A Lipopolysaccharide (LPS) inhalation model to characterise divergent innate cellular responses and presence of alveolar leak, early in the course of acute lung inflammation

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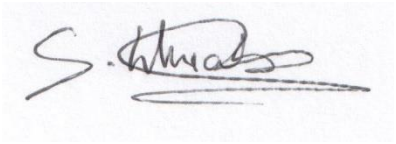
I dedicate this work to my ever-patient husband Gary, and to my beautiful and funny children Libby and James. Without whom, this thesis would have been submitted on time, but I would be a lesser person for it.

Declaration

This thesis is my own work. I carried out the studies described, and the specific contributions of other individuals and members of my research group are clearly acknowledged where appropriate.

This work has not been submitted on candidature for any other degree or professional qualification.



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Abstract

Acute respiratory distress syndrome (ARDS) is a common condition presenting to the intensive care unit (ICU) and is associated with high morbidity and mortality. Experimental models in humans using bacterial lipopolysaccharide (LPS, delivered by nebulised inhalation or bronchial instillation) create reproducible acute lung inflammation and can be used to model early stages of the pathological process leading to ARDS. A significant body of evidence already exists from animal and human studies suggesting LPS inhalation results in rapid release of pro-inflammatory cytokines and movement of innate immune cells (neutrophils and monocytes) into the alveolar space. The functional status of neutrophils in response to this stimulus is largely unknown, based on circumstantial evidence provided by predominant cytokines, chemokines and cell surface protein expression. Most studies rely on invasive assessment of the alveolar space using bronchoalveolar lavage (BAL), and imaging modalities have been poorly explored in LPS respiratory models.

This thesis aimed to test the hypothesis that, following inhalation of LPS, neutrophils circulating within peripheral blood increase their capacity for phagocytosis and generation of reactive oxygen species (ROS), and that dynamic contrast-enhanced magnetic resonance imaging (DCE MRI) detects early increases in pulmonary vascular permeability.

Forty-nine healthy human volunteers were recruited to an LPS inhalation study. Volunteers underwent inhalation of 60μg of LPS or Saline via a nebuliser dosimeter, with peripheral blood sampling. A subset underwent DCE MRI scans and bronchoscopy with BAL. Functional assays of phagocytosis and respiratory burst activity were performed on isolated neutrophils from blood.

Neutrophils demonstrated a trend towards increased phagocytosis following LPS inhalation (change from baseline of 3.6% versus 1.2% in control subjects, p=0.058). This was not supported by any change in respiratory burst activity or flow cytometry assessment of cell surface protein expression. Analysis of DCE MRI of the lungs proved difficult and was complicated by significant artefact from surrounding structures and respiratory motion.

In conclusion, LPS inhalation did not significantly affect phagocytosis or respiratory burst activity of neutrophils in the systemic circulation. DCE MRI was unable to detect changes in vascular permeability following LPS inhalation above the background noise.

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Abbreviations

ACTH	Adrenocorticotropic hormone
AE	Adverse event
AIF	Arterial input function
Akt/PKB	Protein kinase B
ALI	Acute lung inflammation
ALT	Alanine transaminase
AM	Alveolar macrophage
ActPC	Activated protein C
APC	Allophycocyanin
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BMI	Body mass index
BTS	British thoracic society
Bw	Body weight
CCRE	Clinical Centre Reference Endotoxin
COPD	Chronic obstructive pulmonary disease
CPAP	Continuous positive airway pressure
CRF	Clinical research facility
CRP	C-reactive protein
CT	Computer tomography
CTU	Clinical trials unit
CXR	Chest x-ray
DAPI	4,6-diamidino-2-phenylindole
DCE	Dynamic contrast-enhanced
DLCO	Diffusing capacity for carbon monoxide
ECG	Electrocardiogram
ECP	Eosinophil cationic protein
EDTA	Ethylenediamine tetraacetic acid
EES	Extravascular extracellular space
ENA-78	Epithelial-derived neutrophil activating peptide-78
eNO	Exhaled nitric oxide

ERS	European respiratory society
FACS	fluorescence-activated cell sorting
FBC	Full blood count
FDG	2-fluoro-2-deoxy-D-glucose
FEV1	Forced expiratory volume in 1 second
FH	Freeman hospital
FiO_2	Fraction of inspired oxygen
FITC	Fluorescein isothiocyanate
fMLP	N-formyl methionyl leucyl phenylalanine
FOV	Field of view
FSC	Forward scatter
FVC	Forced vital capacity
G-CSF	Granulocyte colony-stimulating factor
GGT	Gamma-glutamyl transpeptidase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	General practitioner
GPI	Glycosylphosphatidylinositol
Hb	Haemoglobin
HBSS	Hanks' balanced salt solution
HNE	Human neutrophil elastase
ICM	Institute of cellular medicine
ICU	Intensive care unit
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
IMDM	Iscove's Modified Dulbecco's Medium
IRAK	IL-1 receptor associated kinase
IV	Intravenous
JRE	Joint research executive
Kdo	2-keto-3-deoxyoctonoic acid
KGF	Keratinocyte growth factor
Ki	Mean influx constant
K*trans*	Volume transfer constant
LABA	Long acting B_2-adrenoreceptor agonist
LBP	LPS binding protein

LFTs	Liver function tests
LOS	Lipooligosaccarides
LPS	Lipopolysaccharide
MAP(K)	Mitogen-activated protein (kinase)
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence intensity
MIF	Macrophage inhibitory factor
MIP-1ß	Macrophage inflammatory protein -1ß
MIP-2	Macrophage inflammatory protein-2
MMP	Matrix metalloproteinase
MN	Mononuclear
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NAC	N-acetylcysteine
NAG	N-acetylglycosamine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute for Health
NIHR	National Institute for Health Research
NMRC	Newcastle magnetic resonance centre
NuTH	Newcastle Upon Tyne Teaching Hospitals NHS Foundation Trust
PAF	Platelet activating factor
PaO_2	Partial pressure of arterial oxygen
PAWP	Pulmonary arterial wedge pressure
PBS	Phosphate buffered solution
PE	Phycoerythrin
PEEP	Positive end expiratory pressure
PET	Positron emission tomography
PFC	Perfluorocarbons
PI3-K	Phosphatidylinositol-3-kinases
PIS	Participant information sheet
PMN	Polymorphonuclear
PPI	Protein permeability index (BAL protein: plasma protein ratio)

R&D	Research and development
RCT	Randomised-controlled trial
REC	Regional ethics committee
ROI	Region of interest
ROS	Reactive oxygen species
RVI	Royal Victoria infirmary
SABA	Short-acting B_2-adrenoreceptor agonists
SAE	Serious adverse event
SAPS	Simplified acute physiology score
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
SP-D	Surfactant protein D
SPECT	Single-photon emission computer tomography
SSC	Side scatter
TGF-ß	Transforming growth factor beta
TLR-4	Toll-like receptor-4 protein
TNF-R	Tumour necrosis factor-receptor
TNFα	Tumour necrosis factor-α
Tollip	Toll interacting protein
TR	Time to repetition
TRAF-6	TNF-receptor associated factor 6
Tregs	Regulatory T cells
TXB2	Thromboxane B2
UK	United Kingdom
USA	United states of America
V*e*	Extravascular extracellular volume fraction
V*p*	Plasma fraction
VTR	Variable time to repetition
vWF	von Willebrand factor
WCC	White cell count

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1. Introduction

1.1 Overview of introduction chapter

Here I will present a review of the literature relevant to the research project and required to address the research hypothesis. This predominantly relates to the clinical syndrome of acute respiratory distress syndrome (ARDS), with particular focus on the underlying pathological features and the role of neutrophils within it. I will also review evidence from clinical research regarding aspects such as vascular permeability and imaging modalities currently used in research trials and clinical practice.

I will then review the literature regarding models of acute lung inflammation (ALI) focussing mainly on human studies utilising respiratory challenge with lipopolysaccharide (LPS). I will review what we have learnt from these models, especially the underlying pathological processes and the role of neutrophils, but also imaging and interventional studies. I will highlight ongoing questions and areas requiring further study. Towards the end of the chapter, I state my research hypothesis.

1.2 Literature review

1.2.1 Acute respiratory distress syndrome (ARDS)

1.2.1.1 Definitions of ARDS

ARDS is a common, serious pulmonary condition associated with high mortality, morbidity and substantial costs to healthcare systems. It is characterised by acute onset, uncontrolled inflammation and increased permeability of the alveolar-capillary membrane, resulting in hypoxaemia refractory to supplementary oxygen (Bernard et al, 1994). In 1994, the American-European consensus committee suggested a standardised definition for ARDS and it's less severe form, at this time known as 'acute lung injury', based on timing, x-ray

appearances and the level of hypoxaemia, in the absence of evidence for cardiogenic pulmonary oedema (see table 1.1).

Acute Lung Injury	Acute Respiratory Distress Syndrome (ARDS)
1. Acute onset (< 7 days) 2. Bilateral infiltrates on frontal chest X-ray consistent with pulmonary oedema (these may be patchy, asymmetric and pleural effusions may be present) 3. Absence of left atrial hypertension (PAWP ≤ 18mmHg where measured) 4. Hypoxaemia - PaO_2: FiO_2≤300mmHg (irrespective of PEEP)	1. Criteria 1-3 as for Acute Lung Injury 2. Severe hypoxaemia - PaO_2: FiO_2≤ 200mmHg (irrespective of PEEP)

Table 1.1 Definitions of acute lung injury and acute respiratory distress syndrome (ARDS). Adapted from Bernard et al, 1994. PEEP: Positive end expiratory pressure, PAWP: Pulmonary arterial wedge pressure

The strength of these criteria was that they recognised a range of severity in lung injury, with the same pathological process sometimes, but not always, progressing to the more severe ARDS. Also these criteria were simple and easily applied to the clinical situation and their widespread acceptance led to greater standardisation and coordination across research and clinical trials (Ware and Matthey, 2000). Criticisms of these criteria have been that their simplicity did not therefore take account of the underlying cause or any presence of multiorgan failure, both of which may have an impact on mortality and outcome. Also, standardised ventilator settings were not required in the diagnosis meaning that some patients could be converted from categorisation as ARDS into acute lung injury or even missed completely by changes in ventilation pressures, particularly increased positive end-expiratory pressure (PEEP). Similarly, the radiological criteria for diagnosis were highly non-specific and present in large numbers of patients admitted to the intensive care unit (ICU) and, when coupled with the substantial variability in physicians' interpretation of radiographs, could lead to over- or under-diagnosis of ARDS in the ICU (Wheeler and Bernard, 2007).

As such, a panel of experts convened to create the 'Berlin definition' of ARDS, published in 2012 (summarised in table 1.2). According to this latest definition, diagnosis is based on the development of hypoxaemia and bilateral chest opacifications within 1 week of a known risk factor. Respiratory failure must not be fully explained by cardiac failure or fluid overload. In this definition, there is removal of the term 'acute lung injury' and instead classification of ARDS into mild, moderate or severe based on severity of impairment in gas exchange at a predetermined level of PEEP (ARDS Definition Task Force, 2012). These criteria strengthen some of the radiographic diagnostic criteria, have been linked to mortality outcomes and have led to greater standardisation in subsequent clinical trials.

	Acute respiratory distress syndrome
Timing	Within 1 week of a known clinical insult or new or worsening respiratory symptoms
Chest imaging[a]	Bilateral opacities – not fully explained by effusions, lobar/lung collapse, or nodules
Origin of oedema	Respiratory failure not fully explained by cardiac failure or fluid overload Need objective assessment (e.g. echocardiography) to exclude hydrostatic oedema if no risk factor present
Oxygenation[b]	
Mild	200mmHg < PaO_2/FiO_2 ≤ 300mmHg with PEEP or CPAP ≥ 5cmH_2O[c]
Moderate	100mmHg < PaO_2/FiO_2 ≤ 200mmHg with PEEP ≥ 5cmH_2O
Severe	PaO_2/FiO_2 ≤ 100mmHg with PEEP ≥ 5cmH_2O

Table 1.2 Berlin definition of ARDS. (Adapted from ARDS Definition Task Force, 2012)**.** PaO_2: Partial pressure of arterial oxygen, FiO_2: fraction of inspired oxygen, PEEP: positive end expiratory pressure, CPAP: Continuous positive airway pressure, ***a***: chest radiograph or computerised tomography scan, ***b***: if altitude is greater than 1000m above sea level then correction factor should be calculated, ***c:*** This may be delivered non-invasively in the mild ARDS group.

1.2.1.2 Epidemiology of ARDS

ARDS is common and associated with high mortality. A large, prospective multicentre trial in King County, Washington, USA (United States of America) found the incidence of acute lung injury in patients requiring assisted ventilation on the ICU to be 78.9 cases per 100,000 person-years (Rubenfeld et al, 2005). The corresponding incidence of ARDS was 58.7 per 100,000-person years. This is the equivalent of 1 in 8 ventilated patients for ARDS. Incidence increased with age to a peak of 306 cases per 100,000 person-years in those over 75 years. Extrapolating these data to the age-adjusted ICU population in the USA estimates approximately 190,000 cases of ARDS per year. In-hospital overall mortality was 41.1%. Mortality also increased substantially with age, with the lowest in-patient mortality of 24% in those under 20 years, rising to 60% in those over 85 years. This translates to annual deaths of approximately 74,500 from ARDS. Average lengths of stay in ICU and hospital were 7 days and 13 days respectively, with only one-third of patients able to be discharged straight home. The authors estimated ARDS accounts for 2.2 million days in the ICU and 3.6 million in-hospital days per year in USA.

These results were largely replicated for a UK and European population by the ALIVE study, which found that ARDS occurred in 7% of all patients admitted to the ICU, and 16% of those requiring mechanical ventilation (Brus-Buisson et al, 2004). Of those initially presenting with what was classed as acute lung injury at the time, 55% progressed within 3 days to ARDS. The crude in-hospital mortality rates were 32.7% and 57.9% for acute lung injury and ARDS respectively. Factors associated with increased mortality were increasing age, immune incompetence, SAPS II (simplified acute physiology score) score, degree of organ dysfunction, blood pH<7.30 and pneumothorax. More than 90% of patients with ARDS had failure of ≥2 organs, with respiratory, renal and cardiovascular being the most common systems affected, but all systems had potential to become involved. Average ICU stay was longer than in the USA series at 16 days, with in-hospital stay an average of 25 days.

Over the last three decades mortality from ARDS has fallen (often reported as between 70-90% all-cause mortality when first recognised). Since no therapeutic intervention has

consistently been shown to alter the underlying pathological process or overall prognosis, this fall is considered largely attributable to advances in supportive care. The best evidence for this comes from protective ventilatory strategies, which are associated with improved survival but also careful attention to fluid management, nutritional support and prudent antibiotic usage (McIntyre et al, 2000).

In a very recent review of epidemiology, mortality and ventilatory strategies in 50 countries worldwide, the prevalence of ARDS was 10.4% among all-comers in an ICU patient population and represented 23.4% of those requiring invasive mechanical ventilation. In-hospital mortality remained at between 35-46% (mild versus severe ARDS). Despite recent evidence, less than two-thirds of patients received ventilation with tidal volumes of ≤ 8mls/kg predicted body weight although around 83% of patients received a PEEP of ≤ 12cmH_2O (Bellani et al, 2016).

In many studies, outcome from ARDS is determined at the end of 28-days or their hospital admission (McAuley at el, 2014; Rubenfeld et al, 2005). However, approximately 10% of patients demonstrate a slow recovery, requiring more than one month of ventilatory support (Wheeler and Bernard, 2007). In the majority of survivors, lung function returns to normal over approximately 6-12months. However, this may not be the most important regulator of morbidity as survivors have repeatedly been shown to demonstrate functional disability 1-2 years following discharge from the ICU. This is most often due to other complications of severe illness and intensive support such as muscle wasting, weakness, entrapment neuropathy and neuropsychiatric problems (Herridge et al, 2003), which require ongoing support and result in delayed return to previous function and work.

1.2.1.3 Risk factors for ARDS

Common risk factors for ARDS are often classified according to those directly affecting the lung and those causing indirect lung injury through a systemic process (table 1.3). The most common risk factor for ARDS is severe sepsis (>75% of all cases) with a pulmonary source accounting for approximately 45% of cases. Other causes include multiple trauma, pancreatitis, transfusion, cardiopulmonary bypass, drug overdose, pulmonary contusion, fat embolism and reperfusion injury following transplantation or pulmonary embolectomy. Mortality also varies with cause, being highest in those with aspiration or pulmonary sepsis and probably lowest in those with trauma (40% vs. 24% in the USA study [Rubenfeld et al, 2005]). Patient characteristics are also a factor. Chronic alcoholism increases risk and there is evidence that genetic predisposition may be important in the development of ARDS (Marshall et al, 2002).

Direct Injury (pulmonary)	Indirect Injury (extrapulmonary)
Pneumonia	Severe sepsis
Gastric aspiration	Severe trauma
Pulmonary contusion	Shock
Alveolar haemorrhage	Transfusion-related
Near drowning	Salicylate or narcotic overdose
Smoke and toxic gas inhalation	Cardiopulmonary bypass
Reperfusion injury (e.g. following embolectomy, lung transplantation)	Pancreatitis
Fat or amniotic fluid embolization	

Table 1.3 Causes of ARDS.

1.2.2 Pathogenesis of ARDS

The early, exudative phase of ARDS is characterised by disruption to the alveolar-capillary barrier, with increased permeability allowing influx of protein-rich fluid into the alveolar space. This is accompanied by an intensive neutrophilic infiltrate and activation of a complex

network of inflammatory cytokines and chemokines. Release of proteases such as human neutrophil elastase (HNE) from neutrophils may inactivate available surfactant and damage the structural framework of the lung, exacerbating alveolar flooding, impairing fluid clearance and contributing to widespread atelectasis. A procoagulant tendency is set up, leading to platelet-fibrin microthrombi and impaired fibrinolysis within the small vessels of the lung (Wheeler and Bernard, 2007). These changes are summarised in the now-famous figure published by Ware and Matthey (2000), see figure 1.1. It is widely accepted that ARDS often then progresses through an 'organising phase' and a 'fibrotic' or 'resolution phase' with features of these being more variable depending on cause, severity and superimposed impact of treatment and its complications.

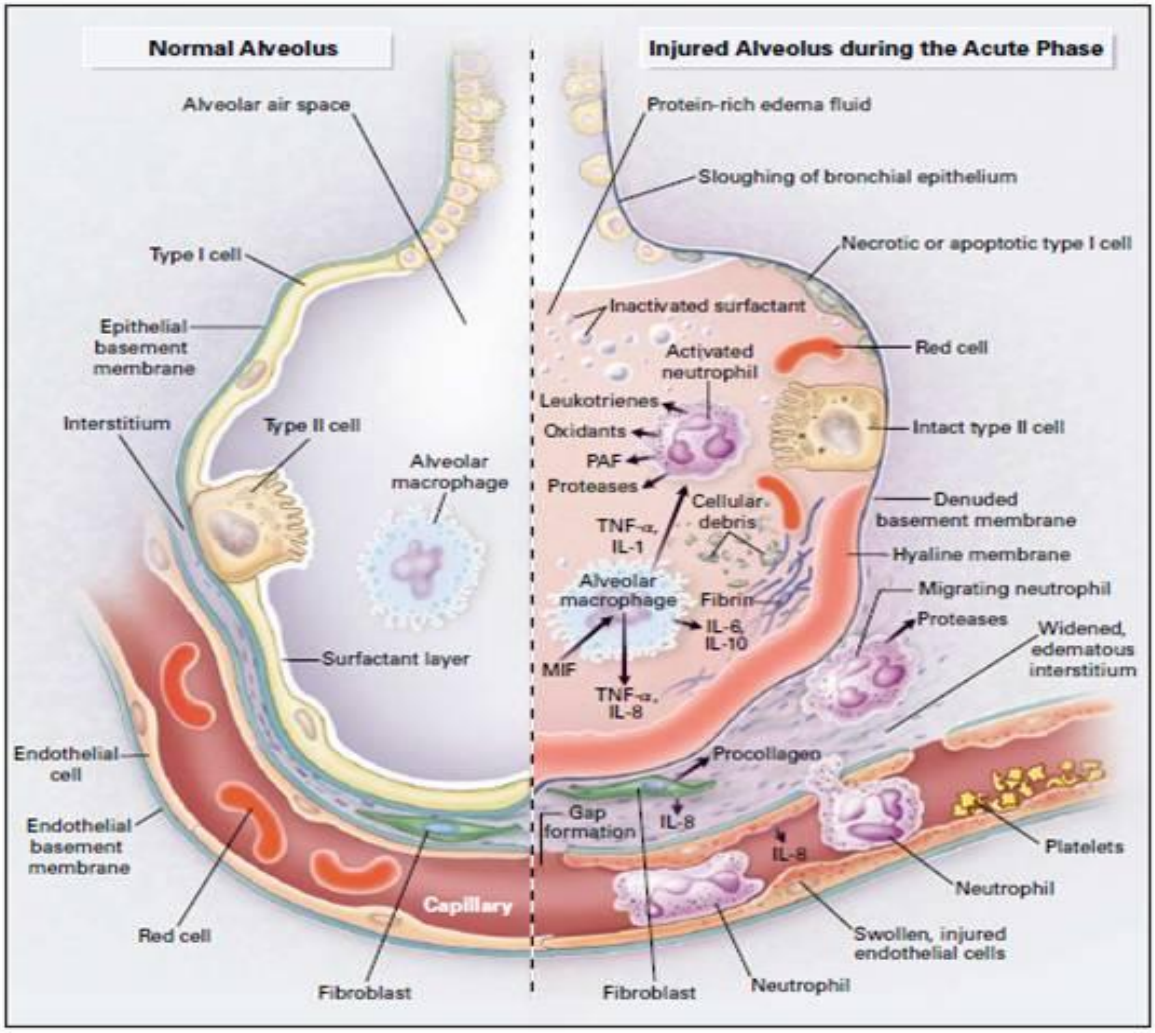


Figure 1.1 Summary of the pathological changes seen in the damaged alveolus in the acute phase of ARDS. (Taken from Ware and Matthey, 2000)

1.2.2.1 Disruption to the alveolar-capillary barrier in ARDS

There is no single uniform response to the variable conditions that may result in ARDS and it is not yet clear what the initiating and establishing factors are in the pathological processes. From an early stage there is loss of integrity of the endothelial basement membrane, resulting in increased vascular permeability. This is particularly the case in animal models of ALI where, following exposure to endotoxin the vascular endothelium appears more sensitive than the epithelium (Wiener-Kronish et al, 1991; Matute-Bello et al, 2008). However, in humans it is likely that the degree of epithelial damage is more critical to the development and subsequent recovery from ARDS. The epithelium usually forms a tight barrier resisting movement of fluid and proteins from the interstitium into the alveolar space even in the presence of significant vascular disruption. Studies examining those who die early from ARDS found a greater proportion of type 1 alveolar epithelial cells disrupted, reduced in size and with evidence of DNA fragmentation (Bachofen and Weibel, 1982; Bellingan et al, 2002). Cuboidal type 2 alveolar epithelial cells actively transport sodium and chloride ions from the alveolar space with subsequent movement of water through specialised aquaporins in the basement membrane. Damage to type 2 alveolar cells reduces this clearance of alveolar oedema and also impairs synthesis and recycling of surfactant leading to loss of surface tension within the alveoli. Furthermore, since type 2 cells proliferate and promote differentiation into type 1 cells following injury, their loss may lead to disordered repair and fibrosis at a later stage in the disease process (Martin et al, 2003). Activation of the Fas-Fas ligand pathway has been shown to be important in triggering significant and premature apoptosis of alveolar epithelial cells in response to inflammatory stimuli. Increased expression of Fas membrane receptor (CD95) on alveolar epithelial cells has been detected in response to bacterial endotoxin (Kitamura et al, 2001) and soluble Fas ligand is present in the bronchoalveolar lavage (BAL) of patients with ARDS and at higher concentrations in those who die early (Matute-Bello et al, 1999). The degree of alveolar epithelial disruption is an important determinant of outcome in ARDS with intact epithelial function associated with better prognosis (Ware and Matthay, 2001). With the loss of the alveolar epithelium, the basement membrane becomes denuded and there is accumulation of cell debris. Later there is formation of protein-rich hyaline membranes within the alveolar

space and, in some patients, this leads to non-reversible scarring and fibrosis of distal air spaces.

1.2.2.2 Neutrophils in ARDS

Neutrophils are the dominant inflammatory cell involved in the pathogenesis of ARDS. They are found in large quantities in the bronchoalveolar lavage fluid (BALF) of patients with ARDS and histological samples taken early in the exudative phase show marked accumulation of neutrophils within the swollen interstitium and alveolar space (Weiland et al, 1986; Park et al, 2019). Clearly neutrophils are the key regulator cell of the early innate immune response to bacterial invasion involved in killing and phagocytosis of foreign material.

Many animal models of ALI are neutrophil-dependent (Parsey et al, 1998; Matute-Bello et al, 2008) and depletion of neutrophils significantly attenuates ALI in response to endotoxin (Heflin and Brigham, 1981; Dhaliwal et al, 2012). Although ARDS has been documented in neutropenic ICU patients (Ognibene et al, 1986) there are also case series documenting increased risk of ARDS and deterioration in lung function on recovery from neutropenia (Azoulay et al, 2002). In humans, neutrophils rapidly enter the lung parenchyma in response to septic shock, haemorrhage, reperfusion and endotoxin-induced ALI (Hierholzer et al, 1998; Abraham, 2003, Lomas-Neira et al, 2006).

At the onset of sepsis, neutrophils undergo changes within the cytoskeleton that reduce deformability and they become trapped in the pulmonary microcirculation (Park et al, 2019). They undergo shape change, with upregulation of surface adhesion molecules, particularly the β2-integrins (CD18-CD11b complex), bind to the endothelium and migrate into the interstitium and alveolar space (Bellingan, 2002). This response is triggered by chemokine gradients, most notably those involving Interleukin-8 (IL-8), leukotriene B4, tumour necrosis factor-α (TNFα), epithelial-derived neutrophil activating peptide-78 (ENA-

78), CCL2 and CCL7 released by alveolar macrophages (AM) and epithelial cells in response to hypoxia, endotoxin and stress (Martin et al, 1989; Martin et al, 2003; Williams et al, 2016). Macrophage inhibitory factor (MIF) is secreted by the anterior pituitary gland in response to injury and increases production of IL-8 and TNFα thus potentially promoting neutrophil recruitment although its complete role in ARDS is currently unclear (Donnelly et al, 1997).

On recruitment to the alveolar space, many authors suggest that neutrophils become functionally activated and undertake their core functions of respiratory burst activity, phagocytosis and programmed cell death in response to cell debris and high levels of endotoxin. In localised infection these functions are normally tightly regulated with minimal release of toxic by-products and tissue damage. However, in ARDS the 'over-activation' of neutrophils is suggested to result in excessive release of reactive oxygen species (ROS), free radicals and proteases, increasing oxidative stress and inactivation of free surfactant adding to widespread atelectasis. Abundant amounts of HNE and collagenases additionally damage the surrounding architecture of the lung and contribute further to loss of alveolar and endothelial integrity (Abraham et al, 2000; Wheeler and Bernard, 2007). Neutrophil activation may release further cytokines and chemokines that coordinate and perpetuate the inflammatory response, particularly IL-1β (Loams-Neira et al, 2006), platelet activating factor (PAF), macrophage inflammatory protein-2 (MIP-2), leukotrienes and additional IL-8 and TNFα (Chollet-Martin et al, 1996; Ware and Matthey, 2000). This increased release of pro-inflammatory cytokines appears dependent on NF-κB (*nuclear factor* kappa-light-chain-enhancer of activated B cells) transcriptional factors. Studies in murine models demonstrate increased nuclear concentration of NF-κB in pulmonary neutrophils compared to those in blood in response to haemorrhage or endotoxin (Shenkar and Abraham, 1999; Abraham, 2003). Furthermore, the action of antioxidants such as N-acetylcysteine (NAC) on NF-κB attenuates pro-inflammatory cytokine release and further neutrophilic accumulation in response to endotoxin (Blackwell et al, 1996).

There is evidence of prolonged neutrophil lifespan within the alveolar space in ARDS. For example, Matute-Bello at el demonstrated that the median percentage of apoptotic

neutrophils present in BALF from patients with ARDS on day 1 was low at 1.5%, increasing to only 3% 14 days following diagnosis (Matute-Bello et al, 1997). Furthermore, they demonstrated that incubation of normal peripheral blood neutrophils with BALF from the above patients greatly reduced apoptosis and this was associated with high levels of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Immunodepleting BAL of G-CSF and GM-CSF almost completely reversed this observation, suggesting these two factors promote neutrophil lifespan. Survival from ARDS is associated with higher levels of G-CSF/GM-CSF in the early stages (Matute-Bello et al, 2000; Pinder et al, 2018). It is likely that delayed neutrophil apoptosis may be beneficial in the early stages and reflect enhanced microbicidal activity, particularly in the context of sepsis. However, it may then become harmful, by prolonging the release of tissue-damaging ROS and proteases, as discussed above (Martin et al, 2003).

Clearance of apoptotic neutrophils by AMs is important for the resolution of ARDS. Many authors have documented that this starts to occur very rapidly and in great numbers (Hussain et al, 1998; Brittan et al, 2014). However, the sheer volume of neutrophils invading the airways can overload macrophage phagocytic mechanisms, leading to accumulation of insoluble proteins and apoptotic neutrophils, and further impairing gas exchange in the early stages, providing a framework for abnormal repair in the later stages (Martin et al, 2003).

1.2.2.3 The role of alveolar macrophages in ARDS

There are two main states of AMs characterised by their responses to environmental stimuli: the classically activated phenotype (M1) and the alternatively activated phenotype (M2) (Sica and Mantovani, 2012). In the normal state, long-lived resident AMs predominantly express M2 phenotypic features acting as a uniform, quiescent and immunosuppressive population (Duan et al, 2012). Upon stimulation in ALI/ARDS, resident AMs rapidly shift to the predominant M1 phenotype in response to infection-induced activation of Toll-like receptors (TLR) (Higgins et al, 2008). Additionally, peripheral blood monocytes are recruited to the

alveolar space and differentiate into macrophages with the M1 phenotype (Herold et al, 2013). These AMs act as the first coordinator and promoter of the acute inflammatory response, releasing various potent proinflammatory mediators such as IL-1ß, IL-6 and IL-18. As detailed above, release of these factors results in rapid and significant recruitment of neutrophils from the intravascular space into the lung interstitium and alveoli. Eyal et al (2007) demonstrated that depletion of AMs led to decreased recruitment of neutrophils to the lung and reduced pulmonary oedema in ventilator-associated lung injury in rats. Koay et al (2002) found that AM depletion attenuated neutrophilic alveolitis and proinflammatory cytokines resulting in reduced evidence of lung injury 4 hours after administration of LPS to mice.

However, in contrast, in a study by Broug-Holub et al (1997) at 48 hours after infection with Klebsiella pneumoniae, AM-depleted mice showed increased neutrophil recruitment and decreased bacterial clearance. In addition, experiments by Narasaraju et al (2011) have shown that influenza infection led to excessive recruitment of neutrophils, extensive alveolar damage, and increased viral load in an AM-depleted group at 5 days after infection. These results suggest that whilst depletion of AMs at an early stage may offer some protective effect against neutrophilic alveolitis it exacerbates lung injury at a later stage.

This is likely due to the subsequent shift of resident and recruited AMs from the M1 phenotype to the anti-inflammatory M2 phenotype, once pathogenic factors are eliminated. M2 macrophages help produce anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL1-RA). What is more, the phagocytosis of apoptotic neutrophils by macrophages appears a key step in limiting the proinflammatory cycle. Fadok at el, demonstrated that phagocytosis of apoptotic neutrophils actively inhibited production of IL-1β, IL-8, GM-CSF, TNFα, leukotriene C4 and thromboxane B2 by human macrophages. Instead, it promoted an anti-inflammatory phenotype with increased production of transforming growth factor beta (TGF-β1) and prostaglandin E2 (Fadok et al, 1998) and IL-10 (Lomas-Neira et al, 2006). Therefore, M2 polarisation and phagocytosis functions of AMs alleviate inflammation.

M2 phenotypic cells are also involved in regulating fibrotic responses in the lungs. Persistence of M2 macrophages at the site of injury is a hallmark of the development of fibrosis, and the steady expression of IL-4 and IL-13 can promote collagen deposition through TGF-ß and arginase 1 pathways (Mora et al, 2006). It is not clear the factors that promote or regulate this aspect of M2 function in response to ALI/ARDS.

1.2.2.4 The balance between pro- and anti-inflammatory factors in ARDS

It is a common supposition that it is inappropriate and prolonged 'over-activation' of neutrophils within the alveolar space that results in much of the tissue damage associated with ARDS. However, some authors question if neutrophilic inflammation is the cause or result of other factors (Ware and Matthey, 2000). In humans, ARDS can develop in the presence of significant neutropenia (Ognibene et al, 1986) and some animal models of ALI are neutrophil-independent (Matute-Bello et al, 2008). Mechanical stress caused by ventilation with high tidal volumes has been shown to result directly in alveolar epithelial necrosis, haemorrhage, oedema and heightened cytokine release (Tremblay et al, 1997). This observation led to significant interest in ventilator strategies employed in patients with ARDS and the landmark ARDS-Net study demonstrated that ventilation with smaller tidal volumes and reduced plateau pressures was associated with decreased mortality and reduced number of days requiring mechanical ventilation (ARDS-Network, 2000).

It may be that neutrophils are initially appropriately activated by epithelial injury and cytokines, but then become 'deactivated' in the alveolar space and therefore unable to perform their core functions. This leads to ongoing accumulation of numbers, abnormal apoptosis and impaired clearance from the alveolar compartment.

It is likely that the balance between pro- and anti-inflammatory factors may be a more important indicator of disease outcome than the presence of individual pro-inflammatory

cytokines alone. There is now increasing evidence that higher levels of IL-1RA, autoantibodies against IL-8 and 'anti-inflammatory' cytokines such as IL-10 are powerful down-regulators of the inflammation in ARDS and may be associated with recovery (Pittet et al, 1997; Bellingan, 2002).

1.2.3 Imaging in ARDS

Plain chest x-ray (CXR) is usually the first imaging modality in patients with potential ARDS. It is fast, inexpensive and safe in unstable patients on the ICU. It can be useful in detecting the malposition of lines or endo-tracheal tubes and subsequent complications (Zompatori et al, 2014). CXR changes have been recognised as an integral part of the diagnostic criteria since the first definitions of acute lung injury and ARDS (see table 1.1). However, the finding of bilateral infiltrates on CXR is non-specific with a wide differential diagnosis including ARDS, infection without ARDS, aspiration, cardiogenic pulmonary oedema, diffuse alveolar haemorrhage, acute hypersensitivity pneumonitis, organising pneumonia and acute eosinophilic pneumonia (Elicker et al, 2016). Interobserver agreement in interpretation of plain CXR is often poor, particularly when films are taken supine when pleural effusions layering posteriorly can mimic diffuse lung opacification (Rubenfeld et al, 1999).

In more recent years the focus both clinically and in research has shifted towards imaging via CT (computerised tomography) scans. Compared to CXR it is more sensitive in the detection of early disease and characterisation of abnormalities. CT serves several simultaneous roles in identifying different stages of disease, suggesting alternative diagnoses, and determining underlying cause and complications of treatment. Several authors have proposed the use of CT to predict outcome and guide therapy, particularly ventilation strategies. As such, results of CT scanning in those meeting clinical criteria for ARDS have been shown to yield new information in 66% of patients and change management in approximately 25% of patients (Simon et al, 2015). Simon et al described these management alterations as being change in antibiotic prescribing (12.7%), drainage of pleural effusions (7.8%), correction of misplaced lines or tubes (4.9%), diuresis (2.9%) and

anticoagulation (2.5%). Of course, the diagnostic advantages of CT need to be weighed against the practicalities and risks of transporting often ventilated patients from ICU to the radiology department (knight et al, 2015).

The characteristic CT appearances in the early (exudative) phase of ARDS are heterogenous changes bilaterally. Classically there can be normal or near-normal lung appearances most commonly in the superior and anterior (non-dependent areas when in supine position) areas, with ground glass opacification in the middle lung, progressing to consolidation in the most dependent areas of the lung (posterior and basal in supine position) (Gattinoni et al, 2001). The theory behind the inhomogeneity of appearances in ARDS is that the increased weight of overlying lung tissue causes compressive atelectasis posteriorly resulting in dense opacification. This is supported by the observation that changing patients to prone position rapidly redistributes the density gradient (Sheard et al, 2012). Identification of dense consolidation in non-dependent areas of the lung should raise the suspicion of infective consolidation, either as the cause of ARDS or as a potential complication of management (ventilator-associated pneumonia). It has been reported that ARDS due to a pulmonary cause more often demonstrates this classic heterogenous appearance, whilst indirect or extrapulmonary causes result in a more diffuse, symmetric and uniform parenchymal change, presumed secondary to haematological distribution of mediators (Goodman et al, 1999).

ARDS is a dynamic process which follows a variable course. Some patients show rapid resolution of changes (within the first week) while others follow a protracted course. As the lung moves through the organising phase, in general the CT density of lung change decreases and lung architecture can undergo remodelling. Reticular opacification appears alongside alveolar changes although these do not necessarily represent fibrosis as these can often fully resolve. Subpleural cysts or bullae can be seen and are often associated with prolonged ventilation and ‘barotrauma’. At this stage, the extent of CT involvement (>80% of the lung), presence of secondary bronchial dilatation, honeycombing or presence of pulmonary artery hypertension indicates early fibrosis and predicts mortality (Chung et al, 2011). In the later, fibrotic phase, surviving patients show variable appearances of the

lungs. 70% of patients have abnormalities present at 6 months, most often persistent ground glass change, reduced lung volumes and coarse reticulations. More severe fibrosis is reported in those with a pulmonary cause of ARDS and in those exposed to prolonged ventilation and high PEEP (Zompatori et al, 2014). In these cases, changes are usually seen in the non-dependent areas of the lung and are assumed to be related to ventilator-induced lung injury or oxygen toxicity.

A few authors have suggested CT appearances could be used to guide ventilation strategies or effects. Gattinoni et al (2001) suggested performing limited CT (2-3 slices on inspiration, expiration and at different PEEPs) to differentiate atelectatic from consolidated lung and to assess potential degree of recruitment at different ventilator settings. Constantin et al (2019) used CT imaging to separate patients with ARDS into those with 'focal' and 'non-focal' ARDS to guide ventilator settings (Control group used standardised ARDSnetwork tables versus 'personalised group' with regimen based on CT features). They found no difference in the 90-day mortality between groups. Moreover, that single-slice CT imaging misclassified patients to focal/non-focal groups in 21% of cases and personalised ventilator strategies based on this led to increased mortality in those that were misclassified.

There is very little evidence on other imaging modalities in ARDS. Bedside ultrasound is well established in the recognition and management of pleural effusion and thus helps differentiate this from the diagnosis of ARDS. In skilled hands it may be able to identify other features consistent with cardiogenic pulmonary oedema or the presence of pneumothorax, but these interpretation skills are not widespread within the ICU environment. Positron emission tomography (PET) CT scans have not been widely adopted into clinical practice but have been used in some research studies. Bellani et al (2011) found that in patients with ARDS, metabolic activity (measured by uptake of FDG, 2-fluoro-2-deoxy-D-glucose) of aerated regions of lung was linked to both plateau pressure and tidal volume. To my knowledge, no studies have examined MRI (magnetic resonance imaging) scans in the clinical setting of ARDS, but it has been studied in animal and human models of ALI (see section 1.2.5.10, page 42).

1.2.4 What is Lipopolysaccharide (LPS)?

1.2.4.1 The structure and function of LPS

LPS is a key component of the cell wall of all Gram-negative bacteria. The term 'endotoxin' is sometimes used to refer to all bacterial cell-associated toxins but the more specific term 'lipopolysaccharide' is reserved for the lipid-sugar complex (shown in figure 1.2) present in the outer cell membrane and essential to its stability (Todar, 2013). Several parts of the structure of LPS are well-conserved across species of bacteria, as is the innate immune response triggered by these components. However, various aspects of these immune responses have only recently been identified and/or remain to be fully elucidated.

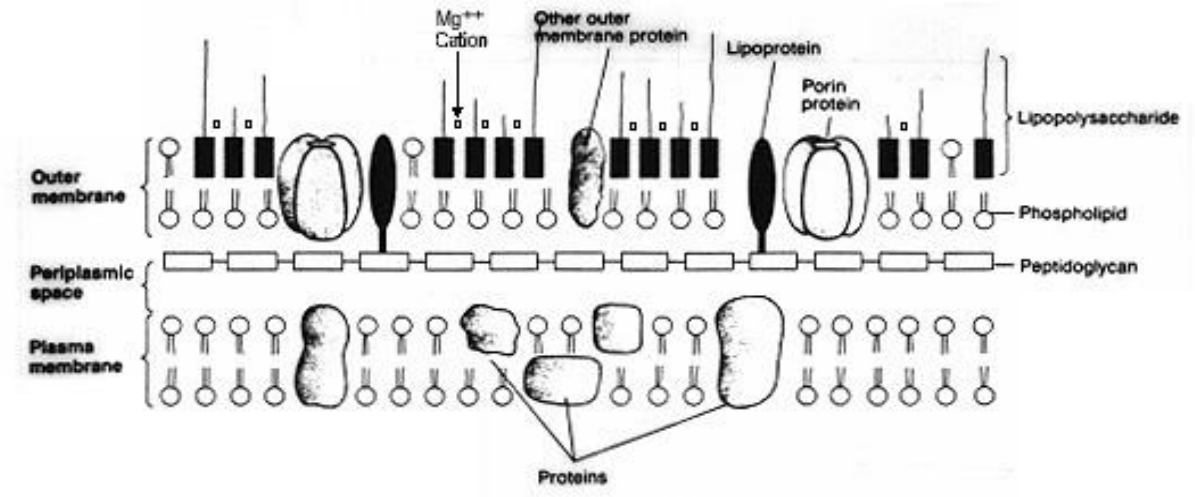


Figure 1.2 Structure of the cell wall of a Gram-negative bacterium showing the position of lipopolysaccharide (LPS). (Taken from Todar, 2013).

The general structure of LPS is shown in figure 1.3. LPS is essential to the stability of the outer cell membrane, establishing a barrier that is only permeable to small hydrophilic molecules. This prevents penetration of the bacterium by bile salts and many antimicrobial agents. LPS may also be involved in adhesion to endothelial surfaces and modulation of the O-polysaccharide chain allows development of different antigenic species which may elude existing immunological responses (Todar, 2013).

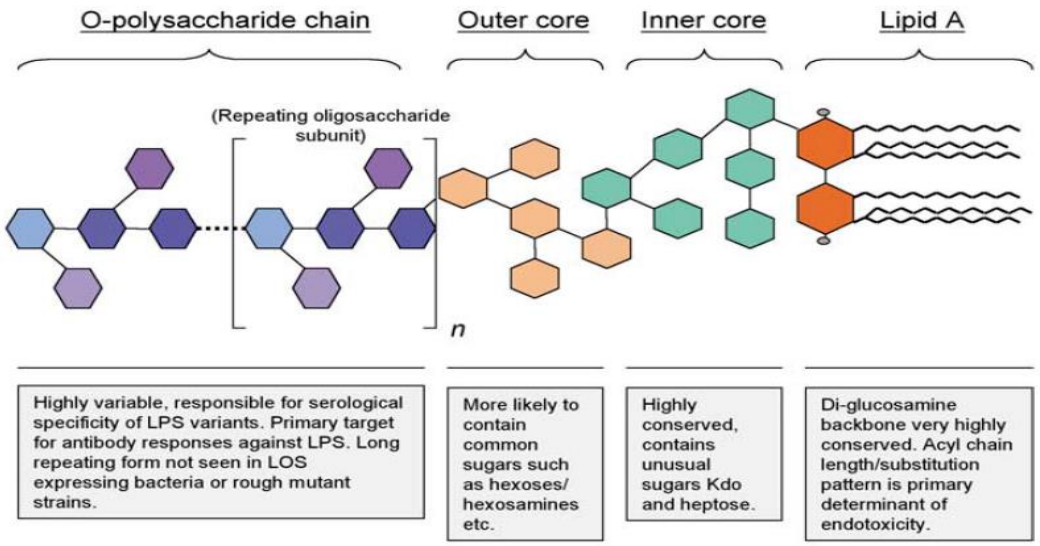


Figure 1.3 General structure of lipopolysaccharide (LPS). LOS=lipooligosaccarides, Kdo=2-keto-3-deoxyoctonoic acid (taken from Erridge et al, 2002)

The central lipid A core is highly hydrophobic and anchors the molecule to the cell membrane. It is responsible for the 'toxin'-mediated effects on immunity. Lipid A consists of an N-acetylglycosamine (NAG) dimer carrying two phosphoryl groups. Four acyl chains (fatty acids) are attached directly by ester or amide linkage with two (sometimes three) additional fatty acid chains attached to the beta-hydroxy group. Unsaturated fatty acids are rarely seen. The structure of lipid A is highly conserved in Gram-negative bacteria, with the structural format of lipid A in *Escherichia coli* widely considered closest to that optimally recognised by human cellular LPS receptors, for example TLR-4 (Erridge et al, 2002). In almost all cases derivations from this structure reduces the biological activity of the molecule.

The core polysaccharide is divided into the 'inner' and 'outer' core segments. The inner core is attached to position 6 of one NAG and consists of a short chain of sugars. Two unusual sugars, heptose and 2-keto-3-deoxyoctonoic acid (Kdo) are invariably present. The outer core usually consists of more common sugar subunits and may be more variable. The core polysaccharide unit together is usually common to Gram-negative bacteria of the same genus.

The O-polysaccharide chain consists of repeating subunits of 3-5 sugars, is highly variable, and can have up to 40 repeat units. It maintains the hydrophilic component of the LPS molecule and is the major site for antibody recognition and binding to the Gram-negative cell wall. The great variation within the O-polysaccharide, particularly in the terminal arrangements, confers the immunological specificity of hundreds of serotypes within a particular Gram-negative bacterial species. The O-polysaccharide chain may also be recognised by the innate immune system through activation of complement, and in some bacterial species it is essential for survival of the host, as loss of the O-chain results in loss of virulence and greater susceptibility to phagocytosis and serum bactericidal defences (Joiner et al, 1984).

1.2.4.2 Cellular signalling in response to LPS

It is likely that small amounts of LPS are released from Gram-negative bacteria during replication which may be important in the development of natural immunity. Otherwise it becomes released in large amounts when bacterial cells undergo autolysis (due to restriction of essential nutrients or growth conditions) or external lysis as a result of complement attack, antimicrobial enzymes or phagocytosis (Todar, 2013). LPS is not intrinsically toxic but potently interacts with myeloid and non-myeloid cells to induce multiple intracellular signalling pathways resulting in conversion to an inflammatory phenotype and release of many of the mediators seen in acute inflammation (Ulevitch and Tobias, 1995). Interaction with LPS receptors in the human body is complex and several steps have only recently been identified.

The discovery in 1986 of an acute phase reactant protein able to directly bind LPS and endow normal serum with properties of acute phase serum (Tobias et al, 1986) led to an upsurge in interest in LPS signalling. This 60-kDa glycoprotein was termed LPS-Binding Protein (LBP) and was later shown to exhibit high affinity with the lipid A portion of the molecule. This was followed by the discovery of a membrane receptor for the LPS-LBP complex required to initiate cellular responses to LPS. This membrane receptor was

identified as CD14, a 55-kDa glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) found on myeloid cells and a key regulator of sensitivity to LPS. It was later identified in soluble form (sCD14), lacking the GPI-anchor, secreted by monocytes and the liver where it is important in activating non-myeloid cells such as endothelium and epithelial cells that lack mCD14 (Frey et al, 1992). However, since CD14 lacks a transmembrane domain with which to affect intracellular signalling the search for a further factor finally revealed that a mutated form of Toll-like receptor-4 protein (TLR-4) conferred intrinsic resistance to LPS in mice (Qureshi et al, 1999). The observation that transfection of TLR-4 into deficient mice was not able to fully restore LPS signalling led to the final piece of the pathway; that the extracellular membrane protein MD-2 was required to interact with TLR-4 and transfection of both of these factors was able to fully restore LPS response in deficient mice (Shimazu et al,1999; Erridge et al, 2002).

It is now understood that the role of LBP is to more efficiently deliver LPS to either mCD14 or sCD14 (Hailman et al, 1994) which, whilst not essential for interaction with the TLR-4/MD-2 membrane complex, is likely to catalyse its insertion into the plasma membrane or the receptor-complex directly (da Silva Correia et al, 2001). Both TLR-4 and MD-2 protein must be present in close proximity in order to trigger dimerisation of TLR-4, which appears to be the key step in initiating intracellular signalling pathways (Erridge et al, 2002).

Intracellular signalling in response to LPS appears to follow very similar pathways to that utilised by the IL-1 receptor but may follow several coexisting pathways. The adaptor protein MyD88 (myeloid differentiation factor 88) is thought to be activated in response to cytosolic dimerisation of TLR-4 and is able to catalyse activation of the IL-1 receptor-associated kinase (IRAK) family. This in turn activates TNF receptor-associated factor 6 (TRAF-6) which upregulates MAP (mitogen-activated protein) kinase and NF-κB cascades (Erridge et al, 2002). However, a number of MyD88-independent pathways have also been identified, where intracellular proteins termed Tollip (Toll interacting protein) and TIRAP may be able to interact directly with IRAK in response to changes in TLR-4 (Horng et al, 2001) which may be inhibitory in order to regulate the system. These pathways are summarised in figure 1.4. There is also evidence that activation of Akt (also known as

protein kinase B) through phosphatidylinositol 3-kinases (PI3-K) occurs in response to changes in TLR-4 and results in nuclear accumulation of NF-κB (Yum et al, 2001). Upregulation and concentration of NF-κB cascades within the nucleus of myeloid cells promotes production of inflammatory cytokines, particularly TNFα, IL-6 and IL-8, as well as modulating chemotaxis and phagocytosis activity (Abraham, 2003).

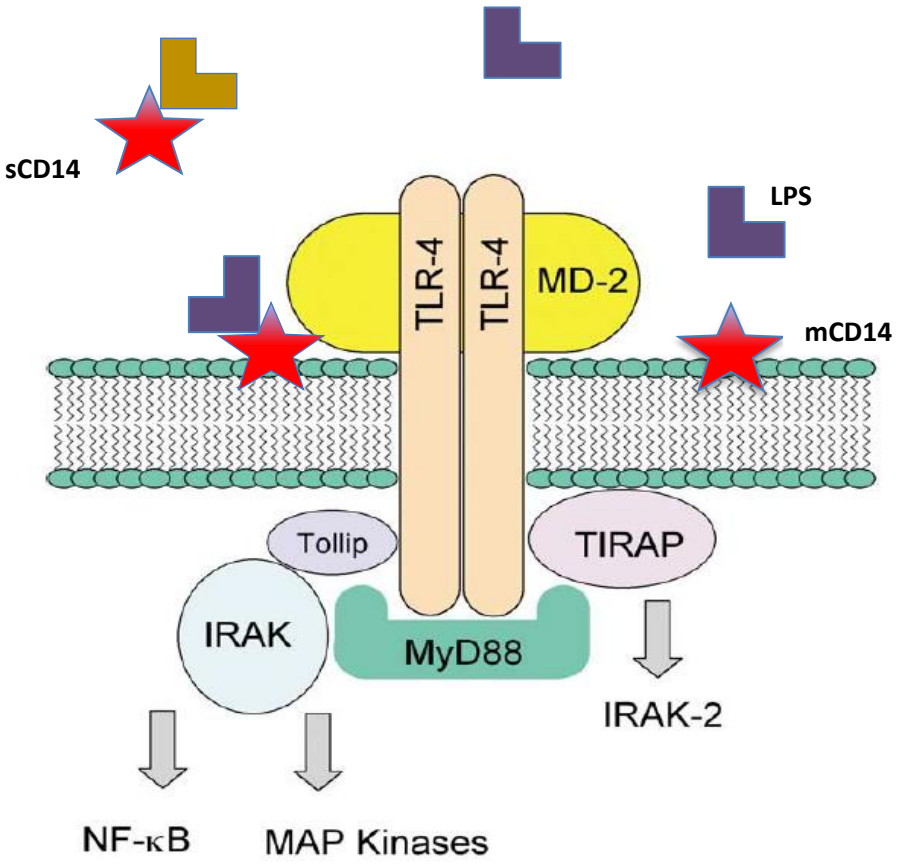


Figure 1.4 Intracellular signalling cascades following LPS-CD14 induced dimerization of the TLR-4/MD2 receptor complex. Adapted from Erridge et al, 2002

1.2.5 Models of acute lung inflammation (ALI)

1.2.5.1 Animal models of ALI

Modelling human ARDS is a difficult task. ARDS is defined by a cluster of hypoxaemia, non-specific clinical features and the requirement for higher level organ support, all in the presence of recognised risk factors. However, the fundamental mechanisms that initiate, and to a certain extent propagate, ARDS in humans are not fully known (Matute-Bello et al, 2008). Detailed information on the early pathological processes is limited as the clinical diagnosis is often made several days after initial onset. Histological samples are usually confined to the later fibrotic stages of disease or those who have ultimately died. Also, we are only really beginning to understand why the same set of risk factors may precipitate ARDS in some individuals but not in others. Finally, a situation unique to the human condition of ARDS is that the ultimate pathological processes leading to its development and its resolution may be affected not only by individual characteristics and the nature of the initial insult but also by attempts at treatment, including a multitude of systemic medications and mechanical ventilation.

Since many research-focused investigations would be extremely hazardous in unwell patients in the ICU, if we are to make significant advances in our understanding of ARDS there is a need to develop appropriate, accurate and reproducible models in which to examine the pathological process and develop further potential treatments. Table 1.4 identifies the key characteristics the 'ideal model' of ARDS (or perhaps in a milder form of ALI) would incorporate.

Unfortunately, no single animal model (or human model for that matter) reproduces all these characteristics. However, animal studies provide a key bridge between laboratory observation and human studies. Simple in vitro hypotheses can be tested to see their relevance within an intact biological system and evidence from human studies can be reliably reproduced for repeated study. Mechanistic studies into cell signalling can be conducted by creating specific genetic knockout variants and animal models may be able to facilitate understanding of the heterogeneity of ARDS through chemical mutagenesis or

gene linkage studies in mice (Matthay et al, 2003). Without animal models there would be no way to assess basic safety and efficacy of new treatments prior to human studies, which to most would be unacceptable.

Features of the model	Model characteristic
Clinical features	Acute onset Bilateral alveolar infiltrates Injury mechanism likely to occur in humans
Physiological changes	Ventilation/perfusion mismatch Severe hypoxaemia Decreased lung compliance Impaired alveolar fluid clearance
Pathological changes	Acute exudative phase Increased endothelial permeability Injury to alveolar epithelium and increased permeability Neutrophilic alveolar infiltrates Increased cytokine signalling Protease activation Intra-alveolar coagulation and fibrin deposition Repair with or without fibrosis

Table 1.4 Key characteristics of the 'ideal model' of ARDS to incorporate into an experimental model. Adapted from (Matute-Bello et al, 2008).

Most animal models have focussed on reproducing a single risk factor for ARDS and these are summarised in table 1.5. As such they produce some, but not all, of the pathological hallmarks of ARDS and are most usefully viewed in the study of one particular feature of the pathway. Similarly, dependent on the level of stimulus they can be considered to often produce a lesser level of change and model the early phases of ALI and not necessarily all the features of ARDS.

There are some generic problems with animal models of ALI. It is well recognised that there are species-specific differences in susceptibility to ALI (Kuida et al, 1958; Venaille et al, 1989; Wheeler et al, 1990). For example the lungs of sheep, pigs and cats have a resident population of pulmonary intravascular macrophages, which tend to localise LPS and particulate matter in the blood to the pulmonary circulation increasing the likelihood of developing ALI in response to a smaller stimuli (Sone et al, 1999). This contrasts with mice, primates and humans who have intravascular macrophages predominantly localised to the liver and spleen. The relative constituents of immune cells in adult blood differs between species, with human blood being neutrophil rich (50-70% neutrophils, 30-50% lymphocytes) compared to mouse blood being lymphocyte predominant (75-90% lymphocytes, 10-25% neutrophils) (Mestas and Hughes, 2004).

There may also be significant differences in receptor activation and cytokine release involved in response to endotoxin. Work by Hajjar et al revealed that TLR-4 in humans was able to recognise and respond to adaptation of LPS by Pseudomonas bacterium, whilst this was not the case in mice (Hajjar et al, 2002). Furthermore, IL-8 (CXCL8), a potent neutrophil chemotactic agent implicated in human ARDS is not produced by mice or rats. Instead they produce two related CXC chemokines (KC and MIP-2) thought to take on the same role of neutrophil recruitment. However, evidence suggests these bind to separate CXC receptors, suggesting that whilst there may be overlap in some functions, the ultimate intracellular signalling may well be different (Fan et al, 2007). Schroder at al (2012) demonstrated 24% divergence in LPS-regulated orthologous gene expression between humans and mice. Divergently regulated orthologues were enriched for genes encoding cellular ‘inputs’ such as cell surface receptors (for example TLR6, IL-7Rα) and functional ‘outputs’ such as inflammatory cytokines and chemokines (for example CCL20, CXCL13).

Animal size clearly also has some impact on modelling ARDS. It is often difficult to monitor physiological parameters in small rodents or to take repeated blood samples, and most cannot be mechanically ventilated for more than 12-24 hours.

Recognising all these limitations, animal models have been instrumental in developing our understanding of ARDS and ALI in humans. In particular, mouse models of endotoxin-induced ALI have led to significant advances in the pathogenesis underpinning ARDS and much of the evidence presented in section 1.2.2 ('Pathogenesis of ARDS') comes from these models. By harnessing the full genetic power of murine studies, it is likely this work will continue to inform research in this complex area.

Risk factor (mechanism) for ALI/ARDS	Pathological hallmarks of model	Advantages to the model	Disadvantages to the model
Sepsis **1. Endotoxin/LPS**	Endothelial cell activation and apoptosis, increased permeability and activation of innate immunity – particularly neutrophilic invasion. Increased cytokine signalling and involvement of coagulation pathways	Highly reproducible. Replicates many of the key features of human ALI in the acute phase. Can be given IV, intratracheally or nebulised. Accurate model of a major component involved in Gram-negative sepsis	Permeability changes are mild. LPS preparations may vary and animal signalling response to LPS may differ from humans. LPS is only one component of toxins involved in Gram-negative sepsis
Sepsis **2. Intravenous live bacteria**	Increased endothelial permeability, interstitial oedema and mild neutrophilic sequestration. Relatively little alveolar epithelial change or hyaline membrane formation	Models a common cause of ALI (although the link between bacteraemia, septic shock and ALI is not fully understood)	Alveolar epithelium is relatively resistant to IV bacteraemia in animals and therefore not associated with the full spectrum of pathological changes seen in ARDS
Sepsis **3. Intrapulmonary live bacteria**	Increased endothelial permeability with greater alveolar epithelium involvement. Neutrophilic inflammation & cytokine release.	Models the biggest single risk factor for development of ARDS	Pathological changes may be more akin to focal pneumonia depending on method of administration and rarely does human ARDS results from single large infective bolus
Sepsis **4. Caecal ligation and puncture (peritonitis)**	Increased endothelial permeability and variable interstitial oedema with neutrophilic infiltrate. Changes occur over several days.	Models a common cause of ARDS. Multiple Gram-negative organisms are often isolated from culture (may be more representative than IV bacteraemia with single organism)	Mild changes of ALI. Requires complex surgery. The major bacterial inoculum is unknown and is likely to differ across species and even strains within a species
Lipid embolism **(Oleic acid)**	Endothelial necrosis and microvascular thrombi followed by epithelial necrosis. Variable interstitial oedema and fibrin	Good model to study the endothelial damage and subsequent physiological	Requires IV injection (can be difficult)

	deposition. Neutrophilic infiltrate but not dependent on neutrophils. Healing is with proliferation of type 2 pneumocytes and fibroblastic foci	changes in gas exchange and mechanical impairment. Reproducible	Very rapid onset and resolution of acute inflammation Lipid embolism only accounts for a very small minority of cases of human ARDS
Gastric aspiration (acid aspiration)	Primary injury to the alveolar epithelium with reduced alveolar fluid clearance. Patchy interstitial oedema and neutrophilic infiltrate. Fibrotic phase follows acute inflammation approximately 1 week later	Good model to study alveolar injury and haemodynamic and physiological changes seen in ALI. Often combined with other injury (e.g. ventilation strategies to make more clinically relevant)	Narrow range for acid concentrations causing injury. Humans aspirate gastric content not purely acid (and usually contents much less acidic than required in animal models to produce ALI)
Hyperoxia	Production of oxygen free radicals causes epithelial and endothelial injury and increased cytokine release with minor neutrophilic inflammation. Healing via type 2 pneumocyte proliferation and scarring	Well defined acute exudative phase and proliferative phases make it a good model to study repair mechanisms	100% oxygen only rarely causes ARDS in human and effects are usually only seen when combined with other factors Requires specialist equipment to deliver oxygen accurately
Surfactant depletion (saline lavage)	Depletion of surfactant facilitating alveolar collapse and loss of type 1 and 2 pneumocytes.	May be combined with other injuries (especially mechanical ventilation strategies) to accurately model multiple simultaneous processes in the human lung.	Animals require general anaesthesia, intubation and ventilation throughout. On its own there is little change in barrier permeability or neutrophilic inflammation
Mechanical ventilation strategies	Epithelial and endothelial stretch resulting in necrosis and denuding of the basement membrane. Neutrophilic inflammation and increased cytokines occur later than in other models and at higher tidal volumes	Models a relevant therapeutic intervention (rather than a risk factor for development). The only animal model that has led to changes in clinical practice and shown to reduce mortality. Can be combined with other models (e.g. sepsis)	Animals require general anaesthesia, intubation and ventilation throughout. Pressures shown as deleterious in animals are in excess of those used in humans, small rodent can only be ventilated for short periods

Bleomycin **(model of pulmonary fibrosis but some acute features may mirror ALI)**	Intratracheal bleomycin results in alveolar epithelial injury. Well defined sequence of neutrophilic inflammation, increased cytokine release, with later lymphocytic infiltration and fibrotic healing	Reproducible and well-defined stages of acute inflammatory infiltrate and fibrosis	Relevance to mechanisms of injury in ARDS unclear
Ischaemia-reperfusion injury **1. Pulmonary** **(e.g. following transplantation or pulmonary embolectomy)**	Increased pulmonary vascular permeability and neutrophilic infiltration. Haemorrhagic interstitial oedema with activation of coagulation.	Models a specific injury relevant to a subset of patients	Multiple variables contribute to the injury (ischaemic time, control of reperfusion and deflation of the lungs). Species-specific differences make it difficult to compare studies
Ischaemia-reperfusion injury **2. Non-pulmonary** **(e.g. following aortic aneurysm repair)**	Increased systemic microvascular permeability and neutrophilic sequestration within the lungs. Injury is usually mild with inflammatory infiltrate limited to the interstitium (minimal epithelial injury)	As above for pulmonary ischaemia-reperfusion injury	As above. Both pulmonary and non-pulmonary mechanisms may only account for a small proportion of human ARDS Requires complex animal surgery

Table 1.5 Summary of animal models of ALI. Their main sites of injury, pathological hallmarks and advantages/disadvantages for use as a model for human ARDS/ALI. Adapted from (Matute-Bello et al, 2008)

1.2.5.2 Overview of human studies using LPS

Recognising the strengths, but also the significant constraints, of information gained purely from animal models led investigators to search for a human model of ALI. Since almost all the stimuli cited in table 1.5 would be totally unacceptable in humans, most of the attention has focussed on administration of endotoxin. In fact, there was already emerging evidence for a role of endotoxin in human disease related to occupational organic dust exposure. Symptoms in workers exposed to cotton (Rylander et al, 1985), poultry and swine-dust (Rylander et al 2006; Kirychuk et al, 2006) such as fever, malaise, progressive shortness of breath throughout the working week and/or asymptomatic chronic impairment of respiratory function had been termed the 'organic toxic dust syndrome' (von Essen et al, 1990). The primary agent responsible for these effects was identified as bacterial lipopolysaccharide (Cavagna et al, 1969; Zhiping et al, 1996). Subsequent exploration of the underlying mechanisms of disease revealed a highly acute inflammatory reaction in response to pure endotoxin, which mirrored many features seen in ALI.

Endotoxin has been administered to humans by several routes including intravenous injection, subcutaneous injection, intra-tracheal or bronchial instillation, and nebulisation. Intravenous administration more accurately models Gram-negative bacteraemia/sepsis rather than ARDS per se. As seen in animal models it is associated with elevation of temperature (Dillingh et al, 2014), increase in peripheral blood total white cell counts, neutrophilia (Copeland et al, 2005; de Kleijn et al, 2012), activation of systemic cytokines (Suffredini et al 1995; Dillingh et al 2014); and coagulation pathways (de Jonge et al, 2000; de Kruif et al, 2007). However, there is little change in respiratory physiology, reflected by only minimal evidence of alveolar epithelial disruption, interstitial oedema, changes in surfactant or fibrin deposition. Therefore, the remainder of this chapter will focus on changes seen in humans in response to LPS administered directly to the lungs (inhalation and instillation) as the more accurate model of ARDS.

1.2.5.3 Human studies of ALI using respiratory challenge with LPS

LPS can be delivered to the respiratory system either via nebuliser ('inhaled', resulting in inflammation of all lobes) or via bronchoscopically-guided instillation to the trachea or lobar bronchus ('instilled', producing localised inflammation in a lobe). Response to respiratory challenge with LPS may be monitored within the systemic circulation (changes in peripheral blood) or within the lungs. Most often samples taken to reflect the alveolar compartment are in the form of BAL, but induced sputum has also been used. Authors have also looked at response to endotoxin in different populations with some studies specifically comparing healthy volunteers to those with an asthmatic tendency (non-atopic and atopic) and 'chronic bronchitis' phenotypes, (Michel et al, 1989; Michel et al, 1992). However, these were associated with increased symptoms and risks for participants. Therefore, most later studies specifically screen out any underlying lung or atopic condition. Dose comparisons are also difficult, with studies using between 20-60µg of LPS from a variety of different commercially available sources, which may exhibit differing potency in inflammatory response. Differences in the exact method of delivery, mode of monitoring, dosing regimen and population studied reflect some of the variety of outcomes seen in human studies in response to respiratory challenge with LPS.

