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Understanding the Mechanisms of Action of Hypercapnic Acidosis and the Therapeutic Potential of Human Mesenchymal Stromal Cells in Diminishing Inflammation and Enhancing Repair in Acute Respiratory Distress Syndrome.

By Claire Masterson, BSc, MSc

A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy (Ph.D.)

Anaesthesia, School of Medicine, National University of Ireland, Galway

September 2013
Supervisor: Professor John G Laffey
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<tr>
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</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<td>Adenosine triphosphate</td>
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<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HMVEC</td>
<td>Human microvascular endothelial cell</td>
</tr>
<tr>
<td>HPAEC</td>
<td>Human pulmonary artery endothelial cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>IkappaB kinase</td>
</tr>
<tr>
<td>IL1R</td>
<td>Interleukin 1 receptor</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IR</td>
<td>Ischemia reperfusion</td>
</tr>
<tr>
<td>IT</td>
<td>Intratracheal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IVK</td>
<td><em>In vitro</em> kinase</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte derived chemokine</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial blood pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein 2 alpha</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>NC</td>
<td>Normocapnia</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-kappa-B essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end expiratory pressure</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly-ethylene glycol</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAEC</td>
<td>Small airway epithelial cell</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>SNARF</td>
<td>Seminaphtharhodafluor</td>
</tr>
<tr>
<td>TAD</td>
<td>Trans-activation domain</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>UCB</td>
<td>Umbilical cord blood</td>
</tr>
<tr>
<td>VALI</td>
<td>Ventilator associated lung injury</td>
</tr>
<tr>
<td>VILI</td>
<td>Ventilator induced lung injury</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
</tbody>
</table>
Acknowledgements

I would firstly like to express my sincere gratitude to Professor John Laffey for his encouragement, advice, and support throughout the years of my PhD. I was given enough free rein to make this project my own, while at the same time receiving all the guidance and advice I needed to make it successful.

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Dedication

This work is dedicated to my daughter Caitlin Jessica
1. Abstract

Introduction: Acute respiratory distress syndrome (ARDS) is a term used to describe severe lung injury characterised by uncontrolled inflammatory response and resultant damage to endothelial and epithelial layers leading to eventual loss of pulmonary function. ARDS can be aggravated by the only therapy currently available to prolong survival – mechanical ventilation. To date many attempts have been made to alter ventilation protocols to reduce the over-distension and cycle of atelectasis and shear stresses associated with artificial gas delivery to the lung. Thus far the most effective therapeutic strategy overall has been to reduce the tidal volumes used which can lead to the build-up of CO$_2$ – this is termed ‘permissive hypercapnia’. Experimental studies recommend avoiding buffering the resultant acidosis as there is no evidence of benefit. In fact, both the decrease in pH and the elevated CO$_2$ may confer their own beneficial effects during lung inflammation and injury, but may also have adverse effects such as slowing repair and inhibiting the host response to infection. The anti-inflammatory effects of hypercapnic acidosis (HCA), appears to be mediated, at least in part, by the suppression of NF-$\kappa$B, a key transcription factor in inflammation, injury and repair. however the exact mechanisms by which HCA suppresses activation of the NF-$\kappa$B pathway remain to be elucidated. A greater understanding of these mechanisms may provide opportunities to develop strategies to harness the benefits of hypercapnia while minimising any potential for harm.

The investigation of the therapeutic potential of mesenchymal stem/stromal cells (MSCs) is a rapidly escalating, including recent application to the area of lung disease and injury. The safety, and in some cases, efficacy of hMSCs has been established in disease states such as skeletal muscle injury, myocardial infarction, stroke, and graft versus host disease. In fact, an initial phase 1 dose escalation efficacy and safety study of MSCs has recently commenced in patients with ARDS. However, much remains to be understood in regard to the efficacy and mechanisms of action of MSCs before we can move forward to definitive clinical testing.
Therefore these studies, in a continuation of previous studies from our laboratory are aimed at determining the precise mechanism of action of HCA on the pulmonary NF-κB pathway and following this, to provide critical pre-clinical data that will enable the safe and effective use of human MSCs in ARDS.

**Methods:** *In vitro* models of lung injury were used to determine the effects of HCA on the NF-κB pathway. Pulmonary cell lines were transfected or transduced with an NF-κB luciferase reporter and subjected to TNF-α, IL-1β or endotoxin injury. Therapeutic HC was administered by increasing CO₂ levels in cell culture environments for 24 hours. NF-κB activation was assessed based on luciferase production in cell lysates and IL-8 concentration in cell culture media. Effects of HCA on intracellular proteins were analysed in cellular fractions which were prepared using nuclear extraction kits and the proteins were analysed using Western blotting, ELISA, binding assays, kinase assays and transcription, translation assays.

*In vivo* experiments were conducted to determine the optimal route, therapeutic window and the benefits of using purified sub-populations of MSCs in a rodent model of recovery following VILI. VILI was induced using high pressure ventilation until a severe lung injury, as evidenced by a 50% drop in lung compliance, was induced. Following establishment of the injury, the animals were then treated with BM-MSCs (4x10⁶ cells/kg; IV, IT or IP), either immediately post injury or 0, 6 or 24 hours post injury, depending on the specific experimental protocol. The ORB-1⁺ and ORB-1⁻ sub-populations of MSCs were compared to heterogeneous ‘parental’ MSCs. At 24 or 48 hours following VILI, animals were harvested and analysis performed to determine the extent of repair of the lung injury. Key indices of injury and recovery assessed included arterial oxygenation, lung compliance, lung inflammation, cytokine response and histologic morphology.

**Results:** HCA was demonstrated to reduced NF-κB activation in pulmonary and systemic cell lines as indicated by both luciferase assay and IL-8 ELISA. HCA also suppressed NF-κB pathway activation following over-expression of key pathway proteins. HCA was shown to affect protein activation by inhibiting
phosphorylation and kinase activity as demonstrated using ELISA and *in vitro* kinase assays. Further to this HCA was shown to decrease NF-κB dimer activity and the binding of NF-κB to its consensus sequence, but not the formation of these dimers *in vitro*. HCA was shown to inhibit transcription factor to DNA binding and subsequent transcription and translation.

*In vivo*, it was determined that an optimal dose of $4 \times 10^6$ cells/kg was effective in the attenuation of VILI when administered IT or IV up to 24 hours post injury. This was demonstrated by improvements in arterial blood oxygenation, static lung compliance, cytokine profiles and histological morphology compared to controls. In addition to this the use of a particular, more rigorously defined, sub-population of cells was almost as effective as mixed, parental populations of MSCs.

**Conclusions:** The application of therapeutic HCA to injured pulmonary and systemic cell lines suppress NF-κB activation by acting at multiple points in the pathway. Further to this, HCA reduces the ability of NF-κB complexes to translocate to the nucleus, bind to DNA and potentially decreases their ability to initiate transcription.

The administration of hMSCs IV or IT demonstrates superior efficacy to IP administration to an animal model of VILI. In addition, these cells can be administered up to 24 hours post injury without significant loss in efficacy toward repair and recovery. The use of ORB-1 hMSC sub-populations confers no additional benefit in the repair and recovery following VILI.
2. Introduction

2.1 Lung Structure and Function

The primary function of the lungs is gas exchange which is a critical process to maintain life. The respiratory system facilitates oxygenation of the blood and removal of carbon dioxide as a waste product from the circulation. The respiratory system also functions to filter substances from the circulation, to metabolise certain compounds, maintain acid-base equilibrium and as a reservoir of blood. The human lungs consist of three right and two left lobes and contain conducting airways and a respiratory zone. The conducting airways include the trachea, bronchi and bronchioles, the respiratory zone contain the alveoli.

![Image 1: Structure of the lungs](image)

The epithelium in conducting airways changes in morphology as the airways reduce in size toward the alveoli with six different epithelial cells found in the airways; ciliated columnar cells, goblet cells, Clara cells, basal cells, brush cells and neuroendocrine cells. As well as lung resident lymphocytes and mast cells, these can also migrate to the lung from surrounding connective tissue. Gas exchange occurs at the blood-gas barrier, which consists of networks of thin capillaries surrounding the alveoli. The area of this barrier is between 50 and 100 square meters in adults, and consists of roughly 500 million alveoli yielding a
total volume of about 4 litres. The surface of the blood-gas barrier is extremely thin and even a slight increase in capillary pressure can cause damage. The lungs contain two distinct circulatory pathways, namely the pulmonary arteries and pulmonary veins. The pulmonary veins deliver oxygenated blood from the lungs to the heart for delivery to the body by the left ventricle.

The lungs are highly vulnerable to infection due to exposure from inhaled agents and particles. The lung possesses its own defence mechanisms against inhaled particles, in that large particles are trapped in mucous, particles in the alveoli are engulfed by macrophages and removed by blood or lymph, glands in the bronchial tree prevent dehydration, goblet cells respond to local irritation, and antiviral and antibacterial substances have been found in fluid from bronchial glands and lymphoid nodules in the bronchial lining giving local immunological protection against infection.

Like other organs, the lungs can be affected by a wide number of diseases stemming from a range of injurious sources. However, unlike other organs the lungs are more exposed to the environment and can therefore be highly susceptible to injuries from infections and physical insults.

Diseases of the lungs can be divided into three categories. *Airway diseases* include asthma, allergies, chronic obstructive pulmonary disease (COPD), bronchitis and emphysema. *Alveolar diseases* include pneumonia, tuberculosis (TB), pulmonary oedema, cancer and acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) and finally *interstitial lung disease* which can include sarcoidosis, fibrosis, autoimmune disease, pneumonia and edema.
2.2 Acute Respiratory Distress Syndrome

Acute Respiratory Distress Syndrome (ARDS) is a syndrome of hypoxia and loss of respiratory system compliance, resulting from disruption of the alveolar-capillary barrier, and a resultant inflammatory pulmonary oedema. (1). It is a leading cause of death and disability in critically ill adults and children (2).

2.2.1 Definition and Diagnosis of ARDS

ARDS was originally known as adult respiratory distress syndrome, a phrase first coined by Ashbaugh et al in 1967 when collectively describing respiratory failure in a group of adult patients (3). The criteria for diagnosis were vague and a more precise description was laid out in 1988 by Murray and colleagues (4) which had a four point lung injury scoring system examining the level of positive end expiratory pressure (PEEP), the PaO₂:FiO₂ ratio, static compliance and chest x-ray changes.

<table>
<thead>
<tr>
<th>Extended definition of ALI/ARDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Course of the lung injury: acute or chronic</td>
</tr>
<tr>
<td>- Severity of physiological lung injury as determined by a composite lung injury score</td>
</tr>
<tr>
<td>- Lung injury caused by or associated with known risk factors for ARDS (direct or indirect lung injury)</td>
</tr>
</tbody>
</table>

Table 1: Extended three part definition of ALI/ARDS proposed by Murray et al (4). Adapted with permission from (5).
Chapter 2

<table>
<thead>
<tr>
<th>Calculation of the lung injury score (LIS)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chest radiograph</strong></td>
<td></td>
</tr>
<tr>
<td>• No alveolar consolidation</td>
<td>0</td>
</tr>
<tr>
<td>• Alveolar consolidation confined to 1 quadrant</td>
<td>1</td>
</tr>
<tr>
<td>• Alveolar consolidation confined to 2 quadrants</td>
<td>2</td>
</tr>
<tr>
<td>• Alveolar consolidation confined to 3 quadrants</td>
<td>3</td>
</tr>
<tr>
<td>• Alveolar consolidation extended to 4 quadrants</td>
<td>4</td>
</tr>
<tr>
<td><strong>Hypoxaemia score</strong></td>
<td></td>
</tr>
<tr>
<td>• $P_aO_2/FiO_2 \geq 300$</td>
<td>0</td>
</tr>
<tr>
<td>• $P_aO_2/FiO_2 225-299$</td>
<td>1</td>
</tr>
<tr>
<td>• $P_aO_2/FiO_2 175-224$</td>
<td>2</td>
</tr>
<tr>
<td>• $P_aO_2/FiO_2 100-174$</td>
<td>3</td>
</tr>
<tr>
<td>• $P_aO_2/FiO_2 &lt; 100$</td>
<td>4</td>
</tr>
<tr>
<td><strong>PEEP score (when mechanically ventilated)</strong></td>
<td></td>
</tr>
<tr>
<td>• $\leq 5 \text{ cmH}_2\text{O}$</td>
<td>0</td>
</tr>
<tr>
<td>• 6-8 cmH$_2$O</td>
<td>1</td>
</tr>
<tr>
<td>• 9-11 cmH$_2$O</td>
<td>2</td>
</tr>
<tr>
<td>• 12-14 cmH$_2$O</td>
<td>3</td>
</tr>
<tr>
<td>• $\geq 15 \text{ cmH}_2\text{O}$</td>
<td>4</td>
</tr>
<tr>
<td><strong>Lung compliance (when available)</strong></td>
<td></td>
</tr>
<tr>
<td>• $\geq 80 \text{ ml/cmH}_2\text{O}$</td>
<td>0</td>
</tr>
<tr>
<td>• 60-79 ml/cmH$_2$O</td>
<td>1</td>
</tr>
<tr>
<td>• 40-59 ml/cmH$_2$O</td>
<td>2</td>
</tr>
<tr>
<td>• 20-39 ml/cmH$_2$O</td>
<td>3</td>
</tr>
<tr>
<td>• $\leq 19 \text{ ml/cmH}_2\text{O}$</td>
<td>4</td>
</tr>
<tr>
<td><strong>The score is calculated by adding the sum of each component and dividing by the number of components used</strong></td>
<td></td>
</tr>
<tr>
<td>• No lung injury</td>
<td>0</td>
</tr>
<tr>
<td>• Mild to moderate lung injury</td>
<td>0.1-</td>
</tr>
<tr>
<td>• Severe lung injury</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>&gt; 2.5</td>
</tr>
</tbody>
</table>

Table 2: Lung injury score (LIS) describing the severity of the disease; and the identification of the cause or associated medical conditions of ALI/ARDS. Adapted with permission from (5).
These criteria were revised in 1994 by the American-European Consensus Conference (AECC) on ARDS (6) and separate definitions for ALI and ARDS were outlined. The AECC on ARDS group defined the condition as a rapid onset of diffuse bilateral pulmonary infiltrates, a hypoxemic ratio (\(\text{PaO}_2/\text{FiO}_2\)) of less than 300 for ALI or less than 200 for ARDS and a pulmonary artery wedge measurement of less than, or equal to 18mmHg.

In essence, the term ‘ALI’ was used to define the overall condition, and included all patients fulfilling the AECC criteria, while the term ‘ARDS’ referred specifically to the subset of patients within the ALI spectrum with the more severe disease, as evidenced by a P:F ratio of \(\leq 200\) mmHg. This has subsequently led to some confusion with some considering ALI to be a distinct, and less severe entity than ARDS, rather than ALI encompassing the ARDS subset, as was originally envisaged.

The main limitations of the AECC definition included: (1) the lack of exact description of the meaning of “acute”; (2) the sensitivity of \(\text{P}_a\text{O}_2/\text{FiO}_2\) ratio in the context of varying PEEP and \(\text{FiO}_2\); (3) the poor reliability of chest radiography due to inter-observer variability and difficulties in distinguishing between hydrostatic and permeability pulmonary oedema. In 2012, an expert panel initiative of the European Society of Intensive Care Medicine (ESICM), the American Thoracic Society (ATS) and the Society of Critical Care Medicine (SCCM) convened to redefine ARDS based on a robust meta-analysis of 4188 patients with ARDS (7). The consensus panel developed updated criteria that have since become known as “The Berlin Definition of ARDS” (7). The revised definition incorporates the following changes:
### The Berlin definition of ARDS

**Timing**
- Onset of ARDS is within 1 week of a known clinical insult or new or worsening respiratory symptoms.

**Chest imaging (Chest radiograph or computed tomography scan)**
- Bilateral opacities – not fully explained by effusion, lobar/lung collapse, or nodules

**Origin of oedema**
- Respiratory failure not fully explained by cardiac failure or fluid overload.
- Need objective assessment (e.g. ECHO) to exclude hydrostatic oedema if no risk factor present.

**Oxygenation**
- Mild: $200 \, \text{mmHg} < \frac{\text{PaO}_2}{\text{FiO}_2} \leq 300 \, \text{mmHg}$ with PEEP or CPAP $\geq 5 \, \text{cmH}_2\text{O}$
- Moderate: $100 \, \text{mmHg} < \frac{\text{PaO}_2}{\text{FiO}_2} \leq 200 \, \text{mmHg}$ with PEEP $\geq 5 \, \text{cmH}_2\text{O}$
- Severe: $\frac{\text{PaO}_2}{\text{FiO}_2} \leq 100 \, \text{mmHg}$ with PEEP $\geq 5 \, \text{cmH}_2\text{O}$

*Table 3*: The Berlin definition of ARDS (7).

A “rapid onset” has been defined as within one week from the known insult or worsening of the condition, chest radiograph criteria is now more explicit, pulmonary wedge measurement has been removed and PEEP of a minimum of 5cmH₂O has been factored into the measurement of the hypoxemic ratio. The term ALI has been removed and there are now three categories into which ARDS patients fall (mild, moderate and severe) based on their hypoxemic ratio.
2.2.2 Aetiology and Epidemiology of ARDS

Several clinical conditions are associated with the development of ARDS, but the majority of patients develop the syndrome in the presence of an established pulmonary or non-pulmonary infection. The other important major causes of ARDS include aspiration of gastric contents; haemorrhage and shock following major trauma; and several other less common causes such as severe acute pancreatitis, transfusion-associated lung injury, and drug reactions. (8).

2.2.2.1 Sepsis Induced ARDS

Sepsis is defined as a systemic inflammatory response in the presence of a known or suspected microbial infection. Sepsis-associated ARDS (either pulmonary or systemic in origin) is the most common cause of ARDS. (9). Severe sepsis is the risk factor most frequently associated with the development of ARDS, and is the leading cause of death in critically ill patients (2). The incidence of sepsis-induced critical illness is 150 per 100,000 person-years in the United States alone (10). Evidence suggests that approximately 40% of patients with severe sepsis develop ARDS (11), and it has been estimated that sepsis-associated ARDS has an incidence of 45–63 cases per 100,000 person-years (12). Pulmonary sepsis is characterised by a neutrophilic inflammatory response and increased vascular permeability. Endothelial dysfunction, induced by host immune mediators, leads to fluid leak to the intravascular space and hypotension and shock, clinical features of severe sepsis (13, 14). In sepsis related ARDS there is a higher severity of disease, while the duration of mechanical ventilation is longer, ICU stay is longer and mortality is higher than non-sepsis induced ARDS (15). As well as this, compared to non-sepsis induced ARDS, biochemical markers of disease severity are more severely deranged; for example; protein C levels are lower and pro-calcitonin, neopterin, vWF, ICAM-1, E-Selectin, IL-6, IL:-8, IL1-0 are higher (16-19)

2.2.2.2 Non-septic Causes of ARDS

ARDS may also be caused by non-infectious aetiologies, including aspiration of gastric contents, lung contusion, haemorrhage, drug overdose, pancreatitis and
smoke inhalation (8). The aetiology of ARDS, and individual patient characteristics, have important implications in terms of disease course and outcome. Several studies suggest that non-septic causes of ARDS, particularly trauma (20), are associated with lower mortality than ARDS that is of infectious origin (2, 10, 11). Also patients who are critically ill for some reason other than sepsis have less risk of developing ARDS than patients with sepsis (11). Furthermore, patient demographic characteristics vary among specific ARDS aetiologies (2, 21). As a specific example, trauma associated ARDS is more common in younger patients who tend to have fewer coexisting medical conditions, whereas patients with ARDS induced by pulmonary aspiration or sepsis are generally older and have more co-morbidities (20, 21). Patients with trauma related ARDS demonstrate greater hemodynamic stability (while septic patients have lower blood pressures with higher prevalence of vasopressor use) (20, 21) and also tend to be less sick, with lower illness severity scores (e.g. APACHE III), and overall lower mortality rates compared to sepsis or other non-trauma causes of ARDS (20-22).

2.2.3 Morbidity and Mortality of ARDS

The disease burden conferred by ARDS is evident, it is the leading cause of death in paediatric and adult critical care, exerting a substantial strain on the public health care system (2, 12, 23). It is estimated that 5% of hospitalised, mechanically ventilated patients have ARDS (24) and of these cases, 75% are classified as moderate to severe (2, 25) with a third of the remaining 25% eventually developing moderate or severe ARDS. The incidence of ARDS varies widely, with higher rates reported in US cohort studies (78.9 cases per 100,000 persons per year) (2), than in European studies (31.4 cases per 100,000 persons per year) (26) (reviewed in (27)). This can perhaps be attributed to under recognition of the condition (1), differences in patient demographics, and/or healthcare systems (28). It is thought that these reported figures will double in the next 25 years due to rapid population growth (2). Despite the high reported incidence, Ferguson et al., demonstrated that only 48% of patients with ARDS were diagnosed pre-mortem which demonstrates the difficulty in clinical diagnosis (1). Mortality rates in ARDS are seen to increase from 27% in mild
cases to 45% in patients with severe ARDS (25) and significant morbidity is evident in 50-70% of survivors according to long term follow-up studies (12, 29). In a further study Herridge and colleagues evaluated ARDS patients up to 5 years after discharge from the ICU. They found that there were ongoing decreases in the quality of the patient’s lives and increased financial burden on healthcare systems as a result of increased use due to physical, mental and quality of life impairments (30).

2.2.4 Pathogenesis of ARDS

2.2.4.1 Role of the Inflammatory Response:
Alveolar-capillary barrier damage and the resultant pulmonary oedema are key features of ALI/ARDS. The accompanying endothelial and epithelial cell injury occurs as a result of recruited inflammatory cells and mediators (31). Neutrophils are the first responders in injury and inflammation and play a key role in the initiation and progression of ARDS (32). Neutrophil recruitment is mediated by several steps including chemo-attraction, interactions with the endothelium (termed rolling adhesion), followed by tight adhesion and eventual movement into the airspace. In ARDS patients the concentration of neutrophils in bronchoalveolar lavage (BAL) fluid directly correlates with the severity and outcome of the condition (33). However, neutrophil recruitment alone is not enough to cause injury - neutrophil activation is required. In ARDS, neutrophil function is dysregulated leading to lung damage from the production of cytotoxic agents, cytokines, and reactive oxygen species (ROS) (31).
In ARDS, numerous chemokines, cytokines and lipid mediators promote neutrophil activation and recruitment, and decreased apoptosis of neutrophils is seen. The mechanism of this reduced apoptosis is not clear, however it is thought to be NF-κB related or attributed to the release of granulocyte macrophage colony stimulating factor (GM-CSF) from the pulmonary epithelium (35). Alveolar macrophages (AM) are a major source of chemokines responsible for further neutrophil recruitment to the lung. AM produce interleukin-8 (IL-8), a potent neutrophil chemo-attractant (36) and increased BAL concentrations of IL-8 are associated with increased lung neutrophil influx (37, 38). Keratinocyte derived chemokine (KC), also known as CXCL-1 (rat homolog CINC-1) and MIP-2, also known as CXCL-2, are also key chemokines associated with neutrophil recruitment (39) and their levels in BAL fluid correlate with injury severity.

Tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) are early response cytokines in ALI (40) and have been identified, along with their antagonists, in the BAL of ARDS patients (41). BAL levels of IL-1β are correlated with increased severity and poor outcome in ARDS, but increases of BAL TNF-α are not (42). In endotoxin induced ALI activated lung neutrophils are the main source of IL-1β and contribute to the production of TNF-α, MIP-2, IL-8 and other pro-inflammatory mediators (43-45).
Neutrophil elastase (NE) levels have been found to be increased in ALI/ARDS patients and also correlate with the severity of injury (46, 47). In ALI, NE plays a role in basement membrane degradation and endothelial and epithelial damage by proteolytic cleavage of cadherins (48, 49). Neutrophil serine proteases such as proteinase 3, cathepsin G and elastase degrade anti-inflammatory proteins in ALI and therefore prolong inflammation (50, 51) and also bind cell surface receptors activating signalling pathways. For example, NE induction of lung epithelial apoptosis is mediated via activation of the NF-κB pathway (52).

Increased levels of interleukin-8 (IL-8), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) in plasma are strongly predictive of mortality in ARDS (53), along with alterations in coagulation and fibrinolysis (54).

**2.2.4.2 Epithelial and Endothelial Injury in ARDS**

ARDS is characterised at a cellular level by a loss of alveolar-capillary membrane integrity, which results from diffuse epithelial and endothelial injury and dysfunction (34, 55). Loss of alveolar-epithelial integrity allows the influx of protein rich, highly cellular fluid containing activated neutrophils and macrophages which initiate the inflammatory process (56). As a result of inflammation and injury, alveolar cells lose the ability to produce surfactant leading to further dysregulation of the fluid transport system (56). Injury to the lung endothelium can occur by several mechanisms, although neutrophil-dependent lung injury is probably the best-documented pathway. Platelets may also play an important role in neutrophil-mediated endothelial injury (57). In addition to the effects of neutrophils on the endothelium, some inflammatory mediators act directly on the lung endothelium, resulting in the increased expression of chemokines as well as cell-surface molecules that are important for leukocyte adhesion (58). Thus, a key hypothesis in pathogenesis rests on the concept that endothelial activation is central to lung injury (59). For example microvascular endothelial injury increases the release of von Willebrand factor (vWF) (60) and intercellular adhesion molecule 1 (ICAM-1) (61) (both markers of ARDS which can predict morbidity and mortality) both of which increase leukocyte adhesion to the endothelium and the accumulation of neutrophils in the
microcirculation, which in turn leads to inflammatory lung injury and further accumulation of other leukocytes in the lung.

2.2.4.3 Clinicopathological ‘Phases’ of ARDS

ARDS can be classified according to three phases of damage and repair (reviewed in (62)). Phase I is known as the acute phase with proteinaceous fluid influx, damage to type I and type II cells, ventilation-perfusion mismatch and the release of inflammatory mediators. This is classified in the clinical setting by acute respiratory failure, hypoxemia and chest sounds indicative of pulmonary oedema. Phase II, the resolution phase, depends on fluid clearance and epithelial repair of type I and type II alveolar cells. This phase normally occurs approximately 7 days after the initial onset of ALI. Clinically there is an improvement in lung compliance and hypoxemia is resolved. Phase III, the fibrotic phase, occurs when resolution is delayed or abnormal, and is characterized by extracellular matrix (ECM) protein deposition, leading to a decrease in compliance and continued hypoxemia (63). The presence of fibrotic tissue is indicated by continued, persistent impairment of gas exchange and decreased compliance and in severe cases, pulmonary hypertension.

2.2.5 Management of ARDS

2.2.5.1 Pharmacologic Therapies for ARDS:

Despite intensive research efforts over four decades, there are no pharmacologic therapies for ARDS. Management of ARDS currently focuses on good supportive care in terms of diagnosis, treatment and prevention of further injury, whilst maintaining adequate oxygenation. Numerous pharmacological therapies have been investigated with the majority of outcomes demonstrating negative or no effect.

β2-adrenergic agonists have also recently been investigated in the treatment of ARDS due to their ability to enhance alveolar fluid clearance by up regulation of active Na⁺ transport (64) and possible reduction in lung vascular permeability (65). After promising phase 1 and 2 trials which demonstrated reduction in extra vascular lung water (66), the use of β2 agonists were taken to phase 3 large scale
multicentre clinical trials where the outcomes demonstrated worsening of outcome with intravenous administration (67) or no improvement in clinical outcomes with inhaled administration (68).

Elevated levels of neutrophil elastase (NE) have been implicated in the pathogenesis of ARDS as high levels can lead to basement membrane degradation. A phase 3 trial using NE inhibitors in ARDS in an attempt to attenuate this damage was stopped early due to adverse outcomes in 128 day mortality (69).

Despite much interest in the use of corticosteroids due to their anti-inflammatory properties, few definitive positive results have been obtained from their use in ARDS. The safety and efficacy of corticosteroids in the treatment of ARDS, at high and moderate doses, over prolonged periods, has been investigated without clear outcomes (70). Further studies are planned which may give clearer insight into the potential use of steroids in the treatment of ARDS.

A systematic review with meta-analysis and trial sequential analysis was conducted on the use of inhaled nitric oxide (iNO) in patients with ARDS (71). The outcome of this study was that despite evidence of some improvement in oxygenation, iNO did not reduce mortality and in fact may be harmful.

Neuromuscular blockers may facilitate lung protective mechanical ventilation by limiting patient ventilator dyssynchrony, and by reducing transpulmonary pressures, which, together with low tidal volumes, may decrease overall alveolar distention (72). A multicentre randomised trial was conducted to examine the effects of the administration of neuromuscular blockers in early ARDS on mortality rates with an overall improvement in 90 day survival rates compared to placebo (73). The exact mechanism by which this occurs however, warrants further study.

HMG CoA-reductase inhibitors (statins) have demonstrated promise in a pre-clinical study in humans subjected to LPS inhalation injury. Volunteers who were given Simvastatin had decreased concentrations of pre-inflammatory cytokines in BAL versus placebo controls (74). A phase 1 study also demonstrated clear promise for statin therapy in ARDS in centres across Ireland and the U.K. A phase 2 trial (HARP-2) is currently underway (75).
2.2.5.2 Supportive Therapies for ARDS

Advances in the management of ARDS have relied on improvements in supportive measures, such as ‘protective’ mechanical ventilation strategies (76), restrictive intravenous fluid management approaches (77), and prone positioning of severely hypoxemic patients (78). While these and other improvements in supportive care have decreased mortality (79), the failure of pharmacologic therapies suggests the need to consider novel approaches for ALI/ARDS.

2.2.6 Mechanical Ventilation in ARDS

As mentioned, thus far the most effective strategy to reduce mortality rates in mechanically ventilated ARDS patients is the use of protective ventilation (76). This entails the use of lower tidal volumes (6-8mL/kg) compared to more traditional tidal volumes of 10-15mL/kg thus reducing over-distension of the lung and further injury.

2.2.6.1 ‘Conventional’ Mechanical Ventilation in ARDS

Mechanical ventilation is vital to maintain adequate gas exchange in patients with ARDS and other forms of respiratory failure, and also for patients with diminished levels of consciousness, such as following acute brain injury. As such, mechanical ventilation constitutes a life-sustaining intervention, and is frequently used for beneficial effect in the critically ill. Traditionally, most patients, including those with ARDS, that require mechanical ventilation received ventilation with tidal volumes of 10-15 ml/kg, in order to ‘normalize’ gas exchange, minimize atelectasis, and achieve normocapnia (80-82). Peak inspiratory pressure of 50 cmH$_2$O was considered acceptable quite often in the absence of gross evidence of barotrauma, i.e. the development of pneumothoraces.

2.2.6.2 ARDS and the ‘Baby Lung’

The potential for the ventilator to injure the lung was known from preclinical studies since the 1970’s. In fact, as early as 1974 Webb and Tierney demonstrated in an in vivo rodent model that high inflation pressures damage the lung and PEEP, can attenuate this injury (83). From the mid 1980s onwards, clinicians
increasingly began to recognize that these approaches to ventilation might actually cause harm in patients with ARDS (80-82). In the 1980’s the use of computed tomography in ARDS clearly demonstrated that lung injury was heterogeneous and not uniformly affecting the entire lung. It became clear that in ARDS a large part of the lung does not take part in gas exchange and that the actual volume of the healthy lung is very small (84, 85). In fact, in patients with ARDS it has been shown that the amount of normally aerated tissue is equivalent to that of a small child – this has been termed the “baby lung” (86). By using ‘normal’ tidal volumes during mechanical ventilation in these smaller functional lungs, we can cause over-distension – this is termed VILI.

**2.2.6.3 Ventilator Induced Lung Injury**

Mechanical ventilation, while necessary to sustain life in patients with ARDS, can also cause or worsen lung injury. In fact, VILI is a significant contributor to mortality and morbidity in ARDS patients (76). There are several potential mechanisms of injury in VILI. Lung injury may occur in response to high peak airway pressures (83) (what is known as barotrauma) as a result of high tidal volumes (volutrauma) and the accompanying excessive lung stretch. Either or both of these injurious ventilation strategies can lead to further lung and end organ injury due to the release of pro-inflammatory cytokines and chemokines (biotrauma) (87). The repeated opening and collapse of alveoli (atelectrauma) may also contribute to physical injury and biotrauma (88). Volutrauma has been implicated as the major cause of VILI and some of the effects of high tidal volume ventilation were shown to be attenuated by the use of PEEP (89). The use of PEEP prevented the collapse of alveoli and the shear force caused by re-inflation thereby reducing injury and fluid leak.

The volutrauma hypothesis is strengthened by the finding that the risk of VILI is particularly high in ARDS patients where the amount of lung available for inflation is greatly reduced and the volumes delivered to the ‘baby lung’, cause further injury due to cyclic over-inflation (86, 90). Biotrauma is an attractive hypothesis, but has thus far been demonstrated only in rat lungs subjected to high tidal volume ventilation (87) which was accompanied by significant increases in TNF-α, IL-1β, IL-6 and macrophage inflammatory protein (MIP)-2. Biotrauma is
more severe in conjunction with another injury such as hemorrhagic shock or ischemia reperfusion (91, 92).

Many attempts have been made to avoid or overcome the damage caused by mechanical ventilation. To date the most significant clinical trial of a protective ventilation strategy was conducted in 2000 by the ARDS Network (76), which randomised 861 patients to either protective low tidal volume ventilation of 6mL/kg with a plateau pressure of less than or equal to 30cmH$_2$O, or ‘traditional’ levels of 12mL/kg with a plateau pressure of less than or equal to 50cmH$_2$O. The results of this study demonstrated a significant decrease in mortality in the low tidal volume group and formed the basis for routine alteration of ventilatory tidal volumes in patients with ALI/ARDS from 12mL/kg to 6mL/kg.

VILI may also occur in patients with normal or near-normal lungs who are receiving mechanical ventilation for other reasons, such as those undergoing surgery or following neurologic injury, or trauma. Gajic and colleagues have shown that high stretch mechanical ventilation can induce injury in healthy lungs (93).
2.3 Hypercapnia, Acidosis and ARDS

2.3.1 Role of Hypercapnia and Acidosis in ARDS
The use of lower tidal volumes as a method of protective ventilation in patients with ARDS has been documented to show a significant reduction in mortality rates (76). This method of ventilation leads to an increase in arterial CO₂ (hypercapnia, HC), and the associated drop in pH resulting from this is termed hypercapnic acidosis (HCA). Typical CO₂ partial pressures in arterial blood (PaCO₂) range from approximately 35 to 45mmHg and in permissive HCA are usually a mean maximum of 67mmHg and a mean pH of 7.2 (82). From studies spanning the last 30 years HCA has been associated with improvement in the outcome of patients with ALI/ARDS (82, 94-98). However in various in vivo, ex vivo and in vitro models of ALI there has been some evidence for harmful effects of HCA, even when they seem to be outweighed by the beneficial effects (reviewed in (99)).

2.3.2 Physiologic Effects of Hypercapnia and Acidosis

2.3.2.1 Systemic CO₂
Carbon dioxide can be transported in blood by dissolving in solution, combining with water or binding to proteins (100). CO₂ has a high solubility and diffusion capacity with arterial blood containing approximately 2.5mL CO₂ and venous blood containing approximately 3mL CO₂ per 100mL blood (101). CO₂ forms carbonic acid when combined with water which can then freely dissociate to hydrogen and bicarbonate ions. Unlike CO₂, hydrogen ions cannot move freely through membranes, but can bind haemoglobin, removing them from solution. CO₂ can bind to proteins, including haemoglobin, at their terminal, uncharged, amino acid group facilitating its systemic transport (101).

