.

b) This Agreement and the Application attached hereto constitutes the entire agreement between the parties hereto relating to the subject matter hereof, and supersedes any prior agreements, written or oral, regarding the subject matter hereof.

c) This Agreement will be binding upon and inure to the benefit of the parties hereto and their respective heirs, successors and assignees; provided, however, that neither party shall transfer or assign this Agreement or their rights and obligations hereunder, including by merger or operation of law, with a change of control of APPLICANT constituting an assignment for purposes of this Agreement, without the prior written consent of the other party.

IN WITNESS WHEREOF, the parties have executed and delivered this Agreement.

RESEARCHER:

MUSCULOSKELETAL TRANSPLANT

FOUNDATION:

Print name/title: _____

Print name/title: _____

Signature: _____ Date: _____

Signature: _____ Date: _____

RESPONSIBLE DEPARTMENT HEAD:

Print name/title: _____

Signature: _____ Date: _____

Exhibit A- Synopsis of Research

(to be completed by Researcher/Applicant)

SUMMARY:

Despite technological advances and substantial investment, current respiratory drug development pipelines have a high attrition rate. This, in part, relates to limitations of current animal models of respiratory disease which fail to recreate many of the pathophysiological hallmarks of human disease. Novel preclinical models and preclinical research based on human tissues is therefore required.

Ex vivo lung perfusion (EVLP) is a technique used successfully in human transplantation to evaluate and regenerate donor lungs previously thought to be unsuitable for transplantation. Outside the transplant arena, the potential of human *ex vivo* lung research as a novel translational model in respiratory medicine is now being realised. *Ex vivo* modelling is functionally superior to tissue and cell-based assays. It provides preserved physiologic functions and conditions, inherent in the tissues and organs being intact. Refinement and development of human *ex vivo* lung models and *ex vivo* disease modelling has the potential to create more relevant, robust preclinical data.

SPECIFIC AIM:

We aim to establish whether an *ex vivo* lung perfusion model is a viable, predictable and reproducible translational research model.

Our laboratory has established *in vitro* models of lung injury using human tissue from lung resection specimens. In addition, we have developed and refined an EVLP circuit using porcine lung, confirming structural lung viability at twelve hours.

Data gained from *ex vivo* human study will build on the results of current *in vivo* animal and *in vitro* human work, enhancing our understanding of the pathogenesis of lung disease and hopefully reveal novel therapeutic targets

LONG TERM OBJECTIVE:

Establishment of an EVLP research model will facilitate pharmacodynamic and pharmacokinetic study of novel agents prior to phase I study. EVLP has the potential to offer a new gold standard for preclinical testing of novel therapeutics in lung disease.

IIAM Biomaterials Request Form-Exhibit B (Organ Division)

Date of Request **02/09/10**_ Requested by **Dr A Proudfoot**_____ Application # (IIAM Use Only)

SHIP TO:

Adult Intensive Care Unit room 3078,

Level 3 Royal Brompton Hospital,

Sydney Street

City: **London** State: _____ Zip: **_SW3 6NP_**_____

Attention: **_Alastair Proudfoot (Bleep 1109 / Ext 8528)_** Phone Number:

Courier preferred: **Please circle one:** **Sterling** AirNet Freight Services

Accept Weekend Deliveries? Yes ____ or **No** ____ Accept Sunday referral for Monday delivery? **Yes** ____ or
No ____

BIOMATERIALS REQUESTED: Whole human lungs with intact trachea, bronchi, pulmonary arteries and cuff of left atrium sufficient to attach a perfusion cannula (as large as possible). Intact vagus nerve. 1 unit (250mL) of autologous whole blood.

Donor Criteria: (i.e. Age, Race, Gender) **Age >16 yrs <50 years, no race specification, male OR female**

Contraindications: (i.e. IV Drug Use, ETOH abuse) **_Free from communicable diseases as previously**

Quantity (Please note "singles" or "pairs"): **whole lungs & trachea** _____ Frequency: **As required by researchers – likely weekly / bi-weekly**_____

DISPOSAL OF MEDICAL WASTE: Disposed of by Applicant's Designated Medical Waste Agent:

In accordance with Local Trust Guidelines and complying with Human Tissue Act (UK; 2004)

Exhibit C-Fee Schedule

Whole human Lung:

\$ 857 per specimen

Service Fee:

\$ 50 per shipment (to cover costs for

the shipping materials and follow-

up forms including the Donor

Demographic Form with all

pertinent medical info.)

Shipping:

Shipping costs vary depending on

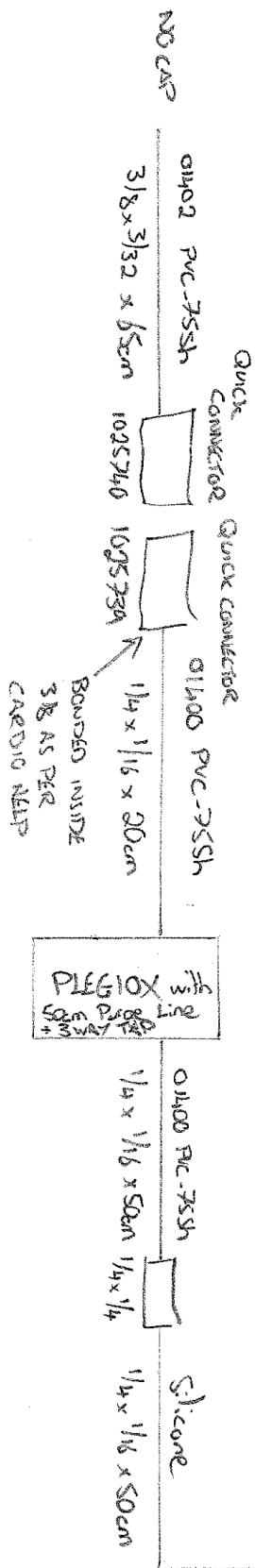
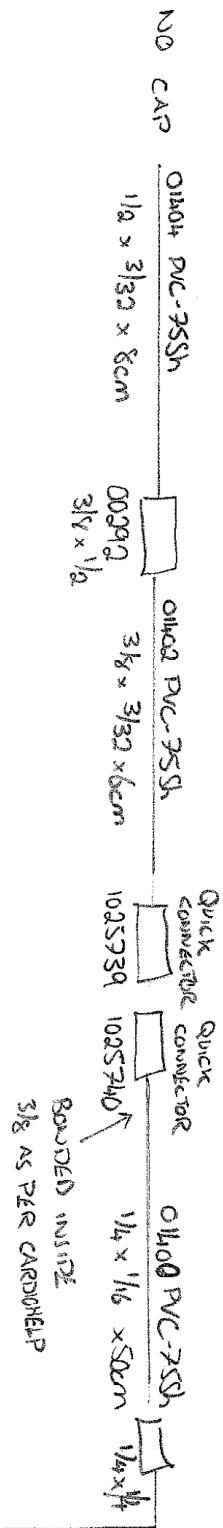
weight, courier and destination.

Estimates available upon request.

Agreed as roughly \$800

① LINE ① AS PREVIOUS DRAWING REF H19702

②



③ AS PER LINE ④ PREVIOUS DRAWING REF H19702

ROYAL BROMPTON
LUNG RESEARCH PUCK

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