1.2.5.4 Clinical and physiological changes in response to respiratory challenge with LPS

The most common symptoms reported by human subjects are 'flu-like' symptoms such as dry cough, dyspnoea, lethargy, muscle aches and headaches (Thorn and Rylander, 1998; Sundblad et al, 2009; Korsgren et el, 2012). Fever is often reported, associated with a documented elevation in temperature by 0.5-1.5 °C, peaking at 6-8 hours following exposure (Michel et al, 2001; Shyamsundar et al, 2009). Changes in heart rate, blood pressure, respiratory rate and oxygen saturations are uncommon. (Michel et al 2001; Loh et al, 2006). There appears to be a significant dose-response with lower doses of between 5-20µg pure LPS not inducing symptoms while a threshold of 50µg induces symptoms in approximately 40-50% of subjects (Michel et al, 1997). The threshold for onset of symptoms appears lower in those with asthma and is likely to be related to the degree of bronchial

constriction. There are also considerable differences in onset of symptoms reported depending on whether subjects were exposed to pure LPS or organic dusts containing an estimated concentration of endotoxin. Early studies almost invariably showed that direct inhalation of the organic dust (cotton or swine) produced symptoms at an apparently lower dose of endotoxin, approximately 2-10µg endotoxin (Larsson et al, 1994). However, this is likely due to a combination of poor extraction techniques, inaccurate concentration measurement and/or presence of other contaminants within the dusts also causing toxic effects. Interestingly, later studies showed that the average cotton mill worker was likely to inhale approximately 30-60µg of pure endotoxin over an 8-hour shift, roughly equivalent to the threshold for producing symptoms (Clapp et al, 1993).

Low doses of inhaled endotoxin (up to 20µg) have no appreciable effect on lung function when measured by spirometry (Thorn and Rylander, 1998). Doses of 50-60µg are associated with a slight, but usually non-significant, reduction in FEV1 (forced expiratory volume in 1 second) in healthy subjects (usually a fall of between 1-4%) (Michel et al, 1997; Kitz et al, 2006; Shyamsundar et al, 2009). This is in contrast to subjects known to be asthmatic who demonstrate a detectable fall in FEV1 on challenge with 20µg of inhaled LPS (Michel et al, 1992; Michel et al, 2001). This fall of between 5-11% becomes apparent approximately 30 minutes after inhalation, lasting up to 6 hours. Michel et al (1992) went on to show that this corresponds to an increase in bronchial hyperresponsiveness to histamine, maximal at 5-6 hours following inhalation of 20µg LPS in asthmatic subjects. Bronchial hyperresponsiveness was unchanged in normal subjects (Michel et al, 1989; Michel et al, 1992). In a single study, inhalation of 70µg of LPS resulted in a statistically significant reduction in diffusing capacity for carbon monoxide (DLCO) of between 7-18% from baseline to 2-4 hours following inhalation. Results were lower than in participants inhaling control at 8 and 24 hours, but this did not reach statistical significance (Herbert et al, 1992).

1.2.5.5 Total leukocyte and neutrophil response to respiratory challenge with LPS

a) Peripheral blood compartment

A multitude of studies have demonstrated an increase in total circulating blood leukocytes in response to inhaled/instilled LPS. This is overwhelmingly due to an increase in peripheral blood neutrophils (Herbert et al, 1992; Clapp et al, 1993; Michel et al, 1995; Michel et al, 1997; Thorn and Rylander, 1998; Michel et al, 2001; Loh et al, 2006; Brittan et al, 2012) which becomes apparent approximately 3-4 hours following exposure, is maximal at approximately 6-10 hours, and is falling by 24 hours (see figure 1.5). The level of stimulus required to commence this process is extremely low, with immune changes demonstrated following inhalation of 0.5µg LPS (Michel et al, 1997). The threshold for immune changes is certainly well below that required to precipitate symptoms in most individuals (Kline et al, 1999).

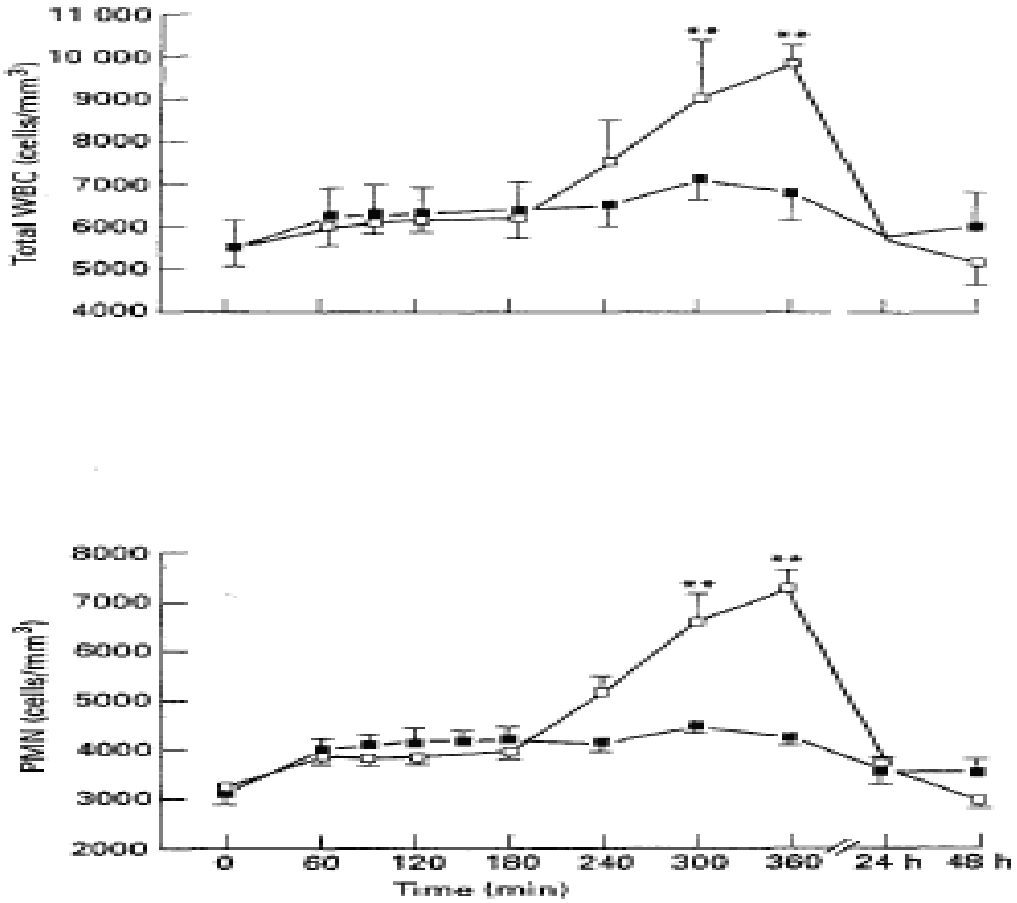


Figure 1.5 Timeline of classical changes in peripheral blood total leukocytes and neutrophils in response to inhaled LPS. Taken from Michel at el, 1995. White boxes represent changes after inhaling 20µg LPS (Escherichia coli 026:B6), black boxes after control (saline).

Despite this well documented blood neutrophilia little is known about the functional status of these cells in response to inhaled LPS. Activation status has been assessed by ROS-induced chemoluminescence with Michel et al showing this is initially reduced in low dose (0.5µg) inhalation of LPS, suggesting this may reflect margination/extravascular sequestration of neutrophils (Michel et al, 1997). At higher doses (20µg and 50µg) ROS-chemoluminescence was shown to increase (Michel et al, 1995). Peripheral blood neutrophils have been shown to increase expression of surface adhesion molecules such as E-Selectin (CD62E) (Michel et al, 2001) but otherwise functional status has mostly been inferred from changes observed in cytokine release and evidence from animal models.

In a key paper by Alexis et al (2003), the authors investigated changes in circulating blood phagocytes in individuals with atopic asthma following inhalation of 5µg of LPS. They demonstrated a significant reduction in blood neutrophil and monocyte phagocytic function, at 6 hours following inhalation (Alexis et al, 2003). This was assessed by flow cytometric measurement of phagocytosis of opsonised zymosan particles (see figure 1.6) and to my knowledge is the only study examining blood neutrophil phagocytosis in a human model of inhaled LPS. This interesting study is in accordance with findings regarding ROS-chemoluminescence by Michel et al (1995), that low dose LPS inhalation causes an initial margination of circulating neutrophils with perhaps a decrease average in function within the blood as the 'activated' cells are trapped and migrate into the pulmonary parenchyma, leaving immature cells in the blood. However, it is difficult to know if this reflects what may be occurring in human ARDS or what changes may occur on challenge with a larger stimulus of LPS.

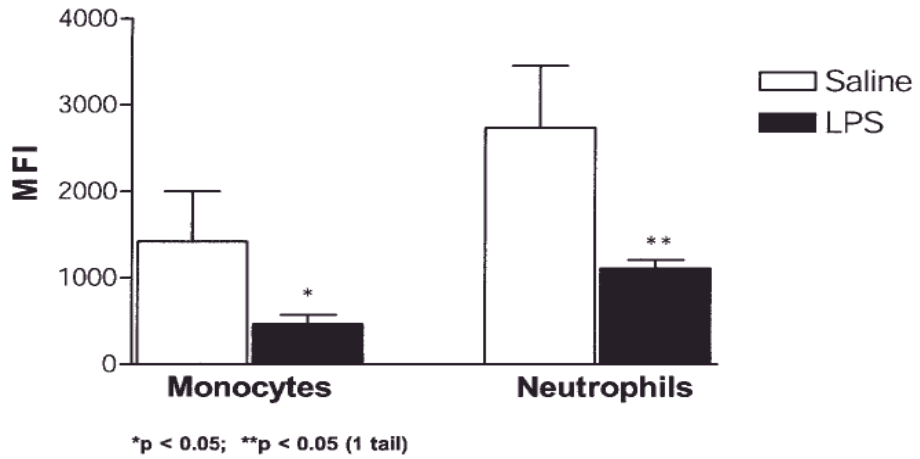


Figure 1.6 Phagocytosis of opsonised zymosan particles of circulating blood monocytes and neutrophils, (measured by mean fluorescence intensity, MFI). Taken from Alexis et al, 2003. Subjects had atopic asthma and responses were measured 6 hours after 5µg LPS or saline inhalation. Bars represent mean, error bars represent standard error of the mean (SEM).

b) Alveolar compartment

In 1992, Sandstrom et al demonstrated a 100-fold increase in BAL neutrophils following nebulisation of 100µg LPS (Sandstrom et al, 1992). This has subsequently been reproduced by a number of other authors, with increases in BALF neutrophils of between 2 and 24 times baseline or control, which is apparent approximately 90 minutes following exposure, peaks at 6-10 hours and persists at 24 hours (O'Grady et al, 2001; Wallin et al, 2004; Shyamsundar et al, 2009; Barr et al., 2010). Increases in neutrophil population in the order of 3-5 times have been detected in induced sputum (Michel et al, 1997; Thorn and Rylander, 1998; Alexis et al, 2003). Differences are dependent on the magnitude of the LPS stimulus, mode of delivery, and the timing of sample retrieval, with maximal effects observed between 6-8 hours following inhalation, although neutrophil counts are usually still elevated at 24 hours.

This increase in alveolar neutrophils has often been linked to biochemical evidence of cellular activation with neutrophil enzymes, myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP9) and HNE peaking at 6 hours in BALF (Loh et al, 2006; Michel et al, 2007: Singh et al, 2015). It is difficult to isolate live neutrophils from BALF/sputum for

functional assays, with the additional significant problem that cells may become artificially activated by the process of separation. In a study by Coldren et al (2006), microarray analysis of isolated alveolar neutrophils demonstrated a dramatic difference in gene expression, with altered expression for 15% of genes encoding inflammatory, chemotaxis and antiapoptotic pathways compared to blood circulating neutrophils following LPS instillation. Functional analysis of air space neutrophils showed increased superoxide release and diminished apoptosis. There were no observed changes in the functional status of circulating blood neutrophils. The excellent paper by Alexis et al (2003), is one of the only other examples to explore functional status of neutrophils within the airspace following LPS challenge. The authors were able to demonstrate reduced phagocytic capacity of neutrophils following the small dose of inhaled LPS in individuals with atopy (see figure 1.7).

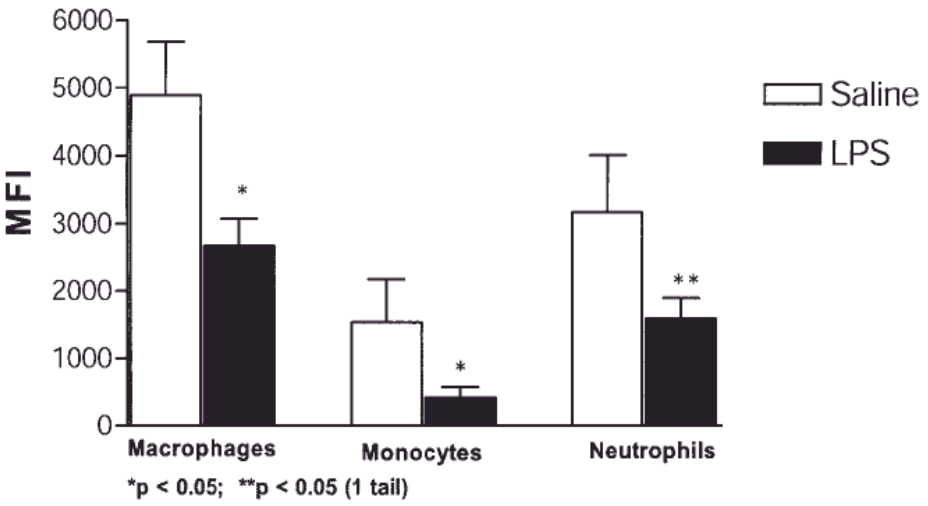


Figure 1.7 Phagocytosis of opsonised zymosan particles of recovered sputum macrophages, monocytes and neutrophils (as assessed by median fluorescence intensity, MFI). Taken from Alexis et al, 2003. Subjects had atopic asthma, and assessments were performed 6 hours after challenge with 5µg or saline. Bars represent mean, error bars represent standard error of the mean (SEM).

1.2.5.6 Other innate immune cellular responses to respiratory challenge with LPS

Changes in a variety of other innate immune cells in response to inhaled LPS have also been studied in humans. In peripheral blood it is commonly observed that there is no change in the circulating monocyte or lymphocyte populations (Michel et al, 1992; Barr et al, 2010).

However, this does not reflect large shifts in cell populations within the alveolar compartment. Michel et al (1997) reported changes in induced sputum after inhalation of 50µg LPS, showing an approximate 4-fold increase in monocytes and doubling of lymphocytes with eosinophil and alveolar macrophage (AM) populations unchanged. O'Grady et al (2001) reported a similar trend in BALF following bronchial instillation with a 5-fold increase in monocytes, trebling of lymphocytes and no change in AMs. It is noticeable that, while neutrophil influx occurs rapidly, the increase in monocytes is maximal at approximately 24 hours, and changes in lymphocytes occur later still, increasing at 48 hours following inhalation and normalising within 7 days (see figure 1.8). The lymphocytosis is generally composed of a greater proportion of effector $CD4^+$ and $CD8^+$ T cells and regulatory T cells (Tregs) than in peripheral blood (Ronit et al, 2015).

Interestingly, although AM numbers show little overall change, AMs isolated 6 hours after inhaled LPS challenge express reduced levels of TLR-4 and MD2 messenger RNA (mRNA) compared to saline controls (Maris et al, 2006). Sandstrom's early paper suggested a reduction in macrophage capacity for phagocytosis of opsonised yeast particles (Sandstrom et al, 1992). The authors suggested that the early BAL at only 3 hours following inhalation probably reflected the activation of alveolar macrophages, resulting in prioritisation of increased cytokine release and adherence to the alveolar epithelial cell wall. They postulated that a later BAL at 8-24 hours would show an increase in phagocytosis by these cells. However, the paper by Alexis et al (2003) also showed that phagocytosis of opsonised zymosan by macrophages and monocytes was significantly reduced in induced sputum (figure 1.7). This was in response to a low dose inhalational stimulus in individuals with atopy.

Interestingly, the study by Brittan et al (2012) identified subpopulations of pulmonary monocytes in the alveolar response to inhaled LPS. The authors suggested the increase in monocytes was due to an 'inducible' pulmonary monocyte population displaying a more 'inflammatory phenotype' with high CD14 but low CD16 expression. There was little change in the 'resident' pulmonary monocytes (expressing high CD14 and CD16). There has also been recent interest in whether these observed changes in AM and monocyte populations may actually reflect a dendritic-cell precursor (Alexis et al, 2005; Schaumann et al, 2008).

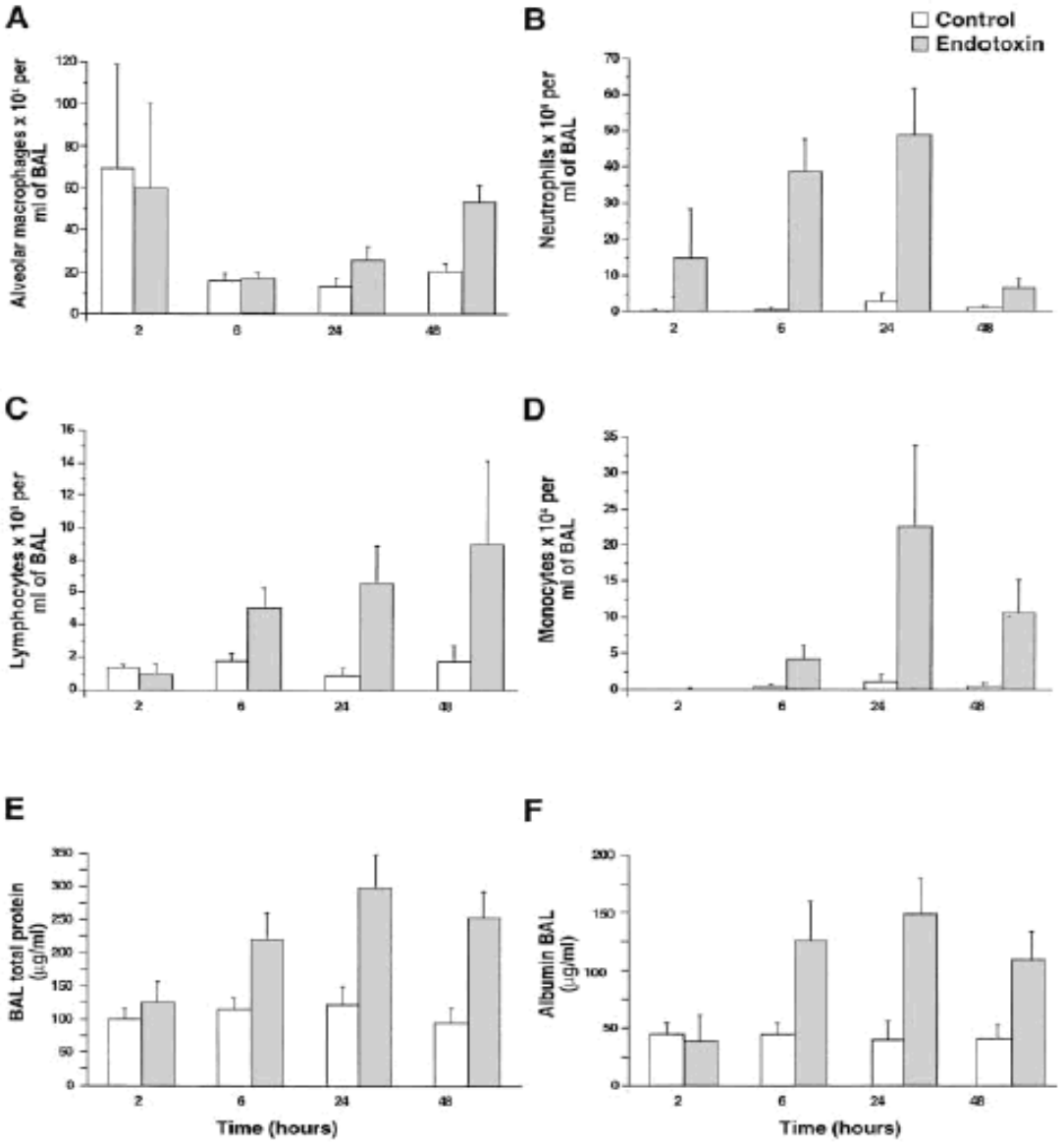


Figure 1.8 Changes in other innate immune cellular responses in bronchoalveolar lavage fluid (BALF) at 2, 6, 24 and 48 hours after saline and LPS instillation. Taken from O'Grady et al, 2001. Bars represent means, error bars represent SEM.

1.2.5.7 Endothelial-epithelial injury and increased permeability in response to respiratory challenge with LPS

Given that disruption of the alveolar capillary-epithelial barrier is a hallmark of pathological ARDS this has received surprisingly little attention within human models of respiratory challenge with LPS. This may be because early studies failed to detect any significant differences suggestive of increased permeability. For example, Sandstrom et al were able to show elevated fibronectin in BALF but no change in albumin (Sandstrom et al, 1992). However, since then, more sensitive methods have resulted in several authors reporting an approximate doubling in total protein and albumin detected in BALF following LPS inhalation, (O'Grady et al, 2001). More recently, Shyamsundar et al (2009) were able to show more specific evidence for epithelial and endothelial activation in humans. They demonstrated a significant increase in BALF surfactant protein-D levels (SP-D, a marker of alveolar type 2 cell injury) and von Willebrand Factor (vWF, an endothelial cell biomarker) following inhalation of 50μg LPS. The protein permeability index (PPI, BAL protein: plasma protein ratio) was also significantly elevated suggesting a reduction in barrier integrity in response to LPS (Shyamsundar et al, 2009).

1.2.5.8 Cytokine and chemokine release in response to respiratory challenge with LPS

Changes in inflammatory mediators within peripheral blood and airways (either sputum or BALF) have been extensively studied in human LPS inhalation. In the blood, increased TNFα is detectable by sensitive methods at approximately 60 minutes following LPS inhalation, lasting up to 6 hours (Michel et al, 1995; Jagielo et al, 1996). Elevated levels of circulating blood LBP (Michel et al, 2001; Kitz et al, 2006; Michel et al, 2007), IL-6 (Jagielo et al, 1996), neutrophil adhesion markers such as E-selectin (CD62E) (Michel et al, 2001; Michel et al, 2007), neutrophil degranulation markers such as MPO (Thorn and Rylander, 1998) and adrenocorticotropic hormone (ACTH) (Michel et al, 1995) (suggested as a surrogate marker of increased IL-1), have been detected. Several of these mediators are able to upregulate hepatic acute phase proteins, and elevated serum C-reactive protein (CRP) concentration has been repeatedly detected as a late response (24-48 hours) to inhaled LPS (Michel et al,

1995; Michel et al, 1997; Michel et al, 2001; O'Grady et al, 2001; Kitz et al, 2006; Michel et al, 2007).

In sputum and BALF TNFα, largely produced by alveolar macrophages, is significantly elevated at an early stage (Michel et al, 1997; Michel et al, 2001). Similarly, levels of IL-6 and IL-8, stimulated through p38 MAPK (mitogen-activated protein kinase) signalling, increase early and peak at 6 hours in induced sputum (Nightingale et al, 1998; Singh et al, 2015). Isolated studies have also detected increases in sputum MMP-9 (Michel et al, 2007) and the chemotactic marker MIP-1ß (macrophage inflammatory protein -1ß) (Singh et al, 2015). Exhaled nitric oxide (eNO) has been studied by some authors, demonstrating increased levels approximately 6 hours following LPS inhalation, particularly in subjects with asthma (Rolla et al, 1997; Kitz et al, 2006).

Many of the same markers have been identified in BALF after LPS challenge. TNFα, along with TNFα receptor (TNFα-R) are increased in BALF at 90 minutes and stay elevated at 24 hours (O'Grady et al, 2001; Maris et al, 2005; Hecker at, 2015). MPO and ECP (Eosinophil cationic protein) are consistently found within BALF from 2-24 hours following inhalation (Jagielo et al, 1996; Maris et al, 2005; Shyamsundar et al, 2009; Brittan et al, 2012). Other markers of neutrophil 'activation' such as IL-1β (Jagielo et al, 1996; O'Grady et al, 2001), L-selectin (CD62L), lactoferrin (O'Grady et al, 2001) and HNE (Maris et al, 2005) have been detected in BALF, usually approximately 6-8 hours following inhalation. Other potent cytokines such as IL-6 (Jagielo et al, 1996; O'Grady et al, 2001; Maris et al, 2005; Barr et al, 2010) and proteases such as MMP-7, -8 and -9 (Shyamsundar et al, 2009) have also been found. As expected from the resultant movement of cell populations, neutrophil and monocytes chemoattractants are also commonly observed following LPS inhalation. These include IL-8 (Jagielo et al, 1996; O'Grady et al, 2001; Maris et al, 2005), ENA-78 (epithelial-derived neutrophil activating peptide-78) (O'Grady et al, 2001; Maris et al, 2005), monocyte chemoattractant protein-1 (MCP-1)(O'Grady et al, 2001; Barr at el, 2010), and MIP-1α and -1β (O'Grady et al, 2001; Maris et al, 2005). Isolated studies have also looked in more depth at macrophage activation showing increased cell surface expression of HLA-DR and CD71 (Maris et al, 2005) and increased nuclear translocation of NF-κB (Shyamsundar et al, 2009).

The balance between these pro-inflammatory cytokines and the presence of possible anti-inflammatory factors has been poorly investigated in human models of ALI. Some authors have noted the presence of IL-1RA (Interleukin 1 receptor antagonist) in BALF at 24 hours following LPS instillation, but found no difference in IL-10 from baseline (O'Grady et al, 2001).

1.2.5.9 Therapeutic studies in respiratory challenge with LPS

Human respiratory models using LPS give a unique opportunity to study the effect of pharmacological intervention in the early pathological stages of ALI, providing an important therapeutic bridge to clinical intervention in ARDS. Several agents have shown biochemical promise and have led onto large therapeutic trials.

Van der Poll et al have extensively studied the activation of coagulation pathways within an LPS instillation model. Consequently, they demonstrated that pre-administration of intravenous activated protein C (ActPC) inhibited LPS-induced local activation of coagulation as reflected in thrombin-antithrombin complexes and soluble tissue factor levels in BALF (van der Poll, 2005). Conversely, however, they also demonstrated that bronchial instillation of ActPC resulted in unexpected pro-coagulant and pro-inflammatory responses following LPS challenge (Kager et al, 2013).

Salmeterol, a long acting B_2-adrenoreceptor agonist (LABA) has been shown to reduce neutrophil influx, and concentrations of MPO and TNFα, when inhaled prior to LPS in a respiratory model (Maris et al, 2005). Short-acting B_2-adrenoreceptor agonists (SABA e.g. salbutamol) initially showed potential therapeutic action in patients with ARDS (Perkins et al, 2006), however a subsequent randomised-controlled trial (RCT) of intravenous salbutamol showed increased mortality in a study of all-comers with ARDS (Gao Smith et al, 2012). These trials demonstrate the complexity of the evolving acute inflammatory process

and that mode of drug delivery as well as timing is likely to be important in therapeutic intervention in that process.

Oral corticosteroids (prednisolone) have been shown to attenuate the LPS-induced CRP rise, but had no effect on induced sputum neutrophilia or levels of pro-inflammatory cytokines (TNFα, MMP-9) (Michel et al, 2007; Michel et al 2014). McAuley's group demonstrated that deployment of statins (e.g. simvastatin) prior to LPS inhalation effectively reduced BALF neutrophilia, MPO, TNFα, MMP-7, -8 and -9 and CRP as well as plasma CRP (Shyamsundar, 2009). Similarly, levels of inflammation measured by PETCT were also attenuated by pre-treatment with lovastatin (Chen et al, 2009). However, a multicentre, RCT of simvastatin versus placebo in ARDS demonstrated that, although safe and well tolerated in this patient population, simvastatin did not improve clinical outcomes (McAuley et al, 2014).

Treatment with both high and low dose aspirin was shown to reduce BALF neutrophilia, TNFα, MMP-8 and -9 and markers of platelet activation (TXB2 – Thromboxane B2) following LPS inhalation (Hamid et al, 2017). This supports the findings of a recent meta-analysis of cohort studies examining the effect of antiplatelet therapy on mortality in ARDS. Treatment was associated with a lower incidence of ARDS in critically ill patients and reduced mortality in those who did go on to develop the disease (Wang et al, 2016).

Attempts have been made to alter recruitment of inflammatory cells to the alveolar space in early ALI. An RCT of peripheral blood mononuclear cell depletion following inhalation of LPS showed reduced total, and proportion of, mononuclear cells in BALF but no effect on neutrophil recruitment or release of inflammatory cytokines (Barr et al, 2013). Hecker et al (2015) demonstrated that pre-infusion with fish-oil based lipid emulsion reduced neutrophil recruitment, pro-inflammatory cytokines and monocytes adhesion markers following inhalation of LPS. A recent study by Proudfoot et al (2018) showed promising results using a novel anti-TNF receptor-1 (TNF-R1) domain antibody. Inhalation of the selective antagonist ameliorated post-LPS BALF neutrophilia as well IL-6, IL-8, IL-1ß, MCP1, MIP-1α and MIP-1ß.

A further approach may be to target the protective or recovery factors associated with resolution of early acute lung inflammation. In the study described above (Proudfoot et al, 2018) the selective inhibition of TNF-R1 allowed the sparing or possible potentiation of TNF-R2 activity, known to be important in the downregulation of Treg activity and tissue regeneration (Yang et al, 2018). Shyamsundar et al (2014) demonstrated that infusion of keratinocyte growth factor (KGF) prior to LPS inhalation resulted in increased BALF concentration of surfactant protein D (SP-D), IL-1RA and GM-CSF, with the biological effects of inhibiting pulmonary fibroblast proliferation and increasing alveolar epithelial proliferation.

1.2.5.10 Imaging modalities in respiratory challenge with LPS

As respiratory challenge with LPS (either by instillation or inhalation) models the early stages of ALI, little or no change is expected to be detectable on imaging with CXR. Given the lack of sensitivity and the high inter-interpreter variability in CXR reporting, early studies necessarily focussed on cellular responses in induced sputum or BALF and how this related to the clinical syndromes of ALI and ARDS. However, bronchoscopy is clearly invasive and comes with some risks, even in healthy volunteers, and BAL also results in a degree of lung inflammation in itself. BAL is therefore not an easily repeatable measure. Induced sputum does not sample the alveolar space and gives variable results depending on technique. Therefore, more recent developments in imaging modalities, particularly PETCT and MRI, have led to a renewed interest in finding non-invasive imaging biomarkers of lung inflammation within the LPS respiratory model.

Chen et al (2009) were among the first to study the use of PETCT before and after segmental challenge with LPS in an interventional study examining the effect of statins and human recombinant ActPC. Placebo-treated participants showed the greatest change in FDG uptake (calculated as the mean influx constant: Ki) post-LPS instillation, correlating with BAL neutrophilia. Those pre-treated with lovastatin showed reduced FDG uptake, which matched a small decrease in BAL total leukocyte count and neutrophilia (compared to

placebo), although this was not statistically significant. Neither BAL neutrophilia nor FDG uptake were significantly different in the ActPC arm, strengthening the case that PETCT findings matched biological markers of intervention. Barr et al (2013) undertook PETCT at 24 hours following LPS inhalation in participants subsequently undergoing mononuclear cell depletion or 'sham' leukapheresis (placebo). They demonstrated no difference in Ki between placebo and treatment arms, which matched outcomes observed in BALF neutrophils, protein and cytokines. Most recently Tregay et al (2019) were able to show the utility of single-photon emission computer tomography/CT (SPECT/CT) in assessing neutrophilic inflammation in the lung. Autologous, radiolabelled neutrophils showed differential uptake in placebo versus LPS inflammation versus COPD (chronic obstructive pulmonary disease). Changes reflected those seen in neutrophilia and IL-6 and were reproducible on repeat scanning in those with COPD.

Several authors have investigated the utility of MRI in detecting the acute pulmonary inflammation following LPS respiratory challenge, but only in animal models. In a rat model of intratracheal instillation, an initial diffuse, high intensity signal was observed within the first 48 hours corresponding to neutrophilic inflammation and proinflammatory cytokine release. Changes were still observed up to 8 days following challenge and were accompanied by a second more irregular and weak signal that the authors associated with mucus hypersecretion (Beckman et al, 2002). The strengths of the approach included the repeatability of measures within the same individual, creating a time course of pulmonary inflammation (often difficult to study by other techniques). However, the doses of LPS administered (between 0.03mg/kg – 1mg/kg) were at least 30 times those routinely administered in human LPS respiratory challenge. Ebner et al (2010) demonstrated that conventional MRI was only able to detect modest changes in signal intensity, and only after 48 hours, in a mouse model of LPS instillation using much lower doses (0.06 – 1.5µg/gbw, where bw = body weight). In this model, the authors incorporated infusion with emulsified perfluorocarbons (PFCs) which are readily phagocytosed by monocytes and macrophages and easily visible by ^{19}F-MRI. This allowed ^{19}F-MRI to detect changes at 24 hours following LPS challenge, and signal intensity strongly correlated with LPS dose.

1.2.6 Dynamic contrast-enhanced magnetic resonance imaging (DCE MRI) and its role in assessing vascular permeability

The lung is not typically assessed by MRI due to sparse proton density owing to large amounts of air and relatively small amounts of tissue. Also, the many air-tissue interfaces in lung parenchyma substantially reduce the T2 relaxation time giving rise to pronounced artefacts. Given the additional problems of respiratory and cardiac motion artefact, conventional proton-based MRI adds little in clinical practice over standard CXR of the lung (Ebner et al, 2010).

MRI can measure the magnetic property of molecules in tissues called T1. Administration of a paramagnetic contrast agent (typically gadolinium chelate) causes reduction in this property to produce a more intense 'bright' signal on T1-weighted images. Dynamic contrast-enhanced MRI (DCE MRI) involves serial T1-weighted images before, during and after intravenous administration of contrast agent. As the contrast agent is delivered through blood flow to the tissues it may stay predominantly within the plasma (vasculature) or move to the extravascular extracellular space (EES). Therefore, contrast changes the native T1 tissue properties by different degrees depending on concentration and volume of its distribution (Barnes et al, 2014). Images obtained can create a signal intensity time course, producing a 4-dimensional data set (figure 1.9).

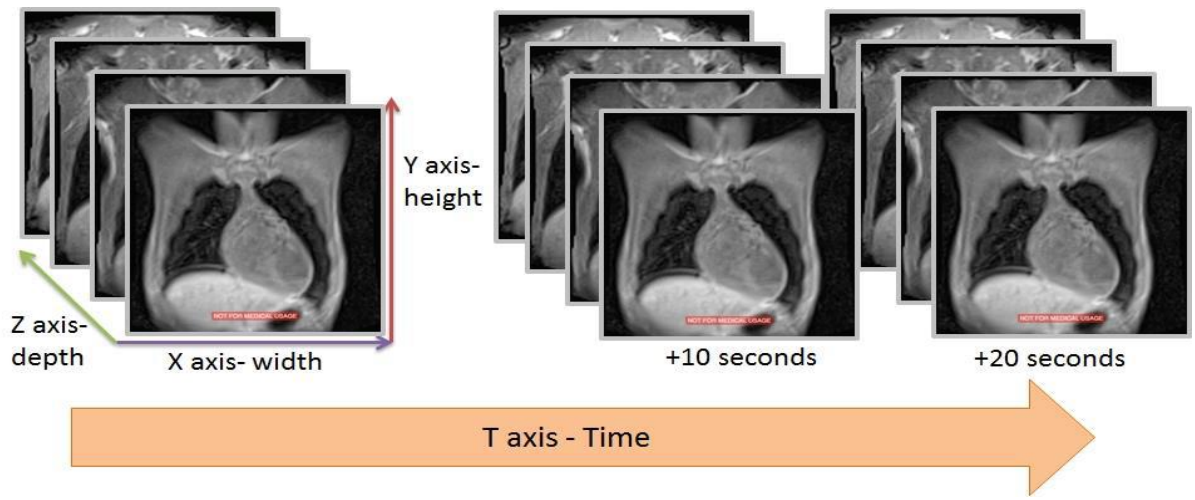


Figure 1.9 Orientation of the 4-dimensions of the dataset of DCE MRI. X axis = width, y axis = height, z axis depth, t axis = time, resulting in a new 3D data set for each time point (Reproduced with kind permission of Dr Pete Thelwall)

Applying mathematical models (such as the Extended Tofts model) to a DCE MRI data set can produce quantitative measures that relate to vascular properties and permeability. Commonly reported include the K*trans* (the volume transfer constant), which represents the movement of contrast from the vasculature into the EES (see figure 1.10) and indicates how permeable the blood vessels are to contrast agent. V*e* (the extravascular extracellular volume fraction) is the volume of the tissue signal, as a fraction that is within the EES, and V*p* (the plasma fraction) represents the volume of the tissue signal, as a fraction that is within the plasma (see figure 1.10) (Tofts et al, 1999).

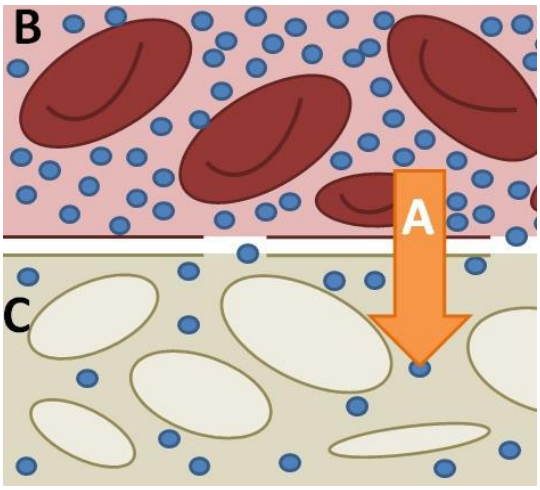


Figure 1.10 Kinetic parameters of DCE MRI. A: K*trans*, the volume transfer constant, B: V*p*, the plasma fraction, C: V*e*, the extravascular extracellular fraction (Tofts et al, 1999)

In order to undertake this modelling, 3 key entities are required. 1) an accurate baseline map of the tissue's native T1 values, 2) the rate of change over time of the concentration of contrast agent both within the tissue of interest and within a feeding artery (called the arterial input function, AIF) and 3) a pharmacokinetic model to analyse the data (usually now supplied by various computer software programmes e.g. Osirix DCE tool) (Barnes et al, 2014). The pre-contrast T1 map is created by repeatedly measuring T1 for each pixel (voxel) at different flip angles. A flip angle is a scanning parameter that changes the intensity of images produced and adjusts the sensitivity of the scanner to the contrast. For the kinetic model to be accurate the AIF input must be the same for each data set. If the curves are not the same, model results will not be representative of the true value (Heisen et al, 2010). Therefore, the dataset must have an accurate T1 map and AIF for the kinetic model to

produce accurate quantitative values that describe the vascular properties of the tissue of interest.

DCE MRI has found clinical utility in the assessment of microvascular and tissue perfusion properties in a number of cancers and inflammatory conditions. It has been used in the early detection of breast and prostate cancer and been shown to predict response to neoadjuvant chemotherapy in the former (Padhani et al, 2006). It is useful in the accurate staging and differentiation of head and neck tumours, particularly brain gliomas where it has been used to assess treatment response and predict prognosis (Zhang et al, 2017). It has been used in the assessment of vascularity of liver tumours and in therapy monitoring in renal rejection after transplantation (Khalifa et al, 2014).

The role of DCE MRI in the assessment of lung disease is just beginning to be explored. Naish et al (2008) demonstrated regional elevation in K*trans* and V*e* in current smokers with normal lung function versus non-smokers. Differences were greatest in those with more than a 20 pack-year smoking history. The authors suggested the findings on DCE MRI were able to detect the vascular endothelial permeability and early inflammation seen in smokers prior to physiological impairment. The same group demonstrated significantly different V*e* in patients with asthma compared to healthy controls. V*p* was unaffected between groups. K*trans* was not different between cohorts as a whole, however, those with more severe asthma showed elevated K*trans* when compared to mild asthma or healthy controls, implying increased vascular permeability and/or increased blood flow in more severe disease (Zhang et al, 2014).

1.2.7 Unanswered questions in ARDS and human respiratory challenge with LPS

Some of the many gaps in our understanding of both pathological ARDS in the clinical setting and models of ALI using respiratory challenge with LPS in humans have been

discussed in the literature above. Here I highlight a few pertinent points relevant to my subsequent research hypothesis, aims and objectives.

As discussed, neutrophil 'overactivation' in the alveolar space has been suggested as being partly responsible for injury to alveolar epithelium, deactivation of surfactant, promotion of inappropriate pro-inflammatory signals and the resultant extremely poor alveolar-perfusion matching. It appears likely that there is increased recruitment of neutrophils to the alveolar compartment in response to LPS-induced injury with increased expression of cytokines and neutrophil chemokines most likely released by alveolar macrophages and pulmonary alveolar epithelium. Neutrophil surface adhesion markers are upregulated on pulmonary vascular endothelium, and neutrophils migrate into the interstitium and alveolar space. What occurs at this stage is then difficult to determine. The evidence for overactivation is circumstantial, based on cytokine patterns. The few studies incorporating functional assays of these cells suggest a decrease in phagocytic capacity (suggesting impairment of key functions in this environment) although this was in response to low dose endotoxin in individuals with atopy. Further direct study of neutrophil status and function, both systemically and in the alveolar space, would be useful in the context of the human model of inhaled LPS.

There also remain significant questions regarding the role of monocytes and monocyte-like cells within the inflamed lung. This includes, the nature of subsets of monocytes migrating to the lung in response to inhaled endotoxin, their role in regulating inflammation and their ultimate fate (including the possibility of differentiation into macrophage or mature dendritic cells).

Finally, there has been little published specifically examining barrier integrity and increased permeability in response to respiratory challenge with LPS, despite this being a hallmark of inflammation seen in ARDS. Imaging techniques have rarely been applied to the LPS model and currently have a limited role in clinical cases of ARDS.

1.3 Research objectives, hypothesis and aims

1.3.1 Research objectives

Based on the information presented so far, the objectives of this project were to determine the activation status of innate immune cells (with a particular focus on neutrophils) in peripheral blood and in the lungs of humans exposed to an acute inflammatory stimulus, and to establish the usefulness of DCE MRI as a tool for assessing lung inflammation without the requirement for ionising radiation.

1.3.2 Research Hypothesis

The overarching hypothesis is that**, following inhalation of LPS, neutrophils circulating within peripheral blood increase their capacity for phagocytosis and generation of reactive oxygen species (ROS), and that DCE MRI will detect early increases in pulmonary vascular permeability.**

1.3.3 Research aims

The aims of the study were designed to test the stated hypothesis and are laid out below.

1. To set up and deliver a healthy human volunteer model of ALI using inhaled LPS and Saline.

2. To compare the function of peripheral blood neutrophils in healthy volunteers inhaling either LPS or saline, with particular emphasis on phagocytic function and generation of ROS, but also assessing cell surface expression of adhesion markers and activation.

3. To assess alveolar leak/vascular permeability using DCE MRI and establish whether there are detectable differences when comparing healthy volunteers inhaling either LPS or saline.

4. To perform a preliminary analysis of the cellular and chemical composition of BALF, comparing samples from healthy volunteers inhaling either LPS or saline.

1.4 Summary of introduction chapter

In this introductory chapter I have described the relevant scientific literature as a background to my field of research. This led to the formation of research objectives, hypothesis and aims as stated above. In the next chapter I will describe the various methods I have used during this period of study in order to answer my research questions.

2. Methods and materials

2.1 Overview of methods and materials chapter

In this chapter I will describe the methods used to conduct an LPS inhalation study and the laboratory procedures used to analyse the resulting samples and data. I will detail the study design, inclusion and exclusion criteria, preparation of LPS or saline and inhalation via the dosimeter nebuliser. The study design utilised separate study groups (see table 2.1, page 51) and a summary of what each group involved will be presented, followed by the details of each individual study intervention.

All laboratory materials will be listed. Laboratory procedures will be outlined including isolation of neutrophils by Percoll gradient and functional assays of phagocytosis and superoxide anion burst. Technique for preparation and analysis of whole blood and BALF by flow cytometry are detailed. Finally, I will report the method used for acquisition and analysis of DCE MRI in the LPS inhalation model. The statistical strategy for handling all results is discussed.

2.2 Study design

Table 2.1 summarises the original study design with the main interventions for each study group and number of study visits. The details of each study group will be outlined in more detail in section 2.2.11 (page 60).

Study Group	N	Visit 1	Visit 2		Visit 3
		t = > -1 day	t = 0 hours	t = 6 hours	t = 24 hours
Group 1	8		Saline	Blood	Blood
	8		LPS	Blood	Blood
Group 2	5	MRI			
Group 3	8	MRI	Saline	Blood + MRI + BAL	Blood
	8	MRI	LPS	Blood + MRI + BAL	Blood
Group 4	8	MRI	Saline	Blood + MRI	MRI + Blood + BAL
	8	MRI	LPS	Blood + MRI	MRI + Blood + BAL
Total	**53**				

Table 2.1 Original study design showing main interventions and number of study visits per group. n = number of study participants per group, Saline = Saline inhalation, LPS = LPS inhalation, Blood = blood sampling at 0,2,4,6 and 24 hours following inhalation, MRI = Dynamic contrast-enhanced magnetic resonance imaging scan, BAL = Bronchoscopy and bronchoalveolar lavage

2.2.1 Setting

All healthy volunteers were recruited from advertisements within Newcastle University. Volunteers were screened at a dedicated Clinical Research Facility (CRF) at the Royal Victoria Infirmary (RVI) Newcastle upon Tyne Hospitals NHS Foundation Trust. If found to be eligible for recruitment, participants were given time to consider (minimum 24 hours) and invited to attend on a set day. Participants allocated to study group 1 attended study visits at the CRF, RVI. Specialist MRI scanning took place on the Siemens Espree 1.5T scanner at the Freeman

Hospital (FH) also Newcastle Upon Tyne Hospitals NHS Foundation Trust. Therefore, participants in groups 2-4 attended study visits at the dedicated Sir Bobby Robson Clinical Trials Unit (CTU) at FH. Bronchoscopy was located within the dedicated endoscopy suite within the same building at FH. LPS/Saline for inhalation was prepared within a sterile fume cupboard within the Simpson lab (Institute of Cellular Medicine [ICM], Newcastle University) or within the Institute of Transplantation lab, in FH. Processing of screening bloods and study full blood counts (FBCs) were undertaken in Newcastle upon Tyne Hospitals clinical labs. Further analysis of blood and BALF samples generated by the study were processed within the Simpson lab and flow cytometry facilities (ICM and Centre for Life, both Newcastle University). For analysis of DCE MRI, images were transferred to software within the Newcastle Magnetic Resonance Centre (NMRC, Newcastle University).

2.2.2 Sponsorship and funding

The study was jointly funded by the Joint Research Executive Scientific Committee (JRE, which oversees the Newcastle Health Care Charity and Newcastle Upon Tyne Hospital NHS Charity) and the National Institute for Health Research (NIHR) Newcastle Biomedical Research Centre based at Newcastle upon Tyne Hospitals NHS Foundation Trust and Newcastle University. The study was sponsored by the Newcastle Upon Tyne Hospitals NHS Foundation Trust.

2.2.3 Ethical approval

Prior to study commencement ethical approval was gained in May 2012 from NRES committee North East – County Durham and Tees Valley, REC Ref number: 12/NE/0196. A substantial amendment was approved in March 2013 to accommodate increased volume of blood sampling at time points 2 and 4 hours, and reduced observation period following bronchoscopy. A minor amendment was approved in February 2015 to extend the study completion date to December 2015.

2.2.4 Study population

Volunteers for all study groups were recruited using the same inclusion and exclusion criteria. The study had broad inclusion criteria. However, a comprehensive list of exclusion criteria was incorporated to ensure that subjects had no history of prior respiratory disease, were able to safely undertake MRI scanning and bronchoscopy, and to minimise risk to volunteers of LPS inhalation. Exclusion criteria were in keeping with previous LPS inhalation studies (Michel et al, 1997; Barr et al, 2013).

2.2.4.1 Inclusion criteria

- Healthy adult volunteers between 18 and 40 years of age.
- Able to give informed consent.

2.2.4.2 Exclusion criteria

- Age <18 or >40 years.
- Past history of chronic respiratory disease (e.g. asthma, COPD, bronchiectasis, tuberculosis).
- Past or current history of conditions known to affect immunity or cardiac function (e.g. diabetes, ischaemic heart disease, congenital heart disease, valvular heart disease, chronic renal impairment, recurrent urinary tract infection).
- History of an acute intercurrent cardiorespiratory illness (with particular reference to upper and lower respiratory tract infection).
- Any current medication (excepting oral contraceptive pill).
- Current history of smoking.
- Past smoking history amounting to >2 pack-years.
- Any history of smoking in the last 12 months.
- Reported alcohol intake >21 units per week.
- Pregnant or lactating women.

- Abnormal physical signs detected at cardiorespiratory examination.
- Temperature >37.3 ℃
- Oxygen saturation <95% breathing room air.
- Haemoglobin outside the laboratory reference range.
- Platelet count less than 100 $x10^9$/L.
- Total white cell count and neutrophil count outside the laboratory reference range.
- Blood sodium, potassium, creatinine or alanine aminotransferase outside the laboratory reference range.
- Blood urea greater than 10mg/dL.
- Bilirubin greater than 30umol/L.
- Forced expiratory volume in one second (FEV1) or forced vital capacity (FVC) <80% predicted.
- FEV1:FVC ratio <0.7.
- Standard exclusion to undergoing MRI scanning (cardiac pacemakers, cochlear implants, aneurysm clips, intra-ocular metallic foreign bodies, prior history of allergic reaction to contrast agent, [in those undergoing MRI scanning]).
- History of significant claustrophobia (in those undergoing MRI scanning).

2.2.5 Identifying potential study participants

Study adverts were placed on public notice boards within Newcastle University. The adverts were also emailed to all students on distribution lists for the medical, dental and biomedical sciences schools. Volunteers were asked to contact the study team if they considered themselves to be healthy and were interested in taking part. At this first contact, volunteers were sent a copy of the participant information sheet (PIS) for the study group open for recruitment, and a copy of the screening visit consent form (see appendices B, C and D). Volunteers were asked to re-contact the study team if they wished to proceed in order to arrange a screening visit, or to decline participation.

2.2.6 Screening visit

Volunteers were screened on the basis of the inclusion and exclusion criteria stated above. Screening tests were specifically designed to identify any risk for adverse events associated with study interventions. Particularly, LPS inhalation followed by bronchoscopy is known to be safe in healthy volunteers in the absence of pre-existing lung disease and normal physiological examination (Sandstrom et al, 1992). Furthermore, MRI is considered extremely safe providing volunteers have undergone routine MRI contraindication screening.

After the volunteer received the PIS and screening visit consent form, a date and time for the screening visit was arranged after an interval of at least 24 hours. Due to the number of assessments and the need for taking blood, volunteers were asked to sign a separate consent form for the screening visit, which was completed prior to any interventions. I undertook all screening visits for study entry, each taking approximately 1 hour to complete. The assessment included:

- a short medical history
- vital signs measurement (temperature, pulse, blood pressure, respiratory rate)
- measurement of oxygen saturation breathing room air
- cardiorespiratory examination
- blood sample for full blood count
- blood sample for urea & electrolytes assay and liver function tests
- spirometry
- urinary pregnancy test in women
- standard questionnaire for contraindications to MRI scanning
- 'practice inhalation' of saline

'Practice inhalation' with sterile saline using the dosimeter nebuliser was undertaken to ensure all volunteers understood the inhalation procedure and had the chance to ask questions. Identical equipment and protocols were used for practice inhalation at screening and in study visits (see section 2.2.14, page 68).

The study adverts did not mention any payment for study involvement. There was no payment for completion of the screening visit. If participants progressed to study entry, they were paid £250 as a token of reimbursement for time and inconvenience. This was the same for all study inhalation groups (groups 1, 3 and 4). Group 2 (single MRI scan) were offered £50.

2.2.7 Ineligible and non-recruited volunteers

In volunteers found to be ineligible at screening, or eligible but not wishing to proceed, the reason for ineligibility (or non-recruitment) was kept on a screening log. Only anonymised information was entered with a screening number linked to gender, age and the reason for ineligibility or non-recruitment.

If ineligible, volunteers were contacted with the reason for screen failure. If the reason for ineligibility was felt to be 'reversible' and transient, the protocol allowed for a targeted re-screening visit within 30 days and volunteers were given this option. The outcome of repeat screening was kept in the screening log.

If clinically relevant results were identified as part of the screening visit, this was discussed with the volunteer and permission sought to contact their general practitioner (GP) with the results and any further recommended action.

2.2.8 Randomisation and blinding

Plain white cards were marked with either 'LPS' or 'Saline' and placed in identical plain white, opaque envelopes. There were equal numbers of LPS and Saline cards for each study group involving inhalation, to allow 1:1 randomisation. Prior to study visit 1, an administrative member of the research team drew out a single envelope, recorded the allocation on the study log against the participant number, and informed a single member of the research team (Dr Jim MacFarlane or Dr Emma Browne) to prepare either LPS or saline for inhalation as per the protocol.

All study participants were 'blinded' to study inhalation. For study group 1, I was also blinded to inhalation. This was to avoid bias in recording of clinical parameters during study visits and in the analysis of phagocytosis of blood neutrophils. Therefore, another member of the study team (named above) prepared LPS or Saline for inhalation the evening prior to inhalation visit and checked the automated cell count results at the end of each study visit.

At the end of study group 1, once phagocytosis of zymosan had been analysed, results were unblinded and FBC results examined for the group. Unfortunately, this demonstrated that several participants randomised to receive LPS had not shown the expected change in peripheral blood neutrophils or total white cell count (WCC). After examining the clinical record for symptoms (these participants also reported no symptoms expected in relation to LPS) it was concluded that the LPS had not been adequately delivered to the airway in some participants. After discussion with other research groups experienced in LPS inhalation studies the protocol for randomisation was continued but blinding was changed.

For subsequent inhalation study groups (3 and 4), I was unblinded. I made up the LPS and Saline just prior to inhalation and kept it in constant suspension. I also examined the FBC results at the end of each participant's involvement to ensure the expected change in blood neutrophils and total WCC were observed. This allowed any further problems to be picked up immediately and resulted in no further loss of data. Nursing staff at the CRF/CTU kindly undertook all observations and recorded information on symptoms experienced by

volunteers. Phagocytosis plates were labelled with participant number only and batch counted by me at the end of each study group, but also by a second 'blinded' investigator to ensure the robust assessment of one of the key outcome measures.

2.2.9 Preparation of LPS or Saline for inhalation

2.2.9.1 LPS for inhalation

The dosimeter nebuliser delivers a highly accurate volume of fluid for inhalation within a closed system. For our LPS model the desired dose for inhalation was 60µg over 5 inhalations. This dose was selected in order to reliably elicit a reproducible inflammatory response within the systemic circulation and lung compartments whilst minimising risk of adverse events to healthy volunteers (Brittan et al, 2012). The settings of the dosimeter were calibrated to deliver a volume of 9.6µl per inhalation. However, the nebuliser pot requires a minimum of 1.5mls volume of fluid to allow the end of the tubing to be fully submerged in solution for nebulisation. The following calculations were made:

60µg in 5 inhalations = 9.6µl x 5 = 60µgin 48µl (1.25µg/µl)

1.25x 1600 = 2000µg in 1600µl = 2mg in 1.6mls

LPS for inhalation was sourced from 1mg vials of lyophilised lipopolysaccharide from *Escherichia coli* 026:B6 (L2654, Sigma-Aldrich, Poole, UK). Preparation of solution was undertaken in a sterile fume cupboard in a lab separate to the CRF. 0.8ml of endotoxin-free sterile saline was added to a 1mg vial of LPS 026:B6. The lid was replaced on the vial and carefully agitated for 2 minutes to fully dissolve the LPS. The contents of the vial were transferred using a pipette to a sterile, 2ml plastic tube (Eppendorf) labelled with the participant study number. This procedure was repeated for a second vial, giving 2mg LPS in 1.6mls endotoxin-free saline. Since LPS dissolves fully in saline, the fluid appeared as a clear, colourless liquid (the same as sterile saline alone). The tube containing LPS solution was removed from the fume cupboard and mixed using a vortex machine for 30 seconds. It was placed on ice and transferred to the CRF for inhalation. Five minutes prior to inhalation the

solution was removed from ice to warm to room temperature and the fluid was mixed again by vortex for 30 seconds to encourage even suspension.

Following preparation of LPS for inhalation, both empty vials of LPS were labelled with the participant number and study visit date and stored in case of adverse reaction.

2.2.9.2 Sterile saline for inhalation

Preparation of saline was undertaken in a sterile fume cupboard in a lab separate to the CRF. 1.6ml of endotoxin-free sterile 0.9% saline was transferred into a sterile 2ml plastic tube (Eppendorf) labelled with the participant's study number. The tube containing saline solution was removed from the fume cupboard and placed on ice to transfer to the CRF for inhalation. Five minutes prior to inhalation, the solution was removed from ice to warm to room temperature and the fluid was mixed by vortex for 30 seconds to ensure blinding of participants.

As discussed above, for study group 1, the initial preparation of LPS or sterile saline took place the evening prior to inhalation. The samples were stored on ice in a -5°C fridge. Samples were removed from ice 5 minutes prior to inhalation and mixed via vortex. In all subsequent study groups undergoing inhalation (groups 3 and 4) the LPS and saline were made up on the morning of inhalation and delivered via nebulised dosimeter to the participants within 30 minutes.

2.2.10 Consent for study enrolment

All participants were adult volunteers and able to give informed consent. All volunteers were given written information on study involvement and a copy of the study consent form

a minimum of 24 hours prior to enrolment (see appendix C and D). All relevant study interventions were also discussed during the screening visit with participants given time to consider and ask any questions. Consent for study enrolment was taken at the beginning of study visit 1, prior to commencing any study interventions.

2.2.11 Summary of study visits

2.2.11.1 Study group 1

Study group 1 participants underwent 2 study visits. At visit 1 (inhalation visit), volunteers attended the CRF at 8am on a planned date. Informed consent for study entry was taken. A brief history regarding illness symptoms, exposure to upper and lower respiratory tract infections, any changes to medical history, and new medications (including those over-the-counter) was taken. Cardio-respiratory examination and physiological observations (temperature, pulse rate and rhythm, blood pressure, oxygen saturations on room air and respiratory rate) were recorded. Spirometry was performed before and after 'practice' inhalation with sterile saline. A 20-gauge (pink) cannula was placed in a vein and 35mls of blood drawn. These assessments (prior to inhalation) were termed 'baseline'.

The relevant inhalation solution was removed from ice and allowed to warm to room temperature for 5 minutes. Participants received either 60µg LPS or sterile saline (control) over five inhalations, using an automatic inhalation-synchronised dosimeter nebuliser (see section 2.2.14, page 68). Inhalation took place at approximately 9am, with time of inhalation indicating time 0 (t=0) for all subsequent study assessments.

Following inhalation participants were monitored continuously for 6 hours by clinical staff (myself and research nurses). Emergency equipment was always available in case of severe reaction to LPS, along with simple symptomatic treatments such as nebulised bronchodilators and oral paracetamol should they have been required. Observations were taken and recorded every hour until 6 hours and volunteers could discuss symptoms with

the study team at any point. Further blood samples of 15mls were taken at 2 hours and 4 hours post inhalation from the same cannula placed at baseline. At 6 hours, participants were specifically asked about symptoms, observations were recorded, and cardiorespiratory examination was undertaken. Spirometry was repeated. If FEV1 or FVC had fallen by more than 10% from baseline the participant was required to stay for further observation and/or treatment. Thirty-five milliliters of blood were taken from the same cannula before it was removed.

Volunteers were given a 24-hour contact telephone number to contact me, if they felt unwell through the evening or overnight, and allowed home from the CRF. In the event that a participant did not feel well they were offered a return visit to the CRF and were assessed by me with notification to Professor Simpson, if required. All necessary equipment for further observation and simple symptomatic relief was available. If a participant wished to stay at home, I arranged further phone call assessments as required until the participant felt back to full health. All adverse events were recorded and actioned (see section 2.2.19, page 75).

Study visit 2 took place at 8.30am the following morning in the CRF. A brief history regarding symptoms, cardiorespiratory examination findings and observations were recorded. Spirometry was repeated and compared to baseline. A final blood sample of 35mls was taken (t=24 hours) via peripheral venepuncture with a 21-gauge (green) needle. If spirometry results were within 10% of baseline and if observations and symptoms were found to be satisfactory the study visit was considered complete.

Participants were telephoned later the same day (approximately 36 hours following inhalation) to ensure they remained well and that any symptoms present at study visit 2 had resolved. This concluded the participant's study involvement. Information regarding study involvement and any adverse events were communicated to the participant's GP as per the consent procedure. Participants were advised to keep my telephone number and email address should they have any further questions or concerns.

2.2.11.2 Study group 2

Participants in study group 2 underwent a single study visit. Volunteers meeting the same entry criteria and screening requirements attended the CTU on a planned date at approximately 2pm. The consent form for study entry was signed. The same baseline assessments as for study group 1, visit 1 were undertaken, i.e. a brief history, cardio-respiratory examination, physiological observations and spirometry. A 20-gauge (pink) cannula was placed but no blood samples taken. A repeat screening questionnaire for MRI contraindications was completed.

Participants were accompanied to the research scanner at FH to undergo a single MRI scan. Data from these participants were used to optimise the MRI scanning protocol for subsequent study groups. Pilot scans were acquired to confirm correct volunteer positioning, followed by non-invasive perfusion and respiratory motion measurements. DCE imaging was then performed using intravenous administration of MRI contrast agent (Gadoteric acid, Dotarem) at doses between 0.1mls/kg - 0.2mls/kg actual body weight. Scanning took place over approximately 1 hour with the study team in contact with the volunteer throughout. All symptoms and adverse events in relation to MRI scanning were recorded and actioned. Provided the participant remained well at the end of scanning, the cannula was removed, and this was considered the end of study involvement. Participants were given my contact telephone number and email address should they experience symptoms or have further questions.

2.2.11.3 Study group 3

Participants in study group 3 underwent 3 study visits. The first study visit was to complete 'baseline' assessments and undergo the 'baseline' DCE MRI scan. This visit therefore took exactly the same format as the single study visit for group 2 (see section 2.2.11.2, above). I.e. Informed consent, history, examination, observations, spirometry, cannula (no blood

sampling) and single DCE MRI scan. The first study visit usually took place 5 days prior to study visit 2 (inhalation) but could take place anytime between 1 and 21 days prior to inhalation visit.

Study visit 2 was the inhalation visit for this group. Participants attended the CTU at 8am on a planned date. The first 6 hours of this study visit were exactly the same as the 'inhalation visit' for those in study group 1. I.e. History, examination, observations, spirometry, practice inhalation with sterile saline, repeat spirometry, cannula and baseline 35mls blood sampling. LPS or Saline inhalation was completed according to the same protocol (see section 2.2.14, page 68) at approximately 9am (time 0). Further hourly observations, blood sampling at 2, 4 and 6 hours, history, examination and spirometry were completed, up to and including the 6-hour time point. Participants were fasted from 12pm (noon) in preparation for bronchoscopy (minimum 4 hours). If spirometry (FEV1 or FVC) had fallen by greater than 10% from baseline all subsequent study interventions (MRI and bronchoscopy) were not performed.

If the participant remained well and met safety criteria, they were accompanied to the MRI scanner. They underwent their second DCE MRI scan, post-inhalation, using the same protocol as for baseline scanning (see section 2.2.15, page 72). Following completion of the MRI scan, the participant was accompanied to the Endoscopy Suite for bronchoscopy. They were checked in by nursing staff according to usual clinical practice and repeat observations were taken. All participants were given supplementary oxygen and had continuous monitoring of heart rate, oxygen saturations and ECG (electrocardiogram) throughout. Local anaesthetic throat spray was applied to the airway and used to denote start time of bronchoscopy. No participants requested sedation for the test (all were offered). Participants underwent research bronchoscopy and BAL as per protocol (see section 2.2.16, page 74).

Study participants were monitored for 30-60 minutes following completion of bronchoscopy. Observations were taken immediately at the end of the procedure and at 30

minutes afterwards. Clinical examination was repeated. If the participant was well, with satisfactory observations/examination, the study visit was concluded and they were allowed home. Participants were given written information on the time they were able to commence eating and drinking (2 hours following administration of throat spray) as well as my 24-hour contact telephone number if they felt unwell overnight. All adverse events were recorded and actioned.

The third study visit for group 3 took place at 8.30 am the next day at the CTU. A brief history regarding symptoms, cardiorespiratory examination and observations were recorded. Participants often reported symptoms in the evening/overnight which were considered mild, and typical following routine bronchoscopy. Spirometry was repeated and compared to baseline. The final blood sample of 35mls was taken (t=24 hours). If spirometry values were within 10% of baseline, and if observations and symptoms were found to be satisfactory the study visit was considered complete.

Participants were telephoned later the same day (approximately 36 hours) to ensure they remained well and that any symptoms present at study visit 3 had resolved. This concluded study involvement. Information regarding study involvement and any adverse events were communicated to the GP at the end of study involvement. Once again, participants were advised to keep my contact telephone number and email address should they have any further questions or concerns.

2.2.11.4 Study group 4

The original study had planned for study group 4 to undergo 3 DCE MRI scans and to move the correlation time point with bronchoscopy/BAL to 24 hours following inhalation. An interim analysis of MRI results at the end of study group 3 was planned and completed prior to commencing study group 4. Full analysis, including attempts at kinetic modelling and review of the raw data, demonstrated no detectable difference in MRI scans between

subjects inhaling LPS versus saline (see results chapter section 3.3.6, page 123). Since this time point would be expected to show the greatest inflammatory response to LPS inhalation and there was no signal change present, it was felt unlikely that further study at 24 hours post inhalation would yield positive results. Therefore, the research team made the decision not to pursue further DCE MRI within the LPS model.