2.3.2.2 Hypercapnia and the Respiratory System
Small increases in CO₂ produce large increases in ventilation and decreases in CO₂ below normal can decrease ventilation (102). Central chemoreceptors are
responsible for the changes in CO₂ levels indirectly, through alterations in brain pH changes in the brain. These pH changes lead to increases or decreases in minute ventilation in an attempt to maintain arterial PaCO₂ within steady state ranges. However, central chemoreceptors alone do not account for the ventilator response to hypercapnia. Carotid body, or peripheral chemoreceptors are located in the carotid artery and have been shown to be necessary for the rapid response to changes in alveolar PCO₂, responsible for 37% of the sensitivity to changes in PCO₂ seen (103).

2.3.2.3 Hypercapnia and the Cardiovascular System

Increases in PaCO₂ have been shown in various in vivo pre-clinical and clinical studies to directly reduce the force and rate of contraction of the heart; cardiac output is maintained or increased as a result of blood vessel relaxation and increased sympathetic nervous system activity (reviewed in (104)). Adequate oxygenation of tissues is vital for the oxidative eradication of pathogens by neutrophils (105, 106). HCA has been shown to increase cardiac index (107) which is the major determinant of tissue oxygen availability. A study conducted by Akça et al. (108) demonstrated the effects of HCA on tissue oxygenation in healthy volunteers. HCA was induced by reducing respiratory rate and maintaining HCA levels for 45 minutes. Tissue oxygenation was measured in the upper arm using a subcutaneous tonometer. Results demonstrated a linear increase in cardiac index with increasing HCA. This study hypothesised that increases in cardiac index were seen due to the release of catecholamines and, or local vasodilation (108). In the myocardium HCA has been shown to be protective (109) and hypoxic and ischemic injury in the brain has been shown to be attenuated. HCA has also been shown to be protective and therapeutic by preserving microvascular mucosal oxygenation in canine models of splanchnic haemorrhage (110).

2.3.2.4 Hypercapnia and the Immune System

Innate immunity constitutes the first line of defence in the body against infection and injury and in the lung it includes leukocytes (neutrophils and macrophages) and alveolar and bronchial epithelial cells (111). The initial phase of host response is macrophage recognition of pathogens and subsequent, rapid release of
cytokines triggering inflammation (112). HCA has been shown to attenuate the innate immune response to mechanical ventilation in mice as evidenced by reductions in IL-1β, TNF-α, IL-6, and IL-10 compared to mice ventilated using normocapnic conditions (113). HCA has been shown to inhibit TNF and IL-6 expression in LPS stimulated human and mouse macrophages, giving further insight into its effects on innate immunity (114). It has been shown that varying CO₂ levels leads to changes in IL-8 production and intracellular superoxide generation in neutrophils, with the major contributor being intracellular pH changes (115). The effects of HCA on neutrophil adhesion in human microvascular endothelial cells (HMVECs) and animal models of LPS induced injury have been demonstrated (116). Both models showed that HCA increased neutrophil adhesion and enhanced NF-κB activity in the HMVECs and, without injury, HCA had the same effect as an inflammatory stimulus by increasing IL-8 production. The authors acknowledge that their results conflict with many other studies and attribute this to differences in cell lines and experimental protocols used. Billert et al. reported a significant increase in neutrophil numbers after permissive HCA in an uninjured rabbit model of ventilation (117), and Lang and colleagues showed that HCA amplified the inflammatory response in LPS induced lung injury in rabbits (118). The effect of HCA on bacterial clearance was examined in a mouse model of Pseudomonas aeruginosa infection with increased mortality evident due to impaired defence against infection (119).

**Adaptive Immunity:** Little is known of the effects of HCA in the adaptive immune system. In a review by Curley et al. important lessons from cancer studies were examined (97). Due to poor blood supply, the tumour microenvironment is both hypoxic and acidotic. During metabolic acidosis, some components of the adaptive immune system, such as natural killer cells (120) and T-lymphocytes (121) are functionally impaired, whereas dendritic cells have enhanced antigen presenting capacity (122).

**2.3.3 Insights from Pre-clinical Studies of HCA in ARDS**

Various *in vitro* and *in vivo* experiments have been conducted to demonstrate the beneficial effects of HC in a range of injury conditions (reviewed in (123)). HC
has been shown to diminish inflammation induced injury in heart, lung and brain, and in a variety of sterile and infectious animal models of organ injury. As deaths in ARDS patients usually occur due to multi-organ failure (124), HC may have benefit over and above its effect in ARDS.

2.3.3.1 Ischemia Reperfusion Induced Lung Injury
Ischemia reperfusion (IR) lung injury is a significant cause of mortality after lung transplant and also contributes to the pathogenesis of ARDS. It is characterised by oxidative tissue damage, oedema and hypoxemia (125). In an in vivo model of IR and oxidative damage HCA was shown to prevent microvascular permeability and reduce pulmonary artery pressure (126). The most significant mechanism by which CO₂ is protective in IR induced lung injury has been suggested to be the stabilisation of the iron-transferrin complex which prevents iron ions being involved in free radical reactions (127). The iron transferrin complex is composed of an iron ion and a bicarbonate anion, necessary for the iron binding. Therefore iron binding requires bicarbonate anions which depends on bicarbonate concentration and therefore on pCO₂. In mechanically ventilated dogs it was shown that both permissive HCA (128) and prolonged therapeutic HCA (129) increased the O₂ carrying capacity of blood due to increases in haemoglobin concentration.

2.3.3.2 Ventilator Induced Lung Injury
Various studies have been conducted to demonstrate the effects of HCA on VILI in vitro, in vivo and ex vivo. HCA was shown to improve microvascular permeability, oedema and protein concentration in BAL fluid in an isolated lung model of VILI (94). The effects of HCA in VILI were further investigated in animal models with more evidence for the protective effects of HCA demonstrated (95) with improvements in physiological variables such as gas exchange and fluid and cell infiltration to the lungs. Subsequent studies have demonstrated the protective effects of HCA in in vivo models of non-septic ARDS, showing decreases in levels of inflammatory cytokines compared to NC (113, 130) in a dose responsive, time dependent manner (130). In a recent study conducted by Contreras et al. (131), the benefits of therapeutic HC were outlined

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both *in vivo* and *in vitro*. Rodent models of moderate and severe VILI were treated with CO\(_2\) added to inspired gas (FiCO\(_2\) 0.05) and indices of inflammation and repair were shown to be improved. *In vitro* as part of the same experimental series, A549 cells were injured using cyclic mechanical stretch, exposed to 10% and 15% CO\(_2\), and NF-κB activation was demonstrated to be attenuated.

### 2.3.3.3 Septic Lung Injury

Most patients with severe sepsis require mechanical ventilation and up to half of these patients will develop ARDS. These patients have a high susceptibility to VILI if subjected to injurious ventilatory strategies (132). Protective ventilatory strategies using low tidal volumes and the resultant HCA has been demonstrated to be beneficial in various models of ALI. However the effects of HCA in pre-clinical models of sepsis induced ALI are more complex. Our group has demonstrated that HCA is protective in the early phase of *E. coli* induced pneumonia in rats with improvements in arterial oxygenation and static lung compliance compared to NC (133). Our group also examined the effects of HCA in a more established model of pneumonia (6 hours post *E. coli* inoculation) with similar results (133).

In contrast, in our study into the effects of sustained HCA on *E. coli* induced ALI, we demonstrated that prolonged exposure to HCA worsened the overall lung injury, increased the bacterial load, and impaired the ability of BAL neutrophils to engage in phagocytosis (134). More recent studies have reported that HCA suppresses innate immunity in Drosophila (135), and reduces phagocytosis in the macrophage after LPS injury (114). The effects of HCA on neutrophil adhesion in human microvascular endothelial cells (HMVECs) and animal models of LPS induced injury have been demonstrated (116). Both models showed that HCA increased neutrophil adhesion and enhanced NF-κB activity in the HMVECs and, without injury, HCA had the same effect as an inflammatory stimulus by increasing IL-8 production. The authors acknowledge that their results conflict with many other studies and attribute this to differences in cell lines and experimental protocols used. Billert *et al.* reported a significant increase in neutrophil numbers after permissive HCA in an uninjured rabbit model of
ventilation (117) and Lang and colleagues showed that HCA amplified the inflammatory response in LPS induced lung injury in rabbits (118). The seeming adverse effect of HCA in these models may be compounded by the inhibition of innate immunity (114), the ability of metabolic acidosis to enhance bacterial growth (136) and the reduction in repair seen under conditions of HCA (137).

2.3.4 Concerns Regarding Effects of HCA in ARDS
Experiments have shown that HCA confers a protective effect in a range of disease states. However, where does the CO₂ have its effect and is it globally beneficial, or does the good merely outweigh the bad? VILI is a condition of concern in ARDS patients whereby the over distension of the lung leads to alveolar wounding. These wounds have been demonstrated in in vitro models of VILI (138). Harmful effects of HCA seem to be mediated by suppression of the host’s immune defence. HCA was shown to worsen pneumonia and increase the bacterial load in an experiment which tested both prophylactic and therapeutic HCA (134) by exposing animals to 5% CO₂ before or after E.coli instillation. HCA was shown to increase bacterial load, attributed to the reduction in neutrophil phagocytosis. The concerning effects of HCA can also be attributed to the impaired clearance of alveolar fluid (139, 140), the impaired innate immunity seen in Drosophila (135) and the decrease in host defence cytokine expression in macrophages (114) as reviewed by Vadasz et al. (141).

2.3.4.1 Effects on Healing and Repair Process
An important study conducted by Doerr et al. (137) demonstrated the effect of HCA on plasma wound resealing both in vitro and ex vivo. Using propidium iodide to fluorescently label injured cells ex vivo, and wound assays in vitro they were able to determine that HCA decreased the cells ability to repair after plasma membrane tear. In a similar study, the effects of buffering HCA on the repair of plasma membranes were investigated (142). Normalising the pH attenuated the overall injury in this study despite the presence of oedema. The effects of HCA on gross pulmonary wounds were examined in vitro in a study by O’Toole et al.
(143). Using pulmonary epithelial cells it was demonstrated that HCA impaired wound healing by a NF-κB dependant mechanism.

2.3.4.2 Effects on Alveolar Fluid Clearance
A recent review by Vadasz et al. (141) sheds some light on the mechanism of action of CO₂ at a cellular level, specifically in relation to alveolar fluid clearance. Independently of pH, CO₂ activates the ERK pathway (139) which leads to AMP-activated kinase (AMPK) induced activation of PKC-ζ which in turn phosphorylates the α₁ subunit of the Na⁺/K⁺-ATPase transporter leading to its endocytosis (140). This therefore decreased the activity of the fluid transport system in the lung leading to impaired fluid clearance.

2.3.5 Mechanisms of Action of Hypercapnia
Although HCA has been shown to be beneficial in a range of in vitro and in vivo experiments, its mechanism of action is not entirely clear. Various in vitro experiments have been conducted to determine the precise mechanism by which HC confers its protective effects.

2.3.5.1 Acidosis versus Hypercapnia
Many groups, including ours, have attempted to ascertain whether it is the CO₂ molecule or the resultant drop in pH that is responsible for the effects seen in HCA. These experiments are usually conducted in vitro or ex vivo by either buffering the drop in pH resulting from exposure to HCA to show the effects of CO₂, or by the addition of acid to show the effects of acidosis without CO₂. In vivo, physiological buffering systems such as renal buffering take place which can be difficult to mimic in an experimental model.

To date, experiments conducted to determine the exact mechanism of action of HCA (CO₂ vs. pH) have had mixed outcomes. Buffering HCA in an ex vivo model of IR was shown to abolish the protective effects of HCA (144), and gross wound resealing was impaired in an in vitro scratch wound model buffered with sodium bicarbonate (143). Higgins et al. (338) conducted a study to examine the effects of buffered and unbuffered HC in a rat model of cecal ligation and puncture (CLP).
It was shown that HCA attenuated sepsis induced lung injury whereas buffered HC did not.

Plasma membrane injury was shown to be attenuated by buffering *ex vivo* and *in vitro* models of VILI (142), and metabolic acidosis was shown to be as effective as hypercapnic acidosis in an *ex vivo* model of VILI (145). Briva *et al.* (343) demonstrated that the impaired clearance of alveolar fluid in rat lungs in short-term, severe HCA via Na⁺/K⁺-ATPase was not due to the acidosis associated with increased CO₂ but rather the CO₂ itself.

These conflicting results can likely be attributed to the different experimental conditions imposed, i.e. buffering vs. induction of metabolic acidosis. Further detailed study is required to reveal insights into a consistent mechanism of action.

### 2.3.5.2 Hypercapnia and the NF-κB Pathway

In 2003 Takeshita and colleagues attempted to address the molecular mechanisms of HCA during endotoxin-induced inflammation in human pulmonary artery endothelial cells (HPAEC) (146). Results implicated the involvement of NF-κB in the process of injury attenuation by demonstrating that HCA decreased levels of NF-κB bound to its consensus sequence DNA, and decreased both ICAM-1 and IL-8 expression after LPS stimulation. They attributed this to HCA preventing IκBα protein degradation and subsequent NF-κB protein release. Despite the considerable quantity of data, the exact mechanism, or mechanisms, by which HCA modulates the NF-κB pathway is still unknown.
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2.4 The Nuclear Factor-Kappa B Signalling Pathway

The Nuclear Factor-Kappa B (NF-κB) family of transcription factors play critical roles in initiating and coordinating immune responses, proliferation, differentiation and repair of cells. The NF-κB proteins found in mammals are RelA/p65, RelB, c-Rel, p50 and p52 which, as homo or heterodimers, bind DNA κB sites and confer positive or negative effects on gene transcription.

The NF-κB pathway is central to the inflammatory response of cells to stress or other injurious stimuli. Since its discovery in 1986 (147), NF-κB has become a pivotal molecule in studies of injury and repair. The roles, regulations, activations and interactions of the various NF-κB proteins have been extensively reviewed throughout the years and special attention given in 2011 to mark 25 years since its discovery (148).

Image 3: Schematic diagram of the NF-κB pathway
2.4.1 Key Components of the NF-κB Signalling Pathway

The basic activation cascade of the NF-κB pathway is generally accepted as follows; in its resting state, NF-κB proteins consist of dimers which are retained in the cytoplasm by IκBα. NF-κB activation occurs with the phosphorylation of IκB proteins (on the destruction box serine residues), poly-ubiquitination and degradation of IκBα then occurs leading to NF-κB release, translocation, DNA binding and initiation of transcription. The core elements of the NF-κB pathway are the IκB kinase (IKK) proteins, the IκB proteins, and the NF-κB dimers. The NF-κB pathway is divided into the “canonical” or “classical” pathway and the “non-canonical” or “alternative pathway”. These pathways were distinguished based on the activators, receptors and subsequent proteins involved however, due to emerging evidence for pathway cross-over and linking, the pathways are now distinguished based on their dependence on the NF-κB essential modulator (NEMO) protein (149, 150). The traditional canonical pathway is characterised by ligand binding to the tumour necrosis factor receptor (TNFR), interleukin-1 receptor (IL1R), pattern recognition receptors (PRRs), antigen receptors etc. which trigger a signalling cascade leading to IKK-β phosphorylation and subsequent IκB protein phosphorylation. The non-canonical pathway is activated by a number of members of the TNF family (151) and is dependent on IKK-α whilst independent of NEMO. The NF-κB inducing kinase (NIK) protein remains constitutently active and is regulated by TRAF-3. NIK directly phosphorylates IKK-α which leads to p100 phosphorylation and the formation of p52/RelB complexes (Image 3). Canonical pathway activation can augment non-canonical signalling by p100 activation through IKK-α, an example of the cross talk which occurs between the pathways (150).

The Rel homology domain (RHD) present in the NF-κB proteins is responsible for their ability to form dimers and also make contact with DNA binding sites. Following dimerisation, the NF-κB proteins interact with the IκB proteins at the ankyrin repeat domain at the dimeric interface (152). IκB proteins associated with NF-κB are targeted by a canonical IKK mechanism for degradation via the 20S proteasome pathway (153). Activation of the canonical pathway via TNFR, IL-1βR or TLR ligand binding induces phosphorylation of the NEMO/IKK complex.
which in turn phosphorylates the IκBα protein leading to its proteasomal degradation (154).

The p65, c-Rel and RelB proteins contain c-terminal transactivation domains (TADs) which confer the ability to initiate transcription. Conversely, these domains are absent in p50 and p52. However, these proteins can still regulate transcription by dimerising with other proteins which do contain transactivation abilities. These dimers can negatively regulate transcription by competing with the TAD dimers. Therefore it can be deduced that due to the large number of possible protein-protein interactions and DNA binding and transcription there is a huge opportunity to target the NF-κB subunits to either positively or negatively regulate gene transcription (152, 155). The physiological role of p50-p50 homodimers is unclear; however some NF-κB regulated genes are maintained in an inactive state by p50 homodimer complexes which occupy DNA binding sites (156).

2.4.2 Role of the NF-κB Pathway in the Pathogenesis of ARDS
The NF-κB pathway has been implicated development, immunity, cell proliferation and repair (150). The importance of this pathway in the pathogenesis of ARDS has not gone unnoticed.

2.4.2.1 Role of NF-κB in Pulmonary Inflammation
Inflammation is a major characteristic in the pathogenesis of ARDS, as described earlier in this chapter (section 2.2.4) and the cytokine profile of BAL fluid from ARDS patients can predict the severity and outcome of patients (42). NF-κB mediates the production of cytokines commonly found in the BAL fluid of ARDS patients such as TNF-α, IL-1β, IL-6 and IL-8 (53). Schwartz and colleagues demonstrated the activation of NF-κB in alveolar macrophages in the BAL fluid of ARDS patients, implicating the NF-κB pathway as a key mediator of inflammation and repair in ARDS. Alveolar macrophages are rapid responders to lung injury and produce IL-8, a potent neutrophil chemo-attractant (38) and a product of gene transcription by NF-κB (157). NF-κB activation in the lungs is increased due to injury (158, 159) and the link between neutrophil activation and NF-κB accumulation in the nucleus was shown.
in an *in vivo* study where neutropenia in murine lungs decreased NF-κB activation (158). NF-κB in pulmonary neutrophil nuclei was found to be increased in injury compared to that of peripheral blood neutrophils and correlated with cytokine release from lung neutrophils after endotoxemia or haemorrhage (44).

### 2.4.2.2 Role of NF-κB in Injury Resolution and Repair

NF-κB plays a role in the repair process of pulmonary epithelial wounds by the control of cell migration. This has been demonstrated by our group in *in vitro* experiments using pharmacological NF-κB inhibitors and gene therapy (143) which showed the inhibition of the NF-κB pathway slowed cell migration. NF-κB also plays a role in the immune response to bacterial infection by increasing neutrophil recruitment, as shown in an experiment using p65 over expression (160).

### 2.4.2.3 Targeting NF-κB as a Therapeutic Strategy

The NF-κB signalling cascade is composed of a wide range of proteins (Image 1). Specific proteins in the pathway have been highlighted as playing specific roles in certain conditions. Lipopolysaccharide (LPS) induced ALI leads to the activation of the serine threonine kinase AKT, also known as protein kinase B (PKB), which leads to NF-κB accumulation in the nucleus and enhanced expression of NF-κB dependent cytokines (161). It was demonstrated that blocking the activation of PI3-K, in particular PI3-Kγ, prevented the activation of AKT and decreased LPS induced NF-κB accumulation and cytokine production. PI3-Kγ has been shown to play a role in the apoptosis of neutrophils, as PI3-Kγ knockout mice had enhanced neutrophil apoptosis and clearance in endotoxin induced ALI (162).

Another protein of the NF-κB pathway centrally involved in pulmonary neutrophil mechanisms is p38 MAPK. p38 has been shown to be involved in neutrophil adhesion, chemotaxis, chemokine synthesis, apoptosis and NF-κB translocation in response to LPS (163, 164). *In vivo* studies have shown blocking activation of p38 decreases accumulation of neutrophils in alveoli after LPS inhalation (165) pointing towards a possible therapeutic for early stage ALI. Lee *et al.* demonstrated that the inhibition of p38 phosphorylation partially improved
alveolar fluid clearance by alteration of the gene and protein expression of the main ion transporters in alveolar cells (166).

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are implicated in the progression of ARDS (167). MMPs, specifically MMPs 2 and 9, can break down the ECM and cell junctions contributing to the fluid influx seen in ARDS. MMP-2 and 9 are also implicated in the repair of wounds via cell detachment and migration (168). The regulation of MMPs and TIMPs seems to be governed by the NF-κB pathway (143, 169). NF-κB is linked to the expression of MMP-2 and -9 promoting cell migration (170, 171). However NF-κB could also inhibit this process by TIMP-1 up regulation as seen in other cell types (172), and in the decrease in wound healing following HCA (137, 143).

The IκBα protein is a central, key protein in NF-κB pathway activation. The prevention of IκBα degradation stops the release and translocation of the NF-κB protein, blocking NF-κB DNA binding and further production of inflammatory cytokines (150). In a sepsis model of ARDS, our group has shown that the over-expression of IκBα in the lungs via administration of a viral vector, dose dependently decreased indices of lung injury in acute short term pneumonia, however worsened the outcome of prolonged pneumonia. This would suggest that NF-κB activation is an important process in the recovery of sepsis induced ARDS.

2.4.3 Hypercapnia and the NF-κB Pathway – Key Unknowns

A review on the regulation of gene expression by CO₂ brings together many years of evidence pointing toward the likelihood that the majority of CO₂ sensing occurs via the NF-κB pathway (173). One of the first attempts made to determine the mechanism of action of the protective effects seen with the use of HCA in ARDS was that of Takeshita and colleagues (146). The key finding in this study was that application of HCA to an in vitro system of endotoxin induced injury prevented the degradation of IκBα and subsequent translocation and DNA binding of the NF-κB transcription factor.

One of the more important questions posed by the use of HCA is whether the anti-inflammatory effects are attributable to the presence of intracellular acidosis due
to CO₂ elevation, or the CO₂ molecule itself. Numerous studies have attempted to address this with various models. The study performed by Takeshita et al. also attempted to determine if it was the resulting acidosis or the CO₂ which caused the decreased NF-κB binding to consensus sequence DNA. Positive results were seen for both, however neither was as effective as HCA (146). In a study by Cummins et al. (174) cells were exposed to 0% or 10% CO₂, with or without buffering. The group also conducted graded pH experiments to compare effects of HCA and metabolic acidosis on NF-κB activity. Results demonstrated that nuclear translocation of IKK-α was independent of O₂ but dependent on CO₂ concentration and rapidly reversible. HC was also shown to inhibit p65 translocation in mouse embryonic fibroblast (MEF) cells in response to LPS injury. In a further study by the same group (175), RelB was shown to be cleaved and to translocate to the nucleus under HC conditions, independent of pH changes induced by buffering cell culture media.

Despite the considerable quantity of data, the exact mechanism by which HCA modulates inflammation via the NF-κB pathway is still unknown. A more complete understanding of the exact mechanisms involved will give us a better understanding of the benefits and risks of HCA in ARDS, and may bring us closer to identifying a therapeutic agent incorporating therapeutic hypercapnia and/or NF-κB.
2.5 Mesenchymal Stromal Cells

2.5.1 Identification and Classification
Mesenchymal stromal cells (MSCs) were first identified by Friedenstein as clonogenic fibroblast precursor cells in the bone marrow (BM) of adult mice (176, 177). MSCs have also been isolated from other tissue such as fat, umbilical cord blood (UCB), peripheral blood, and organs such as the lung and heart (178). Those isolated from BM remain the most frequently investigated. MSCs are isolated by adherence of cells to tissue culture plastics from BM aspirates and surface marker expression. BM-MSCs are positive for CD105, CD73, CD44, CD90 and negative for CD45, CD34, CD14 (haematopoietic markers), CD11b, CD79α/CD19 and HLA-DR and will differentiate down the chondrogenic, osteogenic and adipogenic lineages (179). Following isolation of BM-MSCs, correct culture conditions must be implemented. MSCs reside in hypoxic environments in vivo and exposure to normoxic conditions (21% O₂) leads to ROS generation, premature senescence, loss of stemness (180) and a reduction in population doublings (181). MSCs are known to be immunosuppressive in a range of conditions (182, 183) and are also partly immunoprivileged as they do not express HLA class II cell surface proteins (184), and also perhaps partly due to their ability to be immunosuppressive (185). Thus, MSCs are an attractive option as a cell therapy for the treatment of disease and injury.

2.5.2 Sources of MSCs

2.5.2.1 Bone Marrow Derived MSCs
Bone marrow derived stromal cells (BM-MSCs) are the most extensively studied type of stem cell. The popularity in using BM-MSCs is due to successes seen when bone marrow transplantations were carried out in leukemia patients following full body irradiation (186, 187). The bone marrow is obtained from aspiration of iliac crests and is composed of a heterogeneous mix of cell types of which the actual ‘stem cell’ population constitutes roughly 0.001% - 0.01% of the nucleated cells (188). MSCs were initially identified as plastic adherent, colony forming cells with the ability to differentiate along the osteogenic lineage (176,
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177, 189) and subsequent studies demonstrated their multi-lineage differentiation potential (188) igniting great interest for their use in regenerative medicine.

2.5.2.2 Adipose Derived MSCs

Adipose-derived stromal cells (ASCs) derived from subcutaneous adult adipose tissue show promise for use in a range of disease and injury states. They are easily accessible, ethically acceptable and can be prepared in large quantities (190). ASCs have been used in cigarette smoke induced ALI (190) as well as elastase induced emphysema (191) and more recently in a rat model of LPS-induced ALI (192) with promising results. ASCs are thought to function in a paracrine fashion largely due to their secretion of multiple pro-angiogenic and anti-apoptotic growth factors (193) and have also been shown to be superior to BM-MSCs in terms of senescence (194).

2.5.2.3 Umbilical Cord Blood Derived MSCs

Umbilical cord blood (UCB) contains three types of stem cell; hematopoietic stem cells (HSCs) which can be differentiated to specific hematopoietic lineages in vitro (195), MSCs which are of high similarity to BM-MSCs but demonstrate higher proliferation abilities (196) and differentiate to osteogenic, chondrogenic, hepatic and neural lineages (197-200), and finally multipotent non-hematopoietic stem cells which express embryonic stem cell markers and differentiate into tissues from the three germ layers (reviewed in (195)). UCB is one of the most abundant stem cell sources with various advantages over other stem cell types and their sources. There are no ethical issues regarding collection of these cells (201) which is relatively simple to do without any impact on the mother or newborn. Compared to BM-MSCs these stem cells are less immunogenic, have a lower infection risk and have a higher proliferation rate (reviewed in (195)). However, to date, they are less studied than BM-MSCs. UCB-MSCs are currently being investigated in clinical trials for brain injury, marrow transplant failure, hematologic malignancies, cerebral palsy, spinal cord injury and diabetes (202). Safety and efficacy of UCB-MSCs have been demonstrated in a rat model of
bronchopulmonary dysplasia (203) and has also been shown to decrease systemic inflammation and attenuate LPS-induced ALI in a rat model (204).

2.5.3 Clinical Trials Using MSCs
Due to the success of pre-clinical experiments, numerous clinical trials in the use of MSCs are ongoing. These include investigation in the areas of diabetes, Parkinson’s and heart failure (202). Numerous small clinical studies have been conducted using MSCs in auto-immune diseases such as multiple sclerosis, Crohn’s disease, scleroderma and lupus (205). The majority of these studies demonstrated positive outcomes; however, differences in experimental techniques and small patient numbers mean it has been difficult to establish definitive efficacy. Due to the success of phase 1 safety trials, phase 2 efficacy studies are underway or completed for myocardial infarction, diabetes and COPD, and larger scale phase 3 studies have been authorised by the FDA in Crohn’s disease and graft vs. host disease (202).

BM-MSCs are the most extensively studied MSC to date and have been successfully used in a range of in vivo models of lung injury and disease (reviewed in (206, 207)) including bleomycin induced fibrosis, hypoxia, hyperoxia, ischemic-reperfusion, VILI, and endotoxin induced lung injury. Currently the only completed BM-MSC clinical trial for lung disease has been that of Weiss et al. (208) in which patients with COPD were treated intravenously (IV) using Prochymal MSCs provided by Osiris Therapeutics Inc. (Columbia, MD, USA). The trial was conducted over a 2 year period and comprised of four monthly infusions of $1 \times 10^8$ cells per dose. Safety of MSC administration in COPD was confirmed, although the study failed to demonstrate any efficacy ((202) NCT00683722). Currently active clinical trials to assess safety and efficacy of MSCs in pulmonary repair include the use of ASCs in COPD (NCT01559051), BM-MSCs in severe emphysema (NCT01849159), BM-MSCs in ARDS (NCT01775774) and ASCs in ARDS (NCT01902082) (202).
2.5.4 Insights from Pre-clinical Studies of MSC Therapy in ARDS

2.5.4.1 Effects of MSCs in Sepsis Induced ARDS Models

Sepsis induced ARDS is characterised by severe microbial infection and associated inflammation. The use of MSCs has been shown to be beneficial in pre-clinical sepsis models (209) by enhancing bacterial clearance and reducing inflammation (210, 211). MSCs have been shown to produce prostaglandin E2 which acts on macrophages inducing their production of IL-10, responsible for their anti-inflammatory effects (212). MSCs are known to be anti-bacterial in pneumonia and sepsis models of ALI (212, 213) and this has been shown to be modulated in part by the secretion of LL-37 (214). LL-37 is a human cathelicidin peptide which is antibacterial, antifungal and antiviral (reviewed in (215)). MSCs have also been shown to be anti-bacterial by their production of lipocalin-2 in response to LPS stimulation (213). Lipocalin-2 is a protein which is known to mediate the innate immune response to bacterial infections (216).

Image 4: Biological effects and potential mechanisms of MSCs in sepsis-induced ARDS. Reproduced with permissions (209).
2.5.4.2 Effects of MSCs in Recovery following VILI

Even with the evolution of protective mechanical ventilation strategies it is difficult to avoid the occurrence of VILI in ARDS patients (217, 218). The use of stromal cell therapy has become a rapidly expanding field of research with much success both pre-clinically and in clinical trials (reviewed in (219)). An important pre-clinical trial conducted in the use of MSCs in a VILI repair model (220) demonstrated that recovery of lung structure and function was enhanced by MSCs and this was due in part to a paracrine mechanism of action mediated by the secretion of keratinocyte growth factor (KGF) from the MSCs. In a further study by the same group the route of administration was investigated by administration of MSCs IV or IT, and the paracrine mechanism further examined (221), with results indicating comparable efficacy despite route of administration chosen. The pre-treatment of VILI using IV or IT MSCs has been investigated with promising results (222) in rodent models, with typical VILI injury indices significantly reduced. Recently, autologous adipose derived MSCs have been investigated in rodent models of VILI repair (223) with significant improvements in BAL protein levels, oedema and histological injury. Overall these studies show promising results for the use of MSCs in the treatment of VILI.

2.5.4.3 Effects of MSCs in the Human Lung

An ex vivo human lung model of sepsis induced ARDS has been developed and used to demonstrate the therapeutic effects of human MSCs on both endotoxin induced ARDS(224), and live bacterial infection (225). MSCs were shown to be anti-microbial, decreasing inflammation, increasing bacterial clearance and reducing pulmonary oedema. In this study the importance of KGF was demonstrated with a role in the anti-microbial activity of the MSCs. Studies such as these are important steps toward the use of MSCs in human clinical trials of sepsis induced ARDS, and as such have formed the basis for an actively recruiting phase 1 clinical trial (NCT01775774 (202)).
2.5.5 Effects of MSC Therapy in ARDS

2.5.5.1 MSC Homing and Migration
It is has been established that MSCs have the ability to home to sites of injury (226), however how this occurs is less clear. It has been demonstrated that MSCs navigate to sites of injury in response to chemo-attractive substances from injured cells (227) or that MSCs themselves express chemokine receptors to enable response to the release of inflammatory cytokines (228). Despite the ability of MSCs to migrate, the systemic infusion of MSCs in any injury state is subject to a phenomenon known as the “pulmonary first pass effect” (229) whereby cells introduced systemically become trapped in the pulmonary capillary bed. Where this is an obstacle in the systemic application of MSC therapy to other injured organs such as the brain (230) and heart (231), it makes systemic infusion an attractive option in the treatment of ARDS.

2.5.5.2 Effects of MSCs on Immune system and Inflammation
It is well documented that MSCs have the ability to modulate the immune response through the action of soluble factors on immune cells responding to injury (232-234). MSCs have been shown to regulate both the innate and adaptive immune systems (235) as suggested by their effects on TLR signalling in sepsis (236). Acute inflammation is a key characteristic in the pathogenesis of ARDS and MSCs play a crucial role in the reduction of expression of many pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (219, 235), and the production of anti-inflammatory cytokines such as IL-10 and IL-13 (237). In ARDS, neutrophils play a key role in the initiation and progression of the condition (32) and MSCs have been shown to improve the outcome in a number of ARDS studies by reduction of neutrophil migration and subsequent inflammation (224, 238, 239). Lung resident macrophages also play an important role in the pathogenesis of ARDS contributing to the inflammatory responses and also the resolution of these responses (240). MSC transplantation in spinal cord injury has been shown to promote an alternative, anti-inflammatory macrophage phenotype (M2) correlating with increased production of anti-inflammatory IL-10 and reductions in pro-inflammatory TNF-α and IL-6 levels (241). It has been suggested that the reprogramming of M1 macrophages to M2 phenotype is
involved in the resolution of lung injury (240), and therefore the interaction of alveolar macrophages with MSCs is an important issue with further investigation required.

2.5.5.3 Effects of MSCs on Alveolar Epithelial Fluid Clearance

As well as inflammation, ARDS patients have characteristic alveolar fluid accumulation due to impaired vascular endothelial integrity (242). Therefore, a defining factor in the resolution of injury is the clearance of this fluid and MSCs have been shown to do this in models of ALI (221, 224). The main mechanism by which fluid re-absorption occurs is via alteration in the activity of sodium and Na⁺/K⁺-ATPase channels. This is thought to be mediated by MSC paracrine factors such as KGF (243, 244). Other mechanisms of MSC action on alveolar fluid in the lungs include the action of other paracrine substances such as angiopoietin-1 in the maintenance of endothelial integrity (245).

2.5.6 Mechanisms of Action of MSC Therapy in ARDS

The complete mechanism of action of MSCs in the repair and attenuation of ARDS has yet to be ascertained. A number of experiments point toward specific mechanisms and there have been some conflicting theories put forward. For many years it was thought that the MSC mechanism of action was to home to the site of injury, engraft and differentiate to replace the damaged tissues (246). This theory has since been abandoned due to mounting evidence of a paracrine mechanism; however there are still studies which maintain that MSC contact without engraftment or differentiation is the basis for the beneficial outcomes seen (247).

2.5.6.1 Cell Contact Dependent Effects

The initial attraction of MSCs was their ability to differentiate down various lineages and potentially replace injured cells in different organs. Mounting evidence points toward a paracrine mechanism of action in MSCs as has been demonstrated in ARDS models (224, 248). Islam and colleagues (247) have shown BM-MSCs given to mouse models of LPS induced ALI confer their protective effects by attaching and transferring mitochondria to the alveolar
epithelium. An earlier study demonstrated that BM-MSCs could engraft in the lung and trans-differentiate into cells with the morphological and molecular phenotype of type I pneumocytes (246); however, further studies have indicated that engraftment rates are below 5% (249, 250).

