Therapies for Acute Respiratory Distress Syndrome: Determination of the Mechanisms of Action of Hypercapnia & the Therapeutic Efficacy of Mesenchymal Stromal Cells

By

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Abstract

Rationale: Acute Respiratory Distress Syndrome (ARDS) causes severe respiratory failure and is associated with high mortality. Pharmacological therapies are non-existent and supportive ventilation treatments are limited. High ventilator induced stretch, termed Ventilator Induced Lung Injury (VILI), is a common propagator of ARDS development and causes severe inflammation and death of the lung parenchyma. Hypercapnia with its associated acidosis (HCA) appears to play a role in ARDS modulation by regulating inflammation but the mechanisms of this are not fully understood in the context of high stretch injury. Furthermore, Mesenchymal Stem/Stromal cells (MSCs) are promising contenders for ARDS therapy but issues with source, batch and donor variability, culture conditions and delivery to the clinic have not been fully resolved.

Aims: **(1)**: Determine the mechanisms by which HCA attenuates NF-κB activation and epithelial injury following high mechanical stretch *in vitro*. **(2)**: Determine the mechanisms by which the MSC "secretome" attenuates lung injury *in vitro* and the potential for enhancement by MSC pre-activation. **(3)**: Re-establish a relevant VILI animal model of ARDS and elucidate the potential for different MSC populations to enhance recovery. **(4)**: Determine the efficacy of umbilical cord (UC) versus bone marrow (BM) derived MSCs. **(5)**: Determine the impact of cryo-preservation and **(6)** culture in xeno-free (XF) conditions on the efficacy of MSC repair; and **(7)** the potential to enhance efficacy of XF and/or cryo-preserved MSCs by pre-stimulation.

Methods: **(1)**: Pulmonary alveolar epithelial cells were subjected injurious mechanical stretch injury and the potential for HCA to attenuate stretch induced cell inflammation and death was assessed. The interaction of the NF-κB pathway in HCA mediated effects was also assessed, as was the role of acidosis versus CO_2. **(2)**: The pulmonary alveolar epithelium was subjected to oxidative stress injury, inflammatory activation, wound injury and mechanical stretch injury and the efficacy of the MSC secretome to attenuate

damage and promote repair was assessed. The potential to enhance the MSC secretome efficacy by pre-activation with inflammatory cytokines was also determined. **(3)**: Animals were subjected to high pressure injurious ventilation followed by intravenous MSC administration. The efficacy of a defined $S2^+$ MSC subpopulation to enhance recovery and repair following VILI was assessed. **(4)**: The efficacy of UC derived MSCs to promote recovery post VILI was compared to standard BM derived MSC therapy and the effect of cryo-preservation on this efficacy was also determined. **(5-7)**: The efficacy of cryo-preserved XF MSCs to promote recovery and repair at a time point of maximal VILI was determined, as was the potential for enhancement of efficacy by pre-activation.

Results: **(1)** HCA attenuates high stretch induced inflammation and injury in the pulmonary epithelium through inhibition of the canonical NF-κB pathway and by way of generating an acidosis. **(2)**: Pre-activation of MSCs enhances the protective effects the secretome in the injured pulmonary epithelium. **(3)**: An $S2^+$ subpopulation of MSCs enhances resolution and repair post VILI. **(4)**: UC derived MSCs promote recovery post VILI with similar efficacy to BM MSCs and **(5)** this efficacy is retained after cryo-preservation. **(6)** XF cultured MSCs retain efficacy, while **(7)** pre-activation enhances the efficacy of cryo-preserved, XF expanded MSCs in lung recovery and repair following ventilator injury.

Conclusion: **(1)**: HCA modulates inflammation and may contribute to the benefit seen with protective ventilation in ARDS patients. **(2)**: MSCs possess protective paracrine mechanisms of action and the secretome may be a viable alternative to the cell treatment. **(3)**: $S2^+$ MSCs may represent a more defined and less variable therapy for ARDS, and needs further investigation in other pre-clinical ARDS models. **(4)**: UC derived MSCs are efficaciously comparable to standard BM MSCs, **(5)** even after cryo-preservation and may be a more readily available and less variable MSC therapy for the ARDS patient. **(6)**: XF MSCs are also therapeutic, while **(7)** pre-activation enhances the protective and immunomodulatory functions of cryo-preserved XF MSCs and may provide a more efficacious treatment in the clinical setting of ARDS.

List of Abbreviations

3, 3′, 5, 5′-Tetramethylbenzidine	TMB
Acute Respiratory Distress Syndrome	ARDS
Adipose	AD
Alpha Minimum Essential Eagle Medium	MEM-α
Alveolar Epithelial Cells	AECs
Analysis Of Variance	ANOVA
Angiotensin-Converting Enzyme 2	ACE-2
Bone Marrow	BM
Bovine Serum Albumin	BSA
Bronchoalveolar Lavage Fluid	BAL
Buffered Hypercapnia	BHA
Conditioned Medium	CM
Cytokine-Induced Neutrophil Chemoattractant 1	CINC-1
Dendritic Cells	DCs
Dermal Fibroblast	DF
Empty Vector	EV
Enzyme Linked Immunosorbent Assay	ELISA
Eosin	E
Ethanol	EtOH
Ethylenediamine Tetra Acetic Acid	EDTA
Fibroblast Growth Factor	FGF
Graft vs. Host Disease	GVHD
Haematoxylin	H
Hepatocyte Growth Factor	HGF
Human Leukocyte Antigen	HLA
Hydrogen Peroxide	H_2O_2
Hypercapnia	HCA
Indoleamine 2, 3-Dioxygenase	IDO
Interferon Gamma	IFN-γ

Interleukin-6	IL-6
Intra-Peritoneal	IP
Intra-Tracheal	IT
Intravenous	IV
Keratinocyte Growth Factor	KGF
Lactate Dehydrogenase	LDH
Lipopolysaccharide	LPS
Lymphotoxin-Beta	LT-β
Macrophage Inflammatory Protein 2	MIP-2
Major Histo-Compatibility Complex	MHC
Matrix Metalloproteinase-2	MMP-2
Mesenchymal Stem/Stromal Cells	MSCs
Microvesicles	MVs
Minutes	m
Natural Killer Cells	NKs
NF-κB-Inducing Kinase	NIK
Nitric-Oxide	NO
Nuclear Factor Kappa B	NF-κB
Ovalbumin	OVA
Paraformaldehyde	PFA
Partial pressure of arterial oxygen & fraction of inspired oxygen	PaO_2/FiO_2
Peak Inspiratory Pressure	PIP
Phosphate Buffered Saline	PBS
Polymorphonuclear Neutrophil	PMN
Positive End-Expiratory Pressure	PEEP
Prostaglandin E_2	PGE_2
Standard Deviation	SD
Super-Oxide	SO
Super-Repressor	SR
Syndecan-2	S2
Thiazolyl Blue Tetrazolium Bromide	MTT

TNF-α-Stimulated Gene 6	TSG-6
Transforming Growth Factor	TGF
Tumour Necrosis Factor-Alpha	TNF-α
Umbilical Cord	UC
Vascular Endothelial Growth Factor	VEGF
Ventilator Induced Lung Injury	VILI
Xeno-Free	XF
Xylene	Xy

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1.0 Introduction

1.1 Acute Respiratory Distress Syndrome

1.1.1 Definition and Diagnosis

Acute Respiratory Distress Syndrome **(**ARDS), first described by Ashbaugh *et al*., in 1967 [1] is a prominent condition of severe respiratory failure that has a high mortality rate of about 40% [2, 3]. Patients with ARDS usually present with signs of dyspnoea, chest pain and cyanosis, and the disorder is clinically characterised by an acute onset (usually within one week of a known clinical insult) with bilateral pulmonary infiltrates, oedema and hypoxemia **(Table 1.1)** [4-7].

In accordance with the recent Berlin definition of ARDS, patients are classified into three severity categories known as mild, moderate or severe ARDS [7]. These categories are based on the severity of hypoxemia, which is measured by the ratio of partial pressure of arterial oxygen and fraction of inspired oxygen (PaO_2/FiO_2 ratio); in essence to standardise the level of oxygen in the blood by dividing it by the inspired fraction of oxygen **(Table 1.1)** [7]. This definition also highlights that for severe ARDS, patients must show "severe oedema as evidenced by the pulmonary infiltrates on chest radiograph examination" [7]. Additional diagnostic criteria for severe ARDS were proposed, such as "compliance of $\leq$ 40mL/cmH_2O, corrected expired air volume of $\geq$ 10L/min and positive end-expiratory pressure (PEEP) of $\geq$ 10cmH_2O" but these ultimately were not included in the Berlin criteria [7].

Table 1.1: The Berlin Definition of ARDS [7]	
Category	*Description*
Timing/Onset of ARDS	• Within one week of a known clinical insult or new or worsening respiratory symptoms
Chest Imaging (Chest radiograph or computed tomography scan)	• Bilateral opacities that are 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 ARDS – 200 mmHg < PaO_2/FiO_2 ≤ 300 mmHg with PEEP or CPAP ≥ 5 cmH_2O • Moderate ARDS – 100 mmHg < PaO_2/FiO_2 ≤ 200 mmHg with PEEP ≥ 5 cmH_2O • Severe ARDS – PaO_2/FiO_2 ≤ 100 mmHg with PEEP ≥ 5 cmH_2O

1.1.2 Aetiology and Epidemiology of ARDS

The development of ARDS is associated with several conditions, including pneumonia, systemic infection, aspiration of gastric contents or shock and haemorrhage following major surgery or trauma to the lung [2-4] with pneumonia and extra-pulmonary sepsis being the main causes of ARDS [3, 8]. In fact, a recent study in a cohort of intensive care units across 50 countries highlighted that pneumonia was the risk factor in 59.4% of ARDS patients and systemic sepsis preceded ARDS in 16% of cases [3]. It is also important to highlight that patients with ARDS can usually have more than one risk factor [3].

It is estimated that there are 190,000 cases of ARDS every year in the US, which result in 74,500 deaths [2]. It has also been estimated that patients with ARDS account for 10.4% of intensive care unit admissions and 23.4% of all patients that require mechanical ventilation [3]. More worryingly, only approximately 62% of ARDS cases

were correctly recognised in the clinical setting [3]. Of these, ~ 70% of patients presented with moderate to severe ARDS and as mentioned previously the mortality rate was high, at approximately 40% [3]. Furthermore, patients that survive ARDS continue to suffer from functional and neuropsychological impairment up to five years after follow up, and as such there is a heavy disease burden on both the families of these patients and healthcare systems [9].

1.1.3 ARDS Pathogenesis

1.1.3.1 Epithelial and Endothelial Injury

ARDS is characterised by severe epithelial and endothelial damage and cell death leading to impaired barrier function, altered fluid clearance and hence increased vascular permeability [5, 10, 11]. As a result, there is an accumulation of protein rich fluid in the lungs which contains activated neutrophils and macrophages that in turn facilitate severe inflammation **(Figure 1.1)** [4, 5, 10, 12, 13].

1.1.3.2 Inflammatory Response

Inflammation is one of the key features in the pathogenesis of ARDS. As mentioned, lung membrane damage is caused by inflammation of the lung parenchyma, which is arbitrated by an influx of neutrophils and macrophages [4, 10, 12, 13]. Neutrophil and macrophage activation leads to the unbalanced release of pro-inflammatory cytokines and chemokines, including interleukin-6 (IL-6), IL-1β, IL-8, tumour necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ), which in turn facilitate further inflammatory cell recruitment and cytokine release into the lung **(Figure 1.1)** [5, 10, 13-15]. Furthermore, the release of anti-inflammatory molecules such as IL-10 is severely inhibited in ARDS patients [16].

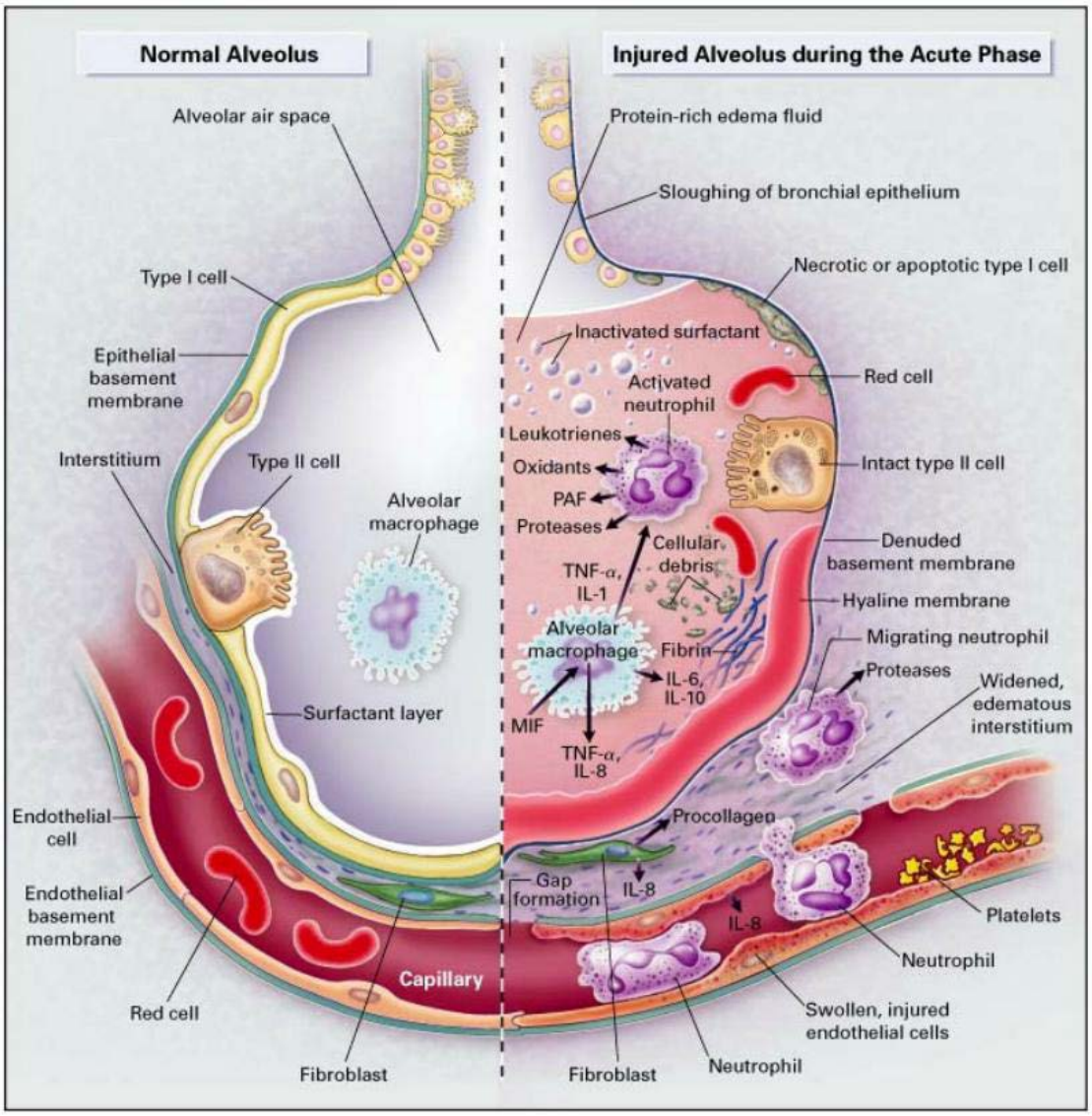

Figure 1.1: The Uninjured and Injured Alveolus in ARDS. Reproduced with Permission from Matthay *et al*., 2011 [4]. Copyright Massachusetts Medical Society.

Inflammatory cell infiltration, also causes the production of reactive oxygen and nitrogen species, such as super-oxide (SO) and nitric-oxide (NO) radicals, which causes extensive cell death and consequent reduced surfactant production, leading to even further impairment of endothelial and epithelial barrier function and prominent vascular oedema **(Figure 1.1)** [4, 5, 11, 13, 17, 18]. Of interest, studies have shown that the activation of the nuclear factor kappa B (NF-κB) inflammatory signalling pathway is prominent in alveolar macrophages of ARDS patients [19], while numerous different

experimental studies using high mechanical stretch have linked injury directly to the activation of NF-κB [20-22].