However, interesting results were being explored within the blood and BAL samples and therefore the study group was changed to complete the planned numbers undergoing blood and BAL samples up to 6 hours post-inhalation (see table 2.2, next page)

Therefore, participants in study group 4 underwent 2 study visits. The first was their 'inhalation visit' and the first 6 hours was the same as all other 'inhalation' study visits. Informed consent was taken, followed by baseline assessments (history, examination, observations, spirometry, practice inhalation with sterile saline, repeat spirometry, insertion of cannula and baseline blood sampling). Participants underwent inhalation of either LPS or saline as per the protocol. Hourly observations and blood sampling at 2, 4 and 6 hours were performed followed by history, examination and spirometry at 6 hours. Providing the participant was well and met safety criteria, they were accompanied to bronchoscopy. Participants underwent the same monitoring, observations and protocol for research bronchoscopy and BAL as participants in study group 3. Study participants were monitored for 30-60 minutes following bronchoscopy and discharged if they remained well, with the contact telephone number and written information on when to commence oral intake.

Study Group	N	Visit 1	Visit 2		Visit 3
		t = > -1 day	t = 0 hours	t = 6 hours	t = 24 hours
Group 1	8		Saline	Blood	Blood
	8		LPS	Blood	Blood
Group 2	5	MRI			
Group 3	8	MRI	Saline	Blood + MRI + BAL	Blood
	8	MRI	LPS	Blood + MRI + BAL	Blood
Group 4	**6**		**Saline**	**Blood + BAL**	**Blood**
	6		**LPS**	**Blood + BAL**	**Blood**
Total	**49**				

Table 2.2 Final study design showing main interventions and number of study visits per group. n = number of participants per study group, Saline = Saline inhalation, LPS = LPS inhalation, Blood = blood sampling at 0,2,4,6 and 24 hours following inhalation, MRI = Dynamic contrast-enhanced magnetic resonance imaging scan, BAL = Bronchoscopy and bronchoalveolar lavage. Changes to the original study design are highlighted in red.

The second study visit for group 4 took place at 8.30 am the next day and followed the same procedures as the final visit for all study participants (i.e. history, examination, observations, spirometry, t=24-hour blood sampling). If spirometry values were within 10% of baseline, and if observations and symptoms were felt to be satisfactory the study visit was considered complete.

Participants were telephoned later the same day (approximately 36 hours) to ensure they remained well and that any symptoms present at study visit 2 had resolved. This concluded study involvement and their GP was informed. Once again, participants were advised to keep my contact telephone number and email address should they have any further questions or concerns.

2.2.12 Blood sample collection and intravenous (IV) access

As discussed above, all participants in study groups 1, 3 and 4 underwent blood sample collection at baseline, 2 hours, 4 hours, 6 hours and 24 hours following inhalation. All participants were asked to drink a glass of water and to recline on a couch to reduce risk of vasovagal syncope prior to all blood sampling. Sterile, non-touch technique was used throughout. At baseline, a 20-guage (pink) cannula was placed into a large vein, usually in the antecubital fossa of the participant's non-dominant arm. Thirty-five millilitres of blood was immediately, gently drawn using a syringe for testing. The cannula was secured with an opsite dressing (unless allergic, when surgical tape was used). The cannula was not flushed but kept patent for subsequent blood sampling by use of a sterile stylet.

At 2- and 4-hours following inhalation, the stylet was removed and a syringe used to gently draw back 15mls of blood. A new stylet was placed into the cannula. At 6 hours 35mls were drawn. In the event that blood could not be drawn from the cannula the protocol allowed separate peripheral venepuncture providing the participant was happy with this at the time. This was rarely needed (only 2 separate occasions).

For those participants in study groups 3 and 4 who went on to complete an MRI scan and/or bronchoscopy, the cannula placed at baseline was used for IV administration of contrast agent at MRI and for safety during bronchoscopy. No further blood samples were taken via the cannula following administration of intravenous contrast. The cannula was removed, and a simple dressing applied at the end of the 'inhalation visit'.

Blood samples taken at 24 hours were taken via a separate venepuncture of a large vein (usually antecubital fossa of dominant arm) using a 21-gauge (green) needle and syringe to draw a final 35mls. Again, the participants were semi-recumbent and given a drink of water before and after blood sampling. A simple dressing was applied. In total, 135mls of blood was taken over all timepoints.

2.2.13 Measurement of spirometry

At the screening visit the participant's height, age, sex and ethnicity were recorded. The combined European Respiratory Society (ERS)/British Thoracic Society (BTS) predicted lung function calculator was used to predict the patients' expected FEV1 and FVC. Spirometry was undertaken using a Vitalograph volumetric spirometer 2150. Participants were asked to stand, wear a nose clip and form a tight seal around the mouthpiece. Participants underwent tidal breathing to get used to the mouthpiece. They were then asked to complete full expiration, followed by full inspiration and forced and full expiration ('blowing as hard and fast' as they could). I undertook a demonstration if necessary, to help with optimal technique. FEV1 and FVC were read from the resultant traces. The recorded measurement was the best of a minimum of 3 attempts at the full manoeuvre. Any member of the research team supervising study visits was trained in undertaking spirometry. Acceptable quality standards of spirometry were in keeping with published guidelines (Miller et al, 2005) particularly with respect to maximal inspiration and expiration, no coughs or glottis closure and meeting end-of-test criteria (exhaling for ≥6 seconds, with <50 mL being exhaled in the last 2 seconds). For reproducibility criteria, the best 2 results for FEV1 and FVC had to be within 5% or 150mls of each other.

Participants had a chance to 'practice' spirometry at the screening visit to ensure they were able to reproducibly undertake measurements prior to study enrolment. Spirometry was then measured at 'baseline', following practice inhalation with sterile saline, and at 6 hours and 24 hours following inhalation. It was also repeated if the participant complained of any respiratory symptoms following inhalation, MRI or bronchoscopy. A fall of greater than 10% from the participant's baseline was considered significant and warranted further action/assessment.

2.2.14 Inhalation of LPS or Saline by dosimeter nebuliser

Inhalation of either LPS or sterile saline (control) took place using a Spira automatic inhalation-synchronised dosimeter nebuliser (Spira, Hameenlinna, Finland, 08TSM202, see

figure 2.1). The dosimeter was connected to a power supply and driven by a compressed air supply (size E medical gas air cylinder) fitted with a customised pressure regulator set at 2 bar (30psi). The air supply was connected to the dosimeter via the input pressure tube. A flow meter was connected to the dosimeter to guide controlled inspiration. The nebuliser onset was set at 50mls with nebuliser duration of 0.6 seconds.

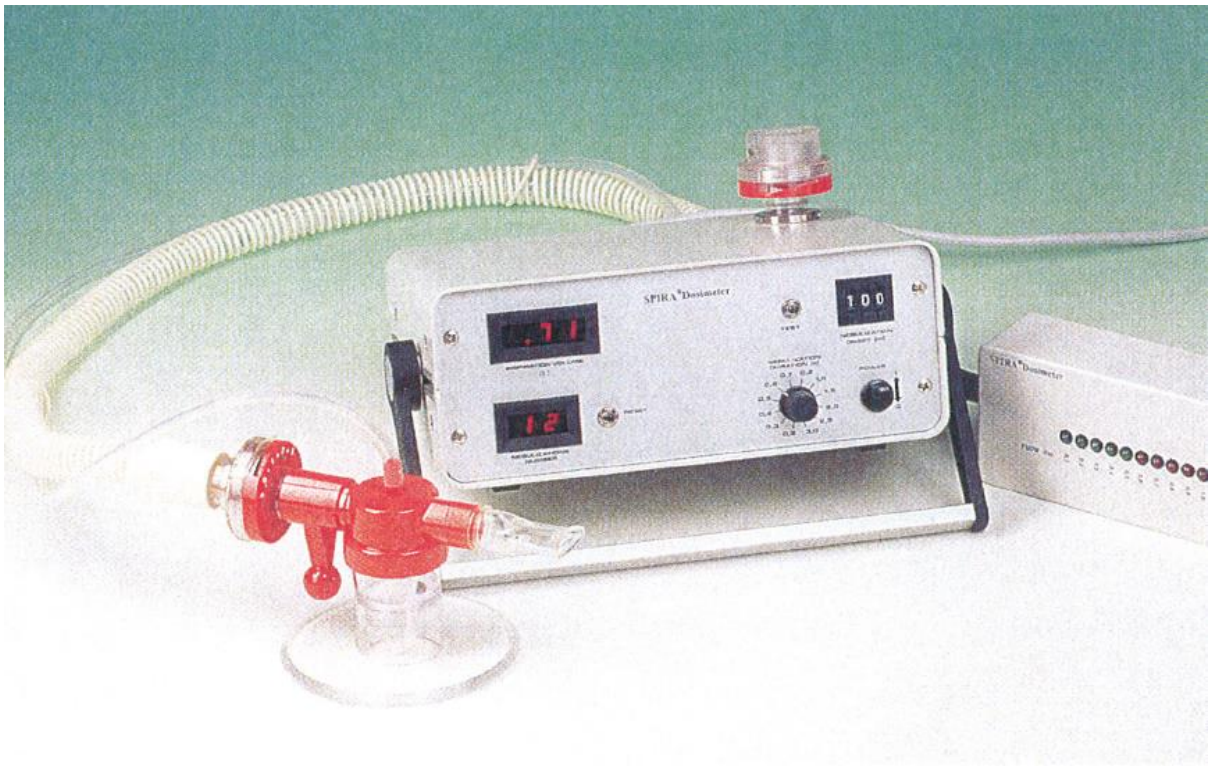


Figure 2.1 Spira dosimeter nebuliser, with nebuliser equipment, flow meter and connection to power supply.

The nebuliser equipment was set up as shown in figure 2.2 The participant tube and the nebuliser pressure tube were connected to the back of the dosimeter. All nebuliser equipment was sterilised between each participant's use and only opened a few moments prior to inhalation. 'Practice' inhalation was performed with sterile saline prior to study inhalation (saline or LPS). The nebuliser mouthpiece and chamber were substituted with sterilised parts just prior to study inhalation. Parts were replaced as needed at any sign of wear.

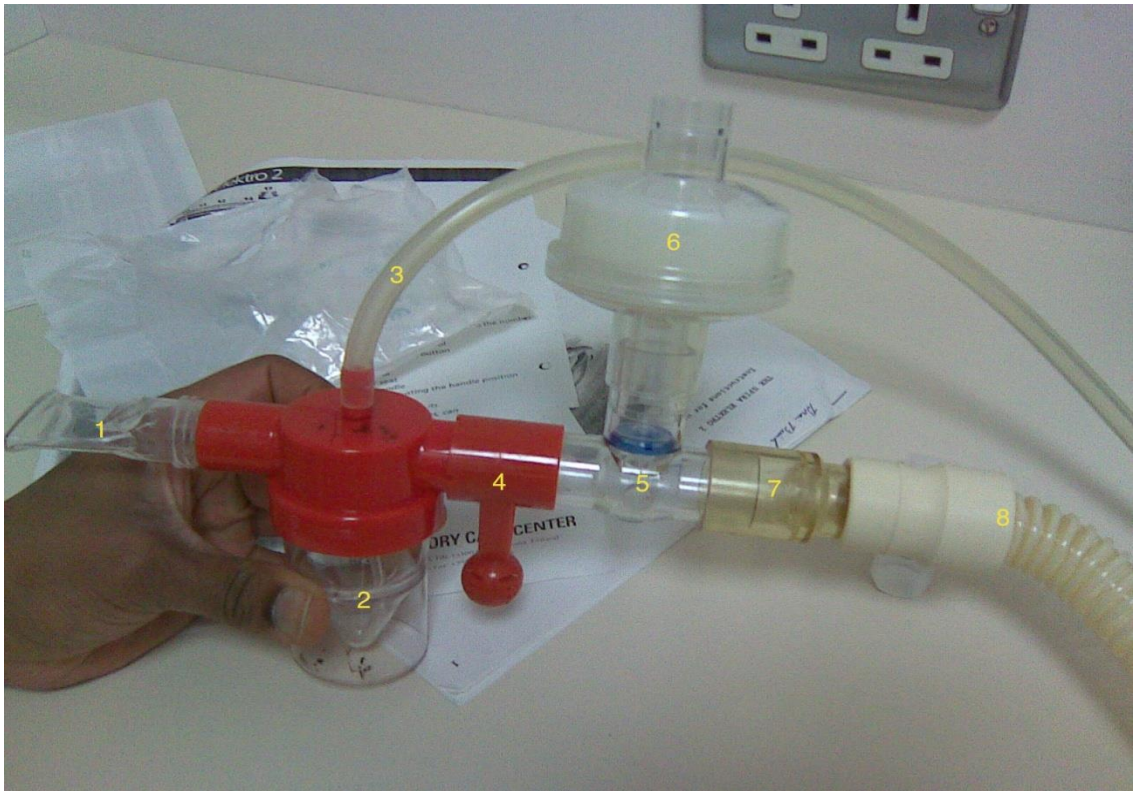


Figure 2.2. Set up of nebuliser equipment. 1 = mouthpiece, 2 = nebuliser chamber, 3 = nebuliser pressure tube, 4 = holder arm, 5 = 'T' piece (exhalation valve), 6 = single-use filter, 7 = tube connector, 8 = participant tube (adapted from L.Barr, monocytes working document 4.0, 2010)

1.6mls of sterile, endotoxin-free saline or 2mg of LPS suspended in 1.6mls sterile saline was emptied into the nebuliser pot. Participants were asked to use a nose clip and then breathe out to full expiration (residual volume). When ready, they placed the nebuliser mouthpiece just beyond their teeth and used their lips to create a tight seal around the mouthpiece. The participant then took a 'slow and steady' breath-in up to full inspiration (vital capacity) triggering automatic nebulisation of study solution. The flow meter was used to guide inspiration to ensure adequate triggering, with the participant asked 'to light up all 5 green lights and none of the red lights'. At maximum inspiration, the participant was asked to hold their breath while the researcher counted 5 seconds (to allow alveolar deposition). The participant was then instructed to breathe out until completely empty (residual volume). The procedure for inhalation was repeated to a total of 5 times without removing the mouthpiece. The dosimeter counter recorded the number of triggered nebulisation events. The volunteer could then remove the mouthpiece and breathe normally.

2.2.14.1 Cleaning the dosimeter nebuliser equipment

Separate sets of nebuliser equipment (numbers 1-8, figure 2.2, page 70) were used for LPS inhalation, saline inhalation and screening/calibration/practice inhalation. There was no cross-over between sets of equipment. Once inhalation was complete, excess liquid from the nebuliser chamber was emptied onto paper towels and wiped clean. All nebuliser equipment (except the single use bacterial filter) was then placed within an orange clinical waste bag and sealed. The bacterial filter was discarded directly into a clinical waste bin.

To sterilise equipment between use by participants, and to remove all traces of LPS, the same sterilisation technique was used for all kits. Milton fluid (<5%, Milton, UK, 23571) was diluted in deionised water at 50mls/L. All nebuliser equipment was submerged in the cleaning solution for a minimum of 15 minutes (up to 1 hour) along with a plastic container. The nebuliser equipment and container were then rinsed in deionised water to remove excess cleaning solution and moved to a sterile hood to dry. Once dry and whilst still within the sterile hood, the nebuliser equipment was placed within the container and sealed. The container was then removed from the hood and stored until next use.

2.2.14.2 Calibration of the dosimeter nebuliser

The dosimeter nebuliser was calibrated to deliver 9.6µl of fluid from the nebuliser chamber per inhalation. The nebuliser equipment was assembled, connected to the dosimeter, power supply and air cylinder with the same settings as for all inhalations (see section 2.2.14, page 68).

The nebuliser pot was filled with 1.6mls sterile saline and weighed (with lid but no further attachments, scales accurate to within 0.5mg). I followed the same procedure for inhalation as in section 2.2.14 but with the important exception that the mouthpiece was removed for each exhalation to prevent water droplets re-entering the nebuliser pot and altering weight measurement. The difference in weight before and after 10 inhalations was calculated. The

expected weight difference was 96mg (9.6µl x 10 inhalations). The nebuliser duration was adjusted, if necessary, until 96mg ± 10% weight loss was achieved. Calibration was repeated following every 20 uses.

2.2.15 DCE MRI scans

All MRI scans took place on the Siemens Espree 1.5T MRI scanner, FH. Volunteers arrived at the MRI scanner with IV access in place. Participants were checked into the MRI department and MRI safety checks were completed as per usual clinical practice (i.e. safety checks to undergo MRI were done in triplicate; at screening, at each study visit prior to attending the MRI department, and just prior to entering the MRI scan room).

The participant was positioned on the scanner bed, with arms raised above the head and a surface coil over the thorax. The participant and the centre of the surface coil (B0/B1) were positioned in line with the centre of bed coils S2 and S3 and the volunteer moved to ensure the whole thorax was covered (bottom of the neck to bottom of the ribs, see figures 2.3 and figures 2.4).

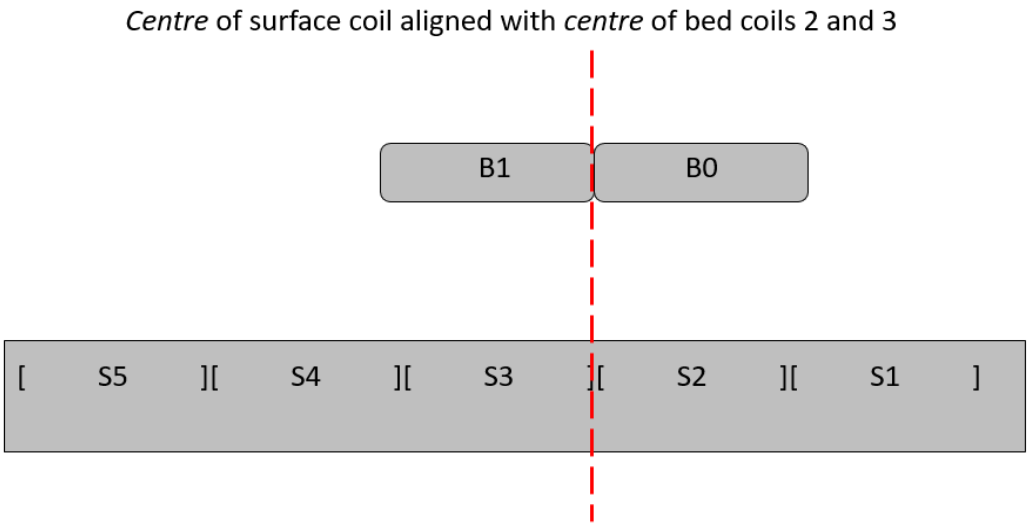


Figure 2.3 Alignment of surface coil with bed coils for DCE MRI scan

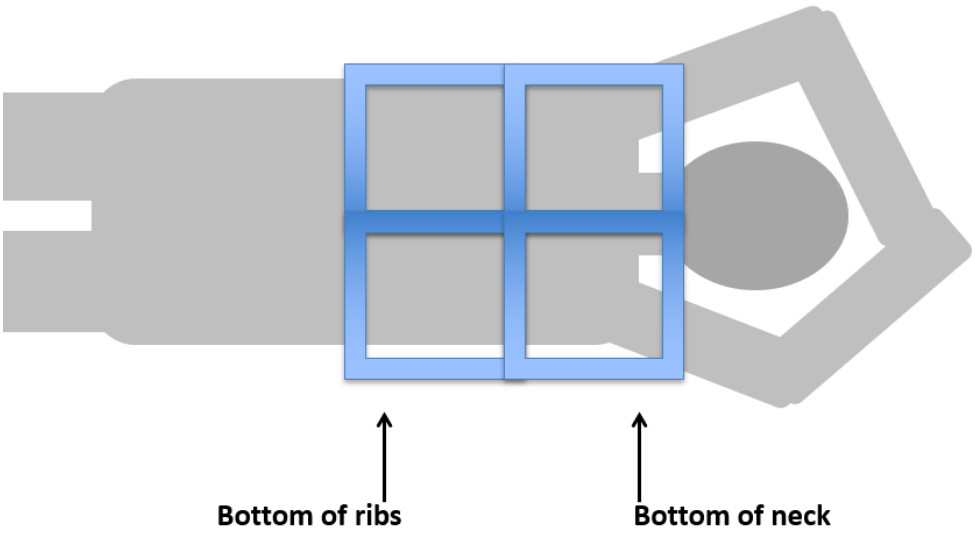


Figure 2.4 Position of surface coil to cover whole thorax for DCE MRI scan

The LPS study scan protocol was loaded and a localiser scan was performed to confirm the volunteer's position. Auto-coil select and coils S1 and S4 were disabled. The field of view (FOV) was positioned centrally in relation to coils S2 and S3 and angled along the descending aorta. This resulted in image capture for majority of the lung parenchyma, heart and descending aorta (see figure 2.5)

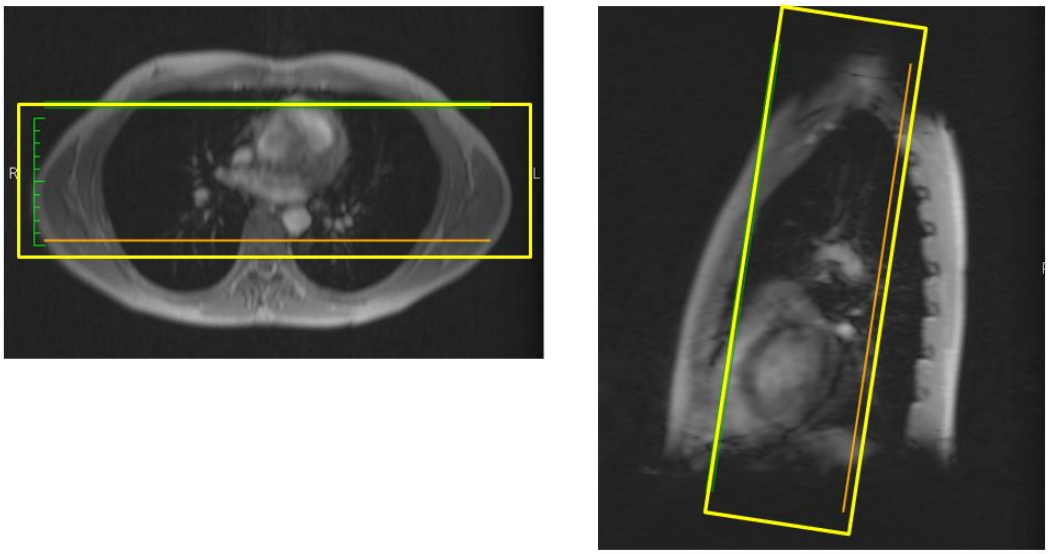


Figure 2.5. Field of view (FOV) for DCE MRI covering majority of lung parenchyma, heart and descending aorta.

The volunteer was connected to the injector with 'keep vein open' enabled. The Dotarem dose was calculated at 0.2mls per kg, with an injection rate of 4mls per second. The saline flush was set at 20mls, with an injection rate of 4mls per second.

VTR (variable time to repetition) scans were undertaken at TRs (time to repetition) of 3.14, 10, 20, 35 and 50 seconds. T1 scans for T1 mapping were carried out at flip angles of 2°, 5°, 10°, 20° and 30° and repeated 6 times. DCE images were acquired under free breathing with TR 3.14 (repetition time), TE 0.91ms (echo time), flip angle 18.4° and 5mm slice thickness. Contrast agent was administered mechanically with the trigger controlled by the radiographer to commence at the start of the 6th Dynamic. Scanning continued for 8 minutes after contrast infusion in order to capture 'wash out' from the lung parenchyma. The scanning protocol was then considered complete and the participant assisted from the scanner. Any adverse symptoms or events were recorded and actioned (see section 2.2.19, page 75).

2.2.16 Bronchoscopy and BAL

Bronchoscopy and BAL took place in a dedicated Endoscopy Suite at FH. All bronchoscopies were performed by 2 senior members of the research team experienced in undertaking research BAL (myself and Dr Ian Forrest, consultant respiratory physician). Participants arrived at the Endoscopy Suite with IV access in situ. They were checked into the unit, consent was checked (as per study consent), observations were recorded and standard monitoring was applied (oxygen saturations, heart rate, blood pressure, ECG). Supplementary oxygen was applied at 2L/min via nasal cannula. 200mls of sterile saline was warmed prior to commencing bronchoscopy.

All participants were offered sedation for bronchoscopy, which was discussed at the screening visit, at consent for study participation and at the time of attending bronchoscopy. All participants declined. Topical, local anaesthetic throat spray (Xylocaine,

10mg lignocaine in 50mls, AstraZeneca) was applied to the upper airway, between 4-10 applications. The time of local anaesthetic throat spray was used to denote 'start time of bronchoscopy'. A flexible bronchoscope was introduced by either the nasal or oral route, depending on the bronchoscopist's preference and the participant's anatomy. 2mls aliquots of 1% local anaesthetic (lignocaine hydrochloride, various brands) were sequentially applied to the upper airway and vocal cords via the bronchoscope until instillation elicited no further coughing response (typically between 8-14mls). The bronchoscopist navigated through the vocal cords to a segment of choice for sampling (usually the medial segment of the right middle lobe) taking care to cause minimal trauma to the central airways. 20mls of warmed sterile saline was instilled, gently aspirated and discarded as the bronchiolar sample. 150mls of warmed sterile saline was then instilled (in 50mls aliquots) and gently aspirated into sterile specimen containers. If the volunteer's oxygen saturations dropped to below 92% on 2L oxygen per minute, the protocol allowed supplementary oxygen to be increased to 4L/min if necessary and/or for no further saline to be instilled (this action was rare, 1 participant with increased oxygen, 1 participant only 100mls saline instilled). The total volume instilled and the volume of return were recorded.

The bronchoscope was removed and the participant sat upright. Observations were taken at the end of the examination and the participant moved to the recovery area. If observations were satisfactory, continuous monitoring and IV access were removed. The participant underwent a final cardiorespiratory examination and further observations 30 minutes following bronchoscopy. Provided these were satisfactory, they were allowed home. Participants were given written information on when they could resume oral intake (2 hours following throat spray) and my 24-hour contact telephone number to call in the event they felt unwell. The time and place for the study visit the following morning were confirmed.

2.2.19 Adverse event reporting

Despite this study not being a formal clinical trial, the group's previous experience with the LPS inhalation model led us to feel it was important to explicitly monitor and classify any

adverse events in relation to LPS inhalation and/or study involvement. Adverse events (AE) were defined as any untoward medical occurrence in a study participant. A serious adverse event (SAE) was defined as any untoward medical occurrence in a study participant or effect that a) results in death, b) is life threatening (i.e. the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe), c) requires hospitalisation or prolongation of existing hospitalisation, d) results in persistent or significant disability or incapacity or e) is a congenital anomaly or birth defect. In the event of an adverse event being detected, a member of the research team made an assessment of the seriousness (as defined above) of the event. In the event of a SAE, a member of the research team then considered if the circumstances were 'Related' to the study – that is, it resulted from administration of research procedures and/or 'Unexpected' **–** that is a type of event that is not identified as an expected occurrence.

All AEs and SAEs were recorded from the time a participant consented to join the study until 24 hours after completing the final study assessments. I asked about the occurrence of AEs/SAEs during the study and on the surveillance phone call after the volunteer completed his/her study visits. Information collected included the type of event, the onset date, assessment of implications (if any) for the safety of participants and how this was addressed, the date of resolution (and treatment required), any investigations needed, and outcome. All information was recorded in the participant's study file. If an SAE had occurred, the protocol dictated that the research team must report the information to Newcastle Upon Tyne Teaching Hospitals NHS Foundation Trust, Research and Development (NuTH R&D) office within 24 hours. An SAE form was to be completed as thoroughly as possible with all available details of the event, signed by the Investigator or designee. The SAE form was to be transmitted by fax or by hand to the office. NuTH R&D was then responsible for reporting SAEs that were considered to be related and unexpected as described above to the Research Ethics Committee (REC) that approved the study within 15 days of becoming aware of the event. As per usual practice, the Co-ordinator of the main REC would then acknowledge receipt of a related, unexpected safety report within 30 days.

2.3 Study assessments

2.3.1 Laboratory materials

The materials described below were used to undertake all laboratory experiments and analysis. Materials and reagents are listed by supplier, alphabetically and in size order with the catalogue number in brackets. Antibodies are listed by the protein of interest by supplier with the catalogue number in brackets.

2.3.1.1 Plastics

The following plastics were supplied by Beckton Dickinson (BD) Biosciences (Oxford, U.K.):

- 50ml polypropylene conical Falcon tubes (352070)
- 15ml polypropylene conical Falcon tubes (352096)
- Ethylenediamine tetraacetic acid (EDTA)-coated 3ml blood tubes (367835)
- Rapid serum gel tubes (368774).

The following plastics were supplied by Fisher Scientific (Loughborough, Leicestershire, U.K.)

- 2ml Eppendorf tubes (FB74111)
- 1.5ml Eppendorf tubes (FB74031)
- 0.5ml Eppendorf tubes (FB74023).

The following plastics were supplied by Greiner Bio-one Limited (Stonehouse, Gloucestershire, U.K.):

- 50ml polypropylene conical tubes (227261)
- 15ml polypropylene conical tubes (188271)
- 5ml serological pipettes (606180)
- 10ml serological pipettes (607180)
- 96-well sterile flat bottomed microplates (655101).

The following plastics were supplied by Scientific Laboratory Supplies Limited (Nottingham, U.K.):

- 24-well multi-well plate with lid (sterile) (S3526).

The following plastics were supplied by Starlab U.K. Limited (Milton Keynes, U.K.):

- 3ml graduated Pasteur pipettes (E1414-0311)
- 1000µl pipette tips (S1111-2721)
- 200µl pipette tips (S1111-1700)
- 0.1-10µl natural pipette tips (S1111-3700).

2.3.1.2 Glassware

The following glassware was supplied by Sigma-Aldrich (Gillingham, Dorset, U.K.):

- Corning microscope slides (CLS294875x25-1440E).

The following glassware was supplied by VWR (Pennsylvania, U.S.):

- Microscope cover slips (631-0150).

2.3.1.3 Reagents

The following reagents were supplied by BD Biosciences (Oxford, U.K.):

- Pharmlyse (10x concentrate) (555899).

The following reagents were supplied by GE Healthcare Lifescience (Little Charlefont, Buckingham, U.K.):

- Percoll Plus (GZ17544501).

The following reagents were supplied by Invitrogen (Paisley, U.K.):

- EDTA (AM9912)
- Iscove's Modified Dulbecco's Medium, IMDM (21980065).

The following reagents were supplied by Pharmacosmos (Holbaek, Denmark):

- Dextran T500, 6% (5510050090070).

The following reagents were supplied by Sigma-Aldrich (Gillingham, Dorset, U.K.):

- Agarose 2%, high resolution (A4718)
- Albumin from bovine serum (A7906)
- Calcium chloride solution (Ca Cl2) (21114)

- Citrate concentrated solution, sodium citrate 3.8% (S5770)
- Cytochrome C (C2037)
- Dulbecco's phosphate buffered solution (PBS) 10x (without Ca2+/Mg2+) (D1408)
- Dulbecco's phosphate buffered solution (PBS) 1x (without Ca2+/Mg2+) (D8537)
- fMLP (N-formyl methionyl leucyl phenylalanine) (47729)
- Gelatin 2% solution (G1393)
- Giemsa (48900)
- Hanks' balanced salt solution with Ca2+/Mg2+, HBBS+ (55037)
- Hanks' balanced salt solution without Ca2+/Mg2+, HBSS- (H6648)
- Methanol (179957)
- Paraformaldehyde (P6148)
- Platelet activating factor, PAF (P260)
- Sodium chloride solution (0.9%), NaCl (S5886)
- Superoxide dismutase (SOD) (S5395)
- Trypan blue (T6146)
- Tween 20 (P1379)
- Zymosan A from *Saccharomyces cerevisiae* (Z4250).

The following reagents were supplied by Sysmex Partec (Milton Keynes, UK):

4,6-diamidino-2-phenylindole (DAPI)

2.3.1.4 Antibodies

Those applied in whole blood:

- Fluorescein isothiocyanate (FITC)-conjugated murine IgG1 recognising human CD11b (Biolegend, 301404)
- FITC-conjugated Mouse IgG1 isotype control (Biolegend)
- Allophycocyanin (APC)-conjugated IgG1 recognising human CD62L (Biolegend, 304810)
- APC-conjugated Mouse IgG1 isotype control (Biolegend)
- Phycoerythrin (PE)-conjugated IgG1 recognising human CD88 (Beckton Dickinson, 550494)

- PE-conjugated Mouse IgG1 isotype control (BD)

Those applied in BALF:

- V500-conjugated CD45 (Beckton Dickinson, 560777)
- APC-conjugated – APC (Biolegend, 301309)
- PE/Dazzle-594-conjugated CD16 (Biolegend, 302054)

2.3.2 Laboratory procedures

2.3.2.1 Isolation of neutrophils from whole blood

Neutrophils were isolated from whole blood by dextran sedimentation and discontinuous Percoll gradient separation (Haslett et al, 1985; Ruchaud-Sparagano et al, 2013). Immediately after sampling from study participants whole blood was transferred to a 50mls Falcon tube pre-filled with sodium citrate 4% (1ml per 9mls of participant blood). Falcon tubes were labelled with the participant's study number and the visit date and transferred to the lab at room temperature. On arrival at the Simpson lab, citrated blood was centrifuged at 300g for 20 minutes at room temperature, without a break. During centrifugation, 90% Percoll was diluted with 1% ± 10% PBS (without Ca/Mg) to create 81%, 70% and 55% concentrations. Following centrifugation of citrated whole blood, the upper layer of platelet-rich plasma was removed to a glass tube. Calcium chloride was added ($CaCl_2$ 220µl per 10mls plasma) and warmed in a 37°C water bath. Warmed, filtered 6% dextran solution was added to the remaining blood cell pellet at a volume of 2.5mls per 10mls of cell pellet. The solution was then made up to the original blood volume with the addition of warmed 0.9% saline (i.e. approximately 30mls). The sample was gently inverted several times to ensure the solution was fully mixed. The Falcon tube lid was loosened, and the solution left to sediment for 30 minutes at room temperature without disturbing.

After sedimentation, the leukocyte-rich upper layer of cells was pipetted into a separate Falcon tube and made up to 50mls with warmed normal saline solution. This was then centrifuged at 200g for 5 minutes at room temperature. Following centrifugation, the

supernatant was discarded and the cell pellet re-suspended in 2.5mls of 55% Percoll solution. 2.5mls of 70% Percoll was carefully layered onto 2.5mls of 81% Percoll solution. Finally, the cell-containing 55% Percoll solution was carefully layered onto the 70% to create the complete Percoll gradient. The gradient was then centrifuged at 700g for 20 minutes at room temperature without a break.

Following centrifugation, cells separated as shown in figure 2.6. Percoll supernatant and the mononuclear cell (MN) layer were carefully removed and polymorphonuclear (PMN) cell harvested from the 81%/70% interface. Cells were transferred to a new 50mls Falcon tube and washed with HBSS without calcium and magnesium (HBSS-) made up to the original blood volume (approximately 30mls). The solution was centrifuged at 200g for 5 minutes at room temperature. Following centrifugation, supernatant was removed and the neutrophil cell pellet resuspended in a small volume of HBSS- ready for cell counting and further dilution for use in subsequent functional assays.

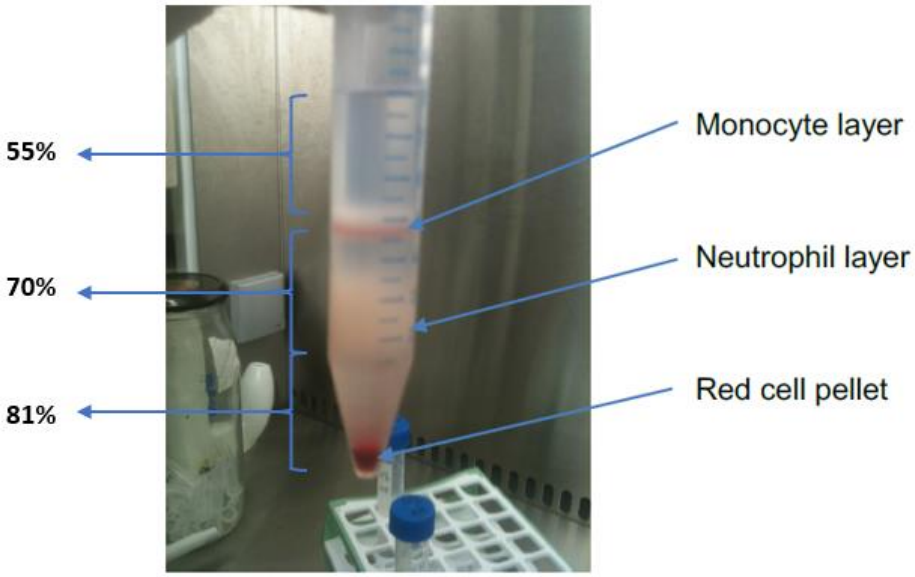


Figure 2.6. Percoll gradient and separation of neutrophils and monocytes following centrifugation.

2.3.2.2 Cell counts and cytospins

Following isolation by the above method, 100µl of suspended PMNs were removed to a 1.5mls Eppendorf tube and stained with 5µl of trypan blue. 100µl of stained cell solution was then placed in the chamber of a haemocytometer and a cell count performed using light microscopy. Using the known dilution factor, the total number of PMNs isolated from the whole blood sample was calculated.

150µl of unstained PMNS were added to a cytospin chamber along with a glass slide and centrifuged for 3 minutes at 300g in the cytospin. Slides were removed and allowed to dry before fixing in acetone for 10 minutes. Review of these confirmed the PMNs were overwhelmingly neutrophils (>96%, see section 3.3.2, page 114) and therefore from this point on these cells will be referred to neutrophils.

2.3.2.3 Measurement of phagocytosis by adhered neutrophils

Isolated neutrophils in HBSS- were recentrifuged (200g for 5 minutes at room temperature) and the supernatant discarded. The cell pellet was suspended in IMDM at a concentration of 1 million cells per ml, and 500µl (0.5 million cells) pipetted into 4 wells of a 24-well plate. 1% autologous serum was added to each well and the cells were adhered to the plate by incubation at 37°C in a 5% CO_2 incubator for 30 minutes. Zymosan was opsonised in autologous serum (2% zymosan, 50% serum, 48% IMDM) at 37°C in a water bath for 30 minutes. Following opsonisation, the zymosan was microfuged (3000g for 2 minutes at room temperature), washed twice in warmed IMDM and resuspended in 200µl of IMDM. 50µl (0.01mg zymosan) was added to all but one of the 4 wells containing adhered neutrophils. The plate was then incubated for a further 30 minutes at 37°C in a 5% CO_2 incubator to allow phagocytosis to occur.

After incubation, cells were gently washed 3 times with PBS and allowed to air dry. Cells were fixed with methanol (300µl per well for 10 minutes) and then stained with Giemsa

(300µl of 10% Giemsa for 15 minutes). Giemsa was aspirated from the plate and the cells washed with deionised water until running clear and the plate allowed to air dry.

The plate was then assessed under light microscopy at a power of x40. The 'control' well (no zymosan) was used to assess adequate adherence/staining of the neutrophils by this method. For all other wells, a field of approximately 100 neutrophils was chosen. The number of neutrophils demonstrating phagocytosis of 2 or more zymosan particles was counted and expressed as a percentage of the 100 cells (see figure 2.7). This was repeated 4 times for each well and the average percentage taken as a measure of phagocytosis in that well. To ensure accurate counting and to avoid investigator bias, a sample of wells (20%) were re-counted by a second 'blinded' investigator. Data were only included if counts showed less than 5% inter-investigator variability.

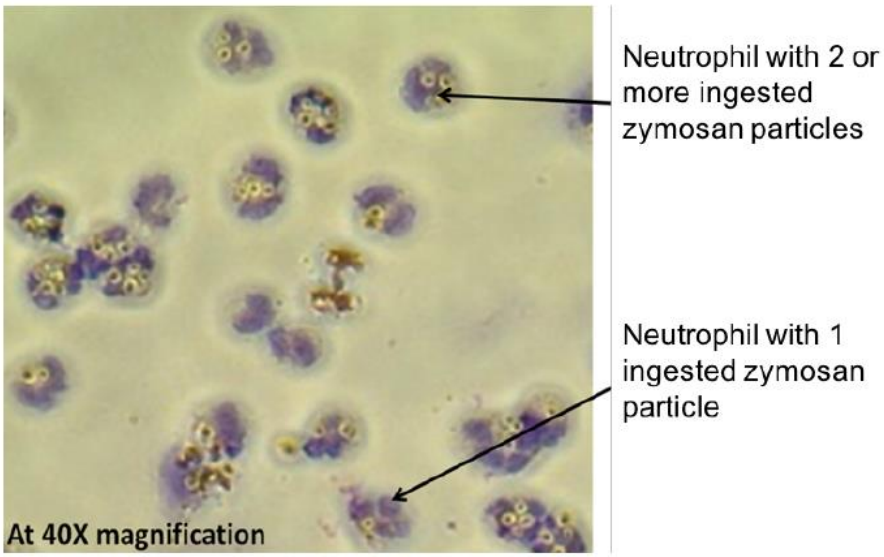


Figure 2.7. Example of assessing neutrophil phagocytosis under light microscopy. 100 cells were counted and the number of neutrophils containing ≥2 zymosan particles expressed as a percentage (repeated four times)

2.3.2.4 Measurement of superoxide anion release by neutrophils

Measurement of superoxide release by neutrophils was determined by calculating the amount of superoxide dismutase (SOD)-inhibitable reduction of cytochrome C. Isolated neutrophils from whole blood (see section 2.3.2.1, page 80) were re-suspended in HBSS with calcium and magnesium (HBSS+) at a concentration of 1 million cells/ml and mixed well. Eight 2mls Eppendorf tubes were labelled as per table 2.3 and 50µl of neutrophil suspension (0.5 million cells) were added to each tube. Cells were then 'primed' (P) with 1µl platelet activating factor (PAF, 200nM) or left unprimed (H) with the equivalent volume of HBSS+ and placed in a shaking water bath at 37°C for 10 minutes.

Following priming, cells were removed from the shaking water bath. 25µl of SOD was added to four tubes marked 'S' and 25µl of HBSS+ added to the other four tubes to keep overall volume the same. This was followed by 375µl cytochrome C (1mg/ml) added to all 8 tubes. Finally, cells were either 'stimulated' with fMLP ('F', 50µl per tube, 100nM concentration) or made up to the equivalent volumes with HBSS+ (50µl). Cells were placed in the shaking water bath at 37°C for 15 minutes.

Following stimulation, the reaction was stopped by plunging the cell suspension into ice for 5 minutes. All tubes were then microfuged at 10,000g for 3 minutes at 4°C. Supernatants (200µl) were transferred into a 96-well plate (appropriately labelled) and read immediately at 550nm using a plate reader. The generation of superoxide anion was determined by the amount of superoxide dismutase-inhibitable reduction in cytochrome C which occurred. Results were expressed as nanomoles of superoxide anion per 10^6 neutrophils (nmol/10^6 neutrophils).

Eppendorf label	Primed (PAF)	Stimulated (fMLP)	Superoxide dismutase (SOD)
H	No	No	No
HS	No	No	SOD
P	PAF	No	No
PS	PAF	No	SOD
HF	No	fMLP	No
HFS	No	fMLP	SOD
PF	PAF	fMLP	No
PFS	PAF	fMLP	SOD

Table 2.3 Layout of Eppendorf tubes for measurement of superoxide anion release by suspended neutrophils. PAF=platelet activating factor, fMLP= formyl methionine leucine phenylalanine, SOD=superoxide dismutase.

2.3.2.5 Measurement of neutrophil cell surface expression by flow cytometry on whole blood

Expression of CD11b, CD62L and CD88 on the cell surface of neutrophils was measured by flow cytometry. Prior to isolation of neutrophils, 1ml of citrated whole blood was removed for flow cytometry and stored at 4°C until analysis. 50µl of blood was transferred into each of three 2mls Eppendorf tubes, which remained unlabelled or contained antibody or isotype controls. Cells were incubated for 30 minutes at 4°C in the dark.

Following incubation, 1.5mls of 10% Pharmlyse solution (Pharmlyse 1:10 dilution with deionised water, pH 7.3) was added to each Eppendorf, mixed via vortex and placed in the dark at 4°C for 20 minutes to allow red cell lysis. Following lysis, samples were transferred to labelled fluorescence-activated cell sorting (FACS) tubes and washed 3 times using the FACS wash machine, ready for analysis.

Flow cytometry was performed on a FACS Canto II machine in the ICM, Newcastle University. Neutrophils were identified by forward scatter and side scatter characteristics and gated appropriately. Median fluorescence was recorded for each antibody and FCS Express, version 6 used for analysis (see example, figure 2.8)

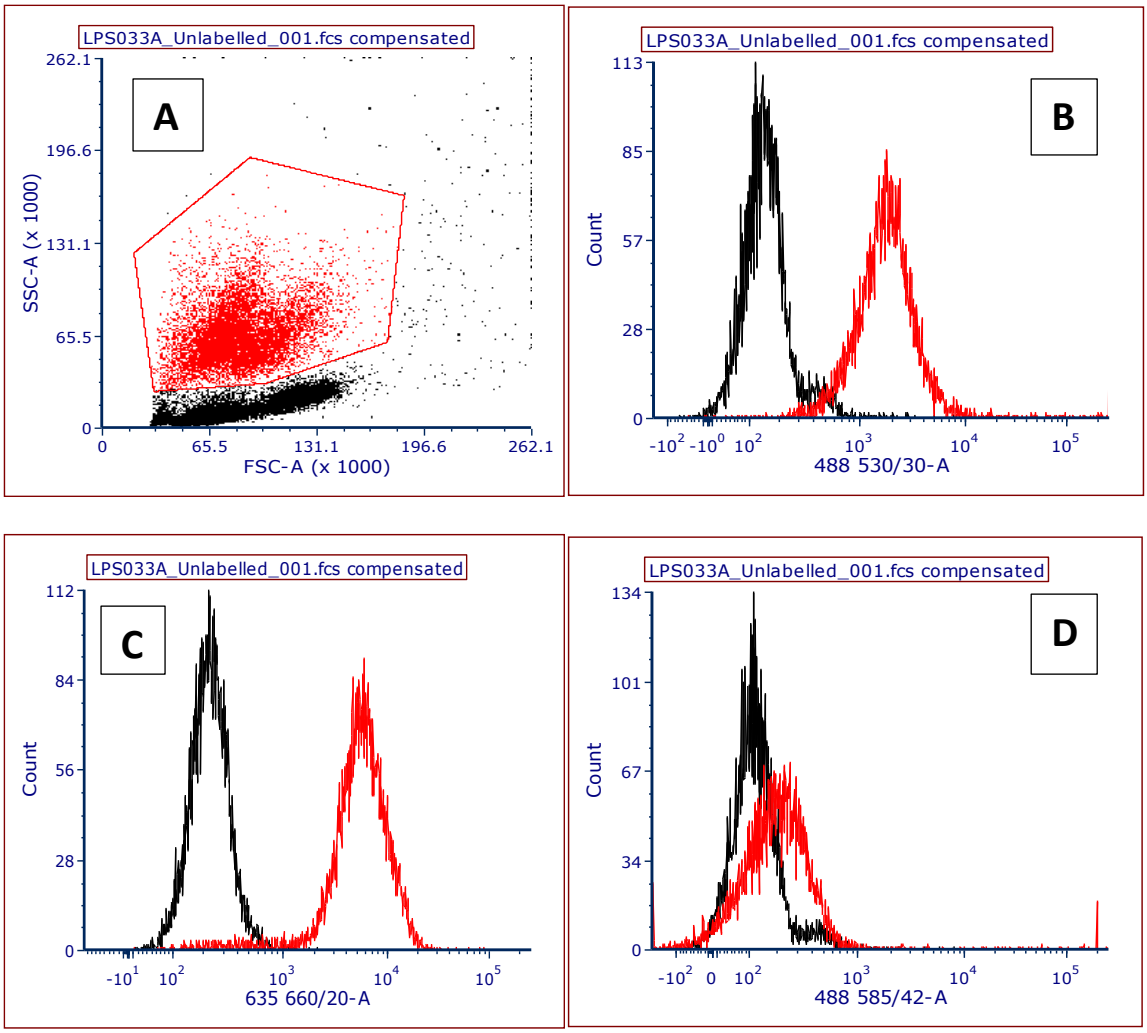


Figure 2.8. Assessing expression of CD11b, CD62L and CD88 on neutrophils by flow cytometry. A: Neutrophils identified by forward scatter (FSC-A) and side scatter (SSC-A) characteristics and gated. B: Neutrophil expression of CD11b C: Neutrophil expression of CD62L D: Neutrophil expression of CD88. Black = isotype control, red = stained neutrophils. All traces are taken from example LPS033 (baseline sample for LPS-treated participant).

2.3.2.6 Analysis of bronchoalveolar lavage fluid (BALF)

BALF was kept at room temperature for 60-90 minutes before processing at 4°C. BALF was passed through a 100µm filter into new 50mls Falcon tubes. Samples were centrifuged at 700g for 10 minutes at 4°C. Cell-free supernatant was removed and stored at -80°C and cells were prepared for immediate flow cytometry analysis. Cell pellets were washed in cold PBS with the addition of 2% foetal calf serum and 2mM EDTA. Antibodies were applied for 20 minutes at 4°C. Flow cytometry analysis was performed on a BD FACS Canto II or BD Fortessa X20, Centre for Life, Newcastle University. Dead cells were excluded with DAPI. CD45-positive cells were used to differentiate populations of leukocytes and neutrophils gated by CD11b and CD16 characteristics. Consistent instrument performance was ensured by running Cytometer setup and tracking beads. Doublets were excluded with an SSC-H vs. SSC-A plot. FlowJo version 9.6.7 was used for analysis.

All preparations of BALF, following initial filtering and centrifugation, were undertaken by my collaborator Dr Laura Jardine (Newcastle University) who performed all subsequent analysis. A large body of work as a result of BALF findings within the LPS inhalation model, including detailed methods, is presented in appendix E. A small section of BAL results referring to neutrophil populations in BALF of LPS- and saline-treated subjects is included within the results chapter and is done so with Dr Jardine's permission.

2.3.3 Acquisition DCE MRI images

As per section 2.2.11.2 (page 62), study group 2 consisted of 5 volunteers recruited to undergo a single DCE MRI scan, in order to set up the study protocol for pre- and post-LPS inhalation in later study groups. These volunteers were not chosen at random but taken from within the lab study group as is common practice among the MRI group when setting up new protocols. However, all volunteers underwent the same consent procedures and screening visit to ensure they met the same study and safety criteria (particularly renal function and MRI safety questionnaire) as all other groups.

Scans were carried out on a 1.5T Espree Siemens scanner (Siemens Medical Solutions, Erlangen, Germany). Participants were positioned on the scanner bed and a surface coil applied over the thorax. A localiser scan was conducted and the participant moved, autocoil select and FOV altered as required (see section 2.2.15, figures 2.3, 2.4 and 2.5 page 72).

Scanning set up was adapted from successful DCE MRI scanning protocols from colleagues in Manchester (Dr J. Naish and colleagues, with permission). During set up (study group 2) slight alterations were trialled with dose and rate of infusion of contrast, coil use and FOV in order to acquire the best image quality. Images and study protocols were verified with collaborators in Manchester prior to commencing study group 3 (see example figure 2.9). Identical scanning protocols were used throughout study group 3 at baseline scan (pre-inhalation) and post-inhalation (approximately 6.5 hours following inhalation).

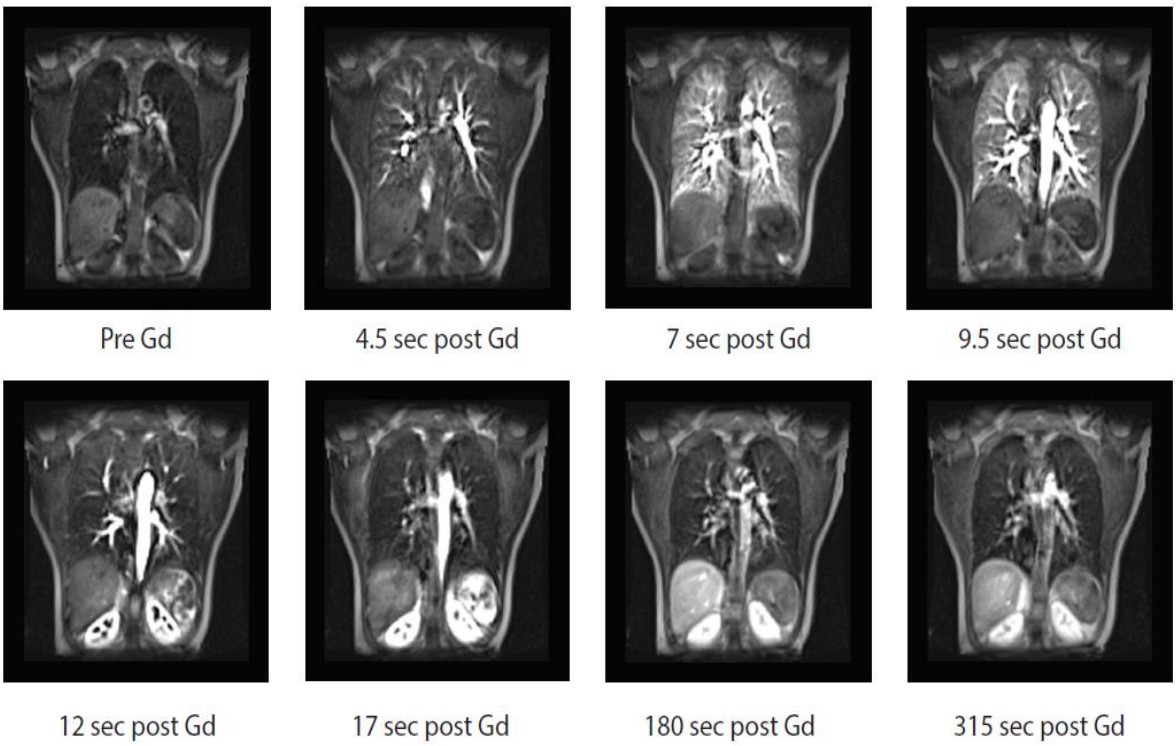


Figure 2.9. Example raw data images of DCE MRI post-gadolinium contrast agent and wash out period (example from LPS017, study group 2)

2.3.3.1 Analysis of DCE MRI

DCE data were analysed in OsiriX MD using a region of interest (ROI)-based approach. A T1 map was created by importing T1 images from all flip angles and opening images in the 4D viewer. The appropriate flip angle data were incorporated and the DCE tool plug-in used to automatically create the T1 map. Measurements of T1 were taken from 3 separate areas of the lung (right lung apex, left lung apex and centre of left lung). An initial review of results found measurements of T1 to be highly variable between ROI and at different depths within lung tissues (see results chapter section 3.3.6.1, page 124).

Therefore, raw DCE data were used for subsequent analysis. A ROI was drawn on an image acquired after contrast injection at a depth slice with a lower T1 value (usually avoiding images encompassing the heart). The ROIs were all drawn on the top half of the right lung, away from the pleura (minimising respiratory motion artefact and excess noise) and avoiding major arteries (see figure 2.10). On the same slice, a ROI was drawn within a major artery and used to provide the data for the arterial input function (AIF). Using a ROI enhancement plug-in, the raw signal intensity was measured in both the lung parenchyma and pulmonary artery. The data were exported into Excel. ROIs for both lung and arterial measurements were kept the same for both scans (pre- and post-inhalation) for an individual volunteer, and measurements repeated over time.

The final 100 slices (80 seconds to 8 minutes) for each MRI were analysed separately to determine the wash out slope. The signal time course was found for the same ROI for both visits and then plotted on a scatter graph. The curve was treated linearly, and the equation of the line found following $\boldsymbol{y = mx = c}$,where m is the gradient.

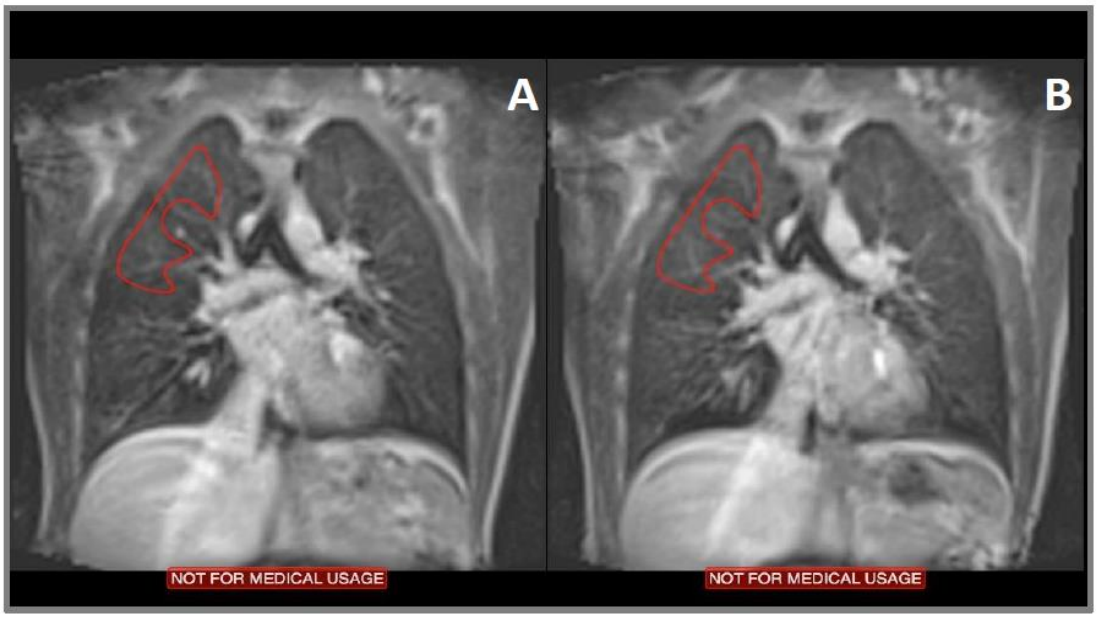


Figure 2.10. Example images from DCE MRI showing regions of interest (ROIs). A = pre-inhalation and B = post-inhalation scans, ROI were kept the same for each study participant

2.3.4 Storage of samples and data

Samples of whole blood were centrifuged as soon as possible following collection for preparation of serum and plasma. Plasma and/or serum was aspirated carefully using a Pasteur pipette and transferred in 1ml aliquots to new 1.5mls Eppendorf tubes for storage. All tubes were labelled with the participant study number, the date of collection and the nature of the sample and stored in a locked -80°C freezer for subsequent analysis. Cytospin slides and phagocytosis plates were labelled with the participant study number and date of collection and stored at room temperature within the Simpson lab in ICM, Newcastle University, for later counting and second observer verification. Superoxide plates and flow cytometry samples were labelled with the participant study number and date during analysis and discarded on completion at the end of study day. BALF supernatant was carefully removed by Pasteur pipette and stored in 10mls aliquots in universal containers. Samples were labelled with the participant study number and the date of collection and stored in a locked -80°C freezer for further analysis. DCE MRI images were anonymised at collection (using only participant study number and date of collection) and the data burnt

onto a CD. Anonymised images were transferred for analysis and storage to the network drive of NMRC, Newcastle University.

Identifiable participant personal data (including name, sex, date of birth, contact telephone number and GP details) were kept on a single sheet of paper, linked to both the screening number and the study number. For the duration of screening and study visits this was kept with the participant's hospital record and, on completion of each visit, removed and kept in a locked file within the chief investigator's administrative office. All clinical research forms for screening and study visits were completed on paper and each page labelled with the participant study number and date. All observation charts and bronchoscopy forms were labelled with the participant number and date and filed within the participant's hospital notes at the end of the study. At the end of study involvement, the participant's GP was contacted with information regarding the study (+/- any adverse events) and all participant identifiable data were kept in the locked administrative file.

All biological samples, clinical research forms, stored images and thus participant details will be stored in the above secure manner for a maximum of 5 years following completion of the study as per the original ethical approval. At this time, participant identifiable data will be destroyed in a confidential manner and biological samples either destroyed or be subject to separate ethical applications to undergo further study.

2.4 Statistical analysis

The primary outcome measures and key time points were set a priori. Raw data were anonymised to participant number and transferred into Excel files (Office 365, version 16001). On completion of each study group (following analysis of phagocytosis) data were unblinded and arranged into saline and LPS inhalation groups. Basic descriptors, by inhalation group, were analysed with means and standard deviations unless otherwise stated. Symptoms and adverse events are presented as simple frequencies.

All statistical analysis was conducted using SPSS (IBM, SPSS Statistics, version 24). Many of the results were measured over 3 time points of baseline, 6 hours and 24 hours following inhalation. The key outcomes were those measured at 6 hours following inhalation and, due to the study design, there was comparable 'control' data at 6 hours. However, participants could also act as their own control with change from their own baseline. Each data set was assessed for normality using the Shaprio-Wilk test and the outcome used to guide the appropriate statistical test. Independent samples t-tests were used to compare results from separate inhalation groups and paired samples t-tests to compare change from baseline to 6 hours where appropriate.

Changes in clinical parameters throughout study visits utilised data from 8 time points, and changes in automated cell counts over 5 time points. Groups were compared for difference over time using a two-way mixed ANOVA. Data were assessed for normality using the Shapiro-Wilk test. Homogeneity of variance was tested using Levene's test and if Mauchly's test of sphericity indicated that the assumption of sphericity was violated for the two-way interaction, the Greenhouse-Geisser correction was applied for all subsequent effects. A value of $p < 0.05$ was considered to indicate statistical significance.

2.5 Summary of methods and materials chapter

In this chapter I have described the methods and materials I used to undertake an LPS inhalation study of healthy volunteers. In particular, I have outlined the funding and ethical approval, inclusion and exclusion criteria, and appropriate procedures for screening and consenting individuals for study involvement. I have also discussed the delivery of LPS or saline by dosimeter nebuliser and outlined the details of each study group and all study interventions.

I have then described the laboratory procedures used to isolate neutrophils from whole blood and to undertake measures of phagocytic function and respiratory burst activity. I have discussed the methods used for flow cytometric analysis of whole blood and BALF obtained from study participants. I have also described the experimental procedures used to acquire and analyse DCE-MRI data before and after inhalation of LPS or saline. Lastly, I have outlined the storage of samples and statistical strategy used to analyse data.

3. Results

3.1 Overview of results chapter

In this chapter I will describe the results of conducting the LPS inhalation study. I will outline the number and outcomes of screening visits and numbers of study visits undertaken. I will describe the baseline characteristics of study participants, changes in physiological parameters and lung function in response to inhaled saline (control) and LPS. I will report the safety data of the study in terms of symptoms reported by participants and all adverse events.

I will also report experimental data focussed on neutrophils within the blood compartment in response to inhaled LPS. This includes automated cell counts (as proof of LPS delivery) and functional assays of phagocytosis, superoxide anion release and also flow cytometry cell characteristics. I will report in detail the results and analysis of DCE MRI data within the LPS inhalation model. Finally, I will demonstrate delivery of LPS to the airway as evidenced by initial analysis of BALF.

3.2 Record of the study

3.2.1 Screening visits

The first round of advertising for study groups 1 and 2 commenced in September 2012, followed by further rounds of advertising in September 2013 and October 2014 (group 3) and February 2015 (group 4).

A total of 391 individuals expressed an interest in the study in response to adverts, comprising 98 for recruitment to group 1, 6 for group 2, 185 for group 3 and 102 for group 4.

A total of 84 screening visits were undertaken for recruitment to all study groups. Figure 3.1 summarises the outcome of all screening visits. Approximately 45% of individuals screened were not eligible to proceed into the study by our exclusion criteria. The most common reason for ineligibility was an incidental finding on blood testing. This was usually abnormal liver function tests (LFTs) in young males (isolated elevation in Alanine transaminase [ALT] or Gamma-glutamyl transpeptidase [GGT]) or mild anaemia in young females. Some participants had total white cell or neutrophil counts marginally outside the laboratory's normal reference range. A past medical history raising a potential diagnosis of asthma (usually in childhood) was also common, as was risk of syncope/vasovagal on blood taking.

A number of volunteers were offered re-screening visits if the reason for screen failure was felt to be 'reversible' and they were keen to participate. Of note however, when the reason for screen failure was mildly abnormal LFTs, repeat screen within the 30-day time frame did not result in any volunteer meeting study criteria.