2.5.6.2 Key Paracrine Mediators

Rodent BM-MSCs have been successfully used in a rodent model of VILI (221) and the use of conditioned medium pointed toward a paracrine mechanism of action for recovery and repair. The production of paracrine factors by MSCs is believed to involve the PI3K/AKT pathway (251). Over-expression of the AKT gene was shown to lead to the up-regulation of genes for VEGF, FGF-2, IGF-1, HGF and thrombospondin-4 (252). Numerous studies have indicated that keratinocyte growth factor (KGF) is an important factor in the prevention and treatment of lung injury (244, 253) and further studies have indicated its importance as an MSC paracrine factor in ARDS (224, 248). KGF is currently being investigated in a clinical trial for ALI (254). Other paracrine factors implicated in the repair process of MSCs include hepatocyte growth factor (HGF) and epithelial growth factor (EGF).

2.5.6.3 Effects of Microvesicles

Following the observations that MSCs confer their beneficial effects through the release of paracrine factors (255), further study of these ‘factors’ has lead to the investigation of microvesicles (256). These vesicles were previously shown to be protective in kidney injury (257) with a suggested mechanism of action involving mRNA and miRNA transfer (258). A recent study by Zhu and colleagues (259) demonstrated the effects of isolated microvesicles in a sepsis induced ALI ex vivo model. Reduction in lung water, protein levels in BAL, neutrophil influx, and inflammatory cytokine levels were evident after IT administration of microvesicles. KGF mRNA in the vesicles appeared to be important for these beneficial effects.
2.5.7 Key Translational Barriers to the Testing of MSC Therapy in ARDS

Trounson et al. (260) outlined the possibility that variances in procedures may impact the outcome for patients undergoing stem cell treatment and so the timing, delivery, dose and cell type need to be optimised for each procedure. In the in vivo studies reviewed by Lee et al. (255) and Sinclair et al. (207) the majority of cells were administered IV and the remainder were administered IT. In addition, the dose and method of injury varied between studies. These parameters are important factors to take into consideration when comparing the safety and efficacy of MSC therapy.

2.5.7.1 Understanding MSC Dose-Response

In the in vitro, in vivo and ex vivo studies outlined, of MSCs administration for lung and other organ injury, consistent dose, route and timing of administration is lacking. The optimal dosing of MSCs is not fully known, nor is it known how many doses must be administered. Indeed, this will likely vary between conditions; however for safety and efficacy reasons a general dose response curve must be determined. A study conducted by our group aimed to establish a dose-response curve for the IV administration of human BM-MSCs to a rodent model of VILI (Data not shown). The lowest significantly effective dose was shown to be 2x10^6 cells/kg body weight with efficacy remaining comparable with doses up to 1x10^7 cells/kg. The maximum tolerated dose has not been established; however studies have been conducted with doses of up to 1x10^7 cells/kg bodyweight safely tolerated in a clinical trial of Hurler syndrome (261) and up to 2.5x10^7 cells/kg have been used in mouse models of pneumonia without adverse effects (262).

2.5.7.2 Optimising MSC Route of Administration

As with the application of all therapies, route of administration is an important point to be considered. Numerous studies have been conducted to determine the distribution pattern of systemically infused MSCs in both injured and non-injured models using a range of labelling and detection techniques (Reviewed in (263)). The main findings were that administered MSCs initially accumulated in the lungs.
(264, 265) followed by “homing” of the cells to sites of injury (264) or in the case of uninjured models, deposition to the spleen, liver, kidney and bone marrow (265). The fate of MSCs appears to depend on the route of administration chosen and it has been shown in many studies that the majority of systemically infused MSCs are found in the lungs (229, 264). This can be attributed to MSC size (20-60µm) (266) in comparison to the diameter of pulmonary capillaries (5-10µm).

### 2.5.7.3 Understanding MSC Distribution Following Administration

The majority of MSC distribution studies used polymerase chain reaction (PCR), fluorescence, luciferase or bioluminescent DNA labelling to track MSCs after systemic administration and subsequently found that after initial accumulation in the lungs, the cells were found in the liver, spleen and other organs (267). However a study by Liu et al. (268) has shown that the majority of MSCs become apoptotic after administration. In a study by Eggenhofer et al. (267) cell viability was taken into account and the results indicated that viable MSCs were identified in the lung up to 24 hours post administration, beyond which they were not found anywhere else in both un-injured and injured animal models. The authors suggest that previous studies may have detected phagocytosed MSC label and cell debris in systemic organs. Further studies by this group attributed this MSC disappearance to immune cells, most likely macrophages (267).

### 2.5.7.4 Optimising the Timing of Administration of MSC Therapy

In patients with ALI/ARDS it is difficult to establish the precise time-course of the condition and it will undoubtedly vary from patient to patient and with time of presentation to the clinic. A question which must be answered is at what stage of ALI/ARDS are MSCs most or least effective. Approaching this subject using pre-clinical models is a difficult process due to the strict control over the initiation and progression of the condition. To date there has been very little work completed or purposely performed to determine the therapeutic window of MSC administration in ALI/ARDS (206).
2.5.7.5 Optimising the Therapeutic Effect of MSCs

Bringing promising therapies from bench to bedside in a time conscious fashion is a crucial process in medicine. However, a number of factors must be addressed in this process. Experimental studies are vital to determine the optimum dosage, route of administration and therapeutic window of the therapy in question to optimise its efficacy. Many factors must be considered in this process including the injury model as a representation of the human situation, the source of the therapeutic agent, the time course as a reflection of real-life situations and so forth. Taking these factors into account, we designed a series of experiments to determine the optimal dose, route of administration and time of administration of human bone marrow derived MSCs in a rat model of VILI.

2.5.7.6 Optimising MSC Population Purity

Studies which refer to the use of mesenchymal ‘stem’ cells actually refer to a general population of stromal cells with plastic adherent properties isolated from bone marrow aspirates. The actual ‘stem’ cell population of these cells is as low as 1 per 100,000 cells. Attempts to advance prospective isolation of MSC began in the late 80’s when Simmons and Torok-Storb identified the first reagent that could prospectively isolate MSC from BM; an IgM antibody called Stro-1 (269). Since then, significant advances have been achieved over the last few years with regard to identification and culture of human MSC populations. MACS separation of cells can now enrich populations to 1 MSC per 300 bone marrow mononuclear cells (MNC) using anti CD271. FACS separation has further enhanced this, yielding a ratio of 1:13 MSC:MNC (270).

A major bottleneck in advancing beyond MACS-based technologies is the current absence of cGMP-compliant multi-parameter FACS technologies that permit the isolation of highly defined cell populations for clinical use. Currently available commercial FACS instruments are extremely expensive, open and complex mechanical devices that require fastidious cleaning regimens and are still prone to microbial infections. These attributes do not lend themselves to the extremely rigorous validation protocols required to obtain cGMP compliance.
2.6 Models of the Lung for Research

In the development of intervention strategies a hypothesis must be tested before it reaches a clinical setting. *In vitro* studies only allow for a limited number of variables to be examined while *in vivo* animal studies provide an intact biological system in which to give a clearer understanding of the functions and outcomes of an experimental procedure. The characteristics of ALI in humans should be closely replicated in the animal model to derive useful conclusions relevant to clinical setting. These characteristics can be divided into four groups; clinical, physiological, biological and pathological changes (271). The time frame for the evolution of injury and repair should also be similar. Due to the limited understanding of all underlying complexities of ALI in humans and of individual patient profiles, it is difficult to reproduce an exact clinical representation in an animal model. Therefore, good understanding of model characteristics as well as competent interpretation of results within the limits of the model can give excellent insight to the key elements of ALI (271).

### 2.6.1 Choosing a Model for Research

A large amount of species specific factors should be taken into account when choosing an animal model.

#### 2.6.1.1 Differences in Biological Indices

Differences between humans and animals exist in proteomic profiles of BAL fluid, airway and pleural anatomy, epithelial fluid transport rates and immune responses. Specifically, differences exist in TLRs (272) and therefore LPS recognition, macrophage activation and infection response (273, 274). Species specific differences exist in chemokines and their receptors. IL-8 is an important chemokine in ALI as it is produced in response to injury and is a potent neutrophil chemotactic factor (38, 275). Although the true mechanisms are not clear, KC and MIP-2 are considered functional homologs of CXCL8 in rodents due to their neutrophil chemotactic properties even though, in terms of protein sequence, they...
are more similar to human CXCL 1-3 (276). One must also consider the availability of species specific enzyme-linked immunosorbent assay (ELISA) kits. Animal size must be considered for measurement of physiological parameters; for example arterial oxygen tension measurements require numerous blood samples to be taken, and also for visualisation of lung injury and availability of tissue samples for post-mortem analysis.

2.6.1.2 Animal Models of ARDS

In a clinical setting the manifestation of ARDS in patients may involve multiple parameters and be potentiated by underlying conditions. Animal models have been developed and used to reproduce the characteristic indices of injury seen in a wide variety of cases; Oleic acid injury is used to represent lung injury due to lipid embolism caused by the release of bone marrow oleic acid due to long bone fracture (277), LPS is used to mimic sepsis induced injury (278), acid aspiration is used to reproduce ARDS due to aspiration of gastric contents (279). There is no conclusive evidence for the role of hyperoxia in ALI in humans, it is merely suspected as a causative element (280), however it has been used as an injury disease model in animals with or without underlying injury (281). Saline lavage has been used to reproduce surfactant depletion (282), mechanical ventilation to produce VILI (283, 284). These experiments have been conducted in mice, rats, dogs, sheep and rabbits.

2.6.1.3 Human Models of ARDS

The ideal models of ALI are, of course, humans and Proudfoot and colleagues discuss this in their review (56). Trials have been conducted in human models of ALI induced by LPS inhalation (74) and in established ALI (75) to test HMGCoA reductase inhibitors, in COPD patients to test safety and efficacy of MSCs (208) and recently recruitment has begun to examine MSC efficacy in ARDS patients. Models of lung injury such as LPS inhalation and certain surgeries which predispose patients to ARDS only display the initial stages of injury with low level inflammation. Performing clinical trials where the disease condition is induced in the subjects is obviously under intense scrutiny regarding clinical
priorities and ethical considerations. *Ex vivo* research using human lungs offers opportunity for research to create and experimental model of more severe ALI and also allows for continuous invasive analysis and manipulation of the lung tissue not possible in human subjects. A range of experiments have been conducted using *ex vivo* human lungs including a model of ALI associated oedema (285) and endotoxin induced ALI (224). Human lungs are available for use in research due to rejection from use in organ transplant and therefore are most likely damaged, unlikely to be homogenous with variation in ischemia times and donor co-morbidities. The supply of lungs for research is also, understandably, extremely limited and further so by legal, ethical and cultural considerations. *Ex vivo* models of ALI do possess advantages over animal models and in *in vitro* studies but it must be remembered that lungs studied *ex vivo* are not part of a whole body system and this must be taken into consideration when analysing results (56).

### 2.6.1.4 Using an Established Rodent Model of VILI

In this and other studies conducted by our research group we have used a rat VILI repair model of ARDS. This model was developed by our group and the resolution and repair responses are documented and discussed (286). Briefly; high stretch ventilation was used to induce lung injury using an inspiratory pressure of 35 cmH₂O until compliance had decreased by 50%. This resulted in a severe injury characterised by worsening of pulmonary physiological and histological indices and early inflammatory response. Increases in pro-inflammatory cytokines correlated with an increase in neutrophil and lymphocyte infiltration to the lungs. Concentrations of BAL monocytes/macrophages increased in early injury and levels remained above baseline up to 7 days post injury. Increases in MMPs, IL-10 and TGF-β were evident in early phases and BAL KGF levels were shown to increase toward the latter stages, mediating the repair and resolution of the injury which had largely resolved by 96 hours without evidence of fibrosis.

In our study, we used this VILI model to demonstrate the effects of human MSCs on the inflammatory and reparative responses in ARDS after the initial injury. In using this model we are able to demonstrate the effect of MSC therapy on the severe inflammation characteristically seen with the use of mechanical ventilation in ARDS patients.
This model has its limitations. While it does demonstrate an appropriate response following injury to healthy lungs using high pressure ventilation, its clinical relevance is limited by the fact that in ARDS patients, VILI is rarely an isolated injury. Other co-morbidities, such as sepsis, in mechanically ventilated ARDS patients may contribute to the dysfunction of repair leading to further tissue damage, fibrosis and long-term morbidity. However, demonstrating the beneficial effects of MSC therapy in the restoration of functionality following VILI is an important step in the understanding of the mechanisms of action of MSCs in ARDS treatment.
3. Aims and Hypothesis

3.1 The Overall Aim

The overall aim of these studies was to understand the effects of hypercapnic acidosis (HCA) on the NF-κB pathway, a key interaction in the setting of ventilator induced lung injury (VILI) in patients with ARDS, and to investigate the potential for mesenchymal stromal cells, to enhance repair following VILI induced ARDS.

3.2 The Specific Aims

The first set of specific aims related to the investigation of the effects of HCA, a frequent consequence of current approaches to mechanical ventilation, in patients with ARDS, on the NF-κB pathway, a key transcription pathway central to lung inflammation, injury and repair. Our specific aims were:

1. To demonstrate the central role of inhibition of the NF-κB pathway in mediating the anti-inflammatory effects of HCA in the pulmonary epithelium.
2. To elucidate the specific mechanism(s) by which HCA inhibits the NF-κB activation pathway.
3. To elucidate the specific mechanism(s) by which HCA inhibits the activity of the NF-κB heterodimer

These and other studies form our group demonstrated that hypercapnic acidosis diminished pulmonary inflammation but also inhibiting repair, both via NF-κB dependent mechanisms. These insights prompted a search for strategies to enhance repair following injury, either as a sole therapy, or in combination with hypercapnia as an anti-inflammatory strategy. Consequently we focused on evaluating the potential of human mesenchymal stromal cells (hMSCs) to enhance repair and recovery following VILI. Our specific aims were:

4. To determine the optimal route of administration of hMSCs to enhance recovery and resolution of VILI in an in vivo rodent model.
5. To determine the ‘hMSC therapeutic window’, i.e. the efficacy of later administration, of hMSCs to enhance recovery following VILI.

6. To compare the efficacy of specific sub-populations of hMSCs isolated using a proprietary cell surface marker to heterogeneous MSCs in enhancing repair and recovery following VILI.

3.3 Overall Hypothesis

That by generating insights into the mechanisms by which hypercapnic acidosis attenuates the Nuclear Factor Kappa B signalling pathway, we can develop approaches to diminish inflammation while enhancing repair for patients with ARDS.

3.4 Specific Hypotheses

The specific hypotheses were that;

1. That HCA diminishes pulmonary epithelial inflammation via inhibition of the NF-κB pathway.

2. That HCA inhibits activation of the canonical NF-κB activation pathway at multiple activation steps.

3. That HCA inhibits the activity of the NF-κB heterodimer in the pulmonary epithelium.

4. That hMSCs will enhance recovery and resolution of VILI when administered by the intravenous, intra-tracheal and intra-peritoneal routes, in an in vivo rodent model.

5. That hMSC demonstrate a ‘therapeutic window’, i.e. they are effective when administered at later time points, in enhancing recovery following VILI.

6. That specific sub-populations of hMSCs isolated using a proprietary cell surface marker are as effective as heterogeneous MSCs in enhancing repair and recovery following VILI.
4. *In vitro* Materials and Methods

4.1 Cell Cultures

4.1.1 Pulmonary Epithelial Cell Lines

4.1.1.1 Pulmonary Type II Alveolar A549 Cell Line

Pulmonary type II alveolar A549 cells were purchased from The European Collection of Cell Cultures (ECACC, Porton Down, UK) as cryopreserved 90-passage culture and used at passages 91-95. A549 cells are adenocarcinomic human alveolar basal epithelial cells and are categorised under the squamous subdivision of epithelial cells, associated with the diffusion of water, electrolytes, and other substances. Cells were cultured and expanded in 175 cm$^2$ tissue culture flasks (SARSTEDT AG & Co., Nümbrecht, Germany) with RPMI growth medium (RPMI-1640, Sigma-Aldrich, St.Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/ml) and streptomycin (100 μg/ml) solution (Sigma-Aldrich), and then sub-cultured with 0.025% trypsin-0.05 mM ethylenediamine tetra acetic acid (EDTA; GIBCO®, Invitrogen Corporation, NY, USA). For reporter assays A549 cells were seeded at 6x10$^4$ cell/cm$^2$ in 96 well flat bottomed tissue culture plates (SARSTEDT) and for nuclear extract experiments A549 cells were seeded at 5x10$^5$ cells/cm$^2$ in 150mm tissue culture dishes (SARSTEDT) and allowed to reach confluence.

4.1.1.2 Human Bronchial Epithelial Cell Line

Human bronchial 16HBE14o-transformed epithelial (HBE) cells were a kind gift from D. Gruender (University of Vermont, Burlington, VT, USA) as cryopreserved passage-1 culture and used at passages 4-14. HBE cells are virally immortalised normal human bronchial epithelial cells which form correctly polarized cell layers *in vitro* (287). Cells were cultured and expanded in 175 cm$^2$ tissue culture flasks using Minimal Essential Media, alpha modification growth medium (Sigma-Aldrich), supplemented with 10% fetal calf serum, 1% penicillin G (100 U/ml) and streptomycin (100 μg/ml) and then sub-cultured with 0.025% trypsin-0.05 mM EDTA.
4.1.1.3 Small Airway Epithelial Cell Line

Small Airway Epithelial cells (SAECs) were purchased from Lonza (Lonza Group Ltd, Basel, Switzerland) as a cryopreserved 2nd passage culture and used at passages 2-4. SAECs are obtained from the distal airspace and used in toxicology and pulmonary inflammatory response studies. Cells were cultured using EBM supplemented with the small airway epithelial cell medium BulletKit™ (Clonetics™ SAGM™ BulletKit™, Lonza Group Ltd, Basel, Switzerland). For reporter assays SAECs were seeded at 1x10^5 cells/cm^2 in 96 well flat bottom tissue culture plates and transduced with a Lentiviral Luciferase reporter virus.

4.1.1.4 Transformed Human Bronchial Epithelial Cells

Transformed Human Bronchial Epithelial Cells (BEAS-2B) were purchased from The European Collection of Cell Cultures (ECACC) as cryopreserved passage 1 and used at passages 2-14. BEAS-2B cells are stably transduced bronchial epithelium cells commonly used for the screening of chemical and biological agents and in the study of carcinogenesis. Cells were cultured and expanded using Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (Sigma-Aldrich) supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml). Cells were expanded in 75 cm^2 tissue culture flasks and then sub-cultured for luciferase assay in 96 well, flat bottomed tissue culture plates at a density of 6x10^4 cell/cm^2.

4.1.2 Systemic and Control Cell Lines

4.1.2.1 Human Embryonic Kidney 293T Cell Line

Human embryonic kidney (HEK) 293T cells were given as a kind gift from Prof. T. O’Brien with the assistance of Ms. Martina Harte (REMEDI, NUI Galway, Ireland) as cryopreserved passage 4 culture and used at passages 5 to 10. 293T cells are stably transformed human embryonic kidney cells commonly used for the production of therapeutic proteins and viruses for gene therapy. HEK293T cells were cultured in high glucose Dulbecco’s Modified Eagles Medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml). These cells were expanded in 175 cm^2 tissue culture
flasks and then sub-cultured for transfection in 150mm tissue culture dishes or 96 well, flat bottomed tissue culture plates at a density of $6.6 \times 10^4$ cell/cm$^2$.

4.1.2.2 T84 Human Colonic Adenocarcinoma Cell Line

T84 human colonic adenocarcinoma cells were received as a kind gift from Dr. Leo Quinlan (Department of Physiology, NUI Galway, Ireland) as a growing passage 8 culture and used at passages 9 to 12. T84 cells were originally derived from a lung metastasis of colon carcinoma and are commonly used as a model in the in vitro study of epithelial chloride secretion and contain receptors for a range of peptide hormones and neurotransmitters (288). T84 cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100μg/ml). Cells were expanded in 75 cm$^2$ tissue culture flasks (SARSTEDT) and then sub-cultured for luciferase assay in 96 well, flat bottomed tissue culture plates.

4.1.2.3 Immortalised Human Cervical Cancer (HeLa) Cell Line

HeLa cells were given as a kind gift from Prof. T. O’Brien as cryopreserved passage 7 culture and used at passages 8 to 12. HeLa cells are an immortal human cervical carcinoma cell line which is widely used in research. HeLa cells were cultured in high glucose Dulbecco’s Modified Eagles Medium supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml). These cells were expanded in 175 cm$^2$ tissue culture flasks and then sub-cultured for transduction in 6 well tissue culture plates (SARSTEDT) or 96 well, flat bottomed tissue culture plates.

4.1.2.4 Human Monocytic (THP-1) Cells

THP-1 cells were received as a kind gift from Prof. Matt Griffin (REMedI, NUI Galway, Ireland) as a growing passage 8 suspension culture and used at passages 9 to 12. THP-1 cells are a human acute monocytic leukemia cell line derived from peripheral blood. For luciferase reporter assays THP-1 cells were seeded at $6 \times 10^4$ cell/cm$^2$ in 96 well flat bottomed tissue culture plates in RPMI growth medium,
supplemented with 10% fetal calf serum and 1% penicillin G (100 U/ml) and streptomycin (100 μg/ml) solution.

4.1.2.5 Mesenchymal Stromal Cells

Rodent mesenchymal stromal cells were isolated from the long bones of adult male Sprague Dawley rats as previously described (289) and provided for use at culture passages 3 to 5 (kindly provided by Georgina Shaw, REMEDI, NUI Galway, Ireland). Cells were cultured in low glucose Dulbecco’s Modified Eagles Medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml). These cells were then sub-cultured with 0.025% trypsin-0.05 mM EDTA, counted and resuspended for in vivo administration in 300μL PBS.

Human mesenchymal stromal cells were provided by Orbsen Therapeutics (NUI Galway, Ireland) as passage 2 to 5 resuspended cell solutions.

4.1.2.6 Sub-populations of Mesenchymal Stromal Cells

Sub-populations of MSCs were provided by Orbsen Therapeutics Ltd. and isolated as follows; Parental hMSCs were initially isolated from bone marrow aspirates using plastic adherence. Cells were expanded using the recommended cell culture media under conditions of 37°C, 95% humidity, 5% CO₂ and hypoxic conditions of 2% O₂. Orbsen Therapeutics Ltd. identified the ORB-1 protein by comparing the Affymetrix Gene Expression Microarray (GEM) profiles derived from human BM MSC and human MRC-5 lung fibroblasts. ORB-1 was selected for further investigation as the protein is expressed on the surface of human MSC, but not human MRC-5 fibroblasts. Additionally, using flow cytometry, anti-ORB-1 antibody was shown to label CD45⁻CD271⁺bright MNC. This indicated that the ORB-1 protein may represent a novel isolation marker for human BM MSC.

4.1.3 Generation of Normocapnic and Hypercapnic Environments

All cell cultures were incubated under normal tissue culture conditions of 5% CO₂ (Normocapnia, NC), 20% O₂, 95% humidity at 37°C in Series 8000 Water-Jacketed CO₂ Incubators (Thermo Fisher Scientific Ltd., Waltham, MA, USA).
For generation of HCA, cells were cultured in the same conditions except with a CO₂ setting of 15%. HCA conditions were confirmed in medium using a blood gas analyser (ABL 705; Radiometer, Copenhagen, Denmark).

### 4.2 Cell Transfection and Transduction

<table>
<thead>
<tr>
<th>Seeding Density (cm²)</th>
<th>DNA/Well (ng)</th>
<th>Lipofectamine™ or JetPEI™/well (μL)</th>
<th>Total volume added/well (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well</td>
<td>6x10⁴</td>
<td>150</td>
<td>25</td>
</tr>
<tr>
<td>24 well</td>
<td>5x10⁴</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>6 well</td>
<td>3x10⁴</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>150mm dish</td>
<td>3x10⁴</td>
<td>25000</td>
<td>4000</td>
</tr>
</tbody>
</table>

**Table 4**: Seeding densities, DNA quantities and Lipofectamine 2000/jetPEI reagent used in cell transfections

#### 4.2.1 Lipofectamine™ 2000

Lipofectamine™ 2000 (Invitrogen Corporation, NY, USA) is a liposome formulation that complexes with nucleic acids to allow them to be taken up by the cell despite electrostatic repulsion by the cell membrane (290). Cells were transfected following the recommended protocol, briefly as follows; 24 hours after cells were seeded and the transfection reagent prepared by combining the indicated amounts of DNA and Lipofectamine™ (Table 3) with OPTI-MEM I Reduced Serum Medium (Invitrogen Corporation) separately and incubating for 5 minutes at room temperature. After incubation the Lipofectamine™ 2000 reagent was added to the DNA mix and allowed to incubate for up to 25 minutes at room temperature. The indicated amounts were added to each well and the cells further incubated for 24-48 hours.

#### 4.2.2 JetPEI™

JetPEI™ Reagent (Polyplus-transfection SA, BIOPARC, France) is a linear polyethylenimine derivative which functions in a similar fashion to
Lipofectamine™ 2000 reagent, enveloping negatively charged DNA into positively charged particles allowing for their cellular uptake. Transfection using jetPEI™ was performed in 150mm dishes and 96 well plates using the amounts indicated in Table, using similar guidelines for Lipofectamine™ 2000 transfection with the exception that 150mM sterile NaCl was used in place of OPTI-MEM and samples were mixed by vortexing for 30 seconds separately and again after addition of the jetPEI solution to the DNA.

### 4.3 A549 Nuclear Extracts

Cells were seeded in 150mm tissue culture dishes and allowed to reach 90% confluency. Cells were then pre-treated in NC or HCA conditions at 37°C for 1 hour and then treated for 30 minutes under the same incubation conditions with either TNF-α (20ng/mL) or phosphate buffered saline solution (PBS, Sigma-Aldrich). Cells were immediately placed on ice and the media aspirated. After washing twice in ice-cold PBS the cells were then harvested by scraping them into ice-cold PBS and centrifuging at 500 x g for 5 minutes. Supernatant was discarded. Nuclear extraction was performed on cells using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Thermo Fisher Scientific Ltd., Waltham, MA, USA) following the recommended protocol, briefly as follows; for a packed cell volume of 50μL, 500μL of CER I was added and the sample mixed using a vortex at the highest setting for 15 seconds. The sample was then incubated on ice for 10 minutes. 27.5μL of CER II was added and the sample was mixed by vortexing again for 15 seconds before incubation on ice for 1 minute. After incubation, vortexing was repeated and the sample was centrifuged for 5 minutes at 16,000 x g. The supernatant (cytoplasmic extract) was transferred to clean 0.2mL microcentrifuge tubes in 20μL aliquots and stored at -80°C. The insoluble pellet was re-suspended in 250μL NER and vortexed every 10 minutes for 15 seconds for a total of 40 minutes. The sample was then centrifuged at 16,000 x g for 10 minutes. The supernatant (nuclear extract) was then transferred to 0.2μL microcentrifuge tubes in 10μL aliquots. All samples were stored at -80°C until use.
4.4 BCA Protein Assay

Protein in samples was quantified using the Pierce BCA protein assay kit (Pierce Thermo Fisher Scientific Ltd.) following the recommended protocol as follows; Working solution of 1:50 ratio of reagent A and reagent B was prepared as follows; 
((number of standards + number of unknowns) x replicates) x amount of working reagent to be added (100µL) = Total amount needed. 2µL of nuclear or cytoplasmic extract or standard was added to 48µL PBS in a round bottom 96 well plate in triplicate. 100µL of working solution was added to each well and the plate incubated at 37°C for 30 minutes. The plate was then read at 562nm using a Victor luminometer (Perkin Elmer, Waltham, MA, USA). Protein was quantified using the line equation from the standard curve and the quadratic equation 
y=ax²+bx+c, where y is the concentration and x is the absorbance. $x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$

4.5 Luciferase Assay

Luciferase assays were used to measure the activity of NF-κB in response to over expression of a gene of interest and in the presence or absence of HCA.

Image 5: Schematic diagram of the pGL3 Basic vector system used for luciferase assays. (Promega)
4.5.1 Reporter Plasmids
The NF-κB reporter used consists of 5 copies of the NF-κB consensus sequence (5'GGGACTTTCC-3') cloned into the pGL3-Basic vector (Promega Corporation, Madison, WI, USA). All results were normalized for a co-transfected TK-Renillin internal control (pRL-TK Vector, Promega Corporation). Briefly, A549 cells were seeded at a density of 2x10⁴ cells per well of a 96 well plate and 24 hours later, 40ng/well of each reporter and 100ng of the plasmid carrying a gene of interest was transfected into the cells using Lipofectamine™ 2000 (as described in Section 4.2.1) and then incubated for a further 24 hours.

4.5.2 Lentivirus Reporter
A NF-κB pGreenFire transcription reporter system (System Biosciences, Inc., CA, USA) used to produce a Lentivirus reporter (See Section 4.17) which was used to transduce cell lines which were resistant to transfection. A Multiplicity Of Infection (MOI) ratio of 100 was used in a cell suspension of 3x10⁵ cells/mL of which 100μL was added per well in a 96 well plate. Due to the uniform viral infection of cells a control plasmid was deemed unnecessary.

4.5.3 Substrates
Following incubation, the cell culture medium was aspirated and 100μL of 1x Reporter Lysis Buffer (Promega Corporation) was added to each well for transfected samples and 50μL to the transduced samples. Samples were then subjected to a freeze thaw cycle, following which 40μL of the lysate was mixed with Bright-Glo luciferase substrate (Promega Corporation) or coelenterazine (Sigma-Aldrich) and assessed in a Victor luminometer (Perkin Elmer). Results were expressed as a fold change compared to samples transfected with Null plasmid, or samples that were non-transduced, and treated with PBS and NC.
4.6 Cell Viability Assay

4.6.1 MTT Assay
The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) viability assay was performed on 96 well plates of cells to assess cell viability in response to various experimental conditions. Media was aspirated from the cells and discarded. 100µL of fresh cell culture media was added along with 10µL of 10mg/mL MTT. Cells were incubated at 5% CO₂, 37°C for 2 hours following which the media and MTT was replaced with 100µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich) per well. The plate was then incubated at room temperature on a plate rocker. After incubation signal was read in a Victor luminometer (Perkin Elmer) at 595nm.

4.7 Intracellular pH Assay

4.7.1 SNARF-1 Assay
Seminaphtharhodafluor carboxylic acid, acetate, succinimidyld ester (SNARF-1) is a dye which changes fluorescent intensity in accordance to pH levels. For this experiment SNARF-1 (Invitrogen Corporation) was used to detect intracellular pH changes in response to HCA treatment. Cells were seeded at 2x10⁵ cells per well in 96 well plates and allowed to reach 90% confluence. Wells were designated as no SNARF-1, Control, Buffered and Nigericin (Sigma-Aldrich) at n=6. 10µL of 1mg/mL SNARF-1 was diluted 1:1000 in cell culture media and all cells were re-fed with SNARF-1 except the wells designated No SNARF-1 which were re-fed with fresh RPMI. Cells were incubated for 1 hour at 5% CO₂, 37°C in a cell culture incubator. After incubation cells were washed three times in PBS. 100µL RPMI was added to the No SNARF-1 and Control wells. 100µL of RPMI containing 0.5M NaHCO₃ was added to the ‘buffered’ wells and 100µL of 0.1µg/mL Nigericin resuspended in cell culture medium was added to the ‘Nigericin’ wells. Nigericin is used to open the membrane allowing H⁺ to cross freely allowing calibration of the experiment. Plates were incubated for 1 hour at 5% or 15% CO₂. After incubation media was replaced with PBS and plates were read immediately at 550 nm/585nm using a Victor luminometer (Perkin Elmer).
4.8 Enzyme Linked Immunosorbent Assay (ELISA)

4.8.1 Phospho-\(\text{IκB}_\alpha\) Sandwich ELISA

For detection of phosphorylated \(\text{IκB}_\alpha\) levels from the \textit{in vitro} kinase reaction (Section 4.10), a sandwich ELISA antibody pair for phospho-\(\text{IκB}_\alpha\) was purchased from Cell signalling Technology (Danvers, MA, USA). Samples were analysed according to manufacturer’s instructions as follows;

A 96 well microplate (NUNC, Thermo Fisher Scientific Ltd.) was rinsed in dH\(_2\)O by adding 200μL/well and discarding liquid. The plate was then blotted dry on a paper towel. The capture antibody was diluted 1:100 in PBS and 100μL added per well. The plate was then incubated for 17 to 20 hours at 4°C. After incubation, the capture antibody was discarded and the wells washed four times with 200μL per well of wash buffer (1x PBS, 0.5% Tween-20 (Sigma-Aldrich). Excess wash buffer was removed by striking the plate on paper towel. Following this the plates were blocked by adding 150μL of blocking buffer per well (1xPBS, 0.5% Tween-20, 1% bovine serum albumin (BSA)) and incubated at 37°C for 2 hours. Following blocking, plates were washed four times as previously described. 100μL of the samples per well were added (diluted 3 in 10 in blocking buffer) to the plate and incubated at 37°C for 2 hours. Following sample incubation plate was washed four times as previously described. The detection antibody was diluted 1:100 in blocking buffer and 100μL added per well. The plate was incubated at 37 °C for 1 hour. The plate was then washed four times and secondary antibody (anti-rabbit horse radish Peroxidase (HRP)) was diluted 1:1000 in blocking buffer. 100μL of secondary antibody was added and the plate incubated at 37°C for 30 minutes. The plate was washed as described and 100μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich,) was added per well. Following incubation for 10 minutes at room temperature, 100μL of stop solution (2N H\(_2\)SO\(_4\), Sigma-Aldrich) was added per well. Samples were then read at an absorbance of 450nm with a wavelength correction at 570nm using a Victor luminometer. Results were expressed as a fold change compared to NC untreated samples.
4.8.2 Human CXCL8/IL-8 DuoSet ELISA

A human CXCL8/IL-8 DuoSet ELISA kit (R&D Systems Inc., MN, USA) was utilised to quantify the levels of IL-8 production in cell culture media as an indicator of NF-κB activity. Samples were analysed according to manufacturer’s instructions as follows;

Capture antibody was diluted to 4μg/mL in 1x PBS and 100μL added per well of a 96 well MaxiSorp® microplate (NUNC, Thermo Fisher Scientific Ltd.). The plate was sealed and incubated overnight at room temperature. Following incubation, the wells were aspirated and washed three times with 300μL per well of wash buffer (1x PBS, 0.5% Tween-20). Plates were then blocked using 300μL per well of blocking buffer (1% BSA, in PBS with 0.5% NaN₃) and incubated for 1 hour at room temperature. The wash steps were repeated. Samples were diluted 1:10 in reagent diluent (0.1% BSA, 0.5% Tween-20 in Tris-buffered Saline (50Mm Tris-hydrochloric acid (HCl) pH 7.4, 150mM NaCl)), 100μL added per well and incubated for 2 hours at room temperature. The wells were washed as described. Detection antibody was diluted to 20ng/mL in reagent diluent and 100μL added per well. The plate was then incubated for 2 hours at room temperature. The wells were washed as described and 100μL of streptavidin-HRP (diluted 1:200 in reagent diluent) added per well. The plate was incubated for 20 minutes at room temperature in the dark. The wash step was repeated and 100μL of TMB substrate solution was added to each well. The plate was incubated for 20 minutes at room temperature in the dark following which 50μL of stop solution (2N H₂SO₄) was added. The samples were read immediately at 450nm with a wavelength correction at 570nm. Levels of IL-8 were quantified against a 7 point standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 2000pg/mL.

