Mechanisms of alveolar macrophage activation during ventilator-induced lung injury

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ABSTRACT

Mechanical ventilation is a life-saving intervention, but through exacerbating inflammation, it can contribute to mortality, known as ventilator-induced lung injury (VILI). Patients with acute respiratory distress syndrome (ARDS) are particularly susceptible to VILI, however, it is recognised that over-distension of the lung should also be avoided in patients with healthy lungs, whom are also at risk of VILI. It is important to minimise the adverse effects of mechanical ventilation, yet our understanding of how over-distension promotes the devastating inflammatory response in VILI remains poor.

In this project, the kinetics of cellular activation were investigated within the lungs of mice following short periods of high tidal volume ventilation using phospho-flow cytometry. The results revealed that the alveolar epithelium is activated prior to alveolar macrophages, suggesting that the epithelium could be responsible for stimulating their activation. The activation observed was very rapid, leading me to hypothesise that communication between these two cell types could either occur via the release of soluble mediators, or through a direct-contact dependent mechanism. Subsequent experiments led to the theory that alveolar macrophages may become activated in response to stretch through the detection of the damage-associated molecular pattern ATP, which is rapidly released by the alveolar epithelium in response to cyclic stretching.

These experiments also revealed that pulmonary endothelial cells may be the most sensitive pulmonary cell type to mechanical ventilation *in vivo*, indicating that stretch-induced inflammation could be immediately disseminated to the systemic circulation. This concept is in contrast to the current 'decompartmentalisation' model in which inflammatory mediators released within the alveolar space leak into the vasculature only following the breakdown of the alveolar-capillary barrier. This discovery has important implications for our understanding of the pathogenesis of multiple organ failure which is the most common cause of death in ARDS patients and therefore warrants further research.

DECLARATIONS

Declaration of Originality

I declare that all of the work submitted within this thesis is my own and that those that have assisted me with this work have been duly recognised within the Acknowledgements.

Some of the data presented in Chapters 3 and 4 of this thesis have been submitted in abstract form and presented at the European Respiratory Society's 24th International Congress (Woods et al. 2014). Some of the data presented in Chapters 3 and 4 also formed the basis of a manuscript published in the American Journal of Physiology: Lung Cellular and Molecular Physiology (Woods et al. 2015).

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ABBREVIATIONS

AECC	American-European Consensus Conference
AECs	Alveolar epithelial cells
ALI	Acute lung injury
AMs	Alveolar macrophages
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BPM	Breaths per minute
COX	Cyclooxygenase
CT	Computed tomography
DMSO	Dimethyl sulfoxide
ECMO	Extracorporeal membrane oxygenation
ERK1/2	Extracellular signal-regulated kinase 1/2
gMFI	Geometric mean fluorescence intensity
ICAM-1	Intercellular adhesion molecule-1
ICU	Intensive Care Unit
IT	Intratracheal
LPS	Lipopolysaccharide
MAP kinase	Mitogen-activated protein kinase
MK2	Mitogen-activated protein kinase activated protein kinase 2
MOF	Multiple organ failure
NF-κB	Nuclear factor kappa B
NMBA	Neuromuscular blocking agent
PaO2/FiO2	Arterial partial pressure of oxygen to fraction of inspired oxygen
PBS	Phosphate-buffered saline
PEEP	Positive end-expiratory pressure
PIP	Peak inspiratory pressure
ROS	Reactive oxygen species
TACE	TNFα converting enzyme
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
VAP	Ventilator-associated pneumonia
VILI	Ventilator-induced lung injury
V_T	Tidal volume

CHAPTER ONE

INTRODUCTION

Mechanical ventilation is a life-saving intervention utilised in the intensive care unit (ICU) to provide respiratory support to critically ill patients commonly suffering from acute respiratory distress syndrome (ARDS). The characteristic accumulation of non-cardiogenic pulmonary oedema and hypoxaemia in these patients necessitates mechanical ventilation as a vital aspect of their care, though it does not treat the cause of the condition. Conversely, inevitable overstretching of the lungs can exacerbate the underlying injury and increase mortality, a condition referred to as ventilator-induced lung injury (VILI).

A considerable amount of research has focused on altering mechanical ventilation strategies in an attempt to reduce the effects of VILI; however only reducing the tidal volume has proved successful in reducing mortality. Beyond this, it is widely accepted that research into the inflammatory responses induced by over distending the lung, will most likely prove beneficial to patient care in the future. So far, studies aimed at attenuating the inflammatory response have been largely unsuccessful, probably due the complex interplay of factors such as timing, patient heterogeneity, and immunosuppression. A more targeted approach is essential to attenuate the injury caused by mechanical ventilation.

1.0 Acute Respiratory Distress Syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) may develop within days of exposure to an array of pulmonary and non-pulmonary insults. It is characterised by the acute onset of a substantial inflammatory response that leads to pulmonary oedema and severe hypoxaemia. The condition is responsible for high rates of morbidity and mortality within the ICU and has been the subject of many studies and reviews (Matthay, Ware, and Zimmerman 2012; Ware and Matthay 2000).

Definition

ARDS comprises a collection of respiratory derangements that can arise from a variety of insults, and is therefore not simple to define. A reliable diagnosis of ARDS is not only important in being able to provide appropriate treatment to patients but also for the understanding of its burden and pathogenesis within the population. ARDS was first described by Ashbaugh and colleagues in 1967, who reported a similar pattern of acute onset of dyspnoea, hypoxaemia and reduced respiratory compliance amongst 12 patients under their care (Ashbaugh et al. 1967). Since then, several definitions of ARDS have been proposed which have attempted to identify suitable diagnostic criteria. Definitions include the Murray lung injury score (Murray et al. 1988), the American-European Consensus Conference (AECC) definition (Bernard et al. 1994) and the Delphi definition (Ferguson, Davis, et al. 2005), each of which have been evaluated in a review by Thompson et al. (Thompson and Moss 2013).

The diagnostic criteria set out by the AECC in 1994 was the standard definition used in clinical practise for a long time (Bernard et al. 1994). The AECC definition classified patients with mild hypoxaemia, an arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) of ≤300mmHg, as having acute lung injury (ALI). They classified patients with severe hypoxaemia (PaO_2/FiO_2 ≤200mmHg) as having ARDS. Whilst these criteria recognised the need to identify disease severity, they did not take into account the use of a peak end-expiratory pressure (PEEP) which can have a considerable effect on oxygenation (Bernard et al. 1994).

The diagnostic criteria currently in practise are those outlined by the Berlin definition proposed in 2012 (ARDS Definition Task Force 2012). This present definition addresses a number of limitations of the AECC definition by providing the timescale that should be considered an 'acute onset' as within one week of a known insult or worsening of respiratory function. Importantly, this deterioration in respiratory function should not be the result of hydrostatic oedema with a purely cardiogenic origin. The Berlin definition also further categorise patients into three severity levels based on the degree of hypoxaemia. A PaO_2/FiO_2 of between 200-300mmHg is categorised as mild ARDS, replacing the previous ALI term, though both ALI and ARDS are still used. A PaO_2/FiO_2 of between 100-200mmHg is classified as moderate ARDS and a PaO_2/FiO_2 of less than 100mmHg is described as severe ARDS. Furthermore, the Berlin definition specifies that these measurements must be taken in the presence of an applied PEEP of at least 5.0cmH_2O.

When the Berlin definition criteria was applied to existing clinical data, the severity categories were found to correlate with rates of mortality, mild (27%), moderate (32%) and severe (45%), offering

improved predictive power over previous definitions. This study also revealed that the majority of patients (50%) commonly present with moderate ARDS, whilst 22% of patients present with mild ARDS and 28% present with severe ARDS (ARDS Definition Task Force 2012). Further studies have since concluded that the Berlin definition is indeed the most reliable set of diagnostic criteria for ARDS developed to date (Kushimoto et al. 2013; Thille et al. 2013; Thille, Vuylsteke, and Bersten 2015) and it is the current standard within the clinical setting.

The broad definition of ARDS covering all aetiologies of the condition has probably been helpful in raising awareness of the disease both in the ICU and as a therapeutic research area. However, it may be more advantageous to consider them as separate entities, particularly when identifying the appropriate course of treatment. There are numerous events including trauma, sepsis and aspiration that all lead to the same collection of symptoms defined as ARDS, though they are likely to have different pathologies. It is essential to treat the specific underlying cause, which may lead to different phenotypes of ARDS; however the ARDS definition does not take this factor into account (Raghavendran and Napolitano 2011).

Epidemiology

The difficulty in diagnosing ARDS has undoubtedly made it challenging to establish reliable incidence and mortality rates, which can appear to vary considerably between studies (Ferguson, Frutos-Vivar, et al. 2005). These differences are considered to result not only from the sometimes ambiguous and continuously evolving diagnostic criteria, but also from differences in methodology, geographical area, age of patients, and the size of ICUs with respect to the size of the population that they serve (Sweatt and Levitt 2014). There have also been no studies looking at the incidence of ARDS in the developing world where diagnosis would be more difficult due to a lack of resources to assess arterial gases and to perform radiographs in patients (Buregeya et al. 2014).

Early studies examining the incidence of ALI/ARDS reported high rates of incidence and mortality. Goss et al. performed a prospective cohort study on patients admitted to 20 ICUs in the United States (US) between 1996 and 1999 (Goss et al. 2003). They extrapolated their findings to the entire US population and estimated an incidence of 64.2 cases per 100,000 person-years (Goss et al. 2003). Another prospective cohort study performed in the US looked at patients admitted to 21 hospitals over a period of 15 months between 1999 and 2000 (Rubenfeld et al. 2005). This study reported an incidence of ALI/ARDS of 78.9 cases per 100,000 person-years and an in-hospital mortality rate of 38.5%. It was also noted that the incidence and mortality rates associated with ALI/ARDS increased significantly with age (Rubenfeld et al. 2005).

More recent studies suggest that rates of incidence and mortality from ARDS have decreased and this is thought to be partly due to advances in treatment of the underlying injury, as well as the use of less injurious mechanical ventilation strategies as a result of the ARDSNet trial, published in 2000 (ARDS Network 2000), which will be discussed later in this chapter. Li et al. reported a decrease in the incidence of ARDS between 2000 and 2008 in 2 US hospitals from 82.4 to 38.9 cases per 100,000 person-years. However, this was found to be entirely due to a decline in hospital-acquired ARDS and was not accompanied by a decrease in mortality, which ranged between 33-48% (Li et al. 2011). A prospective multicentre study conducted in Spain between 2008 and 2009 reported an incidence of 7.2 cases per 100,000 person-years with a mortality of 42.7% (Villar et al. 2011). An epidemiology study conducted in France that used the Berlin criteria to diagnose ARDS, reported an incidence of 32 cases per 100,000 person-years with a 28-day mortality of 35% (Hernu et al. 2013). In Brazil, a 15-month prospective study reported an incidence of 10.1 cases per 100,000 person-years and a 28-day mortality of 38.5% (Caser et al. 2014).

It would appear from these reports that the incidence of ARDS is much higher in the US than in Europe and South America. However this may be due to the number of large-scale clinical trials on ARDS that have been conducted in the US, such as the ARDS Network trial. This may have led to increased awareness of the condition and increased diagnoses. Whereas, in Europe and South America, where fewer trials have been performed, health-care practitioners may be less familiar with the ARDS diagnosis criteria and patients may therefore be recorded as having pneumonia for example. Rates of incidence may also vary between community hospitals and academic centres, which are more likely to be involved in such clinical trials.

On the other hand, mortality rates seem to be consistent across continents at around 30-40%. A meta-analysis by Zambon et al. concluded that mortality from ARDS averaged at 43% but has decreased over time between 1994 and 2006 by 1.1% per year (Zambon and Vincent 2008). In contrast, a systemic review by Phua et al., which looked at the same period, concluded that mortality rates had not decreased since 1994, the year that the AECC definition was published, but have remained constant ranging between 35-45% (Phua et al. 2009). These reviews suggest that there is a lack of compelling evidence to suggest that mortality from ARDS is improving. These estimates of mortality may even be an underestimate of the true impact of ARDS as many patients do not actually die of respiratory failure, but in fact most frequently die as a result of multiple organ failure (MOF) (Villar et al. 2011; Hernu et al. 2013). In such cases, ARDS may not be recorded as the cause of death for many patients.

For those patients that do recover from ARDS, a long-term outcomes study found that reduced pulmonary function can persist for up to 6 to 12 months and patients experience reduced physical capabilities due to persistent muscle weakness that can last for up to 5 years (Herridge et al. 2003; Herridge et al. 2011). Therefore, even following discharge from hospital, ARDS is associated with significant morbidity and survivors face a long recovery process.

Pathogenesis

The most common predisposing factor for ARDS is pneumonia, which accounts for 35-45% of cases and non-pulmonary sepsis, which accounts for 30-35% of cases (Caser et al. 2014; Hernu et al. 2013; Villar et al. 2011). Other common risk factors include aspiration, trauma, high-risk surgery, multiple blood transfusions and pancreatitis (Gajic et al. 2011).

The inflammatory injury resulting from these insults can lead to an acute onset of hypoxaemia due to the accumulation of pulmonary oedema (Han and Mallampalli 2015; Sharp, Millar, and Medford 2015). Immune cells within the lung including alveolar macrophages, interstitial monocytes, marginated neutrophils and dendritic cells release pro-inflammatory cytokines such as TNFα and IL-1β as well as the chemokine CXCL8 (IL-8). High levels of pro-inflammatory cytokines within the bronchoalveolar lavage (BAL) fluid of patients have been found to correlate with poor outcomes in ARDS patients (Meduri et al. 1995). The initiation of inflammation and diffuse alveolar damage seen in the early stages of ARDS is referred to as the exudative phase.

A well-studied event in the complex inflammatory response is the infiltration of neutrophils into the alveolar space in response to high local concentrations of chemoattractants such as CXCL8. Neutrophils contribute to the disruption of the epithelial-endothelial barrier and release a host of pro-inflammatory mediators such as proteases, reactive oxygen species (ROS) and cytokines that promote apoptosis and autophagy in surrounding cells. The infiltration of immune cells including neutrophils and monocytes into the lung is known as the proliferative phase.

The apoptosis of the lung parenchymal cells leads to further destruction of the alveolar-capillary barriers that can no longer prevent the movement of protein-rich fluid into the distal airspaces. The presence of fluid within the alveoli impairs gas exchange and reduces lung compliance. The apoptosis of type II alveolar epithelial cells (AECs), which in a healthy lung produce surfactant and play an active role in fluid transport, makes the alveoli susceptible to collapse (atelectasis). In response to damage, the type II AECs begin to proliferate and differentiate to replace the damaged type I AECs, which constitute the majority of the alveoli surface area in a healthy lung. Fibrosis may also occur as part of

the reparative process that can actually lead to a further reduction in lung compliance and decreased respiratory function. This stage of ARDS is known as the fibro-proliferative phase.

Management of ARDS

There have been a multitude of animal studies and clinical trials that have aimed to reduce the impact of ARDS through pharmacological intervention (McIntyre et al. 2000; Spieth and Zhang 2014; Modrykamien and Gupta 2015). Trialled therapies range from treatment with neuromuscular blocking agents to improve patient-ventilator synchrony (Arroliga et al. 2005), corticosteroids to reduce inflammation (Steinberg et al. 2006), exogenous surfactant therapy to improve gas exchange (Zhang et al. 2013), and stem cell therapy to promote regeneration of the lung (Wang, Li, and Wang 2013). Some of these treatments have had small beneficial effects on hypoxaemia; however, they have all had either none or a very limited effect on mortality. With no effective or proven treatment for ARDS, patients are simply given respiratory support with mechanical ventilation, and possibly treated for the underlying cause if known. Unfortunately, mechanical ventilation can cause mechanical stress and exacerbate inflammation, an iatrogenic condition referred to as ventilator-induced lung injury (VILI).

1.1 Ventilator-induced Lung Injury (VILI)

Mechanical ventilation supports ARDS patients experiencing respiratory failure with the aim of improving gas exchange. However, it has the potential to exacerbate injury through several mechanisms including volutrauma, barotrauma and atelectrauma. In animal models, mechanical ventilation alone induces substantial lung injury similar to that seen in ARDS patients. These models have demonstrated that mechanical ventilation of previously healthy lungs can cause damage to the alveolar-capillary barrier, permitting the accumulation of pulmonary oedema, with a detrimental effect on gas exchange (Tsuno, Prato, and Kolobow 1990; Cilley, Wang, and Coran 1993; Dreyfuss, Martin-Lefevre, and Saumon 1999).

Mechanical ventilation not only induces direct physical damage to the lung parenchyma but also initiates stretch-induced inflammation through mechanotransduction and the release of pro-inflammatory mediators into the alveolar space, known as biotrauma (Slutsky 2005). The resident alveolar macrophages are the first line of defence within the lung and are soon accompanied by infiltrating neutrophils and monocytes, which further exacerbate inflammatory processes and lung injury (Imanaka et al. 2001).

Volutrauma and Barotrauma

Volutrauma is considered to be the major contributing factor leading to VILI; overstretching of the lung through the application of high tidal volumes. Dreyfuss et al. were the first to differentiate that injury to the mechanically ventilated lung is induced by high volumes rather than high airway pressures (Dreyfuss et al. 1988). In their experiments, rats were subjected to the high tidal volume of 40-44ml/kg, produced by either positive pressure or negative pressure ventilation (iron lung). In further experiments, rats were exposed to ventilation with a lower tidal volume (19ml/kg) but at a high pressure of 45cmH_2O, achieved through thoraco-abdominal strapping. The experiments demonstrated that high tidal volumes induced widespread alveolar oedema within just 20 minutes of ventilation regardless of the peak airway pressure. However, ventilation with the lower tidal volume in combination with large cyclic changes in airway pressure did not induce any detectable pulmonary oedema (Dreyfuss et al. 1988). Whilst this study did demonstrate the importance of over-stretching the lungs, it used very extreme settings that induced physical damage through over-stretching and death within 20 minutes. This is not likely to reflect the level of stretch experienced by patients, which even at low levels not sufficient to cause direct physical damage, can result in significant injury through biotrauma (Uhlig 2002).

Dreyfuss et al. demonstrated that volutrauma rather than barotrauma is responsible for the pulmonary oedema associated with VILI. However, barotrauma can still carry a small but significant risk in patients with underlying diseases such as chronic obstructive pulmonary disease (COPD) or asthma (Hsu and Sun 2014; Hsu et al. 2014). In patients with ARDS, the risk of pneumothorax increases with duration of mechanical ventilation and its incidence is associated with increased mortality (Gattinoni et al. 1994; Terzi et al. 2014).

Functional residual capacity

ARDS patients are particularly at risk of volutrauma due to the presence of pulmonary oedema, which causes heterogeneity within the lung. This means that some areas of the lung are normally aerated, some are poorly aerated, and some are not aerated at all. This leads to the normally aerated regions, which are small relative to the whole lung, receiving the majority of the tidal volume applied. Therefore, the regions unaffected by pulmonary oedema are subjected to hyperinflation and overstretching (Grasso et al. 2004; Borges et al. 2014). The reduced volume of aeratable lung available for ventilation in patients with significant pulmonary oedema, can be compared with the size of a child's lung and has been termed 'baby lung' (Gattinoni and Pesenti 2005).

Analysis of computed tomography (CT) scans of 68 patients with ARDS revealed that the volume of non-recruitable lung was on average 24%, but varied considerably between patients from a negligible fraction to more than half of the entire lung volume, even when airway pressure was held at 45cmH_2O (Gattinoni et al. 2006). Another study found that the average aeratable lung volume was reduced to 59% in a group of ARDS patients, whilst some demonstrated remaining volumes of as little as 29% (Mattingley et al. 2011).

Lowering the tidal volume

Amato et al. reported the first clinical trial examining the effect of tidal volume on clinical outcomes in 53 patients with ARDS (Amato et al. 1998). Patients were assigned to either conventional ventilation with 12ml/kg with the lowest PEEP necessary to maintain good oxygenation, or to protective ventilation with 6ml/kg and the PEEP set at above the lower inflection point on the pressure-volume curve. They found that after 28 days, 71% of patients on the conventional settings had died compared with just 38% of patients on the protective settings. The protective strategy was also associated with reduced barotrauma in spite of the use of a higher PEEP and peak inspiratory pressures. Despite this promising decrease in mortality risk at 28 days, survival to hospital discharge was not significantly different between the two strategies (Amato et al. 1998). Another clinical trial published not long after, looked at 116 patients with ARDS who were ventilated at 7 or 10ml/kg with a peak inspiratory pressure of 25cmH_2O (Brochard et al. 1998). The reduction in tidal volume was not found to significantly decrease mortality.

Shortly after, a large scale multicentre trial was published by the ARDS Network that investigated responses to mechanical ventilation in 861 patients with ARDS over 3 years (ARDS Network 2000). Patients were ventilated with the conventional tidal volume of 12ml/kg and a maximum plateau pressure of 50cmH_2O, or with the lower tidal volume of 6ml/kg and a maximum plateau pressure of 30cmH_2O. The lower tidal volume settings were associated with significantly reduced mortality (31.0%), compared with that observed in the conventionally ventilated group (39.8%), a reduction of 22%. In addition, the lower tidal volume group had more ventilator-free days and days without non-pulmonary organ failure. The results of this interim analysis led to the trial being stopped early (ARDS Network 2000).

This investigation clearly demonstrated in a large population that the lower tidal volume reduced mortality when compared with the traditional ventilator settings. However, the traditional tidal volume used in this trial was actually higher than was used routinely by physicians at the time. Therefore, it was not possible to conclude that the lower tidal volume was favourable compared with

current practice. It has also been argued that the beneficial effect of lower tidal volumes was actually due to an increase in mortality in the traditionally ventilated group, rather than a decrease in mortality with the lower tidal volume (Eichacker et al. 2002). Nevertheless, the importance of lowering tidal volumes and airway pressures in ARDS patients has been well accepted.

The ARDS Network trial included a broad range of patients with ARDS severity ranging from mild to severe (PaO_2/FiO_2 <300mmHg). Since then, further studies have specifically shown that the application of lower tidal volumes is effective in reducing mortality even in severe cases of ARDS (PaO_2/FiO_2 <200mmHg) (Villar et al. 2006). In this trial by Villar et al., 95 patients diagnosed with severe ARDS from 8 ICUs across Spain were ventilated with standard ventilator settings of 9-11ml/kg and a PEEP of above 5cmH_2O, or with protective settings of 5-8ml/kg and a PEEP set above the lower inflection point on the pressure-volume curve. The protective ventilation strategy was found to reduce mortality from 53.3% to 32.0%, increase the number of ventilator-free days, and reduce the occurrence of organ failure. This study demonstrated that lower tidal volumes are beneficial in patients with severe ARDS and that a higher PEEP can help maintain oxygenation in these patients (Villar et al. 2006).

The application of lower tidal volumes evidently reduces mortality in ARDS patients; however, the heterogeneous nature of the disease means that these patients can still be susceptible to VILI. In a group of 30 patients ventilated at 6ml/kg and a PEEP of 9-13cmH_2O, CT scanning revealed that one-third were experiencing tidal hyperinflation (Terragni et al. 2007). Therefore, even low tidal volumes can still over distend the lungs of patients with large non-aerated compartments. A prospective study, which included 482 ARDS patients, revealed that an increase in tidal volume by just 1ml/kg over the recommended 6ml/kg was associated with an increase in mortality by 15% (Needham et al. 2015).

Mechanical ventilation of patients without ARDS

The study by Needham et al. recommended a switch to lower tidal volume ventilation upon noticing signs of ARDS (Needham et al. 2015), however patients may benefit from protective ventilation strategies from the onset of mechanical ventilation. This theory arises from the finding that high tidal volumes are also an important risk factor in the development of ARDS in critically ill patients without signs of the condition when ventilator support is started. In a retrospective cohort study, which included 332 patients admitted to an ICU with underlying risk factors including aspiration or multiple blood transfusions, 24% developed ARDS within 5 days of being mechanically ventilated and high tidal volumes were associated with an increased risk (Gajic et al. 2004). In a follow-up prospective

multicentre study by the same group, 6.2% of 3261 patients with risk factors such as pneumonia and trauma developed ARDS within 2 days of being mechanically ventilated (Gajic et al. 2005). Once again, high tidal volumes were associated with an increased risk of developing ARDS and the mortality rate of patients who did develop the condition was nearly double (63%).

In these two studies, the incidence of ARDS in ventilated patients without pre-existing lung injury seemed to increase with length of ventilation. A subsequent study that retrospectively analysed 789 patients ventilated for at least 2 days without ARDS at the onset, revealed a similarly high incidence of 19% (Jia et al. 2008). As before, the risk factors were determined to be the use of high tidal volumes and high peak inspiratory pressures (PIP), which were better predictors of ARDS development than underlying illness. These studies demonstrate that the development of ARDS in critically ill patients without lung injury at the onset of mechanical ventilation is common within the ICU.

Meta-analyses have consequently investigated the benefits of applying a protective ventilation strategy to patients without pre-existing lung injury. A review of 7 trials that together included 2184 patients, found that patients that had been ventilated with a tidal volume of 6ml/kg or less were more likely to be breathing unassisted by day 28 (Serpa Neto, Simonis, et al. 2014). This review also noted that the use of lower tidal volumes was not associated with increased dose or duration of sedatives, opioids or neuromuscular blocking agents. Another meta-analysis by the same group revealed that the use of lower tidal volumes in critically ill patients without pre-existing lung injury reduced the incidence of complications (Serpa Neto, Nagtzaam, and Schultz 2014).

These studies revealed the importance of low tidal volumes and pressures in patients at risk of developing ARDS. Previous attitudes had been that the utilisation of low tidal volumes was only necessary in patients with established lung injury. However, it is now recognised that the use of low tidal volumes in healthy lungs could prevent the development of lung injury.

The potential benefits of a protective ventilation strategy have also spread to the operating room where patients are subjected to mechanical ventilation for a relatively short period compared with a patient in the ICU. A meta-analysis of 22 studies that included 2822 patients with previously healthy lungs who were mechanically ventilated within the ICU or during surgery, revealed that a protective ventilation strategy reduced the incidence of lung injury in these patients by around 8% (Serpa Neto et al. 2012). Protective ventilation also improved survival rates and reduced the incidence of pulmonary infection (Serpa Neto et al. 2012). Another study looking purely at a population of 4420 surgery patients found that 5.4% developed post-operative respiratory failure of which ALI was the

most common cause (Fernandez-Perez et al. 2009). A patient's risk of developing ALI increased with the use of high airway pressures and those that did develop ALI had reduced survival rates (Fernandez-Perez et al. 2009).

There have been many clinical trials and meta-analyses that have demonstrated that the use of lower tidal volumes improves clinical outcomes in patients undergoing elective surgery in the absence of pre-existing lung injury (Severgnini et al. 2013; Hemmes, Serpa Neto, and Schultz 2013; Futier et al. 2013; Serpa Neto, Hemmes, et al. 2014; Serpa Neto et al. 2015). These studies demonstrate that mechanical ventilation, and in particular the use of high tidal volumes, can promote the development ARDS even in the absence of pre-existing lung injury, therefore we should be aiming to give protective ventilation to all patients requiring respiratory support.

Atelectrauma

With the use of low tidal volumes becoming a common feature of mechanical ventilation strategies, a new aspect of VILI has been identified. Atelectrauma describes the injury caused by repetitive opening and closing of unstable alveoli at low pressures. The shear stress imposed on the alveoli causes structural damage to the epithelial and endothelial barriers, leading to the leakage of fluid into the alveolar space. In addition, where some regions of the lung become completely collapsed and no longer re-inflate during inspiration, the remaining open lung receives the entire tidal volume and may be subjected to over distension. This may explain the injury seen with low tidal volume ventilation, which is still capable of inducing VILI.

PEEP

The application of a positive end-expiratory pressure (PEEP) aims to maintain a certain level of pressure at the end of expiration to prevent alveolar collapse. A study of 10 patients suggested that those suffering from ARDS are especially at risk of atelectasis due to increased hydrostatic pressure (Gattinoni et al. 1993). However, the benefits of PEEP in combination with lower tidal volumes have also been recognised in the operating theatre where patients undergoing mechanical ventilation do not have pre-existing lung injury (Severgnini et al. 2013; Hemmes, Serpa Neto, and Schultz 2013; Futier et al. 2013). To overcome alveolar derecruitment, the PEEP must be set above the lower inflection point of the pressure-volume curve, which is specific to each patient, and depends on the severity of lung injury (Richard et al. 2001).

Dreyfuss et al. were the first to demonstrate the beneficial effects of PEEP on permeability oedema in experiments comparing the ventilation of rats with zero or 10cmH_2O PEEP (Dreyfuss et al. 1988).

Subsequent studies in animals and patients have all identified improvements in oxygenation as the main advantage associated with the application of PEEP (Halter et al. 2003; Mercat et al. 2008; Santa Cruz et al. 2013; Huh et al. 2009; Ko et al. 2008).

PEEP must be applied with caution as it results in an overall increase in peak airway pressure that could lead to hyperinflation of aerated lung, especially if the PEEP is not sufficient to fully recruit collapsed lung. Increasing the level of PEEP has been linked to higher incidences of barotrauma (Eisner et al. 2002). A higher PEEP is also known to decrease cardiac output and increase pulmonary oedema. These factors may explain why, despite improvements in oxygenation, the application of higher levels of PEEP have been shown to have little impact on rates of mortality in patients with ARDS (Brower et al. 2004; Mercat et al. 2008; Meade, Cook, Guyatt, et al. 2008).

A meta-analysis of 3 trials that categorised 2299 patients based on disease severity, found that patients with ARDS had improved chance of survival if they were ventilated with a higher PEEP, whilst patients with ALI (mild ARDS) demonstrated increased mortality with higher PEEP (Briel et al. 2010). This suggests that mechanical ventilation requirements depend on the individualities of the patient. The combination of tidal volume and PEEP that provides protective ventilation differs between patients and becomes more restricted as VILI progresses (Seah et al. 2011; Martin-Lefevre et al. 2001). For these reasons, the appropriate level of PEEP remains unclear and it is difficult for guidelines to set a level that would be suitable for all patients.

Recruitment manoeuvres

Another technique used to maintain an open lung are recruitment manoeuvres which involve increasing the pressure within the lung up to around 45cmH_2O, where it is held for up to 1 minute before it is released. Such recruitment manoeuvres may be used at regular intervals but are only effective in reducing atelectasis if the PEEP is high enough to prevent alveoli collapse following the manoeuvre (Ko et al. 2008; Halter et al. 2003).

Recruitment manoeuvres have been found to improve oxygenation but only very transiently, probably the result of recurring compressive atelectasis at the end of expiration (Oczenski et al. 2004; Hodgson et al. 2009). Recruitment manoeuvres have also been suggested to combat re-absorptive atelectasis, which describes alveoli that have succumbed to flooding (Pestana et al. 2005). Flooded alveoli require higher pressures to overcome their opening threshold and in reversing atelectasis in these dependent regions, the non-dependent regions are inevitably over distended (Gomez-Laberge et al. 2013; Mertens et al. 2009).

Indeed, an increased risk of barotrauma in patients with ARDS has been associated with the application of recruitment manoeuvres (Fan et al. 2012; Meade, Cook, Griffith, et al. 2008). Conversely, a study in an *ex vivo* rabbit model revealed that there was a considerable difference between the pressure required to recruit the lungs and that which would induce barotrauma (Garcia-Fernandez et al. 2013). However, it must be noted that these experiments were performed in healthy rabbit lungs in the absence of the thorax, and it would be expected that the 'safe' range of applied pressure would be substantially reduced in an injured lung.

In addition to the risk of barotrauma, the impact of recruitment manoeuvres on haemodynamics has been found to be considerable. A study that examined the use of recruitment manoeuvres in 11 patients with ARDS found that cardiac output was transiently decreased by 23% and that systolic blood pressure dropped significantly during the procedure (Kanoore Edul, Maskin, and Dubin 2010). A similar effect was found in mice subjected to two recruitment manoeuvres per minute, which caused lung vascular dysfunction (Mekontso Dessap et al. 2012).

In light of these potential adverse effects, the use of recruitment manoeuvres as a regular intervention in clinical practise has been debated. Despite their clear protective effect on oxygenation and respiratory mechanics (Allen et al. 2006; Cannizzaro et al. 2009; Reiss, Kowallik, and Uhlig 2011), there has been a lack of evidence to suggest they decrease mortality in patients with ARDS (Hodgson et al. 2009). Recruitment manoeuvres are therefore advised only to be used as a rescue therapy following events such as disconnection from the ventilator, whilst their scheduled use during mechanical ventilation has not been recommended (Fan et al. 2012).

Biotrauma

In addition to the detrimental impact on lung function described above, mechanical ventilation is also known to promote inflammatory responses. This ventilator-induced inflammation has been well studied in animal models, which provide a simplified model of the critically ill patient being cared for in the ICU. Using such models, mechanical ventilation has been shown to be capable of initiating inflammation in the absence of other injuries or confounding factors.

The role of inflammation in VILI was initially controversial as reports of cytokine detection in BAL fluid were inconsistent between studies, probably due to differences in ventilator settings and sampling times (Tremblay et al. 1997; Ricard, Dreyfuss, and Saumon 2001). Wilson et al. published the first definitive study showing that high tidal volume ventilation induced the release of cytokines into the BAL fluid of mice with previously healthy lungs (Wilson et al. 2003). These experiments revealed that

whilst some cytokines such as CXCL2 (MIP-2) appeared to be constantly present in the BAL fluid during continuous ventilation, others such as TNFα displayed a transient pattern of release (Wilson et al. 2003), explaining the apparent inconsistencies of previous studies.

Since then, many *in vivo* studies have reported increased concentrations of several cytokines in both BAL fluid and in plasma in response to injurious ventilation (Yoshikawa et al. 2004; Hoegl et al. 2008). The use of high tidal volumes has been most frequently linked to the release of cytokines, however atelectasis may induce inflammatory responses too (Ko et al. 2008). The impact of recruitment manoeuvres is less clear with some studies suggesting that they can attenuate inflammatory responses (Allen et al. 2006; Reiss, Kowallik, and Uhlig 2011), whilst others suggest they promote inflammation by over stretching the lung (Cannizzaro et al. 2009; Mekontso Dessap et al. 2012). A meta-analysis of 20 animal studies concluded that lung strain, the degree of alveolar deformation related to the tidal volume and PIP, was the most important determinant in the development of VILI (Caironi et al. 2011).

High tidal volume ventilation has been frequently reported to induce cytokine release; however, several studies have also detected cytokine release even in response to low tidal volume ventilation in healthy murine lungs (Vaneker et al. 2007; Wolthuis et al. 2009; Hegeman et al. 2010). These studies suggest that a completely protective ventilation strategy that does not promote VILI does not exist. Hauber et al. similarly found that ventilation of mice with low tidal volumes prompted inflammatory responses and suggested that atelectasis and poor oxygenation may be the cause (Hauber et al. 2010). However, in this study, low stretch ventilation was compared with spontaneously breathing controls and therefore the experiments did not control for a possible inflammatory response caused by surgery, anaesthesia, or ventilation in the supine position.

Jaecklin et al. performed a set of experiments that suggested that soluble mediators released into the systemic circulation during mechanical ventilation were pathogenic (Jaecklin et al. 2011). In an isolated perfused mouse lung, they found that recirculation of the perfusate worsened the injury caused by high tidal volume ventilation. They also perfused lungs exposed to low tidal volume ventilation to perfusate from the injurious model and found that it induced similar injury (Jaecklin et al. 2011).

Cannizzaro et al. attempted to identify a link between the release of specific cytokines into the BAL fluid and the development of specific derangements in lung function and suggested that IL-6 and TNFα were associated with atelectasis (Cannizzaro et al. 2011). This study identified a relationship

between inflammatory responses to mechanical ventilation and distinct changes in lung mechanics, further supporting a pathogenic role of inflammation in VILI.

In addition to initiating inflammation in previously healthy lungs, mechanical ventilation can also exacerbate existing injury. Injurious ventilation strategies have been shown to increase cytokine expression and release within the alveolar space synergistically in animal models previously exposed to endotoxin (Tremblay et al. 1997; Altemeier et al. 2004). Even low tidal volume ventilation can elevate inflammatory responses in mice inoculated with pneumonia compared with those left breathing spontaneously (Bem et al. 2009).

Inflammation in patients

This finding in animal models that mechanical ventilation can promote inflammation supports data from clinical trials. Patients with ARDS that present with persistent inflammation, characterised by high plasma concentrations of several inflammatory mediators, are less likely to survive (Meduri et al. 1995). Mechanical ventilation is associated with the exacerbation of inflammation in these patients with ARDS whom are frequently already suffering from infection. An early trial by Ranieri et al. enrolled 44 patients onto 'standard' or 'protective' ventilation strategies, and found that the protective settings which included a lower tidal volume, higher PEEP and lower PIP significantly attenuated both local and systemic inflammation (Ranieri et al. 1999). The protective strategy was accompanied by reduced neutrophil infiltration into the BAL fluid along with lower levels of several soluble inflammatory mediators that were also decreased in the plasma (Ranieri et al. 1999).

The ARDS Network trial also found a reduction in plasma levels of IL-6 and a higher number of distal organ failure free days in patients ventilated with protective settings in a much larger cohort of 861 patients (ARDS Network 2000). Terragni et al. suggested that mechanical ventilation might exacerbate inflammation through over distension of regions of the lung, which they found to occur in a high proportion of patients with ARDS despite the use of low tidal volumes. They noted that patients that experienced over-distension were found to have higher levels of several inflammatory mediators in their BAL fluid (Terragni et al. 2007).

Protective ventilation strategies have also been shown to reduce the incidence of infection and inflammation in critically ill patients without ARDS (Serpa Neto et al. 2012; Determann et al. 2010). Furthermore, even patients undergoing elective surgery without pre-existing lung injury have been shown to benefit from attenuated inflammatory responses with protective ventilation (Michelet et al. 2006; Wrigge et al. 2005; Wolthuis et al. 2008).

Multiple organ failure (MOF)

Non-protective (injurious) ventilation strategies are thought to promote multiple organ failure (MOF), which is the most common cause of death in patients with ARDS, with respiratory failure responsible for the death of only a minority of cases (Hernu et al. 2013; Villar et al. 2011). The transfer of inflammatory material between the alveolar space and the systemic circulation is a phenomenon known as 'decompartmentalisation', which is thought to be the cause of MOF (Plotz et al. 2004; Kuebler 2010; Del Sorbo and Slutsky 2011). Mechanical ventilation can cause substantial structural damage to the lung including the disruption of the alveolar-capillary cell wall leading to its increased permeability to inflammatory products (Mandava et al. 2003; von Bethmann et al. 1998).

This can even include the translocation of bacteria or endotoxin from the lung into the circulation as demonstrated in two animal models (Nahum et al. 1997; Murphy et al. 2000). Injurious mechanical ventilation has been found to augment cytokine release into the perfusate of an isolated perfused lung model of chronic MOF (Uhlig et al. 2006). Mechanical ventilation has also been shown to exacerbate pulmonary and systemic cytokine release following inoculation of mice with *Staphylococcus aureus*, a common cause of ventilator-associated pneumonia (VAP). In this study, biochemical markers of hepatic and renal dysfunction were detected in serum suggesting that mechanical ventilation contributed to distal organ failure (Dhanireddy et al. 2006).

Other studies have similarly demonstrated injury in distal organs following mechanical ventilation. High tidal volume ventilation has been shown to increase the permeability of the gut within just 2 hours in rats, which was prevented by administration of anti-TNFα antibody (Guery et al. 2003). Hegeman et al. reported *de novo* synthesis of several adhesion molecules in the lung as well as the liver and kidney in ventilated mice. This was accompanied by increased expression of cytokines and chemokines in these tissues as well as elevated granulocyte numbers (Hegeman et al. 2009). Another group reported increased apoptosis of epithelial cells in the kidneys and small intestine following injurious ventilation of rabbits and biochemical markers of renal dysfunction (Imai et al. 2003). The induction of apoptotic cascades in distal organs could be propagated through the release of soluble Fas and Fas ligand (FasL) from the lungs of ARDS sufferers, which can also be detected in their plasma, and are found at particularly high concentrations in patients who die from the condition (Albertine et al. 2002). In mice treated with adrenomedullin, the permeability increase induced by high tidal volume ventilation was attenuated and there was a decrease in systemic levels of creatinine, a marker of renal dysfunction (Muller et al. 2010). Liu et al. discovered that the kidney showed high levels of apoptotic changes when they ventilated rats with 40ml/kg for 6 hours. They concluded that the kidney is more vulnerable than other organs such as the heart and liver, to the spill over of

inflammatory mediators into the systemic circulation associated with VILI (Liu et al. 2014). Collectively, these studies present convincing evidence that injurious ventilation strategies can augment the development of extra-pulmonary organ failure and thereby increase mortality of patients with ARDS.

Application of low tidal volumes in practise

Despite the abundance of evidence that proves the benefits of a protective ventilation strategy, which employs low tidal volumes, a substantial PEEP and low peak inspiratory pressures, this strategy is not always applied in practise. An investigation into ventilation practises in three teaching hospitals, which are typically known to rapidly implement medical advances, were found to ventilate patients with an average tidal volume of 10ml/kg in the 2 years following the publication of the ARDS Network trial (Weinert, Gross, and Marinelli 2003). This study highlighted how slowly practises change in spite of proven mortality benefits of low tidal volume ventilation.

A survey of experienced ICU nurses and respiratory therapists revealed that the initiation of low tidal volume ventilation was often prevented by a lack of recognition of ARDS and concerns of adverse effects. They also reported that barriers to continuing low tidal volume ventilation included hypercapnia and hypoxaemia (Rubenfeld et al. 2004). It is widely accepted however that it is almost impossible to maintain 'normal' gas exchange in these patients without inflicting substantial ventilator-induced lung injury and instead 'adequate' gas exchange should be the goal (Villar, Kacmarek, and Hedenstierna 2004).

A prospective observational study of 88 patients ventilated between 2000 and 2002 within medical and surgical ICUs found that patients were only switched to low tidal volume ventilation once significant signs of ARDS had developed whilst mild signs of ARDS were not recognised (Kalhan et al. 2006). Another study compared ventilation practises within 107 ICUs that had been involved in clinical trials in 1998 and 2004 that included 1675 patients. The average applied tidal volume was found to have decreased from 9.1 to 7.4ml/kg and PEEP had increased slightly from 7.7 to 8.7cmH_2O (Esteban et al. 2008). This study showed that randomised trials have been successful in advancing mechanical ventilation practises within some ICUs.

The implementation of protective ventilation within the operating room has been slower. A study of 2161 patients undergoing surgery over a 6-month period in 2006 reported that 18% patients were ventilated with a tidal volume above 10ml/kg (Jaber et al. 2012). Another observational study conducted between 2006 and 2011 that included 45,575 patients, revealed that higher than

recommended tidal volumes are still used in 16% of patients and zero PEEP in 18% (Hess et al. 2013). However, the application of high tidal volumes had decreased from 28.5% of patients in 2006. High tidal volumes were found to cause ARDS in 13.5% of these surgical patients and were associated with an increased risk of MOF (Hess et al. 2013).

The importance of protective ventilation strategies on patient outcome is clear and the application of low tidal volumes in practise has increased. However, there remain a significant number of patients receiving higher tidal volumes than are recommended, and lung injury remains a risk to all patients requiring mechanical ventilation.

1.2 Preventing VILI

ARDS is associated with a rapid deterioration of respiratory function that can be provoked by an array of initial insults prior to admission to the ICU. However, the development of the iatrogenic condition VILI can be pre-empted as patients become at risk upon being intubated. This offers an opportunity to administer preventative treatment against the development of VILI, which in turn could provide more time for diagnosis and treatment of the underlying cause of disease. Protective treatment could also be useful in patients without pre-existing lung injury who have also been shown to be susceptible to VILI. Despite being the focus of many studies, the only effective method for reducing the impact of VILI has been to use a protective ventilation strategy consisting of a low tidal volume, high PEEP and a limited peak airway pressure. Unfortunately, even a 'protective' ventilation strategy can promote considerable inflammation and VILI remains a significant contributor to patient mortality.

Alternative ventilation strategies

Respiratory Rate

A ventilation setting that is often not controlled for in clinical trials is respiratory rate, and only a handful of animal studies have specifically addressed its impact on the development of VILI. A higher respiratory rate may be employed in the ICU to attenuate hypercapnia, an issue that can arise with the use of low tidal volumes. However, an increased respiratory rate can elevate intrinsic PEEP (Richard et al. 2002), which is associated with the same issues of extrinsically applied PEEP that include increased peak pressures and the potential for over-distension. This may explain the negative impact of increasing respiratory rates on the development of VILI in animal models (Vaporidi et al. 2008; Mandava et al. 2003; Retamal et al. 2015), though its effect has not been directly examined in

patients. However, a high frequency oscillation ventilation (HFOV) technique that delivers small tidal volumes (1-2ml/kg) at a rate of up to 15 breaths per second has been trialled in patients with no effect on mortality (Ferguson et al. 2013; Young et al. 2013; Gu et al. 2014).

Extracorporeal membrane oxygenation (ECMO)

For very severe cases of ARDS with refractory hypoxia, extracorporeal membrane oxygenation (ECMO) can be offered to patients at specialised centres. ECMO is an invasive therapy that involves the artificial oxygenation and removal of carbon dioxide from the blood outside of the body. Complications such as bleeding from sites other than the head and intracranial bleeding or infarction have high incidence rates (39% and 8% respectively) due to the use of anti-coagulants (Gray et al. 2015). During ECMO, the lungs may be unventilated, held at a constant pressure, or ventilated with a low tidal volume, with the aim of resting the lungs and allowing them to recover. The optimal ventilator settings to avoid further VILI are unclear since adequate gas exchange is achieved extracorporeally. Survival of patients receiving ECMO has been reported to reach up to 60%, which is thought to be higher than if they had not received ECMO intervention (Schmidt et al. 2013; Michaels et al. 2013; Roch et al. 2014). A study that matched ARDS severity of patients receiving or not receiving ECMO revealed a lower 6-month mortality rate in the ECMO treated patients (Tsai et al. 2015). However, there is not yet enough evidence to recommend ECMO despite its use as a salvage therapy in cases of severe ARDS (Leligdowicz and Fan 2015).

Prone positioning

Prone positioning has been shown to attenuate the development of VILI in several animal models including in dogs (Broccard et al. 2000). In this particular study, prone positioning reduced the accumulation of oedema and histologic injury scores, and injury was more homogeneously distributed across dependent and non-dependent regions of the lung. Prone positioning increases chest wall elastance and alveolar inflation is redistributed to previously dependent regions, resulting in lung recruitment. Several clinical trials in patients have reported improved oxygenation as the main benefit of prone positioning although it has been found to have little effect on mortality (Gattinoni et al. 2001; Guerin et al. 2004). Post-hoc analyses revealed that patients with severe hypoxia are the most likely to benefit from prone positioning, and in this group of patients, mortality may be reduced. Indeed, in a trial that only enrolled patients with severe ARDS, mortality rates were found to improve from 58% to 43% with prone positioning (Mancebo et al. 2006). The patients in this trial received prone ventilation for an average of 17 hours per day, which was at least double the time reported in previous trials (Mancebo et al. 2006).

Another clinical trial investigated the effect of combining prone positioning with high frequency oscillatory ventilation (HFOV) in a small cohort of patients with severe ARDS. This study found that prone positioning improved oxygenation, regardless of ventilation strategy, whilst HFOV could improve oxygenation of patients in the supine position. However, HFOV, both in the supine and prone positions, was associated with increased lung inflammation, as determined by increased CXCL8 levels in the BAL fluid (Papazian et al. 2005).

The debate on the effectiveness of prone positioning in patients with severe ARDS has continued with some trials demonstrating no survival benefit (Taccone et al. 2009) and others reporting a halving of the mortality rate to as low as 16% (Guerin et al. 2013). Meta-analyses have concluded that whilst prone positing has little effect on mortality of ARDS patients overall, it can reduce mortality in severely hypoxemic patients (Sud et al. 2014; Gattinoni et al. 2010). The associated risks of pressure sores, catheter disconnection and airway obstruction (Benson and Albert 2014) may explain the decrease in the use of prone positioning between 1998 and 2004 (Esteban et al. 2008).

Non-ventilatory interventions

Fluid management

It has been suggested that a conservative fluid strategy could be effective in reducing pulmonary oedema in patients with ARDS. The theory proposes that hypovolaemia will result in a decrease in capillary hydrostatic pressure and an increase in oncotic pressure that will reduce the amount of fluid moving into the lungs. In a clinical trial that included 1000 patients, a conservative fluid strategy improved oxygenation, reduced lung injury, and increased the number of ventilator-free days, but importantly, only resulted in a non-significant trend towards reduced mortality (Wiedemann et al. 2006). Although a conservative fluid strategy appeared to reduce morbidity in this study, the use of conservative fluid management must be applied with caution since fluid restriction can worsen haemodynamics and promote organ dysfunction (Roch, Guervilly, and Papazian 2011).

Surfactant replacement therapy

Injurious mechanical ventilation is known to disrupt the structure and function of surfactant resulting in reduced surface tension and decreased compliance (Veldhuizen et al. 2002). It was therefore hypothesised that the administration of exogenous surfactant may be beneficial in ARDS patients, particularly given its success in reducing mortality of premature neonates with respiratory distress syndrome (Hoekstra et al. 1991; Corbet et al. 1991). Unfortunately, surfactant therapy was not found to increase survival of adult patients with ARDS, despite improvements in oxygenation (Spragg et al.

2004), and a recent meta-analysis concluded that surfactant treatment of adult ARDS patients does not reduce mortality (Zhang et al. 2013). In animal models, surfactant replacement has been found to limit over-distension of terminal alveoli (Wu et al. 2010) and improve oxygenation, but has also been shown to exacerbate inflammatory responses following acid aspiration (Zambelli et al. 2015). The usefulness of surfactant therapy in the prevention or treatment of VILI therefore remains unclear.

Neuromuscular blocking agents

Neuromuscular blocking agents (NMBAs) are commonly given to ARDS patients with the aim of increasing oxygenation through improving the ventilation-to-perfusion ratio and reducing patient-ventilator asynchrony (Hraiech, Forel, and Papazian 2012). Several patient trials have been performed and have concluded that NMBAs can reduce mortality and morbidity in severe ARDS patients with early administration (Papazian et al. 2010; Needham and Brindley 2012). Meta-analyses have also found improved survival that is not accompanied by an increase in ICU-acquired muscle weakness as a result of NMBA use (Neto et al. 2012; Alhazzani et al. 2013). However, benefits with NMBAs have only been found in the most severely hypoxic ARDS patients and should not be used in patients with mild ARDS, in whom NMBA use increases the risk of ICU-acquired weakness and diaphragmatic dysfunction (Hraiech, Yoshida, and Papazian 2015).

β-adrenergic agonists

β-adrenergic agonists were considered a potential therapy for ARDS due to their role in the modulation of active transport of sodium across the alveolar epithelial cell wall, a process that is essential for the clearance of pulmonary oedema. An aerosolised β-adrenergic agonist salbutamol sulphate has been trialled in patients with ARDS but was not found to improve clinical outcomes (Matthay et al. 2011). A clinical trial of intravenously administered salbutamol sulphate even reported increased mortality (34%) compared with the placebo group (23%) (Gao Smith et al. 2012) and therefore their use in mechanically ventilated patients with ARDS is not recommended.

Corticosteroids

The use of corticosteroids in the treatment of patients with ARDS has yielded inconsistent results concerning their effect on clinical outcomes. A study in rats revealed that methylprednisolone reduced neutrophil activation and recruitment into the lungs following injurious ventilation (Ohta et al. 2001). However, clinical trials administering the same drug to patients at risk of ARDS, found that corticosteroid therapy actually increased the development of the condition and enhanced patient susceptibility to infection (Weigelt et al. 1985). Another trial reported that whilst corticosteroid

therapy did not increase survival, it did improve gas exchange and reduce the incidence of MOF (Varpula et al. 2000). One clinical trial reported an increase in mortality associated with starting methylprednisolone in persistent ARDS that has been present for more than 2 weeks (Steinberg et al. 2006). The benefits of corticosteroid therapy may be reserved for early ARDS if therapy can be initiated within 3 days of onset (Meduri et al. 2007). The timing of corticosteroid therapy and its efficacy remain unclear perhaps suggesting that a generalised approach to reducing inflammation is not sufficient.

Targeted therapies

Dampening the inflammatory response is considered the best target for reducing the mortality and morbidity associated with VILI. However, as we have discovered in clinical trials of corticosteroids, it is important that we target inflammatory responses that are specific to VILI, as a general weakening of the immune system could leave patients susceptible to infection. Animal models provide the opportunity to examine inflammatory consequences specific to VILI in the absence of the multiple co-morbidities that patients admitted to the ICU present.

Permeability

Derangement of the alveolar-capillary barrier and subsequent increase in permeability not only leads to the accumulation of pulmonary oedema, but may also enable the release of local inflammatory mediators into the systemic circulation. This theory of 'decompartmentalisation' has not been well studied, but offers a possible explanation as to how mechanical ventilation often leads to MOF, which is the most common cause of death in patients with ARDS. Preservation of this barrier has therefore been the focus of several studies. Huang et al. reported that enhancing activation of plasminogen, which degrades fibrin, reduced BAL fluid protein and the histological lung injury score in rats that had been subjected to 2 hours of injurious ventilation (Huang et al. 2012). They concluded that preventing fibrin deposition within the alveolar space avoided surfactant dysfunction and attenuated derangements to the alveolar capillary barrier (Huang et al. 2012).

Another group studied the effects of the peptide intermedin, which signals via the calcitonin receptor-like receptor (CRLR), previously shown to stabilise the endothelial barrier (Muller-Redetzky et al. 2012). They found that continuous infusion of intermedin did indeed prevent deterioration of the endothelial barrier as demonstrated by a reduction in BAL fluid protein following ventilation of mice at 12ml/kg for 6 hours, but had no effect on neutrophil recruitment or plasma cytokine levels (Muller-Redetzky et al. 2012). Finally, metformin, commonly used to treat type 2 diabetes mellitus,

has been suggested to exhibit pleiotropic properties including anti-inflammatory and endothelial barrier protection. This led to its being trialled in an isolated perfused rabbit lung ventilated injuriously for 1 hour; in which metformin was found to reduce BAL fluid protein and histological lesions, suggesting it preserved the integrity of the alveolar-capillary barrier (Tsaknis et al. 2012).

Apoptosis

Several studies have attempted to reduce apoptotic signalling in VILI with the aim of preserving the parenchymal cells that are vital in maintaining the alveolar barrier and the surface for gas exchange. Pre-treatment of rats with captopril, an angiotensin-converting enzyme inhibitor, was found to inhibit the apoptosis of alveolar epithelial cells (AECs) in response to injurious ventilation and reduce BAL fluid cytokine levels (Jiang et al. 2007). This signalling pathway has also been targeted with Losartan, which was administered to rats prior to 2 hours of injurious ventilation, and was found to attenuate BAL fluid protein and neutrophil infiltration (Yao et al. 2008). Another group pre-treated mice with sivelestat, a neutrophil elastase inhibitor, which was similarly found to reduce apoptosis of AECs and prevented neutrophil recruitment following 4 hours of injurious ventilation (Sakashita et al. 2007).

Inflammatory signalling

The inhibition of VILI-specific inflammatory signalling whilst preserving inflammatory responses to infection could enable time to treat the underlying cause of ARDS, and could prevent further lung injury leading to disruption of the alveolar-capillary barrier and MOF. Uhlig et al. inhibited activity of phosphoinositide 3-OH kinase and found that it prevented nuclear translocation of NF-κB and subsequent release of cytokines in isolated perfused mouse lungs subjected to injurious ventilation (Uhlig et al. 2004). They also demonstrated that inhibiting this pathway did not prevent the nuclear translocation of NF-κB in response to endotoxin exposure, thereby demonstrating that it is possible to prevent ventilator-induced inflammation whilst preserving immune responses to pathogens.

1.3 Role of leukocytes in VILI

Neutrophils

Neutrophils are recruited to the lung by chemokines including CXCL8, CXCL1 (KC) and CXCL2, which are released by alveolar macrophages and AECs (Belperio et al. 2002). Neutrophil stiffening causes them to move slowly through pulmonary capillaries where selectins mediate 'rolling' of the neutrophils along the endothelial surface (Choudhury et al. 2004). Integrin connections promote the

migration of neutrophils through the endothelial barrier into the interstitium and subsequently into the alveolar space.

It was suggested that the process of neutrophil migration itself was the cause of injury to the alveolar-capillary barrier, however; there are circumstances where neutrophils can enter the lung without accompanying injury or prior to the development of physiological signs of injury (Walker, Behzad, and Chu 1995; Choudhury et al. 2004). Conversely, the alveolar-capillary barrier can become damaged in the absence of neutrophil migration (Steinberg et al. 2004; Kaynar et al. 2008).

The recruitment of neutrophils to the lung can be associated with the severity of injury (Steinberg et al. 1994), and due to their proficient release of copious amounts of injurious mediators, neutrophils were once considered to be the cause of ARDS (Martin 2002). Indeed, neutrophil depletion can attenuate the development of ARDS in animal models (Kawano et al. 1987). However, ARDS has been shown to manifest in the absence of neutrophils, as demonstrated in neutropenic patients whom can still develop ARDS (Ognibene et al. 1986; Mokart et al. 2012).

Once in the alveolar space, neutrophils may become activated by pro-inflammatory mediators and consequently make their own contribution to the pro-inflammatory milieu. Neutrophils release a combination of cytokines, reactive oxygen species (ROS) and proteinases including elastase and matrix metalloproteinases (MMPs) that mediate epithelial damage.

Alveolar Macrophages

Whilst the recruitment and activation of neutrophils clearly has an aggravating effect on VILI, their infiltration into the lung is a secondary response to the release of chemokines from the lung. Therefore, ventilator-induced inflammatory signalling must be initiated by local pulmonary cells, such as AECs, pulmonary endothelium, and alveolar macrophages. The epithelium and endothelium have both been found to be capable of pro-inflammatory cytokine and chemokine release; however, it is assumed that the alveolar macrophages, as the resident immune cell specifically adapted to the lung environment, would be the main source of inflammatory mediators.

Several studies have demonstrated that alveolar epithelial and endothelial cells can release chemokines such as CXCL8 in response to cyclic stretch (Pugin et al. 1998; Iwaki et al. 2009). However, alveolar macrophages have been shown to be much more prolific producers of such mediators (Pugin et al. 1998), though it remains unclear whether they would be directly subjected to stretch *in vivo*. Nevertheless, the alveolar macrophages are considered the most likely pulmonary cell to orchestrate the inflammatory response that leads to the recruitment of neutrophils to the lung.

Several studies have suggested that alveolar macrophages become more adhesive in response to short lengths of high tidal volume ventilation, an indicator of macrophage activation. Imanaka et al. ventilated rats with high-pressure (45cmH_2O) ventilation for 40 minutes and found that alveolar macrophages isolated by bronchoalveolar lavage, displayed increased expression of the adhesion molecules CD11b and ICAM-1 (Imanaka et al. 2001). They also noted that the number of alveolar macrophages that could be extracted by bronchoalveolar lavage decreased significantly within this period. They suggested that the timescale did not allow for apoptosis to have occurred and that the most likely explanation, taking into account their up-regulation expression of adhesion molecules, was that they had become adhered to the alveolar wall (Imanaka et al. 2001).

Subsequent studies have reported the same phenomenon of decreasing alveolar macrophage counts in BAL fluid following injurious ventilation under even shorter timescales. Frank et al. ventilated rats with 30ml/kg and found that alveolar macrophage numbers had begun to decrease within just 10 minutes, and became significantly decreased (by 45%) after 20 minutes of injurious ventilation (Frank et al. 2006). Eyal et al. ventilated rats at 40ml/kg and discovered that alveolar macrophage counts decreased almost immediately following initiation of injurious ventilation. This reduction in macrophage recovery was evident within 1 minute and was reduced by 50% after 5 minutes (Eyal, Hamm, and Parker 2007). Both of these studies came to the same conclusion that under such a short timescale, the only explanation for decreased alveolar macrophage recovery in BAL fluid is that they become tightly adhered to the alveolar wall.

Further experiments by these research groups have involved depleting alveolar macrophages from the lung with the use of clodronate liposomes to highlight their importance in VILI. Frank et al. reported that depletion of alveolar macrophages significantly attenuated the increase in permeability and pulmonary oedema and reduced the deterioration in oxygenation caused by 4 hours of high tidal volume ventilation (Frank et al. 2006). In the absence of alveolar macrophages, the release of the neutrophil chemoattractant CXCL1 into the systemic circulation was also significantly reduced and neutrophil migration into the BAL fluid was diminished (Frank et al. 2006). Eyal et al. similarly demonstrated that depletion of alveolar macrophages attenuated decreases in oxygenation, accumulation of pulmonary oedema, and compliance associated with injurious ventilation (Eyal, Hamm, and Parker 2007).

These studies confirmed that alveolar macrophages do indeed play a key role in the development of VILI and their presence seems to influence many aspects of lung injury. However, alveolar macrophages are also important in mediating the resolution of lung injury through the phagocytosis of neutrophils and apoptotic cells, as well as being the lungs defence against pathogens. Therefore,

their depletion from the lungs of human patients is not a viable therapy, as it would leave patients susceptible to infection and persistent inflammation. Instead, a potential target may be to prevent alveolar macrophage activation in response to high tidal volume ventilation; however, this mechanism remains unknown.

Possible mechanisms of alveolar macrophage activation

Despite evidence that alveolar macrophages when stretched *in vitro* become activated and release inflammatory cytokines (Pugin et al. 1998), it seems unlikely that they would be directly subjected to stretch *in vivo*. Therefore, they must become activated through communication with the lung parenchymal cells, which would undoubtedly be exposed to stretching during high tidal volume ventilation. This could occur through direct contact with the alveolar wall, or through the release of soluble mediators. Studies described previously have shown that alveolar macrophages become more adherent in response to just minutes of high tidal volume ventilation (Eyal, Hamm, and Parker 2007), suggesting that the means by which they become activated must be very rapid.

Cell-to-cell contact

Alveolar macrophages demonstrate enhanced adherence following stimulation and this could enable direct communication between them and the alveolar epithelium. It remains unclear in this situation, whether alveolar macrophages would become more adherent in response to soluble mediator release by the AECs, or due to increased expression of adhesion molecules on the alveolar surface. Inflation of alveoli during high tidal volume ventilation could result in a thinning of the surfactant layer, bringing alveolar macrophages into close contact with the epithelium. Alternatively, it has been suggested that a sub-population of alveolar macrophages may be in contact with the alveolar wall under normal conditions and therefore could be already positioned to detect over-distension (Westphalen et al. 2014). Adhered alveolar macrophages may be able to sense stretching of the alveolar wall through forces applied to adhesion molecules, or through gap junctions that allow transfer of signalling molecules between cells.

The importance of adhesion molecules in the recruitment of leukocytes into the lung during lung injury has been well studied (Ridger et al. 2001; Burns et al. 2001), but their role within the alveolar space during VILI remains unknown. An *in vitro* study demonstrated that ICAM-1 expression increased in A549 epithelial cells in response to cyclic stretching that increased with strain, frequency and duration (Hu et al. 2008). Increased ICAM-1 expression has been correlated with the need for prolonged mechanical ventilation and mortality in paediatric patients (Flori et al. 2003).

In animal models, increased expression of ICAM-1 and CD11b has been reported on alveolar macrophages of rats ventilated with high tidal volume for 40 minutes (Imanaka et al. 2001; Ohta et al. 2001). ICAM-1 expression is also enhanced on the alveolar surface following 6 hours of ventilation at a moderate tidal volume of 15ml/kg in an isolated perfused rat lung (Miyao et al. 2006). Anti-CD11c antibody has been shown to attenuate cytokine release into BAL fluid following 2 hours high tidal volume ventilation of rats (Pan et al. 2014). These studies all demonstrate the up-regulation of adhesion molecules in response to stretch, although within relatively long time scales, though it is possible that they might become up-regulated at earlier time points than have been studied. If these adhesion molecules are capable of rapid up-regulation, they may provide a plausible mechanism for alveolar macrophages to sense deformation of the alveolar wall.

Gap junctions are another mechanism by which alveolar macrophages could be alerted to over-distension of the alveolar epithelium. Gap junction proteins have been shown to be expressed by both the alveolar macrophages and epithelial cells, but there has been conflicting evidence regarding whether they play an anti- or pro-inflammatory role following LPS exposure (Westphalen et al. 2014; Sarieddine et al. 2009). Gap junctions have also been implicated in communication of stretch in tracheal airway epithelial cells (Boitano and Evans 2000). These gap junctions could provide a potential mechanism for immediate communication of stretch within the alveolar space.

Soluble mediators

An alternative mechanism by which alveolar macrophages may become activated in response to injurious ventilation could be through the release of soluble mediators by the alveolar epithelium. The epithelium has been shown to be capable of releasing pro-inflammatory mediators such as cytokines in response to stretch (Vlahakis et al. 1999; Oudin and Pugin 2002), though most cytokines are not available for rapid release. Instead, more likely candidates for a rapidly released soluble mediator are those that are already present within the cell and do not require transcription or translation into protein for secretion. Frank et al. ventilated rats with high tidal volume ventilation for 10 or 20 minutes and found that their BAL fluid induced nitrite production in naïve primary macrophages (Frank et al. 2008). These experiments clearly demonstrated a role for soluble mediators in the activation of alveolar macrophages in VILI but did not identify the cell source, or indeed the responsible mediators.

Potential soluble mediators could be DAMPs such as ATP, which could be rapidly released from intracellular stores (Rich et al. 2003). Other alternatives are prostanoids, which are lipid mediators whose synthesis is controlled post-translationally (Mbonye and Song 2009); or TNFα, which, although

a cytokine, is expressed on the cell surface where it can be rapidly cleaved and released into the extracellular space (Imai et al. 1999). Whilst each of these mediators has been implicated in the development of VILI, studies have focussed on their roles in the later stages of VILI. It remains to be determined whether any of these mediators could be responsible for the initial activation of alveolar macrophages in response to injurious mechanical ventilation.

Mechanotransduction in Parenchymal Cells

Both of the hypotheses above that describe how alveolar macrophages may become stimulated in response to injurious mechanical ventilation; require prior activation of the alveolar epithelium. The epithelial layer may be subjected to physical damage when subjected to extreme ventilator settings; very high tidal volumes can rupture alveoli and a lack of PEEP can cause alveoli to collapse on expiration (Carrasco Loza, Villamizar Rodriguez, and Medel Fernandez 2015). However, we have seen that more moderate tidal volumes can also induce VILI and initiate inflammatory responses in the absence of significant physical damage to the epithelial barrier (Vaneker et al. 2007). Under these moderate stretch conditions, mechanical ventilation stimulates AECs through deformation of the plasma membrane, which can trigger intracellular signalling, a process known as mechanotransduction.

Mechanical ventilation with positive pressures applies atypical mechanical forces on the lung, and this effect may be amplified in heterogeneously injured lungs since the forces applied to a single alveolus is dependent on the surrounding alveoli, which could be collapsed or over-distended. The majority of research into the mechanisms of mechanosensation and mechanotransduction has been performed *in vitro*, which cannot entirely replicate the complexity of the whole lung. Therefore, these mechanisms remain to be fully elucidated, although several events have been proposed to be involved in the sensing of stretch by AECs (Spieth et al. 2014).

The cytoskeleton, which links integrins on the cell surface to intracellular proteins, is considered an integral component in the mechanotransduction of stretch (Taniguchi et al. 2010). These cell surface integrins are constituents of adherens junctions which mediate interactions with adjacent cells and focal adhesion plaques, facilitating contact with the extracellular matrix. The cytoskeleton is composed of a network of filaments, microtubules, intermediate filaments and actin microfilaments which can rapidly rearrange themselves in response to tension, altering the cell shape to suit the environment (Wang, Butler, and Ingber 1993). Such changes in cell shape could be involved in the increased permeability of the lung induced by mechanical ventilation (Dudek and Garcia 2001).

Stretch-activated ion channels have also been frequently suggested to be involved in initiating intracellular signalling in response to mechanical strain. Possible channels include mechanosensitive K^+ channels, non-selective cation channels that permit influxes of Ca^{2+} and voltage-gated Na^+ channels. Gadolinium chloride, an inhibitor of stretch-activated cation channels has been shown to prevent increases in microvascular permeability following high tidal volume ventilation of rat lungs, suggesting that an increase in intracellular calcium concentration initiates this cellular response (Parker, Ivey, and Tucker 1998).

The sensing of stretch leads to the activation of intracellular signalling pathways that involve common signalling machinery. Mechanical stimulation leads to the up-regulation of adhesion molecules that may be involved in direct communication between AECs and alveolar macrophages, or the expression of soluble inflammatory mediators that stimulate alveolar macrophages upon their release into the alveolar space.

1.4 Intracellular inflammatory signalling pathways

It is clear that cellular responses to over distension caused by mechanical ventilation are initiated well before physical damage to the lung can be detected, and before hypoxaemia develops. This has been demonstrated in several animal models where inflammatory responses can be detected prior to the development of significant injury to the alveolar-capillary barrier (Vaneker et al. 2007). Derangements to the respiratory mechanics require some time to develop, but the inflammatory process that leads to these downstream effects must occur much earlier.

Some studies have detected signs of alveolar macrophage activation within just 5 minutes exposure to injurious ventilation as described earlier. However, the mechanism by which these resident immune cells become activated remains unknown. Furthermore, the role of the alveolar epithelium in this process is also unclear. One way to study the kinetics of cellular activation of the major pulmonary cell types following stimulation, is through utilisation of phospho-flow cytometry to examine activation of intracellular signalling pathways. The advantage of this method is that activation can be localised to specific cell populations.

Phosphorylation is one of many types of post-translational modification that provides fast and precise regulation over the activity, localisation and interactions of proteins. Catalysed by kinases, phosphorylation involves the transfer of a phosphate group onto serine, threonine or tyrosine amino acid residues of the target protein. Phosphorylation sites are highly specific and can be identified by a conserved sequence of flanking residues. In most cases, phosphorylation promotes a conformational

change in the proteins structure, which results in it being switched 'on' or activated through altering the accessibility to active sites, localisation sequences or interaction domains. Once activated, proteins may go on to activate other proteins as part of a signalling cascade, which may lead to the activation of transcription factors and changes in gene expression. This process is readily reversible and proteins may be rapidly dephosphorylated or inactivated by phosphatases, which play a key role in modulating the strength and duration of activation.

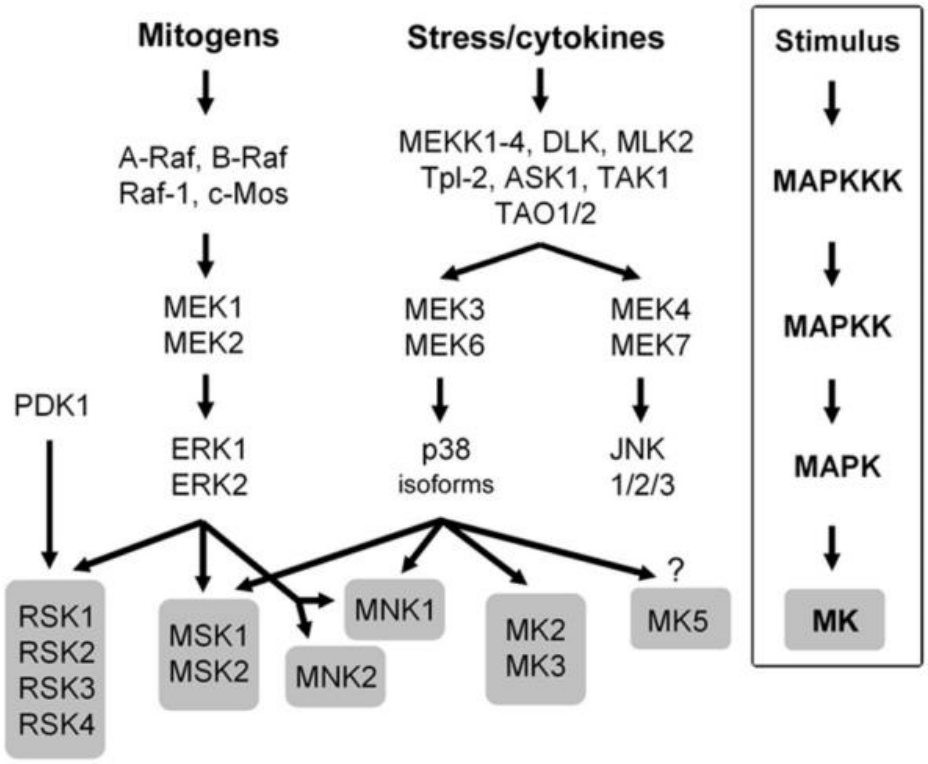
Commonly measured signalling molecules that can be quantified by phospho-flow cytometry include the mitogen-activated protein (MAP) kinases p38 and ERK1/2, p38's immediate downstream substrate MK2, and the transcription factor NF-κB. These markers are frequently reported to be activated following injurious ventilation *in vivo* and their inhibition has been shown to be effective in attenuating inflammation in animal models of VILI. However, all of these activation markers are involved in multiple signalling cascades and therefore their pharmacological inhibition in patients may be associated with significant adverse effects such as immunosuppression and carcinogenesis. Whilst they may not be viable targets for therapy, they serve as excellent markers of general cell activation in mechanistic studies.

MAP Kinases

Mitogen-activated protein (MAP) kinase signalling cascades are involved in transducing external stimuli into a wide range of finely controlled cellular responses. They are activated in response to myriad stress stimuli through multi-tiered cascades in which MAP3Ks phosphorylate MAP2Ks, which then go on to activate MAPKs (Figure 1.1). There are several classes of MAP kinase including the extracellular signal-regulated kinases (ERK1/2), c-Jun amino (N)-terminal kinases (JNK1/2/3), p38 isoforms (α, β, γ and δ), and ERK5.

The ERK1/2 pathway is activated in response to growth factors and can lead to cell responses such as cell proliferation or differentiation (Lenormand et al. 1993). The JNK1/2/3 pathway is activated by stress stimuli (Bogoyevitch et al. 2010) and its downstream effects can converge with those of the p38 pathway, which is also induced by stress stimuli and inflammatory cytokines (Ono and Han 2000). The ERK5 pathway is important in early embryonic development, particularly in the development of the vascular system (Regan et al. 2002). The MAP kinases are ubiquitously expressed across all cell types and tissues, making them ideal indicators of cellular activation.

Figure 1.1 Overview of MAPK Signalling Pathways



Mitogens and stress stimuli lead to the activation of ERK1/2 and p38 signalling cascades. Figure adapted from (Roux and Blenis 2004).

p38 & MK2

p38 was first identified as a kinase that becomes rapidly phosphorylated and activated in response to LPS stimulation as well as other stresses such as hyperosmolarity (Han et al. 1994). In these initial studies, p38 was found to be involved in the production of pro-inflammatory mediators including IL 1B and TNFα (Lee et al. 1994). The mechanism of p38 activation was soon discovered to be mediated through the dual phosphorylation of specific amino acid residues Thr-180 and Tyr-182 within the Thr-Gly-Tyr motif (Raingeaud et al. 1995). These regulatory phospho-sites are phosphorylated by the two MAP2Ks MKK3 and MKK6 (Raingeaud et al. 1996; Keesler et al. 1998).

Once activated, p38 can activate several other protein kinases and the most well-known downstream substrates of p38, are MK2 and MK3, which phosphorylate a number of substrates including small heat shock protein 27 HSP27 (Rouse et al. 1994). p38 also activates p38 regulated/activated kinase PRAK, which can also phosphorylate and activate HSP27 (New et al. 1998). p38 is additionally known to activate MAP kinase interaction protein kinase MNK1, which is involved promoting the initiation of translation (Waskiewicz et al. 1997). p38 has also been reported to phosphorylate mitogen- and

stress-activated kinase MSK, which phosphorylates the transcription factor cAMP response element-binding protein CREB (Ono and Han 2000; Deak et al. 1998).

In addition, p38 can also activate several other transcription factors, including the activating transcription factors ATF-1 and ATF-2 (Tan et al. 1996) , SRF accessory protein 1 Sap1 (Whitmarsh et al. 1997), and growth arrest and DNA damage inducible gene 153 GADD153, which is important in regulating cell proliferation and differentiation (Wang and Ron 1996). p38 is also known to activate p53, which is involved in regulating apoptosis (Huang et al. 1999), and monocyte enhancer factors MEF2A and MEF2C (Yang, Galanis, and Sharrocks 1999; Zhao et al. 1999). Through the activation of all these transcription factors, activation of the p38 pathway leads to increased expression of pro-inflammatory cytokines, the induction of enzymes such as COX-2 and iNOS (Werz et al. 2000; Guan et al. 1998; Badger et al. 1998), and increased expression of adherence proteins amongst other inflammatory molecules (Pietersma et al. 1997). In this way, initiation of p38 signalling is amplified through various downstream signalling events to produce a co-ordinated response to stress stimuli.

In order to activate these downstream signalling events, p38, which normally resides in the cytoplasm under resting conditions, translocates into the nucleus upon phosphorylation by MKK3/6 (Gong et al. 2010). It is unclear exactly how this is regulated but since p38 does not contain a nuclear localisation sequence (NLS) itself, it has been suggested that p38 may interact with a NLS-containing chaperone protein (Gong et al. 2010). Once activated p38 has entered the nucleus, it is able to activate various transcription factors and protein kinases including MK2, which resides in the nucleus under resting conditions and has been reported to be present in excess of p38 (Ben-Levy et al. 1998). Activation of MK2 requires phosphorylation of three specific residues Thr-222, Ser-272 and Thr-334, which is mediated by p38, and involves the formation of a heterodimer (Ben-Levy et al. 1995).

Following phosphorylation, MK2 assumes an open conformation that exposes a nuclear export signal (NES) located at its C-terminus, resulting in the export of the p38:MK2 complex from the nucleus (Ben-Levy et al. 1998; Neininger, Thielemann, and Gaestel 2001; Meng et al. 2002; ter Haar et al. 2007). Therefore, p38 phosphorylation of MK2 not only induces its activation, but also regulates the localisation of p38 within the cell. Once in the cytoplasm, MK2 plays an important role in the pro-inflammatory cytokine response. MK2 has been found to increase the translation of pro-inflammatory mRNAs through a stabilising post-transcriptional mechanism involving AU rich elements (AREs) (Kotlyarov et al. 1999; Winzen et al. 1999).

The p38 MAP kinase pathway has been shown to be specifically activated in pulmonary cells subjected to stretch *in vitro* (Abdulnour et al. 2006; Cohen et al. 2010; Boudreault and Tschumperlin

2010; Iwaki et al. 2009; Li et al. 2003). p38 has also been found to be activated in several *in vivo* models of VILI (Chiang et al. 2012; Park et al. 2012; Dolinay et al. 2004; Kotani et al. 2004; Chiang et al. 2011; Uhlig et al. 2002).

Furthermore, inhibition of p38 signalling has even been shown to attenuate VILI in animal models. Dolinay et al. mechanically ventilated mice lacking MK3, which is a downstream protein kinase substrate of p38 that has similar activity to MK2 (Ronkina et al. 2007). They found that these knockout mice demonstrated significantly reduced inflammatory responses to injurious ventilation compared with wild type mice, implicating the p38 pathway in the induction of inflammation in VILI (Dolinay et al. 2008). Damarla et al. found that inhibition of p38/MK2 signalling in mice ventilated with injurious settings attenuated phosphorylation of HSP25, which was accompanied by a decrease in barrier dysfunction (Damarla et al. 2009). Siegl et al. concluded that p38 signalling was responsible for the induction of pro-inflammatory gene expression in response to injurious mechanical ventilation in their model of ventilated and perfused mouse lungs (Siegl and Uhlig 2012).

It is clear that the p38/MK2 pathway plays an important role in regulating the expression of pro-inflammatory cytokines in response to cell stress. This has been demonstrated in models of VILI where inhibition of this pathway attenuates symptoms. Whilst the blockade of this pathway in patients is likely to be associated with a dangerous dampening of the general immune response, the quantification of p38 and MK2 activation in mechanistic studies provide a reliable indication of early cellular activation.

ERK1/2

The ERK1/2 pathway was the first MAP kinase cascade to be discovered and has been the most thoroughly studied. The two isoforms ERK1 and ERK2 are translated from unique genes but demonstrate almost identical regulation, substrate specificity and subcellular localisation. ERK1/2 signalling is central to many cellular responses including proliferation, differentiation, and survival. ERK1/2 was first identified as a kinase that was capable of very rapid activation, within minutes of exposure to mitogens such as α-thrombin, platelet-derived growth factor and foetal calf serum (Meloche, Pages, and Pouyssegur 1992). ERK1/2 is phosphorylated by the MAP2Ks MEK1 and MEK2, on the threonine and tyrosine residues within the Thr-Xaa-Tyr regulatory motif (Seger et al. 1992).

Activated ERK1/2 has been reported to activate hundreds of substrates in both the cytoplasm and the nucleus (Chuderland and Seger 2005). These include the activation of protein kinases such as ribosomal s6 kinases RSKs, which regulate transcription factors involved in the expression of immediate early genes (Anjum and Blenis 2008), MSK, which regulates activation of the transcription

factor CREB (Deak et al. 1998), and MNKs, which phosphorylate the translation initiation factor eIF4E (Waskiewicz et al. 1997). ERK1/2 signalling is also responsible for the activation of transcription factors Elk-1 (Marais, Wynne, and Treisman 1993) and c-Fos (Gille, Sharrocks, and Shaw 1992). Activation of all these substrates prepares the cell proliferation, which requires up-regulation of the biosynthesis of membranes, organelles, nucleic acids and proteins.