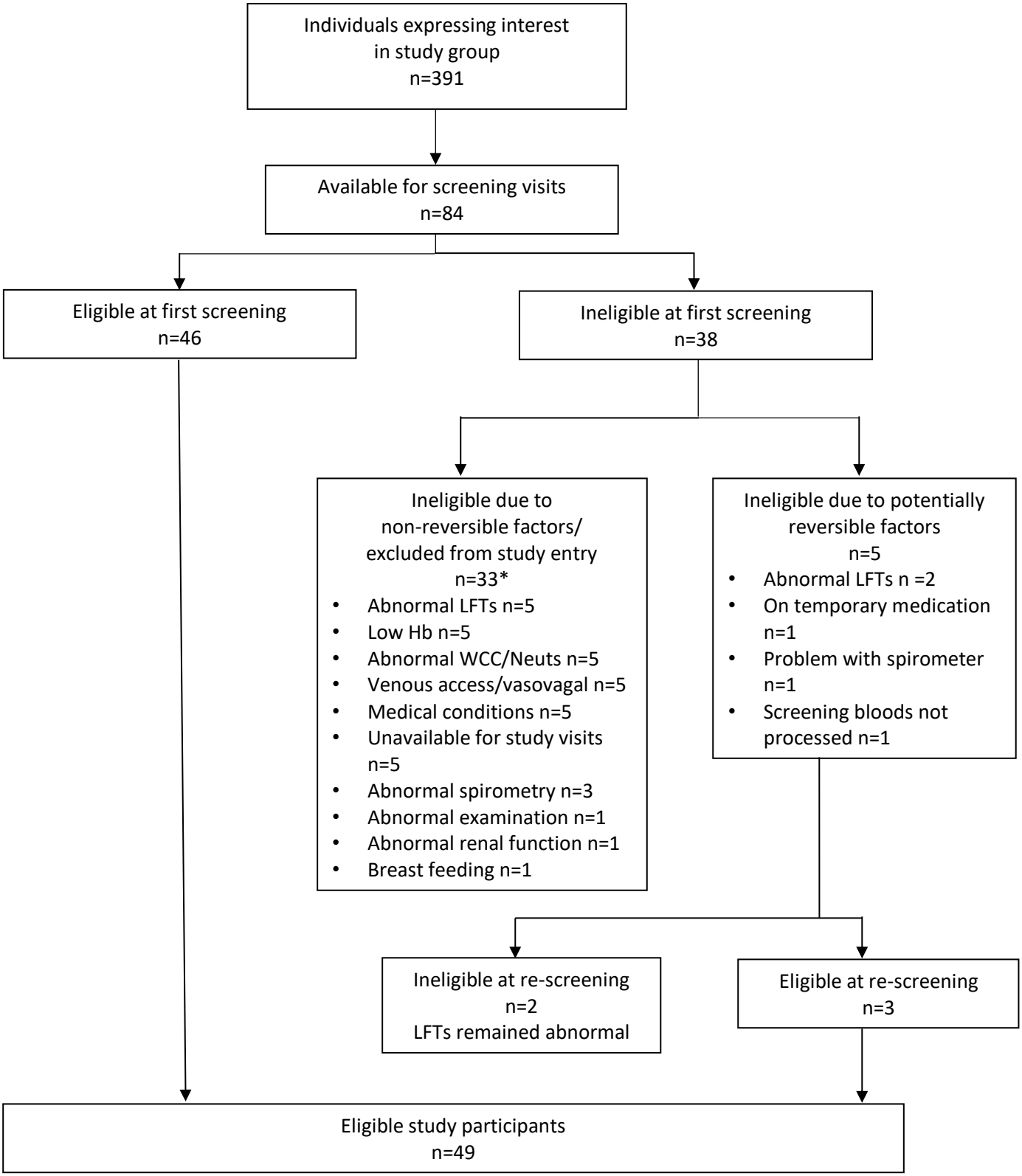


Figure 3.1 Summary of screening visits and reasons for ineligibility. LFT=liver function tests, Hb=haemoglobin, WCC=total white cell count, Neuts=total neutrophil count. *Some participants with more than one exclusion criterion at screening.

3.2.2 Study visits

A total of 107 study visits were conducted for 49 study participants. See figures 3.2 to 3.5. This comprised 32 for study group 1, 5 for study group 2, 50 for study group 3 and 20 for study group 4. Forty-seven of 49 participants completed all study visits as per protocol for their respective study group. Two participants in study group 3 only completed visit 1 (baseline assessments and single MRI). This was due to damage to the valve on the inhalator equipment and separately a personal emergency for the participant resulting in changes to the planned study dates and the participant being unable to re-attend. In both cases, as neither volunteer had undergone inhalation of either LPS or saline, a replacement eligible participant was found to meet the group size of 16 undergoing inhalation.

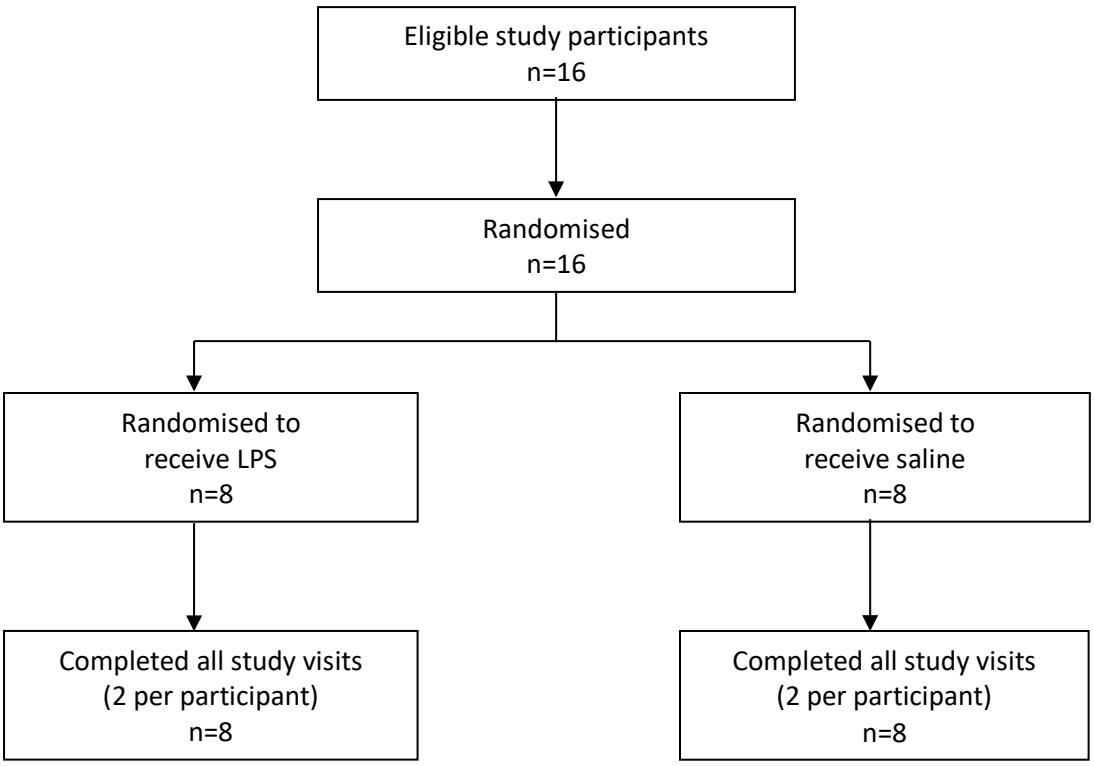


Figure 3.2 Consort diagram showing study visits completed by group 1

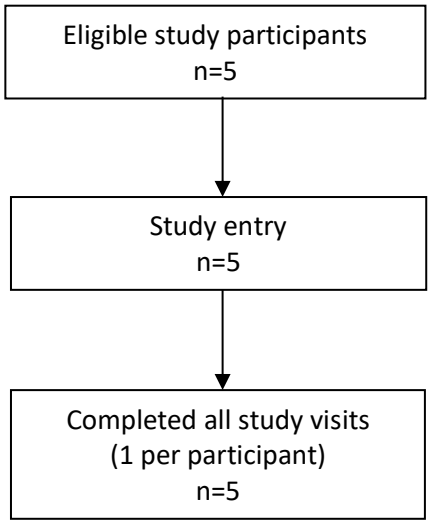


Figure 3.3 Consort diagram showing study visits completed by group 2

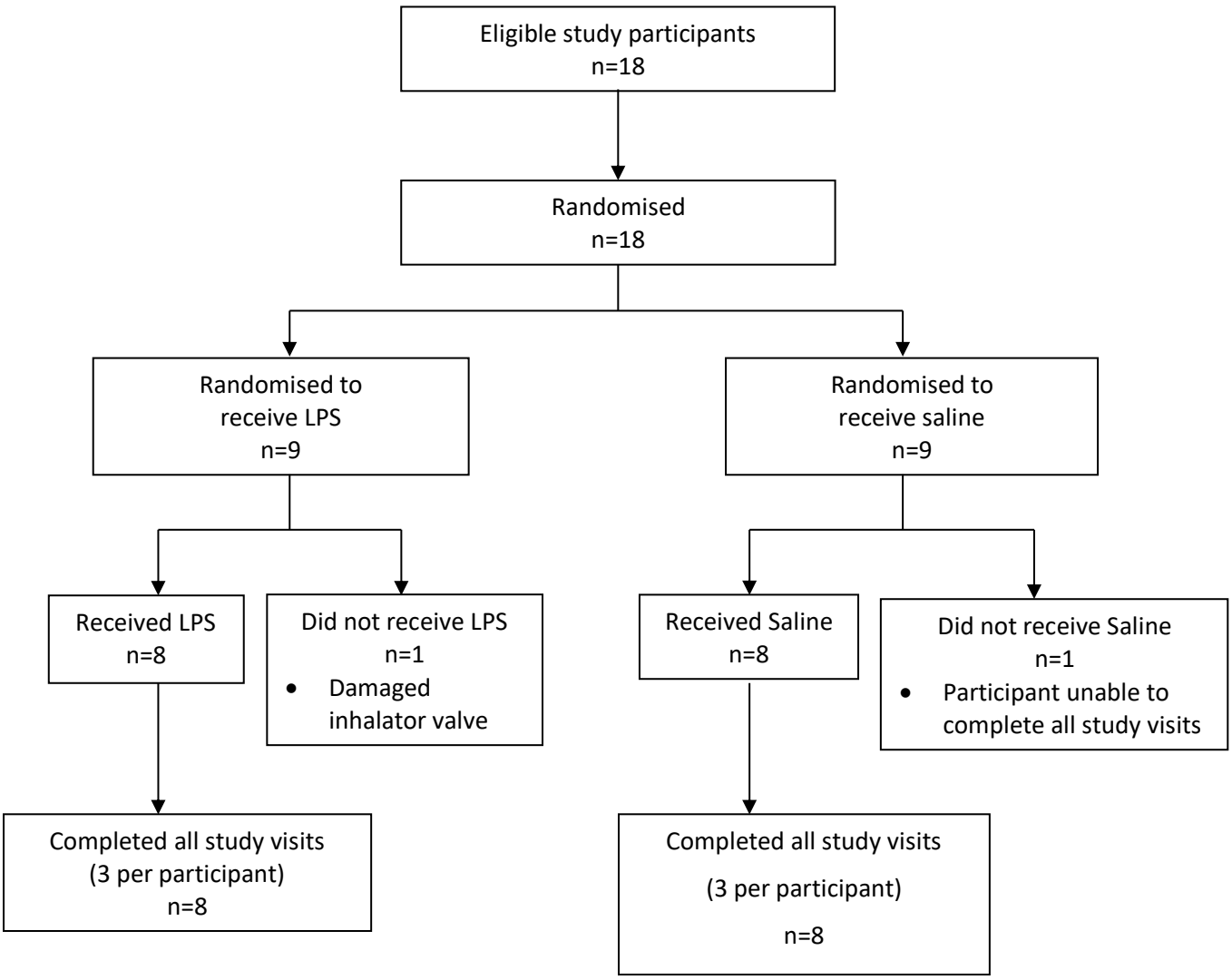


Figure 3.4 Consort diagram showing study visits completed by group 3

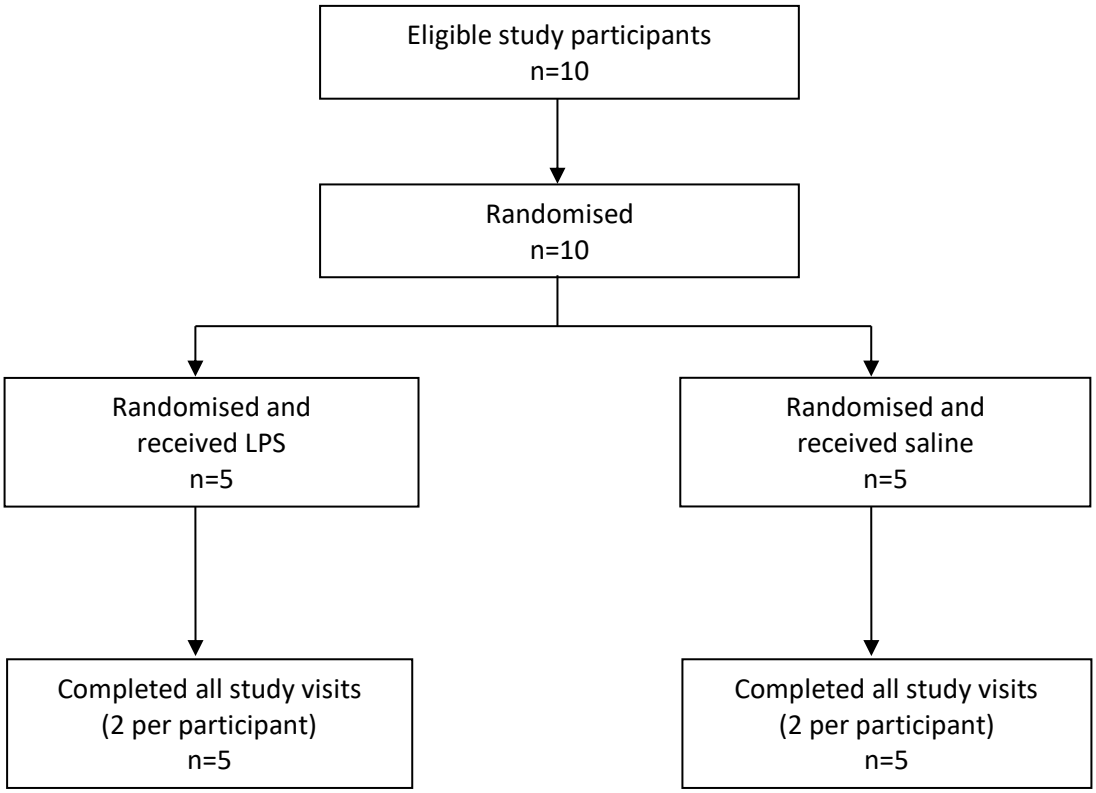


Figure 3.5 Consort diagram showing study visit completed for group 4

3.2.3 Study participant characteristics

3.2.3.1 Baseline demographics

Baseline demographic data for study participants by group and overall are summarised in Table 3.1.

Baseline Characteristic	Group 1	Group 2	Group 3	Group 4	Overall
Number of participants	16	5	18	10	49
Sex (%) Male Female	 4(25) 12 (75)	 3 (60) 2 (40)	 9 (50) 9 (50)	 4 (40) 6 (60)	 20 (41) 29 (59)
Age years	22.5 (4.74)	31.2 * (5.45)	21.3 (2.17)	20.8 (1.14)	22.6 (4.52)
Ethnicity (%) Caucasian Other	 13 (81) 3 (19)	 5 (100) 0 (0)	 15 (83) 3 (17)	 8 (80) 2 (20)	 41 (84) 8 (16)
BMI kg/m^2	22.0 (2.00)	23.1 (2.48)	22.9 (2.59)	21.9 (2.83)	22.4 (2.43)
Absolute FEV1 litres	3.76 (0.93)	4.24 (1.28)	4.07 (1.04)	3.99 (0.82)	3.97 (0.97)
FEV1 % predicted	105.2 (13.28)	114.7 (7.53)	103.1 (11.88)	97.8 (9.31)	103.9 (12.11)
Absolute FVC litres	4.37 (1.22)	5.17 (1.88)	4.87 (1.32)	4.56 (1.07)	4.67 (1.29)
FVC % Predicted	 103.5 (14.03)	 114.1* (14.17)	 106.1 (12.31)	 95.7 (8.77)	 103.9 (13.15)

FEV1/FVC ratio %	86.5 (4.66)	83.5 (5.82)	84.0 (6.04)	88.4 (5.39)	85.7 (5.60)
Temperature °C	36.3 (0.51)	36.9 (0.22)	36.9 (0.29)	35.9 (0.76)	36.5 (0.62)
Heart rate beats per minute	72.7 (12.76)	73.4 (5.94)	73.1 (9.45)	76.9 (11.18)	73.8 (10.56)
Systolic blood pressure mmHg	106.8 (8.81)	134.6 * (11.50)	119.9 (11.11)	119.5 (10.46)	117.0 (13.09)
Diastolic blood pressure mmHg	62.5 (6.73)	69.6 (5.68)	67.9 (9.16)	73.1 (7.87)	67.4 (8.58)
Respiratory Rate breaths per minute	13 (12-14)	13 (12-14)	15 (13-16)	12 (11-13)	14 (12-15)
Oxygen saturations %	100 (98-100)	100 (98-100)	99 (98-100)	99 (98-100)	99 (98-100)

Table 3.1 Baseline characteristics of participants by study group and overall. All values are mean (standard deviation) excepting sex/ethnicity (where frequency [percentage] are reported) and respiratory rate/oxygen saturations (where median [interquartile range] are reported). BMI=body mass index, FEV1=forced expiratory volume in 1 second, % predicted=percentage of predicted value, FVC=forced vital capacity. *significant difference between groups

Table 3.1 shows that overall the groups appeared well matched at baseline, considering the small sample size. There are differences in the proportion of males and females in each study group. Study group 2 showed some significant differences in baseline characteristics in relation to other study groups. Age was significantly higher than in all other study groups,

(mean 31.2 years versus 22.5 years, 21.3 years and 20.8 years for groups 1, 3 and 4 respectively, $p<0.001$). Absolute FEV1 and FVC were the highest in this group although the only measure that reached statistical significance was percentage predicted FVC (in comparison to study group 4, $p=0.048$). Systolic blood pressure was also significantly higher in study group 2 ($p<0.001$).

Table 3.2 summarises baseline characteristics of study participants by inhalation group (saline or LPS). There were no significant differences observed at baseline in any characteristics.

Baseline Characteristic	Saline	LPS
Number of participants	21	16
Sex (%) Male Female	 7 (33) 14 (67)	 9 (56) 7 (44)
Age years	21.3 (1.85)	22.3 (4.58)
Ethnicity (%) Caucasian Other	 17 (81) 4 (19)	 14 (88) 2 (12)
BMI kg/m^2	21.7 (2.19)	23.6 (2.37)
Absolute FEV1 Litres	3.86 (0.99)	4.31 (0.82)
FEV1 % predicted	103.6 (12.31)	104.0 (11.50)
Absolute FVC Litres	4.53 (1.30)	5.06 (1.08)
FVC % predicted	103.8 (14.31)	103.6 (11.28)
FEV1/FVC ratio %	86.2 (6.67)	85.6 (4.94)
Temperature °C	36.5 (0.57)	36.4 (0.76)
Heart rate beats per minute	72.6 (11.72)	74.2 (10.67)
Systolic BP mmHg	112.9 (11.21)	120.1 (10.95)
Diastolic BP mmHg	65.9 (8.10)	68.1 (8.96)
Respiratory Rate breaths per minute	14 (13-15)	14 (13-15)
Oxygen saturations %	98 (98-99)	98 (97-99)

Table 3.2 Baseline characteristics of participants by inhalation group. All values are mean (standard deviation) excepting sex/ethnicity where frequency (percentage) and respiratory rate/oxygen saturations where median (interquartile range). BMI=body mass index, FEV1=forced expiratory volume in 1 second, % predicted=percentage of predicted value, FVC=forced vital capacity

3.2.3.2. Changes in clinical parameters throughout study visits

In order to monitor the physiologic response to inhaled LPS or saline and to monitor the safety of all study participants, volunteers were assessed prior to inhalation and every hour for 6 hours following inhalation for temperature, heart rate, blood pressure, oxygen saturations and respiratory rate. The same parameters were measured at the final study visit, 24 hours following inhalation. Table 3.3 shows the mean physiological measurements for LPS and saline inhalation groups over time.

Few participants showed overt change in physiological parameters following inhalation. There were no differences between groups in heart rate, systolic blood pressure, diastolic blood pressure, oxygen saturations or respiratory rate at any time points. There was a significant difference in temperature observed in those inhaling LPS and saline at 6 hours following inhalation (mean 37.2 °C versus 36.8 °C respectively), two-way mixed ANOVA, p=0.002, figure 3.6). Furthermore, in those undergoing bronchoscopy (study groups 3 and 4) there was often a further rise in temperature in observations taken prior to bronchoscopy (average 7.5 hours following inhalation, data not shown due to small numbers).

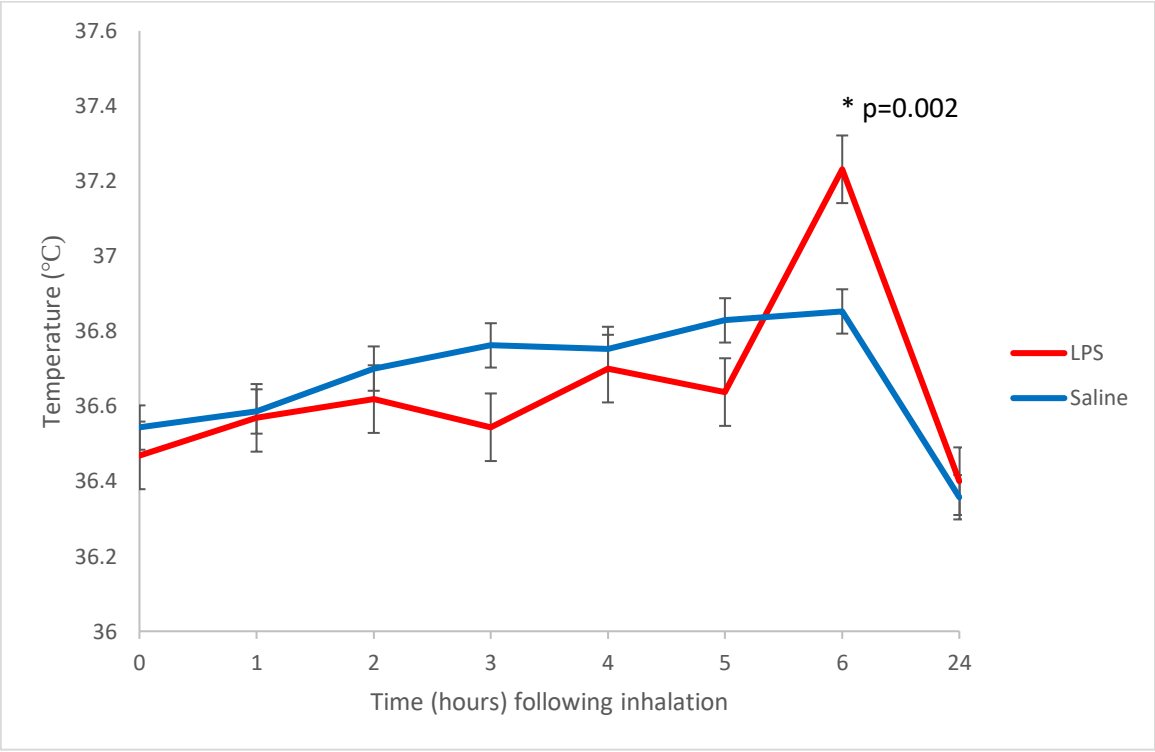


Figure 3.6 Change in temperature following inhalation of either saline or LPS. Points indicate means, error bars represent standard deviation.

	Baseline		1 hour		2 hour		3 hour		4 hour		5 hour		6 hour		24 hour	
	Saline	**LPS**	**Saline**	**LPS**	**Saline**	**LPS**	**Saline**	**LPS**	**Saline**	**LPS**	**Saline**	**LPS**	**Saline**	**LPS**	**Saline**	**LPS**
Temperature °C	36.5 (0.56)	36.5 (0.59)	36.6 (0.48)	36.6 (0.31)	36.7 (0.36)	36.6 (0.30)	36.8 (0.37)	36.5 (0.47)	36.8 (0.38)	36.7 (0.32)	36.8 (0.39)	36.6 (0.55)	36.8* (0.32)	37.2* (0.38)	36.4 (0.54)	36.4 (0.66)
Heart rate	73 (11.7)	74 (9.6)	68 (13.1)	69 (10.2)	67 (12.3)	69 (12.5)	69 (12.6)	68 (9.5)	69 (13.0)	70 (8.7)	66 (12.1)	71 (8.9)	67 (12.2)	71 (11.1)	77 (11.9)	78 (9.8)
Systolic blood pressure mmHg	114 (11.1)	122 (9.4)	111 (11.1)	114 (8.4)	114 (9.7)	120 (15.2)	112 (13.0)	115 (9.3)	115 (11.8)	116 (9.9)	112 (9.3)	117 (7.2)	112 (11.3)	121 (11.9)	112 (14.6)	118 (9.4)
Diastolic blood pressure mmHg	67 (8.3)	68 (7.0)	67 (8.1)	66 (9.5)	69 (6.8)	70 (9.5)	67 (8.0)	66 (7.7)	66 (7.0)	66 (6.6)	65 (5.7)	66 (6.3)	68 (7.6)	67 (4.9)	66 (7.3)	67 (6.6)
Oxygen saturations %	99 (1.2)	98 (1.3)	100 (0.7)	98 (1.4)	100 (0.5)	99 (0.8)	99 (0.7)	98 (1.1)	99 (0.9)	98 (1.4)	99 (0.9)	99 (1.3)	99 (1.0)	99 (1.5)	98 (1.0)	98 (1.4)
Respiratory rate	14 (1.3)	14 (1.7)	15 (2.2)	15 (2.0)	15 (1.9)	16 (2.1)	16 (2.0)	15 (2.1)	16 (2.1)	16 (2.2)	16 (2.0)	17 (2.2)	16 (2.3)	17 (2.6)	14 (1.5)	14 (1.5)

Table 3.3 Physiological changes in participants by inhalation group over time. All figures are mean (standard deviation). LPS n= 16, saline n= 21 *significant difference $p<0.05$

3.2.3.3. Changes in lung function throughout study visits

Spirometry was measured prior to inhalation as a baseline and at 6 hours and 24 hours post-inhalation. Table 3.4 summarises mean lung function in the LPS and saline groups at each time point.

Mean change in percent predicted FEV1 from baseline to 6 hours following inhalation was -1.1% in the LPS group and +1.3% in the Saline group (p=0.130, independent t-test). Mean change in percent predicted FVC from baseline to 6 hours was -0.9% in the LPS group and -0.5% in the Saline group (p=0.850). Lowest measured values for spirometry for both groups was usually at the 24-hour timepoint, but individual (and averages) were well within 10% difference from baseline as specified in the protocol. Mean change in percent predicted FEV1 from baseline to 24 hours following inhalation was -2.4% in the LPS group and -4.3% in the Saline group (p=0.472) and mean change in percent predicted FVC from baseline to 24 hours was -2.2% in the LPS group and -4.3% in the Saline group (p=0.360). There were no significant differences observed within any group from baseline, or between the LPS and Saline groups, at any time points.

	Baseline		6 hours		24 hours	
	Saline	**LPS**	**Saline**	**LPS**	**Saline**	**LPS**
FEV1 Litres	3.85 (0.96)	4.32 (0.82)	3.92 (0.98)	4.23 (0.87)	3.70 (0.99)	4.18 (0.82)
FEV1 % predicted	103.5 (12.13)	104.1 (10.84)	104.8 (12.71)	103.0 (10.69)	99.2 (14.93)	101.7 (10.91)
FVC Litres	4.51 (1.25)	5.08 (1.10)	4.56 (1.26)	4.99 (1.19)	4.33 (1.26)	4.93 (1.08)
FVC % predicted	103.5 (13.69)	103.9 (11.47)	104.0 (14.47)	103.0 (12.09)	99.2 (14.96)	101.7 (10.79)
FEV1/VC ratio %	86.4 (6.35)	85.6 (5.01)	86.8 (6.01)	85.6 (5.44)	86.1 (6.25)	85.3 (6.83)

Table 3.4 Lung function for LPS and Saline groups at each time point. Data shows mean (standard deviation). LPS n= 16, saline n= 21

3.2.4 Symptoms and adverse events

All participants were well and reported no physical symptoms when they attended for baseline assessment at their first study visit. Those volunteers undergoing a single MRI scan (study group 2) were monitored during scan acquisition and given a contact number to report any symptoms for 24 hours following the study visit. For all others, following inhalation volunteers were kept under constant surveillance and could report symptoms to investigators at any point, should they arise. Volunteers were specifically asked about symptoms at 6 hours following inhalation. In those undergoing an additional MRI scan and/or bronchoscopy, further observations and questions regarding symptoms were also undertaken following MRI scan and post-bronchoscopy. At this point all participants could return to their usual place of residence with a 24-hour telephone number to contact should they feel unwell or be concerned regarding symptoms. Table 3.5 summarises symptoms reported by participants throughout the study.

Twenty-three of 49 (46%) study subjects reported no symptoms during study visits or at home overnight. Few participants reported symptoms during the study visits themselves. Of those reported during study visits, 2 participants had a non-symptomatic pyrexia documented, 4 had pre-syncopal symptoms in relation to placing of intravenous access/blood taking (none had syncope) and 2 had skin irritation or required replacement of the venflon dressing. One participant reported cough at 6 hours (control group) and 1 participant reported cough and chest ache at 1 hour and 6 hours following inhalation of LPS, which was not associated with any change in observations, examination findings or change in spirometry.

The majority of symptoms were experienced at home on the evening of inhalation, and in those who had additionally undergone bronchoscopy. They were reported to the study team at the 24-hour visit, at which point some symptoms were ongoing but the majority had resolved. No participants contacted the out-of-hours telephone number to report symptoms overnight.

Symptoms	Saline n=21	LPS n=21	No inhalation n=7
None	8 (38%)	9 (43%)	6 (86%)
Cough	11 (52%)	10 (48%)	0 (0%)
Sore throat	6 (29%)	4 (19%)	0 (0%)
Tiredness/malaise	3 (14%)	4 (19%)	0 (0%)
Chest ache	3 (14%)	4 (19%)	0 (0%)
Sputum/clear secretions	3 (14%)	3 (14%)	0 (0%)
Wheeze	2(10%)	2 (10%)	0 (0%)
Light-headed/pre-syncopal (in relation to blood taking)	1 (5%)	2 (10%)	1 (14%)
Pyrexia (overall) - During study visits - Reported overnight	1 (5%) 0 (0%) 1 (5%)	6 (29%) 2 (10%) 4 (19%)	0 (0%) 0 (0%) 0 (0%)
Flu-like symptoms	1 (5%)	3 (14%)	0 (0%)
Rash/skin irritation in relation to venflon dressing	0 (0%)	2 (10%)	0 (0%)
Other	1 (5%)	0 (0%)	0 (0%)

Table 3.5 Summary of symptoms reported by study participants, by inhalation group. All data are reported as frequency (%). 'No inhalation' group represents 5 participants undergoing single MRI in study group 2 and 2 participants who only attended the baseline study visit in group 3 due to problems with the inhalator valve and a personal emergency.

The most commonly reported symptom was cough, which was experienced by a large proportion of volunteers following bronchoscopy. In addition to the pyrexia found during study visits, several candidates reported feeling feverish at home and several took their own temperature. All were afebrile by 24 hours. Sore throat, general tiredness and some mild chest ache were also common. Surprisingly, very few participants reported 'flu-like' symptoms (which is commonly described following LPS inhalation) though it remains possible that individual symptoms that might contribute to "flu-like illness" were conveyed in the component parts as listed above.

Two participants required unscheduled further assessment due to symptoms. The first reported ongoing cough at the 24-hour study visit but remained well. Prior to the 36-hour phone call, the volunteer rang the study telephone number and asked for further assessment as they felt generally unwell. Due to the time of day and symptoms reported, the decision was taken to assess the participant in Accident & Emergency (A&E). The volunteer reported cough with purulent sputum, fever, chest pain and fatigue. Observations revealed pyrexia, but otherwise normal measurements. Examination revealed no abnormality and CXR was normal. A course of antibiotics was given as a precaution. A further telephone assessment was undertaken the next day and the participant reported no further pyrexia and symptoms resolved (48 hours following inhalation).

In the second case, the participant reported being well at 24 hours and at the 36-hour phone call. The volunteer re-contacted the study team 9 days later with symptoms on an upper respiratory tract infection and sought reassurance that it was unrelated to the study intervention. The participant was invited for review and history, examination and observations were all normal. No further action was required. All other participants reported that symptoms had completely resolved at the 36-hour phone call.

3.3 Experimental results

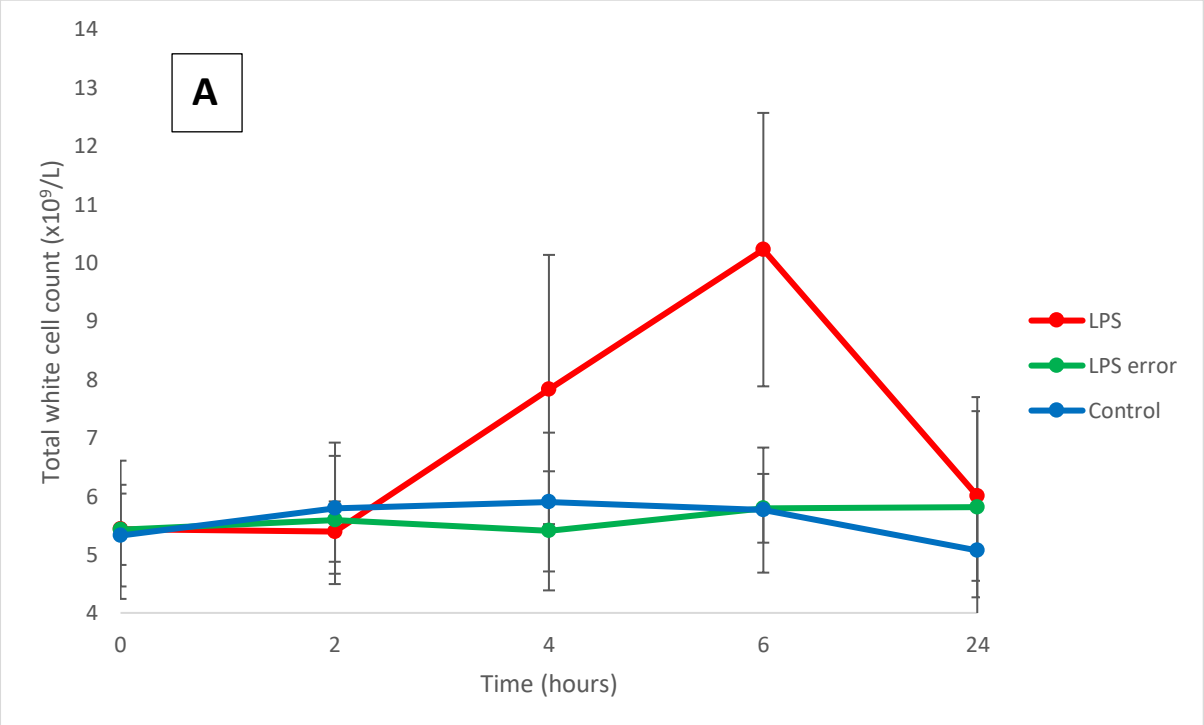
3.3.1 The effects of LPS inhalation as measured in automated cell counts

Inhalation of LPS at doses of approximately 50-60µg is associated with peripheral blood neutrophilia and a corresponding increase in total blood WCC (Michel et al, 2001; Loh et al, 2006; Brittan et al, 2012). As such, this can be used as a measure that LPS has been successfully delivered to the airway. In this study we were interested, not only in the increased numbers of cells, but measures of their functionality.

A sample of blood was sent for automated FBC analysis on all participants at baseline (prior to inhalation), and at 2 hours, 4 hours, 6 hours and 24 hours following inhalation. In study

group 1, it was decided that the LPS suspension for inhalation could be made up the evening before by a different investigator and that I would remain 'blinded' to inhalation in order to evaluate the phagocytosis of zymosan. At the end of study group 1, once phagocytosis had been counted, the group was unblinded and the FBC data examined.

Unfortunately, this demonstrated that several participants randomised to receive LPS, had not shown the expected change in peripheral blood neutrophils or total WCC. After examining the clinical record for symptoms (these participants also reported no symptoms expected in relation to LPS) we concluded that the LPS had not been adequately delivered to the airway. After discussion with other research groups experienced in LPS inhalation studies we decided that participants must show a minimum increase in 2 x10^9/L in peripheral blood neutrophils by 6 hours following inhalation (and the same for total WCC) in order to be considered to have adequately received LPS. Only 3 of the 8 participants randomised to receive LPS met this criterion from study group 1 (figure 3.7). As such, all subsequent data for those 5 participants showing inadequate response in peripheral blood neutrophils and total WCC were discounted as they could not reliably be attributed to either LPS or true 'Control'.



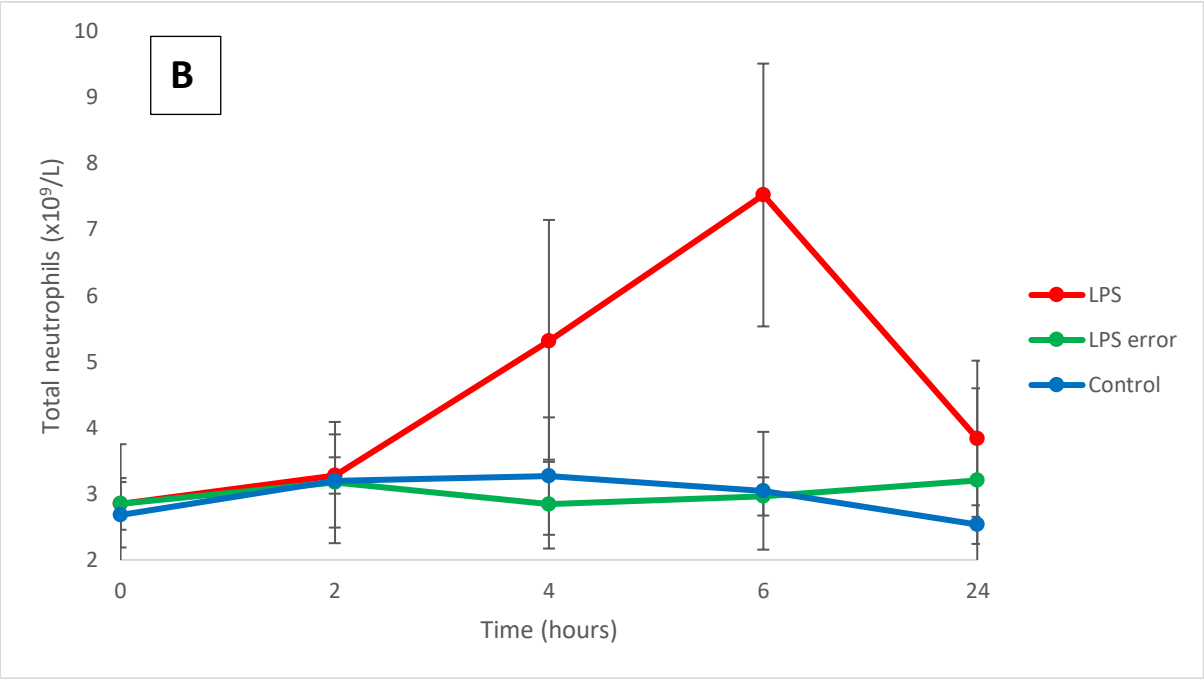


Figure 3.7 Mean change in total white cell count (A) and neutrophils (B) in peripheral blood following inhalation of LPS or Control in study group 1. Also showing the lack of response in those inadequately delivered LPS (i.e. same response as control). LPS n=3, LPS error n=5, Control n=8. Data points are means; error bars represent standard deviation.

Changes were made to the delivery of LPS for study groups 3 and 4. The LPS suspension was made up in the morning, just prior to inhalation, and kept in suspension. Changes were made to the way the inhalation kits were cleaned between participants to ensure it was not due to cleaning product residue inactivating the LPS. I took responsibility for making up the LPS or saline and was no longer 'blinded' to inhalation in order to examine the FBC results at the end of each participants involvement. This allowed any further problems to be picked up immediately and confirmed the expected response in peripheral blood neutrophils and total WCC according to inhalation and resulted in no further loss of data.

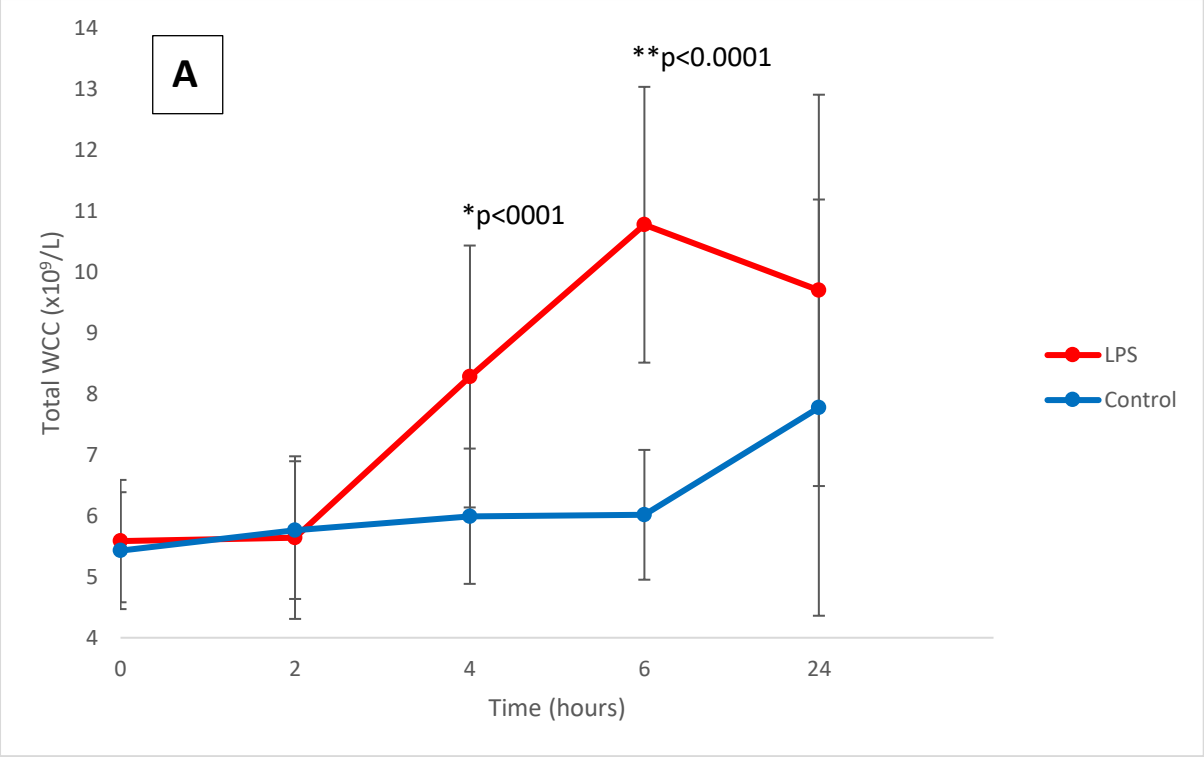
A further 26 volunteers were randomised and received LPS or saline (1:1 ratio, 13 in each group). All subsequent participants met the criteria for appropriate peripheral blood response to LPS. With the addition of the reliable data from study group 1 this resulted in LPS n= 16 and Control n=21 for all consequent functional assays. Figure 3.8 demonstrates

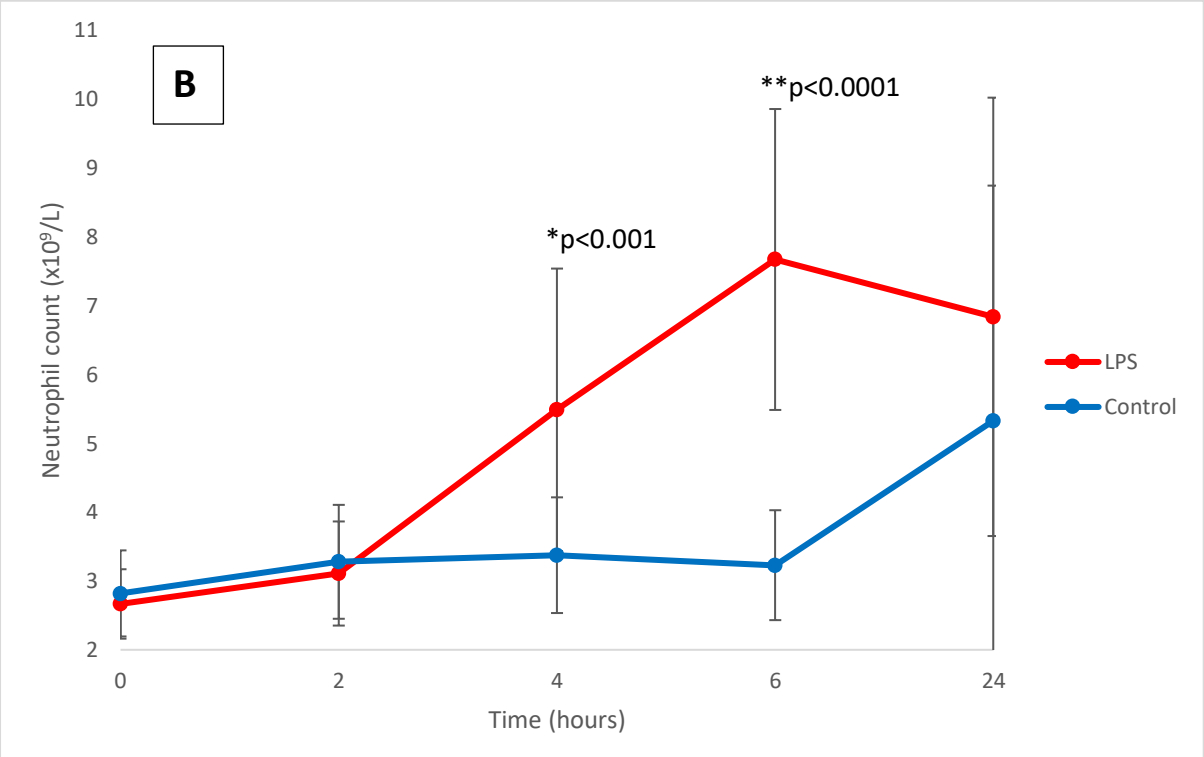
mean change in total WCC and neutrophils in peripheral blood in response to LPS and Saline.

Mean total WCC in those inhaling LPS rose from 5.59 $x10^9$/L (± 1.00) at baseline to 10.78 $x10^9$/L (± 2.26) at 6 hours following inhalation ($p < 0.001$, paired samples t-test). In Controls, total WCC did not significantly change between baseline and 6 hours following inhalation (mean 5.43 $x10^9$/L ± 0.96 at baseline and 6.02 $x10^9$/L ± 1.07 at 6 hours). At baseline and 2 hours following inhalation there was no significant difference in total WCC between inhalation groups. There was a significant difference in total WCC observed at 4 hours ($p<0.001$ independent samples t-test) and 6 hours ($p<0.0001$, independent samples t-test).

The same pattern was observed for peripheral blood neutrophils. Mean blood neutrophil count rose from 2.67 $x10^9$/L (± 0.50) at baseline to 7.67 $x10^9$/L (± 2.19) at 6 hours following inhalation with LPS ($p<0.0001$, paired samples t-test). Blood neutrophil count did not significantly change in those inhaling control (mean 2.82 $x10^9$/L ± 0.63 at baseline and 3.23 $x10^9$/L ± 0.80 at 6 hours). There was a significant difference in blood neutrophil count at 4- and 6-hours following inhalation according to inhalation group ($p<0.001$ at 4 hours and $p<0.0001$ at 6 hours, independent sample t-test).

Figure 3.8 also demonstrates that total WCC and neutrophils have increased in participants at 24 hours following inhalation of control and have not fully resolved to baseline in those inhaling LPS. Most of the participants also underwent bronchoscopy and BAL following blood taking at 6 hours (13 of 16 in LPS group and 13 of 21 in Control group). This procedure itself, is known to cause minor blood neutrophilia (and thus changes in total WCC), as shown in previous studies (Huang et al, 2006).





Figures 3.8 Mean change in total white cell count (A) and neutrophils (B) in peripheral blood following inhalation of LPS or Control in all study groups. LPS n=16, Control n=21. Error bars represent standard deviation.*p<0.001, **p<0.0001

3.3.2. Purity of isolated neutrophils for subsequent functional assays

Cytospins were taken following separation of PMNs by Percoll gradient. These were counted visually at light microscopy to monitor purity of cells for subsequent functional assays. Data are shown in table 3.6. Overall purity of PMNs was 96.8% and this was comparable across inhalation group and all time points.

	Time following inhalation (hours)			
	0	**6**	**24**	**Total**
LPS	97.1 (1.8	98.0 (1.1)	96.9 (0.8)	97.3 (1.5)
Control	96.6 (1.2)	97.0 (1.3)	96.4 (1.0)	96.6 (1.2)
Total	96.7 (1.4)	97.3 (1.3)	96.5 (1.0)	**96.8 (1.3)**

Table 3.6 Percentage purity of neutrophils by cytospin analysis following separation by Percoll gradient. Data represents means (standard deviation)

3.3.3. Phagocytosis of zymosan by adhered neutrophils

A larger volume of blood was taken at baseline (time 0), 6 hours and 24 hours following inhalation in all participants. This was to undertake separation of blood neutrophils for assessment of functional assays. Percentage phagocytosis of zymosan by adhered neutrophils was a key outcome measure of the study, with the hypothesis that inhaled LPS may 'activate' peripheral blood neutrophils and result in higher rates of phagocytosis. Table 3.7 and figure 3.9 compare phagocytosis of zymosan by adhered neutrophils at each time point, by inhalation group.

	Time (hours) following inhalation		
	0	**6**	**24**
LPS	85.6% (5.1)	89.2% (4.4)	87.3% (7.1)
Control	86.1% (3.5)	87.3% (3.4)	85.7% (6.2)

Table 3.7 Phagocytosis of zymosan by adhered neutrophils by inhalation group over time. Data represents mean percentage phagocytosis (standard deviation) for LPS n=15, Control n=15, p > 0.05 independent t-test at all time points.

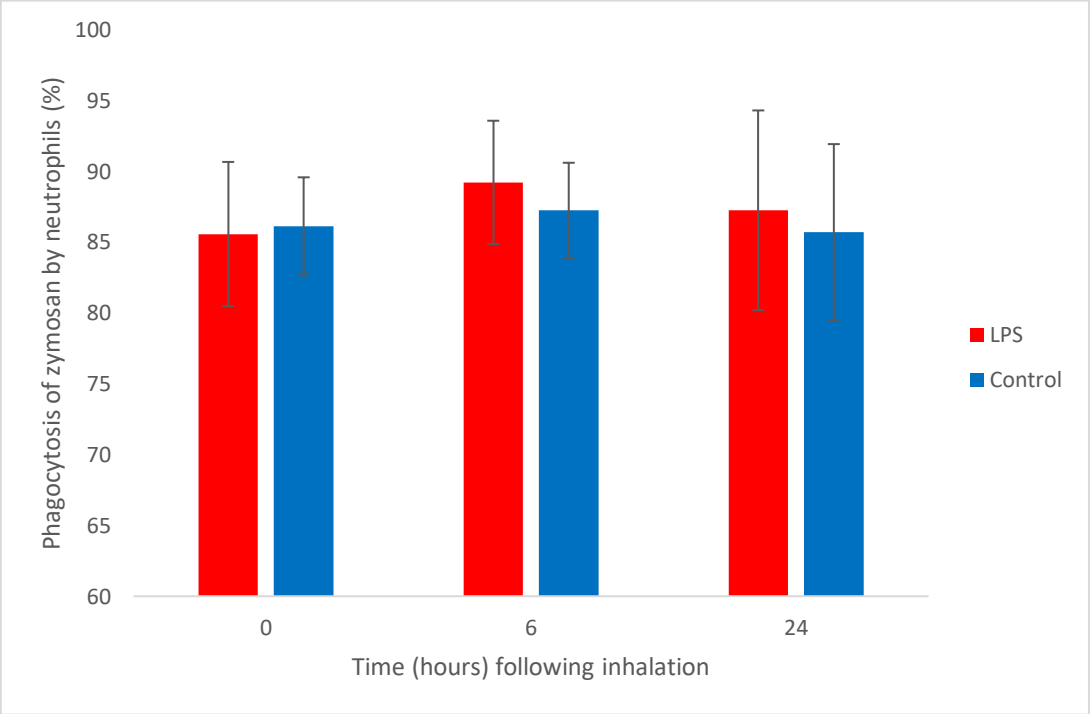


Figure 3.9 Phagocytosis of zymosan by adhered neutrophils by inhalation group over time. Data represents mean percentage phagocytosis; error bars represent standard deviation. LPS n=15, Control n=15, p > 0.05 independent t-test at all time points.

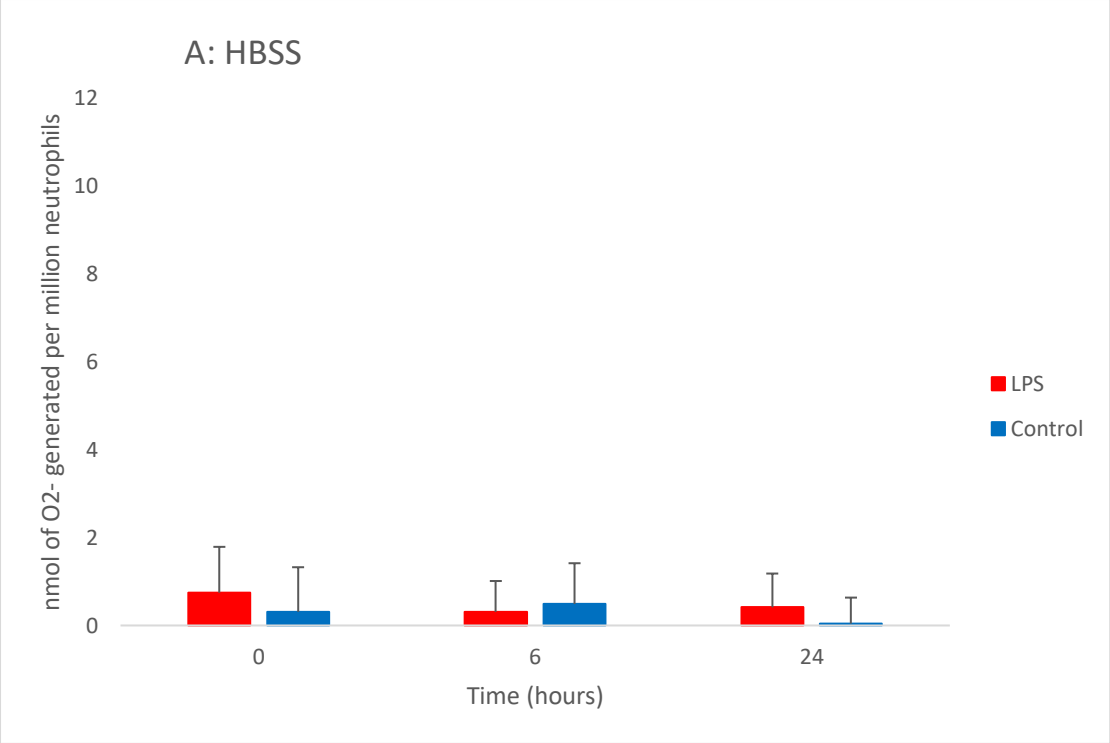
Baseline (time 0) phagocytosis of zymosan by neutrophils was high in both groups (mean 85.6% LPS versus 86.1% Control). This was expected and is in keeping with healthy volunteers' populations of circulating neutrophils. There were no differences observed between LPS and Control groups at baseline (p=0.73, independent t-test) or at any other time points (p=0.17 6 hours, p=0.53 24 hours). There appeared to be a slight increase in phagocytosis from baseline to 6 hours in the LPS group (mean change 3.6% ± 4.31 vs control

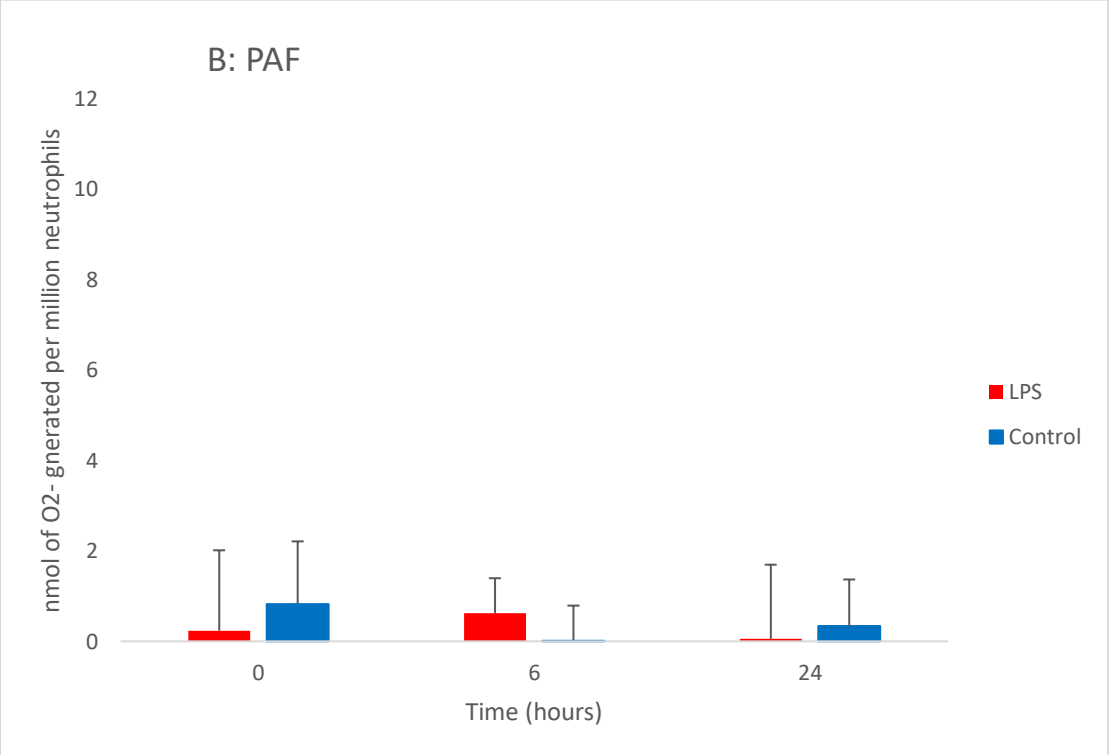
1.2% ± 2.90). Independent samples t-test revealed p = 0.058 suggesting a possible trend toward change from baseline in those inhaling LPS against controls in this small sample size (n=15 in each group).

3.3.4. Superoxide anion release by neutrophils in suspension

Neutrophils separated from whole blood at baseline (time 0), 6 hours and 24 hours following inhalation were also used to measure superoxide anion release by neutrophils suspended in HBSS. Cells were left in HBSS alone, 'primed' with PAF, 'stimulated' with fMLP or both primed and stimulated with PAF and fMLP together. This was done for all those undergoing LPS and saline inhalation in groups 1 and 3 (n=11 LPS, n=16 control) at each time point. Figure 3.10 shows superoxide release for LPS and saline groups for each experimental condition over time.

Neutrophils left in HBSS alone showed low level release of superoxide anion in all volunteers (Figure 3.10, Panel A:HBSS) suggesting very little 'artificial' activation from the process of blood taking or separation by Percoll gradient. The pattern of changes within neutrophils was as expected for both groups with a minor increase in superoxide release in response to PAF, moderate increase in response to fMLP and the greatest increase in response to both.


A: HBSS
nmol of O2- generated per million neutrophils
12
10
8
6
4
2
0
0
6
24
Time (hours)
LPS
Control


B: PAF
nmol of O2- gnerated per million neutrophils
12
10
8
6
4
2
0
0
6
24
Time (hours)
LPS
Control

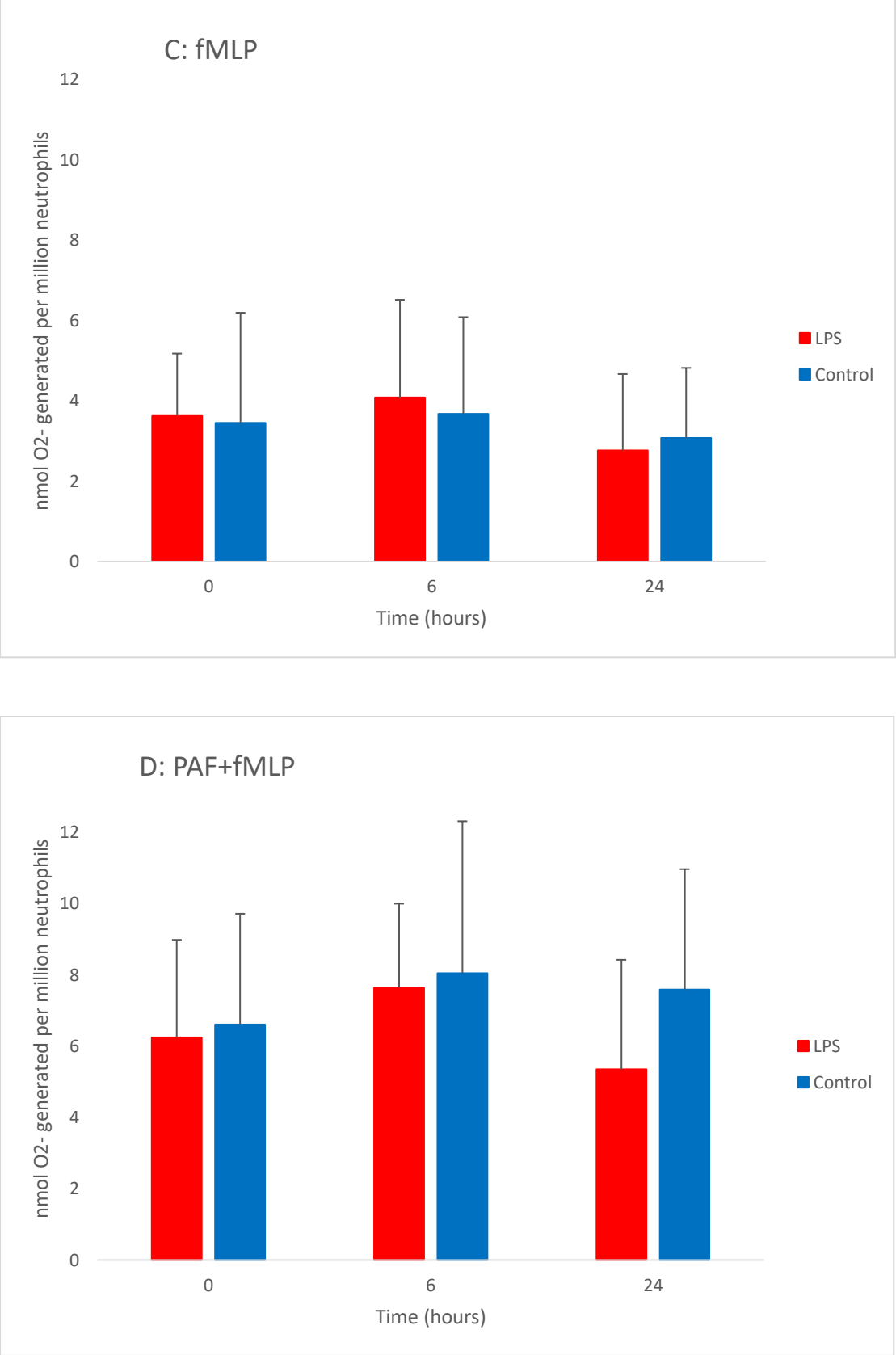


Figure 3.10 Superoxide anion release by neutrophils in suspension, by inhalation group, over time. A: HBSS, B: PAF, C: fMLP, D: PAF+fMLP. Data represents mean and error bars represent standard deviation.

A series of independent t-tests was conducted. There were no significant differences between LPS and saline at baseline or any other time points for any experimental condition. There looked to be a possible difference between LPS and saline at 6 hours in cellular response to PAF (figure 3.10, panel B), p=0.054 (independent t-test). But this was not supported by any suggestion of difference between groups in response to fMLP (p=0.671) or PAF+fMLP (p=0.751).

Using the participant as their own control it is possible to look at change from baseline to 6 hours following inhalation (maximum expected time point for effect of LPS) and compare LPS and Control. Table 3.8 summarises the mean change from baseline to 6 hours following inhalation of LPS and Saline under each experimental condition. Independent sample t-tests were used to determine any differences between groups. Results demonstrated the same pattern with a borderline difference at 6 hours in response to PAF, which was not corroborated by any suggested difference between groups in the other experimental conditions.

	LPS	Control	t.test
HBSS	-0.435 (1.189)	0.181 (1.269)	p=0.217
PAF	0.388 (1.796)	-0.899 (1.271)	p=0.058
fMLP	0.455 (2.118)	0.156 (1.520)	p=0.695
PAF+fMLP	1.393 (2.419)	1.261 (2.685)	p=0.897

Table 3.8 Change in superoxide anion generation between baseline and 6 hours, by inhalation group, under different experimental conditions. Data represents mean change (standard deviation), with results of independent sample t-tests.

3.3.5. Cell surface marker expression of neutrophils measured by flow cytometry

Neutrophil cell surface expression of CD11b, CD62L and CD88 was measured on samples from whole blood. The tests were repeated at baseline (t=0), 6 hours and 24 hours following inhalation of either LPS or saline (control). Neutrophils were identified by forward scatter and side scatter characteristics and gated appropriately. Median fluorescence of gated neutrophils was recorded for each antibody and compared over time for each participant. Figures 3.11 and 3.12 show example plots for a Control and LPS subject respectively.