1.1.3.3 NF-κB Signalling in Lung Injury

NF-κB is a transcription factor which regulates the inflammation response by initiating or enhancing the release of numerous pro-inflammatory molecules such as IL-1β, TNF-α, IL-8 and cytokine-induced neutrophil chemoattractant 1 (CINC-1) [23] and is implicated in numerous disease states including diabetes, arthritis and sepsis [24-26]. Of importance to ARDS, the NF-κB pathway has been observed to be intimately involved in regulating lung inflammation, injury and death specifically by promoting inflammation, oxidative stress and cell death [15, 19, 23, 27-31].

NF-κB is made up of five subunits NF-κB1 (p50/105), NF-κB2 (p52/100), Rel A (p65), Rel B, and c-Rel that can form a number of homodimers and heterodimers [32-34]. Under normal physiological conditions, NF-κB dimers are sequestered in the cytoplasm by inhibitory IκB proteins (IκBα, IκBβ, and IκBε) which are regulated by the IKK complex (made up of active IKKα and IKKβ and regulatory IKKγ) [32, 33]. NF-κB activation can occur by classical/canonical activation or by alternative/non canonical activation **(Figure 1.2)**.

Canonical NF-κB activation is a rapid and transient process [35], and can be induced by numerous exogenous stimuli such as lipopolysaccharide (LPS), a bacterial endotoxin, cytokines (IL-1β or TNF-α) and oxidative stress molecules [32, 33]. The IKK complex is then specifically activated, which leads to the phosphorylation of IκBα, which is then targeted for degradation, that then causes the translocation of mostly the p50/p65-containing heterodimers into the nucleus **(Figure 1.2)** [32, 33, 36]. There, NF-κB can up-regulate inflammatory gene transcription, including that of IL-1β and IL-8 [32, 33].

The non-canonical pathway is activated by TNF-family cytokines such as lymphotoxin-β (LT-β) and other ligands but not by TNF-α [32, 33, 37]. Furthermore, this activation occurs through NF-κB-inducing kinase (NIK) dependent, IKKα-mediated

phosphorylation of Rel B which leads to the generation of active p52-RelB dimers that can translocate to the nucleus **(Figure 1.2)** [32, 33, 37]. Non-canonical NF-κB pathway activation is slow and persistent, and regulates the gene transcription of chemokines including SLC and ELC which are involved in lymphoid organogenesis and T cell trafficking [35, 37, 38]. Deregulation of this pathway is associated with severe inflammation, autoimmunity and cancer development [35].

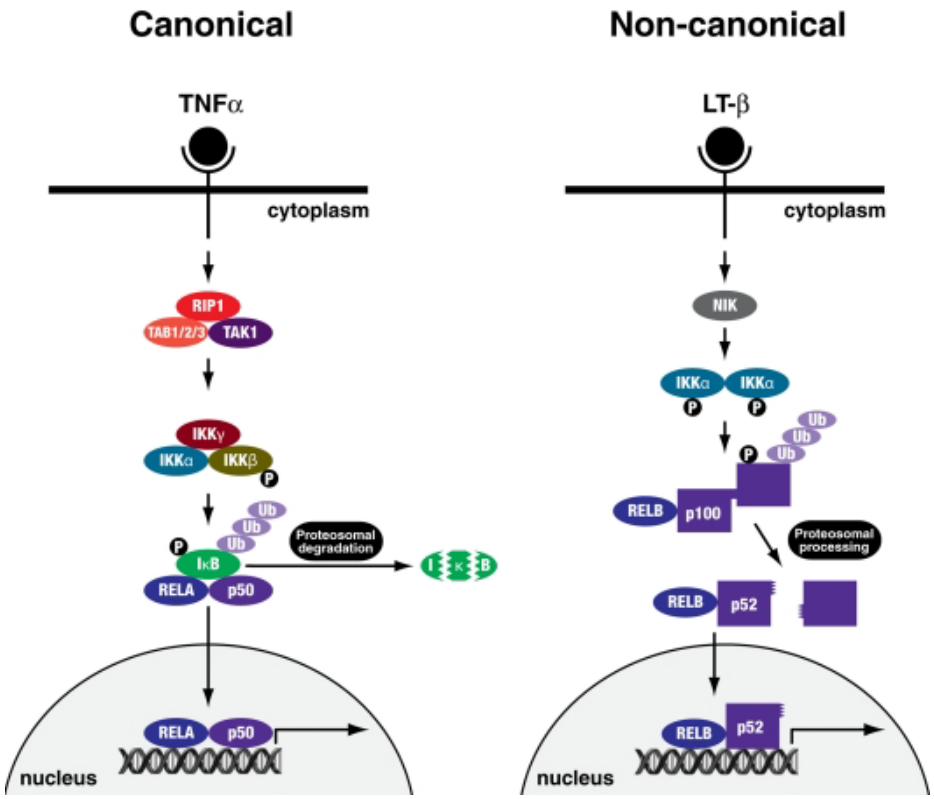

Figure 1.2: The Canonical and Non-Canonical Pathways of NF-κB Activation. Reproduced with Permission from Vaughan *et al*., 2011[39]. Copyright 2011 Vaughan and Jat.

1.1.3.4 Resolution of ARDS

The late phase of ARDS is known as the fibrotic phase and occurs for many reasons including, (1) when inflammation and alveolar cell death is prolonged, (2) alveolar fluid clearance is not restored and (3) abnormal repair occurs due to an imbalance between the release of pro-coagulant and anti-coagulant molecules (such as tissue factor and protein C respectively), as well as collagen deposition and extracellular matrix damage [40-44]. As such, fibrotic tissue development leads to a further decrease in static lung

compliance and patients continue to suffer from hypoxemia and this is associated with poor outcome [41-43].

1.2 Mechanical Ventilation in ARDS

1.2.1 Traditional Approaches to Ventilation

Mechanical ventilation is a necessary life saving measure for patients in intensive care with respiratory failure or other severe e.g. neurologic conditions [3]. In order to maintain the patient at regular values of arterial oxygen, carbon dioxide and pH, and to keep the lungs fully expanded and avoid the development of oedema, high volume and high pressure ventilator settings are frequently utilised [40, 45-47]. Traditionally, tidal volumes on ventilation were typically kept between 10-15mL/kg, which is significantly higher than the normal tidal volumes of the lung spontaneously breathing at rest (7mL/kg) [46, 48]. This was done to minimize alveolar collapse, termed atelectasis, a significant risk with mechanical ventilation [4, 48-50].

1.2.2 Ventilator Induced Lung Injury

Mechanical ventilation can also exacerbate ARDS or even induce it and this is termed Ventilator Induced Lung Injury (VILI) [46, 47, 49, 51, 52]. In fact, it has been shown that the proportion of patients with ARDS increased when high tidal volumes and airway pressures were used [47]. Trauma, due to the high lung volumes (volutrauma) and high lung pressures (barotrauma) can occur and lead to over distension of the lungs that in turn can cause alveolar shearing or rupture, and result in complications including pneumothorax [16, 40, 45, 53-55]. An intense inflammatory response or biotrauma is the end result in the injured lung [5, 13, 40, 56, 57]. This is accompanied by altered fluid clearance due to reduced surfactant production by alveolar type II cells and endothelial and epithelial (alveolar type I cell death) membrane damage and the influx of protein rich fluid [5, 10, 18, 58, 59]. Numerous pre-clinical studies of VILI have also investigated and demonstrated these findings [49, 51, 60]. As mentioned previously, injurious stretch has also been shown to activate the NF-κB pathway of inflammation [20-22]. The mechanism by which a mechanical stress is converted to a biochemical

response, termed mechanotransduction, is still largely unknown. In relation to VILI it is believed that plasma membrane disruption caused by stretch results in mechanosensor receptor activation which transmits a stress signal across the membrane though the involvement of integrins and adhesion molecules which consequently cause NF-κB translocation, in fact NF-κB itself contains a stress response element [61]. Furthermore, in ARDS, it has been shown that damage-associated molecular patterns which are host derived molecules that can be released from damaged cells can activate NF-κB through toll-like receptor signalling cascades [62].

1.3 ARDS Therapy

Despite a growing understanding of the injurious mechanisms of ARDS, there are no pharmacological therapies for the condition. Pharmacological agents, such as inhaled NO, were shown to slightly improve oxygenation but didn't improve outcome, while treatment with corticosteroids and beta agonists were shown to be either ineffective or overall were more harmful to patients [63-65]. Therefore, management of the disease relies on improvements in supportive measures such as protective/low tidal mechanical ventilation and fluid management approaches [45, 66-68].

1.4 Role of Hypercapnia in Protective Lung Ventilation

1.4.1 Permissive Hypercapnia

Protective ventilation strategies with low tidal volumes often result in insufficient CO_2 clearance in patients, a development which has become known as permissive hypercapnia (a tolerated increase in arterial CO_2 that permits lower tidal volume mechanical ventilation) [67]. At first it was believed that hypercapnia with its associated acidosis (HCA) did not have any active role in the lung injury process but some studies suggest that HCA may beneficially contribute to the survival of ARDS patients [67]. This is further supported by pre-clinical evidence showing that HCA inhibits inflammation and lung injury **(Table 1.2)**. Interestingly, the effects of HCA appear to be predominantly mediated by the generation of an acidosis due to the rise in CO_2 in the cases of pre-clinical ischemia-reperfusion injury and systemic sepsis [69, 70].

Table 1.2: HCA Efficacy in Pre-Clinical and Clinical ARDS		
Model	*Intervention*	*Effects*
VILI in Rabbits [71]	HCA ($PaCO_2$ 80 – 100 mmHg)	Decreased pulmonary oedema Decreased bronchoalveolar lavage fluid (BAL) protein and cell count Decreased lung histological injury
VILI in Rabbits [72]	HCA ($PaCO_2$ 70 – 100 mmHg)	Decreased pulmonary oedema Decreased BAL protein Decreased plasma nitrate
E.coli Pneumonia Injury in Rats [73]	HCA ($PaCO_2$ 55±1 – 62±2 mmHg)	Improved oxygenation and compliance Decreased BAL neutrophils, protein and TNF-α
LPS Induced Lung Injury in Rats [74]	HCA pre and post injury ($PaCO_2$ 45±2.5 – 73 ± 2.7 mmHg)	Improved oxygenation and compliance Decreased neutrophil infiltration Post injury HCA decreased lung histological injury
Lung Ischemia-Reperfusion Injury in Rabbits [75]	HCA ($PaCO_2$ 30 – 40 mmHg)	Decreased pulmonary oedema Decreased BAL protein and TNF-α Decreased nitrogen radical mediated apoptosis
Patients with Severe ARDS [67]	Peak Inspiratory Pressure (PIP) 30-40 cmH_2O Tidal volume 4-7 mL/kg Permissive HCA	Improved survival
Patients with ARDS [45, 76]	Tidal volumes of 12 mL/kg versus 6 mL/kg HCA	HCA associated with improved survival in patients with high tidal volumes

1.4.2 HCA and Lung Injury

The mechanisms by which HCA protects against lung injury are believed to be due to its antioxidant properties, whereby it was shown to attenuate ischemia-reperfusion injury by inhibiting free radical mediated injury as induced by xanthine oxidase [77] and VILI that correlated with reduced plasma nitrate levels [72]. More interestingly, recent evidence has demonstrated that HCA exerts its protective effects via inhibition of the NF-κB pathway. One such study observed that HCA mitigated the degradation of IκBα by inhibiting IKK activation thus inhibiting NF-κB to exert its protective effects against pneumonia injury in rats [78].

In vitro, HCA was shown to attenuate NF-κB activation and subsequent IL-8 secretion as induced by short term mechanical cyclic stretch injury [20]. While in an animal model of moderate and severe VILI, HCA was shown to enhance recovery and modulate inflammation in rats [20]. In the case of moderate VILI injury, HCA decreased the concentrations of NF-κB and upregulated the NF-κB inhibitory protein IκBα in the cytoplasm [20]. This was not significant in the severe VILI injury which the authors concluded may have been due to a timing issue whereby the window to examine NF-κB activation had lapsed [20]. HCA has also previously been shown to be associated with improved survival in clinical cases where high tidal volume ventilation was used [45, 76].

In contrast, other evidence suggests that HCA may exert some detrimental effects pre-clinically. For example, *in vitro* it has been shown to attenuate wound healing, an effect also mediated by NF-κB inhibition [79]. Furthermore, while HCA was shown to be therapeutic in a study of acute pneumonia lung injury [73], prolonged environmental HCA exposure worsened the pneumonia infection in another study [80]. These findings suggest that that the potent anti-inflammatory response of HCA may inhibit the lungs long term ability to fight infection [80] and that the effects of HCA may be time dependent. Therefore an understanding of the effects – both beneficial and detrimental – of HCA, and its key mechanisms of action is important in the context of ARDS.

1.5 Cell Based Therapies – the Future for ARDS?

As mentioned previously, there are currently no effective therapies for ARDS, a disorder characterised by inflammation. However, in recent years there has been increased interest in the role of stem cell based therapy for the treatment of this disorder. The interest in stem cells arose from the findings that lung resident stem cells were involved in endogenous tissue repair [81, 82]. These stem cells are found in niches along the airway epithelium and have the ability to self renew and to differentiate into numerous other lung cell types, as well as promote repair after injury under strict feedback regulation [81, 83]. However, it has been difficult to isolate a homogenous lung cell population with the ability to repair the entire lung after injury [81, 84]. Thus interest has grown in the potential for exogenous stem/progenitor cells, such as Mesenchymal Stem/Stromal cells (MSCs) as a therapy for ARDS. MSCs in particular are emerging as a promising contender for application in numerous disorders including myocardial infarction [85, 86], graft vs. host disease (GVHD) [87, 88] and ARDS [89-91].

1.6 MSCs for ARDS

1.6.1 Identification, Classification and Source

MSCs are multi-potent adult progenitor cells that were first identified by Friedenstein and colleagues in the early 1970's [92]. MSCs were originally derived from bone marrow (BM) and have the ability to self renew and to differentiate into a variety of cells from the mesodermal germ layer including fat, muscle and cartilage [93, 94]. The characterisation of MSCs includes adherence to plastic, positive expression of surface markers CD105, CD73 and CD90, negative expression of CD45, CD34, CD14 (these are hematopoietic markers), CD11b, CD79α/CD19 and human leukocyte antigen (HLA), and differentiation down the chondrogenic, osteogenic and adipogenic lineages [95]. MSCs are easily isolated and have high expansion potential *ex vivo* and since their identification have been isolated from diverse tissues including adipose tissue, Wharton's jelly, umbilical cord blood and tissue, the placenta and the pancreas [96-98].

MSCs lack immune co-stimulatory molecules such CD80, CD86 and CD40 and they lack major histo-compatibility complex (MHC) class II and have low levels of MHC class I and HLA, making allogeneic MSC therapy a real possibility [99, 100]. MSCs are also of particular interest as a potential therapeutic because they have been shown to home toward areas of damage where they attenuate the inflammatory response and promote tissue regeneration and repair [101-104]. At first, their reparative nature was thought to be due to their "stemness" however studies have confirmed that MSCs show little engraftment after transplantation in both pre-clinical and clinical disorder settings [104, 105]. In fact, it has been shown that they provide support to the injured environment by releasing immunomodulatory molecules and growth factors [104, 106-108]. Hence, they have become known as a stem/stromal cell population. Their stem cell potential allows for expansion while their stromal characteristics provide support in an injury setting.

1.6.2 MSC Efficacy in Pre-Clinical ARDS Models

ARDS has a multifaceted pathology and is rarely encountered without co-morbidities [3] which can be difficult to model *in vivo*. That being the case many studies have undertaken to investigate the effects of MSCs in a number of different animal models that focus on one or more aspects of the causes or pathological processes involved in ARDS **(Table 1.3)**. These include sepsis induced models of ARDS, and injury and repair models such as VILI, which allow for the examination of the resolution of injury after an initial inflammatory response as observed with ARDS.

1.6.2.1 Effects of MSCs in Pneumonia Induced ARDS Models

Several studies using LPS induced lung injury observed that MSCs reduced histological injury and prevented pulmonary oedema as well as the infiltration of immune cells, thus enhancing survival when compared to vehicle treated animals [102, 109-111] **(Table 1.3)**. Furthermore, MSCs attenuated BAL inflammatory cytokine levels [102, 109-111] and enhanced anti-inflammatory IL-10 release [102, 109, 111] as well as the expression of the IL-1 receptor antagonist [102].