4.8.3 Human phospho-MKK6 DuoSet ELISA

A human phospho-MKK6 DuoSet ELISA kit was purchased (R&D Systems Inc.) to detect levels of phosphorylated dual specificity mitogen-activated protein kinase kinase 6 (MAP2K6/MKK6) in cell lysates in response to over expression of transforming growth factor (TGF)-beta-activated kinase-1 (TAK1) and TGF-beta-activated kinase-1 binding protein (TAB1). Cells were harvested by scraping
into ice-cold PBS and re-suspending in 1x lysis buffer (Cell Signaling Technology, Danvers, MA, USA) composed of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM ethylene glycol tetra acetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and 1mM phenylmethanesulfonylfluoride (PMSF). Re-suspended cells were incubated on ice for 5 minutes and then briefly sonicated and centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatant was transferred to clean tubes on ice or stored at -80°C. Samples were analysed according to manufacturer’s instructions as follows;

The capture antibody was diluted to 4µg/mL in PBS and 100µL per well was added to a 96 well microtitre plate. The plate was then sealed and incubated overnight at room temperature. Following incubation each well was aspirated and washed with 300µL per well wash buffer (0.5% Tween-20 in PBS, pH 7.2 - 7.4) 3 times. The wells were then blocked by adding 300µL per well of block buffer (1% BSA, in PBS, pH 7.2 - 7.4) and incubating for 1 to 2 hours at room temperature. The wash step was repeated and 100µL of sample or positive control added per well. Samples were incubated for 2 hours at room temperature. The wash step was repeated before addition of the detection antibody. A working concentration of 1µg/mL of detection antibody was prepared using sterile filtered block buffer, as diluent. 100µL was added to each well and the plate was incubated for 2 hours at room temperature. After incubation, the wash step was repeated. Streptavidin-HRP was diluted 1:200 using sterile filtered block buffer. 100µL was added per well and the plate incubated for 20 minutes at room temperature in the dark. The wells were washed as described and 100µL TMB substrate solution added to each well for 20 minutes at room temperature in the dark. 50µL of stop solution (2N H₂SO₄) was added to each well and the samples read immediately at 450nm with a wavelength correction at 570nm.

4.9 Immunoprecipitation

Immunoprecipitation (IP) was performed on cell lysates following treatment for enhanced detection of the protein being precipitated and its phosphorylated form. Lysates were prepared as follows; cells from 150mm tissue culture dishes were
washed once with ice cold PBS and harvested by scraping into 10mL PBS. Cell suspensions were centrifuged and the supernatant discarded. 1mL of ice cold non-denaturing lysis buffer (20mM Tris-HCl (pH8), 137mM NaCl, 10% glycerol, 1% Nonidet P-40 (NP-40), 2mM EDTA), supplemented with Complete, EDTA-free; Protease Inhibitor Cocktail Tablets (Roche Diagnostics Corporation, IN, USA) was added and the cells re-suspended. Samples were then incubated at 4°C with constant agitation for 30 minutes. Following incubation the samples were centrifuged at 16,000 x g for 20 minutes at 4°C and the supernatant transferred to a fresh tube. On ice, 1µL of primary antibody directed against the protein of interest was added to the samples and incubated overnight at 4°C with constant agitation. Following incubation 50µL of Protein A Agarose beads (Sigma-Aldrich) were added to the samples and they were incubated for a further 4 hours at 4°C with constant agitation.

Samples at this point were either used directly in Western blot protocols (Section 4.11), in which case samples were boiled in 50µL Laemmli sample buffer (2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromphenol blue, 0.0625M Tris-HCl), or used to perform in vitro kinase assays.

4.10 In vitro Kinase Reaction

An in vitro kinase (IVK) reaction was performed on samples which had been previously immunoprecipitated (Section 4.9). Protein A beads conjugated to the protein of interest were washed 3 times in 250µL IVK buffer (20mM HEPES pH 7.4, 10mM MgCl₂, 20mM βGP, 10mM NaF, 0.2mM NaVO₃, 1mM Dithiothreitol (DTT)). 0.5µg/µL substrate and 100µM adenosine triphosphate (ATP) (100mM stock) in 30µL IVK buffer was equilibrated to 30°C and 5% (NC) or 15% CO₂ (HCA) for 10 minutes. This reaction mix was then added to the samples and incubated for 30minutes at 30°C. Samples were then divided and used in Western blotting (Section 4.11) or ELISA (Section 4.8) For Western blotting, an equal volume of Laemmli sample buffer was then added and the samples run on SDS-polyacrylamide gel electrophoresis (PAGE) gel (Section 4.11). Samples were immune-blotted for phosphorylated protein (substrate) then total protein (substrate) run in conjunction with lysate samples which were blotted for
phosphorylated protein (transfected in) and total protein (transfected in). For ELISA, phosphorylation reactions were stopped after incubation by the addition of 3mM Sodium Orthovanadate. Samples were added to the ELISA plate as described (Section 4.8.4).

4.11 Western Blot

Western blots were performed on whole cell lysates, immunoprecipitated proteins or in vitro kinase reactions. Samples were boiled in Laemmli sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromphenol blue, 0.0625M Tris-HCl) prior to being loaded into a 4-20% polyacrylamide gels (Pierce Biotechnology, Rockford, IL). Proteins were separated at 100Volts for approximately 1 hour in Tris-HEPES-SDS Running Buffer (100mM Tris, 100mM HEPES, 3mM SDS) and then transferred at 30 to 40 Volts overnight to nitrocellulose membrane by wet transfer. Membranes were blocked and then probed using primary and secondary antibodies diluted in either PBS or tris buffered saline (TBS).

4.11.1 Phosphorylated Protein Antibodies

Phosphorylated IKK-α and, or IKK-β protein levels were detected using either Anti-IKK alpha + IKK beta (phospho S180 + S181) antibody (Abcam Plc., Cambridge, UK), phospho-IKK-α/β (Ser176/180) rabbit mAb (Cell Signaling Technology) or Anti-IKK alpha (phospho S176 + S180) antibody (Abcam). Levels of phosphorylated IκBα and phosphorylated p65 were detected using phospho-IκBα (Ser32) rabbit mAb and phospho-NF-κB p65 (Ser276) antibody (Both Cell Signaling Technology). All aforementioned antibodies were diluted in 5% BSA-TBST (TBS, 0.5% Tween-20) at a concentration of 1:2000 and incubated with the nitrocellulose membrane overnight at 4°C with constant gentle agitation. Following incubation and three 10 minute wash steps with TBS-T, the membrane was incubated for 2 hours with anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology) which was diluted 1:2000 in 5% w/v BSA-TBST. The membrane was then washed three times as described and incubated for 5
minutes in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) and imaged in a Florchem imager (Alpha Innotech Corporation, CA, USA) for 20 to 30 minutes.

### 4.11.2 Total Protein Antibodies

Levels of total IKK-β and IκBα protein were detected using antibodies purchased from Cell signalling Technology (#2684 and #4814 Cell Signaling Technology) and used diluted 1:2000 in 5% w/v skimmed, dried milk powder in PBS. Proteins labelled with the FLAG-peptide tag were detected in Western blots using a monoclonal anti-FLAG® M1 antibody produced in mouse (Sigma-Aldrich, St.Louis, MO, USA) and anti-mouse IgG, HRP-linked antibody (#7076 Cell Signaling Technology). Nuclear and cytoplasmic levels of p-65 protein were detected using the NF-κB p65 (C-20) antibody (#sc-372 Santa Cruz Biotechnology Inc., Bergheimer Str. 89-2, 69115 Heidelberg, Germany) at a dilution factor of 1:1000. The primary antibodies for total protein were incubated with the nitrocellulose membranes for 2 to 4 hours at room temperature. Respective secondary antibodies were incubated with membranes for 1 to 2 hours following the necessary wash steps. HRP-linked β-Actin antibody (Monoclonal anti-b-Actin-Peroxidase; Sigma-Aldrich) was used as an internal control for total protein quantitation.

### 4.12 p50/p65 Dimerisation

Recombinant p50 protein (Cayman Chemical Company, Ann Arbor, MI, USA) and p65 protein (OriGene Technologies Inc., Rockville, MD) homo- and hetero-dimers were produced as previously described (291) in the presence of 5% or 15% CO₂ briefly as follows; 3μg of each protein was incubated in 7μL dialysis buffer (20mM HEPES pH 7.9, 1mM EDTA, 0.1mM ZnCl₂, 0.1% Nonidet P40, 10% Glycerol, 0.1mM PMSF, 0.5mM dithiothreitol, 50mM NaCl) which was pre-incubated for four minutes at 37°C. Reactions were incubated for 40 minutes at 37°C at either 5% or 15% CO₂ and then stopped by the addition of 10x Non-denaturing loading buffers (250mm Tris-HCl pH 7.5, 0.2% Bromophenol Blue, 40% glycerol). 12μL of the samples and 0.1μL of
standard (MagicMark™ XP Western Protein Standard (20-220 kDa, Invitrogen Corporation), were run on a 4-20% TBE Ready Gel (#161-1235, Bio-Rad Laboratories Ltd., Hertfordshire, UK) at 100Volts for 1 hour at 4°C in 0.5x TBE buffer (10x Stock; 107.8g Tris Base, 55g Boric acid, 7.44g EDTA). Proteins were visualised using the PageSilver™ Silver Staining Kit (Fermentas GmbH, Thermo Fisher Scientific Ltd.) and images acquired using a Florchem imager.

### 4.13 Electrophoretic Mobility Shift Assay

P50 and p65 dimers (Section 4.12) and A549 nuclear extracts (Section 4.3) produced under conditions of 5% and 15% CO₂ were used in an electromobility shift assay (EMSA) to assess NF-κB binding in a non-radio labelled experiment. A biotin-labelled NF-κB consensus sequence nucleotide dimer was produced using the following sequences; sense: 5’-biotin-AGTTGAGGGGACTTTCCCAGGC-3’, antisense: 5’-GCCTGGGTTTGAGGGCTCAACT-3’. Sequences were annealed by incubating 480 picomoles of each sequence in 100μL of buffer (10mM Tris HCl, 20mM NaCl) at 95°C for 5 minutes and allowing to return to room temperature. Protein dimers were allowed to bind to this DNA probe in a reaction consisting of 2μL of the protein sample, 2μL of 5x Binding buffer (20% glycerol, 5mM MgCl₂, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl (pH 7.5)), 1μL poly(dI-dC)•poly(dI-dC) (1mg/mL), 3μL H₂O. For nuclear extract experiments 10μL of 350μg/mL nuclear extract was combined with 4μL 5x binding buffer, 2μL poly (dI-dC) •poly (dI-dC) (1mg/mL), 2μL H₂O. After a 5 minute room temperature equilibration and a further 5 minute equilibration at 5% or 15% CO₂, 2μL of the nucleotide probe was added and the reaction incubated for 20 minutes under 5% or 15% CO₂. 1μL of 10x non-denaturing loading buffer was then added and the samples electrophoresed on a 4-20% TBE Ready Gel at 100Volts for 1 hour at 4°C in 0.5x TBE buffer. The DNA probes were then transferred from the gel to a positively charged Nytran® SPC, 0.45 μm nylon blotting membrane (#10416294 Whatman plc, Kent, UK) in 0.5x TBE buffer at 100Volts for 30 minutes. Probes were visualised using the Chemiluminescent Nucleic Acid Detection Module (Pierce Thermo Fisher Scientific Ltd.) following the
manufacturer’s protocol, briefly as follows; 16mL of the pre-warmed blocking buffer was incubated with the membrane for 15 minutes at room temperature with gentle agitation. The streptavidin –HRP conjugate was prepared and incubated with the membrane for 15 minutes with gentle agitation. 1x was solution was prepared and the membrane washed for 5 minutes 4 times using 20mL each time. 30mL of substrate equilibration buffer was incubated with the membrane for 5 minutes with gentle agitation. Substrate working solution was prepared and the membrane submerged in it, nucleic acid side down, for 5 minutes without shaking. The membrane was then imaged in a Florchem Imager.

4.14 p50 and p65 Binding Assays

The NF-κB (p65) Transcription Factor Assay Kit (#10007889, Cayman Chemical Company) was used to determine the levels of p65 protein binding to NF-κB consensus sequence DNA under conditions of NC and HCA. A549 nuclear extracts prepared from cells grown under NC and HC conditions and treated with PBS or TNF-α (Section 4.3) were used in this experiment. The manufacturer’s protocol was followed and slightly altered to incorporate conditions of NC and HC, briefly as follows; The provided plate and buffers were equilibrated to room temperature and the complete transcription factor binding assay buffer prepared (73μL ultra pure
water, 25μL 4x Transcription factor binding assay buffer, 1μL reagent A, 300mM DTT to a total volume of 100μL per well). The plate was then prepared by adding 100μL of this complete buffer alone for blank samples, 90μL per well for experimental samples and positive control wells. 10μL nuclear extract (1mg/mL) and 10μL provided positive control (clarified cell lysate) per well was aliquoted. The plate and sample aliquots were incubated in either NC or HCA separately for 10 minutes and then samples and positive control added to the plate which was allowed to incubate for a further 1 hour at NC or HCA. Following incubation the wells were washed 5 times with 200μL per well of the provided wash buffer. The primary antibody was diluted 1:100 in 1x antibody binding buffer and 100μL added to all wells except blank. The plate was sealed using an adhesive cover and incubated for 1 hour at room temperature. Following incubation the wells were washed 5 times as previously described. Goat anti rabbit HRP conjugate antibody was diluted 1:100 in 1x antibody binding buffer and 100μL added to all wells except blank. Plate was sealed as before and incubated for 1 hour at room temperature. The wash step was repeated and 100μL of developing solution added to all wells for 15 to 45 minutes at room temperature with gentle agitation in the dark. 100μL stop solution was then added after sufficient colour development and the plate was immediately read at 450nm with a wavelength correction at 570nm using a VICTOR™ multi-label plate reader (Perkin Elmer).

4.15 In vitro Transcription/Translation Assays

The effect of HCA on transcription/translation was assessed using the TNT® Coupled Transcription/Translation System (Promega Corporation). This kit enables single-tube, coupled transcription/translation reactions for eukaryotic in vitro translation using ribonucleic acid (RNA) synthesized in vitro from T7 RNA polymerase. Reactions were carried out in accordance with the manufacturer’s instructions with slight alterations to incorporate conditions of NC and HCA. Briefly, a master mix consisting of 10μL TNT® Rabbit Reticulocyte Lysate, 0.4μL Amino Acid Mixture (minus leucine, 1mM), 0.4μL Amino Acid Mixture (minus methionine, 1mM), 0.4μL TNT® Reaction Buffer was prepared for each
sample. The master mix was equilibrated at 30°C in either 5% or 15% CO₂ for 10 minutes after which the reaction initiator (nuclear extract samples or control T7 RNA polymerase) was added and the reaction allowed to proceed for 60 minutes at 30°C in either 5% or 15% CO₂. Samples were then mixed 1:1 with Bright-Glo luciferase substrate (Promega Corporation) and luminescence assessed in a VICTOR™ multilabel plate reader (Experiment 1, Image 7). These samples were then recovered and RNA extraction performed using TRIzol (Invitrogen Corporation). 1μL of the isolated RNA from NC sample was added to equilibrated master mix and the reaction repeated. Samples were then mixed with Bright-Glo luciferase substrate and assessed in a VICTOR™ luminometer (Experiment 2, Image 7).

Image 7: Schematic diagram of in vitro transcription/translation assay procedure.

4.16 Statistical Analysis

Statistical analysis was performed on scientific data using GraphPad Prism software. All data is expressed as mean (n=3) with error bars representing standard deviation (SD). Statistical analyses were performed using the Student's t-test to compare data sets. P values less than 0.05 were considered to be significant.
5. The Effects of Hypercapnic Acidosis on the Pulmonary NF-κB Signalling Pathway

5.1 Abstract

Introduction: Current ‘protective’ mechanical ventilation approaches for patients with ARDS mandates the use of low tidal and minute volumes, which results in the accumulation of CO₂ – this is termed ‘permissive hypercapnia’. NF-κB is a pivotal transcription factor, which is activated in the setting injury, and which activates the transcription of genes pivotal to the pathogenesis of inflammation, injury, and repair. Hypercapnic acidosis (HCA) suppresses NF-κB activation, but the precise mechanism(s) by which this effect is mediated is uncertain.

Objectives: To demonstrate the central role of inhibition of the NF-κB pathway in mediating the anti-inflammatory effects of HCA in the pulmonary epithelium, and elucidate the specific mechanism(s) by which HCA inhibits the NF-κB activation pathway.

Methods: Pulmonary epithelial cells, and cells from systemic organs, were co-transfected with a kappa-B luciferase reporter and TK-renillin internal control, and activated by TNFα and/or IL-1β and LPS under normocapnic (5% CO₂) and hypercapnic (15% CO₂) conditions. The effect of HCA on the activity of specific components of the canonical pathway was assessed via over expression of these components and in vitro kinase activity assays, immunoblotting and ELISA of the NF-κB activated cytokine IL-8 in cell supernatant.

Results: HCA inhibited canonical pathway activation of NF-κB. HCA decreased IκBα degradation, and reduced phosphorylative activation and intrinsic activity of the IκB kinase-β (IKK-2). Interestingly, HCA also increased the activation of IκB kinase-α (IKK-1) by NF-κB inducing kinase (NIK), which can activate NF-κB via a separate non-canonical pathway.
Conclusion: Hypercapnic acidosis inhibits NF-κB canonical signaling in human lung and other organ cells by acting at multiple points in the pathway proximal to, and including, IκBα degradation.
5.2 Introduction

In cases of acute lung injury mechanical ventilation is necessary to sustain life. However, mechanical ventilation can result in over distension of the lung tissue contributing to an overall worsening of the initial condition by leading to alveolar oedema, cytokine release and granulocyte infiltration. Lowering tidal volumes to facilitate ‘protective’ mechanical ventilation results in a build up of CO$_2$ in the system leading to HCA, and this build up has been shown to be beneficial in the setting of inflammatory pulmonary injury (96, 98, 95) by reducing indices of injury in the lung (Reviewed in (296)). A deliberate allowance of build-up of CO$_2$ is termed ‘Permissive Hypercapnia’ and the resulting drop in extracellular pH is known as HCA (297).

NF-κB was first described in 1986 by Sen and Baltimore (147) and since has become a major target of study in cell injury and repair. The nuclear factor κB (NF-κB) pathway is known as a rapid responder to injury as shown by the immediate degradation of IκBα and subsequent release of active NF-κB (298). The pathway is activated in response to injurious stimulants such as TNF-α, IL-1β and endotoxin. Activation of this pathway leads to the further production of inflammatory cytokines such as IL-6 and IL-8. The NF-κB pathway is activated in response to numerous stimuli which leads to an activation of either the “canonical” or “non-canonical” pathway. In activation of the canonical pathway, TGF-β associated kinase (TAK1) and TAK1 binding protein (TAB1/2) complex phosphorylates IKK-β which in turn phosphorylates the inhibitory κB (IκBα) protein leading to its dissociation from the p50/p65 NF-κB complex. In the non-canonical pathway NIK phosphorylates IKK-α which leads to the proteasomal processing of the p100/RelB complex to p52/RelB NF-κB which translocates to the nucleus. It is established that HCA exerts a protective effect in lung injury by, in part, inhibiting the NF-κB pathway (143).

HCA has been demonstrated to exert protective effects in the setting of inflammatory injury to the lungs and other organs. For example the advantageous effects of HCA have been shown in acute ischemic myocardial and brain injury
(reviewed in (299)), and endotoxin induced gut mucosal injury in rabbits (300). HCA has also been shown to inhibit IL-6 and TNF-α expression in the macrophage (114). Consequently, our group has previously advocated evaluation of the therapeutic potential of hypercapnia – termed ‘therapeutic hypercapnia’ – in the setting of lung and systemic organ injury in the critically ill (123). However HCA can also have adverse effects, including during wound healing (301) and prolonged untreated pneumonia (134). Consequently, a greater understanding of the mechanisms of action of HCA is needed if we are to harness its benefits, while minimizing the potential for harm.

In 2003, Takeshita et al. (146) showed that HCA attenuated endotoxin-induced NF-κB activation by suppressing IκBα degradation in human pulmonary artery endothelial cells (HPAEC). More recently, we have demonstrated that HCA reduces mechanical stretch induced injury (131), and decreases pulmonary epithelial wound healing (301) by inhibiting NF-κB. However, the precise mechanisms by which HCA suppresses NF-κB activation, and the potential for HCA to exert effects at points in the pathway other than by suppressing IκBα degradation, is not known.

We therefore hypothesized that HCA directly inhibits NF-κB activation via: (1) inhibition of discrete steps on the canonical NF-κB pathway. In these experiments we examined the potential for HCA to modulate the activation state and/or activity of key components of the canonical signaling pathway, including the cytosolic inhibitor IκBα, the IκB kinase (IKK) complexes, and the NF-κB inducing kinase (NIK) complex.

### 5.3 Aims

To demonstrate the central role of inhibition of the NF-κB pathway in mediating the anti-inflammatory effects of HCA in the pulmonary epithelium, and in systemic organ tissues, and to elucidate the specific mechanism(s) by which HCA inhibits the NF-κB activation pathway.
5.4 Methods

5.4.1 Cell Culture
This first part of this experimental study was conducted using human small airway epithelial cells (SAECs), human A549 cells and HBE cells, the properties and culture conditions of which are described (Section 4.1) Systemic cell lines used in this experiment included HEK293 cells, human colonic T84 cells and human monocytic THP-1 cells. HEK293T, T84 and THP-1 cell lines were obtained from The European Collection of Cell Cultures (Porton Down, UK) and cultured as described (Section 4.1.2). Cells were prepared for luciferase assay and I-8 ELISA by seeding cells at a density of $2 \times 10^4$ cells per well in 96 well tissue culture plates and the cells either transfected with a NF-κB Luciferase reporter plasmid and TK-Renillin reporter plasmid or transduced with an NF-κB luciferase VSVG-pseudotyped Lentiviral reporter vector as described (Section 4.5). 100ng per well of a CMV-driven plasmid containing the gene of interest was transfected into cells for study of the effects of over expression. Transfected cells were incubated under normal cell culture conditions (37°C, 5% CO₂, 20% O₂, 95% humidity) for 24 hours, and transduced cells for 72 hours to allow for integration and protein production. Samples were pre-incubated before injury by incubating the cell cultures in their experimental environments of 5% and 15% CO₂, with other culture parameters remaining unchanged, for 1 hour. Cells were then subjected to injury by the addition 10ng/mL TNF-α and/or IL-1β, or 50ng/mL endotoxin (LPS) and incubated under experimental conditions for a further 24 hours. For Western blotting, cells were seeded at $6 \times 10^5$ cells per well in 6 well plates and transfected with 500ng of a plasmid encoding the gene of interest. Pre-incubation and injury were performed in the same fashion as described. A549 pulmonary adenocarcinoma cells were seeded at $3 \times 10^5$ cells per cm² in 150mm tissue culture dishes. 24 hours later cells were transfected with 25g per plate of a plasmid encoding the IKK-β FLAG tagged protein using Lipofectamine as described (Section 4.2.1). After 48 hours cells were subjected to either 1 hour in NC or HCA.
5.4.2 Cell Harvesting and Luciferase Assay
Cells were harvested by removing the culture medium and adding 1X reporter lysis buffer (Promega Corporation) directly to the cells. THP-1 monocyte suspension cultures were centrifuged at 500xg for 5 minutes, the supernatant aspirated and 1X Reporter lysis buffer added directly to the cells. After three freeze-thaw cycles, cells were briefly assessed for lysis under a light microscope. Cell lysates were combined with Bright-Glo Luciferase assay buffer (Promega) or coelenterazine (Sigma-Aldrich, St.Louis, MO, USA), as described (Section 4.5.3) and luciferase production assessed using a Victor Luminometer (Perkin Elmer, Waltham, MA). Results were graphed as fold change as compared with uninjured cells treated with vehicle incubated under NC conditions.

5.4.3 Western Blotting
Western Blots were performed on whole cell lysates harvested from 6 well plates. At the appropriate time point cells were harvested by aspirating the media and performing two wash steps with ice cold PBS. Cells were harvested in PBS by using rubber cell scrapers and samples centrifuged at 500 x g for 5 minutes at 4°C. Samples were immediately resuspended and boiled in Laemmli sample buffer (2% SDS (w/v), 10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), 0.002% bromphenol blue (w/v), 0.0625M Tris-HCl) prior to being loaded into a 4-20% polyacrylamide gel (Pierce Biotechnology, Rockford, IL). Proteins were separated at 100V for approximately 1 hour in Tris-HEPES-SDS Running Buffer (100mM Tris, 100mM HEPES, 3mM SDS) and then transferred at 30-40V overnight onto nitrocellulose membrane by wet transfer. Ponceau stain was used to visually assess transfer efficiency. Membranes were blocked and then probed using primary and secondary antibodies diluted in either 5% (w/v) milk-PBS or 5% (w/v) BSA in TBS as described (Section 4.11). Membranes were then treated in chemiluminescent substrate (Pierce Thermo Fisher Scientific Ltd.) and imaged in a Florchem imager.
5.4.4 IKK-β *in vitro* Kinase Assay

Following pre-incubation, cells were immediately harvested into ice-cold PBS using rubber cell scrapers as described and the FLAG tagged IKK-β protein isolated by immunoprecipitation (Section 4.9). The isolated protein was then used in an *in vitro* kinase assay with recombinant IκBα protein as substrate (Section 4.10). Briefly, the isolated protein and recombinant protein were added to equilibrated reaction mix along with ATP and the reaction allowed to proceed for 30 minutes at 30°C in either HC or NC environments. Following the reaction, sodium orthovanadate was added to the samples to a final concentration of 0.3mM. Samples were then added to previously prepared phospho-IκBα ELISA plates and levels of phosphorylated IκBα assessed as described (Section 4.8).

5.4.5 ELISA

Levels of phosphorylated recombinant IκBα from the *in vitro* kinase reaction were analyzed using the Pathscan Sandwich ELISA Antibody Pair for Phospho-IκBα (Cell Signalling Technology) as per manufacturer’s instructions as described (Section 4.8). Production of IL8 in cell culture media was assessed using a commercially available Human CXCL8/IL-8 ELISA DuoSet (R&D Systems Inc., Minneapolis, MN) as described (Section 4.8.2). Results were graphed as picogram of IL8 per milliter of cell culture media.
5.5 Results

5.5.1 HCA Reduces NF-κB Activation in SAECs
In small airway epithelial cells HCA significantly reduced both TNF-α and endotoxin induced NF-κB activation as assayed using NF-κB driven luciferase accumulation (Figure 5-1 A). Corresponding with luciferase data, IL-8 secretion was shown to be significantly reduced under hypercapnic conditions compared to NC as measured using ELISA (Figure 5-1 B).

5.5.2 HCA Reduces NF-κB Activation in HBE Cells
HBE cells transduced with the NF-κB lentiviral luciferase reporter virus and injured with TNF-α and endotoxin followed a similar trend to that of SAECs in that NF-κB activation was significantly reduced under hypercapnic conditions when compared to NC (Figure 5-2).

5.5.3 HCA Reduces NF-κB Activation in A549 Cells
A significant NF-κB activation is shown in A549 cells which have undergone TNF-α stimulation. HCA significantly reduced TNF-α induced NF-κB activation. Inhibition of NF-κB was also seen in uninjured cells, although not significant (Figure 5-3 A).
IL-8 production significantly increased with TNF-α stimulation, corresponding to the luciferase assay data. This increase in IL-8 production was also significantly inhibited under conditions of HCA (Figure 5-3 B).

5.5.4 HCA Reduces NF-κB Activation in Systemic Cells
HCA is seen to inhibit NF-κB activation in non-pulmonary cells. HCA decreased TNF-α induced NF-κB activation in HEK 293T cells (Figure 5-4, Panel A), in T84 colonic cells (Figure 5-4, Panel B), and in THP-1 monocytes (Figure 5-4, Panel C).
5.5.5 HCA Modulates TAK/TAB Complex Signalling

Over expression of the TAK1/TAB1/2 complex directly activated NF-κB in A549 cells (Figure 5-5 Panel A), although not as remarkable as that seen with certain other intermediary protein over-expression. This increase in activity is significantly inhibited by an increase in CO₂.

5.5.6 HCA Modulates NIK

Over-expression of NIK directly activated NF-κB in the presence and absence of external stimulation by TNF-α in pulmonary A549 cells (Figure 5-5 Panel B). HCA reduced NF-κB activation at baseline and following TNF-α stimulation in the presence and absence of NIK.

5.5.7 HCA Inhibits IKK-β Phosphorylation and Kinase Activity

Over-expression of the IKK-β subunit in A549 cells activated NF-κB in the presence and absence of external stimulation (Figure 5-5 Panel C). HCA is then shown to reduce NF-κB activation at baseline and following TNF-α stimulation in the presence and absence of IKK-β over-expression. HCA inhibits the phosphorylation, and therefore activation, of IKK-β under baseline conditions and following TNF-α stimulation in A549 cells (Figure 5-6 Panel A). HCA did not modulate the overall concentration of IKK-β at baseline or following TNF-α stimulation but HCA directly inhibits IKK-β kinase activity. IKK-β extracted from cells incubated under conditions of HCA has decreased IκBα kinase activity (Figure 5-6 Panel B). HCA directly reduces the ability of IKK-β to phosphorylate IκBα when the reaction is carried out under HCA (Figure 5-6 Panel C).

5.5.8 HCA Exerts Contrasting Effects on IKK-α and IKK-β Activation

HCA appeared to increase phosphorylation of the IKK-β component of the IKK complex (Figure 5-6). Subsequent experiments demonstrated that HCA specifically enhanced the phosphorylation of the IKK-α subunit by NIK in the presence and absence of TNF-α (Figure 5-7). Levels of FLAG tagged protein
remained unchanged and were not detected in cells transfected with empty vector control samples. β-Actin protein levels were used as a gel loading reference and remained consistent.

**5.5.9 HCA Inhibits IκBα Breakdown**

TNF-α stimulation of HBE cells decreased IκBα concentration at 60 minutes which was restored at 240 minutes under NC conditions (Figure 5-8 Panel A). Hypercapnic acidosis prevented the TNF-α induced degradation of IκBα at 60 minutes. HCA did not decrease or increase protein concentration at other time points (Figure 5-8 Panel A). TNF-α induced IκBα gene transcription which was decreased under HCA conditions (Figure 5-8 Panel B). Further to this, HCA’s effects on TNF-α induced NF-κB activation was not altered by over-expression of IκBα at different concentrations. Cells were exposed to a fixed concentration of TNF-α and increasing concentrations of IκBα (Figure 5-8 Panel C). Similarly in the presence of a fixed IKK-β concentration and increasing IκBα concentrations the effects of HCA are not diminished (Figure 5-8 Panel D).
5.6 Discussion

5.6.1. ARDS, Hypercapnia and the NF-κB Pathway
In the clinical setting, over distension of lung tissue by mechanical ventilation can cause injury and NF-κB activation appears to play a crucial role in mediating this stretch induced lung inflammation and injury (131). One possible mechanism of overcoming this injury is to reduce tidal volumes used in mechanical ventilation (76). This leads to a build up of CO$_2$, known as HCA, which has been shown to be beneficial in the setting of free radical (126), ischemia-reperfusion (302), endotoxin (96), and high stretch ventilation induced (94, 95) lung injury. However HCA can also have adverse effects, including during wound healing (301) and prolonged untreated pneumonia (134). Consequently, a greater understanding of the mechanisms of action of HCA is needed if we are to harness its benefits, while minimizing the potential for harm.

NF-κB is a rapid responder to lung injury leading to release of factors contributing to the initiation of inflammation and further injury in the surrounding tissues (303). The potential for key effects of HCA – both beneficial and deleterious – to be mediated via inhibition of the NF-κB pathway is clear. Takeshita et al. (146) first demonstrated that HCA attenuated endotoxin-induced NF-κB activation by suppressing ΙκBα degradation. More recently, we have demonstrated that HCA reduces mechanical stretch induced injury (131), and decreases pulmonary epithelial wound healing (301) by inhibiting NF-κB. These studies were designed to determine the precise mechanism(s) by which HCA suppresses NF-κB activation.

5.6.2. Hypercapnic Acidosis attenuates NF-κB Activation
Hypercapnic Acidosis attenuates NF-κB activation in the pulmonary epithelium. Specifically, HCA decreased IL-1β and endotoxin induced NF-κB activity, in small airway epithelial cells (SAECs). HCA also decreased TNF-α and endotoxin induced secretion of IL-8 in small airway epithelial cells. These effects were replicated in human bronchial epithelial (HBE) cells and also in alveolar A549
cells, suggesting that this effect of HCA is seen throughout the pulmonary epithelium.

The potential for HCA to attenuate NF-κB activation is not limited to the pulmonary epithelium. We selected three cell lines on which to test the effects of HCA which we have seen to inhibit NF-κB activation in pulmonary cell lines. HEK293 cells are stably transformed human embryonic kidney cells commonly used for the production of therapeutic proteins and viruses for gene therapy. Due to their rapid growth, and the fact that they can be transfected with relative ease, they were used in this experiment. Hypercapnic Acidosis attenuated both baseline and TNF-α induced NF-κB activation in HEK293 cells.

Human colonic T84 cells were originally derived from a lung metastasis of colon carcinoma and are commonly used as a model in the in vitro study of epithelial chloride secretion and contain receptors for a range of peptide hormones and neurotransmitters (288). THP-1 cells are a human acute monocytic leukemia cell line derived from peripheral blood. Both cell lines activated NF-κB in response to TNF-α stimulation, and in both cell types, NF-κB activation was suppressed by HCA. This suggests that, the ability of HCA to suppress NF-κB activation is a fundamental property seen in a wide variety of cell types. In addition, we can further conclude that in this broad selection of cell types that HCA does not activate NF-κB in uninjured cells. This is important in the setting of ventilation strategies where the use of HCA as a therapeutic in lung injury could be counterproductive if it caused injury to other organ systems.

5.6.3. Mechanisms by which Hypercapnic Acidosis attenuates NF-κB Activation

NF-κB has become a major focal point in the setting of injury and repair due to its numerous stimuli, complex activation of canonical and non-canonical pathways and wide range of proteins and DNA binding sites which makes it an ideal candidate for counteracting the progress of inflammation and further injury (304). A key aim of these studies was to determine the precise mechanism(s) by which HCA suppresses NF-κB activation. While the potential for HCA to modulate NF-κB activation via inhibition of IκBα breakdown was already known (146), the potential for HCA to exert effects at other points in the NF-κB activation pathway
had not been examined. In our studies, over-expression of both NIK and IKK-β potently activated the NF-κB pathway, an effect enhanced by TNF-α or IL-1β stimulation. These results show that over-expression of these genes activates NF-κB but they do not necessarily conclude that HCA exerts its inhibitory effect on these particular proteins, merely that the effects occur downstream of these proteins.

We then examined the activity of the IKK complex, specifically focusing on IKK-2, which phosphorylates IκBα, tagging it for proteasomal digestion. We found that HCA decreased the phosphorylation and activation of IKK-2, but did not affect total IKK-2 protein expression. HCA also directly attenuated the kinase activity of the activated IKK-2, decreasing its ability to phosphorylate IκBα. These findings suggest that the inhibition of IκBα degradation by HCA is a result of impaired IKK-2 activity rather than any alteration in proteasome activity.

The phosphorylation of IκBα subunit of the inhibitor of kappa B (IκB) complex, is the final step in the canonical NF-κB signaling pathway, and leads to the unbinding and activation of the NF-κB heterodimer. The potential for HCA to inhibit the decrease in cytoplasmic IκB has previously been described (305). Our data confirm and extend these findings. HCA maintains cytoplasmic IκBα by reduces its breakdown and cellular turnover. HCA does not enhance the generation of ‘new’ IκBα; in fact, HCA decreased IκBα mRNA. The reduced cellular turnover of IκBα appears largely mediated via the HCA induced reduction in IKK-2 activation and function, which phosphorylates and inactivates the IκBα. Taken together with our earlier findings, these data suggest that the IKK-complex is a major locus of control for HCA on the NF-κB signaling pathway.