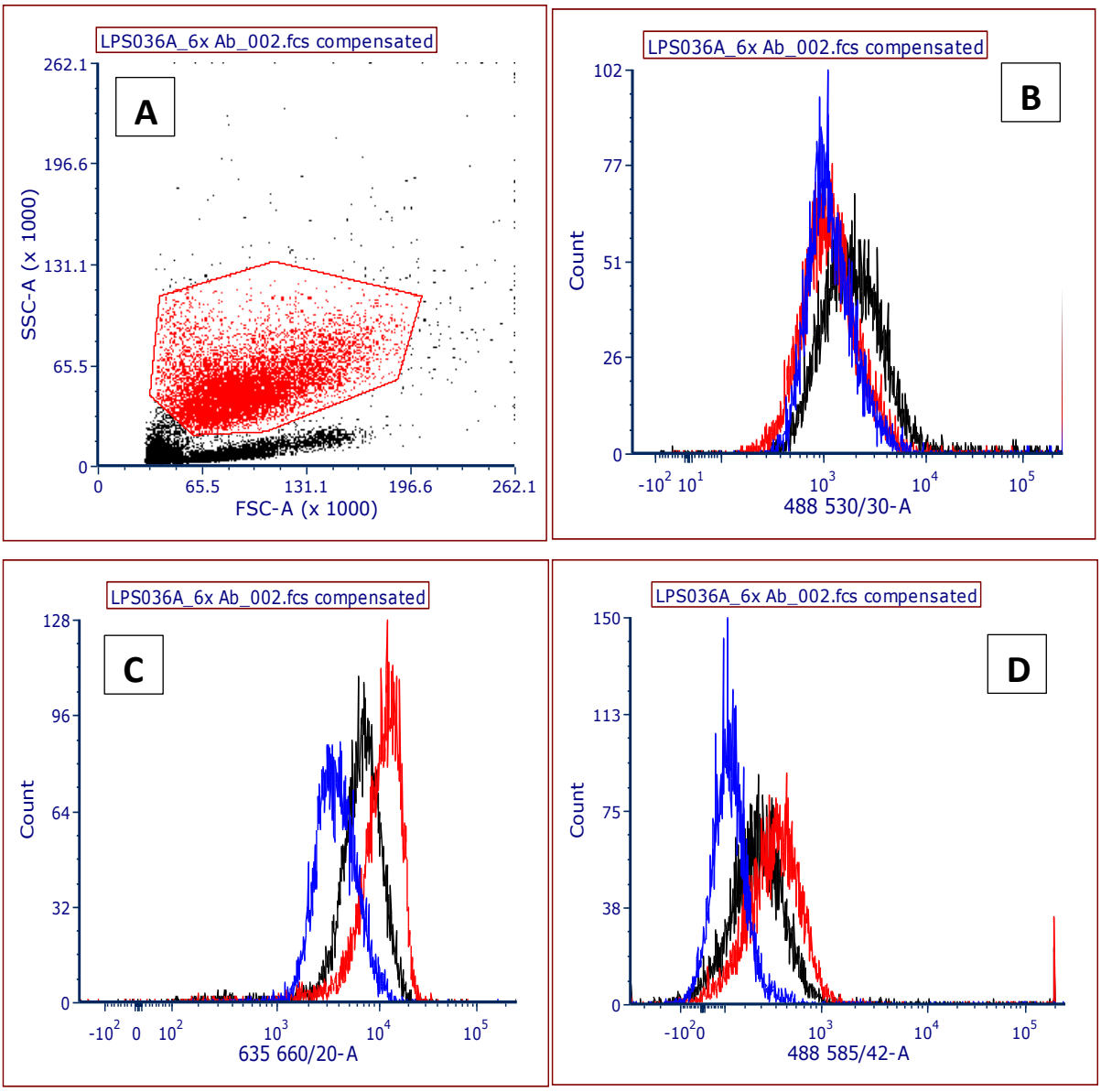


Figure 3.11 Expression of CD11b, CD62L and CD88 on neutrophils assessed by flow cytometry. A: Neutrophils were identified by forward scatter (FSC-A) and side scatter (SSC-A) characteristics. B: Neutrophil expression of CD11b (488 530/30) C: Neutrophil expression of CD62L (635 660/20) D: Neutrophil expression of CD88 (488 585/42) . Black = baseline, red = 6 hours post inhalation, blue = 24 hours post inhalation. All traces, LPS036 (Control).

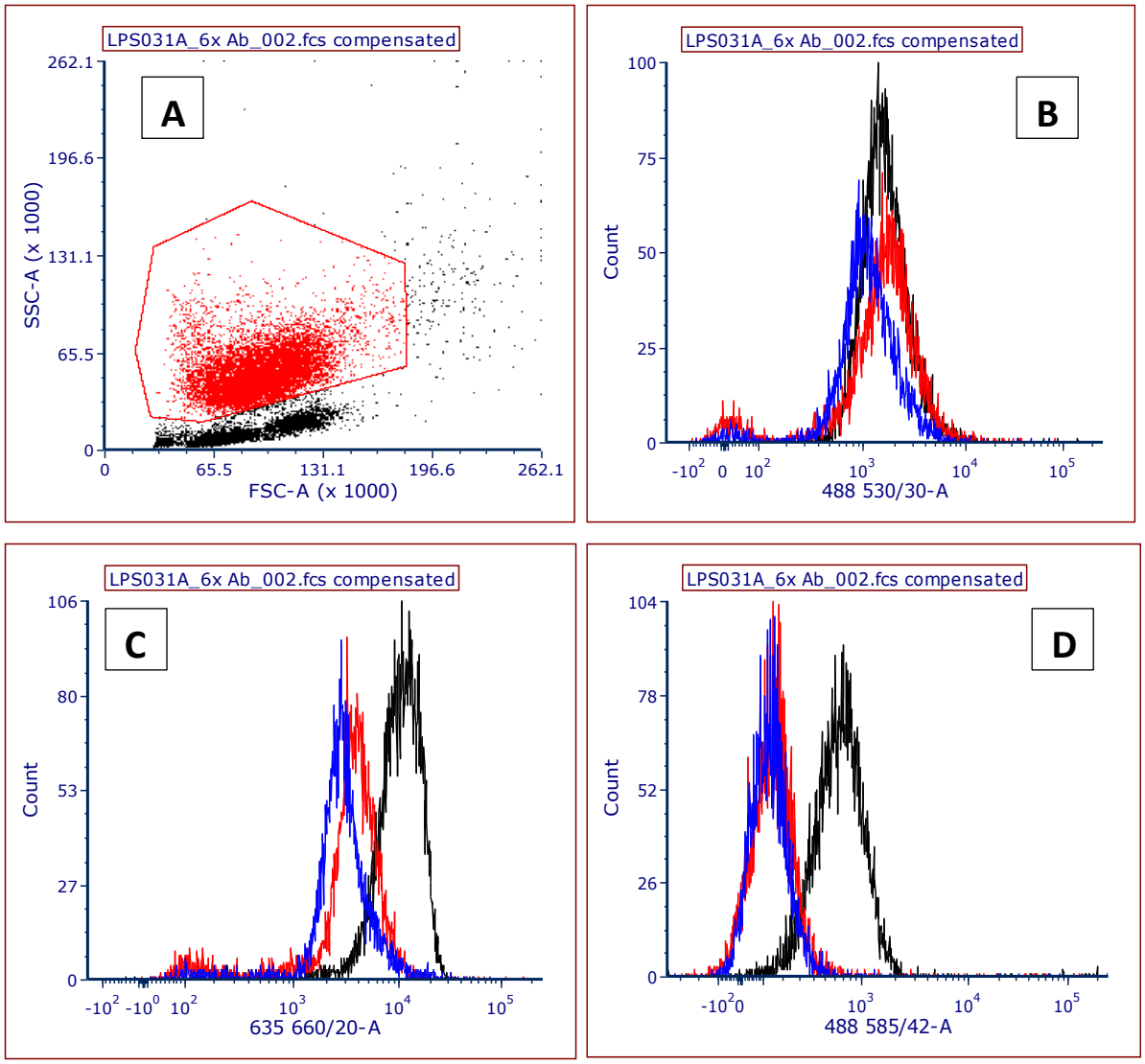
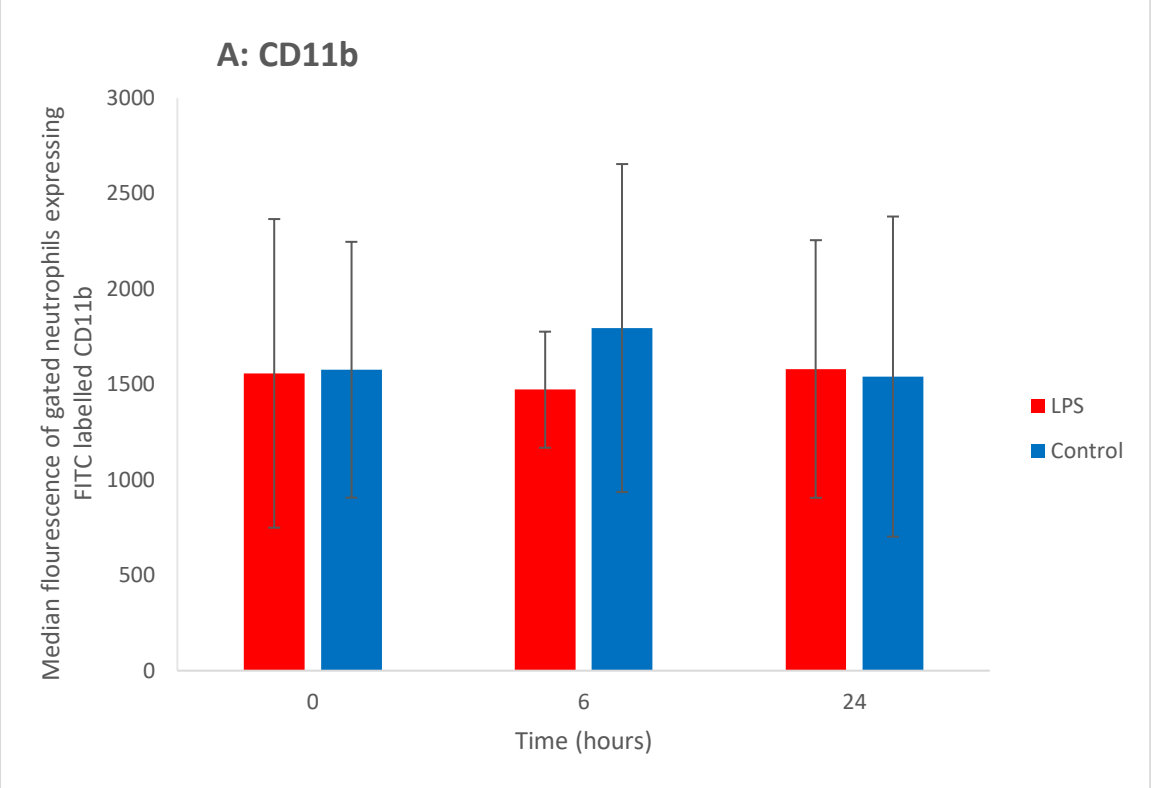
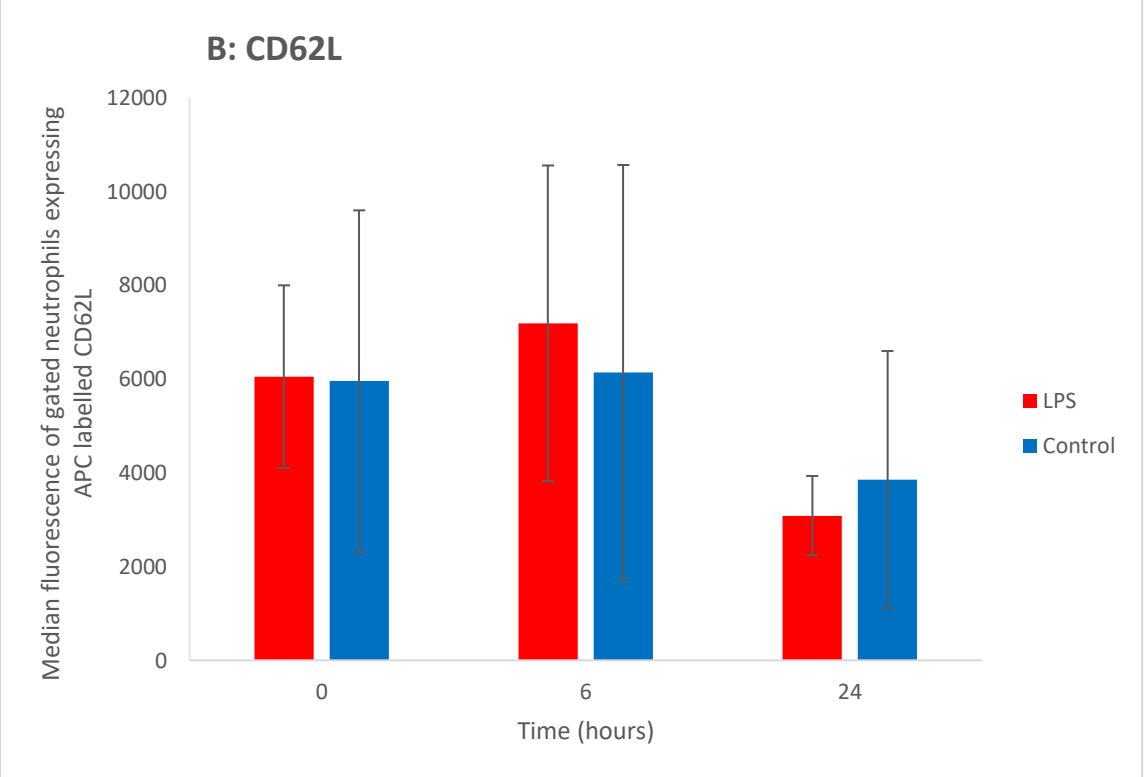


Figure 3.12 Expression of CD11b, CD62L and CD88 on neutrophils assessed by flow cytometry. A: Neutrophils were identified by forward scatter (FSC-A) and side scatter (SSC-A) characteristics. B: Neutrophil expression of CD11b (488 530/30) C: Neutrophil expression of CD62L (635 660/20) D: Neutrophil expression of CD88 (488 585/42) . Black = baseline, red = 6 hours post inhalation, blue = 24 hours post inhalation. All traces LPS031 (LPS subject).

Data were then analysed according to inhalation group. Mean and standard deviation of fluorescence at each time point was calculated by LPS or saline inhalation group. This was repeated for each antibody. Data was obtained from participants in study groups 1 and 3 (final data represents n=9 LPS, n=14 Saline) and results are presented in figure 3.13 (panels A, B and C). Independent samples t-tests were used to look for any difference between groups. There were no significant differences between LPS and saline at baseline, 6 hours or 24 hours for any antibody expression.


A: CD11b
Median flourescence of gated neutrophils expressing FITC labelled CD11b
3000
2500
2000
1500
1000
500
0
0
6
24
Time (hours)
LPS
Control


B: CD62L
Median fluorescence of gated neutrophils expressing APC labelled CD62L
12000
10000
8000
6000
4000
2000
0
0
6
24
Time (hours)
LPS
Control

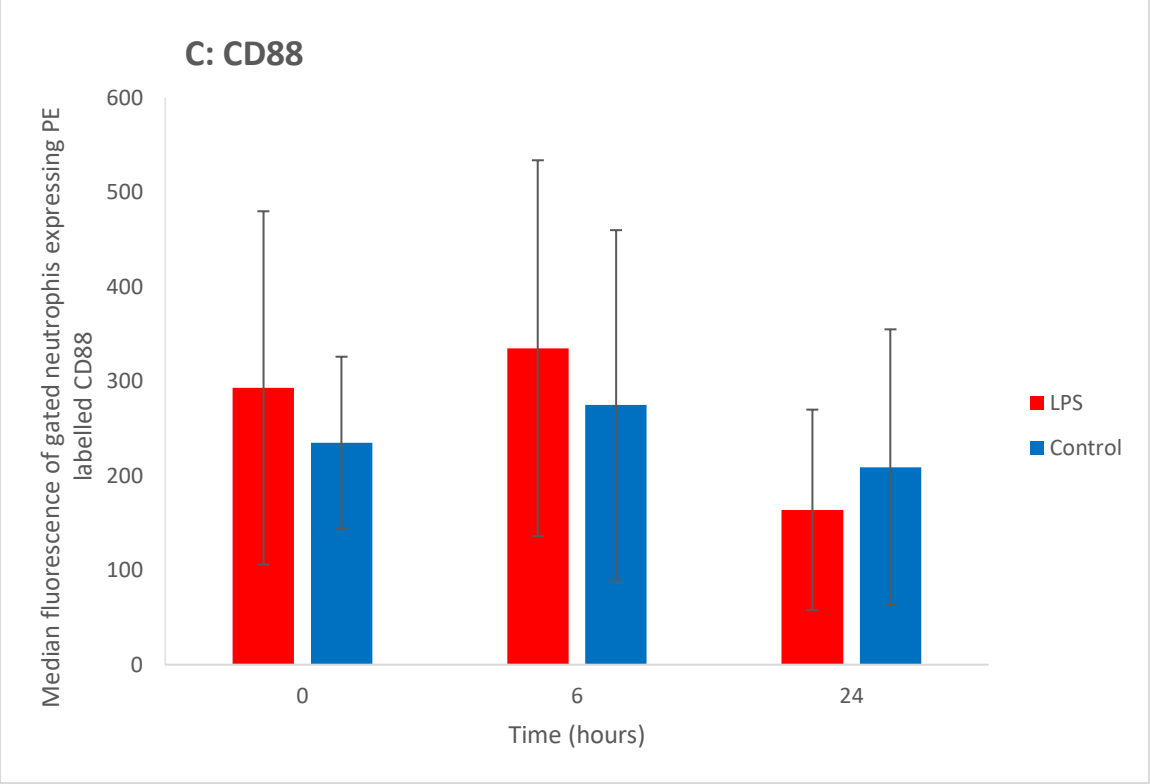


Figure 3.13 Antibody expression of gated neutrophils by inhalation group over time. A: FITC labelled CD11b, B: APC labelled CD62L, C: PE labelled CD88. Bars represent mean of median fluorescence for each group, error bars represent standard deviation. LPS n=9, Control n=14

3.3.6 Results of DCE MRI

Study group 3 (18 volunteers) attended an extra study visit on a day prior to inhalation to undergo a 'baseline' DCE MRI scan. Immediately after the 6-hour blood sampling following inhalation of either LPS or control, the volunteer then proceeded for their repeat DCE MRI. Two volunteers only completed the baseline scan and had to withdraw prior to inhalation (see section 3.2.2, page 97). A further subject had problems with data acquisition/transfer at the time of the second scan resulting in very few images for analysis. Therefore 15 complete sets of volunteer data were analysed. The average time from inhalation to start of MRI scanning was 397mins (6hrs and 37mins ± 15mins). There was no difference for those undergoing LPS inhalation versus Control (397 ± 15mins vs 398 ± 18mins respectively, p=0.91).

3.3.6.1 T1 Mapping

Gadolinium-enhanced T1 measurements were acquired from the data. This was to be used in quantitative analysis to assess contrast agent concentration in lung tissues and define vascular permeability. It was expected that T1 measurements within a standardised ROI would be similar throughout the lung.

However, initial analysis showed that T1 measurements had high variability depending on position of the ROI and depth slice within the lung tissue. The first was likely due to respiratory motion having greater effect at the lung bases and to a certain extent was anticipated. I also found that moving from the anterior regions of the lungs to the posterior regions showed T1 measurements started at low levels, then demonstrated two periods of increased measurements and two subsequent decreases (figure 3.14). The highest measurement of T1 was almost always in slices also encompassing the heart. The lowest mean value measured for T1 was 800 ± 382ms and the peak was 2982 ± 1137ms.

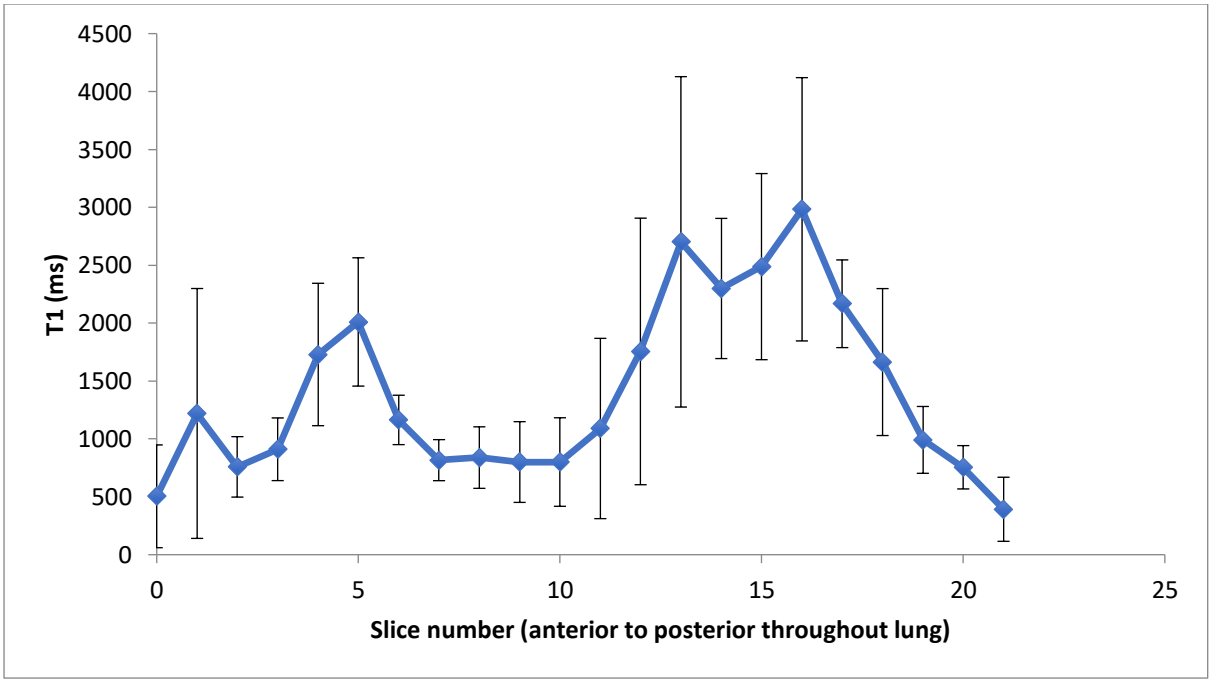


Figure 3.14 Variance in T1 measurement as move anteriorly to posteriorly through the lungs. Data points represent means, error bars represent standard deviation (original analysis by Emily Fitzgerald, BSc Biomedical Sciences 2015).

Since T1 should be uniform throughout the lungs, it was hypothesised that an MRI image artefact was affecting the T1 measurements. Data suggested that signal from surrounding fat from outside the measurement field (subcutaneous and mediastinal) was folding inwards and contaminating signal in the lung images. In support of this theory, the T1 lung signal in female volunteers was higher than in men (1956 vs 1652 ms, p = 0.027). This supports the theory due to the presence of additional subcutaneous fat in breast tissue in female volunteers.

It is possible to suggest the areas of the lungs likely to be most and least affected by surrounding areas of subcutaneous fat deposits. The T1 measurements were expected to be in the range of 400-800ms (personal communication Dr Pete Thelwall). Examining areas of the lung with least chance of contamination revealed mean T1 values of 534 and 505ms (females and males respectively), further supporting the theory of corruption of the T1 measurements by surrounding subcutaneous fat. The mean difference between females and males in the least contaminated lung T1 measurements was found to be statistically insignificant (p=0.441, figure 3.15).

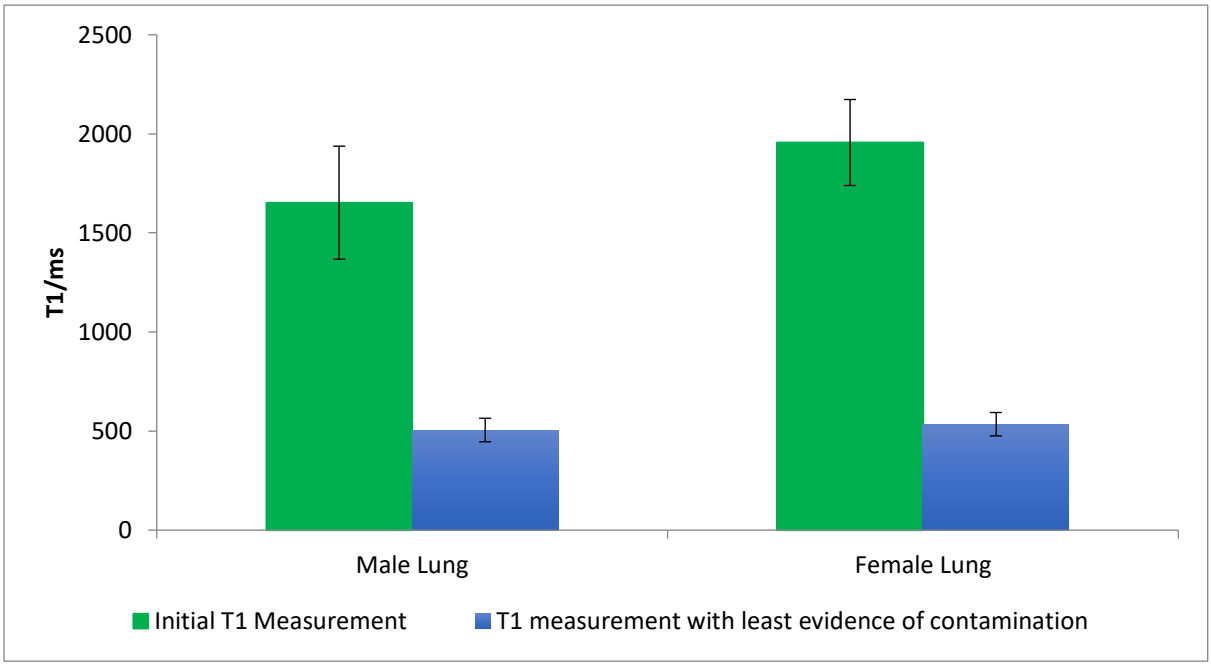


Figure 3.15 T1 value by sex, comparing overall mean and measurements taken from slices with least signal contamination. Bars represent mean, error bars represent standard deviation (original analysis by Emily Fitzgerald).

Therefore, the T1 measurements available in the dataset had shown that they could not be used within the kinetic model with confidence as an individual's results were likely to be confounded by variable artefact from surrounding fat.

An alternative approach to allow quantitative modelling would be to use a fixed T1 measurement in DCE MRI analysis. The potential advantage of this included the ability to acquire quantitative kinetic parameters for the data to enable comparison between datasets. However, using a fixed T1 may not accurately represent the true T1 and therefore might not yield representative results. Therefore, it was felt that any data acquired using this method could result in inaccurate or misleading results (high risk of type 1 error) and this approach was abandoned.

3.3.6.2. Arterial input function (AIF)

A ROI was drawn over a major vessel (pulmonary artery) for each volunteer at each study visit to measure AIF. This signal was charted over time. It shows peaks at the first, second and third pass of contrast agent through the vasculature and then a 'wash out' period. Figure 3.16 shows typical curves for an individual Control and LPS subject at study visit 1 and 2 (pre and post inhalation).

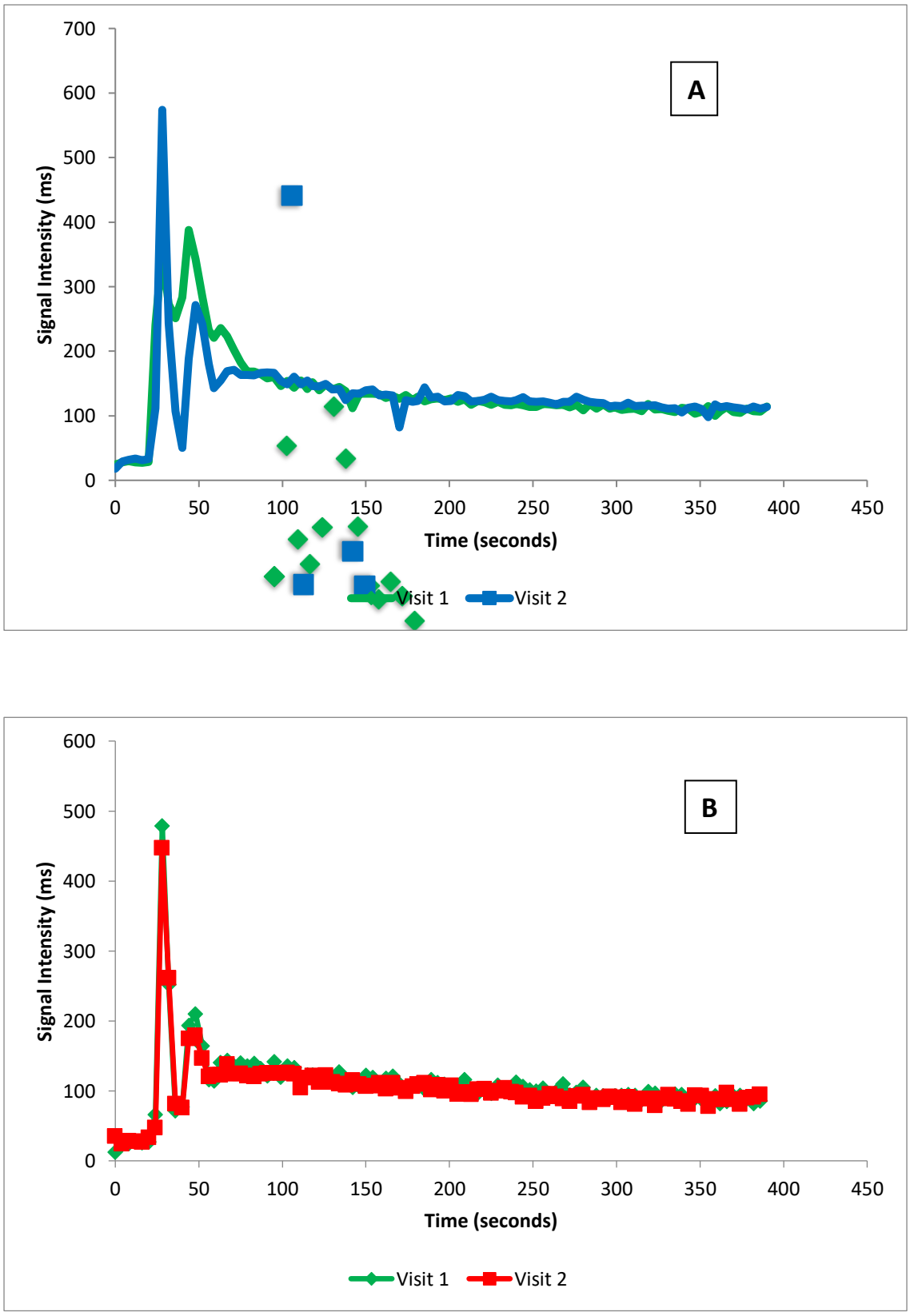
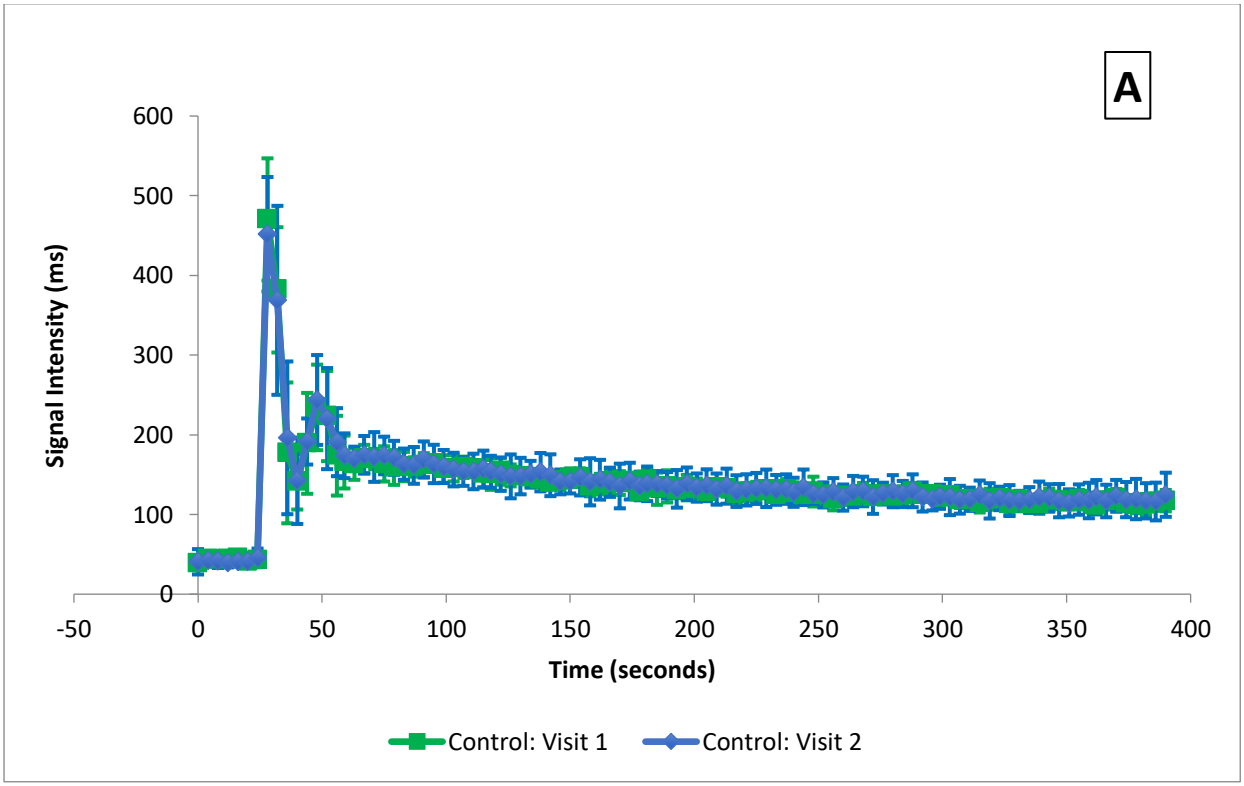
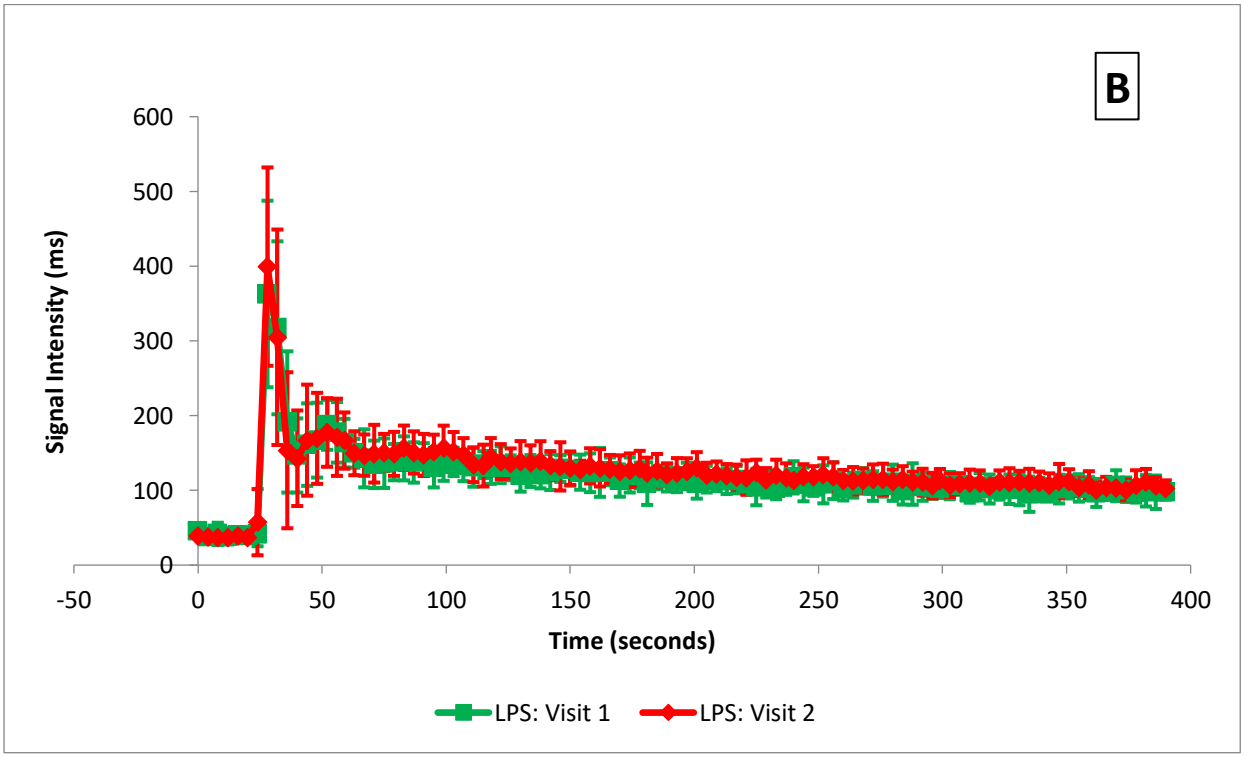


Figure 3.16 Change in signal intensity over major artery in a single volunteer inhaling Control (A) and LPS (B) measured at DCE MRI

The mean signal intensity for the control and LPS groups was calculated and plotted over time and is shown in figure 3.17. T-tests showed no significant difference in signal intensity for AIF in the control or LPS groups, either pre- and post-inhalation or between study visits.





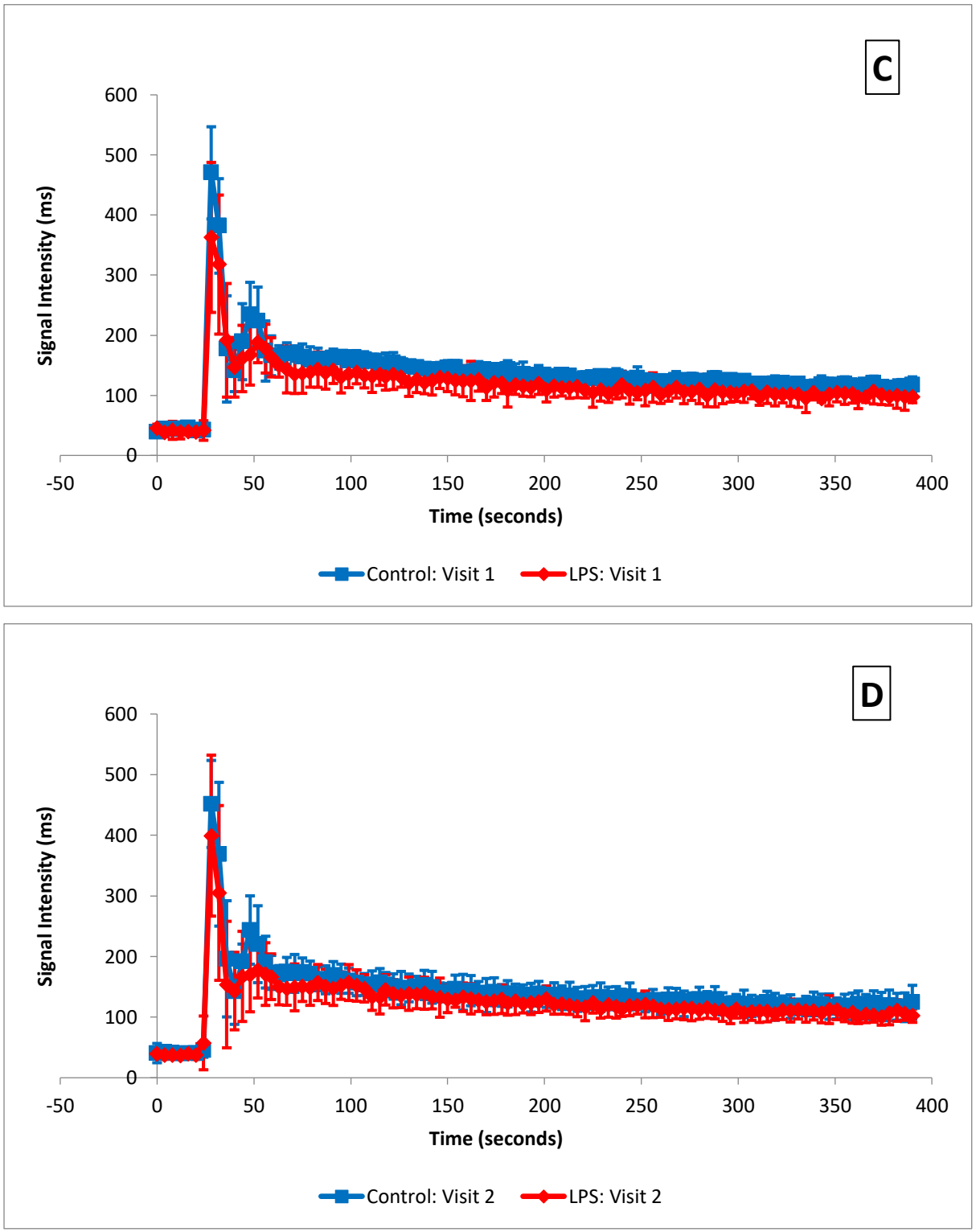
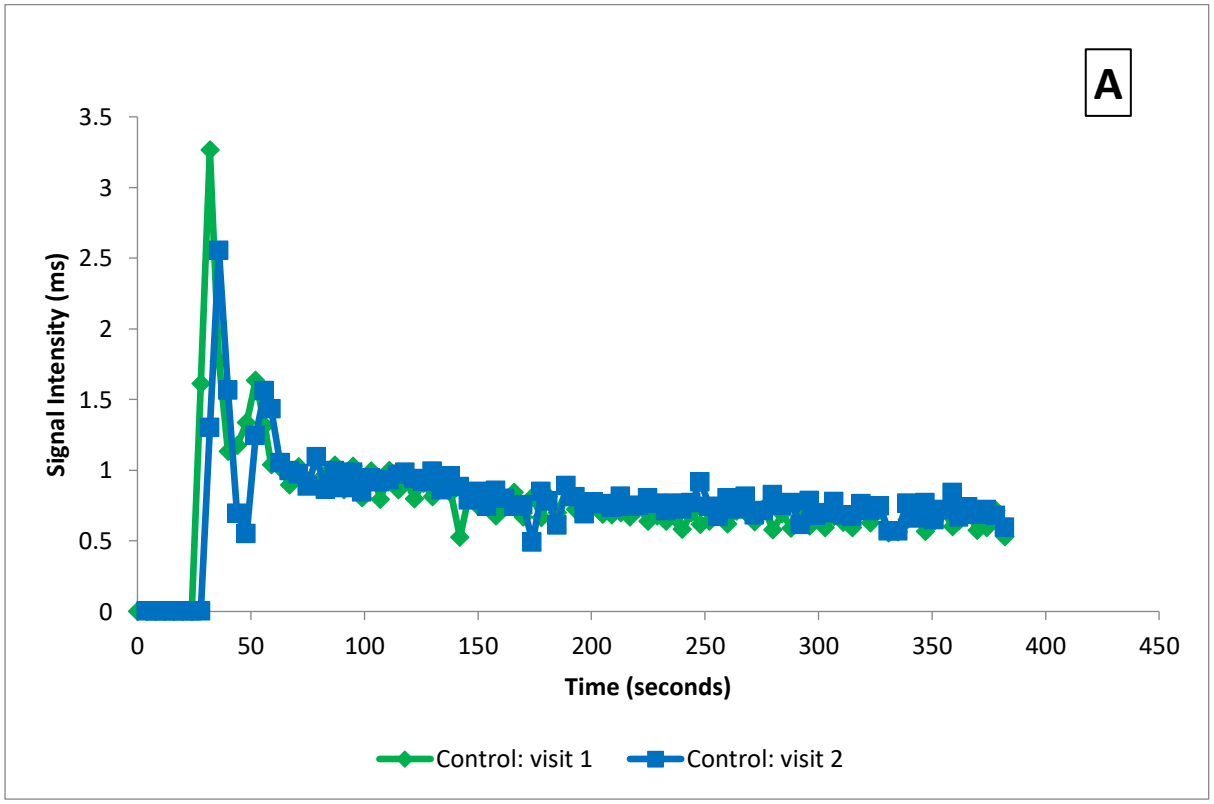


Figure 3.17 Signal intensity for an arterial ROI over time by different groups. Data points represent mean, error bars show standard deviation. A: Comparing pre- and post-saline inhalation (no difference). B: Comparing pre- and post-LPS inhalation (no difference). C: Comparing Control and LPS groups at baseline (no difference) and D: Comparing Control and LPS groups post-inhalation (no difference. LPS n=7, Saline n=8).

Due to the signal intensity being much greater in the arterial ROI it is likely that these images were less affected by artefact from surrounding adipose tissue. Although the AIF data could be used in the model, concerns over accuracy of the measured T1 and likelihood that fixed T1 would result in false outcomes it was decided that the original kinetic model could not be applied to the dataset. Therefore, a decision was made to look at the raw data to see if any change was visible in association with inhalation of LPS prior to any parametric analysis.

3.3.6.3 Raw data analysis of lung parenchymal signal

A ROI was drawn in the same area for each volunteer (right apex of lung) on a slice selected that had the least evidence of signal contamination. The raw data were taken and plotted over time. Each volunteer had their visit 1 and 2 data (pre- and post-inhalation) compared and a three-period moving average was taken for each visit to account for noise, and comparisons were made. Results are shown in figure 3.18.



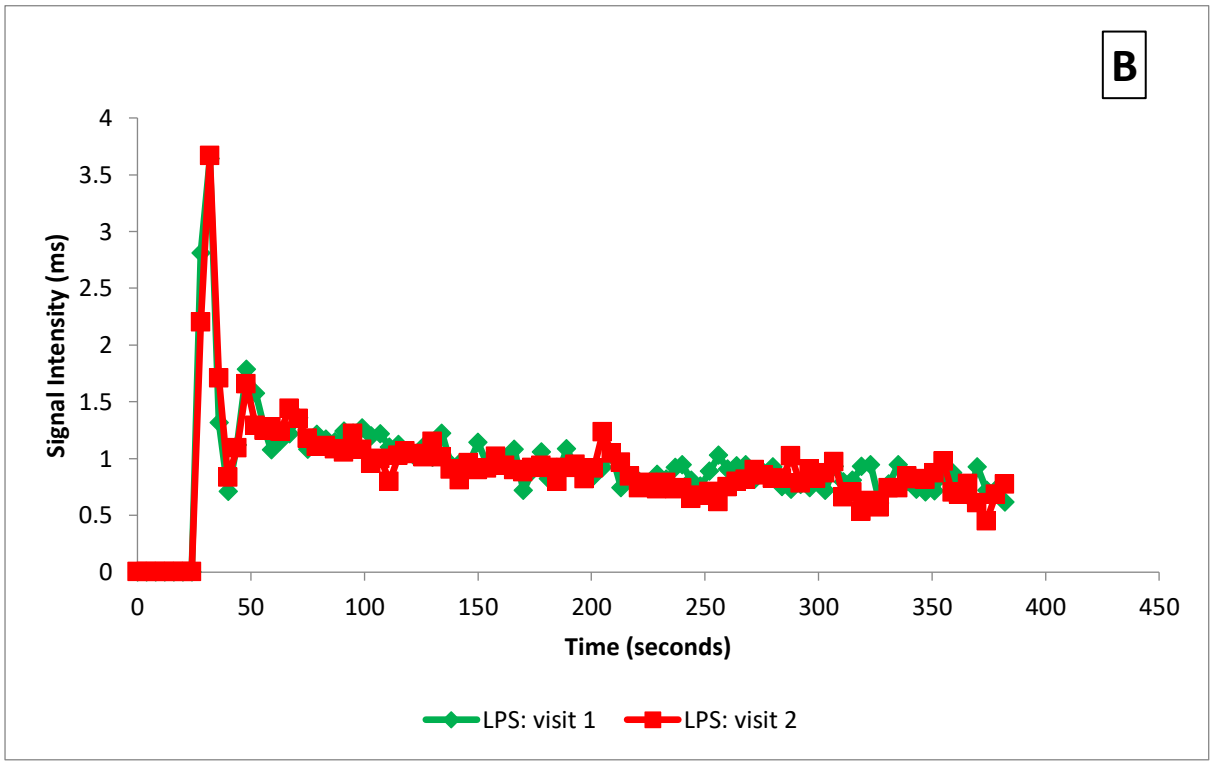
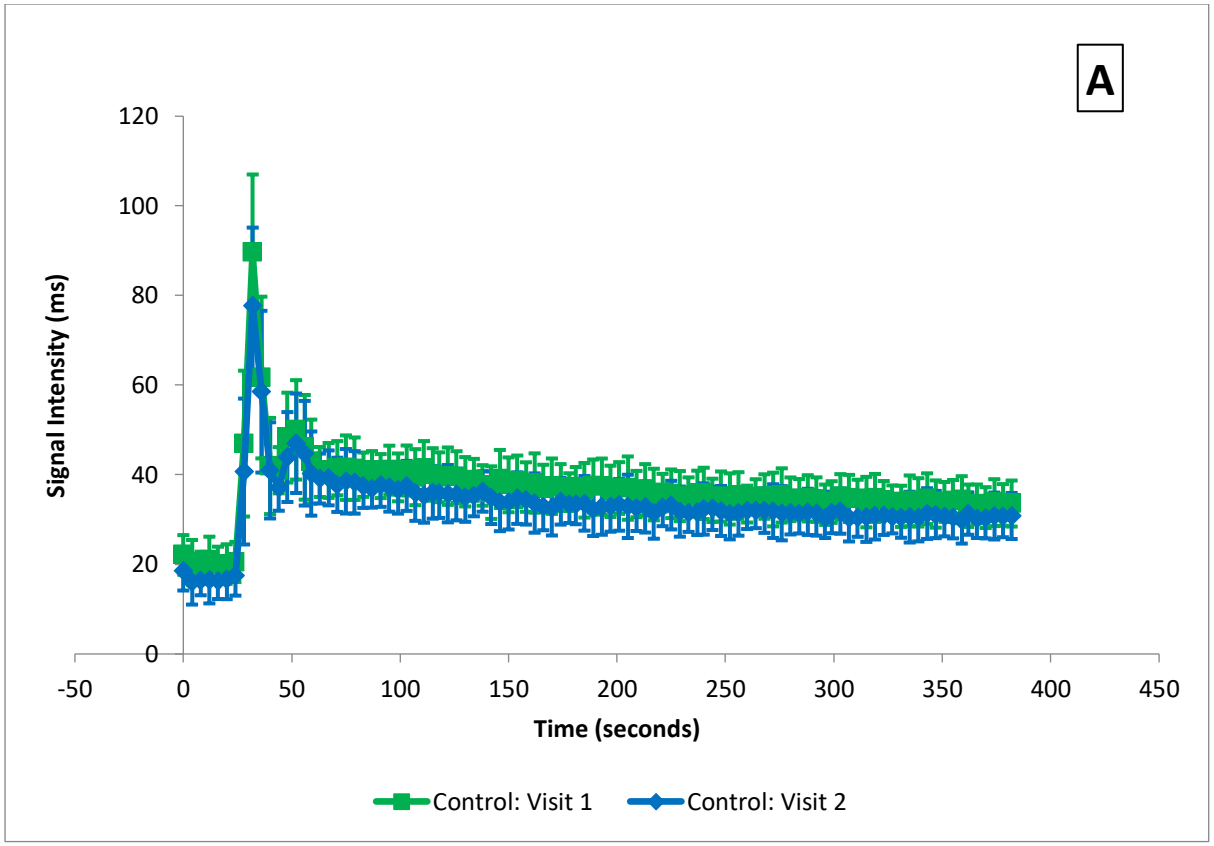


Figure 3.18 Example plots showing raw data DCE signal intensity over ROI of lung tissue. Single participant inhaling (A) Control and (B) LPS

Mean DCE signal intensity was plotted by study group and compared pre- and post-inhalation and at each study visit by inhalation group (figure 3.19). There was a small non-significant difference between Control and LPS at study visit 1 (36.74 ± 5.89 and 31.33 ± 7.46 respectively p=0.054, independent t-test). Both groups had lower mean signal intensity at visit 2 (Control 33.33 ± 8.93 and LPS 29.67 ± 7.34). Due to the higher baseline the difference was greater in the Control group than the LPS group. Mean difference between pre- and post-inhalation for the Control group was -3.40 ± 1.28 compared with -1.67 ± 1.04 for the LPS group. T-test was used to compare visits 1 and 2 in the LPS group, with a p value of 0.229 suggesting no significant difference. The greater difference occurring in the Control group also supports that any differences seen were due to the natural physiological variations in lung signal with this technique.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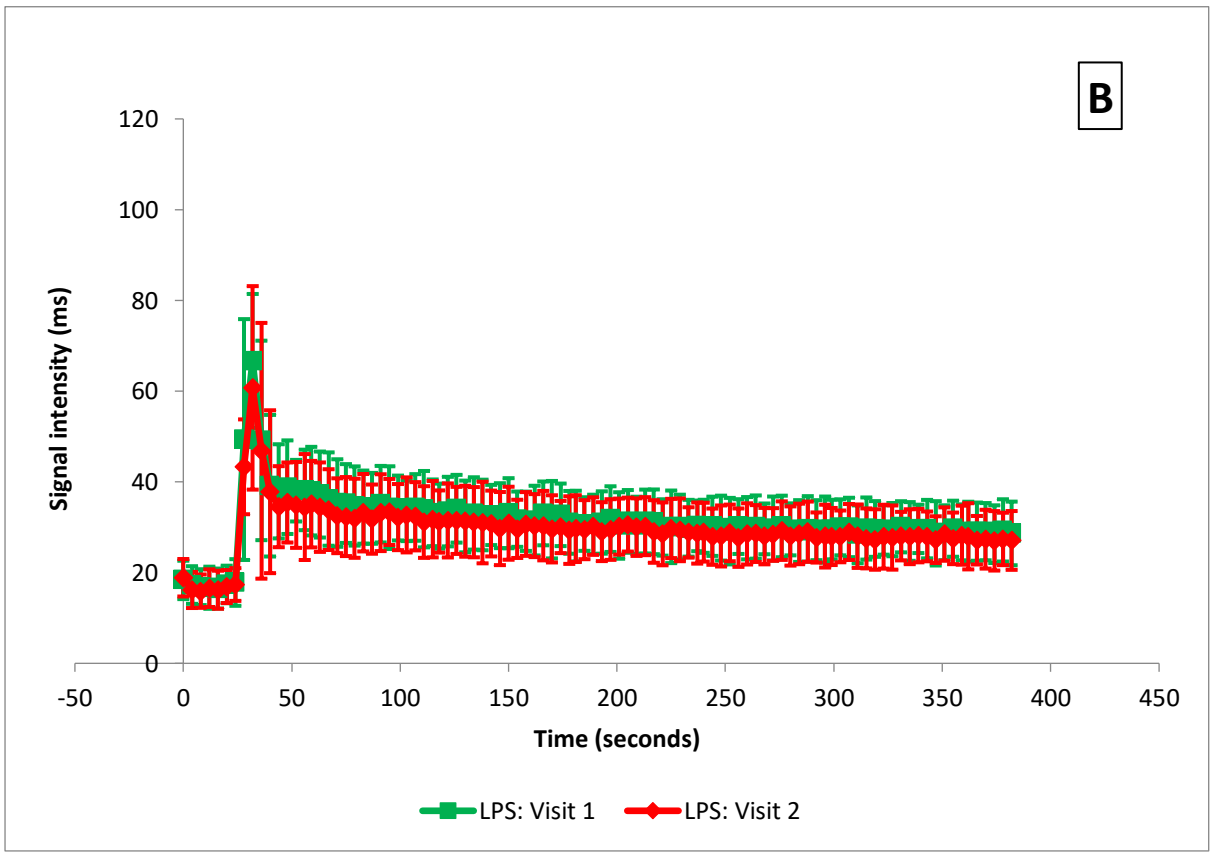
Time (seconds)
Control: Visit 1
Control: Visit 2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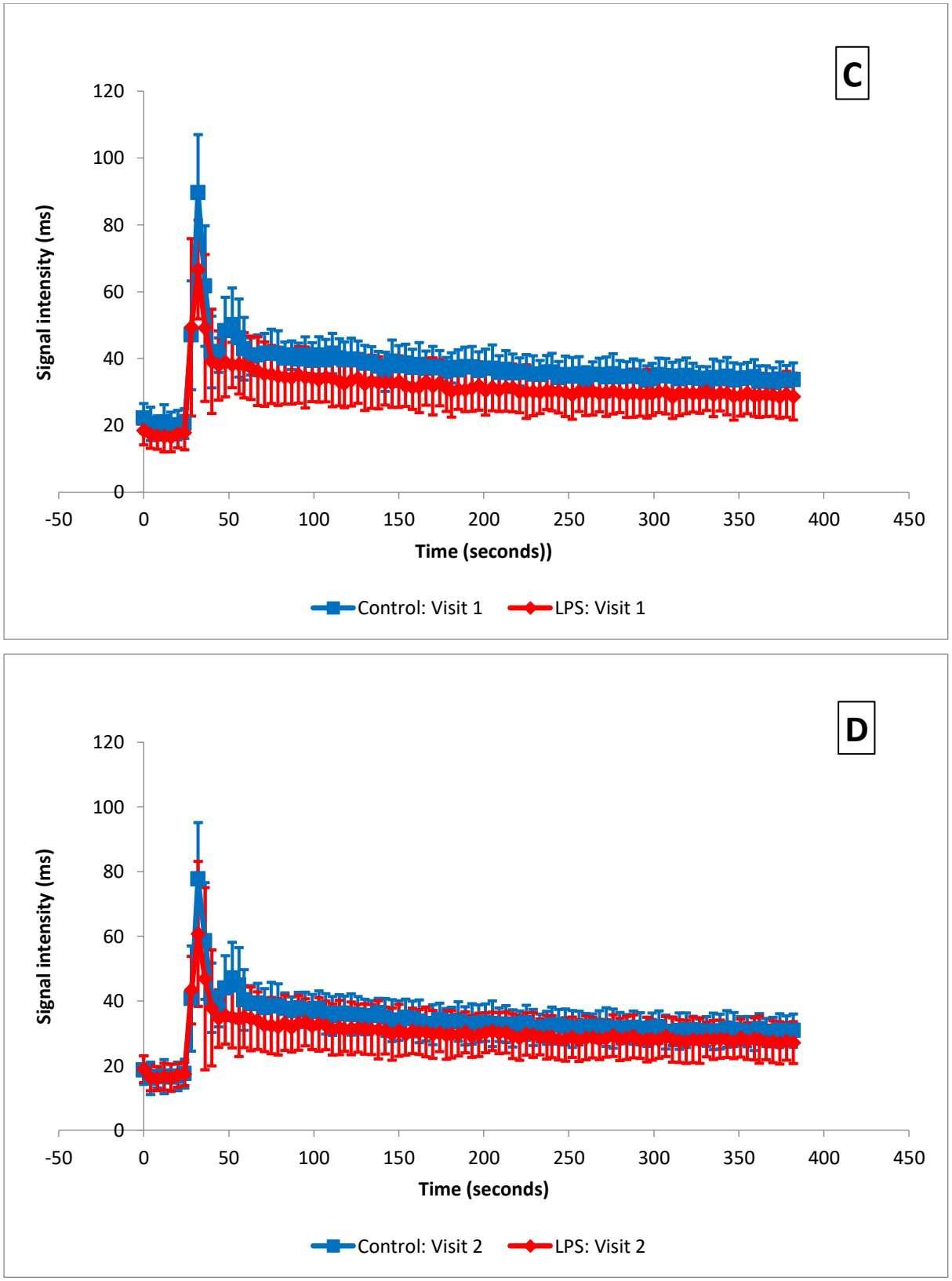
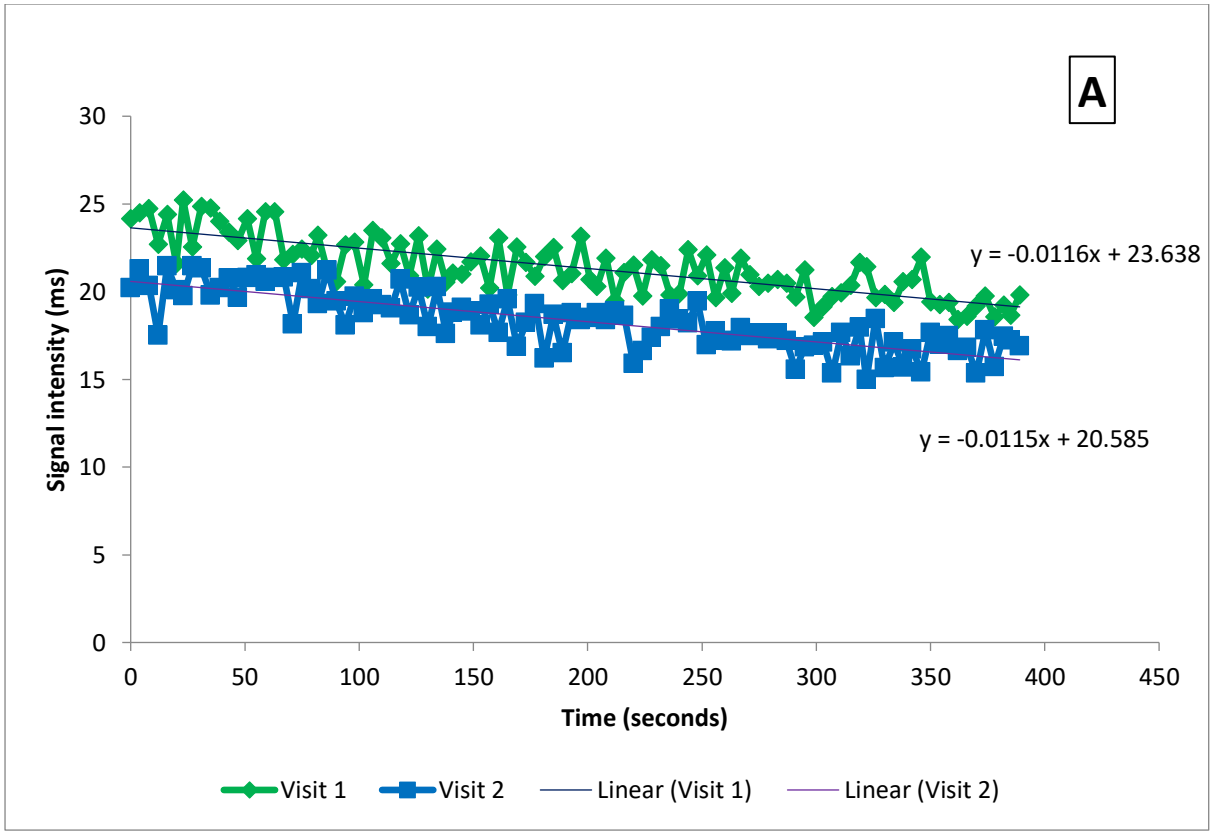


Figure 3.19 Mean signal intensity for ROI of lung tissue over time comparing (A) Pre and post inhalation in Control group; (B) Pre and post inhalation in LPS group; (C) Pre inhalational in Control and LPS groups; (D) Post inhalation in Control and LPS groups. Points represent means, error bars represent standard deviations. LPS n=7, Saline n=8.

3.3.6.4 Washout gradient

It was hypothesised that the washout curve of the LPS-treated group would demonstrate a shallower gradient than the Control group. If there is a greater degree of vascular permeability in response to LPS inhalation, a greater amount of contrast agent would be expected to enter the extracellular space and also take a longer time to leave, thus appearing brighter for longer on DCE MRI. Other studies have shown that a washout slope between 40 seconds and 5 minutes is most sensitive to differences in K*trans* and V*e* (Chen et al, 2012).

Therefore, the final 100 slices of each MRI were analysed separately to calculate the washout gradient (figure 3.20). Data was used from 80 seconds to 8 minutes, avoiding the first, second and third pass peaks. The signal intensity at the same lung ROI for both visits was found and plotted against time for each participant. The curve was treated linearly, and the equation of the line found following the equation **y = mχ + c** where m is the gradient.



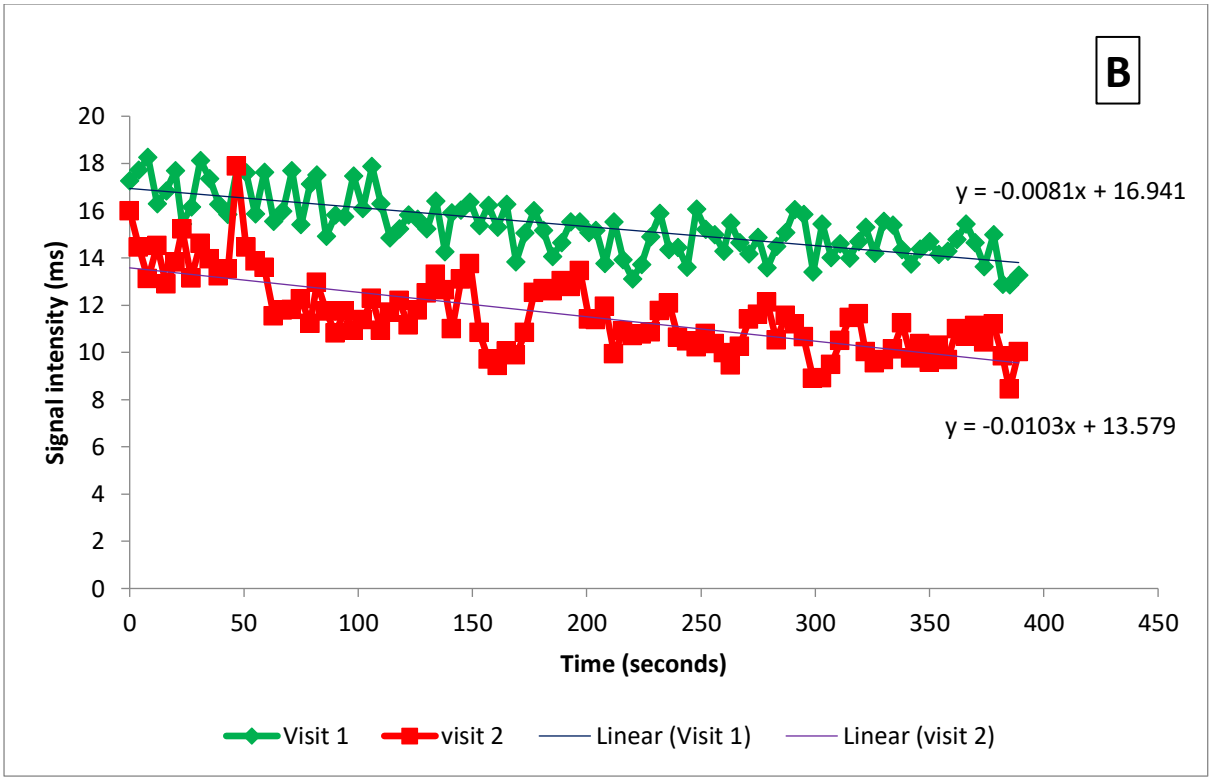


Figure 3.20 Example plots showing gradient of the curve for the washout period of a single Control participant (A) and a single LPS participant (B). 0 = 80 second timepoint to end of measurement at 8 minutes

The mean gradient (x1000) and standard deviation for each group were calculated for study visits 1 and 2 (figure 3.21). The Control group showed no difference in gradient of the slope pre- and post-inhalation (16.08 ± 6.11 versus 15.89 ± 3.82). The mean for the LPS group showed a slight increase following inhalation (13.73 ± 5.02 versus 14.90 ± 6.38). Whilst the mean value for the LPS group post inhalation was lower than that of the Control group post inhalation, the other results suggest again that any difference observed was due to natural variation in the measurement among individuals. p value for the difference between mean gradient for the Control group and LPS groups at study visit 2 was p=0.630.