In the study by Danchuk *et al*., MSCs were observed to potently induce the expression of TNF-α-induced protein 6 and inhibition of this protein appeared to reverse their anti-inflammatory effects [109]. Furthermore, the study by Mei *et al*., showed MSCs over-expressing the vasculoprotective gene, angiopoietin 1, had enhanced anti-inflammatory properties and prevented excessive alveolar permeability [110]. Finally, Gupta *et al*., showed that MSCs abrogated the levels of BAL macrophage inflammatory protein (MIP)-2, further indicating their anti-inflammatory action [111].

A more recent study by Liu *et al*., demonstrated that mouse MSCs attenuated lung injury produced by both intra-tracheal (IT) and intravenous (IV) LPS [112]. IV administration of BM derived MSCs was able to ameliorate lung injury and inflammation in both models but these MSCs were more effective in the IT injured mice, possibly as a result of greater MSC migration to the lungs in these animals [112]. Finally, in the study by Rojas *et al*., BM MSCs were shown to improve oxygenation and pulmonary oedema as well as lung histology score in a LPS induced lung injury sheep model [113]. More importantly the MSCs were shown to be well tolerated and there were no adverse effects or organ toxicity [113].

Investigations have also shown that MSCs enhance the survival of animals following *E.coli* administration by reducing lung injury in terms of conserving vascular permeability and alveolar air space [114]. Specifically, studies have observed MSCs to reduce bacterial proliferation and enhance bacterial clearance following *E.coli* administration **(Table 1.3)** [114, 115]. In fact the study by Krasnodembskaya *et al*., showed that BAL from mice that received MSCs had greater anti-bacterial efficiency when compared to BAL from control animals [115]. Furthermore, this study observed that LL-37 (anti-microbial peptide) release was proportional to the anti-bacterial activity of the MSCs [115]. MSCs were also shown to attenuate neutrophil infiltration as well as inflammatory cytokine and MIP-2 release [114].

Recently, Devaney *et al*., demonstrated that human BM MSCs improved survival, arterial oxygen and compliance in rats with *E.coli* induced pneumonia with the lowest

therapeutic dose being 10 million cells per kg and IT administration being the optimum route [116]. These cells also reduced inflammation by decreasing BAL neutrophils and *E.coli* load while increasing BAL IL-10 and keratinocyte growth factor (KGF) levels (plays a pivotal role in tissue repair) [116]. Interestingly, this study also showed that thawed cryo-preserved MSCs retain their beneficial and therapeutic potential, which is important from a clinical perspective [116]. Finally, this study observed that MSC administration caused a significant increase in the release of LL-37 [116]. Further *in vitro* investigation then determined that MSCs enhance the phagocytic action of macrophages indicating a possible mechanism of action for their therapeutic efficacy [116]. Human BM MSCs have also shown efficacy in a sheep model of *Pseudomonas aeruginosa* pneumonia [117].

1.6.2.2 Effects of MSCs in Lung Models of Fibrosis

Bleomycin induced lung injury leads to severe inflammation and fibrosis in the lung [118]. Studies have shown that MSCs enhance survival, ameliorate inflammatory cytokine levels and reduce collagen deposition following this type of injury **(Table 1.3)** [118-122]. Furthermore, MSCs were shown to enhance the expression of osteopontin, a cytokine which plays a key role in immune cell modulation, aids cell migration and promotes wound healing [120]. In a study by Moodley *et al*., MSCs were also shown to increase matrix metalloproteinase (MMP)-2 which aids in collagen degradation [121].

1.6.2.3 Effects of MSCs in Recovery Following VILI

A number of studies have also investigated the potential of MSCs to enhance recovery and repair in pre-clinical models of VILI **(Table 1.3)**. These studies showed that IV and IT administration of allogeneic rat MSCs restored lung function by restoring blood oxygenation and respiratory static compliance, as well as resolving lung vascular permeability, when compared to vehicle controls [89, 123]. Histological analysis further revealed that MSCs enhanced lung repair by restoring airspace volume [89, 123]. IT administration of MSCs also caused a significant increase in KGF, although the source of KGF was not identified [89]. Furthermore, MSCs resolved neutrophil and macrophage infiltration while significantly attenuating BAL inflammatory cytokine

levels and restoring anti-inflammatory cytokine release [89]. Systemic and direct application of MSCs had comparable results, suggesting that the beneficial effects of MSCs may be caused by paracrine mechanisms [89]. Studies by the same group also revealed comparable results using human MSCs in the same rat VILI model [90, 123].

Lai *et al*., recently reported that rat BM MSCs were efficacious in recovery post VILI in rats at a dose of 20 million cells/kg [57]. The study also showed that VILI caused an increase in the activation of polymorphonuclear neutrophil (PMN) predominant inflammation (indicated by an increase in elastase activity and reactive oxygen species development) which was inhibited by the MSCs and more extensively so if MSCs were administered prior to injury [57]. These rat BM MSCs also restored oxygenation and compliance and alleviated BAL inflammatory cytokine levels as well as recovering the lung airspace volume [57]. A similar study by Hayes *et al*., used human BM MSCs instead and observed that an IV or IT dose of 2 million/kg of human BM MSCs was sufficiently efficacious in promoting repair and modulating inflammation in a rat VILI model [90]. The study also showed MSC administration either at 6 or 24 hours after injury also enhanced later recovery though some loss of efficacy was observed at 24 hours [90], which could suggest that MSCs may have a limited therapeutic window.

1.6.2.4 Effects of MSCs in the Human Lung

Finally, a recent study by Lee *et al*., showed that human MSC administration reduced vascular fluid accumulation, restored endothelial barrier function and maintained fluid clearance by alveoli in an *ex-vivo* perfused human lung injured with *E. coli* endotoxin **(Table 1.3)** [124]. These effects seemed to be dependent on the release of KGF by MSCs [124]. Other studies have observed that MSCs were able to re-establish alveolar fluid clearance in human lungs rejected for transplantation [125] and improve the compliance in lungs from non-heart-beating donors in pigs [126], thus indicating that MSCs may have the ability to improve lung function before and after transplantation.

Table 1.3: MSC Therapy in Pre-Clinical Studies (↓= decreased, ↑= increased/restored)			
Cell type	*Injury Model*	*Outcome*	*Reference*
Human MSCs	Murine LPS Induced Lung Injury	↓BAL lymphocytes and neutrophils, ↓IL-1β, TNF-α and ↑IL-10	Bustos *et al.,* [102]
Human MSCs	*Pseudomonas Aeruginosa* Peritonitis Mouse Model	↑Bacterial clearance ↑Phagocytic activity	Krasnodembskaya *et al.,* [127]
Human MSCs	Murine Model of *E. coli* Pneumonia	↑Bacterial clearance	Krasnodembskaya *et al.,* [115]
Human MSCs	Murine LPS Induced Lung Injury	↓IL-1β, IL-6 and IL-17, ↓MIP-2 and ↑TNF-α-Induced Protein 6	Danchuk *et al.,* [109]
Human MSCs	LPS Induced Lung Injury in Sheep	↑Oxygenation ↓Pulmonary oedema ↓Histologic injury	Rojas *et al.,* [113]
Human MSCs	Rat Model of VILI	↓BAL TNF-α and IL-6	Curley *et al.,* [123]
Human MSCs	Rat Model of VILI	↓BAL neutrophils ↓BAL IL-1β and IL-6	Hayes *et al.,* 2015 [128]
Human MSCs	Rat Model of VILI	↑Arterial O_2 and compliance ↓IL-6 and CINC-1	Hayes *et al.,* [90]
Human MSCs	Bleomycin Induced Lung Injury	↓TNF-α	Moodley *et al.,* [121]
Human MSCs	Rat Model of *E. coli* Pneumonia	↓BAL neutrophil and E.coli ↑IL-10, KGF and LL-37	Devaney *et al.,* [116]
Human MSCs	*Pseudomonas Aeruginosa* Pneumonia in Sheep	↑Oxygentaion ↓Pulmoanry oedema	Asmussen *et al.,* [117]
Human MSCs	Ex Vivo Human Lung Perfusion Model	↑Alveolar fluid clearance	McAuley *et al.,* [125]
Human MSCs	Lung Transplantation Non-Heart-Beating-Donors in Pigs	↑Dynamic lung compliance	Wittwer *et al.,* [126]
Mouse MSCs	Murine Sepsis Model	↓Il-1β, IL-6 and neutrophils ↑Bacterial clearance	Mei *et al.,* [129]
Mouse MSCs	Murine LPS Induced Lung Injury	↓BAL neutrophil, ↓TNF-α and IL-6	Mei *et al.,* [110]
Mouse MSCs	Bleomycin Induced Lung Injury	↓Neutrophil infiltration ↓IL-1 and TNF-α	Ortiz *et al.,* [118]

Mouse MSCs	Bleomycin Induced Lung Injury	↓IL-1β, IL-2, IL-4 and IFN-γ	Rojas *et al.*, [119]
Mouse MSCs	Murine LPS Induced Lung Injury	↓TNF-α, MIP-2 and ↑IL-10	Gupta *et al.*, [111]
Mouse MSCs	Murine Model of *E. coli* Pneumonia	↑Bacterial clearance, ↓BAL MIP-2 and TNF-α and ↓Neutrophil infiltration	Gupta *et al.*, [114]
Mouse MSCs	Murine LPS Induced Lung Injury	↓Both intra-pulmonary and extra-pulmonary injury	Liu *et al.*, [112]
Rat MSCs	Rat Model of VILI	↓BAL neutrophil, ↓TNF-α secretion and ↑IL-10 release	Curley *et al.*, [123]
Rat MSCs	Rat Model of VILI	↓Neutrophil, ↓Macrophage and ↓BAL TNF-α and IL-6	Curley *et al.*, [89]
Rat MSCs	Rat Model of VILI	↓IL-6, TNF-α, and MIP-2 ↓PMN inflammation	Lai *et al.*, [57]

1.6.3 Mechanisms of Action of MSCs

1.6.3.1 Effects of MSCs on the Cellular Immune Response

Of importance to ARDS, the mechanism by which MSCs are thought to be immunomodulatory is through the induction of anti-inflammatory macrophage phenotypes which have enhanced expression of IL-10 and reduced levels of TNF-α [130, 131] **(Table 1.4)**. MSCs have also been observed to induce monocytes/macrophages to exhibit enhanced phagocytic action [110, 111, 127, 130, 132]. These effects are thought to be mediated by prostaglandin E_2 (PGE_2) and KGF release [131-133]. Recent studies have shown that MSCs alter the polarisation of alveolar macrophages *in vivo* by up-regulating CD206, CD71 and Arginase1 expression which are markers of M2 (immuno-modulatory) macrophages [134, 135].

Other research has shown that MSCs decrease MIP-2 release which is a potent chemo-attractant for neutrophils [109]. In the study by Nemeth *et al.*, it was observed that IL-10 produced from macrophages in an animal model of sepsis that received MSCs inhibited neutrophil migration into tissues, preventing oxidative tissue damage and leading to

their accumulation in the blood stream which aided the clearance of bacteria [131, 136]. Inducing enhanced bacterial clearance is an important MSC function because, as mentioned previously, bacterial pneumonia is a major cause of ARDS.

Recent evidence suggests MSCs can also directly alleviate the bacterial burden following infection. Recent studies observed that human MSCs decreased bacterial growth in a mouse model of pneumonia and furthermore the BAL fluid of these animals had enhanced anti-bacterial properties when compared to controls [115, 116]. They also observed that this effect was caused in part by the release of human LL37, a potent anti-microbial from the human MSCs [115, 116].

Numerous studies have also observed MSCs to elicit immunomodulatory effects, which are thought to be due to their induction of tolerant T cell, B cell, natural killer cell (NKs) and dendritic cell (DC) phenotypes [102, 106, 137-139] **(Table 1.4)**. Specifically, it was noted that MSCs interact with immune cells to decrease the release of pro-inflammatory cytokines such as TNF-α and IL-1β and enhance the release of anti-inflammatory cytokines such as IL-10 [102, 106, 137, 138, 140]. Studies have observed that MSCs can inhibit T cell allogenic responses [141] as well as reduce T lymphocyte and NK cell proliferation [100, 137, 142, 143] and this is due in part to the release of transforming growth factor (TGF) and indoleamine 2,3-dioxygenase (IDO) [142-144]. Meanwhile, TGF-β and hepatocyte growth factor (HGF) production by MSCs has been directly linked to suppressing responder T cells [142].

MSCs have also been observed to enhance the expansion of regulatory T cells which are important suppressors of immune reactions [145]. This was the case in the study by Gore *et al*., whereby MSCs that were administered to rats that had received a unilateral lung contusion injury followed by hemorrhagic shock were shown to significantly increase the number of regulatory T cells in the peripheral blood [146]. This resulted in the promotion of wound healing and subsequent alleviation of pulmonary oedema and pulmonary inflammation associated with this injury model [146]. Other studies have observed that MSCs inhibit DC proliferation and maturation and cause these cells to

release anti-inflammatory IL-10 [147, 148] **(Table 1.4)**. This effect is mediated by PGE_2 release which also inhibits the release of pro-inflammatory cytokines (IL-1β, IL-8 and TNF-α) from DCs and T lymphocytes [149, 150].

Table 1.4: MSC Interaction with Immune Cells (↓= decreased, ↑= increased)

Cell Type	*Effect of MSCs*	*Reference*
Mature Dendritic Cells Type 1	↓Ability to release TNF-α	Aggarwal *et al.*, 2005 [106]
Mature Dendritic Cells Type 2	↑Release of IL-10	Aggarwal *et al.*, 2005 [106]
T Helper 1 Cells	↓IFN-γ, TNF-α and IL-2 secretion	Aggarwal *et al.*, 2005, Chinnadurai *et al.*, 2014 [106, 144]
T Helper 2 Cells	↑IL-4 secretion	Aggarwal *et al.*, 2005 [106]
Natural Killer Cells	↓IFN-γ secretion ↓NK cell proliferation	Aggarwal *et al.*, 2005, Spaggiari *et al.*, 2006 [106, 137]
Monocytes/Macrophages	↑Expression of IL-10 ↓Expression of TNF-α ↑Phagocytic action	Kim *et al.*, 2009, Krasnodembskaya *et al.*, 2012 [127, 130]

1.6.3.2 The MSC Secretome

One potential alternative to MSC cell therapy is the MSC secretome, particularly because MSCs provide their therapeutic action at least in part, through the release of soluble molecules in the MSC secretome. Soluble factors such as IDO, PGE_2, HGF, KGF and TGF-β, as mentioned previously, play a major role in the immunomodulatory and reparative functions of MSCs [102, 106, 137-139, 151].

A recent study showed that MSC secretome i.e. the conditioned medium (CM) from cultured MSCs, prevented increased epithelial permeability and injury by restoring sodium channel function in rat alveolar epithelial cells injured by hypoxia and inflammatory activation [152]. MSC CM has also shown efficacy in pre-clinical

settings. One study highlighted that MSC CM protected rats from bleomycin injury by alleviating inflammatory markers and apoptosis in the lung [153]. MSC CM also reduced collagen deposits and fibrosis [153]. Another study showed that the MSC secretome was comparable in effect to the MSC cell treatment in a rat model of VILI [89]. Specifically, the secretome significantly recovered static lung compliance and alveolar airspace, modulated inflammatory cell infiltration, decreased TNF-α and IL-6 release and restored serum IL-10 concentrations [89]. Finally another study observed the MSC secretome to attenuate endotoxin-induced lung injury specifically by improving alveolar fluid clearance in an *ex vivo* perfused lung in a comparable manner to MSC cell treatment [124].

Interestingly other studies have shown that MSC CM was not as effective as the cell treatment in modulating VILI when animals were assessed 4 hours after injury induction [128], suggesting that there is variable efficacy with the secretome at different phases of the injury/recovery process. Furthermore, in an animal model of pneumonia, MSC CM enhanced survival but failed to attenuate any parameters of lung injury in comparison to the MSC cell treatment [116]. These studies highlight that the therapeutic MSC cell effect may not be fully recapitulated by the MSC secretome.

Other studies have gone on to further investigate and to identify the soluble factors that may contribute to MSC efficacy. A recent study has shown that MSCs can inhibit LPS induced lung injury in mice partly by the release of lipoxin A_4, a molecule derived from arachidonic acid [154]. This molecule is heavily involved in the resolution of inflammation by inhibiting chemotaxis, superoxide generation and NF-κB activation [154]. MSCs administered to this injury model showed increased levels of BAL lipoxin A_4 as well as decreased BAL TNF-α and MIP-2. Interestingly, an antagonist of lipoxin A_4 partially blocked these MSC effects [154].