We found that HCA significantly inhibited the activation of NF-κB by the NIK, a potent activator of the canonical (and non-canonical) pathway. Interestingly, further experiments conducted to determine whether this was a specific effect of HCA on NIK or simply an effect of HCA on the distal pathway showed that HCA enhanced NIK induced phosphorylation of the IKK-1 subunit of the IKK complex. NIK activates the ‘non-canonical’ NF-κB pathway via
phosphorylation of IKK-1 which subsequently phosphorylates p100 (306), targets it for degradation to form the p52 subunit, which then heterodimerises with RelB. This p52/RelB dimer has diverse effects in the nucleus, where it can modulate canonical NF-κB promoter expression. In addition, the activated IKK-1 can translocate to the nucleus, where it may exert inhibitory effects on NF-κB. Cummins et al recently demonstrated that HCA increases IKK-1 and RelB nuclear translocation, providing an additional mechanism by which HCA may modulate NF-κB signaling (174, 307). Our studies support this finding, and suggest that the HCA induced nuclear translocation of IKK-1 may be initiated via a phosphorylation event.

5.6.4. Implications of effects of Hypercapnic Acidosis on the NF-κB Pathway

Our work is supports and extends the work conducted by Takeshita et al. (146) who showed that HCA attenuated endotoxin-induced NF-κB activation by suppressing IκBα degradation in human pulmonary artery endothelial cells (HPAEC) but expands on their point by showing the effect of HCA on pulmonary cell lines. By demonstrating that HCA has a beneficial effect on three separate pulmonary cell lines in vitro this highlights the potential therapeutic advantages of HCA in the setting of lung injury. While it has been established by various in vitro, ex vivo and in vivo models (99) that this build up of CO₂ is well tolerated, and, indeed, therapeutic in the setting of ALI, it is less studied in other organ systems. Multiple organ dysfunction syndrome is often a detrimental outcome of ARDS (308), which is poorly understood and therefore needs to be addressed in the setting of therapeutic and permissive HCA. HCA in other organ injury has been assessed in injured myocardial tissue and in the brain with positive outcomes (reviewed in (309)) and also in hepatocytes and kidney cells (reviewed in (99)).

5.6.5. Summary

In summary, the current findings provide evidence to suggest that the interaction between CO₂ and the NF-κB pathway occurs at multiple points in the canonical signaling pathway.
5.7 Figures

**Figure 5-1:** The Effects of Hypercapnic Acidosis on NF-κB Activation in Small Airway Epithelial Cell. HCA reduced TNF-α and LPS induced NF-κB activity, in small airway epithelial cells (SAECs) compared to NC (Panel A). HCA decreased TNF-α and LPS induced secretion of IL-8 in SAECs (Panel B).

Columns represent mean values (n=3). Error bars represent standard deviation.

* = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student's t-Tests, p≤0.05)
Figure 5-2: The Effects of Hypercapnic Acidosis on NF-κB Activation in Human Bronchial Epithelial Cells. HCA decreased TNF-α and LPS induced NF-κB activation in HBE cells.

Columns represent mean values (n=3). Error bars represent standard deviation.

* = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student’s t-Tests, p≤0.05)
Figure 5-3: The Effects of Hypercapnic Acidosis on NF-κB Activation in A549 Pulmonary Cells. HCA reduced baseline and TNF-α induced NF-κB activity (Panel A) & TNF-α induced IL-8 secretion (Panel B) in A549 pulmonary cells.

Columns represent mean values (n=3). Error bars represent standard deviation. * = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student’s t-Tests, p≤0.05)
Figure 5-4: The Effects of Hypercapnic acidosis on NF-κB Activation in Systemic Cell Lines. HCA significantly reduces NF-κB activation in HEK293 cells (Panel A), human colonic T84 cells (Panel B) and human monocytic THP-1 cells (Panel C).

Columns represent mean values (n=3). Error bars represent standard deviation. * = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student’s t-Tests, p≤0.05)
Figure 5-5: The Effects of Hypercapnia on NF-κB Activation in the Presence of Over-Expressed Pathway Activators. HCA significantly reduces NF-κB activation in A549 cells over expressing TAK1/TAB1, 2 complexes (Panel A), NIK (Panel B) and IKK-β (Panel C).

Columns represent mean values (n=3). Error bars represent standard deviation. * = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student’s t-Tests, p≤0.05)
Figure 5-6: The Effects of Hypercapnia on IKK-β Kinase Activity. HCA inhibits phosphorylation of IKK-α/β but does not alter total IKK-β concentrations (Panel A). IKK-β protein isolated from A549 cells treated with HCA has decreased IκBα kinase activity compared to NC (Panel B). In vitro experiments using isolated IKK-β from NC cells also show decreased kinase activity when exposed to HCA compared to NC (Panel C).

Columns represent mean values (n=3). Error bars represent standard deviation. * = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student's t-Tests, p≤0.05)
Figure 5-7: The Effects of Hypercapnia on the IKK-α and IKK-β Subunits in the Presence of NIK Over-Expression. HCA induces IKK-α/β phosphorylation in the presence of NIK over expression. This increase is due to increased phosphorylation of IKK-α rather than β. Levels of FLAG tagged NIK protein remained unchanged and were not detected in cells transfected with empty vector (EV) control samples. β-Actin protein levels were used as a gel loading reference and remained consistent. Image representative of three independent experiments.
Figure 5-8: The Effects of Hypercapnia on IκBα Degradation. HCA attenuates TNF-α induced decrease in IκBα (Panel A) and decreases TNF-α induced transcription of the IκBα gene (Panel B).

Columns represent mean values (n=3). Error bars represent standard deviation.

* = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student’s t-Tests, p≤0.05)
Figure 5-8: The Effects of Hypercapnia on IκBα Degradation (Continued).

Over expression of IκBα inhibits NF-κB activation by TNF-α where A549 cells were exposed to a fixed concentration of TNF-α and increasing concentrations of IκBα (Panel C) and in the presence of a fixed IKK-β concentration and increasing IκBα concentrations the effects of HCA are not diminished (Panel D). This inhibition is dose dependent and does not abolish the effect of HCA on NF-κB activation.

Columns represent mean values (n=3). Error bars represent standard deviation. * = Significant inhibition with respect to Normocapnia, † = significant injury with respect to Normocapnia, 0ng sample (Student's t-Tests, p≤0.05)
6. The Effects of Hypercapnic Acidosis on the Activity of the NF-κB Dimeric Protein

6.1 Abstract

**Introduction:** Following the activation and degradation of the cytosolic inhibitor IκBα, the NF-κB hetero- or homo-dimer is released, translocates to the nucleus and binds to its consensus DNA sequences to initiate gene transcription. We have demonstrated the potential for HCA to inhibit activation of NF-κB via the canonical pathway. Our findings suggested the possibility that HCA might suppress the activity of the NF-κB heterodimer. We wished to examine the potential for HCA to (a) inhibit nuclear translocation of NF-κB; (2) decrease NF-κB binding to its consensus sequences; and/or (3) inhibit NF-κB directed DNA transcription and/or protein translation.

**Objectives:** To investigate the potential for HCA to decrease the activity of the NF-κB Heterodimer, and elucidate the mechanism underlying this effect.

**Methods:** Nuclear extracts were prepared from cells treated with NC or HCA and Western blotting used to assess p65 nuclear translocation. P50 and P65 homo and heterodimers were formed under NC and HCA conditions and visualised using PAGE and silver staining. EMSA was used with NF-κB consensus sequence DNA, reacted with these dimers and nuclear extracts. P65 binding assays were also used to assess the direct effects of HCA on binding activity of activated nuclear extracts. Finally a transcription/translation assay was performed under NC and HCA conditions to determine the effects of increased CO₂ on gene transcription and translation.

**Results:** HCA suppressed the nuclear translocation and DNA binding of NF-κB in cytokine activated pulmonary cells. HCA had no effect on the formation of homo or heterodimers. HCA decreased the affinity of the NF-κB dimer to bind to the NF-κB consensus DNA sequence. CMV driven gene transcription was also
significantly decreased under conditions of HCA. HCA did not affect protein translation.

**Conclusion:** HCA suppresses the activity of the NF-κB heterodimer via (1) inhibiting nuclear translocation of the NF-κB p65 protein; and (2) decreasing p65 NF-κB binding to its consensus sequence. HCA may also (3) inhibit DNA transcription but has no effect on protein translation.
6.2 Introduction

The potential for key effects of HCA – both beneficial and deleterious – to be mediated via inhibition of the NF-κB pathway is clear. Takeshita et al. (146) first demonstrated that HCA attenuated endotoxin-induced NF-κB activation by suppressing IκBα degradation. More recently, we have demonstrated that HCA reduces mechanical stretch induced injury (131), and decreases pulmonary epithelial wound healing (301) by inhibiting NF-κB. These studies were designed to determine the precise mechanism(s) by which HCA suppresses NF-κB activation. In chapter 5, we demonstrated that HCA inhibits several discrete steps on the canonical NF-κB pathway, by modulating the activation state and/or activity of key components of the cytosolic inhibitor IκBα, the IκB kinase (IKK) complexes, and the NF-κB inducing kinase (NIK) complex.

Five proteins which share a Rel homology domain (RHD) make up the NF-κB family of proteins, combinations of which form homo- or heterodimers to yield over a dozen transcription factor complexes (310). Upon activation of NF-κB, several of the protein dimers are activated and bind cis-regulatory regions of NF-κB target genes which have multiple κB sites (311). As well as this, different NF-κB complexes are activated in different cells in response to a range of signals. Therefore, predicting which NF-κB dimer will bind which κB sequence remains a challenge (312). Of the potential dimers that can form p50 and p52 homodimers as well as p50-p52 heterodimers bind DNA but do not possess transcriptional activity. RelB homodimers, p65-RelB and cRel-RelB dimers do not bind DNA (313).

As discussed in Chapter 5, NF-κB dimers are retained in an inactive form in the cell cytoplasm by the IκB proteins. Upon phosphorylation of the IκB protein, the dimer is released and allowed to translocate to the nucleus for DNA binding. In the canonical NF-κB pathway inactive p50-p65 heterodimers are predominantly found bound to IκBα and p50 proteins are present in their inactive forms as precursor p105 proteins. p105, along with the p52 precursor protein p100, can also dimerise with NF-κB proteins preventing translocation and DNA binding (314, 315). Due to the ability of the precursor proteins to bind NF-κB complexes,
activation of the non-canonical pathway, leading to proteasomal processing of these proteins, releases the NF-κB dimers and gives rise to canonical signalling (316). The p100 gene promoter is NF-κB dependent (317). Therefore, theoretically, activation of the canonical pathway increases levels of p100 protein which leads to increased levels of p52 and non-canonical signalling (318).

Formation of NF-κB dimers is governed by several biochemical reactions. Rel homology domains must be synthesised and these must then be dimerised to avoid degradation. Following dimerisation, the NF-κB proteins interact with the IκB proteins at the ankyrin repeat domain at the dimeric interface (152). IκB proteins associated with NF-κB are targeted by a canonical IKK mechanism for degradation via the 20S proteasome pathway (153). Activation of the canonical pathway via TNFR, IL-1βR or TLR ligand binding induces phosphorylation of the NEMO/IKK complex which in turn phosphorylates the IκBα protein leading to its proteasomal degradation (154).

The physiological role of p50-p50 homodimers is unclear; however some NF-κB regulated genes are maintained in an inactive state by p50 homodimer complexes which occupy DNA binding sites (156). Our findings in Chapter 5 suggested the possibility that HCA might suppress the activity of the NF-κB heterodimer. We hypothesized that HCA would: (a) inhibit nuclear translocation of NF-κB; (2) decrease NF-κB binding to its consensus sequences; and/or (3) inhibit NF-κB directed DNA transcription and/or protein translation.

6.3 Aims

To examine the potential for HCA to (a) inhibit nuclear translocation of NF-κB; (2) decrease NF-κB binding to its consensus sequences; (3) inhibit NF-κB directed DNA transcription and/or (4) inhibit protein translation.
6.4 Methods

6.4.1 Cell Culture
Nuclear extracts were prepared as described (Section 4.3). Briefly, A549 cells were seeded in 150mm tissue culture dishes and allowed to reach 90% confluence. Cells were pre-incubated for 1 hour and TNF-α added for a further 1 hour. Cells were immediately harvested on ice and nuclear extraction performed using the NE-PER nuclear extraction kit (Pierce Thermo Fisher Scientific Ltd., Waltham, MA, USA) as per manufacturer’s instructions. Samples were aliquoted and stored at -80°C until use. BCA assay was performed on the nuclear and cytoplasmic fractions (Section 4.4) and protein concentrations normalised.

6.4.2 Western Blotting
A549 nuclear and cytoplasmic extracts were separated on polyacrylamide gels as described (Section 4.11). Membranes were probed with p65 protein antibody and imaged using a Florschim imager.

6.4.3 Protein Dimerisation and EMSA
p50 and p65 homo and heterodimers were prepared as described (Section 4.12) in the presence and absence of HCA. Samples were loaded onto non-denaturing 10% acrylamide gels and visualised using silver stain (Fermentas) (Section 4.12). Dimers were also used in EMSA to assess NF-κB binding by incubating them with a biotin labelled NF-κB consensus sequence nucleotide dimer under conditions of NC and HCA (Section 4.14). Samples were loaded onto non-denaturing gels and transferred to nylon membranes. Membranes were probed using streptavidin HRP and imaged by chemiluminescence. Previously prepared A549 nuclear extracts were also incubated with the NF-κB consensus sequence and assessed in the same manner.

6.4.4 p50 and p65 Binding Assays
Effects of HCA on transcription factor binding were assessed using a NF-κB (p50 or p65) transcription factor assay kit (Cayman) (Section 4.14). 10µg of nuclear
extract was added to the provided plate and allowed to bind under NC or HCA. P50 and p65 binding was assessed as per manufacturer’s instructions.

### 6.4.5 In vitro Transcription/Translation Assay

The effect of HCA on transcription/translation was assessed using the TNT® Quick Coupled Transcription/Translation System (Promega) as described (Section 4.15). The master mix was equilibrated at 30°C in either NC or HCA for 10 minutes after which the T7 RNA polymerase was added and the reaction allowed to proceed for 60 minutes. Samples were then mixed with Bright-Glo luciferase substrate (Promega) and luminescence assessed in a VICTOR™ multilabel plate reader (Perkin Elmer). Samples were then harvested and RNA extraction performed using TRIzol (Section 4.15). 1μL of the isolated RNA from NC sample was added to equilibrated master mix and the reaction repeated. Samples were then mixed with Bright-Glo luciferase substrate and assessed in a VICTOR™ luminometer.
Chapter 6

6.5 Results

6.5.1 HCA does not affect NF-κB Subunit Dimerisation
Following release from IκBα, NF-κB is activated and the p50 and p65 subunits translocate to the nucleus as dimers. The mechanism of formation of these subunit dimers is not yet fully understood, but it is known that p50/p65 heterodimers promote the most powerful induction of pro-inflammatory genes such as IL-6 or MIP-2α. In contrast p50/p50 homodimers are often considered to be inhibitory to gene transcription. The formation of p50/50 and p50/p65 dimers was not altered by HCA (Figure 6-1, Panel A).

6.5.2 HCA Decreases NF-κB Dimer Activity
HCA did not modulate the binding of p50/p65 heterodimers to its DNA consensus sequences in vitro (Figure 6-1, Panel B). HCA abolished the increase in binding of NF-κB to its DNA consensus sequences in A549 monolayers in response to TNF-α stimulation (Figure 6-1, Panel C). HCA may decrease binding of NF-κB to its DNA consensus sequences by decreasing the amount of nuclear NF-κB and/or by decreasing the affinity of NF-κB for its consensus sequence. HCA decreased nuclear translocation of p65 following TNF-α stimulation (Figure 6-1, Panel D) compared to NC.

6.5.3 HCA Inhibits p65, but not p50, Nuclear Accumulation and Binding to the NF-κB Consensus Sequence
Transcription factor binding ELISA studies demonstrated that. HCA ablated the nuclear accumulation of the NF-κB p65 subunit following TNF-α activation of A549 cells compared to NC (Figure 6-2, Panel A). In contrast, HCA only modestly decreased the binding of exogenous p65 to its consensus sequence compared to NC (Figure 6-2, Panel B). The nuclear accumulation of the NF-κB p50 protein following TNF-α stimulation in A549 cells was not affected by HCA (Figure 6-3, Panel A). Similarly, the binding of exogenous p50 protein to its consensus sequence was not affected (Figure 6-3, Panel B)
6.5.4 HCA Inhibits DNA Transcription and Translation

Following binding of NF-κB to its consensus sequence, transcription of NF-κB dependent genes is initiated, followed by translation of the mRNA to produce the effector molecules. Using a commercially available \textit{in vitro} transcription/translation assay it was shown that reactions performed under HCA showed reduced production of a (non-NF-κB specific) T7 promoter dependent luciferase protein compared to NC \textit{in vitro} (Figure 6-4 Panel A). RNA was then isolated from samples which had undergone the reaction in NC conditions. This RNA was used in a further translation assay performed under NC and HCA conditions. It was demonstrated that HCA conditions significantly decreased RNA translation to luciferase protein (Figure 6-4, Panel B).
6.6 Discussion

6.6.1 Actions of the Activated NF-κB Dimer
Activation of the NF-κB pathway leads to the dissociation and subsequent proteasomal degradation of the IκB proteins which retain the NF-κB dimers in the cytoplasm of the cell (291). Following the unbinding, the NF-κB dimers undergo translocation to the nucleus for DNA binding at κB sites of NF-κB inducible genes. The formation of NF-κB dimers, their interaction with IκB proteins, nuclear translocation and DNA binding all rely on the RHD of the NF-κB proteins (315, 319). Of the twelve possible dimeric combinations of Rel proteins, the p50/p65 heterodimer is known to be the most potent NF-κB protein complex (320).

The p50 protein is synthesized in cells as p105 and undergoes proteasomal processing to p50. It lacks a TAD and either forms homodimers or binds p65, c-Rel or RelB (321) thereby giving it transcriptional activity. As a homodimers it is thought to play a role in negative regulation of transcription and it blocks κB binding sites from TAD containing dimers (156).

6.6.2 Effects of HCA on the Activated NF-κB Dimer
The formation of p50/p50 homodimers and p50/p65 heterodimers was not altered by the application of HCA in vitro. Following this we sought to investigate if HCA plays a role beyond the formation of NF-κB dimers. HCA did not alter the binding of recombinant NF-κB dimers to consensus sequence DNA. This experiment was repeated using nuclear extracts from A549 cells which were stimulated with TNF-α under NC and HCA conditions. HCA reduced NF-κB binding to the consensus sequence extracts from in TNF-α stimulated cells. To demonstrate that this was not solely due to decreased translocation of the NF-κB proteins, the binding reaction was performed under conditions of HCA using cell extracts which had been cultured in NC and activated with TNF-α and compared to vehicle control. Equal quantities of nuclear protein were examined for p65 protein content by Western blot. It was shown that cells treated with TNF-α and HCA had decreased nuclear translocation of the p65 protein. Because it has been
demonstrated here that HCA does not affect the binding of recombinant proteins in an *in vitro* model but effects are seen in cell extracts it can be deduced that HCA acts elsewhere to decrease binding. To concentrate more definitely on the p65 protein, a transcription factor binding ELISA was utilised to show that translocation of p65 was reduced following TNF-α activation of A549 cells. When this reaction was carried out under HCA conditions using extracts prepared in NC, it was shown that HCA significantly reduced the binding ability of the p65 protein. Application of HCA to the binding reaction of p50 to its consensus sequence had little to no effect on either translocation or binding. It can now be deduced from this result that the protein binding seen in the EMSA is more likely to be p65 rather than p50. Once an activated transcription factor binds the κB consensus sequence, transcription and translation of NF-κB proteins ensues. It remained to be determined if HCA also affected the process of the NF-κB pathway at this point. We had seen that the p50 protein remains relatively unchanged whereas the p65 protein seems to be affected at numerous steps. To determine if HCA had an effect on the transcription and translation of mRNA and proteins respectively we utilized a cell free mammalian expression system. HCA inhibited T7 promoter driven luciferase production compared to NC. In contrast the effects of HCA on translation, if any, were relatively modest. This suggests that effects of HCA on the transcription/translation process – which are not confined to NF-κB driven genes - are mediated mainly via effects on DNA transcription.

**6.6.3 Summary and Conclusions**

In summary, the current findings provide evidence to suggest that HCA can inhibit the activity of the NF-κB dimer. The mechanisms by which this suppression is mediated includes: (a) inhibition of nuclear translocation of NF-κB; and (2) decreased NF-κB binding to its consensus sequences. HCA may also (3) inhibit DNA transcription but has no effect on protein translation.
6.7 Figures

![Figure 6-1: The Effects of Hypercapnia on NF-κB Subunit Dimer Formation.](image)

The formation of p50/50 and p50/p65 dimers was not altered by HCA (Panel A) as visualized by silver staining. HCA did not modulate the *in vitro* binding of recombinant NF-κB dimers to the DNA consensus sequences (Panel B). p50/p50 and p50/p65 dimers were examined for NF-κB consensus sequence binding ability under NC and HCA by EMSA. HCA decreased intracellular binding of NF-κB to its DNA consensus sequences (Panel C). Nuclear extracts prepared from TNF-α stimulated A549 cells under HCA conditions had decreased binding to the NF-κB consensus sequence compared to cells incubated under NC conditions. HCA decreased translocation of NF-κB/p65 to the nucleus (Panel D). Nuclear extracts prepared from TNF-α stimulated A549 cells under HCA conditions, following standardisation for protein content, were Western blotted for NF-κB/p65. TNF-α induced accumulation of p65 in the nucleus, which was inhibited by HCA. Images representative of 3 experiment repeats.
Figure 6-2: The Effects of Hypercapnia on Nuclear p65 Translocation and Binding to the NF-κB Consensus Sequence. HCA ablated the nuclear accumulation of the NF-κB p65 subunit following TNF-α activation of A549 cells compared to NC (Panel A). A549 cells were grown to confluence and incubated with TNF-α for 30m. Under conditions of HCA there was decreased binding to NF-κB consensus DNA sequences compared to cells incubated in NC. In contrast, HCA only modestly decreased the binding of exogenous p65 to its consensus sequence compared to NC (Panel B).

Columns represent mean values (n=3). Error bars represent standard deviation.
* = Significant inhibition with respect to Normocapnia, † = significant injury with respect to vehicle (Student’s t-Tests, p≤0.05)
Figure 6-3: The Effects of Hypercapnia on Nuclear p50 Translocation and Binding to the NF-κB Consensus Sequence. HCA did not affect the translocation of p50 to the nucleus or binding of the p50 protein to its consensus sequence.

Columns represent mean values (n=3). Error bars represent standard deviation.
Figure 6-4: The Effects of Hypercapnia on DNA Transcription and Translation. HCA decreased the *in vitro* transcription and translation of luciferase protein from a T7-driven luciferase reporter construct compared to NC (Panel A). When mRNA isolated from the reaction performed under NC was used in a further assay, it was shown that HCA significantly decreased its translation to protein (Panel B).

Columns represent mean values (n=3). Error bars represent standard deviation. * = Significant inhibition with respect to Normocapnia, (Student's t-Tests, p≤0.05)
Chapter 7

7. *In vivo* Materials and Methods

7.1 Approval and Ethical Issues

All experiments were conducted under license from the Department of Health, Government of Ireland. Approval was received from the Animal Care Research Ethics Committee of National University of Ireland, Galway. All *in vivo* experiments were carried out using specific pathogen free adult male CD® Sprague Dawley rats, obtained from Charles River Laboratories, Kent, United Kingdom.

7.1.2 Animal Health and Distress Scoring

All animals were continuously monitored for pain, distress, illness, morbidity or mortality with the necessary actions taken to avoid such circumstances. Animal welfare was priority and it was ensured that no unnecessary distress or harm was suffered. Prior to the commencement of *in vivo* experimentation and following experimental procedures, animal models underwent distress scoring to ascertain their health and well being. Distress scoring was performed both before injury and 24 hours following injury and treatment according to table 1 (Appendix). Animals reaching a distress score of 12 or more were euthanized.
7.2 Injury Model

7.2.1 Oro-Tracheal Intubation

Animals were anaesthetised using an isoflurane inhalation gas anaesthesia system (3-5% isoflurane in 100% O₂ at 2L·min⁻¹) and intra-peritoneal (IP) injection of ketamine (40mg·kg⁻¹) (Pfizer, Kent, United Kingdom). Depth of anaesthesia was confirmed using paw clamp. IV cannulation was performed using a 22G IV catheter (BD Insyte®; Becton Dickinson Ltd., Oxford, United Kingdom) via the tail vein. Orotracheal intubation was performed using a method described previously (322). After confirmation of depth of anaesthesia, the animal was positioned on an inclined metal plate by hooking a Mersilene ribbon under the upper incisors. An otoscope was used to visualise the vocal cords and a guide wire introduced to the trachea. A 14G IV catheter was placed over the guide wire and inserted to the trachea. The guide wire was carefully removed and correct position of the tube confirmed by connecting the ventilator and inspecting the movement of the thorax and absence of stomach distention.

7.2.2 Pre-Injury Ventilation

Animals were ventilated using small animal ventilators (CWE SAR 830 AP, CWE Inc, Pennsylvania, USA) with a respiratory rate of 80 breaths per minute, flow rate of 500mL/min, tidal volume of 6mL/kg, a positive end-expiratory pressure of 2cm H₂O with an inspired gas mixture of FIO₂ 0.3. Recruitment manoeuvres were performed every 10 minutes for 20 to 30 breaths using a positive end-expiratory pressure of 10cm H₂O. Anaesthesia was maintained throughout the ventilation procedure via IV injection of Alfaxan® (Alfaxadone 0.9% and alfadolone acetate 0.3%; Vétoquinol S A, BP 189, 70204 Lure Cedex, France) at 5–20 mg/kg/h. Cisatracurium besilate (6mg·kg⁻¹·h⁻¹; Nimbex, GlaxoSmithKline, Dublin, Ireland) was administered IV to produce muscle relaxation. Body temperature was maintained at approximately 37°C using a homoeothermic blanket system and monitored using a rectal probe (Harvard Apparatus, Holliston, MA).
7.2.3 Assessment of Baseline Lung Compliance

After 20 minutes of pre-injury ventilation, baseline static compliance was measured by administering 1mL volumes of room air, 3 seconds apart, for a total of 5mL via the intubation tube and pressure changes recorded from ventilation trace. Baseline compliance greater than 0.5mLs/cm H₂O was required before proceeding with injurious ventilation.

7.2.4 Injury Ventilation

The injurious ventilation protocol was implemented once baseline criteria were met. Ventilator settings were altered to allow for a pressure controlled system with a respiratory rate of 18 breaths per minute, flow of 550cc/min, a peak inspiratory pressure of 35cm H₂O and a positive end-expiratory pressure of 0cm H₂O. These ventilator settings produced a tidal volume of approximately 40mL/kg. Anaesthesia, temperature and inspired gas mixture criteria remained unchanged. The injurious ventilation strategy was continued until static compliance had decreased by 50% from baseline measurements. Once a 50% decrease was confirmed, ventilation settings were returned to pre-injury settings.

7.2.5 Recovery and Extubation

Once the desired decrease in compliance had been reached, ventilation settings were returned to that of pre-injury settings with a respiratory rate of 80 breaths per minute, flow rate of 500cc/min, a positive end-expiratory pressure of 2cm H₂O with FIO₂ 1.0 inspired gas. At this point anaesthesia was ceased and ventilation continued until spontaneous breathing occurred. Animals were extubated once full consciousness had returned and limb and head movement were demonstrated. Animals were then placed in individual ventilation cages (Tecniplast Inc., Buguggiate, Italy) and allowed to fully recover from anaesthesia.
7.3 Assessment of the Extent of Recovery Following VILI

7.3.1 Surgical Tracheostomy
Following the indicated recovery times, animals were re-anaesthetised and subjected to surgical tracheostomy and carotid artery canulation as previously described (286). Briefly, animals were anaesthetised via IP injection of ketamine 80 mg·kg\(^{-1}\) (NARKETAN-10 100mg/ml, Vétoquinol, Dublin, Ireland) and xylazine 8 mg·kg\(^{-1}\) (Xylapan, Vétoquinol, Dublin, Ireland). Depth of anaesthesia was confirmed by a lack of reaction to paw clamp. IV canulation was performed on the tail vein using a 22G IV catheter and anaesthesia maintained using Alfaxan (Alfaxadone 0.9% and alfadolone acetate 0.3%; Schering-Plough, Welwyn Garden City, United Kingdom) at 5–20 mg·kg\(^{-1}\)·h\(^{-1}\). Cisatracurium besilate (6mg·kg\(^{-1}\)·h\(^{-1}\); Nimbex, GlaxoSmithKline, Dublin, Ireland) was administered IV to produce muscle relaxation. Tracheostomy was performed by removing fur and performing blunt dissection using arterial forceps to reveal the trachea. A tracheostomy tube with an internal diameter of 2mm was inserted between the 4\(^{th}\) and 5\(^{th}\) tracheal rings and secured. Muscles and tissue to the right of the trachea were separated and retracted to reveal the carotid artery. Following dissection of the vagus nerve off the artery, intra-arterial access was gained using a 22G IV canula. 250U heparin was given intra arterially, diluted to 1mL in Hartmann’s IV solution. Airway pressures, arterial pressures and temperature were continuously monitored throughout the experimental procedure.

7.3.2 Harvest Ventilation Parameters
Animals were ventilated using small animal ventilators (CWE SAR 830 AP, CWE Inc, Pennsylvania, USA) with a respiratory rate of 80 breaths per minute, flow rate of 500cc/min, a positive end-expiratory pressure of 2cm H\(_2\)O with an inspired gas mixture of FIO\(_2\) 0.3. Recruitment manoeuvres were performed every 10 minutes for 20 to 30 breaths using a positive end-expiratory pressure of 10cm H\(_2\)O. Anaesthesia was maintained throughout the ventilation procedure via IV infusion of Alfaxan (Alfaxadone 0.9% and alfadolone acetate 0.3%; Schering-Plough, Welwyn Garden City, United Kingdom) at 5–20 mg/kg/h, titrated to mean arterial pressure. Cisatracurium besilate (6mg·kg\(^{-1}\)·h\(^{-1}\); Nimbex,
GlaxoSmithKline, Dublin, Ireland) was administered IV to produce muscle relaxation. Body temperature was maintained at approximately 37°C using a homoeothermic blanket system and monitored using a rectal probe (Harvard Apparatus, Holliston, MA).

7.3.3 Physiological Parameter Measurements
After 20 minutes of baseline ventilation, an arterial blood sample was obtained and examined using a blood gas analyser (ABL 705; Radiometer, Copenhagen, Denmark). Static compliance was then measured via the tracheostomy tube and pressure changes recorded from ventilation. Following measurement of arterial blood gas and static compliance the FIO$_2$ 0.3 inspired gas mixture was exchanged for FIO$_2$ 1.0 for 15 minutes to calculate the alveolar-arterial oxygen gradient. A further blood gas sample was taken at the end of the 15 minute ventilation period and analysed as described.

7.3.4 Euthanasia
At the end of the harvest ventilation protocol animals were given 400U.kg$^{-1}$ IV heparin (CP Pharmaceuticals, Wrexham, United Kingdom, and sacrificed by exsanguinations under anaesthesia. Death was verified by observation of permanent cessation of the circulation.
7.4 Sample Collection

Following confirmation of death the heart lung block was dissected from the thorax and the carcass disposed of in accordance with in-house procedures. A note of the physical appearance of the lungs was taken before sample collection commenced.

7.4.1 Wet:Dry Ratio

To measure the Wet:Dry ratio, the right postcaval lobe was isolated and removed. The wet lobe was weighed and then incubated at 40°C for 72 hours. After 72 hours the lobe was re-weighed and the Wet:Dry ratio calculated.

7.4.2 Bronchoalveolar Lavage

BAL fluid collection was performed by injecting 15mL of sterile saline (0.9% NaCl) in 5mL increments. Each increment was allowed to flow back from the lung and collected and pooled in sterile 15mL tubes. Volume of collected BAL fluid was noted and 1mL removed for total and differential cell counts. The remaining BAL fluid was centrifuged at 1500 x g for 10 minutes and the clear supernatant stored at -80°C in 1mL aliquots.

7.4.2.1 Total BAL Cell Count

1mL of the total BAL fluid was centrifuged at 4000xg for 3 minutes and the supernatant removed. The remaining cell pellet was resuspended in 250mL of sterile 0.9% NaCl solution and 50µL of this diluted 1:1 in Trypan blue vital stain (0.4% solution, Sigma-Aldrich, St. Louis, MO, USA). 20µL of this solution was visualised on a haemocytometer using a light microscope. All enucleated cells were counted in the five corners and centre square of the haemocytometer. Cell number per millilitre was calculated by dividing the total number counted by 5 and multiplying by 10,000 with a dilution factor of 0.5.
7.4.2.2 Differential Cell Count

1mL of the total BAL fluid was centrifuged at 4000xg for 3 minutes and the supernatant removed. The remaining cell pellet was resuspended in 250mL of sterile 0.9% NaCl solution and 150µL of this added to a cytospin cartridge (Thermo-Fisher Scientific) assembled with a clean glass slide in the cytospin apparatus. The sample was then centrifuged at 200 RPM for 3 minutes and the slide removed from the chamber. Differential staining was performed using a variant of the Romanowsky stain as follows; after air drying, the slide was fixed by immersing 6 times in methanol and stained with 5 immersions in Eosin Y and 3 in methylene blue. After drying, stained cells were visualised using a light microscope and inflammatory cells counted. Total inflammatory cells were counted to 300 and neutrophils expressed as a percentage of the total. The total cell count (Section 7.4.2.1) was used to determine the neutrophil count per millilitre of BAL fluid.

7.4.3 Whole Lung Homogenate and Right Lobe Fixation

Following bronchoalveolar lavage fluid collection, the right superior, middle and inferior lobes were isolated and removed for whole lung homogenate analysis. Lobes were crudely homogenised and stored in two separate aliquots at -80°C until use. The left lobe was perfused and inflated via the tracheal catheter using a 4% w/v Paraformaldehyde in phosphate buffered saline solution. Once the lobe was completely inflated, it was isolated and the trachea and heart removed. The lobe was then submerged in paraformaldehyde solution and stored.
7.5 Sample Analysis

7.5.2 Protein Assay

Protein in samples was quantified using the Pierce BCA protein assay kit (Pierce Thermo Fisher Scientific Ltd., Waltham, MA, USA) using the recommended protocol as follows:

Working solution of 1:50 ratio of reagent A and reagent B was prepared as follows;

\((\text{number of standards} + \text{number of unknowns}) \times \text{Replicates} \times \text{amount of working reagent to be added} (100 \mu\text{L}) = \text{Total amount needed})\)

Standards were prepared using BSA diluted in PBS with a top value of 2000 µg/mL. BAL fluid was diluted 1 in 20 using PBS and 50 µL of this added to a round bottom 96 well plate in triplicate. 100 µL of working solution was added to each well and the plate incubated at 37°C for 30 minutes. The plate was then read at 562 nm using a Victor luminometer (Perkin Elmer, Waltham, MA). Protein was quantified using the line equation from the standard curve and the quadratic equation:

\[ x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \]

7.5.3 Enzyme-Linked Immunosorbent Assay

Samples were analyzed according to manufacturer’s instructions as follows;

Capture antibody was diluted to 4 µg/mL in 1X PBS and 100 µL added per well of a 96 well microplate. The plate was sealed and incubated overnight at room temperature. Following incubation, the wells were aspirated and washed three times with 400 µL per well of wash buffer (1X PBS, 0.5% Tween-20). Plates were then blocked using 300 µL per well of blocking buffer (1% BSA, in PBS with 0.5% NaN₃) and incubated for one hour at room temperature. The wash steps were repeated. Samples were diluted 1:10 in reagent diluent (0.1% BSA, 0.5% Tween-20 in Tris-buffered Saline; 50Mm Tris-HCl pH 7.4, 150 mM NaCl)), 100 µL added per well and incubated for 2 hours at room temperature. The wells were washed as described. Detection antibody was diluted to 20 ng/mL in reagent diluent and 100 µL added per well. The plate was then incubated for 2 hours at room temperature. The wells were washed as described and 100 µL of
Streptavidin-HRP (diluted 1:200 in reagent diluent) added per well. The plate was incubated for 20 minutes at room temperature in the dark. The wash step was repeated and 100μL of TMB substrate solution (Sigma-Aldrich, St.Louis, MO, USA) was added to each well. The plate was incubated for 20 minutes at room temperature in the dark following which 50μL of stop solution (2N H2SO4) was added. The samples were read immediately at 450nm with a wavelength correction at 570nm. Levels of cytokines were quantified against a seven point standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 2000pg/mL.