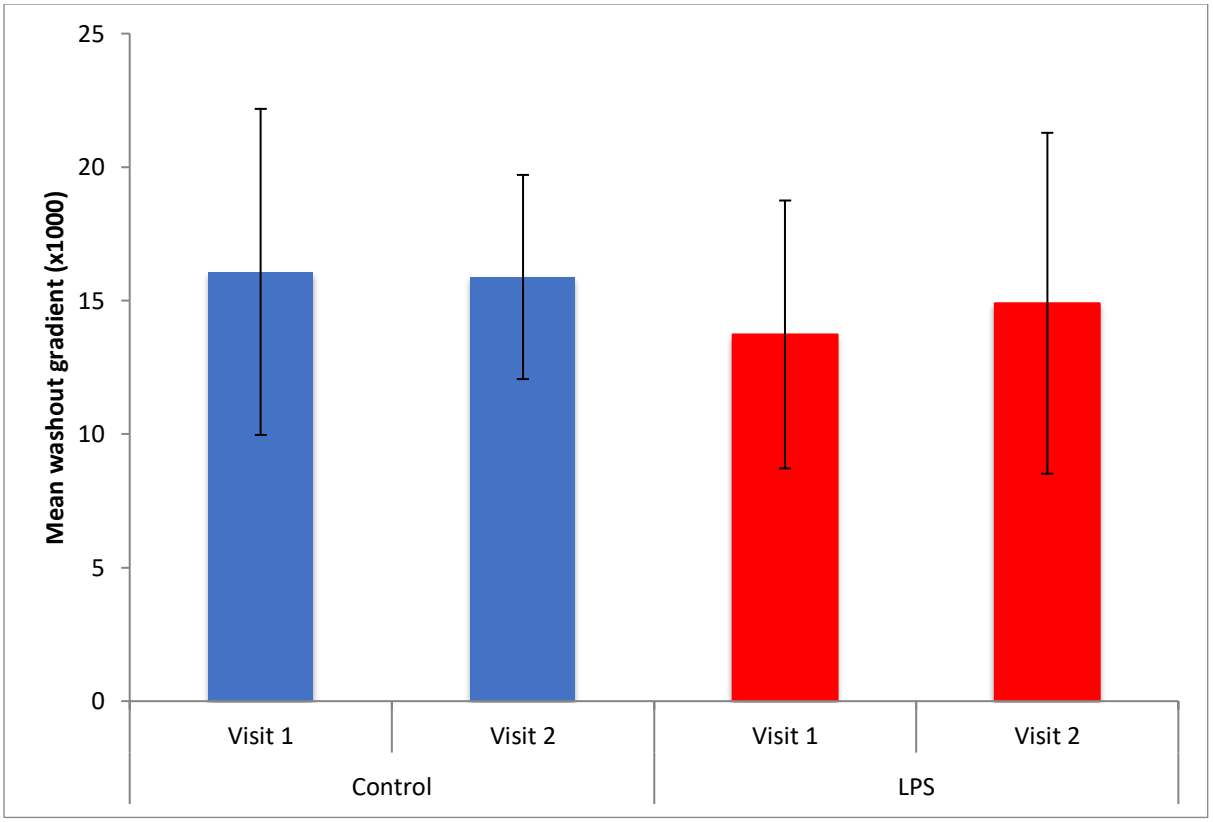


Figure 3.21 Mean washout gradient comparing Control and LPS pre and post inhalation. Error bars represent standard deviation. LPS n=7, Saline n=8

3.3.7 Effects of LPS inhalation measured in BALF

Participants in study group 3 underwent bronchoscopy immediately following DCE MRI scanning (n=16). At the end of study group 3, analysis of DCE MRI found no discernible difference with this technique between LPS and Control (see above). Therefore, the protocol was amended for study group 4 (n=10) to complete the planned blood sampling and proceed with bronchoscopy but omit further DCE MRI measurement.

The average time from inhalation to commencing bronchoscopy (defined as the time local anaesthetic throat spray was administered) was 470mins (7hours and 50mins ± 19mins). There was no difference in time to bronchoscopy for LPS or Control (464 ± 11mins and 475 ± 25mins respectively, p=0.17). The average amount of 2% lignocaine administered to the

airways during bronchoscopy was 12mls (± 4.5mls) and the average return from the 150mls lavage was 90mls (± 24.3mls).

3.3.7.1 Flow cytometry analysis of BALF

Analysis of BALF samples was undertaken by Dr Laura Jardine and is included here with her kind permission. Dead cells were excluded with DAPI. CD45 positive cells were used to gate different populations of leukocytes. Neutrophils were gated by CD11b and monocytes identified by CD14/CD16 characteristics. Figures 3.22 and 3.23 show example cell populations in BALF from a saline and LPS participant respectively.

Figures 3.22 versus 3.23 clearly demonstrates the expected increase in BALF neutrophils in response to inhalation of LPS in an individual participant. This was accompanied by significant increase in alveolar monocytes. Table 3.9 demonstrates the mean cell populations by inhalation group. As expected BALF neutrophil population increased by over 20 times following inhalation of LPS, compared to saline (mean LPS 122,719 ± 38,268 vs saline 5,222 ± 2258, $p<0.001$). Alveolar macrophage (AM) population was largely unchanged and populations of both classic and non-classic monocytes were also significantly increased in response to inhaled LPS.

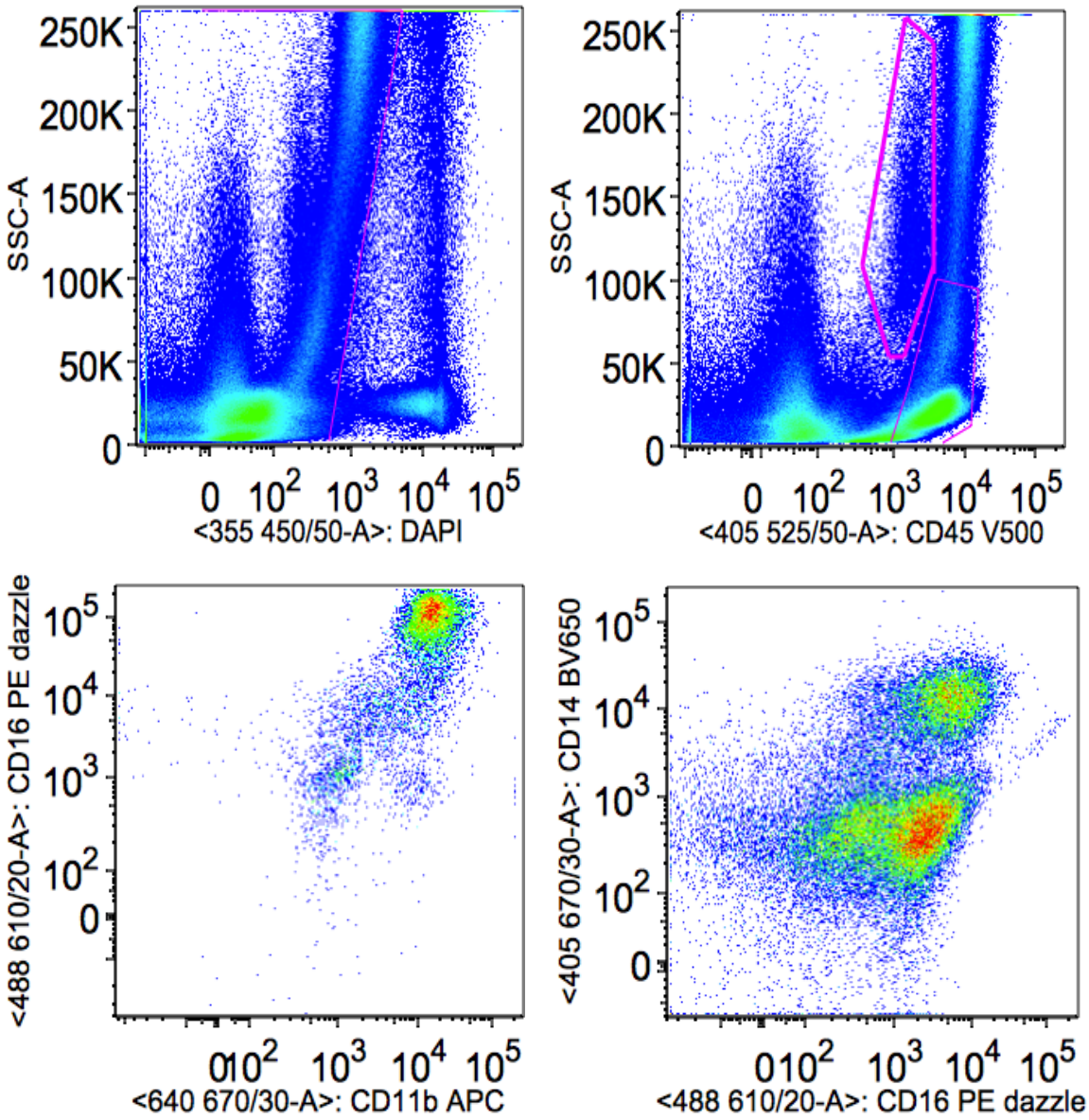


Figure 3.22 Cell populations in BALF identified on flow cytometry from LPS037 (Saline). (A) Dead cells excluded by DAPI, (B) CD45 gated leukocytes, (C) Neutrophil population identified by CD11b/CD16 positive cells, (D) Monocyte populations identified by CD14/CD16 characteristics

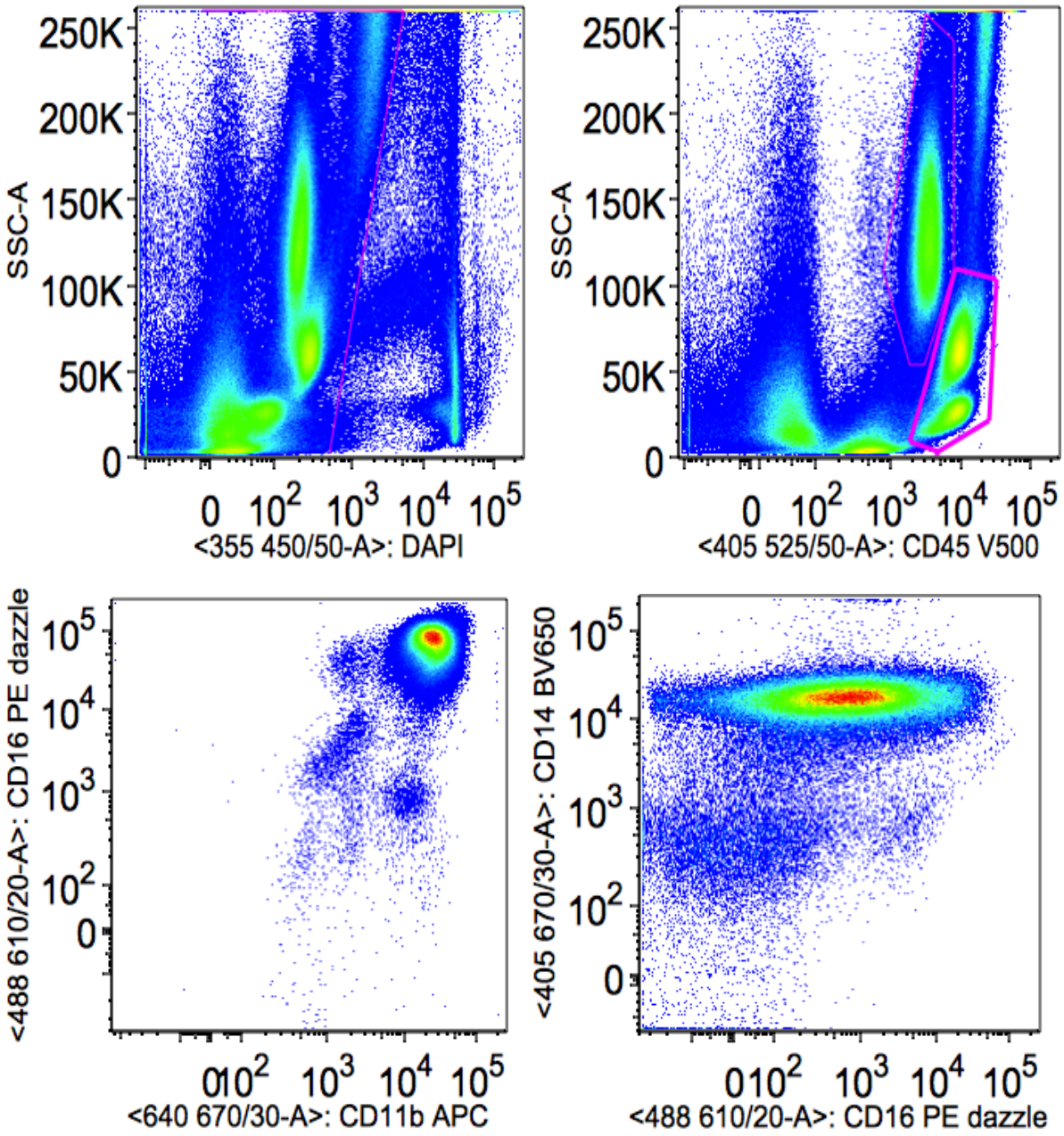


Figure 3.23 Cell populations in BALF identified on flow cytometry from LPS029 (LPS).
(A) Dead cells excluded by DAPI, (B) CD45 gated leukocytes, (C) Neutrophil population identified by CD11b/CD16 positive cells, (D) Monocyte populations identified by CD14/CD16 characteristics

	Neutrophils	Alveolar macrophages	CD14+ (classical) monocytes	CD14+/CD16+ (non-classical) monocytes
Saline	5,222 (2,258)	189,095 (98,992)	436 (168)	8,235 (2,218)
LPS	122,719 (38,268)	119,468 (76,696)	51,930 (25,779)	34,052 (11,283)
t-test (p=)	<0.001	0.15	<0.01	<0.01

Table 3.9 Cell populations in BALF by inhalation group. n=6 for both LPS and saline BALF. Numbers represent mean (stand deviation), statistical significance measured by independent samples t-test.

A large body of subsequent analysis on the BALF of LPS and saline group participants was undertaken by Dr Jardine. This focussed on the further differentiation of monocyte subsets and dendritic cells in response to inhaled LPS and is presented in the nature communications publication (Jardine L and Wiscombe S, et al. 2019), see appendix E. It demonstrates that the increase in classical monocytes and 2 dendritic cell subsets (DC 2/3 and DC5) are predominantly blood derived but on recruitment to the alveoli, cells rapidly adopt airspace cell type characteristics. The recruitment of monocytes and dendritic cells to the airway is coordinated by upregulation of cytokines released by resident alveolar macrophages (cytokine data is presented within the paper).

3.4 Summary of results chapter

In this chapter I have described the results of undertaking an LPS inhalation study in healthy volunteers. This includes the screen failure rate, physiological response to LPS and adverse events.

I have also described the experimental results achieved during this LPS inhalation study. Particularly focussing on functional status of neutrophils within the peripheral blood and flow cytometry analysis. I have described the results of DCE MRI scans and the difficulties of undertaking and applying this technique to a lung model of perfusion. Finally, I have shown some preliminary data from BALF samples as evidence of active inflammation within the alveolar space. In the next chapter I will interpret these results in the context of current literature and clinical practice and discuss their implications and limitations.

4. Discussion and conclusion

4.1 Summary of key findings

The results that form this thesis can be subdivided into 3 sections - a) delivery of an LPS inhalation study in healthy volunteers, b) function of neutrophils from the peripheral circulation within an LPS inhalation model, and c) the role of DCE MRI in assessing vascular permeability within an LPS inhalation model.

With respect to the first, our eligibility and ineligibility criteria led to an approximate 45% screen failure. Once recruited, study completion was extremely high with only 2 of 49 participants not completing all study interventions. Inhalation of LPS was well-tolerated with a good safety profile (a minimal increase in temperature at 6 hours was the only significant change in safety parameters) with no significant associated change in lung function. Symptoms reported were as expected for this dose (60µg) of inhaled LPS and there were no serious untoward incidents.

With respect to peripheral blood compartment results, the lack of inflammatory effect of LPS (and subsequent loss of value of the blood data) in the first study group was disappointing. However, having made changes to the protocol for delivery of LPS inhalation, further monitoring of automated WCC demonstrated appropriate changes for all other participants inhaling LPS. There was no observed difference demonstrated in phagocytosis of neutrophils at 6 hours in the LPS and control groups. There were also no differences seen in superoxide anion release by neutrophils or cell characteristics measured by flow cytometry.

The analysis of DCE MRI in the LPS inhalation model was novel and challenging. Although a minor difference was observed between LPS and control groups in the raw data at 6 hours following inhalation, having a baseline for both groups also showing variation strongly

suggested that any differences observed were due to experimental technique. Finally, the BALF results presented show evidence that, although blood compartment changes were negative and MRI data were unable to detect changes between groups, the expected response to LPS inhalation was seen within the alveoli.

4.2 Delivery of an LPS inhalation model

One of the aims of the research was to establish the delivery of an LPS inhalation model in Newcastle University for use in a number of studies over time. Professor John Simpson (supervisor) had experience of this technique in Edinburgh but I needed to establish the necessary clinical links in order to safely deliver key aspects of a study like this. i.e. screening and study visits within an area equipped for clinical monitoring and intervention if necessary, processing of blood samples and undertaking research BAL. Although it was not a clinical trial, ethics committees, R&D and other regulatory bodies were reassured that the participant information, consenting, clinical report forms and adverse event reporting were essentially being conducted to the standards expected of a clinical trial.

Our eligibility and ineligibility criteria led to a screen failure rate of approximately 45%. As discussed, the most common reason for screen failure was an isolated abnormality on blood testing. It is important to appropriately counsel potential volunteers regarding this possibility and to ensure robust systems are in place for informing volunteers and their respective GPs of results. Future studies should take this into account when planning expected numbers and time to recruitment. Although not often reported within publications we know from collaborative work with others who currently undertake research using an LPS inhalation model that our recruitment rate is in line with other investigators (Barr/Brittan Edinburgh, Shyamsundar/McAuley Belfast).

Our study specifically screened out any individuals with a history of atopy, asthma, significant smoking history or any abnormal signs on cardiorespiratory clinical examination.

Early in the development of LPS respiratory models (by which I continue to refer to both instilled and inhaled LPS), it was noted that patients with asthma demonstrated evidence of increased bronchial hyperresponsiveness, even on low dose LPS challenge. Michel et al demonstrated that inhalation of 20μg LPS resulted in rapid development of an obstructive deficit on spirometry that was associated with ongoing increased histamine responsiveness at 5 hours post-challenge (Michel et al, 1989; Michel et al, 1992). It is unclear if this is explained by any exaggerated or different signalling response to LPS systemically or rather by the effect of added neutrophilic inflammation on the background of an already oedematous and reactive airway. Therefore we, in alignment with most other recent investigators (Maris et al, 2006; Shyamsundar et al, 2009; Barr et al, 2013), sought to avoid LPS inhalation in subjects with atopy or asthma. This ensures minimal risk to participants and allows study of the 'pure' response to LPS without confounding factors as a result of other inflammatory pathways.

Our study did allow recruitment of healthy female volunteers and those of any ethnic background. This led to a 60% female participation which is uncommon, with most published studies recruiting exclusively male subjects. This may have some impact on scientific results as females have been shown to demonstrate increased TNFα, IL-6 and cortisol in response to LPS (van Eijk et al, 2007; Engler et al, 2016). It has some practical implications with absolute FEV1 and FVC values expected to be lower in females. This led me to reduce the volume of our routine research BAL from 200mls to 150mls to avoid risk of desaturation in young female volunteers.

16% of our participants were from an ethnic group other than Caucasian, which included Asian-Indian, Asian-Chinese, Black-African and mixed race. Little evidence exists regarding ethnic difference in response to LPS challenge and ours, like most other studies, was underpowered to examine these differences. However, the GENE study (Genetics of Evoked Responses to Niacin and Endotoxemia) specifically looked to examine this within an IV LPS model. Authors demonstrated that participants of African ancestry demonstrated higher levels of pre-LPS cytokines (e.g. IL-6) but lower levels of evoked inflammatory response to LPS (most significantly in IL-1RA, and CRP) compared to participants of European ancestry

(Ferguson et al, 2013). Better studied is the presence of genetic mutations resulting in alteration of the TLR-4 receptor and its impact on LPS signalling. Presence of the co-segregating mis-sense mutations 'Asp299Gly' and 'Thr399Ile', results in significantly lower circulating levels of CRP, LBP and WCC in response to inhaled LPS (Michel et al, 2003). However, no differences were observed between presence or absence of mutations following IV LPS challenge (Calvano et al, 2006). Presence of TLR polymorphisms have been widely studied in a number of different diseases and in different countries, but not widely compared across ethnic groups (Medvedev, 2013).

Overall, study groups undergoing inhalation with LPS and saline were well matched at baseline. Excepting the differences noted above, clinical characteristics reflect those of other published studies within the field (Michel et al, 1995; Van der Poll, 2005; Brittan et al, 2012). This strengthens the case for valid comparison between groups, and for results and conclusions being interpretable within a large body of published evidence.

The participants used to set up the protocol for DCE MRI (study group 2) were different in several characteristics to those who eventually went on to have DCE MRI following inhalation (study group 3). Although all volunteers met criteria for study enrolment and underwent the same screening and physiological testing, study group 2 had proportionately more males, significantly higher age, percent-predicted FVC and systolic blood pressure. This occurred due to the routine practice in MRI set up at the time, of using healthy members of the study team to set up protocols. Although very difficult to say conclusively, I wonder now if this contributed to why I did not foresee the problems with T1 mapping until the end of study group 3. The initial datasets looked encouraging but if these were predominantly undertaken on males with higher FVC, this may have underestimated the image artefacts from surrounding subcutaneous tissue.

Changes in clinical and lung function parameters were as expected for this dose of inhaled LPS. The only significant change was a mean rise in temperature of 0.7°C from baseline following LPS versus 0.3°C following Saline. This is in accordance with published literature

with Loh et al (2006) reporting a mean rise of 1.0°C and Michel et al (2001) 0.6°C following inhalation of 50µg LPS. There were no other significant changes in physiological measures between groups, which again is widely reported in healthy subjects at 50-60µg (Nightingale et al, 1998; Loh et al, 2006). Changes in FEV1 following LPS inhalation have been widely reported, with differing results. Early studies suggested definite reduction in FEV1 in those with clinical asthma (Michel et al, 1992) but also significant variability in healthy volunteers (Kline et al, 1999; Michel et al, 2001). However, most recent studies report minimal or no change in lung function parameters following inhalation of 40-60µg LPS if asthma/atopy is appropriately screened out (Wallin et al, 2004: Loh et al, 2006; Shyamsundar et al 2009) in keeping with my findings.

Symptoms and adverse events were as expected for this dose of inhaled LPS. 23 of 49 participants (46%) reported no symptoms at all. The addition of DCE MRI brought no additional burden of symptoms. The majority occurred in those additionally undergoing bronchoscopy and BAL and peaked at 12-15 hours following inhalation (approximately 9pm - midnight). Symptoms most commonly reported were cough, sore throat, malaise and pyrexia, in accordance with the published literature (Sandstrom et al, 1992; Wallin et al, 2004; Brittan et al, 2014). Symptoms were managed by paracetamol and rest. Participants in this study were at home during this peak in symptoms and none contacted me overnight despite being strongly encouraged to do so. This shows the safety and utility of delivering this model without the need for overnight observation, which has implications for costs in the research arena.

Two participants had a fall in oxygen saturation during bronchoscopy (<92% on 2L/min supplementary oxygen). The first was our first participant to undergo bronchoscopy and BAL who desaturated following 150mls of instillation (plus the 20mls discarded as a bronchiolar sample) and supplementary oxygen was increased to 4L/min. The decision was made to stop bronchoscopy at this point and oxygen levels quickly recovered and she remained well in recovery and at the study visit the next day. Since the participant was a young female of small height, this led to discussions and the later decision to reduce routine research BAL in all volunteers to 150mls. One other volunteer desaturated to < 92% on oxygen at 2L/min

after 100mls BAL. Although oxygen levels recovered following an increase in oxygen to 4L/min, the participant indicated he did not want to continue with BAL and the test was terminated. This was the only participant not to complete the full protocol for bronchoscopy and BAL again suggesting this is a safe and acceptable test for healthy volunteers in our hands. There were no serious untoward incidents.

A common criticism of LPS models (both respiratory and intravenous) is that they do not fully embody the heterogeneity of patient populations experiencing ARDS or sepsis (some of the features of which they aim to model). As already discussed, female and different ethnicities are often poorly represented. Participation by older subjects is also limited, despite good evidence that immune biology changes with advancing age (Montecino-Rodriguez et al, 2013). One study by Krabbe et al (2001) demonstrated that, following an IV LPS infusion, elderly subjects displayed a more prolonged fever, a more pronounced inflammatory response (TNFα, IL-6) and a more rapid increase in CRP. Blood monocyte levels dropped to a greater degree than in younger controls and took longer to recover after IV challenge. Similarly, the effect of BMI on outcomes for ARDS is complex with some authors reporting an 'obesity paradox' with BMI > 30 kg/m^2 linked to lower mortality (Zhi et al, 2016). Other authors argue that, if adjusted for confounding factors, mortality is probably the same for obese subjects as for those with ideal body weight, with some evidence that morbidity is higher. Most reviews conclude that low BMI (< 19kg/m^2) is more strongly associated with increased mortality (Ball et al, 2017). Most LPS studies include volunteers with a BMI within the normal range (20 – 25 kg/m^2) although some studies have specifically looked to address this question. Van Eijk et al (2014) measured blood cytokine response following IV LPS infusion in 112 males with BMIs ranging from 18.3 to 33.6 kg/m^2. The authors found no relationship between inflammatory response and BMI, although clearly the range of abnormal BMIs was small.

When and how LPS is delivered to the lung also effects the results and biology studied. As already stated, segmental instillation may more accurately model bronchial pneumonia rather than ALI or ARDS. My study, like most others, administered LPS challenge in the early morning in order to then perform investigations within working hours and monitor

participants safely. However, it must be remembered that healthy humans exhibit diurnal variations in physiological parameters. For example, total WCC is highest at midnight and secretion of several cytokines in response to LPS has been shown to be higher at night (Alamili et al, 2014).

Finally, we chose to use commercially available LPS from *Escherichia coli* 026:B6 (L2654, Sigma-Aldrich, Poole, UK) delivered via the Spira dosimeter-nebuliser. Previous studies using this combination had safely delivered this dose and shown the appropriate inflammatory response to allow further study. However, some authors are now suggesting that (like IV administration) LPS inhalation should consider using Clinical Centre Reference Endotoxin (CCRE), obtained from the National Institute for Health (NIH) and derived from the original *E.Coli* 0:113 source. This is likely to contain less contaminants (e.g. lipopeptides) and be more potent, with standardisation of potency allowing direct comparison of dose-response across studies and mitigating the risk of inter-batch effects using commercial LPS. Similarly, standardisation of nebuliser equipment or instillation strategy allows better modelling and consistency of experimental findings (Doyen et al, 2016).

4.3 Discussion of experimental results

The laboratory work's main focus was on assessing change in the functional status of neutrophils circulating within the peripheral blood compartment in response to LPS inhalation. With respect to phagocytosis of zymosan by neutrophils, my results show that baseline (pre-inhalation) function in normal healthy individuals was high (86.1% in the saline group and 85.6% in LPS group). This is consistent with findings published by our group in other disease areas. For example, my colleagues Ruchaud-Sparagano et al (2013) found phagocytosis of zymosan by healthy matched controls versus patients with stable bronchiectasis (i.e. free of current exacerbations) to be 81% and 80% respectively. These controls had an average age of 58 years. Our research group has also found that mean phagocytosis of zymosan by neutrophils isolated from young patients with idiopathic pulmonary arterial hypertension did not significantly differ from healthy controls at 83.1%

(Ruchaud-Sparagano et al, 2016). This is in direct contrast to findings in patients recruited from ITU with systemic inflammatory response syndrome (SIRS) and requiring at least 1 organ support, where two-thirds of patients had phagocytosis levels below 50% using the same method (Pinder et al, 2018). Other authors investigate phagocytosis by neutrophils with other methods such as flow cytometry. Whilst it is difficult to directly compare these results to our own, Alexis et al (2003) demonstrated high peripheral blood neutrophil phagocytosis (indicated by mean fluorescence intensity at flow cytometry of cells ingesting FITC-labelled zymosan) in those inhaling control.

My research hypothesis was that in response to LPS inhalation peripheral blood neutrophils would increase their capacity for phagocytosis. One concern of the study design was that, as demonstrated, baseline function would be high in control healthy volunteers. Therefore, demonstrating a 'statistical' difference would be difficult by this method. My results demonstrated a trend towards increased phagocytosis following inhalation of 60µg LPS, with a 3.6% increase versus 1.2% increase following LPS and saline respectively. This involved n=15 for both groups and was not statistically significant (p=0.058). There would also be an argument regarding whether this level of change is of biological significance. Whilst 60µg LPS is a small stimulus (in comparison to likely size of insult commonly linked to clinical ARDS) and therefore might only prompt a small measurable change in phagocytosis, we have also shown that investigator variability in interpreting phagocytosis by this method is in the region of 1.5-3% (Ruchaud-Sparagano et al, 2013; Pinder et al, 2018).

My findings are not consistent with those reported by Alexis et al (2003) who found phagocytosis by blood neutrophils was decreased following low dose LPS inhalation in individuals with asthma. My argument is that there is likely to be a dose-response change in function in relation to LPS exposure, as demonstrated by previous authors (Michel et al, 1995; Michel et al 1997). At lower doses of LPS inhalation there is rapid margination of neutrophils into the alveolar space, with these being likely to display the greatest 'activated' status, leaving more immature cells within the blood, with overall less efficient function. However, I hypothesised that the higher dose would be enough to prompt true up-

regulation of neutrophil function within the blood in keeping with the changes in cytokines often measured. Overall, my results did not support my hypothesis.

My data also showed no difference in ROS generation by neutrophils in peripheral blood after LPS inhalation, which did not support the research hypothesis, and was broadly in keeping with the phagocytosis data. However, it is perhaps inconsistent with the circumstantial evidence of increased cytokine and chemokine release seen in blood following LPS inhalation (increases in TNFα, IL-6, LBP and MPO). It is discordant with evidence from Michel et al (1995), in which ROS-induced chemoluminescence was increased in response to inhaled LPS at a similar dose used in my study. However, this was still an indirect measure of function, at a time when dose-delivery in LPS studies may not have been as robust as present methods. I propose that the still moderate dose of inhaled LPS in my study was not sufficient to change the function of circulating blood neutrophils. This could potentially be a protective effect, in that whilst there is an increase in cytokine and chemokine release to encourage early neutrophil migration to the affected organ, neutrophils display a degree of tolerance to these stimuli to prevent overactivation systemically to a small stimulus.

My results also showed no demonstrable difference in CD11b, CD62L or CD88 expression by circulating blood neutrophils following inhalation of LPS. This is consistent with my other blood findings. The majority of reports regarding increased expression of CD11b and loss of CD62L in response to LPS have been in isolated cells in vitro, in animal models exposed to high pulmonary doses, or within an IV LPS models (Condliffe et al, 1996: Marsik et al, 2003; Calvano et al, 2012). Alexis et al (2003) demonstrated that baseline CD11b expression by blood neutrophils correlated well with the extent of subsequent LPS-induced neutrophilia. However, CD11b expression fell on blood monocytes and was presumably unchanged on neutrophils (not reported). Conway Morris et al (2009) reported a strong correlation between impaired neutrophil phagocytosis and reduced expression of C5a receptor type 1 (CD88) in blood. However, this was in very unwell patients on ITU with ventilator-associated pneumonia where median phagocytosis was 48% (using the same method as ours).

Therefore, I think it is not surprising that I was unable detect a difference at this level of stimulus.

4.4 Discussion of DCE MRI results

The use of DCE MRI to assess vascular permeability in a human inhaled LPS model had not been done before. Prior evidence suggesting that the technique was able to detect vascular change between healthy smokers and non-smokers (Naish et al, 2008) led me to believe that it would be able to quantify a low level of change. Although this increase was anticipated to be small, the research hypothesis was that DCE MRI would be able to detect the early increase in pulmonary vascular permeability following inhalation of LPS.

Set up of the technique was built into the study protocol with group 2 participants used to acquire images under a range of different protocols. This was done in collaboration with (and based on protocols used by) Dr Jo Naish and colleagues (University of Manchester) in their studies of smokers and patients with asthma (Zhang et al, 2014). An interim analysis after the first 6 study participants in study group 3 looked promising as a blinded investigator experienced in MRI felt able to predict inhalation with either LPS or saline based on the shape of the washout curve.

However, at the next analysis point (completion of study group 3) it became clear there were difficulties creating the T1 map due to high variability in T1 values throughout the lung, for the reasons discussed in the DCE MRI results chapter (see section 3.3.6, page 123). This meant I could not fit a kinetic model to the dataset to quantify levels of change due to high likelihood of false results. Therefore, the raw data were used, as if there was a true experimental signal it should have been visible in the raw data. The research hypothesis contended that mean signal intensity following LPS inhalation should be higher, reflecting more contrast entering the EES, resulting in a 'brighter' T1 signal. Both groups showed lower mean signal intensity post-inhalation, although the difference was greatest in the control

group. The variation observed within the control group pre- and post-inhalation suggests that any changes observed simply reflected normal physiological variance between different people and different scan sessions. Thus, DCE MRI failed to detect any difference in vascular permeability in this human model of inhaled LPS. There may be two explanations for this - 1) no significant change occurred in vascular permeability following LPS inhalation or 2) there was a change, but it was too small to be detected by this technique.

Addressing explanation 1, my results show the expected increase in BALF neutrophils in response to LPS inhalation. Through the work of my collaborators, TNFα was also shown to be significantly increased in BALF supernatant, as expected (see appendix E). I have not specifically measured BALF total protein or albumin in order to support or refute this argument. Whilst disruption to the alveolar-capillary barrier is a hallmark of ARDS, its presence within the LPS inhalation model is variable. Evidence for increased vascular permeability is suggested by release of cytokines such as TNFα, which is detected rapidly and significantly in BAL and blood in response to inhaled LPS (O'Grady et al, 2001; Barr et al, 2010; Hecker et al, 2015). In acute or chronic inflammation TNFα classically results in recruitment of phagocytes and Tregs, vasodilatation and increased vascular permeability (Parameswaran et al, 2011). However, in the homeostatic state, it plays an important role in localising infection through macrophage signalling, but also promoting clot formation in small vessels, local vessel occlusion and promoting fluid drainage into lymphatics (Murphy 2012). As neutrophils are found in only minimal amounts in the resting lung, the rapid increase in BALF neutrophils following LPS inhalation seen in many studies is often cited as supporting the extravasation through 'leaky' blood vessels into the alveoli. However, the process of neutrophil migration is still under investigation and the association with barrier permeability is likely to be complex (Williams and Chambers, 2014). Many studies report the neutrophilia and increased BALF total protein or albumin (Sandstrom et al, 1992; Wallin et al, 2004). However, other studies are conflicting, with Martin et al (1989) showing neutrophil recruitment with no change in protein permeability. More recently several authors have demonstrated that depleting neutrophils in oleic-acid or hyperoxia-induced lung injury does not prevent capillary-alveolar leak (Hill et al, 2004; Perkowski et al, 2006). Because of the conflicting evidence between different animal and human models it remains

unclear if the actual process of neutrophil recruitment to the lungs is dependent on (or indeed causes) a degree of endothelial-epithelial barrier disruption (Williams and Chambers, 2014).

The alternative explanation for my results is that DCE MRI failed to detect an increase in vascular permeability because the level of change was below the resolution of this technique. It is likely within the LPS inhalation model that the predominant disruption to barrier integrity is within the alveolar epithelium (Shyamsundar et al, 2009). This promotes release of TNFα from resident alveolar macrophages and allows influx of protein from the interstitium and marginated neutrophils from within the pulmonary microvasculature (Williams and Chambers, 2014). In a healthy volunteer human model of LPS challenge the stimulus was perhaps not enough to cause significant disruption to the pulmonary vascular endothelium and therefore, contrast agent administered systemically does not enter the EES in sufficient concentration to be detectable.

The final potential explanation is that there was in fact disruption to the vascular endothelium and subsequent change in the volume of distribution of contrast agent, but that the artefacts from respiratory motion and signal from surrounding subcutaneous tissue overshadowed the ability of the DCE MRI to detect this change. As discussed in the introduction (section 1.2.5.10, page 42) MRI has been able to detect change with respiratory instillation of LPS in animal models. However, the doses used in animals far exceed those which would be ethically acceptable in a human research model. My results are consistent with those found by Barr et al (2013) where PETCT was unable to detect a difference post LPS inhalation at the same dose.

4.5 Limitations to the research

There are some limitations to the research presented here. First, I chose to discount 5 out of 8 participant's results from study group 1 who were designated to have inhaled LPS. This was due to their peripheral blood total WCC and neutrophils failing to show 'the expected' response to inhaled LPS. However, it is known that the LPS-stimulated inflammatory response from circulating blood leukocytes varies greatly from individual to individual. As discussed above, (page 145) specific genetic mutations resulting in changes to TLR-4 can result in a significantly attenuated response to inhaled LPS (Michel et al, 2003). Human subjects carrying the T399I polymorphism either exhibit a milder LPS-hyporesponsive phenotype or do not manifest it at all (Arbour et al, 2000). Wurfel et al (2005) measured LPS-induced cytokine production ex vivo in whole blood from 102 healthy human subjects and found that TNFα response varied by more than three orders of magnitude, and values for IL-1β spanned a 300-fold range. Within their cohort they identified individuals who consistently showed either very high or very low responses to LPS (often referred to as LPS-responders or non-responders). More recently, Peden and Alexis (2020) examined gene signatures in LPS-responders and non-responders at baseline and following LPS inhalation. They identified 13 genes differentiated groups at baseline where responders displayed 10 upregulated genes relative to non-responders, including neutrophil-modifying genes. Therefore, an argument might have been that these 5 participants were LPS non-responders and their results should have been included. However, TLR-4 polymorphisms are thought to occur with a frequency of approximately 5% - 10% in Caucasians and are relatively rare in Asian populations (Vogel et al, 2005). Therefore, the likelihood that 5 consecutive individuals would be non-responders was felt to be low.

Instead, it seemed more plausible that LPS had not been appropriately delivered to the alveoli in those individuals resulting in a loss of blood response. Initially, I failed to recognise the importance of making up LPS on the morning of inhalation and keeping it in suspension to enable deposition to the alveoli and subsequent inflammation. I was also keen that I was fully blinded to participant inhalation in order to record participant observations/symptoms and assess phagocytosis. Having made changes to the protocol for delivery of LPS inhalation, further monitoring of automated WCC demonstrated the expected, appropriate changes for

all other participants inhaling LPS. This, in association with characteristic changes in BALF, provided evidence that for all subsequent subjects the expected responses were seen in both LPS and saline groups.

The functional assay used here to assess phagocytosis has a subjective element. There is first a quality control element to ensure there has been adequate adherence of isolated neutrophils to the plate. For accurate results, the investigator must then examine the remaining wells and ensure counting of fields of cells that represent the overall picture of phagocytosis. By performing the counts 4 times per well (and in my study I repeated this again in a separate well, i.e. 8 counts) and then taking the mean value, it makes the technique more representative of the true value. We have shown that in experienced hands, investigators show close agreement in their interpretation of phagocytosis rates (approximately 1.5-3%). Due to the changes to LPS preparation for study groups 3 and 4, I was unblinded to inhalation group and I examined FBC results at the end of each participant's involvement to ensure they were appropriate. I was then the main investigator assessing the phagocytosis results and this unblinding is a weakness of the study design. To avoid bias in the assessment of phagocytosis, all samples were labelled with study participant only and batch counted at the end of each study group (i.e. many months following inhalation). Therefore, in reality, at the time of counting I could not remember whether LPS039 (for example) had inhaled LPS or saline. A proportion of samples in each study group (approximately 20%) were counted by a second investigator, blinded to both inhalation and my counts. Results were discarded if counts were not within 5% congruence and also if they did not follow the same trend from baseline, 6 hours and 24 hours.

Most other published LPS studies used surrogate markers to assess innate immune cellular changes within LPS models. i.e. the change in cytokines, chemokines or protein expression markers in serum or plasma. I undertook whole cell functional assays of phagocytosis and respiratory burst activity to directly assess neutrophil function within the model. These are hard to do well, avoiding artificial activation of cells on isolation from the blood or during the assay. The superoxide results suggest that there was very little false activation of neutrophils during isolation. It was always going to be hard to prove the research

hypothesis of increased phagocytosis by neutrophils in blood, due to the baseline being high in healthy volunteers. In retrospect, I think I should have incorporated some other measures of functional status, particularly chemotaxis, as this might be expected to be among the first changes that systemic neutrophils undergo in response to insult in a specific organ. Also, I have not presented any data on cytokine changes, which is commonly done in LPS studies. If there had been a change in phagocytosis, I might have gone on to do this to link functional status to cytokine signalling. As phagocytosis was unchanged and the changes have been reported many times, I chose not to investigate these parameters in our samples.

Clearly, the functional assays were undertaken on neutrophils isolated from whole blood, in a model of *inhaled LPS*. I think the evidence in LPS challenge increasingly suggests that IV LPS infusion accurately models some elements of sepsis and produces changes within the systemic circulation, while inhaled/instilled LPS models some features of early ARDS, with the majority of changes taking place within the alveolar compartment. The research hypothesis was that the techniques used would be able to detect the very early changes that take place in systemic neutrophils in response to LPS inhalation. I suspect that provocation with a larger dose of inhaled LPS would have been enough to provoke measurable changes in functional status of neutrophils within the systemic circulation. Previous studies have used cumulative doses up to 100µg (Kitz et al, 2008) but now that stimulus with between 50 - 60µg is accepted practice I do not think higher doses could be ethically justified in healthy volunteers.

DCE MRI was unable to detect a change in pulmonary vascular permeability although the reasons for this are unclear. I have presented some basic data on neutrophil influx as evidence that the expected changes were seen within the alveolar space. I did not analyse BALF protein or albumin levels which may have provided evidence of protein 'leak' into the alveolar space. Since I did not test the BALF samples for changes in biochemical markers I do not know if barrier function was impaired in our LPS-inhalation model. As such, the MRI results may have been unchanged between groups because there was no disturbance to barrier function in our model (i.e. there was no difference to detect) or because there was a difference but the MRI was unable to detect it. However, as the research aimed to address if

DCE MRI could offer a non-invasive method of monitoring response following LPS inhalation, when the results were negative the decision was made not to examine these samples.

There are some limitations to the DCE MRI technique itself, most notably the variability in T1 measurement. This led to the inability to create a T1 map and apply the mathematical model to allow quantitative analysis. Analysis of the DCE MRI was overseen by Professor Pete Thelwall (Professor of Magnetic Resonance Physics, Newcastle University) and Dr Jo Naish (Honorary Lecturer, University of Manchester). After attempts at creating a T1 map were unsuccessful, we decided to undertake analysis on the raw signal data. Since this simply meant analysis of the 'brightness' of the T1 signal, if there was a true experimental difference between the two groups it should have been visible in the raw data. This meant the risk of a false positive in the MRI data was avoided. Due to the infolding of signal from subcutaneous tissues we selected measurements from areas of the lung least affected by artefact (upper lobes). We reflected that this method may have ignored data from areas of the lungs most affected by changes in perfusion/permeability (lower lobes) which could have resulted in a false negative result.

Set up of the DCE MRI was built into the study protocol. 5 participants underwent DCE MRI scans with variations to the protocol to ensure appropriate image capture. However, this process did not pick up the later problems experienced with image artefact. According to common practice within the MR research centre, volunteers for MRI set up were taken from within the research staff. Although all these participants underwent a screening visit and met all inclusion and exclusion criteria, this led to study group 2 having significantly different baseline characteristics to the rest of the study population. Particularly, there were more male volunteers, they were older (age 31.2 years vs. 22.6 years) and had better lung function (percentage-predicted FVC 114.1% vs. 103.9%). These factors may have led to a reduction in artefact on the setup scans meaning it did not become apparent until the later study group was completed. In retrospect, we should have used volunteers from within my pool of 'student volunteers' who responded to adverts meaning the group would likely have reflected the other study groups more accurately.

There are, of course, further limitations to the LPS inhalation model itself. My study, like most others, limited recruitment to non-smokers, of young age (<40 years) and most had an 'ideal' body weight (average BMI 22.4). This is in direct contrast to those at higher risk of developing ARDS (discussed in more detail on page 147). In addition to the limited patient populations, and variations in when and how LPS is delivered to the airspace, there is also an argument that LPS inhalation may only model one subtype of ARDS, i.e. the pro-inflammatory, endotoxin-mediated state. Most studies deliver LPS as a single bolus, in contrast to the more likely sustained or repeated hits leading to ARDS. As the changes likely represent the early stages of acute lung inflammation, models do not incorporate the effects of treatment commonly used in the more severe forms of human disease, particularly the use of antibiotics, fluid resuscitation and ventilation strategies. Finally, interventional studies in LPS models tend to have given the therapeutic agent prior to LPS insult which, whilst demonstrating biological plausibility, does not reflect clinical presentation. This may, in part, explain the failure to translate therapies from showing action in LPS models into successful treatments in critically unwell patients.

4.6 Areas for further study

In terms of this work, there could be merit in examining the BALF to measure protein levels and markers of epithelial injury to conclude the arguments around whether DCE MRI failed to detect a change due to lack of actual change in the model or lack of sensitivity of the test. For DCE MRI, I recommend other investigators consider protocols that allow better assessment of T1 measurement and consider incorporating breath-hold or regimented breathing patterns for a set period of time to help reduce noise. In an LPS inhalation model at doses now commonly used, my opinion is that scientific focus should be on the alveolar space rather than the systemic circulation (this remains appropriate for IV LPS models). Whilst isolation of neutrophils from BALF is extremely difficult to do without inducing activation several authors have demonstrated the technique successfully. Whole cell functional assessment of neutrophils in BALF has not been done at a 'standard' higher dose

for LPS inhalation (Alexis et al used 5µg in individuals with atopy) and this would significantly improve our understanding of the role of neutrophils in early lung inflammation. Examining the changes at different time points following LPS inhalation would also be useful to look at the factors that aid resolution of inflammation.

LPS models will never fully replicate all the changes experienced in critically ill patients, but they provide an important link between basic cellular research and clinical practice. Future research may benefit from adjustments to the way in which LPS inhalation studies are currently conducted. Standardisation of LPS source, dosing and delivery device would allow improved comparison across research groups. As discussed, wider inclusion to incorporate female participants, older participants, and participants of different ethnicity, plus perhaps those with underlying chronic diseases (diabetes and COPD) would better represent the 'real world' of patients managed with ARDS on ICU. Similarly, models should consider incorporating a second or repeated hit delivery of LPS and investigate subsequent effects on innate immune response. The scientific benefits of this would need to be carefully evaluated against potentially increased symptom burdens and risks to study participants. Finally, researchers should aim to show the effect of interventions delivered following LPS challenge, in order to increase the chance of successful translation into clinical practice.

4.7 Conclusion

In a healthy human-volunteer study, LPS inhalation did not affect phagocytosis or respiratory burst activity of neutrophils in the systemic circulation. DCE MRI is subject to technical artefact when applied to assessment of the pulmonary parenchyma and was unable to detect significant changes in vascular permeability following LPS inhalation.

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Study Protocol

A lipopolysaccharide (LPS) inhalation model to characterise divergent cellular innate immune responses and presence of alveolar leak early in the course of acute lung inflammation

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INTRODUCTION

Acute lung injury (ALI) is a severe condition characterised by acute onset, new pulmonary infiltrates on chest x-ray, and profound hypoxaemia in the absence of left atrial hypertension.[1] ALI may arise from direct insult to the lung (e.g. due to pneumonia) or from extrapulmonary causes such as trauma or pancreatitis. ALI exerts a considerable burden of mortality, morbidity and cost on modern healthcare systems. Approximately 10% of patients admitted to European intensive care units (ICUs) develop ALI, and a large epidemiological study estimated that 190,000 American citizens develop ALI each year.[2,3]

Despite the wide range of conditions causing ALI, a consistent pathological finding is intense infiltration of the alveolar space by neutrophils, with disruption of the alveolar-capillary membrane resulting in flooding of the alveoli with a protein-rich exudate (alveolar leak).[4] Monocytes also consistently enter the alveolar space.[4] This led to the concept that ALI represents a pro-inflammatory state. However it is increasingly recognised that 'deactivation' of neutrophils and monocytes to a relatively anti-inflammatory phenotype occurs in ALI,[5] and may predict an adverse outcome.

A greater understanding of ALI in humans is hampered by the fact that patients are already critically ill when they develop the disease. It is therefore exceptionally rare to be able to study early evolution of the disease in man, and most of the available data have to be extrapolated from animal studies, despite clear limitations of this approach. Similarly, clinical definition of ALI rests on chest x-ray (CXR) information, which is highly non-specific in the detection of particular pathological entities (like alveolar leak) in the ICU. Other more specific measurements of alveolar leak have employed detection of extravasated compounds (usually proteins) in alveolar fluid via bronchoscopy and bronchoalveolar lavage (BAL) or via radiolabelling of blood proteins (e.g. ^{67}Ga-labelling of blood transferrin) with detection of label accumulation in the lungs.[6] However, BAL involves an invasive procedure in this already precarious population and radiolabelling studies necessitate exposure to ionising radiation while providing poor spatial resolution. No pharmacological intervention has impacted significantly on outcome for ALI and mortality remains unacceptably high at approximately 20-

30%.[7] Whilst several factors may contribute to this, principal among these are an incomplete understanding of the complex pathogenesis, including ability to study the early stages of the human acute inflammatory response to lung inflammation, and absence of reliable, repeatable non-invasive imaging to identify alveolar leak. Our study seeks to address these elements, using an inhaled lipopolysaccharide (LPS) model of experimental lung inflammation to study early inflammatory responses, and using the novel modality 'dynamic contrast-enhanced magnetic resonance imaging' (DCE-MRI) to quantitatively image alveolar leak.

LPS is a component of the outer membrane of Gram-negative bacterial cells. Animal studies of respiratory injury have widely employed LPS inhalation/instillation, and this approach has been translated to human research studies to induce a subclinical alveolar inflammatory response in healthy subjects. LPS produces a brisk but safe and self-limiting condition characterised by rapid accumulation of neutrophils and monocytes in the bloodstream and alveolar space, with clear evidence of alveolar inflammation.[8,9] These features appear to mimic the early stages of ALI closely, and allow improved insights into pathogenesis.
DCE-MRI allows lung extravascular fluid and vascular permeability to fluid to be quantified without recourse to ionising radiation. The use of a low molecular weight contrast agent (eg gadodiamide or gadoteric acid) allows the rate of extravasation to be safely quantified. Recent data have illustrated that this technique accurately delineates alveolar leak in cigarette smokers[10]. The significance of this finding stems from the traditional difficulties in imaging 'air-liquid' interfaces in the lung, as well as in the safety and repeatability of the procedure.
Current concepts suggest that immune cell 'over-activation' damages lung tissue during acute lung inflammation. However we and others have suggested that immune cell 'deactivation' may predict for adverse outcomes[5]. With specific regard to circulating monocytes, it has been demonstrated that both profound 'deactivation' and abnormal processing/regulation of mitochondrial DNA are associated with sepsis. We propose that effective resolution of inflammatory responses requires a delicate balance whereby inflammatory cells released into the circulation retain the capacity to access tissues rapidly, but 'deactivate' in a programmed, controlled manner. We propose that such 'controlled deactivation' serves to minimise inadvertent damage as leukocytes pass through capillary beds in tissues unaffected by the inflammatory process. The LPS model allows us to test this theory, while simultaneously evaluating a) the role of deactivated neutrophils and monocytes b) the regulation of mitochondrial DNA in human leukocytes (a process that remains poorly understood), and c) the utility of DCE-MRI in detecting alveolar leak induced by inhaled LPS.
The importance of this work lies in the potential to inform future studies in ALI. Two findings would be particularly relevant in this regard. Firstly, support for the concept of 'programmed deactivation' would suggest novel therapeutic approaches for ALI, depending on the inflammatory phenotype of patients' cells. Secondly, a role for DCE-MRI in reliably detecting alveolar leak would provide an invaluable end-point for studies investigating efficacy of new treatments in ALI. Such end points are distinctly lacking and most ALI studies rely on 'surrogate' end points such as oxygenation indices, duration of mechanical ventilation, or cytokine levels in BAL.

We therefore intend to conduct studies in healthy volunteers exposed to either LPS or inhaled saline (control). Timed blood samples will be used to isolate innate immune cell populations and to assess their relative deactivation. For example, in the case of neutrophils this is to establish their capacity for phagocytosis and superoxide generation and in monoctyes to quantify extent of HLA-DR expression and alteration in mitochondrial DNA. We will also determine the corresponding profile of pro- and anti-inflammatory mediators in serum. Subjects will then undergo bronchoscopy and BAL to compare markers of alveolar inflammation and leak. Subjects will undergo DCE-MRI scanning at different time points in relation to LPS administration in order to establish the optimum protocol for detection of alveolar leak via this method.

SCIENTIFIC BACKGROUND TO THE STUDY

Safety of the Lipopolysaccharide (LPS) model

Inhalation of LPS sets up a reproducible, mild and self-limiting inflammation creating a model for ALI that incorporates neutrophil recruitment, cytokine and chemokine production, and resultant alveolar leak[11]. Mild systemic effects are also induced, including increased white cell count and elevated inflammatory cytokines in the blood[12]. As such, LPS inhalation is capable of recreating many of the pathological hallmarks of early ALI, albeit with short-term inflammation, and its suitability for carefully monitored healthy volunteer studies is reflected in these effects.

Our group has recently administered 60μg inhaled LPS to 36 healthy male volunteers with no serious adverse events recorded[9]. All had evidence of lung inflammation without respiratory compromise. McAuley's group has recently administered inhaled LPS (50μg) to 44 volunteers, in which similar findings were observed[8]. Inhalation of 50μg and 60μg have been reported to cause transient pyrexia (0.7°C) and mild symptoms (malaise, myalgia, shivers, fatigue, headache and cough) lasting less than 4 hours with no reduction in lung function as measured by FEV1 and FVC.[12,13] In a previous study, inhalation of 100μg did not induce clinically significant adverse signs or symptoms and was not associated with significant changes in FEV1 and FVC.[14] LPS doses greater than 100μg have been tolerated well by healthy subjects.[15]

60μg LPS will be administered by nebulised inhalation in this study because of our experience with this dose, the good safety profile, and the consistent transient pulmonary and systemic inflammation produced.

The role of neutrophils and monocytes after LPS inhalation

The model used in our recent studies produces a consistent peripheral blood neutrophilia at 4-8 hours post-inhalation, with a corresponding neutrophilia in bronchoalveolar lavage fluid (BALF). Interestingly, while *circulating* monocyte subpopulations did not change in number after LPS inhalation we demonstrated rapid recruitment to the *lung* of an inflammatory monocyte-like population[9]. One interpretation could be that, during appropriately regulated, self-limiting, resolving inflammation, the blood compartment remains appropriately quiescent.

Evidence for 'deactivation' of innate immune cells

We have recently described the mechanisms underlying deficient neutrophil phagocytosis in the ICU.[16] We demonstrated that mechanically ventilated patients with new lung infiltrates have elevated levels of neutrophil-recruiting cytokines (interleukin (IL)-1β and IL-8) in bronchoalveolar lavage fluid (BALF).[17] BALF from these patients also contained an excess of cytotoxic mediators (e.g. human neutrophil elastase) released from neutrophils. Interestingly, blood neutrophils from these patients are activated but simultaneously appear 'deactivated' in that they are deficient in their capacity for phagocytosis and superoxide generation.[18] This maladaptive scenario suggests inappropriate, prolonged recruitment to the lung of dysfunctional, cytotoxic neutrophils. However it remains completely unclear whether monocyte and neutrophil 'deactivation' is a purely pathological phenomenon. Data from patients with sepsis increasingly suggests that controlled, synchronised pro- and anti-inflammatory systemic cytokine responses are required for resolution and return of organ function.[19] It remains plausible that a similar scenario exists for cellular innate immunity. For example it remains feasible that effective resolution of lung inflammation invokes 'deactivation' of circulating monocytes and neutrophils to prevent inappropriate inflammation in capillary beds in unaffected tissues. Whether immune cell deactivation occurs as part of self-limiting, appropriately resolving inflammation remains unclear and is the focus of this project. We

postulate that it does, thus limiting inflammatory responses to the affected organ. In contrast, and in keeping with our work in critically ill patients,[16,17,18] we suggest that overwhelming and/or prolonged inflammation produces a sustained, inappropriate and ultimately maladaptive deactivation of circulating innate immune cells. While these findings suggest novel therapies for critically ill patients, we believe far more research is required before reaching the conclusion that deactivation of neutrophils and monocytes is maladaptive in all acute settings.

Development of Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI)
LPS inhalation provides an 'early ALI' model that we will use to validate MRI measures of alveolar leak. This will in turn look to provide a much needed non-invasive method for diagnosis and monitoring of lung inflammation, delivering novel tools for research and clinical use. The inability of alveoli to clear extravasated fluid from air spaces perturbs lung function[4] and redistributes pulmonary blood flow away from oedematous regions[20]. Alveolar leak acts as a predictor of outcome in ALI[21]. Current measures of lung structure (e.g. computed tomography (CT)) and function (e.g. spirometry, lung volumes) provide little or no specific information about vascular leak. Thermodilution methods of extravascular lung water content aid delineation of lung injury extent and monitoring of therapy response[22], but the method is highly invasive and prone to errors when oedema and/or perfusion is heterogeneous. Other measurements of alveolar leak typically employ detection of extravasated compounds, either by assay of protein or exogenous compounds in BALF or via radiolabelling of blood proteins with detection of label accumulation in the lungs[6]. However, this is invasive or involves exposure to ionising radiation while providing poor spatial resolution.
Magnetic resonance imaging (MRI) offers significant potential for investigating lung inflammatory processes, both in patients and in healthy volunteers. It avoids use of ionising radiation, and is repeatable and safe. Recent advances have yielded novel methods with great clinical potential. Dynamic contrast-enhanced-MRI (DCE-MRI) enables non-invasive, quantitative imaging of pulmonary perfusion and capillary permeability[23-26]. The method involves intravenous injection of a contrast agent with concurrent cine imaging to record the changing parenchymal contrast agent concentration over time. Analysis of these data provide images of perfusion, blood volume and transit time and show that contrast agents (e.g. gadoteric acid) are able to leak out of the capillary bed and into the surrounding extravascular space. Measuring *the rate of this leak* over several minutes can provide a measurement of vascular permeability. Such calculation of capillary permeability and the volume of extravascular extracellular space is well established in oncology, where DCE-MRI provides assessment of tumour microvasculature properties that report on effects of tumour therapy[27,28]. Application to lung tissue is more challenging due to low tissue proton density and respiratory motion over a long scan, but this can be overcome using optimised MR acquisition protocols and post-processing motion correction. Our collaborators in Manchester recently provided the first demonstration of elevated vascular permeability due to smoking-induced inflammation using DCE-MRI. The study compared asymptomatic smokers with healthy volunteers[10], demonstrating significantly elevated vascular permeability to contrast agent and elevated fluid in the lungs. Thus DCE-MRI can provide non-invasive measurements of lung perfusion, oedema and vascular leak: key markers in ALI. We will further develop the lung DCE-MRI approach via the LPS model of lung inflammation, with the aim of establishing the role of DCE-MRI for diagnosis and monitoring treatment response in acute lung inflammation. Only by understanding the key steps in the complex pathogenesis of acute lung inflammation and by developing new diagnostic tools can we hope to identify future therapeutic remedies and have the necessary skills to evaluate their impact in clinical studies.

OBJECTIVES

Primary Objective

The primary objective is to test the hypothesis that ***deactivation of circulating monocytes and neutrophils occurs early in the course of self-limiting, acute pulmonary inflammation.***

Secondary objectives

1. To characterize neutrophils and specific monocyte subsets in the blood and bronchoalveolar fluid (BALF, alveolar space) of volunteers exposed to LPS or control

2. To examine the relative activation and deactivation of neutrophil populations in blood and BALF in those exposed to LPS or control

3. To examine the relative activation and deactivation of monocyte subsets in blood and BALF in those exposed to LPS or control

4. To determine the status of mitochondrial DNA in monocytes from individuals exposed to LPS or control

5. To determine the corresponding profile of pro- and anti-inflammatory mediators in blood and BALF of those exposed to LPS or control

6. To test DCE-MRI data acquisition protocols in healthy volunteers to demonstrate ability to measure alveolar leak

7. To correlate DCE-MRI derived measures of alveolar leak with established BALF measures of alveolar leak in healthy volunteers exposed to LPS or control.

STUDY DESIGN

Summary

Healthy volunteers will be randomly assigned to receive inhaled LPS or vehicle (saline) control. Using this template, 5 broad determinations will be made, assessing:

- activation/deactivation status of blood neutrophils and monocytes
- pro- and anti-inflammatory mediators
- mitochondrial DNA and function in monocytes
- optimum MRI scanning protocols
- correlation between DCE-MRI measurements of alveolar leak, and alveolar inflammation assessed by BAL.

Group	n	Visit 1	Visit 2		Visit 3
		t = > -1 day	t = 0 hours	t = 6 hours	t = 24 hours
Group 1A	8		Neb saline	Blood	Blood
Group 1B	8		Neb LPS	Blood	Blood
Group 2	**10**	MRI			
Group 3A	8	MRI	Neb Saline	Blood + MRI + BAL	Blood
Group 3B	8	MRI	Neb LPS	Blood + MRI + BAL	Blood
Group 4A	8	MRI	Neb Saline	Blood + MRI	Blood + MRI + BAL
Group 4B	8	MRI	Neb LPS	Blood + MRI	Blood + MRI + BAL
Total	**58**				

Neb = nebulised, Blood = blood sampling, MRI = indicates single MRI scan at each time point, BAL = bronchoscopy and bronchoalveolar lavage

Table 1: Summary of volunteer groups and main study assessments

Screening Visit (all volunteers)

Healthy volunteers will be recruited from within Newcastle University **and Northumbria University**. Volunteers will be screened and assessed for eligibility based on inclusion and exclusion criteria used in recent studies (see inclusion/exclusion criteria). Screening is designed specifically to identify any risk for adverse events associated with procedures to be undertaken. In particular, LPS inhalation and bronchoscopy/BAL is considered extremely safe under the conditions used here if there is no evidence for pre-existing lung disease. Therefore volunteers will have vital signs measured (pulse, blood pressure and temperature) and will undergo spirometry, quantification of oxygen saturation and cardiorespiratory history and examination. Furthermore, MRI is considered extremely safe and all participants will have undergone screening for routine MRI contraindications. Unlike CT or X-ray, it does not involve a radiation dose and use of MRI contrast agents for DCE-MRI is considered very safe if volunteers have normal renal function, therefore serum urea and electrolytes will be measured.

Visit 1 (volunteers having DCE-MRI)

Volunteers satisfying study criteria, wishing to proceed with the study, and providing written, informed consent will attend the dedicated clinical research facility (CRF) at Freeman Hospital on an agreed day. A brief history (regarding symptoms/exposure to upper and lower respiratory tract infections), respiratory examination and spirometry will be performed prior to scanning.

Volunteers assigned to Group 2 (n=**10**) will undergo one DCE-MRI scan only (with no subsequent LPS or control saline exposure, nor bronchoscopy). Data from these volunteers will be used to optimise the MRI scanning protocol for studies of Groups 3 and 4. DCE-MRI will be performed on the Siemens Espree 1.5T MRI scanner at the Freeman Hospital. A cannula will be placed into a vein prior to the scan for infusion of contrast agent during imaging. Volunteers will enter the MR scanner and pilot scans will be acquired to confirm correct volunteer positioning, followed by non-invasive perfusion and respiratory motion measurements (quantitative arterial flow scans and respiratory-gated cine imaging). Dynamic

contrast-enhanced imaging (DCE-MRI) will then be performed with intravenous administration of MRI contrast agent (Gadoteric acid [Dotarem, **standard dose 0.2mls/kg body weight or half dose 0.1mls/kg]) with concurrent acquisition of lung images.**

The data from this group will be used to confirm optimal MRI data acquisition parameters on this scanner system. Volunteers in Groups 3 and 4 will be studied once the MRI protocol has been proven successful in **Group 2**.

Visit 2

Volunteers who have had DCE-MRI will be asked to return to the CRF **between 1 and 21 days after baseline DCE-MRI**, for the inhalation stage of the study (for those in group 1 undergoing blood tests only, this will be their first visit and they will have the option of attending the CRF at either Freeman Hospital or the Royal Victoria Infirmary, both Newcastle Upon Tyne Hospitals NHS Trust). On this morning, a brief history and respiratory examination will be repeated. Spirometry will be performed before and after 'practice' inhalation with saline. Blood will be drawn at baseline (30mls), this will indicate time 0 (t=0).
LPS or Saline will be prepared by research staff **in the 24 hours prior to the study visit**. Since LPS dissolves fully in normal saline both LPS and saline will appear as identical clear colourless, same-viscosity fluid, and following preparation, the sample will only be marked with the individual's study number.
Volunteers will receive either 60µg LPS or sterile 0.9% saline over five-inhalations, using an automatic inhalation-synchronised dosimeter nebuliser (Spira, Hameenlinna, Finland), as used in our previous studies. All LPS/saline inhalations will be carried out within a CRF of Newcastle Upon Tyne Hospitals NHS Trust .
Further blood samples (of **between 14mls and 35mls**) will be drawn at 2 (t=2), 4 and 6 hours following inhalation and volunteers will be monitored continuously throughout the day by staff at the CRF. As per Table 1, volunteers may proceed to no further interventions, MRI alone or MRI + bronchoscopy (and BAL) at approximately 6 hours post inhalation (t=6).
Spirometry will be repeated 6 hours after inhalation in all groups and immediately before proceeding to MRI +/- bronchoscopy (or sooner if there are any symptoms of bronchoconstriction). If FEV_1 has fallen by >10% at the 6-hour time-point, relative to baseline, then neither bronchoscopy nor MRI will be performed. Following the blood test and spirometry at t = 6 hours participants in groups 3 and 4 will undergo repeat DCE-MRI scanning following the same protocol as in visit 1.