The release of HGF has also been implicated in the therapeutic action of MSCs. Chen *et al.,* showed that MSC interaction with endothelial cells *in vitro* released more HGF than the MSC alone group following LPS activation [108]. This treatment group also had

significantly inhibited endothelial permeability and this was attributed to the release of HGF which was mainly derived from the MSCs [108]. Furthermore, HGF released from MSCs restored endothelial cell integrity and increased endothelial cell proliferation [108]. Finally, anti-HGF neutralizing antibodies suppressed the therapeutic MSC effects [108].

1.6.3.3 Effects of MSC Microvesicles

Microvesicles (MVs) are fragments of plasma membrane that are shed by numerous cell types, including MSCs, and play a role in cell-cell communication [155]. They are also able to carry and transfer cellular contents, including mitochondria, mRNA and miRNA [155]. Monsel *et al*., showed that MVs derived from human MSCs significantly enhanced survival and alleviated lung injury by decreasing the bacterial burden in mice injured with *E.coli* [155]. MSC MVs also enhanced the phagocytic action of macrophages; this was further enhanced by MSCs with a toll-like receptor 3 agonist [155]. These effects were mediated in part by the release of KGF [155].

In vitro, monocyte and alveolar epithelial MSC MV uptake was increased following inflammatory activation via a CD44 (involved in cell adhesion and migration) dependent mechanism [155]. A study by Zhu *et al*., found similar MV therapeutic efficacy in LPS injured mice and also showed that KGF mRNA expression by MVs in the injured alveolus is at least partially required for these therapeutic benefits [156]. Finally, in an *ex vivo* lung perfusion model, human MSC derived MVs improved alveolar fluid clearance and lung compliance, effects which were blocked by anti-CD44 treatment [157]. These combined results suggest that KGF and CD44 receptor expression may be important for the therapeutic efficacy of MSC derived MVs. Furthermore, recent studies have observed that MSC MVs release mitochondria which are taken up by alveolar macrophages *in vivo* and these macrophages show enhanced phagocytic activity and thus maybe an important mechanism by which MSC MVs provide therapeutic benefit [158, 159].

1.6.4 Clinical Translation of MSCs for ARDS

MSCs have already been shown to be safe in early phase clinical studies for multiple diseases, and are in clinical use for steroid resistant GVHD (NTR4228), which provides further reassurance regarding their safety and translatability for ARDS.

1.6.4.1 Key Early Phase Clinical Studies

An important case study observed that two patients with ARDS who received $2x10^6$ cells per kg showed improved respiratory function and reduced multi-organ failure which correlated with dampened inflammation and improved alveolar fluid clearance [160] suggesting possible benefits with MSC therapy which require clinical evaluation. Recently, Wilson *et al*., demonstrated that human BM MSCs were safe in doses of up to 10 million cells/kg, in a phase 1b dose escalation study, and this group are now conducting a phase II efficacy study [91]. While Zhang *et al.,* have demonstrated that a dose of 1 million adipose derived MSCs was well tolerated in their phase 1b study [161]. However, this study also observed that MSCs at this dose did not ameliorate BAL inflammatory IL-8 and IL-6 concentrations [161]. The patients enlisted into the trial had a baseline PaO_2/FiO_2 of 122.4 ± 42.0, and in accordance with the Berlin Definition were representative of moderate to severe ARDS [7, 161]. Though the lack of efficacy may have been dose related and may be resolved by higher doses in later phase assessment, it does highlight potential efficacy issues with MSCs at the clinical stage particularly because the enrolled patients had varying severities of ARDS [161].

1.7 Addressing Barriers to Clinical Testing of MSC Therapy in ARDS

1.7.1 Understanding the Key Mechanisms of Action

Many mechanisms, some of which are overlapping, have been demonstrated for the action of MSCs, but the key mechanisms and how they vary depending on the specific injury or repair mechanism is unclear. This has certainly been highlighted for the variability seen with the MSC therapeutic window [90] and the MSC secretome efficacy at different stages of the injury and recovery process [128] and in different pre-clinical

models of ARDS [89, 116]. Therefore a greater understanding of the mechanisms of MSC therapeutic action in the context of ARDS is crucial.

1.7.2 Reducing MSC Heterogeneity

One challenge in translating MSCs to clinical use in ARDS is the issue of MSC heterogeneity. Within a heterogeneous MSC population there are an assortment of distributions of stromal and stem cells that differ in surface marker expressions, differentiation capability and immunomodulatory roles [97, 162]. Furthermore, MSCs from different batches and donors also show variability [163]. Therefore there is a need for a more defined subpopulation of MSCs with distinct function and potency assays that can predict efficacy.

A study by Lee *et al*., showed that over-expression of TNF-α-stimulated gene 6 (TSG-6) (anti-inflammatory molecule that also plays a role in extracellular matrix remodelling) in MSCs enhanced their anti-inflammatory effects in bleomycin injured mice [163]. Interestingly the study showed that TSG-6 expression in MSCs varied among patient MSC donors (higher in female donors), with those that have higher expression performing more therapeutically *in vivo* [163] . TSG-6 could potentially be used as a marker to predict MSC effects in different settings but also used to reduce variability among MSCs from different donors [163]. MSC donor age may also be important. MSCs from aging murine donors demonstrated lower migration ability and reduced their therapeutic potential in murine endotoxemia *in vivo* [103]. These findings suggest that MSC heterogeneity is an issue, and the identification of biomarkers to predict MSC potency and quantify batch-to-batch variability would of great benefit for the clinical translation of MSCs in ARDS.

Finally, recent studies have examined the potential of isolating more immunomodulatory and reparative MSCs based on their expression of certain surface markers. One such study demonstrated that $CD271^+$ selected MSCs showed similar differentiation capacity to plastic adherence alone isolated MSCs but caused enhanced release of PGE_2 and were more immunosuppressive *in vitro* and improved engraftment

of hematopoietic stem cells *in vivo* [164]. Another group showed that MSCs selected based on higher positive expression for CD90 (anti-inflammatory and repair functions) were more immunosuppressive and upregulated the release of IL-10 when compared to MSCs with lower positivity for CD90 [165].

Another subpopulation of MSCs positive for CD362 (syndecan-2, (S2)) have recently been identified (and patented) and show promise for therapy [166]. S2 is a surface marker for MSC isolation with functions related to cell proliferation, migration and cellular matrix development and is a membrane protein expressed in MSCs undergoing chondrogenesis [167]. A study recently demonstrated that CM from hypoxic pre-conditioned MSCs showed higher concentrations of S2 which was associated with enhanced wound repair in human dermal fibroblasts [168]. Another study observed that topical treatment with BM derived $S2^+$ MSCs [166] also improved wound healing and angiogenesis in a diabetic ulcer model in rabbits [169]. Finally administration UC $S2^+$ MSCs augmented inflammation in type 2 diabetic mouse model with kidney disease [170]. This highlights the potential for therapy of these subpopulations of MSCs for numerous disease states including ARDS.

1.7.3 Determining the Optimal MSC Tissue Source

Interest has grown in the isolation and use of MSCs from alternative, less invasive, cheaper and more readily available sources and as such, pre-clinical investigations have sought to elucidate any potential differences in their therapeutic effects. Fikry *et al*., showed that BM and adipose (AD) derived MSCs protected rats against injury from methotrexate induced pulmonary fibrosis in a comparable manner [171]. Furthermore, both of these cell types inhibited the expression of BAX, a pro-apoptotic molecule, and increased the level of superoxide dismutase activity (anti-oxidant) and were both superior when compared to dexamethasone treatment [171].

AD MSCs have also shown therapeutic efficacy in other models of lung injury. A study by Sun *et al*., observed that rats with ischemia-reperfusion lung injury had reduced inflammation and oxidative stress damage following treatment with AD MSCs [172].

Jiang *et al.*, also showed that AD MSCs protected rats from lung injury following radiation insult, by decreasing cytokine release (IL-1, IL-6 and TNF-α) and reducing collagen levels, while also inhibiting the expression of apoptotic markers [173]. Furthermore, Mao *et al.*, showed that mouse AD MSCs protected mice from *Pseudomonas aeruginosa* pulmonary infection by reducing lung bacterial load, neutrophils and MIP-2 levels [174]. AD MSCs also enhanced the phagocytic and bactericidal ability of mouse BM derived macrophages *in vitro* by inhibiting PGE_2 signalling [174]. Interestingly it was observed that when PGE_2 was administered to AD MSCs their protective effects were negated [174]. This is somewhat contrasting to those effects observed with mouse BM derived MSCs. Previous studies have suggested BM MSCs release PGE_2 which enhances phagocytic ability and bacterial clearance by macrophages and stimulates them to produce anti-inflammatory IL-10 [131, 133]. These studies show obvious differences between alternative sources of MSCs but may also indicate that there may be other mediators of macrophage activity involved.

The AD MSC secretome has also shown efficacy *in vivo* by abrogating LPS induced lung injury and cytokinemia, reducing oxidative stress and apoptosis [175]. Finally, pre-activation of AD MSCs, as observed with BM MSCs, can also enhance their therapeutic efficiency. Serum deprived AD MSCs showed enhanced anti-inflammatory effects (lower TNF-α expression and NF-κB activation) and increased anti-oxidant action (increased glutathione peroxidase expression) in the lungs of rats with sepsis [176].

Umbilical Cord (UC) derived MSCs have also shown efficacy in pre-clinical models of ARDS. Chang *et al.*, showed that IT administration of UC MSCs decreased BAL cytokines (IL-1β and TNF-α) and ameliorated the impaired alveolarization and angiogenesis in neonatal hyperoxic injured rats [107]. The study also observed that vascular endothelial growth factor (VEGF) knockdown negated UC MSCs therapeutic effects, suggesting that UCs have a paracrine mechanism of action dependent on VEGF release [107]. Furthermore, pre-activation of UC MSCs can enhance their therapeutic effects in models of lung injury. Min *et al.*, observed that angiotensin-converting enzyme 2 (ACE-2) over-expression in UC MSCs was best at alleviating bleomycin

induced pulmonary fibrosis in mice compared to ACE-2 or UC MSCs alone [177]. ACE2-UC-MSCs decreased collagen deposition and prevented oxidative injury via increased super oxide dismutase in lung tissue [177]. These cells also decreased inflammatory cytokine concentrations (TNF-α and IL-6) and caused an increase in anti-inflammatory IL-10 release [177].

Similar results were observed with ACE2-UCs in an animal model of acute lung ischemia-reperfusion injury. Again ACE2-UC-MSCs attenuated injury and were also better at decreasing the inflammatory response (lower CD68, inflammatory markers) and enhancing anti-oxidant action (increased glutathione reductase and glutathione peroxidase expression) [178]. Zhang *et al*., also observed enhanced anti-inflammatory (decreased TNF-α expression and NF-κB activation) and anti-oxidant (increased anti-oxidant protein, heme oxygenase 1) effects with ACE2-UC treatment in the same animal model [179].

Though all BM, AD and UC derived MSCs show potential for therapy in ARDS other studies have highlighted that there are certain difference between these populations of cells in regards to differentiation, proliferation and levels of expression of surface markers that could alter their reparative and immune function [97, 180-182]. As a result, studies should directly compare these different sources of MSCs to elucidate any potential differences in efficacy for ARDS.

1.7.4 Enhancing Safety – Xenogeneic-Free Culture of MSCs

Other issues for MSC use in the clinical setting are due to MSC expansion and culture conditions. MSCs are usually expanded in MSC culture medium that uses animal product supplements. Concerns outlined by EMA and FDA regulations highlight that the use of animal products in MSC cultures may alter MSC function due to batch-to-batch variability and pose the risk of disease transmission [183]. Thus research efforts have prompted the production of xeno-free (XF) culture supplements that would eliminate these issues. One of these being a recently patented XF supplement (WO2015121471 A1) which has been demonstrated to retain all of the same

differentiation and proliferation capacities, as well as the immunogenicity of MSCs cultured in serum containing medium [184]. As such, pre-clinical studies of ARDS may still need to determine whether these XF cells are comparably efficacious to serum cultured MSCs.

1.7.5 Enhancing Feasibility – Cryo-Preservation of MSCs

Another consideration to clinical efforts is the constant culture of high volumes of fresh cultured MSCs required for patient doses. This method is inefficient, not only due to the increased risk of contamination but also due to the potential of cell variability and the high costs involved. Cryo-preservation of MSCs would reduce variability between batches, allow for long term storage and more efficient delivery to the patient. Fortunately, cryo-preserved MSCs have been shown to retain their proliferative and functional properties *in vitro* and therapeutic efficacy in animal models of injury including sepsis [116, 185-187]. Conversely, other studies have demonstrated that MSCs do lose function after cryo-preservation. One such study observed that the biodistribution of cryo-preserved MSCs is significantly altered post thaw due to alterations in their cytoskeleton [188]. While another study showed that immediately thawed MSCs showed reduced suppression of T cell proliferation, did not respond to IFN-γ stimulation and showed reduced release of IDO and these effects were only restored after 24 hours of re-culture [189]. Therefore, studies to compare the effect of cryo-preservation on MSC efficacy in pre-clinical ARDS injury models are imperative.

1.7.6 Enhancing the Therapeutic Effect of MSCs

One way to possibly enhance the effect of MSCs and resolve any efficacy concerns, stems from the evidence that MSCs can behave differently depending on the environment to which they are exposed [190-192], thus allowing for potential priming to more immunomodulatory phenotypes. For example, one study observed that MSCs exposed to the injured lung showed altered proliferation and immune function [190]. This is of huge importance as it is known that MSCs get lodged in the lung after administration [193] and as such are highly exposed to the ARDS lung inflammatory micro-environment. Another study demonstrated that IFN-γ stimulation of MSCs

upregulated IDO and hence reduced T cell and NK cell proliferation [191]. Of relevance to ARDS, one study even demonstrated that pre-conditioning of MSCs with BAL from ARDS patients enhanced their anti-inflammatory effects [102]. Therefore recent studies have focused on strategies to enhance MSC efficacy **(Table 1.5)**. These approaches centre on methods to 'activate' the MSCs or to over-express specific therapeutic genes.

1.7.6.1 Culture Modification

Hypoxic pre-conditioning of MSCs is a promising approach **(Table 1.5)**. Lan *et al.*, showed that hypoxic pre-conditioning (1.5% oxygen for 24 hours) enhanced MSC survival, and increased their efficacy in murine bleomycin induced lung injury, reducing inflammation, injury and fibrosis to a greater extent than normoxia conditioned MSCs [194]. Another study by Li *et al*., found that pre-conditioning MSCs in anoxia for 60 minutes enhanced their efficacy in reducing murine endotoxin induced lung injury [195]. Furthermore, exosomes isolated from these pre-conditioned MSCs were comparable in their protective effects in this injury model [195].

1.7.6.2 Gene Over-Expression

Over-expression of ACE-2 by MSCs also appears a promising strategy **(Table 1.5)**. Studies have shown that ACE-2 over-expressing MSCs were more effective than naïve MSCs in protecting mice subjected to bleomycin induced lung injury [196]. Another study showed that ACE-2 over-expression enhanced the MSC capacity to decrease inflammation and maintain pulmonary endothelial function in mice subjected to endotoxin lung injury when compared to naïve MSCs [197, 198]. The mechanism of action appeared to be mediated by MSC produced ACE-2 preventing the activation of the rennin-angiotensin, thereby decreasing angiotensin II, and increasing Ang(1-7) production [198]. Angiotensin II is potent pro-inflammatory mediator which can cause endothelial and epithelial injury, inflammation and cell death and is implicated in ARDS development [199-203].

A potentially interesting approach is the over-expression of β-catenin, an activator of the canonical Wnt signaling pathway which is involved in cell proliferation and differentiation [204]. Intriguingly, data from Cai *et al*., suggest that mouse MSCs over-expressing β-catenin differentiated into type II alveolar epithelial cells (AECs) and more effectively reduced endotoxin induced injury and inflammation compared to naïve MSCs [204]. MSCs pre-activated with salvianolic acid B, which activates Wnt-1 and Wnt-3a signaling, enhanced MSC expression of type I AEC markers and hence improved the resolution of alveolar edema *in vitro* [205].