The outcome of ERK1/2 signalling can depend on the duration of activation, by regulating the expression of cell cycle proteins. ERK1/2 displays biphasic activity, with a rapid and transient peak in activation at 5 to 10 minutes following mitogen stimulation that is followed by a less intense but prolonged second wave of activation (Meloche, Pages, and Pouyssegur 1992). This sustained activation of ERK1/2 has been found to be required for advancement through G_1 phase and entry into S phase of the cell proliferation cycle (Meloche and Pouyssegur 2007). Signal duration is sensed by the immediate early gene product c-Fos, which fails to accumulate when ERK1/2 activation is transient (up to 30 minutes), but when ERK1/2 activation is sustained for several hours, c-Fos becomes phosphorylated and stably expressed (Murphy et al. 2002). The progression of the cell cycle into S phase is associated with the continued expression of cyclin D1, which is up-regulated by ERK1/2 activity, and promotes the translation of proteins required for DNA replication through activation of retinoblastoma (Rb) proteins (Bracken et al. 2004). Inhibition of ERK1/2 signalling has been shown to decrease levels of cyclin D1 and is accompanied by growth arrest (Weber et al. 1997).

As the for the p38/MK2 pathway, ERK1/2 signalling is also partly regulated by subcellular localisation (Pouyssegur, Volmat, and Lenormand 2002). It is thought that under unstimulated conditions, ERK1/2 resides in the cytoplasm where it is anchored to its activator MEK1. Upon mitogen stimulation, phosphorylation of residues within ERK1/2's activation loop results in a conformational change that allows it to dissociate from MEK1 (Wolf et al. 2001). Phosphorylation of the Thr-Xaa-Tyr motif on ERK1/2 is necessary, but not sufficient for nuclear localisation, with reports suggesting that translocation requires association with other proteins, which have not yet been identified (Caunt et al. 2012; Caunt and McArdle 2012).

Once in the nucleus, ERK1/2 may phosphorylate its many substrates that lead to the up-regulation of protein biosynthesis and other processes involved in cell proliferation. ERK1/2 activity may be terminated by the MKP1/2 phosphatases, which are localised within the nucleus (Volmat et al. 2001). It has been reported that MEK1 is capable of shuttling between the nucleus and the cytoplasm, entering the nucleus by passive diffusion, and exiting by NES-dependent active transport (Adachi, Fukuda, and Nishida 2000). It has been suggested that MEK1 is responsible for the exportation of

inactive ERK1/2 from the nucleus, and therefore not only regulates ERK1/2 activity but also its subcellular localisation (Adachi, Fukuda, and Nishida 2000).

Whilst activation of ERK1/2 signalling may not appear to be directly involved in promoting inflammatory responses, it has been found to be activated in response to LPS stimulation. Schuh et al. demonstrated that the MEK1/2 inhibitor U0126 significantly attenuated lung neutrophilia and levels of pro-inflammatory cytokines in the BAL fluid of LPS treated mice (Schuh and Pahl 2009). In response to mechanical ventilation, activation of ERK1/2 signalling would seem more likely given that stretch is a common trigger for cell proliferation and differentiation in AECs (Sanchez-Esteban et al. 2004; Chess, Toia, and Finkelstein 2000; Edwards 2001).

ERK1/2 phosphorylation has been frequently detected in pulmonary cells stretched *in vitro* (Cohen et al. 2010; Boudreault and Tschumperlin 2010; Li et al. 2003; Correa-Meyer et al. 2002). ERK1/2 activity is also commonly reported in *in vivo* models of VILI (Li et al. 2007; Abdulnour et al. 2006; Kotani et al. 2004; Uhlig et al. 2002). Furthermore, a few studies have reported that inhibition of ERK1/2 signalling is protective against the development of VILI. Li et al. reported that the ERK1/2 inhibitor PD98059 attenuated ventilator-induced fibrosis and levels of BAL fluid CXCL2 in a two-hit murine model of bleomycin and VILI (Li et al. 2008). A study by Kaniaris et al. found that mice treated with a Tpl2 inhibitor, a MAP3K that activates ERK1/2, were protected against the development of VILI (Kaniaris et al. 2014). ERK1/1 is therefore a very useful indicator of cellular activation following mechanical stimulation.

NF-κB

The nuclear factor kappa B (NF-κB) family of transcription factors regulate the expression of many pro-inflammatory genes in response to various stimuli including cytokines and infectious material. Members of the NF-κB family include p65 (RelA), RelB, c-Rel, p50 and p52, which all share a conserved DNA binding domain known as the Rel homology region (RHR). The NF-κB proteins exist as homo- and heterodimers, mediated by their RHR, and in which form they bind target DNA. Only p65, RelB and c-Rel harbour a C-terminal transactivation domain (TAD) that is able to activate transcription of target genes.

Under resting conditions, NF-κB dimers are sequestered in the cytoplasm through interaction of their ankyrin repeat domains with an inhibitor of NF-κB (IκB) protein (IκBα, IκBβ, and IκBε). Upon stimulation of the cell, the IκB kinase (IKK) complex becomes activated and releases NF-κB from this inhibitory interaction (Hinz and Scheidereit 2014). The IKK complex consists of a combination of two kinases IKKα and IKKβ that become activated by phosphorylation of two serine resides, located in

their activation loops, as well as a regulatory subunit NF-κB essential modulator (NEMO/IKKγ). The activated IKK complex phosphorylates the IκB protein on specific serine residues, which marks it for ubiquitination and degradation by the proteasome (Traenckner et al. 1995). This permits the NF-κB dimer to translocate into the nucleus, driven by its NLS that had previously been hidden in the closed conformation induced by its interaction with IκB (Figure 1.2). These events that lead to the translocation of the NF-κB dimer are relevant to complexes of p50-p65 with IκBα. However, p65 homodimers complexed with IκBβ have been reported to continuously shuttle between the nucleus and cytoplasm due to one of the NLS sequences remaining accessible (Malek et al. 2003).

Figure 1.2 Activation of NF-κB Signalling

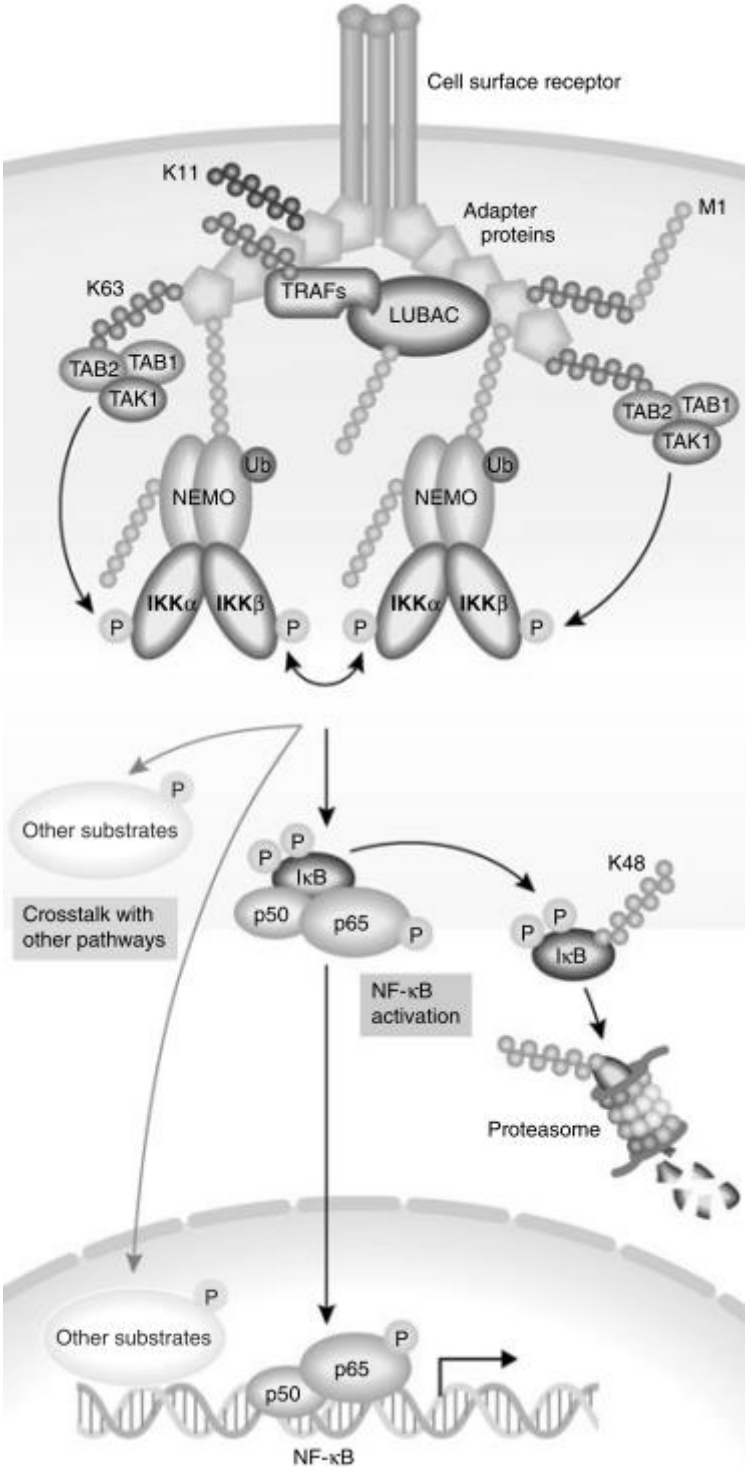


Figure adapted from (Hinz and Scheidereit 2014).

The release of NF-κB dimers from their cytoplasmic anchor permits them to enter the nucleus, but they also require post-translational modifications at specific sites in order to become fully transcriptionally active. Several kinases have been identified that are able to induce this modification

including cyclin-dependent kinase 6 CDK6 (Buss et al. 2012), the IKK complex proteins IKKα and IKKβ (Buss et al. 2004), casein kinase II CKII (Wang et al. 2000), and protein kinase C PKC (Leitges et al. 2001). Phosphorylation of p65 NF-κB at Ser-276 and Ser-539 has been found to be necessary for interaction with transcriptional co-activator CBP/p300 (Zhong, Voll, and Ghosh 1998). The importance of phosphorylation on the transcriptional activity NF-κB makes it an ideal event to measure as an indicator of cellular activation.

NF-κB signalling is further regulated by the tertiary structure of its target genes. Some genes that contain the κB binding site may be inaccessible to NF-κB activation due to the compact arrangement of DNA and chromatin. The chromatin environment is regulated through post-translational modifications to the histone proteins, which can be cell type specific. Therefore, NF-κB signalling may lead to different downstream effects depending of the type of cell stimulated. However, in some circumstances, NF-κB can modify chromatin organisation through the recruitment of chromatin modifying co-activator complexes (Bhatt and Ghosh 2014). Such remodelling is suggested to explain the transcriptional activation of some genes immediately following stimulation of NF-κB signalling, and others at later time points (Saccani, Pantano, and Natoli 2001).

Activation of NF-κB signalling in the literature is most frequently measured in terms of nuclear accumulation, increased binding activity, or degradation of NF-κBs cytoplasmic anchor IκB protein. In such studies, NF-κB has been found to rapidly translocate into the nucleus in response to cyclic stretching of pulmonary cell lines (Ning and Wang 2007b; Li et al. 2003; Kumar, Knox, and Boriek 2003; Pugin et al. 1998). NF-κB activation has also been frequently detected in *in vivo* models of injurious mechanical ventilation (Liu et al. 2009; Ko et al. 2013; Dolinay et al. 2004). Several studies have found that pharmacological attenuation of VILI is often associated with a dampening of NF-κB signalling (Held et al. 2001; Lopez-Alonso et al. 2013; Xia et al. 2015; Li et al. 2014). Furthermore, a couple of studies have reported that direct inhibition of NF-κB activity is protective in animal models of VILI. Hayes et al. reported that overexpression of IκBα, thus inhibiting NF-κB activity, was protective in a two-hit model of infection and VILI (Hayes et al. 2014). Anti-NF-κB antibody has also been shown to be protective in an isolated perfused lung model of VILI (Chiang, Pai, and Liu 2008).

1.5 Aims & Hypotheses

In this project, the first aim was to develop a phospho-flow cytometry protocol that enabled the quantification of cellular activation within the lung during the initiation of VILI. Most of the current literature has focused on the downstream effects of injurious mechanical ventilation, but this project aimed to investigate the initiation of inflammatory signalling within the lung prior to the infiltration of

extra-pulmonary immune cells. In order to examine the kinetics of cellular activation, it was essential to be able to quickly process the lungs in order to measure the phosphorylation levels of intracellular activation markers within specific cell populations following exposure to short periods of injurious mechanical ventilation. An inherited phospho-flow cytometry protocol was adapted to investigate the activation of several pulmonary cell types in addition to alveolar macrophages and AECs (Waite 2011).

This technique was used to test the hypothesis that AECs become activated prior to the alveolar macrophages in response to over-distension of the lung. Although this is a widely held assumption, given that the AECs would be directly exposed to over-stretching whilst the alveolar macrophages would not, it has never actually been proven. We hypothesised that the mechanism by which alveolar macrophages become activated so rapidly following stretch must occur either via the release of soluble mediators by the AECs, or through direct contact between the epithelium and alveolar macrophages. The list of potential soluble mediators is limited by the rapid release that would be required to activate alveolar macrophages within short periods of exposure to over-stretching. On the other hand, direct interactions may be a more feasible means of communication under such short time scales as suggested by studies that demonstrate increased adherence of alveolar macrophages within minutes of exposure to mechanical ventilation *in vivo*. The aim of this project was to use phospho-flow cytometry to investigate these potential mechanisms in the presence of specific inhibitors *in vivo*.

CHAPTER TWO

METHODS & MATERIALS

2.0 Animals

The protocols used in this project were approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act, 1986. Experiments have been reported in line with the Animals in Research: Reporting *in vivo* experiments (ARRIVE) guidelines (Kilkenny et al. 2010). Male C57BL/6 mice were sourced from Charles River Laboratories (Margate, UK) whilst TNF receptor knockout mice were sourced from The Jackson Laboratory (Maine, USA). Genetically modified mice were bred on site and their phenotypes were confirmed by flow cytometric analysis of their TNF receptor expression. Mice were housed in groups of up to 5 in individually ventilated cages (IVCs) with free access to food and water. Male mice used in experiments were aged 8-12 weeks and weighed 20-30g.

2.1 *In vivo* model of ventilator-induced lung injury (VILI)

In vitro studies are very valuable in answering specific mechanistic questions; however the use of animal models in studying VILI is crucial to understanding the multicellular responses to extreme alveolar strain. The multi-dimensional forces experienced during ventilation are difficult to replicate *in vitro* due to the highly intricate and interdependent structure of the lung. In addition, the most clinically important consequence of VILI is the inflammatory reaction in which alveolar macrophages play a vital role, as well as monocytes and neutrophils that are recruited from the systemic circulation. The complexity of the lungs structure, combined with intercellular communication via local and systemic inflammatory propagators, mean that the study of VILI is most meaningful and physiologically relevant in an *in vivo* setting.

Relevance of the model

The *in vivo* model of VILI used within this project is well established in our group (Wilson et al. 2009; Wilson, Patel, and Takata 2012) and models using similar ventilation protocols are also commonly used within the field (Uhlig et al. 2002; Vaporidi et al. 2012; Tremblay et al. 2002). It is necessary to use a higher tidal volume was than would be used in human patients because the murine lung is inherently more compliant than human lungs and therefore require higher tidal volumes to induce over-distension (Wilson, Patel, and Takata 2012; Soutiere and Mitzner 2004). We also are able to observe advanced VILI within 2 to 3 hours using a higher tidal volume, a timescale which is more applicable to murine experiments than the development of VILI over several days in the clinical setting. This single-hit approach of injury induced purely by mechanical ventilation may be considered less clinically relevant to the patient with ARDS compared with two-hit models, but it has enabled the investigation of the specific mechanisms of VILI in the absence of confounding factors.

Some clinical data suggest that patients without pre-existing lung injury, such as those undergoing surgery, or critically ill patients without ARDS, may be at risk of VILI (Serpa Neto et al. 2012; Wolthuis et al. 2008). However, our model better replicates the injury induced by the mechanical ventilation of patients suffering from ARDS, who are the most susceptible to VILI. The lungs of these patients are dominated by large regions of oedema that reduce the volume of lung that can be ventilated. Consequently, the set tidal volume which is calculated based on height and weight is applied to this smaller region of aeratable lung, known as the 'baby lung' which results in extreme deformation even with moderate or low tidal volumes (Gattinoni and Pesenti 2005).

Ventilation protocols

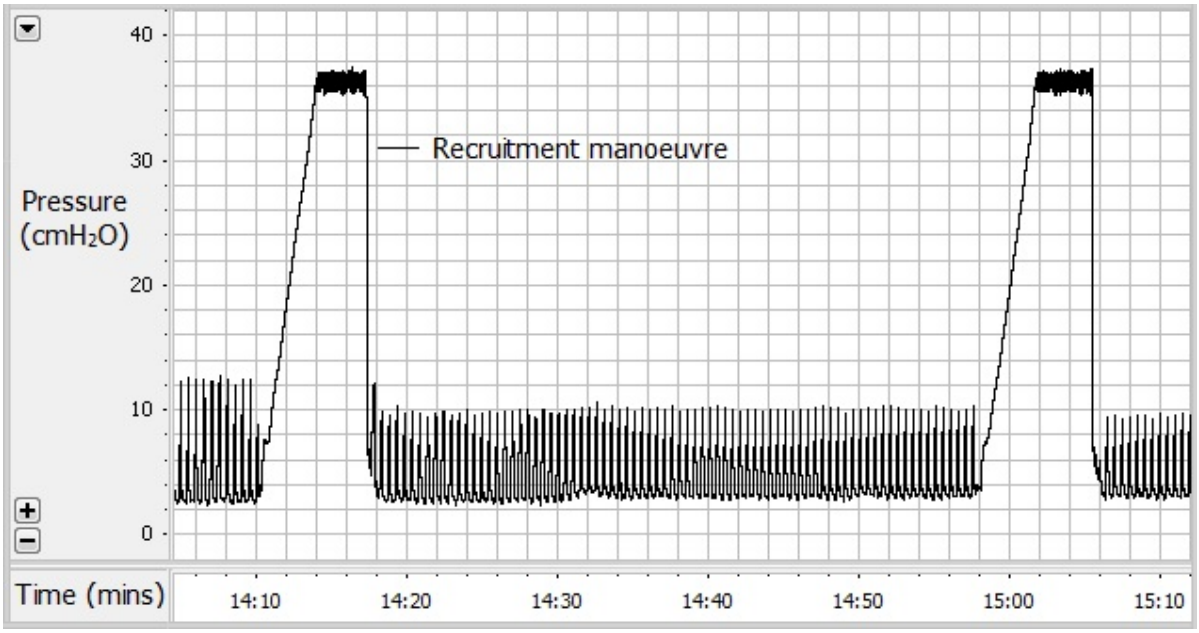
General anaesthesia was induced by an intraperitoneal injection of 80mg/kg ketamine (Ketaset, Pfizer, Kent, UK) and 8mg/kg xylazine (Xylacare, Animalcare Ltd, UK). Once the mice had lost consciousness, usually within 2-3 minutes, they were transferred from their IVC onto a heat pad with a blanket to maintain a constant body temperature for the duration of the experiment. Surgery began once the mice no longer demonstrated pedal and righting reflexes (usually about 10 minutes after administration of anaesthetic). A surgical incision was then made to access the trachea, into which an endotracheal tube of a standard size and length was inserted and tied into place before connection to the ventilator apparatus. A mark on the endotracheal tube ensured that it was not inserted too far into the trachea so as to only ventilate one side of the lung.

The custom-made ventilator used electronic solenoid-controlled valves to control the gas flow rates, the respiratory rate, and to maintain an inspiratory: expiratory ratio of 1:2. These and other

ventilation parameters were continuously monitored and recorded by the data acquisition system PowerLab (ADInstruments Ltd, Oxford, UK).

In all ventilation protocols, mice were initially ventilated with low stretch settings, defined by a tidal volume (V_T) of 8ml/kg, a respiratory rate of 120 breaths per minute (bpm) and a positive end-expiratory pressure (PEEP) of 3cmH_2O, using 100% oxygen. Two recruitment manoeuvres, in which a sustained pressure of 35cmH_2O was held for 5 seconds within the lung, separated by a 30-second break, were performed to re-open alveoli that had become derecruited under anaesthesia, thereby standardising volume history (Figure 2.1). These recruitments were followed by an inspiratory pause which was used to calculate the tidal volume (Figure 2.2). At this point, some mice remained on the low stretch settings for control experiments, whilst others were switched to the high stretch settings. For those mice transferred onto the high stretch strategy, the ventilation gas was switched to oxygen with 4% CO_2, the respiratory rate was gradually decreased to 80bpm, and the flow rate steadily increased to reach a tidal volume of 40ml/kg, calculated from another inspiratory pause (see Table 2.3 for a comparison of the two ventilation strategies).

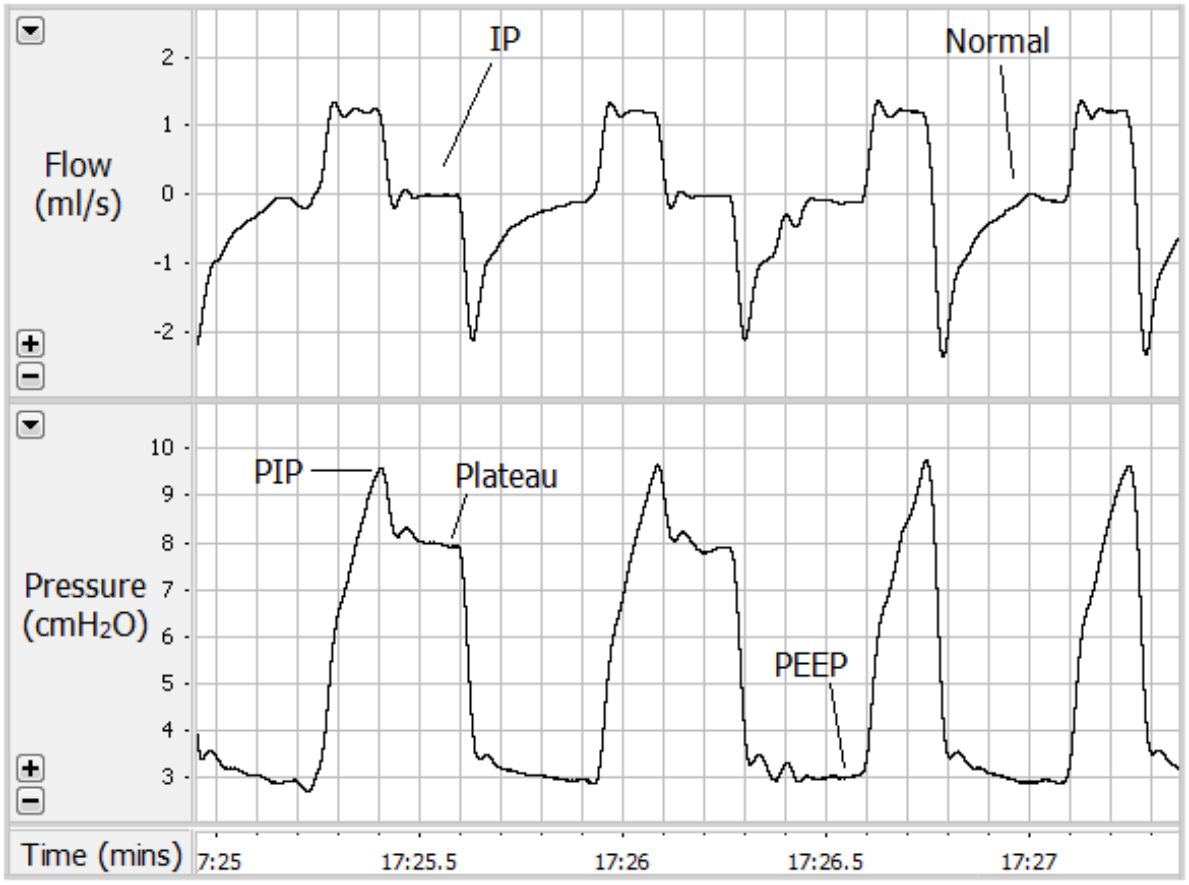
Figure 2.1 Recruitment manoeuvres



Recruitment manoeuvres involve applying a sustained pressure of 35cmH_2O for 5 seconds and this was repeated twice with a 30-second break between holds. The aim was to recruit alveoli that may have collapsed during anaesthesia induction and surgery time (around 15 minutes) and may also be performed every 30 minutes in longer protocols under low stretch settings to prevent atelectasis. Avoidance of atelectasis was important in our model of VILI as we aimed to specifically study the

effects of stretch-induced injury. The result is a decrease in pressure which can be seen particularly clearly following the first recruitment manoeuvre in the figure where the peak inspiratory pressure falls from 12cmH_2O to around 10cmH_2O.

Figure 2.2 Positive pressure ventilation



The two traces represent the airflow and pressure during an inspiratory pause (IP) (first two breaths) and during normal breathing (final two breaths). During an IP the inspired gas is held within the lungs for a short period during which the pressure plateaus before being released. This enables the calculation of tidal volume, compliance and resistance. The peak inspiratory pressure (PIP) is the highest pressure applied to the lungs during inspiration, and in longer ventilation experiments can indicate the extent of pulmonary oedema. A PEEP is applied to ensure that the pressure does not fall below 3.0cmH_2O, thereby preventing complete alveoli collapse.

The following equations illustrate how ventilation parameters including tidal volume, resistance, compliance and elastance are calculated.

$$\text{Tidal volume (ml/kg)} = \frac{\text{Volume (ml)}}{\text{Body weight (kg)}}$$

$$\text{Resistance (Rrs) (cmH}_2\text{O/ml/s)} = \frac{\left(\text{Peak (cmH}_2\text{O)} - \text{Plateau (cmH}_2\text{O)}\right)}{\text{Flow rate (Qaw ml/s)}}$$

$$\text{Compliance (Crs) (ml/kg/H}_2\text{O)} = \frac{\text{Tidal Volume (ml/kg)}}{\left(\text{Plateau (cmH}_2\text{O)} - \text{PEEP (cmH}_2\text{O)}\right)}$$

$$\text{Elastance (Ers) (cmH}_2\text{O/ml/kg)} = \frac{\left(\text{Plateau (cmH}_2\text{O)} - \text{PEEP (cmH}_2\text{O)}\right)}{\text{Tidal Volume (ml/kg)}}$$

Table 2.3 Low and high stretch ventilation strategies

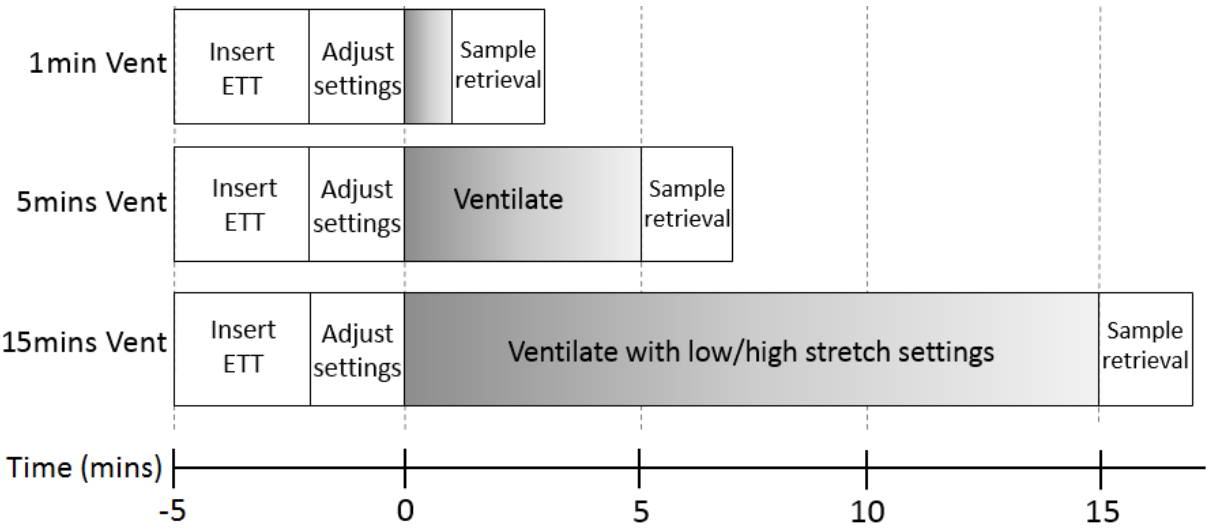
Ventilation Settings	Low Stretch	High Stretch
Tidal volume (V_T)	8 ml/kg	40 ml/kg
Breaths per minute (bpm)	120	80
Positive end-expiratory pressure (PEEP)	3.0 cmH_2O	3.0 cmH_2O
Ventilation gas	100% O_2	O_2 with 4% CO_2

The two ventilation strategies used in these experiments are referred to as 'low stretch' and 'high stretch'. The increased tidal volume used during the high stretch ventilation settings is known to cause hypocapnia and therefore we ventilated with oxygen supplemented with 4% CO_2. The high stretch protocol used a reduced respiratory rate of 80bpm to avoid incomplete expiration before the next breath is inhaled, which can lead to a progressive increase in alveolar pressure at the end of expiration, referred to as intrinsic PEEP.

Ventilation experiment timescales

The experiments in this project were short timescale experiments which aimed to investigate the initiation of VILI. Such experiments lasted 1, 5 or 15 minutes following calculation of the tidal volume. After the timed period, the mouse was exsanguinated and the lungs retrieved and processed as described in detail later in Section 2.4. The timings of the short ventilation experiments are illustrated in Figure 2.4.

Figure 2.4 Timings of ventilation experiments



After the tracheostomy, which takes approximately 3 minutes ('insert ETT'); mice were connected to the ventilator under low stretch settings. Recruitment manoeuvres were performed followed by an inspiratory pause, and tidal volume set to low or high ('adjust settings'). At the end of the ventilation exposure (grey), the experiment was terminated and the lungs were removed and homogenised, yielding a fixed single cell suspension within 2 minutes of terminating the experiment ('sample retrieval').

2.2 *In vivo* model of LPS-induced lung injury

Relevance of the model

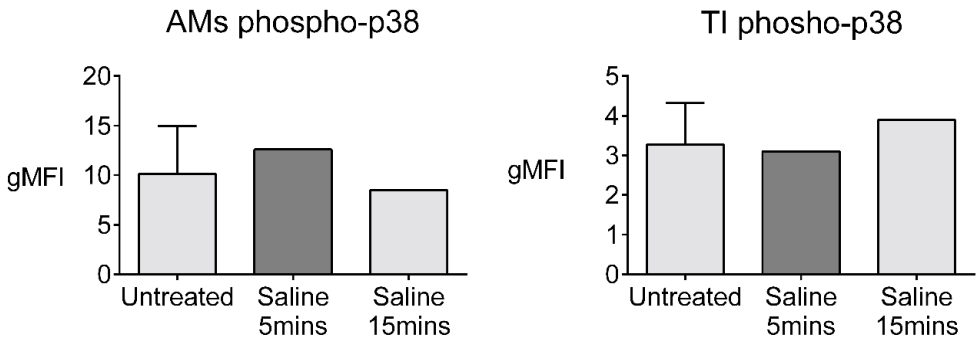
LPS-induced lung injury is used to model intra- or extra-pulmonary bacterial infection, the most common cause of ARDS. The delivery of lipopolysaccharide (LPS) intratracheally is typically used to model bacterial infection with a pulmonary source, such as pneumonia, whilst LPS administered intravenously models bacterial infection with a non-pulmonary source. Both of these models of LPS-induced lung injury are well established within our group (Dorr et al. 2011; O'Dea et al. 2011; O'Dea et al. 2009). The LPS endotoxin is a component of the cell wall of gram-negative bacteria that stimulates strong immune responses via the TLR-4 signalling pathway (Poltorak et al. 1998; Jeyaseelan et al. 2005). The intratracheal LPS model was initially used in this project as a positive control to validate the complex protocol for measuring intracellular markers of cell activation. However, the model also provided an interesting comparison of lung cellular responses to a bacterial and mechanical insult.

Intratracheal LPS instillation

General anaesthesia was induced by an intraperitoneal injection of ketamine (60mg/kg) and xylazine (6mg/kg). Once the mouse had lost consciousness and no longer demonstrated pedal and righting reflexes, it was then secured to a board and held for a short period of time almost vertical during the instillation process. A microscope and an external light source were used to visualise the airways. The tongue was held out of the way and blunt forceps were used to gently open the epiglottis for a clearer view of the vocal chords. A fine catheter (0.28mm ID x 0.61mm OD) was briefly passed through the vocal chords and a mark on the catheter at 1cm from the tip ensured that it did not go beyond the carina where the trachea branches into two bronchi. A syringe was connected to the other end of the catheter which contained the 50µl fluid bolus that was instilled into the lungs followed by a small volume of air (300µl) to ensure delivery of the entire dose.

Mice were given 20µg 'Ultrapure' LPS (E. coli O111:B4; InVivoGen, San Diego, CA) diluted in a final volume of 50µl saline. As the LPS model was initially used as a positive control, it was not important to determine whether the instillation procedure itself was inducing inflammatory signalling activation. Saline instillations performed for 5 and 15 minutes did not demonstrate increased activation of any marker or in any cell type compared with untreated controls (Figure 2.5). In the interest of reducing numbers, a set of untreated mice subjected only to the anaesthesia and tracheostomy served as controls to both the 5- and 15-minute LPS exposures. The 1-minute LPS experiments have their own set of untreated controls as these experiments were conducted at a later stage. A 50µl bolus is a commonly used bolus size for instillation into the lungs and should be well received by a 20-30g mouse whose total lung volume is approximately 1000-1200µl (Soutiere and Mitzner 2004). Following instillation, breathing became rapid and deep indicative of successful dosing into the lungs.

Figure 2.5 Effect of saline instillation versus untreated on cellular activation

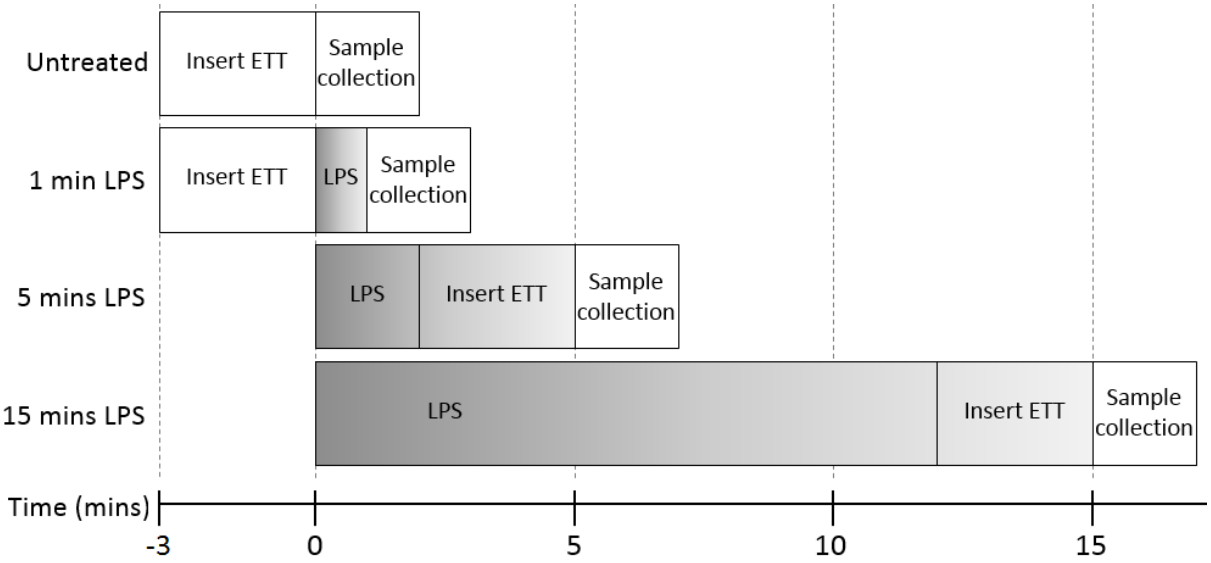


Intratracheal instillation of saline did not induce activation of p38 in alveolar macrophages (AMs) or type I AECs (TI) compared with untreated controls. These results are representative of the other activation markers and cell types, none of which responded to instillations of saline. N=3 for untreated, N=1 for saline 5mins, N=1 for saline 15mins.

After instillation of the bolus, the catheter was promptly removed and the mouse was held upright on the board for a further 30 seconds before being laid down on a heat pad and under a blanket for the remaining exposure time (up to 15 minutes) whilst breathing spontaneously. For LPS exposure times of 30 and 60 minutes, mice were transferred to an 'intensive care box' which was warmed with a heat pad and contained 40% supplementary oxygen. Mice did not regain consciousness but were given a top-up of anaesthesia prior to insertion of an endotracheal tube.

For LPS experiments, which lasted for either 5, 15, 30 or 60 minutes, an endotracheal tube was inserted via tracheostomy a few minutes before termination of the experiment, to facilitate immediate instillation of inhibitors. To ensure accurate timing in LPS experiments lasting only 1 minute, the endotracheal tube was inserted first, before administering the LPS bolus by passing the fine catheter through the endotracheal tube (see Figure 2.6 for an illustration of the timings of these protocols).

Figure 2.6 Timings of LPS experiments



Control mice were subjected to the same anaesthesia and surgery protocols as LPS-treated mice, involving the insertion of an endotracheal tube followed by immediate termination of the experiment and retrieval of the lungs. For 1-minute LPS treatments, LPS was administered via the endotracheal tube to ensure accurate timings since the insertion of an endotracheal tube requires 3 minutes. The 5- and 15-minute LPS treatments involved administering the LPS intratracheally, and insertion of the endotracheal tube only during the last 3 minutes of the exposure period.

Intravenous LPS administration

In a set of experiments examining the effect of systemic administration of LPS, the jugular vein was cannulated to deliver a 20µg dose of LPS (the same dose as was given intratracheally) in a total volume of 120µl saline. A number of studies by our research group have previously administered this same dose systemically, and found that it caused neutrophil and monocyte sequestration into the lung within a couple of hours (Ghosh et al. 2005; O'Dea et al. 2005; O'Dea et al. 2009). Control mice were given 120µl saline via the same route. A 27G needle tip glued into place was used to cannulate the vein, and was connected to a syringe via a short piece of thin tubing (0.28mm ID x 0.61mm OD) filled with the dose to be delivered. Administration via the jugular vein ensured that the LPS would quickly reach the lungs. The jugular vein is also easily accessed during the surgery performed during the insertion of an endotracheal tube, making it an ideal route of administration when the pre-treatment time required is very short. In this set of experiments the LPS was administered just 5 minutes before terminating the experiment to assess cellular activation within the lung.

2.3 Pre-treatments to *in vivo* experiments

Some mice were pre-treated with drugs or appropriate control vehicle from 15 minutes to up to 24 hours prior to ventilation experiments. Some drugs were inhaled directly into the lungs via the intranasal route, whilst others were given systemically via intravenous or intraperitoneal injection.

Intranasal Route

With this route of administration, it is inevitable that the bolus will come into contact with the upper airways leading to some of it not reaching the lungs. Miller et al. investigated the effects of bolus volume size and type of anaesthesia on the efficiency of intranasal dosing in mice. They concluded that doses of 50µl or more were required for efficient delivery into the lower respiratory tract and that delivery was more efficient in mice anaesthetised with inhaled isoflurane compared with parenteral ketamine/xylazine. They suggested that the increased efficiency seen with isoflurane is associated with episodes of transient hypoxia associated with inhalational anaesthetics, which promotes an irregular but deep Cheyne-Stokes breathing pattern that draws larger volumes into the lungs compared with the shallow regular breathing observed under ketamine/xylazine anaesthesia (Miller et al. 2012).

Mice were put into an anaesthetic induction box filled with oxygen and 3.5% isoflurane (Abbott Animal Health, Illinois, USA), an inhalational anaesthetic used to induce short-lasting anaesthesia. After 3 minutes, mice were briefly removed from the box and a 25µl dose was placed in a continuous stream onto the nares and was instantly inhaled. Mice were returned to the induction box for a further 1 minute prior to administration of the second 25µl dose. Mice remained in the induction box for a further 30 seconds after receiving the full 50µl dose before being returned to their cage where they were regularly monitored until the ventilation experiment. Mice regained consciousness and returned to normal behaviour within a few minutes and displayed no signs of distress following this procedure.

In one set of experiments, 50µg (1mg/ml) Gap26 peptide (Alfa Aesar, UK) or saline control was given intranasally in a total volume of 50µl and mice were monitored for the following 90 minutes until surgery was performed. In another set of experiments, 50µg (1mg/ml) anti-CD18 antibody (GAME-46, BD Pharmingen) or its isotype control (Rat IgG1 κ, BD Pharmingen) were given intranasally in a total volume of 50µl, and mice were regularly monitored for the next 24 hours until surgery. Antibodies were administered to mice at their stock concentration of 1mg/ml, therefore purified antibodies were used that were diluted in 0.2µm sterile filtered aqueous buffer containing no preservatives or azide

and endotoxin levels of less than 0.001ng/µg of protein as determined using the LAL assay by the manufacturer.

Intravenous Route

Intravenous administration via the tail vein was performed using a 29G micro-fine insulin syringe (BD Biosciences, Oxford, UK). Mice were warmed in a mouse incubator for approximately 10 minutes prior to the injection to help dilate blood vessels. Mice were given 400µg of the p38 inhibitor SB 203580 hydrochloride (Tocris Bioscience, Bristol, UK) in a total volume of 140µl of water (approximately 16mg/kg). This solution had the same osmolarity as saline, which was given to control mice. Mice were returned to their cage and monitored for the duration of the pre-treatment time of 30 minutes.

Intraperitoneal Route

Another method of pre-treatment used in this project was the intraperitoneal route. This was used when the drug to be delivered was dissolvable only in dimethyl sulfoxide (DMSO), a solvent that is known to cause irritation. The intraperitoneal route therefore ensures that the drug is delivered in a slow and gradual manner. It also allows the drug to be diluted in a large volume of saline for administration.

The $P2X_7$ receptor antagonist A-839977 (Tocris Bioscience) was given to mice 30 minutes prior to ventilation experiments. A dose of 2mg was dissolved in 50µl of DMSO and diluted in a total volume of 500µl of saline (approximately 80mg/kg) to give a DMSO content of 10%. Control mice were given 50µl DMSO in a total volume of 500µl saline intraperitoneally.

2.4 Processing of *in vivo* samples

At the end of experiments, mice were exsanguinated under deep general anaesthesia by lacerating the carotid artery and the caudal vena cava prior to collecting samples.

Lung – protocol development

Several months were spent refining and validating a protocol for the processing of the lungs in order to yield a fixed and permeabilised single cell suspension for phospho-flow cytometry. The phosphorylation of proteins can be rapidly reversed by phosphatases and therefore it was vital to be able to fix the intracellular contents of the cells as soon as possible after terminating the experiment.

It was therefore critical to homogenise the lungs into a single cell suspension to allow the fixing and permeabilising agents to access each individual cell.

Using a protocol inherited from a previous student (Waite 2011), early experiments showed flow cytometry histograms that indicated that not all of the alveolar macrophages were reacting to the same degree following LPS stimulation. This was illustrated by the majority of cells showing increased phosphorylation of the activation markers, whilst a smaller population remained within the same activation level of the untreated controls (Figure 2.7). The percentages of these populations varied between experiments and sometimes appeared as very broad peaks suggesting high variability. One possible explanation for this was uneven distribution of the LPS dose within the lungs.

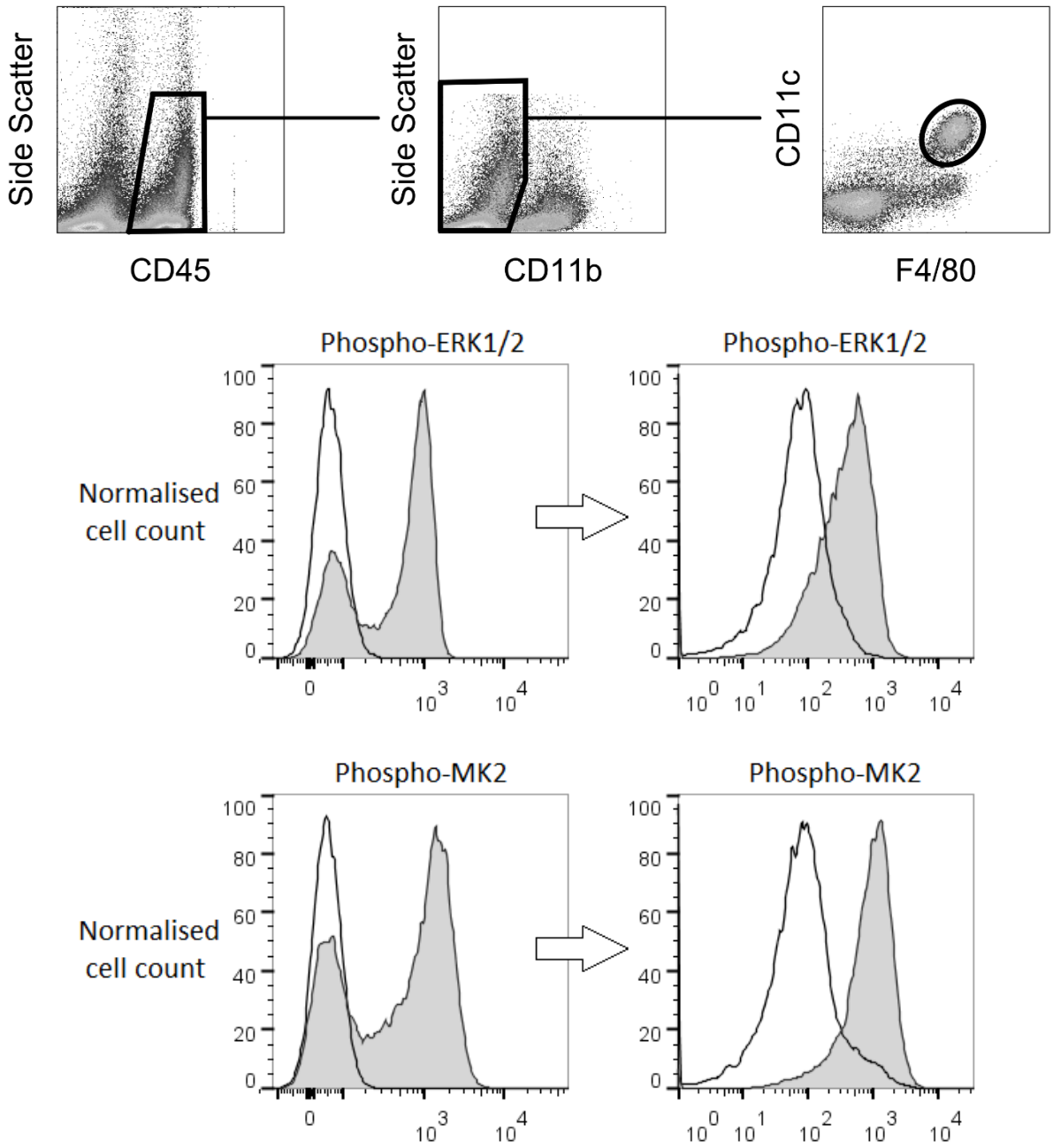
To try to resolve this potential issue, an endotracheal tube was inserted to visualise how far the catheter entered the lungs, ensuring that it was not going past the carina and delivering the LPS dose to only one side of the lungs. The individual lobes were also processed individually to exclude this possibility. A further tactic used to ensure even distribution was to ventilate the mouse following LPS instillation and to perform recruitment manoeuvres to prevent collapse of certain areas of the lung. The final approach was to study the effects of LPS instillation after the longer time of 15 minutes as opposed to 5 minutes, to see if the results were a phenomenon only seen at early time points. However, even after all of these interventions, a 'double population' was still observed in the histograms, suggesting that this phenomenon could be due to inefficient fixing.

The inherited protocol had instructed that following termination of the experiment, the lungs should be removed and added to IC fixation buffer (eBioscience), followed by mechanical homogenisation and incubation at 37^{o}C for ten minutes before washing. The first alteration to the protocol was to use a stronger fixation buffer (BD Cytofix/Cytoperm) which also permeabilised cells for access to the cytoplasm. The fixation buffer was also warmed to 37^{o}C before receiving the lungs to help fix cells in a physiological state. To eliminate the delay in getting the lungs into the fixative, it was instead instilled directly into the lungs via an endotracheal tube that was inserted during the final 3 minutes of the exposure period. However, this meant the fixative was introduced to the intact lung for an increased length of time prior to homogenisation, which hindered the homogenisation process presumably by cross-linking cells together.

As an alternative, a phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany) was instilled into the lungs that specifically preserved the phosphorylation status of proteins without delay and did not affect the generation of a single cell suspension. It was probably a combination of all of these

changes to the protocol and my improving technique and speed that led to the elimination of the 'double population' (Figure 2.7).

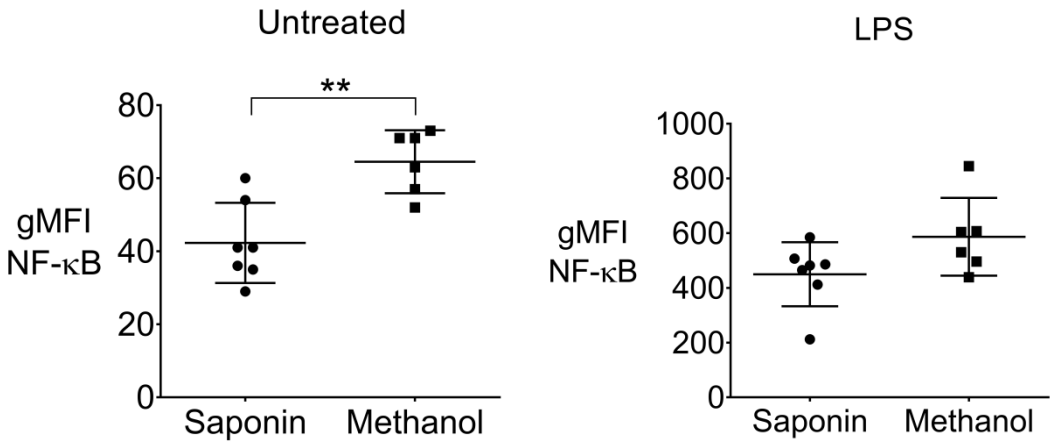
Figure 2.7 Improving the efficiency of the fixation protocol



Flow cytometric histograms showing levels of phosphorylated ERK1/2 and MK2 in alveolar macrophages following intratracheal instillation of 50µg LPS (grey) versus untreated (white). The original protocol revealed two populations of alveolar macrophages that responded differently to LPS; however, refinement of the protocol indicated that this was due to an inefficient fixing process.

The final hurdle in the development of this protocol was the level of permeabilisation required to measure the intracellular activation markers. The cells were maintained in a PBS-based buffer containing 2mg/ml saponin (perm-wash buffer) known to be capable of permeabilising cell membranes. It is commonly reported in the literature that methanol, which is a much stronger permeabilising agent, is needed to permeabilise the nuclear membrane in order to measure NF-κB (Krutzik and Nolan 2003). However, methanol is not compatible with multi-parameter flow cytometry as it disrupts protein fluorophores including PE and its derivatives PE-CF594, PerCP and PE-Cy7 (Krutzik, Clutter, and Nolan 2005). Methanol permeabilisation therefore could not be used if discrete cell populations were to be identified, as without PE-based fluorophores, there would only be two fluorescent channels remaining, excluding one for the measurement of phospho-epitopes. We therefore compared the effects of permeabilising with saponin and methanol on the measurement of phosphorylated NF-κB in alveolar macrophages, which can be easily identified even with few channels, and found a small but significant increase in phosphorylated NF-κB with methanol in untreated samples (Figure 2.8). However, the measurement of NF-κB activity with saponin was still very good (10-fold increase with LPS compared with untreated), and importantly, enabled us to measure NF-κB in several specific cell types.

Figure 2.8 Saponin versus methanol permeabilisation for NF-κB staining

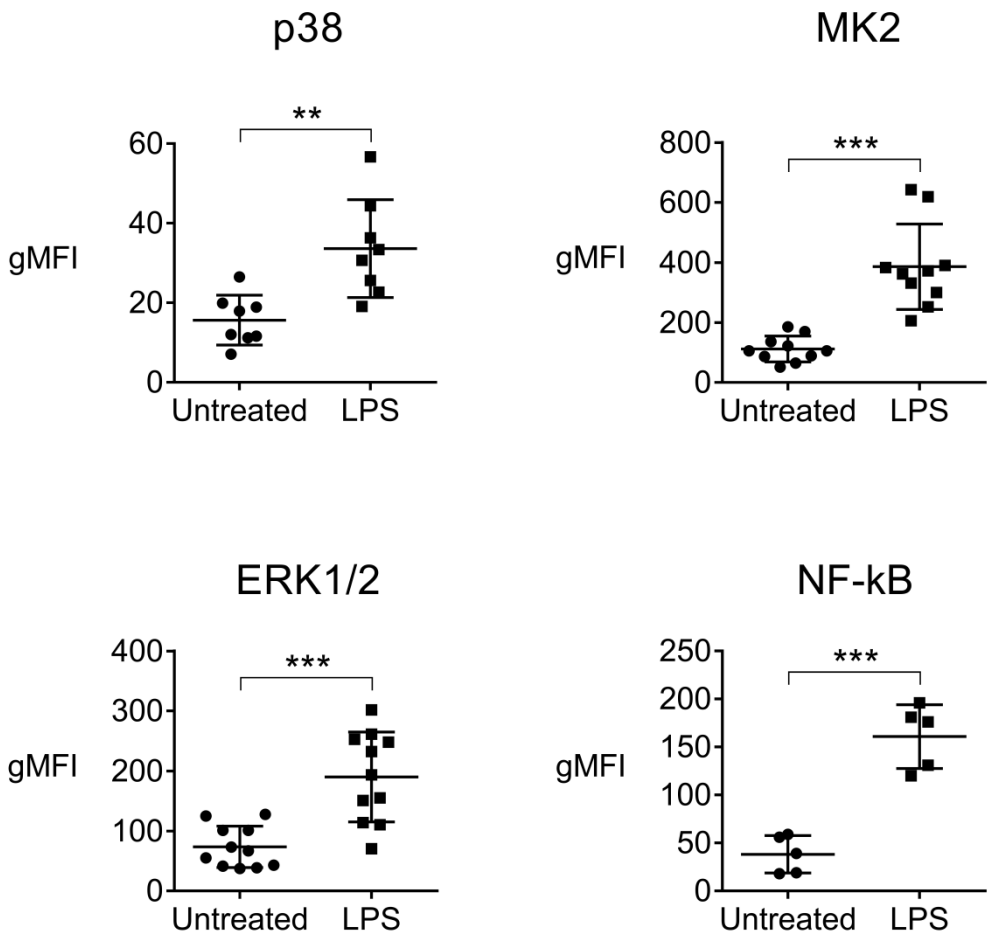


*There was a small but significant change in the gMFI of NF-κB with the different permeabilising agents. In the 5 minute 20µg LPS treated lung, alveolar macrophages displayed a non-significant trend (p=0.083) for a difference between the two permeabilising agents. N=6-7, *p<0.05, **p<0.01. Data were analysed by t-test and are displayed as mean ± SD.*

Using the refined protocol, we were able to study the cellular activation within alveolar macrophages following 5 minutes exposure to 50µg LPS. These preliminary experiments used a very high dose of LPS (50µg) to ensure that the phospho-flow cytometry system was capable of detecting an increase in

gMFI as was expected with LPS stimulation. Indeed, significant activation was detected within alveolar macrophages 5 minutes after instillation of the 50µg LPS dose (Figure 2.9). Each of the four activation markers p38, MK2, ERK1/2 and NF-κB demonstrated significantly increased phosphorylation.

Figure 2.9 Alveolar macrophage activation following intratracheal instillation of 50ug LPS for 5 minutes



*Within alveolar macrophages, each of the four intracellular markers of activation demonstrated significantly increased phosphorylation (geometric mean fluorescence intensity (gMFI)) following 5 minutes exposure to 50µg intratracheal LPS. N=5-11, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

Lung – optimised protocol

Following termination of experiments, 500µl of a cell-permeant phosphatase inhibitor cocktail set V (Calbiochem, Darmstadt, Germany) was instilled directly into the lungs via the endotracheal tube. This cocktail targets a broad-spectrum of phosphatase enzymes and was used to preserve the

phosphorylation status of the intracellular activation markers under investigation. The lobes of the lung were then rapidly removed and mechanically disrupted in 2ml of warm (37^{o}C) fixation/permeabilisation buffer (Cytofix/Cytoperm, BD Biosciences) using a GentleMACS dissociator (Miltenyi Biotec, Surrey, UK). This process yielded a fixed single cell suspension of the lung in less than two minutes from the time of terminating the experiment.

The lung cell suspension was then incubated in the fixative at 37°C for 10 minutes. The active ingredient in the fixation buffer was formaldehyde (4.2%) which forms cross-links within and between proteins, thereby creating a chain of immobile proteins within the cell that prevents further signalling. Warming the fixative to 37^{o}C increases the speed of fixation and promotes fixation of the sample in a physiologically relevant conformation. Following 10 minutes of incubation in the fixative, the fixing process was stopped by the addition of ice-cold perm-wash buffer which contained the permeabilising agent saponin (phosphate-buffered saline, 2mg/ml saponin, 25mg/ml bovine serum albumin and 1mg/ml sodium azide). Saponin forms complexes with cholesterol in cell membrane bilayers to form large pores that permit antibodies access to intracellular markers. The sample was filtered through a 40µm cell strainer and washed twice by centrifugation (450g for 5mins at 4^{o}C) to remove remaining fixative. After sample processing, the cell suspension was prepared for analysis by flow cytometry.

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) was performed by instilling 750µl PBS into the lungs via the endotracheal tube, then withdrawing that fluid to analyse the contents of the alveolar space. Contaminating surfactant was removed from the top of the BAL fluid sample prior to centrifuging at 200g for 5 minutes at 4^{o}C to pellet cells, which were then used for *in vitro* studies (described in detail in Section 2.5). Alternatively, the cell-free supernatant was tested for ATP content (described in Section 2.6).

Blood

Blood samples were retrieved via cannulation of the caudal vena cava and withdrawal of blood into a syringe containing the anti-coagulant heparin. The blood sample was stained for analysis of TNF receptor expression of immune cells in knockout mice by flow cytometry. Erythrocytes were lysed prior to running the samples through the flow cytometer using Lysing Solution (BD Biosciences).

2.5 *In vitro* experiments

Cell cultures

All cell cultures were grown in 150cm^2 culture flasks with vented caps (BD Biosciences) in incubators maintained at 37^{o}C and 5% CO_2. Media was changed every 2 to 3 days and cells were split when confluent using trypsin-EDTA (Life Technologies, Paisley, UK) for epithelial and endothelial cell lines, and cell dissociation solution (Sigma-Aldrich, MO, USA) for monocyte cell lines.

Stretch experiments were conducted using the human lung epithelial A549 carcinoma cell line (ATCC® CCL-185™, VA, USA). This cell type was chosen for their regular growth pattern which was expected to enable them to form a uniform layer that would enable them to respond uniformly to stretch. They were also used to confirm that findings in mouse cells were relevant to human cells. A549 cells were grown in RPMI-1640 medium containing buffering agent HEPES (Sigma-Aldrich) and supplemented with 6mg/ml newborn calf serum (Sigma-Aldrich) and 0.5mg/ml penicillin-streptomycin-glutamine (PSG) (Life Technologies).

The murine lung epithelial MLE 12 SV40 transformed cell line (ATCC® CRL-2110™) was also used in *in vitro* stretch experiments as being more relevant to the *in vivo* studies in this project. MLE 12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 3mg/ml new-born calf serum and 0.5mg/ml PSG.

As no murine pulmonary endothelial cell line was available, the murine brain endothelial bEnd5 cell line (ATCC®) was used to represent the endothelial barrier. bEnd5 cells were grown in RPMI-1640 medium supplemented with 6mg/ml protein new born calf serum, 0.5mg/ml PSG, 1mM sodium pyruvate (Sigma-Aldrich), 1% 100X MEM non-essential amino acid solution (Sigma-Aldrich) and 2.25mg/ml D-(+)-glucose solution (Sigma-Aldrich).

Similarly, as no alveolar macrophage cell line exists, the murine monocyte/macrophage RAW 264.7 tumour cell line (ATCC® TIB-71™) was used to gain insight into macrophage responses. RAW cells were grown in DMEM supplemented with 3mg/ml new-born calf serum and 0.5mg/ml PSG.

Primary cells

Alveolar macrophages were isolated from mice and studied under *in vitro* conditions and in the absence of other cell types. Mice were euthanised by overdose with 100mg pentobarbital (Merial Animal Health Ltd, Essex, UK) given IP followed by exsanguination. Then a BAL was performed using

warm calcium-free PBS with 5mM EDTA (Life Technologies). Calcium is suspected to enhance alveolar macrophage adherence to the alveolar walls and as a result it is common practise to eliminate calcium from the BAL medium when harvesting primary alveolar macrophages. A total of 750µl of this solution was instilled into the lungs and 500µl was withdrawn and re-instilled twice before finally withdrawing as much as possible. This process was repeated with fresh calcium-free PBS solution. Surfactant was removed from the top of the sample before it was centrifuged at 200g for 5 minutes to pellet the alveolar macrophages. The pellet was re-suspended in calcium-containing PBS with added calcium chloride (5mM). This process was repeated three times in total to remove any traces of EDTA. The alveolar macrophages were split into two wells of a 24-well plate and left to settle for 45 minutes before experiments were conducted.

ATP stimulation

ATP stimulation experiments were conducted with the RAW cell line to assess cellular activation by phospho-flow cytometry. RAW cells were counted using a haemocytometer and seeded at an approximate density of 4×10^5 per well into 12 well plates (BD Biosciences). Cells were stimulated with 100µM ATP disodium salt (Tocris Bioscience) for time lengths of 5, 15 and 20 minutes. At the end of the stimulation period, the ATP solution was removed from wells and replaced with warm fixation buffer (BD Cytofix/Cytoperm), in which the cells were incubated for 10 minutes at 37^{o}C. The cells were then recovered from the wells using a CytoOne Cell Scraper (Starlab, Milton Keynes, UK) and were centrifuged twice at 450g for 5 minutes at 4^{o}C before being re-suspended in perm-wash buffer for analysis by flow cytometry.

Cell stretching *in vitro*

The cell lines A549, MLE 12 and bEnd5 cells were counted using a haemocytometer and seeded at an approximate density of 5×10^5 per well into type I collagen coated BioFlex® 6-well culture plates (Flexcell International, NC, USA) around 36 hours prior to stretching. Normal cell culture media (described above) was replaced with serum-starved media containing 0.6-1.2mg/ml new-born calf serum, 12 hours prior to stretching. The serum-starved media was used to slow cell proliferation and maintain a confluence of around 90% for the experiment. Immediately prior to stretching, media was gently replaced with 1ml of fresh serum-starved media. The stretched plate was fitted into a rubber gasket and then inserted into the baseplate, situated within an incubator. A Plexiglas sheet was placed on top of the culture plates to ensure that the setup was airtight. Non-stretched control cells were also grown onto the BioFlex plates and were placed into the same incubator for the same 15-minute exposure time, which was maintained at 37^{o}C and 5% CO_2. The bottoms of the wells in the

BioFlex plates were made of a flexible silicone elastomer membrane that was deformed using a Flexercell FX-4000™ Tension System (Flexcell International). This system uses a vacuum to pull the flexible membranes down reaching a maximum strain of 13% at 20 cycles per minute.

Cell stretching - Sample collection

Following on from the *in vitro* ATP stimulation experiments, a more effective technique was developed to harvest the cells from the culture plate wells, and this was utilised in cell stretch experiments. Plates were immediately put onto ice following stretching or control treatment. The 1ml of media was aspirated and divided into two Eppendorf tubes kept on ice; one of which contained an ecto-ATPase inhibitor ARL 67156 trisodium salt (250µM) to preserve the ATP content. Media samples were centrifuged at 200g for 5 minutes to remove any contaminating cells and the supernatant was then frozen at -80^{o}C in aliquots for later use in assays. After collection of media, 1ml of ice-cold phosphatase inhibitor cocktail (Calbiochem), diluted in PBS without Ca^{2+}, was added to each well. The immediate cooling of cells was designed to help lift them from the surface of the well, whilst the phosphatase inhibitor cocktail preserved the phosphorylation levels of intracellular activation markers. The cells were then gently scraped from the bottom of the well and added to ice-cold fixation-permeabilisation buffer (Cytofix/Cytoperm, BD Biosciences). Following a 10-minute incubation in the fixative, the cells were washed twice by centrifugation at 200g for 5 minutes and re-suspended in perm-wash buffer for analysis by flow cytometry.

2.6 ATP assay

The ATP content of samples was measured using an ATPlite Luminescence ATP Detection Assay System (Perkin Elmer, USA), sensitive down to 5 cells in 100µl medium. The light produced by the ATP-dependent conversion of D-luciferin to oxyluciferin by luciferase is proportional to the ATP concentration within the sample. To measure the ATP content in BAL fluid, BALs were performed with 550µl PBS (without Ca^{2+}) containing 250µM ARL 67156 trisodium salt (Tocris), an ecto-ATPase inhibitor. BAL fluid samples were centrifuged at 200g for 5 minutes at 4^{o}C to remove contaminating cells and then kept on ice for up to one hour before they were analysed. ATP content was also measured in the media from cells stretched *in vitro*, which was harvested immediately following stretching. ARL, an ecto-ATPase inhibitor, was added to these samples at 250µM, samples were centrifuged to remove cells and kept on ice for up to two hours before being stored at -80^{o}C until analysed.

Solutions of known ATP concentrations made up in the appropriate diluents were used to create a standard curve. These standard solutions plus the samples were added to a white 96 well microplate (Greiner Bio-One GmbH, Austria) in duplicate. ATP stabilisation solution (ATPlite) was added to each well and the plate was shaken on a plate shaker for 5 minutes at 250rpm whilst covered with foil to protect from light. Next, substrate solution (ATPlite) was added to each well before the plate was shaken again. The plate was then dark adapted for 10 minutes prior to analysing with a microplate fluorescence/luminescence reader FL_X800 (BioTek, Swindon, UK). All samples were measured at the same sensitivity setting of 170 which was found to give the best signal: noise ratio. Luciferase emits light across a broad spectrum of visible wavelengths and therefore detection was not performed at a specific wavelength.

2.7 ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed on supernatant samples to measure their TNFα content using a Mouse TNF-alpha DuoSet ELISA (R&D Systems, USA) (sensitivity range 31.20 to 2000pg/ml) and IL-6 content using a Mouse IL-6 DuoSet ELISA (R&D Systems) (sensitivity range 15.60 to 1000pg/ml). As per the manufacturer's instructions, a 96-well microplate (NUNC-Immuno Maxisorp, Thermo Fisher Scientific, USA) was coated with capture antibody against the target molecule TNFα by incubating overnight at room temperature, covered with a plate sealer. The next morning, the capture antibody (DuoSet) was aspirated and wells were washed using an auto-washer three times with wash buffer (0.05% TWEEN 20 in PBS). The remaining protein binding sites in the wells were then blocked by incubating for 1 hour with reagent diluent (1% BSA in PBS). The plate was washed as before, and then samples and standard solutions were added to wells in duplicate. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature. The plate was washed as described previously, and then biotinylated detection antibody (DuoSet) against TNFα was added to each well and incubated at room temperature for 2 hours. The plate was washed once again before streptavidin-HRP (DuoSet) was added to each well and incubated in the dark for 20 minutes at room temperature. The streptavidin protein binds to biotin on the detection antibody. After washing, substrate solution (3, 3', 5, 5' tetramethylbenzidine (TMB), Sigma-Aldrich) was added to the wells and incubated for another 20 minutes at room temperature in the dark. The substrate solution provides the colour change that represents the concentration of the target molecule. The HRP (horseradish-peroxidase) conjugated to streptavidin cleaves the hydrogen peroxide from the substrate, a reaction which is coupled to the oxidation of TMB which acts as a hydrogen donor, and changes colour during the reaction. Stop solution (2M sulphuric acid 2N H_2SO_4) (Sigma-Aldrich) was added to each well and the plate was gently tapped

to ensure even mixing. The sulphuric acid stops the reaction and converts the pale blue colour into yellow, which is then measured. The optical density of each well was measured by a microplate reader (Dynex MRX II) set at 450nm.

2.8 Flow cytometry

Flow cytometry enables the identification of individual cell populations from a mixed cell suspension of whole tissue based on cell size, granularity and characteristic surface markers. Our group has extensive experience in the detection of lung parenchymal cells (Patel et al. 2013; Bertok et al. 2011), and leukocytes (Bertok et al. 2012; O'Dea et al. 2011) via flow cytometry. This technique is particularly useful in studying small sub-populations of cells. Fluorophore-conjugated antibodies are used to bind to specific surface antigens permitting their detection on a single cell when passed through a laser beam. The detection of a combination of surface antigens enables the identification of a specific cell type. The CyAn ADP Analyser flow cytometer (Beckman Coulter, High Wycombe, UK) uses two lasers and filters to measure fluorescence in 7 different wavelength ranges (Table 2.10).

Table 2.10 CyAn ADP flow cytometer channels and fluorophores

Channel	Fluorophores	Excitation Wavelength (nm)	Emission Wavelength (nm)
Blue laser (488nm)			
FL 1	FITC/AF-488	494/495	520/519
FL 2	PE	496	578
FL 3	PE-CF594	496	612
FL 4	PerCP	482	678
FL 5	PE/Cy7	496	785
Red laser (642nm)			
FL 8	AF-647/APC	650/650	668/660
FL 9	APC-eFlour-780	650	780

The CyAn ADP flow cytometer used in this project had two lasers, blue and red, which along with filters, provided seven channels. This means that up to seven different fluorophores may be used in combination. Listed in this table are the most common fluorophores that were used throughout this project, and their respective maximum excitation and emission wavelengths.

Each whole lung sample collected was divided into several samples to study a range of cell types and a number of activation markers. A 50µl aliquot of the sample was added to 50µl of the common antibody cocktails used to identify ranges of cell populations. A final additional antibody was added to each staining mixture which specifically targeted the phosphorylated activation markers. The

samples were incubated at room temperature for 30 minutes in the dark before unbound antibody was washed away by centrifugation (450g for 5mins at 4°C). The samples were then re-suspended in 300µl perm-wash buffer and run in turn on the flow cytometer.

Separate antibody cocktails were used to identify parenchymal cell populations (Table 2.11) and leukocyte populations (Tables 2.12 and 2.13). The parenchymal antibody cocktail was used to identify parenchymal cells of the lung including type I and II AECs (Figure 2.14), and endothelial cells (Figure 2.16). The leukocyte antibody cocktail I was used to identify leukocytes localised within the lung including alveolar macrophages (Figure 2.18), neutrophils and Ly6C low and high monocytes (Figure 2.19). The leukocyte antibody cocktail II was used to identify further leukocytes including dendritic cells (DCs) and to further differentiate Ly6C low monocytes into vascular and interstitial sub-types (Figure 2.20).

Table 2.11 Parenchymal antibody cocktail

Antigen	Clone	Fluorophore	Expression	Conc. (ng/ml)	Source
CD31 (PECAM-1)	MEC13.3	FITC	Endothelium	1.25	BioLegend
EpCAM (CD326)	G8.8	PE	Epithelium	0.5	eBioscience
CD45	30-F11	PerCP	Leukocytes	0.5	BioLegend
T1-alpha (Podoplanin)	8.1.1	PE/Cy7	Type I AECs	1.25	BioLegend

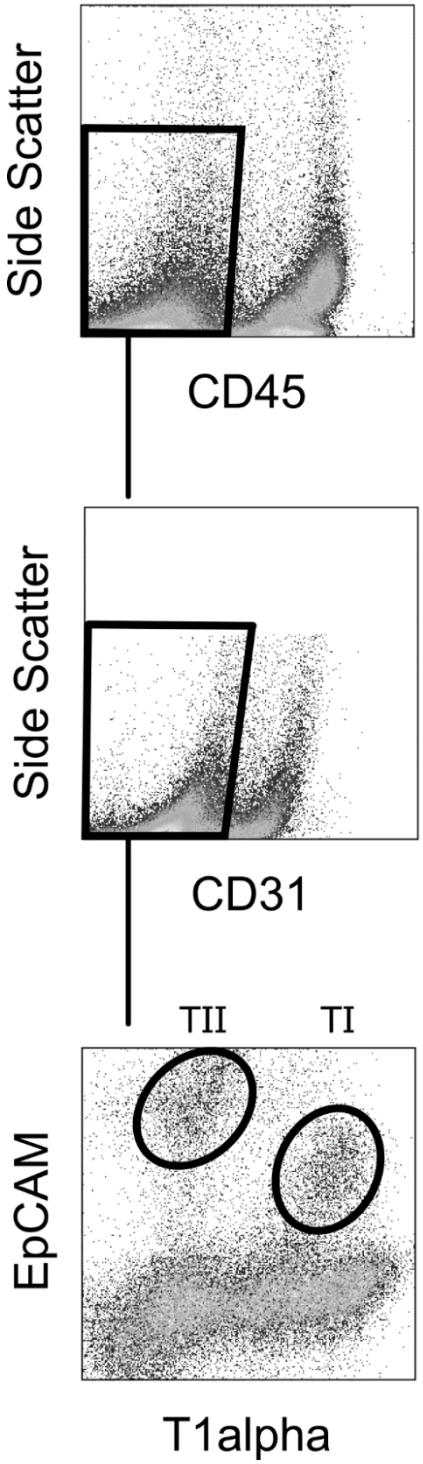
Table 2.12 Leukocyte antibody cocktail I

Antigen	Clone	Fluorophore	Expression	Conc. (ng/ml)	Source
F4/80	BM8	FITC	Macrophages ++, monocytes +	1.25	eBioscience
Ly6G	1A8	PE	Neutrophils	0.5	BD Pharmingen
CD45	30-F11	PerCP	Leukocytes	0.5	BioLegend
CD11b	M1/70	PE-CF594	Neutrophils, monocytes	0.5	BD Horizon
Ly6C	HK1.4	PE/Cy7	Ly6C high monocytes	0.5	BioLegend
CD11c	N418	APC-eFluor® 780	Alveolar macrophages	0.2	eBioscience

Table 2.13 Leukocyte antibody cocktail II

Antigen	Clone	Fluorophore	Expression	Conc. (ng/ml)	Source
Ly6C	HK1.4	AF-488	Ly6C high monocytes	1.25	BioLegend
Ly6G	1A8	PE	Neutrophils	0.5	BD Pharmingen
MHCII	M5/114.15.2	PerCP	Dendritic cells	0.5	BioLegend
CD11b	M1/70	PE-CF594	Neutrophils, monocytes	0.5	BD Horizon
CD45	30-F11	PE/Cy7	Leukocytes	0.5	BioLegend
CD11c	N418	APC-eFluor® 780	Alveolar Macrophages	0.2	eBioscience

Figure 2.14 Identification of alveolar epithelial cells from a whole lung sample by flow cytometry



Type I alveolar epithelial cells (AECs) (TI) were identified as CD45 negative (a leukocyte marker), CD31 negative (an endothelial cell marker), and positive for both EpCAM and T1-alpha. Type II AECs (TII) were identified as CD45 negative, CD31 negative, positive for EpCAM but negative for T1-alpha.

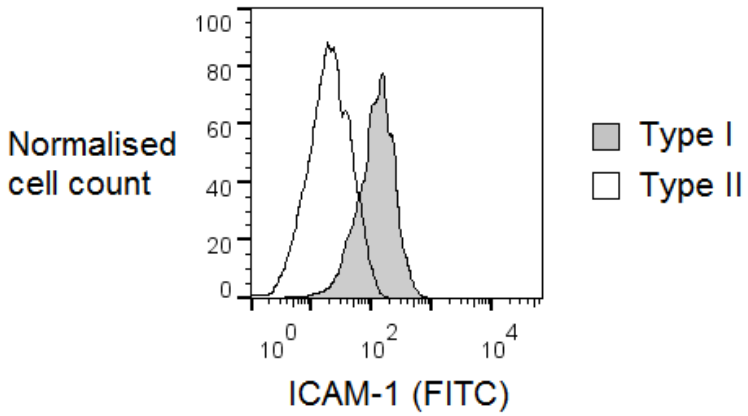
AECs were identified by excluding the two other main cell populations in the lung sample, which are CD45 positive leukocytes and CD31 positive endothelial cells. The two types of AECs can then be separated based on their cell surface expression of epithelial cell adhesion molecule (EpCAM or CD326) and T1-alpha (podoplanin). Type I AECs, the squamous shaped cells which form the majority of the gas-exchange surface area of the alveoli, were identified as positive for both EpCAM and T1-alpha. Type II AECs, the cuboidal shaped cells which synthesise and secrete surfactant into the alveolar space, were identified as positive for EpCAM and negative for T1-alpha.

T1-alpha is used universally as a marker of differentiation of type II AECs into type I AECs, a process which occurs within days when type II AECs are cultured *in vitro* (Borok et al. 1998; Gutierrez, Gonzalez, and Dobbs 1998). T1-alpha is a transmembrane protein expressed on the apical surface of type I AECs, however its role remains unknown despite its abundance within the lung (Williams et al. 1996) and its vital role in alveolar development at birth (Ramirez et al. 2003) . EpCAM on the other hand has been better characterised due to it being implicated in the development of carcinomas stemming from its connections to cell signalling, migration, proliferation and differentiation (Trzpis et al. 2007; Maetzel et al. 2009). EpCAM is a homophilic intercellular adhesion molecule that is widely expressed across all types of epithelial cell (Litvinov et al. 1994; Kasper et al. 1995).

A study by Fujino et al. which focused specifically on the identification of AEC types from a whole lung by flow cytometry used the same two main markers, EpCAM and T1-alpha (Fujino et al. 2012). Having separated the two types of AEC, Fujino et al. then confirmed further phenotypic characteristics, finding that the EpCAM(+) T1-alpha(+) type I population expressed the water channel aquaporin 5, another type I maker (Kreda et al. 2001). They also found evidence of bronchial epithelial cell markers within their type I AEC population; however these cells are not likely to be present in our population as the upper airways were avoided when sampling the lobes of the lungs. Fujino et al. also confirmed that the EpCAM(+) T1-alpha(-) type II population contained pro-surfactant protein-C (pro-SP-C), a marker of type II AECs since they exclusively synthesise and secrete surfactant.

To further validate the phenotypes of these AEC populations, the expression of intercellular adhesion molecule-1 was measured (ICAM-1) (CD54-FITC; 3E2; BD Biosciences, San Jose, CA). The type I AECs were found to express ICAM-1 whilst the type II AECs were found not to which is supported by the literature (Wang and Hubmayr 2011; Christensen et al. 1993) (Figure 2.15).

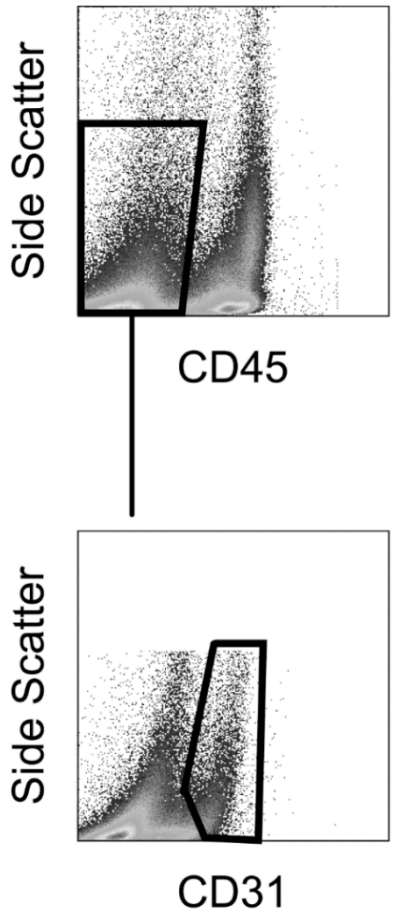
Figure 2.15 Comparison of ICAM-1 expression on type I and type II AECs



Expression of ICAM-1 was found to be higher in type I AECs (grey) compared with type II AECs (white).

Despite their abundance within the lung as the main components of alveoli, the numbers of alveolar epithelial cells collected by flow cytometry was proportionally low compared with the numbers of leukocytes collected. This is most likely due to a number of physical limitations including strong adhesion between cells, their single cell thick organisation, and the squamous shape of the type I AECs in particular. These factors probably lead to damage of parenchymal cells during the fixation and homogenisation process, which might explain the 'noise' at the bottom of the final trace in Figure 2.14. This large EpCAM negative population probably arise from broken cell material and cells which have lost their cell surface markers. Nevertheless we were able to collect enough cells to be able to analyse them reliably.