7.6 Histology

7.6.1 Preparation of Lung Tissue for Histological Analysis
After animal harvest the lungs were removed and prepared for further analysis (Section 7.4.3). The left lobe was chemically fixed by perfusion with 4% w/v Paraformaldehyde in phosphate buffered saline solution. The lobe was stored in paraformaldehyde solution. After 48 hours the lung was removed from the fixative and cut into 5 large sections, labelled A to E representing top to bottom along the vertical axis. Sections were enclosed in labelled histo-cassettes (Sigma) and processed over-night using a Leica ASP 300 Tissue processor (Leica Microsystems Nussloch GmbH, Heidelberger Strasse 17-19, D-69226 Nussloch Germany). Following this the tissue sections were removed from the cassettes and immediately embedded in paraffin using a Heated Paraffin Embedding Module (Leica). Sections were allowed to set on a cold plate before slide preparation.

7.6.2 Tissue Sectioning and Histological Staining
Sectioning of the paraffin embedded tissue was carried out using the Leica RM2235 Microtome (Leica). Sections were cut at a 7μm thickness and carefully placed onto the surface of water maintained at 45°C. Sections were examined on the water surface for imperfections before being lifted onto Superfrost Plus microscope slides (Thermo Scientific) and allowed to drain. Sections were then
stored at room temperature before staining using Haematoxylin and Eosin as follows; Slides were immersed in xylene for 15 minutes, twice to de-wax the sections. Sections were then brought to water through baths of 100%, 95%, 70% and 50% alcohol and rinsed for 2 minutes with running tap water. Slides were then stained with haematoxylin for 6 minutes and rinsed for 4 minutes through running tap water. Sections were checked for adequate staining at this point. Eosin staining occurred for 2 minutes followed by dehydration through 50%, 70%, 95% and 100% alcohol baths for 30 seconds each. Slides were then immersed in two xylene baths for 15 minutes each. One drop of DPX mountant (VWR International, Orion Business Campus, Northwest Business Park, Ballycoolin, Dublin 15, Ireland) was placed on each section and a cover slip (Thermo Scientific Thickness: Nr. 1) placed on the slide. The DPX was allowed to spread between the section and the cover slip and the slide was then incubated at 65°C overnight. Slides were removed from the incubator and stored in plastic slide boxes.

7.6.5 Stereological Analysis
Lung sections were examined using an Olympus BX51 Upright Brightfield Microscope (Olympus, Mason Technologies, Dublin 8, Ireland) at 20X magnification and using bright field view. Two images were taken at random from different locations on each slide with a 300ms exposure. A 10x10 grid (50μm x 50μm) was overlaid on each image using Cell B Software (Image 8). Each intersection of the grid was examined for airspace (A), alveolar (acinar) tissue (B), or non-acinar tissue (C). Intersections with acinar and non-acinar tissue were counted to calculate percentage airspace. Non-acinar tissue was subtracted from the overall tissue to calculate percentage alveolar tissue.
7.7 Flow Cytometry and FACS Analysis

In an attempt to determine the fate of MSCs delivered to rodent models of VILI (Section 7.2), an experiment was designed to track cells delivered at predetermined time-points following injury. Fluorescently labelled rodent MSCs (Section 4.1) were delivered intravenously and intra-tracheally as part of a larger experimental series (221). Organs were harvested and analysed by FACS analysis to determine distribution following administration.

7.7.1 MSC Labelling for FACS analysis

Rat Mesenchymal Stromal Cells were cultured to passage 4 and harvested as described (Section 4) using 0.025% trypsin-0.05 mM ethylenediamine tetra acetic acid (EDTA; #25200-056, GIBCO®, Invitrogen Corporation, NY, USA). Cells were quantified using a haemocytometer chamber and 4 x 10^6 cells per animal model isolated. Cells were labelled using the PKH26 Red Fluorescent Cell Linker Kit (PKH26GL; Sigma-Aldrich Co. LLC., St. Louis, MO, USA) as per the manufacturer’s instructions. Briefly, cells were washed with serum free media and centrifuged at 400 x g for 5 minutes. The supernatant was discarded and the pellet resuspended using a pipette in 1mL of the provided diluent C. A 2X dye solution was prepared by adding 4µL of the PKH dye to 1mL diluents C (This is sufficient
for $2 \times 10^7$ cells). The dye and cell solutions were then combined and mixed by pipetting. Cells were centrifuged at 400 x g for 10 minutes and the supernatant discarded. Cell pellets were washed 3 times in PBS and resuspended in aliquots of $4 \times 10^6$ cells in 300µL.

### 7.7.2 Preparation of Single Cell Lung Tissue Suspension

Following injury and administration of labelled MSCs, the animal was anaesthetised via IP injection of ketamine 80 mg.kg$^{-1}$ (Ketalar, Pfizer, Cork, Ireland) and xylazine 8 mg.kg$^{-1}$ (Xylapan, Vétoquinol, Dublin, Ireland). Anaesthesia was confirmed by reaction to paw clamp and observation of breathing pattern. The animal was sacrificed by decapitation using a Rodent and Small Animal Guillotine (Kent Scientific Corporation, USA). The heart lung block was removed and the lung homogenised using spring scissors. Lung homogenate was placed in a 15mL polypropylene centrifuge tube (SARSTEDT AG & Co., Nümbrecht, Germany) and 3 ml of 200U/mL Collagenase type I in DMEM (C9891; Sigma-Aldrich Co. LLC., St. Louis, MO, USA). Samples were incubated for 2 hours at 37°C and vortexed every 20 minutes. At the end of the protocol the samples were further homogenised using a P1000 pipette tip. Samples were filtered through 100µm nylon mesh (NY1H04700; Millipore, MA, USA) to remove debris. 10mL of DMEM was added to the filter membrane to wash through remaining cells before samples were centrifuged at 400 x g for 10 minutes. The cell pellet was resuspended in 3mL Ammonium-Chloride-Potassium (ACK) red blood cell lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.5M EDTA, All Sigma-Aldrich Co. LLC., St. Louis, MO, USA, pH 7.2-7.4) for not longer than 2 minutes. The ACK buffer was then neutralised using 10mL DMEM and samples were centrifuged at 400 x g for 10 minutes. If complete red blood cell lysis had failed to occur, this step was repeated. The resulting pellet was resuspended in 10mL FACS buffer (2% foetal calf serum in 1X PBS, both Sigma-Aldrich) and allowed to incubate on ice for 10 minutes. The samples were then further filtered through 100µm nylon mesh and samples were returned to ice until use.
7.7.3 FACS analysis using the Accuri C6

Samples were prepared as follows to 300μL volumes with the following controls to establish the appropriate cytometry gates; FACS buffer alone, FACS buffer plus 1μL of a 10μM Sytox solution to exclude dead cells (Invitrogen), Spike control which consisted of 4x10⁶ PKH labelled MSCs. Samples were loaded into a round bottom 96 well plates (SARSTEDT) and analysed using either the BD FACSCanto or the BD Accuri C6 Flow Cytometer (BD Biosciences, 2350 Qume Drive, San Jose, California, USA).

7.8 Statistical Analysis

Statistical analysis was performed on scientific data using SPSS software. The majority of results were presented as mean ± standard deviation (SD). Data distribution was examined for normality using Kolmogorov-Smirnov tests. Unpaired, two tailed student T-Tests were used to compare two experimental data sets and one way ANOVA analysis followed by the Newman-Keuls method to compare all pairs of means was performed on data from experiments comparing three or more experimental groups. The significance threshold was set to p < 0.05.
8. The Optimal Route of MSC Administration in an *in vivo* Model of Ventilator Induced Lung Injury

8.1 Abstract

**Introduction:** Previous studies performed by our research group indicated that intravenously delivered human MSCs (hMSCs) can enhance recovery and repair of the lung following VILI. Other studies by our group have demonstrated these effects using rat MSCs (rMSCs) administrated IV and IT in a rat model of VILI.

**Objectives:** We sought to investigate the optimal route of administration of human MSCs (hMSCs) in a rat model of VILI.

**Methods:** Human MSCs (4x10^6 cells/kg) were administered IV, IT or IP with corresponding Vehicle controls in a rat model of VILI. FACS analysis was used to determine the distribution profile of labelled MSCs when administered IV or IT following VILI.

**Outcomes:** Results indicate that cells administered via both IV and IT routes confer beneficial effects on physiological and molecular parameters of injury, whereas no significant improvement is seen in animals which received cells via IP administration. For example, PaO_2 in arterial blood was significantly increased in the IV MSC group (123.93 mmHg ±8.85) compared to the IV vehicle control (100.95 mmHg ±24.66) and in the IT MSC group (121.33 mmHg ±13.89) compared to the IT vehicle control group (69.65 mmHg ±14.88), whereas no significant difference was observed in the IP MSC group compared to the IP vehicle control (115.5 mmHg ±11.17 Vs 103.95 mmHg ±15.84). Distribution data demonstrates that MSCs are not equally distributed following IT versus IV delivery.

**Conclusions:** Administering hMSCs either IV or IT demonstrates greater efficacy than IP administration 24 hours post VILI.
8.2 Introduction

Ventilator induced lung injury is an unavoidable consequence of mechanical ventilation - a life-saving procedure to support respiration. In such cases, an underlying condition, usually severe pulmonary disease, or the necessity of surgery leads to the scenario whereby a patient needs to be artificially ventilated. Many clinical strategies have been put in place since the advent of mechanical ventilation to reduce the over-distension necessary to provide adequate oxygenation and prevent the occurrence of atelectasis.

The most influential study conducted to date was by the ARDS Network in 2000 which used lower tidal volumes and low PEEP and found mortality rates were decreased in mechanically ventilated ARDS Patients (76). However, it has been shown that using low tidal volumes can contribute to atelectasis and derecruitment (324, 325), and this can further contribute to lung injury progression (326, 327). All of these strategies harbour their own advantages and disadvantages, the desired outcome of all would be the maintenance of adequate oxygenation and inflation while avoiding over distension and injury. An alternative or co-operative therapeutic approach would be highly desirable.

Mesenchymal stem/Stromal cells (MSCs) have emerged as a highly promising therapeutic strategy in numerous fields of injury and disease. They are an attractive option for the treatment of lung injury due to the “pulmonary first pass effect” (229). This phenomenon is an obstacle in the systemic application of MSC therapy to other organs such as the brain (230) and heart (231), where direct application is possible but systemic administration is desirable. Numerous studies have been conducted using MSCs in various models of lung injury (Reviewed in (207, 219)). All MSC intervention was administered either IV or IT in the studies reviewed and covered various animal models, disease conditions and dose sizes. Curley et al. examined the efficacy of rodent MSCs and also stem cell conditioned medium in a rodent model of VILI after IV and IT administration (221). Results generated by the use of conditioned medium in this study pointed to a paracrine mechanism of action and cell studies showed comparable results for cells given IV and IT. In an earlier study by the same group (248), 2x10⁶ cells per animal
were shown to be effective when given IV. In a further study a dose response curve was generated with cell doses of 1, 2, 5, 10x10^6 cells per kilogram used. The outcome of this was that efficacy was not seen at 1x10^6 cells per kilogram, but doses of 2-10x10^6 cells per kilogram showed significant improvements compared to control groups (Data not yet published). Taking data from these studies we aimed to determine the most clinically effective route of administration. Three routes were studied; IV and IT which were shown to be comparable as mentioned and IP. IP injection is a minimally invasive, quick, simple procedure, ideal in a clinical situation for the administration of MSCs. Due to the experimental nature of this experiment, a median dose of MSCs was chosen. Curley et al. (221) used 4x10^6 cells per animal, which roughly translates as 1x10^7 cells/kg. With the knowledge that MSCs are effective in doses as low as 2x10^6 cells/kg IV, we chose a median dose of 4x10^6 cells/kg. This study, in a continuation of the previously mentioned series of experiments, sought to directly compare three routes of administration of human MSCs in a rodent model of VILI; IV, IT and IP delivery using 4x10^6 cells per kilogram. We hypothesised that MSCs would be equally effective via each route due to their paracrine mechanism of action.
8.4 Methods

8.4.1 Induction of VILI
Animals were anaesthetised and injured as previously described (Section 7.2). Briefly, anaesthesia was induced by isoflurane gas and a tail vein was canulated. Following further anaesthesia using isoflurane, oro-tracheal intubation was performed by passing a 14 gauge canula past the vocal cords. Animals were ventilated on a baseline protocol of 80 breaths per minute, with a flow rate of 500cm$^3$ per min of 30% O$_2$ gas in air mixture and a PEEP of 2cm H$_2$O. Anaesthesia was maintained using IV Alfaxan and Nimbex. Recruitment manoeuvres were performed every 7 to 10 minutes for 20 to 30 breaths.

After 20 minutes of baseline ventilation, static compliance was measured and the ventilation protocol switched to injurious as follows; respiratory rate was decreased to 18 breaths per minute, settings were switched from volume to pressure with PIP of 35cm H$_2$O and flow increased to 550cm$^3$ air per minute. Injurious ventilation proceeded until a 50% decrease in static compliance was observed. The animal was then returned to baseline ventilation parameters with 100% O$_2$.

8.4.2 Administration of hMSCs
The MSC dose was administered following the return of the animal to baseline ventilation parameters with 100% O$_2$.
For IV administration the cells were given via the existing tail vein canula in 50µL increments followed by a 100µL vehicle flush and the animal was recovered as normal. For IP administration cells were given into the peritoneal cavity in the lower right-hand quadrant of the body in a single dose. For IT administration the animal remained fully anaesthetised, the ventilator was removed and the animal was positioned on an inclined metal plate by hooking a Mersilene ribbon under the upper incisors. Cells were given in two doses followed by 2mL of air in a 1mL syringe. After each dose animals were placed laterally and the ventilator reconnected. Two 5 second recruitment manoeuvres were performed to disperse the cells. After both doses were administered the animal was returned to supine position and allowed to recover from anaesthesia.
8.4.3 Assessment of Recovery following VILI
Animals were assessed for recovery post VILI 24 hours after hMSC administration as described previously (Section 7.3). Briefly, animals were anaesthetised by IP injection of ketamine and xylazine and surgical tracheostomy performed. Baseline ventilation was performed for 20 minutes with a respiratory rate of 80 breaths per minute with a PEEP of 2 cm H$_2$O, flow rate of 500 cm$^3$ per minute with 30% O$_2$. After baseline ventilation arterial blood gas concentration was measured and static compliance recorded. Ventilation proceeded with the same parameters with 100% O$_2$ for a further 20 minutes. After this, another blood sample was taken and the animal was sacrificed by exsanguination. Blood, and BAL were collected and the lung tissue harvested and preserved as previously described (Section 7.4).

8.4.4 MSC Distribution Analysis following VILI
To try to determine the fate of administered MSCs in a rodent model of VILI, an experiment was conducted using labelled MSCs and FACS analysis (Section 7.7). MSCs were fluorescently labelled using PKH26 Red Fluorescent Cell Linker Kit as described (Section 7.7.1) before IV and IT administration to rodent models of VILI. 30 minutes, 4 hours or 24 hours following administration the animals were sacrificed and the organs were processed to produce single cell suspensions which were analysed by FACS for the presence of labelled MSCs.
8.5 Results

Overall, 45 rodents were entered into the experimental study. All animals survived the induction of VILI and treatment administration. 5 animals were entered into each vehicle control group and 10 were entered into each hMSC experimental group. Great care was taken to ensure that animals endured no excessive or unnecessary distress or pain.

8.5.1 MSCs Improve Lung Physiological Structure and Function Following VILI

Following the induction of VILI, human MSCs were administered via the IV, IT or IP routes. Each of these groups was compared to controls which were given vehicle via IV, IT or IP routes. Groups that received MSCs by IV and IT showed significant improvements in arterial oxygenation (Figure 8-1, Panel A) and alveolar-arterial oxygen gradient levels (Figure 8-1, Panel B). IV and IT MSCs also demonstrated significant improvements in respiratory static compliance (Figure 8-2, Panel A) and IT delivered therapy significantly reduced Wet:Dry ratios after 24 hours when compared to vehicle controls (Figure 8-2, Panel B). Groups which received MSCs via the IP route all trended toward improvement, however none of the parameters measured (aside from the wet:dry ratio) reached statistical significance.

8.5.2 MSCs Reduce Alveolar Cellular Infiltrations and Modulate Cytokine Profiles Following VILI

The total white cell numbers in IT treated animals was significantly decreased 24 hours following injury compared to vehicle control (Figure 8-3, Panel A), however the percentage of BAL neutrophils was significantly reduced in all treatment groups 24 hours following injury (Figure 8-3, Panel B). Concentrations of BAL protein were significantly decreased in IV and IT therapy groups (Figure 8-4, Panel A). Both IV and IT delivered therapy significantly decreased the levels of the inflammatory cytokines IL-6 and IL-1β present in the lungs when compared to vehicle controls (Figure 8-4, Panels B & C).
8.5.3 MSCs Enhance Histologic Recovery following VILI

The degree of recovery of histological structure following injury was determined by quantitative stereological analysis, which demonstrated that there was significant recovery in IV and IT MSC treated rats in terms of alveolar tissue volume fraction or alveolar air-space volume fraction (Figure 8-5).

8.5.4 MSCs are not Equally Distributed Following IV and IT Administration in a Rodent Model of VILI.

The route of MSC administration affects the extent and time course of their accumulation within the injured lung. Immediately following VILI, PKH26 labelled MSCs were given IV or IT and the animals were harvested at different time-points. Lungs were harvested, digested and analysed by flow cytometry to determine the percentage of labelled MSCs present. After 30 minutes the majority of cells (64%) were found in the lungs after IT with only 16% found after IV administration (Figure 8-6, Panel A). By 4 hours these numbers had decreased to 23% and 8% respectively (Figure 8-6, Panel C).
8.6 Discussion

8.6.1 ARDS – A Devastating Disease with No Treatment
Numerous studies have been conducted to test the efficacy of various methods of ventilation and other supportive therapies, pharmacological or otherwise (reviewed in (242)) to prevent the onset or worsening of ALI and ARDS due to VILI. Ventilator strategies concentrate on lung protective ventilation by lowering tidal volumes, altering levels of PEEP, ventilation frequency or patient positioning ((76, 328-330). Other therapies include the use of surfactant (331), β₂-Agonists (68), fluid management systems (77) and antioxidants (332). These studies have shown varying levels of efficacy and have given valuable insights into the management and treatment of ARDS, however results were either not conclusive enough to recommend these procedures as primary treatments or had worsened the outcome of the condition. The aims of ventilation are to maintain oxygenation and prevent atelectasis whilst attempting to avoid over-distension. It is generally accepted that localised over distension of alveoli cannot be avoided and therefore a strategy to attempt to counteract these adverse effects of ventilation is necessary.

8.6.3 MSC Therapy – Promise for ARDS
A therapy which can be given concurrently with promising effects is MSC therapy. Numerous research groups have shown the beneficial effects of MSCs in ALI (reviewed in (207). In a study of the effects of rodent MSCs on VILI, 2x10⁶ rodent MSCs or concentrated conditioned medium (MSC-CM) was administered IV at 0 hours and again 24 hours later with resulting improvements in physiological and histological parameters (248). A more recent study by our group examined the effects of 4x10⁶ rodent MSCs and rodent MSC-CM on VILI when given IV or IT. Results concluded that the MSCs and MSC-CM were effective via both routes and based on this data our study graduated to the use of human MSCs in VILI. A dose response study was conducted with MSCs administered in doses from 1x10⁶ to 1x10⁷ cells/kg bodyweight via the IV route (Data in press). Results concluded that the lowest significantly effective dose of MSCs which could be given IV to a rodent model of VILI was 2x10⁶ cells/kg with
slight improvements seen in arterial oxygen pressures seen at 5x10^6 cells/kg and a slight decrease at 1x10^7 cells/kg. This is encouraging given that the average dose instilled in mice during early phase ALI is 29.9±20.4x10^6 cells/kg and in rats is 20.3±22.5x10^6 cells/kg (206).

8.6.3 MSC Therapy – Optimising the Route of Administration
Once the effective dose has been established, delivery route must be decided. Studies conducted using MSCs in ALI have been mainly via the IV route of administration (207), with more recent studies looking at intra-pleural (333) or IT delivery (221). The aim of these studies was to determine if localised delivery held any significant advantages over systemic delivery for the treatment of ALI. Intra-pleural delivery demonstrated MSC survival of at least one month with the cells remaining localised to the pleurae and pleural cavity (333). A subsequent study by the same group found that the MSCs delivered intra-pleurally attenuated LPS induced lung injury parameters compared to vehicle control and were not found in the lung parenchyma indicating a paracrine or endocrine mechanism of action (334). Curley et al. investigated the role of IT rodent MSCs compared to IV MSCs and conditioned medium (221). The study deduced that both routes were equally effective and that MSCs also conferred their benefits via a paracrine mechanism of action.

Following the finding that MSCs work, at least partially, by a paracrine mechanism the option of using IP administration became an option. IP injection is a minimally invasive, quick, simple procedure which would be ideal in a clinical situation for the administration of MSCs in critically ill patients with ARDS.

8.6.4 MSC Therapy Following VILI – Importance of Delivery Route
In this study MSCs administered via the IV or IT routes significantly improved lung function as evidenced by arterial blood-gas analysis and alveolar-arterial oxygen gradient. The delivery of MSCs IP was not as effective as the IV and IT routes but did show a trend toward improvement in the majority of parameters measured. Total white cell counts were decreased in all routes, however
significance was only reached in the IT MSC group. The percentage of these cells that were neutrophils was significantly reduced in all treatment groups. These results, coupled with improvements in BAL protein levels and compliance point toward the MSCs’ role in restoring epithelial barrier integrity in the alveoli. This restoration of integrity leads to a restoration in function as indicated by the improved blood gas and alveolar-arterial oxygen gradient measurements. It was also observed that the lung injury appeared to be less severe in the IP control group. This indicates that perhaps the bolus of fluid administered as vehicle controls in the IV and IT group controls contribute to some aspects of lung injury. Further to this it appeared that the IT injury was slightly more severe than that of the IV or IP groups which can be attributed to the application of fluid directly to the lungs. This enhanced injury was most apparent in the total protein, IL-6 and IL-1β levels in the BAL fluid. This observation was not made in previous studies using IT MSCs (221) and might possibly be attributable to the shorter recovery period in this study (24 hours versus 48 hours in previous studies by Curley et al. (221, 248)). The additional 24 hours is likely to be sufficient for the adaptation to and clearance of this fluid.

8.6.5 The Impact of Delivery Route on MSC Distribution
With the knowledge that MSCs tend to home to sites of injury (226) and taking into account the “pulmonary first pass effect” (229) we decided to investigate the distribution of MSCs when delivered IT or IV to and animal model of VILI. Results show that 30 minutes after administration of MSCs 64% of the cells were found after IT administration and 16% of the cells were found after IV administration in the lung tissue (Figure 8-6). By 4 hours these numbers had decreased to 23% and 8% respectively. A study by Eggenhofer and colleagues (267) demonstrated the distribution of radioactively labelled MSCs in a liver ischemia mouse model. Findings were that over 60% of the total radioactivity was seen in the lungs within 1 hour following IV administration. This had significantly diminished at 24 hours.

A study conducted by Gao and colleagues (265) demonstrated the distribution of $^{111}$In-oxine labelled MSCs when given IV, intra-arterially (IA) (both femoral) and
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IP to an uninjured rodent model. Distribution was examined immediately after administration and also 48 hours later using a gamma camera. Radioactivity was detected in the liver, lungs, kidney, spleen and long bones (in order of intensity). The study found that the use of vasodilators decreased the amount of MSCs found in the lung by 15% after IV and IA administration due to the fact that MSCs are large ranging in size from 20 to 30μm (compared to, for example, red blood cells which are approximately 8μm), whereas the pulmonary capillaries are approximately only 14μm in diameter (335). It is therefore important to consider any concurrent therapy being administered and the possible effects that it may have.

8.6.6 Summary and Conclusions

In summary, the efficacy of three different routes of administration of MSCs was examined in an established rodent model of VILI in the enhancement of recovery and repair. The IV and IT routes of administration were comparable in their effects on the indices measured including arterial PaO₂, inflammatory cell infiltration and cytokine concentration in BAL fluid. The IP route was shown only to be mildly effective, if at all, with the majority of indices measured not reaching significance. Despite results from IV and IT administration being comparable, it has been previously shown that systemic delivery may have a greater impact on modulation of inflammation (221). It is therefore suggested that the IV route of administration be of choice in the future administration of MSCs to treat VILI.
Figure 8-1: The Effect of Route of hMSC Administration on Physiological Function Following VILI. IV and IT MSC therapy significantly increased arterial pO\(_2\) (Panel A) and decreased the alveolar-arterial O\(_2\) gradient (Panel B), 24 hours following induction of VILI compared to their vehicle control groups. * (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (T-Test)
Figure 8-2: The Effect of Route of hMSC Administration on Pulmonary Structure and Function Following VILI. IT and IV MSC therapy each significantly increased lung static compliance (Panel A). IT therapy reduced lung Wet:Dry weight ratios (Panel B).

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (T-Test)
Figure 8-3: The Effect of Route of hMSC Administration on Pulmonary Inflammatory Cell Infiltrates Following VILI. Only IT MSC therapy decreased BAL total cell count (Panel A), while IT, IV and IP MSCs decreased BAL neutrophils 24 hours following induction of stretch induced lung injury, compared to the control groups (Panel B).

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (T-Test)
Figure 8-4: The Effect of Route of hMSC Administration on Pulmonary Protein Profiles Following VILI. Both IV and IT routes of administration of MSCs significantly improved lung cytokine profiles by reducing levels of protein (Panel A), IL-6 (Panel B) and IL-1β (Panel C) when compared to vehicle controls. Changes were not seen in the IP groups.

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (T-Test)
Figure 8-5: The Effect of Route of hMSC Administration on the Resolution of Structural Lung Injury Following VILI. MSC therapy decreased alveolar lung tissue (Panel A) and increased alveolar airspace fraction (Panel B) compared to vehicle controls 24 hours after injury. Representative photomicrographs of lung from a vehicle treated (Panel C, E, G), and MSC treated (Panel D, F, H) animal demonstrate greater resolution of lung injury at 24 hours when administered via each route.

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (T-Test)
Figure 8-6: The Effect of Route of MSC Administration on the Distribution of Labelled MSCs Following VILI. Representative scatter plots for PKH-labelled MSCs in lung homogenate. $4 \times 10^6$ PKH labelled MSCs were administered to VILI injured lungs via IT or IV after VILI. 1 hour (Panel A), 4 hours (Panel B) and 24 hours (Panel C) later, the lungs were harvested and digested to a single cell suspension for flow cytometry. Percentage of labelled MSCs in each sample was determined using the BD FACSCanto flow cytometer. “Blank Control” indicates no labelled cells administered to the lungs, “Spike Control” indicates $4 \times 10^6$ PKH labelled MSCs were added to the homogenate prior to analysis, “IV Administration” and “IT Administration” indicate lung homogenates from animals that received $4 \times 10^6$ PKH labelled MSCs via the IV or IT routes respectively.

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<tr>
<th>MSC Delivery Route</th>
<th>Cell Accumulation in the Lung (% delivered dose)</th>
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<td>IV delivery</td>
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9. The Therapeutic Window of Mesenchymal Stromal Cell Administration in an *in vivo* Model of Ventilator Induced Lung Injury

9.1 Abstract

**Introduction:** MSCs are a promising therapeutic approach for the treatment of ALI and ARDS and have shown potential in numerous pre-clinical models of lung injury. When any new therapy is developed and efficacy is being evaluated, a series of pre-clinical experiments must be conducted.

**Objectives:** This experiment aimed to establish the therapeutic window of MSC administration to a rodent model of VILI. We wanted to determine the longest period of time following VILI that hMSCs can be administered and still retain the ability to enhance recovery and repair.

**Methods:** VILI was induced in rodents using high pressure ventilation until a 50% decrease in static compliance was observed. Human MSCs (4x10^6 cells/kg) were administered intravenously 0 hours, 6 hours or 24 hours following the induction of VILI.

**Outcomes:** Results indicate that administration up to 24 hours post injury allows the MSCs to exert their beneficial effects on physiological and molecular parameters of injury. Arterial PaO_2 and BAL white cell counts were significantly improved after cells were administered at each time-point and compared to PBS control.

**Conclusions:** hMSCs can be administered up to 24 hours post VILI injury without loss in efficacy toward repair and recovery.
Chapter 9

9.2 Introduction

In developing therapies for clinical use a number of questions must be answered in a pre-clinical setting. In a recent review by Zhu (206), the remaining questions to be answered regarding the use of adult stem cells in ALI were outlined. In summary we need to understand more about the optimal hMSC dose and dosing regimen, route of hMSC administration, and specific type of cells to be used. Overall it was emphasised that in determining the answers to these questions no harm must be done in the development of this therapy and the dangers must be kept in mind at all times. The use of pre-clinical models is an invaluable tool in the demonstration of the safety and efficacy of new and emerging therapies.

Using a rodent model of VILI, this series of studies were performed to further demonstrate the efficacy of human bone marrow MSCs. We have addressed both the optimal dose and optimal route of administration of these cells in this model of ALI (Section 8) and to further address some of the un-answered questions in this area, this study aimed to determine the therapeutic window of MSC administration within a known time period following induced ALI. The exact period of time that has elapsed since the onset of ALI will vary from patient to patient and knowing that MSC therapy could be administrated significant periods of time post injury would indicate that MSC therapy for ALI/ARDS is a flexible solution to this clinical situation.

9.3 Aims and hypothesis

To determine how long after the initial VILI insult can hMSC therapy be administered and maintain their beneficial effects. We hypothesised that administering hMSCs up to 24 hours following VILI would still demonstrate the beneficial effects seen in previous experiments when cells were administered immediately post injury.
9.4 Methods

9.4.1 Induction of VILI
Animals were anaesthetised and injured as previously described (Section 7.2). Briefly, anaesthesia was induced by isoflurane gas and a tail vein was canulated. Following further anaesthesia using isoflurane, oro-tracheal intubation was performed by passing a 14 gauge canula past the vocal cords. Animals were ventilated on a baseline protocol of 80 breaths per minute, with a flow rate of 500cm³ per min of 30% O₂ gas in air mixture and a PEEP of 2cm H₂O. Anaesthesia was maintained using IV Alfaxan and Nimbex. Recruitment manoeuvres were performed every 7 to 10 minutes for 20 to 30 breaths.

After 20 minutes of baseline ventilation, static compliance was measured and the ventilation protocol altered to injurious ventilation which proceeded until a 50% decrease in static compliance was observed. The animal was then returned to baseline ventilation parameters with 100% O₂.

9.4.2 hMSC Administration
At this point animals were either administered MSCs at 0 hours or allowed to recover for 6 or 24 hours before administration of MSCs. All cells were given IV via tail vein canula in 50µL increments followed by a 100µL vehicle flush and the animal was recovered as normal.

9.4.3 Assessment of Recovery following VILI
Animals were harvested 48 hours after induction of VILI as described previously (Section 7.3). Briefly, animals were anaesthetised by IP injection of ketamine and xylazine and surgical tracheostomy performed. Baseline ventilation was performed for 20 minutes with a respiratory rate of 80 breaths per minute with a PEEP of 2cm H₂O, flow rate of 500mL/minute with 30% O₂. After baseline ventilation arterial blood gas concentration was measured and static compliance recorded. Ventilation proceeded with the same parameters with 100% O₂ for a further 20 minutes. After this, another blood sample was taken and the animal was sacrificed by exsanguination. Blood and BAL were collected and the lung tissue harvested and preserved as previously described (Section 7.4).
9.5 Results

Overall, 30 rodents were entered into the experimental study. All animals survived the induction of VILI and treatment administration. 6 animals were entered into the vehicle control group and 8 were entered into each hMSC time-point experimental group. Great care was taken to ensure that animals endured no excessive or unnecessary distress or pain.

9.5.1 MSCs Improve Lung Physiological Structure and Function When Administered 0, 6 or 24 Hours Following VILI

Following the induction of VILI, human MSCs were administered 0, 6 or 24 hours later. Each of these groups was compared to a vehicle control which was given vehicle via the IV route immediately post injury. Groups that received MSCs at each time-point showed significant improvements in arterial oxygenation (Figure 9-1, Panel A) and alveolar-arterial oxygen gradient levels were significantly reduced at 0 and 6 hours (Figure 9-1, Panel B). MSC groups demonstrated significant improvements in respiratory static compliance (Figure 9-2, Panel B). However no effect was demonstrated on Wet: Dry ratios in any group after 48 hours when compared to vehicle controls (Figure 9-2, Panel A). Groups which received MSCs 24 hours post injury showed significant improvement in almost all parameters, however not to the same extent as groups which received cells either immediately or 6 hours post injury.

9.5.2 MSCs Improve Pulmonary Cell Infiltrates and Cytokine Profiles When Administered 0, 6 or 24 Hours Following VILI

The total white cell numbers in all MSC treated animals were significantly decreased 48 hours following injury compared to vehicle control (Figure 9-3, Panel A) and the percentage of BAL neutrophils was significantly reduced in all treatment groups 24 hours following injury (Figure 9-3, Panel B). Levels of BAL total protein were significantly decreased in the groups which received MSC therapy at 0 and 6 hours (Figure 9-4, Panel A). All treatment groups decreased the levels of CINC-1 inflammatory cytokine present in the lungs.
when compared to vehicle controls (Figure 9-4, Panels B & C), however only the 6 hour group reached significance.

**9.5.3 MSCs Decrease Histologic Injury When Administered 0, 6 or 24 Hours Following VILI**

The degree of histological injury was determined by quantitative stereological analysis, which demonstrated that there was significant recovery when MSCs were administered 0, 6 and 24 hours following injury in terms of alveolar tissue volume fraction or alveolar air-space volume fraction (Figure 9-5). Visual analysis shows a decrease in alveolar wall thickening and cellular infiltrates after MSC therapy (Figure 9-5, Panels C-F).
9.6 Discussion

9.6.1 MSCs – Therapeutic Promise for ARDS
The therapeutic potential of MSCs for the treatment of ALI is well established in animal models (207), however as in the development of all therapies a series of preclinical experiments must be conducted prior to their proposed use in humans. The development of MSCs as a therapy should be comparable to that of pharmacological drug development (178) with an established optimal dose, route of administration and timing of dose. In recent preclinical trials, MSC therapy was administered up to 6 hours post injury (206), however these models consisted of sepsis or bleomycin induced ALI. In VILI, preclinical datasets are the result of immediate administration of MSCs post injury (221, 248) and form the basis for the work conducted in previous parts of this study.