Over-expression of CXCR-4, a chemokine involved in migration, proliferation and differentiation, enhanced the efficacy and lung accumulation of exogenous MSCs in LPS injured rats compared to naïve MSCs [206]. In contrast, over-expression of CXCR-4 by MSCs activated the Wnt signalling pathway, but did not produce benefit, resulting in increased differentiation of MSCs into myofibroblasts in a rodent acid aspiration model [207]. Clearly, a greater understanding of the MSC Wnt/ β-catenin signaling pathway, and its response to the specific injury micro-environment, is needed to ensure it can be effectively modulated to enhance MSC efficacy.

Other studies chose to modify MSCs to over-express commonly released soluble factors known to be secreted by these cells. Chen *et al*., showed that KGF over-expression in MSCs significantly enhanced their ability to ameliorate endotoxin induced lung injury in mice [208]. These effects were attributed to KGF causing enhanced type II AEC proliferation and surfactant protein release [208]. Another study found similar results with over-expression of fibroblast growth factor 2 (FGF2) in the same animal model [209].

1.7.6.3 Cytokine Activation

Of relevance to ARDS, evidence shows the potential of the use of inflammatory cytokines that are commonly released in ARDS pathogenesis for MSC pre-activation. One such study observed that hypoxic pre-conditioning along with IL-1β, TNF-α, and IFN-γ (cytomix) stimulation enhanced the MSC CM effects to reduce pulmonary

epithelial permeability after inflammatory injury [152]. In other animal models of disease, MSCs pre-activated with IFN-γ (a potent activator of macrophages and other inflammatory cells) were shown to improve survival in mice with GVHD [210] while another study demonstrated that IFN-γ pre-activated MSCs enhanced therapy in mice with colitis [211] **(Table 1.5)**. *In vitro*, studies have shown that IFN-γ pre-activated MSCs caused the upregulation of anti-inflammatory PGE_2 and IDO, a possible mechanism of their effects *in vivo* [191, 212]. Furthermore, pre-activation with TNF-α, IL-1β and NO together significantly improved the anti-inflammatory effect of the MSC secretome and enhanced repair in a rodent model of radiation induced intestinal injury [213]. This pre-activation strategy shows promise for enhancing MSC therapy but needs to be further investigated in pre-clinical ARDS models.

Table 1.5: MSC Pre-Conditioning *Note*: ↑= Enhanced				
Cell Type	***Injury***	***Conditioning***	***Effect***	***Reference***
Mouse BM MSCs	Murine Bleomycin Injury	Hypoxic Pre-conditioning	↑Reduction of inflammation and fibrosis	Lan *et al.*, [194]
Human BM MSCs	Murine Endotoxin Injury	Ischemic Pre-conditioning	↑Reduction of injury and inflammation	Li *et al.*, [195]
Mouse BM MSCs	Murine LPS Injury	ACE-2 Over-expression	↑Reduction of inflammation and improvement in endothelial function	He *et al.*, [197]
Human UC MSCs	Murine Bleomycin Injury	ACE-2 Over-expression	↑Reduction of apoptosis	Zhang *et al.*, [196]
Mouse BM MSCs	Murine LPS Injury	β-catenin Over-expression	↑ MSC engraftment and differentiation into AEC II cells ↑Reduction of alveolar permeability and histologic injury	Cai *et al.*, [204]
Rat BM MSCs	Rat LPS Injury	CXCR-4 Over-expression	↑ MSC homing and reduction of inflammation	Yang *et al.*, [206]
Mouse BM MSCs	Murine Endotoxin Injury	KGF Over-expression	↑Reduction in lung injury and inflammation ↑Improvement in vascular permeability	Chen *et al.*, [208]
Mouse BM MSCs	Murine LPS Injury	FGF2 Over-expression	↑Reduction in lung injury and inflammation	Zhao *et al.*, [209]
Human MSCs	Hypoxia and inflammation in rat alveolar epithelial cell	Hypoxia and IL-1β, TNF-α, and IFN-γ pre-conditioning	MSC CM ↑restoration of epithelial integrity	Goolaerts *et al.*, [152]
Mouse BM MSCs	GVHD Mouse Model	IFN-γ Pre-activation	↑Animal survival	Polchert *et al.*, [210]
Human and Mouse BM MSCs	Colitis Mouse Model	IFN-γ Pre-activation	↑Body weight, lowered colitis injury score, ↑survival and reduced pro-inflammatory cytokines	Duijvestein *et al.*, [211]
Rat BM MSCs	Rat radiation induced intestinal injury	TNF-α, IL-1β and NO Pre-activation	Reduced injury, improved survival, decreased IL-1β, IL-6 and TNF-α and ↑IL-10	Chen *et al.*, [213]

1.8 Current Status and Key Issues

1.8.1 HCA – Mechanism of Direct Anti-Inflammatory Effects in the Lung

HCA offers protection in pre-clinical models of ARDS [72, 78] and has been associated with improved survival of ARDS patients in the clinical setting [76]. However, HCA negates wound healing *in vtiro* [79] and prolonged exposure worsens lung infection *in vivo* [80]. Furthermore, the evidence of the contribution of the acidosis versus rise in CO_2 to the mechanism of action of HCA is conflicting, whereby some studies attribute therapy to the acidosis and others to the buffering of HCA [45, 67, 69, 76]. Finally, the mechanism of action of HCA appears to be mediated by inhibition of NF-κB inflammatory pathway activation [20, 78], which is up-regulated in ARDS patients [19] and can be induced by mechanical ventilation and stretch injury [20-22]. Therefore it is important to elucidate the mechanisms of HCA in terms of length of exposure and NF-κB pathway interaction in the context of a specific stretch injury.

1.8.2 MSCs for ARDS – Current Status

MSCs may provide an immunomodulatory and pro-repair therapy for ARDS and have certainly shown efficacy in pre-clinical models of the disease [116, 123] and safety in clinical assessment [91, 161]. However, a number of translational obstacles must be overcome before the full potential of MSC therapy can be realised in the clinical setting. Firstly, key mechanisms of action of MSCs need to be identified and targeted and interest has grown in the use of the MSC secretome. However, the effects of the secretome *in vivo* have been conflicting [89, 116, 124, 128] therefore it has become important to elucidate the effects of the secretome in different but specific injury settings related to ARDS.

The ability to predict MSC efficacy is another obstacle that needs to be overcome. MSC heterogeneity and variability due to donor and batch differences may hinder their therapeutic efficacy [97, 162, 163] therefore it is important to identify more specific and defined, MSC subpopulations for use in the treatment of ARDS. Animal derived products in the culture media of MSCs also introduces variability and possible health

risks with MSC therapy [183] therefore it is important to determine whether XF cultured MSCs retain their therapeutic efficacy in pre-clinical models of ARDS. Furthermore, the identification of efficacious MSCs from alternative, readily available and less costly sources would provide a more accessible treatment for the ARDS patient.

Cryo-preserved delivery of MSCs to the clinical setting of ARDS could also potentially reduce variability and enhance accessibility, but studies have been conflicting in regards the effect of cryo-preservation on MSC efficacy [187-189] and this therefore, needs to be elucidated further. Finally, MSC efficacy and paracrine mechanism of action have been shown to be variable at different stages of the injury and recovery process [90, 128] or may have limited therapeutic windows, therefore strategies are needed to ensure MSC efficacy. Studies have observed that pre-activation of MSCs with hypoxic conditioning or inflammatory cytokines for example, enhances therapy in a number of different injury settings [194, 210, 211], however, these pre-stimulation strategies for MSCs have not been fully clarified in the pre-clinical setting of ARDS.

2.0 Aims and Hypotheses

2.1 Overall Aim Part 1

The first overall aim of this thesis was to investigate the effects and mechanisms of action of HCA exposure on cell inflammation and death in the pulmonary epithelium after injurious mechanical stretch injury.

2.1.1 Specific Aims Part 1

The first set of specific aims was:

1. To determine the efficacy of HCA exposure in attenuating short term and prolonged stretch induced cell inflammation and cell death in the pulmonary alveolar epithelium.
2. To establish the importance of HCA interaction with IκBα in inhibiting NF-κB pathway activation and consequent inflammation.
3. To elucidate the function of pH and CO_2 in mediating the effects of HCA on inflammation and cell death induced by high stretch.

2.1.2 Hypothesis of Study Part 1

The first hypothesis of this study is that HCA inhibits mechanical stretch induced NF-κB activation to protect against epithelial inflammation and cell death.

2.2 Overall Aim Part 2

The second overall aim of this thesis was to determine MSC paracrine mechanisms of action by investigating the effects of the MSC secretome in attenuating injury and promoting repair in the pulmonary epithelium.

2.2.1 Specific Aims Part 2

The second set of specific aims was:

1. To determine the efficacy of the MSC secretome in attenuating oxidative stress, inflammation and stretch injury, and in promoting wound repair in the injured pulmonary alveolar epithelium.
2. To examine the potential to enhance the efficacy of the MSC secretome by inflammatory pre-activation.

2.2.2 Hypothesis of Study Part 2

The second hypothesis of this study is that pre-activation of MSCs enhances the efficacy of the MSC secretome in attenuating oxidative stress, inflammation and stretch injury and in promoting repair *in vitro*.

2.3 Overall Aim Part 3

The third overall aim of this thesis was to determine the efficacy of human MSCs to enhance recovery and promote repair in an established rodent model of VILI.

2.3.1 Specific Aims Part 3

The third set of specific aims was:

1. To re-establish a rat model of high pressure VILI, a relevant pre-clinical model of ARDS, and assess consequential lung injury and inflammation.
2. To determine the efficacy of human BM derived heterogeneous MSCs in promoting repair and enhancing recovery post VILI.
3. To determine and compare the therapeutic efficacy of a defined MSC subpopulation, a UC derived $S2^+$ MSC.
4. To directly compare BM and UC derived MSC efficacy in modulating inflammation and enhancing repair following VILI.
5. To determine the efficacy of UC MSCs after cryo-preservation.
6. To elucidate the efficacy of cryo-preserved, XF expanded MSCs in modulating the inflammatory response and enhancing recovery post VILI at a time point of maximal injury.
7. To examine the potential to improve cryo-preserved XF MSC efficacy by pre-activation.

2.3.2 Hypotheses of Study Part 3

The hypotheses of this study were that:

1. A defined MSC subpopulation, UC $S2^+$ MSCs, would enhance repair and recovery post VILI with similar efficacy to heterogeneous BM MSCs.

2. BM derived and UC derived MSCs would be similarly effective in promoting resolution of injury post VILI.
3. MSC efficacy is retained after cryo-preservation.
4. XF cultured MSCs, both fresh and thawed cryo-preserved, maintain efficacy to modulate inflammation and enhance recovery following VILI.
5. Pre-activation of MSCs enhances their therapeutic efficacy in restoring function and modulating inflammation following VILI.

3.0 Materials and Methods

3.1 *In Vitro* Methods

3.1.1 A549/NF-κB-luc Stable Cell Line Culture

A549/NF-κB-luc cells (Panomics, Fremont, CA, USA) were purchased as cryo-preserved 3-passage culture and used at passages 4-10. These cells have an integrated chromosomal luciferase reporter construct that is regulated by NF-κB and are used for examining NF-κB transcription factor activity *in vitro*. A549 cells (ATCC P/N CCL-185) were co-transfected with pNFκB-luc (Panomics) and pHyg followed by hygromycin selection. Hygromycin-resistant cell clones were selected using a TNF-α functional assay which causes luciferase activation. A549/NF-κB-luc were passaged in RPMI-1640 growth medium (Sigma-Aldrich Ireland Ltd. Wicklow, Ireland) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich), 1% penicillin G (100 U/mL) and streptomycin (100 µg/mL) solution (Sigma-Aldrich), 1% L-glutamine (0.2mg/mL) (Sigma-Aldrich) and hygromycin (50µg/mL final) (Roche Life Science, USA). These cells were maintained in a humidified (95%) tissue culture incubator saturated with a gas mixture containing 5% CO_2 and 20% O_2 in air at 37°C. These cells were sub-cultured with 0.025% trypsin-0.05 mM ethylenediamine tetra acetic acid (EDTA) (GIBCO®, Invitrogen Corporation, NY, USA) and cryopreserved in CryoStor cell preservation medium (Sigma-Aldrich) (200µL per 1 million cells).

3.1.2 A549/NF-κB-luc IκBα Super-repressor Cell Line Culture

For some experiments the A549/NF-κB-luc cell line was stably transfected with an IκBα super-repressor (SR) regulated by a chicken beta actin promoter (kindly gifted from Dr. Aideen Ryan, REMEDI, NUI Galway, Ireland) and then selected with G418 (500µg/mL) (Sigma-Aldrich). This SR, as previously described [214, 215], ensures that IκBα cannot be phosphorylated and hence canonical NF-κB activation is inhibited. Control cells were transfected with an empty vector (EV) and the transfection control was CMV eGFP (kindly gifted from the REMEDI lab, NUI Galway, Ireland). For transfection, A549/NF-κB-luc cells were seeded at 20,000 cells per cm^2 in three 75cm^2 culture dishes (Sarstedt AG & Co., Nümbrecht, Germany) and left in tissue culture incubator for 24 hours. 10µg of EV/SR DNA or 1µg of GFP were then added to three

tubes of 500µL of OPTI-MEM I Reduced Serum Medium (Invitrogen Corporation) to make up the DNA mix. Another set of three tubes with 500µL of Opti-MEM® in each received Lipofectamine™ 2000 (Invitrogen Corporation) (1µL of lipofectamine per 1µg of DNA). The contents of each of these tubes were then added to their respective DNA mix tubes to make the transfection mix. The transfection mix was gently mixed and left at room temperature for ten minutes before being added to the three culture dishes of cells. 24 hours later the cells were washed and refed with complete medium and G418 selective agent. GFP images were taken to confirm transfection. 72 hours later a single cell suspension was made and single cells with G418 resistance were seeded in 96 well (Sarstedt) plates (1 cell per well). One week later, EV and SR cell colonies were trypsinised and cultured in the selective agent as described in the previous section. IκBα SR function in these cells was assessed using inflammatory cytokine IL-1β and TNF- α activation (both 20ng/mL final) **(Figure 3.1)**.

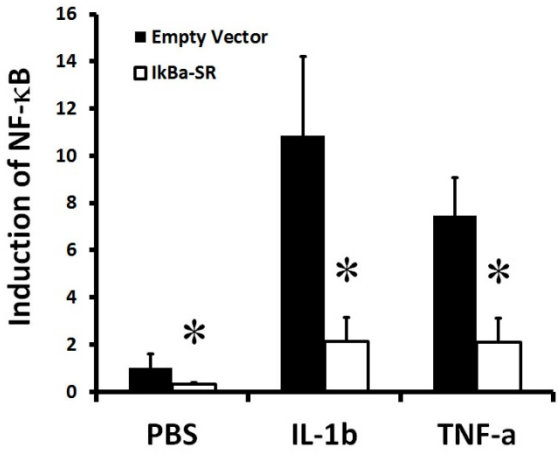

Figure 3.1: A549/NF-κB-luc IκBα SR and EV Cell Lines Activated with IL-1β and TNF-α. IL-1β and TNF-α activation caused NF-κB induction in the EV cell line, but this effect was shown to be ameliorated in the SR cell line. ***Note***: * = $P < 0.05$ versus EV control. Reproduced with permission from Horie *et al*., 2016 [216]. (http://creativecommons.org/licenses/by/4.0/). Copyright Horie *et al*., 2016.

3.1.3 MSC and Dermal Fibroblast (DF) Cell Culture

Human BM and UC MSCs were isolated as described previously **(Table 3.1)** and used at passages 1-3 for all experiments. MSCs were cultured in Alpha Minimum Essential Eagle Medium plus Glutamax (MEM-α) (GIBCO®) supplemented with 10% FCS, penicillin G (100 U/mL) streptomycin (100 µg/mL) and FGF-1 (10ng/mL) (PeproTech EC Ltd., London, UK). Cells were maintained in 95% humidity, 5% CO_2 and 2% O_2

(hypoxia) at 37°C. These cells were sub-cultured with 0.025% trypsin-0.05 mM EDTA and cryopreserved in CryoStor cell preservation medium (200µL per 1 million cells). In some experiments, MSCs were cultured as described above but in XF medium conditions (previously described by Barry *et al*., 2015 [184]) i.e. with growth medium devoid of FCS but with a XF supplement. DFs (kindly provided by Dr. Katya McDonagh, REMEDI, NUI Galway, Ireland) were used as control cells. Briefly DFs were derived from skin punch biopsies (3mm), secured and cultured in 6 well plates (Sarstedt) until 80-90% confluent and then expanded. In our experiments, these control cells were cultured in the same media and conditions (described above) as the MSC cells. For animal dosing, cells were reconstituted in 1mL of phosphate buffered saline (PBS) (Sigma-Aldrich).