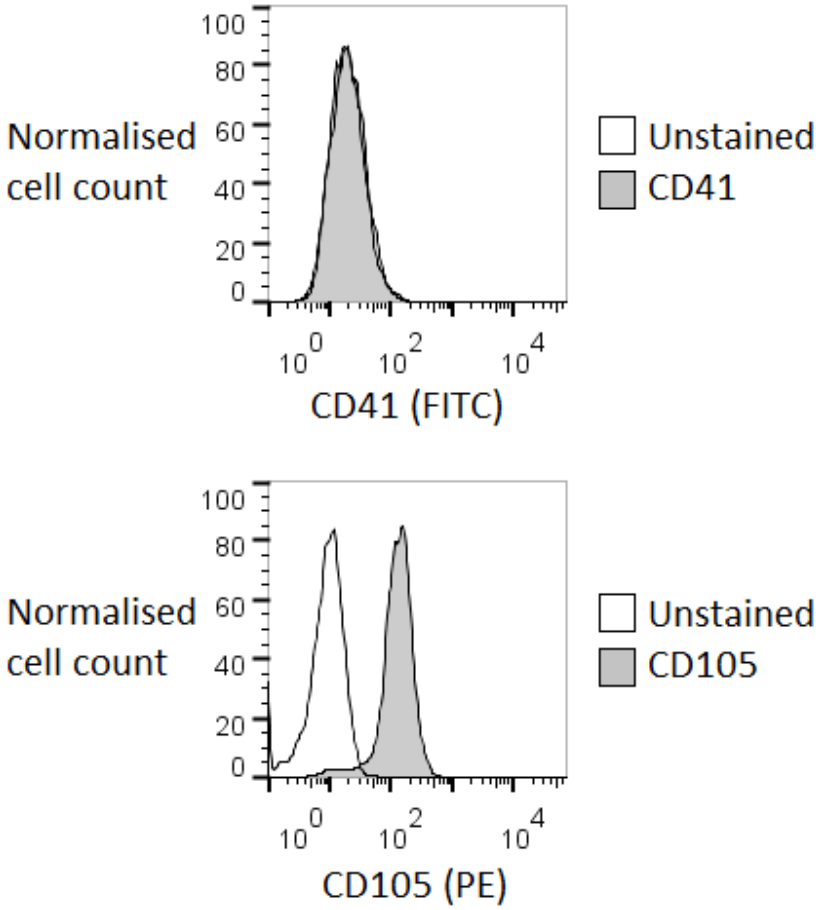
Figure 2.16 Identification of endothelial cells from a whole lung sample by flow cytometry



Pulmonary endothelial cells were identified as CD45 negative and CD31 positive.

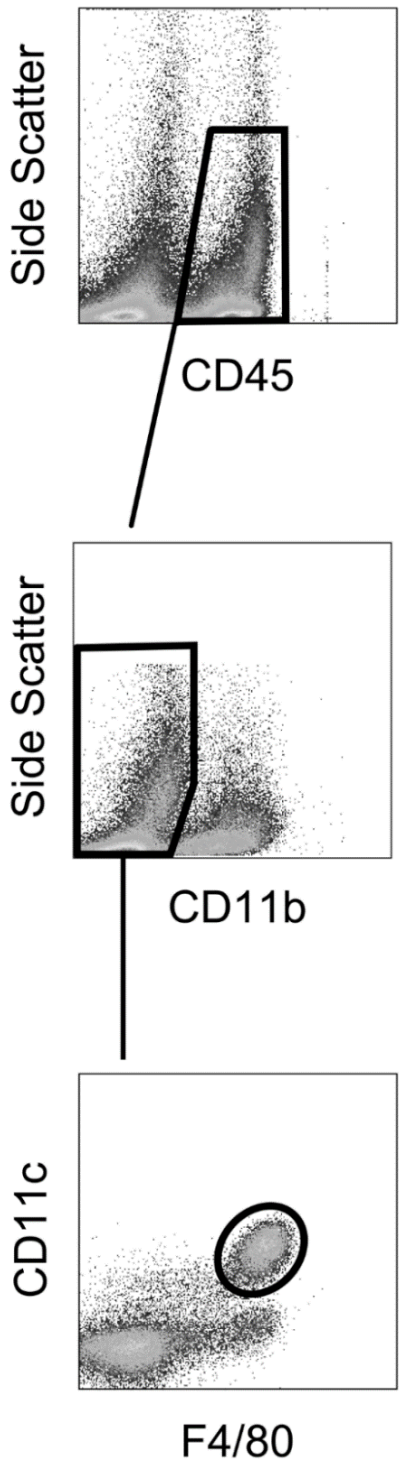
Endothelial cells share few similarities to other pulmonary cell types and can therefore be identified by flow cytometry with relatively few markers. Endothelial cells were separated by first excluding leukocytes by gating on CD45 negative cells, and second by gating on CD31 positive cells (Figure 2.16) (Bertok et al. 2011). CD31 is a transmembrane receptor involved in leukocyte migration and trafficking (Ma et al. 2012). As well as endothelial cells, platelets also express some CD31, as suggested by its alternative name platelet endothelial cell adhesion molecule-1 (PECAM-1). To check that this population did not include platelets we measured the expression of CD41 (CD41-FITC; MWReg,30; Serotec, Oxford, UK). This confirmed that the endothelial population did not express the transmembrane glycoprotein CD41, which is expressed on platelets and is involved in their adhesion and aggregation (Figure 2.17). Further validation of the endothelial population was conducted by checking for expression of CD105, which (CD105-PE; MJ7/18; eBioscience, San Diego, CA). CD105 (endoglin) is expressed on vascular endothelial cells as part of the TGF-β receptor complex and plays a role in adhesion and angiogenesis (Warrington et al. 2005). CD105 was found to be expressed on the endothelial population as was expected (Figure 2.17).

Figure 2.17 CD41 and CD105 expression on endothelial cells



The CD45(-), CD31(+) population was found to express CD105 but not CD41 as was expected for endothelial cells.

Figure 2.18 Identification of alveolar macrophages from a whole lung sample by flow cytometry

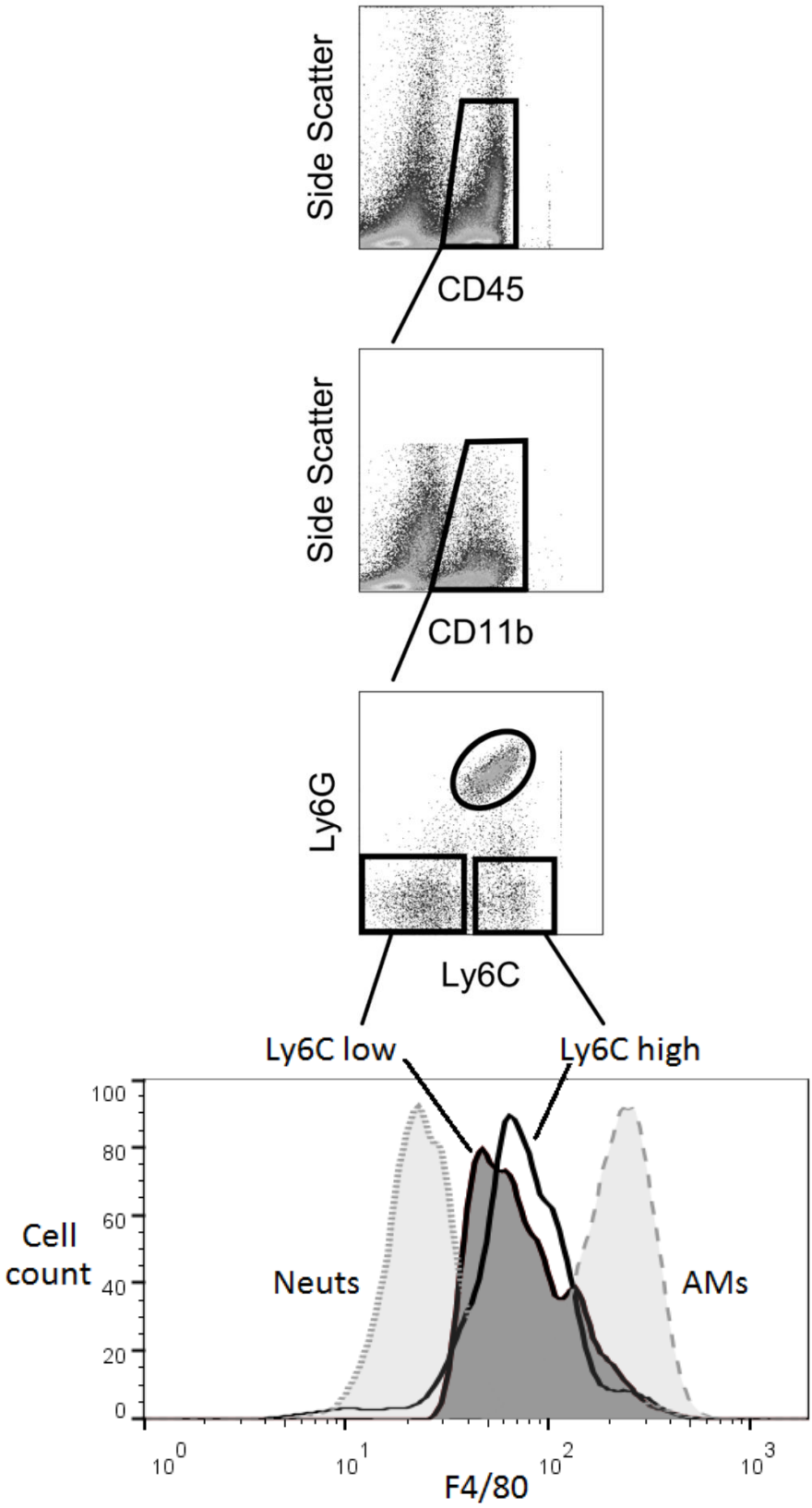


Alveolar macrophages were identified by first selecting for CD45 positive cells, then selecting for CD11b negative cells. Finally alveolar macrophages were identified as positive for both F4/80 and CD11c.

The phenotype of alveolar macrophages has been well characterised, and they are highly auto-fluorescent, making their identification by flow cytometry relatively straightforward (Misharin et al. 2013). Throughout this project their identification has mostly been achieved by detecting positive CD45 staining, negative CD11b staining, and finally high expression of both CD11c and F4/80 (Figure 2.18). CD45 is a protein tyrosine phosphatase that regulates the immune response through affecting leukocyte adhesion, recruitment and cytokine signalling (Saunders and Johnson 2010; Germena et al. 2015). It is a pan-leukocyte marker that is expressed on all hematopoietic cells except erythrocytes and platelets. The second gate used to identify alveolar macrophages separates leukocytes based on their CD11b expression. CD11b is a receptor for intercellular adhesion molecules including ICAM-1 and is expressed on monocytes, macrophages, granulocytes, activated lymphocytes and some natural killer cells. Alveolar macrophages however, differ from macrophages found in all other tissues in that they express very low levels of CD11b under resting conditions (Albert et al. 1992; Dorger et al. 2001; Viksman et al. 1994).

The final gate used to identify alveolar macrophages selected for cells that were positive for CD11c and F4/80. CD11c is another receptor for adhesion molecules whose expression also differs greatly between alveolar macrophages and all other macrophages. Alveolar macrophages show very high expression levels of expression of CD11c, whilst macrophages in other tissues show very low levels of CD11c expression (Albert et al. 1992; Guth et al. 2009). F4/80, an adhesion-G-protein-coupled receptor (GPCR) involved in the differentiation of T regulatory cells (Lin et al. 2005), was used to identify alveolar macrophages as a broad macrophage marker that is constitutively expressed on most tissue resident macrophages (Gordon et al. 2011). Together these four cell surface markers allow for the reliable identification of alveolar macrophages from a whole lung sample.

Figure 2.19 Identification of neutrophils and monocytes within a whole lung sample

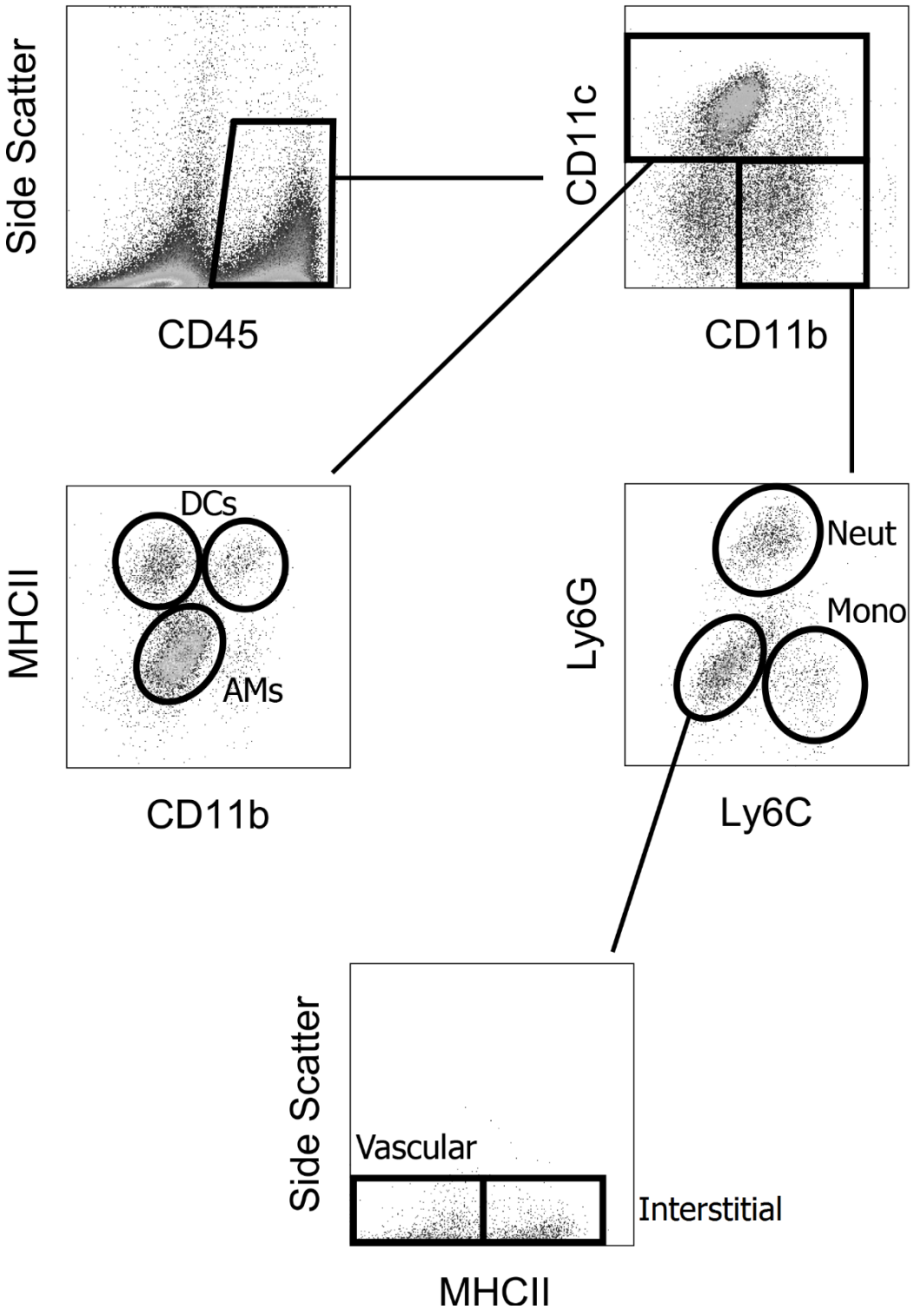


Neutrophils and monocytes were initially gated on their high levels of CD45 and CD11b. This population was then separated based on Ly6G and Ly6C staining from which neutrophils were

identified as positive for both Ly6G and Ly6C. Monocytes lack expression of Ly6G but can be divided into 'Ly6C low' and 'Ly6C high' monocytes, and further validated by checking for low F4/80 expression. The histogram compares low expression of F4/80 on monocytes, high expression of F4/80 on alveolar macrophages and a complete lack of expression on neutrophils.

The flow cytometric analysis of neutrophils and monocyte subsets within the lung has been well documented in the literature (Rose, Misharin, and Perlman 2012; Misharin et al. 2013; Barletta et al. 2012; O'Dea et al. 2009; O'Dea et al. 2011; Zaynagetdinov et al. 2013). The function of many of the Ly6 group of membrane-bound proteins remains largely unclear, however their lineage-specific expression has made them useful markers of lymphocyte subsets (Lee et al. 2013). Ly6G is specifically expressed by neutrophils, mediating their migration into inflamed sites (Wang, Bair, et al. 2012). Neutrophils also express high levels of Ly6C, which is thought to play a role in CD8 T cell adhesion to endothelium and migration (Jaakkola et al. 2003). The expression of Ly6C on monocytes can be used to distinguish between their two subsets: 'Ly6C low' and 'Ly6C high'. The Ly6C low subset are recognised as the resident monocytes that are the precursors of tissue macrophages, whilst the Ly6C high subset are considered to be the inflammatory monocytes that are recruited to sites in response to infection (Geissmann, Jung, and Littman 2003; O'Dea et al. 2011).

Figure 2.20 Identification of neutrophils, dendritic cells and monocytes within a whole lung sample



This gating strategy was used to identify dendritic cells, and to differentiate between Ly6C low monocytes located within the interstitium and the vasculature.

A slightly more complicated gating strategy was used for some experiments to further delineate monocyte and dendritic cell populations (Figure 2.20). Ly6C low monocytes can be further differentiated based on their expression of major histocompatibility complex class II (MHCII) (Patel et al. 2015). Ly6C low MHCII(-) cells are found located in the vasculature. Whilst the Ly6C low MHCII(+) cells represent those that have infiltrated into tissue and are often referred to as interstitial macrophages within the lung (Misharin et al. 2013).

Dendritic cells (DCs) were identified as positive for CD45, CD11c and MHCII. They were then identified as either CD11b positive or negative, the latter of which are commonly referred to as CD103 positive DCs though this marker was not used here (Misharin et al. 2013; Kopf, Schneider, and Nobs 2015). DCs are potent antigen presenting cells, hence their high expression of MHCII. This allows them to be separated from alveolar macrophages which express much lower levels of this complex (Zaynagetdinov et al. 2013). CD11b(-) DCs make up the majority of CD11c(+) DCs within the interstitial space between the alveoli and capillaries, where they tightly adhere through CD103 to the basolateral side of bronchial epithelial cells, with extensions reaching into the airway lumen. CD11b(+) DCs predominantly reside in the peribronchial and perivascular spaces where they interact with infiltrating leukocytes (Sung et al. 2006).

2.9 Phospho-flow cytometry

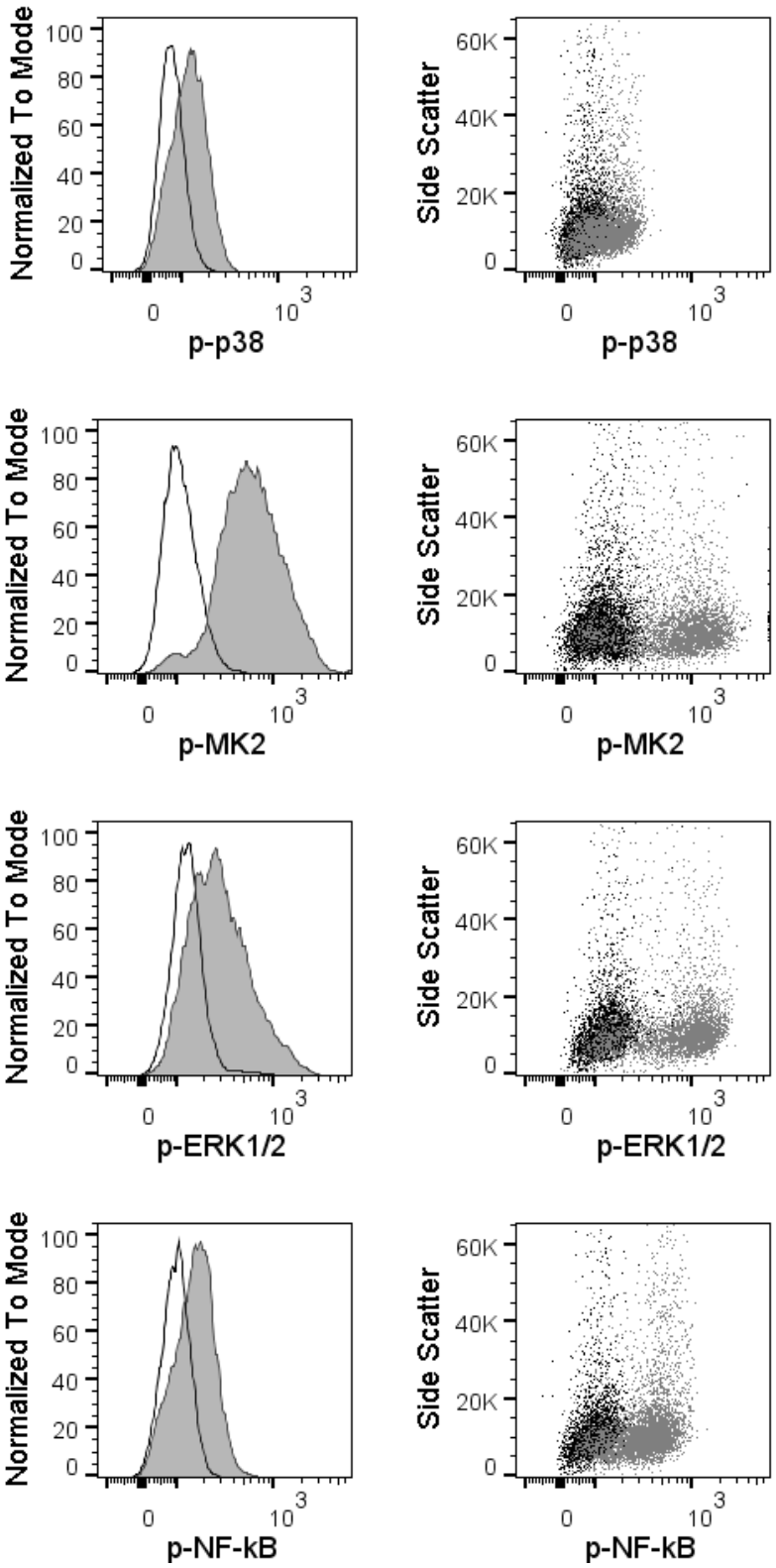
Phospho-flow cytometry involves the detection of phosphorylated residues on intracellular proteins, particularly kinases that are involved in cell signalling. The method has been well described in the literature (Perez and Nolan 2002; Perez, Krutzik, and Nolan 2004; Krutzik, Hale, and Nolan 2005; Oberprieler and Tasken 2011) and has been previously utilised within our group (Scott et al. 2011; O'Dea et al. 2011). The phosphorylation of specific amino acid residues is a post-translational modification that involves the covalent addition of a phosphoryl group onto specific serine, threonine and tyrosine residues. In most cases, the phospho-epitope induces an active state which prompts a cascade of downstream effects. Phosphorylation thereby provides a highly specific and reversible way of regulating complex cell signalling cascades that can lead to a myriad of cellular responses including changes in protein expression, cell proliferation, differentiation or apoptosis.

In order to measure such transient and reversible changes in activation state, a highly efficient method of fixation and permeabilisation is required to take a snapshot of the phosphorylation states of proteins, and allow access to their phospho-epitopes by antibodies. As described earlier, following exsanguination, lungs were filled with a cell permeant phosphatase inhibitor cocktail to prevent dephosphorylation of proteins. Whilst the use of phosphatase inhibitors may artificially increase

phosphorylation states, their use improves the signal to noise ratio, thereby enabling the detection of small differences in activation levels. The lungs were then quickly removed and homogenised in a fixing and permeabilising buffer, containing the cross-linking agent formaldehyde, to further preserve phosphorylation. Despite permeabilisation of the membrane to permit the entry of phospho-antibodies, protein leakage was not an issue as the fixation process conjugated the entire intracellular contents of the cell. This protocol enabled the quantification of phosphorylation levels of intracellular cell signalling proteins within a range of pulmonary cell populations following exposure to precise periods of *in vivo* stimulation.

The identification of individual cell populations within a heterogeneous sample and the localisation of activation to those populations is one of the main advantages phospho-flow cytometry has over Western blotting. The results from phospho-flow cytometry have been shown to correlate well with those obtained by Western blot (Krutzik and Nolan 2003), however, Western blotting can only localise activation to a specific cell type if it is cultured in isolation *in vitro*, or if it is purified from a primary sample, which is often a very expensive and time-consuming process. In addition, it may be impossible to analyse rare cell populations due to low cell counts, whereas flow cytometry can reliably analyse cell types that make up only small fractions of the total cell population. *In vivo* studies that utilise Western blots tend to describe global changes in phosphorylation within the whole lung. Immunohistochemistry on the other hand, can localise activation to specific cell types but only looks at a very limited number of cells and produces qualitative results. Another advantage of phospho-flow cytometry is its capability to examine each cell individually, thereby providing information regarding the percentage of cells responding and to what degree. Compare this with Western blots, which pool the protein from all cells in the sample, and therefore cannot distinguish between a small change occurring in every cell and a large change occurring in few cells (Krutzik et al. 2004). These kinds of characteristics can be visualised by overlaying flow cytometry histograms to compare two samples from a single experiment (Figure 2.21).

Figure 2.21 Representative histograms of alveolar macrophage responses to 15 minutes exposure to intratracheal LPS



Alveolar macrophages were identified from a whole lung suspension using CD45, CD11b, CD11c and F4/80 markers. Using FlowJo software, flow cytometry histograms can be overlaid for a visual

comparison of marker activation within these endothelial cells following 1 minute of low or high stretch ventilation. Corresponding dot plots show an almost complete shift in the entire population.

Flow cytometry data were analysed using FlowJo software V10 (TreeStar, Ashland, OR). The phosphorylation level of proteins within the cells analysed (1000-10,000) was quantified as the geometric mean fluorescence intensity (gMFI).

The flow cytometer automatically compensates signals by measuring background fluorescence (background light and electronic noise) when no cells are present and subtracting this value from the signal recorded for each cell. This and poor resolution of signals towards the low end of the fluorescent scale can lead to negative values when the fluorescent signal is absent or very dull. A compensation protocol, based on data from singly-stained microspheres, is also applied to the collected data that estimates the amount of spectral spill-over in each channel and subtracts this from the detected signals to yield the actual amount of each fluorescent dye (Tung et al. 2007).

Manually subtracting the gMFI of an unstained sample from each stained sample compensated for auto-fluorescence of cells, this technique is called 'fluorescence minus one' (FMO). The unstained sample contains all of the fluorophore-conjugated antibodies in the cocktail, excluding the one to be measured. The unstained sample is gated in the same way as the other samples to obtain the individual background fluorescence level for each cell type (Herzenberg et al. 2006).

This is considered the most effective way to normalise data as the use of isotype controls for intracellular markers assumes that the isotype has the same non-specific stickiness as the staining antibody, and can give an erroneous threshold for separating cells with low levels of fluorescence from unstained cells. The recommended method for intracellular staining is to always compare a stimulated sample to an unstimulated sample as a negative control.

These compensation techniques and controls must be used in every experiment as the fluorescence detection is very sensitive to external factors such as changes to the sheath fluid and laser alignment. For this reason, it is difficult to directly compare gMFI data that have been collected several months apart.

The phospho-specific antibodies used in this project targeted the MAP kinase p38 and its immediate downstream substrate MK2, the MAP kinase ERK1/2 and the transcription factor NF-κB (Table 2.22). Phospho-specificity of the antibodies used was validated by LPS stimulation experiments which provided a positive control. Further validation was also performed by assessing changes in total protein levels.

Table 2.22 Phospho-specific MAP kinase and NF-κB antibodies

Antigen	Clone	Phospho-residues	Fluorophore	Dilution
Phospho-p38	28B10	Thr-180/-182	AF647	1/100
Phospho-MK2	27B7	Thr-334	AF647	1/800
Phospho-ERK1/2	D13.14.4E, 197G2	Thr-202/Tyr-204	AF647	1/200
Phospho-NF-κB	93H1	Ser-536	AF647	1/50

All phospho-specific antibodies were conjugated to the Alexa Fluor 647 (AF647) fluorophore and purchased from Cell Signaling (Danvers, MA). The AF647 fluorophore in channel 8 is considered to be the optimal fluorophore for the detection of phospho-epitopes as it is, extremely bright, photo-stable, and small in size which enables easy access to intracellular proteins, and is not affected by auto-fluorescence, which is increased with the use of formaldehyde fixation. During this project, the phospho-ERK1/2 antibody was discontinued by Cell Signaling and replaced with an alternative clone (197G2) which recognised the same phosphorylated residues.

2.10 Statistical analysis

Comparisons between two datasets were performed using t-tests. Comparisons between three or more datasets were performed using ANOVA with Tukey HSD (comparing mean of each treatment group with the mean of every other treatment group) or Dunnet t test (comparing the mean of each treatment group with the mean of the control group). Statistical significance was defined as $p<0.05$. Data were analysed and graphed using IBM SPSS (V20) and Prism software (V6).

CHAPTER THREE

INITIATION OF INFLAMMATORY SIGNALLING IN LPS-INDUCED LUNG INJURY

3.0 Chapter summary

The LPS-induced lung injury model is commonly used to study ARDS with a bacterial aetiology. In the experiments presented in this chapter, intratracheal LPS was found to induce significant activation of both the NF-κB and MAP kinase signalling pathways in alveolar macrophages within just 5 minutes. The type I and II AECs displayed a distinct pattern of activation, demonstrating NF-κB activation after 5 minutes, and slower activation of the MAP kinases after 15 minutes. Finally, endothelial cells demonstrated activation only after 15 minutes of exposure to intra-alveolar LPS. The original aim of these LPS-induced lung injury experiments was to act as a positive control for the validation of the phospho-flow cytometry technique to be used throughout the project. However, the experiments additionally provided a novel insight into the way in which different pulmonary cell types respond to LPS, which served as an interesting comparison to the cellular activation induced by injurious mechanical ventilation.

3.1 Background

LPS-induced lung injury is the second most commonly used animal model of ARDS reported in the literature after the VILI model (Matute-Bello, Frevert, and Martin 2008). In such models, LPS may be administered directly into the lungs via intratracheal or intranasal routes to imitate pulmonary infection such as pneumonia, which is responsible for almost half of ARDS cases (Rubenfeld et al. 2005). Alternatively, LPS may be administered indirectly via intraperitoneal or intravenous routes to

imitate severe sepsis with a non-pulmonary source, which accounts for almost a third of ARDS cases (Rubenfeld et al. 2005).

Models of LPS-induced lung injury reproducibly replicate some of the main symptoms associated with ARDS in humans. In particular, LPS promotes considerable neutrophil infiltration into the lung in response to cytokines, where they release damaging molecules such as reactive oxygen species (ROS); however LPS is less effective at disrupting the alveolar-capillary barrier (Matute-Bello, Frevert, and Martin 2008; Faffe et al. 2000). It is difficult for an animal model to fully replicate ARDS in humans, which is complicated by the interplay of several risk factors and individual differences in immune responses. Nevertheless, animal models of ARDS are valuable to the investigation of specific concepts within complete biological systems (Chen, Bai, and Wang 2010).

The model of LPS-induced lung injury was used in this project as a positive control to develop a protocol for the quantification of phosphorylated cell signalling proteins using flow cytometry. LPS is known to induce a reproducible inflammatory response and therefore provided not only a robust system to validate our phospho-flow methodology, but also provided an interesting comparison to the mechanical insult of mechanical ventilation.

LPS (lipopolysaccharide) is a major component of the outer membrane of gram-negative bacteria, estimated to constitute up to 75% of the bacterial cell surface, with one bacterial cell harbouring approximately 3.5×10^6 LPS molecules (Rietschel et al. 1994; Gioannini et al. 2014). The structure of LPS consists of a hydrophobic lipid A component, which is the immunogenic portion of the LPS molecule, a conserved core region consisting of bacteria-specific carbohydrate residues, and finally the O-specific chain of repeating disaccharide residues (Rietschel et al. 1994).

The presence of bacterial toxin in the circulation of patients is thought to be associated with the development of sepsis in cases of both gram-negative and gram-positive infections (Cohen 2002; Ramachandran 2014). LPS is thought not only to be released as a result of cell lysis, but also in a highly controlled manner in the form of outer membranes vesicles, the exact function of which is unclear (Schertzer and Whiteley 2013). Aggregates of LPS may also form spontaneously when present in the circulation above the critical micellar concentration due to its amphiphilic structure. LPS on the surface of these vesicles and on whole bacteria are recognised by soluble LPS binding protein (LBP) in the plasma and transferred to CD14 on the surface of monocytes, which presents monomeric LPS molecules to the TLR-4-MD-2 complex (Martin et al. 1992; Wright et al. 1989; Tobias et al. 1995).

CD14 is a co-receptor to toll-like receptor-4 (TLR-4) that is predominantly expressed on the surface of monocytes and macrophages in the form of a glycophosphatidylinositol (GPI)-anchored glycoprotein (Ziegler-Heitbrock and Ulevitch 1993). It also exists as a soluble protein released through proteolytic cleavage of its membrane-bound form and through constitutive secretion (Bufler et al. 1995; Bazil and Strominger 1991). Soluble CD14 was once thought to enable non-expressing cells such as epithelium and endothelium to respond to LPS (Pugin et al. 1993; Goldblum et al. 1994; Arditi et al. 1995). However, it is now known that these non-myeloid lineage cells do express CD14 (Thorley et al. 2011; Jersmann 2005) and that the role of soluble CD14 is actually in facilitating binding of LPS to the TLR-4 complex when present at low concentrations. Conversely, TLR-4 signalling can be initiated in the absence of CD14 when the concentration of LPS is sufficiently high (Jeyaseelan et al. 2005; Perera et al. 1997).

The discovery of TLR-4 as a critical receptor for LPS was made when a mutation in the TLR-4 protein carried by C3H/HeJ and C57BL/10ScCr mice was found to be responsible for their resistance to LPS (Poltorak et al. 1998). TLR-4 was subsequently confirmed to be involved in LPS-induced NF-κB activation in HEK 293 cells transfected with TLR-4 (Chow et al. 1999), LPS-induced cytokine release (Tapping et al. 2000), and LPS-induced neutrophil recruitment (Calkins et al. 2002).

The extracellular domain of the transmembrane protein TLR-4 is associated with MD-2 (myeloid differentiation protein 2), which serves as the main binding site for LPS and is crucial for TLR-4 signalling (Maeshima and Fernandez 2013; Park and Lee 2013; Ren et al. 2011). Binding of LPS induces homodimerisation of the TLR-4-MD-2 complex, which brings intracellular Tolle/IL-1β receptor (TIR) domains together, providing a scaffold for the recruitment of adaptor proteins. Dimerization of the TLR-4-MD-2 complex initiates two signalling pathways, which have been summarised in a number of reviews (Banerjee and Gerondakis 2007; Barton and Medzhitov 2003; Kawasaki and Kawai 2014). The MyD88-dependent pathway leads to NF-κB and MAP kinase activation resulting in the production of inflammatory cytokines. The TRIF-dependent pathway similarly causes activation of NF-κB and MAP kinases that results in production of type I interferons (IFN) and inflammatory cytokines (Figure 3.1).

Figure 3.1 TLR Signalling Pathways

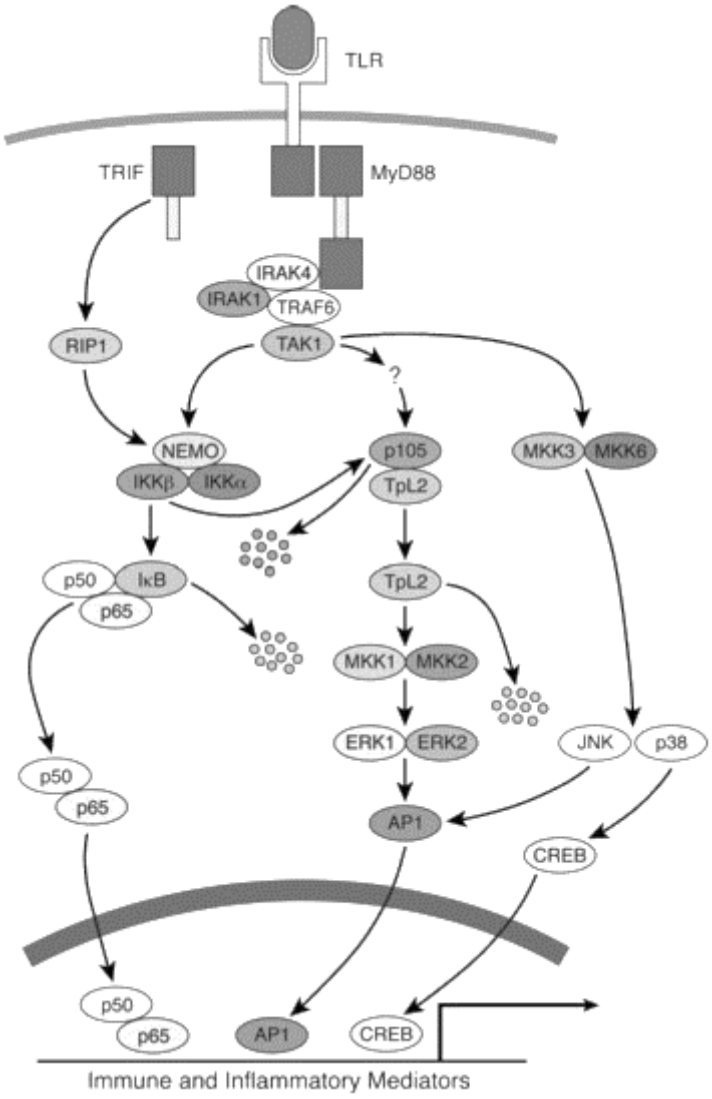


Figure adapted from (Banerjee and Gerondakis 2007).

The MyD88-dependent pathway involves the recruitment of adaptor protein MyD88 (myeloid differentiation factor 88) via TIRAP. Through interaction of the death domain of MyD88 with IL-1β receptor kinases (IRAK)-1 and IRAK-4, TRAF6 (tumour necrosis factor receptor associated factor 6) becomes activated which in turn activates an ubiquitin ligase complex that activates TAK1 (transforming growth factor β-activated kinase). TAK1 is responsible for the phosphorylation of the IKK (IκBα kinase) complex that subsequently phosphorylates IκBα, an inhibitor of NF-κB. Phosphorylation of this IκBα protein leads to its degradation, thereby releasing NF-κB to translocate into the nucleus where it up-regulates many pro-inflammatory genes including TNFα, IL-1β, and IL-6. In addition to initiating the NF-κB signalling pathway, TAK1 is also involved in the phosphorylation of

MKK3 and MKK6 which leads to activation of the MAP kinases p38, ERK1/2 and JNK (Kawasaki and Kawai 2014).

The TRIF-dependent pathway involves the recruitment of the TRIF (TIR-domain containing adaptor inducing interferon-β) adaptor protein via TRAM. TRIF interacts with TRAF3 and TRAF6, with the latter going on to recruit the kinase RIPK-1 (receptor-interacting serine/threonine kinase 1), another activator of TAK1. As in the MyD88-dependent pathway, activation of TAK1 leads to the activation of NF-κB and MAP kinases eventually resulting in the production of inflammatory cytokines. TRAF3, following interaction with TRIF, promotes the phosphorylation of IRF3 (interferon regulatory factor 3) via the IKK kinases inducing its dimerization and translocation into the nucleus where it up-regulates type I IFN genes (Kawasaki and Kawai 2014). Together, these two signalling pathways initiated by TLR-4 stimulation promote the up-regulation of a range of inflammatory cytokines. The induction of both NF-κB and MAP kinase activation in response to LPS made the LPS-induced lung injury model an ideal positive control to develop the phospho-flow cytometry protocol.

The expression of TLR-4 was thought to be predominantly expressed by monocytes, neutrophils and dendritic cells due to their substantial responses to LPS (Muzio et al. 2000). However, TLR-4 expression has also been detected on the surface of endothelial and alveolar epithelial cells, which are now also known to respond directly to LPS (Faure et al. 2000; Schulz et al. 2002; Armstrong et al. 2004).

There have been several studies examining the activation of the MAP kinase and NF-κB signalling pathways within the lung in response to LPS *in vivo*, utilising Western blots to measure global phosphorylation changes. Most *in vivo* studies have examined cellular activation after long periods of LPS exposure; reporting increased phosphorylation of p38 and ERK1/2 within periods of 2 to 12 hours following intratracheal or intranasal instillation of between 10 to 20μg LPS (Schuh and Pahl 2009; Hoogendijk et al. 2013; Chi et al. 2013). Other studies have also found evidence of activation of the NF-κB signalling pathway in addition to the MAP kinases under similar experimental conditions (Kim et al. 2006; Kuo et al. 2011; Sun et al. 2012; Liu, Sun, et al. 2013; Zhong et al. 2013). Schnyder-Candrian et al., who found increased phospho-MK2 after just 5 minutes reported the earliest detection of cellular activation following LPS instillation *in vivo* (Schnyder-Candrian et al. 2005).

LPS clearly induces activation within the lung but previous *in vivo* studies have only reported increased activation within the lung as a whole. Most previous research looking at the activation of MAP kinases have used the Western blotting technique, which only reveals localisation of activation when a sample contains only a single cell type. Nevertheless, *in vitro* work has provided insights into

the sensitivity of particular cell types including macrophages and epithelial cells to the bacterial endotoxin LPS.

The assessment of macrophage activation in response to LPS through the detection of MAP kinase phosphorylation *in vitro* has been well cited in the literature. One study by Srivastava et al. used phospho-flow cytometry to assess p38 and ERK1/2 phosphorylation in the murine macrophage cell line RAW 264.7 following LPS stimulation and reported transient activation of p38 and ERK1/2 peaking at 5 and 10 minutes respectively (Srivastava et al. 2009). Min et al. utilised Western blotting to detect increased ERK1/2 phosphorylation after just 3 minutes stimulation of the human macrophage-like cell line THP-1 that returned to baseline within 10 minutes (Min, Suk, and Lee 2013). There are also several other studies that have demonstrated very rapid activation (within 5 to 10 minutes) of macrophage cell lines in response to LPS stimulation *in vitro* through the detection of MAP kinase phosphorylation (Chen and Wang 1999; Sanghera et al. 1996; Beinke et al. 2004; Kim et al. 2006). Similar timescales of activation have been found in primary human alveolar macrophages stimulated with LPS *in vitro*, with severeal reports of p38 and ERK1/2 activation after 5 minutes (Carter, Monick, and Hunninghake 1999; Monick et al. 1999; Thorley et al. 2007).

The majority of studies looking at NF-κB signalling have monitored activity through the detection of IκB degradation, an event that releases NF-κB for translocation into the nucleus, or through detecting increased binding activity of NF-κB to nucleic acid. In response to LPS stimulation of RAW macrophages, Beinke et al. noted degradation of IκBα within 7.5 minutes by Western blotting (Beinke et al. 2004), and Chen & Wang observed NF-κB translocation into the nucleus within 10 minutes by electrophoretic mobility shift assay (EMSA) (Chen and Wang 1999). However, NF-κB activity may also be measured by its phosphorylation, which is essential for its transactivation capacity by enabling the recruitment of co-activators. Increased phosphorylation of IκBα and NF-κB has been detected by Western blotting in RAW macrophages following stimulation with LPS for 5 and 10 minutes respectively (Kim et al. 2006).

Whilst macrophages are commonly studied, there have been fewer studies looking at cellular activation of epithelial cells in the presence of LPS. One study using the human lung epithelial cell line A549, reported increased p38 phosphorylation following 30 minutes stimulation with LPS (Wei, Matsumoto, and Yamaguchi 2013). However, a study by Thorley et al. in which primary human type II AECs were stimulated with LPS was not able to detect activation of p38 or ERK1/2 (Thorley et al. 2007). Activation of NF-κB on the other hand, has been demonstrated within 10 minutes of LPS stimulation of primary rat type I and type II AECs through the measurement of increased phosphorylation of IKKα/β by fluorescent microscopy (Wong and Johnson 2013). Phosphorylation of

IKKα/β is an indirect marker of NF-κB activation since it leads to phosphorylation and degradation of the IκBα complex, which results in the release of NF-κB to translocate into the nucleus.

Despite the small number of studies that have detected epithelial cell sensitivity to LPS via the activation of the MAP kinase and NF-κB signalling pathways, numerous studies have reported significant responses by other means. Thorley et al. reported that primary human type II AECs were capable of releasing the chemokines CCL2 (MCP-1) and CXCL8 at higher levels than primary human alveolar macrophages in response to 24 hours of LPS exposure, despite their finding of a lack of MAP kinase activation (Thorley et al. 2007). Thorley et al. went on to report that both primary human type I and type II AECs express low levels of CD14, TLR-4 and MD-2, but their expression is increased by LPS exposure, particularly within the type II AECs (Thorley et al. 2011). Other studies have shown that both primary rat type I and type II AECs produce significant quantities of CCL2, CXCL2 and nitrite when stimulated with LPS for 18 hours (Manzer et al. 2006), and that primary mouse epithelial cells release significant amounts of CXCL1 and IL-6 with 4 hours of LPS exposure (Raoust et al. 2009).

As the cellular activation induced by the instillation of LPS into the lungs *in vivo* had not previously been localised to specific pulmonary cell types, the experiments presented in this chapter were expected to reveal a novel insight into which pulmonary cell types respond to LPS and in what order, under a short timescale *in vivo*.

3.2 Aims & hypotheses

The first aim of this chapter was to use the LPS-induced lung injury model as a positive control for the development of the phospho-flow cytometry protocol. The second aim was to investigate the effects of intratracheal LPS instillation on the activation of pulmonary cells during the very early stages of LPS-induced lung injury. Studies so far have investigated cellular activation of particular cell types in isolation *in vitro*, or have examined global activation of the lung as a whole *in vivo*. The current experiments in which the cellular activation of specific cell populations were examined from lungs that had been exposed to an LPS insult were expected to provide a novel insight into the response to LPS *in vivo*.

I predicted that the pattern of cellular activation in response to LPS would be different to that induced by high tidal volume mechanical ventilation. We hypothesised that in response to LPS the alveolar macrophages would be activated first, followed by AEC activation. However, we expected to find the opposite direction of activation in response to stretch.

3.3 Protocols

Intratracheal LPS instillation

Following the protocol described previously in Chapter 2: Methods & Materials, a 20µg in 50µl LPS bolus was administered intratracheally via a fine catheter. The mice were held in the vertical position on a board for 30 seconds after the instillation to ensure that the full bolus entered the lungs. For LPS exposures of 1 minute, insertion of an endotracheal tube was performed prior to instilling the LPS. For exposures lasting 5 and 15 minutes, the LPS dose was instilled and the mouse laid down and kept warm on a heat pad. Three minutes before the end of the 5- or 15-minute exposure, the process of inserting an endotracheal tube was started. For LPS exposures lasting 30 and 60 minutes, mice were transferred to an 'intensive care box' that was warm and contained 40% supplementary oxygen. Mice did not regain consciousness but were given a top-up of anaesthesia prior to insertion of the endotracheal tube.

LPS stimulated samples were compared with untreated control samples from mice that had undergone the anaesthetic and tracheostomy procedure, but not fluid instillation. Mice treated with instillations of saline alone did not differ from untreated mice and so one set of untreated mice served as controls to both the 5 and 15 minute LPS exposures. Other sets of LPS experiments were not conducted at the same time, and so each set of experiments has its own set of controls to avoid a drifting effect of baseline levels.

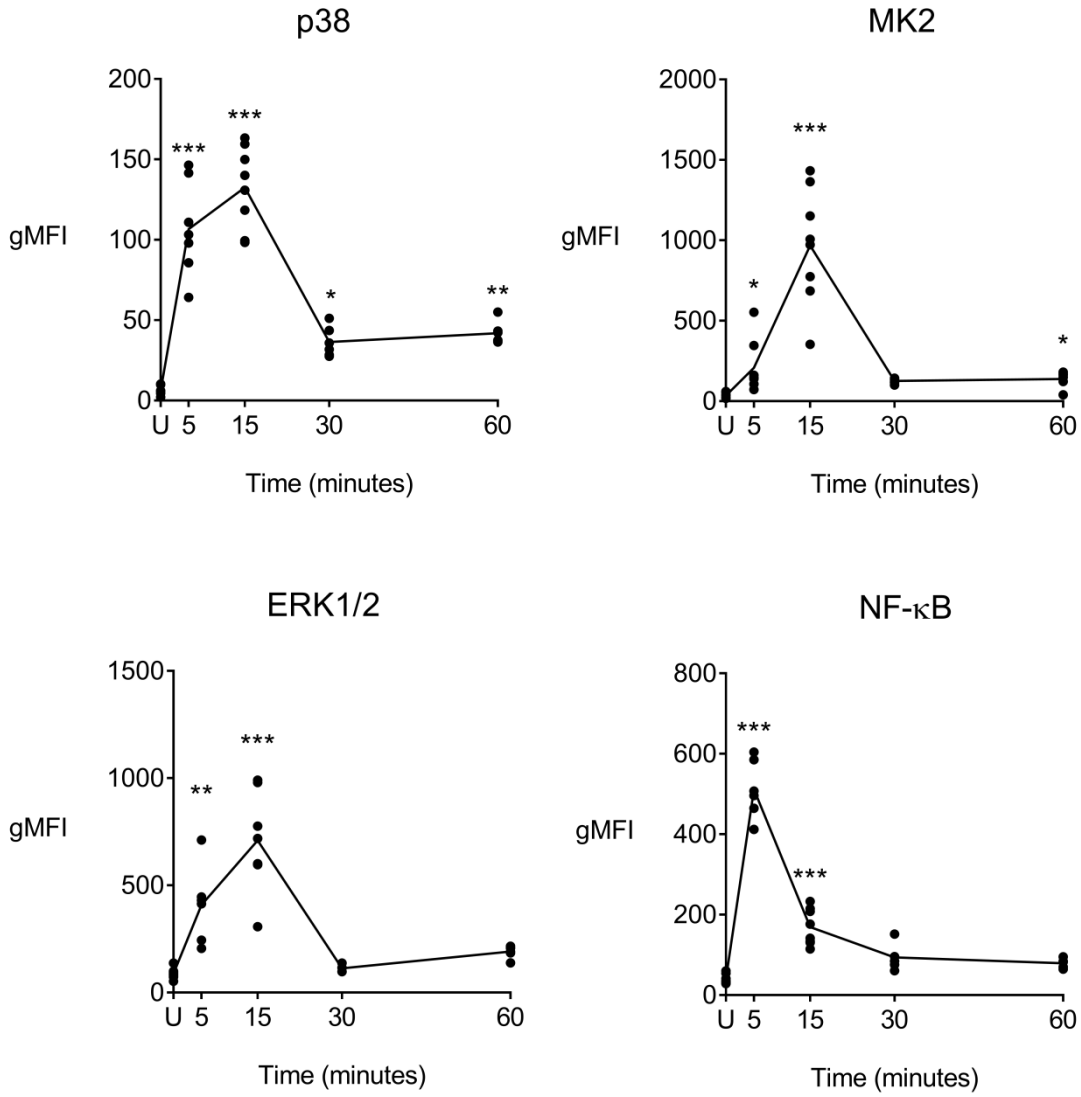
Sample collection

At the end of the timed exposure period, the mice were exsanguinated by lacerating the carotid artery and the caudal vena cava. The endotracheal tube was used to insert 500µl of cold phosphatase inhibitor directly into the lungs before their removal and dissociation in warm fixation buffer for 60 seconds. The sample was incubated in the fixation buffer for 10 minutes at 37°C, filtered through a 40µm cell strainer in order to collect a single cell suspension, and finally washed twice in a perm-wash buffer to remove excess fixative. The sample was then stained with a panel of antibodies to identify specific cell populations and with phospho-specific antibodies for the detection of phosphorylated forms of p38, MK2, ERK1/2 and NF-κB.

3.4 Results

A time course of alveolar macrophage activity was performed, examining the phosphorylation level of each marker after 5, 15, 30 and 60 minutes exposure to intratracheal instillation of LPS (Figure 3.2). These experiments revealed that p38 activation peaked at around 5 to 15 minutes and subsequently decreased but remained significantly elevated compared with its baseline phosphorylation level for up to 60 minutes. This time frame correlated with the activation of MK2, which lies directly downstream of p38. MK2 showed a slightly later peak in activation at 15 minutes, followed by a large fall in activation by 30 minutes but like p38, remained significantly activated at 60 minutes compared with baseline. ERK1/2 demonstrated a peak in activation between 5 to 15 minutes that had decreased to baseline by 30 minutes, and remained at this baseline level for up to 60 minutes. Activation of NF-κB peaked at 5 minutes, and was the only marker to have already shown a considerable decrease by 15 minutes, returning almost to baseline at 30 and 60 minutes (Figure 3.2). All four markers demonstrated transient activation, with significant decreases in phosphorylation levels by 30 minutes and showed continued low levels of activity at the 60-minute time point.

Figure 3.2 Phosphorylation time course of activation markers in alveolar macrophages following intratracheal instillation of 20ug LPS

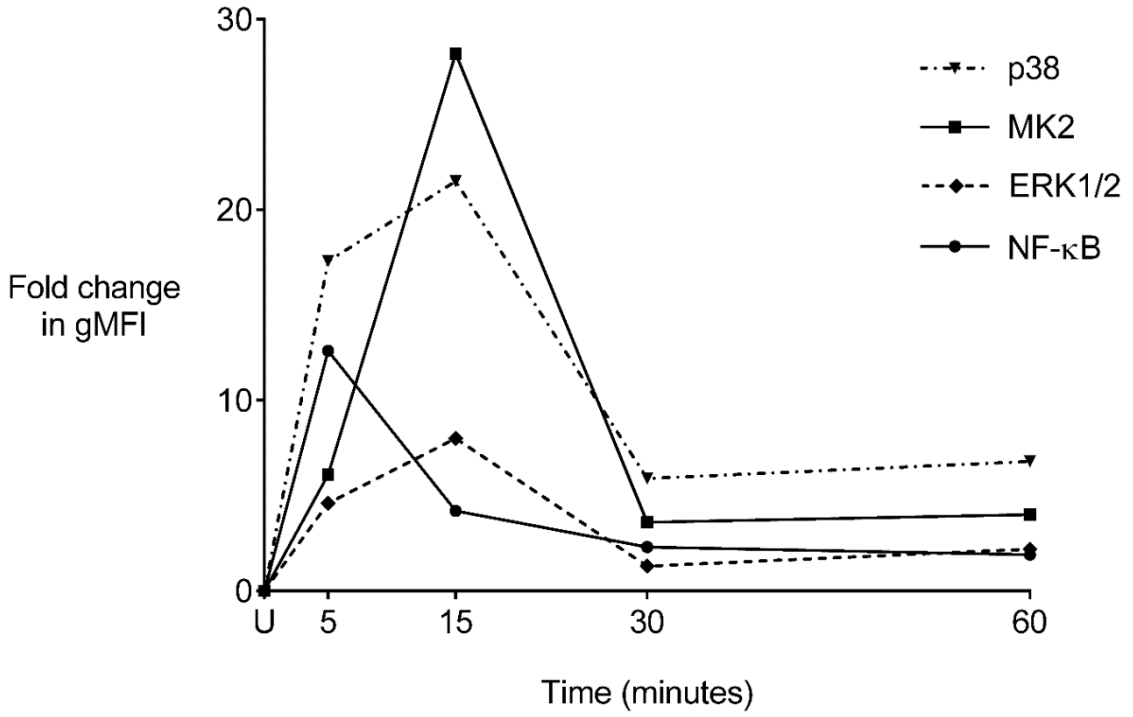


*Alveolar macrophages demonstrated rapid activation of the four intracellular markers that all peaked around 5 to 15 minutes. Phosphorylation levels then returned close to baseline for ERK1/2 and NF-κB whilst p38 and MK2 demonstrated continued low levels of activation. N=5-8, *p<0.05, **p<0.01, ***p<0.001. The mean of each treatment group (5, 15, 30 or 60 minutes) was compared with the untreated group (U). Data was analysed by ANOVA and Dunnett t test. Data are displayed as mean ± SD.*

Comparing fold changes in the data from Figure 3.2 allows for a comparison of the activation patterns of the four markers (Figure 3.3). p38 showed a 17.3-fold increase in phosphorylation level after 5 minutes which peaked after 15 minutes at 21.5-fold. This corresponded with a 6.1-fold increase in MK2 phosphorylation by 5 minutes, which escalated to a 28.2-fold increase by 15 minutes. MK2 lies directly downstream of p38 and its higher fold-increase suggests that the signal is amplified. ERK1/2

displayed the lowest fold increase peaking at 8.0-fold at 15 minutes. NF-κB showed the earliest peak in activation at 5 minutes with a 12.6-fold increase.

Figure 3.3 Fold changes in phosphorylation of activation markers in alveolar macrophages following instillation of 20ug LPS



This figure combines the data from the four graphs from Figure 3.2 represented as average fold changes from baseline (untreated) phosphorylation levels. N=5-8.

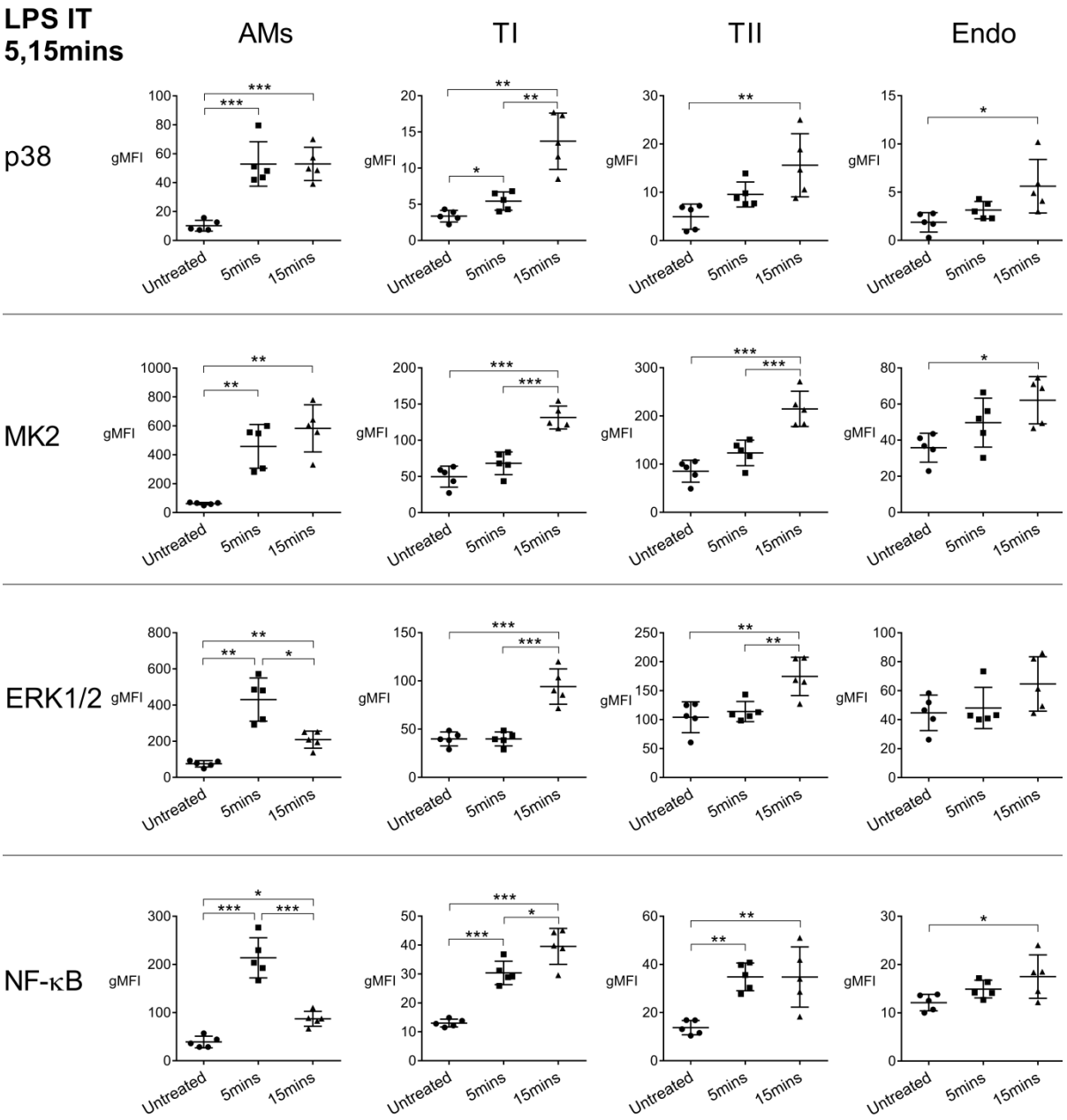
Having refined the protocol for detecting cellular activation by phospho-flow cytometry in alveolar macrophages following an *in vivo* insult, a more in-depth investigation into the activation induced by LPS in the lung was conducted. This involved not only examining cellular activation within alveolar macrophages, but also within type I and II AECs, and endothelial cells. Given that cellular activation had returned to baseline by 30 minutes in the previous experiments, the two earlier time points of 5 and 15 minutes were focused on for future experiments. These early time points were also of particular interest in investigating the initiation of LPS-induced lung injury and in the remaining chapters, to compare to the initiation of VILI.

In accordance with previous results, we found once again that the alveolar macrophages demonstrated significant phosphorylation of all four markers after just 5 minutes exposure to intratracheal LPS (Figure 3.4). p38 and MK2 both demonstrated sustained activation at the 15-minute time point with five-fold and seven- to nine-fold increases in phosphorylation respectively. ERK1/2

and NF-κB displayed transient activation, with both showing considerable decreases in phosphorylation by 15 minutes, though both were still significantly activated compared with their baseline levels. ERK1/2 activation had peaked at 15 minutes in the previous set of data compared with 5 minutes in the current experiments, which probably suggests that the true peak in activation for ERK1/2 lies in between the 5- and 15-minute time points.

The type I and II AECs both demonstrated rapid activation of NF-κB within just 5 minutes, which was just as fast as the alveolar macrophages (Figure 3.4). However whilst NF-κB showed a transient increase in phosphorylation in alveolar macrophages, the type I and II AECs both demonstrated sustained activation between 5 and 15 minutes. The type I and II AECs both showed little activation of the MAP kinase signalling proteins at 5 minutes, with just a small increase in phospho-p38 detected in the type I AECs. However after 15 minutes, the AECs displayed significant activation of all the MAP kinase markers. The endothelial cells seemed much less responsive to intratracheal LPS than the AECs. They displayed no signs of activation within 5 minutes, but did demonstrate significantly increased phosphorylation of p38, MK2 and NF-κB after 15 minutes (Figure 3.4).

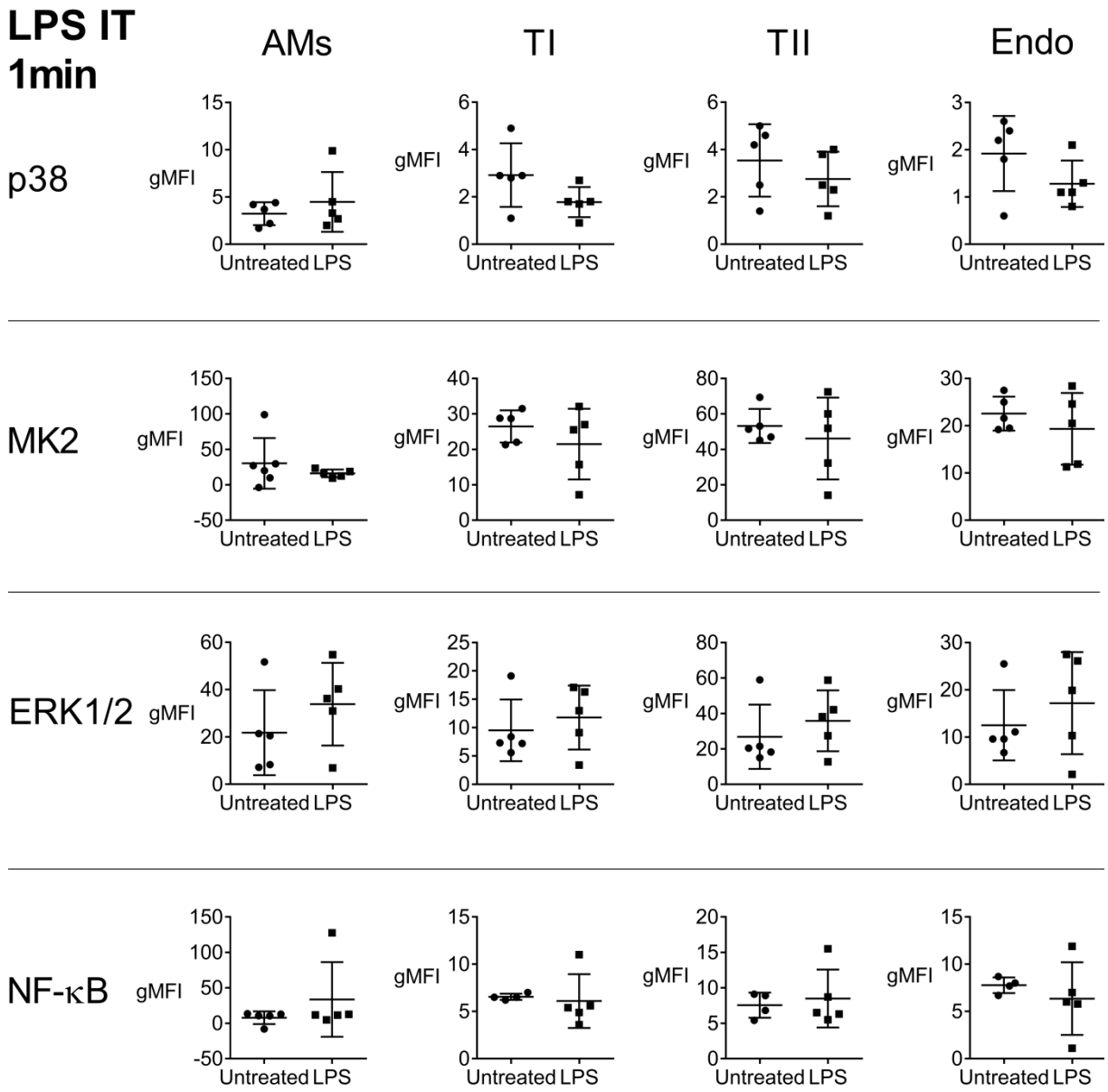
Figure 3.4 Phosphorylation of activation markers in alveolar macrophages, alveolar epithelium and pulmonary endothelium following exposure to intratracheal LPS for 5 or 15 minutes



Alveolar macrophages (AMs) displayed activation of all four markers following 5 minutes of in vivo *LPS exposure whilst type I and II alveolar epithelial cells (TI and TII) displayed only NF-κB activation at this time point. Endothelial cells (Endo) demonstrated significant activation following 15 minutes of intra-alveolar LPS exposure. Results from the 5- and 15-minute LPS exposure experiments were compared with phosphorylation levels in untreated control lungs. N=5, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by ANOVA with Tukey HSD. Data are displayed as mean ± SD.*

Intratracheal LPS induced substantial activation of both alveolar macrophages and both the type I and II AECs after just 5 minutes. In an attempt to distinguish which cell type is the first to respond to LPS, a further set of experiments were conducted in which mice were exposed to intratracheal LPS for just 1 minute. Unfortunately, these experiments did not yield any clues as to which cell type reacts the fastest to LPS, since no significant signs of activation were detected (Figure 3.5).

Figure 3.5 Phosphorylation of activation markers in alveolar macrophages, alveolar epithelium and pulmonary endothelium following exposure to intratracheal LPS for 1 minute



No significant signs of activation were detected after 1 minute of intratracheal LPS. N=4-6. Data were analysed by t-test and are displayed as mean ± SD.

3.5 Discussion

Intratracheal LPS was found to induce substantial and rapid activation within the lung. The rapidity of this response is consistent with previous observations by Schnyder-Candrian et al. who detected a significant increase in the phosphorylation level of MK2 within the whole lung just 5 minutes after intranasal instillation of 20µg LPS in mice (Schnyder-Candrian et al. 2005). Our data offers a novel insight into which pulmonary cell types are actually responsible for this global increase in activation.

As expected, the alveolar macrophages were found to be one of the main contributors, demonstrating strong activation of both the NF-κB signalling pathway and the MAP kinases within just 5 minutes of LPS exposure. At 5 minutes, the alveolar macrophages were sole source of MAP kinase activation within the lung. Of course, alveolar macrophages are the resident immune cell of the lung and are well known to be capable of generating substantial inflammatory reactions. The transient activation of ERK1/2 and NF-κB that was observed within the alveolar macrophages is a characteristic feature of signalling pathway kinetics (Thompson et al. 1995; Covert et al. 2005; Murphy et al. 2002; Weber et al. 1997; Murphy and Blenis 2006). Phosphorylation of the signalling proteins induces their active state, in which they go on to modulate the activity of other proteins, with their own activity regulated by phosphatases (Owens and Keyse 2007). This means that these signalling pathways are switched on just as easily as they are switched off. Similarly rapid and transient activation of these signalling proteins has been seen in alveolar macrophages stimulated with LPS *in vitro* (Srivastava et al. 2009; Min, Suk, and Lee 2013; Thorley et al. 2007; Shinohara et al. 2005; Beinke et al. 2004).

The type I and II AECs were also found to be significant contributors to the activation detected within whole lungs exposed to LPS. In contrast to alveolar macrophages through, they only displayed activation of the NF-κB pathway after 5 minutes, with MAP kinase signalling requiring 15 minutes to become activated. These results correspond with those found *in vitro* by Thorley et al., where epithelial cells were shown to respond to LPS through activation of the NF-κB pathway, in the absence of MAP kinase signalling. In the same study, MAP kinase signalling was not detected in isolated epithelial cells for up to 4 hours following LPS exposure (Thorley et al. 2007). This perhaps suggests that the NF-κB pathway is activated within epithelial cells through direct ligation of LPS with the TLR-4 complex, whilst another cell type initiates the MAP kinase activation observed after 15 minutes in our experiments. This could occur via a secondary mediator released by alveolar macrophages or even through direct contact.

There is evidence to suggest that communication between alveolar macrophages and the epithelium may involve direct contact. A study by Wong et al. found that LPS-induced cytokine release from

epithelial cells and alveolar macrophages was highest when in co-culture, whilst the transfer of supernatant had a reduced effect (Wong and Johnson 2013). Further evidence for the possibility of contact-dependent communication arises from studies that have reported LPS-induced adherence of alveolar macrophages to the alveolar surface. Significant decreases in the number of alveolar macrophages recovered by BAL have been reported after 30 minutes exposure to intratracheal LPS (Hirano 1996) and after 4 hours of intravenous LPS (Edelman, Cardozo, and Lesser 1989). *In vitro*, adherence of primary rabbit alveolar macrophages to the surface of the culture plate dramatically enhances their MAP kinase and NF-κB activation, and TNFα production in response to LPS stimulation, suggesting that adherence may have a priming effect (Bauer et al. 2000).

In contrast, a more recent a study by Westphalen et al. showed that a subset of alveolar macrophages that were in contact with the epithelium were less responsive to LPS and suggested that their adherence may in fact be protective (Westphalen et al. 2014). They reported that alveolar macrophages are attached to the epithelium via gap junctions that enable communication with alveolar macrophages located in different alveoli. MyD88 signalling was found to induce intercellular communication via Ca^{2+} waves that began 4 hours after LPS instillation, and was found to be immunosuppressive (Westphalen et al. 2014).

Our results did not determine whether a particular cell type is activated first in response to LPS. However, our experiments do confirm that in addition to rapid activation of alveolar macrophages, both the type I and II alveolar epithelial cells are likely to play a major role in the pulmonary response to LPS. Our results also suggest that there may be rapid communication between the epithelium and the endothelium, which displayed activation within just 15 minutes of intratracheal LPS instillation.

Endothelial cells are not thought to express CD14 and therefore require its soluble form, or alternatively very high levels of LPS to activate TLR4 signalling (Faure et al. 2000; Jeyaseelan et al. 2005; Perera et al. 1997). It has previously been shown in our lab that a very large dose of intravenous LPS (200µg) is required to promote pulmonary endothelial cell activation (O'Dea et al. 2005). It therefore seems unlikely that the endothelial response observed in the current experiments was due to direct LPS ligation, which would have required the LPS to travel across the epithelial barrier, which unless injured is not normally permeable. The remaining explanation for this phenomenon then is that endothelial cells became activated through communication with the epithelium.

The alveolar epithelium has been shown to communicate inflammatory signals to endothelial cells within minutes *in situ* (Kuebler et al. 2000). In these experiments, intra-alveolar TNFα was found to

induce increases in intracellular calcium and $cPLA_2$ activation in epithelium, which led to increased intracellular calcium and P-selectin expression in endothelium (Kuebler et al. 2000). A similar method of communication could be responsible for the rapid endothelial activation observed in our experiments in response to intra-alveolar LPS.

Model of LPS-induced lung injury

The intratracheal LPS-induced lung injury model used in this chapter involved the instillation of a concentrated bolus of LPS. This insult is not very similar to that which would be experienced during a live bacterial infection, where the release of LPS into the alveolar space would be much more gradual. The intratracheal bolus may even cause focal lesions or haemorrhage if the LPS solution is not dispersed evenly throughout the lung. We believe however that the LPS dose was disseminated equally around the lung in our experiments as the histograms obtained through flow cytometry demonstrated that all cells within a given population responded to the LPS to approximately the same degree. Westphalen et al. have recently shown that the detection of LPS within the lung is communicated between AMs across several alveoli via gap junctions on epithelial cells (Westphalen et al. 2014). This method of communication could explain how the whole lung appeared to respond in unison to the LPS bolus, even if not all AMs were directly exposed.

Conclusions

In spite of any limitations of the LPS-induced lung injury model, it enabled the validation and refinement of a complex phospho-flow cytometry protocol that was to be utilised throughout the remainder of the project. The results showed that intratracheal LPS induced significant activation within alveolar macrophages as well as the type I and II AECs, with distinct patterns of activation. These results of these experiments not only demonstrated that the phospho-flow technique could successfully detect changes in cellular activation, but would also serve as an interesting comparison to results obtained from future experiments using the mechanical ventilation model.

CHAPTER FOUR

INITIATION OF INFLAMMATORY SIGNALLING IN VENTILATOR-INDUCED LUNG INJURY

4.0 Chapter summary

The experiments presented in this chapter revealed that just 1 minute of high stretch ventilation induced significant activation of MAP kinase signalling proteins in endothelial cells, which displayed increased levels of phosphorylated p38, MK2 and ERK1/2 at this early time point. The type I and II AECs were also activated within just 1 minute, with both demonstrating significant p38 activation. Cellular activation of alveolar macrophages was apparently slower and was not observed until after 5 minutes of high stretch ventilation. These results suggest that the parenchymal cells of the lung are responsible for sensing stretch directly, whilst the resident leukocytes may become activated via a secondary mechanism possibly involving a soluble secondary mediator, or through physical interactions with the parenchymal cells. The apparently direct activation of endothelial cells by stretch suggests that inflammation could be propagated within the systemic circulation almost immediately following the initiation of high tidal volume ventilation, and this finding could have a major impact on the theory behind the development of multiple organ failure (MOF) in ARDS and VILI patients.

4.1 Background

Ventilator-induced lung injury (VILI) results from over-stretching of the lungs during mechanical ventilation, which can exacerbate inflammation in patients with pre-existing lung injury and can induce inflammation in patients with previously healthy lungs. Several studies have examined the effects of two-hit models combining mechanical ventilation and systemic LPS and found a synergistic

increase in inflammation (Altemeier et al. 2004; Whitehead et al. 2004). It could be argued that these models are likely to be more clinically relevant to ARDS than a one-hit model of VILI, however in order to investigate the mechanism of stretch-induced inflammation, the insult must be studied in the absence of confounding factors. On the other hand, our one-hit model could be said to have relevance to those patients undergoing surgery or critically ill patients without previous lung injury, whom have also been shown to be susceptible to VILI (Serpa Neto, Nagtzaam, and Schultz 2014; Serpa Neto et al. 2015). The one-hit model uses high tidal volumes to replicate the degree of over-distension experienced by the oedematous ARDS lung in the absence of the underlying cause and therefore focuses exclusively on the mechanism of stretch-induced inflammation.

VILI is a significant contributor to mortality in the ICU, and as a result has been the focus of a lot of research, yet the mechanism of stretch-induced inflammation remains unknown. Many cell types have been shown to be crucial to the development of VILI including resident leukocytes alveolar macrophages and lung-marginated monocytes (Eyal, Hamm, and Parker 2007; Frank et al. 2006; Wilson et al. 2009). In addition, parenchymal epithelial and endothelial cells have also been implicated in VILI for their role in cytokine release, mediating leukocyte infiltration, and the impact of barrier dysfunction on pulmonary oedema (Ning and Wang 2007b; Tremblay et al. 2002; Bhattacharya et al. 2003).

A study that deformed primary human alveolar macrophages and monocyte-derived macrophages *in vitro* found that they released a number of cytokines, but predominantly CXCL8 after several hours of cyclic stretching. The same study demonstrated NF-κB activation after 30 minutes of cyclic stretching within the monocyte-derived macrophages (Pugin et al. 1998). However, given that alveolar macrophages and monocytes are not structural cells, it seems unlikely that they would respond directly to stretch within the lung. It is possible though that alveolar macrophages may be exposed to stretch *in vivo* if in contact with the alveolar epithelium. Alveolar macrophages are known to be closely associated with the alveolar wall and a subset has recently been shown to be adhered to the epithelium via gap junctions (Westphalen et al. 2014). Other studies have suggested that AMs very quickly become adherent in response to stretch (Frank et al. 2006; Eyal, Hamm, and Parker 2007; Imanaka et al. 2001). There is no evidence to suggest whether monocytes would be subjected to stretch during high tidal volume ventilation, but it seems feasible that those located within the interstitial space or within the vasculature, may be compressed or stretched as the alveoli expand.

In contrast to these resident leukocytes, it is fairly obvious that the epithelium and endothelium would be directly exposed to stretch during high tidal volume ventilation (Figure 4.1). Indeed, epithelial cells are known to respond to physiological stretching with responses including surfactant

secretion and cell differentiation (Wirtz and Schmidt 1992; Wirtz and Dobbs 1990; Ashino et al. 2000; Sanchez-Esteban et al. 2004). Furthermore, AECs have frequently been shown to release pro-inflammatory cytokines following stimulation with stretch *in vitro* (Vlahakis et al. 1999; Tremblay et al. 2002), and pro-inflammatory signalling, in terms of MAP kinase activation, has been detected in epithelial cell lines within just minutes of exposure to stretch.

Sanchez-Esteban et al. reported the fastest known MAP kinase activation in response to stretch in foetal rat lung cells which displayed increased ERK1/2 phosphorylation after just 2 minutes exposure to 5% strain. In this study, ERK1/2 activation peaked at 15 minutes, and had returned to baseline by 30 minutes (Sanchez-Esteban et al. 2004). Another group detected ERK1/2 activation within 5 minutes of exposure to 30% strain in primary type II AECs that demonstrated a peak in activation at 10 minutes and subsequent decrease back to baseline by 30 minutes. Interestingly, 5% strain was found to induce the same level of ERK1/2 activation, though at a delayed time point of 15 minutes. This study also showed that a single stretch at 30% strain for 10 seconds caused an increase in the levels of phospho-ERK1/2 that was observed 10 minutes later (Correa-Meyer et al. 2002). A number of other studies have similarly detected rapid activation of MAP kinase signalling in response to stretch *in vitro* (Chess, Toia, and Finkelstein 2000; Cohen et al. 2010) and some have additionally detected NF-κB activation (Ning and Wang 2007b; Li et al. 2003).

Forming the capillaries which spread over the entire surface of every alveoli, pulmonary endothelial cells may also be subjected to considerable stretching during high tidal volume ventilation. They are certainly known to play an active role in controlling vascular tone in response to changes in blood flow and blood pressure. Furthermore, stretching of pulmonary endothelial cells *in vitro* has revealed rapid and transient activation of MAP kinase signalling after just 5 minutes (Kito et al. 2000; Abdulnour et al. 2006; Iwaki et al. 2009).

Figure 4.1 Activation of Endothelial cells, TII AECs and Alveolar Macrophages by Stretch

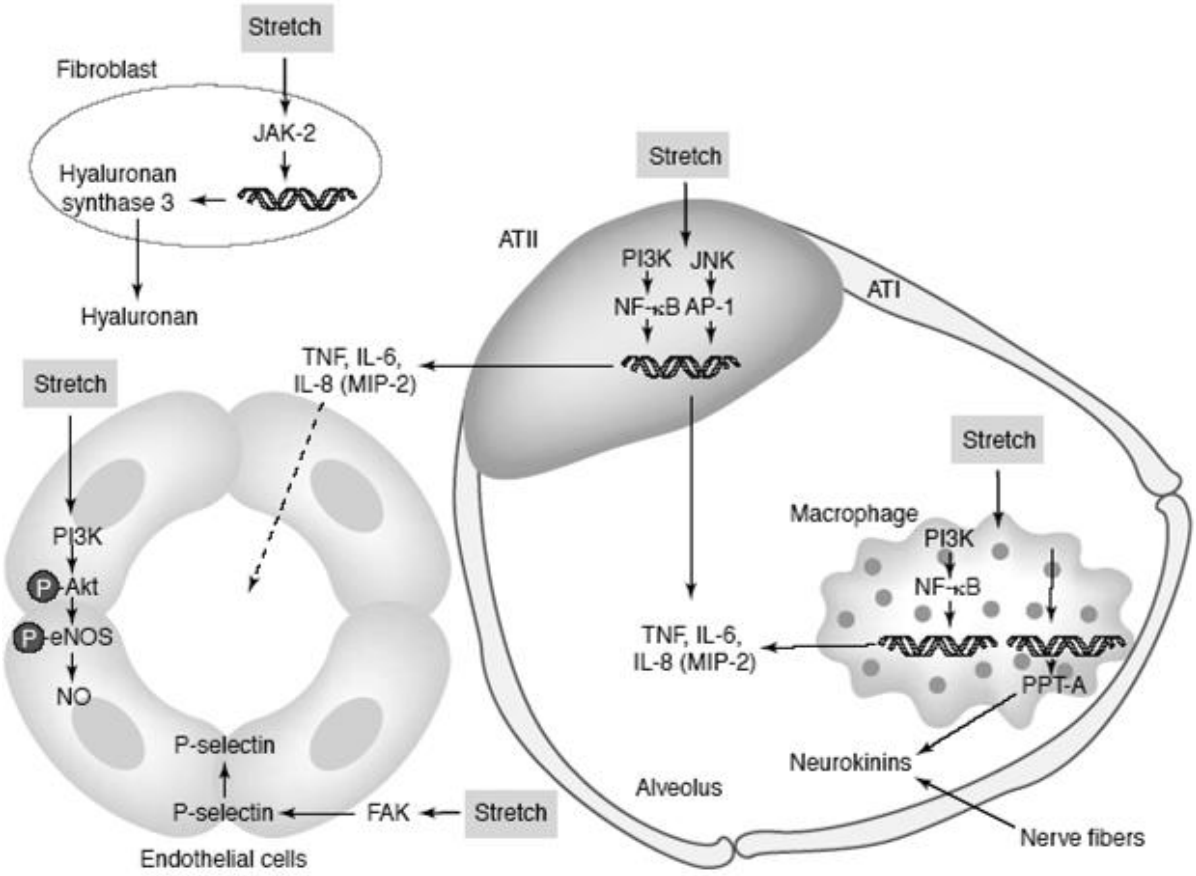


Figure adapted from (Uhlig and Uhlig 2004).