9.6.2 Evaluating ‘True’ Therapeutic Potential of MSCs
In the clinical setting, patients with ARDS have a severe injury at the time of admission to the Intensive Care Unit (ICU) for treatment. Therefore, any treatment must be effective even when started well after the onset of the injury process. To date very little work has been conducted on the timing of MSC dose in ALI (206) and more specifically in VILI; however it is an important parameter which needs to be considered. Given these issues, we wished to establish the extent of the ‘therapeutic window’ for hMSC therapy, i.e. the potential for them to be effective later in the disease process. While it would be feasible to conduct a long term safety, efficacy and toxicology study, in our pre-clinical model of VILI the most severe symptoms are seen between 6 and 48 hours (286) with a resolution of the injury at 96 hours. Therefore to test the timing effect of MSCs in this model we remained within these time frames. Doses were given at 0, 6 and 24 hours with the animals harvested 48 hours post injury.

9.6.3 hMSCs Demonstrate an Extended ‘Therapeutic Window’
Results from these experiments clearly demonstrate that hMSCs enhance recovery from VILI even when delivered up to 24 hours post injury. This is evidenced by
increases in arterial blood oxygen levels and decreases in the alveolar-arterial oxygen gradient when compared to a vehicle control. It is noted here that effects of MSCs are seen to be beneficial in a step-wise fashion correlating with the timing of dose administration. Improvements in fluid clearance as measured by wet to dry ratios are not evident at any of the time-points and this can be attributed to the 48 hour time-point harvest (286) used in this experiment. Highly significant improvements in respiratory static compliance were seen at 0 and 6 hours, with significance also seen at 24 hours. Total white cell counts were decreased at all time-points, with an increasing step-wise pattern from 0 to 24 hours evident. The percentage of these white cells that were visualised as neutrophils was significantly reduced in all treatment groups in the same pattern. BAL protein infiltration was shown to be significantly decreased when cells were administered either 0 or 6 hours post injury. No significance was seen when cells were administered 24 hours post injury (P Value = 0.1625). Analysis of cytokine profiles demonstrated at significant decrease in CINC-1 when cells were given 6 hours post injury, but not at 0 or 24 hours.

9.6.4 Limitations of These Studies

Due to constraints in this injury model a 48 hour post harvest time was selected to remain within the time frame for significant injury post insult (286). One must then consider that animals that received cells immediately post injury were allowed 48 hours for their therapy to take effect, and animals that received cells at 24 hours post injury merely had 24 hours for therapy to take effect. These conditions pose a number of important questions bringing to light the limitations which are encountered when using pre-clinical models of ALI.

For a complete understanding of the efficacy of MSCs when administered at different time points, a full, comprehensive profile of the MSC secretome in response to ALI must be established. Questions remain as to the ideal activation of MSCs in vitro to more rapidly produce the factors which are most beneficial to ALI, what is the factor or combination of factors secreted by the MSCs in response to this injury and how long does it take for these factors to conduct their
regeneration and repair, and overall, how can the MSC secretome be exploited for the enhanced treatment of ALI.

9.6.5 Summary and Conclusions
The findings in this study would indicate an extended window of opportunity for intervention in ALI/ARDS using MSC based cell therapy. However such therapy is more likely to be of overall benefit when administered as soon as possible after diagnosis of lung injury.
9.7 Figures

Figure 9-1: The Effect of Administration of MSCs at Different Time-points on Physiological Function Following VILI. MSC therapy significantly increased arterial pO$_2$ at 0, 6 and 24 hours after injury (Panel A) and decreased the alveolar-arterial O$_2$ gradient at 0 and 6 hours (Panel B), following induction of VILI compared to the vehicle control group.

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (ANOVA)
Figure 9-2: The Effect of Administration of MSCs at Different Time-points on Pulmonary Structure and Function Following VILI. No significant differences were seen in lung Wet:Dry weight ratios (Panel A) 48 hours after injury in any injury group. MSC therapy at 0, 6 and 24 hours post injury each significantly increased lung static compliance (Panel B).

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (ANOVA)
Figure 9-3: The Effect of Administration of MSCs at Different Time-points on Pulmonary Inflammatory Cell Infiltrates Following VILI. MSC therapy decreased BAL total cell count (Panel A) at all time-points following injury, MSC administration at all time-points also decreased the percentage of BAL neutrophils 48 hours following induction of stretch induced lung injury, compared to the control group (Panel B).

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (ANOVA)
Figure 9-4: The Effect of Administration of MSCs at Different Time-Points on Pulmonary Cytokine Profiles Following VILI. Administration of MSCs immediately and 6 hours following VILI injury demonstrated significant improvements in BAL total protein concentration (Panel A) and a significant decrease was seen in CINC-1 concentrations when MSCs were administered 6 hours after VILI (Panel B) when compared to vehicle groups.

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (ANOVA)
**Figure 9-5: The Effect of Administration of MSCs at Different Time-Points on the Resolution of Structural Lung Injury Following VILI.** Administration of MSCs at all time-points decreased alveolar lung tissue (Panel A) and increased alveolar airspace fraction (Panel B) compared to the vehicle control group 48 hours after injury. Representative photomicrographs demonstrate a clear resolution of injury compared to vehicle treated animals (Panel C) at 0, 6 and 24 hours (Panels D-F respectively). * (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from corresponding vehicle control group (ANOVA).
10. The Effects of Sub-Populations of MSCs on Recovery and Repair Following Ventilator Induced Lung Injury *in vivo*

**10.1 Abstract**

**Introduction:** Methods to isolate more defined populations of MSCs are being pursued with the goal of producing a routine method for isolation of MSCs. The fact that perhaps only a subset of the overall MSC population harbours the beneficial effects demonstrated in numerous disease and injury states to date would possible concentrate these benefits in lower cell doses.

**Objectives:** This experiment examined a subpopulation of cells provided by Orbsen Therapeutics Ltd., isolated using the ORB-1 cell surface marker, in a rodent model of VILI to compare efficacy to that of heterogeneous populations of MSCs.

**Methods:** VILI was induced in rodents using high pressure ventilation until a 50% decrease in static compliance was observed. Human parental populations or specific ORB-1-positive or negative sub-populations of MSCs ($4 \times 10^6$ cells/kg) were administered intravenously following the induction of VILI. Both vehicle and fibroblast controls were used. Animals were assessed for indices of recovery and repair 24 hours following injury.

**Outcomes:** Results concluded that beneficial effects were seen with ORB-1$^+$ cells comparable to that of parental MSCs, however significant improvement over parental MSCs was not shown.

**Conclusions:** The use of specific sub-populations of hMSCs containing the ORB-1 surface marker confer no additional benefit in the repair and recovery following VILI.
10.2 Introduction

The use of MSCs has emerged as a promising therapy in many disease models such as neurological (336), inflammatory (337), as well as other injury models and their efficacy in ALI has been well established in numerous preclinical models (Summarised in (207)). Recently, a clinical trial has been completed demonstrating the apparent safety of systemically delivered allogenic MSCs in patients with COPD 2 years after infusion (208). Following promising results in these areas, methods to potentiate the beneficial effects of MSCs are being sought.

One approach is to focus on specific MSC sub-populations. This approach may have a number of advantages. Firstly, it is well known that MSC populations are a heterogeneous mix of progenitors, lineage restricted precursors and fibroblasts (338). It is a topic of much debate as to whether the beneficial effects of MSCs are derived from a certain sub-population of this heterogeneous mix, from paracrine factors secreted by the MSCs (151), from direct MSC-cell contact and engraftment (247) or by a combination of all these methods. Secondly, using a specific sub-population would result in a better characterised therapeutic, which would simplify clinical translation. Attempts have been made to isolate sub-populations of MSCs based on cell surface markers, however it is known that the putative markers used (STRO-1, CD29, CD44, CD73, CD90, CD105, CD166, and MHC-1) are not unique to MSCs (339) which necessitates the need for a marker or markers to isolate potentially superior sub-populations of MSCs.

This study was performed in collaboration with Orbsen Therapeutics Ltd. who provided us with their own patented sub-population of cells derived from human bone marrow MSCs. Orbsen Therapeutics identified a series of antibodies that enhance the isolation and purity of human MSC from BM beyond the MSC/MNC purity ratio of 1/13 (Figure 10-6). The lead candidate is a cell-surface sulfated proteoglycan, designated ORB-1. ORB-1 has been codified due to ongoing Intellectual Property filings by Orbsen Therapeutics.

Using the ORB-1 marker they were able to produce ORB-1 positive (ORB-1+) and ORB-1 negative (ORB-1−) populations of cells from parental populations of MSCs which were then tested for their efficacy in a rodent model of VILI.
10.3 Aims and Hypothesis

To determine if a subpopulation of MSCs isolated using the ORB-1 marker can reproduce the efficacy of parental MSCs in VILI or could provide enhanced effects compared to heterogeneous populations of MSCs. We hypothesised that ORB-1 positive sub-populations would be at least as effective as parental populations of MSCs and would be far superior to ORB-1 negative sub-populations in the recovery and repair following VILI.
10.4 Methods

10.4.1 Induction of VILI
Animals were anaesthetised and injured as previously described (Section 7.3). Briefly, anaesthesia was induced by isoflurane gas, a tail vein was canulated and oro-tracheal intubation was performed by passing a 14 gauge cannula past the vocal cords. Animals were ventilated using baseline settings of 80 breaths per minute, with a flow rate of 500 cm$^3$ per min of 30% O$_2$ gas in air mixture and a PEEP of 2 cm H$_2$O. Recruitment manoeuvres were performed every 7 to 10 minutes for 20 to 30 breaths.

Following baseline ventilation, static compliance was measured and injurious ventilation settings were applied as follows; respiratory rate was decreased to 18 breaths per minute, settings were switched from volume-controlled to pressure-controlled injurious ventilation with PIP of 35 cm H$_2$O and flow increased to 550 cm$^3$ air per minute. Ventilation proceeded until a 50% decrease in static compliance was observed. The animal was then returned to baseline ventilation parameters with 100% O$_2$.

10.4.2 hMSC Sub-Population Isolation
Parental and sub-populations of MSCs were provided by Orbsen Therapeutics Ltd. (Section 4.1) briefly as follows; hMSCs were initially isolated from bone marrow aspirates using plastic adherence. Cells were expanded using the recommended cell culture media and conditions. Sub-populations were isolated using the anti-ORB-1 antibody and flow cytometry. The ORB-1 protein is expressed on the surface of hMSCs, but not human MRC-5 fibroblasts. The ORB-1 protein may represent a novel isolation marker for human BM MSC.

10.4.3 hMSC Administration
Cells were used in animal studies between passages 3 and 6 at 4x10$^6$ cells/kg. Animals were randomised to receive either ORB-1$^+$, ORB-1$^-$ or heterogeneous MSCs in experimental groups, and either vehicle or MRC-5 human lung fibroblasts in control groups.
10.4.4 Assessment of Recovery following VILI

Animals were harvested 24 hours after stromal cell administration as described previously (Section 7.3). Briefly, animals were anaesthetised and surgical tracheostomy performed. Baseline ventilation was performed for 20 minutes as described. After baseline ventilation arterial blood gas concentration was measured and static compliance recorded. Ventilation proceeded with 100% O₂ for a further 20 minutes. After this, another blood sample was taken and the animal was sacrificed by exsanguination. Blood and BAL were collected and the lung tissue harvested and preserved as previously described (Section 7.4).
10.5 Results

10.5.1 ORB-1+/- MSCs Improve Lung Physiological Structure and Function Following VILI

Following the induction of VILI, animals were randomised to receive mixed population MSCs (PA-MSCs), ORB-1 positive or negative MSCs. Groups were compared to both a vehicle control group, and a MRC-5 fibroblast control group which received 4x10⁶ cells/kg of human lung MRC-5 fibroblasts immediately post injury. Groups that received MSCs populations showed significant improvements in arterial oxygenation (Figure 10-1, Panel A) and alveolar-arterial oxygen gradient levels were significantly ameliorated in all MSC groups (Figure 10-1, Panel B). All MSC populations also demonstrated significant improvements in respiratory static compliance (Figure 10-2, Panel B), and Wet:Dry ratios after 24 hours when compared to vehicle and MRC-5 controls (Figure 10-2, Panel A). Groups which received ORB-1 positive or negative MSC sub-populations 24 hours post injury showed significant improvement in all the aforementioned physiological parameters.

10.5.2 ORB-1+/- MSCs Improve Pulmonary Cell Infiltrates and Cytokine Profiles Following VILI

The total white cell numbers in all MSC treated animals were significantly decreased 24 hours following injury compared to vehicle and MRC-5 controls (Figure 10-3, Panel A), the percentage of BAL neutrophils was also significantly reduced in all MSC treatment groups 24 hours following injury (Figure 10-3, Panel B).

Levels of BAL protein were significantly decreased in the group which received PA-MSC therapy (Figure 10-4, Panel A), but not those that received ORB-1+ or ORB-1- h MSCs, in comparison to both vehicle and MRC-5 controls. All MSC groups showed decreased the levels of CINC-1 with PA-MSC and ORB-1+ groups showing significance compared to MRC-5 control group. TNF-α and IL-1β inflammatory cytokines levels present in the lungs after PA-MSC therapy were significantly decreased when compared to vehicle controls (Figure 10-4, Panels C & D).
10.5.3 ORB-1⁺/⁻ of MSCs Decrease Histological Injury Following VILI

The degree of histological injury was determined by quantitative stereological analysis, which demonstrated that there was significant recovery when MSCs were administered following injury in terms of alveolar tissue volume fraction or alveolar air-space volume fraction (Figure 10-5) compared to both vehicle and MRC-5 control groups.
10.6 Discussion

10.6.1 Evolution of hMSC therapy
With the emergence of any new therapies, new techniques and methods to enhance this therapy also tend to be developed. In the case of MSC therapy, an insight into the mechanism of action opens new doors into the development of MSC derived treatments for a range of diseases. In ALI it has been shown that KGF is an important factor in the resolution of VILI (253) and it is known that this factor is secreted from MSCs (224). Lee and colleagues contend that the action of MSCs in ALI is in large part via a paracrine mechanism of action with KGF being the most responsible factor (151). However, Islam and colleagues have shown that MSCs confer their reparative effects in ALI by alveolar cell attachment and mitochondrial transfer (247). Indeed, with our own study (Section 8) we have shown that the beneficial effects of MSC therapy are enhanced after either IV or IT administration compared to IP implying that MSCs require some form of contact or co-localisation with injured cells.

10.6.2 Isolation of a ‘Pure’ hMSC Population
Considering the heterogeneity of MSC populations, it is assumed that only a small proportion of the cell population is responsible for the therapeutic effects of MSCs. Methods to isolate pure populations of cells from bone marrow yield 38 MSCs for every 500 MNCs sorted, increasing the MSC/MNC purity ratio from ~1/300 as seen in MACS isolations to 1/13 and above (270). In this experiment we sought to investigate if a sub-population of MSCs isolated by Orbsen Therapeutics Ltd. using ORB-1/CD271 cell surface markers was as or more effective than using heterogeneous MSCs in VILI.

10.6.3 ORB-1+ hMSCs Demonstrate Efficacy in VILI
Using our established model of VILI we administered populations of MSCs that were positive or negative for the ORB-1 cell surface marker. These cells were directly compared to heterogeneous MSC populations and MRC-5 Fibroblast
controls. The sub-populations of MSCs were able to significantly restore lung function as evidenced by arterial blood-gas analysis, however not to the same degree as parental MSCs (P value $\leq 0.0001$ vs. Vehicle). Only PA-MSCs were shown to be significantly effective in decreasing alveolar-arterial oxygen gradient. All MSC populations similarly improved respiratory static compliance and wet to dry ratios compared to control groups. Total white cell count and neutrophil percentages present in BAL fluid were all significantly improved with a step-wise trend seen.

Protein and cytokine profiles demonstrate a preference for the use of PA-MSCs compared to the sub-populations of MSCs. BAL protein leak, CINC-1, TNF-α and IL-1β are all significantly decreased when using PA-MSCs compared to control groups. ORB-1$^+$ cells reduced levels of IL-1β compared to MRC-5 controls and ORB-1- reduced CINC-1 compared to MRC-5 controls. Stereological analysis of lung tissue showed significant improvements in tissue and airspace fraction in MSC treated lungs.

### 10.6.4 Summary and Conclusions

We had hoped with the use of a more homogeneous and clearly defined population of MSCs to see an enhanced level of repair and restoration of function following VILI. The ORB-1$^+$/CD271$^+$MNC fraction represents ~0.001% of the total bone marrow MNC fraction (or 1/100,000 MNC). Whereas, the ORB-1$^-$ /CD271$^+$ MNC fraction represents ~0.1% of the total BM-MNC. Notably, the CFU-F activity is significantly enhanced in the ORB-1$^+$/CD271$^+$MNC fraction, with ~1/20 MNC forming CFU-F, representing a ~5000-fold enrichment over existing PA-methods. It is thought that despite this some properties of the MSC population may be harboured by other cell populations in the bone marrow cell combination. The ability of ORB-1 positive cells to confer comparable efficacy to PA-MSCs in VILI shows promise for the use of a more clinically defined therapy such as these sub-populations of cells.
10.7 Figures

Figure 10-1: The Effect of Sub-Populations of MSCs on Physiological Function Following VILI. Treatment with all MSC populations significantly increased arterial pO$_2$ compared to both vehicle and MRC-5 control groups (Panel A). Only treatment with PA-MSCs significantly improved alveolar-arterial O$_2$ gradient compared to both sets of control groups (Panel B).

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from vehicle control (ANOVA)

° (P < 0.05), °° (P<0.01), °°° (P<0.001) significantly different from MRC-5 control (ANOVA)
Figure 10-2: The Effect of Sub-Populations of MSCs on Pulmonary Structure and Function Following VILI. All MSC populations significantly improved respiratory static compliance (Panel A) and wet:dry ratio (Panel B) 24 hours following VILI injury.

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from vehicle control (ANOVA)

° (P < 0.05), °° (P<0.01), °°° (P<0.001) significantly different from MRC-5 control (ANOVA)
Figure 10-3: The Effect of Sub-populations of MSCs on Pulmonary Inflammatory Cell Infiltrates Following VILI. All MSC treated groups showed significant improvements in both total white cell count (Panel A) and neutrophil percentage (Panel B) in BAL fluid 24 hours following the induction of stretch induced lung injury compared to control groups.

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from vehicle control (ANOVA)

°(P < 0.05), °° (P<0.01), °°° (P<0.001) significantly different from MRC-5 control (ANOVA)
Figure 10-4: The Effect of Sub-populations of MSCs on Pulmonary Cytokine Profiles Following VILI. Administration of PA-MSCs following VILI injury demonstrated significant improvements in BAL total protein concentration compared to MRC5 controls (Panel A) and a significant decrease was seen in CINC-1 concentrations when PA-MSCs and ORB-1+ sub-populations of MSCs were administered after VILI (Panel B) when compared to MRC5 control group.

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from vehicle control (ANOVA)° (P < 0.05), °° (P<0.01), °°° (P<0.001) significantly different from MRC-5 control (ANOVA)
Figure 10-4: The Effect of Sub-populations of MSCs on Pulmonary Cytokine Profiles Following VILI (Continued). Administration of PA-MSCs following VILI injury demonstrated significant improvements in inflammatory TNF-α concentration compared to vehicle controls (Panel C) and a significant decrease was seen in IL-1β concentrations when PA-MSCs and ORB-1+ sub-populations of MSCs were administered after VILI (Panel D) when compared to MRC5 control group.

* (P < 0.05), ** (P < 0.01), *** (P < 0.001) significantly different from vehicle control (ANOVA)
° (P < 0.05), °° (P < 0.01), °°° (P < 0.001) significantly different from MRC-5 control (ANOVA)
Figure 10-5: The Effect of Sub-populations of MSCs on the Resolution of Histological Injury Following VILI. All MSC therapy decreased alveolar lung tissue fraction (Panel A) and increased alveolar airspace fraction (Panel B) significantly (P<0.0001) compared to both vehicle and MRC-5 control groups. Representative photomicrographs of sectioned lung tissue demonstrate resolution of injury 24 hours after administration of MSCs. Vehicle and MRC-5 control groups (Panels C & D), ORB-1⁺, ORB-1⁻ and MSC groups (Panels E-F).

* (P < 0.05), ** (P<0.01), *** (P<0.001) significantly different from vehicle control (ANOVA)

° (P < 0.05), °° (P<0.01), °°° (P<0.001) significantly different from MRC-5 control (ANOVA)
Figure 10-6: Isolation and Characterisation of ORB-1-MSC Sub-populations. ORB-1 MSC isolation and characterisation: Flow cytometry of antibody-labelled ORB-1 protein on the surface (blue line) of human MSC and rodent MSC isolated from Dark Agouti or Lewis rat, and C57/Bl6 mice (Panel A). FACS isolation of ORB-1+CD271+ CD45-CD235- MNC from 3x10^7 human marrow cells MNC produces a ~3000-fold enrichment in CFU-F compared to PA-method of MSC isolation (Panel B). Flow cytometry of CFSE-labelled CD4+ T cells, reveal CD3/CD28-mediated stimulation of proliferation is inhibited by human PA-MSC and ORB-1-MSC when plated at a MSC/PBMC ratio of 1:200 (Panel C).
11. Discussion

11.1 Acute Respiratory Distress Syndrome

Acute Respiratory Distress Syndrome (ARDS) is a syndrome of acute respiratory failure that presents with progressive arterial hypoxemia, severe dyspnea, and a marked increase in the work of breathing. The disease is associated with loss of integrity of the alveolar-capillary barrier, together with infiltration of inflammatory cells into the airspaces and the development of pulmonary edema (1). Since the original description of the syndrome in 1967 (3), considerable effort has been expended in characterizing the pathophysiology of the disease, and in the search for a therapy. Although progress has been made in supportive care (79), no pharmacological intervention has proven effective, suggesting that novel approaches must be considered for ARDS treatment. Data from a meta-analysis study in 2008 showed that the pooled mortality rate in patients with ALI/ARDS was 43%, despite this number falling yearly during the previous decade by approximately 1.1%. Statistics such as these demonstrates that ARDS is a severe condition with a high mortality rate which needs to be addressed.

11.1.1 Need for Novel Therapies for ARDS

Treatments investigated for ARDS include NO therapy (340), prone positioning (341), extracorporeal membrane oxygenation (ECMO) (342) and pharmacological treatments such as inhaled surfactant, IV antibodies, ketoconazole and ibuprofen (343). All these investigated therapies have either conflicting, inconclusive results or have been proven to be ineffective in the treatment of ARDS. Conservative fluid management has shown to be effective at improving lung function and decreasing the duration of ventilation (77), however this is subject to strict conditions depending on the patient (344). Other studies have investigated the use of high or low PEEP in patients with ALI/ARDS (345), with no demonstrable beneficial effects. Thus far the most effective intervention for the treatment of ARDS has been the use of lower tidal volumes which demonstrated a significant decrease in mortality rates when compared to conventional ventilation settings (76).
11.2 Ventilator Induced Lung Injury

11.2.1 Importance of VILI
The use of mechanical ventilation in ARDS patients is a crucial component of basic life support; however it has been shown that conventional ventilation procedures can contribute and further worsen lung injury (76). The risk of VILI occurrence is increased in ARDS patients where healthy uninjured lung volume is greatly reduced and tidal volumes which impose no risk in healthy lungs are now severely injurious (86).

11.2.2 Traditional versus Protective Ventilation in ARDS
The most significant mechanical ventilation trial conducted was a randomised clinical trial involving 861 patients (76). Protective low tidal volume ventilation of 6mL/kg with a plateau pressure of less than or equal to 30cmH\(_2\)O was compared to traditional levels at 12mL/kg with a plateau pressure of less than or equal to 50cmH\(_2\)O. A significant decrease in mortality was shown and formed the basis for routine alteration of ventilatory tidal volumes in ARDS patients.

11.2.3 Role of Hypercapnia in Protective Ventilation
In the use of lower tidal volumes a patient is allowed to become hypercapnic as a result of reduced removal of waste CO\(_2\), a process termed ‘permissive’ hypercapnia. Arguments have been made for and against the permissive use of HCA in the setting of lung injury (99). It was initially shown to be protective in patients with ARDS undergoing low tidal volume mechanical ventilation (76) and since then it has been tested in various \textit{in vitro}, \textit{ex vivo} and \textit{in vivo} models of lung injury such as IR (98, 302), VILI (95, 131), endotoxin induced ALI (146) and CLP induced sepsis (346). HCA has been shown to decrease the production of free radicals by blood phagocytes and alveolar macrophages (347), reduce apoptosis and inflammation in an \textit{in vivo} and \textit{ex vivo} rabbit IR model (144, 302), be therapeutic in an \textit{in vivo} rabbit VILI model (95), to reduce inflammation in an \textit{ex vivo} rabbit endotoxin model (146), reduce reactive nitrogen species production and neutrophil infiltration in endotoxin induced ALI in the rat (96), decrease
injury in models of early and prolonged sepsis (346) and reduce the injury seen in moderate and severe VILI in a rodent model (131). A recent study by Jung and colleagues (348) demonstrated the beneficial effects of using prolonged, moderate CO₂ in a piglet model of ventilator-induced diaphragmatic dysfunction.

Contrary to these beneficial effects, reports have been published which demonstrate some potentially harmful effects of HCA. It was shown that HCA increased NO production in rat foetal alveolar epithelial cells (349), decreased alveolar epithelial cell function by the endocytosis of Na⁺/K⁺-ATPase (350) and increased bacterial load and worsened lung injury in *E.coli* injured rat lungs (134).

**11.3 ARDS and the NF-κB Pathway**

11.3.1 Role in Pathogenesis of ARDS

Almost every cell type expresses NF-κB and therefore has a functional NF-κB pathway (149). NF-κB mediated inflammation has been implicated in the pathogenesis of ARDS. This was due to the high levels of NF-κB regulated inflammatory cytokines in the BAL fluid of ARDS patients (53). The role of NF-κB in the pathogenesis of ARDS involves regulation of inflammation and the immune response to injury. The NF-κB pathway has been shown to be implicated in the production of inflammatory cytokines commonly found in the BAL fluid of ARDS patients (53) by activation in alveolar macrophages and neutrophils in injury (158). Thus, in this manner, NF-κB is engaged in the progression of the inflammatory process seen in ARDS.

NF-κB has been shown to be involved in the repair process of wound healing in models of lung injury by the control of cell migration. (143). Due to the central role of NF-κB in neutrophil influx, it appears that NF-κB plays a role in the immune response to bacterial infection, contributing to host defence against *P. aeruginosa* (160). The NF-κB pathway can be down-regulated as a therapeutic strategy to reduce the inflammation associated with pneumonia induced lung injury as demonstrated by Devaney *et al.* (351) by the over expression of IκBα.
11.4 Mechanism of Action of HCA on the NF-κB Signalling Pathway

11.4.1 Evidence for the Role of NF-κB in Mediating the Effects of HCA

Many researchers have attempted to determine the mechanism by which HCA exerts its therapeutic (and sometimes potentially harmful) effects in pulmonary injury and disease. It is well known that the NF-κB pathway is one of the major inflammatory pathways in tissue injury including lung injury. Takeshita et al. demonstrated that HCA decreased the activation of NF-κB in human pulmonary artery endothelial cells (352) and further expanded on this theory by demonstrating that HCA does indeed attenuate the LPS induced activation of NF-κB by influencing the function of the IκBα molecule (146). By inhibiting the degradation of the IκBα protein, HCA prevented the release of the NF-κB protein, its subsequent translocation and DNA binding.

Hypercapnic acidosis has been demonstrated to slow wound healing by reducing NF-κB activation, and therefore cell migration, in airway epithelial cell models of injury (143). Elevated levels of CO₂ have been shown to alter gene expression in mammalian cells via the NF-κB pathway (174), reduce the expression of NF-κB dependent cytokines in macrophages (114), and act on the RelB NF-κB protein to protect cells against LPS injury (175).

More recently, Contreras and Ansari (131) demonstrated that HCA acts via the NF-κB pathway to attenuate VILI using rodent models and in vitro in cell stretch models demonstrating similar findings. In these studies, HCA was induced by the use of FiCO₂ 0.05 (which yielded PaCO₂ values of 52-78mmHg) in vivo and 10% or 15% CO₂ in vitro. Results demonstrated that, in vivo, HCA decreased BAL cytokines, improved arterial PaO₂, and increased alveolar airspace fraction. In vitro the use of HCA demonstrated improved cell viability, decreased NF-κB reporter activity and subsequent IL-8 production in pulmonary epithelial cell lines.
11.4.2 Understanding the Mechanisms by Which HCA Modulates the NF-κB Pathway

In an attempt to pin-point the location of HCA interaction on the NF-κB pathway, Cummins et al. (174) demonstrated a rapid, reversible translocation of IKK-α in response to HCA as a possible mechanism of LPS injury attenuation. Experiments were conducted in MEF and A549 cells which were exposed to 0.03% CO₂ as NC and 10% CO₂ as HC conditions. Findings from this study, along with those by Takeshita et al. demonstrating the attenuation of IκBα degradation after LPS injury and HCA exposure (146), provide further evidence that HCA acts at more than one point on the NF-κB pathway, independently of pH. Further studies (175) demonstrate that the attenuation of LPS injury by HCA can also be attributed to the cleavage and translocation of RelB which is also independent of pH (175). Further to these experiments, very little work has been completed to demonstrate the mechanism of action of HCA in pulmonary NF-κB signalling.

11.4.2.1 Effects on NF-κB Pathway Activation

In pulmonary cells, the effects of HCA on pulmonary cell types has been reviewed by Ijland et al. (296) and Takeshita et al. reported that HCA acted on the NF-κB pathway in HPVECs, preventing IκBα degradation (146). It has been reported that the immune response can be hampered by HCA’s actions on the NF-κB pathway seen by the reduction in pro-inflammatory cytokines from alveolar macrophages (114). In this study, using primary airway epithelial cells, alveolar basal epithelial cells, and bronchial epithelial cells, we demonstrated a significant induction of NF-κB after injury with significant reduction in NF-κB signalling following exposure to HCA.

Multiple organ dysfunction syndrome (MODS), including Acute Kidney Injury, heart failure and hepatic dysfunction, occur frequently with or as a consequence of ARDS. In other organs and cell types, the beneficial effects of HCA are clear - in acute ischemic myocardial and brain injury (reviewed in (299)), and endotoxin induced gut mucosal injury in rabbits (300). It is important to demonstrate that the HCA, which is shown to be protective in pulmonary epithelial cells, does not
injure cells from other organs. Using embryonic kidney cells, colon cancer cells, and a monocyctic cell line, injured with TNF-α, we demonstrated HCA was similarly beneficial to what was seen with pulmonary cell lines.

11.4.2.2 Effects of HCA on Specific Components of the Canonical and Non-canonical Pathways

After establishing that HCA does indeed decrease the activation of the NF-κB pathway induced by TNF-α or endotoxin on pulmonary and other cell lines, the study proceeded to determine the point of action of HCA on the pathway. Specific elements of the activation pathway were selected based on observation of their ability to induce NF-κB activation in previous experiments, and included the TAK1/TAB1/2 complex, IKK-β and NIK (Image 9). In other studies, TAK1/TAB1/2 is thought to act as an up-stream activator of IKK- β (353) but its deletion in further reports did not lead to alterations in NF-κB signalling (354). Here, the over expression of these proteins gave a significant NF-κB induction and HCA was shown to significantly decrease this signal demonstrating the ability of HCA to attenuate signalling in the presence of potent pathway activators.
Next we demonstrated the effects of HCA on the activation of the IKK-β protein. Western blotting of lysates from cells transfected to over-express the IKK-β protein was used to detect phosphorylated IKK protein. HCA was shown to decrease IKK phosphorylation in response to injury. IKK proteins are pivotal proteins in NF-κB signalling pathway activation and we have demonstrated here that HCA acts on diminishing their activation.

To determine if the ability of proteins to activate their downstream target protein was impacted by exposure to HCA, an *in vitro* kinase assay was used. This experiment demonstrated that when IKK-β was isolated from cells and used to modulate recombinant IκBα protein, the levels of phosphorylation were decreased under conditions of HCA. When the IKK-β protein was isolated from cells exposed to NC and used in a reaction performed under conditions of HCA, the HCA was shown to directly decrease the phosphorylation of IκBα. IκBα is a key protein in the NF-κB pathway cascade (154) as its activation and degradation leads to the release of NF-κB dimers. By demonstrating that HCA acted on the
reducing the activation of the IKK-β protein, and also on the ability of IKK-β to induce the activation of the IκBα protein.

Similar experiments were attempted with isolated NIK protein to demonstrate if its effects on the IKK-β protein were also altered. Multiple attempts failed due to the low concentrations of NIK isolated from cells, most likely due to the regulation of NIK levels by up-stream proteins, which subject the protein to constant degradation and prevents build-up in the cytoplasm (355, 356). It is possible to show the effect of NIK over-expression on NF-κB activity using luciferase assay due to the accumulation of the luciferase protein in the cytoplasm. However this is more difficult using Western blot as the NIK protein is degraded and the effects on down-stream proteins are greatly diminished. Despite this, using controlled conditions, we were able to demonstrate the phosphorylation of IKK by NIK in the presence of HCA using Western blotting. The increased IKK phosphorylation seen was shown to be mainly that of the IKK-α protein, which is considered a key protein of the non-canonical pathway. As we have shown the effect of HCA on the phosphorylation of IKK without NIK over-expression, and we know NIK can activate the NF-κB pathway, probably through IKK-β phosphorylation (357), further experimentation is needed to demonstrate the exact mechanism.

So far, we have demonstrated that HCA exerts it effects at almost every interaction in the canonical and non-canonical pathways, which suggests an overall decrease in pro-inflammatory pathways. The activation of IKK-α seems to be enhanced in response to NIK over-expression during HCA exposure, which indicates an enhanced non-canonical response. This correlates with previously published data by Cummins et al. (174) who demonstrated that nuclear accumulation of IKK-α in response to elevated CO₂ was associated with decreased NF-κB signalling. On the other hand we have seen a significantly reduced canonical response with the application of HCA to a number of injury and protein over-expression in vitro cell models (Section 5). So the investigation continued down-stream to one of the most significant and extensively studied NF-κB pathway proteins – IκBα.
11.4.2.3 IκBα – A Pivotal Protein of the NF-κB Pathway

It is known that NF-κB is a rapid response pathway when cells are exposed to numerous injurious stimuli (303) and this activation culminates in the degradation of IκBα, followed by the release and translocation of NF-κB and DNA binding. Experiments were conducted to determine the time-course of NF-κB release from the IκBα protein by injuring cells exposed to HCA and examining protein levels at intervals following injury. It was shown, after injury for 30 to 60 minutes, that IκBα had degraded under NC conditions. HCA exposure prevented this degradation, demonstrating another mechanism by which HCA decreases NF-κB activation.

Activation of NF-κB leads to the transcription of numerous proteins including the IκBα protein, leading to fluctuation in NF-κB function with sustained injury (313). The effect of HCA on the production of “new” IκBα protein was examined by real-time PCR and it was shown that HCA decreased IκBα mRNA levels after injury. Following this it was examined if the HCA effect in response to injury was dependent on IκBα concentration. Cells over expressing increasing amounts of IκBα and exposed to injury and HCA were assessed for NF-κB activation. Even though NF-κB induction was decreased in a dose dependent manner by increasing concentrations of IκBα protein, HCA further decreased this signal. In the presence of consistent upstream NF-κB activation and increasing IκBα concentrations, the effects of HCA on pathway activation remained consistent with results seen without upstream activation. This not only demonstrates the effectiveness of HCA at preventing degradation of IκBα, but it shows that the degradation of the IκBα protein is central to the induction of NF-κB signalling.
11.4.3 Understanding the Mechanisms by Which HCA Modulates the NF-κB Dimer

In demonstrating the effects of HCA on IκBα mRNA levels, we may have demonstrated that HCA affects the potency of the NF-κB subunit or the effect may be at the level of transcription. Therefore the next section of our study concentrated on the effects of HCA on the NF-κB proteins after dissociation from IκBα.