Table 3.1: MSC Type and Source (Note: All experiments utilised MSCs from different donors, donor information is not currently available)

MSC Type	*Isolation Methods*	*Source*
Human Bone Marrow Derived Heterogeneous MSCs	As previously described by Wagner *et al*., 2005 [217]	1: Orbsen Therapeutics Ltd., NUI Galway, Ireland 2: Centre for Cell Manufacturing, Ireland (CCMI) 3: Georgina Shaw, REMEDI, NUI Galway, Ireland
Human Umbilical Cord Derived Heterogeneous MSCs	As previously described by Sarugaser *et al*., 2005 [218]	2: Tissue Regeneration Therapeutics Inc., Toronto, Canada
Human Umbilical Cord Derived MSC Subpopulation termed Cyndacel-C ™	As previously described by Elliman, 2015 [166]	1: Orbsen Therapeutics Ltd., NUI Galway, Ireland

3.1.4 Pre-Activation Protocol for DF/MSC Cells and CM

Cells were seeded (Day 1) at $1x10^5$ cells/cm^2 in a T175 culture flask (Sarstedt) and left to reach confluence for 48 hours. The cells were then washed (Day 3) in PBS solution and re-fed with serum-free medium. Fibroblast/MSC CM was collected 48 hours later (Day 5). For pre-activation CM experiments, the cells on Day 3, were re-fed with

complete medium and cytokine activators **(Table 3.2)** were added (for control CM, PBS was added). 24 hours later (Day 4), the cells were washed in PBS and re-fed with serum free medium and 24 hours later the CM was harvested **(Figure 3.2)**. The pre-activation protocol was the same for the pre-activated cell delivery experiments. In those experiments cells were delivered freshly after harvest or cryo-preserved (as described above), thawed and re-suspended in PBS and then delivered. Cryo-preserved MSCs were preserved for up to two months and cell viability after thaw was between 95-97%.

Table 3.2: List of Activators for Pre-activated MSC Experiments	
Activator	*Concentration*
Human Recombinant IL-1β (ImmunoTools, Germany)	10ng/ mL
Human Recombinant IFN-γ ImmunoTools)	50ng/ mL
Human Recombinant TNF-α ((ImmunoTools)	50ng/ mL
Cytomix (IL-1β + TNF-α + IFN-γ)	10 + 50 + 50 ng/mL

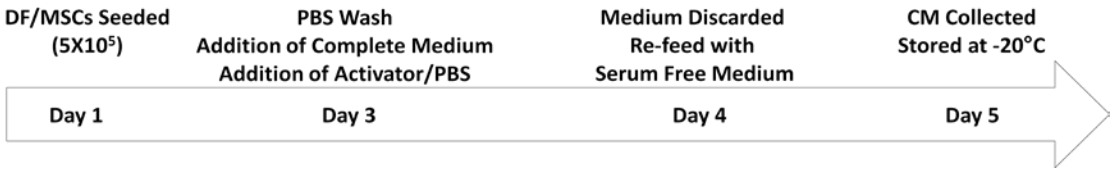

Figure 3.2 Pre-Activated CM Protocol.

3.1.5 Production of Hypercapnic, Acidosis and Buffered Conditions

Normocapnic conditions in cell culture, were produced using 5% environmental CO_2, and hypercapnic conditions produced, using 15% CO_2 **(Table 3.3)**. These were confirmed using pH and PCO_2 analysis. Acidosis was produced by adding strong acid (0.01M Hydrochloric Acid final) (Sigma-Aldrich) to titrate media pH to 7.1 (i.e. to the pH as seen with HCA) when incubated in normocapnia. Buffered hypercapnia (BHA) was produced by using sodium bicarbonate (0.04M final) (Sigma-Aldrich) to buffer the medium pH to normal when under hypercapnic conditions. Finally, sodium chloride (either 0.04M or 0.01M final) (Sigma-Aldrich) was added to all groups to ensure that all groups were equi-osmolar **(Table 3.3)**. pH was confirmed using a blood gas analyser (ABL 705; Radiometer, Copenhagen, Denmark).

Table 3.3: Cell Environmental Treatment Conditions			
Condition	*CO_2*	*Additions*	*pH*
Normocapnia	5%	Sodium Chloride (0.04M and 0.01M final)	7.2
HCA	15%	Sodium Chloride (0.04M and 0.01M final)	7.1 (Note: Patients with permissive HCA reach pH levels of 7.15)
Acidosis	5%	Hydrochloric Acid (0.01M final) + Sodium Chloride (0.04M final)	7.1
BHA	15%	Sodium Bicarbonate (0.04M final) + Sodium Chloride (0.01M final)	7.2

3.1.6 Mechanical Cyclic Stretch Injury and Harvest Procedure

A549/NF-κB-luc cells were seeded to laminin coated 6-well Bioflex plates (Flexcell International, NC, USA) at $1x10^5$ cells/cm², incubated for 48 hours and re-fed with fresh complete RPMI medium. They were then mounted onto the Flexcell FX-4000T® Tension Plus® baseplate (Flexcell International) where they were pre-conditioned in their respective conditions/treatment for 1 hour before being subjected to 22% equibiaxial stretch at a frequency of 0.1Hz for 24, 72 or 120 hours. The Flexcell FX-4000T® applies a vacuum to the Bioflex plates that applies equibiaxial tension or mechanical strain to cells in culture **(Figure 3.3)**.

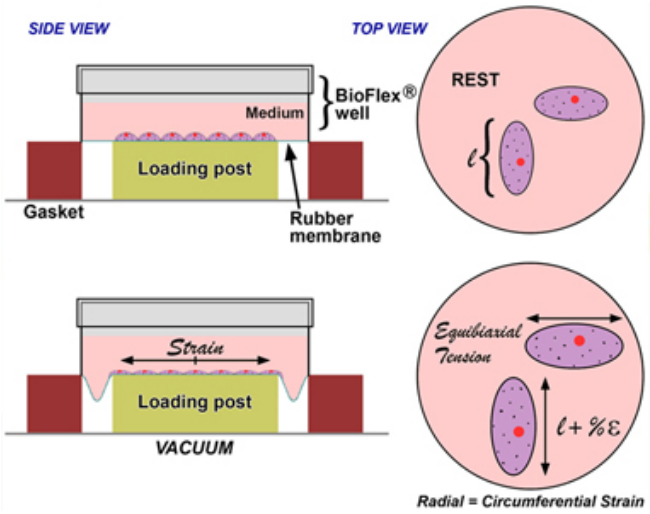

Figure 3.3: Equibiaxial Tension Being Applied to Cells in Culture. Image Adapted from Flexcell International Customer Website [219].

Non-stretched cells were used as control cells and this was based on the demonstration that stretch under normal physiological conditions (10%) does not produce any evidence

of cell inflammation or injury when compared to high stretch (22%) (**Figure 3.4 A + 3.4 B**). Cells and medium were then harvested for analysis. Medium was removed for cytokine IL-8 and lactate dehydrogenase (LDH) analysis. Cells were then scraped by a 1mL pipette tip in 1mL of PBS and placed into a 2mL eppendorf. Cells were then centrifuged at 400 x g for 5 minutes. Cells were reconstituted in 1mL of PBS and 50µL was taken for the viability assay and 50µL taken for the protein quantification assay. The cells were pelleted again and used for the luciferase assay.

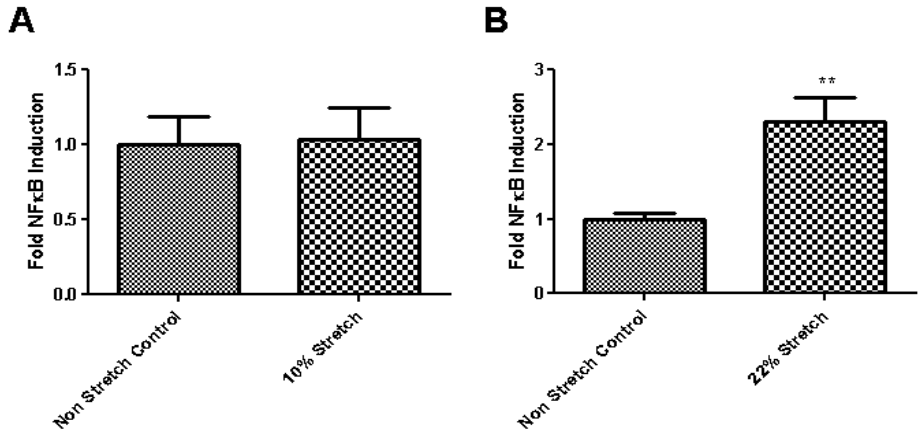

Figure 3.4: Physiological and Injurious Mechanical Stretch. A549/NF-κB-luc cells underwent 24 hours of 10% physiological stretch and this did not cause any significant increase in NF-κB activity (A). However, there was a significant activation of NF-κB induced by 22% stretch (B). ***Note***: ** = $P < 0.01$ versus non stretch control.

3.1.7 Cytokine Inflammatory Activation Injury and Harvest Procedure

A549/NF-κB-luc cells were seeded at $1x10^5$ cells per cm^2 in a 96 well plate and left to reach confluence for 48 hours. Cells were usually pre-conditioned in their respective treatment for 1 hour prior to IL-1β additions (final concentrations of 0-10ng/mL). Control cell received PBS additions. 24 hours later the medium was removed and analysed for cytokine IL-8 and the cell pellet was analysed for luciferase activity **(Figure 3.5)**.

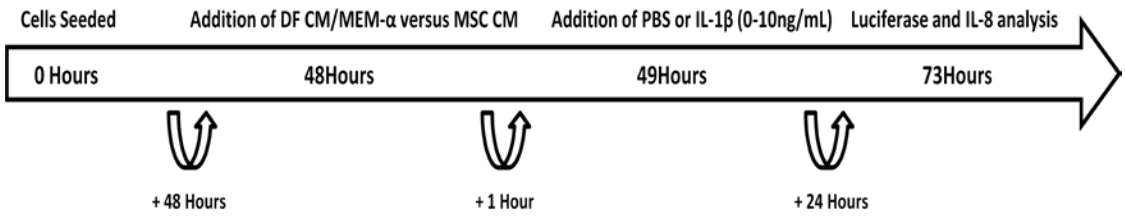

Figure 3.5 Inflammatory Cytokine Activation Protocol.

3.1.8 Oxidative Stress Injury and Harvest Procedure

A549/NF-κB-luc cells were seeded at 1x105 cells per cm2 in a 96 well plate and left to reach confluence for 48 hours. Cells were usually pre-conditioned in their respective treatment for 1 hour prior to hydrogen peroxide (H2O2) (Sigma-Aldrich) additions (concentration ranges of 0-10mM). Control cells received PBS additions only. 24 hours later the medium was removed and the cells were analysed for cell viability.

3.1.9 Scratch Wound Injury and Harvest Procedure

A549/NF-κB-luc cells were seeded at 1x105 cells per cm2 in a 24 well plate (Sarstedt) and left to reach confluence for 48 hours. Single scratch wounds were generated with a 1mL pipette tip (Sarstedt). The cells were washed with PBS and their respective treatments were added. Wound restitution was assessed over 48 hours using light microscopy imaging and edge finding software (Photoshop; Adobe Inc., San Jose, CA, USA).

3.1.10 Luciferase Assay

NF-κB activity in all cell stretch experiments was measured by mixing full pellet of intact harvested cells with 50μL of SolarGlow SuperBright (Molecutools, Dublin, Ireland) luciferase assay substrate for 5 minutes. The luminescence was assessed in a VICTOR™ X plate reader (Perkin Elmer, Waltham, MA, USA). For the inflammatory activation experiments the media was removed from the 96 well plate of cells and 50μL of the substrate was added to each well. A pipette with a 200μL tip was used to mix cells and substrate and luminescence was measured as above.

3.1.11 Viability Assay

Metabolic/mitochondrial activity was assessed by the thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) assay, as previously described [220]. This commonly used assay measures total mitochondrial activity which can be related as a measure of total cell viability [220]. In this study it is assumed that all cells have equal metabolic activity therefore the MTT assay may be used as a measure of viability. For cell stretch experiments, 50μL of intact harvested cells were added to a 96 well plate followed by

100µL of MTT solution (100µg/mL final) in complete RPMI medium. The plate was then incubated in a tissue culture incubator (5% CO_2) for 2 hours. After two hours the media and MTT solution was removed at which stage the cells will have adhered and remain in the well. 50µL of DMSO was then added to each well, and the plate was incubated in room temperature on an orbital mixer for 30 minutes. Absorbance values were read in a VICTOR plate reader at a 550nm wavelength. For 96 well experiments, the medium is removed from the adhered cells and the MTT solution is added directly.

3.1.12 LDH Assay

Epithelial membrane integrity was assessed by measuring medium LDH using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega Corporation, WI, USA). LDH is a stable intracellular enzyme that is released into the medium following epithelial cell membrane damage [221, 222]. The amount of LDH released correlates with the amount of membrane damage and/or membrane resealing and later cell lysis [222]. In a 96 well plate 50µL of harvested medium was mixed with 50µL of CytoTox reagent and incubated at room temperature for 30 minutes. 50µL of the stop solution was then added and the absorbance values were read in a VICTOR plate reader at a 490nm wavelength.

3.2 *In Vivo* Methods

3.2.1 Anaesthesia, Intubation and Ventilation

All animal work was conducted with approval from the Animal Care Research Ethics Committee of National University of Ireland, Galway and under license from the Department of Health, Government of Ireland. Animal injury was performed in accordance with a VILI protocol previously described in our group [123]. Briefly, adult male Sprague-Dawley rats (Charles River Laboratories, Kent, United Kingdom) were anaesthetised under isoflurane gas (5% in 100% O2 at 2L per min) in a gas anaesthesia machine until the animal stopped moving and breathing slowed down. The animal tail vein was then cannulated using a 22 gauge catheter (BD Insyte®, Becton Dickinson

Ltd., Oxford, UK) and a 1mL syringe containing Alfaxan® (Alfaxadone 0.9% (w/v) and alfadolone acetate 0.3% (w/v); Vétoquinol S A, Lure Cedex, France) was attached.

The animal was then intubated as previously described [223] using an otoscope and guide wire which was passed through the vocal cords. A 14 gauge cannula (BD Insyte®) was then passed over the guide wire and the guide wire was then removed. The animal was then weighed quickly and attached to the small animal ventilator (CWE SAR 830 AP, CWE Inc, PA, USA) and ventilated under protective settings **(Table 3.4)**. Baseline ventilation was continued for 20-30 minutes and anaesthesia was maintained with Alfaxan® (0.2ml at 5 to 20 mg/kg/hour) followed by a muscle relaxant, Cisatracurium besilate (Nimbex, GlaxoSmithKline, Dublin, Ireland) (0.1mL at 6 mg/kg/hour) at ten minute intervals. A clamp was also used at ten minute intervals to perform the recruitment manoeuvre which ensures the lungs remain inflated by increasing the PEEP to $10cm^2H_2O$ for 20-30 breaths. A homoeothermic blanket and rectal probe (Harvard Apparatus, MA, USA) were used to maintain and monitor animal body temperature at 37°C.

Table 3.4: Ventilator Settings for Baseline Protective Ventilation

Baseline Protective Ventilation	*Settings*
Machine Mode	Volume
Respiration Rate	90 breaths per minute
PEEP	$2cm^2H_2O$
Flow of Air	500cc air/min
FiO_2	0.3

3.2.2 Measurement of Baseline Function

Following baseline ventilation, static lung compliance was measured using a 5mL syringe which pushes 5 x 1mL increments of room air into the lung through the intubation tube every 3 seconds. The change in pressure was monitored on the trace and the 0mL pressure value was subtracted from 5mL pressure value and then divided by 5. This is known as 100% compliance or baseline compliance. Normal healthy animals should have a compliance of approximately $1mL/cmH_2O$. Once compliance was

measured and a 50% decrease was calculated the animal was put on injurious ventilation.