In contrast to the short timescales used in *in vitro* studies, detection of MAP kinase and NF-κB signalling *in vivo* has only been performed after much longer lengths of exposure to high tidal volume ventilation. The earliest detection of MAP kinase activation reported *in vivo* was a two-fold increase in phospho-ERK1/2 following 30 minutes of ventilation at 48ml/kg in rats. This study also detected increased phosphorylation of IκB and translocation of NF-κB into the nucleus within 30 minutes (Uhlig et al. 2002). Other studies have detected MAP kinase and NF-κB signalling within 1 hour of exposure to high stretch ventilation in mice using tidal volumes ranging between 20 to 32ml/kg (Abdulnour et al. 2006; Damarla et al. 2009; Li et al. 2007; Liu et al. 2009; Held et al. 2001; Huang et al. 2011).

These studies have all utilised Western blotting for the detection of phospho-epitopes or electrophoretic mobility shift assays (EMSA) for assessing NF-κB binding activity. Using these methods it is not possible to identify which cell populations are showing responses to stretch, as cells must be lysed to release the protein of interest. However, a few studies have utilised immunohistochemistry to qualitatively localise cellular activation mainly to the alveolar epithelium

and pulmonary endothelium (Uhlig et al. 2002; Abdulnour et al. 2006; Liu et al. 2009; Liu, Li, et al. 2013).

The current literature suggests that high tidal volume ventilation is capable of activating MAP kinase and NF- κB signalling in several pulmonary cell types. However the existing *in vivo* data, looking only as early as 30 minutes after the initiation of high tidal volume ventilation, is not early enough to determine which cell types are responsible for starting the inflammatory process. We hope to gain a novel insight into which cell types are the initial responders to stretch within the lung through examining cellular activation of inflammatory signalling pathways following very short lengths of exposure to high tidal volume ventilation *in vivo*.

4.2 Aims

The aim of the experiments in this chapter was to identify which cell type was the first to respond to high stretch ventilation, and which cells became activated thereafter. We hoped that the detection of a sequence in which cell types became activated, would indicate a direction of communication within the alveolar space. The second aim of this chapter was to determine the most sensitive cell type to stretch. The high tidal volume of 40ml/kg corresponding to approximately 40cmH_2O is approaching the highest level of pressure expected to be experienced in lungs of patients with ARDS (Gattinoni and Pesenti 2005) so we investigated whether cellular activation could also be detected at lower tidal volumes and pressures.

We hypothesised that the AECs would be the first cell type to respond to over-stretching as they make up the structural alveolar walls and would therefore definitely be subjected to substantial stretching forces. We predicted that the alveolar macrophages would then become activated soon after.

4.3 Protocol

Under general anaesthesia, a tracheotomy was performed and an endotracheal tube inserted that was used to connect the mouse to the ventilator. For all treatment groups, ventilation began with low stretch settings and two recruitment manoeuvres that were performed with a 30-second gap between them. Mice either remained on the low stretch settings (7-8ml/kg), or were switched to the high stretch settings (40ml/kg) that were confirmed by performing an inspiratory pause before the timer was started. In some experiments, a moderate stretch strategy was used and is compared with the other strategies in Table 4.2.

Table 4.2 Ventilation strategies

Ventilation Settings	Low Stretch	Moderate	High Stretch
Tidal volume (V_T)	8 ml/kg	30ml/kg	40 ml/kg
Breaths per minute (bpm)	120	120	80
Positive end-expiratory pressure (PEEP)	3.0 cm H_2O	3.0 cm H_2O	3.0 cm H_2O
Ventilation gas	100% O_2	O_2 with 4% CO_2	O_2 with 4% CO_2

The moderate stretch strategy employed a tidal volume of 30ml/kg, respiratory rate of 120bpm and used oxygen plus 4% CO_2, whilst the level of PEEP was the same as for the high stretch strategy. Blood gas analysis was performed to ensure the use of appropriate respiratory rate and ventilation gas for the moderate settings.

Each set of data was compared with its own set of low stretch controls that controlled for the effects of the anaesthesia and surgery protocol. For the first set of experiments (Figure 4.3) the 5 and 15 minute high stretch conditions were both compared with 5 minutes of low stretch as the controls.

Intravenous LPS administration

In one set of experiments, mice were ventilated with low stretch settings and LPS was delivered systemically. The jugular vein was cannulated to deliver a 20µg dose of LPS (the same as was given intratracheally in previous experiments) in a total volume of 120µl saline. LPS or saline vehicle alone in control experiments was allowed to circulate for 5 minutes prior to termination of the experiment and the collection of samples. In these experiments, the activation of neutrophils and Ly6C low and high monocytes were also examined alongside the usual pulmonary cell types to see if the 20µg dose of LPS was sufficient to activate cells systemically within 5 minutes, given that the LPS would be distributed in a larger volume than when administered intratracheally.

4.4 Results

Following 5 minutes of high stretch ventilation, the alveolar macrophages demonstrated significant activation of MK2 and ERK1/2 compared with that observed after 5 minutes of low stretch. After 15 minutes of high stretch, the AMs no longer demonstrated significantly increased MK2, and ERK1/2 was actually significantly decreased from the activation level detected after 5 minutes of high stretch. The alveolar macrophages did not display any significant signs of p38 or NF-κB activation within 15 minutes of mechanical ventilation (Figure 4.3).

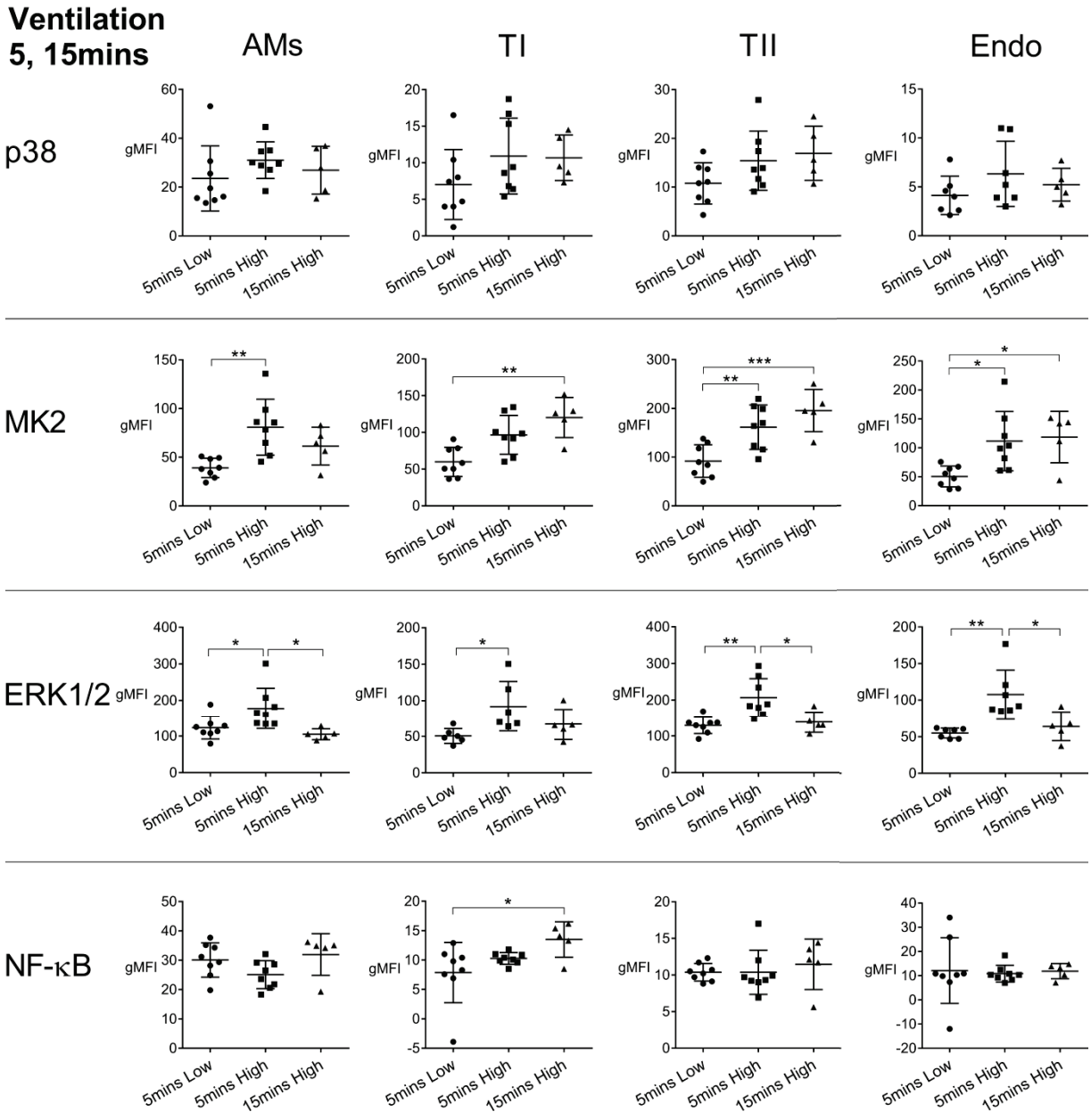
The type I AECs demonstrated a significant increase in ERK1/2 activation within 5 minutes of high stretch ventilation that had decreased again by 15 minutes. A gradual increase in MK2 phosphorylation was apparent at 5 minutes that became significantly increased by 15 minutes and NF-κB also became significantly activated after 15 minutes. p38 demonstrated a 1.6-fold increase in phosphorylation, that was identical to the increase observed for phospho-MK2, however, the increase in p38 did not reach statistical significance (Figure 4.3).

The type II AECs demonstrated significant activation of both MK2 and ERK1/2 after just 5 minutes of high stretch ventilation compared with low. Whilst MK2 showed sustained activation after 15 minutes, ERK1/2 as for all the other cell types, displayed transient activation, actually showing a significant decrease compared with 5 minutes of high stretch. The type II AECs did not show any significant signs of p38 or NF-κB activation within 15 minutes of high stretch ventilation (Figure 4.3).

The endothelial cells demonstrated significant activation of both MK2 and ERK1/2 after 5 minutes of high stretch ventilation compared with low. They displayed a similar pattern of activation to the AECs, with sustained activation of MK2 and transient activation of ERK1/2. The endothelial cells did not show a significant change in the phosphorylation of p38 or NF-κB (Figure 4.3).

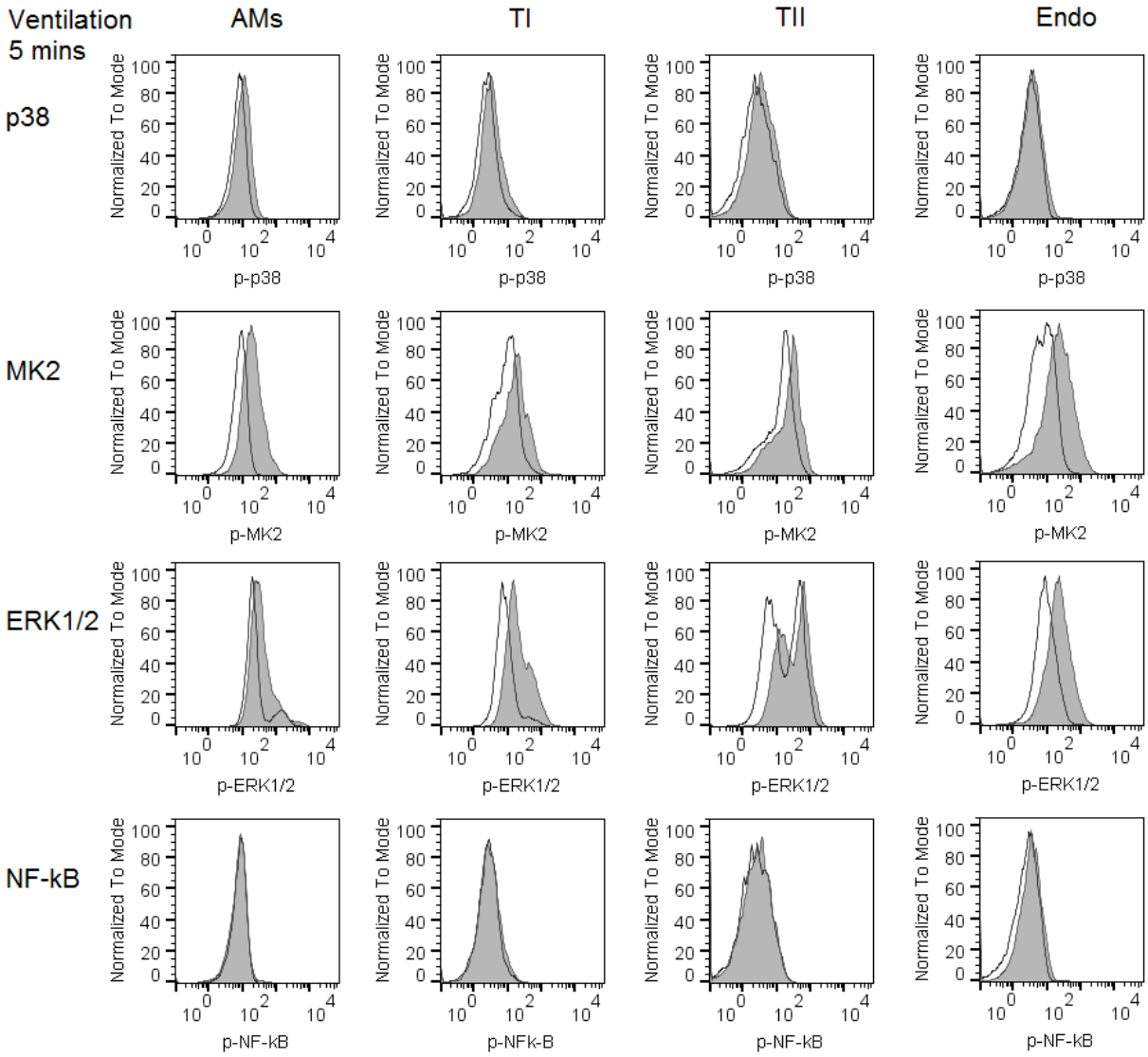
Representative histograms from one experiment that compared 5 minutes of low stretch ventilation with 5 minutes of low stretch is displayed in Figure 4.4.

Figure 4.3 Phosphorylation of activation markers in alveolar macrophages, epithelium and endothelium following exposure to 5 or 15 minutes of high stretch ventilation compared with 5 minutes of low stretch



*Each cell type demonstrated significant activation of markers following just 5 minutes of high stretch ventilation, mostly MK2 and ERK1/2. N=5-8, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by ANOVA with Tukey HSD and are displayed as mean ± SD. A post-hoc power calculation based on the alveolar macrophage phospho-MK2 means (39 versus 89 gMFI) and standard deviation (27 gMFI) suggested that an sample size of 7 was required in each treatment group to be able to detect a difference of 40 gMFI with 80% power and a 0.05% significance level.*

Figure 4.4 Representative histograms for alveolar macrophage activation following exposure to 5 minutes of low versus high stretch ventilation



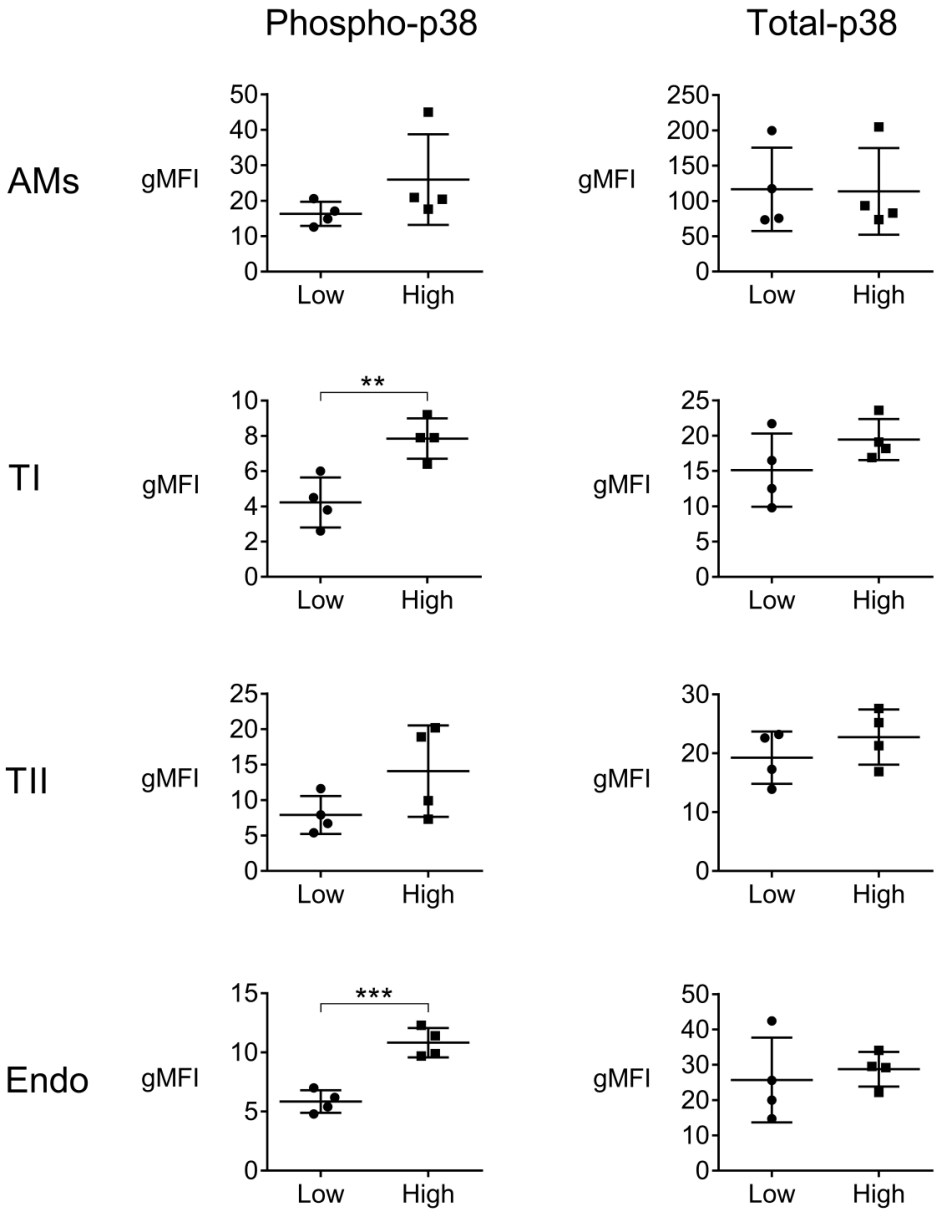
Representative histograms from one experiment displayed in Figure 4.3, showing phosphorylation levels of the four activation markers in alveolar macrophages, TI AECs, TII AECs and endothelial cells following exposure to 5 minutes of low stretch (white) compared with 5 minutes of high stretch ventilation (grey).

Phosphorylation changes in response to high tidal volume ventilation were much more subtle compared with those induced by intratracheal LPS seen in the previous chapter. We therefore sought to validate these phosphorylation level changes by confirming that they were not simply the result of a general increase in the overall levels of the measured proteins. The levels of both the phosphorylated and total protein were quantified within the same lungs subjected to 5 minutes of low or high stretch ventilation. These experiments were performed looking at p38 (AF-647; clone A-

12, Santa Cruz Biotechnology, US) and ERK1/2 (AF-647; clone 137F5, Cell Signalling). MK2 and NF-κB were not studied since there was no commercially available fluorophore conjugated antibody for MK2, and we had detected little NF-κB activation in response to stretch. After MK2, ERK1/2 was the most consistently activated and p38 demonstrated non-significant trends for activation in response to 5 minutes of high stretch ventilation within most cell types as well as being the kinase that is responsible for MK2 activation.

In this set of experiments, significant increases in the phosphorylation of p38 were detected in type I AECs and endothelial cells whilst non-significant trends for increased activation were seen in the AMs and type II AECs. p38 activation in this set of experiments reached statistical significance, which had not been the case in the previous experiments, perhaps due to more variability within the previous data sets. The levels of total p38 protein detected were not altered after 5 minutes of high stretch ventilation compared with low (Figure 4.5).

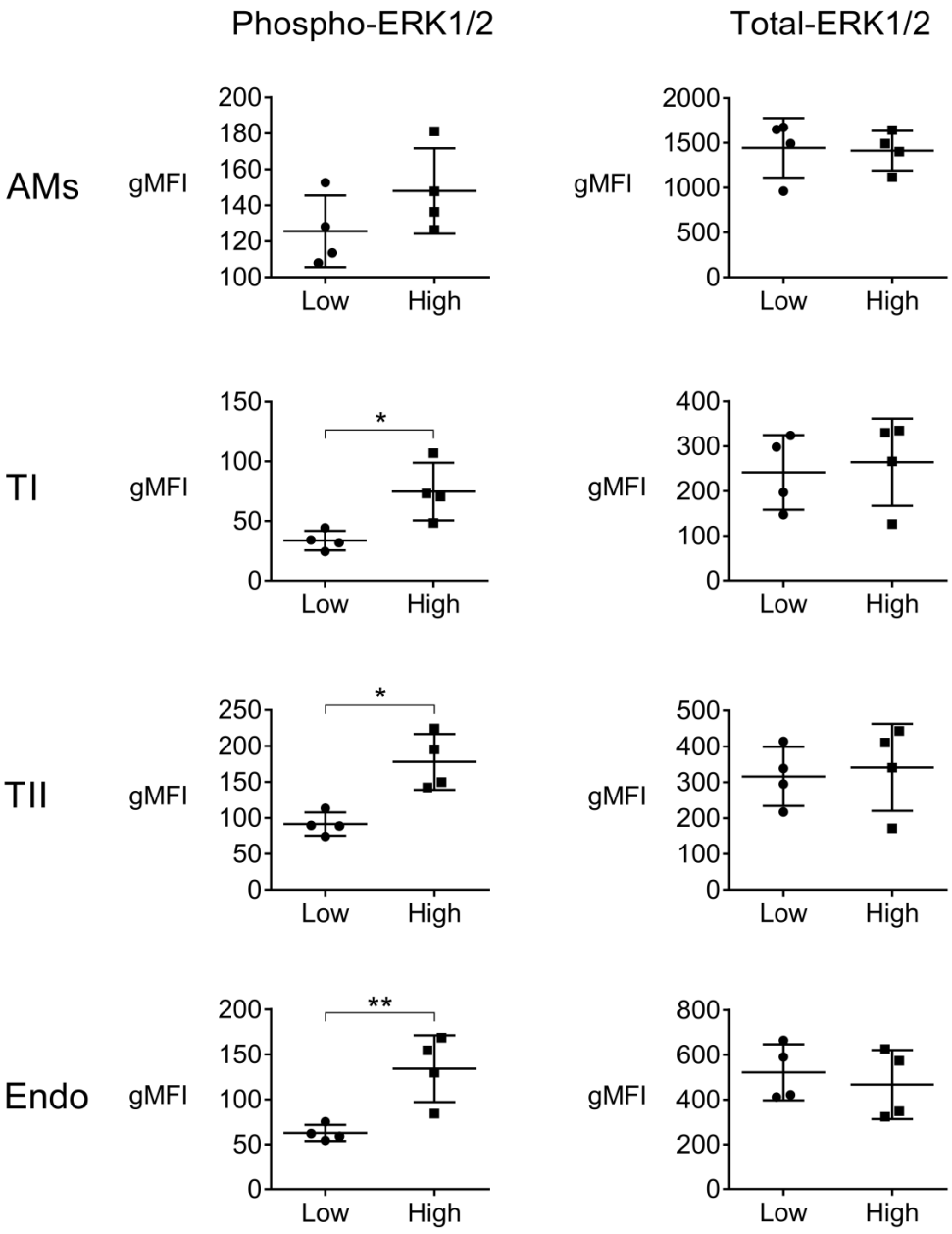
Figure 4.5 Levels of phosphorylated and total p38 protein within lungs subjected to 5 minutes of low or high stretch ventilation



*Type I AECs (TI) and endothelial cells (Endo) demonstrated significant p38 phosphorylation following 5 minutes of high stretch ventilation, whilst the total amount of p38 protein within the same lungs were unaltered. N=4, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

In the set of validation experiments for ERK1/2, as in the previous set of experiments, ERK1/2 was found to be significantly phosphorylated within 5 minutes of high stretch ventilation in the type I and II AECs and endothelial cells. However, the total amount of ERK1/2 protein remained unaltered (Figure 4.6).

Figure 4.6 Levels of phosphorylated and total ERK1/2 protein within lungs subjected to 5 minutes of low or high stretch ventilation

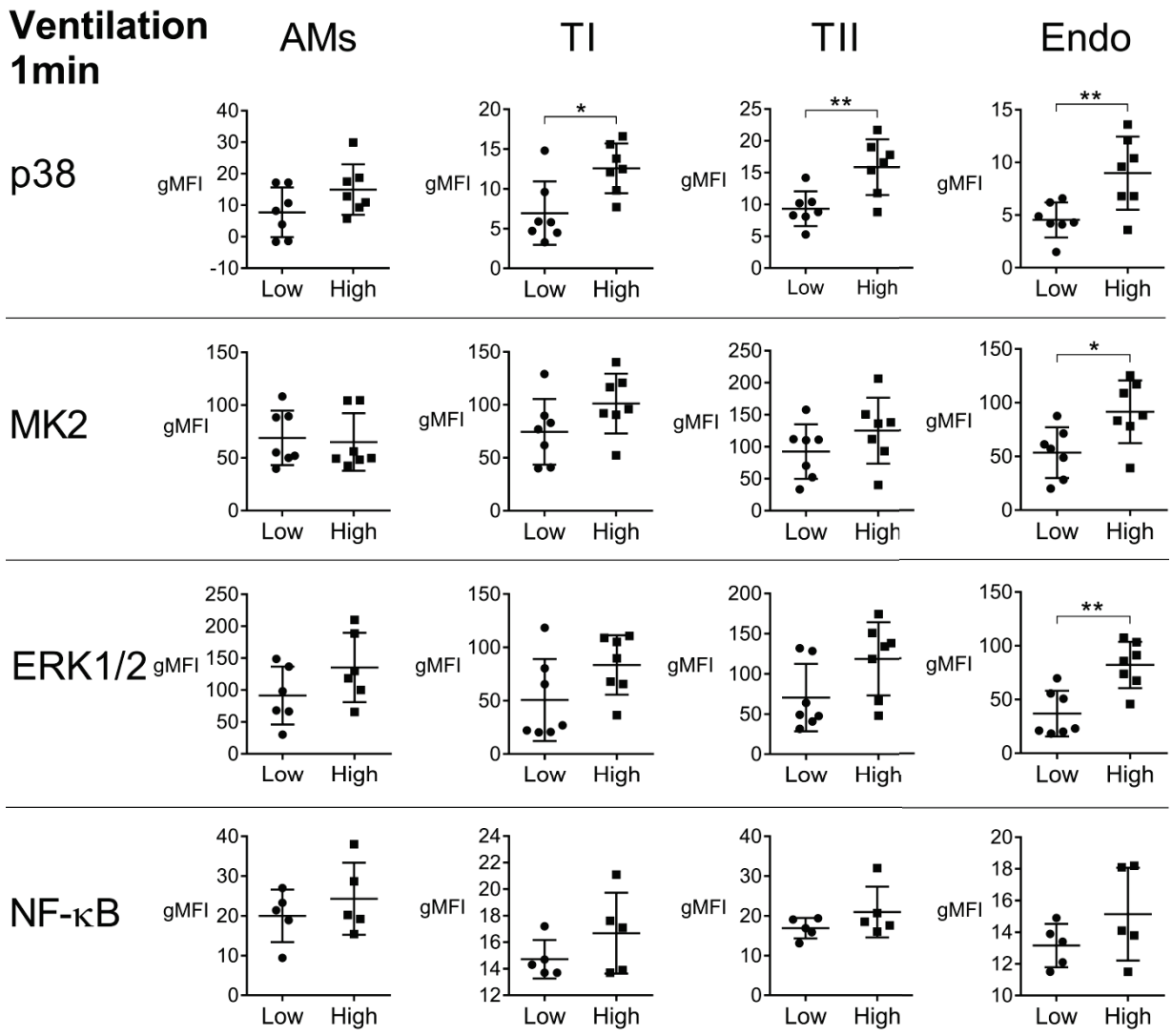


*Consistent with the data presented in Figure 4.3, the levels of phospho-ERK1/2 clearly increased with high stretch ventilation compared with low stretch, whilst the levels of total ERK1/2 protein within the same lungs were unchanged. N=4, *p<0.05, **p<0.01. Data were analysed by t-test and are displayed as mean ± SD.*

Our results showed signs of significant activation within all cell types following just 5 minutes of high stretch ventilation and so it was not possible to determine which cell type might be activated first in response to stretch. We therefore conducted another set of experiments in which mice were

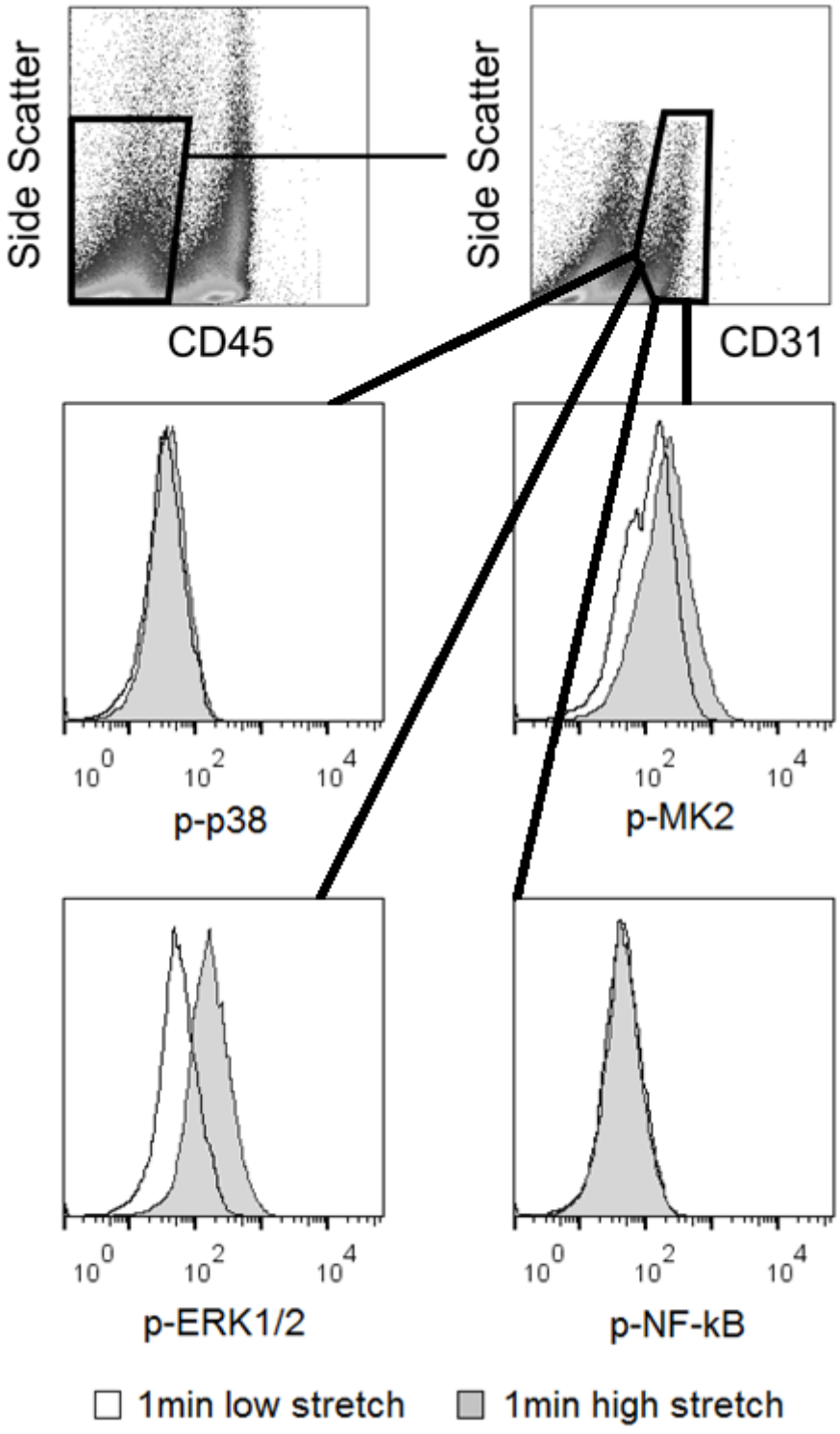
ventilated with low or high stretch ventilation for just 1 minute prior to lung sampling. At this very early time point, no signs of alveolar macrophage activation were detected, however significantly increased p38 phosphorylation was found in both type I and II AECs. Interestingly, the endothelial cells appeared to be the most responsive to stretch at this early stage, displaying significant increases in phosphorylation (approximately two-fold) of all the MAP kinase signalling proteins p38, MK2 and ERK1/2 (Figure 4.7 and 4.8).

Figure 4.7 Phosphorylation of activation markers in alveolar macrophages, epithelium and pulmonary endothelium after exposure to 1 minute of low or high stretch ventilation



*The endothelial cells displayed activation of the most markers after just 1 minute of high stretch ventilation. Type I and II alveolar epithelial cells both demonstrated significant increases in p38 phosphorylation. N=5-7, *p<0.05, **p<0.01. Data were analysed by t-test and are displayed as mean ± SD.*

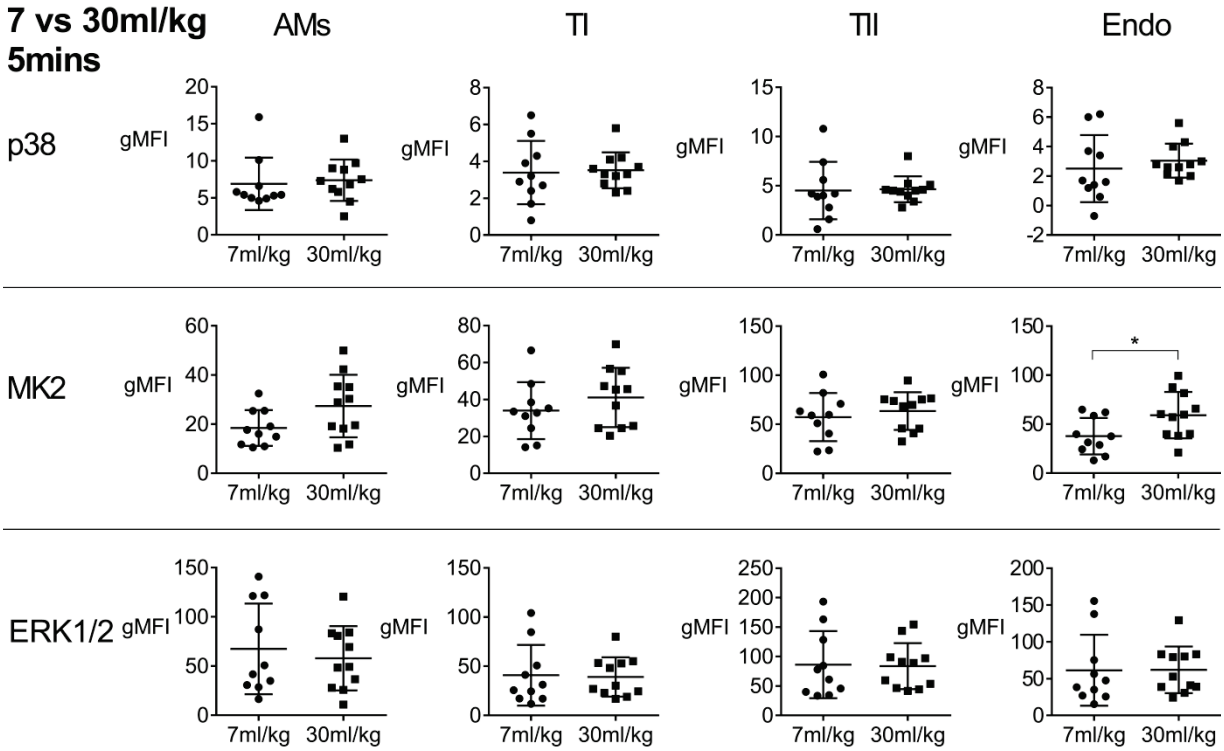
Figure 4.8 Representative histograms displaying increases in phosphorylation of four intracellular proteins in endothelial cells following 1 minute of high stretch ventilation



Representative dot plots identifying endothelial cells and histograms displaying phosphorylation levels of the activation markers following 1 minute of low versus high stretch ventilation.

We have previously discussed the value of studying the very high tidal volume of 40ml/kg. However we wanted to investigate which cell type is the most sensitive to stretch by looking for cellular activation at a more moderate tidal volume. We compared 5 minutes of ventilation at the low tidal volume of 7ml/kg to 5 minutes of ventilation at the moderate tidal volume of 30ml/kg. Using this ventilation strategy, only significant activation of MK2 in endothelial cells was detected (Figure 4.9).

Figure 4.9 Phosphorylation of activation markers in alveolar macrophages, epithelium and endothelium after 5 minutes of ventilation at 30ml/kg compared with 5 minutes of low stretch ventilation



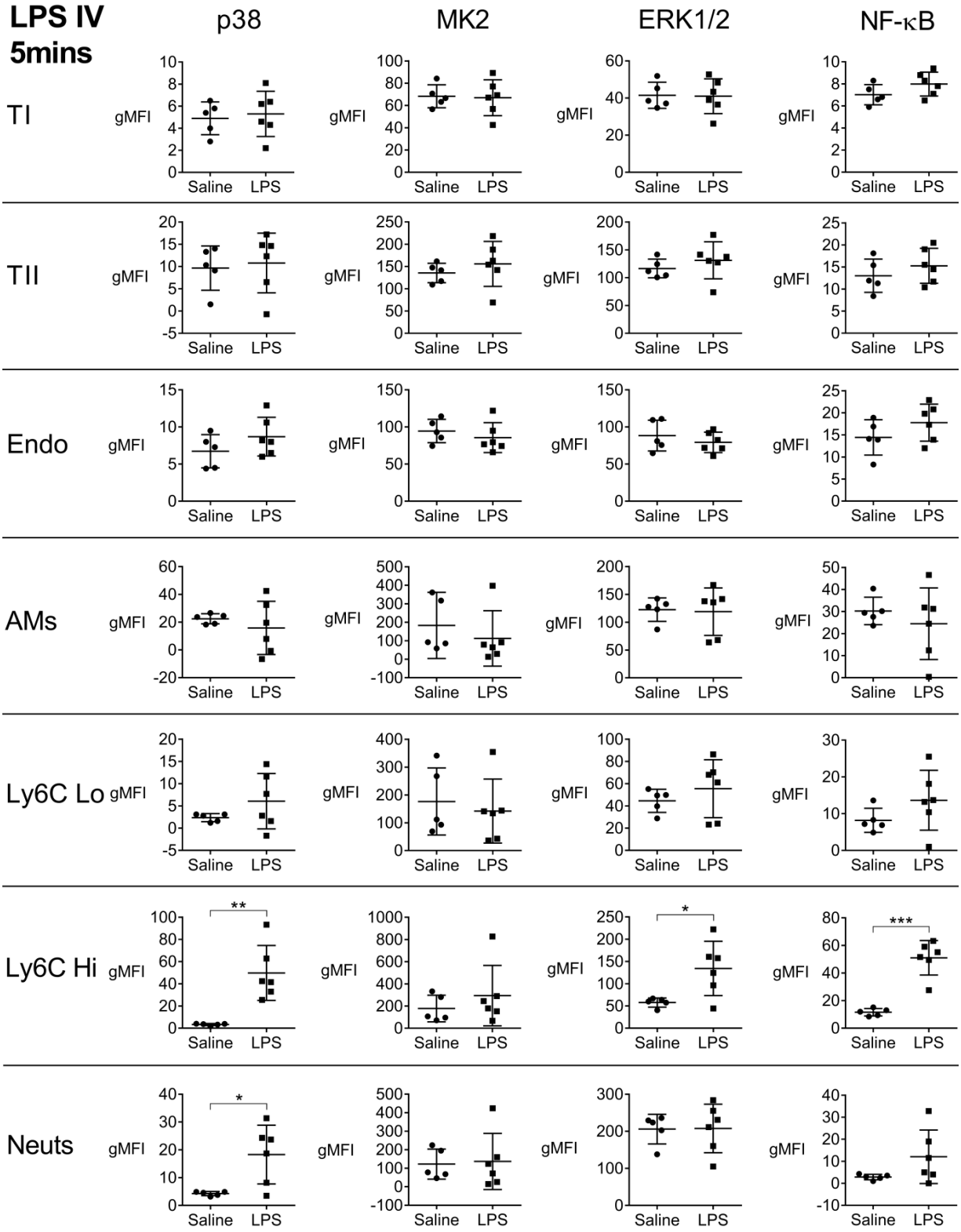
*Only the endothelial cells (Endo) demonstrated sensitivity to the moderate tidal volume of 30ml/kg. N=7-11, *p<0.05. Data were analysed by t-test and are displayed as mean ± SD.*

Having found such rapid activation of endothelial cells in response to high stretch ventilation, we wanted to investigate whether they could respond as quickly to LPS stimulation. In the previous chapter, endothelial activation was only detected 15 minutes after intratracheal instillation of LPS. We therefore examined activation of the pulmonary endothelium in response to systemic LPS, which would be expected to be able to activate the endothelium directly.

Neutrophils, as well as Ly6C low and high monocytes were included in the range of cells analysed by flow cytometry in this set of experiments. The same dose of LPS was given intratracheally (20µg) as

was given intravenously via the jugular vein and left to circulate for 5 minutes before collecting samples. The results showed that alveolar macrophages and type I and II AECs did not respond to such a short exposure to systemic LPS and neither did endothelial cells. Neutrophils on the other hand demonstrated a significant increase in p38 phosphorylation, whilst Ly6C high monocytes showed significant activation of p38, ERK1/2 and NF-κB (Figure 4.10).

Figure 4.10 Phosphorylation of activation markers in pulmonary cells and leukocytes following exposure to systemic LPS for 5 minutes



The parenchymal cells of the lung (TI, TII and Endo) did not demonstrate activation, and neither did alveolar macrophages (AMs). Of the leukocytes most likely localised within the pulmonary

*vasculature, neutrophils (Neuts) demonstrated p38 activation, whilst 'Ly6C Hi' monocytes displayed activation of p38, ERK1/2 and NF-κB. N=5-6, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

4.5 Discussion

The results presented in this chapter show that the lungs are very sensitive to stretch. Alveolar macrophages, type I and II AECs, and endothelial cells all demonstrated significant cellular activation within just 5 minutes of exposure to high stretch ventilation compared with low. This led us to investigate an even earlier exposure time of 1 minute, at which time, the alveolar macrophages appeared inactive, whilst the AECs both demonstrated significant p38 activation, and the endothelial cells displayed significant activation of p38, MK2 and ERK1/2.

Theory of AM activation in VILI

These results suggest that the parenchymal cells of the lung, are the most likely to initiate inflammatory signalling in response to stretch. Within the alveolar space, the epithelial cells are probably responsible for sensing stretch and communicating with the alveolar macrophages which then go on to propagate the inflammatory response. The nature of communication between AECs and alveolar macrophages in response to stretch remains unclear. According to our data, the possible mechanisms are limited to those which could occur within 5 minutes.

One means of rapid communication within the alveolar space could arise from direct communication between the alveolar epithelium and alveolar macrophages. Direct contact of these cells could enable alveolar macrophages to sense deformation of the alveolar wall. In this way, alveolar macrophages may sense forces applied to adhesion molecules via the breaking or stretching of intercellular connections, or through gap junctions that allow the transfer of signalling molecules between cells.

Another means of communication between the AECs and alveolar macrophages could occur via the release of a soluble mediator. One previous study has shown that BAL fluid from rats ventilated at 30ml/kg without PEEP for 20 minutes, could induce nitric oxide synthase activity in primary rat alveolar macrophages *in vitro* (Frank et al. 2006). Unfortunately, it was not possible to determine which cell type was responsible for the release of the unknown mediator. The group were able to show that the release of activating mediator(s) into the supernatant of alveolar epithelial and

macrophages cultured *in vitro* required them to be in co-culture, also indicating the importance of cellular interactions (Frank et al. 2006).

A potential mediator of rapid communication between stretched AECs and alveolar macrophages is TNFα. TNFα is an unusual cytokine in that it can be expressed in a membrane-bound form that is readily cleaved by TNFα converting enzyme (TACE) to yield soluble TNFα. Tremblay et al. found increased TNFα mRNA expression in AECs when rat lungs were injuriously ventilated *ex vivo* for 30 minutes (Tremblay et al. 2002). This and other studies have implicated TNFα in the pathogenesis of VILI with a focus on the later stages of VILI, though it remains unclear whether TNFα could be involved in the initiation of inflammatory signalling.

Another rapidly released soluble mediator that could be responsible for the activation of alveolar macrophages is ATP, which is not only known to be released in response to stretch (Belete et al. 2011) but is also involved in inflammatory signalling (Ferrari et al. 1997). ATP has been detected in the BAL fluid of injuriously ventilated animals within 30 minutes, and ATP instillation into the lungs induces pulmonary oedema and inflammation (Rich et al. 2003; Matsuyama et al. 2008). These studies suggest that ATP has the potential to be involved in the initial communication of mechanical stress within the alveolar space.

Prostanoids and leukotrienes are lipid mediators that can be rapidly released in response to stretch. Inhibition of the enzyme 5-lipoxygenase, which is involved in the synthesis of leukotrienes from arachidonic acid, has been shown to attenuate VILI in rats ventilated for 10 hours with high tidal volume ventilation (Caironi et al. 2005). A couple of studies have reported release of several prostanoids from foetal rat lung epithelial cells exposed to cyclic stretch *in vitro* within 10 to 15 minutes (Copland et al. 2006; Skinner, Somervell, and Olson 1992). This study examined 10 minutes as their earliest time point, but prostanoid release by lung epithelium could be released even earlier. Prostanoids synthesis is controlled by post-translational modifications as opposed to at the transcriptional level which is the case for cytokines, and this permits them to be rapidly produced and secreted (Mbonye and Song 2009). Prevention of prostanoid synthesis with cyclooxygenase (COX) inhibitors has been shown to be beneficial in reducing inflammation in rat models of VILI (Niitsu et al. 2011; Jin et al. 2013; Huang et al. 2014). The current evidence suggests that the involvement of these lipid mediators in the initiation of inflammation in VILI is worth investigating.

Transforming growth factor (TGF)-β is involved in wound healing, inflammation, epithelial apoptosis, collagen synthesis and fibroblast proliferation. TGF-β is also known to play a major role in both the early pathogenesis and late-stage resolution of ARDS. TGF-β has been found to be significantly

increased in the BAL fluid of patients with ARDS within 1 day of diagnosis (Dhainaut, Charpentier, and Chiche 2003), and in animal studies, TGF-β mRNA expression is increased in the lungs of rats ventilated for 40 minutes with high tidal volume ventilation (Imanaka et al. 2001). Early TGF-β release into the alveolar space could be responsible for ventilator-induced pulmonary endothelial permeability through a mechanism involving integrin αvβ5 and interaction with the actin cytoskeleton (Su et al. 2007). TGF-β is stored as inactive protein bound to the extracellular matrix via LAP and αvβ5 and primed for rapid activation (Goodwin and Jenkins 2009). It is unclear how quickly TGF-β may be released in response to stretch but it could be a viable soluble mediator for communication of stretch to the alveolar macrophages.

Endothelium is initial responder to stretch

Interestingly, our data showed that pulmonary endothelial cells were not only the first cell type to demonstrate MAP kinase signalling in response high stretch ventilation, but also appeared to be the most sensitive to lower tidal volumes. Pulmonary endothelial cells have been shown to demonstrate rapid activation of the p38 and ERK1/2 pathways within 5 minutes when stretched *in vitro* (Kito et al. 2000; Abdulnour et al. 2006; Iwaki et al. 2009). Yet it was still unexpected that these cells, in the context of a whole lung, would be seemingly more responsive than AECs to the stretch induced by high tidal volume ventilation. Work by Dreyfuss and Saumon performed nearly twenty years ago revealed endothelial blebbing by electron microscopy within just 5 minutes exposure to high stretch ventilation of rats with a PIP of 45cmH_2O (Dreyfuss and Saumon 1998). Another study showed that in an isolated perfused mouse lung, high stretch ventilation induced Akt phosphorylation within 15 minutes that was localised to the endothelium but not to the AECs or alveolar macrophages (Kuebler et al. 2003). Therefore, there are some suggestions in the literature that endothelial cells are very sensitive to stretch caused by high tidal volume ventilation. In the experiments presented in this chapter, both type I and II AECs also demonstrated p38 activation after just 1 minute of high stretch, but the endothelial cells additionally demonstrated MK2 and ERK1/2 activation, perhaps indicating that they were activated prior to AECs, particularly as MK2 lies downstream of p38. To the best of our knowledge, this data is the first to demonstrate such rapid endothelial activation in an *in vivo* model of VILI.

Of course endothelial cells are known to be sensitive to stretch under normal physiological conditions. They are able to sense shear stress generated by blood flow, to sense circumferential stretch caused by vascular transmural pressure; and respond by altering vascular tone (Ando and Yamamoto 2011). These forces would be reduced with the application of high tidal volume ventilation, as capillaries are compressed in a perpendicular direction to alveolar expansion, thereby restricting blood flow.

However, pulmonary endothelial cells would be subject to other mechanical forces induced by high alveolar pressure. The network of pulmonary capillaries is tightly connected to the alveolar wall via the basement membrane and form a mesh comprehensively surrounding each alveoli. This arrangement means that upon expansion of the alveoli, the endothelial cells of capillaries would too be subjected to significant stretch in a longitudinal direction, as suggested in the theory of capillary stress failure proposed by West (Fu et al. 1992; West 2000; West et al. 1991).

Endothelium is more sensitive to stretch than LPS

Interestingly, the pulmonary endothelium appeared to be more sensitive to stretch than to LPS. Systemic administration of LPS significantly activated neutrophils and Ly6C high 'inflammatory' monocytes localised within the lung vasculature, but did not activate the parenchymal cells or alveolar macrophages within the short timescale studied.

It was not surprising that systemic LPS did not activate alveolar macrophages or the type I and II AECs within 5 minutes, given the physical barrier that would need to be crossed. The endothelial cells also did not show any signs of activation in response to systemic LPS, despite being well positioned to encounter LPS circulating in the blood. Endothelial cells may be expected to respond to systemic LPS given that they play an important role in leukocyte trafficking. Previous data from our group has shown that 20µg LPS administered systemically can induce changes in p55 TNF receptor expression on pulmonary endothelial cells within a couple of hours (Bertok et al. 2011). However, our group has also shown that a much larger dose of LPS (200µg) is required to induce up-regulation of cell adhesion molecules on pulmonary endothelial cells (O'Dea et al. 2005). These studies have examined the effects of systemic LPS on the endothelium several hours after the delivery of LPS and so it remains unclear as to whether pulmonary endothelial cells respond directly to systemic LPS.

Given their large surface area within the body, it may be undesirable for endothelial cells to respond directly to LPS in such a rapid manner. Their activation in response to LPS may be regulated through interactions with monocytes or neutrophils, which did demonstrate activation after 5 minutes exposure to systemic LPS in the current experiments. Endothelial cells do not express CD14, and therefore require its soluble form. However, CD14 shedding from human monocytes has been shown to take up to 60 minutes following LPS stimulation which would be too slow to allow rapid endothelial activation (Bazil and Strominger 1991). In the presence of soluble CD14, endothelial cells have been shown to be capable of responding to LPS through increased phosphorylation of p38 and ERK1/2 within 5 to 15 minutes (Arditi et al. 1995).

Neutrophils, which demonstrated a significant increase in p38 phosphorylation, are known to respond to systemic LPS by marginating within the pulmonary vasculature (Wilson et al. 2009; Calkins et al. 2002; Hu, Malik, and Minshall 2010; Schnyder-Candrian et al. 2005). The neutrophil and monocyte populations detected in our experiments had probably not infiltrated into the interstitium or alveolar space, particularly given the timescale of the experiment, but were most likely simply located within the pulmonary vasculature at the time of sampling. The Ly6C high monocytes demonstrated a stronger response to LPS with three out of the four markers significantly activated. The Ly6C high monocytes are characterised as the 'inflammatory' subset of monocytes that are recruited to sites of inflammation (O'Dea et al. 2011; Geissmann, Jung, and Littman 2003) and therefore it is expected that they would demonstrate activation in response to systemic LPS.

Propagation of systemic inflammation in VILI

The favoured 'decompartmentalisation' theory states that inflammatory mediators, apoptotic factors or bacteria leak from the lung into the systemic circulation, an effect that is enhanced by the disruption of the alveolar-capillary barrier (Plotz et al. 2004; Jaecklin, Otulakowski, and Kavanagh 2010). However our data which revealed rapid endothelial cell activation, suggest a different mechanism by which systemic inflammation could be propagated prior to any physical damage to the epithelial-endothelial barrier. Endothelial cells, upon being stretched, may initiate systemic inflammation directly through the release of soluble mediators into the blood stream.

In support of this theory, there is evidence that endothelial cells can be directly involved in inflammatory responses through the release of chemokines such as CXCL8 and CCL2 in response to several hours of cyclic stretching *in vitro* (Iwaki et al. 2009; Okada et al. 1998). CXCL8 and CCL2 are well known chemotactic factors for neutrophils and monocytes that promote the migration and margination of these leukocytes into sites of inflammation. Stretched endothelial cells have also been shown to up-regulate xanthine oxidoreductase (XOR) (Abdulnour et al. 2006), an enzyme involved in the generation of reactive oxygen species, which have been shown to induce p38 activation in monocytes (Scott et al. 2011).

In combination with the literature, our data suggest that endothelial activation in response to high tidal volumes could play an important role in the development of systemic inflammation, which very often leads to multiple organ failure (MOF) and increased mortality in mechanically ventilated patients (Plotz et al. 2004; Slutsky and Tremblay 1998; Villar et al. 2011).

Conclusions

The data presented here indicate that the parenchymal AECs and pulmonary endothelial cells are the initial responders to high tidal volume ventilation. We hypothesised that within the alveolar space, the AECs initially sense stretch and communicate with alveolar macrophages, to induce their activation and amplify the inflammatory response. Whilst on the other side of the alveolar-capillary barrier, the endothelial cells are responsible for sensing stretch induced by high stretch ventilation and their communication or interaction with leukocytes in the circulation could lead to the immediate propagation of systemic inflammation, possibly leading to MOF.

CHAPTER FIVE

EXPLORATION OF DIRECT CONTACT-DEPENDENT MECHANISMS IN VILI

5.0 Chapter summary

Evidence suggests that some alveolar macrophages within the lung may be directly adhered to the alveolar wall. This chapter aimed to investigate whether a direct-contact dependent mechanism of communication is responsible for the initiation of inflammatory signalling in VILI. An intranasal dosing method was used under inhalational anaesthetic to administer Gap26 peptide or anti-CD18 antibody aimed at disrupting intercellular interactions via adhesion molecules or gap junction channels respectively. Unfortunately, the instillation procedure itself appeared to influence the responsiveness of alveolar macrophages to subsequent exposure to high stretch ventilation. This effect may have been caused by the volatile anaesthetic isoflurane which has been reported to attenuate LPS- and ventilator-induced lung injuries with long periods of exposure. The few minutes of exposure to isoflurane used in the current experiments made this explanation seem unlikely, but the effect regardless of the cause, was that it was difficult to interpret the results of these experiments. Another model was required to identify the exact mechanism of communication within the alveolar space that leads to inflammatory signalling in VILI.

5.1 Background

Based on the findings from the previous chapter, subsequent experiments investigated the hypothesis that the initial communication of stretch-induced lung injury may occur through direct contact between the epithelium and alveolar macrophages. This theory stems from studies that have reported significant decreases in the recovery of macrophages in BAL fluid after just minutes of high

stretch ventilation *in vivo* (Frank et al. 2006; Eyal, Hamm, and Parker 2007). This decrease in alveolar macrophage counts is thought to occur too quickly for them to have undergone apoptosis, or to have translocated out of the alveolar space. Therefore, this phenomenon is thought to be explained by the adherence of alveolar macrophages to the alveolar surface. This interaction with the epithelium could enable cell-to-cell communication that leads to alveolar macrophage activation. This theory led to the hypothesis that inhibiting such cell-to-cell interactions within the alveolar space could prevent alveolar macrophage activation and the initiation of inflammatory signalling that leads to VILI.

Gap junctions

Gap junction channels are present in most cells types and provide a direct route for the diffusion of various small molecules between the cytoplasm of connected cells. The transfer of molecules such as cyclic AMP, inositol 1,4,5-triphosphate, and glutamate permits a rapid and effective means of communication that allows a group of cells to respond as a single unit. The gap junction channel consists of two hemichannels, one provided by each cell, which when not involved in a gap junction have been shown to play a role in the release of paracrine mediators into the extracellular space. These hemichannels are comprised of six connexin (Cx) proteins that form a large pore with extracellular loops that determine the gating properties of the channel and control interactions with other hemichannels. There are many different connexin proteins that all confer different selectivity properties on the pores, and this selectivity is additionally regulated through the docking of hemichannels consisting of either a single connexin (homotypic), different connexins (heterotypic), or combination of different connexins within one hemichannel (heteromeric) (Koval 2002).

The formation of hemichannels is highly regulated both at the level of connexin protein expression and through trafficking to the plasma membrane. Different cell types display different patterns of connexin expression, and additionally, specific connexin proteins are only compatible with certain other connexin proteins. Connexin mimetic peptides that bind to the extracellular loop of connexins within hemichannels are used to inhibit the formation of gap junction channels (Evans and Leybaert 2007). The most commonly used mimetic peptides, Gap26 and Gap27, are 11-13 amino acid residues long and their sequences correspond to the extracellular loops 1 and 2 respectively on connexin 43 (Cx43) (Evans and Boitano 2001). These inhibitors are highly specific and have been used to demonstrate the propagation of calcium signalling through gap junction channels between neighbouring cells (Desplantez et al. 2012; Leybaert et al. 2003; Wang, De Bock, et al. 2012).

Within the lung, AECs have been shown to express several connexins that change as type II AECs differentiate into type I AECs in culture (Lee et al. 1997; Abraham et al. 1999; Abraham et al. 2001).

Alveolar macrophages have also been shown to express connexins and have been found to communicate with alveolar macrophages in distant alveoli via calcium signalling through the alveolar epithelium. This method of communication was thought to be immunosuppressive in a model of LPS-induced lung injury since inhibition of gap junction channels using connexin mimetic peptides Gap26 and Gap27 increased neutrophil recruitment and production of pro-inflammatory cytokines (Westphalen et al. 2014).

Other studies however, have suggested that gap junction channels can enhance inflammation within the lung. Sarieddine et al. found that Cx43 knockout mice demonstrated reduced neutrophil recruitment as well as reduced cytokine levels within BAL fluid following LPS instillation. They also observed that mice with a mutation in the Cx43 protein that increased channel permeability, demonstrated augmented neutrophil recruitment and mortality (Sarieddine et al. 2009). Another study noted that calcium waves triggered in pulmonary endothelium of an isolated perfused lung, travelled up to 150μM along capillaries and caused up-regulation of P-selectin, an effect that was inhibited using Gap26 and Gap27 peptides (Parthasarathi et al. 2006).

Mechanical stimulation of primary rabbit tracheal airway epithelial cells has also been shown to induce immediate propagation of calcium signalling to approximately 15 neighbouring cells which again could be inhibited by incubation with Gap26 peptide (Boitano and Evans 2000). These studies suggest that gap junction channels could be involved in responses to mechanical forces, and in the propagation of inflammatory signalling. It was therefore believed that they may represent a viable means of communication between alveolar macrophages and AECs in the initiation of VILI.

Adhesion molecules

Intracellular adhesion molecule-1 (ICAM-1/CD54) has been frequently studied in the context of lung injury. ICAM-1 is structurally related to the immunoglobulin supergene family and is a ligand for the β_2-integrin CD18 which forms heterodimeric structures with CD11a-d found on the surface of leukocytes. ICAM-1 is predominantly expressed on type I AECs and alveolar macrophages within the lung but is also constitutively expressed on most pulmonary parenchymal cells and leukocytes at low levels (Stent et al. 1995; Kang et al. 1993). In response to a variety of pulmonary insults, many cells demonstrate clear up-regulation of the ICAM-1 protein and accompanying enhanced cellular adhesion. Exposure of primary rat AEC to hypoxic conditions (5% O_2) resulted in a significant increase in ICAM-1 mRNA that peaked at 30 to 60 minutes, and adhesion of primary alveolar macrophages to these cells was augmented by 118% (Beck-Schimmer et al. 2001). LPS has been shown to increase ICAM-1 mRNA and protein expression within 4-6 hours in rat type II AECs that led to a 3-fold increase

in neutrophil adhesion, and was inhibited by 54% after pre-treatment with anti-ICAM-1 antibody (Madjdpour et al. 2000). An increase in soluble levels of ICAM-1 has also been shown to correlate with poor outcome in both mice with pulmonary inflammation and in human patients with ARDS (Mendez et al. 2011; Calfee et al. 2009; Schmal et al. 1998).

Research has largely focussed on the role of ICAM-1 interactions within the pulmonary vasculature, where endothelial ICAM-1 is involved in the adherence of neutrophils and their migration into the lung (Mizgerd et al. 1999; Li, Miyasaka, and Issekutz 1998). Burns et al. demonstrated neutrophil infiltration into the lungs of rats 6 hours following intratracheal instillation of LPS that was inhibited by simultaneous systemic administration of anti-integrin antibodies (Burns et al. 2001). Ridger et al. similarly demonstrated in mice that neutrophil infiltration following intratracheal LPS could be inhibited by simultaneous systemic administration of anti-CD18 antibody (Ridger et al. 2001). Administration of anti-CD18 intratracheally alongside hydrochloric acid, was found to not alter neutrophil counts, but reduced lung injury overall in rabbits (Folkesson and Matthay 1997).

A few studies have administered anti-ICAM-1 intratracheally and some have similarly noted a reduction in neutrophil infiltration into the lungs following LPS stimulation (Beck-Schimmer et al. 2002), whilst others have found no effect on neutrophil counts. This study reported that CD18 blockade in mice did not reduce neutrophil recruitment to the lung in response to cytokine insufflation, but did reduce injury to the AECs (Parmley et al. 2007). Previous studies have all looked at the role of ICAM-1 interactions in neutrophil recruitment and activity; however the role of ICAM-1 interactions between alveolar macrophages and AECs in response to mechanical ventilation has not been investigated.

ICAM-1 interactions have not only been shown to be important in intercellular adhesion, but also in the regulation of alveolar macrophage and neutrophil function during inflammation. One study demonstrated that alveolar macrophages and neutrophils showed enhanced phagocytic activity for *K. pneumoniae* when in contact with AECs expressing ICAM-1, an effect that was inhibited in the presence of an ICAM-1 blockade (O'Brien et al. 1999). Another study demonstrated that phagocytic activity of alveolar macrophages was reduced in ICAM-1 knockout mice, and further studies *in vitro* revealed that ICAM-1 blockade also severely reduces mobility of alveolar macrophages in response to chemotactic signals (Paine et al. 2002).

In addition to such data, there is also evidence that ICAM-1 is involved specifically in VILI. A study of paediatric patients found that levels of soluble ICAM-1 were highest amongst patients requiring prolonged mechanical ventilation and in non-survivors (Flori et al. 2003). *In vitro*, cyclic stretch has

been shown to induce ICAM-1 secretion by an endothelial cell line and up-regulation of ICAM-1 mRNA production within 3 hours of exposure to continued cyclic strain. This study also reported that adhesion of a monocyte cell line to the endothelial cells was enhanced following stretch, an effect that was reduced by anti-ICAM-1 treatment (Cheng et al. 1996). Another study, which stretched A549 epithelial cells, showed increases in ICAM-1 protein and mRNA levels that was strain, frequency and duration dependent (Hu et al. 2008).

In vivo, increased expression of ICAM-1 and CD11b on alveolar macrophages and neutrophils from rats has been detected following just 40 minutes of high stretch ventilation compared with low (Imanaka et al. 2001; Ohta et al. 2001). In an isolated-perfused lung (IPL) model of rat lungs, expression of ICAM-1 was significantly enhanced on the surface of alveoli following 6 hours of ventilation at 15ml/kg. The group also observed increased margination of neutrophils and monocytes in the pulmonary vasculature that was prevented by ICAM-1 blockade (Miyao et al. 2006).

The current literature suggests an important role for ICAM-1 in the development of lung injury. Whilst the majority of studies have investigated the role of this adhesion molecule under much longer timescales, an *in vitro* study showed that cross-linking of ICAM-1 on A549 epithelial cells induced phosphorylation of ERK1/2 that peaked after 10 minutes and returned to baseline by 30 minutes (Krunkosky and Jarrett 2006). Together these data indicate that ICAM-1 linking between alveolar macrophages and epithelial cells could be a viable mechanism for the communication of stretch-induced lung injury and the rapid initiation of inflammatory signalling in VILI.

5.2 Aims

The aim of the experiments in this chapter was to investigate whether a contact-dependent mechanism was responsible for the activation of alveolar macrophages following AEC activation in response to stretch. We hoped to find reduced alveolar macrophage activity after blocking cell surface molecules potentially involved in intercellular interactions. If the blocking experiments were successful, a reduction in MAP kinase activity would be observed in alveolar macrophages following 5 minutes exposure to high stretch ventilation. If a reduction in alveolar macrophage activity was found then the next step would be to perform ventilation experiments under longer timescales to see if the blockade would prevent the development of VILI in terms of cytokine secretion and cell infiltration into the alveolar space, as well as changes in respiratory mechanics.

The first target was gap junctions which were blocked using a connexin mimetic peptide Gap26. The second target was the CD18 adhesion molecule which was blocked using an anti-CD18 antibody. We

hypothesised that if a contact-dependent mechanism was responsible for alveolar macrophage activation in response to stretch, then based on the current literature these two targets would be the most likely candidates.

5.3 Protocol

Mice were pre-treated intranasally prior to the mechanical ventilation protocol as described in previous chapters. Mice were put into an anaesthetic induction box filled with oxygen and 3.5% isoflurane. After 3 minutes, mice were briefly removed and a 25µl dose of either 1mg/ml Gap26 peptide inhibitor (or saline control) or 1mg/ml anti-CD18 antibody (or isotype control) was placed onto the nares and was instantly inhaled. Mice were returned to the induction box for a further 1 minute prior to administering the second 25µl dose. Mice were once again put into the induction box for a final 30 seconds before returning to their cage where they regained consciousness within 2 minutes.

Mice were pre-treated with Gap26 or saline 90 minutes prior to ventilation. Two saline-treated controls were subjected to 5 minutes of low and high stretch ventilation, whilst the Gap26-treated mice were subjected to 5 minutes of high stretch. Studies that have used the peptide under *in vivo* or *ex vivo* conditions have found the drug to be most effective after 1 hour (Desplantez et al. 2012; Parthasarathi et al. 2006; Westphalen et al. 2014). We pre-treated mice for 90 minutes to minimise any lasting effects of the fluid bolus on the ventilation mechanics.

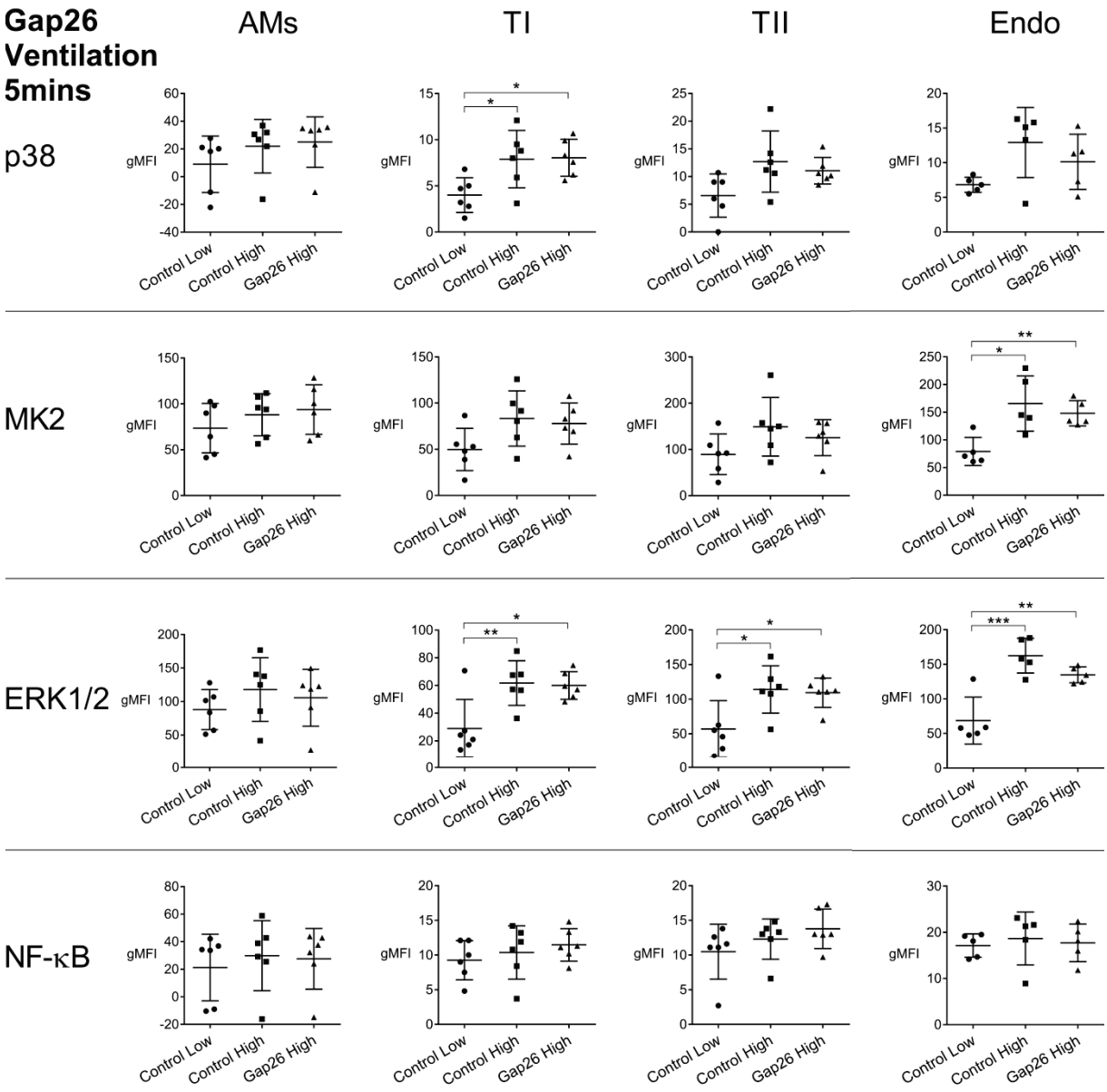
Mice were pre-treated with anti-CD18 or isotype antibody for 24 hours prior to ventilation. The isotype-treated control mice were subjected to 5 minutes of low or high stretch ventilation, whilst the anti-CD18 treated mice were subjected to 5 minutes of high stretch. A large percentage of antibody (54%) has been shown to be retained within the murine lung for up to 24 hours following inhalation (Pereira 2012).

5.4 Results

Following saline instillation, activation of the AECs and pulmonary endothelial cells corresponded closely with the previous results reported in Chapter 4, suggesting that the 50µl bolus instilled 90 minutes earlier did not significantly impact on their responsiveness to high stretch ventilation (Figure 5.1). Each of the parenchymal cell types demonstrated significant ERK1/2 activation after 5 minutes, and this was found to still be significantly activated in the Gap26 pre-treated mice. As before, the endothelial cells also demonstrated significantly increased MK2 activation that again was

not affected by Gap26 pre-treatment. The AECs demonstrated trends for MK2 activation, which in previous experiments had reached statistical significance in the type II AECs. p38 demonstrated trends for increased phosphorylation across the parenchymal cells that correlated with previous results. However, rather disappointingly, the alveolar macrophages did not display activation following 5 minutes high stretch ventilation in the saline pre-treated controls (Figure 5.1). Therefore, it was impossible to determine the effect of the Gap26 peptide on the possible communication between alveolar macrophages and AECs in response to stretch.

Figure 5.1 Phosphorylation of activation markers in pulmonary cell types after intranasal pre-treatment with saline or Gap26 peptide 90 minutes prior to 5 minutes of low or high stretch ventilation



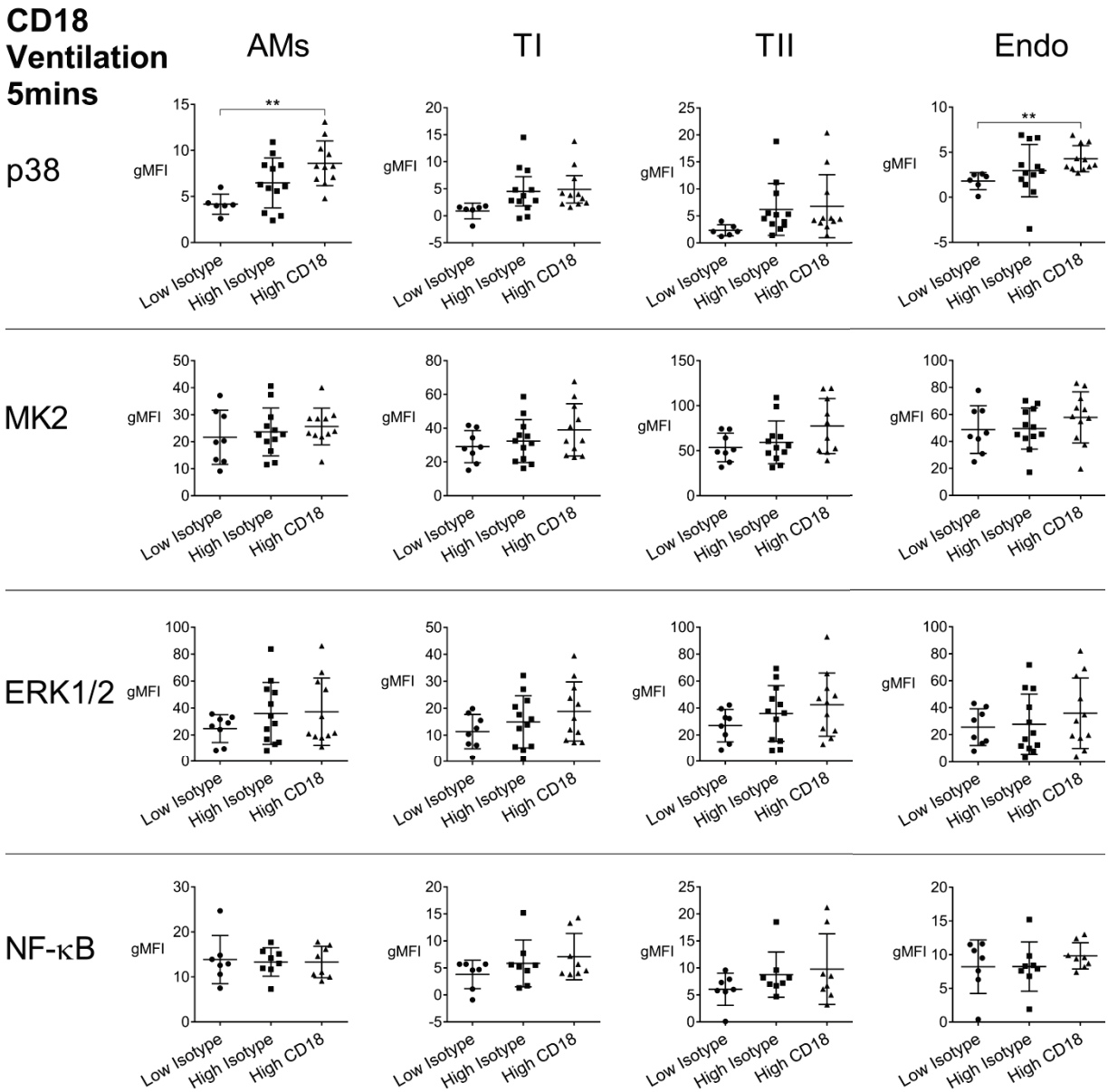
*The pre-treatment procedure appeared to reduce the cellular activation of alveolar macrophages in response to high stretch, and so the potential protective effects of the Gap26 peptide could not be determined. N=5-9, *p<0.05, **p<0.01, ***P<0.001. Data were analysed by ANOVA with Tukey HSD and are displayed as mean ± SD.*

It appeared that the short pre-treatment time of 90 minutes that was necessary for effective gap junction inhibition was influencing the sensitivity of the alveolar macrophages to stretch. The most likely explanation for this may be that the alveolar macrophages were activated by the instillation of

the fluid bolus which made them less responsive to further stimulation for a period afterwards. However, inhibition with the Gap26 peptide was thought not to be compatible with longer pre-treatment times, given the rapid turnover rate of gap junctions and the loss of the small peptide from the lung.

Therefore, we moved on to investigate the effects of CD18 blockade on the activation of alveolar macrophages in response to high stretch ventilation. The anti-CD18 antibody was expected to remain within the alveolar space for longer (Pereira 2012) and had been previously shown to work following 24 hour pre-treatment. Unfortunately, it was not possible to determine the effect of CD18 blockade on the initiation of VILI as no activation of any of the cell types was detected (Figure 5.2). In the experiments from the previous chapter we found significant activation of MK2 and ERK1/2 in alveolar macrophages following 5 minutes of high stretch ventilation. However, 5 minutes of high stretch ventilation in the current experiments, in which controls were treated with isotype antibody, did not demonstrate alveolar macrophage activation compared with low stretch ventilation. These results made it impossible to determine whether CD18 blockade reduced cellular activation in response to stretch. Even the AECs and pulmonary endothelial cells showed completely diminished activation (Figure 5.2) compared with the substantial activation of these cell types observed in previous experiments.

Figure 5.2 Phosphorylation of activation markers in pulmonary cell types after intranasal pre-treatment with isotype or anti-CD18 antibody 24 hours prior to 5 minutes of low or high stretch ventilation

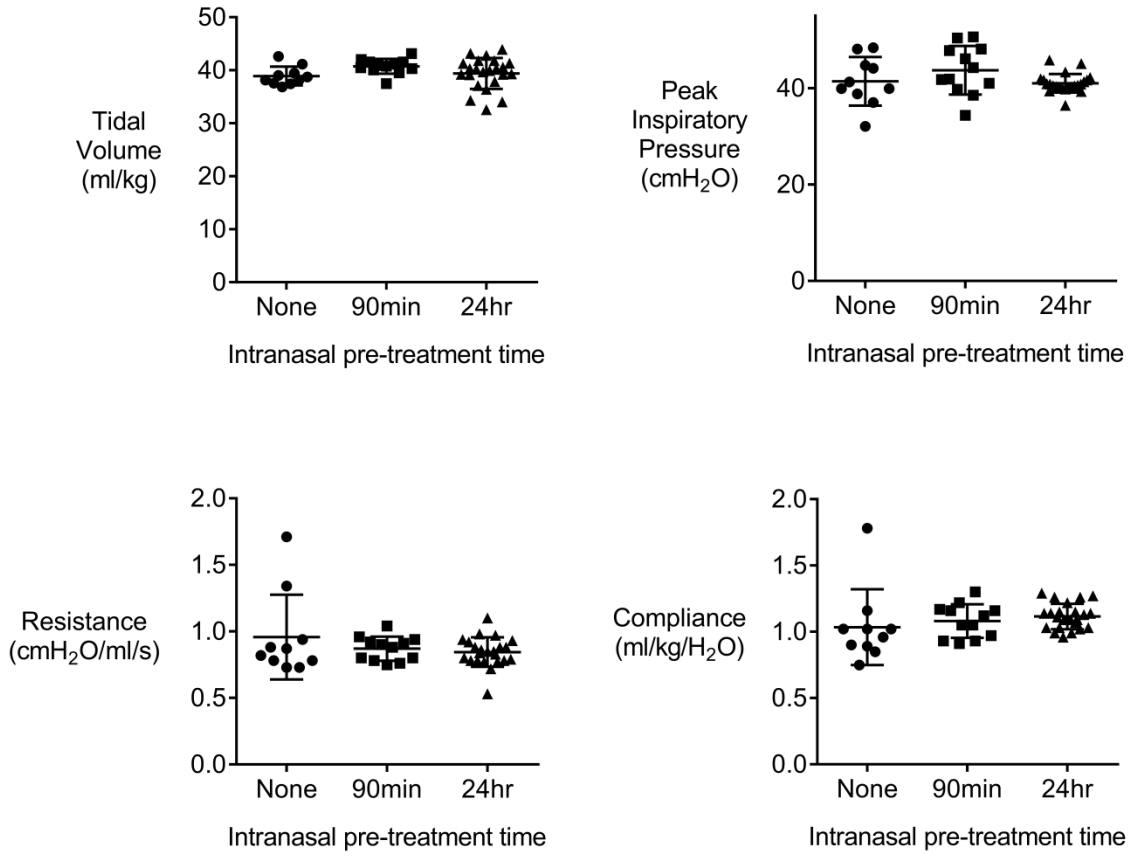


*Some aspect of the pre-treatment procedure itself appeared to influence cellular responses to high stretch ventilation with even isotype-treated controls displaying reduced activation. N=6-12, *p<0.05, **p<0.01. Data were analysed by ANOVA with Tukey HSD and are displayed as mean ± SD.*

The data from this chapter indicated that some technical aspect of the 'pre-treatment protocol' was having a major impact within the lung that increased with time. One possible explanation was that the administration of fluid was influencing the respiratory mechanics such that the animals were not being ventilated with a high enough tidal volume to stimulate a response. Analysis of the respiratory

mechanics revealed that there was no effect on resistance or compliance, with no changes in tidal volume or PIP between the pre-dosing regimens (Figure 5.3).