Participants in group 3 will then proceed directly to bronchoscopy and BAL (within 2 hours of completion of DCE-MRI scanning) in the endoscopy/bronchoscopy suite at Freeman Hospital, Newcastle Upon Tyne. If the volunteer is undergoing bronchoscopy on visit 2, they will have been fasted for a minimum of 4 hours. Bronchoscopy and BAL will be performed using a standardised protocol and procedure employed throughout our work thus far.[29] Local anaesthesia will be applied topically to the throat. Sedation (eg with intravenous midazolam) will be optional. Electrocardiogram (ECG) trace and oxygen saturations will be monitored continuously. A flexible fibreoptic bronchoscope will be passed per-orum or per-nasum. 20ml of sterile saline will be instilled into a single segment of the lung (typically the medial segment of the right middle lobe), gently aspirated and discarded (as the 'bronchiolar' sample). 200ml of warmed saline will then be instilled in aliquots and gently aspirated. Approximately, two to three hours after the procedure participants can eat and drink.
Volunteers will be **monitored for approximately 30 minutes to 1 hour following bronchoscopy** and if participants have normal observations and cardiorespiratory examination they will be allowed home and supplied with a number to contact should they feel unwell. Patients who develop minor symptoms after LPS +/- bronchoscopy/BAL will be treated according to clinical indication (eg symptomatic pyrexia or headache may be treated with

paracetamol, bronchospasm may be treated with bronchodilators, low oxygen saturations may be treated with supplemental oxygen etc). If volunteers have elected to undergo bronchoscopy with sedation they will be required to be accompanied home by a responsible adult and advised to abstain from driving, cycling, working, operating machinery or signing any legal documentation for 24 hours. Volunteers who feel unwell will be invited to return for clinical review.
If volunteers are undergoing only MRI at visit 2 (group 4) they will be monitored for approximately 30 minutes following completion of MRI and if they have normal observations and cardiorespiratory examination, they will be allowed home and supplied with a contact number to call if they feel unwell. **Volunteers will also undergo a final blood sample of 2mls for Full Blood Count (FBC) measurement, following DCE-MRI, prior to the cannula being removed.**

Visit 3
All participants (excluding group 2) will be asked to return the following morning to the CRF. All will undergo brief respiratory history, examination and repeat spirometry. A final blood sample **(35mls)** will be taken (t=24). Participants in groups 1 and 3 will then be allowed home and study investigators will telephone the volunteer later that day to ensure they remain well.

Volunteers in group 4 will undergo MRI and bronchoscopy at approximately 24 hours. Procedures for DCE-MRI and bronchoscopy/BAL will be exactly as described for visit 2. Volunteers will be **monitored for approximately 30 minutes to 1 hour following bronchoscopy** and if they have normal observations and examination they will be allowed home. Contact numbers will be provided and study investigators will telephone volunteers later that day to ensure they remain well.

In the event that participants do not feel well they will be offered a return visit to the clinical research facility and be assessed by a medical member of the research team. All necessary equipment for further observation and simple symptomatic relief will be available. If participants wish to stay at home, a member of the research team will arrange a further phone call assessment as indicated until the participant is feeling back to full health. All adverse events will be recorded and actioned as noted in sections below.

SETTING

All volunteers will be recruited through advertisement within Newcastle **and Northumbria Universities**. Volunteers will be screened at the dedicated CRFs at either Freeman Hospital or the Royal Victoria Infirmary (both Newcastle Upon Tyne Hospitals NHS Trust). If found eligible for the study, they will be given time to consider if they wish to continue to study entry (minimum 24 hours) and invited to re-attend on a set day. Participants allocated to group 1 (LPS and blood tests only) will be given the option to attend the CRF at Freeman Hospital or the Royal Victoria Infirmary (for their convenience). All specialist MRI scanning will take place on the Siemens Espree 1.5T scanner at the Freeman Hospital. Therefore participants in groups 2-4 will be asked to attend the CRF at Freeman Hospital for LPS inhalation to be performed, adjacent to the MRI scanner and **endoscopy/bronchoscopy suites** located in the same buildings. LPS or saline for inhalation will be prepared in the Simpson lab, Institute of Cellular Medicine, Newcastle University. Downstream preparation and analysis of samples generated by the study will be performed in the Simpson lab, in the flow cytometry facilities (Centre for Life and Institute of Cellular Medicine), in the Chinnery lab (Centre for Life, eg for assessment of mitochondrial DNA) **and within the musculoskeletal research lab (micro RNA species), all Newcastle University. Processing of screening blood tests and prothrombotic markers (e.g. thromboelastography) will take place in Newcastle Upon Tyne hospitals clinical labs.**

STUDY POPULATION

Number of participants

58 healthy participants will be recruited (see table 1).

Inclusion criteria

- Healthy adult volunteers aged between 18 and 40 years of age
- Able to give informed consent

Exclusion criteria

Volunteers will not be eligible for inclusion in the study if any of the following criteria apply at entry:

1. Age <18 or >40 years
2. Past history of chronic respiratory disease (e.g. asthma, chronic obstructive pulmonary disease, bronchiectasis, tuberculosis)
3. Past or current history of conditions known to affect immunity or cardiac function (e.g. diabetes, ischaemic heart disease, congenital heart disease, valvular heart disease, chronic renal impairment, recurrent urinary tract infection)
4. History of an acute intercurrent cardiorespiratory illness (with particular reference to upper and lower respiratory tract infection)
5. Any current medication (excepting oral contraceptive pill)
6. Current history of smoking
7. Past smoking history amounting to >2 pack-years
8. Any history of smoking in the last 12 months
9. Reported alcohol intake >21 units per week
10. Pregnant or lactating women
11. Abnormal physical signs detected at cardiorespiratory examination
12. Temperature >37.3 degrees Celsius
13. Oxygen saturation <95% breathing room air
14. Haemoglobin outside the laboratory reference range.
15. Platelet count less than 100 $x10^9$/L
16. Total white cell count and neutrophil count outside the laboratory reference range
17. Blood sodium, potassium, creatinine, alanine aminotransferase, outside the laboratory reference range
18. Blood urea greater than 10mg/dL
19. Bilirubin greater than 30umol/L
20. Forced expiratory volume in one second (FEV1) or forced vital capacity (FVC) <80% predicted
21. FEV1:VC ratio <70%
22. Standard exclusion to undergoing MRI scanning (pacemakers, cochlear implants, aneurysm clips, intra-ocular metallic foreign bodies, prior history of allergic reaction to contrast agent, [in those undergoing MRI scanning])
23. History of significant claustrophobia (in those undergoing MRI scanning)

PARTICIPANT ENROLMENT AND SELECTION

Identifying potential participants

An advert will be placed on Newcastle **and Northumbria** University email lists and on Newcastle **and Northumbria** University notice boards. Potential participants will be asked to make contact with the research team only if they consider themselves to be healthy. Interested individuals will be invited to contact the research team who will send out information on the study (participant information sheets and screening consent form). Participants sent such information will be invited to contact the research team to arrange a screening visit (see below) or to decline participation. If no reply is received after 2 weeks, the research team will send a reminder.

Screening for eligibility

Volunteers who contact the research team will be invited to attend for a screening visit at the CRF at either Freeman Hospital or the Royal Victoria Infirmary, both Newcastle Upon Tyne. This will take the form of:

- a short history
- vital signs measurement (temperature, pulse rate, blood pressure)
- measurement of oxygen saturation breathing room air
- cardiorespiratory examination
- blood sample for full blood count
- blood sample for urea & electrolytes assay and liver function tests
- spirometry
- urinary pregnancy test in women
- standard questionnaire for contraindications to MRI scanning
- 'practice inhalation' of saline

These are performed with the sole intention of identifying potential exclusion criteria as above. The history, examination and investigations will be performed by a trained member of the research team.

Consenting for the study

All eligible volunteers will be given written and verbal information regarding study participation. All will be asked to give written consent with a minimum 24 hours to consider entering the study. Consent will be taken by an experienced member of the research team.

Ineligible and non-recruited participants

For volunteers found to be ineligible at screening, or eligible but not subsequently entered into the study, the reason for ineligibility or non-recruitment will be entered on the database. Only anonymised data will be entered on to the database and this will include gender, age, “ineligible” or “non-recruitment” and the associated reason. If found ineligible, permission will be sought from participants to contact their GP with the results of screening tests and any further action required.

STUDY ASSESSMENTS

Processing and analysis of blood samples

'Screening visit' blood samples will be sent to the hospital laboratories for assessment of full blood count, urea and electrolytes and liver function tests.
Blood samples will be taken at 0, 2, 4, 6, 7 and 24 hours after exposure to LPS or saline control (visits 2 & 3). **Blood sample volume will be approximately 14-15mls at 2 and 4 hours and 35mls at 0, 6 and 24**. The first 10mls of all samples will be used for full blood count (~2mls), preparation of serum (~4mls), and for flow cytometric quantification of leukocyte markers (~4mls) such as monocyte HLA-DR and neutrophil CD88 (at the dedicated flow cytometry service at Newcastle University, run by Dr Ian Dimmick). CD88 is the neutrophil surface receptor for activated complement factor 5 (the anaphylatoxin C5a), and correlates strongly with neutrophil phagocytic capacity.[16,18] Serum will be stored at -80ºC for later measurement of pro- and anti-inflammatory markers (such as IL-1β, IL-8, IL-10, IL-12, tumour necrosis factor alpha (TNFα) and secretory leukocyte protease inhibitor (SLPI)). **The sample at 7 hours following inhalation will be 2mls and sent for full blood count measurement only in group 4**.
Approximately 5mls at each timepoint will be prepared in PAXgene tubes for the preparation of microRNA species relevant to the inflammation pathway and in citrated blood for estimation of corresponding prothrombotic markers (such as thromboelastography and plasma concentration of tissue factor). The remaining 20mls of samples taken at 0, 6 and 24 hours will be used for preparation of granulocyte and mononuclear samples by dextran sedimentation and Percoll gradient extraction, according to standard techniques with which the Simpson group has longstanding experience. Granulocyte layers will be used if they contain >95% neutrophils. Phagocytosis by neutrophils will be assessed using standard techniques (for example by adhering cells to plastic then adding serum-opsonised zymosan and quantifying the number of cells containing ≥2 zymosan particles).[18] Superoxide generation will be assessed using techniques such as a cytochrome c reduction assay.[18] Monocytes will be isolated from the mononuclear cell fraction by positive or negative selection columns. Monocyte populations will be adhered to plastic and then stimulated (eg by the addition of TNFα). Cell-free conditioned medium will be collected and the cytokine profile assessed. Using protocols developed in our lab, $CD14^{hi}$,$CD16^{lo}$ monocytes will be flow sorted and cultured singly or in co-culture with other relevant cells (eg human pulmonary vascular endothelial cells). Cell-free conditioned medium may be used to quantify markers of endothelial cell activation, including von Willebrand factor and tissue-type plasminogen activator. Nuclear and mitochondrial DNA will be extracted from monocytes and relative levels measured by RT-PCR using standard protocols developed in the Chinnery lab. Monocyte bioenergetics, including mitochondrial oxidative phosphorylation and ATP production, will be determined using standardised protocols on an extracellular flux analyser in the Chinnery lab.

Processing and analysis of Bronchoalveolar lavage fluid (BALF)

The total cell count in BALF will be determined (eg using a haemacytometer) and differential cell counts established (eg using stained cytospins or flow cytometry). BALF will be centrifuged and the supernatant frozen at -80ºC for later estimation of pro-/anti-inflammatory mediators and markers of alveolar-capillary membrane disruption (eg total protein, MCP-1, MIP-1α, IL-1β, IL-8, TNFα, IL-17, SDF-1, RAGE, SP-D, vWF and t-PA). Fractions of the cellular pellet will also be subjected to flow cytometry (eg to estimate expression of CD11b, CD11c, CD14,

CD16, CXC3R1 and CCR2 on monocytes). Phagocytosis of neutrophil fractions will be determined by exposure to particles such as opsonised zymosan.

Processing and analysis of magnetic resonance images (MRI)

MRI data will be processed and analysed to determine measures of perfusion, vascular permeability, extracellular extravascular space, relative regional compliance and quantitative arterial blood flow rate. Data will be stored on a password-protected Newcastle University network datastore and on password-protected computers within the Newcastle Magnetic Resonance Centre, Newcastle University. Access to these data will be limited to study investigators.

Data Analysis

Data from analysis of blood samples, BALF and DCE-MRI will be compared between those receiving LPS and those inhaling control, for all time points along with changes from baseline (where appropriate). Participants inhaling LPS will be expected to demonstrate an increase in peripheral blood neutrophil count, maximal at 6 hours, which acts as a reliable marker that LPS has been delivered appropriately to the airways. As such, data will only be included in analysis for those inhaling LPS if full blood counts show an increase in peripheral blood neutrophil count from baseline to 6 hours of > 2.00 x10^9/L.

RISK ASSESSMENTS

Blood Sampling

Blood sampling is occasionally accompanied by discomfort or by vasovagal symptoms. Risks are minimised through all samples being taken in a fully-supported medical facility. Blood will be taken on a self-reclining chair or bed. Volunteers feeling syncopal will be positioned in the supine position and venepuncture discontinued.

Inhalation of LPS or 0.9% saline (control)

Sterile 0.9% saline will be used for practice inhalation and then for control inhalation in half of the study participants. The LPS is also dissolved in sterile saline to make up the LPS solution for nebulisation. Nebulised saline is not anticipated to cause any adverse effects.
LPS inhalation will be undertaken in the CRF under medical supervision. All the necessary facilities to safely undertake this procedure are available. Participants will then be observed following LPS/control inhalation and medications required to treat any adverse effects will be readily available (e.g. paracetamol in the event of significant symptoms of fever, salbutamol in the event of bronchospasm, oxygen in the event of hypoxia). Standard spirometric measures of forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) will be undertaken at baseline (to exclude those with possible subclinical airway obstruction) and prior to bronchoscopy. If lung function falls by >10% from baseline, MRI, bronchoscopy and BAL will not be undertaken.
Vital signs will be measured at least hourly over the course of the day following inhalation of LPS/control, until allowed home. The only exceptions are that monitoring will be suspended during MRI scanning for those undergoing MRI at 6 hours. In this case, the volunteer can be

observed directly during MRI scanning and monitoring can be re-initiated immediately if there are any signs of clinical distress.

Bronchoscopy and Bronchoalveolar Lavage (BAL)

Bronchoscopy and BAL will be undertaken in a dedicated **endoscopy/bronchoscopy suite**. All the necessary facilities to safely undertake this procedure are available. During bronchoscopy and bronchoalveolar lavage, participants will be continuously monitored by ECG trace and oxygen saturation.
Bronchoscopy and BAL can rarely be associated with low oxygen levels. Prior to bronchoscopy subjects will be given supplemental oxygen. If oxygen levels as measured by pulse oximetry fall to <90% for 1 minute on supplemental oxygen, bronchoscopy and BAL will be stopped.
Experience from our previous study showed that volunteers commonly have transient low oxygen saturations when sitting up after removal of the bronchoscope. This is presumed to be due to redistribution of ventilation perfusion matching. Where appropriate, supplemental oxygen will be given after bronchoscopy and subjects transferred once oxygen saturations are 95% or greater.
If the subject chooses to receive light sedation, he/she will need to be accompanied home and will be instructed that he/she must not work, drive, cycle, operate moving machinery (e.g. DIY tools), drink alcohol or sign legal documents for the remainder of the day (because of the amnesic effects of midazolam and effects on wakefulness and coordination).
Other risks of bronchoscopy include aspiration (in the rare event of vomiting while there are significant gastric contents) and arrhythmia if too much local anaesthetic is used. We will minimise the risk of vomiting firstly by offering 'non-sedated' bronchoscopy (thereby eliminating midazolam, the principal cause of nausea), and secondly by ensuring at least 4 hours between eating and bronchoscopy. The risk of arrhythmia will be minimised by limiting airway anaesthesia to ≤20mls 2% lignocaine and by the fact that we study young healthy volunteers (in whom arrhythmia is extremely rare).
Subjects can experience cough and fever within 24 hours following LPS inhalation and/or bronchoscopy and BAL. Subjects will be advised how to manage symptoms (paracetamol as anti-pyretic and analgesia) and given a telephone number to contact a member of the study team if any symptoms develop after leaving hospital.

Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI)

Risks at the time of DCE-MRI are considered very low. Staff can directly observe the subject throughout the procedure, and can communicate with the subject easily.
The most common adverse effect is a sensation of claustrophobia in susceptible individuals. This is minimised by the MRI suite being a spacious, well-lit room such that the individual neither feels enclosed nor 'in the dark'. Individuals can also feel discomfort due to loud noise of the scanner and vertigo-like sensations from movements of the scanner. Subjects will be offered head phones and music to listen to, to minimise discomfort.
Risks associated with MRI are related to the presence of non-MRI compatible or loose metalwork within the body. As such, standard assessment tools for all patients and participants undergoing MRI will be undertaken at screening. If doubt remains regarding potential metalwork within the body or orbit, participants will be excluded from the streams of the project involving MRI scanning.

The injection of contrast agent (gadoteric acid) will only be carried out through a functioning venous cannula thus minimising any risk of extravasation. As with all contrast agents there is a very small risk of anaphylaxis however several large reviews have demonstrated that anaphylaxis to Gadolinium-based MRI contrast agents is extremely rare (less than 1 in 3000

or 0.03%)[30,31]. All necessary safety equipment will be available to recognise and treat anaphylaxis.

In extremely rare cases gadoteric acid has been associated with a condition known as nephrogenic systemic fibrosis (NSF). This is a skin, joint and renal condition characterised by increase in the dermal deposition of fibroblast-like cells associated with collagen remodelling and can cause permanent renal impairment. To date, over 200 million patients worldwide have been exposed to gadolinium-based contrast agents and in total 250 cases of NSF have been reported[32]. To our knowledge, there has never been a case of NSF reported in a patient will normal renal function, with the vast majority occurring in those with end-stage renal dysfunction/dialysis dependency. All of our participants will be screened and excluded if any abnormality in serum creatinine is found at screening.

ADVERSE EVENTS

Our research group has experience with the LPS model. Our previous study successfully administered 60µg of LPS to 36 young, healthy participants (same population) with no significant adverse events recorded.[9] Other groups have used higher doses (100µg) without precipitating clinical signs or symptoms and with no adverse change in lung function[14]. Over the last 20 years data to support the safe use of LPS in carefully controlled studies has been growing. Nevertheless, we feel that we must remain vigilant in detecting and recording any adverse events as a result of exposure to LPS or other procedures undertaken. Whilst recognising that this current work is not a clinical trial, our groups' previous work (which included a clinical trial) benefited from classifying and monitoring adverse events in the manner described below, and we have elected to continue using this terminology in this research.

Definitions

An ***adverse event*** (AE) is any untoward medical occurrence in a study participant.
A ***serious adverse event*** (SAE) is any untoward medical occurrence in a study participant or effect that:

- results in death
- is life threatening (i.e. the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe)
- requires hospitalisation or prolongation of existing hospitalisation
- results in persistent or significant disability or incapacity
- is a congenital anomaly or birth defect

If an adverse event is detected, a member of the research team will make an assessment of seriousness as defined by the above definitions. If the event is deemed to be serious (SAEs) a member of the research team will then consider if the event was:

- **Related** – that is it resulted from administration of research procedures and/or
- **Unexpected –** that is a type of event that is not identified as an expected occurrence

Detecting and reporting AE and SAEs

All AEs and SAEs will be recorded from the time a participant consents to join the study until 24 hours after completing the final study assessments. A medically qualified member of the research team will ask about the occurrence of AEs/SAEs during the study and on a surveillance phone call after the volunteer completes visit 3. Information to be collected includes type of event, onset date, researcher assessment of implications, if any, for safety of participants and how these will be addressed, date of resolution as well as treatment required, investigations needed and outcome. All information will be recorded in the participants study file.

An AE/SAE may necessitate discontinuation of a given part of the study (but progression through the remainder of the study) or complete and immediate discontinuation of any further participation. All participants will maintain the right to discontinue or completely withdraw from the study at any time for any reason, or without stating a reason. The reason and circumstances for premature discontinuation (where known) will be documented in the participant's study file.

If a SAE has occurred, the research team must report the information to NUTH R&D within 24 hours. The SAE form must be completed as thoroughly as possible with all available details of the event, signed by the Investigator or designee. The SAE form should be transmitted by fax or by hand to the office.

NUTH R&D is responsible for reporting SAEs that are considered to be related and unexpected as described above to the Research Ethics Committee (REC) that approved the study (main REC) within 15 days of becoming aware of the event using the NRES Reporting of SAE Form. The Co-ordinator of the main REC should acknowledge receipt of related, unexpected safety report within 30 days.

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A lipopolysaccharide (LPS) inhalation model to characterise divergent cellular innate immune responses and presence of alveolar leak early in the course of acute lung inflammation

Consent form for Screening Visit

SCREENING NUMBER | | | | | | |

TO BE COMPLETED BY THE PARTICIPANT (please initial each box):

1. I Confirm that I have read and understood the Participant Information Sheet Study Group ..... dated ..... version..... for the above study. I have had the opportunity to consider information, ask questions and have had those questions answered fully. [ ]

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected [ ]

3. I understand that information gathered for the purpose of screening will be kept in a secure confidential file. I agree that this file may be looked at by researchers involved in this study or, where relevant, by regulatory authorities overseeing the research. I understand that my personal data will be processed and stored in compliance with the 1998 Data Protection Act. I understand that my blood results for screening will be kept on the secure NHS results system only accessible by NHS staff. [ ]

4. I understand the purpose of the screening visit is to determine whether I am eligible to participate further in the study [ ]

5. I agree that the research team may take a medical history, examine my cardiovascular and respiratory systems, take a blood sample, assess my oxygen levels and vital signs and perform a breathing test [ ]

6. I agree to provide a urine sample so that the research team may ensure I am not pregnant (female participants only) [ ]

7. I agree to 'practice' inhalation with nebulised saline [ ]
8. I agree that the research team may inform me of any abnormal or clinically relevant results [ ]

9. I agree that the research team may inform my general practitioner of my participation in screening for this study and of any abnormal results [ ]

_____	_____	_____
Participant's Name	Signature	Date
_____	_____	_____
Researcher's Name	Signature	Date

Chief Investigator:
Professor John Simpson
Institute of Cellular Medicine
4th Floor, William Leech Building
Medical School, Newcastle University
NE2 4HH, Tel: 0191 222 7770
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Newcastle University

A lipopolysaccharide (LPS) inhalation model to characterise divergent cellular innate immune responses and presence of alveolar leak early in the course of acute lung inflammation

Consent Form: Study Group 3 Trial Participation

PARTICIPANT NUMBER

TO BE COMPLETED BY THE PARTICIPANT (please initial each box):

1. I Confirm that I have read and understood the Participant information sheet Study Group 3, dated 12/03/2013 version 1.3 for the above study. I have had the opportunity to consider the information, ask questions and have had those questions answered fully. [ ]

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. [ ]

3. I understand that information gathered for the purpose of the study will be kept in a secure confidential file. I agree that this file may be looked at by researchers involved in this study or by regulatory authorities overseeing the research. I understand that my personal data will be processed and stored in compliance with the 1998 Data Protection Act. I understand that my MRI scan will also be kept on the Radiology Department's secure system, which is accessible only to health professionals. [ ]

4. I agree to a clinical history and examination at each study visit and if clinically indicated [ ]

5. I agree to inhalation of lipopolysaccharide (LPS) or control (normal saline)

6. I agree to have blood tests, as outlined in the participant information sheet, and I am aware that while this includes some DNA and RNA analysis none of that testing is about specific medical conditions [ ]

7. I agree to have two Magnetic resonance imaging scans with administration of intravenous contrast [ ]

8. I agree to a bronchoscopy test and bronchoalveolar lavage

9. I agree that my blood, lung fluid samples and MRI scans may be stored for up to 5 years after completion of the study and that these will be stored in a secured, confidential way [ ]

10. I agree that my samples may be used in future studies, on condition that I cannot be identified from my samples, and that new ethical approval is granted for those studies [ ]

11. I agree that the research team may inform my general practitioner of my participation in the study and of any abnormal results or adverse events. [ ]

_____	_____	_____
Participant's Name	Signature	Date
_____	_____	_____
Researcher's Name	Signature	Date

Chief Investigator:
Professor John Simpson
Institute of Cellular Medicine
4th Floor, William Leech Building
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NE2 4HH

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PARTICIPANT INFORMATION SHEET
STUDY GROUP 3

HELPING YOU DECIDE WHETHER TO ENTER THIS STUDY

INTRODUCTION

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study, outlines what will happen to you if you take part, and informs you about the conduct of the study.

- Part 2 gives you more detailed information about what will happen to you if you take part.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1

What is the purpose of the study?

Many critically ill patients in the intensive care unit (ICU) develop a condition known as 'acute lung injury' (ALI) whereby the lung becomes 'inflamed'. This condition can have serious consequences for the patient. Despite intensive research in the past few decades, little is known about the complex processes leading to a person developing ALI. Also, despite current x-rays and scanning techniques it is very difficult to accurately diagnose this condition and tell it apart from other complications that can arise in the ICU. As such, no medicine has made any difference to patients' outcomes with ALI over the last 3 decades.

Accurate ways to help diagnose the condition are urgently needed to improve understanding of the early stages of acute lung inflammation.

In response to this need, our research group and others have developed a 'model' of early lung inflammation in healthy volunteers. This involves inhaling (breathing in) lipopolysaccharide (LPS) which is a component of bacteria, but is not infectious. This produces a safe, mild and temporary form of lung inflammation that mimics the early stages of ALI. This allows us to study early stages of lung inflammation in a controlled and safe way.

In the current study we aim to use this model to work out what happens to function of the body's white blood cells and the blood vessels of the lungs after inhaling LPS. We hope this improved understanding may suggest new ways of treating ALI in the future and help us to develop safe, improved scan techniques using Magnetic Resonance Imaging (MRI) to help more accurate diagnosis of ALI.

Why have you been chosen?

Because you are a healthy young person.

What will happen to you if you take part?

You will attend for a screening visit. If the screening visit is satisfactory and you want to proceed to the study you will then be asked to return for 3 separate study visits, over a maximum of 3 weeks. The procedures we would like you to have are:

- Two MRI (Magnetic Resonance Imaging) scans
- Blood samples
- Inhalation (breathing in) of LPS, aiming to cause mild, temporary inflammation in the lung (or inhalation of a saline 'placebo' instead of LPS, though you will not be told whether you will receive LPS or saline)
- A 'camera' investigation of the lung (called bronchoscopy) and bronchoalveolar lavage (using saline to 'wash' cells from a small segment of the lungs)

Greater detail about each of these is found in Part 2.

Do you have to take part?

No. It is up to you to decide whether or not to take part.

You are free to withdraw from the study at any time and without giving a reason. A decision not to take part, or withdraw at any time, will not affect the health care you receive at any stage, now or in the future.

How long can you take to decide?

You should take as much time as you wish

Do you have to complete all of the tests described?
No. While we obviously prefer to obtain all of the samples described, you are under no obligation to have any of the tests. You can complete all of the tests, or you can decline as many of the tests as you wish.

Involvement of your GP
With your permission, we shall inform your GP by letter that you have taken part in our study. With your permission, should any abnormal clinical results emerge during your tests, we would inform you and your GP.

Will any genetic tests be done?
Yes. Part of our research aims to determine how 'mitochondrial DNA' behaves in response to inflammation. Mitochondria are the 'engine of cells', providing the cell with energy. Some research studies have suggested that mitochondrial DNA in white blood cells may respond differently during inflammation. We will also test for the presence of 'micro-RNA' in your blood. RNA is related to DNA and is made when cells produce essential proteins for the body. By looking for small segments of RNA produced in response to inflammation we can make judgements about the activity of the cells producing these proteins. We shall not, at any point, be testing DNA or RNA for genes associated with specific medical conditions.

What are the potential benefits of taking part?
There is no direct benefit to you.

Is there any reimbursement for taking part?
Yes. We shall reimburse any travel costs. There is no reimbursement for attending the screening visit, but we will provide £250 for completing the subsequent study days.

Can you access the results of the research?
Yes. We will be happy to send you a summary of the overall results of the study after its completion, should you so wish. If you would like us to do this, please contact Professor Simpson at the address shown below.

What will happen to the results of the research study?
We intend for the results to be published in medical/scientific journals and presented at medical/scientific meetings. All information in the public domain will be anonymous (i.e. you cannot be identified from these publications/presentations). We intend that the results of the study will inform the design of future studies and treatments for people with ALI.

What if there is a problem?
If you have a concern about your treatment by members of staff during the study, you should ask to speak with the researchers who will do their best to answer your concerns (see contact details for Professor Simpson given at the head of this sheet). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from your hospital.

In the unlikely event that something goes wrong and you are harmed during the study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the NHS/Newcastle University but you may have to pay for your legal costs. The normal NHS complaints mechanisms will still be available to you.

If you should lose the mental capacity to give informed consent during the study you would no longer be able to take part in the study but we would keep any identifiable data collected about/from you before that point.

Will any material be stored?
Yes, but only with your permission. We propose to store the liquid portion from blood samples (plasma/serum), the mitochondrial DNA, and fluid (lavage fluid) obtained from your lungs. In addition we shall retain slides containing cells from your blood and lungs and copies of your MRI images. These will be kept in an anonymised form (i.e. you cannot be identified from the samples). We propose to keep your samples for up to 5 years after completion of the study.
At 5 years we propose to destroy the samples and images. However, as medicine advances and new information becomes available, we occasionally find good reasons to perform additional tests on stored samples in the future. Should this situation arise we may use your samples/images again, but this would be on the strict condition that you give permission for this, that the information would be anonymous (i.e. you could not be identified from them) and that we obtain new and separate permission from an ethics committee.

Will your taking part in this study be kept confidential?
Yes. Your information and samples will be given a study identity number, and all the information would remain strictly confidential. Your name will not appear in any report or publication that arises as the result of this study.

Who is organising and funding the research?
The research team is made up of clinicians and staff from Newcastle University and Newcastle Upon Tyne Teaching Hospitals NHS Trust. The research is funded by the Joint Research Executive Scientific Committee (Newcastle Health Care Charity and Newcastle Upon Tyne Hospitals NHS Charity, an independent registered charity that funds medical research) and Newcastle NIHR Biomedical Research Centre, Newcastle University.

Who has reviewed the study?
The scientific basis of the study was independently reviewed by doctors/scientists at Newcastle University. The study has also been independently reviewed and approved by a local Research Ethics Committee and the regional Research and Development Office.

Is there an independent doctor you can approach for further information?
If you would like to discuss any aspect of this research with an experienced researcher who is not linked in any way to this study, please feel free to contact:
Dr Bernard Higgins
Consultant Respiratory Physician
Cardiothoracic Centre
Freeman Hospital
Freeman Road
NE7 7DN
Tel: 0191 2137693
bernard.higgins@nuth.nhs.uk

This completes Part 1 of the Participant Information

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making your decision.

THANK YOU FOR TAKING THE TIME TO READ THIS INFORMATION AND FOR CONSIDERING TAKING PART

A lipopolysaccharide (LPS) inhalation model to characterise divergent cellular innate immune responses and presence of alveolar leak early in the course of acute lung inflammation

PARTICIPANT INFORMATION SHEET
STUDY GROUP 3

PART 2

What will happen to you if you take part?
You will attend for a ***screening visit*** to see if you satisfy all the necessary conditions for the study. If you continue to take part you would come back at a later date for 3 separate ***study visits.***

Screening visit
On a day suitable to you, you would come to the Clinical Research Facility at the Royal Victoria Hospital, Newcastle. The purpose of this visit is to check that you do not have any features which might exclude you from our study, and to answer any questions you may have. Pease also note that you should not be taking part in any other research studies at the time.
A doctor from the research team will take a short medical history and examine your heart and lungs. You would also have:

- Simple measurements of heart rate, blood pressure and temperature
- Measurement of the oxygen level in the blood (this involves placing a probe on your finger and does not involve needles)
- A blood sample taken to check your 'full blood count' (which tells us about your white blood cells) and blood biochemistry
- Spirometry (a test of lung function which involves taking a deep breath and breathing out as hard and fast as you can into a mouthpiece)
- For female participants a urine test (to exclude pregnancy)
- 'Practice' inhalation using saline (so you can get used to the nebuliser machine)

The information obtained will be kept confidentially by the research team. We shall inform you if we find any unexpected abnormalities and, with your permission, inform your general practitioner. If we are satisfied that there are no reasons to prevent you taking part, and if you still wish to participate in the study, you will be asked to return for the actual study.

Study visits in Summary

Study Visit 1:
Venue: Clinical Research Facility and MRI scanner at Freeman Hospital, Newcastle
Time: 1-2 hours

- Consent
- Brief medical history and examination of heart and lungs
- First MRI scan

Study visit 2: Between 1-21 days from Study Visit 1
Venue: Clinical Research Facility, MRI scanner and Bronchoscopy suite, all Freeman Hospital, Newcastle
Time: Approximately 9 hours (commencing approx 8am)

- Brief medical history and examination of heart and lungs
- Spirometry (simple breathing test)
- Practice inhalation of saline
- Baseline blood test
- Inhalation of lipopolysaccharide (LPS) or saline
- Observation over the following 6 hours, within the Clinical Research Facility (including measurement of blood pressure, heart rate, temperature and oxygen level)
- Blood tests taken at 2 hours, 4 hours and 6 hours after inhalation (blood will be taken from a 'cannula' placed in your vein at the baseline blood test, such that we can take blood more than once from a single use of a needle)
- Fasting (nothing to eat or drink) for 4 hours in preparation for bronchoscopy
- Second MRI scan (approximately 6 hours after inhalation)
- Bronchoscopy and lavage ('wash' of the lungs; approximately 7 hours after inhalation)
- Observation after bronchoscopy

Study visit 3: The day after study visit 2
Venue: Clinical Research Facility, Freeman Hospital, Newcastle
Time: Approximately 30 minutes (commencing approx. 8am)

- Brief medical history and examination of heart and lungs
- Spirometry (simple breathing test)
- Blood test

- Telephone call later that day (to check that you still feel well)
  At the end of this Telephone call your involvement in the study will be complete.

A more detailed explanation and potential symptoms associated with each procedure are described in the next section.

MRI (magnetic resonance imaging) scan

This will be performed at the MRI scanner at Freeman Hospital.

An MRI scan is a routine radiology technique that uses magnets, radio-waves and computers to create images of the body's structures. Unlike x-rays or CAT scans, no radiation is used. The MRI scanner is a tube surrounded by a large circular magnet. The person lies flat on a bed that can move in and out of the scanner. The scanner creates a strong magnetic field and radiowaves that allows us to get a magnetic signal from water in the body. These signals are detected by a receiver in the MRI scanner and processed by a computer to produce an image. The image and resolution produced by MRI is very detailed and can detect tiny changes of structures within the body. We also use 'contrast agents' when performing the MRI scans. A contrast agent is injected into a vein during a scan, and can be seen on MRI scans, allowing us to measure blood flow and improve images of blood vessels in certain parts of the body (e.g. the lungs).

A 'cannula' will be placed into a vein in your hand or arm. A cannula is a thin plastic tube introduced by a needle, commonly used in medicine to allow us to take blood or give a person medicines. In this case it will be used to give a contrast agent. On study visit 2, you will already have a cannula in place (see blood sampling below). You will be asked to lie flat on the moveable bed. Because the MRI scanner can be noisy you will be given headphones with noise-reduction technology built-in and also offered music to listen to (you can bring your own choice of music if you would like). The bed will move in and out of the scanner. The room and scanner are well lit at all times. You will be asked to lie still and sometimes hold your breath for a few seconds. Contrast agent will be injected through the vein part way through the scan. Although staff are in a different room, you will be able to talk to research staff or tell us if you are uncomfortable and we will be able to talk to you.

The magnetic tube can be quite close to the body and you will need to lie still for approximately 40 minutes to 1 hour. Some people find this uncomfortable or claustrophobic and for this reason we exclude volunteers with known claustrophobia. It is important to recognise that the scan can be discontinued at any point – i.e. in the unlikely event that you experienced significant claustrophobic symptoms, the scan can be stopped. Some patients describe a feeling of warmth during the scan.

MRI is considered an extremely safe medical investigation. Any risk is almost exclusively associated with having metallic material or medical devices within the body. You will therefore complete a routine questionnaire to check for this at your screening visit.

Contrast agents are used to measure blood flow by MRI. As with injection of all contrast agents, there is a very small risk of allergic reaction (estimated at less than 1 in 3000 injections). Medical supervision and treatment will be immediately available if this reaction occurs. There is also an extremely rare kidney condition (called nephrogenic systemic fibrosis, NSF) caused by injection of gadolinium-based contrast agents. This causes scarring of the kidneys and loss of kidney function. In over 200 million patients worldwide given the contrast agents that we will be using only 250 cases have been reported and only in cases where the patients' kidneys were not working properly due to disease. To our knowledge there has never been a case reported in a person with normal kidney function, and we will screen your kidney function in the blood test taken at your screening visit for this specific reason.

Blood sampling

At the screening visit will shall take a single blood sample using a needle. The amount of blood on the screening day will be approximately 10mls (i.e. equivalent to one third of a tablespoonful). On Study visit 1, you will have a cannula placed for injection of contrast agent but no blood sample will be taken at this time. At the end of the MRI scan the cannula will be removed.

On study visit 2, we take multiple blood samples, through a cannula. In this way, several samples can be taken over time, but we should only require to use a needle once. We propose to take 4 blood samples over the course of the day (i.e. at baseline, 2, 4 and 6 hours). Two samples will be approximately 35mls (i.e. a tablespoonful), the other 2 will be approximately 15mls**.** The cannula will remain in place and will be used for your second MRI scan and bronchoscopy (thus avoiding more needles). It will be removed after a period of observation following bronchoscopy.

The blood sample taken on study visit 3 (next day) will also be a single blood test taken with a needle. This will be approximately 35mls. Therefore, the maximum amount of blood that a volunteer can be asked to provide over the course of this study (including screening visit) is approximately 145mls. To place this in context, a standard blood donation to the Blood Transfusion Service is 2 times this amount.

Inhalation of LPS or saline

A doctor in the research team will ask you a few medical questions and briefly examine your heart and lungs. You will also perform a breathing test (spirometry) which involves taking a maximal deep breath in, then breathing out as fast and hard as you can, into a tube. You will then proceed to the inhalation. You will not be informed whether you are to breathe the LPS or saline (this is called 'blinding' and is routinely used in clinical science). Before the actual inhalation you will practice breathing in through the specialised nebuliser machine. The spirometry test will be repeated.

You will then be asked to breathe in the LPS or saline through the nebuliser machine you have just practiced on. The actual test simply involves 5 slow, controlled breaths through a mouthpiece. LPS is a product made by certain bacteria and is just one small component of the bacterium – i.e., if you are allocated to receive LPS, you would not be inhaling actual bacteria that may cause an infection but a small, non-infectious component of the bacterium. The dose of LPS we use is 60 micrograms. This dose has proved to be extremely informative and safe in our previous studies. We would be pleased to supply you with further literature from such studies if you wish. Your pulse, blood pressure, temperature and oxygen levels will be recorded after the inhalation.

The LPS is used to cause a mild inflammation in the lung lasting a few hours only. Up to a third of volunteers may feel slightly tired or have a warm, flushed or 'flu-like' feeling a few hours afterwards, but not all of these volunteers actually have fever. Rarely a transient sensation of tightness in the chest can develop but it is unusual for this to be associated with any change in lung function tests. Nebulised saline is unlikely to cause any symptoms.

After the nebulised LPS/saline you will rest in the clinical research facility for approximately 6 hours. During this period you will have blood samples (as described above) and intermittent recording of your pulse, blood pressure, temperature and oxygen levels. You will proceed to MRI scan, followed by bronchoscopy.

Bronchoscopy and Bronchoalveolar Lavage (BAL)

Bronchoscopy is a routine medical diagnostic procedure, in which a thin, soft, flexible plastic tube is passed into the lung. The tube carries a light source (fibre-optic bundles) and a channel through which we can introduce fluid and/or suck fluid from the lung. This test allows us to visualise your lungs directly (images are projected to a television screen in the room), and to 'wash' a small part of one of your lungs, allowing us to obtain cells for study in the lab. The procedure is carried out in a dedicated 'endoscopy or bronchoscopy suite'.

You will be asked to 'Fast' (go without food or fluid) for 4 hours prior to the procedure. This is to empty your stomach to prevent any food entering your lungs while the main windpipe is anaesthetised (see below). Preparation for the procedure takes about 20 minutes. You will be given a low dose of oxygen to breathe through your nose, an oxygen detection probe will be placed on your finger and your heart trace will be recorded throughout (as is standard practice with all diagnostic bronchoscopies in the NHS). Some local anaesthetic spray will be sprayed into your mouth (to numb the back of the throat so that it is not sore during the procedure). You will be offered a sedative, in the form of a medicine called midazolam (given through the cannula) which has the combined functions of making you sleepy and relaxed. It is up to you to decide if you would like to take this sedative or not so please discuss this option with us at the time, or before, if you want to. You will be asked to wear a small mouth guard (to prevent you biting the bronchoscope). When you are comfortable, the bronchoscopy and lavage will commence.

The procedure itself usually takes 10-20 minutes. The tube is passed through your mouth (or nose if you prefer) and to your vocal cords ('voice box'). We spray some local anaesthetic on the vocal cords (in order to numb them and limit coughing). About a minute later we pass the telescope through the vocal cords into the windpipe and lungs. This transiently induces coughing. Once this has settled, we perform the **bronchoalveolar lavage.** This involves introducing some sterile saline (salty water) through the telescope into a single 'segment' of the lung (the lungs have over 20 segments, and so saline enters only a small proportion of the lung). We then gently suck the fluid back through the telescope. The salty water now contains cells from deep inside your lungs that we can analyse. We then remove the telescope.

Bronchoscopy and BAL are considered extremely safe procedures, and even more so in healthy volunteers. The continuous oxygen monitoring and cardiac trace are part of the safety checks. In the extremely unlikely event that your oxygen levels cause concern, we remove the scope and administer extra oxygen until oxygen levels are satisfactory again. Although bronchoscopy is very safe, some elements may be transiently unpleasant.

a) the local anaesthetic spray tastes bitter and makes your eyes water for a few minutes. It then leaves the sensation that the mouth is 'swollen' (which it is not) and a sensation that it is difficult to swallow – you may have experienced this sensation if you have had dental work with local anaesthetic. The spray wears off after about 2-3 hours. If saliva pools in your mouth because of the spray, we suck it away with a suction catheter (again, you may have experience of this from having dental work).

b) in a very small minority of patients the natural 'gag reflex' is particularly strong and makes it hard for us to reach the voice box. It is impossible for us to predict whether you will be in this small minority until we do the test. If you are, and you find this particularly unpleasant, we simply stop the procedure.

c) almost all people have a bout of coughing and sometimes a sensation of choking, immediately after the scope has entered the lung for the first time. In nearly all cases this is accompanied by normal oxygen levels throughout, and lasts less than 10 seconds. We talk to you throughout the procedure and during this period you will hear us confirming that your oxygen levels are satisfactory and counting slowly to 10 – in our previous studies this has proved an effective means of reassurance that this period is safe and transient.

d) All volunteers cough at c) above – the majority have only minor cough after that. A small minority have more frequent cough – again this is unpredictable.
Most patients who have bronchoscopy have a mild sore throat for a few hours after the anaesthetic spray has worn off. Some volunteers have mild feverish symptoms for a few hours after bronchoscopy, particularly those who inhale LPS earlier (this is because the BAL, like the LPS, can cause mild feverish symptoms of itself). Some people have a cough that disappears within 24 hours – occasionally tiny specks of blood can be seen in the sputum, but this is entirely normal after bronchoscopy. The majority of patients relax for the rest of the day and resume full normal activities the following day.

After bronchoscopy and bronchoalveolar lavage you will rest and be observed. Your pulse, blood pressure, temperature and oxygen levels will be monitored. Two-to-three hours after administration of the local anaesthetic spray you will be able to eat. You will be seen by a doctor from the medical team and allowed home unless any unexpected findings arise.

If you have not been given sedative medicine, you may go home unaccompanied, where we recommend you rest for the remainder of the day. ***If you have accepted sedation (midazolam) you must be taken home by a friend or relative***, as the sedation means that you must rest for the remainder of the day and ***NOT*** return to work, drive, cycle, operate moving machinery (e.g. DIY tools), or sign any legal documents. This is because the sedation has subtle effects on your coordination, judgement and memory for a few hours afterwards.

You will be provided with a 24-hour telephone number to contact in the unlikely event that you feel unwell.

Contact Details
Should you wish any further information, please contact Professor John Simpson (contact details at the head of part 1) or:

Dr Sarah Wiscombe
Clinical Research Associate
Institute of Cellular Medicine, 4th Floor William Leech Building
Medical School, Newcastle University,
Framlington Place, NE2 4HH
Tel: 0191 222 6998 / 07782251915
Email: sarah.wiscombe@newcastle.ac.uk

If we have not heard anything from you within two weeks of sending you this we intend to send you a reminder about the study. If you would prefer not to be sent this reminder then please write, email or phone to tell me that and I will make sure you are not sent one.

This completes Part 2 of the Participant Information

THANK YOU FOR TAKING THE TIME TO READ THIS INFORMATION AND FOR CONSIDERING TAKING PART

nature COMMUNICATIONS

Appendix E

ARTICLE

https://doi.org/10.1038/s41467-019-09913-4 OPEN

Lipopolysaccharide inhalation recruits monocytes and dendritic cell subsets to the alveolar airspace

Laura Jardine[1,2,4], Sarah Wiscombe[1,2,4], Gary Reynolds[1,2], David McDonald[1], Andrew Fuller[1], Kile Green[1], Andrew Filby[1], Ian Forrest[2], Marie-Helene Ruchaud-Sparagano[1], Jonathan Scott[1], Matthew Collin[1,2], Muzlifah Haniffa[1,3,4] & A. John Simpson[1,2,4]

Mononuclear phagocytes (MPs) including monocytes, macrophages and dendritic cells (DCs) are critical innate immune effectors and initiators of the adaptive immune response. MPs are present in the alveolar airspace at steady state, however little is known about DC recruitment in acute pulmonary inflammation. Here we use lipopolysaccharide inhalation to induce acute inflammation in healthy volunteers and examine the impact on bronchoalveolar lavage fluid and blood MP repertoire. Classical monocytes and two DC subsets (DC2/3 and DC5) are expanded in bronchoalveolar lavage fluid 8 h after lipopolysaccharide inhalation. Surface phenotyping, gene expression profiling and parallel analysis of blood indicate recruited DCs are blood-derived. Recruited monocytes and DCs rapidly adopt typical airspace-resident MP gene expression profiles. Following lipopolysaccharide inhalation, alveolar macrophages strongly up-regulate cytokines for MP recruitment. Our study defines the characteristics of human DCs and monocytes recruited into bronchoalveolar space immediately following localised acute inflammatory stimulus in vivo.

[1] Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne NE2 4HH, UK. [2] Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne NE7 7DN, UK. [3] Department of Dermatology and NIHR Newcastle Biomedical Research Centre, Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne NE2 4LP, UK. [4] These authors contributed equally: Laura Jardine, Sarah Wiscombe, Muzlifah Haniffa, A. John Simpson. Correspondence and requests for materials should be addressed to L.J. (email: Laura.Jardine@ncl.ac.uk) or to M.H. (email: M.A.Haniffa@ncl.ac.uk)

The alveoli of the lung present a large but fragile surface area to the environment. Maintaining the integrity of the alveolar-capillary membrane is critical to effective gas exchange. Immune regulation at this interface must control infection and limit immunopathology. Mononuclear phagocytes (MPs), comprising monocytes, macrophages, and dendritic cells (DCs) have a critical role at any environmental interface as innate immune effectors equipped to shape the adaptive immune response through antigen presentation, co-stimulation, and cytokine production.

Leukocytes from the alveolar airspace can be readily isolated by bronchoscopy and bronchoalveolar lavage (BAL). BAL has been less extensively characterized than lung tissue but presents a number of advantages as a window to the lung's immune system: BAL yields a cell suspension free from contaminating blood leukocytes with minimal processing requirement thus preserving surface antigens and native activation status.

MPs in any anatomical compartment are a heterogeneous group of leukocytes. Establishing the steady-state repertoire of a compartment is crucial to understanding infiltrates seen in inflammation. Most tissues contain embryonically-derived macrophages with variable contributions from circulating monocytes depending on how available the tissue niche is[1,2]. Monocyte-derived cells are observed adopting a spectrum of macrophage or DC features depending on the tissue. While steady state monocyte-derived DCs are observed in mouse skin and gut[3–5], their existence in human tissues has not been convincingly established. DCs are broadly divided into plasmacytoid DCs (pDC) that characteristically produce IFN-α and conventional DCs (cDC) that effectively stimulate T cell proliferation[6]. Two subsets of cDCs with homology across species have been clearly identified. cDC1 expresses CD141, CLEC9A, and XCR1 in humans and is adept at cross-presenting antigen[7–11]. cDC2 expresses CD1c in humans and is important for activation of CD4 T cells, induction of regulatory T cells and activation of Th2 and Th17 responses[12,13].

A number of recent studies have capitalized on multi-parameter flow cytometry to define subsets of MPs across human lung compartments[14–18], but differences in approach have led to continued debate about whether rare DC subsets (pDC and cDC1) exist in BAL or have been inadvertently excluded during analysis. As blood is a source of tissue-recruited leukocytes, a logical approach would use blood MP definitions to classify tissue MPs. Recent insights from single cell RNA sequencing have revealed additional complexity in our understanding of blood MPs, including the presence of previously undiscovered DC subsets, and heterogeneity within existing subsets[19]. Briefly, this confirmed the presence of cDC1 (DC1) and pDC (pDC or DC6). It revealed two subdivisions within cDC2 (DC2, DC3) and identified additional DCs subsets: $Axl^{+}Siglec^{-}6^{+}$ DCs (AS DC or DC5) and $CD1c^{-}CD141^{-}$ DCs (DC4). To date, this revised classification has not been tested in non-lymphoid tissue.

As our understanding of the BAL MP repertoire in steady state develops, it becomes tangible to address the question of what happens during inflammation. In mice, inflammatory macrophages and DCs have been described in numerous infection and sterile inflammation models[20–25]. Monocytes are thought to be the source of inflammatory DCs, based on studies in CCR2 and Flt3-deficient animals and adoptive transfer of monocytes[20–24]. Distinct inflammatory macrophages and DCs have also been identified in human chronic inflammatory exudates[26]. These inflammatory DCs are proposed to arise from monocytes based on transcriptional similarity to in vitro monocyte-derived DCs[26]. Early time-points of inflammation have not been adequately explored.

Here, we use inhaled lipopolysaccharide (LPS) as an experimental inflammatory stimulus to reproducibly study the earliest events in human lung inflammation. Eight MP subsets can be identified in steady state BAL following sterile saline inhalation (SS-BAL), in line with subsets described in blood. Monocytes and two myeloid DC subsets (DC2/3 and DC5) are recruited as early as 8 h following LPS inhalation (LPS-BAL) and rapidly adopt gene expression profiles characteristic of airspace MP residence. The cytokine and chemokine profile of BAL implicates AMs as the likely instigator of blood monocyte and DC recruitment into the alveolar airspace during acute inflammation.

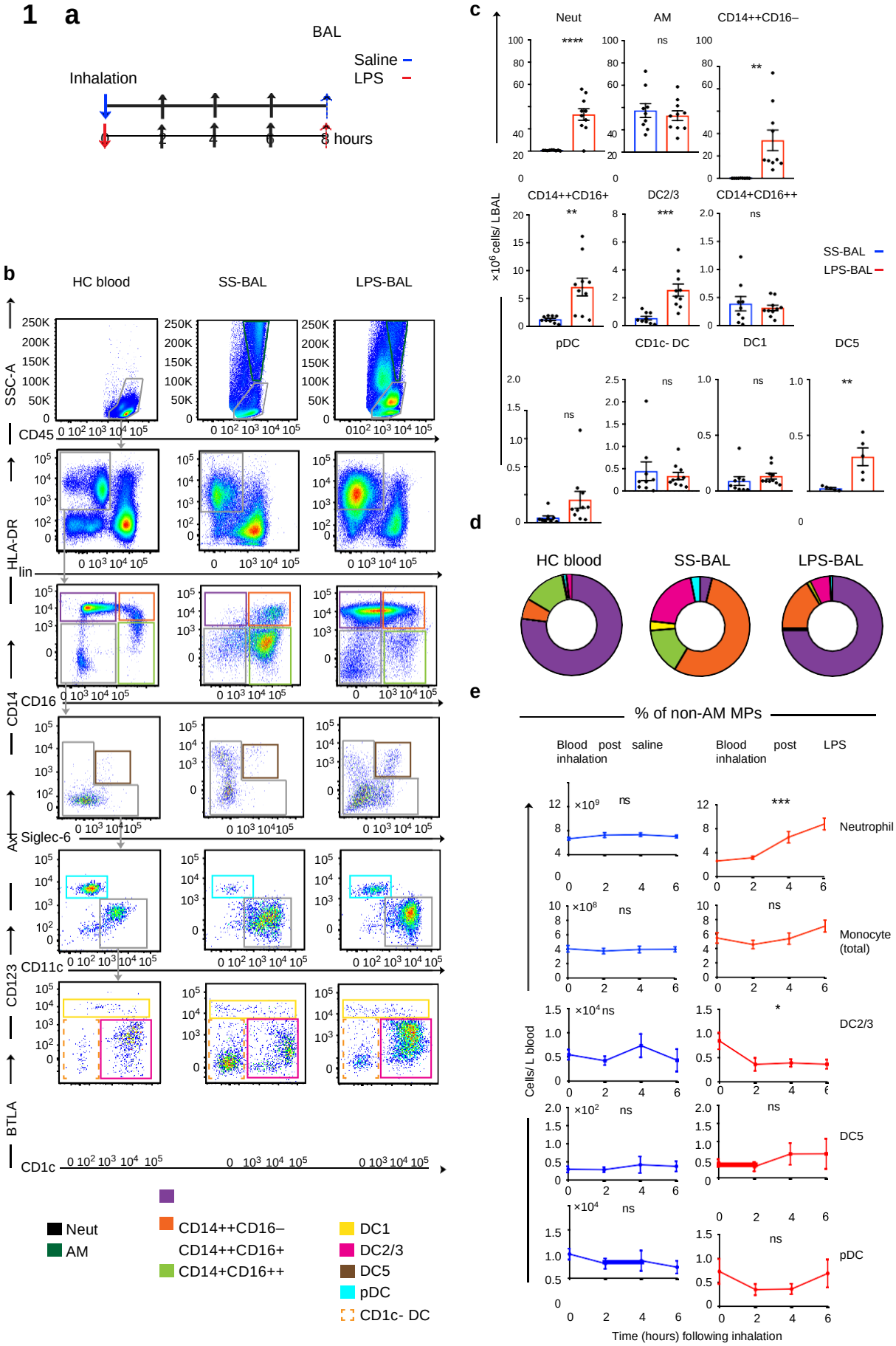
Results

Accumulation of alveolar neutrophils, monocytes, and DCs. The steady state MP repertoire of BAL was defined by flow cytometry in healthy individuals 8 h after inhalation of isotonic saline (Fig. 1a, Supplementary Fig. 1). We used a recent description of human blood MP diversity based on single cell RNA-sequencing as a template for identifying BAL MP popula- tions[19]. As an adaption for BAL analysis, we first used side scatter and CD45 expression to exclude $CD45^{lo}SSC^{mid}$ neutrophils and identify alveolar macrophages (AM) as $CD45^{+}SSC^{hi}$ cells (Sup- plementary Fig. 1). BAL $CD45^{+}SSC^{lo}$ cells were then comparable to peripheral blood mononuclear cells (PBMC) (Fig. 1b). Per- ipheral blood was sourced from healthy controls (HC) that had not received inhalation challenge. $CD45^{+}SSC^{lo}$ cells that expressed HLA-DR but not lineage markers (CD3, 19, 20, 56) were gated into $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}$ $CD16^{++}$ fractions analogous to blood classical, intermediate and non-classical monocytes, respectively. The $CD14^{-}CD16^{-}$ frac- tion containing DCs was first probed for the rare $Axl^{+}Siglec6^{+}$ population (DC5) as its varied expression of CD123 and CD11c would otherwise place it within pDC and cDC gates. After DC5 exclusion, $CD123^{+}CD11c^{lo}$ cells identified pDC and further segregation of the $CD11c^{+}$ cells using a combination of BTLA and CD1c identified DC1 and DC2/3. While DC1 is most typi- cally defined by its expression of CD141 or CLEC9A, immune gene expression by cells sorted from the $BTLA^{hi}$ gate expressed the expected gene profile of DC1, confirming the validity of this approach (Supplementary Fig. 1). We did detect a population of $CD11c^{+}CD1c^{-}BTLA^{-}$ cells within our DC gate, which are likely to correspond to DC4 described in Villani et al., but can only refer to these as $CD11c^{+}CD1c^{-}$ cells without further characterization.

The dominant MP subset in SS-BAL was the AM (Table 1). Comparing SS-BAL $CD45+SSC^{lo}$ cells with PBMC, MPs were richer in SS-BAL (Table 1). Of MPs, the most abundant subset was a $CD14^{++}CD16^{+}$ population (Table 1; Fig. 1d). $CD14^{++}$ $CD16^{-}$ cells, analogous to classical monocytes in blood, were comparatively rare in SS-BAL and $CD14^{+}CD16^{++}$ cells, analogous to non-classical blood monocytes, were virtually absent. All DC subsets, especially CD1c-expressing DC1 and DC2, were enriched in SS-BAL relative to blood.

Following LPS inhalation, the greatest leukocyte expansion was in $CD14^{++}CD16^{-}$ MPs (400-fold difference in mean concentration between SS-BAL and LPS-BAL), followed by neutrophils and DCs (Fig. 1c). Amongst DCs, the concentrations of $CD1c^{+}$ DCs (DC2/3) and DC5 were selectively increased (Fig. 1c).

To examine the impact of acute lung inflammation on peripheral blood MP populations, their concentrations were tracked at 2-h intervals following inhalation of LPS or saline (Fig. 1e). Neutrophils, which were abundant in LPS-BAL and pDCs, which were not enriched in LPS-BAL, were tracked for comparison with monocytes and DCs. Following saline inhalation, no differences in leukocyte concentrations occurred compared with baseline. Following LPS inhalation, blood neutrophil concentration rose progressively, but pDC and


1
a
BAL
Saline
LPS
Inhalation
0
2
4
6
8 hours
b
HC blood
SS-BAL
LPS-BAL
SSC-A
CD45
HLA-DR
lin
CD14
CD16
Siglec-6
CD11c
CD123
BTLA
CD1c
Neut
AM
CD14++CD16–
CD14++CD16+
CD14+CD16++
DC1
DC2/3
DC5
pDC
CD1c- DC
c
Neut
AM
CD14++CD16–
CD14++CD16+
DC2/3
CD14+CD16++
×10^6 cells/ LBAL
SS-BAL
LPS-BAL
pDC
CD1c- DC
DC1
DC5
d
HC blood
SS-BAL
LPS-BAL
e
% of non-AM MPs
Blood post saline inhalation
Blood post LPS inhalation
Neutrophil
Monocyte (total)
DC2/3
DC5
pDC
Cells/ L blood
Time (hours) following inhalation

monocyte concentrations remained static. Blood DC2/3 concentration fell significantly within 2 h of LPS inhalation.

Alveolar $CD14^{++}CD16^{-}$ MPs are recruited blood monocytes. As $CD14^{++}CD16^{-}$ MPs were particularly enriched in LPS-BAL, we focused on this population to interrogate the molecular changes of tissue adaptation in vivo following acute inflammatory challenge. The surface antigen phenotype of LPS-BAL $CD14^{++}$ $CD16^{-}$ MPs by flow cytometry resembled that of blood monocytes. LPS-BAL $CD14^{++}CD16^{-}$ cells expressed genes characteristic of classical monocytes including *CD14*, *CCR2* and *SELL* but did not express *FCGR3A/B* (CD16), *CD79b*, and *CX3CR1*,

Fig. 1 Neutrophils and mononuclear phagocytes are expanded in the alveolar airspace following LPS inhalation. a Schematic overview of study design. Solid arrows denote LPS inhalation (red) or saline inhalation control (blue). Black arrows denote blood sampling. Dashed arrows denote BAL. b Flow cytometry of leukocyte preparations from SS-BAL, LPS-BAL, and HC blood. The CD45 versus SSC plot was used to define $CD45^{+}SSC^{hi}$ AM, $CD45^{lo}SSC^{mid}$ neutrophils and $CD45^{+}SSC^{lo}$ mononuclear cells (see also Supplementary Fig. 1). Monocyte/macrophages and DCs were negative for lineage markers CD3, CD19, CD20, and CD56 and expressed HLA-DR. Monocyte/macrophages were divided into $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{-}CD16^{++}$ populations analogous to blood classical, intermediate and non-classical monocytes. DCs within the $CD14^{-}CD16^{-}$ gate were divided into subsets: Axl^{+} $Siglec6^{+}$ DC5s, $CD11c^{lo}CD123^{+}$ pDCs, $CD1c^{+}BTLA^{lo\text{-}mid}$ DC2/3s, and $BTLA^{hi}$ DC1s. Plots are representative of $n = 9$ SS BAL and $n = 10$ LPS BAL. c Concentrations of neutrophil, monocyte/macrophage and DC subsets in SS-BAL and LPS BAL. Bars represent mean and lines SEM. *p*-values from unpaired *t*-tests of SS versus LPS are shown: "ns" $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. d Monocyte/macrophage and DC frequency in HC blood, SS-BAL and LPS-BAL as a proportion of SSC^{lo} MHC class II-expressing cells (not-including CD1c- DCs). e Concentration of selected leukocyte populations in peripheral blood at 2-h intervals following inhalation of saline (blue line) or LPS (red line). Data points show mean ± SEM for 3–5 participants. *p*-values from one-way ANOVA are shown. *p*-value representation is described in c

Table 1 Frequency of MP subsets in BAL and blood

MP subset		AM	$CD14^{++}$ $CD16^{-}$	$CD14^{++}$ $CD16^{+}$	$CD14^{+}$ $CD16^{++}$	DC1	DC2/3	$CD1c^{-}$	DC5	pDC
Antigen expression by flow cytometry	CD45	+	+	+	+	+	+	+	+	+
	SSC	hi	lo	lo	lo	lo	lo	lo	lo	Lo
	Lineage	−	−	−	−	−	−	−	−	−
	HLA-DR	+	+	+	+	+	+	+	+	+
	CD14	+	++	++	+	−	−	−	−	−
	CD16	+	−	+	++	−	−	−	−	−
	CD11c					+	+	+	-/+	−
	CD123					−	−	−	−/+	+
	BTLA					++	−/+	−	+	+
	CD1c					+/−	+	−	-/+	−
	Axl					+/−	+/−	+/−	+	−
	Siglec 6					−	−	−	+	−
% of leukocytes mean (SD) *% of MPs mean (SD)*	HC whole blood	0	8.01 (2.04) *75.0 (7.94)*	0.47 (0.22) *6.41 (2.29)*	1.29 (0.85) *13.8 (6.11)*	0.02 (0.01) *0.23 (0.22)*	0.30 (0.1) *1.07 (0.75)*	0.07 (0.07) *0.77 (0.72)*	0.02 (0.02) *0.22 (0.20)*	0.23 (0.05) *1.93 (0.93)*
	SS BAL	60.1 (14.3)	1.06 (0.14) *2.92 (1.88)*	2.38 (0.95) *43.7 (8.00)*	0.64 (0.56) *11.4 (7.31)*	0.12 (0.10) *2.41 (1.91)*	0.82 (0.35) *16.1 (4.24)*	0.69 (0.72) *12.3 (13.9)*	0.04 (0.02) *1.06 (0.36)*	0.13 (0.07) *2.57 (1.53)*
	LPS BAL	23.1 (13.5)	18.7 (9.13) *64.3 (17.8)*	4.51 (3.23) *18.2 (16.6)*	0.23 (0.16) *0.91 (0.67)*	0.09 (0.04) *0.33 (0.15)*	1.63 (0.61) *6.12 (2.59)*	0.2 (0.11) *0.69 (0.37)*	0.14 (0.05) *0.45 (0.11)*	0.22 (0.16) *0.76 (0.49)*

MP subsets present in BAL. MP subsets are defined by the flow cytometry gates in Fig. 1b. Expression of defining antigens is indicated as "++" bright expression, "+" positive expression, "+/−" low-level expression or "−" no expression. Where no symbol is given, expression was not measured on that subset. Frequencies of each MP subset in whole blood, SS BAL and LPS BAL are given. In standard type, frequencies are given as a percentage of total leukocytes (defined as $CD45^{+}$ cells). In italic type, frequencies are given as a percentage of MPs (defined as $CD45^{+}SSC^{lo}$, CD3,19,20,56^{-} $HLA\text{-}DR^{+}$ cells)

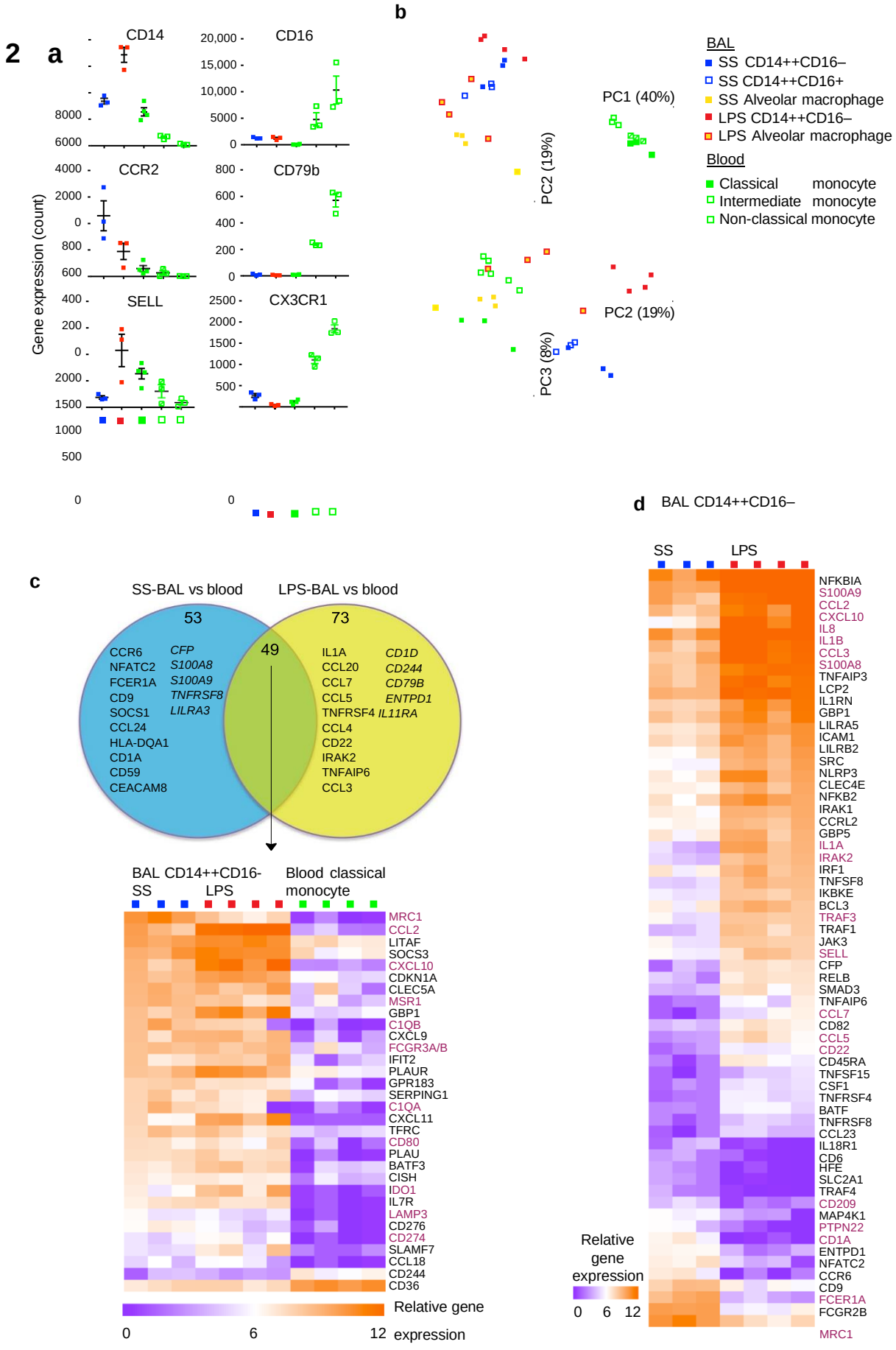
which are characteristic of non-classical monocytes[27] (Fig. 2a). However, unsupervised transcriptome analysis revealed distinct changes as a consequence of recruitment into the alveolar airspace.