3.2.3 Induction of VILI

For injurious ventilation, anaesthesia was continued as before but the recruitment procedure was no longer performed, and injurious ventilation settings were instituted **(Table 3.5)**. Once a noticeable change or flattening in the ascending curve of the trace was observed, compliance was checked every 5 to 10 minutes. When a 50% decrease was observed (50% increase in cmH_2O) injurious ventilation was ceased and the animal was returned to protective ventilation and a FiO_2 of 1.0. The anaesthesia was stopped, the treatment/intervention was administered in 0.1mL increments over a 2 minute period and the cannula was then removed. The animal remained ventilated until breathing became spontaneous and noticeable movements were observed (blinking, whisker movements and limb jerking). The animal was weaned off the ventilator and the tracheal cannula was removed. Sham animals received 80 minutes of protective baseline ventilation and were not placed on injurious ventilation settings.

Table 3.5: Ventilator Settings for High Pressure Injurious Ventilation

High Pressure Injurious Ventilation	*Settings*
Machine Mode	Pressure ($35cmH_2O$)
Respiration Rate	18 breaths per minute
PEEP	$0cm^2H_2O$
Flow of Air	500cc air/min
FiO_2	0.3

3.2.4 Assessment of Recovery from VILI

The animal was allowed a 24 hour recovery period before recovery from injury was assessed. The animal was anaesthetised with a 0.3mL intra-peritoneal (IP) injection of 80 mg/kg of Ketamine (Narketan-10 100mg/mL, Vétoquinol, Dublin, Ireland) and 8 mg/kg Xylazine (Xylapan, Vétoquinol). A paw pinch reflex technique was performed to ensure animal was completely under anaesthesia and the tail vein was then cannulated (22G catheter) for subsequent anaesthesia purposes. The fur between the sternum and

the chin was removed using surgical scissors and a dissection was performed to reveal the trachea. A tracheostomy tube (2mm) was inserted into the trachea and tied into place with surgical thread. The carotid artery was then revealed and cannulated with a 24G catheter. 400U/kg of Heparin (CP Pharmaceuticals, Wrexham, UK) diluted to 1mL in Hartmann's solution (Baxter Healthcare Ltd., Dublin, Ireland) was flushed into the artery. The animal was then placed onto the ventilator under the protective ventilation settings and procedure outlined above and anaesthesia was also maintained with Alfaxan and Nimbex as previously described. The animal was monitored for 20 minutes and an arterial blood sample was then collected using a capillary tube and passed though the blood gas analyser (Radiometer). Static lung compliance was also measured at this time. The animal was then switched to the inspired gas FiO_2 of 1.0 for 15 minutes. Another blood sample was taken and analysed.

3.2.5 Animal Sacrifice and Sample Collection

The animal was flushed with another dose of heparin (400U/kg) and sacrificed by exsanguination.

3.2.5.1 Blood and Serum Collections

The blood was collected in a 15mL tube (Sarstedt) and was centrifuged at 4000g for 15 minutes. The serum was aspirated, aliquoted and stored at -80°C.

3.2.5.2 Lung Wet:Dry Ratio

To assess lung pulmonary oedema, wet:dry ratio analysis was performed as previously described [123]. The heart–lung block was dissected from the thorax and the lowest lobe of the right lung was tied off, removed, weighed (on a piece of tinfoil) and placed into a 40°C oven. 168 hours later the lung was weighed again. Calculation of ratio was as follows; (Wet weight - tinfoil weight) / (Dry weight - tinfoil weight).

3.2.5.3 BAL Fluid Collection

BAL fluid was collected for inflammatory cell infiltration and cytokine profile analysis by methods previously described [123]. 15mL of sterile saline (0.9% NaCl) (Sigma-

Aldrich) was then flushed into the remaining lobes and collected as BAL fluid. 1mL of BAL was taken for total and differential cell counts and the rest centrifuged at 4000g for 10 minutes and the clear supernatant stored at -80°C for later cytokine analysis.

3.2.5.4 Tissue for Histological Analysis

To assess lung histological damage, the left lobe was re-suspended and perfused with 4% w/v Paraformaldehyde (PFA) (Sigma-Aldrich) in PBS and then stored whole in this solution for 1 week prior to further procedures.

3.2.6 BAL Total and Differential Cell Counts

1mL of previously collected BAL fluid was centrifuged at 4000g for 10 minutes and was concentrated by re-suspension in 250µL of PBS. 10µL of this was added to 10µL of Trypan Blue solution (Sigma-Aldrich) and 10µL of the mix was added to a haemocytometer for total cell counts and viability. Total cell count calculation was as follows; the number of cells in a 4x4 haemocytometer grid (1mm per side) was counted and this equalled the number of cells per 0.1µL. This number was divided by 4 (to adjust for concentration above) and multiplied by 2 (to adjust for Trypan Blue dilution). This result was then multiplied by 10,000 and equalled the amount of cells per 1mL. For the differential cell counts 150µL of the re-suspended cells were placed into a cytospin cartridge (ThermoFisher Scientific) with attached Superfrost Plus microscope slide (ThermoFisher Scientific) and centrifuged at 200 RPM for 6 minutes. The cells were allowed to dry before being stained with the Hema ‘Gurr®’ (VWR International, PA, USA) staining kit **(Table 3.6)**. The stained cells were observed under a light microscope (40x) and the inflammatory cells were counted up to 300. The neutrophils were expressed as a % of total inflammatory cell count. Neutrophil counts were calculated based on total cell counts above and represented as neutrophil counts per mL of BAL.

Table 3.6: Staining Protocol for Differential Cell Counts	
Staining	*Time*
Gurr® Fixing solution	30 seconds
Gurr® Staining reagent 1	6 seconds
Gurr® Staining reagent 2	4 seconds
Buffer solution pH 7.2	45 seconds
Rinse	45 seconds
Drying	3 minutes

3.2.7 Assessment of Histologic Injury

Histologic injury assessment was performed as described previously [51]. The left lobe was removed from the PFA and cut vertically into 5 sections (labelled A-E). Each section was then placed into histology cassettes (Sigma-Aldrich) with the lateral cut facing upwards. The tissue was processed in a Leica ASP 300 Tissue processor (Leica Microsystems, Germany) and then paraffin embedded using a heated paraffin dispensing module (Leica Microsystems). The embedded tissues were then sectioned into 7μm thick sections using a microtome (Leica Microsystems) and placed onto Superfrost Plus microscope slides for Haematoxylin (H) (Sigma-Aldrich) and Eosin (E) (Sigma-Aldrich) staining **(Table 3.7)**. The slides were then fixed with DPX mountant (VWR International), cover slip (VWR International) applied and then left to dry overnight at room temperature. An Olympus camera attached to a BX43 Olympus microscope was used to take images (20x) of these slides. Total lung tissue and airspace was determined by scoring the number of cross sections that marked tissue or airspace on the slide in a 100 square grid (each grid covers an area of 50μm^2).

Table 3.7: H and E Staining Protocol for Lung Histology Sections **Note: Xy = xylene, EtOH = Ethanol, m = minutes**											
Xy	Xy	100% EtOH	100% EtOH	95% EtOH	70% EtOH	50% EtOH	H	E	50% EtOH	Xy	Xy
10m	10m	2m	2m	2m	2m	2m	6m	2m	1m	15m	15m

3.3 Protein Quantification Assay

Protein concentrations of samples were measured using the Pierce BCA protein assay kit (Pierce, Thermo Fisher) as per manufacturers guide lines. A 12 point, 2-fold serial dilution of the BCA standard was performed with the highest point value at 2000µg/mL. 25µL of standards and samples were added to a 96 well plate in duplicate and 150µL of the BCA assay solution (made up of 1:50 ratio of reagent A and reagent B mixture) was added to each well. The plate was left to incubate at room temperature on an orbital mixer for 30 minutes. The absorbance values were read in a VICTOR plate reader at a 550nm wavelength and the protein concentrations of the samples was quantified against the standard curve.

3.4 Cytokine Measurement Using Enzyme Linked Immunosorbent Assay (ELISA)

Epithelial A549/NF-κB-luc inflammation was assessed by epithelial secretion of the NF-κB dependent cytokine IL-8, using an IL-8 sandwich ELISA DuoSet kit (R&D Systems Inc., Minneapolis, MN, USA) as per manufacturer's instructions. A 96 well MaxiSorp® microplate (NUNC, Thermo Fisher Scientific Ltd.) was coated with 100µL (per well) of the capture antibody (diluted in PBS to a working concentration of 4µg/mL), sealed with parafilm and incubated overnight at 4°C. The plate was then washed three times with wash buffer (1% Tween-20 in PBS) (with 300µL per well per wash). Plates were then blocked with the reagent diluent (1% bovine serum albumin (BSA) in PBS) (100µL per well) and incubated for 1 hour at room temperature. The wash step was then repeated.

The IL-8 standard was reconstituted in deionised water and a standard curve made with the highest value reading at 2000pg/mL and a seven point, 2-fold serial dilution of this was made in reagent diluent. Standards were added in duplicate to the plate (100µL per well). At this time the cell stretch samples and inflammatory activation samples were diluted 1 in 20 in reagent diluent and also added to the plate (100µL per well) and incubated overnight at 4°C. The wash step was then repeated. The detection antibody was diluted in reagent diluent to a working concentration of 20ng/mL and 100µL was added to each well and incubated for 2 hours at room temperature. The wash step was

again repeated. The streptavidin-HRP was diluted 1 in 200 in the reagent diluent and added to the plate (100µL per well to give 50ng/mL final) for 30 minutes at room temperature. The wash step was again repeated and 100µL of 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added to the plate (100µL per well). The plate was incubated at room temperature in the dark. 50µL of stop solution (2N H_2SO_4, Sigma-Aldrich) was then added. The optical density of the samples was read on a plate reader at 450nm with a wavelength correction at 550nm. IL-8 levels were quantified against the standard curve.

The same protocol was used for BAL fluid cytokine ELISA analysis **(Table 3.8)**. For the IκBα ELISA intact harvested cells were suspended in 1mL of PBS and centrifuged at 500 x g for 5 minutes. The pellet was then resuspended in 100 µL of 1% Triton (v/v) (Sigma-Aldrich) in PBS with protease inhibitors (Pierce protease inhibitor mini tablets, EDTA-free; Fisher Scientific Ireland Ltd, Dublin, Ireland). A protein assay was then performed and equal amounts of protein were added to a PathScan® Total IκBα Sandwich ELISA (Cell Signaling Technology, Danvers, MA, USA) as per manufacturer's guidelines. Total IκBα present was expressed as a fold of the non stretch control group.

Table 3.8: BAL Fluid ELISA Protocol

ELISA	Source	Detection Limit	Peak Standard Curve Point	Dilution of BAL
Rat CINC-1	R&D Systems	15.6 pg/mL	1000pg/mL	1 in 10 Dilution
Rat IL-1β	R&D Systems	62.5 pg/mL	4000 pg/mL	Neat
Rat IL-6	R&D Systems	125 pg/mL	8000 pg/mL	Neat
Rat IL-10	R&D Systems	62.5 pg/mL	4000 pg/mL	Neat
Human KGF	R&D Systems	31.2 pg/mL	2000 pg/mL	Neat
Rat TNF-α	R&D Systems	62.5 pg/mL	4000 pg/mL	Neat

3.5 Data Presentation and Statistical Analysis

All data was analysed using a one way analysis of variance (ANOVA) and a post hoc Student-Newman-Keuls test was utilised for analysing statistical differences. Data is presented as mean +/- standard deviation (SD) values and a P value of < 0.05 was considered statistically significant.

4.0 Results One: Hypercapnic Acidosis Attenuates Stretch Induced Injury in the Pulmonary Epithelium through Inhibition of the Canonical NF-κB Pathway

4.1 Introduction

Mechanical ventilation is a necessary life saving measure for patients with severe acute respiratory failure, including patients with ARDS, a disorder characterised by pulmonary hypoxemia, oedema, and severe inflammation [7, 45]. However, mechanical ventilation with its repetitive cyclic mechanical stretch can cause further injury to the lung [47]. This injury, which is termed VILI, can potentially exacerbate the effects of ARDS or even induces ARDS in some patients receiving mechanical ventilation for other reasons [224, 225]. VILI occurs because large tidal volume ventilation is often required to maintain patients at the normal ranges of arterial oxygen, carbon dioxide and pH and to prevent atelectasis, and this can lead to volutrauma (high lung stretch/over-distension causing alveolar shearing) [45, 47, 225]. Furthermore, high airway pressures are also usually required in ventilated ARDS patients to prevent the further development of atelectasis and pulmonary oedema and can lead to barotrauma [45, 47, 53]. Finally the inflammatory response, known as biotrauma, in the injured lung intensifies as a result of the severe lung endothelial and epithelial damage [19, 31, 225]. Such instances have also been confirmed in numerous pre-clinical models of VILI and ARDS [20, 226].

Protective ventilator strategies such as low tidal volume ventilation and/or low inflation measures have been introduced to combat the lung damage associated with VILI and ARDS [3, 45]. This however was observed to cause insufficient CO_2 clearance in patients, and is known as “permissive hypercapnia” i.e. whereby the hypercapnia with its associated acidosis (HCA) is “tolerated” in order to permit reduction of the intensity of mechanical ventilation [67]. However, HCA can exert potent biologic effects, and may even have direct therapeutic potential in certain settings [67, 75]. Previous studies have observed HCA to be protective in numerous pre-clinical injury models including lung ischemia reperfusion injury [75], endotoxin induced lung injury [74] and VILI [71].

In contrast, other studies have observed potentially deleterious effects of HCA, such as hindering of wound healing *in vitro* [79]. Of concern, prolonged exposure to environmental HCA has been shown to worsen pulmonary bacterial infection *in vivo* [80]. These effects may be attributed to the potent anti-inflammatory effects of HCA

which may hinder the ability of the lung to fight infection [80]. This also suggests that HCA effects may be time dependent, as HCA has been shown to be therapeutic in an acute pneumonia lung injury model [73]. Furthermore, studies have observed that the therapeutic efficacy of HCA in systemic sepsis is credited to the acidosis initiated as a result of the rise in CO_2 levels [69].

In relation to high stretch injury, the inflammatory response is mainly mediated by a neutrophil and macrophage influx into the lung that causes a consequent release of pro-inflammatory cytokines [14, 19, 31]. This has been shown to occur through the activation of the NF-κB signalling pathway which has been observed to be intimately involved in regulating lung inflammation, lung injury and lung repair [19, 29-31]. Certainly, previous experimental studies have linked mechanical cyclic stretch injury directly to NF-κB activation [20-22]. As such, more recent studies have shown that *in vivo*, HCA modulates the NF-κB pathway by inhibiting the degradation of IκBα (the endogenous inhibitor of NF-κB) to exert its protective effects against sepsis induced lung injury and VILI [20, 78]. Furthermore, NF-κB activation has been shown to be up-regulated in the lungs of ARDS patients [19, 31].

The hypothesis of this study is that HCA inhibits mechanical stretch induced NF-κB activation to protect against epithelial inflammation and cell death *in vitro*. The objectives of this study are to decipher the mechanisms by which HCA modulates NF-κB to exert its protective effects against high stretch induced injury. The specific aims of this study are: (1) to determine the efficacy of HCA to attenuate short term and prolonged stretch induced cell inflammation and cell death of the pulmonary alveolar epithelium; (2) to determine the importance of HCA interaction with IκBα to inhibit NF-κB pathway activation and (3) to decipher the role of pH versus CO_2 in mediating the effects of HCA on inflammation and cell death induced by high stretch.

4.2 Methods

4.2.1 Pulmonary Epithelial Cell Cultures

A549/NF-κB-luc cells, with an integrated luciferase reporter construct for monitoring NF-κB activity, were cultured in complete RPMI medium with hygromycin selection, as previously described **(Section 3.1.1)**. For some experiments these cells were stably transfected with an IκBα SR in complete RPMI medium with additional G418 selection **(Section 3.1.2)**.

4.2.2 Experimental Culture Conditions

Cells were incubated in either normocapnia (5% CO_2) or HCA (15% CO_2). For an acidosis, hydrochloric acid (0.02M final) was added to culture medium and for BHA, 0.04M final of sodium bicarbonate was added to culture medium **(Section 3.1.5)**.