Figure 5.3 Respiratory mechanics following intranasal dosing



There were no changes in respiratory mechanics at 90 minutes or 24 hours after intranasal pre-treatment with isoflurane. Controls (none) did not receive any kind of pre-treatment and respiratory mechanics were taken from 5-minute ventilation experiments reported in Chapter 4. The effect of the time delay between pre-treatment and ventilation was analysed rather than the effect of the individual drugs. Therefore the respiratory mechanics from control and drug-treated mice ventilated with high tidal volume ventilation were combined. N=10-23.

5.5 Discussion

Gap junction blockade

In the gap junction experiments, which involved the instillation of small Gap26 peptide into the lung, parenchymal cell activity demonstrated cellular activity in response to 5 minutes of high stretch ventilation that correlated with previous findings. However, the alveolar macrophage response was attenuated which made it impossible to assess the effectiveness of this treatment on intercellular communication via gap junctions. It appeared that the isotype treated mice that were ventilated with low stretch showed increased activation compared with previous data from Chapter Four rather than the isotype treated mice ventilated with high stretch showing less activation. Despite no significant changes to the respiratory mechanics, we concluded that the fluid bolus must be having an adverse effect within the lung that is still present after 90 minutes. Perhaps the bolus activated the alveolar macrophages which made them more sensitive to the low stretch ventilation.

CD18 blockade

Cellular activation in response to 5 minutes of high stretch ventilation was completely diminished in isotype antibody pre-treated mice in the CD18 blockade experiments. This effect was potentially due to the intranasal instillation of large antibodies into the lungs. Monoclonal antibodies are known to sometimes cross-link receptors and inadvertently activate them as opposed to inhibit them, and in addition, their Fc domains can induce complement activation (Mulligan et al. 1993). Either of these affects may have dampened the lungs responsiveness to further insults. Within the 24 hours between antibody dosing and ventilation, the pre-treatment procedure could have induced changes in gene regulation that made the cells within the alveolar space less responsive to insults such as over-stretching.

It may be possible to overcome the apparent complications induced by the instillations of whole antibodies into the lungs. One option would be to fragment the antibodies to remove the Fc domain (Holt et al. 2003), however, previous attempts to apply this method within our lab have shown that the antibody yield tends to be very low. Another alternative may be to use siRNA delivered in liposomes to knockdown the expression of β_2-intergrins.

The pre-treatment procedure was found to have no effect on the respiratory mechanics at 90 minutes or 24 hours after intranasal dosing. This suggests that the dosing was not having a long-term effect on the 'stretchiness' of the lungs, which could have accounted for the reduced

parenchymal cell activity following injurious ventilation. In comparison to the previous data reported in Chapter 4, the MAP kinase levels seemed to be generally lower across all isotype/anti-CD18 conditions. This may have been caused by the induction of general anaesthesia and intranasal instillation, which may have induced changes in respiratory rate or caused brief hypoxia.

Isoflurane exposure

A possible explanation would seem to be that the isoflurane used during intranasal dosing was having an effect within the lung. This would be rather unexpected as the mice were only exposed to isoflurane for a few minutes and at a time point way before ventilation experiments were conducted. It appeared that only the alveolar macrophages demonstrated reduced activation in response to stretch just 90 minutes following isoflurane exposure, whilst all cell types demonstrated reduced activation 24 hours after exposure. The use of intranasal instillations under brief isoflurane anaesthesia enables quick and easy administration of the blocking agent directly into the lungs without the side effects that accompany general anaesthesia using ketamine and xylazine. However, it may have provided a protective effect itself which was unexpected, but interesting.

Isoflurane preconditioning is reported to prevent inflammation to subsequent pulmonary insults such as sepsis. Li et al. reported that a 30-minute period of isoflurane exposure immediately prior to systemic delivery of LPS significantly reduced hypotension, pulmonary oedema and mortality in rats (Li et al. 2009). Similar effects have been reported in another model of sepsis by Bedirli et al. who found that 30 minutes exposure to isoflurane immediately prior to caecal ligation and puncture reduced levels of TNFα, IL-1β and IL-6 in plasma (Bedirli et al. 2012).

Isoflurane has also proved protective in models of direct administration of LPS into the lungs. Reutershan et al. exposed mice to 30 minutes of isoflurane at different times before LPS inhalation and found that pulmonary oedema and neutrophil infiltration were attenuated when the isoflurane pre-treatment was given 1 or 12 hours (but not 4, 6 or 24 hours) prior to LPS delivery (Reutershan et al. 2006). Another study found that a 60-minute isoflurane exposure period, 60 minutes prior to intratracheal LPS ameliorated the release of pro-inflammatory cytokines including TNFα, IL-1β and IL-6 (Chung et al. 2013). The mechanisms behind the apparent protective effect of isoflurane preconditioning is unclear but has been suggested to involve iNOS signalling, reactive oxygen species (ROS) and inhibition of NF-κB signalling (Pang et al. 2012; Chung et al. 2013). Whilst these studies use isoflurane exposure times that are much longer than the mice in my experiments would have been exposed to, they do indicate that exposure to isoflurane could reduce inflammation in response to an insult given up to 12 hours later.

Another mechanism by which isoflurane is thought to be protective is through the down regulation of adhesion molecules such as ICAM-1 and CD11b, which could explain the often-described effect of reduced infiltration of neutrophils into the alveolar space (de Rossi et al. 2002; Reutershan et al. 2006; Bedirli et al. 2012). These studies suggest that similar effects may apply to alveolar macrophages that would be exposed to high concentrations of the inhaled isoflurane. Reduced adhesiveness of alveolar macrophages to the alveolar wall was the aim of the drugs given in this chapter; however it may be that the isoflurane also reduced adhesion. If this effect was the reason for the reduced activation observed in alveolar macrophages treated only with isotype antibody or saline vehicle in response to high stretch ventilation, then we may conclude that initiation of inflammation in VILI could indeed be mediated by a direct-contact method of communication.

Conversely, a recent study reported that the protective effects of isoflurane in a two-hit model of inhaled endotoxin and mechanical ventilation are mediated through the up-regulation of adhesion molecules (Englert et al. 2015). The group concluded that isoflurane is protective against ARDS by preserving the integrity of the alveolar-capillary barrier.

Isoflurane has also been shown to be protective in one-hit models of VILI. Vaneker et al. demonstrated that mice ventilated for 4 hours with a tidal volume of 8ml/kg showed increased levels of IL-1β and TNFα in their BAL fluid that was reduced when isoflurane was added to the ventilation gas (Vaneker et al. 2009). Faller et al. reported further protective effects in mice ventilated at 12ml/kg in which isoflurane prevented alveolar wall thickening, neutrophil transmigration and secretion of CXCL2 and IL-1β. This group also reported that the effects of isoflurane might be mediated through Akt signalling, as inhibition of Akt phosphorylation prevented isoflurane-induced protection (Faller et al. 2012). Sevoflurane, another volatile anaesthetic which is structurally similar to isoflurane and used more frequently in human patients, has also been shown to offer protection against VILI in animal models (Kim et al. 2015; Xiong et al. 2013; Romero et al. 2015). A study found that the immunomodulatory effects of these volatile anaesthetics was caused by the fluorinated carbon groups, which reduced the expression of inflammatory mediators following LPS stimulation of epithelial and endothelial cells *in vitro* (Urner et al. 2011).

A review of the current literature on the immunosuppressive effects of isoflurane suggests that it may indeed have had a major impact on the present experiments. This effect rendered this intranasal dosing model unusable if we were to determine the mechanism of communication between alveolar macrophages and AECs. Nevertheless, the results potentially suggest that even very short exposure to isoflurane could have long-lasting protective effects on the development of VILI that may be worth exploring further.

CHAPTER SIX

THE ROLE OF TNFα SIGNALLING IN THE INITIATION OF VILI

6.0 Chapter summary

TNFα has been frequently implicated in the development of VILI but so far, studies have only investigated its role following several hours of mechanical ventilation. TNFα signalling is unlike many other cytokines in that it is expressed in a membrane-bound form that enables shedding and release within 30 minutes. The experiments presented in this chapter sought to determine whether TNFα signalling could be involved in the initiation of inflammatory signalling in response to stretch using TNF receptor knockout mice. The role of TNFα signalling in LPS-induced lung injury was also investigated. The results of these experiments indicated that following LPS instillation, TNFα signalling is potentially involved in mediating the activation of MAP kinase pathways within parenchymal cells, which was observed after 15 minutes in wild type mice. However, the impact of TNFα signalling in the early stages of stretch-induced inflammatory signalling was less clear. TNF receptor knockout mice appeared to display reduced activation of alveolar macrophages and type II alveolar epithelial cells, though non-significant trends of activation were still apparent, suggesting that additional signalling pathways may be involved.

6.1 Background

The previous chapter highlighted difficulties in pre-treating mice with intranasal instillations, and so we moved on to examine the role of TNFα in the initiation of inflammatory signalling in VILI using a strain of genetically modified mice. TNFα is a pro-inflammatory cytokine that has been frequently implicated in experimental lung injuries of several aetiologies including LPS (Bertok et al. 2011), acid

(Patel et al. 2013) and VILI (Wilson, Choudhury, and Takata 2005) within our research group. In the clinical setting, increased levels of TNFα in the BAL fluid of patients have been correlated with the development of ARDS (Suter et al. 1992). However, clinical trials examining the effects of anti-TNFα therapy have so far had limited success in reducing mortality in sepsis (Fisher et al. 1996; Abraham et al. 1998; Abraham et al. 1995; Qiu et al. 2013).

This could be due to the existence of two TNF receptors which are now known to induce different and opposing cellular responses (MacEwan 2002). The two TNF receptors have different affinities for the two forms of TNFα which can be presented as membrane-bound or in its soluble form that is generated by the proteolytic cleavage of membrane-TNFα by TNFα converting enzyme (TACE) (Horiuchi 2013). The p55 TNF receptor (TNFR1) is ubiquitously expressed and can be activated by both the soluble and membrane-bound forms of TNFα (Grell et al. 1998), leading to pro-inflammatory signalling. The p75 TNF receptor (TNFR2) is expressed at low levels on immune cells and is predominantly activated by membrane-bound TNFα as the soluble form only binds transiently (Grell et al. 1995). Most studies investigating TNFα signalling have utilised the soluble TNFα as a source of stimulation, and therefore much less is known about the p75 receptor, though it is thought to promote pro-survival signalling.

Cellular responses to membrane or soluble TNFα differ due to their binding to specific TNF receptors, which activate different intracellular signalling pathways. The binding of TNFα to the p55 receptor transiently activates the MAP kinase JNK, whilst p75 activation leads to a more sustained activation profile. Other MAP kinases such as p38 and ERK1/2 however, are thought to be exclusively activated by TNFα binding to the p55 receptor (Jupp et al. 2001). The two receptors also differ in their activation of NF-κB, which is activated within minutes through p55 stimulation that lasts for a few hours whereas NF-κB activation following p75 binding is delayed but can last for up to 24 hours (Marchetti et al. 2004). The p55 receptor complex contains a death domain which promotes signalling that leads to caspase activation and apoptosis. However, there is evidence to suggest that activation of the p75 receptor, despite not containing a death domain, can also promote apoptosis via receptor cross talk (Figure 6.1) (Naude et al. 2011).

Figure 6.1 TNF p55 (TNFR1) and p75 (TNFR2) receptor signalling

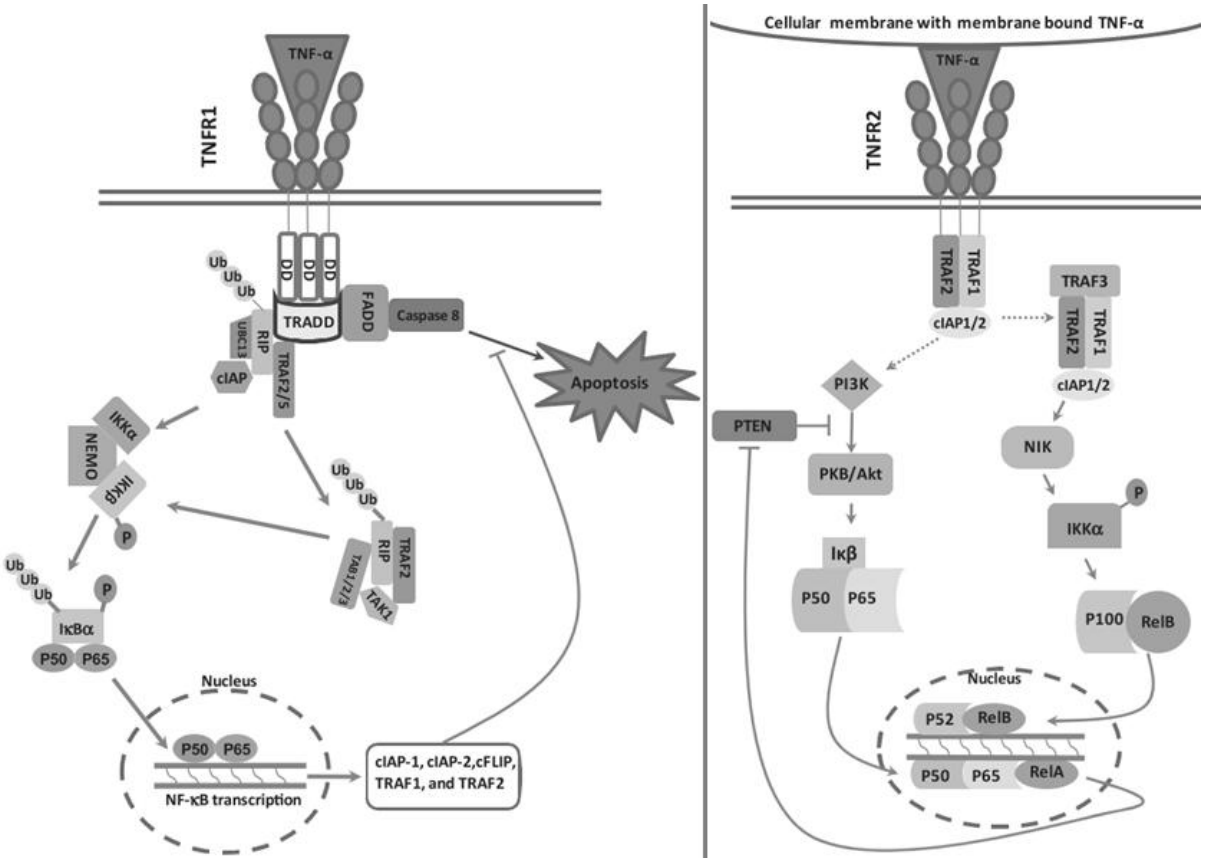


Figure adapted from (Naude et al. 2011).

Both types of TNF receptor may be cleaved from the cell surface, in which form they retain the capability to bind TNFα. These soluble TNF receptors are present under normal conditions but can increase in response to a wide range of stimuli including endotoxin (Spinas, Keller, and Brockhaus 1992). Their function is unclear, though the predominant theory suggests that they act as a buffer, regulating the levels of soluble TNFα in circulation. The binding of soluble TNF receptors to TNFα does not induce signalling as it would on the cell surface, and so their role is thought to be anti-inflammatory. In this instance the binding of TNFα may prevent it from binding cell surface receptors and therefore neutralise its effect (Aderka et al. 1992). Alternatively, binding of TNFα to soluble receptors may preserve its biological activity and prolong the presence of circulating TNFα.

Several studies have shown that signalling via the two TNF receptors leads to different responses *in vivo*. Peschon et al. demonstrated how signalling via the p55 receptor exacerbated injury in knockout mice exposed to intranasal LPS through enhanced neutrophil infiltration into the lungs (Peschon et al. 1998). Whilst another study showed that p55 signalling shortened survival and induced hypothermia in a murine model of sepsis caused by caecal ligation and puncture, whereas

signalling via the p75 receptor was found to provide protection (Ebach, Riehl, and Stenson 2005). A study within our research group has also demonstrated that p55 signalling is injurious in a murine model of acid aspiration in which knockout mice lacking the p55 receptor showed improved barrier function and reduced caspase-8 activity (Patel et al. 2013).

TNFα signalling has been shown to play an important role in the development of VILI in previous research by our group. High tidal volume ventilation has been shown to promote increases in TNFα mRNA in intra-alveolar cells and induce secretion of TNFα protein into the BAL fluid of rabbits (Takata et al. 1997; Imai et al. 1999). Furthermore, anti-TNFα antibody reduced deteriorations in oxygenation and lung compliance as well as reducing neutrophil sequestration in this rabbit model of VILI (Imai et al. 1999). In mouse models of VILI, TNFα signalling has also been implicated in neutrophil infiltration, which was shown to be attenuated in both double knockout mice lacking both TNF receptors by up to 45% and in anti-TNFα antibody treated wild type mice by up to 70% (Wilson, Choudhury, and Takata 2005).

The two TNF receptors have also been demonstrated to have opposing roles in the development of VILI in previous research by our group. In agreement with findings in other models of lung injury, the p55 receptor was found to be responsible for injurious signalling whilst p75 signalling was found to be protective. Mice lacking the p55 receptor were found to be protected from VILI, demonstrating reduced pulmonary oedema, whilst mice lacking the p75 receptor were more susceptible to VILI (Wilson et al. 2007).

These results were further confirmed using domain antibodies targeted against the p55 receptor which were given intratracheally to ventilated mice and led to reduced derangements in respiratory mechanics, gas exchange and protein accumulation within the alveolar space. This effect was not apparent in mice treated with a general anti-TNFα antibody which would have inhibited both the injurious and protective signalling pathways (Bertok et al. 2012). Inhibition of p55 signalling has also been demonstrated to be protective in a two-hit model of LPS and VILI, in which a p55 domain antibody was found to reduce neutrophil recruitment, alveolar macrophage activation and the release of cytokines and chemokines into the BAL fluid. Alveolar macrophage activation was demonstrated by an increase in expression of ICAM-1 which was attenuated by inhibition of TNFα signalling via the p55 receptor (Bertok et al. 2012).

Whilst the work by our group and others clearly implicate TNFα signalling in the pathogenesis of VILI specifically through the p55 receptor, the reported studies all examine the effects of TNFα on lung injury following several hours of high tidal volume ventilation. TNFα signalling has the capacity to be

very quickly activated due to its presence on the surface of cells and through cleavage by TACE; however the role of TNFα signalling in the initiation of VILI remains unknown. The availability of TNF receptor knockout mice maintained by the group enabled the investigation of the role of TNFα without the need for pre-treatment, which had been found in the previous chapter to cause complications. Experiments were conducted in double knockout mice that lack both the p55 and p75 TNF receptors. These double knockout mice have been shown to be equally susceptible to ventilator-induced pulmonary oedema as wild type mice (Wilson et al. 2007), but importantly, recruit fewer neutrophils following VILI (Wilson, Choudhury, and Takata 2005), which suggests that the inflammatory response may be dampened in these animals.

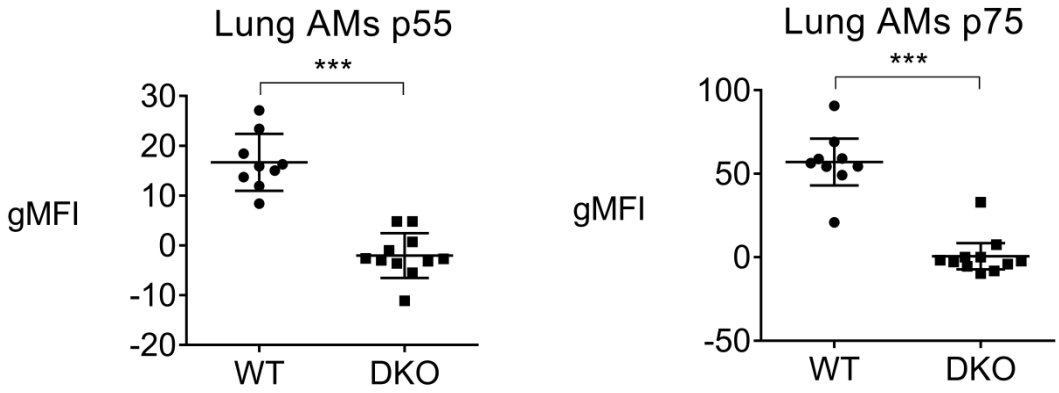
6.2 Aims

The aim of the experiments in this chapter was to investigate the involvement of TNFα in the initiation of inflammatory signalling in VILI. TNFα signalling has been associated with the development of VILI over longer timescales, however we wished to determine whether it was responsible for the initial communication between AECs and alveolar macrophages that leads to their activation in response to stretch. Despite there being no evidence of TNFα release from AECs specifically in response to stretch, they have been shown to release other cytokines following stretch (Vlahakis et al. 1999) and are known to be capable of releasing TNFα when stimulated with LPS (Wong and Johnson 2013; Thorley et al. 2007).

6.3 Protocol

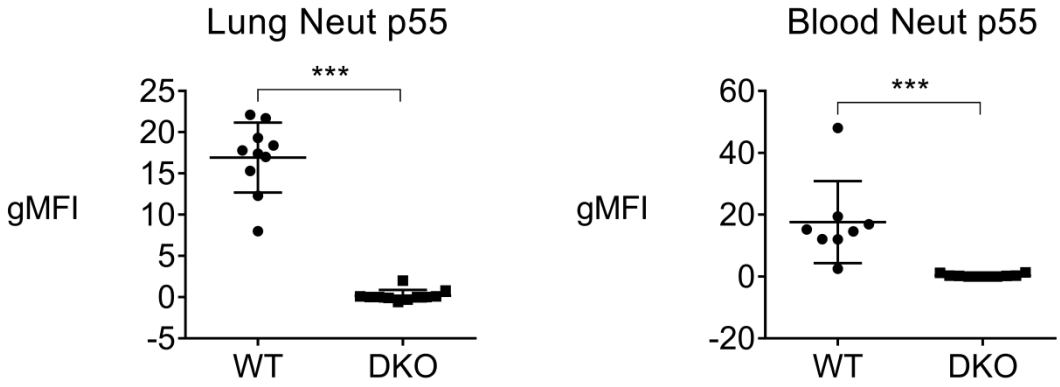
TNF receptor double knockout mice were bred on site and male pups were used for experiments at approximately 8-12 weeks old. The TNF receptor DKO mice have mutations in the two receptors that prevent TNF from binding. The phenotype of the TNF receptor knockout mice was confirmed by flow cytometry using antibodies against both the p55 (PE TNFR1; 55R-289; BioLegend) and p75 receptors (PE TNFR2; TR75-89; BioLegend). The gMFI detected with the appropriate isotype control (PE Armenian Hamster IgG; HTK888; BioLegend) was subtracted and results were compared with wild type mice. Data on wild type TNF receptor expression was provided by Charley Oakley. Expression levels were checked for alveolar macrophages (Figure 6.2), neutrophils (Figure 6.3) and monocytes (Figure 6.4) within the lung and blood for at least one mouse per litter. All cell types that demonstrated expression in wild types showed diminished expression in the double knockout mice.

Figure 6.2 Expression of TNF receptors on alveolar macrophages from wild type and TNF receptor double knockout mice



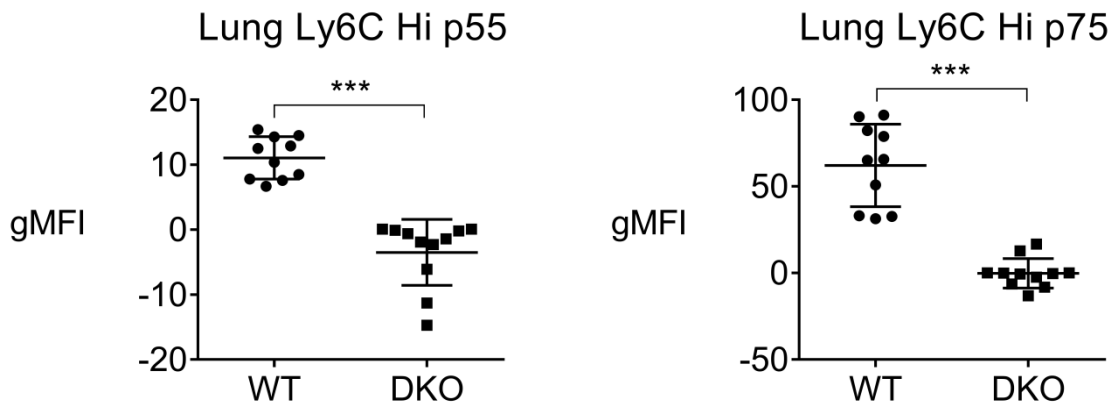
*Alveolar macrophages from wild type (WT) mice demonstrated expression of both TNF receptors p55 and p75 whereas the double knockout (DKO) strain showed no expression. Data on wild type TNF receptor expression was provided by Charley Oakley. N=9-11, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

Figure 6.3 Expression of TNF receptors on neutrophils from wild type and double knockout mice



*Neutrophils displayed diminished p55 expression in both lung and blood samples. Expression of p75 was very low on neutrophils from wild type mice and therefore the data have not been presented. Data on wild type TNF receptor expression was provided by Charley Oakley. N=8-10, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

Figure 6.4 Expression of TNF receptors on Ly6C high monocytes from wild type and double knockout mice



*Expression of p55 on Ly6C high monocytes was significantly reduced in knockout mice in the lungs. p75 expression was significantly reduced. Expression of p55 and p75 was very low on Ly6C low monocytes from wild type mice and therefore the data have not been presented. Data on wild type TNF receptor expression was provided by Charley Oakley. N=10-11, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

As for the previous ventilation experiments, mice were anaesthetised by intraperitoneal injection of ketamine (80ml/kg) and xylazine (8ml/kg). An endotracheal tube was inserted and mechanical ventilation initiated under low stretch settings (8ml/kg). Mice either remained on the low stretch settings or were switched onto the high stretch strategy (40ml/kg). Ventilation experiments were conducted for 5 minutes prior to termination of the experiment and sampling of the lungs for phospho-flow cytometry as previously described.

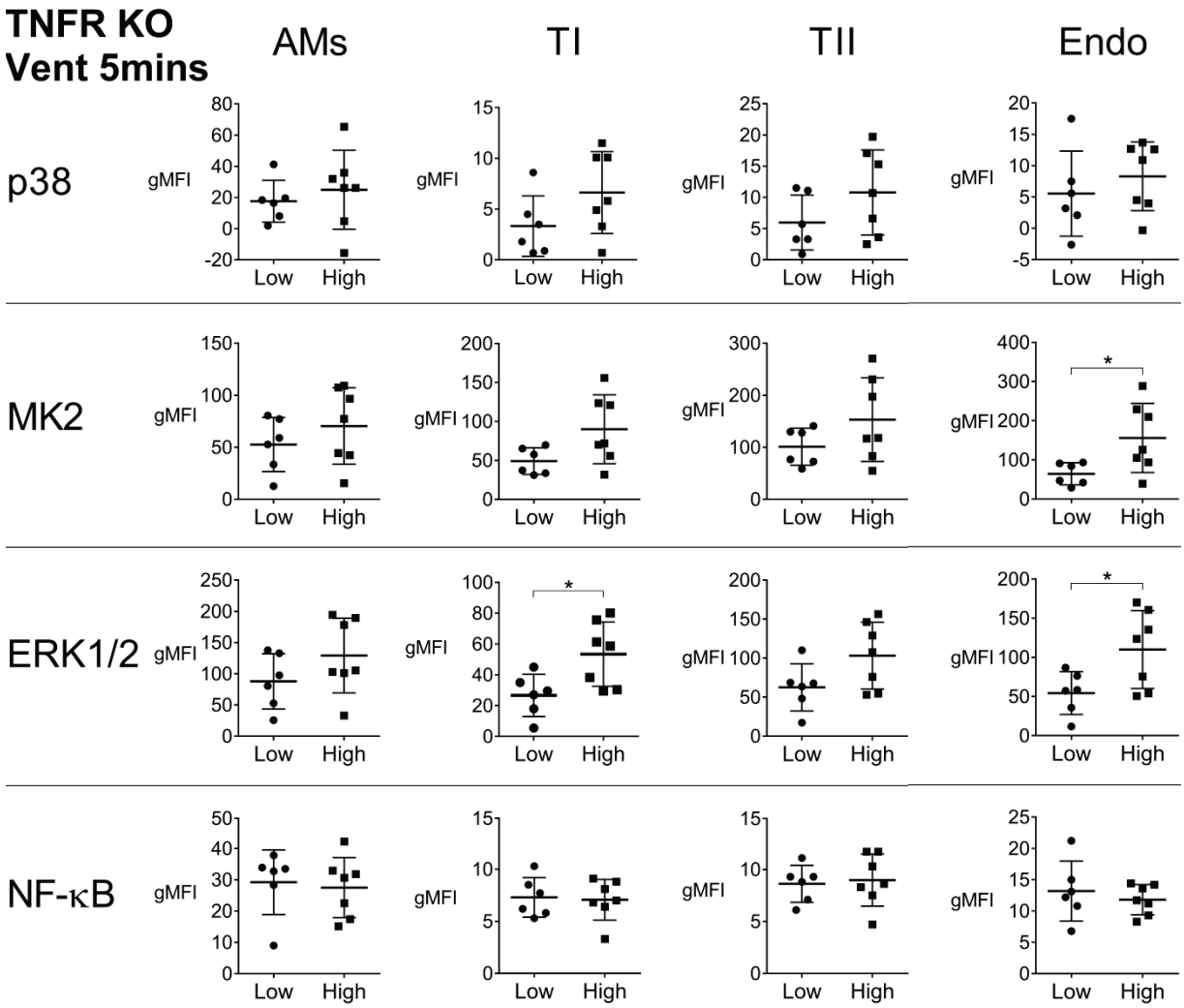
TNF receptor double knockout mice were also subjected to 5 or 15 minutes exposure to intratracheal LPS. Mice were anaesthetised with ketamine and xylazine and then held to a board that was stood almost vertical for the brief duration of the instillation procedure. A microscope and external light source were used to pass a fine catheter through the vocal chords to administer the LPS bolus (20µg in 50µl saline). Mice were held upright for 30 seconds following instillation before being laid down for the insertion of the endotracheal tube for lung sampling.

6.4 Results

The results from the current study showed that double TNF receptor knockout mice exposed to 5 minutes of high stretch ventilation demonstrated similar parenchymal activation to that of wild type

mice as observed in previous experiments reported in Figure 4.3, Chapter 4. The double knockout mice displayed significant MK2 and ERK1/2 phosphorylation within endothelial cells and the type I AECs showed significant ERK1/2 activation and trends for increased MK2 (p=0.057) and p38 (p=0.128) phosphorylation (Figure 6.5). Whilst no significant activation was detected in the type II AECs, there was a non-significant trend for ERK1/2 activation (p=0.078) as well as for p38 (p=0.166) and MK2 (p=0.106). The alveolar macrophages displayed no significant activation following stretch in the knockout mice, whilst the wild types displayed clear MK2 and ERK1/2 activation in macrophages after 5 minutes of high stretch (Figure 4.3, Chapter 4).

Figure 6.5 Phosphorylation of activation markers in pulmonary cell types after exposure of TNF receptor double knockout mice to 5 minutes of low or high stretch ventilation

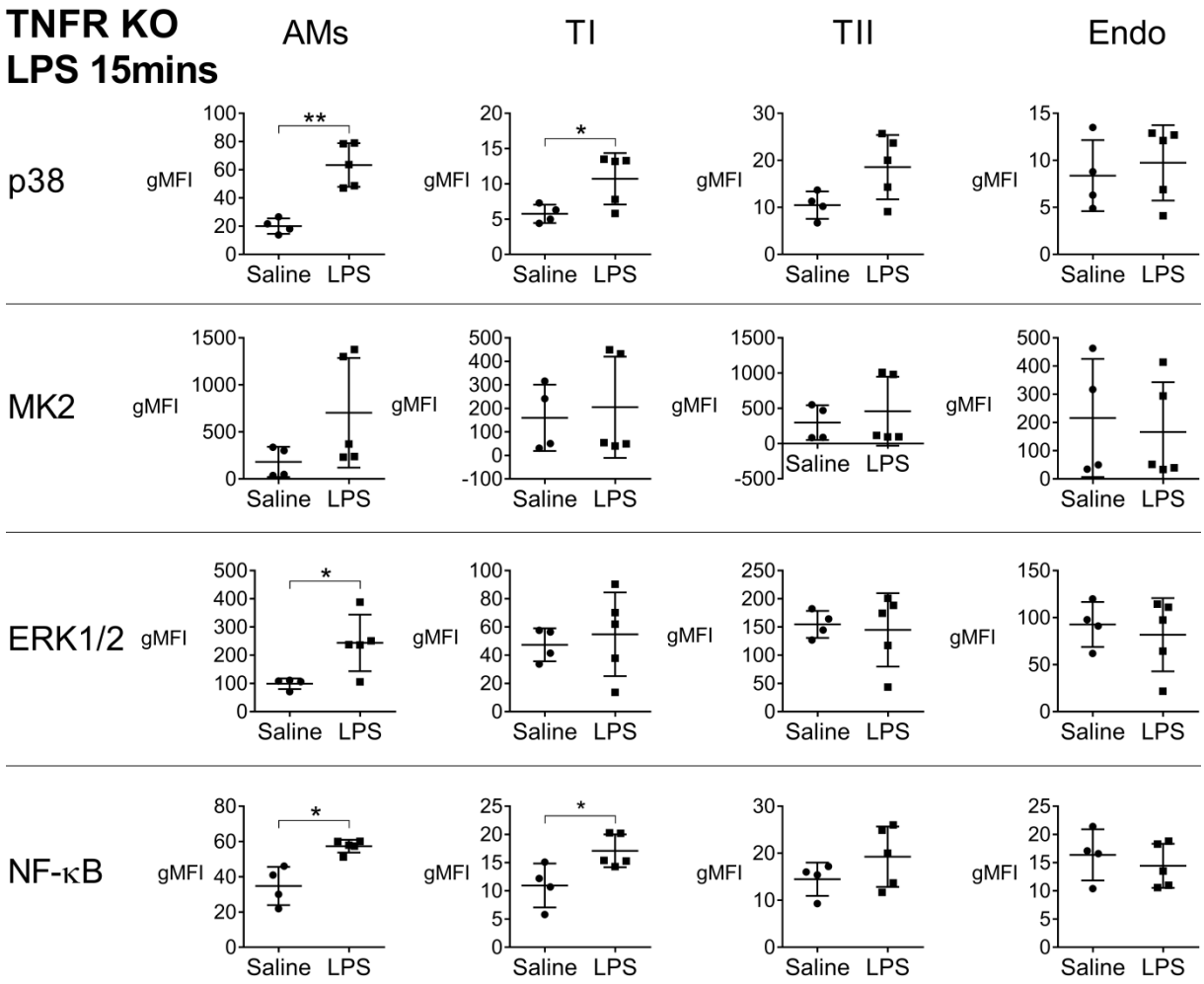


*ERK1/2 was significantly activated in TI and endothelial cells and MK2 was significantly activated in endothelial cells after 5 mins high stretch compared with low in TNF receptor DKOs. N=6-7, *p<0.05. Data were analysed by t-test and are displayed as mean ± SD.*

As well as studying the effect of 5 minutes of high stretch ventilation in mice lacking TNF receptors, we also examined the role of TNFα signalling in LPS-induced lung injury. In previous experiments with wild type mice (Figure 3.4, Chapter 3), significant MAP kinase activation was detected in parenchymal cells after 15 minutes of LPS exposure and significant NF-κB activation after 5 minutes. The role of TNFα in the activation of MAP kinase signalling within the lung parenchyma was examined in the TNF receptor double knockout mice.

The knockout mice were found to display similar levels of alveolar macrophage activation compared with wild types after 15 minutes of LPS (Figure 6.6). They showed significant phosphorylation of p38, ERK1/2 and NF-κB and a non-significant trend for increased MK2 ($p=0.117$) all with very similar fold increases in phosphorylation levels as observed in Figure 3.4, Chapter 3. The parenchymal cells however displayed an obvious lack of activation following LPS exposure for 15 minutes compared with that seen in the wild type mice which displayed significant NF-κB and MAP kinase activity at this time point (Figure 3.4, Chapter 3). In the knockout mice, the type I AECs demonstrated significant activation of p38 and NF-κB, and the type II AECs did not demonstrate any significant activation but showed a trend for increased p38 phosphorylation ($p=0.065$). The endothelial cells also did not display any significant activation following 15 minutes exposure to intratracheal LPS.

Figure 6.6 Phosphorylation of activation markers in pulmonary cell types after exposure of TNF receptor double knockout mice to 15 minutes of intratracheal LPS compared with saline



*AMs displayed significant activation of p38, ERK1/2 and NF-κB, whilst TI AECs displayed significant activation of p38 and NF-κB following 15 mins exposure to LPS compared with saline in TNF receptor DKOs. N=4-5, *p<0.05, **p<0.01. Data were analysed by t-test and are displayed as mean ± SD.*

6.5 Discussion

LPS

The results from the TNF receptor double knockout mice showed that TNFα does not mediate the rapid activation of alveolar macrophages observed within 15 minutes exposure to intratracheal LPS. The alveolar macrophages displayed identical responses to those from wild type mice at 15 minutes after LPS instillation. This is unsurprising since alveolar macrophages express high levels of TLR-4 and are well known to respond directly to LPS ligation.

However, LPS instillation in TNF receptor knockout mice did not induce the same amount of activation in the parenchymal cells as was observed in the wild type mice. Apart from some p38 activation, the knockouts displayed a clear lack of MAP kinase activity and even showed reduced NF-κB activation, which was previously detected within 5 minutes of LPS instillation in wild type mice. These results suggest that TNFα may be responsible for activation of these signalling pathways at 15 minutes in the parenchymal cells.

Alveolar macrophages are likely to be the source of TNFα following detection of LPS, which may then activate MAP kinase signalling in the AECs. Release of soluble TNFα by alveolar macrophages would activate p55 signalling which is associated with MAP kinase activation (Jupp et al. 2001). However, membrane-bound TNFα is capable of initiating both p55 and p75 signalling and so could also initiate signalling through direct contact with the alveolar epithelium.

The alveolar macrophages residing within the alveoli would be unable to directly communicate with the pulmonary endothelial cells however intra-alveolar TNFα has been shown to induce rapid responses in endothelial cells in a blood-perfused rat lung by Kuebler et al. In this study TNFα introduced into the alveolar space caused increases in intracellular calcium and $cPLA_2$ activation in AECs which in turn led to an increase in intracellular calcium and P-selectin expression in endothelial cells within 5 minutes (Kuebler et al. 2000). This could explain the activation of endothelial cells that was observed in wild type mice following LPS exposure for 15 minutes, which was abolished in the TNF receptor knockout mice.

VILI

In the ventilation experiments, the TNF receptor double knockout mice appeared to display slightly reduced cellular activation in some cell types in response to stretch compared with wild type mice. The endothelial cells and type I AECs both displayed identical patterns of activation to that observed

in wild types. On the other hand, the type II AECs displayed reduced activation of MK2 and ERK1/2 that were significantly activated in wild type mice, but displayed only a non-significant trend for increased phosphorylation in the TNF receptor knockouts. Despite this lack of statistical significance the type II AEC response was probably not affected by the lack of TNFα signalling following exposure to high tidal volume ventilation.

The alveolar macrophages also displayed slightly reduced activation in response to high stretch ventilation with diminished MK2 activation in the TNF receptor knockouts. This suggests that rapid TNFα signalling could be responsible for the activation of alveolar macrophages in response to stretch. This could occur through the interaction of membrane-bound TNFα on the alveolar epithelium with p75 receptors on the alveolar macrophage surface, brought into contact through the stretching of the alveolar surface. Alternatively, as discussed earlier, membrane TNFα can be rapidly cleaved by TACE giving TNFα the potential to act as a soluble mediator.

A study from our research group found that TACE expression was not upregulated during a 3-hour LPS stimulation of human monocytes, but importantly TACE activity was significantly increased within 15 minutes (Scott et al. 2011). Although this 15-minute time point was the earliest studied in these experiments, it is feasible that TACE activity could be induced even faster. This study suggested that such rapid regulation could be achieved through post-translational modification mediated by reactive oxygen species (ROS) and the p38-MK2 MAP kinase pathway (Scott et al. 2011). Another study stretched cardiomyocytes at 10% constant strain and detected phosphorylation of TACE at a specific tyrosine residue which promotes activity within just 5 minutes (Niu, Wang, and Li 2015). The same group have also looked at TNFα release from murine myoblasts and found a slight increase in TACE activity after 5 minutes of static stretch that became significant within 30 minutes (Niu et al. 2013).

The results from these studies suggest that TACE activity has the potential to be up-regulated within the timescale of our experiments. This could indicate that the shedding of TNFα from the AEC surface could be a potential mechanism by which alveolar macrophages become activated in response to high stretch ventilation. However, this mechanism remains speculative and probably involves other signalling pathways since the alveolar macrophages still demonstrated a trend for activation in the TNF receptor double knockout mice.

Knockout strains

The use of TNF receptor knockout mice in this chapter allowed us to study a lack of TNFα signalling without the need for pre-treatments with possible confounding factors. The main advantage of

knockout mice is that the target gene can be disrupted specifically and efficiently. However the disadvantage of genetically modified mice is that they can develop compensatory mechanisms whereby alternative signalling pathways may be up-regulated. They may respond to stimuli using such alternative signalling pathways that would not normally be activated and can therefore hide the effects of the knocked-out gene. For these reasons, the results of experiments performed using knockout mice should be interpreted with caution.

Conclusions

The data presented in this chapter suggest a role for TNFα signalling in the rapid activation of parenchymal cells following intratracheal instillation of LPS. The role of TNFα in the initiation of inflammatory signalling in response to high tidal volume ventilation remains unclear despite it being frequently implicated in the longer-term derangements of VILI.

CHAPTER SEVEN

CELL STRETCHING IN VITRO

7.0 Chapter summary

In this chapter, an *in vitro* model of VILI was developed to investigate the involvement of soluble mediators in the activation of alveolar macrophages. In an *in vivo* setting, it is impossible to know which cell types have released the soluble mediators that are detected in BAL fluid following injurious mechanical ventilation. However, an *in vitro* cyclic stretching device was utilised in the experiments presented in this chapter, which enabled the examination of cell types in isolation. Pulmonary epithelium and brain endothelium cells were subjected to stretch *in vitro* and the media was analysed for ATP, TNFα and IL-6 content. Murine lung epithelial (MLE) cells were found to release significant levels of the DAMP ATP during 15 minutes of cyclic stretching. These experiments indicated that ATP is capable of being released very rapidly into the extracellular space in response to cyclic stretching and could therefore play a role in the initial activation of alveolar macrophages in VILI.

7.1 Background

An *in vitro* protocol was developed so that pulmonary cells types could be studied in isolation. This would make it easier to determine which cells types were responsible for the release of any soluble mediators that may be involved in the initiation of inflammatory signalling in VILI. Previous work investigating the activation of intracellular cell signalling pathways in cells cultured *in vitro* has been performed using Western blotting. However we wanted to develop a protocol in which we could recover cells from culture plates whilst retaining their integrity for analysis by flow cytometry. This novel method would provide many advantages over Western blotting, including not only quantitative results but also the possibility of looking at co-cultures of two cell types.

The murine monocyte/macrophage RAW cell line has been previously shown to display activation of the p38 and ERK1/2 MAP kinase pathways as well as the NF-κB pathway in response to LPS (Chen et al. 2013; Srivastava et al. 2009; Beinke et al. 2004; Chen and Wang 1999; Rao, Meighan, and Bowman 2002; Sanghera et al. 1996; Swantek, Cobb, and Geppert 1997; Barbour et al. 1998). This cell line was used in pilot studies using LPS stimulation as a positive control for developing a protocol to recover cells from culture plates and analyse them by phospho-flow cytometry.

The human pulmonary epithelial the A549 cell line has been most frequently used to study signalling in response to stretch in the literature. Li et al. stretched A549 cells at 20 cycles per minute up to 15% strain and noted increased activation of p38, ERK1/2 and NF-κB within 15 minutes (Li et al. 2003). Another study that stretched A549 cells up to 15% strain reported increased NF-κB translocation into the nucleus and degradation of IκB within just 10 minutes (Ning and Wang 2007b). A549 cells were used in the present study to confirm that the findings in murine cells were relevant to human cells. In addition, A549 cells grow more uniformly and have been frequently used in cyclic stretching experiments compared with MLE cells, which to my knowledge have only been reported to have been used in one stretching experiment (Dai et al. 2013).

Although bEnd5 cells specifically have not been used to study intracellular signalling in response to cyclic stretch, other pulmonary endothelial cell lines have been utilised for such experiments. Human pulmonary microvascular endothelial cells (HPMVECs) were found to respond to 20% strain at 50 cycles per minute within 5 to 10 minutes through increased phosphorylation of p38 (Iwaki et al. 2009). In another study, rat pulmonary microvascular endothelial cells (RPMVECs) were shown to demonstrate increased phosphorylation of p38 and ERK1/2 within 5 minutes exposure to 18% strain at 20 cycles per minute (Abdulnour et al. 2006).

There is some evidence that pulmonary endothelial cells are capable of releasing soluble inflammatory mediators when stretched *in vitro*. HPMVECs mentioned above were found to secrete IL-6 and CCL2 when subjected to 20% cyclic strain for 12 hours. This study also reported secretion of CXCL8 within the much shorter timescale of 3 hours that was dependent on time and strain and coincided with an increase in CXCL8 mRNA within 1 hour (Iwaki et al. 2009). Another study showed secretion of CXCL8 and CCL2 by primary human umbilical vein endothelial cells (HUVECs) following 24 hours of 15% cyclic strain, which was accompanied by increases in mRNA of both soluble mediators within 3 hours (Okada et al. 1998).

Many more studies have noted soluble inflammatory mediator release by pulmonary epithelial cells in response to being stretched *in vitro*. CXCL8 production in particular has been frequently studied in

stretched epithelial cells, often A549 cells, and has been detected following various periods of stretching from 1 to 24 hours (Pugin et al. 1998; Vlahakis et al. 1999; Oudin and Pugin 2002; Jafari et al. 2004; Li et al. 2003; Ning and Wang 2007b; Ning and Wang 2007a). Primary rat type II AECs have been shown to release various mediators including nitrite and eicosanoids, and to express COX-2 within 12 hours of exposure to 30% cyclic strain (Hammerschmidt et al. 2005). One of the earliest time points studied was in primary rat foetal lung epithelial cells subjected to 17% cyclic strain. This study analysed the culture medium by mass spectrometry and found 5-fold increases in the levels of prostaglandins and arachidonic acid within just 10 minutes (Copland et al. 2006).

Another potential soluble mediator within the alveolar space is ATP, which has been shown to be released from A549 cells following just a single second 21% stretch with local concentrations reaching up to 10μM within 10 seconds (Grygorczyk, Furuya, and Sokabe 2012). This study describes the fastest release of a soluble mediator by epithelial cells which makes ATP a candidate for the rapid initiation of inflammatory signalling in alveolar macrophages in response to high tidal volume ventilation.

It is clear from the previous work described here that pulmonary epithelial cells are capable of rapidly releasing soluble inflammatory mediators. However it is not known whether these soluble mediators could play a role in the very early stages of VILI in which inflammatory signalling is initiated in alveolar macrophages. To our knowledge, only one group has investigated this theory, with inconclusive results. Frank et al. ventilated rats at 30ml/kg for short lengths of time before collecting BAL fluid and transferring it onto naïve alveolar macrophages for 18 hours (Frank et al. 2006). They found that BAL fluid taken from rats ventilated for 20 minutes induced a significant increase in nitrite production within the primary alveolar macrophages. When they tried to replicate these results *in vitro* they found that culture medium from primary alveolar macrophages or type II AECs stretched in isolation for 30 minutes was not capable of inducing nitrite production in naïve macrophages. However when primary alveolar macrophages and type II AECs were stretched in co-culture, the culture medium induced significant nitrite production by alveolar macrophages (Frank et al. 2006).

The researchers from this study suggested that both soluble mediators and contact-dependent mechanisms may be important in cellular activation during the initiation of VILI. The study of soluble mediators is difficult *in vivo* as it is impossible to determine the cellular source, therefore we developed an *in vitro* model to identify a potential soluble mediator involved in the initiation of inflammatory signalling in VILI.

7.2 Aims

The aim of the experiments in this chapter was to set up an *in vitro* model for the study of soluble mediators in VILI. This model would be used to determine whether isolated pulmonary parenchymal cells are capable of secreting soluble mediators of inflammation in response to a short period of stretching.

7.3 Protocol

Pilot experiments

In pilot experiments, the murine monocyte/macrophage RAW cell line was stimulated with 1μg/ml LPS (E. coli O111:B4) for 15 minutes with the aim of detecting cellular activation via phospho-flow cytometry. The usual method for detecting activation of intracellular signalling pathways *in vitro* is by Western blotting, however we wished to use phospho-flow cytometry, as this is a rapid and more informative method that could also be used to study cell co-cultures. We therefore developed a protocol to fix and recover cells that were attached to the bottom of culture plate wells.

Cell stretching *in vitro*

A549, MLE and bEnd5 cells were plated onto type I collagen coated BioFlex® 6-well culture plates (Flexcell International) approximately 36 hours prior to stretching and were serum starved (0.6-1.2g/ml new-born calf serum) for 12 hours prior to stretching to limit cell proliferation. Cells were stretched at around 90% confluence and in the presence of 1ml fresh low-serum media in an incubator at 37^{o}C and 5% CO_2 for 15 minutes. Cells were stretched at 20 cycles per minute reaching a maximum strain of 13% and were compared with non-stretched cells.

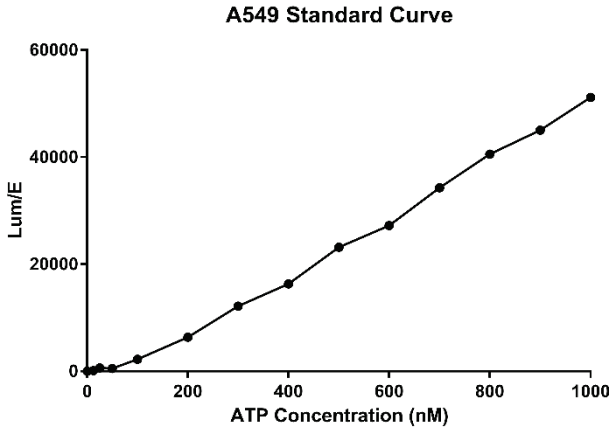
Sample collection

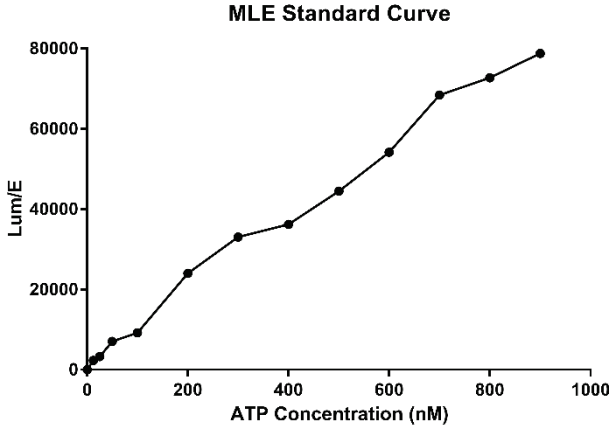
Following the 15 minute stretch protocol, plates were placed directly onto ice and the media was aspirated and divided into two Eppendorf tubes on ice with one containing an ecto-ATPase inhibitor ARL at 250μM (Tocris). Media was immediately replaced with ice-cold phosphatase inhibitor cocktail (Calbiochem) that had also been used in *in vivo* experiments. Then cells were gently scraped from the surface of the well and added to ice-cold BD Cytofix/Cytoperm (BD Biosciences) to incubate for 10 minutes on ice before being washed twice and re-suspended in perm-wash buffer for analysis by flow cytometry. Supernatant samples were washed once to remove any contaminating cells and were stored in aliquots at -80^{o}C for later use in assays.

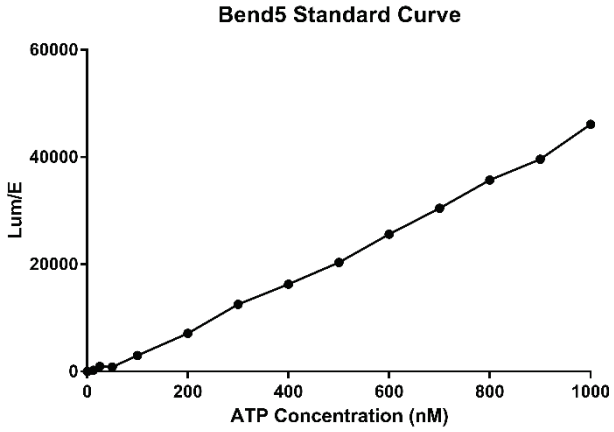
ATP Assay

Samples collected from cells stretched *in vitro* for 15 minutes were analysed using the ATPlite Luminescence ATP Detection Assay System (Perkin Elmer), which is described in more detail in Chapter 2: Methods & Materials. Briefly, samples were stored in the presence of ARL, an ecto-ATPase inhibitor to prevent ATP breakdown. ATP could then be quantified using the ATPlite kit that utilises an ATP-dependent luciferase reaction to produce light that is proportional to the concentration of ATP in the sample. Standard curves for ATP assays are displayed in Figure 7.1.

Figure 7.1 ATP Standard Curves for ATP detection in supernatant from cells stretched for 15 minutes (Figure 7.9)







Different standard curves were required for samples from each cell type due to the use of different culture medium.

ELISA

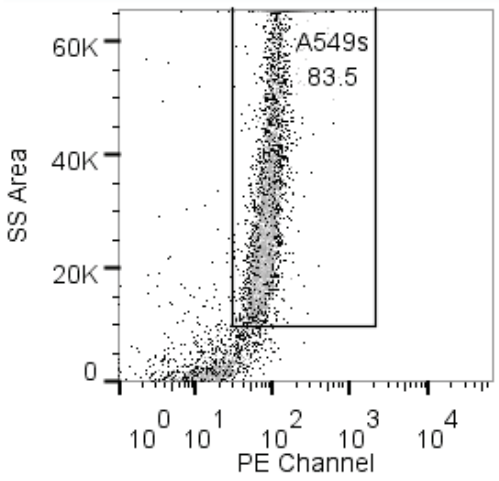
Colorimetric ELISAs were performed on media samples from stretched and non-stretched MLE cells to measure the TNFα and IL-6 content as per the manufacturer's instructions. The optical density of each sample was then measured by a microplate reader set at 450nm.

Flow cytometry of cell cultures

Identification of pure populations such as cell lines from *in vitro* studies by flow cytometry does not the require detection of specific cell surface markers but should exclude noise and cell debris. A549 cells were identified based on their auto-fluorescence and size alone (Figure 7.2), whilst MLE cells were identified based on their expression of EpCAM and T1-alpha (Figure 7.3), and bEnd5 cells were identified based on their expression of CD105 and CD31 (Figure 7.4).

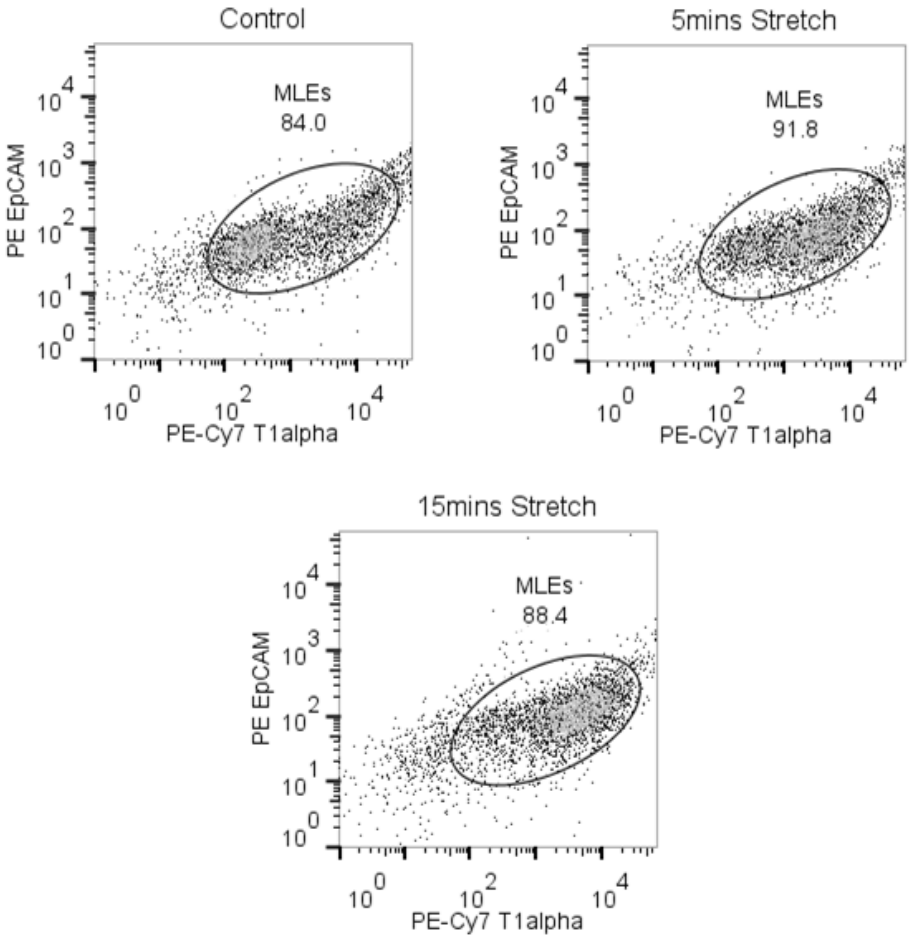
Within these three populations, phosphorylation of MAP kinases p38, MK2 and ERK1/2 and the transcription factor NF-κB were measured. In addition, expression of ICAM-1 protein was also measured (CD54-FITC; 3E2; BD Biosciences) (with unstained control subtracted) as an additional marker of cellular activation.

Figure 7.2 A549 cells identified by auto-fluorescence and size



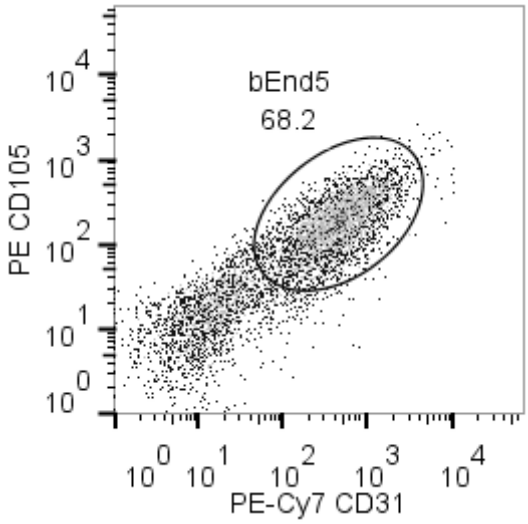
It is possible to not use any cell identification markers when analysing a pure population of cells as they stand out very clearly from noise and cell debris. This method identified 83.5% of the total events as A549 cells in this representative plot, suggesting there was little debris in the sample.

Figure 7.3 MLE cells identified by EpCAM and T1-alpha



This gating protocol identified between 85-90% of the total events as MLE cells in these representative plots, which again suggests little debris within the sample. Interestingly there appeared to be two populations in the control samples (T1-alpha intermediate and high) that seemed to merge into a single population expressing high levels of T1-alpha with increased length of cyclic stretching.

Figure 7.4 bEnd5 identified by CD105 and CD31 staining

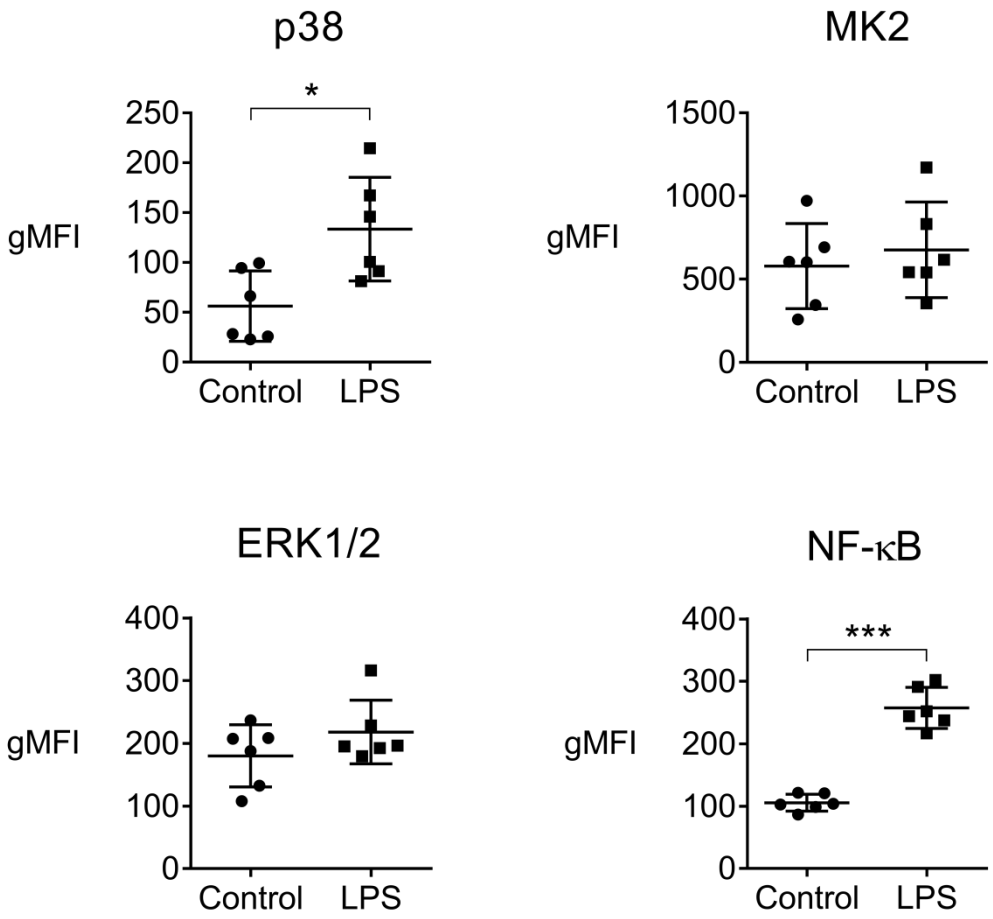


This gating strategy identified 68.2% of the total events to be bEnd5 cells in this representative plot. The increased debris in this sample may be due these being a more fragile cell type or could be due to non-uniform expression of the endothelial markers used.

7.4 Results

Pilot experiments were conducted using RAW cells stimulated with LPS, which was expected to provide a good positive control for the development of a method measuring activation of intracellular signalling by phospho-flow cytometry in cells cultured *in vitro*. Following 15 minutes of LPS stimulation, the RAW cells displayed significant increases in phosphorylation of p38 and NF-κB (Figure 7.5). *In vivo* stimulation of alveolar macrophages following intratracheal LPS also induced activation of MK2 and ERK1/2 at this time point (Chapter 3). However it is very difficult to compare these results given the different doses of LPS, differences in intercellular communication and environment of the two different cell types. Despite this, we could be confident that our protocol was capable of detecting activation of intracellular signalling within cells stimulated *in vitro*.

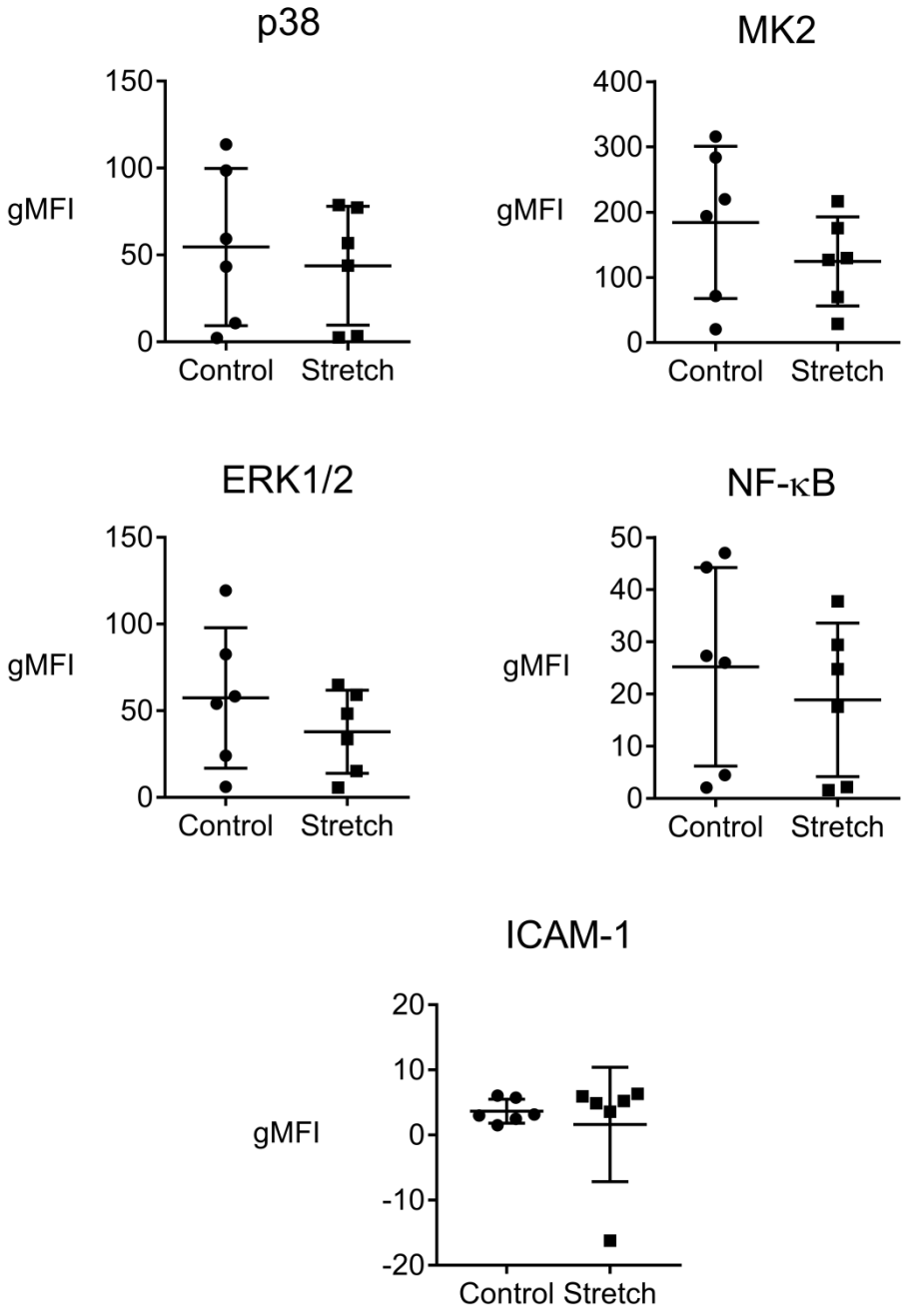
Figure 7.5 Cellular activation of RAW cells following 15 minutes LPS stimulation



RAW cells exposed to LPS for 15 minutes demonstrated significant increases in phosphorylation of p38 and NF-κB. N=6. Data were analysed by t-test and are displayed as mean ± SD.

Having successfully recovered cells from culture plates for phospho-flow cytometric analysis, the effects of stretching isolated pulmonary parenchymal cells *in vitro* was investigated. A549 cells were stretched for 15 minutes, however no significant signs of activation were found in any of the markers (Figure 7.6).

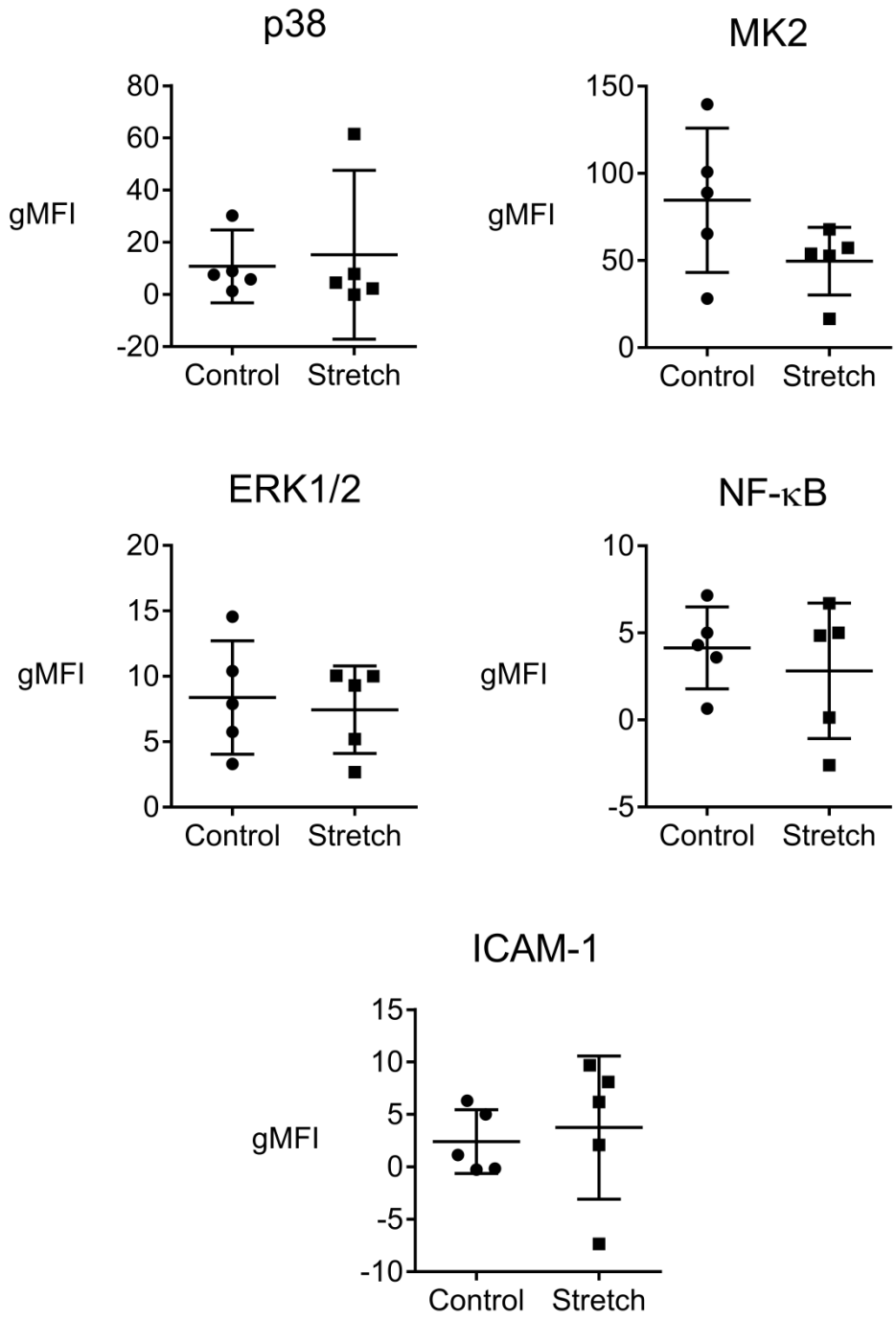
Figure 7.6 Cellular activation of A549 cells following 15 minutes cyclic stretch



A549 cells did not show any significant signs of cellular activation based on the five markers studied following 15 minutes of cyclic stretch. N=6. Data were analysed by t-test and are displayed as mean ± SD.

As for the A549 cells, the MLE cells also did not display any significant signs of activation following 15 minutes of cyclic stretch (Figure 7.7).

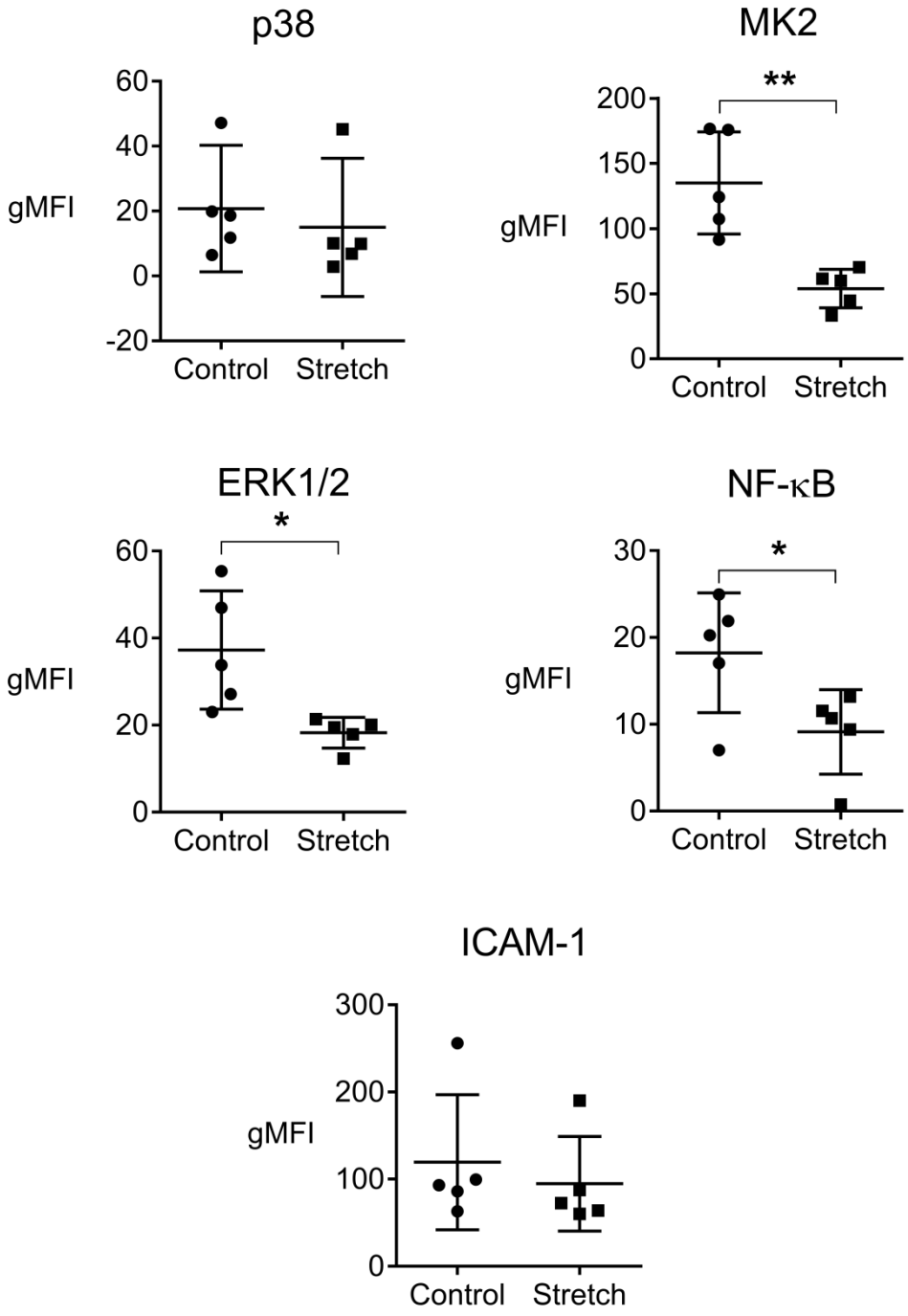
Figure 7.7 Cellular activation of MLE cells following 15 minutes cyclic stretch



MLE cells did not show any signs of cellular activation based on the five markers studied following 15 minutes of cyclic stretch. N=5. Data were analysed by t-test and are displayed as mean ± SD.

The bEnd5 cells did display changes in phosphorylation of some of the activation markers though not in a manner that was expected. MK2, ERK1/2 and NF-κB all demonstrated significant decreases in activation (Figure 7.8). p38 phosphorylation remained unchanged despite the significant decrease in MK2, whilst expression of ICAM-1 also remained unaltered between the non-stretched and stretched bEnd5 cells.

Figure 7.8 Cellular activation of bEnd5 cells following 15 minutes cyclic stretch



*bEnd5 cells showed significant decreases in the phosphorylation levels of MK2, ERK1/2 and NF-kB following 15 minutes of cyclic stretch. N=5, *p<0.05. Data were analysed by t-test and are displayed as mean ± SD.*

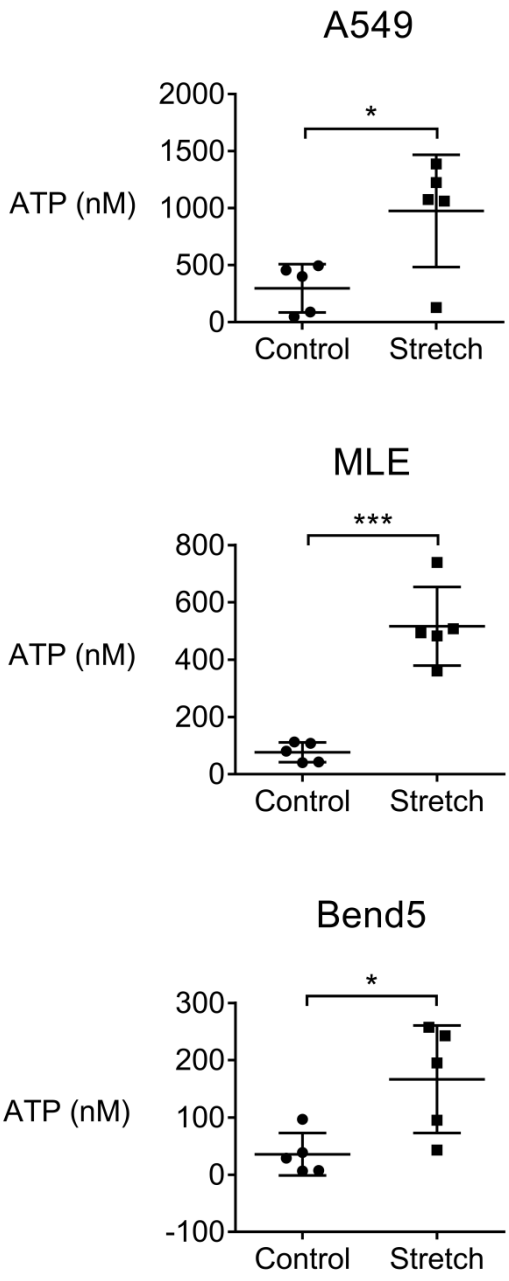
We went on to measure the ATP content within the media samples collected from the stretched and non-stretched cells. Media was exchanged for fresh media immediately prior to the 15-minute exposure period and therefore any ATP detected must have been released during the experiment.

Significant increases in ATP release were found from all 3 cell types in response to cyclic stretch for 15 minutes (Figure 7.9). The A549 cells which were not found to demonstrate activation of MAP kinase or NF-κB signalling nor increased ICAM-1 protein expression; were found to release 975nM ATP into 1ml of media, a 3.3-fold increase compared with baseline. We can estimate that a total of 495ng ATP was released into the media, approximately 0.25pg per cell.

The MLE cells, which were also found not to demonstrate signs of cellular activation by phosho-flow cytometry, released 517nM ATP (6.7-fold increase) within 15 minutes exposure to cyclic stretch. This corresponds to a total of 262ng ATP or 0.13pg per cell. The bEnd5 cells, which demonstrated significant decreases in activation of MAP kinase and NF-κB signalling, were found to release over 160nM ATP (4.7-fold increase) into the supernatant following 15 minutes of cyclic stretch (Figure 7.9). This corresponds to a total of 85ng ATP and 0.04pg per cell.

In addition to ATP, media samples from stretched and non-stretched MLE cells were also analysed by ELISA for the presence of TNFα and IL-6. However, the samples were found to contain no detectable levels of either soluble mediator.

Figure 7.9 ATP in supernatant from cells stretched for 15 minutes



*Significant levels of ATP were detected in the supernatant of stretched cells compared with static controls. N=5, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

7.5 Discussion

In this chapter, a model was developed that enabled the assessment of MAP kinase activation in cells stimulated *in vitro*. Detection of MAP kinase signalling enabled the exploration of how quickly cells become activated as these pathways are involved in the initiation of inflammatory signalling, which is more commonly measured in terms of secreted soluble mediators at much later time points. The effectiveness of this method was demonstrated in RAW cells stimulated with LPS, prior to assessing MAP kinase activation in cells stretched *in vitro*. Another advantage of this *in vitro* model was the ability to study single cell types in isolation, which enabled us to identify the cell source of released soluble mediators that would not be possible *in vivo*.

Unfortunately, none of the cell lines stretched *in vitro* demonstrated MAP kinase or NF-κB activation following 15 minutes of cyclic stretching. This was an unexpected result given that the epithelial and endothelial cells had been very responsive to mechanical ventilation *in vivo*. Cells lines are easier to culture and maintain compared with primary cells, however, it is known that they can behave differently. The A549 cell line in particular, has been used in previous cell stretching experiments, and activation of MAP kinase and NF-κB signalling pathways have been detected within 15 minutes by Western blot (Li et al. 2003; Ning and Wang 2007b). Reports of stretching MLE cells has only been reported once (Dai et al. 2013), whilst there are no reports of bEnd5 cells being subjected to cyclic stretch.