11.4.3.1 Effects of HCA on NF-κB Dimer Formation

Prior to association with IκBα, the NF-κB proteins form dimers, a reaction which is dependent on the RHD of each protein. This domain is also responsible for DNA binding after translocation. Experiments were conducted in this study to examine the effect of HCA on the dimerisation ability of the NF-κB proteins. There was no effect on the ability of the proteins to form either p50/p50 homodimers or p50/p65 heterodimers. We were unable to visualise p65/p65 homodimer complexes in this experiment. It is possible that the time needed for p65/p65 homodimers to form is longer than the time necessary for either p50 homodimers or p50/p65 heterodimers to form, or there may be a requirement for an additional factor or factors in the reaction mixes.

11.4.3.2 Effects of HCA on Translocation and DNA Binding

Western blot for p65 protein in nuclear and cytoplasmic pulmonary cell extracts showed a notable increase in nuclear p65 in injured cells under NC conditions. This translocation is significantly decreased in nuclear extracts of injured cells exposed to HCA. It was then shown that conditions of HCA returned the nuclear levels of p65 protein to baseline compared to NC conditions and the concentration of p65 was also decreased under conditions of HCA. The translocation and binding of the p50 protein present in nuclear extracts was not shown to be affected by HCA.

Using the p50/p65 and p50/p50 dimers produced under conditions of NC or HCA we examined their DNA binding ability. Levels of DNA binding were examined using EMSA detection of the biotin labelled DNA consensus sequence. No
differences were seen in the binding ability of these dimers to the DNA. This may suggest that HCA does not have an effect at the RHD of these proteins.

To determine the effect of HCA on the binding activity of endogenous transcription factors, A549 nuclear extracts were prepared from pulmonary cells exposed to HCA after injury. These extracts were examined for binding levels to biotin labelled κB consensus DNA and the binding of a protein complex approximately the same size as the NF-κB heterodimer was seen. Under NC DNA binding conditions, cells exposed to HCA did not display protein bound to the consensus sequence DNA. When the binding conditions were changed to HCA, a diminished binding potential was seen compared to extracts allowed to bind under conditions of NC.

These findings strongly suggest that HCA has an effect on the activation of the p65 protein acting on its ability to translocate to the nucleus and bind NF-κB consensus sequence DNA, pointing toward another mechanism by which the NF-κB signal is attenuated by HCA in response to injury.

11.4.3.3 Effects on Transcription and Translation

We attempted to demonstrate the potential for the proteins which had bound the κB consensus sequence to initiate transcription and the subsequent translation under conditions of HCA using a cell free in vitro transcription/translation system. Using nuclear extracts containing NF-κB we attempted to examine the effects of HCA on the initiation of transcription and translation of luciferase mRNA and protein using a NF-κB driven luciferase plasmid. Despite numerous attempts to optimise the assay, the use of pulmonary nuclear extracts did not generate detectable signal in this system. Using T7 RNA polymerase and a T7 driven luciferase plasmid construct, we demonstrated a reduction in the luciferase mRNA transcribed. Isolation of this mRNA allowed us to demonstrate its ability to translate to protein under conditions of HCA, which was diminished. We aimed to show the effects of HCA on the transcription and translation of NF-κB driven luciferase mRNA and protein respectively, however despite failure to detect signals from this experiment, subsequent experiments using T7 driven luciferase plasmid and T7 RNA polymerase demonstrated that HCA has a suppressive effect.
on both the transcription and translation of the luciferase gene. This may suggest that HCA would have an overall subduing effect on gene expression.

11.4.4 Limitations and Future Direction of These Studies

Hypercapnia is a common finding in patients with ARDS who are managed with protective lung ventilation approaches. However, the use of permissive HCA in ARDS is not a standardised technique and there is no defined protocol for its use clinically. Levels of CO$_2$ used in experiments to show the effect of HCA have ranged from partial pressures of 45mmHg to 140mmHg for differing periods of time, in a variety of injuries and models, with different outcomes. Our research group has used consistent levels of HC both in vitro and in vivo. Future experiments would require the use of a range of CO$_2$ levels, perhaps with the application of buffering and equi-osmolar conditions. Based on other studies, the effects of HCA on NF-κB may be different with differing levels of HCA (141, 175).

In addition, despite the wide range of pulmonary and other organ cells used in these experiments, the majority of experimental procedures were carried out in pulmonary A549 cells. As initial experiments in each of the cell types yielded similar results, we would expect that the interaction of HCA and the proteins of each of these cells would also be similar. Future experiments could incorporate examination of the interaction of HCA with the NF-κB pathways in immune cells such as alveolar macrophages and neutrophils.

We limited the range of injurious stimuli used in order to demonstrate a clear outcome. The incorporation of different injurious stimuli such as stretch-induced injury or cell disruption could be used in these experiments to show the effects of HCA in those situations.

*In vitro* procedures are in-valuable in determining the specific molecular processes taking place however, perhaps further insight could be gained into the effects of HCA on the NF-κB pathway in the lung by using isolated organ experiments or whole body systems of lung injury. The *in vitro* experiments
conducted here demonstrate the effects of HCA in different pulmonary and systemic cell lines, however it does not demonstrate what happens in vivo.

*In vitro* testing took a significant step forward with the advent of the “lung on a chip”, the closest *in vitro* simulation of the lung to date (358). Composed of a lung epithelial cell layer with an air and blood interface it was demonstrated that the introduction of bacteria to the air-lung interface induced the release of white blood cells into the airspace. Such a system has enormous potential in the development and testing of lung therapies in a more thorough manner before animal studies. The use of such a system may be vital in answering important questions without the need for animal models, such as the effect of HCA on leukocyte infiltration, barrier permeability, bacterial growth, interactions with various injurious stimuli in a controlled, closed system with specific cell types.
11.5 Mesenchymal Stromal Cells as a Therapy for ARDS

Mechanical ventilation is a life-saving therapy for patients with ARDS. Efforts to protect the lung with different modes of delivery of positive pressure ventilation have met with some success. However, despite the widespread adoption of protective mechanical ventilatory strategies, it is clear that even small tidal volumes, can have an injurious effect in ARDS. This is due to the heterogeneous nature of the injury (359). Rather than focus on efforts to further diminish ventilator induced lung injury, it may be more appropriate to try to enhance lung repair.

11.5.1 Insights from the Use of MSCs in Other Diseases

A promising therapeutic strategy has been widely studied whereby stromal cells harvested from human bone marrow are used to repair and protect in situations of disease and injury. These MSCs have shown efficacy in numerous studies regarding skeletal muscle injury (360), myocardial infarction (361), stroke (362), and GvHD (363). It has been known for some time that, among other unique characteristics, MSCs have the potential to be anti-inflammatory, immunomodulatory and exert regenerative effects. MSCs also have the capability to home to sites of injury. Little is known of their regenerative properties following ALI. Previous studies from our group and others have shown that MSCs enhance repair and regeneration whilst attenuating cytokine release in a rat model of ALI (224, 248).

11.5.2 Insights from pre-clinical ARDS models

11.5.2.1 Use of MSCs in the Treatment of Sepsis

Xu et al (209) reported that intravenous administration of MSCs after intraperitoneal endotoxin decreased the influx of neutrophils into the air spaces of the lung and also reduced the quantity of pulmonary edema. In animal models where endotoxin (250) or live Escherichia coli bacteria (213) was administered by the intrapulmonary route, survival was significantly improved with MSC therapy
MSCs reduced the quantity of pulmonary edema, the degree of histologic lung injury, and the concentration of lung pro-inflammatory cytokines. Importantly, MSCs reduced lung bacterial loads compared with saline or fibroblast controls. In a mouse caecal ligation and puncture model of polymicrobial sepsis, Nemeth et al. (212) determined that systemically administered MSCs, given either 24 hours prior to, or 1 hour after surgery, improved survival, reduced organ dysfunction including indices of ALI, reduced neutrophil oxidative injury and increased circulating neutrophils, while lowering bacterial counts in blood. The efficacy of MSCs in the setting of cecal-ligation and puncture induced polymicrobial peritonitis and sepsis was replicated in another study by Mei et al. In this study (211), there was evidence that bacterial phagocytosis by splenic macrophages was enhanced in the MSC treated mice, an interesting finding since the Nemeth study reported that alveolar macrophages can be reprogrammed by the release of prostaglandin E2 from MSCs. The Mei study also found that the MSC effect persisted even in the presence of antibiotic therapy. A study from the Matthay group in gram negative peritoneal sepsis in mice demonstrated that survival was significantly improved with intravenous human MSC given 4 hours after high dose IP injection of Pseudomonas aeruginosa, and the number of bacteria in the blood stream was significantly reduced in the MSC-treated mice compared to PBS or fibroblast-treated controls (262). The percentage phagocytosis by blood macrophages at 12 hours was higher in these experiments than the two control groups (fibroblasts and PBS), providing a mechanism to explain the reduced number of bacteria in the blood of the MSC-treated mice. One of the mechanisms outlined includes increased expression of the complement component C5a (262). MSC administration was associated with moderate (15%) upregulation of C5a plasma levels. C5a complement fragment induces expression of the phagocytosis receptor CD11b on monocytes and macrophages and, by that mechanism, was proposed to enhance their phagocytic activity. This group has also found that human MSCs produce large quantities of the antimicrobial peptide, cathelicidin, LL-37, which inhibits growth of Staphylococcus aureus, a gram-positive bacteria, and two gram negative bacteria, E. coli and P. aeruginosa (214). Finally, in experiments to provide more clinically relevant information regarding the potential of cell therapy in human ALI, Lee et al (224, 225) utilized the established ex vivo perfused human lung preparation to
test the effects of MSC therapy. When the lung was treated with intrabronchial allogeneic human MSCs, 1 h after instillation of endotoxin (224) or E. coli bacteria (225), lung vascular permeability and extravascular lung water returned to normal levels, and the rate of alveolar fluid clearance increased to uninjured levels. Control studies with human fibroblasts showed no effect. This effect was associated with increased bacterial phagocytosis and killing by monocytes and macrophages, was more effective than antibiotic therapy alone during prolonged pneumonia, and was abolished by the administration of anti-KGF antibody (225).

**11.5.2.2 Use of MSCs for the Repair of the Injured Lung**

Recent insight into stem cell biology has generated excitement over the potential of MSCs to regenerate damaged organs, including the lung (206). MSCs residing in local epithelial niches, including in the lung, in close proximity to capillary walls, may provide a regenerative pool of trophic factors such as epidermal growth factor (EGF) and keratinocyte growth factor (364, 365). During epithelial injury in a colitis model, a subset of tissue resident stromal cells was able to sense microbial molecules and respond by increasing expression of the proliferative factor prostaglandin-endoperoxidase synthase 2 (PGE2), which in turn promoted epithelial proliferation (366). Adoptively transferred human BM-MSCs have been shown to restore the homeostasis of the epithelial fluid- and protein-transportation systems in models of injury and repair in the lung (224, 245). MSCs have also been shown to enhance the recovery and repair of ventilator induced injury to rat lungs (221, 223, 248).

**11.5.3 Key Translational Knowledge Gaps**

**11.5.3.1 The need to Optimise hMSC Dosage Regimens**

One of the obstacles to MSC therapy is obtaining adequate numbers of cells for administration. Bone marrow aspirates are in short supply and MSCs successfully harvested from samples are low. This is particularly true if isolation of the MSC requires further processing, such as FACS identification. Numerous passages are required to produce adequate numbers for infusion and it has been shown that the
cells lose their properties after a limiting number of passages (367). The optimal
dose of MSCs in humans is not known and it is likely that it may vary from
condition to condition. In addition to this the number of doses which must be
administrated, the route by which these cells are given, and the period of time
since initial injury will all likely influence the MSC dose given.

11.5.3.2 The Need to Optimise Route of hMSC Delivery

Many studies have examined the effect of MSCs following IV administration
(207). IV administration would seem to be the logical choice in patients with
ARDS given that an already compromised lung airspace may not readily accept a
large dose of MSCs. In the setting of lung injury systemic administration is fully
acceptable due to the tendency for MSCs to travel to, and become trapped in, the
pulmonary capillary bed almost immediately after administration (229). In other
organ studies, local administration is sometimes employed to attempt to overcome
this obstacle (263, 368). As we have seen, the delivery of MSCs IV or IT have
comparable levels of efficacy in a rat model of ALI (221), in terms of arterial
PaO₂, compliance, wet to dry ratio and BAL cell counts. Intra-pleural
administration of MSCs has also been investigated (333) which is an invasive
procedure directed at the local application of MSCs to treat pleural disease. After
intra-pleural delivery, MSCs were not detected in any other organ and viable cells
were recovered from the pleural cavity up to 30 days after administration. LPS
injury in this model of MSC delivery was shown to be attenuated indicative of a
paracrine mechanism of action possibly mediated by an observed increase in IL-
10 (334). Curley et al. (221) also demonstrated that there was a paracrine
mechanism of action at work through the use of conditioned MSC medium.
Results were comparable with those seen when using IV and IT delivered MSCs.
This paracrine action was attributed to increased levels of KGF and IL-10.
As locally administered MSCs in the lung do not significantly enhance therapeutic
effect over systemically administered MSCs, other more distal routes of delivery
may equally provide benefit. IV, IT and intra-pleural administration requires some
form of anaesthesia to be administered, followed by an invasive procedure to
deliver the cells. The option to administer MSCs via the intra-peritoneal (IP) route
would be attractive clinically. Anaesthesia is not necessary for this procedure in
preclinical models and administration can be completed in one injection. IP delivery procedures over-come problems encountered with IT and IV administration such as possible expulsion from the lungs following delivery IT, the formation of clots due to incorrect administration IV and infection or necrosis surrounding the point of entry. Routine piloting and post-mortem analysis ensured all procedures were carried out correctly.

**11.5.3.3 The Importance in Demonstrating True Therapeutic Efficacy**

Despite the extensive body of work involving MSCs, there is a substantial amount of questions that need to be answered. MSC therapy is ever expanding and has been used in human trials, in some cases with encouraging success (208). The numerous publications of pre-clinical data would suggest that MSCs are an untapped resource with vast possibilities in areas of protection, repair and regeneration, however it has been noted that the data presented is not always clear about the source of MSCs and the need for identification of source, species and cell profiles is clear (369). In our study group, efficacy was shown using rodent MSCs in a rodent model of VILI (220). Following this the safety and efficacy of human MSCs isolated from bone marrow was investigated with similar results seen. Following this, experiments to demonstrate the therapeutic efficacy were performed by administering MSCs at later time-points in the progression of VILI.

**11.5.3.4 The Need to Demonstrate Efficacy for Specific hMSC Sub-populations**

To date, the most simple and frequently used method of isolating MSC from marrow is dependent upon the previously noted adherence to tissue culture plastic, according to which the cells are left to incubate for 10-14 days and in the interim MSC will attach and form colonies at a recognised frequency of 1/100,000. At 10-14 days these colony forming units-fibroblasts (CFU-F) are harvested by trypsin digest and re-plated in serum-rich media at a density of 3-8000 MSC per cm². The CFU-F-derived MSC are then propagated *in vitro* until sufficient cell numbers are
obtained. This ‘parental’ (PA) MSC isolation technique was developed in the 1960s and is used by Osiris, Athersys and most academic investigators (177). However, this is increasingly regarded as inadequate for defining or purifying MSC for clinical use, as only 1/100,000 BM-MNC plated are actually MSC, the methods will not comply with future good manufacturing protocols needed for clinical approval of related products.

All other current trials are testing PA-MSC, and these cells may be considered inadequately pure under future EU/UK legislation. Therefore methods to purify MSC populations are currently being investigated with the hope that a highly efficient, legislation compliant method is developed to isolate the sub-population of cells which demonstrate the efficacy seen with heterogeneous populations.

11.6 hMSCs for ARDS – Addressing Barriers to Clinical Translation

11.6.1 Optimisation of hMSC Dosing Regimens

Prior dosing studies were performed by our study group to investigate the optimal dose of human MSCs in a rodent model of VILI (Data not shown). The doses used were based on earlier experiments conducted in our group using rodent MSCs in a rodent model of VILI (221), in which a dose of 1x10^7 cells/kg was used IV or IT. The dose response study aimed to determine the lowest effective dose (ED) in the same model of ALI using doses ranging from 1x10^6 cells/kg to 1x10^7 cells/kg. It was demonstrated that doses as low as 2x10^6 cells/kg produced significant efficacy in improving arterial PaO_2, BAL neutrophil counts and total white cell numbers. It is promising that doses as low as 2x10^6 cells/kg have shown efficacy in this model.

11.6.2 Optimising the Route of Administration of hMSCs

In these studies we used human MSCs and a dose of 4x10^6 cells/kg, chosen to allow for variances in response to administration via IT or IP routes, we aimed to compare three routes of MSC administration for the treatment of ALI in a rodent
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model of VILI. All MSC doses were harvested from ongoing culture and administered no later than 20 minutes post-trypsinisation. Both IV and IT administration of MSCs demonstrated comparable efficacy in the restoration of lung function, epithelial barrier integrity and lung structure. IP administration did not demonstrate the same levels of efficacy, however promising results were shown. A number of reasons may contribute to this; MSCs are known to home to the site of injury (226, 263), and as such the protective effect of MSCs is thought to be enhanced through cell-cell contact (370). Islam and colleagues have demonstrated using live optical studies that MSCs directly transfer microvesicles to cells in the alveolar epithelium of mice during ALI further suggesting that direct cell-cell contact is needed (247). However, results presented in our study demonstrate a trend toward an improvement after IP administration in support of a paracrine mechanism of action of MSCs.

It has been demonstrated in recent studies that MSCs also work via a paracrine mechanism which does not require cell administration (248, 255) and a recent study by Zhu and colleagues have demonstrated the efficacy of isolated MSC microvesicles in the treatment of E.coli induced ALI in mice (259). It has been known for some time that KGF has beneficial effects in ALI (253, 371, 372) and recently it was shown to be secreted by MSCs and implicated in the enhancement of wound repair (248). Zhu et al. have demonstrated that the therapeutic effects shown by MSCs are due to their secretion of microvesicles and the transfer of mRNA by these vesicles to the lung epithelium (259). KGF has been shown to be at least one of the main mediators of repair in this study. These results would suggest that some degree of cell-cell contact or at least access to the pulmonary blood flow is needed for the effects of MSCs to be seen within 24 hours. The aforementioned studies by Zhu et al. (259) and Curley et al. (221, 248) demonstrated results at 48 hour time-points whereas this study used 24 hour time-points.

We may have found an effect with IP administration at 48 hours; however, at 24 hours it would seem that the MSCs have either been compartmentalised or have moved to the other organs. Lavage of the peritoneal cavity would demonstrate if there were MSCs present at 24 hours. A more in depth distribution study including the IP route of administration would also provide more information regarding this. In the treatment of disease it is desirable that a treatment be as
efficient as possible and fast-acting also. Therefore the IP route was disregarded for further study in this series and IV and IT deemed most suitable. However with IT administration the application of the cells directly to the lungs necessitates fluid to be introduced as vehicle and to injured lungs this is additional fluid, counterintuitive to what we try to achieve. Overall in this study and others (221) introduction of a small bolus of fluid did not however, demonstrate any adverse effects.

A key issue in understanding the optimal route of MSC administration is understanding how it affects the distribution of MSCs. MSC distribution in this model of VILI was demonstrated using labelled MSCs and subsequent flow cytometry analysis (Section 7.7). The percentage of MSCs present in the lungs at 30 minutes, 1 hour and 24 hours was shown after IT and IV administration. Immediately after IT administration the majority of the labelled cells (64%) were detected in the lung whereas a much smaller proportion was detected after IV administration (16%). By 4 hours there was 23% of the IT administered cells and 8% of the IV administered cells detected. This had reduced to 10% for IT and 2% for IV administered cells by 24 hours. It is likely that the cells were more difficult to clear from the lungs after administration as a bolus, whereas with IV administration cells were more evenly distributed in the pulmonary circulation and their further distribution from the lung was less restricted. In addition to this, cells may have travelled to other organs from the venous circulation en route to the lungs. Our study was in agreement with other such distribution studies in other injured or uninjured models (265, 267, 268). Significant benefits of using MSCs were shown in this study after the administration of MSCs either IT or IV leading to the conclusion that for this injury, the choice of either route of administration will produce an equally positive outcome.

11.6.3 Demonstrating the Therapeutic efficacy for hMSCs

So far we have investigated dose-response of human MSCs in VILI and the optimal route of administration of these MSCs. Next we looked at the therapeutic window of administration of the MSCs in a rodent model of VILI. This study was conducted to determine if there is a cut-off point for beneficial administration of
MSCs to this model of lung injury. Time points of 0, 6 and 24 hours post injury (corresponding to 100%, 87.5% and 50%, respectively, into the recovery period following VILI) were chosen for investigation using 4x10^6 cells/kg for comparison of efficacy to the route of administration study. Animals were harvested 48 hours post injury and indices of repair and injury examined. It is evident that MSCs can significantly attenuate VILI when administered up to 24 hours after injury. It does seem that if MSCs can be administered within the first 6 hours after injury the greatest benefit will be seen 48 hours later in terms of lung function. However cell infiltrates and histological analysis demonstrate comparable levels of improvement at all time-points. This data would suggest that MSCs could be administered at later time-points following the initiation of injury without significant loss in efficacy, making this a useful point to note in a clinical sense where time of injury onset may not be known.

11.6.4 The ORB-1 Sub-Population of hMSCs Enhance Recovery Following VILI

Our collaborators at Orbsen Therapeutics have identified a series of antibodies that enhance the isolation and purity of human MSC from BM beyond the MSC/MNC purity ratio of 1/13. The lead candidate is a cell-surface sulfated proteoglycan, designated ORB-1. ORB-1 has been codified due to ongoing intellectual property filings by Orbsen Therapeutics. Using our established model of VILI we administered sub-populations of cells positive for the ORB-1 marker (ORB-1^+), negative for the ORB-1 marker (ORB-1^-), Parental populations of MSCs (PA-MSCs), MRC5 fibroblasts or vehicle (PBS) via the tail vein canula immediately post VILI injury. 24 hours later animals were sacrificed and examined for signs of injury and repair. It was clearly shown that both ORB-1^+ and ORB-1^- cells were significantly effective at attenuating VILI in rodent models. Lung function was shown to be restored as well as structural integrity. Influx of proteinaceous fluid and levels of inflammatory cytokines were also shown to be improved in all MSC groups. This shows an overall promising outcome and leads to future promise for the use of purified, more defined populations of MSCs in the treatment of ARDS.
11.7 Future Direction for hMSC Therapy for ARDS

11.7.1 Clinical Trials – Why Wait?

Due to the promise demonstrated by pre-clinical experiments, clinical trials are ongoing or planned in a wide range of diseases for which there is no cure, including ARDS. Important pre-clinical data exists to encourage the use of MSCs in lung injuries (224, 225) (222, 248). While these and other studies are extremely promising, there are still questions which must be answered for the safe and effective use of MSCs in ARDS.

11.7.2 Additional Pre-Clinical Studies are Needed

In designing experiments to ascertain the effect of a therapeutic in a disease state a number of aspects need to be addressed. The model should reflect the condition being treated, the therapy should be of the same preparation and constitution of that which will be used clinically and the end-points should be pre-determined to allow for all possible effects to be examined and for the reproduction of these studies by others. More relevant animal models need to be developed, the dosing and timing need to be clarified and the safety issues must be addressed.

11.7.2.1 Multiple-Hit Injury Models

In clinical incidences of ARDS, the condition can be induced by a range of injuries such as sepsis, aspiration of gastric contents, inhalation of injurious substances or VILI, likely in the presence of pre-existing conditions. Developing an in vitro (or an in vivo) model that adequately reflects the complexities of clinical ARDS is challenging.

At present animal models provide the opportunity to test experimental drugs and intervention strategies before human trials and are a vital opportunity to examine effects in a whole organ system. Two hit animal models are an attempt to mimic two conditions that are commonly found in conjunction (such as ARDS and systemic sepsis); however, the variables, and therefore, results arising in these models are more difficult to pinpoint. Based on this, it is common practice to study one particular aspect of a disease or injury per series. For example, in the
investigation of therapeutic interventions in ARDS, researchers will study sepsis, VILI or fibrosis and have an animal model developed for this. However, the method by which these injuries are induced can also differ between study groups. Sepsis can be induced by administering bacteria or endotoxin, or by performing CLP, and fibrosis by bleomycin, acid aspiration and others. These injurious agents can be administered in different doses and via different routes and can then therefore have different outcomes. However, despite these difficulties in developing and interpreting multiple-hit models, their greater clinical relevance makes them very attractive for additional MSC translational studies.

11.7.2.2 Dosing, Timing and MSC Distribution

The dose of MSCs required for benefit, while minimizing harm, is an important issue including the need for multiple administrations. Despite being a cell therapy, there is still a very real danger of adverse effects as seen with pharmacological interventions. The highest tolerable dose has not been established, nor has the most efficient route or timing, all important clinical factors. Our studies presented in this thesis aimed to tackle some of these parameters with promising results; however, further studies are required and until then caution should be exercised in clinical trials. The mean doses administered in pre-clinical models of ARDS were almost 30x10^6 cells/kg in mice and over 20x10^6 cells/kg in rats without adverse effects (206). In the COPD clinical trial patients were given four doses of MSCs, 10x10^7 cells/infusion, which were much lower than the doses used in many of the preclinical ARDS studies.

The timing of dose administration may be an important factor in the efficacy of MSCs. Most studies of MSCs in ARDS/ALI administer therapy within 6 hours of the induction of injury (206). The present study demonstrated efficacy up to 24 hours post VILI induction which correlates with these studies in terms of efficacy.

As well as this it has been shown that MSCs administered IV first accumulate in the lungs and then home to sites of injury (263), however this has been subject to dispute. Due to variances in models, injury status, MSC labelling techniques and detection methods a conclusive result cannot be drawn. In terms of safety, the fate
of MSCs should be determined after administration. This would require long term pre-clinical studies with a method of persistent MSC labelling and efficient detection methods. The need for longer time-points is evident.

11.7.2.3 Need to address safety concerns regarding MSCs

Despite the undeniable promise of MSCs there are some safety concerns which need to be addressed. It has been shown in pre-clinical models, that rapid administration of murine MSCs IV lead to a significant risk of pulmonary thrombosis in mice (373). Our group observed that administering relatively high doses of $4 \times 10^6$ cells were not tolerated in models of VILI, likely due to increased right ventricular strain. The actual maximum tolerated dose has not been established.

MSCs have been previously reported to undergo malignant transformation in vitro, however these reports have been either unfounded or as a result of cross-contamination of MSC cultures (374). Nevertheless, it is vital that such a scenario is avoided at all costs. MSCs will need to be rigorously defined for clinical use which necessitates the need for new methods of characterisation, isolation, and purification. Producing a more homogeneous population of MSCs will decrease the risk of contamination with other cell types. These methods and the resulting populations yielded will need to be thoroughly tested pre-clinically before advancing to human trials.

11.7.2.4 Homogenous Cell Populations

Current MSC isolation techniques involve the use of plastic adherence (177) which is being increasingly regarded as inadequate for clinical use as it yields 1 MSC per 100,000 MNC. MSCs will need to be purified and clinically defined to comply with future GMP protocols.
11.7.3 Limitations and Future Direction of These Studies

As with any experimental analysis a number of limitations must be addressed before a procedure is considered for clinical use. In this study we used a previously established rat model of VILI (286) and examined the effects of MSC administration via different routes and at different time-points on the recovery profile of the animal. The limitations of this model are as follows;

1: VILI is generally seen in conjunction with other lung disease or injury and rarely found as an isolated condition. Future studies should be carried out in multiple hit models of ARDS to obtain a clearer picture of the effects of MSCs on injury and repair mechanisms in a more clinically relevant injury.

2: Injury parameters seen in this pre-clinical model of VILI return toward normal values at 96 hours making the effects of MSCs in this injury over longer periods harder to determine. Perhaps the use of longer ventilation protocols would alter the outcome and prolong the injury and repair profile giving further insight to both the injury state and the effects of MSCs in ARDS.

3: The airway pressures used in this model to induce VILI are rarely encountered in a clinical setting. However, the model represents pressures that likely occur in regional areas of the ARDS lung, termed ‘baby lung’ (86). Moreover, the model fulfils the American Thoracic Society guidelines on the use of animal models for VILI (375).

Further to this, a number of limitations and future experiments can be outlined with regards to the use of MSCs in the treatment of ARDS.

1. Clinical grade MSCs were not used in this study as the definition of such populations are not clearly defined. We received MSCs and control cells from Orbsen Therapeutics Ltd which ensured the cells were processed to the same standards each time. In an attempt to produce a more clinically defined MSC population this company have produced sub-populations isolated using their patented surface marker ‘ORB-1’. We tested these cells for efficacy compared to heterogeneous populations of MSCs with comparable results, however further analysis in other models of ARDS will need to be conducted.

2. The MSCs used in this study were not pre-activated and this could be an important factor in potentiating the effect of the MSCs by resulting in increased secretion of beneficial proteins of the MSC secretome. A recent study has
demonstrated the increased production of tumour necrosis factor-α-related apoptosis-inducing ligand (TRAIL) and DKK-3 from MSCs when they are pre-treated with TNF-α, and found their tumour suppressive activity to be enhanced as a result (376).

3. Previous studies by our group and others demonstrated the efficacy of conditioned medium from MSCs (214, 221, 224). The present study could be conducted using conditioned media from activated MSCs, parental and sub-populations, given via different routes and at different time-points following injury. Further in vitro studies on the conditioned medium of treated and untreated MSCs would also give further insight to the MSC secretome in response to ARDS.
11.8 Summary and Conclusions

To date many attempts have been made to discover a prevention, treatment or cure for ARDS and ALI. Experiments in this study concentrated both on the mechanism of action of HCA, which exerts potentially important effects – both beneficial and potentially harmful – in patients with ARDS seen to be beneficial and on the investigation of the therapeutic potential of MSCs for ARDS. 

*In vitro* experiments aimed to isolate the locations in the NF-κB pathway where CO₂ plays its protective role. To do this we employed various pulmonary and systemic cell lines and cultured them under conditions of NC and HC. Cells were injured using a range of known NF-κB pathway activators and outcomes were measured using various protein/activity detection methods. 

In this series of experiments it was shown that:

1: HCA appeared to confer an effect at almost all steps in the lead-up to gene transcription and protein translation on the NF-κB pathway. Importantly we have shown that therapeutic levels of HCA do not cause further injury to epithelial cells *in vitro.*

2: *In vivo,* it was demonstrated in an established and well understood rodent model of VILI that an optimal dose of MSCs, previously described by our group was equally effective when administered either IV or IT. A more modest level of efficacy was demonstrated when MSCs were administered IP.

3: Therapeutic efficacy was demonstrated when MSCs were administered up to 24 hours post injury. Importantly no adverse effects of MSC treatment were shown.

4: In a more specific study a highly purified sub-population of MSCs were used and shown, in some aspects, to be as effective as mixed populations of MSCs. 

This study broadens our understanding of MSCs and also opens up the opportunity for new experiments in this field aimed at translating this specific MSC subpopulation towards clinical testing.
12. Publications

12.1 Papers:

12.1.1 Peer Reviewed Published and In Press:

Effects of Intratracheal Mesenchymal Stromal Cell Therapy during Recovery and Resolution after Ventilator-induced Lung Injury.

Over expression of pulmonary extracellular superoxide dismutase attenuates endotoxin-induced acute lung injury.

12.1.2 Peer Reviewed Submitted:
Elevated Carbon Dixoide Inhibits Canonical NF-κB signaling and the Activity of the p50/p65 Heterodimer in Human Cells.

12.3 Abstracts:

Efficacy of intravenous vs intra-tracheal human mesenchymal stromal cells therapy in *E.coli* induced pneumonia. (Poster Discussion)
Poster Presented at the American Thoracic Society Annual Conference 2013, Philadelphia, PA, USA.

Effects and Distribution of human Mesenchymal Stem Cells after Intratracheal, Intra-venous and Intra-peritoneal Administration in a Rat Model of Ventilator Induced Lung Injury. (Poster Discussion)
Poster Presented at the American Thoracic Society Annual Conference 2013, Philadelphia, PA, USA.

**Effects and Distribution of Mesenchymal Stem Cells after Intra-tracheal and Intra-venous Administration in a Rat Model of Ventilator Induced Lung Injury.** *(Poster)*


**The Effect of Hypercapnic Acidosis on the NF-κB Signalling Pathway in Human Pulmonary Epithelial Cell Lines in vitro.** *(Poster)*

C.H. Masterson, D. O’Toole, J.G. Laffey

Poster presented at the Royal Academy of Medicine in Ireland Conference 2012, Galway, Ireland.

**Over Expression Of Secretory Leukocyte Protease Inhibitor (SLPI) And Glutathione Peroxidase-3 (GPX-3) Attenuates Inflammation And Oxidant-Mediated Pulmonary Epithelial Injury.** *(Poster)*

C.H. Masterson, D. O’Toole, J.G. Laffey

Poster Presented at the American Thoracic Society Annual Conference 2012, San Francisco, CA

**Hypercapnic Acidosis Attenuates Stretch-Induced Inflammation In Alveolar Epithelial Cells By An NF-κB Dependent Mechanism.** *(Poster)*

Ansari BM, Curley GF, Masterson CH, McHale PM, O’Toole DP, Laffey JG.

Poster presented at the American Thoracic Society 2011, Denver, CO, USA.

**Hypercapnic Acidosis and the NF-κB pathway - Effects on P65 subunit of NF-κB.** *(Poster)*

Masterson CH, O’Toole DP, Laffey JG.

Poster presented at the American Thoracic Society 2011, Denver, CO, USA.

**Contrasting Effects Of Metabolic Versus Hypercapnic Acidosis On Pulmonary Epithelial Wound Healing.** *(Poster)*

McHale PM, Masterson CH, Ansari BM, O’Toole DP, Laffey JG.

Poster presented at the American Thoracic Society 2011, Denver, CO, USA.

**Hypercapnic Acidosis Inhibits NF-κB Mediated Inflammation in a Rodent Model of Ventilator Induced Lung Injury.** *(Poster)*

Contreras M, O’Toole D, Higgins B, Ansari B, Curley G, Masterson C, Laffey JG

Poster presented at the TERMIS EU Conference 2010, Galway, Ireland.
Inhibition of the NF-κB Inflammatory Signalling Pathway by Carbon Dioxide involves Reduced Kinetics of Multiple Kinases. (Poster)
Masterson C, O’Toole D, Leo AM, Conteras M, Hassett P, Laffey JG.
Poster presented at the TERMIS EU Conference 2010, Galway, Ireland.

Hypercapnic Acidosis Impairs Inflammatory Signalling Via Inhibition Of Multiple Points Of The NF-κB Pathway. (Poster)
O’Toole D, Masterson C, Leo AM, Conteras M, Hassett P, Laffey JG.
Poster presented at the American Thoracic Society 2010, New Orleans, LA, USA.

Hypercapnic acidosis attenuates moderate and severe ventilator induced lung injury by an NF-κB dependent mechanism. (Poster)
Conteras M, O’Toole D, Higgins B, Ansari B, Curley GF, Masterson C, Laffey JG.
Poster presented at the American Thoracic Society 2010, New Orleans, LA, USA.

Investigation of the mechanisms by which hypercapnic acidosis inhibits activation of the NF-kB signalling pathway. (Poster)
O’Toole D, Masterson CH, Leo AM, Hassett P, Laffey JG.
Poster presented at the ISGCT Meeting 2009, Cork, Ireland.
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