Using the NanoString Immunology V2 579-gene panel, we compared the expression profile of HC blood monocyte subsets with SS-BAL and LPS-BAL monocyte-macrophages. By principal component analysis, the first principal component (PC1) segregated cells by tissue compartment (blood versus BAL) and the second (PC2) separated monocyte-macrophages from resident alveolar macrophages (Fig. 2b). PC3 separated the $CD14^{++}CD16^{-}$ cells from SS-BAL and LPS-BAL. The distance between BAL $CD14^{++}CD16^{-}$ MPs and monocytes, even by principal components 2 and 3, suggested adoption of a unique gene expression profile upon entry to the airspace. This was explored further by examination of differentially expressed genes (DEGs).

We performed pairwise comparisons of DEGs of $CD14^{++}CD16^{-}$ cells between tissue compartments (HC blood vs. BAL) and between inflammatory states (SS-BAL vs. LPS-BAL). There was a core signature of 49 DEGs (11% of the 457 genes expressed in these cell types) distinguishing SS-BAL and LPS-BAL $CD14^{++}CD16^{-}$ MPs from HC blood classical monocytes (Fig. 2c, Supplementary Dataset 1). The 36 up-regulated included genes encoding phagocytic receptors (*MRC1*, *FCGR3A/B*, *MSR1*), immunoregulatory proteins (*CD274*), innate immune effectors (*C1QA/B*), chemokines capable of monocyte and lymphocyte recruitment (*CXCL10*), and some genes associated with mature DC function (*CD80*, *LAMP3*). This supported the interpretation that $CD14^{++}CD16^{-}$ MPs are recruited blood monocytes with maturation and adaptation to the alveolar environment.

Comparing $CD14^{++}CD16^{-}$ MPs in SS-BAL and LPS-BAL, 98 genes (21%) were differentially expressed (Fig. 2d, Supplementary Dataset 1). Steady state (SS-BAL) was associated with higher expression genes involved in antigen presentation (*CD1A*, *HLA-DPA1*, *HLA-DMB*, *HLA-DOB*, *CD1D*), pathogen clearance (*CD209*, *MRC1*), control of inflammatory signaling (*PTPN22*), and the ability to activate the allergic responses (*FCEFR1A*). Inflammation (LPS-BAL) saw activation of LPS response genes (*IL1A*, *IL1B*, *IRAK2*, *TRAF3*, and others) retention of monocyte-associated genes (*S100A8/9*, *SELL*), and marked expression of chemokine genes (*CCL2/3/4/5/7*, *CXCL10*, and *IL8*). This suggests that in quiescence, $CD14^{++}CD16^{-}$ MPs have the capacity to present antigen, possibly helping to maintain tolerance, while in inflammation, they are primed to modulate immune functions through chemokine and interleukin production.

Blood $CD1c^{+}$ DC are recruited into the alveolar space. We used a panel of antigens showing divergent expression on tissue and blood CD1c-expressing DCs (DC2/3) to evaluate the flow cytometry phenotype of $CD1c^{+}$ DCs found in SS-BAL, LPS-BAL and HC blood[14,26,28] (Fig. 3a). Blood DC2/3 were $CD11b^{lo}$, Axl^{-}, $CD1a^{-}$, and $CD206^{-}$ while SS-BAL DC2/3 expressed all four of these antigens, confirming that these were genuine tissue DCs and not blood contaminants. However, the expression of these antigens on LPS-BAL DC2/3 paralleled that seen in blood DC2/3. Significant blood contamination of LPS-BAL was excluded by


2
a
CD14
CD16
CCR2
CD79b
SELL
CX3CR1
Gene expression (count)
b
PC1 (40%)
PC2 (19%)
PC3 (8%)
BAL
SS CD14++CD16–
SS CD14++CD16+
SS Alveolar macrophage
LPS CD14++CD16–
LPS Alveolar macrophage
Blood
Classical monocyte
Intermediate monocyte
Non-classical monocyte
c
SS-BAL vs blood
LPS-BAL vs blood
53
49
73
CCR6
NFATC2
FCER1A
CD9
SOCS1
CCL24
HLA-DQA1
CD1A
CD59
CEACAM8
CFP
S100A8
S100A9
TNFRSF8
LILRA3
IL1A
CCL20
CCL7
CCL5
TNFRSF4
CCL4
CD22
IRAK2
TNFAIP6
CCL3
CD1D
CD244
CD79B
ENTPD1
IL11RA
BAL CD14++CD16- SS
LPS
Blood classical monocyte
MRC1
CCL2
LITAF
SOCS3
CXCL10
CDKN1A
CLEC5A
MSR1
GBP1
C1QB
CXCL9
FCGR3A/B
IFIT2
PLAUR
GPR183
SERPING1
C1QA
CXCL11
TFRC
CD80
PLAU
BATF3
CISH
IDO1
IL7R
LAMP3
CD276
CD274
SLAMF7
CCL18
CD244
CD36
Relative gene expression
0
6
12
d
BAL CD14++CD16–
SS
LPS
NFKBIA
S100A9
CCL2
CXCL10
IL8
IL1B
CCL3
S100A8
TNFAIP3
LCP2
IL1RN
GBP1
LILRA5
ICAM1
LILRB2
SRC
NLRP3
CLEC4E
NFKB2
IRAK1
CCRL2
GBP5
IL1A
IRAK2
IRF1
TNFSF8
IKBKE
BCL3
TRAF3
TRAF1
JAK3
SELL
CFP
RELB
SMAD3
TNFAIP6
CCL7
CD82
CCL5
CD22
CD45RA
TNFSF15
CSF1
TNFRSF4
BATF
TNFRSF8
CCL23
IL18R1
CD6
HFE
SLC2A1
TRAF4
CD209
MAP4K1
PTPN22
CD1A
ENTPD1
NFATC2
CCR6
CD9
FCER1A
FCGR2B
MRC1
Relative gene expression
0
6
12

counting erythrocytes: $16.7 \times 10^6 \pm 28.4 \times 10^6$ in SS-BAL and $33.3 \times 10^6 \pm 30.8 \times 10^6$ in LPS-BAL (mean ± SD; $p = 0.04$ by unpaired *t*-test). This amounted to a 6 µl blood leak, permitting approximately 0.4% of the 10^5 DC2/3 found in LPS-BAL to be due to blood contaminants (DC2/3 frequency in peripheral blood is approximately 10 cells µl^{-1}[129]).

While the surface antigen phenotype of LPS-BAL DC2/3 by flow cytometry supported their rapid recruitment from blood

Fig. 2 CD14^{++}CD16^{-} monocyte-macrophage cells in LPS-BAL are recruited blood monocytes with transcriptional adaptations. a Expression of monocyte-subset discriminating genes in CD14^{++}CD16^{-} cells from SS-BAL (blue square) and LPS-BAL (red square) compared with HC blood classical (filled green square), intermediate (divided green square) and non-classical (open green square) monocytes. Gene expression was quantified by NanoString array. The left-hand column contains genes with higher expression in classical than intermediate or non-classical monocytes. The right-hand column contains genes with higher expression in intermediate and non-classical than classical monocytes. Bars represent mean ± SEM. b Principal component analysis of immune gene expression (579-gene NanosString array) by BAL monocyte/macrophages and blood monocyte subsets. c Analysis of differentially expressed genes (DEGs) in BAL CD14^{++}CD16− cells and HC blood monocytes. Comparisons were made by unpaired *t*-test with $p < 0.05$ and >3-fold difference in mean expression. Venn diagram shows DEGs in CD14^{++}CD16^{-} MPs from SS-BAL, LPS-BAL and HC blood. Circles represent DEGs between SS BAL and blood (cyan; 101 genes, 22%) and between LPS-BAL and blood (yellow; 124 genes, 27%). The overlapping circles represents DEGs shared between comparisons (49 genes, 11%). The top 10 upregulated genes (standard type) and top 5 downregulated genes (italic type) in BAL relative to blood are listed. Heatmap shows DEGs common to both SS-BAL and LPS-BAL CD14^{++}CD16^{-} cells relative to HC blood classical monocytes. "Relative gene expression" refers to log2 transformed normalized gene expression count data. Genes with >10-fold difference in mean expression are shown. Genes discussed in text are colored burgundy. d Heatmap showing DEGs between SS-BAL and LPS-BAL CD14^{++}CD16^{-} cells. Comparisons were made by unpaired *t*-test with $p < 0.05$. Genes with >5-fold difference in expression are shown. Genes discussed in the text are colored burgundy

DC2/3, consideration was also given to the possibility that they may have differentiated from recruited blood monocytes. The latter possibility was considered less likely within the eight-hour time window studied. Microarray analysis in human chronic inflammatory exudates aligned inflammatory CD1c-expressing DCs with in vitro monocyte-derived DCs[26]. From a list of conserved genes defining human and mouse monocytes and macrophages compared to DCs[28], 25 genes were evaluable on the Nanostring Immunology V2 panel. Hierarchical clustering the expression of these genes located LPS-BAL DC2/3 closest to SS-BAL DC2/3 and HC blood DC2/3 and remote from HC blood monocytes, BAL macrophages and in vitro monocyte-derived DCs (Fig. 3b). Furthermore, LPS-BAL CD1c-expressing MPs were effective stimulators of allogeneic T cell proliferation, in contrast to LPS-BAL CD14^{++}CD16^{-} MPs and AMs, further supporting their origin from circulating CD1c-expressing blood DCs (Fig. 3c).

We next explored the functional adaptations to airspace residence of recruited blood DCs by comparing the immune gene expression profile of blood DC2/3 with their SS-BAL and LPS-BAL counterparts (Fig. 3d, Supplementary Dataset 1). There was a core signature of 100 DEGs in SS and LPS BAL DC2/3 compared with HC blood DC2/3 (21% of the 466 genes expressed in these cell types): 39 upregulated and 61 downregulated genes. Up-regulated genes were required for mature DC function (*CD80*, *CCR7*, *LAMP3*), involved in immunoregulation (*CISH*, *CD274*, *CD276*, *TNFRSF11A*), monocyte and T cell recruitment (*CCL2*, *CCL22*), and pathogen recognition or scavenging (*CLEC5A*, *MSR1*). The genes most down-regulated in BAL DC2/3 compared with blood DC2/3 included *CD1D*, possibly as an adaptation to the lipoprotein-rich alveolar environment, and the lymphoid-lineage associated genes *CD22 and CD244*.

Relatively few genes were differentially expressed between SS and LPS BAL DC2/3 (Fig. 3e, Supplementary Dataset 1). As with LPS CD14^{++}CD16^{-}MPs, LPS DC2/3 expressed LPS response genes (*IL1B*, *IL8*, *IRAK2)* and chemokine genes (*CCL2,4,5 CXCL10,11)* to a greater extent than SS-BAL DC2/3.

CD1c^{+} DC heterogeneity within the alveolar space. The flow cytometry gating strategy used for parallel examination of BAL and blood revealed heterogeneity in the CD1c-expressing DC gate by BTLA expression (Fig. 1b). In blood, BTLA expression segregates CD1c-expressing DCs into two subsets with distinct gene expression profiles, with BTLA^{+} DC expressing lymphocyte lineage genes including *CD5*, *CD79A/B* and *CD24* and BTLA^{-} DC expressing monocyte/macrophage genes such as *CD14*, *S100A8/9* and *F13A1* (Fig. 4a). Gene expression differences between blood BTLA^{+} and BTLA^{-} DC correlate with gene expression differences between blood DC2 (HLA^{-}class II genes) and DC3 (*BST1*, *CD14*, *CD163*, *S100A8/9*)[19] (Fig. 4a) and with blood CD1c^{+} DC subsets segregated by CD5 expression[30].

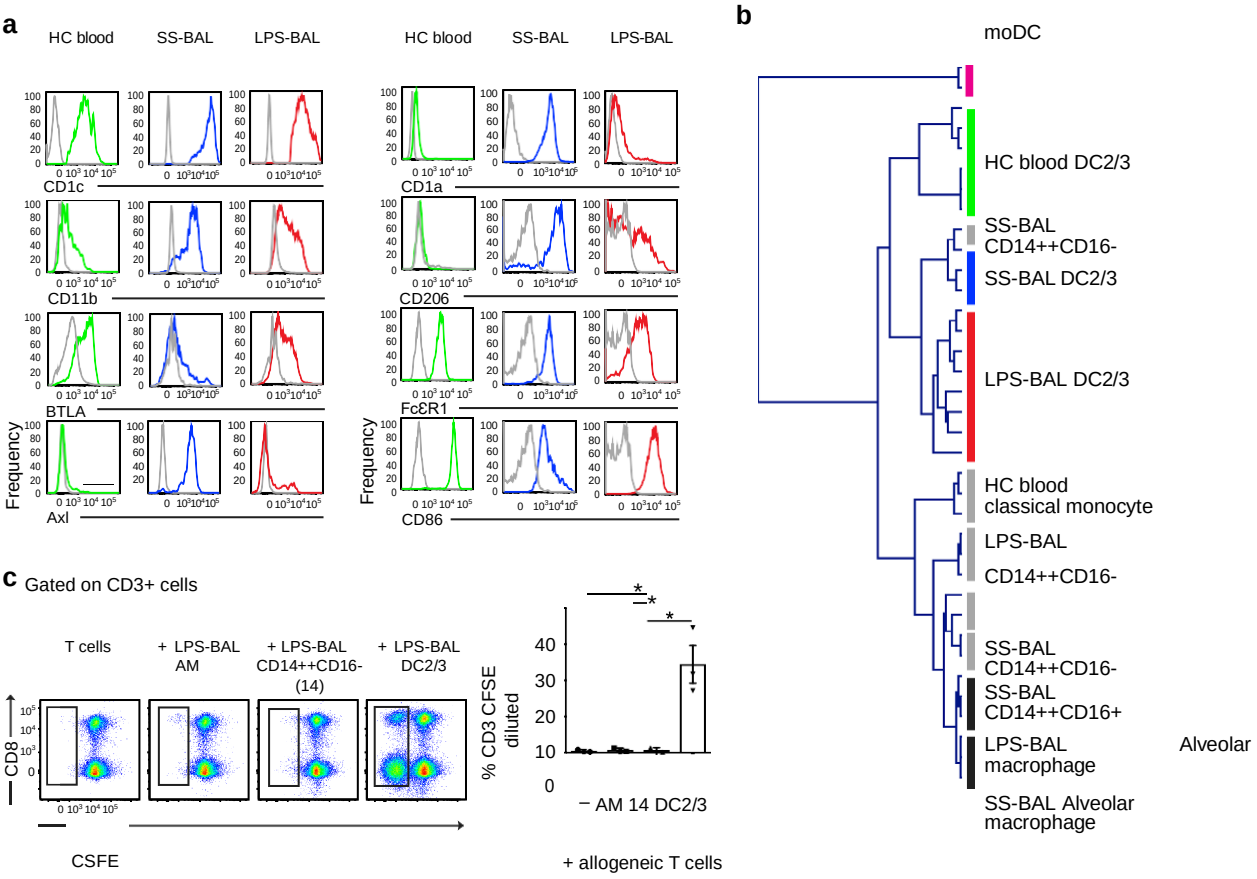
In BAL, BTLA expression discerned two subsets with the mean fluorescence intensity for BTLA^{+} cells at 1×10^{3} (corresponding to DC2) and BTLA^{-} cells at 100 (corresponding to DC3) (Fig. 4b). The ratio of DC2:DC3 in HC blood was 40:60. In contrast, SS-BAL showed marked skewing towards DC3 (20:80). In keeping with our previous findings suggesting that LPS-BAL CD1c^{+} DCs are recruited from circulating DCs, the DC2:DC3 ratio in LPS-BAL approximated that of blood.

The genes differentially expressed between HC blood DC2 and DC3 were not recapitulated between LPS-BAL DC2 and DC3 (Fig. 4c). The convergence in gene expression between LPS-BAL CD1c^{+} DC subsets following recruitment to BAL may be accounted for by inflammation (*SLAMF7*, *TNFRSF9*), as well as change of compartment (*CXCL9*, *FCGR3A/B*, *S100A8/9*).

Both blood CD1c^{+} subsets effectively stimulate allogeneic T cell production[19,30] but have distinct capacity for inducing cytokine production by CD4^{+} T cells, with CD5^{+}CD1c^{+} DCs (representing DC2 or BTLA^{+} DC) inducing IL-10, IL-22, IL-4, and IL-17 production) and CD5^{lo}CD1c^{+} DCs (representing DC3 or BTLA^{-} DC) inducing IFN-γ production. We therefore compared the capacity of LPS-BAL DC2 and DC3 to influence cytokine production by CD4^{+} T cells. We observed that both subsets were equally capable of inducing IFN-γ and IL-17 production without any IL-4 production. The capacity to induce IFN-γ production was significantly greater in LPS-BAL than SS-BAL DC2/3. Yet, even SS-BAL DC2 did not induce IL-4 production, supporting the concept that CD1c+DCs undergo functional alteration upon tissue entry (Fig. 4d).

The Axl^{+}Siglec-6^{+} DC5 population, while enriched in LPS-BAL, remained too small to analyse in functional assays or NanoString arrays. DC5 in peripheral blood exhibit a spectrum of CD123 and CD11c expression, with CD123^{hi}CD11c^{lo} DC5 expressing a pDC-like gene signature and CD123^{lo}CD11c^{hi} DC5 expressing a cDC2-like gene signature[19]. SS-BAL DC5 expressed more CD123, but a greater proportion of LPS-BAL DC5 expressed CD11c (Fig. 4e). This was in keeping with a cDC2 over pDC bias in the inflamed airspace.

Alveolar macrophages orchestrate MP recruitment. We hypothesized that resident AMs orchestrated the recruitment of leukocytes into the alveolar airspace following LPS inhalation. LPS-BAL AMs demonstrated high expression of chemokine genes including *CCL2-4* and *CXCL10-11* (Fig. 5a, Supplementary Dataset 1). Compared with SS-BAL AMs, LPS-BAL AMs expressed 4 to 42-fold higher levels of these chemokine transcripts. Only *CXCL12* (stromal-cell derived factor) was not expressed by AMs. Corresponding protein measurements of secreted chemokines confirmed high expression of CCL2−4,


a
HC blood
SS-BAL
LPS-BAL
HC blood
SS-BAL
LPS-BAL
CD1c
CD1a
CD11b
CD206
BTLA
FcεR1
Axl
CD86
Frequency
Frequency
b
moDC
HC blood DC2/3
SS-BAL CD14++CD16-
SS-BAL DC2/3
LPS-BAL DC2/3
HC blood classical monocyte
LPS-BAL CD14++CD16-
SS-BAL CD14++CD16-
SS-BAL CD14++CD16+
LPS-BAL macrophage
SS-BAL Alveolar macrophage
Alveolar
c
Gated on CD3+ cells
T cells
+ LPS-BAL AM
+ LPS-BAL CD14++CD16- (14)
+ LPS-BAL DC2/3
CD8
CSFE
% CD3 CFSE diluted
− AM 14 DC2/3
+ allogeneic T cells
d
SS-BAL DC2/3 vs blood DC2/3
LPS-BAL DC2/3 vs blood DC2/3
54
MME
PPARG
MARCO
CCL19
ICOS
PDCD1LG2
CEACAM8
IKZF2
EGR2
CD1A
CD45RA
SMAD3
FYN
BATF
LILRA5
81
IRAK2
CCL4
TNFRSF9
IL8
CD40
CXCL2
IL6
CD83
SRC
CCL3
CD79B
C5
KIT
CR1
IL11RA
100
BAL DC2/3
SS
LPS
Blood DC2/3
C1QB
C1QA
SERPING1
MSR1
FCGR3A/B
CCL22
CDKN1A
LAMP3
IL7R
CD80
CXCL9
CD9
IDO1
C2
PLAU
CLEC5A
CXCL10
CCR7
DUSP4
CD276 CISH
TRAF1
CD274
GBP5
TNFRSF11A
CCL2
CXCL11
EBI3
SOCS1
IL1A
CCL20
PDGFB
CSF1
TNFRSF4
TNFRSF8
C4BPA
TNFSF8
BLNK
LTB4R2
CD2
LILRA1
CD244
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CD1D
SELL
FCER1A
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LPS
IL1B
CXCL10
CCL4
CCL2
IRAK2
CXCL11
IL8
CCL5
TNFRSF4
CD45RA
C8B
MME
FN1
C1QA
0
6
Relative gene expression

CXCL10, and CXCL12 in LPS-BAL supernatant (Fig. 5b). mRNA profiling of the cognate chemokine receptors revealed their abundance on blood monocytes and DC2/DC3 (Fig. 5c), in keeping with our findings of their recruitment into BAL following LPS challenge.

Finally, we evaluated the dynamics of pro-inflammatory cytokine secretion by resident AMs and recruited $CD14^{++}CD16^{-}$ monocyte−macrophages isolated from LPS-BAL. Both resident LPS-BAL AMs and recruited LPS-BAL monocyte−macrophages secreted high levels of pro-inflammatory cytokines upon re-

Fig. 3 CD1c$^+$ DCs in LPS-BAL are likely to be tissue-recruited blood CD1c$^+$ DCs. a Expression of surface antigens by flow cytometry on DC2/3 from HC blood (green), SS-BAL (blue) and LPS-BAL (red) relative to isotype control (gray). Antigens predicted to discriminate between blood and tissue CD1c$^+$ DCs were tested. Representative plots from more than three experiments are shown. b Dendrogram showing hierarchical clustering of monocyte/macrophage and DC gene expression by indicated subsets isolated from HC blood and SS/LPS BAL. In vitro monocyte-derived DCs were also included. Clustering used Pearson correlation metric. Monocyte/macrophage and DC identifying genes were taken from McGovern et al.[28]. The 25 genes available on the NanoString Immunology v2 panel were used. c Proliferation of allogeneic peripheral blood T cells measured by CSFE dilution during 7-day co-culture with or without MP subsets isolated from LPS BAL. Flow cytometry plots are gated on CD3$^+$ T cells from a representative experiment. Summary graph shows 2–3 replicates per subset. Bars show mean ± SEM. Means were compared by one-way ANOVA with Dunnett's multiple comparison test of DC2/3 against other subsets. *$p < 0.05$. d Analysis of differentially expressed genes (DEGs) by DC2/3 from SS/LPS-BAL and HC blood. Comparisons were made by unpaired *t*-test with $p < 0.05$ and >3-fold difference in mean expression. Venn diagram shows DEGs in DC2/3 from SS-BAL, LPS-BAL, and HC blood. Circles represent DEGs between SS BAL and blood (cyan; 154 genes, 33%) and between LPS-BAL and blood (yellow; 124 genes, 39%). The overlapping circles represents DEGs shared between comparisons (100 genes, 21%). The top 10 upregulated genes (standard type) and top 5 downregulated genes (italic type) in BAL relative to blood are listed. Heatmap shows DEGs common to DC2/3 in both SS-BAL and LPS-BAL relative to HC blood. Genes with >10-fold difference in mean expression are shown. Genes discussed in the text are colored burgundy. e Heatmap showing DEGs between DC2/3 from SS-BAL and LPS-BAL. Genes with >10-fold difference in mean expression are shown. Genes discussed in the text are colored burgundy

stimulation with LPS in vitro, suggesting a coordinated partnership between early resident AM response which is further amplified by recruited CD14^{++}CD16$^-$ monocyte–macrophages in mediating early acute tissue inflammation (Fig. 5d).

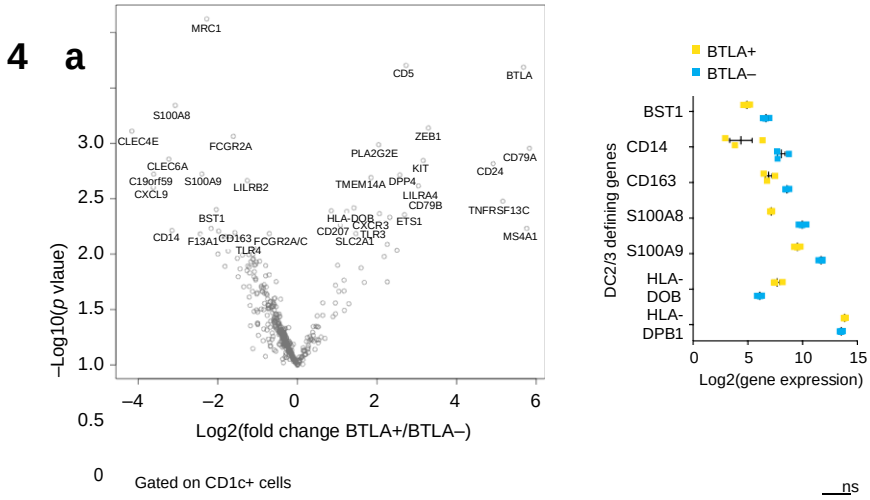
Discussion

The human MP system is a complex repertoire of innate immune cells distributed across distinct anatomical compartments that serve heterogeneous functions in the inflammatory response. Unraveling this complexity is necessary to understand tissue immune surveillance, immunopathology and inform vaccine design.

In keeping with previous reports[15,31,32], we identified alveolar airspace DC subsets (DC1, DC2/DC3, and pDC) corresponding to those previously described in peripheral blood. We established that BTLA distinguishes two CD1c$^+$ DC subsets in BAL with equivalent gene expression profiles to DC2/CD5$^+$ DCs and DC3/CD5^{lo} DCs in blood[19,33]. Separate CD1c$^+$ subsets have also been described in skin and arise independently from haematopoietic precursors, confirming that these are stable subsets and not simply variations in activation state[33]. We showed that BTLA expression is another reliable marker of DC1 in blood and lung and verified the identity of sorted BTLA^{hi} cells through their expression of DC1 defining genes (e.g., *XCR1*, *TLR3*, and *IRF8*). Two other studies[14,18] did not identify DC1 in lung interstitial tissue, which may be explained by the experimental design and gating strategy used for analysis. We identified a distinct pDC population in BAL, consistent with some reports[15,17,34] but not others[18]. The inconsistent finding of pDC in BAL does not arise from their selective vulnerability to cryopreservation[17]. Although Desch et al. did not find a CD123+CD303+pDC population in healthy lung tissue, CD303+ cells were identified in tumor- bearing lung[14], possibly indicating that pDCs in lung are restricted to the alveolar airspace and only present in parenchyma during pathological situations.

By mapping alveolar airspace MP subsets relative to blood populations, we were able to compare MP populations across compartments upon LPS-delivery into the airway. In most previous studies, tissue inflammatory composition has been examined in isolation without reference to blood and origins have been inferred[35–37]. Due to our referencing of blood it appeared logical that CD14^{++}CD16$^-$ MPs infiltrating BAL would be monocytes recruited from blood with tissue adaptation, but the nature of tissue adaptation required investigation. In both mouse and human studies, monocytes recruited to tissues can acquire DC or macrophage characteristics[20,22,26]. Monocytes have also been described entering tissue with minimal adaptation[38]. The type of MP characteristics adopted is highly relevant to the subsequent immune response as macrophages and DCs differ in capacity for migration, phagocytosis, ability to induce adaptive immune responses and in the cytokine/chemokine secretion profile. Through analysis of immune gene expression we established that recruited alveolar CD14^{++}CD16$^-$ cells in LPS-BAL and their counterpart in SS-BAL were distinct from blood monocytes. They expressed a broad array of genes important for mature macrophage function including phagocytic receptors, immunoregulatory proteins and cytokines. Despite the LPS activation signature present in recruited cells, there was commonality in the gene expression profiles of steady state and inflammatory CD14^{++}CD16$^-$ cells, emphasizing the importance and rapidity of the impact of tissue microenvironment on shaping monocyte fate following extravasation.

In addition to the expansion of monocyte–macrophages, we identified several DC subsets expanded in the alveolar airspace. Recruitment of DC from the blood to the gas exchan- ging regions of the previously healthy human lung during early inflammation has never been documented before. This recruitment was observed for specific DC subsets, including CD1c-expressing DC2/3 and Axl$^+$Siglec6$^+$ DC5 but not DC1 and pDCs. Sequential analysis of peripheral blood DC2/3 following LPS inhalation detected significant depletion during the period of accumulation in BAL. Analysis of surface antigens with differential expression between blood and tissue demonstrated that recruited DC had an antigen profile comparable with blood, consistent with recruitment of blood DCs and providing evidence against translocation of DCs from the lung interstitium. In the most comprehensive description of human inflammatory DCs to date, CD1c-expressing DCs in chronic inflammatory exudates were transcriptionally aligned to in vitro monocyte-derived DCs[26]. This fits with numerous observations in mouse that monocyte-derived cells accumulate in tissues under inflammatory conditions and can adopt DC characteristics[3,5,20,23,25]. In vivo data suggests it takes at least 24–48 h for monocytes to differentiate into DCs[39] and in vitro, generation of DCs from monocytes (human) or bone-marrow derived cells (mouse) takes 5–7 days[40,41]. In view of our 8-h time-course, the rapid accumulation of CD1c$^+$ DCs into alveolar airspace following LPS inhalation is most in keeping with recruitment from blood and is consistent with previous observations of CD1c$^+$ DCs accumulating in bronchial (conducting airway) mucosa within 4 h of allergen challenge[35], though detailed analyses of the infiltrating population was not possible in this earlier study. Transcriptome analysis further distinguished in vitro monocyte-derived DCs from LPS-BAL DCs. Our data suggest that in acute tissue inflammation, CD1c$^+$ DCs, including a subset with shared expression of monocyte genes, can be directly recruited from blood and arise independently of monocyte-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ZEB1
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C19orf59
S100A9
LILRB2
TMEM14A
DPP4
CXCL9
LILRA4
CD79B
TNFRSF13C
BST1
HLA-DOB
ETS1
CD14
F13A1
CD163
FCGR2A/C
CD207
CXCR3
TLR3
SLC2A1
MS4A1
TLR4
–Log10(p vlaue)
Log2(fold change BTLA+/BTLA–)
Gated on CD1c+ cells
BTLA+
BTLA–
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DC2/3 defining genes
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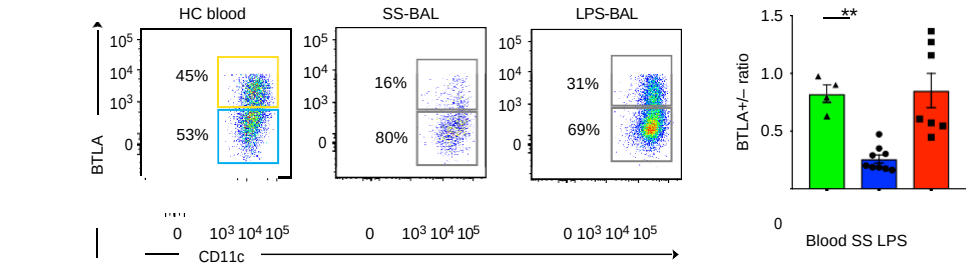
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31%
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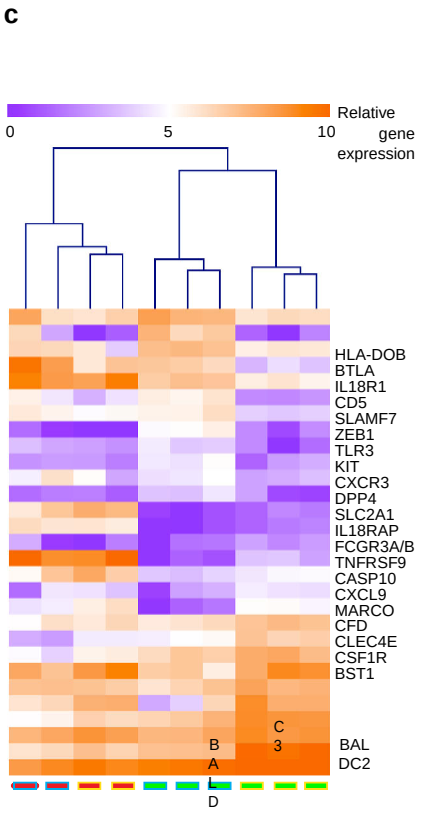
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CD5
SLAMF7
ZEB1
TLR3
KIT
CXCR3
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SLC2A1
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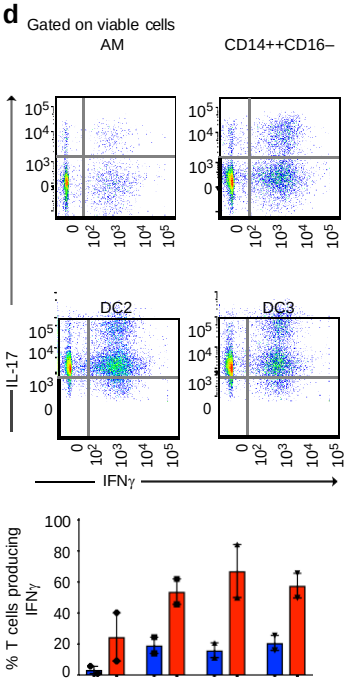
CSF1R
BST1
BAL
DC2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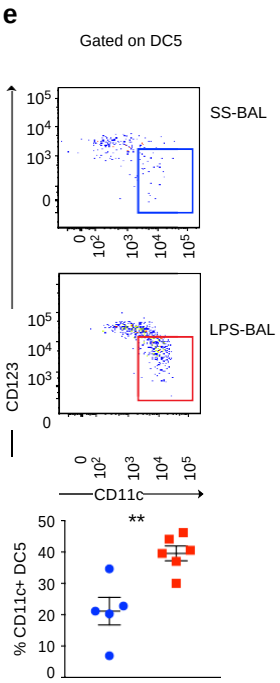
IFNγ
% T cells producing IFNγ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LPS-BAL
CD123
CD11c
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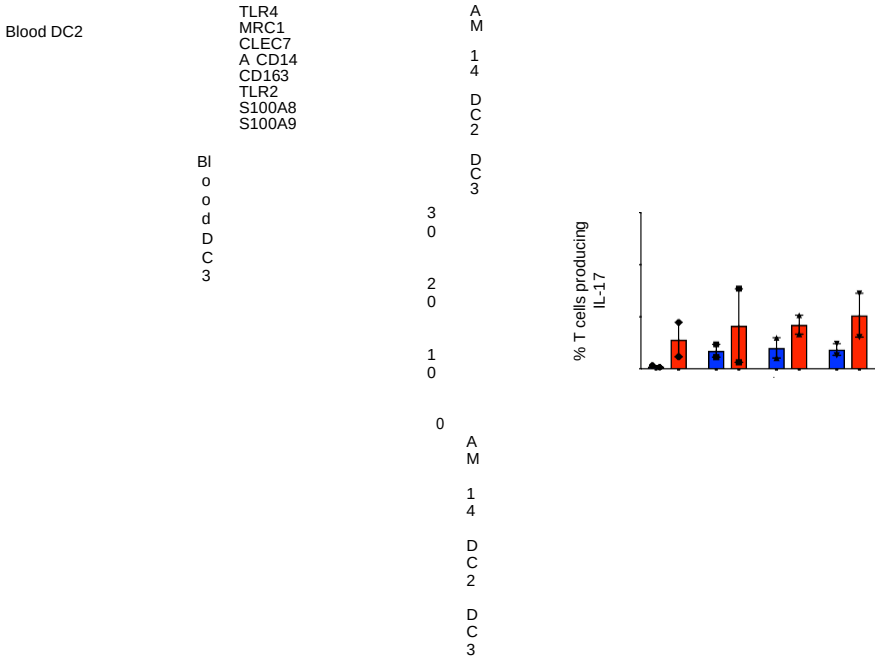
% CD11c+ DC5



differentiation. This finding is likely to reflect the early time-point we were able to study in our experimental model. Later characterization of BAL following experimental inflammatory stimuli and analysis of chronic disease states will reveal whether monocyte-derived DCs arise subsequent to this initial inflammatory DC influx.

We propose that resident AMs operate to recruit DCs into the alveolar airspace, in addition to their role in recruiting neutrophils and monocytes. Activation of AMs by LPS resulted in rapid upregulation of chemokines involved in leukocyte migration and pro-inflammatory cytokine mediators, elucidating how robust neutrophil recruitment and lung inflammation can be

Fig. 4 The pool of recruited LPS-BAL CD1c$^+$ DCs contains DC2 and DC3, which are functionally altered on entry to the inflamed airspace. a Volcano plot shows DEGs between BTLA$^+$ and BTLA$^-$ CD1c$^+$ DCs from HC blood. DEGs were calculated by unpaired *t*-test with *p*-value adjustment using Benjamini-Yekutieli method. Genes with $p<0.05$ are displayed as text. Accompanying scatterplots verifiy that that blood BTLA$^+$ and BTLA$^-$ CD1c$^+$ DCs exhibit the expected gene expression profile of DC2 and DC3 respectively. Plots show expression from $n=3$ samples with bars showing mean ± SEM. b Comparison of BTLA$^+$:BTLA$^-$ ratio in CD1c$^+$ DCs from HC blood, SS-BAL and LPS-BAL by flow cytometry. The gating strategy represented in Fig. 1b was used to define CD1c$^+$ cells. Flow cytometry plots show percentage of BTLA$^+$ and BTLA$^-$ CD1c$^+$ cells in representative examples of blood, SS-BAL and LPS-BAL. Accompanying bar graph summarizes flow cytometry data from n = 4–9 replicates. Statistical comparison by one-way ANOVA with Dunnett's multiple comparisons tests. ns $p>0.05$, $^{**}p<0.01$. c Heatmap showing expression of the 29 genes with differential expression between blood DC2 and DC3 ($p<0.05$ and fold difference >2) in DC2 and DC3 sorted from LPS-BAL and HC blood. Samples were clustered using Euclidian distance metric. d Flow cytometry read-out of a T cell cytokine production assay following 10-day co-culture of allogeneic peripheral blood CD4$^+$ T cells with macrophages and DCs isolated from LPS-BAL. Accompanying bar graphs summarize flow cytometry data showing mean ± SEM IFN-γ and IL-17 production following co-culture of SS-BAL (blue bars) or LPS-BAL (red bars) macrophages and DCs (each $n=2$). Two-way ANOVA of IFN-γ production gives $^{***}p<0.001$ for SS-BAL vs. LPS-BAL and ns $p=0.07$ for MP type. Two-way ANOVA of IL-17 production gives ns $p=0.06$ for SS-BAL vs. LPS-BAL and ns $p=0.67$ for MP type. e Flow cytometry plots show representative examples of CD123 and CD11c expression by Axl$^+$Siglec6$^+$ DC5 in SS-BAL and LPS-BAL. The gating strategy represented in Fig. 1b was used to define DC5. Gates demarcate CD11c-expressing cells. Accompanying scatterplot summarizes flow cytometry data, showing proportions of DC5 expressing CD11c in SS-BAL (blue circles) and LPS-BAL (red squares). $^{**}p<0.01$ by unpaired *t*-test

observed even after monocyte-depletion in a human LPS inhalation model[42]. The acute inflammatory cascade initiated by resident AMs is further amplified by recruited CD14^{++}CD16$^-$ monocyte-macrophages.

There are a number of limitations to this study. LPS inhalation provides the opportunity to study early time points in inflammation but it is not an accurate representation of human disease. Infective stimuli are rarely presented to the immune system as single bolus and will frequently be accompanied by other pathogen-associated molecular patterns. BAL permits effective sampling of the alveolar space, but "leaves behind" alveolar epithelial cells, adherent infiltrating cells, and those migrating through the interstitium. These populations could make important contributions to the regulation of the inflammatory response but cannot be sampled without recourse to biopsies that are unlikely to be ethical in healthy volunteers and may be subject to sampling error. Furthermore, BAL itself is inflammatory to the alveolar region, limiting the opportunity to study serial cell migration in the same individual, or to assess "recovery" BAL samples in this setting.

Our study has permitted detailed phenotypic, transcriptional, and functional analyses of MPs present in the alveolar airspace of healthy volunteers inhaling saline control or LPS to induce acute local inflammation. In LPS-BAL, we observed perturbation of the MP profile, noting significant influx of CD14^{++}CD16$^-$ monocyte−macrophages and selected DC subsets, likely regulated by resident AMs. This finding adds to our understanding of the potential role of blood DCs and the possibility of monocyte-independent inflammatory DCs at early time points in acute inflammation. Understanding the in vivo kinetics and dynamic regulation of MP following local LPS stimulation extends biological insights into the mechanisms of acute inflammation. Dissection of the functional consequences for the host will provide the opportunity to understand both beneficial and detrimental effects of such inflammation.

Methods

Ethical approval. The LPS inhalation study was approved by Newcastle & North Tyneside 2 Research Ethics Committee of the NHS Health Research Authority (REC reference number 12/NE/0196) under the full title "A lipopolysaccharide (LPS) inhalation model to characterize divergent cellular innate immune responses and presence of alveolar leak early in the course of acute lung inflammation". The study was conducted according to the principles expressed in the Helsinki Declaration and informed consent was obtained from all participants.

Study population. The study was advertised to university undergraduates. Potential recruits were invited to a screening interview to assess their suitability for participation. Inclusion criteria were healthy adult volunteers aged 18 to 40 able to give informed consent. Exclusion criteria were age <18 or >40 years; history of chronic respiratory disease, diabetes, heart disease, renal disease or recurrent infections; current respiratory tract infection; taking prescription medication (except oral contraceptives); current smoking or history of smoking 20 cigarettes per day for more than 2 years or any smoking in the past year; alcohol intake of more than 21 units per week; pregnancy or lactation; abnormal examination findings at screening (temperature >37.3 °C or oxygen saturation <95% breathing room air); abnormal blood results at screening (hemoglobin concentration, total white cell count or neutrophil count outside the gender-specific laboratory reference ranges; platelet count $<100\times10^9$ l^{-1}; serum sodium, potassium, creatinine or alanine aminotransferase outside laboratory reference ranges; serum urea >10 mg dl^{-1}; serum bilirubin >30 µmol l^{-1}); abnormal spirometry at screening (FEV1 or FVC <80% predicted or FEV1:FVC ratio <70%). Eligible volunteers were given verbal and written information about the study and at least 24 h to consider their participation before signing a consent form. All primary research documents were anonymised and participant details kept confidentially in accordance with Caldi- cott guidelines. Data from 19 participants are presented (n = 10 LPS, n = 9 saline).

Study interventions. Volunteers were allocated to inhale 54 µl sterile 0.9% sodium chloride with or without 60 µg LPS from *E. coli* 026:B6 (Sigma). Participants were allocated sequentially to receive saline or LPS to give optimal control over downstream experiments and they were not made aware of which intervention they had received. Delivery was targeted at the lower airways using an automatic inhalation-synchronized dosimeter nebulizer (Spira, Hameenlinna, Finland). The test solution was released following inhalation of 50 ml air to ensure that laminar flow was established. Participants performed a 5 s breath hold at vital capacity to promote deposition of LPS in the lower respiratory tract.

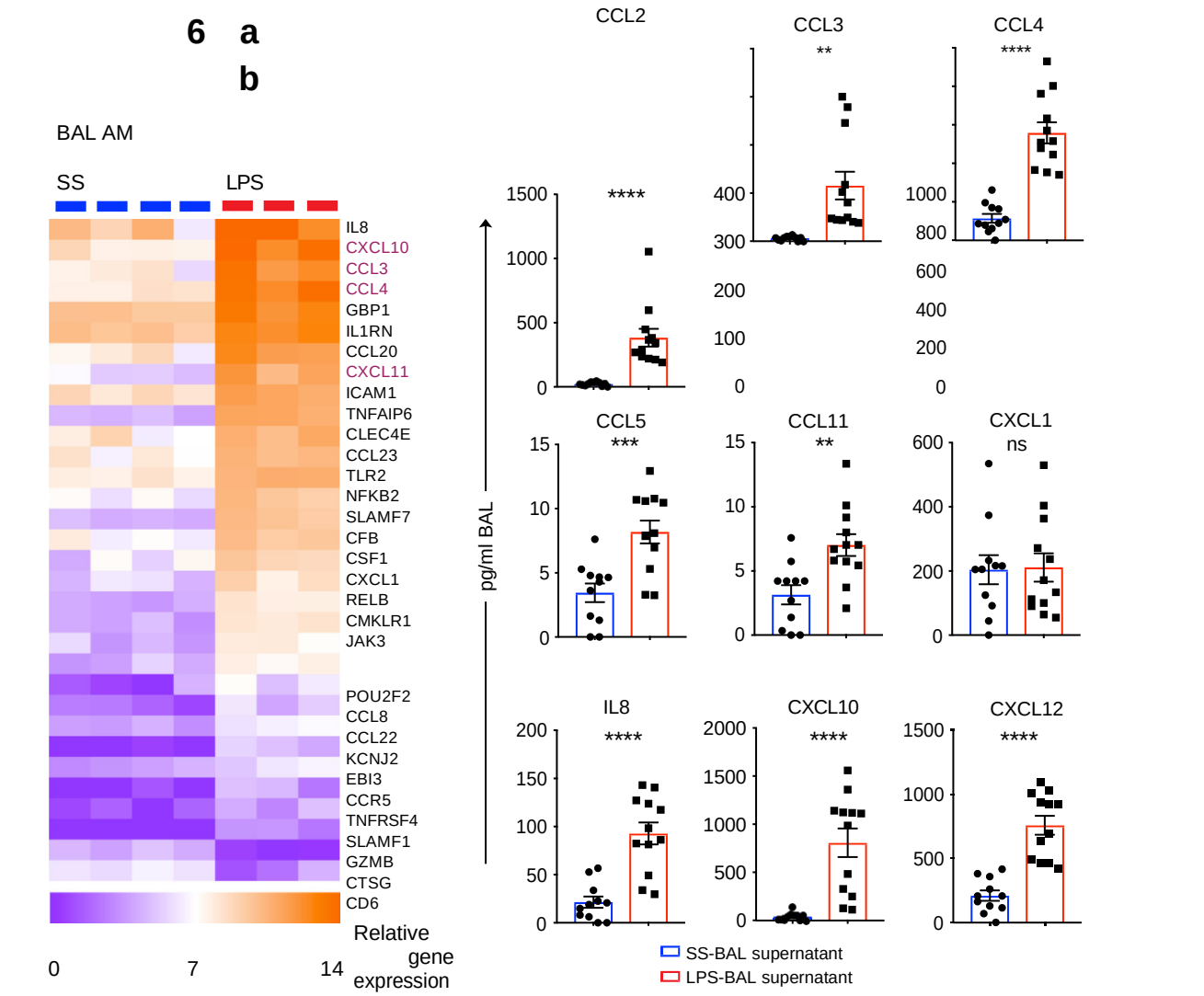
Participants were asked about symptoms (flu-like symptoms, sore throat, cough, wheeze, chest pain, sputum production, nasal secretions, or any other symptom) immediately after inhalation and at 6 h. Body temperature was measured hourly until 6 h. Venous blood samples were obtained at 0, 2, 4, 6, and 24 h after inhalation. We used blood leukocyte counts as the indicator of LPS effect.

Flexible fiber-optic bronchoscopy was performed 8 h after inhalation. Intravenous sedation with midazolam was available but all participants elected for a non-sedated procedure. Participants received topical administration of 1% lignocaine spray to the mouth and pharynx. Bronchial wash of the upper airways was performed with 20 ml warmed 0.9% sodium chloride and discarded. BAL of the right middle lobe was performed with 150 ml warmed 0.9% sodium chloride and retrieved by gentle suction.

Participant safety. History, bedside observations, cardio-respiratory examination, and spirometry were performed immediately before LPS or saline inhalation. Bedside observations were repeated hourly and spirometry repeated at 6 h after inhalation. If FEV1 fell by 10% from baseline, bronchoscopy was canceled. Prior to bronchoscopy, participants were fasted for 4 h. There was constant monitoring of oxygen saturations and electrocardiogram during bronchoscopy. Patients were observed for 30–60 min after bronchoscopy and allowed to leave if bedside observations and cardio-respiratory examinations were normal. Written and verbal advice was given to avoid eating and drinking within two hours of local anesthetic administered to the mouth and pharynx. Participants were informed that LPS inhalation and bronchoscopy may result in temperature, mild headache, shivering, dry cough, and upper airway discomfort.

Cell isolation. Blood was collected into EDTA. PBMC were isolated by density centrifugation using Lymphoprep (Stemcell technologies) according to manufacturers instructions. BAL samples were kept at room temperature for 60–90 min before processing at 4 °C. BAL fluid was passed through a 100 µ filter (Falcon).

Following centrifugation, cell-free supernatant was stored at −80 °C and cells were prepared for immediate analytical flow cytometry and cell sorting.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TLR2
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SLAMF7
CFB
CSF1
CXCL1
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TNFRSF4
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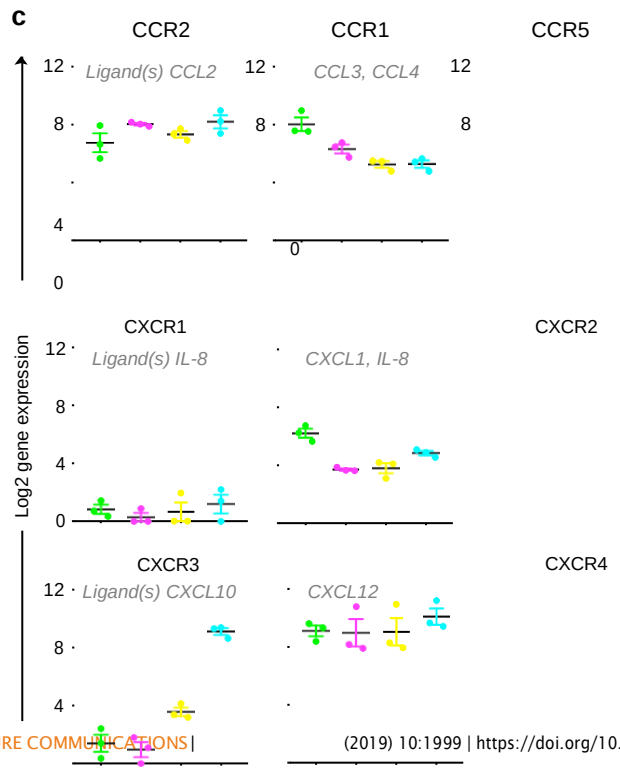
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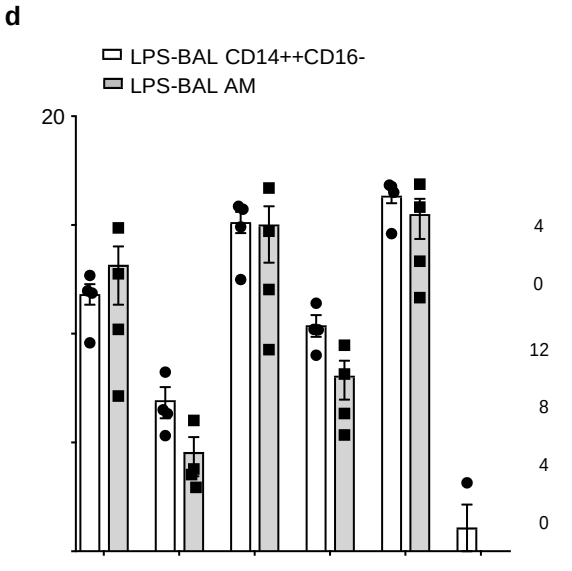
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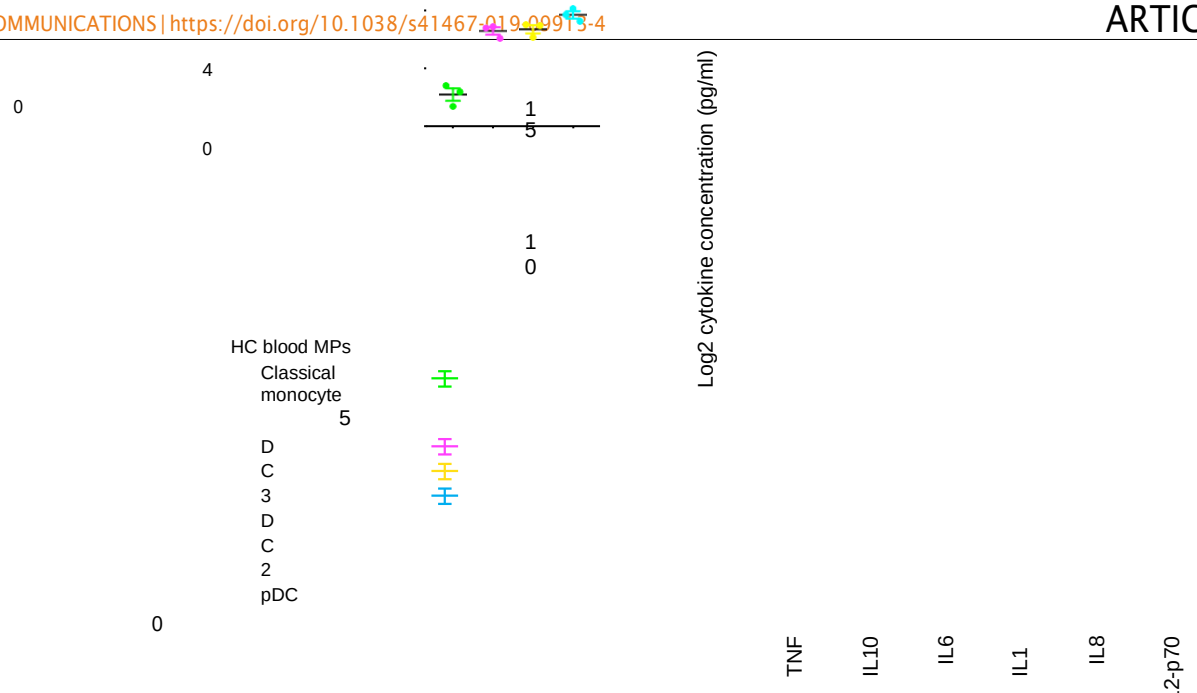

Flow cytometry and sorting. Cell pellets were washed in PBS (Sigma) supplemented with 2% fetal calf serum (Sera lab) and 2 mM EDTA (Invitrogen). The following antibodies were applied for 20 min at 4 °C (clone; supplier; catalog number; final concentration): anti-CD45-V500 (HI30; BD; 560777; 1:25), anti-CD3-FITC (SK7; BD; 345763; 1:25), anti-CD19-FITC (AG7; BD; 347543; 1:25), anti-CD20-FITC (L27; BD; 345792; 1:25), anti-CD56$^-$FITC (NCAM16.2; BD; 345811; 1:25), anti-HLA-DR-BV786 (G46$^-$6; BD; 307642; 1:25); anti-CD14$^-$BV650 (M5E2; Biolegend; 301835: 1:40), anti-CD16-PE/Dazzle-594 (3G8; Biolegend; 302054; 1:33), anti-CD11c-APCCy7 (Bu15; Biolegend; 337218; 1:33), anti-CD123-PERCPCy5.5 (7G3; BD; 558714; 1:25), anti-CD1c-PECy7 (L161; Biolegend;

Fig. 5 Alveolar macrophages recruit monocytes and DCs and secrete pro-inflammatory cytokines upon acute LPS challenge in vivo. a Heatmap of immune genes showing differential expression between AM isolated from saline BAL and LPS BAL. Genes with>5 fold difference in expression and $p<0.05$ are shown. Genes discussed in the text are colored burgundy. b Quantification of chemokines in SS-BAL (blue bars) and LPS-BAL (red bars) supernatant by multiplexed ELISA (Luminex). Bars show mean ± SEM. By unpaired *t*-test, ns, not significant, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$. c Chemokine receptor gene expression on HC blood classical monocytes (green), DC2 (yellow), DC3 (pink) and pDC (cyan) quantified by Nanostring. Bars show mean ± SEM of $n = 3$ for each subset. Log2 gene expression is shown. Ligands for each receptor are listed in gray italic type. d Inflammatory cytokine production by $CD14^{++}CD16^{-}$ cells and AM retrieved from LPS BAL (each $n = 4$) and stimulated ex-vivo with LPS 100 ng ml^{-1} for 10 h. Cytokines were measured by cytometric bead array. Bars show mean ± SEM. $CD14^{++}CD16^{-}$ cells were compared with AM by *t*-tests with Holm-Sidak multiple comparison correction and alpha < 0.05. No comparisons were significant

331506; 1:40), anti-CD11b-APC (ICRF44; Biolegend; 301309; 1:25), anti-BTLA-PE (J168−540; BD; 558485; 1:100), anti-Axl-APC (108724; R&D Systems; FAB154A: 1:33) and anti-Siglec-6-A700 (767329; R&D Systems; FAB2859N; 1:33). $CD1c^{+}$ DC phenotyping experiments additionally used anti-CD1a-AF700 (HI149; Biolegend; 300120; 1:50), anti-CD206-PE (19.2; BD; 555954, 1:25), anti-FcER1-PE (CRA1; eBioscience; 12−5899−42; 1:25) and anti-CD86-PE (FUN-1; BD; 555658; 1:25). Dead cells were excluded with DAPI (Partec). Analytical flow cytometry was performed on a BD Fortessa X20 or BD FACSCanto II and cell sorting with a BD FACS Fusion running FACSDiva version 7. Consistent instrument performance was ensured by running Cytometer Setup and Tracking Beads (BD). PBMC were periodically run through the analysis template as a biological control to ensure gates were capturing the expected populations. Doublets were excluded with a SSC-H vs. SSC-A plot. FlowJo version 9.6.7 was used for analysis. Sorting strategies are given in Supplementary Fig. 3.

Peripheral blood MP quantitation. Quantitation of neutrophils and total monocytes was by complete blood count (CBC) in a UKAS accredited clinical laboratory using the Sysmex XE-2100. For MP subsets not quantitated in the CBC, 1 ml whole blood was lysed using an in-house ammonium chloride lysis buffer and transferred to a Trucount tube (BD). Antibodies were applied as above and flow cytometry performed following a final red cell lysis step. Data were acquired on a BD Fortessa and cell concentrations calculated with reference to bead counts.

Chemokine/cytokine analysis. Chemokines and cytokines in BAL fluid supernatant were quantified using multiplexed ELISA (ProcartaPlexTM 34−plex, EBioscience). Captured analytes were detected on a Qiagen Liquichip 200 running Luminex 100 integrated system software version 2.3. Procartaplex Analyst version 1.0 was used to define standard curves and determine analyte concentrations.

Gene expression analysis. Lysates from 1−2× 10^4 cells per sorted population were prepared using 5µl buffer RLT (Qiagen) with 1% beta-mercaptoethanol (Sigma). Transcripts from 579 immunology genes were detected using a NanoString Immunology v2 panel according to the manufacturers instructions. Experiments using blood and BAL DC2/3 included an additional 30-gene add-on to the Immunology v2 panel, designed to detect DC and monocyte/macrophage genes, including *ASIP*, *DBN1*, *MERTK*, *C19orf59*, *F13A1*, *NDRG2*, *CCL17*, *FGD6*, *PACSIN1*, *CD1C*, *FLT3*, *PPM1N*, *CD207*, *GCSAM*, *PRAM1*, *CLEC10A*, *GGT5*, *S100A12*, *CLEC9A*, *Ki67*, *SIRPA*, *CLNK*, *LPAR2*, *TMEM14A*, *COBLL1*, *LYVE1*, *UPK3A*, *CXCL5*, *MAFF*, *ZBTB46*. Panels included 15 housekeeping genes. Data normalization was performed in nSolver version 3, including a background subtraction step, positive control normalization using synthetic spike-in controls and content normalization using a combination of 15 housekeeping genes.

T cell proliferation assay. T cells were isolated by negative selection from healthy control peripheral blood collected into EDTA using RosetteSep Human T Cell Enrichment Cocktail and Lymphoprep (Stemcell technologies). Aliquots were cryopreserved at −80 °C. Thawed T cells were labeled with 1 µM CSFE (Invitrogen) prior to co-culture. MP populations were sorted from BAL and co-cultured with T cells at a 1:25 ratio in 96 well v-bottom plates containing RPMI 1640 (Lonza) with 100 U ml^{-1} penicillin, 10 µg ml^{-1} streptomycin, 2mM L-glutamine (all Invi- trogen) and 10% heat-inactivated fetal calf serum (Sera Lab). Cultures were maintained for 7 days at 37 °C and 5% CO_2. Outputs were prepared for flow cytometry as described above using anti-CD3-V500 (UCHT1; BD; 561416; 1:50), anti-CD4-PE (RTPA-T4; BD; 555347; 1:50), anti-CD8-APCCy7 (SK1; BD; 557834; 1:50) and analzyed on a FACS Canto II running FACSDIVA version 7. Three experiments were performed on populations sorted from LPS BAL.

T cell cytokine production assay. CD4 T cells were isolated by negative selection from healthy control peripheral blood collected into EDTA using RosetteSep Human CD4 T cell enrichment cocktail. Aliquots were cryopreserved at −80 °C. MP populations were sorted from SS-BAL or LPS-BAL and co-cultured with T cells at a 33:1 ratio in 96-well round-bottom plates containing RF10. On day 6, medium was replenished with RPMI 1640 with supplements as above and 10 U ml^{-1} human recombinant IL-2 (Immunotools). On day 10, cells were stimulated with 10 ng ml^{-1} phorbol 12-myristate 13-acetate (Sigma) and 1 µg ml^{-1} ionomycin (Sigma) for 4 h, with 10 with brefeldin A (Sigma) added after the first hour. Dead cells were stained with a Zombie Aqua (Biolegend) prior to fixation and permeabilization (BD) according to manufacturers instructions. Cells were prepared for flow cytometry as above using anti-IFN-γ-PE/Dazzle-594 (4s.B3; Biolegend; 505845; 1:100), anti-IL17-AF647 (BL168; Biolegend; 512309; 1:100) and anti-IL4-PECy7 (MP4−25D2; Biolegend; 500823; 1:100) and analyzed on a Fortessa X20 (BD) running FACSDIVA version 7.

Ex-vivo macrophage stimulation. Alveolar macrophages and $CD14^{++}CD16^{-}$ monocyte/macrophages were sorted from LPS BAL ($n = 4$ each). 1 × 10^5 sorted cells were cultured in 96-well round bottom plates in 100 µl RPMI 1640 with supplements (as above) containing 100 ng ml^{-1} LPS from *E. coli* (Sigma). After 10 h, supernatants were harvested and stored at −80 °C. Supernatants were batch-analysed by cytometric bead array (BD) to detect IL-10, IL-1β, TNF, IL-6, and IL-8. Bead populations were resolved on a FACS Canto II running FACSDIVA Version 7 and analyzed using FCAP Array software version 3.

Generation of monocyte-derived dendritic cells (moDC). Classical monocytes were isolated from healthy control peripheral blood by density centrifugation and FACS sorting. 5 × 10^5 monocytes were cultured in 500 µl RPMI 1640 with supplements (as above) in 24-well plates for 5 days at 37 °C and 5% CO_2. Medium contained 50 ng ml^{-1} GM-CSF (R&D) and 50 ng ml^{-1} IL-4 (Immunotools).

Medium and cytokines were refreshed on day 3. Cells were harvested and FACS-sorted on day 5, to exclude undifferentiated $CD14^+$ monocytes and include only $CD1c^+$ moDC.

Statistical analysis. Statistical analyses stated in the text were performed using GraphPad Prism version 7.0. Gene expression analyses were performed in Multi- Experiment Viewer version 10.2 and nSolver v4 advanced analysis module.

Data availability

NanoString gene expression data have been deposited in Gene Expression Omnibus (GEO) with the accession code GSE126923. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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Additional information

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