The MLE cells did demonstrate a change in phenotype within the 15 minutes of exposure to cyclic stretching to express more T1-α, a typical marker of type I AECs, which may be an indication that they were activated during this time. The bEnd5 cells actually appeared to demonstrate a decrease in phosphorylation level of a number of markers, but the interpretation of this is not clear. None of the cell lines demonstrated changes to their cell surface ICAM-1 expression, which may have been simply due to the short timescale of the experiments. It may be that events observed *in vivo* may require more time to occur in an *in vitro* setting, and therefore it would be interesting to repeat these experiments under longer timescales.

ATP release

Despite the apparent lack of activation of intracellular signalling pathways, we were able to detect the release of ATP into the media. Each of the cell lines was found to secrete significant levels of the DAMP ATP, which can act as a pro-inflammatory mediator in the extracellular space. ATP release can be an active process in stimulated cells, or can occur as a result of cell death and subsequent release

of ATP from the cytosol. Several mechanisms have been proposed to be involved in the active secretion of ATP including exocytosis of ATP-containing vesicles, or via nucleotide transporters (Schwiebert and Zsembery 2003).

One of the most commonly described mechanisms is via connexin hemichannels, which are uncoupled gap junction channels. Cotrina et al. demonstrated that gap junction deficient cell lines, when forced to express connexins, showed enhanced ability to secrete ATP (Cotrina et al. 1998). Leybaert et al. suggested that connexin hemichannels might be involved in ATP release into the extracellular space in response to activation of intracellular $InsP_3$ signalling (Leybaert et al. 2003). They found that the connexin mimetic peptide Gap26, which interacts with connexin-43, did not affect gap junction coupling, but did prevent ATP secretion in a number of endothelial and epithelial cell lines (Leybaert et al. 2003). This mechanism of active ATP secretion has also been found to exist in human neutrophils, in which ATP release can be inhibited using connexin mimetic peptides (Eltzschig et al. 2006). Pannexin channels, which are similar to connexin hemichannels, have also been associated with active ATP release into the extracellular space (Richter et al. 2014; Ohbuchi et al. 2014).

The concentrations of ATP detected in the media samples following 15 minutes cyclic stretching were within the nanomolar range, corresponding to picomolar amounts of ATP released per cell. Given that a single cell contains ATP within its cytosol in the millimolar range; it seems unlikely that the ATP release observed in the current experiments was the result of cell death. The release of ATP in response to stretch could provide a very rapid means of communication between the epithelium and alveolar macrophages and is explored further in the next chapter.

Conclusions

The *in vitro* experiments reported in this chapter were designed to separate the individual steps in the activation of alveolar macrophages in response to mechanical ventilation. Despite promising results from Chapter 6 which indicated that TNFα could be a potential mediator involved in the early activation of alveolar macrophages, we detected no TNFα release from MLE cells stretched *in vitro* for 15 minutes. However, all three of the cell lines demonstrated significant ATP release within 15 minutes of cyclic stretching, suggesting that ATP is a potential mediator involved in the activation of alveolar macrophages.

CHAPTER EIGHT

ATP SIGNALLING IN THE INITIATION OF VILI

8.0 Chapter summary

Several damage-associated molecular patterns (DAMPs) have been associated with the pathogenesis of inflammatory conditions including VILI. ATP is one such DAMP that has been frequently implicated in inflammation through signalling via the $P2X_7$ receptor, which is activated by high concentrations of extracellular ATP. Increased levels of ATP have been detected in the BAL fluid of mechanically ventilated mice, and have been shown to induce pulmonary oedema. Inhibition of ATP signalling can attenuate certain aspects of VILI; however it remains unknown which cell type is the source of ATP and whether it could be the initial signal responsible for the activation of alveolar macrophages in response to stretch. We found increases in the ATP content of BAL fluid from mice ventilated with a high tidal volume for just 5 minutes, which is the earliest known detection of ATP release in response to mechanical ventilation. We also demonstrated that ATP instilled intratracheally can induce MAP kinase signalling in alveolar macrophages within just 5 minutes. Finally, we attempted to inhibit ATP signalling by pre-treating mice with the A-839977 $P2X_7$ receptor antagonist but found that the vehicle DMSO induced confounding effects that prevented true interpretation of the experiments.

8.1 Background

We have discovered that alveolar macrophages demonstrate cellular activation in response to high tidal volume ventilation *in vivo* within just 5 minutes, following activation of the lung parenchyma which occurs within 1 minute. Soluble mediators are a possible mechanism by which stretch-induced signalling in AECs could be communicated to the non-structural alveolar macrophages. However, the timescale under which we have observed alveolar macrophage activation limits the potential soluble

mediators to those that can be released within just minutes. Such a soluble mediator would therefore need to be already present in considerable quantities within the cell, ruling out several mediators such as most cytokines which require a considerable amount of time to become up-regulated, synthesised and secreted.

A potential group of signalling molecules are damage-associated molecular patterns (DAMPs), which are danger signals released in response to cell injury or death. These DAMPs are endogenous molecules which under normal conditions have intracellular roles in the normal functioning of the cell or may be part of the extracellular matrix. Several DAMPs have been implicated in the pathogenesis of VILI (Kuipers et al. 2011) including a component of the extracellular matrix hyaluronan (Bai et al. 2005; Mascarenhas et al. 2004) and nuclear chromatin-associated protein high-mobility box group-1 (HMGB1) (Ogawa et al. 2006; van Zoelen et al. 2008).

Another DAMP that has been associated with the development of VILI and is also known to be very rapidly released in response to stretch *in vitro* is ATP. Primary human airway smooth muscle and lung fibroblasts have been found to release significant amounts of ATP in response to 10 to 15 minutes of cyclic stretching (Takahara et al. 2014; Murata et al. 2014). In addition, rat lung epithelial (RLE) and A549 cells have also been shown to release ATP into the culture medium with concentrations reaching 30nM within just 10 minutes of exposure to cyclic stretching (Belete et al. 2011). Another study demonstrated even faster ATP release by A549 cells in response to a single 1 second stretch at 21% strain (Grygorczyk, Furuya, and Sokabe 2012). In this study ATP release, observed by real-time imaging, was detected within 1 second and reached a peak concentration within 10 seconds, before decaying after 2 to 3 minutes. Interestingly, ATP was found to be released by only a few cells within the field of vision yet local concentrations often exceeded 10μM and could reach cells located up to 150μm away (Grygorczyk, Furuya, and Sokabe 2012). These studies demonstrate the potential for ATP to be very rapidly released in response to stretch and in high enough concentrations to be able to act in a paracrine manner within the alveolar space.

The primary role of intracellular ATP is in energy metabolism, however once in the extracellular space ATP becomes a potent signalling molecule. ATP has been found to propagate calcium waves in surrounding cells via P2Y receptor signalling (Homolya, Steinberg, and Boucher 2000). Within the healthy lung, ATP is known to play a role in the regulation of surfactant secretion via a paracrine mechanism involving $P2X_7$ receptor signalling (Mishra et al. 2011; Patel et al. 2005). In these studies, extracellular ATP concentrations of up to 50nM were reported to be secreted by type I AECs in response to stretch which was then able to stimulate surfactant secretion by type II AECs.

In an injured lung, ATP is also regarded as a mediator of inflammation via activation of the NLRP3 inflammasome (Vitiello et al. 2012), an effect which is specifically associated with signalling through the $P2X_7$ receptor (Figure 8.1). *In vitro*, extracellular ATP has been shown to induce secretion of IL-1β in LPS-stimulated human macrophages (Ferrari et al. 1997) and this has also been confirmed *in vivo* in mice lacking the $P2X_7$ receptor which could express pro-IL-1β but were unable to release the mature cytokine in response to ATP stimulation (Solle et al. 2001). More recently, increased levels of ATP in the BAL fluid of patients (up to 17nM) have been found to correlate with increased severity of pulmonary fibrosis (Riteau et al. 2010). This study also reported concentrations of up to 1000nM ATP in the BAL fluid of bleomycin-treated mice. These studies show that ATP can be released into the BAL fluid at high concentrations in response to injury under both experimental and clinical settings and is capable of mediating cytokine release.

Figure 8.1 $P2X_7$ Signalling and Inflammation

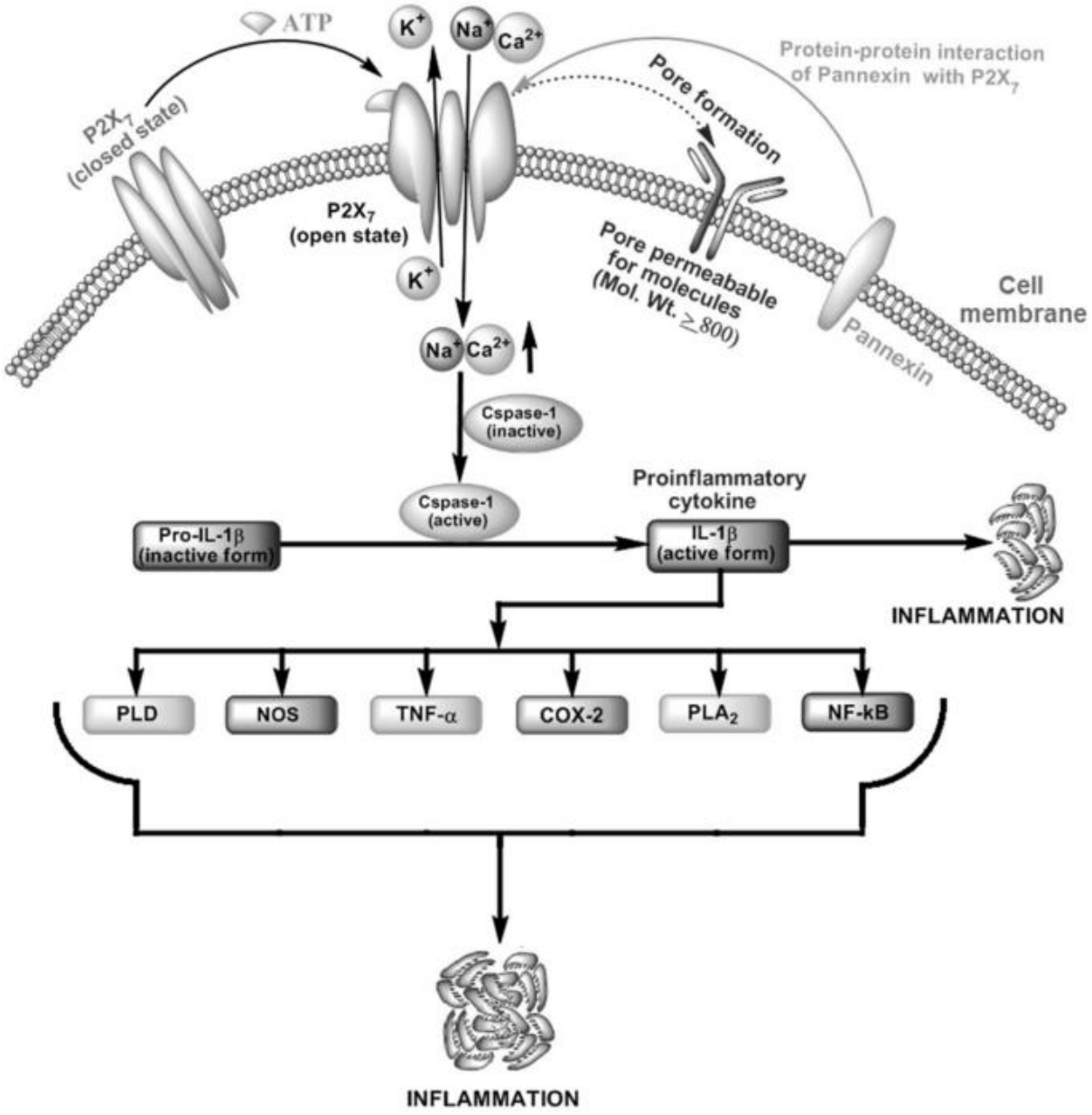


Figure adapted from (Mehta et al. 2014).

Further studies investigating ATP in ARDS models have found that inhibition of ATP signalling is beneficial. Moncao-Ribeiro et al. found that knockout mice lacking the $P2X_7$ receptor demonstrated reduced immune cell migration into the lungs and a reduction in soluble mediators within the BAL fluid, measured 24 hours following intratracheal instillation of LPS (Moncao-Ribeiro et al. 2011). In another mouse model, high concentrations of ATP in the BAL fluid were found to correlate with neutrophil recruitment that peaked at 3 days following intratracheal instillation of LPS and was inhibited by an ecto-ATPase (Shah et al. 2014). This study also showed that instillation of ATP alone could promote neutrophil recruitment to the lungs within 24 hours and suggested that ATP acts as a

chemoattractant for neutrophils to sites of injury. This theory is supported by an earlier study in which systemic administration of the non-hydrolysable ATP analogue ATPγS was said to confuse neutrophils and attenuate their ability to migrate towards the LPS-treated lung (Kolosova et al. 2008). The effect of ATP on neutrophil recruitment could also be due to the disruption of the alveolar barrier, as the potent $P2X_7$ receptor agonist BzATP has been shown to induce cell death in a type I-like AEC line (Guo et al. 2014).

In a study by Rich et al. ATP was found to be released into the BAL fluid of rats at concentrations of up to 39nM following 30 minutes of ventilation at 38ml/kg with no PEEP (Rich et al. 2003). They also found that intratracheal instillation of ATP followed by low tidal volume ventilation resulted in pulmonary oedema leading them to conclude that ATP release during mechanical ventilation is responsible for the accumulation of oedema (Rich et al. 2003). The same group later reported increased BAL fluid ATP levels of up to 90nM following ventilation of rats for 60 minutes at 40ml/kg without PEEP, and also found that intratracheal instillation of the ATPase apyrase reduced the release of IL-6 into the BAL fluid (Douillet et al. 2006).

Matsuyama et al. reported a four-fold increase in relative luminescence units (RLU) for ATP in BAL fluid following 60 minutes of ventilation in mice with 40ml/kg and no PEEP (Matsuyama et al. 2008). This group also showed that administration of a P2 antagonist PPADS reduced IL-6 secretion into the BAL fluid. As had been reported previously, instillation of ATP directly into the lungs induced injury characterised by immune cell infiltration, increased permeability and expression of inflammatory cytokines within 24 hours which could all be attenuated by PPADS, suggesting that ATP was inducing injury via purinergic signalling (Matsuyama et al. 2008).

ATP levels in the BAL fluid of an isolated-perfused rat lung were found to increase after being held at 15cmH_2O for just 15 seconds (Kiefmann et al. 2009). This ATP release induced calcium oscillations in alveolar epithelial and endothelial cells via purinergic receptors. Briva et al. also studied ATP signalling in an isolated perfused rat lung and demonstrated ATP release of up to 24μM into the BAL fluid following ventilation at 35ml/kg with no PEEP for 35 minutes (Briva et al. 2011). The vast differences in ATP concentrations reported in BAL fluid are likely to result from different dilution factors as a result of the BAL procedure and the different methodologies used to quantify ATP.

These studies all show that ATP is released in response to high tidal volume mechanical ventilation *in vivo* and additionally show that ATP release in response to stretch *in vitro* can be very rapid. ATP has also been shown to induce MAP kinase signalling and could therefore be responsible for the early MAP kinase activation we detected in response to stretch. In the murine monocyte/macrophage

RAW cell line, 1mM of ATP has been shown to induce p38 and ERK1/2 activation within 15 minutes (Kawano et al. 2012). Another group have detected MAP kinase activity in the RAW cell line in response to 250µM BzATP, an agonist of the $P2X_7$ receptor with 5 to 10-fold more potency than ATP. This group reported finding p38 activation within just 2 minutes and ERK1/2 activation within 5 minutes (Pfeiffer et al. 2004) whilst another group found ERK1/2 activation within 2 minutes, which they found to be much faster than LPS-induced MAP kinase activation (Aga et al. 2004).

The current literature suggest that ATP is a potential soluble mediator in the initiation of VILI due to its extremely rapid release in response to stretch, being released into the alveolar space at concentrations sufficient for paracrine communication, and finally being implicated in several downstream aspects of lung injury.

8.2 Aims

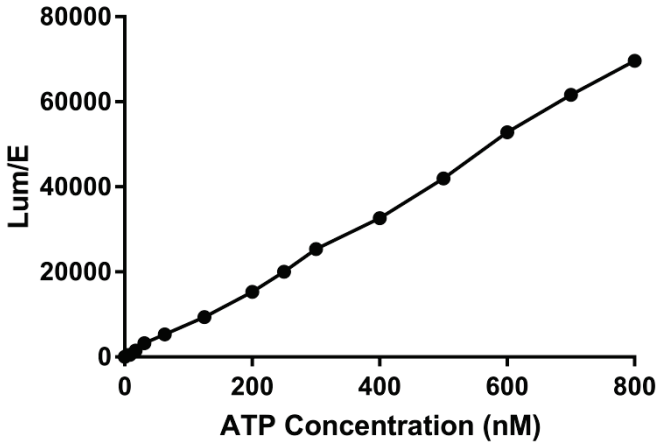
The aim of this chapter was to elucidate the role of ATP signalling in the initiation of inflammation in VILI. Having detected ATP release from pulmonary parenchymal cells stretched *in vitro* in the previous chapter, we wanted to see if these results could be replicated *in vivo*. Further experiments would then be performed to investigate whether this ATP has the potential to induce rapid cellular activation of alveolar macrophages, starting with RAW cells and isolated primary alveolar macrophages to determine the appropriate dose and timings before moving onto *in vivo* experiments. Finally, if these results yielded positive results then the effects of inhibiting ATP signalling on the initiation of inflammatory signalling in VILI would be investigated.

8.3 Protocol

ATP content of BAL fluid

Following 5 minutes of low or high stretch ventilation, a BAL was performed to measure the ATP concentration. The BAL was performed using 550µl ice-cold PBS (without Ca^{2+}) containing 250µM ARL (an ecto-ATPase inhibitor) to preserve the ATP content. Approximately 300µl of BAL fluid was withdrawn and washed to remove contaminating cells and kept on ice for up to one hour before analysis. Various known concentrations of ATP solutions were used to create a standard curve (Figure 8.2) were added alongside samples to a white 96 well plate in duplicate. The ATPlite luminescence assay was run as instructed by the manufacturer's instructions and ATP was measured using a luminometer.

Figure 8.2 ATP standard curve for detection of ATP in BAL fluid following 5 minutes high stretch ventilation



ATP stimulation of RAW cells

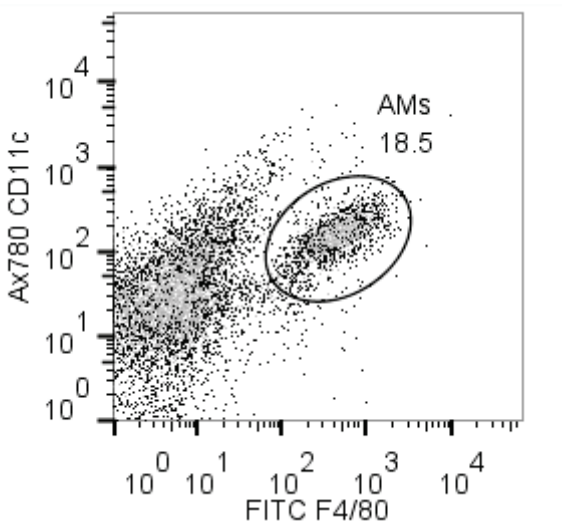
Cells were stimulated with 100µM ATP disodium salt (Tocris Bioscience) for time lengths of 5, 15 and 20 minutes. At the end of the stimulation period, the ATP solution was removed from wells and replaced with warm fixation buffer (BD Cytofix/Cytoperm) in which the cells were incubated for 10 minutes at 37^{o}C. The cells were then recovered from the wells using a CytoOne Cell Scraper (Starlab, Milton Keynes, UK). Cells were centrifuged twice at 450g for 5 minutes at 4^{o}C and re-suspended in perm-wash buffer for analysis by flow cytometry. RAW cells were distinguished from noise and cell debris by gating on CD45 and CD11b positive cells as illustrated in the previous chapter.

Harvesting and stimulation of primary alveolar macrophages

Mice were euthanised by overdose with pentobarbital (Merial Animal Health Ltd, Essex, UK) given intraperitoneally followed by exsanguination. Then a BAL was performed using warm calcium-free PBS with 5mM EDTA (Life Technologies, Paisley, UK). 750µl of this solution was instilled into the lungs and 500µl was withdrawn and re-instilled twice before finally withdrawing as much as possible. This process was repeated with fresh calcium-free PBS plus EDTA solution, which was used to reduce alveolar macrophage adherence. Cells were washed three times to remove EDTA and re-suspended in calcium-containing PBS with added calcium chloride (5mM). The alveolar macrophages were then split into two wells of a 24-well plate and left to settle for 45 minutes before experiments were conducted. Cells were stimulated with 5mM ATP for 5 and 15 minutes or with 100µM for 15 minutes in PBS before recovery for flow cytometry analysis.

The cells obtained by BAL fluid from a healthy murine lung consist of at least 80% alveolar macrophages and therefore the population was expected to be quite pure (Zaynagetdinov et al. 2013). Alveolar macrophages were identified on the flow cytometer by gating on F4/80(+) and CD11c(+) cells, however, the alveolar macrophages only accounted for 18.5% of the events (Figure 8.3). This low proportion may have been due to the high level of electrical noise from the flow cytometer that increases with sample running time, which was necessary for these samples due to the low cell density. Additionally, there was probably a significant amount of cell debris in the sample from the scraping process.

Figure 8.3 Identification of primary alveolar macrophages by flow cytometry



Primary alveolar macrophages made up 18.5% of the total events in this representative dot-plot, suggesting a large amount of noise in the sample, probably a combination of cell debris from the scraping process and noise from the flow cytometer.

Intratracheal ATP instillation

As for the intratracheal LPS instillation, the mouse was held upright for the brief duration of the procedure with an external light source illuminating the vocal chords. A 50µl bolus of ATP (100µM, 5mM or 100mM) was diluted in PBS and was instilled for 5 minutes. Towards the end of the exposure period, an endotracheal tube was inserted to allow for fast instillation of the phosphatase inhibitor cocktail and sampling of the lungs for analysis by flow cytometry.

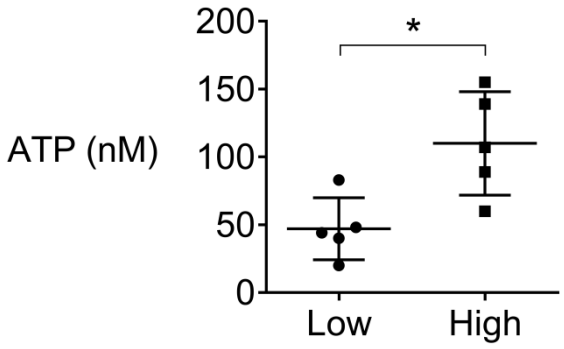
ATP signalling inhibition

The $P2X_7$ receptor antagonist A-839977 (Tocris Bioscience) was dissolved in 50µl DMSO and diluted in a total volume of 500µl saline and administered via the intraperitoneal route (80mg/kg) 30 minutes prior to the experiment. Controls were given 50µl DMSO in a total volume of 500µl saline. Control mice were exposed to 5 minutes of low or high stretch ventilation, and the A-839977 treated mice were exposed to 5 minutes high stretch ventilation.

8.4 Results

The first aim of the experiments in this chapter was to detect ATP release into the alveolar space following 5 minutes of high stretch ventilation compared with low. Following 5 minutes of low stretch ventilation, 47nM of ATP was measured, corresponding to approximately 7.2ng ATP within the 300µl BAL fluid collected (Figure 8.4). Following 5 minutes of high stretch ventilation, the ATP content of the BAL fluid increased up to 110nM, corresponding to 16.7ng ATP, a 2.3-fold increase.

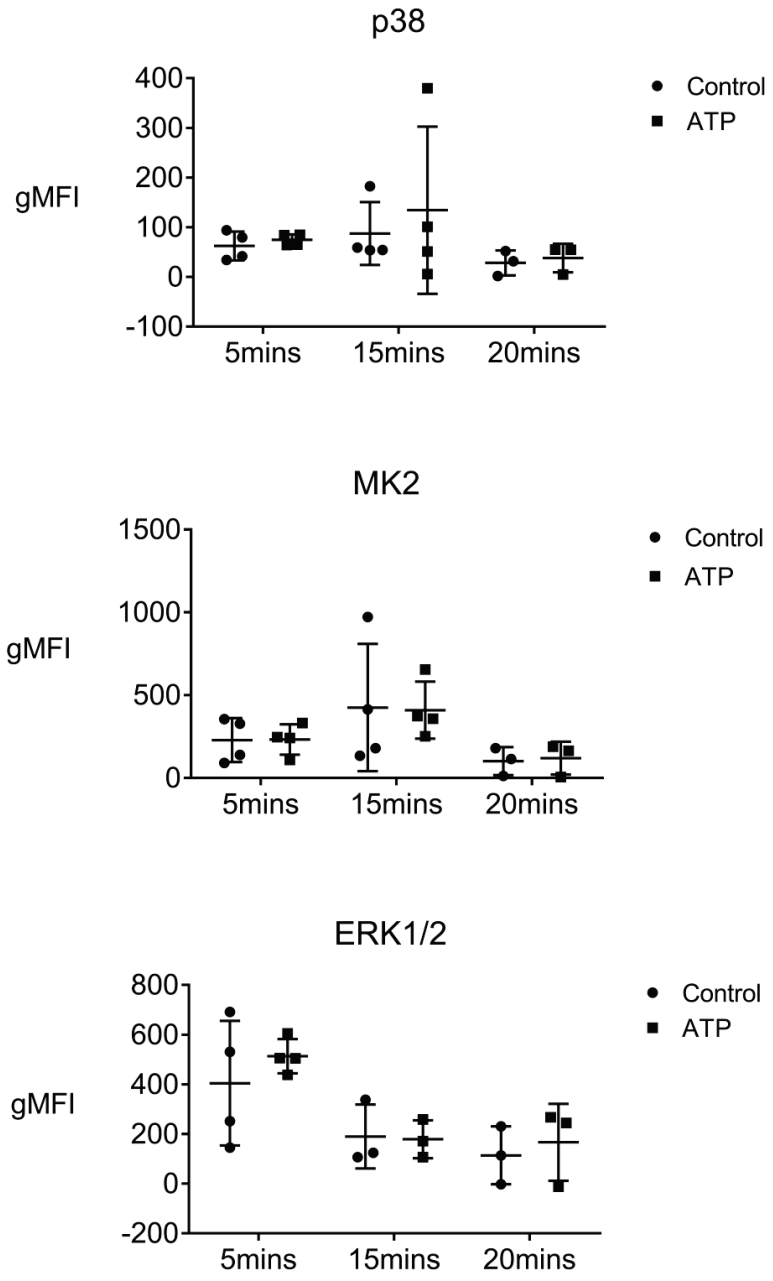
Figure 8.4 ATP in BAL fluid following 5 minutes high stretch ventilation



*ATP levels detected in the BAL fluid of ventilated mice were increased following 5 minutes of high stretch ventilation compared with low stretch. N=5, *p<0.05. Data were analysed by t-test and are displayed as mean ± SD.*

RAW cells were used in *in vitro* studies to identify an approximate length of stimulation time required to induce cellular activation in a macrophage-like cell. This estimate was to be used for *in vivo* studies to investigate whether ATP can stimulate alveolar macrophages when instilled intratracheally. Pilot studies performed in the previous chapter demonstrated that this RAW cell line was capable of demonstrating MAP kinase activity in response to LPS. However, 100µM ATP was found not to induce MAP kinase activation in RAW cells within any of the stimulation times studied (Figure 8.5).

Figure 8.5 Time course for ATP stimulation of RAW cells

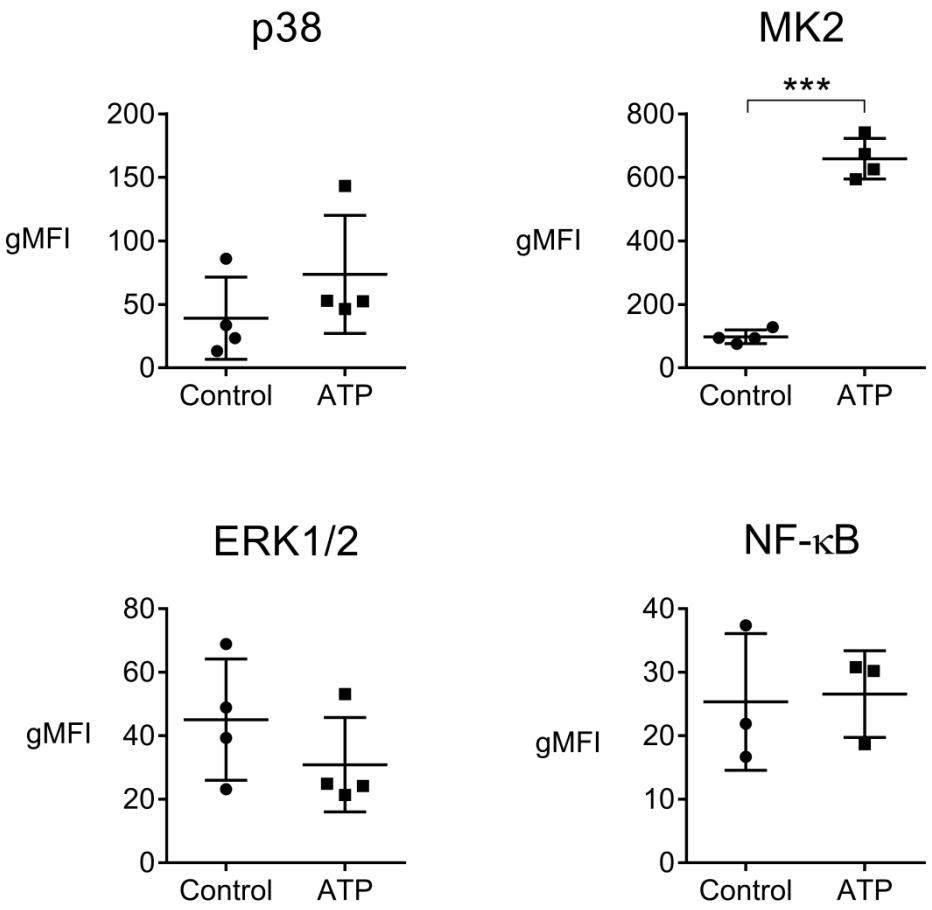


No cellular activation was detected in RAW cells stimulated with ATP (100µM) for 5, 15 or 20 minutes. N=3-4. Data were analysed by t-test, comparing control to ATP for each time point and are displayed as mean ± SD.

Despite varying exposure times, we were unable to detect activation of the RAW cell line in response to ATP. We therefore decided to look at the sensitivity of primary alveolar macrophages which were recovered from BAL fluid and stimulated *in vitro*. We started with the highest ATP concentration recommended by the group (5mM) and found that phosphorylation of MK2 was significantly

increased after 15 minutes. Although p38 did not demonstrate a statistically significant increase in phosphorylation, there was a trend for an increase in activation (Figure 8.6).

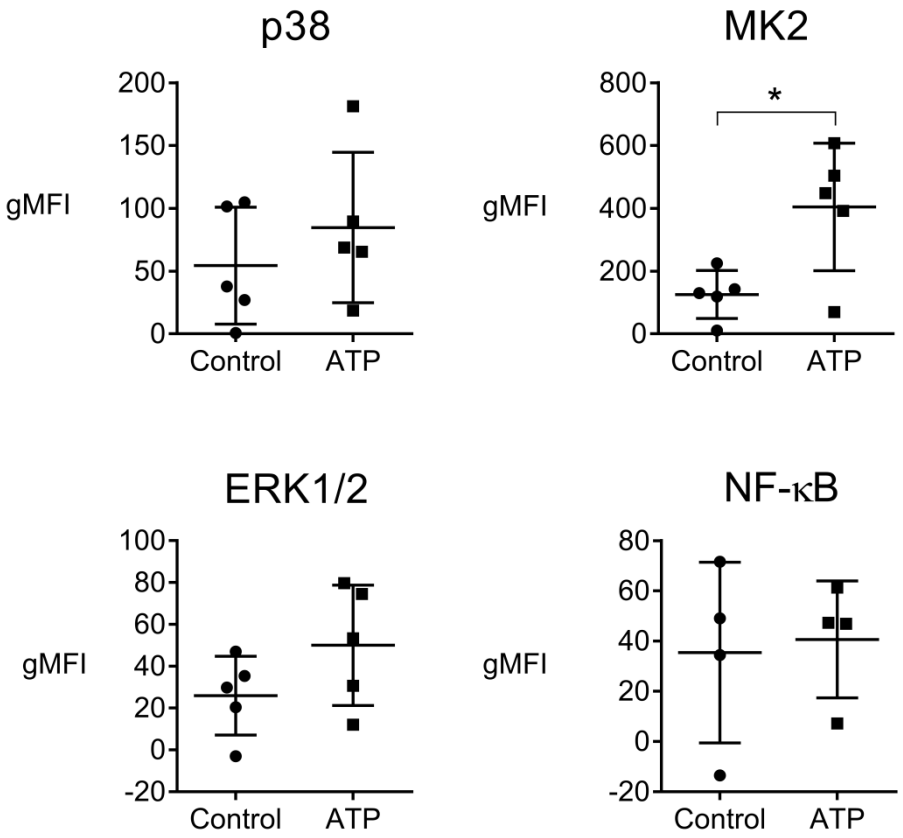
Figure 8.6 Stimulation of primary alveolar macrophages with 5mM ATP for 15 minutes



*Primary alveolar macrophages demonstrated significant activation of MK2 following stimulation with 5mM ATP for 15 minutes. N=3-4, *p<0.05, **p<0.01, ***p<0.001. Data were analysed by t-test and are displayed as mean ± SD.*

Using the same concentration of ATP (5mM), primary alveolar macrophages were stimulated for the shorter time of 5 minutes (Figure 8.7). Following 5 minutes ATP exposure, the primary alveolar macrophages were found to display significantly increased MK2 activation (3.2-fold increase from baseline) though this activation was slightly reduced compared with the level observed following 15 minutes (Figure 8.4).

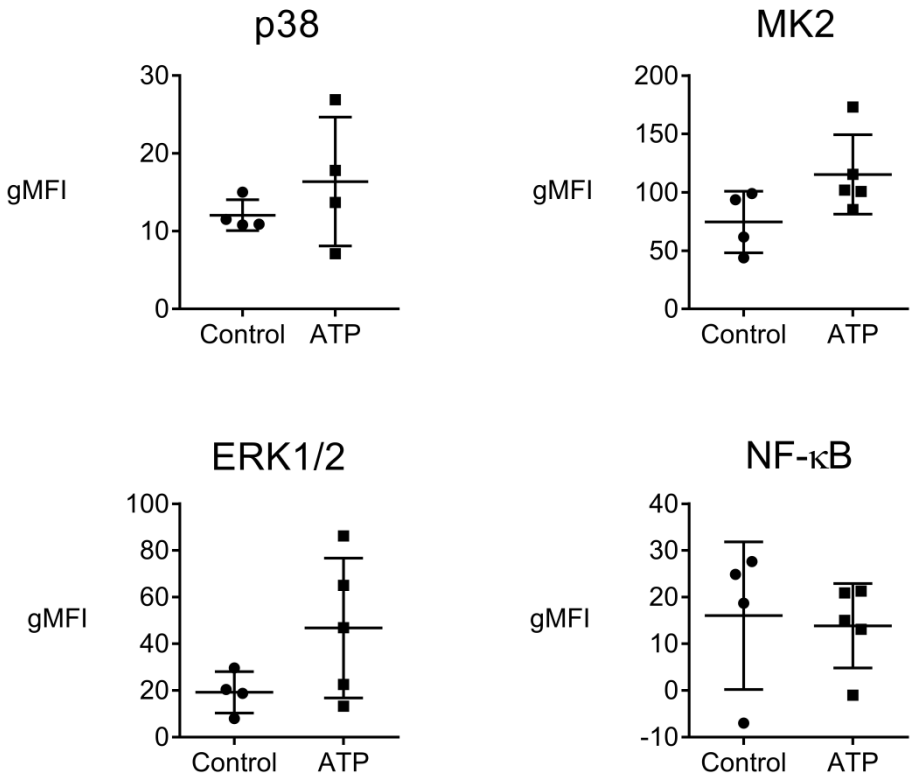
Figure 8.7 Stimulation of primary alveolar macrophages with 5mM ATP for 5 minutes



*Primary alveolar macrophages demonstrated significant activation of MK2 following stimulation with 5mM ATP for 5 minutes. N=4-5, *p<0.05. Data were analysed by t-test and are displayed as mean ± SD.*

The primary alveolar macrophages appeared to be more responsive to ATP than the RAW cell line. It was therefore investigated whether they would be more responsive to a lower dose of ATP. In response to 15 minutes stimulation with 100μM ATP, the primary alveolar macrophages did not display significant signs of activation, but did display non-significant trends for increased MK2 (p=0.091) and ERK1/2 (p=0.122) phosphorylation (Figure 8.8). As for the previous *in vitro* experiments, NF-κB failed to demonstrate activation in response to ATP stimulation.

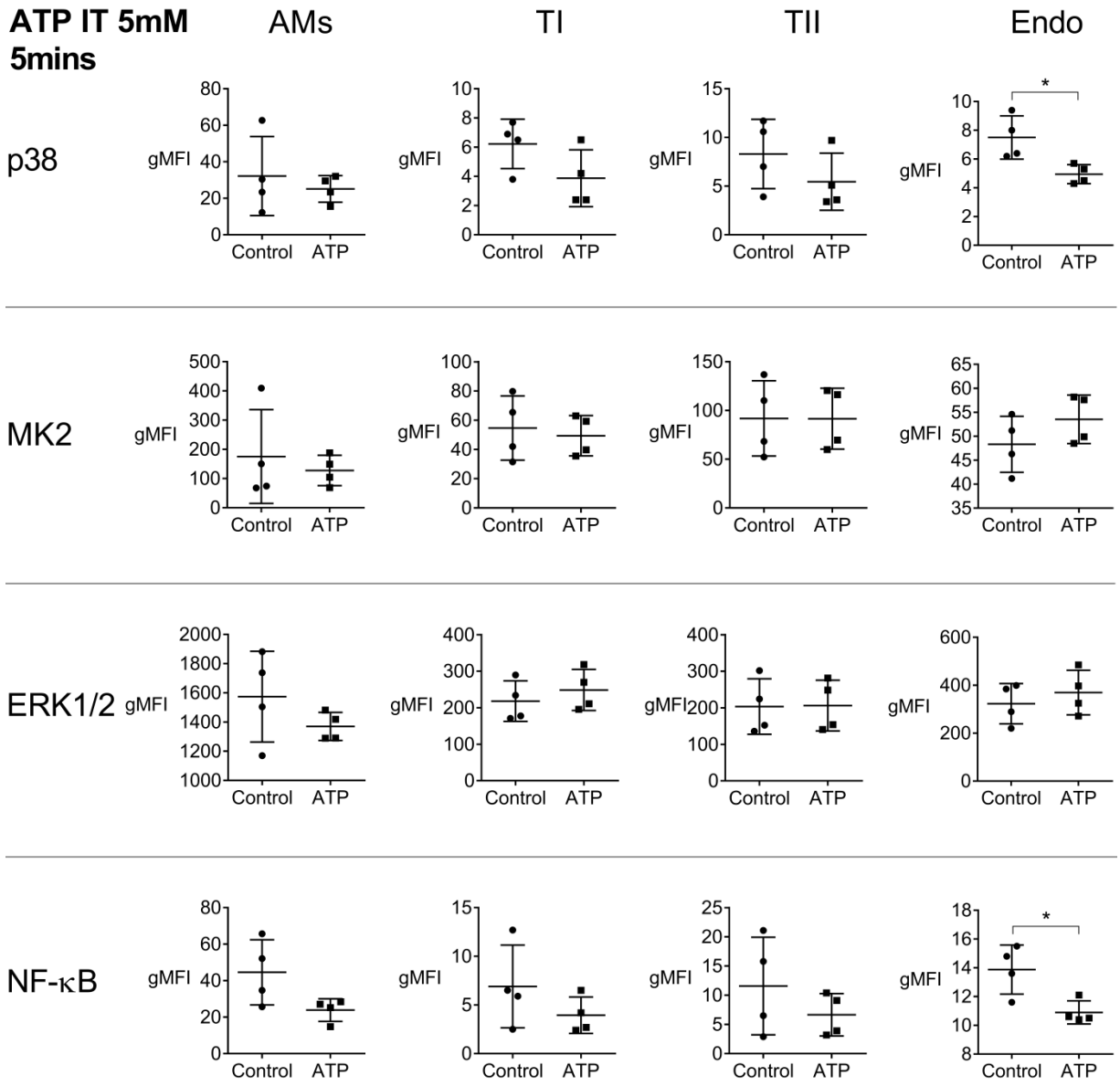
Figure 8.8 Stimulation of primary alveolar macrophages with 100µM ATP for 15 minutes



Primary alveolar macrophages did not demonstrate significant activation following stimulation with 100µM ATP for 15 minutes but did show trends for increased phosphorylation of MK2 (p=0.091) and ERK1/2 (p=0.122). N=4-5. Data were analysed by t-test and are displayed as mean ± SD.

After detecting activation of primary alveolar macrophages when stimulated with 5mM ATP within 5 to 15 minutes *in vitro*, we moved on to look at alveolar macrophage activation following intratracheal ATP instillation. However, no significant signs of cellular activation were detected following instillation of a 50µl bolus of 5mM ATP for 5 minutes (Figure 8.9).

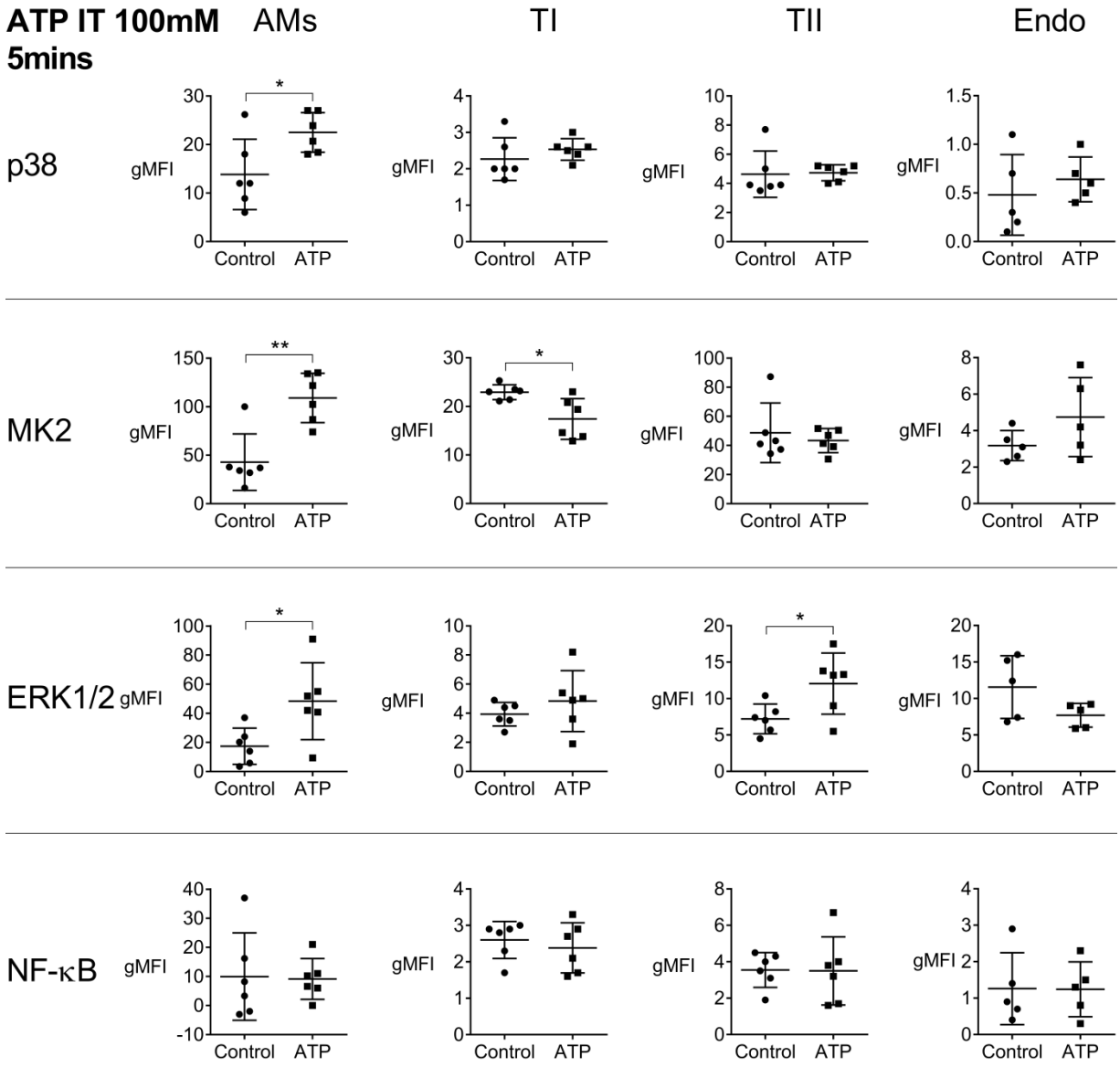
Figure 8.9 Cellular activation following exposure to intratracheal ATP (5mM) for 5 minutes



*No signs of activation were detected following 5 minutes exposure to 5mM ATP instilled intratracheally. N=4, *p<0.05. Data were analysed by t-test and are displayed as mean ± SD.*

Although 5mM was the highest recommended dose of ATP *in vitro*, doses of up to 100mM ATP have been administered *in vivo* via the intratracheal route in mice. Therefore, we examined the activity following intratracheal instillation of the higher dose of 100mM ATP for 5 minutes. In response to this increased dose, the type II AECs displayed a significant increase in ERK1/2 activity. However, the alveolar macrophages displayed the most activation of all cells types with significant activation of p38, MK2 and ERK1/2 (Figure 8.10).

Figure 8.10 Cellular activation following exposure to intratracheal ATP (100mM) for 5 minutes



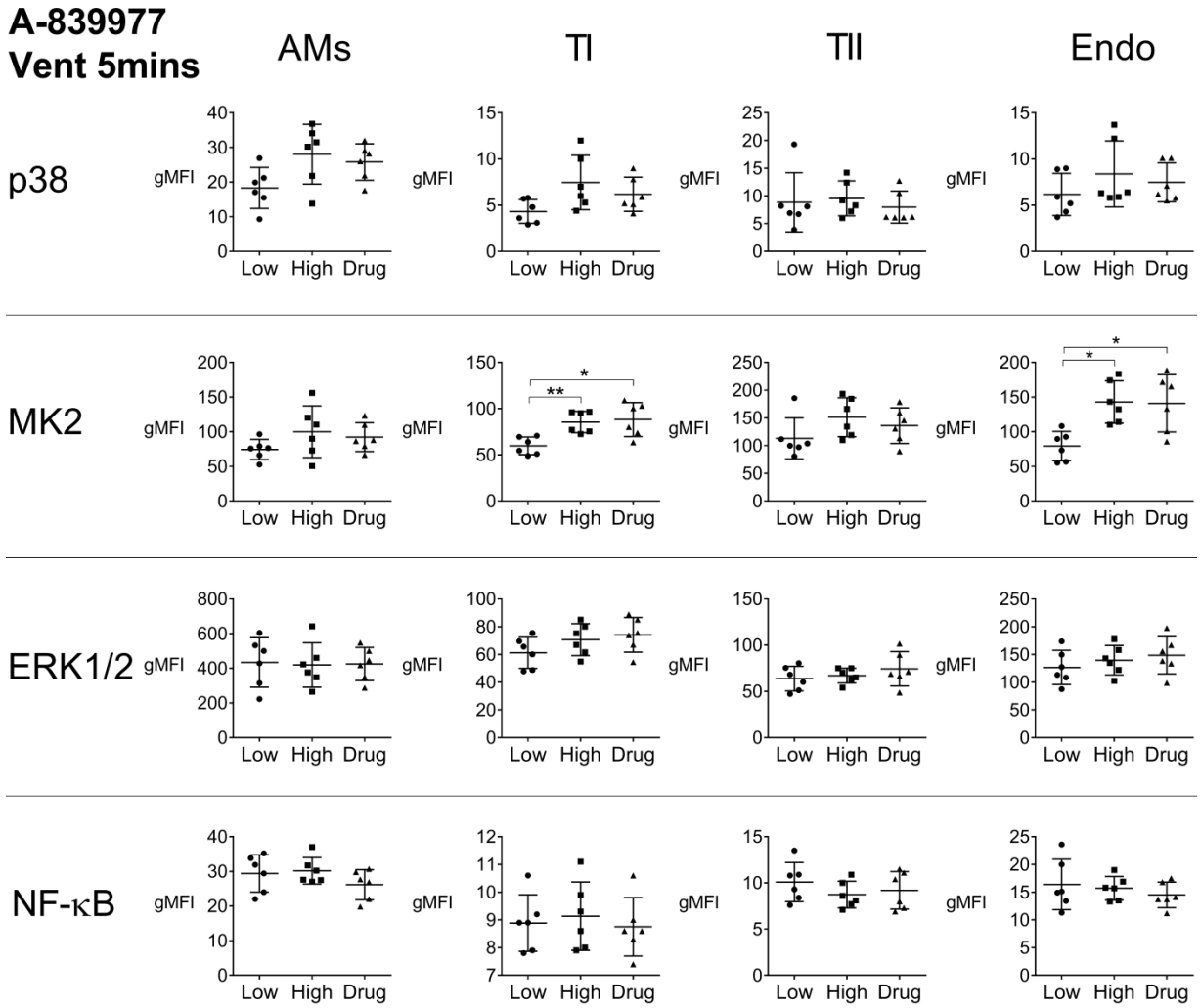
*Considerable activation within alveolar macrophages was detected following 5 minutes exposure to 100mM ATP instilled intratracheally. N=5-6, *p<0.05, **p<0.01. Data were analysed by t-test and are displayed as mean ± SD.*

Our final experiments examined the effect of an ATP signalling inhibitor on the induction of alveolar macrophage activation in response to 5 minutes of high stretch ventilation. This inhibitor was a $P2X_7$ receptor antagonist A-839977 that was delivered systemically and therefore we also examined the cellular activation of other leukocytes within the lung. These cell types were of interest as we had discovered that the bEnd5 endothelial cell line was capable of releasing ATP in response to cyclic stretch.

Our main focus however, was alveolar macrophages since we hypothesised that ATP may be released by the AECs and induce activation in alveolar macrophages. In response to 5 minutes of high stretch ventilation, the alveolar macrophages displayed a non-significant trend for p38 activation (p=0.061) compared with low stretch (Figure 8.11). Following pre-treatment with the A-839977 inhibitor, this p38 activity was less apparent compared with the low stretch control (p=0.168). However, there were no significantly activated markers within the alveolar macrophages to really determine whether the drug had prevented their activation. This may suggest that the DMSO pre-treatment had influenced the responsiveness of the alveolar macrophages to stretch.

Following 5 minutes of high stretch ventilation the type I AECs displayed a non-significant trend for p38 activation (p=0.057) and significant activation of MK2 compared with low stretch which still became significantly activated in the presence of the A-839977 drug (Figure 8.11). The endothelial cells also displayed significantly increased MK2 activation within 5 minutes of high stretch, which was still activated with the administration of $P2X_7$ inhibitor pre-treatment (Figure 8.12).

Figure 8.11 Phosphorylation of activation markers in alveolar macrophages and parenchymal cells after systemic pre-treatment with $P2X_7$ receptor antagonist A-839977 30 minutes prior to 5 minutes of low or high stretch ventilation

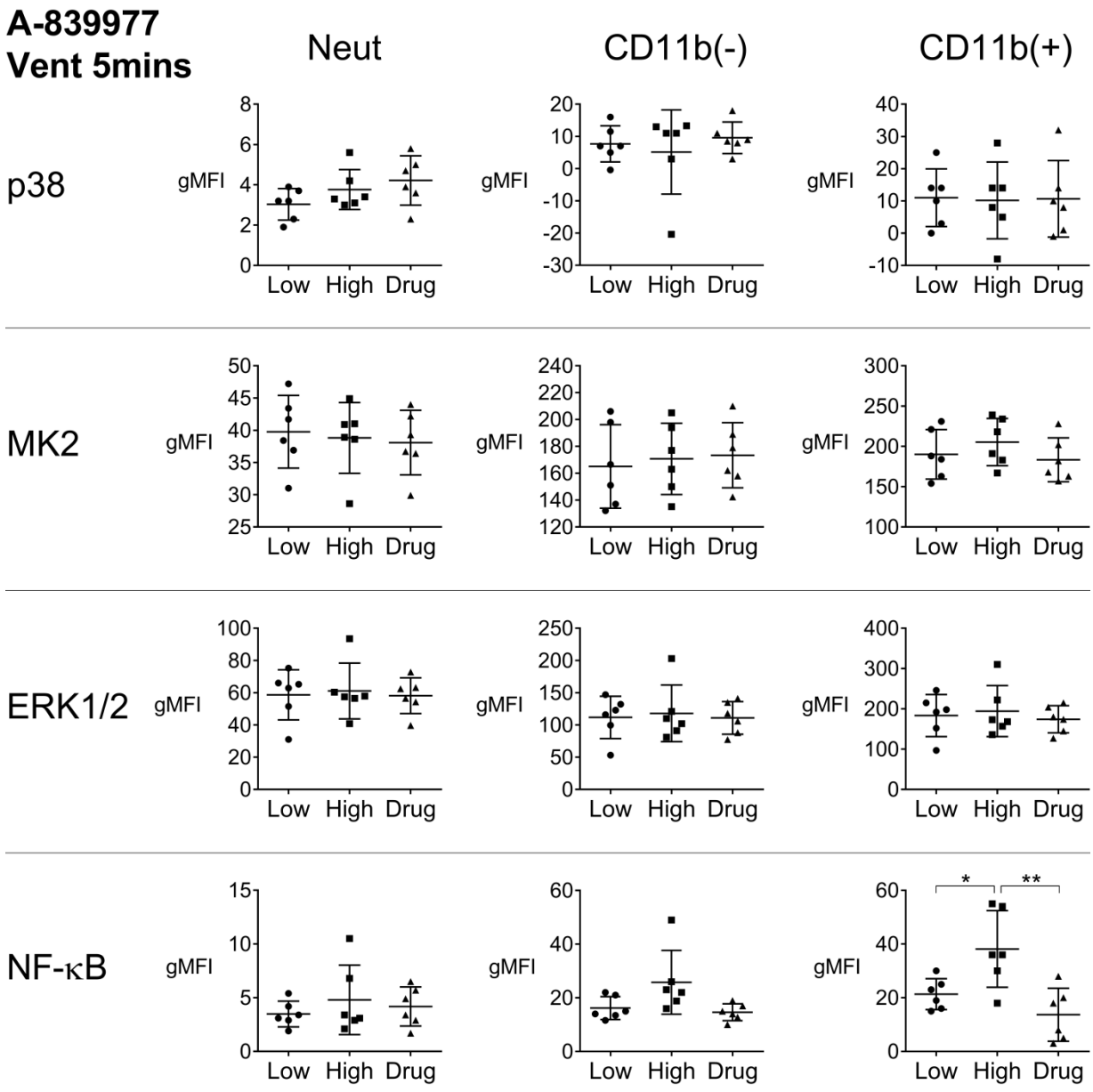


*Alveolar macrophage activation in response to stretch was attenuated in the DMSO pre-treated mice and therefore the effectiveness of the drug was unclear. N=6, *p<0.05, **p<0.01. Data were analysed by ANOVA with Tukey HSD and are displayed as mean ± SD.*

The systemic delivery of this drug meant that it could have had an effect within the pulmonary vasculature where endothelial cells may release ATP in response to high stretch ventilation according to our *in vitro* data. A range of leukocytes including neutrophils and two subtypes of dendritic cells (Figure 8.12) as well as monocytes (Figure 8.13) were additionally analysed in these experiments. No signs of activation were detected in neutrophils following 5 minutes high stretch ventilation (Figure 8.12). However, the CD11b(-) dendritic cells displayed a non-significant trend for NF-κB activation (p=0.064) following stretch that was non-significantly decreased with the A-839977 pre-treatment (p=0.052). Similarly, the CD11b(+) dendritic cells displayed a significant increase in

NF-κB activity following 5 minutes of high stretch, that was significantly decreased by the A-839977 inhibitor (Figure 8.12).

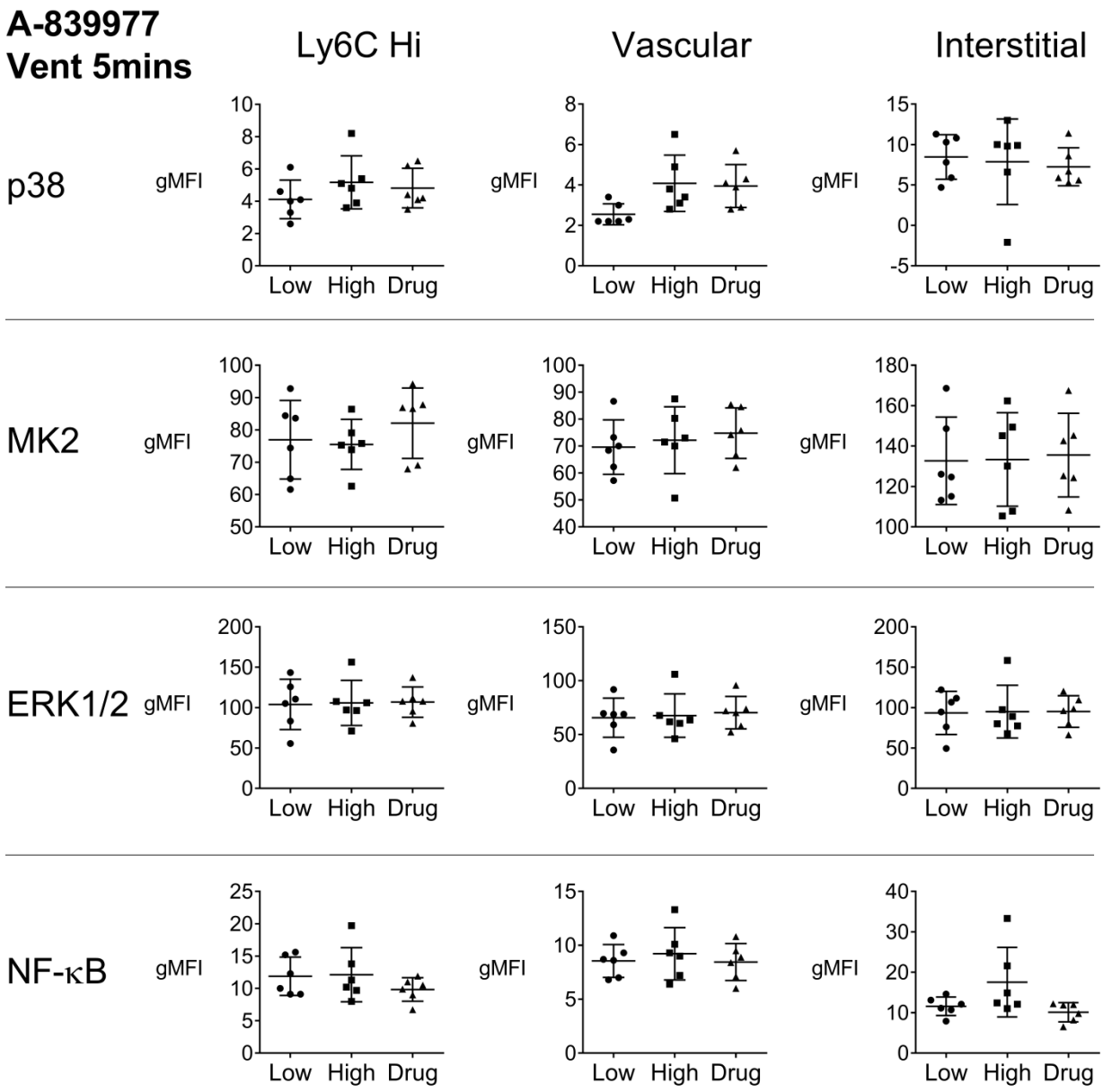
Figure 8.12 Phosphorylation of activation markers in neutrophils and dendritic cells after systemic pre-treatment with P2X7 receptor antagonist A-839977 30 minutes prior to 5 minutes of low or high stretch ventilation



*CD11b(-) and (+) dendritic cells displayed increased NF-κB activation in response to 5 minutes high stretch ventilation that was decreased in the presence of A-839977. N=6, *p<0.05, **p<0.01. Data were analysed by ANOVA with Tukey HSD and are displayed as mean ± SD.*

Ly6C high monocytes displayed no signs of activation following 5 minutes of high stretch ventilation. However, the Ly6C low vascular monocytes displayed a non-significant trend for p38 activation ($p=0.058$) that was also apparent in the presence of A-839977 ($p=0.087$). The Ly6C low interstitial monocytes demonstrated a slight non-significant trend for NF-κB activation between the low and high stretch control conditions ($p=0.163$), which was accompanied by a non-significant decrease in NF-κB activity when ventilated in the presence of the A-839977 inhibitor ($p=0.069$), compared with control DMSO treated high stretch ventilation (Figure 8.13).

Figure 8.13 Phosphorylation of activation markers in monocytes after systemic pre-treatment with P2X7 receptor antagonist A-839977 30 minutes prior to 5 minutes of low or high stretch ventilation



The interstitial monocytes displayed a significant decrease in NF-κB activation following 5 minutes of high stretch ventilation with the A-839977 pre-treatment. N=6. Data were analysed by ANOVA with Tukey HSD and are displayed as mean ± SD.

8.5 Discussion

In this chapter, an increase in ATP content in the BAL fluid was discovered in mice subjected to 5 minutes of high stretch ventilation compared with 5 minutes of low stretch. To my knowledge this is the earliest reported detection of ATP release into the alveolar space in response to high tidal

volume ventilation *in vivo*. The amount of ATP detected was in the 50 to 100 nanomolar range which is consistent with BAL fluid levels reported in the literature following longer periods of mechanical ventilation. Similar levels of BAL fluid ATP have also been reported to be released in response to stretch *in vitro* (Mishra et al. 2011; Patel et al. 2005). Furthermore, similar ATP levels have been found in the BAL fluid of patients with pulmonary fibrosis (Riteau et al. 2010), and in BAL fluid from *in vivo* models of VILI (Rich et al. 2003; Douillet et al. 2006; Matsuyama et al. 2008).

Only a couple of studies have detected higher levels of ATP in BAL fluid, in the micromolar range. A study which detected 1µM ATP in the BAL fluid of mice used a standard luminescence assay, and so we can only speculate that the very high levels of ATP detected were a specific response to the bleomycin-induced lung fibrosis model (Riteau et al. 2010). A study that detected 24µM ATP in the BAL fluid of an isolated perfused rat lung ventilated for 35 minutes at 35ml/kg with no PEEP, used high performance liquid chromatography (HPLC) to quantify ATP levels (Briva et al. 2011) . The high levels of ATP found in this model may be due to a combination of the advanced technique used to measure ATP and due to artefacts of the isolated perfused lung system which might lead to an abnormal accumulation of ATP.

Whilst the majority of studies have detected BAL fluid ATP levels in the nanomolar range, we must not ignore the massive dilution factor caused by instillation of up to a few millilitres of fluid into the lungs to be withdrawn for analysis. It can be predicted that the ATP concentration in the local region in which it is released would be considerably higher than has been detected in very dilute BAL fluid samples. A study investigating adenosine levels in BAL fluid used urea concentrations in the BAL fluid to calculate a dilution factor of 75 to 100-fold caused by the BAL procedure. When this dilution factor was taken into account, they predicted that the adenosine concentration within the normal volume of alveolar fluid would be 60-70µM (Factor et al. 2007). If we approximate that the BAL fluid ATP in our experiment was diluted up to 100-fold then we might predict that the ATP concentration within the surfactant layer could reach 11µM or even higher if the HPLC method provides a more accurate measurement. Indeed a study using real-time imaging without the need for dilution, showed that a single stretched A549 cell could release quantities of ATP which reached up to 10µM in the surrounding area (Grygorczyk, Furuya, and Sokabe 2012).

Beigi et al. developed a method for measuring local concentrations of ATP that involved the attachment of luciferase to the cell surface, using this technique they measured up to 15µM ATP released by stimulated platelets, which was 10- to 20-fold higher than when measured by soluble luciferase (Beigi et al. 1999). Pellegatti et al. had a similar idea and engineered a plasma membrane luciferase that they transfected into HEK293 cells to show that stretch caused by a change in tonicity

of the perfusion buffer induced a 4- to 5-fold increase in ATP release (Pellegatti et al. 2005). These studies all indicate towards a high concentration of ATP release locally, which is greatly diluted by the BAL procedure. Therefore, we may conclude that ATP is released in sufficient quantities to be able to induce activation of other cells.

However, we found that 100µM ATP did not stimulate MAP kinase activation of RAW cells. As a tumour cell line, RAW cells are not expected to respond in the same manner as primary cells and they can also demonstrate high basal levels of activation, preventing them from responding to lower levels of stimulation. A previous study was able to demonstrate p38 and ERK1/2 activation within 15 minutes following stimulation of RAW cells with 1mM ATP; however the RAW cells had been primed by pre-incubation with LPS for 4 hours (Kawano et al. 2012). Other studies have used BzATP, which is an agonist of the $P2X_7$ receptor with 5- to 10-fold more potency than ATP (Aga et al. 2004; Pfeiffer et al. 2004), perhaps suggesting that higher concentrations of ATP than used presently are required to stimulate RAW macrophages.

Primary alveolar macrophages that were isolated by BAL and stimulated *in vitro* were found to be more responsive to ATP than the RAW cell line. This could simply be due to the more physiological responses of primary cells that can be absent in tumour cell lines such as the RAW cells. Nevertheless, these results suggested that ATP would be capable of rapidly inducing activation of alveolar macrophages *in vivo*. Alveolar macrophages stimulated *in vivo* were found to require even higher concentrations of ATP (100mM). This dose does seem high compared with those used *in vitro* conditions but this dose has been previously instilled into the lungs of mice that were then returned to their cages for 24 hours (Matsuyama et al. 2008). The need for the high dose to induce activation *in vivo* could be due to the presence of ecto-ATPases which may quickly neutralise the instilled dose of ATP.

The $P2X_7$ receptor, which has been implicated in inflammatory signalling (Solle et al. 2001; Riteau et al. 2010) is unlike any other purinergic receptor in that it requires an excess of 100µM ATP to be activated. Within milliseconds of exposure to micromolar concentrations of ATP, the $P2X_7$ receptor opens a non-selective cation channel composed of three transmembrane subunits. However, at millimolar concentrations of ATP the channel allows the passage of larger organic cations and anions up to 900 Da. The mechanism for this switch in channel permeability is unknown but evidence for two theories exists, the first suggests that the channel simply dilates whereas the second suggests that an additional channel is involved (Alves et al. 2014).

Despite the need for a high dose of ATP to induce activation *in vivo*, the alveolar macrophages did display increased activation of all the MAP kinases compared with just MK2 when stimulated *in vitro*. Their activation *in vivo* may have been enhanced by the biological environment, which may have provided other mediators or intercellular contacts that may have played a role in their response to ATP stimulation. The primary alveolar macrophages stimulated *in vitro* were additionally subjected to a BAL procedure and the *in vitro* environment of the plastic well which may have activated them and then subsequently influenced their sensitivity to further stimulation.

Interestingly, in neither the *in vitro* nor the *in vivo* experiments, did ATP stimulation induce NF-κB activation in the alveolar macrophages, despite significant activation of the MAP kinases. This reflected the pattern seen in response to high stretch ventilation which failed to induce NF-κB activation in any cell type within 15 minutes. Therefore it could be said that ATP induced a very similar pattern of cellular activation within the lung that supports its role as a soluble mediator involved in activating alveolar macrophages in response to stretch. In line with this theory, ATP instillation did not induce activation of AECs nor endothelial cells that would be expected to be the main source of ATP in response to mechanical ventilation.

So far we have found that ATP is released into the BAL fluid following just 5 minutes of high stretch ventilation and that ATP instilled into the lungs is capable of activating MAP kinase signalling in alveolar macrophages within 5 minutes. This data suggests that ATP is a potential soluble mediator involved in the communication of stretch-induced inflammatory signalling between the AECs and alveolar macrophages. We therefore looked at the effects of a $P2X_7$ receptor competitive antagonist A-839977 on the cellular activation induced by 5 minutes of high stretch ventilation. This drug was suggested in the literature to be able to penetrate the central nervous system (Friedle, Curet, and Watters 2010; Honore et al. 2009) and therefore it was considered likely to also be able to enter the alveolar space if given systemically. This would avoid any negative effect of a fluid bolus on the respiratory mechanics if it were required to be instilled directly into the lungs. Additionally, the A-839977 compound was dissolvable only in DMSO which is an irritant, therefore making intraperitoneal injection the safest route of administration. This drug has been administered to rats via this route 15 to 30 minutes prior to experimentation in studies reported in the literature (Falk et al. 2015; Honore et al. 2009).

All mice used in these experiments were exposed to the same amount of DMSO as it was thought to be a potential confounding factor. Indeed the DMSO pre-treatment did appear to have some effects on the responsiveness of particular cell types to high stretch ventilation. In addition to this extra source of variability, the experiments were performed with an n of 6, but a power calculation

suggested that an n of 7 was required to detect significant changes in cellular activation. This lack of power is evident in the data in which many trends were observed that did not reach statistical significance.

The AECs and endothelial cells displayed normal MK2 activity, but displayed reduced ERK1/2 activation in response to high stretch. As expected, this MK2 activity observed was not reduced by pre-treatment with the A-839977 inhibitor. The parenchymal cells are most likely activated directly in response to stretch rather than through a secondary mediator, and therefore inhibition of ATP signalling via the $P2X_7$ receptor was not predicted to reduce their activation.

The alveolar macrophages displayed diminished activity following DMSO pre-treatment with reduced MK2 activation in response to 5 minutes ventilation. This suggests that the drug did gain access to the alveolar space; however the DMSO had a desensitising effect on the alveolar macrophages. This model was therefore not very useful to study the importance of $P2X_7$ receptor signalling in alveolar macrophage activation following stretching of the lung parenchyma during mechanical ventilation.

Despite the unclear results from the alveolar macrophages, we did find some interesting results in some of the other leukocytes analysed in these experiments. Both the dendritic cell subsets displayed NF-κB activity within just 5 minutes of high stretch ventilation. This was an unexpected finding since NF-κB activity had not previously been detected in any cell type in response to high stretch ventilation within the 15-minute timescale studied, despite many reports of its activity at later time points in the literature. Additionally, the A-839977 drug was found to prevent this activity suggesting that they were activated via purinergic signalling. To my knowledge, dendritic cells have not previously been associated with the pathogenesis of VILI. Dendritic cells are thought to be largely located in the airway tissue and therefore may be subjected to stretch, or may be activated by a secondary mediator released by the airway epithelium such as ATP as indicated by the inhibition experiments.

A trend was also detected for NF-κB activity within the Ly6C low interstitial monocytes that was also significantly decreased with the A-839977 pre-treatment. Interstitial monocytes are located within the interstitial space that is between the AECs and pulmonary endothelial cells, two cell types which are very sensitive to high stretch ventilation. In this location, interstitial monocytes could be directly subjected to stretching, or according to the current results, they may be activated in response to ATP release from either the epithelium or endothelium. Our data seems to suggest that many leukocytes within the lung may be activated in response to stretch through the release of ATP which is maybe released by both the epithelium and endothelium.

CHAPTER NINE

FINAL DISCUSSION

This project investigated the mechanism of alveolar macrophage activation in response to high tidal volume ventilation leading to VILI. Whilst the involvement of alveolar macrophages during established VILI has been very well characterised, the events which actually lead to alveolar macrophage activation in response to an initially structural insult, has not been explored. We hypothesised that the AECs would be the initial responders to over-distension, as they would be directly stretched during high tidal volume ventilation. Once activated, we predicted that the AECs would then communicate with alveolar macrophages to propagate an inflammatory response.

The first aim of the project was to identify how quickly type I and II AECs, pulmonary endothelial cells, and alveolar macrophages, respond to high tidal volume ventilation *in vivo*. By examining which cell types become activated and when, in response to stretch, we hoped to detect a pattern of cellular activation that could indicate a direction of communication. We developed a phospho-flow cytometry protocol that enabled us to quantify the activation of MAP kinase and NF-κB intracellular inflammatory signalling pathways. We were able to detect cellular activation in specific cell populations from whole lung samples that had been exposed to a mechanical ventilation protocol for a precise amount of time. Our initial experiments were conducted using a model of intratracheal LPS instillation as a positive control for the development of the phospho-flow technique. This model also provided an interesting comparison to the VILI model, which we hoped would induce distinct kinetics of cellular activation.

The second aim of the project was to identify the mechanism by which alveolar macrophages become activated in response to high tidal volume ventilation. Our first hypothesis proposed that alveolar macrophages may become activated via a direct contact mediated mechanism such as

communication through intercellular channels, or through macrophages sensing the deformation of AECs via adhesion molecule interactions. Our alternative hypothesis proposed that the AECs may rapidly release a soluble mediator that subsequently activates the alveolar macrophages.

Summary of findings

In the LPS-induced lung injury model, alveolar macrophages were discovered to be the sole source of MAP kinase activation at 5 minutes after LPS instillation. LPS also induced prolific NF-κB signalling in alveolar macrophages and both type I and II AECs within just 5 minutes. However, MAP kinase activation was only detected in epithelial and endothelial cells after 15 minutes of LPS exposure. The speed of NF-κB activation observed in epithelial cells suggested that this was a direct response to LPS stimulation. Whereas the MAP kinase activation observed after 15 minutes in epithelial and endothelial cells, may indicate the involvement of a secondary mediator, probably released by the alveolar macrophages.

We discovered that TNFα may be the mediator responsible for MAP kinase activation in epithelial cells in response to LPS stimulation. In TNF receptor knockout mice, we found that LPS did not induce the MAP kinase activation that was observed at this time point in the epithelial and endothelial cells of wild type mice. There was also evidence of reduced NF-κB activity in the TNF receptor knockouts. This data indicates that TNFα signalling is involved in the activation of inflammatory pathways in the lung parenchyma following LPS instillation into the alveolar space.

In the VILI model, cellular activation was much more widespread following 5 minutes of high stretch ventilation compared with LPS. Each cell type demonstrated significant activation of MK2 and/or ERK1/2 after 5 minutes of high stretch ventilation compared with low. In stark contrast to LPS, mechanical ventilation did not induce NF-κB signalling in any cell type. When LPS and VILI experiments were conducted for just 1 minute, we found that mechanical ventilation induced inflammatory signalling faster than LPS. This very rapid activation was observed in type I and II AECs in terms of p38 phosphorylation, and most significantly in the endothelial cells, which displayed activation of all three MAP kinases. Endothelial cells were also discovered to be the most sensitive to stretch, as they were the only cell type to respond to the moderate tidal volume of 30ml/kg.

These experiments indicated that AECs were activated in response to high tidal volume ventilation prior to alveolar macrophages. This pattern of cellular activation differed to that induced by LPS, which not only appeared to be slower, but also induced the most significant activation of markers in the alveolar macrophages as opposed to the parenchyma. We moved on to investigate our second

aim which was to identify the mechanism which leads to alveolar macrophage activation in response to such a short period of mechanical ventilation.

We first explored the contact-mediated hypothesis based on evidence suggesting that alveolar macrophages can very rapidly become adherent to the alveolar wall following mechanical ventilation *in vivo*. A gap junction inhibitor Gap26 peptide (or saline control) was administered to mice 90 minutes prior to exposure to 5 minutes of low or high stretch ventilation. Unfortunately we were not able to detect the same level of alveolar macrophage activation in the control-treated mice that was observed in the previous experiments. We suspected that this was an adverse effect of the fluid bolus and short pre-treatment time. In another attempt to inhibit AM activation, anti-CD18 antibody was administered, which was expected to be retained within the lung for up to 24 hours, thereby allowing sufficient time to allow for recovery of respiratory mechanics. However, the isotype-treated mice also displayed reduced parenchymal cell activation in response to stretch and changes in respiratory mechanics. We concluded that the anaesthetic protocol and perhaps the isoflurane exposure specifically, were reducing cellular sensitivity to stretch by increasing the compliance of the lung with long-lasting effects.

To avoid the confounding effects of this instillation protocol, we used TNF receptor knockout mice to study the role of TNFα in the initiation of VILI. Whilst these mice showed similar parenchymal activity to that seen in wild type mice, they demonstrated reduced activation of alveolar macrophages. However, the macrophages did still display a trend for activation, perhaps suggesting that TNFα may be involved in the initiation of inflammatory signalling, but probably in combination with other mediators.

In search of these potential mediators, experiments were conducted *in vitro* to study cell types in isolation, which provided a simplified system for identifying the possible cell source. The phospho-flow cytometry protocol for measuring activation of intracellular signalling molecules was adapted for use on cells cultured *in vitro*. A human lung epithelial cell line (A549), murine lung epithelial (MLE) and brain endothelial (bEnd5) cells were exposed to cyclic stretch corresponding to 13% strain for 15 minutes. ATP was found to be released into the supernatant in abundance by all three cell lines, whilst no TNFα or IL-6 could be detected in the media taken from stretched MLE cells. This led to the investigation of ATP as a possible mediator involved in the initiation of inflammatory signalling in VILI.

In correlation with this *in vitro* data, we discovered an increase in ATP content in the BAL fluid of mice ventilated for 5 minutes at a high tidal volume compared with low. We also determined that ATP

instilled intratracheally was capable of inducing MAP kinase activation in alveolar macrophages comparable to that observed in response to mechanical ventilation. Interestingly, intratracheal ATP did not induce activation of NF-κB signalling, which again was similar to the pattern of cellular activation induced by high stretch ventilation. Furthermore, ATP instillation did not induce significant activation of AECs or the endothelium, the cells types that would be expected to be responsible for the rapid ATP release in response to stretch. We attempted to examine the effect of inhibiting purinergic signalling on cellular responses to mechanical ventilation, but unfortunately, the results of these experiments were difficult to interpret due to the confounding effect of the DMSO vehicle. Unfortunately, as these experiments were performed towards the end of this project, it was not possible to investigate this further.

Theory of alveolar macrophage activation

We detected activation of the MK2 and ERK1/2 signalling pathways in alveolar macrophages following just 5 minutes of high stretch ventilation. This activation appeared to occur subsequent to activation of the AECs, which supported our hypothesis that the AECs would be the initial responder to stretch and would then communicate with the alveolar macrophages to propagate an inflammatory response. The timescale in which this interaction occurred limited the possible mechanisms of communication, and excluded the release of most cytokines which can require hours to be up-regulated and secreted as mature protein.

Promising findings from purinergic signalling experiments included detecting high ATP concentrations in BAL fluid after 5 minutes of high stretch ventilation, and the rapid induction of MAP kinase activation following instillation of ATP intratracheally. These results lead us to believe that ATP could be a possible candidate for a soluble mediator that is released very rapidly by AECs in response to stretch, and could instigate inflammatory signalling in alveolar macrophages. Unfortunately, subsequent experiments, in which mice were pre-treated with a purinergic signalling inhibitor, failed to show a reduction in MAP kinase activation in response to high stretch ventilation due to the pre-treatment having a significant effect on the MAP kinase measurements. It was assumed that this effect was due to the DMSO solvent required to dissolve the purinergic inhibitor for administration, which is known to be an irritant.

With more time, it would have been useful to perform further experiments under *in vitro* conditions to gain a better understanding of the role of purinergic signalling before investigating the theory *in vivo*. The *in vitro* experiments that were performed could have been expanded, with a different range of measured outputs to assess activation of inflammatory signalling. It would also have been

interesting to perform cyclic stretch experiments with co-cultures of alveolar macrophages and AECs in the presence of a purinergic signalling inhibitor.

To assess the role of purinergic signalling *in vivo*, it would be necessary to identify a purinergic signalling inhibitor that is soluble in saline, in order to repeat the final experiments performed in Chapter 8, in the absence of DMSO as a confounding factor. If these experiments showed a reduction in alveolar macrophage activation following short periods of high stretch ventilation, then the next step would be to assess whether the purinergic inhibitor could also prevent the downstream effects of high stretch ventilation that are associated with VILI. In such experiments, the ventilation regimen should continue for up to 3 hours, or a time period that would be sufficient to determine whether the pre-treatment was effective in limiting the breakdown of the alveolar-capillary wall, preventing the accumulation of BAL protein, or reducing the infiltration of extra-pulmonary inflammatory cells into the lungs.

Even if ATP proves not to be involved in the initial activation of alveolar macrophages in response to high tidal volume ventilation, our earlier experiments clearly indicate that the AECs are activated prior to alveolar macrophages. Therefore, future research should focus on preventing the communication of stress between these two cell types in order to prevent alveolar macrophage activation. To identify other potential mediators, further *in vitro* experiments where an alveolar epithelial cell line is subjected to cyclic stretch should be performed. Multiplex arrays could then be used to test for the presence of many soluble mediators, and the relative quantities secreted could indicate their importance as a mediator of alveolar macrophage activation. A potential mediator would need to then be tested, by exposing alveolar macrophages to it and assessing their activation.

Activation within the pulmonary vasculature

The most surprising discovery in this project was that the pulmonary endothelial cells appeared to be more sensitive to high tidal volume ventilation than the AECs. The endothelium not only demonstrated activation of the most inflammatory signalling pathways tested following 1 minute of high stretch, but was also the only cell type to demonstrate activation in response to a moderate tidal volume. This data indicates that the development of systemic inflammation and multiple organ failure (MOF) may occur via an alternate mechanism to the popular 'decompartmentalisation' theory. This current model suggests that inflammation localised within the alveolar space leaks into the circulation as a result of damage to the alveolar-capillary barrier. However, our data, which revealed almost immediate activation of the pulmonary endothelium, suggests that injurious ventilation may

be capable of propagating inflammation within the circulation directly, prior to the development of physical derangements to the lung parenchyma.

To further support our findings that dispute the 'decompartmentalisation' theory we would need to demonstrate the activation of extra-pulmonary inflammatory cells before disruption to the alveolar-capillary barrier. Given that the endothelial cells became activated so quickly in our model, it would seem likely that we would be able to detect the activation of neutrophils or monocytes within the timescale under which the alveolar-capillary barrier would begin to show histological damage.

The rapidity of pulmonary endothelial cell activation in response to high stretch ventilation, suggests that it could be difficult to prevent the activation of extra-pulmonary inflammatory cells once injurious ventilation has occurred. Patients may need to receive treatment prophylactically in order to prevent the dissemination of inflammatory responses induced by high stretch ventilation. This kind of preventative measure could be administered in clinical practise alongside the initiation of mechanical respiratory support. A target for the prevention of systemic inflammation in VILI would be mechanisms of communication between the endothelial cells and extra-pulmonary inflammatory cells.

One potential method of communication is through the release of ATP by the pulmonary endothelium. We found that an endothelial cell line exposed to cyclic stretching for 15 minutes, released significant amounts of ATP that could provide a mechanism by which endothelial cells might rapidly activate extra-pulmonary inflammatory cells in response to stretch. Neutrophils are known to express purinergic receptors (Suh et al. 2001), and ATP has been shown to increase their expression of macrophage-1 antigen Mac-1 (Akbar, Mills, and Kunapuli 1997). This could suggest that ATP acts as a chemoattractant for neutrophil migration to the lung during VILI.

This finding requires further investigation to determine the downstream effects of such rapid endothelial activation in response to high stretch ventilation. There is evidence that endothelial cells are capable of releasing inflammatory mediators and can enhance their surface expression of adherence molecules in response to stretch, both of which indicate that the endothelium could have a significant role in the activation of extra-pulmonary leukocytes and could promote their infiltration into the alveolar space. Further research into this hypothesis would have great clinical value especially given that the most common cause of death in ARDS patients is multiple organ failure (MOF).

Phospho-flow cytometry

The application of phospho-flow cytometry to our *in vivo* system permitted us to quantify the cellular activation of individual cells at precise time points following exposure to a stimulus. Assessment of the phosphorylation status of intracellular MAP kinases and transcription factor NF-κB provided the advantage of measuring upstream signalling events that could be localised to specific cell populations. Using this methodology we were able to reveal novel insights and differences in the profiles of cellular activation induced by both intratracheal LPS instillation and high tidal volume mechanical ventilation.

The identification of pulmonary cell populations by flow cytometry has been well documented and validated by both our group and other research groups (Misharin et al. 2013; Zaynagetdinov et al. 2013). In every experiment the whole lung was sampled and processed, which provided a sufficient number of cells for reliable identification. Leukocytes such as the alveolar macrophages and neutrophils were the easiest to identify given their large numbers within the lung and their tendency not to be adhered to other cells, thereby increasing their yield. In comparison, fewer numbers of the parenchymal cell populations were identified despite their abundance within the lung. These cells were probably susceptible to damage during the tissue homogenisation procedure due to their strong attachments to neighbouring cells within the one cell thick alveolar and capillary barriers and their more fragile squamous shape. Finally, fewer numbers of some specific leukocyte subsets were collected that are less abundant within the lung and probably actually located within the pulmonary vasculature. Nevertheless, for the majority of pulmonary cell types we were able to count at least 3000 cells within each stained sample by flow cytometry (each lung was split into 20 samples for staining) and at least 1000 cells even in the least abundant cell types identified. We were therefore confident that we were examining a sufficient number of cells to be representative of the whole population within the lung.

Within these populations, we then measured the phosphorylation status of the MAP kinase p38 and its immediate downstream substrate MK2, the MAP kinase ERK1/2, and the transcription factor NF-κB. In the LPS experiments, the increase in phosphorylation of all four markers was substantial; however, the changes were much more subtle in response to high stretch ventilation. We therefore validated the signals by also measuring the levels of total protein for some of the markers within the same lung samples. We found that the total protein levels of p38 and ERK1/2 remained unaltered following 5 minutes of high stretch ventilation compared with low. We could therefore be confident that the changes in phosphorylation levels that we were detecting were a real response to high stretch ventilation.

The four intracellular activation markers used in this study are involved in three separate signalling pathways that promote inflammation in response to myriad stress stimuli. However, it remains possible that inflammatory signalling pathways other than the ones we measured could have been up-regulated. Many of the intracellular markers also displayed patterns of transient activation, which could mean that activity occurring between the time points studied may have been missed. Transient activation appears to be characteristic of both the MAP kinases and NF-κB, and has been reported many times in response to both LPS and stretch in a variety of pulmonary cell types (Abdulnour et al. 2006; Boudreault and Tschumperlin 2010; Cohen et al. 2010; Schnyder-Candrian et al. 2005). Another explanation for the transiency that we observed may simply be a result of the cycling of these molecules between the cytoplasm and nucleus, which is thought to be less accessible to antibodies with saponin permeabilisation. Regardless, p38, ERK1/2 and NF-κB have all been implicated in the inflammatory response associated with VILI and their inhibition has been found to attenuate cytokine release among other symptoms, suggesting that inflammatory cascades can be initiated even following transient activation.

The MAP kinase signalling system was very sensitive and we frequently found that factors such as pre-treatment under isoflurane or administration of DMSO vehicle influenced cell sensitivity to stretch. For this reason many of the experiments that used a pre-treatment protocol produced results that were difficult to interpret. In these cases, it would have perhaps been useful to measure some other markers of early cellular activation such as nitrous oxide production, though we would not have been able to determine the cell source with measurement of this secreted module.

In vivo model of VILI

The *in vivo* model of LPS-induced lung injury most closely replicates ARDS caused by a respiratory infection such as pneumonia. The LPS endotoxin, which is a constituent of the gram-negative bacterial cell wall, promotes a strong inflammatory response, but does not actually replicate infection and is considered to be a sterile insult. The clinical relevance of this model is limited in this sense, but nevertheless it is widely used. In this thesis, the LPS model was a useful comparison to the VILI model and revealed that the pattern of cellular activation was specific to the given stimuli. This indicated that it might be possible to attenuate cellular activation in response to over-distension caused by mechanical ventilation, without inhibiting responses to infectious agents, which would be an important consideration in the clinical setting.

The *in vivo* model of high tidal volume ventilation used in this project allowed us to study the effects of stretch within the context of a real lung which, due to its complex structure and high level of

interdependence, is very difficult to replicate *in vitro*. Several studies have reported effects of stretching cells *in vitro* such as cytokine release, however it is unclear whether some of these cell types, for example alveolar macrophages, would actually be subjected to stretch *in vivo*. Our study revealed the first insight into which cell types actually demonstrate activation of inflammatory signalling in response to high tidal volume ventilation within the first 15 minutes *in vivo*.

The very high tidal volume used in this model is related to the use of mice which have naturally more compliant lungs compared with humans. The high tidal volume also reflects the use of a one-hit model to specifically study the effects of over-stretching the lung rather than any other injurious aspect of mechanical ventilation such as atelectasis. In addition, the high tidal volume replicates the extreme pressures that ARDS patients are subjected to as a result of substantial pulmonary oedema.

Concluding remarks

Besides the ARDS Network trial published in 2000, which demonstrated that lower tidal volumes are beneficial to patient mortality, there has been no clinical breakthrough in terms of preventing VILI, which remains a major cause of morbidity and contributor to mortality. Experimental studies have shown that over-stretching the lung is a major pathogenic factor that promotes a profound inflammatory response, propagated by the resident alveolar macrophages, yet the mechanism of stretch-induced inflammation has not been fully explored.

This project used an *in vivo* model of VILI to investigate the initial steps that lead to alveolar macrophage activation. This research revealed that the AECs are the most stretch-sensitive cell type within the alveolar space and are likely to be responsible for the subsequent activation of alveolar macrophages, either via the rapid release of a soluble mediator or contact-dependent mechanism. The speed at which these events occurred in response to high tidal volume ventilation was remarkably fast but an even more surprising discovery was that of rapid pulmonary endothelial cell activation. Endothelial cells were not only activated within 1 minute of exposure to high tidal volume ventilation, but were also the most sensitive to stretch at lower tidal volumes.

This data strongly indicate that systemic inflammation may be immediately propagated within the pulmonary vasculature upon exposure to high tidal volume ventilation. Physicians supporting patients with mechanical ventilation should be vigilant in maintaining non-injurious ventilation settings as our data shows that it only takes minutes to initiate inflammatory signalling pathways in response to over-distension of the lungs. Caution should also be applied during recruitment

manoeuvres that involve holding the lungs at a high pressure for an extended length of time, which, as indicated by the present findings, could also initiate inflammatory signalling.

Such rapid inflammatory responses are viable therapeutic targets in an iatrogenic disease such as VILI. Therefore, further research into the initial mechanisms involved in transducing stretch into inflammation both within the alveolar space and within the pulmonary vasculature, has substantial clinical importance.

CHAPTER TEN

REFERENCES

Abdulnour, R. E., X. Peng, J. H. Finigan, E. J. Han, E. J. Hasan, K. G. Birukov, S. P. Reddy, J. E. Watkins, 3rd, U. S. Kayyali, J. G. Garcia, R. M. Tuder, and P. M. Hassoun. 2006. 'Mechanical stress activates xanthine oxidoreductase through MAP kinase-dependent pathways', *Am J Physiol Lung Cell Mol Physiol*, 291: L345-53.

Abraham, E., A. Anzueto, G. Gutierrez, S. Tessler, G. San Pedro, R. Wunderink, A. Dal Nogare, S. Nasraway, S. Berman, R. Cooney, H. Levy, R. Baughman, M. Rumbak, R. B. Light, L. Poole, R. Allred, J. Constant, J. Pennington, and S. Porter. 1998. 'Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. NORASEPT II Study Group', *Lancet*, 351: 929-33.

Abraham, E., R. Wunderink, H. Silverman, T. M. Perl, S. Nasraway, H. Levy, R. Bone, R. P. Wenzel, R. Balk, R. Allred, and et al. 1995. 'Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group', *JAMA*, 273: 934-41.

Abraham, V., M. L. Chou, K. M. DeBolt, and M. Koval. 1999. 'Phenotypic control of gap junctional communication by cultured alveolar epithelial cells', *Am J Physiol*, 276: L825-34.

Abraham, V., M. L. Chou, P. George, P. Pooler, A. Zaman, R. C. Savani, and M. Koval. 2001. 'Heterocellular gap junctional communication between alveolar epithelial cells', *Am J Physiol Lung Cell Mol Physiol*, 280: L1085-93.

Adachi, M., M. Fukuda, and E. Nishida. 2000. 'Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism', *J Cell Biol*, 148: 849-56.

Aderka, D., H. Engelmann, Y. Maor, C. Brakebusch, and D. Wallach. 1992. 'Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors', *J Exp Med*, 175: 323-9.

Aga, M., J. J. Watters, Z. A. Pfeiffer, G. J. Wiepz, J. A. Sommer, and P. J. Bertics. 2004. 'Evidence for nucleotide receptor modulation of cross talk between MAP kinase and NF-kappa B signaling pathways in murine RAW 264.7 macrophages', *Am J Physiol Cell Physiol*, 286: C923-30.

Akbar, G. K., D. C. Mills, and S. P. Kunapuli. 1997. 'Characterization of extracellular nucleotide-induced Mac-1 (alphaM beta2 integrin) surface expression on peripheral blood leukocytes', *Biochem Biophys Res Commun*, 233: 71-5.

Albert, R. K., L. J. Embree, J. E. McFeely, and D. D. Hickstein. 1992. 'Expression and function of beta 2 integrins on alveolar macrophages from human and nonhuman primates', *Am J Respir Cell Mol Biol*, 7: 182-9.

Albertine, K. H., M. F. Soulier, Z. Wang, A. Ishizaka, S. Hashimoto, G. A. Zimmerman, M. A. Matthay, and L. B. Ware. 2002. 'Fas and fas ligand are up-regulated in pulmonary edema fluid and lung

tissue of patients with acute lung injury and the acute respiratory distress syndrome', *Am J Pathol*, 161: 1783-96.

Alhazzani, W., M. Alshahrani, R. Jaeschke, J. M. Forel, L. Papazian, J. Sevransky, and M. O. Meade. 2013. 'Neuromuscular blocking agents in acute respiratory distress syndrome: a systematic review and meta-analysis of randomized controlled trials', *Crit Care*, 17: R43.

Allen, G. B., B. T. Suratt, L. Rinaldi, J. M. Petty, and J. H. Bates. 2006. 'Choosing the frequency of deep inflation in mice: balancing recruitment against ventilator-induced lung injury', *Am J Physiol Lung Cell Mol Physiol*, 291: L710-7.

Altemeier, W. A., G. Matute-Bello, C. W. Frevert, Y. Kawata, O. Kajikawa, T. R. Martin, and R. W. Glenny. 2004. 'Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin', *Am J Physiol Lung Cell Mol Physiol*, 287: L533-42.

Alves, L. A., R. A. de Melo Reis, C. A. de Souza, M. S. de Freitas, P. C. Teixeira, D. Neto Moreira Ferreira, and R. F. Xavier. 2014. 'The P2X7 receptor: shifting from a low- to a high-conductance channel - an enigmatic phenomenon?', *Biochim Biophys Acta*, 1838: 2578-87.

Amato, M. B., C. S. Barbas, D. M. Medeiros, R. B. Magaldi, G. P. Schettino, G. Lorenzi-Filho, R. A. Kairalla, D. Deheinzelin, C. Munoz, R. Oliveira, T. Y. Takagaki, and C. R. Carvalho. 1998. 'Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome', *N Engl J Med*, 338: 347-54.

Ando, J., and K. Yamamoto. 2011. 'Effects of shear stress and stretch on endothelial function', *Antioxid Redox Signal*, 15: 1389-403.

Anjum, Rana, and John Blenis. 2008. 'The RSK family of kinases: emerging roles in cellular signalling', *Nat Rev Mol Cell Biol*, 9: 747-58.

Arditi, M., J. Zhou, M. Torres, D. L. Durden, M. Stins, and K. S. Kim. 1995. 'Lipopolysaccharide stimulates the tyrosine phosphorylation of mitogen-activated protein kinases p44, p42, and p41 in vascular endothelial cells in a soluble CD14-dependent manner. Role of protein tyrosine phosphorylation in lipopolysaccharide-induced stimulation of endothelial cells', *J Immunol*, 155: 3994-4003.

ARDS Definition Task Force. 2012. 'Acute respiratory distress syndrome: the Berlin Definition', *JAMA*, 307: 2526-33.

ARDS Network. 2000. 'Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network', *N Engl J Med*, 342: 1301-8.

Armstrong, L., A. R. Medford, K. M. Uppington, J. Robertson, I. R. Witherden, T. D. Tetley, and A. B. Millar. 2004. 'Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells', *Am J Respir Cell Mol Biol*, 31: 241-5.

Arroliga, A., F. Frutos-Vivar, J. Hall, A. Esteban, C. Apezteguia, L. Soto, and A. Anzueto. 2005. 'Use of sedatives and neuromuscular blockers in a cohort of patients receiving mechanical ventilation', *Chest*, 128: 496-506.

Ashbaugh, D. G., D. B. Bigelow, T. L. Petty, and B. E. Levine. 1967. 'Acute respiratory distress in adults', *Lancet*, 2: 319-23.

Ashino, Y., X. Ying, L. G. Dobbs, and J. Bhattacharya. 2000. '[Ca(2+)](i) oscillations regulate type II cell exocytosis in the pulmonary alveolus', *Am J Physiol Lung Cell Mol Physiol*, 279: L5-13.

Badger, A. M., M. N. Cook, M. W. Lark, T. M. Newman-Tarr, B. A. Swift, A. H. Nelson, F. C. Barone, and S. Kumar. 1998. 'SB 203580 inhibits p38 mitogen-activated protein kinase, nitric oxide production, and inducible nitric oxide synthase in bovine cartilage-derived chondrocytes', *J Immunol*, 161: 467-73.

Bai, K. J., A. P. Spicer, M. M. Mascarenhas, L. Yu, C. D. Ochoa, H. G. Garg, and D. A. Quinn. 2005. 'The role of hyaluronan synthase 3 in ventilator-induced lung injury', *Am J Respir Crit Care Med*, 172: 92-8.

Banerjee, A., and S. Gerondakis. 2007. 'Coordinating TLR-activated signaling pathways in cells of the immune system', *Immunol Cell Biol*, 85: 420-4.

Barbour, S. E., C. Wong, D. Rabah, A. Kapur, and A. D. Carter. 1998. 'Mature macrophage cell lines exhibit variable responses to LPS', *Mol Immunol*, 35: 977-87.

Barletta, K. E., R. E. Cagnina, K. L. Wallace, S. I. Ramos, B. Mehrad, and J. Linden. 2012. 'Leukocyte compartments in the mouse lung: distinguishing between marginated, interstitial, and alveolar cells in response to injury', *J Immunol Methods*, 375: 100-10.

Barton, G. M., and R. Medzhitov. 2003. 'Toll-like receptor signaling pathways', *Science*, 300: 1524-5.

Bauer, G. J., S. Arbabi, I. A. Garcia, I. de Hingh, M. R. Rosengart, and R. V. Maier. 2000. 'Adherence regulates macrophage signal transduction and primes tumor necrosis factor production', *Shock*, 14: 435-40.

Bazil, V., and J. L. Strominger. 1991. 'Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes', *J Immunol*, 147: 1567-74.

Beck-Schimmer, B., C. Madjdpour, S. Kneller, U. Ziegler, T. Pasch, R. P. Wuthrich, P. A. Ward, and R. C. Schimmer. 2002. 'Role of alveolar epithelial ICAM-1 in lipopolysaccharide-induced lung inflammation', *Eur Respir J*, 19: 1142-50.

Beck-Schimmer, B., R. C. Schimmer, C. Madjdpour, J. M. Bonvini, T. Pasch, and P. A. Ward. 2001. 'Hypoxia Mediates Increased Neutrophil and Macrophage Adhesiveness to Alveolar Epithelial Cells', *Am J Respir Cell Mol Biol*, 25: 780-87.

Bedirli, N., C. Y. Demirtas, T. Akkaya, B. Salman, M. Alper, A. Bedirli, and H. Pasaoglu. 2012. 'Volatile anesthetic preconditioning attenuated sepsis induced lung inflammation', *J Surg Res*, 178: e17-23.

Beigi, R., E. Kobatake, M. Aizawa, and G. R. Dubyak. 1999. 'Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase', *Am J Physiol*, 276: C267-78.

Beinke, S., M. J. Robinson, M. Hugunin, and S. C. Ley. 2004. 'Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IkappaB kinase-induced proteolysis of NF-kappaB1 p105', *Mol Cell Biol*, 24: 9658-67.

Belete, H. A., R. D. Hubmayr, S. Wang, and R. D. Singh. 2011. 'The role of purinergic signaling on deformation induced injury and repair responses of alveolar epithelial cells', *PLoS One*, 6: e27469.

Belperio, J. A., M. P. Keane, M. D. Burdick, V. Londhe, Y. Y. Xue, K. Li, R. J. Phillips, and R. M. Strieter. 2002. 'Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury', *J Clin Invest*, 110: 1703-16.

Bem, R. A., J. B. van Woensel, A. P. Bos, A. Koski, A. W. Farnand, J. B. Domachowske, H. F. Rosenberg, T. R. Martin, and G. Matute-Bello. 2009. 'Mechanical ventilation enhances lung inflammation and caspase activity in a model of mouse pneumovirus infection', *Am J Physiol Lung Cell Mol Physiol*, 296: L46-56.

Ben-Levy, R., S. Hooper, R. Wilson, H. F. Paterson, and C. J. Marshall. 1998. 'Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2', *Curr Biol*, 8: 1049-57.

Ben-Levy, R., I. A. Leighton, Y. N. Doza, P. Attwood, N. Morrice, C. J. Marshall, and P. Cohen. 1995. 'Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2', *EMBO J*, 14: 5920-30.

Benson, A. B., and R. K. Albert. 2014. 'Prone positioning for acute respiratory distress syndrome', *Clin Chest Med*, 35: 743-52.

Bernard, G. R., A. Artigas, K. L. Brigham, J. Carlet, K. Falke, L. Hudson, M. Lamy, J. R. LeGall, A. Morris, R. Spragg, and Consensus Committee. 1994. 'Report of the American-European Consensus Conference on Acute Respiratory Distress Syndrome: Definitions, Mechanisms, Relevant Outcomes, and Clinical Trial Coordination', *J Crit Care*, 9: 72-81.

Bertok, S., M. R. Wilson, A. D. Dorr, J. O. Dokpesi, K. P. O'Dea, N. Marczin, and M. Takata. 2011. 'Characterization of TNF receptor subtype expression and signaling on pulmonary endothelial cells in mice', *Am J Physiol Lung Cell Mol Physiol*, 300: L781-9.

Bertok, S., M. R. Wilson, P. J. Morley, R. de Wildt, A. Bayliffe, and M. Takata. 2012. 'Selective inhibition of intra-alveolar p55 TNF receptor attenuates ventilator-induced lung injury', *Thorax*, 67: 244-51.

Bhatt, D., and S. Ghosh. 2014. 'Regulation of the NF-kappaB-Mediated Transcription of Inflammatory Genes', *Front Immunol*, 5: 71.

Bhattacharya, S., N. Sen, M. T. Yiming, R. Patel, K. Parthasarathi, S. Quadri, A. C. Issekutz, and J. Bhattacharya. 2003. 'High tidal volume ventilation induces proinflammatory signaling in rat lung endothelium', *Am J Respir Cell Mol Biol*, 28: 218-24.

Bogoyevitch, M. A., K. R. Ngoei, T. T. Zhao, Y. Y. Yeap, and D. C. Ng. 2010. 'c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges', *Biochim Biophys Acta*, 1804: 463-75.

Boitano, S., and W. H. Evans. 2000. 'Connexin mimetic peptides reversibly inhibit Ca(2+) signaling through gap junctions in airway cells', *Am J Physiol Lung Cell Mol Physiol*, 279: L623-30.

Borges, J. B., E. L. Costa, F. Suarez-Sipmann, C. Widstrom, A. Larsson, M. Amato, and G. Hedenstierna. 2014. 'Early inflammation mainly affects normally and poorly aerated lung in experimental ventilator-induced lung injury*', *Crit Care Med*, 42: e279-87.

Borok, Z., S. I. Danto, R. L. Lubman, Y. Cao, M. C. Williams, and E. D. Crandall. 1998. 'Modulation of t1alpha expression with alveolar epithelial cell phenotype in vitro', *Am J Physiol*, 275: L155-64.

Boudreault, F., and D. J. Tschumperlin. 2010. 'Stretch-induced mitogen-activated protein kinase activation in lung fibroblasts is independent of receptor tyrosine kinases', *Am J Respir Cell Mol Biol*, 43: 64-73.

Bracken, A. P., M. Ciro, A. Cocito, and K. Helin. 2004. 'E2F target genes: unraveling the biology', *Trends Biochem Sci*, 29: 409-17.

Briel, M., M. Meade, A. Mercat, R. G. Brower, D. Talmor, S. D. Walter, A. S. Slutsky, E. Pullenayegum, Q. Zhou, D. Cook, L. Brochard, J. C. Richard, F. Lamontagne, N. Bhatnagar, T. E. Stewart, and G. Guyatt. 2010. 'Higher vs lower positive end-expiratory pressure in patients with acute lung injury and acute respiratory distress syndrome: systematic review and meta-analysis', *JAMA*, 303: 865-73.

Briva, A., C. Santos, L. Malacrida, F. Rocchiccioli, J. Soto, M. Angulo, C. Batthyany, E. Cairoli, and H. Piriz. 2011. 'Adenosine triphosphate-dependent calcium signaling during ventilator-induced lung injury is amplified by hypercapnia', *Exp Lung Res*, 37: 471-81.

Broccard, A., R. S. Shapiro, L. L. Schmitz, A. B. Adams, A. Nahum, and J. J. Marini. 2000. 'Prone positioning attenuates and redistributes ventilator-induced lung injury in dogs', *Crit Care Med*, 28: 295-303.

Brochard, L., F. Roudot-Thoraval, E. Roupie, C. Delclaux, J. Chastre, E. Fernandez-Mondejar, E. Clementi, J. Mancebo, P. Factor, D. Matamis, M. Ranieri, L. Blanch, G. Rodi, H. Mentec, D. Dreyfuss, M. Ferrer, C. Brun-Buisson, M. Tobin, and F. Lemaire. 1998. 'Tidal volume reduction for prevention of ventilator-induced lung injury in acute respiratory distress syndrome. The Multicenter Trail Group on Tidal Volume reduction in ARDS', *Am J Respir Crit Care Med*, 158: 1831-8.

Brower, R. G., P. N. Lanken, N. MacIntyre, M. A. Matthay, A. Morris, M. Ancukiewicz, D. Schoenfeld, and B. T. Thompson. 2004. 'Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome', *N Engl J Med*, 351: 327-36.

Bufler, P., G. Stiegler, M. Schuchmann, S. Hess, C. Kruger, F. Stelter, C. Eckerskorn, C. Schutt, and H. Engelmann. 1995. 'Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants', *Eur J Immunol*, 25: 604-10.

Buregeya, E., R. A. Fowler, D. S. Talmor, T. Twagirumugabe, W. Kiviri, and E. D. Riviello. 2014. 'Acute Respiratory Distress Syndrome in the Global Context', *Glob Heart*, 9: 289-95.

Burns, J. A., T. B. Issekutz, H. Yagita, and A. C. Issekutz. 2001. 'The alpha 4 beta 1 (very late antigen (VLA)-4, CD49d/CD29) and alpha 5 beta 1 (VLA-5, CD49e/CD29) integrins mediate beta 2 (CD11/CD18) integrin-independent neutrophil recruitment to endotoxin-induced lung inflammation', *J Immunol*, 166: 4644-9.

Buss, H., A. Dorrie, M. L. Schmitz, E. Hoffmann, K. Resch, and M. Kracht. 2004. 'Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription', *J Biol Chem*, 279: 55633-43.

Buss, H., K. Handschick, N. Jurrmann, P. Pekkonen, K. Beuerlein, H. Muller, R. Wait, J. Saklatvala, P. M. Ojala, M. L. Schmitz, M. Naumann, and M. Kracht. 2012. 'Cyclin-dependent kinase 6 phosphorylates NF-kappaB P65 at serine 536 and contributes to the regulation of inflammatory gene expression', *PLoS One*, 7: e51847.

Caironi, P., F. Ichinose, R. Liu, R. C. Jones, K. D. Bloch, and W. M. Zapol. 2005. '5-Lipoxygenase deficiency prevents respiratory failure during ventilator-induced lung injury', *Am J Respir Crit Care Med*, 172: 334-43.

Caironi, P., T. Langer, E. Carlesso, A. Protti, and L. Gattinoni. 2011. 'Time to generate ventilator-induced lung injury among mammals with healthy lungs: a unifying hypothesis', *Intensive Care Med*, 37: 1913-20.

Calfee, C. S., M. D. Eisner, P. E. Parsons, B. T. Thompson, E. R. Conner, Jr., M. A. Matthay, and L. B. Ware. 2009. 'Soluble intercellular adhesion molecule-1 and clinical outcomes in patients with acute lung injury', *Intensive Care Med*, 35: 248-57.

Calkins, C. M., K. Barsness, D. D. Bensard, A. Vasquez-Torres, C. D. Raeburn, X. Meng, and R. C. McIntyre, Jr. 2002. 'Toll-like receptor-4 signaling mediates pulmonary neutrophil sequestration in response to gram-positive bacterial enterotoxin', *J Surg Res*, 104: 124-30.

Cannizzaro, V., L. J. Berry, P. K. Nicholls, G. R. Zosky, D. J. Turner, Z. Hantos, and P. D. Sly. 2009. 'Lung volume recruitment maneuvers and respiratory system mechanics in mechanically ventilated mice', *Respir Physiol Neurobiol*, 169: 243-51.

Cannizzaro, V., Z. Hantos, P. D. Sly, and G. R. Zosky. 2011. 'Linking lung function and inflammatory responses in ventilator-induced lung injury', *Am J Physiol Lung Cell Mol Physiol*, 300: L112-20.

Carrasco Loza, R., G. Villamizar Rodriguez, and N. Medel Fernandez. 2015. 'Ventilator-Induced Lung Injury (VILI) in Acute Respiratory Distress Syndrome (ARDS): Volutrauma and Molecular Effects', *Open Respir Med J*, 9: 112-9.

Carter, A. B., M. M. Monick, and G. W. Hunninghake. 1999. 'Both Erk and p38 kinases are necessary for cytokine gene transcription', *Am J Respir Cell Mol Biol*, 20: 751-8.

Caser, E. B., E. Zandonade, E. Pereira, A. M. Gama, and C. S. Barbas. 2014. 'Impact of distinct definitions of acute lung injury on its incidence and outcomes in Brazilian ICUs: prospective evaluation of 7,133 patients*', *Crit Care Med*, 42: 574-82.

Caunt, C. J., and C. A. McArdle. 2012. 'ERK phosphorylation and nuclear accumulation: insights from single-cell imaging', *Biochem Soc Trans*, 40: 224-9.

Caunt, C. J., R. M. Perett, R. C. Fowkes, and C. A. McArdle. 2012. 'Mechanisms of GnRH-induced extracellular signal-regulated kinase nuclear localization', *PLoS One*, 7: e40077.

Chen, C. C., and J. K. Wang. 1999. 'p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages', *Mol Pharmacol*, 55: 481-8.

Chen, H., C. Bai, and X. Wang. 2010. 'The value of the lipopolysaccharide-induced acute lung injury model in respiratory medicine', *Expert Rev Respir Med*, 4: 773-83.

Chen, N., Q. Wu, G. Chi, L. W. Soromou, J. Hou, Y. Deng, and H. Feng. 2013. 'Prime-O-glucosylcimifugin attenuates lipopolysaccharide-induced acute lung injury in mice', *Int Immunopharmacol*, 16: 139-47.

Cheng, J. J., B. S. Wung, Y. J. Chao, and D. L. Wang. 1996. 'Cyclic strain enhances adhesion of monocytes to endothelial cells by increasing intercellular adhesion molecule-1 expression', *Hypertension*, 28: 386-91.

Chess, P. R., L. Toia, and J. N. Finkelstein. 2000. 'Mechanical strain-induced proliferation and signaling in pulmonary epithelial H441 cells', *Am J Physiol Lung Cell Mol Physiol*, 279: L43-51.

Chi, G., M. Wei, X. Xie, L. W. Soromou, F. Liu, and S. Zhao. 2013. 'Suppression of MAPK and NF-kappaB pathways by limonene contributes to attenuation of lipopolysaccharide-induced inflammatory responses in acute lung injury', *Inflammation*, 36: 501-11.

Chiang, C. H., C. H. Chuang, S. L. Liu, C. F. Chian, H. Zhang, and J. H. Ryu. 2012. 'N-acetylcysteine attenuates ventilator-induced lung injury in an isolated and perfused rat lung model', *Injury*, 43: 1257-63.

Chiang, C. H., C. H. Chuang, S. L. Liu, T. S. Lee, Y. R. Kou, and H. Zhang. 2011. 'Apocynin attenuates ventilator-induced lung injury in an isolated and perfused rat lung model', *Intensive Care Med*, 37: 1360-7.

Chiang, C. H., H. I. Pai, and S. L. Liu. 2008. 'Ventilator-induced lung injury (VILI) promotes ischemia/reperfusion lung injury (I/R) and NF-kappaB antibody attenuates both injuries', *Resuscitation*, 79: 147-54.

Choudhury, S., M. R. Wilson, M. E. Goddard, K. P. O'Dea, and M. Takata. 2004. 'Mechanisms of early pulmonary neutrophil sequestration in ventilator-induced lung injury in mice', *Am J Physiol Lung Cell Mol Physiol*, 287: L902-10.

Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. 'Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction', *J Biol Chem*, 274: 10689-92.

Christensen, P. J., S. Kim, R. H. Simon, G. B. Toews, and R. Paine, 3rd. 1993. 'Differentiation-related expression of ICAM-1 by rat alveolar epithelial cells', *Am J Respir Cell Mol Biol*, 8: 9-15.

Chuderland, D., and R. Seger. 2005. 'Protein-protein interactions in the regulation of the extracellular signal-regulated kinase', *Mol Biotechnol*, 29: 57-74.

Chung, I. S., J. A. Kim, J. A. Kim, H. S. Choi, J. J. Lee, M. Yang, H. J. Ahn, and S. M. Lee. 2013. 'Reactive oxygen species by isoflurane mediates inhibition of nuclear factor kappaB activation in lipopolysaccharide-induced acute inflammation of the lung', *Anesth Analg*, 116: 327-35.

Cilley, R. E., J. Y. Wang, and A. G. Coran. 1993. 'Lung injury produced by moderate lung overinflation in rats', *J Pediatr Surg*, 28: 488-93; discussion 94-5.

Cohen, J. 2002. 'The immunopathogenesis of sepsis', *Nature*, 420: 885-91.

Cohen, T. S., G. Gray Lawrence, A. Khasgiwala, and S. S. Margulies. 2010. 'MAPK activation modulates permeability of isolated rat alveolar epithelial cell monolayers following cyclic stretch', *PLoS One*, 5: e10385.

Copland, I. B., D. Reynaud, C. Pace-Asciak, and M. Post. 2006. 'Mechanotransduction of stretch-induced prostanoid release by fetal lung epithelial cells', *Am J Physiol Lung Cell Mol Physiol*, 291: L487-95.

Corbet, A., R. Bucciarelli, S. Goldman, M. Mammel, D. Wold, and W. Long. 1991. 'Decreased mortality rate among small premature infants treated at birth with a single dose of synthetic surfactant: a multicenter controlled trial. American Exosurf Pediatric Study Group 1', *J Pediatr*, 118: 277-84.

Correa-Meyer, E., L. Pesce, C. Guerrero, and J. I. Sznajder. 2002. 'Cyclic stretch activates ERK1/2 via G proteins and EGFR in alveolar epithelial cells', *Am J Physiol Lung Cell Mol Physiol*, 282: L883-91.

Cotrina, M. L., J. H. Lin, A. Alves-Rodrigues, S. Liu, J. Li, H. Azmi-Ghadimi, J. Kang, C. C. Naus, and M. Nedergaard. 1998. 'Connexins regulate calcium signaling by controlling ATP release', *Proc Natl Acad Sci U S A*, 95: 15735-40.

Covert, M. W., T. H. Leung, J. E. Gaston, and D. Baltimore. 2005. 'Achieving stability of lipopolysaccharide-induced NF-kappaB activation', *Science*, 309: 1854-7.

Dai, C. Y., G. F. Dai, Y. Sun, and Y. L. Wang. 2013. 'Loss of p120 catenin aggravates alveolar edema of ventilation induced lung injury', *Chin Med J (Engl)*, 126: 2918-22.

Damarla, M., E. Hasan, A. Boueiz, A. Le, H. H. Pae, C. Montouchet, T. Kolb, T. Simms, A. Myers, U. S. Kayyali, M. Gaestel, X. Peng, S. P. Reddy, R. Damico, and P. M. Hassoun. 2009. 'Mitogen activated protein kinase activated protein kinase 2 regulates actin polymerization and vascular leak in ventilator associated lung injury', *PLoS One*, 4: e4600.

de Rossi, L. W., N. A. Horn, W. Buhre, F. Gass, G. Hutschenreuter, and R. Rossaint. 2002. 'The effect of isoflurane on neutrophil selectin and beta(2)-integrin activation in vitro', *Anesth Analg*, 95: 583-7, table of contents.

Deak, M., A. D. Clifton, L. M. Lucocq, and D. R. Alessi. 1998. 'Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB', *EMBO J*, 17: 4426-41.

Del Sorbo, L., and A. S. Slutsky. 2011. 'Acute respiratory distress syndrome and multiple organ failure', *Curr Opin Crit Care*, 17: 1-6.

Desplantez, T., V. Verma, L. Leybaert, W. H. Evans, and R. Weingart. 2012. 'Gap26, a connexin mimetic peptide, inhibits currents carried by connexin43 hemichannels and gap junction channels', *Pharmacol Res*, 65: 546-52.

Determann, R. M., A. Royakkers, E. K. Wolthuis, A. P. Vlaar, G. Choi, F. Paulus, J. J. Hofstra, M. J. de Graaff, J. C. Korevaar, and M. J. Schultz. 2010. 'Ventilation with lower tidal volumes as compared with conventional tidal volumes for patients without acute lung injury: a preventive randomized controlled trial', *Crit Care*, 14: R1.

Dhainaut, J. F., J. Charpentier, and J. D. Chiche. 2003. 'Transforming growth factor-beta: a mediator of cell regulation in acute respiratory distress syndrome', *Crit Care Med*, 31: S258-64.

Dhanireddy, S., W. A. Altemeier, G. Matute-Bello, D. S. O'Mahony, R. W. Glenny, T. R. Martin, and W. C. Liles. 2006. 'Mechanical ventilation induces inflammation, lung injury, and extra-pulmonary organ dysfunction in experimental pneumonia', *Lab Invest*, 86: 790-9.

Dolinay, T., M. Szilasi, M. Liu, and A. M. Choi. 2004. 'Inhaled carbon monoxide confers antiinflammatory effects against ventilator-induced lung injury', *Am J Respir Crit Care Med*, 170: 613-20.

Dolinay, T., W. Wu, N. Kaminski, E. Ifedigbo, A. M. Kaynar, M. Szilasi, S. C. Watkins, S. W. Ryter, A. Hoetzel, and A. M. Choi. 2008. 'Mitogen-activated protein kinases regulate susceptibility to ventilator-induced lung injury', *PLoS One*, 3: e1601.

Dorger, M., S. Munzing, A. M. Allmeling, K. Messmer, and F. Krombach. 2001. 'Phenotypic and functional differences between rat alveolar, pleural, and peritoneal macrophages', *Exp Lung Res*, 27: 65-76.

Dorr, A. D., M. R. Wilson, K. Wakabayashi, A. C. Waite, B. V. Patel, N. van Rooijen, K. P. O'Dea, and M. Takata. 2011. 'Sources of alveolar soluble TNF receptors during acute lung injury of different etiologies', *J Appl Physiol*, 111: 177-84.

Douillet, C. D., W. P. Robinson, 3rd, P. M. Milano, R. C. Boucher, and P. B. Rich. 2006. 'Nucleotides induce IL-6 release from human airway epithelia via P2Y2 and p38 MAPK-dependent pathways', *Am J Physiol Lung Cell Mol Physiol*, 291: L734-46.

Dreyfuss, D., L. Martin-Lefevre, and G. Saumon. 1999. 'Hyperinflation-induced lung injury during alveolar flooding in rats: effect of perfluorocarbon instillation', *Am J Respir Crit Care Med*, 159: 1752-7.

Dreyfuss, D., and G. Saumon. 1998. 'Ventilator-induced lung injury: lessons from experimental studies', *Am J Respir Crit Care Med*, 157: 294-323.

Dreyfuss, D., P. Soler, G. Basset, and G. Saumon. 1988. 'High inflation pressure pulmonary edema. Respective effects of high airway pressure, high tidal volume, and positive end-expiratory pressure', *Am Rev Respir Dis*, 137: 1159-64.

Dudek, S. M., and J. G. Garcia. 2001. 'Cytoskeletal regulation of pulmonary vascular permeability', *J Appl Physiol*, 91: 1487-500.

Ebach, D. R., T. E. Riehl, and W. F. Stenson. 2005. 'Opposing effects of tumor necrosis factor receptor 1 and 2 in sepsis due to cecal ligation and puncture', *Shock*, 23: 311-8.

Edelman, J., C. Cardozo, and M. Lesser. 1989. 'Lipopolysaccharide stimulates alveolar macrophage adherence in vivo and in vitro', *Agents Actions*, 26: 287-91.

Edwards, Y. S. 2001. 'Stretch stimulation: its effects on alveolar type II cell function in the lung', *Comp Biochem Physiol A Mol Integr Physiol*, 129: 245-60.

Eichacker, P. Q., E. P. Gerstenberger, S. M. Banks, X. Cui, and C. Natanson. 2002. 'Meta-analysis of acute lung injury and acute respiratory distress syndrome trials testing low tidal volumes', *Am J Respir Crit Care Med*, 166: 1510-4.

Eisner, M. D., B. T. Thompson, D. Schoenfeld, A. Anzueto, and M. A. Matthay. 2002. 'Airway pressures and early barotrauma in patients with acute lung injury and acute respiratory distress syndrome', *Am J Respir Crit Care Med*, 165: 978-82.

Eltzschig, H. K., T. Eckle, A. Mager, N. Kuper, C. Karcher, T. Weissmuller, K. Boengler, R. Schulz, S. C. Robson, and S. P. Colgan. 2006. 'ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function', *Circ Res*, 99: 1100-8.

Englert, J. A., A. A. Macias, D. Amador-Munoz, M. Pinilla Vera, C. Isabelle, J. Guan, B. Magaoay, M. Suarez Velandia, A. Coronata, A. Lee, L. E. Fredenburgh, D. J. Culley, G. Crosby, and R. M. Baron. 2015. 'Isoflurane Ameliorates Acute Lung Injury by Preserving Epithelial Tight Junction Integrity', *Anesthesiology*.

Esteban, A., N. D. Ferguson, M. O. Meade, F. Frutos-Vivar, C. Apezteguia, L. Brochard, K. Raymondos, N. Nin, J. Hurtado, V. Tomicic, M. Gonzalez, J. Elizalde, P. Nightingale, F. Abroug, P. Pelosi, Y. Arabi, R. Moreno, M. Jibaja, G. D'Empaire, F. Sandi, D. Matamis, A. M. Montanez, and A. Anzueto. 2008. 'Evolution of mechanical ventilation in response to clinical research', *Am J Respir Crit Care Med*, 177: 170-7.

Evans, W. H., and S. Boitano. 2001. 'Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication', *Biochem Soc Trans*, 29: 606-12.

Evans, W. H., and L. Leybaert. 2007. 'Mimetic peptides as blockers of connexin channel-facilitated intercellular communication', *Cell Commun Adhes*, 14: 265-73.

Eyal, F. G., C. R. Hamm, and J. C. Parker. 2007. 'Reduction in alveolar macrophages attenuates acute ventilator induced lung injury in rats', *Intensive Care Med*, 33: 1212-8.

Factor, P., G. M. Mutlu, L. Chen, J. Mohameed, A. T. Akhmedov, F. J. Meng, T. Jilling, E. R. Lewis, M. D. Johnson, A. Xu, D. Kass, J. M. Martino, A. Bellmeyer, J. S. Albazi, C. Emala, H. T. Lee, L. G. Dobbs, and S. Matalon. 2007. 'Adenosine regulation of alveolar fluid clearance', *Proc Natl Acad Sci U S A*, 104: 4083-8.

Faffe, D. S., V. R. Seidl, P. S. Chagas, V. L. Goncalves de Moraes, V. L. Capelozzi, P. R. Rocco, and W. A. Zin. 2000. 'Respiratory effects of lipopolysaccharide-induced inflammatory lung injury in mice', *Eur Respir J*, 15: 85-91.

Falk, S., S. D. Schwab, M. Frosig-Jorgensen, R. P. Clausen, A. H. Dickenson, and A. M. Heegaard. 2015. 'P2X7 receptor-mediated analgesia in cancer-induced bone pain', *Neuroscience*, 291: 93-105.

Faller, S., K. M. Strosing, S. W. Ryter, H. Buerkle, T. Loop, R. Schmidt, and A. Hoetzel. 2012. 'The volatile anesthetic isoflurane prevents ventilator-induced lung injury via phosphoinositide 3-kinase/Akt signaling in mice', *Anesth Analg*, 114: 747-56.

Fan, E., W. Checkley, T. E. Stewart, J. Muscedere, O. Lesur, J. T. Granton, A. P. Freitag, M. Jacka, N. D. Ferguson, and M. O. Meade. 2012. 'Complications from recruitment maneuvers in patients with acute lung injury: secondary analysis from the lung open ventilation study', *Respir Care*, 57: 1842-9.

Faure, E., O. Equils, P. A. Sieling, L. Thomas, F. X. Zhang, C. J. Kirschning, N. Polentarutti, M. Muzio, and M. Arditi. 2000. 'Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells', *J Biol Chem*, 275: 11058-63.

Ferguson, N. D., D. J. Cook, G. H. Guyatt, S. Mehta, L. Hand, P. Austin, Q. Zhou, A. Matte, S. D. Walter, F. Lamontagne, J. T. Granton, Y. M. Arabi, A. C. Arroliga, T. E. Stewart, A. S. Slutsky, and M. O. Meade. 2013. 'High-frequency oscillation in early acute respiratory distress syndrome', *N Engl J Med*, 368: 795-805.

Ferguson, N. D., A. M. Davis, A. S. Slutsky, and T. E. Stewart. 2005. 'Development of a clinical definition for acute respiratory distress syndrome using the Delphi technique', *J Crit Care*, 20: 147-54.

Ferguson, N. D., F. Frutos-Vivar, A. Esteban, P. Fernandez-Segoviano, J. A. Aramburu, L. Najera, and T. E. Stewart. 2005. 'Acute respiratory distress syndrome: underrecognition by clinicians and diagnostic accuracy of three clinical definitions', *Crit Care Med*, 33: 2228-34.

Fernandez-Perez, E. R., J. Sprung, B. Afessa, D. O. Warner, C. M. Vachon, D. R. Schroeder, D. R. Brown, R. D. Hubmayr, and O. Gajic. 2009. 'Intraoperative ventilator settings and acute lung injury after elective surgery: a nested case control study', *Thorax*, 64: 121-7.

Ferrari, D., P. Chiozzi, S. Falzoni, M. Dal Susino, L. Melchiorri, O. R. Baricordi, and F. Di Virgilio. 1997. 'Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages', *J Immunol*, 159: 1451-8.

Fisher, C. J., Jr., J. M. Agosti, S. M. Opal, S. F. Lowry, R. A. Balk, J. C. Sadoff, E. Abraham, R. M. Schein, and E. Benjamin. 1996. 'Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The Soluble TNF Receptor Sepsis Study Group', *N Engl J Med*, 334: 1697-702.

Flori, H. R., L. B. Ware, D. Glidden, and M. A. Matthay. 2003. 'Early elevation of plasma soluble intercellular adhesion molecule-1 in pediatric acute lung injury identifies patients at increased risk of death and prolonged mechanical ventilation', *Pediatr Crit Care Med*, 4: 315-21.

Folkesson, H. G., and M. A. Matthay. 1997. 'Inhibition of CD18 or CD11b attenuates acute lung injury after acid instillation in rabbits', *J Appl Physiol*, 82: 1743-50.

Frank, J. A., J. F. Pittet, C. Wray, and M. A. Matthay. 2008. 'Protection from experimental ventilator-induced acute lung injury by IL-1 receptor blockade', *Thorax*, 63: 147-53.

Frank, J. A., C.M. Wray, D.F. McAuley, R. Schwendener, and M.A. Matthay. 2006. 'Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury', *Am J Physiol Lung Cell Mol Physiol*, 291: L1191-L98.

Friedle, S. A., M. A. Curet, and J. J. Watters. 2010. 'Recent patents on novel P2X(7) receptor antagonists and their potential for reducing central nervous system inflammation', *Recent Pat CNS Drug Discov*, 5: 35-45.

Fu, Z., M. L. Costello, K. Tsukimoto, R. Prediletto, A. R. Elliott, O. Mathieu-Costello, and J. B. West. 1992. 'High lung volume increases stress failure in pulmonary capillaries', *J Appl Physiol (1985)*, 73: 123-33.

Fujino, N., H. Kubo, C. Ota, T. Suzuki, S. Suzuki, M. Yamada, T. Takahashi, M. He, T. Suzuki, T. Kondo, and M. Yamaya. 2012. 'A novel method for isolating individual cellular components from the adult human distal lung', *Am J Respir Cell Mol Biol*, 46: 422-30.

Futier, E., J. M. Constantin, C. Paugam-Burtz, J. Pascal, M. Eurin, A. Neuschwander, E. Marret, M. Beaussier, C. Gutton, J. Y. Lefrant, B. Allaouchiche, D. Verzilli, M. Leone, A. De Jong, J. E. Bazin, B. Pereira, and S. Jaber. 2013. 'A trial of intraoperative low-tidal-volume ventilation in abdominal surgery', *N Engl J Med*, 369: 428-37.

Gajic, O., O. Dabbagh, P. K. Park, A. Adesanya, S. Y. Chang, P. Hou, H. Anderson, 3rd, J. J. Hoth, M. E. Mikkelsen, N. T. Gentile, M. N. Gong, D. Talmor, E. Bajwa, T. R. Watkins, E. Festic, M. Yilmaz, R. Iscimen, D. A. Kaufman, A. M. Esper, R. Sadikot, I. Douglas, J. Sevransky, and M. Malinchoc. 2011. 'Early identification of patients at risk of acute lung injury: evaluation of lung injury prediction score in a multicenter cohort study', *Am J Respir Crit Care Med*, 183: 462-70.

Gajic, O., S. I. Dara, J. L. Mendez, A. O. Adesanya, E. Festic, S. M. Caples, R. Rana, J. L. St Sauver, J. F. Lymp, B. Afessa, and R. D. Hubmayr. 2004. 'Ventilator-associated lung injury in patients without acute lung injury at the onset of mechanical ventilation', *Crit Care Med*, 32: 1817-24.

Gajic, O., F. Frutos-Vivar, A. Esteban, R. D. Hubmayr, and A. Anzueto. 2005. 'Ventilator settings as a risk factor for acute respiratory distress syndrome in mechanically ventilated patients', *Intensive Care Med*, 31: 922-6.

Gao Smith, F., G. D. Perkins, S. Gates, D. Young, D. F. McAuley, W. Tunnicliffe, Z. Khan, and S. E. Lamb. 2012. 'Effect of intravenous beta-2 agonist treatment on clinical outcomes in acute respiratory distress syndrome (BALTI-2): a multicentre, randomised controlled trial', *Lancet*, 379: 229-35.

Garcia-Fernandez, J., S. Canfran, I. A. de Segura, F. Suarez-Sipmann, D. Aguado, and G. Hedenstierna. 2013. 'Pressure safety range of barotrauma with lung recruitment manoeuvres: a randomised experimental study in a healthy animal model', *Eur J Anaesthesiol*, 30: 567-74.

Gattinoni, L., M. Bombino, P. Pelosi, A. Lissoni, A. Pesenti, R. Fumagalli, and M. Tagliabue. 1994. 'Lung structure and function in different stages of severe adult respiratory distress syndrome', *JAMA*, 271: 1772-9.

Gattinoni, L., P. Caironi, M. Cressoni, D. Chiumello, V. M. Ranieri, M. Quintel, S. Russo, N. Patroniti, R. Cornejo, and G. Bugedo. 2006. 'Lung recruitment in patients with the acute respiratory distress syndrome', *N Engl J Med*, 354: 1775-86.

Gattinoni, L., E. Carlesso, P. Taccone, F. Polli, C. Guerin, and J. Mancebo. 2010. 'Prone positioning improves survival in severe ARDS: a pathophysiologic review and individual patient meta-analysis', *Minerva Anestesiol*, 76: 448-54.

Gattinoni, L., L. D'Andrea, P. Pelosi, G. Vitale, A. Pesenti, and R. Fumagalli. 1993. 'Regional effects and mechanism of positive end-expiratory pressure in early adult respiratory distress syndrome', *JAMA*, 269: 2122-7.

Gattinoni, L., and A. Pesenti. 2005. 'The concept of "baby lung"', *Intensive Care Med*, 31: 776-84.

Gattinoni, L., G. Tognoni, A. Pesenti, P. Taccone, D. Mascheroni, V. Labarta, R. Malacrida, P. Di Giulio, R. Fumagalli, P. Pelosi, L. Brazzi, and R. Latini. 2001. 'Effect of prone positioning on the survival of patients with acute respiratory failure', *N Engl J Med*, 345: 568-73.

Geissmann, F., S. Jung, and D. R. Littman. 2003. 'Blood monocytes consist of two principal subsets with distinct migratory properties', *Immunity*, 19: 71-82.

Germena, G., S. Volmering, C. Sohlbach, and A. Zarbock. 2015. 'Mutation in the CD45 inhibitory wedge modulates integrin activation and leukocyte recruitment during inflammation', *J Immunol*, 194: 728-38.

Ghosh, S., M. R. Wilson, S. Choudhury, H. Yamamoto, M. E. Goddard, B. Falusi, N. Marczin, and M. Takata. 2005. 'Effects of inhaled carbon monoxide on acute lung injury in mice', *Am J Physiol Lung Cell Mol Physiol*, 288: L1003-9.

Gille, H., A. D. Sharrocks, and P. E. Shaw. 1992. 'Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter', *Nature*, 358: 414-7.

Gioannini, T. L., A. Teghanemt, D. Zhang, G. Esparza, L. Yu, and J. Weiss. 2014. 'Purified monomeric ligand.MD-2 complexes reveal molecular and structural requirements for activation and antagonism of TLR4 by Gram-negative bacterial endotoxins', *Immunol Res*, 59: 3-11.

Goldblum, S. E., T. W. Brann, X. Ding, J. Pugin, and P. S. Tobias. 1994. 'Lipopolysaccharide (LPS)-binding protein and soluble CD14 function as accessory molecules for LPS-induced changes in endothelial barrier function, in vitro', *J Clin Invest*, 93: 692-702.

Gomez-Laberge, C., J. S. Rettig, C. D. Smallwood, T. K. Boyd, J. H. Arnold, and G. K. Wolf. 2013. 'Interaction of dependent and non-dependent regions of the acutely injured lung during a stepwise recruitment manoeuvre', *Physiol Meas*, 34: 163-77.

Gong, X., X. Ming, P. Deng, and Y. Jiang. 2010. 'Mechanisms regulating the nuclear translocation of p38 MAP kinase', *J Cell Biochem*, 110: 1420-9.

Goodwin, A., and G. Jenkins. 2009. 'Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis', *Biochem Soc Trans*, 37: 849-54.

Gordon, S., J. Hamann, H. H. Lin, and M. Stacey. 2011. 'F4/80 and the related adhesion-GPCRs', *Eur J Immunol*, 41: 2472-6.

Goss, C. H., R. G. Brower, L. D. Hudson, and G. D. Rubenfeld. 2003. 'Incidence of acute lung injury in the United States', *Crit Care Med*, 31: 1607-11.

Grasso, S., P. Terragni, L. Mascia, V. Fanelli, M. Quintel, P. Herrmann, G. Hedenstierna, A. S. Slutsky, and V. M. Ranieri. 2004. 'Airway pressure-time curve profile (stress index) detects tidal recruitment/hyperinflation in experimental acute lung injury', *Crit Care Med*, 32: 1018-27.

Gray, B. W., J. W. Haft, J. C. Hirsch, G. M. Annich, R. B. Hirschl, and R. H. Bartlett. 2015. 'Extracorporeal life support: experience with 2,000 patients', *ASAIO J*, 61: 2-7.

Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich. 1995. 'The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor', *Cell*, 83: 793-802.

Grell, M., H. Wajant, G. Zimmermann, and P. Scheurich. 1998. 'The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor', *Proc Natl Acad Sci U S A*, 95: 570-5.

Grygorczyk, R., K. Furuya, and M. Sokabe. 2012. 'Imaging and characterization of stretch-induced ATP release from alveolar A549 cells', *J Physiol*.

Gu, X. L., G. N. Wu, Y. W. Yao, D. H. Shi, and Y. Song. 2014. 'Is high-frequency oscillatory ventilation more effective and safer than conventional protective ventilation in adult acute respiratory distress syndrome patients? A meta-analysis of randomized controlled trials', *Crit Care*, 18: R111.

Guan, Z., S. Y. Buckman, A. P. Pentland, D. J. Templeton, and A. R. Morrison. 1998. 'Induction of cyclooxygenase-2 by the activated MEKK1 --> SEK1/MKK4 --> p38 mitogen-activated protein kinase pathway', *J Biol Chem*, 273: 12901-8.

Guerin, C., S. Gaillard, S. Lemasson, L. Ayzac, R. Girard, P. Beuret, B. Palmier, Q. V. Le, M. Sirodot, S. Rosselli, V. Cadiergue, J. M. Sainty, P. Barbe, E. Combourieu, D. Debatty, J. Rouffineau, E. Ezingeard, O. Millet, D. Guelon, L. Rodriguez, O. Martin, A. Renault, J. P. Sibille, and M. Kaidomar. 2004. 'Effects of systematic prone positioning in hypoxemic acute respiratory failure: a randomized controlled trial', *JAMA*, 292: 2379-87.

Guerin, C., J. Reignier, J. C. Richard, P. Beuret, A. Gacouin, T. Boulain, E. Mercier, M. Badet, A. Mercat, O. Baudin, M. Clavel, D. Chatellier, S. Jaber, S. Rosselli, J. Mancebo, M. Sirodot, G. Hilbert, C. Bengler, J. Richecoeur, M. Gainnier, F. Bayle, G. Bourdin, V. Leray, R. Girard, L. Baboi, and L. Ayzac. 2013. 'Prone positioning in severe acute respiratory distress syndrome', *N Engl J Med*, 368: 2159-68.

Guery, B. P., D. A. Welsh, N. B. Viget, L. Robriquet, P. Fialdes, C. M. Mason, G. Beaucaire, G. J. Bagby, and R. Neviere. 2003. 'Ventilation-induced lung injury is associated with an increase in gut permeability', *Shock*, 19: 559-63.

Guo, Y., A. Mishra, T. Weng, N. R. Chintagari, Y. Wang, C. Zhao, C. Huang, and L. Liu. 2014. 'Wnt3a mitigates acute lung injury by reducing P2X7 receptor-mediated alveolar epithelial type I cell death', *Cell Death Dis*, 5: e1286.

Guth, A. M., W. J. Janssen, C. M. Bosio, E. C. Crouch, P. M. Henson, and S. W. Dow. 2009. 'Lung environment determines unique phenotype of alveolar macrophages', *Am J Physiol Lung Cell Mol Physiol*, 296: L936-46.

Gutierrez, J. A., R. F. Gonzalez, and L. G. Dobbs. 1998. 'Mechanical distension modulates pulmonary alveolar epithelial phenotypic expression in vitro', *Am J Physiol*, 274: L196-202.

Halter, J. M., J. M. Steinberg, H. J. Schiller, M. DaSilva, L. A. Gatto, S. Landas, and G. F. Nieman. 2003. 'Positive end-expiratory pressure after a recruitment maneuver prevents both alveolar collapse and recruitment/derecruitment', *Am J Respir Crit Care Med*, 167: 1620-6.

Hammerschmidt, S., H. Kuhn, U. Sack, A. Schlenska, C. Gessner, A. Gillissen, and H. Wirtz. 2005. 'Mechanical stretch alters alveolar type II cell mediator release toward a proinflammatory pattern', *Am J Respir Cell Mol Biol*, 33: 203-10.

Han, J., J. D. Lee, L. Bibbs, and R. J. Ulevitch. 1994. 'A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells', *Science*, 265: 808-11.

Han, S., and R. K. Mallampalli. 2015. 'The Acute Respiratory Distress Syndrome: From Mechanism to Translation', *J Immunol*, 194: 855-60.

Hauber, H. P., D. Karp, T. Goldmann, E. Vollmer, and P. Zabel. 2010. 'Effect of low tidal volume ventilation on lung function and inflammation in mice', *BMC Pulm Med*, 10: 21.

Hayes, M., G. F. Curley, C. Masterson, M. Contreras, B. Ansari, J. Devaney, D. O'Toole, and J. G. Laffey. 2014. 'Pulmonary overexpression of inhibitor kappaBalpha decreases the severity of ventilator-induced lung injury in a rat model', *Br J Anaesth*, 113: 1046-54.

Hegeman, M. A., M. P. Hennus, C. J. Heijnen, P. A. Specht, B. Lachmann, N. J. Jansen, A. J. van Vught, and P. M. Cobelens. 2009. 'Ventilator-induced endothelial activation and inflammation in the lung and distal organs', *Crit Care*, 13: R182.

Hegeman, M. A., M. P. Hennus, M. van Meurs, P. M. Cobelens, A. Kavelaars, N. J. Jansen, M. J. Schultz, A. J. van Vught, G. Molema, and C. J. Heijnen. 2010. 'Angiopoietin-1 treatment reduces inflammation but does not prevent ventilator-induced lung injury', *PLoS One*, 5: e15653.

Held, H. D., S. Boettcher, L. Hamann, and S. Uhlig. 2001. 'Ventilation-induced chemokine and cytokine release is associated with activation of nuclear factor-kappaB and is blocked by steroids', *Am J Respir Crit Care Med*, 163: 711-6.

Hemmes, S. N., A. Serpa Neto, and M. J. Schultz. 2013. 'Intraoperative ventilatory strategies to prevent postoperative pulmonary complications: a meta-analysis', *Curr Opin Anaesthesiol*, 26: 126-33.

Hernu, R., F. Wallet, F. Thiolliere, O. Martin, J. C. Richard, Z. Schmitt, G. Wallon, B. Delannoy, T. Rimmele, C. Demaret, C. Magnin, H. Vallin, A. Lepape, L. Baboi, L. Argaud, V. Piriou, B. Allaouchiche, F. Aubrun, O. Bastien, J. J. Lehot, L. Ayzac, and C. Guerin. 2013. 'An attempt to validate the modification of the American-European consensus definition of acute lung injury/acute respiratory distress syndrome by the Berlin definition in a university hospital', *Intensive Care Med*, 39: 2161-70.

Herridge, M. S., A. M. Cheung, C. M. Tansey, A. Matte-Martyn, N. Diaz-Granados, F. Al-Saidi, A. B. Cooper, C. B. Guest, C. D. Mazer, S. Mehta, T. E. Stewart, A. Barr, D. Cook, and A. S. Slutsky. 2003. 'One-year outcomes in survivors of the acute respiratory distress syndrome', *N Engl J Med*, 348: 683-93.

Herridge, M. S., C. M. Tansey, A. Matte, G. Tomlinson, N. Diaz-Granados, A. Cooper, C. B. Guest, C. D. Mazer, S. Mehta, T. E. Stewart, P. Kudlow, D. Cook, A. S. Slutsky, and A. M. Cheung. 2011. 'Functional disability 5 years after acute respiratory distress syndrome', *N Engl J Med*, 364: 1293-304.

Herzenberg, L. A., J. Tung, W. A. Moore, L. A. Herzenberg, and D. R. Parks. 2006. 'Interpreting flow cytometry data: a guide for the perplexed', *Nat Immunol*, 7: 681-5.

Hess, D. R., D. Kondili, E. Burns, E. A. Bittner, and U. H. Schmidt. 2013. 'A 5-year observational study of lung-protective ventilation in the operating room: a single-center experience', *J Crit Care*, 28: 533.e9-15.

Hinz, M., and C. Scheidereit. 2014. 'The IkappaB kinase complex in NF-kappaB regulation and beyond', *EMBO Rep*, 15: 46-61.

Hirano, S. 1996. 'Interaction of rat alveolar macrophages with pulmonary epithelial cells following exposure to lipopolysaccharide', *Arch Toxicol*, 70: 230-6.

Hodgson, C., J. L. Keating, A. E. Holland, A. R. Davies, L. Smirneos, S. J. Bradley, and D. Tuxen. 2009. 'Recruitment manoeuvres for adults with acute lung injury receiving mechanical ventilation', *Cochrane Database Syst Rev*: Cd006667.

Hoegl, S., K. A. Boost, M. Flondor, P. Scheiermann, H. Muhl, J. Pfeilschifter, B. Zwissler, and C. Hofstetter. 2008. 'Short-term exposure to high-pressure ventilation leads to pulmonary biotrauma and systemic inflammation in the rat', *Int J Mol Med*, 21: 513-9.

Hoekstra, R. E., J. C. Jackson, T. F. Myers, I. D. Frantz, 3rd, M. E. Stern, W. F. Powers, M. Maurer, J. R. Raye, S. T. Carrier, J. H. Gunkel, and et al. 1991. 'Improved neonatal survival following multiple

doses of bovine surfactant in very premature neonates at risk for respiratory distress syndrome', *Pediatrics*, 88: 10-8.

Holt, L. J., C. Herring, L. S. Jespers, B. P. Woolven, and I. M. Tomlinson. 2003. 'Domain antibodies: proteins for therapy', *Trends Biotechnol*, 21: 484-90.

Homolya, L., T. H. Steinberg, and R. C. Boucher. 2000. 'Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia', *J Cell Biol*, 150: 1349-60.

Honore, P., D. Donnelly-Roberts, M. Namovic, C. Zhong, C. Wade, P. Chandran, C. Zhu, W. Carroll, A. Perez-Medrano, Y. Iwakura, and M. F. Jarvis. 2009. 'The antihyperalgesic activity of a selective P2X7 receptor antagonist, A-839977, is lost in IL-1alphabeta knockout mice', *Behav Brain Res*, 204: 77-81.

Hoogendijk, A. J., S. S. Pinhancos, T. van der Poll, and C. W. Wieland. 2013. 'Intrapulmonary administration of a p38 mitogen activated protein kinase inhibitor partially prevents pulmonary inflammation', *Immunobiology*, 218: 435-42.

Horiuchi, K. 2013. 'A brief history of tumor necrosis factor alpha--converting enzyme: an overview of ectodomain shedding', *Keio J Med*, 62: 29-36.

Hraiech, S., J. M. Forel, and L. Papazian. 2012. 'The role of neuromuscular blockers in ARDS: benefits and risks', *Curr Opin Crit Care*, 18: 495-502.

Hraiech, S., T. Yoshida, and L. Papazian. 2015. 'Balancing neuromuscular blockade versus preserved muscle activity', *Curr Opin Crit Care*, 21: 26-33.

Hsu, C. W., and S. F. Sun. 2014. 'Iatrogenic pneumothorax related to mechanical ventilation', *World J Crit Care Med*, 3: 8-14.

Hsu, C. W., S. F. Sun, D. L. Lee, K. A. Chu, and H. S. Lin. 2014. 'Clinical characteristics, hospital outcome and prognostic factors of patients with ventilator-related pneumothorax', *Minerva Anestesiol*, 80: 29-38.

Hu, G., A. B. Malik, and R. D. Minshall. 2010. 'Toll-like receptor 4 mediates neutrophil sequestration and lung injury induced by endotoxin and hyperinflation', *Crit Care Med*, 38: 194-201.

Hu, X., Y. Zhang, D. Cheng, Y. Ding, D. Yang, F. Jiang, C. Zhou, B. Ying, and F. Wen. 2008. 'Mechanical stress upregulates intercellular adhesion molecule-1 in pulmonary epithelial cells', *Respiration*, 76: 344-50.

Huang, C., W. Y. Ma, A. Maxiner, Y. Sun, and Z. Dong. 1999. 'p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389', *J Biol Chem*, 274: 12229-35.

Huang, C. S., T. Kawamura, X. Peng, N. Tochigi, N. Shigemura, T. R. Billiar, A. Nakao, and Y. Toyoda. 2011. 'Hydrogen inhalation reduced epithelial apoptosis in ventilator-induced lung injury via a mechanism involving nuclear factor-kappa B activation', *Biochem Biophys Res Commun*, 408: 253-8.

Huang, L. T., H. C. Chou, L. F. Wang, and C. M. Chen. 2012. 'Tissue plasminogen activator attenuates ventilator-induced lung injury in rats', *Acta Pharmacol Sin*, 33: 991-7.

Huang, L. T., C. H. Lin, H. C. Chou, and C. M. Chen. 2014. 'Ibuprofen protects ventilator-induced lung injury by downregulating Rho-kinase activity in rats', *Biomed Res Int*, 2014: 749097.

Huh, J. W., H. Jung, H. S. Choi, S. B. Hong, C. M. Lim, and Y. Koh. 2009. 'Efficacy of positive end-expiratory pressure titration after the alveolar recruitment manoeuvre in patients with acute respiratory distress syndrome', *Crit Care*, 13: R22.

Imai, Y., T. Kawano, S. Iwamoto, S. Nakagawa, M. Takata, and K. Miyasaka. 1999. 'Intratracheal anti-tumor necrosis factor-alpha antibody attenuates ventilator-induced lung injury in rabbits', *J Appl Physiol (1985)*, 87: 510-5.

Imai, Y., J. Parodo, O. Kajikawa, M. de Perrot, S. Fischer, V. Edwards, E. Cutz, M. Liu, S. Keshavjee, T. R. Martin, J. C. Marshall, V. M. Ranieri, and A. S. Slutsky. 2003. 'Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome', *JAMA*, 289: 2104-12.

Imanaka, H., M. Shimaoka, N. Matsuura, M. Nishimura, N. Ohta, and H. Kiyono. 2001. 'Ventilator-induced lung injury is associated with neutrophil infiltration, macrophage activation, and TGF-beta 1 mRNA upregulation in rat lungs', *Anesth Analg*, 92: 428-36.

Iwaki, M., S. Ito, M. Morioka, S. Iwata, Y. Numaguchi, M. Ishii, M. Kondo, H. Kume, K. Naruse, M. Sokabe, and Y. Hasegawa. 2009. 'Mechanical stretch enhances IL-8 production in pulmonary microvascular endothelial cells', *Biochem Biophys Res Commun*, 389: 531-6.

Jaakkola, I., M. Merinen, S. Jalkanen, and A. Hanninen. 2003. 'Ly6C induces clustering of LFA-1 (CD11a/CD18) and is involved in subtype-specific adhesion of CD8 T cells', *J Immunol*, 170: 1283-90.

Jaber, S., Y. Coisel, G. Chanques, E. Futier, J. M. Constantin, P. Michelet, M. Beaussier, J. Y. Lefrant, B. Allaouchiche, X. Capdevila, and E. Marret. 2012. 'A multicentre observational study of intra-operative ventilatory management during general anaesthesia: tidal volumes and relation to body weight', *Anaesthesia*, 67: 999-1008.

Jaecklin, T., D. Engelberts, G. Otulakowski, H. O'Brodovich, M. Post, and B. P. Kavanagh. 2011. 'Lung-derived soluble mediators are pathogenic in ventilator-induced lung injury', *Am J Physiol Lung Cell Mol Physiol*, 300: L648-58.

Jaecklin, T., G. Otulakowski, and B. P. Kavanagh. 2010. 'Do soluble mediators cause ventilator-induced lung injury and multi-organ failure?', *Intensive Care Med*, 36: 750-7.

Jafari, B., B. Ouyang, L. F. Li, C. A. Hales, and D. A. Quinn. 2004. 'Intracellular glutathione in stretch-induced cytokine release from alveolar type-2 like cells', *Respirology*, 9: 43-53.

Jersmann, H. P. 2005. 'Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells', *Immunol Cell Biol*, 83: 462-7.

Jeyaseelan, S., H. W. Chu, S. K. Young, M. W. Freeman, and G. S. Worthen. 2005. 'Distinct roles of pattern recognition receptors CD14 and Toll-like receptor 4 in acute lung injury', *Infect Immun*, 73: 1754-63.

Jia, X., A. Malhotra, M. Saeed, R. G. Mark, and D. Talmor. 2008. 'Risk factors for ARDS in patients receiving mechanical ventilation for > 48 h', *Chest*, 133: 853-61.

Jiang, J. S., L. F. Wang, H. C. Chou, and C. M. Chen. 2007. 'Angiotensin-converting enzyme inhibitor captopril attenuates ventilator-induced lung injury in rats', *J Appl Physiol*, 102: 2098-103.

Jin, L. D., L. R. Wang, L. Q. Wu, Y. L. Shan, X. Y. Zhao, X. Q. Xiong, J. H. Zhou, L. N. Lin, and L. L. Jin. 2013. 'Effects of COX-2 inhibitor on ventilator-induced lung injury in rats', *Int Immunopharmacol*, 16: 288-95.

Jupp, O. J., S. M. McFarlane, H. M. Anderson, A. F. Littlejohn, A. A. Mohamed, R. H. MacKay, P. Vandenabeele, and D. J. MacEwan. 2001. 'Type II tumour necrosis factor-alpha receptor (TNFR2) activates c-Jun N-terminal kinase (JNK) but not mitogen-activated protein kinase (MAPK) or p38 MAPK pathways', *Biochem J*, 359: 525-35.

Kalhan, R., M. Mikkelsen, P. Dedhiya, J. Christie, C. Gaughan, P. N. Lanken, B. Finkel, R. Gallop, and B. D. Fuchs. 2006. 'Underuse of lung protective ventilation: analysis of potential factors to explain physician behavior', *Crit Care Med*, 34: 300-6.

Kang, B. H., J. D. Crapo, C. D. Wegner, L. G. Letts, and L. Y. Chang. 1993. 'Intercellular adhesion molecule-1 expression on the alveolar epithelium and its modification by hyperoxia', *Am J Respir Cell Mol Biol*, 9: 350-5.

Kaniaris, E., K. Vaporidi, E. Vergadi, E. E. Theodorakis, E. Kondili, E. Lagoudaki, C. Tsatsanis, and D. Georgopoulos. 2014. 'Genetic and pharmacologic inhibition of Tpl2 kinase is protective in a mouse model of ventilator-induced lung injury', *Intensive Care Med Exp*, 2: 16.

Kanoore Edul, V. S., L. P. Maskin, and A. Dubin. 2010. 'Effects of recruitment manoeuvres on haemodynamics, oxygen exchange and oxygen delivery in patients with acute lung injury and acute respiratory distress syndrome', *Crit Care Resusc*, 12: 143-8.

Kasper, M., J. Behrens, D. Schuh, and M. Muller. 1995. 'Distribution of E-cadherin and Ep-CAM in the human lung during development and after injury', *Histochem Cell Biol*, 103: 281-6.

Kawano, A., M. Tsukimoto, T. Noguchi, N. Hotta, H. Harada, T. Takenouchi, H. Kitani, and S. Kojima. 2012. 'Involvement of P2X4 receptor in P2X7 receptor-dependent cell death of mouse macrophages', *Biochem Biophys Res Commun*, 419: 374-80.

Kawano, T., S. Mori, M. Cybulsky, R. Burger, A. Ballin, E. Cutz, and A. C. Bryan. 1987. 'Effect of granulocyte depletion in a ventilated surfactant-depleted lung', *J Appl Physiol*, 62: 27-33.

Kawasaki, T., and T. Kawai. 2014. 'Toll-like receptor signaling pathways', *Front Immunol*, 5: 461.

Kaynar, A. M., A. M. Houghton, E. H. Lum, B. R. Pitt, and S. D. Shapiro. 2008. 'Neutrophil elastase is needed for neutrophil emigration into lungs in ventilator-induced lung injury', *Am J Respir Cell Mol Biol*, 39: 53-60.

Keesler, G. A., J. Bray, J. Hunt, D. A. Johnson, T. Gleason, Z. Yao, S. W. Wang, C. Parker, H. Yamane, C. Cole, and H. S. Lichenstein. 1998. 'Purification and activation of recombinant p38 isoforms alpha, beta, gamma, and delta', *Protein Expr Purif*, 14: 221-8.

Kiefmann, R., M. N. Islam, J. Lindert, K. Parthasarathi, and J. Bhattacharya. 2009. 'Paracrine purinergic signaling determines lung endothelial nitric oxide production', *Am J Physiol Lung Cell Mol Physiol*, 296: L901-10.

Kilkenny, C., W. J. Browne, I. C. Cuthill, M. Emerson, and D. G. Altman. 2010. 'Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research', *PLoS Biol*, 8: e1000412.

Kim, H. J., H. S. Lee, Y. H. Chong, and J. L. Kang. 2006. 'p38 Mitogen-activated protein kinase up-regulates LPS-induced NF-kappaB activation in the development of lung injury and RAW 264.7 macrophages', *Toxicology*, 225: 36-47.

Kim, S. H., M. Li, T. H. Pyeon, K. Y. So, and S. H. Kwak. 2015. 'The volatile anesthetic sevoflurane attenuates ventilator-induced lung injury through inhibition of ERK1/2 and Akt signal transduction', *Korean J Anesthesiol*, 68: 62-9.

Kito, H., E. L. Chen, X. Wang, M. Ikeda, N. Azuma, N. Nakajima, V. Gahtan, and B. E. Sumpio. 2000. 'Role of mitogen-activated protein kinases in pulmonary endothelial cells exposed to cyclic strain', *J Appl Physiol*, 89: 2391-400.

Ko, S. C., H. Zhang, J. J. Haitsma, K. C. Cheng, C. F. Li, and A. S. Slutsky. 2008. 'Effects of PEEP levels following repeated recruitment maneuvers on ventilator-induced lung injury', *Acta Anaesthesiol Scand*, 52: 514-21.

Ko, Y. A., M. C. Yang, H. T. Huang, C. M. Hsu, and L. W. Chen. 2013. 'NF-kappaB activation in myeloid cells mediates ventilator-induced lung injury', *Respir Res*, 14: 69.

Kolosova, I. A., T. Mirzapoiazova, L. Moreno-Vinasco, S. Sammani, J. G. Garcia, and A. D. Verin. 2008. 'Protective effect of purinergic agonist ATPgammaS against acute lung injury', *Am J Physiol Lung Cell Mol Physiol*, 294: L319-24.

Kopf, M., C. Schneider, and S. P. Nobs. 2015. 'The development and function of lung-resident macrophages and dendritic cells', *Nat Immunol*, 16: 36-44.

Kotani, M., T. Kotani, Z. Li, R. Silbajoris, C. A. Piantadosi, and Y. C. Huang. 2004. 'Reduced inspiratory flow attenuates IL-8 release and MAPK activation of lung overstretch', *Eur Respir J*, 24: 238-46.

Kotlyarov, A., A. Neininger, C. Schubert, R. Eckert, C. Birchmeier, H. D. Volk, and M. Gaestel. 1999. 'MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis', *Nat Cell Biol*, 1: 94-7.

Koval, M. 2002. 'Sharing signals: connecting lung epithelial cells with gap junction channels', *Am J Physiol Lung Cell Mol Physiol*, 283: L875-93.

Kreda, S. M., M. C. Gynn, D. A. Fenstermacher, R. C. Boucher, and S. E. Gabriel. 2001. 'Expression and localization of epithelial aquaporins in the adult human lung', *Am J Respir Cell Mol Biol*, 24: 224-34.

Krunkosky, T. M., and C. L. Jarrett. 2006. 'Selective regulation of MAP kinases and chemokine expression after ligation of ICAM-1 on human airway epithelial cells', *Respir Res*, 7: 12.

Krutzik, P. O., M. R. Clutter, and G. P. Nolan. 2005. 'Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry', *J Immunol*, 175: 2357-65.

Krutzik, P. O., M. B. Hale, and G. P. Nolan. 2005. 'Characterization of the murine immunological signaling network with phosphospecific flow cytometry', *J Immunol*, 175: 2366-73.

Krutzik, P. O., J. M. Irish, G. P. Nolan, and O. D. Perez. 2004. 'Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications', *Clin Immunol*, 110: 206-21.

Krutzik, P. O., and G. P. Nolan. 2003. 'Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events', *Cytometry A*, 55: 61-70.

Kuebler, W. M. 2010. 'From a distance: ventilation-dependent extra-pulmonary injury', *Transl Res*, 155: 217-9.

Kuebler, W. M., K. Parthasarathi, P. M. Wang, and J. Bhattacharya. 2000. 'A novel signaling mechanism between gas and blood compartments of the lung', *J Clin Invest*, 105: 905-13.

Kuebler, W. M., U. Uhlig, T. Goldmann, G. Schael, A. Kerem, K. Exner, C. Martin, E. Vollmer, and S. Uhlig. 2003. 'Stretch activates nitric oxide production in pulmonary vascular endothelial cells in situ', *Am J Respir Crit Care Med*, 168: 1391-8.

Kuipers, M. T., T. van der Poll, M. J. Schultz, and C. W. Wieland. 2011. 'Bench-to-bedside review: Damage-associated molecular patterns in the onset of ventilator-induced lung injury', *Crit Care*, 15: 235.

Kumar, A., A. J. Knox, and A. M. Boriek. 2003. 'CCAAT/enhancer-binding protein and activator protein-1 transcription factors regulate the expression of interleukin-8 through the mitogen-activated protein kinase pathways in response to mechanical stretch of human airway smooth muscle cells', *J Biol Chem*, 278: 18868-76.

Kuo, M. Y., M. F. Liao, F. L. Chen, Y. C. Li, M. L. Yang, R. H. Lin, and Y. H. Kuan. 2011. 'Luteolin attenuates the pulmonary inflammatory response involves abilities of antioxidation and inhibition of MAPK and NFkappaB pathways in mice with endotoxin-induced acute lung injury', *Food Chem Toxicol*, 49: 2660-6.

Kushimoto, S., T. Endo, S. Yamanouchi, T. Sakamoto, H. Ishikura, Y. Kitazawa, Y. Taira, K. Okuchi, T. Tagami, A. Watanabe, J. Yamaguchi, K. Yoshikawa, M. Sugita, Y. Kase, T. Kanemura, H. Takahashi, Y. Kuroki, H. Izumino, H. Rinka, R. Seo, M. Takatori, T. Kaneko, T. Nakamura, T. Irahara, and N. Saito. 2013. 'Relationship between extravascular lung water and severity categories of acute respiratory distress syndrome by the Berlin definition', *Crit Care*, 17: R132.

Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, and et al. 1994. 'A protein kinase involved in the regulation of inflammatory cytokine biosynthesis', *Nature*, 372: 739-46.

Lee, P. Y., J. X. Wang, E. Parisini, C. C. Dascher, and P. A. Nigrovic. 2013. 'Ly6 family proteins in neutrophil biology', *J Leukoc Biol*, 94: 585-94.

Lee, Y. C., C. E. Yellowley, Z. Li, H. J. Donahue, and D. E. Rannels. 1997. 'Expression of functional gap junctions in cultured pulmonary alveolar epithelial cells', *Am J Physiol*, 272: L1105-14.

Leitges, M., L. Sanz, P. Martin, A. Duran, U. Braun, J. F. Garcia, F. Camacho, M. T. Diaz-Meco, P. D. Rennert, and J. Moscat. 2001. 'Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway', *Mol Cell*, 8: 771-80.

Leligdowicz, A., and E. Fan. 2015. 'Extracorporeal life support for severe acute respiratory distress syndrome', *Curr Opin Crit Care*, 21: 13-9.

Lenormand, P., C. Sardet, G. Pages, G. L'Allemain, A. Brunet, and J. Pouyssegur. 1993. 'Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts', *J Cell Biol*, 122: 1079-88.

Leybaert, L., K. Braet, W. Vandamme, L. Cabooter, P. E. Martin, and W. H. Evans. 2003. 'Connexin channels, connexin mimetic peptides and ATP release', *Cell Commun Adhes*, 10: 251-7.

Li, G., M. Malinchoc, R. Cartin-Ceba, C. V. Venkata, D. J. Kor, S. G. Peters, R. D. Hubmayr, and O. Gajic. 2011. 'Eight-year trend of acute respiratory distress syndrome: a population-based study in Olmsted County, Minnesota', *Am J Respir Crit Care Med*, 183: 59-66.

Li, H., Z. Wu, D. Feng, J. Gong, C. Yao, Y. Wang, S. Yuan, S. Yao, and Y. Shang. 2014. 'BML-111, a lipoxin receptor agonist, attenuates ventilator-induced lung injury in rats', *Shock*, 41: 311-6.

Li, L. F., S. K. Liao, C. C. Huang, M. J. Hung, and D. A. Quinn. 2008. 'Serine/threonine kinase-protein kinase B and extracellular signal-regulated kinase regulate ventilator-induced pulmonary fibrosis after bleomycin-induced acute lung injury: a prospective, controlled animal experiment', *Crit Care*, 12: R103.

Li, L. F., S. K. Liao, Y. S. Ko, C. H. Lee, and D. A. Quinn. 2007. 'Hyperoxia increases ventilator-induced lung injury via mitogen-activated protein kinases: a prospective, controlled animal experiment', *Crit Care*, 11: R25.

Li, L. F., B. Ouyang, G. Choukroun, R. Matyal, M. Mascarenhas, B. Jafari, J. V. Bonventre, T. Force, and D. A. Quinn. 2003. 'Stretch-induced IL-8 depends on c-Jun NH2-terminal and nuclear factor-kappaB-inducing kinases', *Am J Physiol Lung Cell Mol Physiol*, 285: L464-75.

Li, Q. F., Y. S. Zhu, H. Jiang, H. Xu, and Y. Sun. 2009. 'Isoflurane preconditioning ameliorates endotoxin-induced acute lung injury and mortality in rats', *Anesth Analg*, 109: 1591-7.

Li, X. C., M. Miyasaka, and T. B. Issekutz. 1998. 'Blood monocyte migration to acute lung inflammation involves both CD11/CD18 and very late activation antigen-4-dependent and independent pathways', *J Immunol*, 161: 6258-64.

Lin, H. H., D. E. Faunce, M. Stacey, A. Terajewicz, T. Nakamura, J. Zhang-Hoover, M. Kerley, M. L. Mucenski, S. Gordon, and J. Stein-Streilein. 2005. 'The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance', *J Exp Med*, 201: 1615-25.

Litvinov, S. V., M. P. Velders, H. A. Bakker, G. J. Fleuren, and S. O. Warnaar. 1994. 'Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule', *J Cell Biol*, 125: 437-46.

Liu, F., G. Q. Sun, H. Y. Gao, R. S. Li, L. W. Soromou, N. Chen, Y. H. Deng, and H. H. Feng. 2013. 'Angelicin regulates LPS-induced inflammation via inhibiting MAPK/NF-kappaB pathways', *J Surg Res*.

Liu, Y. Y., C. H. Chiang, C. H. Chuang, S. L. Liu, Y. H. Jheng, and J. H. Ryu. 2014. 'Spillover of cytokines and reactive oxygen species in ventilator-induced lung injury associated with inflammation and apoptosis in distal organs', *Respir Care*, 59: 1422-32.

Liu, Y. Y., L. F. Li, C. T. Yang, K. H. Lu, C. C. Huang, K. C. Kao, and S. H. Chiou. 2013. 'Suppressing NF-kappaB and NKRF Pathways by Induced Pluripotent Stem Cell Therapy in Mice with Ventilator-Induced Lung Injury', *PLoS One*, 8: e66760.

Liu, Y. Y., S. K. Liao, C. C. Huang, Y. H. Tsai, D. A. Quinn, and L. F. Li. 2009. 'Role for nuclear factor-kappaB in augmented lung injury because of interaction between hyperoxia and high stretch ventilation', *Transl Res*, 154: 228-40.

Lopez-Alonso, I., A. Aguirre, A. Gonzalez-Lopez, A. F. Fernandez, L. Amado-Rodriguez, A. Astudillo, E. Batalla-Solis, and G. M. Albaiceta. 2013. 'Impairment of autophagy decreases ventilator-induced lung injury by blockade of the NF-kappaB pathway', *Am J Physiol Lung Cell Mol Physiol*, 304: L844-52.

Ma, L., K. C. Cheung, M. Kishore, S. Nourshargh, C. Mauro, and F. M. Marelli-Berg. 2012. 'CD31 exhibits multiple roles in regulating T lymphocyte trafficking in vivo', *J Immunol*, 189: 4104-11.

MacEwan, D. J. 2002. 'TNF receptor subtype signalling: differences and cellular consequences', *Cell Signal*, 14: 477-92.

Madjdpour, C., B. Oertli, U. Ziegler, J. M. Bonvini, T. Pasch, and B. Beck-Schimmer. 2000. 'Lipopolysaccharide induces functional ICAM-1 expression in rat alveolar epithelial cells in vitro', *Am J Physiol Lung Cell Mol Physiol*, 278: L572-9.

Maeshima, N., and R. C. Fernandez. 2013. 'Recognition of lipid A variants by the TLR4-MD-2 receptor complex', *Front Cell Infect Microbiol*, 3: 3.

Maetzel, D., S. Denzel, B. Mack, M. Canis, P. Went, M. Benk, C. Kieu, P. Papior, P. A. Baeuerle, M. Munz, and O. Gires. 2009. 'Nuclear signalling by tumour-associated antigen EpCAM', *Nat Cell Biol*, 11: 162-71.

Malek, S., D. B. Huang, T. Huxford, S. Ghosh, and G. Ghosh. 2003. 'X-ray crystal structure of an IkappaBbeta x NF-kappaB p65 homodimer complex', *J Biol Chem*, 278: 23094-100.

Mancebo, J., R. Fernandez, L. Blanch, G. Rialp, F. Gordo, M. Ferrer, F. Rodriguez, P. Garro, P. Ricart, I. Vallverdu, I. Gich, J. Castano, P. Saura, G. Dominguez, A. Bonet, and R. K. Albert. 2006. 'A multicenter trial of prolonged prone ventilation in severe acute respiratory distress syndrome', *Am J Respir Crit Care Med*, 173: 1233-9.

Mandava, S., T. Kolobow, G. Vitale, G. Foti, M. Aprigliano, M. Jones, and E. Muller. 2003. 'Lethal systemic capillary leak syndrome associated with severe ventilator-induced lung injury: an experimental study', *Crit Care Med*, 31: 885-92.

Manzer, R., J. Wang, K. Nishina, G. McConville, and R. J. Mason. 2006. 'Alveolar epithelial cells secrete chemokines in response to IL-1beta and lipopolysaccharide but not to ozone', *Am J Respir Cell Mol Biol*, 34: 158-66.

Marais, R., J. Wynne, and R. Treisman. 1993. 'The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain', *Cell*, 73: 381-93.

Marchetti, L., M. Klein, K. Schlett, K. Pfizenmaier, and U. L. Eisel. 2004. 'Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway', *J Biol Chem*, 279: 32869-81.

Martin-Lefevre, L., J. D. Ricard, E. Roupie, D. Dreyfuss, and G. Saumon. 2001. 'Significance of the changes in the respiratory system pressure-volume curve during acute lung injury in rats', *Am J Respir Crit Care Med*, 164: 627-32.

Martin, T. R. 2002. 'Neutrophils and lung injury: getting it right', *J Clin Invest*, 110: 1603-5.

Martin, T. R., J. C. Mathison, P. S. Tobias, D. J. Leturcq, A. M. Moriarty, R. J. Maunder, and R. J. Ulevitch. 1992. 'Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs', *J Clin Invest*, 90: 2209-19.

Mascarenhas, M. M., R. M. Day, C. D. Ochoa, W. I. Choi, L. Yu, B. Ouyang, H. G. Garg, C. A. Hales, and D. A. Quinn. 2004. 'Low molecular weight hyaluronan from stretched lung enhances interleukin-8 expression', *Am J Respir Cell Mol Biol*, 30: 51-60.

Matsuyama, H., F. Amaya, S. Hashimoto, H. Ueno, S. Beppu, M. Mizuta, N. Shime, A. Ishizaka, and S. Hashimoto. 2008. 'Acute lung inflammation and ventilator-induced lung injury caused by ATP via the P2Y receptors: an experimental study', *Respir Res*, 9: 79.

Matthay, M. A., R. G. Brower, S. Carson, I. S. Douglas, M. Eisner, D. Hite, S. Holets, R. H. Kallet, K. D. Liu, N. MacIntyre, M. Moss, D. Schoenfeld, J. Steingrub, and B. T. Thompson. 2011. 'Randomized, placebo-controlled clinical trial of an aerosolized beta(2)-agonist for treatment of acute lung injury', *Am J Respir Crit Care Med*, 184: 561-8.

Matthay, M. A., L. B. Ware, and G. A. Zimmerman. 2012. 'The acute respiratory distress syndrome', *J Clin Invest*, 122: 2731-40.

Mattingley, J. S., S. R. Holets, R. A. Oeckler, R. W. Stroetz, C. F. Buck, and R. D. Hubmayr. 2011. 'Sizing the lung of mechanically ventilated patients', *Crit Care*, 15: R60.

Matute-Bello, G., C. W. Frevert, and T. R. Martin. 2008. 'Animal models of acute lung injury', *Am J Physiol Lung Cell Mol Physiol*, 295: L379-99.

Mbonye, U. R., and I. Song. 2009. 'Posttranscriptional and posttranslational determinants of cyclooxygenase expression', *BMB Rep*, 42: 552-60.

McIntyre, R. C., Jr., E. J. Pulido, D. D. Bensard, B. D. Shames, and E. Abraham. 2000. 'Thirty years of clinical trials in acute respiratory distress syndrome', *Crit Care Med*, 28: 3314-31.

Meade, M. O., D. J. Cook, L. E. Griffith, L. E. Hand, S. E. Lapinsky, T. E. Stewart, K. J. Killian, A. S. Slutsky, and G. H. Guyatt. 2008. 'A study of the physiologic responses to a lung recruitment maneuver in acute lung injury and acute respiratory distress syndrome', *Respir Care*, 53: 1441-9.

Meade, M. O., D. J. Cook, G. H. Guyatt, A. S. Slutsky, Y. M. Arabi, D. J. Cooper, A. R. Davies, L. E. Hand, Q. Zhou, L. Thabane, P. Austin, S. Lapinsky, A. Baxter, J. Russell, Y. Skrobik, J. J. Ronco, and T. E.

Stewart. 2008. 'Ventilation strategy using low tidal volumes, recruitment maneuvers, and high positive end-expiratory pressure for acute lung injury and acute respiratory distress syndrome: a randomized controlled trial', *JAMA*, 299: 637-45.

Meduri, G. U., E. Golden, A. X. Freire, E. Taylor, M. Zaman, S. J. Carson, M. Gibson, and R. Umberger. 2007. 'Methylprednisolone infusion in early severe ARDS: results of a randomized controlled trial', *Chest*, 131: 954-63.

Meduri, G. U., G. Kohler, S. Headley, E. Tolley, F. Stentz, and A. Postlethwaite. 1995. 'Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome', *Chest*, 108: 1303-14.

Mehta, N., M. Kaur, M. Singh, S. Chand, B. Vyas, P. Silakari, M. S. Bahia, and O. Silakari. 2014. 'Purinergic receptor P2X(7): a novel target for anti-inflammatory therapy', *Bioorg Med Chem*, 22: 54-88.

Mekontso Dessap, A., G. Voiriot, T. Zhou, E. Marcos, S. M. Dudek, J. R. Jacobson, R. Machado, S. Adnot, L. Brochard, B. Maitre, and J. G. Garcia. 2012. 'Conflicting physiological and genomic cardiopulmonary effects of recruitment maneuvers in murine acute lung injury', *Am J Respir Cell Mol Biol*, 46: 541-50.

Meloche, S., G. Pages, and J. Pouyssegur. 1992. 'Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44mapk', *Mol Biol Cell*, 3: 63-71.

Meloche, S., and J. Pouyssegur. 2007. 'The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition', *Oncogene*, 26: 3227-39.

Mendez, M. P., Y. K. Monroy, M. Du, A. M. Preston, L. Tolle, Y. Lin, K. L. VanDussen, L. C. Samuelson, T. J. Standiford, J. L. Curtis, J. M. Beck, P. J. Christensen, and R. Paine, 3rd. 2011. 'Overexpression of sICAM-1 in the alveolar epithelial space results in an exaggerated inflammatory response and early death in Gram negative pneumonia', *Respir Res*, 12: 12.

Meng, W., L. L. Swenson, M. J. Fitzgibbon, K. Hayakawa, E. Ter Haar, A. E. Behrens, J. R. Fulghum, and J. A. Lippke. 2002. 'Structure of mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 suggests a bifunctional switch that couples kinase activation with nuclear export', *J Biol Chem*, 277: 37401-5.

Mercat, A., J. C. Richard, B. Vielle, S. Jaber, D. Osman, J. L. Diehl, J. Y. Lefrant, G. Prat, J. Richecoeur, A. Nieszkowska, C. Gervais, J. Baudot, L. Bouadma, and L. Brochard. 2008. 'Positive end-expiratory pressure setting in adults with acute lung injury and acute respiratory distress syndrome: a randomized controlled trial', *JAMA*, 299: 646-55.

Mertens, M., A. Tabuchi, S. Meissner, A. Krueger, K. Schirrmann, U. Kertzscher, A. R. Pries, A. S. Slutsky, E. Koch, and W. M. Kuebler. 2009. 'Alveolar dynamics in acute lung injury: heterogeneous distension rather than cyclic opening and collapse', *Crit Care Med*, 37: 2604-11.

Michaels, A. J., J. G. Hill, W. B. Long, B. P. Young, B. P. Sperley, T. R. Shanks, and L. J. Morgan. 2013. 'Adult refractory hypoxemic acute respiratory distress syndrome treated with extracorporeal membrane oxygenation: the role of a regional referral center', *Am J Surg*, 205: 492-8; discussion 98-9.

Michelet, P., X. B. D'Journo, A. Roch, C. Doddoli, V. Marin, L. Papazian, I. Decamps, F. Bregeon, P. Thomas, and J. P. Auffray. 2006. 'Protective ventilation influences systemic inflammation after esophagectomy: a randomized controlled study', *Anesthesiology*, 105: 911-9.

Miller, M. A., J. M. Stabenow, J. Parvathareddy, A. J. Wodowski, T. P. Fabrizio, X. R. Bina, L. Zalduondo, and J. E. Bina. 2012. 'Visualization of murine intranasal dosing efficiency using luminescent Francisella tularensis: effect of instillation volume and form of anesthesia', *PLoS One*, 7: e31359.

Min, B. K., K. Suk, and W. H. Lee. 2013. 'Stimulation of CD107 affects LPS-induced cytokine secretion and cellular adhesion through the ERK signaling pathway in the human macrophage-like cell line, THP-1', *Cell Immunol*, 281: 122-8.

Misharin, A. V., L. Morales-Nebreda, G. M. Mutlu, G. R. Budinger, and H. Perlman. 2013. 'Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung', *Am J Respir Cell Mol Biol*, 49: 503-10.

Mishra, A., N. R. Chintagari, Y. Guo, T. Weng, L. Su, and L. Liu. 2011. 'Purinergic P2X7 receptor regulates lung surfactant secretion in a paracrine manner', *J Cell Sci*, 124: 657-68.

Miyao, N., Y. Suzuki, K. Takeshita, H. Kudo, M. Ishu, R. Hiraoka, K. Nishio, T. Tamatani, S. Sakamoto, M. Suematsu, H. Tsumura, A. Ishizaka, and K. Yamaguchi. 2006. 'Various adhesion molecules impair microvascular leukocyte kinetics in ventilator-induced lung injury', *Am J Physiol Lung Cell Mol Physiol*, 290: L1059-L68.

Mizgerd, J. P., B. H. Horwitz, H. C. Quillen, M. L. Scott, and C. M. Doerschuk. 1999. 'Effects of CD18 deficiency on the emigration of murine neutrophils during pneumonia', *J Immunol*, 163: 995-9.

Modrykamien, A. M., and P. Gupta. 2015. 'The acute respiratory distress syndrome', *Proc (Bayl Univ Med Cent)*, 28: 163-71.

Mokart, D., T. van Craenenbroeck, J. Lambert, J. Textoris, J. P. Brun, A. Sannini, L. Chow-Chine, S. Hamouda, L. Fouche, F. Ettori, M. Faucher, and J. L. Blache. 2012. 'Prognosis of acute respiratory distress syndrome in neutropenic cancer patients', *Eur Respir J*, 40: 169-76.

Moncao-Ribeiro, L. C., V. R. Cagido, G. Lima-Murad, P. T. Santana, D. R. Riva, R. Borojevic, W. A. Zin, M. C. Cavalcante, I. Rica, A. C. Brando-Lima, C. M. Takiya, D. S. Faffe, and R. Coutinho-Silva. 2011. 'Lipopolysaccharide-induced lung injury: role of P2X7 receptor', *Respir Physiol Neurobiol*, 179: 314-25.

Monick, M. M., A. B. Carter, G. Gudmundsson, R. Mallampalli, L. S. Powers, and G. W. Hunninghake. 1999. 'A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide-stimulated human alveolar macrophages', *J Immunol*, 162: 3005-12.

Muller-Redetzky, H. C., W. Kummer, U. Pfeil, K. Hellwig, D. Will, R. Paddenberg, C. Tabeling, S. Hippenstiel, N. Suttorp, and M. Witzenrath. 2012. 'Intermedin stabilized endothelial barrier function and attenuated ventilator-induced lung injury in mice', *PLoS One*, 7: e35832.

Muller, H. C., M. Witzenrath, T. Tschernig, B. Gutbier, S. Hippenstiel, A. Santel, N. Suttorp, and S. Rosseau. 2010. 'Adrenomedullin attenuates ventilator-induced lung injury in mice', *Thorax*, 65: 1077-84.

Mulligan, M. S., G. P. Wilson, R. F. Todd, C. W. Smith, D. C. Anderson, J. Varani, T. B. Issekutz, M. Miyasaka, T. Tamatani, and et al. 1993. 'Role of beta 1, beta 2 integrins and ICAM-1 in lung injury after deposition of IgG and IgA immune complexes', *J Immunol*, 150: 2407-17.

Murata, N., S. Ito, K. Furuya, N. Takahara, K. Naruse, H. Aso, M. Kondo, M. Sokabe, and Y. Hasegawa. 2014. 'Ca influx and ATP release mediated by mechanical stretch in human lung fibroblasts', *Biochem Biophys Res Commun*.

Murphy, D. B., N. Cregg, L. Tremblay, D. Engelberts, J. G. Laffey, A. S. Slutsky, A. Romaschin, and B. P. Kavanagh. 2000. 'Adverse ventilatory strategy causes pulmonary-to-systemic translocation of endotoxin', *Am J Respir Crit Care Med*, 162: 27-33.

Murphy, L. O., and J. Blenis. 2006. 'MAPK signal specificity: the right place at the right time', *Trends Biochem Sci*, 31: 268-75.

Murphy, L. O., S. Smith, R. H. Chen, D. C. Fingar, and J. Blenis. 2002. 'Molecular interpretation of ERK signal duration by immediate early gene products', *Nat Cell Biol*, 4: 556-64.

Murray, J. F., M. A. Matthay, J. M. Luce, and M. R. Flick. 1988. 'An expanded definition of the adult respiratory distress syndrome', *Am Rev Respir Dis*, 138: 720-3.

Muzio, M., N. Polentarutti, D. Bosisio, P. P. Manoj Kumar, and A. Mantovani. 2000. 'Toll-like receptor family and signalling pathway', *Biochem Soc Trans*, 28: 563-6.

Nahum, A., J. Hoyt, L. Schmitz, J. Moody, R. Shapiro, and J. J. Marini. 1997. 'Effect of mechanical ventilation strategy on dissemination of intratracheally instilled Escherichia coli in dogs', *Crit Care Med*, 25: 1733-43.

Naude, P. J., J. A. den Boer, P. G. Luiten, and U. L. Eisel. 2011. 'Tumor necrosis factor receptor cross-talk', *Febs j*, 278: 888-98.

Needham, C. J., and P. G. Brindley. 2012. 'Best evidence in critical care medicine: The role of neuromuscular blocking drugs in early severe acute respiratory distress syndrome', *Can J Anaesth*, 59: 105-8.

Needham, D. M., T. Yang, V. D. Dinglas, P. A. Mendez-Tellez, C. Shanholtz, J. E. Sevransky, R. G. Brower, P. J. Pronovost, and E. Colantuoni. 2015. 'Timing of low tidal volume ventilation and intensive care unit mortality in acute respiratory distress syndrome. A prospective cohort study', *Am J Respir Crit Care Med*, 191: 177-85.

Neininger, A., H. Thielemann, and M. Gaestel. 2001. 'FRET-based detection of different conformations of MK2', *EMBO Rep*, 2: 703-8.

Neto, A. S., V. G. Pereira, D. C. Esposito, M. C. Damasceno, and M. J. Schultz. 2012. 'Neuromuscular blocking agents in patients with acute respiratory distress syndrome: a summary of the current evidence from three randomized controlled trials', *Ann Intensive Care*, 2: 33.

New, L., Y. Jiang, M. Zhao, K. Liu, W. Zhu, L. J. Flood, Y. Kato, G. C. Parry, and J. Han. 1998. 'PRAK, a novel protein kinase regulated by the p38 MAP kinase', *EMBO J*, 17: 3372-84.

Niitsu, T., S. Tsuchida, V. Peltekova, D. Engelberts, I. Copland, G. Otulakowski, M. Post, and B. P. Kavanagh. 2011. 'Cyclooxygenase inhibition in ventilator-induced lung injury', *Anesth Analg*, 112: 143-9.

Ning, Q. M., and X. R. Wang. 2007a. 'Response of alveolar type II epithelial cells to mechanical stretch and lipopolysaccharide', *Respiration*, 74: 579-85.

Ning, Q., and X. Wang. 2007b. 'Role of Rel A and IkappaB of nuclear factor kappaB in the release of interleukin-8 by cyclic mechanical strain in human alveolar type II epithelial cells A549', *Respirology*, 12: 792-8.

Niu, A., B. Wang, and Y. P. Li. 2015. 'TNFalpha shedding in mechanically stressed cardiomyocytes is mediated by Src activation of TACE', *J Cell Biochem*, 116: 559-65.

Niu, A., Y. Wen, H. Liu, M. Zhan, B. Jin, and Y. P. Li. 2013. 'Src mediates the mechanical activation of myogenesis by activating TNFalpha-converting enzyme', *J Cell Sci*, 126: 4349-57.

O'Brien, A. D., T. J. Standiford, K. A. Bucknell, S. E. Wilcoxen, and R. Paine, 3rd. 1999. 'Role of alveolar epithelial cell intercellular adhesion molecule-1 in host defense against Klebsiella pneumoniae', *Am J Physiol*, 276: L961-70.

O'Dea, K. P., J. O. Dokpesi, K. C. Tatham, M. R. Wilson, and M. Takata. 2011. 'Regulation of monocyte subset proinflammatory responses within the lung microvasculature by the p38 MAPK/MK2 pathway', *Am J Physiol Lung Cell Mol Physiol*, 301: L812-21.

O'Dea, K. P., M. R. Wilson, J. O. Dokpesi, K. Wakabayashi, L. Tatton, N. van Rooijen, and M. Takata. 2009. 'Mobilization and margination of bone marrow Gr-1high monocytes during subclinical endotoxemia predisposes the lungs toward acute injury', *J Immunol*, 182: 1155-66.

O'Dea, K. P., A. J. Young, H. Yamamoto, J. L. Robotham, F. M. Brennan, and M. Takata. 2005. 'Lung-marginated monocytes modulate pulmonary microvascular injury during early endotoxemia', *Am J Respir Crit Care Med*, 172: 1119-27.

Oberprieler, N. G., and K. Tasken. 2011. 'Analysing phosphorylation-based signalling networks by phospho flow cytometry', *Cell Signal*, 23: 14-8.

Oczenski, W., C. Hormann, C. Keller, N. Lorenzl, A. Kepka, S. Schwarz, and R. D. Fitzgerald. 2004. 'Recruitment maneuvers after a positive end-expiratory pressure trial do not induce sustained effects in early adult respiratory distress syndrome', *Anesthesiology*, 101: 620-5.

Ogawa, E. N., A. Ishizaka, S. Tasaka, H. Koh, H. Ueno, F. Amaya, M. Ebina, S. Yamada, Y. Funakoshi, J. Soejima, K. Moriyama, T. Kotani, S. Hashimoto, H. Morisaki, E. Abraham, and J. Takeda. 2006. 'Contribution of high-mobility group box-1 to the development of ventilator-induced lung injury', *Am J Respir Crit Care Med*, 174: 400-7.

Ognibene, F. P., S. E. Martin, M. M. Parker, T. Schlesinger, P. Roach, C. Burch, J. H. Shelhamer, and J. E. Parrillo. 1986. 'Adult respiratory distress syndrome in patients with severe neutropenia', *N Engl J Med*, 315: 547-51.

Ohbuchi, T., F. Takenaga, N. Hohchi, T. Wakasugi, Y. Ueta, and H. Suzuki. 2014. 'Possible contribution of pannexin-1 to ATP release in human upper airway epithelia', *Physiol Rep*, 2: e00227.

Ohta, N., M. Shimaoka, H. Imanaka, M. Nishimura, N. Taenaka, H. Kiyono, and I. Yoshiya. 2001. 'Glucocorticoid suppresses neutrophil activation in ventilator-induced lung injury', *Crit Care Med*, 29: 1012-6.

Okada, M., A. Matsumori, K. Ono, Y. Furukawa, T. Shioi, A. Iwasaki, K. Matsushima, and S. Sasayama. 1998. 'Cyclic stretch upregulates production of interleukin-8 and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in human endothelial cells', *Arterioscler Thromb Vasc Biol*, 18: 894-901.

Ono, K., and J. Han. 2000. 'The p38 signal transduction pathway: activation and function', *Cell Signal*, 12: 1-13.

Oudin, S., and J. Pugin. 2002. 'Role of MAP kinase activation in interleukin-8 production by human BEAS-2B bronchial epithelial cells submitted to cyclic stretch', *Am J Respir Cell Mol Biol*, 27: 107-14.

Owens, D. M., and S. M. Keyse. 2007. 'Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases', *Oncogene*, 26: 3203-13.

Paine, R., 3rd, S. B. Morris, H. Jin, C. E. Baleeiro, and S. E. Wilcoxen. 2002. 'ICAM-1 facilitates alveolar macrophage phagocytic activity through effects on migration over the AEC surface', *Am J Physiol Lung Cell Mol Physiol*, 283: L180-7.

Pan, W. Z., C. X. Shi, M. Tian, and J. G. Yu. 2014. 'Anti-CD11c antibody, Efalizumab attenuate ventilator-induced lung injury', *Eur Rev Med Pharmacol Sci*, 18: 2182-90.

Pang, Y. L., B. S. Chen, S. P. Li, C. C. Huang, S. W. Chang, C. F. Lam, and Y. C. Tsai. 2012. 'The preconditioning pulmonary protective effect of volatile isoflurane in acute lung injury is mediated by activation of endogenous iNOS', *J Anesth*, 26: 822-8.

Papazian, L., J. M. Forel, A. Gacouin, C. Penot-Ragon, G. Perrin, A. Loundou, S. Jaber, J. M. Arnal, D. Perez, J. M. Seghboyan, J. M. Constantin, P. Courant, J. Y. Lefrant, C. Guerin, G. Prat, S. Morange, and A. Roch. 2010. 'Neuromuscular blockers in early acute respiratory distress syndrome', *N Engl J Med*, 363: 1107-16.

Papazian, L., M. Gainnier, V. Marin, S. Donati, J. M. Arnal, D. Demory, A. Roch, J. M. Forel, P. Bongrand, F. Bregeon, and J. M. Sainty. 2005. 'Comparison of prone positioning and high-frequency oscillatory ventilation in patients with acute respiratory distress syndrome', *Crit Care Med*, 33: 2162-71.

Park, B. S., and J. O. Lee. 2013. 'Recognition of lipopolysaccharide pattern by TLR4 complexes', *Exp Mol Med*, 45: e66.

Park, M. S., Q. He, M. G. Edwards, A. Sergew, D. W. Riches, R. K. Albert, and I. S. Douglas. 2012. 'Mitogen-activated protein kinase phosphatase-1 modulates regional effects of injurious mechanical ventilation in rodent lungs', *Am J Respir Crit Care Med*, 186: 72-81.

Parker, J. C., C. L. Ivey, and J. A. Tucker. 1998. 'Gadolinium prevents high airway pressure-induced permeability increases in isolated rat lungs', *J Appl Physiol*, 84: 1113-8.

Parmley, L. A., N. D. Elkins, M. A. Fini, Y. E. Liu, J. E. Repine, and R. M. Wright. 2007. 'Alpha-4/beta-1 and alpha-L/beta-2 integrins mediate cytokine induced lung leukocyte-epithelial adhesion and injury', *Br J Pharmacol*, 152: 915-29.

Parthasarathi, K., H. Ichimura, E. Monma, J. Lindert, S. Quadri, A. Issekutz, and J. Bhattacharya. 2006. 'Connexin 43 mediates spread of Ca2+-dependent proinflammatory responses in lung capillaries', *J Clin Invest*, 116: 2193-200.

Patel, A. S., D. Reigada, C. H. Mitchell, S. R. Bates, S. S. Margulies, and M. Koval. 2005. 'Paracrine stimulation of surfactant secretion by extracellular ATP in response to mechanical deformation', *Am J Physiol Lung Cell Mol Physiol*, 289: L489-96.

Patel, B. V. PhD Mrcp Frca, K. C. Tatham, M. R. Wilson, K. P. O'Dea, and M. Takata. 2015. 'In vivo compartmental analysis of leukocytes in mouse lungs', *Am J Physiol Lung Cell Mol Physiol*: ajplung 00140 2015.

Patel, B. V., M. R. Wilson, K. P. O'Dea, and M. Takata. 2013. 'TNF-induced death signaling triggers alveolar epithelial dysfunction in acute lung injury', *J Immunol*, 190: 4274-82.

Pellegatti, P., S. Falzoni, P. Pinton, R. Rizzuto, and F. Di Virgilio. 2005. 'A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion', *Mol Biol Cell*, 16: 3659-65.

Pereira, C. 2012. 'Kinetics of Inhaled Antibodies by Gamma Scintigraphy', PhD, University of Nottingham.

Perera, P. Y., S. N. Vogel, G. R. Detore, A. Haziot, and S. M. Goyert. 1997. 'CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol', *J Immunol*, 158: 4422-9.

Perez, O. D., P. O. Krutzik, and G. P. Nolan. 2004. 'Flow cytometric analysis of kinase signaling cascades', *Methods Mol Biol*, 263: 67-94.

Perez, O. D., and G. P. Nolan. 2002. 'Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry', *Nat Biotechnol*, 20: 155-62.

Peschon, J. J., D. S. Torrance, K. L. Stocking, M. B. Glaccum, C. Otten, C. R. Willis, K. Charrier, P. J. Morrissey, C. B. Ware, and K. M. Mohler. 1998. 'TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation', *J Immunol*, 160: 943-52.

Pestana, D., C. Hernandez-Gancedo, C. Royo, H. Perez-Chrzanowska, and A. Criado. 2005. 'Pressure-volume curve variations after a recruitment manoeuvre in acute lung injury/ARDS patients: implications for the understanding of the inflection points of the curve', *Eur J Anaesthesiol*, 22: 175-80.

Pfeiffer, Z. A., M. Aga, U. Prabhu, J. J. Watters, D. J. Hall, and P. J. Bertics. 2004. 'The nucleotide receptor P2X7 mediates actin reorganization and membrane blebbing in RAW 264.7 macrophages via p38 MAP kinase and Rho', *J Leukoc Biol*, 75: 1173-82.

Phua, J., J. R. Badia, N. K. Adhikari, J. O. Friedrich, R. A. Fowler, J. M. Singh, D. C. Scales, D. R. Stather, A. Li, A. Jones, D. J. Gattas, D. Hallett, G. Tomlinson, T. E. Stewart, and N. D. Ferguson. 2009. 'Has mortality from acute respiratory distress syndrome decreased over time?: A systematic review', *Am J Respir Crit Care Med*, 179: 220-7.

Pietersma, A., B. C. Tilly, M. Gaestel, N. de Jong, J. C. Lee, J. F. Koster, and W. Sluiter. 1997. 'p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level', *Biochem Biophys Res Commun*, 230: 44-8.

Plotz, F. B., A. S. Slutsky, A. J. van Vught, and C. J. Heijnen. 2004. 'Ventilator-induced lung injury and multiple system organ failure: a critical review of facts and hypotheses', *Intensive Care Med*, 30: 1865-72.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. 'Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene', *Science*, 282: 2085-8.

Pouyssegur, J., V. Volmat, and P. Lenormand. 2002. 'Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling', *Biochem Pharmacol*, 64: 755-63.

Pugin, J., I. Dunn, P. Jolliet, D. Tassaux, J. L. Magnenat, L. P. Nicod, and J. C. Chevrolet. 1998. 'Activation of human macrophages by mechanical ventilation in vitro', *Am J Physiol*, 275: L1040-50.

Pugin, J., C. C. Schurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias. 1993. 'Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14', *Proc Natl Acad Sci U S A*, 90: 2744-8.

Qiu, P., X. Cui, J. Sun, J. Welsh, C. Natanson, and P. Q. Eichacker. 2013. 'Antitumor necrosis factor therapy is associated with improved survival in clinical sepsis trials: a meta-analysis', *Crit Care Med*, 41: 2419-29.

Raghavendran, K., and L. M. Napolitano. 2011. 'ALI and ARDS: challenges and advances', *Crit Care Clin*, 27: xiii-xiv.

Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, R. J. Ulevitch, and R. J. Davis. 1995. 'Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine', *J Biol Chem*, 270: 7420-6.

Raingeaud, J., A. J. Whitmarsh, T. Barrett, B. Derijard, and R. J. Davis. 1996. 'MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway', *Mol Cell Biol*, 16: 1247-55.

Ramachandran, G. 2014. 'Gram-positive and gram-negative bacterial toxins in sepsis: a brief review', *Virulence*, 5: 213-8.

Ramirez, M. I., G. Millien, A. Hinds, Y. Cao, D. C. Seldin, and M. C. Williams. 2003. 'T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth', *Dev Biol*, 256: 61-72.

Ranieri, V. M., P. M. Suter, C. Tortorella, R. De Tullio, J. M. Dayer, A. Brienza, F. Bruno, and A. S. Slutsky. 1999. 'Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial', *JAMA*, 282: 54-61.

Rao, K. M., T. Meighan, and L. Bowman. 2002. 'Role of mitogen-activated protein kinase activation in the production of inflammatory mediators: differences between primary rat alveolar macrophages and macrophage cell lines', *J Toxicol Environ Health A*, 65: 757-68.

Raoust, E., V. Balloy, I. Garcia-Verdugo, L. Touqui, R. Ramphal, and M. Chignard. 2009. 'Pseudomonas aeruginosa LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells', *PLoS One*, 4: e7259.

Regan, C. P., W. Li, D. M. Boucher, S. Spatz, M. S. Su, and K. Kuida. 2002. 'Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects', *Proc Natl Acad Sci U S A*, 99: 9248-53.

Reiss, L. K., A. Kowallik, and S. Uhlig. 2011. 'Recurrent recruitment manoeuvres improve lung mechanics and minimize lung injury during mechanical ventilation of healthy mice', *PLoS One*, 6: e24527.

Ren, W., L. Hu, F. Hua, J. Jin, Y. Wang, and L. Zhu. 2011. 'Myeloid differentiation protein 2 silencing decreases LPS-induced cytokine production and TLR4/MyD88 pathway activity in alveolar macrophages', *Immunol Lett*, 141: 94-101.

Retamal, J., J. B. Borges, A. Bruhn, X. Cao, R. Feinstein, G. Hedenstierna, S. Johansson, F. Suarez-Sipmann, and A. Larsson. 2015. 'High respiratory rate is associated with early reduction of lung edema clearance in an experimental model of ARDS', *Acta Anaesthesiol Scand*.

Reutershan, J., D. Chang, J. K. Hayes, and K. Ley. 2006. 'Protective effects of isoflurane pretreatment in endotoxin-induced lung injury', *Anesthesiology*, 104: 511-7.

Ricard, J. D., D. Dreyfuss, and G. Saumon. 2001. 'Production of inflammatory cytokines in ventilator-induced lung injury: a reappraisal', *Am J Respir Crit Care Med*, 163: 1176-80.

Rich, P. B., C. D. Douillet, S. A. Mahler, S. A. Husain, and R. C. Boucher. 2003. 'Adenosine triphosphate is released during injurious mechanical ventilation and contributes to lung edema', *J Trauma*, 55: 290-7.

Richard, J. C., L. Brochard, L. Breton, J. Aboab, P. Vandelet, F. Tamion, S. M. Maggiore, A. Mercat, and G. Bonmarchand. 2002. 'Influence of respiratory rate on gas trapping during low volume ventilation of patients with acute lung injury', *Intensive Care Med*, 28: 1078-83.

Richard, J. C., S. M. Maggiore, B. Jonson, J. Mancebo, F. Lemaire, and L. Brochard. 2001. 'Influence of tidal volume on alveolar recruitment. Respective role of PEEP and a recruitment maneuver', *Am J Respir Crit Care Med*, 163: 1609-13.

Richter, K., K. P. Kiefer, B. A. Grzesik, W. G. Clauss, and M. Fronius. 2014. 'Hydrostatic pressure activates ATP-sensitive K+ channels in lung epithelium by ATP release through pannexin and connexin hemichannels', *FASEB J*, 28: 45-55.

Ridger, V. C., B. E. Wagner, W. A. Wallace, and P. G. Hellewell. 2001. 'Differential effects of CD18, CD29, and CD49 integrin subunit inhibition on neutrophil migration in pulmonary inflammation', *J Immunol*, 166: 3484-90.

Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahringer, U. Seydel, F. Di Padova, and et al. 1994. 'Bacterial endotoxin: molecular relationships of structure to activity and function', *FASEB J*, 8: 217-25.

Riteau, N., P. Gasse, L. Fauconnier, A. Gombault, M. Couegnat, L. Fick, J. Kanellopoulos, V. F. Quesniaux, S. Marchand-Adam, B. Crestani, B. Ryffel, and I. Couillin. 2010. 'Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis', *Am J Respir Crit Care Med*, 182: 774-83.

Roch, A., C. Guervilly, and L. Papazian. 2011. 'Fluid management in acute lung injury and ards', *Ann Intensive Care*, 1: 16.

Roch, A., S. Hraiech, E. Masson, D. Grisoli, J. M. Forel, M. Boucekine, P. Morera, C. Guervilly, M. Adda, S. Dizier, R. Toesca, F. Collart, and L. Papazian. 2014. 'Outcome of acute respiratory distress syndrome patients treated with extracorporeal membrane oxygenation and brought to a referral center', *Intensive Care Med*, 40: 74-83.

Romero, A., A. Moreno, J. Garcia, C. Sanchez, M. Santos, and J. Garcia. 2015. 'Effects of sevoflurane on ventilator induced lung injury in a healthy lung experimental model', *Rev Esp Anestesiol Reanim*.

Ronkina, N., A. Kotlyarov, O. Dittrich-Breiholz, M. Kracht, E. Hitti, K. Milarski, R. Askew, S. Marusic, L. L. Lin, M. Gaestel, and J. B. Telliez. 2007. 'The mitogen-activated protein kinase (MAPK)-activated protein kinases MK2 and MK3 cooperate in stimulation of tumor necrosis factor biosynthesis and stabilization of p38 MAPK', *Mol Cell Biol*, 27: 170-81.

Rose, S., A. Misharin, and H. Perlman. 2012. 'A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment', *Cytometry A*, 81: 343-50.

Rouse, J., P. Cohen, S. Trigon, M. Morange, A. Alonso-Llamazares, D. Zamanillo, T. Hunt, and A. R. Nebreda. 1994. 'A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins', *Cell*, 78: 1027-37.

Roux, P. P., and J. Blenis. 2004. 'ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions', *Microbiol Mol Biol Rev*, 68: 320-44.

Rubenfeld, G. D., E. Caldwell, E. Peabody, J. Weaver, D. P. Martin, M. Neff, E. J. Stern, and L. D. Hudson. 2005. 'Incidence and outcomes of acute lung injury', *N Engl J Med*, 353: 1685-93.

Rubenfeld, G. D., C. Cooper, G. Carter, B. T. Thompson, and L. D. Hudson. 2004. 'Barriers to providing lung-protective ventilation to patients with acute lung injury', *Crit Care Med*, 32: 1289-93.

Saccani, S., S. Pantano, and G. Natoli. 2001. 'Two waves of nuclear factor kappaB recruitment to target promoters', *J Exp Med*, 193: 1351-9.

Sakashita, A., Y. Nishimura, T. Nishiuma, K. Takenaka, K. Kobayashi, Y. Kotani, and M. Yokoyama. 2007. 'Neutrophil elastase inhibitor (sivelestat) attenuates subsequent ventilator-induced lung injury in mice', *Eur J Pharmacol*, 571: 62-71.

Sanchez-Esteban, J., Y. Wang, P. A. Gruppuso, and L. P. Rubin. 2004. 'Mechanical stretch induces fetal type II cell differentiation via an epidermal growth factor receptor-extracellular-regulated protein kinase signaling pathway', *Am J Respir Cell Mol Biol*, 30: 76-83.

Sanghera, J. S., S. L. Weinstein, M. Aluwalia, J. Girn, and S. L. Pelech. 1996. 'Activation of multiple proline-directed kinases by bacterial lipopolysaccharide in murine macrophages', *J Immunol*, 156: 4457-65.

Santa Cruz, R., J. I. Rojas, R. Nervi, R. Heredia, and A. Ciapponi. 2013. 'High versus low positive end-expiratory pressure (PEEP) levels for mechanically ventilated adult patients with acute lung injury and acute respiratory distress syndrome', *Cochrane Database Syst Rev*, 6: CD009098.

Sarieddine, M. Z., K. E. Scheckenbach, B. Foglia, K. Maass, I. Garcia, B. R. Kwak, and M. Chanson. 2009. 'Connexin43 modulates neutrophil recruitment to the lung', *J Cell Mol Med*, 13: 4560-70.

Saunders, A. E., and P. Johnson. 2010. 'Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45', *Cell Signal*, 22: 339-48.

Schertzer, J. W., and M. Whiteley. 2013. 'Bacterial outer membrane vesicles in trafficking, communication and the host-pathogen interaction', *J Mol Microbiol Biotechnol*, 23: 118-30.

Schmal, H., B. J. Czermak, A. B. Lentsch, N. M. Bless, B. Beck-Schimmer, H. P. Friedl, and P. A. Ward. 1998. 'Soluble ICAM-1 activates lung macrophages and enhances lung injury', *J Immunol*, 161: 3685-93.

Schmidt, M., E. Zogheib, H. Roze, X. Repesse, G. Lebreton, C. E. Luyt, J. L. Trouillet, N. Brechot, A. Nieszkowska, H. Dupont, A. Ouattara, P. Leprince, J. Chastre, and A. Combes. 2013. 'The PRESERVE mortality risk score and analysis of long-term outcomes after extracorporeal membrane oxygenation for severe acute respiratory distress syndrome', *Intensive Care Med*, 39: 1704-13.

Schnyder-Candrian, S., V. F. Quesniaux, F. Di Padova, I. Maillet, N. Noulin, I. Couillin, R. Moser, F. Erard, B. B. Vargaftig, B. Ryffel, and B. Schnyder. 2005. 'Dual effects of p38 MAPK on TNF-dependent bronchoconstriction and TNF-independent neutrophil recruitment in lipopolysaccharide-induced acute respiratory distress syndrome', *J Immunol*, 175: 262-9.

Schuh, K., and A. Pahl. 2009. 'Inhibition of the MAP kinase ERK protects from lipopolysaccharide-induced lung injury', *Biochem Pharmacol*, 77: 1827-34.

Schulz, C., L. Farkas, K. Wolf, K. Kratzel, G. Eissner, and M. Pfeifer. 2002. 'Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549)', *Scand J Immunol*, 56: 294-302.

Schwiebert, E. M., and A. Zsembery. 2003. 'Extracellular ATP as a signaling molecule for epithelial cells', *Biochim Biophys Acta*, 1615: 7-32.

Scott, A. J., K. P. O'Dea, D. O'Callaghan, L. Williams, J. O. Dokpesi, L. Tatton, J. M. Handy, P. J. Hogg, and M. Takata. 2011. 'Reactive oxygen species and p38 mitogen-activated protein kinase mediate tumor necrosis factor alpha-converting enzyme (TACE/ADAM-17) activation in primary human monocytes', *J Biol Chem*, 286: 35466-76.

Seah, A. S., K. A. Grant, M. Aliyeva, G. B. Allen, and J. H. Bates. 2011. 'Quantifying the roles of tidal volume and PEEP in the pathogenesis of ventilator-induced lung injury', *Ann Biomed Eng*, 39: 1505-16.

Seger, R., N. G. Ahn, J. Posada, E. S. Munar, A. M. Jensen, J. A. Cooper, M. H. Cobb, and E. G. Krebs. 1992. 'Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells', *J Biol Chem*, 267: 14373-81.

Serpa Neto, A., S. O. Cardoso, J. A. Manetta, V. G. Pereira, D. C. Esposito, O. Pasqualucci Mde, M. C. Damasceno, and M. J. Schultz. 2012. 'Association between use of lung-protective ventilation with lower tidal volumes and clinical outcomes among patients without acute respiratory distress syndrome: a meta-analysis', *JAMA*, 308: 1651-9.

Serpa Neto, A., S. N. Hemmes, C. S. Barbas, M. Beiderlinden, M. Biehl, J. M. Binnekade, J. Canet, A. Fernandez-Bustamante, E. Futier, O. Gajic, G. Hedenstierna, M. W. Hollmann, S. Jaber, A. Kozian, M. Licker, W. Q. Lin, A. D. Maslow, S. G. Memtsoudis, D. Reis Miranda, P. Moine, T. Ng, D. Paparella, C. Putensen, M. Ranieri, F. Scavonetto, T. Schilling, W. Schmid, G. Selmo, P. Severgnini, J. Sprung, S. Sundar, D. Talmor, T. Treschan, C. Unzueta, T. N. Weingarten, E. K. Wolthuis, H. Wrigge, M. Gama de Abreu, P. Pelosi, and M. J. Schultz. 2015. 'Protective versus Conventional Ventilation for Surgery: A Systematic Review and Individual Patient Data Meta-analysis', *Anesthesiology*.

Serpa Neto, A., S. N. Hemmes, C. S. Barbas, M. Beiderlinden, A. Fernandez-Bustamante, E. Futier, M. W. Hollmann, S. Jaber, A. Kozian, M. Licker, W. Q. Lin, P. Moine, F. Scavonetto, T. Schilling, G. Selmo, P. Severgnini, J. Sprung, T. Treschan, C. Unzueta, T. N. Weingarten, E. K. Wolthuis, H. Wrigge, M. Gama de Abreu, P. Pelosi, and M. J. Schultz. 2014. 'Incidence of mortality and

morbidity related to postoperative lung injury in patients who have undergone abdominal or thoracic surgery: a systematic review and meta-analysis', *Lancet Respir Med*, 2: 1007-15.

Serpa Neto, A., L. Nagtzaam, and M. J. Schultz. 2014. 'Ventilation with lower tidal volumes for critically ill patients without the acute respiratory distress syndrome: a systematic translational review and meta-analysis', *Curr Opin Crit Care*, 20: 25-32.

Serpa Neto, A., F. D. Simonis, C. S. Barbas, M. Biehl, R. M. Determann, J. Elmer, G. Friedman, O. Gajic, J. N. Goldstein, J. Horn, N. P. Juffermans, R. Linko, R. P. de Oliveira, S. Sundar, D. Talmor, E. K. Wolthuis, M. G. de Abreu, P. Pelosi, and M. J. Schultz. 2014. 'Association between tidal volume size, duration of ventilation, and sedation needs in patients without acute respiratory distress syndrome: an individual patient data meta-analysis', *Intensive Care Med*, 40: 950-7.

Severgnini, P., G. Selmo, C. Lanza, A. Chiesa, A. Frigerio, A. Bacuzzi, G. Dionigi, R. Novario, C. Gregoretti, M. G. de Abreu, M. J. Schultz, S. Jaber, E. Futier, M. Chiaranda, and P. Pelosi. 2013. 'Protective mechanical ventilation during general anesthesia for open abdominal surgery improves postoperative pulmonary function', *Anesthesiology*, 118: 1307-21.

Shah, D., F. Romero, W. Stafstrom, M. Duong, and R. Summer. 2014. 'Extracellular ATP mediates the late phase of neutrophil recruitment to the lung in murine models of acute lung injury', *Am J Physiol Lung Cell Mol Physiol*, 306: L152-61.

Sharp, C., A. B. Millar, and A. R. Medford. 2015. 'Advances in understanding of the pathogenesis of acute respiratory distress syndrome', *Respiration*, 89: 420-34.

Shinohara, H., A. Inoue, N. Toyama-Sorimachi, Y. Nagai, T. Yasuda, H. Suzuki, R. Horai, Y. Iwakura, T. Yamamoto, H. Karasuyama, K. Miyake, and Y. Yamanashi. 2005. 'Dok-1 and Dok-2 are negative regulators of lipopolysaccharide-induced signaling', *J Exp Med*, 201: 333-9.

Siegl, S., and S. Uhlig. 2012. 'Using the one-lung method to link p38 to pro-inflammatory gene expression during overventilation in C57BL/6 and BALB/c mice', *PLoS One*, 7: e41464.

Skinner, S. J., C. E. Somervell, and D. M. Olson. 1992. 'The effects of mechanical stretching on fetal rat lung cell prostacyclin production', *Prostaglandins*, 43: 413-33.

Slutsky, A. S. 2005. 'Ventilator-Induced Lung Injury: From Barotrauma to Biotrauma', *Respir Care*, 50: 646-59.

Slutsky, A. S., and L. N. Tremblay. 1998. 'Multiple system organ failure. Is mechanical ventilation a contributing factor?', *Am J Respir Crit Care Med*, 157: 1721-5.

Solle, M., J. Labasi, D. G. Perregaux, E. Stam, N. Petrushova, B. H. Koller, R. J. Griffiths, and C. A. Gabel. 2001. 'Altered cytokine production in mice lacking P2X(7) receptors', *J Biol Chem*, 276: 125-32.

Soutiere, S. E., and W. Mitzner. 2004. 'On defining total lung capacity in the mouse', *J Appl Physiol*, 96: 1658-64.

Spieth, P. M., T. Bluth, M. Gama De Abreu, A. Bacelis, A. E. Goetz, and R. Kiefmann. 2014. 'Mechanotransduction in the lungs', *Minerva Anestesiol*, 80: 933-41.

Spieth, P. M., and H. Zhang. 2014. 'Pharmacological therapies for acute respiratory distress syndrome', *Curr Opin Crit Care*, 20: 113-21.

Spinas, G. A., U. Keller, and M. Brockhaus. 1992. 'Release of soluble receptors for tumor necrosis factor (TNF) in relation to circulating TNF during experimental endotoxinemia', *J Clin Invest*, 90: 533-6.

Spragg, R. G., J. F. Lewis, H. D. Walmrath, J. Johannigman, G. Bellingan, P. F. Laterre, M. C. Witte, G. A. Richards, G. Rippin, F. Rathgeb, D. Hafner, F. J. Taut, and W. Seeger. 2004. 'Effect of recombinant surfactant protein C-based surfactant on the acute respiratory distress syndrome', *N Engl J Med*, 351: 884-92.

Srivastava, N., J. S. Brennan, R. F. Renzi, M. Wu, S. S. Branda, A. K. Singh, and A. E. Herr. 2009. 'Fully integrated microfluidic platform enabling automated phosphoprofiling of macrophage response', *Anal Chem*, 81: 3261-9.

Steinberg, J. M., H. J. Schiller, J. M. Halter, L. A. Gatto, H. M. Lee, L. A. Pavone, and G. F. Nieman. 2004. 'Alveolar instability causes early ventilator-induced lung injury independent of neutrophils', *Am J Respir Crit Care Med*, 169: 57-63.

Steinberg, K. P., L. D. Hudson, R. B. Goodman, C. L. Hough, P. N. Lanken, R. Hyzy, B. T. Thompson, and M. Ancukiewicz. 2006. 'Efficacy and safety of corticosteroids for persistent acute respiratory distress syndrome', *N Engl J Med*, 354: 1671-84.

Steinberg, K. P., J. A. Milberg, T. R. Martin, R. J. Maunder, B. A. Cockrill, and L. D. Hudson. 1994. 'Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome', *Am J Respir Crit Care Med*, 150: 113-22.

Stent, G., L. Irving, S. Lewin, and S. M. Crowe. 1995. 'The kinetics of surface expression of CD11/CD18 integrins and CD54 on monocytes and macrophages', *Clin Exp Immunol*, 100: 366-76.

Su, G., M. Hodnett, N. Wu, A. Atakilit, C. Kosinski, M. Godzich, X. Z. Huang, J. K. Kim, J. A. Frank, M. A. Matthay, D. Sheppard, and J. F. Pittet. 2007. 'Integrin alphavbeta5 regulates lung vascular permeability and pulmonary endothelial barrier function', *Am J Respir Cell Mol Biol*, 36: 377-86.

Sud, S., J. O. Friedrich, N. K. Adhikari, P. Taccone, J. Mancebo, F. Polli, R. Latini, A. Pesenti, M. A. Curley, R. Fernandez, M. C. Chan, P. Beuret, G. Voggenreiter, M. Sud, G. Tognoni, L. Gattinoni, and C. Guerin. 2014. 'Effect of prone positioning during mechanical ventilation on mortality among patients with acute respiratory distress syndrome: a systematic review and meta-analysis', *Cmaj*, 186: E381-90.

Suh, B. C., J. S. Kim, U. Namgung, H. Ha, and K. T. Kim. 2001. 'P2X7 nucleotide receptor mediation of membrane pore formation and superoxide generation in human promyelocytes and neutrophils', *J Immunol*, 166: 6754-63.

Sun, J., G. Chi, L. W. Soromou, N. Chen, M. Guan, Q. Wu, D. Wang, and H. Li. 2012. 'Preventive effect of Imperatorin on acute lung injury induced by lipopolysaccharide in mice', *Int Immunopharmacol*, 14: 369-74.

Sung, S. S., S. M. Fu, C. E. Rose, Jr., F. Gaskin, S. T. Ju, and S. R. Beaty. 2006. 'A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins', *J Immunol*, 176: 2161-72.

Suter, P. M., S. Suter, E. Girardin, P. Roux-Lombard, G. E. Grau, and J. M. Dayer. 1992. 'High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis', *Am Rev Respir Dis*, 145: 1016-22.

Swantek, J. L., M. H. Cobb, and T. D. Geppert. 1997. 'Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK', *Mol Cell Biol*, 17: 6274-82.

Sweatt, A. J., and J. E. Levitt. 2014. 'Evolving epidemiology and definitions of the acute respiratory distress syndrome and early acute lung injury', *Clin Chest Med*, 35: 609-24.

Taccone, P., A. Pesenti, R. Latini, F. Polli, F. Vagginelli, C. Mietto, L. Caspani, F. Raimondi, G. Bordone, G. Iapichino, J. Mancebo, C. Guerin, L. Ayzac, L. Blanch, R. Fumagalli, G. Tognoni, and L. Gattinoni. 2009. 'Prone positioning in patients with moderate and severe acute respiratory distress syndrome: a randomized controlled trial', *JAMA*, 302: 1977-84.

Takahara, N., S. Ito, K. Furuya, K. Naruse, H. Aso, M. Kondo, M. Sokabe, and Y. Hasegawa. 2014. 'Real-time imaging of ATP release induced by mechanical stretch in human airway smooth muscle cells', *Am J Respir Cell Mol Biol*, 51: 772-82.

Takata, M., J. Abe, H. Tanaka, Y. Kitano, S. Doi, T. Kohsaka, and K. Miyasaka. 1997. 'Intraalveolar expression of tumor necrosis factor-alpha gene during conventional and high-frequency ventilation', *Am J Respir Crit Care Med*, 156: 272-9.

Tan, Y., J. Rouse, A. Zhang, S. Cariati, P. Cohen, and M. J. Comb. 1996. 'FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2', *EMBO J*, 15: 4629-42.

Taniguchi, L. U., E. G. Caldini, I. T. Velasco, and E. M. Negri. 2010. 'Cytoskeleton and mechanotransduction in the pathophysiology of ventilator-induced lung injury', *J Bras Pneumol*, 36: 363-71.

Tapping, R. I., S. Akashi, K. Miyake, P. J. Godowski, and P. S. Tobias. 2000. 'Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides', *J Immunol*, 165: 5780-7.

ter Haar, E., P. Prabhakar, X. Liu, and C. Lepre. 2007. 'Crystal structure of the p38 alpha-MAPKAP kinase 2 heterodimer', *J Biol Chem*, 282: 9733-9.

Terragni, P. P., G. Rosboch, A. Tealdi, E. Corno, E. Menaldo, O. Davini, G. Gandini, P. Herrmann, L. Mascia, M. Quintel, A. S. Slutsky, L. Gattinoni, and V. M. Ranieri. 2007. 'Tidal hyperinflation during low tidal volume ventilation in acute respiratory distress syndrome', *Am J Respir Crit Care Med*, 175: 160-6.

Terzi, E., K. Zarogoulidis, I. Kougioumtzi, G. Dryllis, I. Kioumis, G. Pitsiou, N. Machairiotis, N. Katsikogiannis, S. Lampaki, A. Papaiwannou, T. Tsiouda, A. Madesis, T. Karaiskos, B. Zaric, P. Branislav, and P. Zarogoulidis. 2014. 'Acute respiratory distress syndrome and pneumothorax', *J Thorac Dis*, 6: S435-42.

Thille, A. W., A. Esteban, P. Fernandez-Segoviano, J. M. Rodriguez, J. A. Aramburu, O. Penuelas, I. Cortes-Puch, P. Cardinal-Fernandez, J. A. Lorente, and F. Frutos-Vivar. 2013. 'Comparison of the Berlin definition for acute respiratory distress syndrome with autopsy', *Am J Respir Crit Care Med*, 187: 761-7.

Thille, A. W., A. Vuylsteke, and A. Bersten. 2015. 'Does the Berlin definition for acute respiratory distress syndrome predict the presence of diffuse alveolar damage?', *Intensive Care Med*, 41: 342-4.

Thompson, B. T., and M. Moss. 2013. 'A new definition for the acute respiratory distress syndrome', *Semin Respir Crit Care Med*, 34: 441-7.

Thompson, J. E., R. J. Phillips, H. Erdjument-Bromage, P. Tempst, and S. Ghosh. 1995. 'I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B', *Cell*, 80: 573-82.

Thorley, A. J., P. A. Ford, M. A. Giembycz, P. Goldstraw, A. Young, and T. D. Tetley. 2007. 'Differential regulation of cytokine release and leukocyte migration by lipopolysaccharide-stimulated primary human lung alveolar type II epithelial cells and macrophages', *J Immunol*, 178: 463-73.

Thorley, A. J., D. Grandolfo, E. Lim, P. Goldstraw, A. Young, and T. D. Tetley. 2011. 'Innate immune responses to bacterial ligands in the peripheral human lung--role of alveolar epithelial TLR expression and signalling', *PLoS One*, 6: e21827.

Tobias, P. S., K. Soldau, J. A. Gegner, D. Mintz, and R. J. Ulevitch. 1995. 'Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14', *J Biol Chem*, 270: 10482-8.

Traenckner, E. B., H. L. Pahl, T. Henkel, K. N. Schmidt, S. Wilk, and P. A. Baeuerle. 1995. 'Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli', *EMBO J*, 14: 2876-83.

Tremblay, L. N., D. Miatto, Q. Hamid, A. Govindarajan, and A. S. Slutsky. 2002. 'Injurious ventilation induces widespread pulmonary epithelial expression of tumor necrosis factor-alpha and interleukin-6 messenger RNA', *Crit Care Med*, 30: 1693-700.

Tremblay, L., F. Valenza, S. P. Ribeiro, J. Li, and A. S. Slutsky. 1997. 'Injurious ventilatory strategies increase cytokines and c-fos m-RNA expression in an isolated rat lung model', *J Clin Invest*, 99: 944-52.

Trzpis, M., P. M. McLaughlin, L. M. de Leij, and M. C. Harmsen. 2007. 'Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule', *Am J Pathol*, 171: 386-95.

Tsai, H. C., C. H. Chang, F. C. Tsai, P. C. Fan, K. C. Juan, C. Y. Lin, H. Y. Yang, K. C. Kao, J. T. Fang, C. W. Yang, S. W. Chang, and Y. C. Chen. 2015. 'Acute Respiratory Distress Syndrome With and Without Extracorporeal Membrane Oxygenation: A Score Matched Study', *Ann Thorac Surg*, 100: 458-64.

Tsaknis, G., Siempos, II, P. Kopterides, N. A. Maniatis, C. Magkou, M. Kardara, S. Panoutsou, A. Kotanidou, C. Roussos, and A. Armaganidis. 2012. 'Metformin attenuates ventilator-induced lung injury', *Crit Care*, 16: R134.

Tsuno, K., P. Prato, and T. Kolobow. 1990. 'Acute lung injury from mechanical ventilation at moderately high airway pressures', *J Appl Physiol*, 69: 956-61.

Tung, J. W., K. Heydari, R. Tirouvanziam, B. Sahaf, D. R. Parks, L. A. Herzenberg, and L. A. Herzenberg. 2007. 'Modern flow cytometry: a practical approach', *Clin Lab Med*, 27: 453-68, v.

Uhlig, S. 2002. 'Ventilation-induced lung injury and mechanotransduction: stretching it too far?', *Am J Physiol Lung Cell Mol Physiol*, 282: L892-6.

Uhlig, S., and U. Uhlig. 2004. 'Pharmacological interventions in ventilator-induced lung injury', *Trends Pharmacol Sci*, 25: 592-600.

Uhlig, U., D. Dromann, T. Goldmann, H. Dombrowsky, E. Vollmer, P. Zabel, and S. Uhlig. 2006. 'Pulmonary responses to overventilation in late multiple organ failure', *Anesthesiology*, 105: 1192-200.

Uhlig, U., H. Fehrenbach, R. A. Lachmann, T. Goldmann, B. Lachmann, E. Vollmer, and S. Uhlig. 2004. 'Phosphoinositide 3-OH kinase inhibition prevents ventilation-induced lung cell activation', *Am J Respir Crit Care Med*, 169: 201-8.

Uhlig, U., J. J. Haitsma, T. Goldmann, D. L. Poelma, B. Lachmann, and S. Uhlig. 2002. 'Ventilation-induced activation of the mitogen-activated protein kinase pathway', *European Respiratory Journal*, 20: 946-56.

Urner, M., L. K. Limbach, I. K. Herrmann, B. Muller-Edenborn, B. Roth-Z'Graggen, A. Schlicker, L. Reyes, C. Booy, M. Hasler, W. J. Stark, and B. Beck-Schimmer. 2011. 'Fluorinated groups mediate the immunomodulatory effects of volatile anesthetics in acute cell injury', *Am J Respir Cell Mol Biol*, 45: 617-24.

van Zoelen, M. A., A. Ishizaka, E. K. Wolthuls, G. Choi, T. van der Poll, and M. J. Schultz. 2008. 'Pulmonary levels of high-mobility group box 1 during mechanical ventilation and ventilator-associated pneumonia', *Shock*, 29: 441-5.

Vaneker, M., F. J. Halbertsma, J. van Egmond, M. G. Netea, H. B. Dijkman, D. G. Snijdelaar, L. A. Joosten, J. G. van der Hoeven, and G. J. Scheffer. 2007. 'Mechanical ventilation in healthy mice induces reversible pulmonary and systemic cytokine elevation with preserved alveolar integrity: an in vivo model using clinical relevant ventilation settings', *Anesthesiology*, 107: 419-26.

Vaneker, M., J. P. Santosa, L. M. Heunks, F. J. Halbertsma, D. G. Snijdelaar, V. A. N. Egmond J, Van Den Brink IA, Van De Pol FM, Van Der Hoeven JG, and G. J. Scheffer. 2009. 'Isoflurane attenuates pulmonary interleukin-1beta and systemic tumor necrosis factor-alpha following mechanical ventilation in healthy mice', *Acta Anaesthesiol Scand*, 53: 742-8.

Vaporidi, K., E. Vergadi, E. Kaniaris, M. Hatziapostolou, E. Lagoudaki, D. Georgopoulos, W. M. Zapol, K. D. Bloch, and D. Iliopoulos. 2012. 'Pulmonary microRNA profiling in a mouse model of ventilator-induced lung injury', *Am J Physiol Lung Cell Mol Physiol*, 303: L199-207.

Vaporidi, K., G. Voloudakis, G. Priniannakis, E. Kondili, A. Koutsopoulos, C. Tsatsanis, and D. Georgopoulos. 2008. 'Effects of respiratory rate on ventilator-induced lung injury at a constant PaCO2 in a mouse model of normal lung', *Crit Care Med*, 36: 1277-83.

Varpula, T., V. Pettila, E. Rintala, O. Takkunen, and V. Valtonen. 2000. 'Late steroid therapy in primary acute lung injury', *Intensive Care Med*, 26: 526-31.

Veldhuizen, R. A., B. Welk, R. Harbottle, S. Hearn, K. Nag, N. Petersen, and F. Possmayer. 2002. 'Mechanical ventilation of isolated rat lungs changes the structure and biophysical properties of surfactant', *J Appl Physiol (1985)*, 92: 1169-75.

Viksman, M. Y., M. C. Liu, R. P. Schleimer, and B. S. Bochner. 1994. 'Application of a flow cytometric method using autofluorescence and a tandem fluorescent dye to analyze human alveolar macrophage surface markers', *J Immunol Methods*, 172: 17-24.

Villar, J., J. Blanco, J. M. Anon, A. Santos-Bouza, L. Blanch, A. Ambros, F. Gandia, D. Carriedo, F. Mosteiro, S. Basaldua, R. L. Fernandez, and R. M. Kacmarek. 2011. 'The ALIEN study: incidence and outcome of acute respiratory distress syndrome in the era of lung protective ventilation', *Intensive Care Med*, 37: 1932-41.

Villar, J., R. M. Kacmarek, and G. Hedenstierna. 2004. 'From ventilator-induced lung injury to physician-induced lung injury: why the reluctance to use small tidal volumes?', *Acta Anaesthesiol Scand*, 48: 267-71.

Villar, J., R. M. Kacmarek, L. Perez-Mendez, and A. Aguirre-Jaime. 2006. 'A high positive end-expiratory pressure, low tidal volume ventilatory strategy improves outcome in persistent acute respiratory distress syndrome: a randomized, controlled trial', *Crit Care Med*, 34: 1311-8.

Vitiello, L., S. Gorini, G. Rosano, and A. la Sala. 2012. 'Immunoregulation through extracellular nucleotides', *Blood*, 120: 511-8.

Vlahakis, N.E., M.A. Schroeder, A.H. Limper, and R.D. Hubmayr. 1999. 'Stretch induces cytokine release by alveolar epithelial cells in vitro', *Am J Physiol Lung Cell Mol Physiol*, 277: L167-L73.

Volmat, V., M. Camps, S. Arkinstall, J. Pouyssegur, and P. Lenormand. 2001. 'The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases', *J Cell Sci*, 114: 3433-43.

von Bethmann, A. N., F. Brasch, R. Nusing, K. Vogt, H. D. Volk, K. M. Muller, A. Wendel, and S. Uhlig. 1998. 'Hyperventilation induces release of cytokines from perfused mouse lung', *Am J Respir Crit Care Med*, 157: 263-72.

Waite, A.A.C. 2011. 'The crucial roles of mononuclear phagocytic cells during ventilator-induced lung injury', Imperial College London.

Walker, D. C., A. R. Behzad, and F. Chu. 1995. 'Neutrophil migration through preexisting holes in the basal laminae of alveolar capillaries and epithelium during streptococcal pneumonia', *Microvasc Res*, 50: 397-416.

Wang, D., S. D. Westerheide, J. L. Hanson, and A. S. Baldwin, Jr. 2000. 'Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II', *J Biol Chem*, 275: 32592-7.

Wang, J. X., A. M. Bair, S. L. King, R. Shnayder, Y. F. Huang, C. C. Shieh, R. J. Soberman, R. C. Fuhlbrigge, and P. A. Nigrovic. 2012. 'Ly6G ligation blocks recruitment of neutrophils via a beta2-integrin-dependent mechanism', *Blood*, 120: 1489-98.

Wang, N., J. P. Butler, and D. E. Ingber. 1993. 'Mechanotransduction across the cell surface and through the cytoskeleton', *Science*, 260: 1124-7.

Wang, N., M. De Bock, G. Antoons, A. K. Gadicherla, M. Bol, E. Decrock, W. H. Evans, K. R. Sipido, F. F. Bukauskas, and L. Leybaert. 2012. 'Connexin mimetic peptides inhibit Cx43 hemichannel opening triggered by voltage and intracellular Ca2+ elevation', *Basic Res Cardiol*, 107: 304.

Wang, S., and R. D. Hubmayr. 2011. 'Type I alveolar epithelial phenotype in primary culture', *Am J Respir Cell Mol Biol*, 44: 692-9.

Wang, X. Z., and D. Ron. 1996. 'Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase', *Science*, 272: 1347-9.

Wang, Y. Y., X. Z. Li, and L. B. Wang. 2013. 'Therapeutic implications of mesenchymal stem cells in acute lung injury/acute respiratory distress syndrome', *Stem Cell Res Ther*, 4: 45.

Ware, L. B., and M. A. Matthay. 2000. 'The acute respiratory distress syndrome', *N Engl J Med*, 342: 1334-49.

Warrington, K., M. C. Hillarby, C. Li, M. Letarte, and S. Kumar. 2005. 'Functional role of CD105 in TGF-beta1 signalling in murine and human endothelial cells', *Anticancer Res*, 25: 1851-64.

Waskiewicz, A. J., A. Flynn, C. G. Proud, and J. A. Cooper. 1997. 'Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2', *EMBO J*, 16: 1909-20.

Weber, J. D., D. M. Raben, P. J. Phillips, and J. J. Baldassare. 1997. 'Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase', *Biochem J*, 326 (Pt 1): 61-8.

Wei, L., H. Matsumoto, and H. Yamaguchi. 2013. 'Propofol attenuates lipopolysaccharide-induced monocyte chemoattractant protein-1 production through p38 MAPK and SAPK/JNK in alveolar epithelial cells', *J Anesth*, 27: 366-73.

Weigelt, J. A., J. F. Norcross, K. R. Borman, and W. H. Snyder, 3rd. 1985. 'Early steroid therapy for respiratory failure', *Arch Surg*, 120: 536-40.

Weinert, C. R., C. R. Gross, and W. A. Marinelli. 2003. 'Impact of randomized trial results on acute lung injury ventilator therapy in teaching hospitals', *Am J Respir Crit Care Med*, 167: 1304-9.

Werz, O., J. Klemm, B. Samuelsson, and O. Radmark. 2000. '5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases', *Proc Natl Acad Sci U S A*, 97: 5261-6.

West, J. B. 2000. 'Invited review: pulmonary capillary stress failure', *J Appl Physiol (1985)*, 89: 2483-9;discussion 97.

West, J. B., K. Tsukimoto, O. Mathieu-Costello, and R. Prediletto. 1991. 'Stress failure in pulmonary capillaries', *J Appl Physiol (1985)*, 70: 1731-42.

Westphalen, K., G. A. Gusarova, M. N. Islam, M. Subramanian, T. S. Cohen, A. S. Prince, and J. Bhattacharya. 2014. 'Sessile alveolar macrophages communicate with alveolar epithelium to modulate immunity', *Nature*, 506: 503-6.

Whitehead, T. C., H. Zhang, B. Mullen, and A. S. Slutsky. 2004. 'Effect of mechanical ventilation on cytokine response to intratracheal lipopolysaccharide', *Anesthesiology*, 101: 52-8.

Whitmarsh, A. J., S. H. Yang, M. S. Su, A. D. Sharrocks, and R. J. Davis. 1997. 'Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors', *Mol Cell Biol*, 17: 2360-71.

Wiedemann, H. P., A. P. Wheeler, G. R. Bernard, B. T. Thompson, D. Hayden, B. deBoisblanc, A. F. Connors, Jr., R. D. Hite, and A. L. Harabin. 2006. 'Comparison of two fluid-management strategies in acute lung injury', *N Engl J Med*, 354: 2564-75.

Williams, M. C., Y. Cao, A. Hinds, A. K. Rishi, and A. Wetterwald. 1996. 'T1 alpha protein is developmentally regulated and expressed by alveolar type I cells, choroid plexus, and ciliary epithelia of adult rats', *Am J Respir Cell Mol Biol*, 14: 577-85.

Wilson, M. R., S. Choudhury, M. E. Goddard, K. P. O'Dea, A. G. Nicholson, and M. Takata. 2003. 'High tidal volume upregulates intrapulmonary cytokines in an in vivo mouse model of ventilator-induced lung injury', *J Appl Physiol*, 95: 1385-93.

Wilson, M. R., S. Choudhury, and M. Takata. 2005. 'Pulmonary inflammation induced by high-stretch ventilation is mediated by tumor necrosis factor signaling in mice', *Am J Physiol Lung Cell Mol Physiol*, 288: L599-607.

Wilson, M. R., M. E. Goddard, K. P. O'Dea, S. Choudhury, and M. Takata. 2007. 'Differential roles of p55 and p75 tumor necrosis factor receptors on stretch-induced pulmonary edema in mice', *Am J Physiol Lung Cell Mol Physiol*, 293: L60-8.

Wilson, M. R., K. P. O'Dea, D. Zhang, A. D. Shearman, N. van Rooijen, and M. Takata. 2009. 'Role of lung-marginated monocytes in an in vivo mouse model of ventilator-induced lung injury', *Am J Respir Crit Care Med*, 179: 914-22.

Wilson, M. R., B. V. Patel, and M. Takata. 2012. 'Ventilation with "clinically relevant" high tidal volumes does not promote stretch-induced injury in the lungs of healthy mice', *Crit Care Med*, 40: 2850-7.

Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann. 1999. 'The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism', *EMBO J*, 18: 4969-80.

Wirtz, H. R., and L. G. Dobbs. 1990. 'Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells', *Science*, 250: 1266-9.

Wirtz, H., and M. Schmidt. 1992. 'Ventilation and secretion of pulmonary surfactant', *Clin Investig*, 70: 3-13.

Wolf, I., H. Rubinfeld, S. Yoon, G. Marmor, T. Hanoch, and R. Seger. 2001. 'Involvement of the activation loop of ERK in the detachment from cytosolic anchoring', *J Biol Chem*, 276: 24490-7.

Wolthuis, E. K., G. Choi, M. C. Dessing, P. Bresser, R. Lutter, M. Dzoljic, T. van der Poll, M. B. Vroom, M. Hollmann, and M. J. Schultz. 2008. 'Mechanical ventilation with lower tidal volumes and positive end-expiratory pressure prevents pulmonary inflammation in patients without preexisting lung injury', *Anesthesiology*, 108: 46-54.

Wolthuis, E. K., A. P. Vlaar, G. Choi, J. J. Roelofs, N. P. Juffermans, and M. J. Schultz. 2009. 'Mechanical ventilation using non-injurious ventilation settings causes lung injury in the absence of pre-existing lung injury in healthy mice', *Crit Care*, 13: R1.

Wong, M. H., and M. D. Johnson. 2013. 'Differential response of primary alveolar type I and type II cells to LPS stimulation', *PLoS One*, 8: e55545.

Woods, S. J., A. A. Waite, K. P. O'Dea, P. Halford, M. Takata, and M. R. Wilson. 2015. 'Kinetic profiling of in vivo lung cellular inflammatory responses to mechanical ventilation', *Am J Physiol Lung Cell Mol Physiol*, 308: L912-21.

Woods, Samantha, Kieran O'Dea, Masao Takata, and Michael Wilson. 2014. 'Rapid activation of pulmonary endothelial cells during high stretch ventilation in vivo', *European Respiratory Journal*, 44.

Wrigge, H., U. Uhlig, G. Baumgarten, J. Menzenbach, J. Zinserling, M. Ernst, D. Dromann, A. Welz, S. Uhlig, and C. Putensen. 2005. 'Mechanical ventilation strategies and inflammatory responses to cardiac surgery: a prospective randomized clinical trial', *Intensive Care Med*, 31: 1379-87.

Wright, S. D., P. S. Tobias, R. J. Ulevitch, and R. A. Ramos. 1989. 'Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages', *J Exp Med*, 170: 1231-41.

Wu, H., T. Kobayashi, Q. Wan, W. Shi, H. Qian, X. Cui, and W. Li. 2010. 'Effects of surfactant replacement on alveolar overdistension and plasma cytokines in ventilator-induced lung injury', *Acta Anaesthesiol Scand*, 54: 354-61.

Xia, Y. F., J. H. Zhang, Z. F. Xu, and X. M. Deng. 2015. 'Pycnogenol, a compound isolated from the bark of pinus maritime mill, attenuates ventilator-induced lung injury through inhibiting NF-kappaB-mediated inflammatory response', *Int J Clin Exp Med*, 8: 1824-33.

Xiong, X. Q., L. N. Lin, L. R. Wang, and L. D. Jin. 2013. 'Sevoflurane attenuates pulmonary inflammation and ventilator-induced lung injury by upregulation of HO-1 mRNA expression in mice', *Int J Nanomedicine*, 6: 1075-81.

Yang, S. H., A. Galanis, and A. D. Sharrocks. 1999. 'Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors', *Mol Cell Biol*, 19: 4028-38.

Yao, S., D. Feng, Q. Wu, K. Li, and L. Wang. 2008. 'Losartan attenuates ventilator-induced lung injury', *J Surg Res*, 145: 25-32.

Yoshikawa, S., J. A. King, R. N. Lausch, A. M. Penton, F. G. Eyal, and J. C. Parker. 2004. 'Acute ventilator-induced vascular permeability and cytokine responses in isolated and in situ mouse lungs', *J Appl Physiol (1985)*, 97: 2190-9.

Young, D., S. E. Lamb, S. Shah, I. MacKenzie, W. Tunnicliffe, R. Lall, K. Rowan, and B. H. Cuthbertson. 2013. 'High-frequency oscillation for acute respiratory distress syndrome', *N Engl J Med*, 368: 806-13.

Zambelli, V., G. Bellani, M. Amigoni, A. Grassi, M. Scanziani, F. Farina, R. Latini, and A. Pesenti. 2015. 'The effects of exogenous surfactant treatment in a murine model of two-hit lung injury', *Anesth Analg*, 120: 381-8.

Zambon, M., and J. L. Vincent. 2008. 'Mortality rates for patients with acute lung injury/ARDS have decreased over time', *Chest*, 133: 1120-7.

Zaynagetdinov, R., T. P. Sherrill, P. L. Kendall, B. H. Segal, K. P. Weller, R. M. Tighe, and T. S. Blackwell. 2013. 'Identification of myeloid cell subsets in murine lungs using flow cytometry', *Am J Respir Cell Mol Biol*, 49: 180-9.

Zhang, L. N., J. P. Sun, X. Y. Xue, and J. X. Wang. 2013. 'Exogenous pulmonary surfactant for acute respiratory distress syndrome in adults: A systematic review and meta-analysis', *Exp Ther Med*, 5: 237-42.

Zhao, M., L. New, V. V. Kravchenko, Y. Kato, H. Gram, F. di Padova, E. N. Olson, R. J. Ulevitch, and J. Han. 1999. 'Regulation of the MEF2 family of transcription factors by p38', *Mol Cell Biol*, 19: 21-30.

Zhong, H., R. E. Voll, and S. Ghosh. 1998. 'Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300', *Mol Cell*, 1: 661-71.

Zhong, W. T., Y. C. Wu, X. X. Xie, X. Zhou, M. M. Wei, L. W. Soromou, X. X. Ci, and D. C. Wang. 2013. 'Phillyrin attenuates LPS-induced pulmonary inflammation via suppression of MAPK and NF-kappaB activation in acute lung injury mice', *Fitoterapia*, 90: 132-9.

Ziegler-Heitbrock, H. W., and R. J. Ulevitch. 1993. 'CD14: cell surface receptor and differentiation marker', *Immunol Today*, 14: 121-5.