4.2.3 Pulmonary Epithelial Cyclical Stretch Injury Model

A549/NF-κB-luc cells were seeded to laminin coated 6 well Bioflex plates at $1x10^5$ cells/cm² and were incubated for 48 hours until they reached confluence. The cells were then re-fed with fresh complete medium and mounted onto the Flexcell FX-4000T® Tension Plus® baseplate. They were pre-conditioned in their respective environments and were then subjected to 22% equibiaxial stretch at a frequency of 0.1Hz for 24, 72 and 120 hours. Non stretched cells were used as experimental controls **(Section 3.1.6)**.

4.2.4 Experimental Protocol

4.2.4.1 Series 1

A549/NF-κB-luc cells were pre-conditioned in their respective environments for 1 hour before being subjected to 24 hours of 22% stretch in normocapnia or HCA.

4.2.4.2 Series 2

A549/NF-κB-luc cells were pre-conditioned in their respective environments for 1 hour before being subjected to 72 or 120 hours of 22% stretch in normocapnia or HCA.

4.2.4.3 Series 3

A549/NF-κB-luc cells were subjected to 24 hours of 10% stretch.

4.2.4.4 Series 4

A549/NF-κB-luc cells were subjected to 120 hours of 22% stretch in normocapnia/HCA pre and post stretch, HCA pre stretch or HCA post stretch.

4.2.4.5 Series 5

A549/NF-κB-luc IκBα SR cells were pre-conditioned in their respective environments and subjected to 120 hours of 22% stretch in normocapnia or HCA.

4.2.4.6 Series 6

A549/NF-κB-luc cells were pre-conditioned in their respective environments for 1 hour before being subjected to 72 hours of 22% stretch in normocapnia, HCA, acidosis or BHA.

4.2.5 Assessment of Inflammation and Cell Death

At the end of each experiment the medium and the cells were harvested for assessment of inflammation and cell viability.

4.2.5.1 Luciferase Assay

NF-κB activity was assessed using the luciferase assay. Intact harvested cells were mixed with 50μL of SolarGlow SuperBright substrate and luminescence was measured **(Section 3.1.10)**.

4.2.5.2 IL-8 ELISA

NF-κB dependent inflammatory IL-8 secretion into the medium was analysed using an IL-8 sandwich ELISA kit and optical density was measured on a plate reader **(Section 3.4)**.

4.2.5.3 MTT Assay

For cell viability, intact harvested cells were incubated in fresh complete medium containing MTT for two hours. The supernatant was removed and cells then incubated in DMSO for half an hour at room temperature. The optical density was then measured on a plate reader **(Section 3.1.11)**.

4.2.5.4 LDH Assay

Epithelial membrane damage associated medium LDH levels were assessed using a cytoTox 96 Non-Radioactive Cytotoxicity Assay Kit and optical density measurements were taken on a plate reader **(Section 3.1.12)**.

4.2.5.5 IκBα ELISA

To measure the amount of IκBα present, cell total protein was quantified and equal amounts were loaded to an IκBα Sandwich ELISA plate. IκBα levels were expressed as a fold of the non-stretch control group **(Section 3.4)**.

4.2.6 Data presentation and Statistical Analysis

All data was analysed with a one way ANOVA and a post hoc Student-Newman-Keuls test was employed for measuring statistical differences. Data is presented as mean +/- SD and a *P* value of < 0.05 was considered statistically significant. Graphs depicted are representative of single experiments and show technical variation (by statistical analysis) within that experiment however statistically significant differences discussed within the study are based on the statistical analysis of two independent experiments pooled together (biological replicate of N=2).

4.3 Results

4.3.1 The Effect of HCA on Early Alveolar Epithelial Stretch Injury

Confluent alveolar epithelial A549/NF-κB-luc cell layers were pre-conditioned for 1 hour in NC or HCA and then subjected to injurious cyclic stretch (22%) for 24 hours. High cyclic stretch caused a significant induction of NF-κB which was attenuated by HCA **(Figure 4.1 A)**. HCA also ameliorated stretch induced alveolar epithelial IL-8 release **(Figure 4.1 B)**. Finally HCA preserved cell viability **(Figure 4.1 C)** and membrane integrity **(Figure 4.1 D)** following injurious stretch injury.

4.3.2 The Effect of HCA on Moderate and Prolonged Alveolar Epithelial Stretch Injury

Confluent alveolar epithelial A549/NF-κB-luc cell layers were pre-conditioned for 1 hour in NC or HCA and then subjected to injurious cyclic stretch (22%) for 72 and 120 hours. High cyclic stretch caused a significant induction of NF-κB which was attenuated by HCA **(Figure 4.2 A & 4.3 A)**. HCA also attenuated stretch induced alveolar epithelial IL-8 release after moderate and prolonged stretch **(Figure 4.2 B & 4.3 B)**. Finally HCA preserved cell viability **(Figure 4.2 C & 4.3 C)** and membrane integrity **(Figure 4.2 D & 4.3 D)** following moderate and prolonged injurious stretch injury.

4.3.3 The Effect of Physiological Stretch on the Pulmonary Epithelium

Confluent A549/NF-κB-luc cell layers were exposed to physiological stretch conditions (10% equibiaxial stretch for 24 hours) to justify the use of non stretch controls in experiments. Physiological stretch did not induce inflammatory activation **(Figure 4.4 A + B)** in pulmonary epithelial cells and was comparable to the non stretch control cells. Physiological stretch also did not cause pulmonary cell death **(Figure 4.4 C)** or membrane damage **(Figure 4.4 D)**.

4.3.4 The Effect of HCA Pre-Conditioning Versus HCA Post-Conditioning

Confluent A549/NF-κB-luc monolayers were assessed for injury after 22% stretch for 120 hours in the following conditions 1: “Normocapnia + Stretch”, 2: “HCA Pre

Stretch", 3: "HCA Pre and Post Stretch" and 4: "HCA Post Stretch". HCA pre and post stretch was more effective in maintaining viability **(Figure 4.5 C)** and reducing medium LDH **(Figure 4.5 D)** and IL-8 secretion **(Figure 4.5 B)** compared to pre stretch HCA. However, pre stretch HCA decreased NF-κB activation similarly to pre and post stretch HCA **(Figure 4.5 A)**. Finally, HCA incubation post cell stretch commencement was equally effective as pre and post stretch HCA incubation.

4.3.5 The Effect of IκBα Over-Expression on HCA Function

Stably transfected A549/NF-κB-luc cells over-expressing IκBα SR were exposed to high stretch (22%) for 120 hours and showed a significant attenuation of the induction of NF-κB **(Figure 4.6 A)** and consequent release of inflammatory IL-8 **(Figure 4.6 B)** (represented as a change from baseline). Furthermore, there was no further inflammatory inhibition in the presence of HCA. Stretch also caused a significant decrement in cell viability **(Figure 4.6 C)** and increase in membrane damage **(Figure 4.6 D)** which was ameliorated in the SR cells. Furthermore these cells showed no further protection in the presence of HCA. Finally, the cytosolic concentrations of IκBα in A549/NF-κB-luc cells exposed to high stretch (22%) were measured. Cells were preconditioned in NC or HC and stretched for 0-120 minutes. HCA significantly attenuated the decrease of IκBα concentrations in the cytosol induced by 30 and 60 minutes of high stretch **(Figure 4.6 E)**. At 2 hours IκBα concentrations are returned to normal in both NC and HC as the protein is reconstituted **(Figure 4.6 E)**.

4.3.6 The Effect of pH Versus CO_2 on Stretch Induced Epithelial Injury Confluent alveolar epithelial A549/NF-κB-luc cell layers were pre-conditioned in normocapnia, HCA, acidosis or BHA before being subjected to high stretch (22%) for 72 hours. Cyclic stretch caused significant NF-κB activation **(Figure 4.7 A)** and IL-8 secretion **(Figure 4.7 B)** which was attenuated by both HCA and the acidosis (at a pH comparable to that observed in patients) but the BHA group was comparable to the ineffective normocapnia treatment. Mechanical stretch also caused significant cell death **(Figure 4.7 C)** and membrane damage **(Figure 4.7 D)** which both HCA and the acidosis rescued but against which, BHA was ineffective.

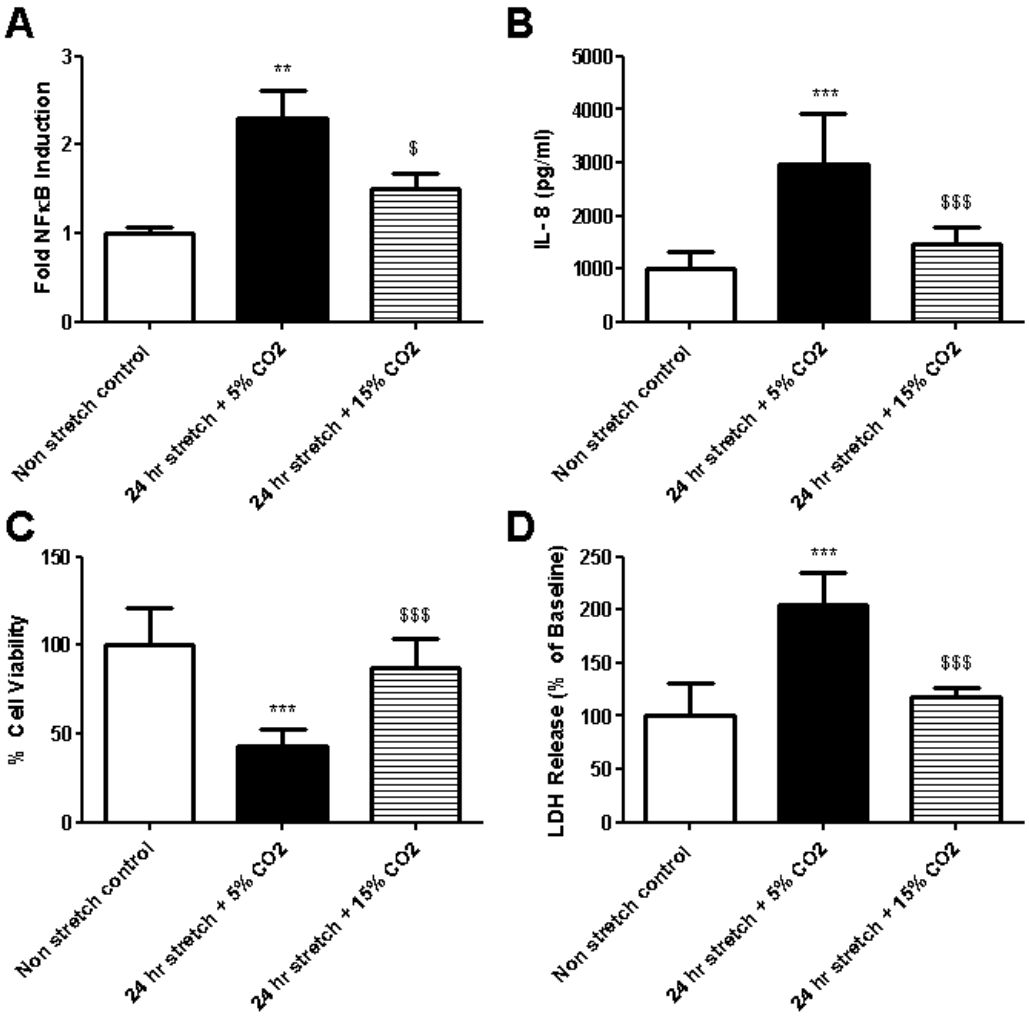

Figure 4.1: HCA Ameliorates Alveolar Epithelial Injury Following Short Term (24 Hour) Cyclic Mechanical Stretch. HCA (15%) reduced the activation of NF-κB (A) and IL-8 secretion (B). HCA also maintained cell viability (C) and attenuated LDH release (D). ***Note***: **, *** = $P < 0.01$, 0.001 versus non stretch control and $, $$$ = $P < 0.05$, 0.001 versus normocapnia (5% CO_2) stretch. n=6 per group per graph (**Section 4.2.6**).

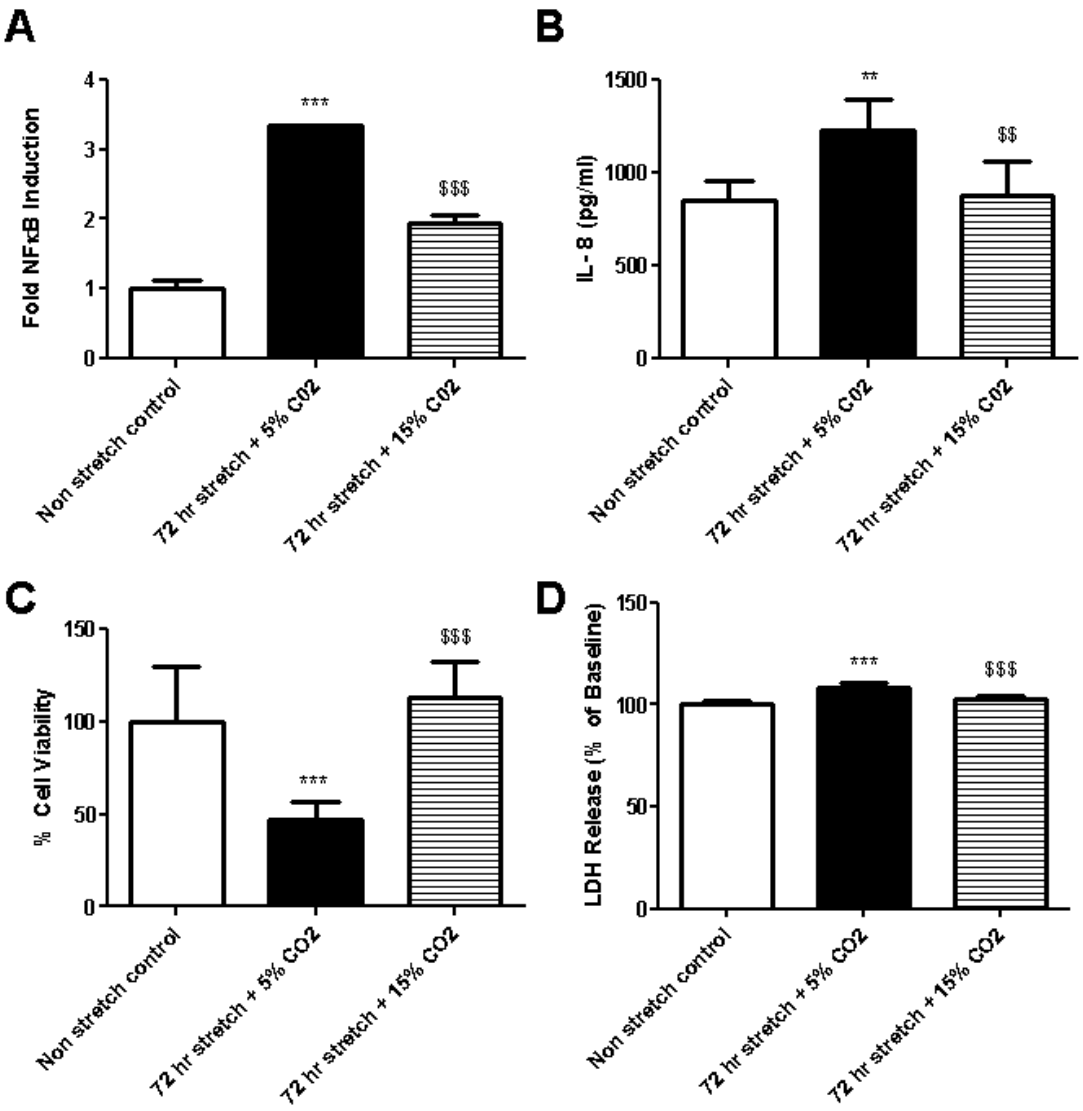

Figure 4.2: HCA Attenuates Alveolar Epithelial Injury Following Moderate (72 Hour) Cyclic Mechanical Stretch. HCA (15%) ameliorated NF-κB activation (A) and epithelial IL-8 secretion (B). HCA also preserved cell viability (C) and reduced medium LDH (D). ***Note***: **, *** = $P < 0.01, 0.001$ versus non stretch control and $$, $$$ = $P < 0.01, 0.001$ versus normocapnia (5% CO_2) stretch. n= 3-6 per group per graph (**Section 4.2.6**).

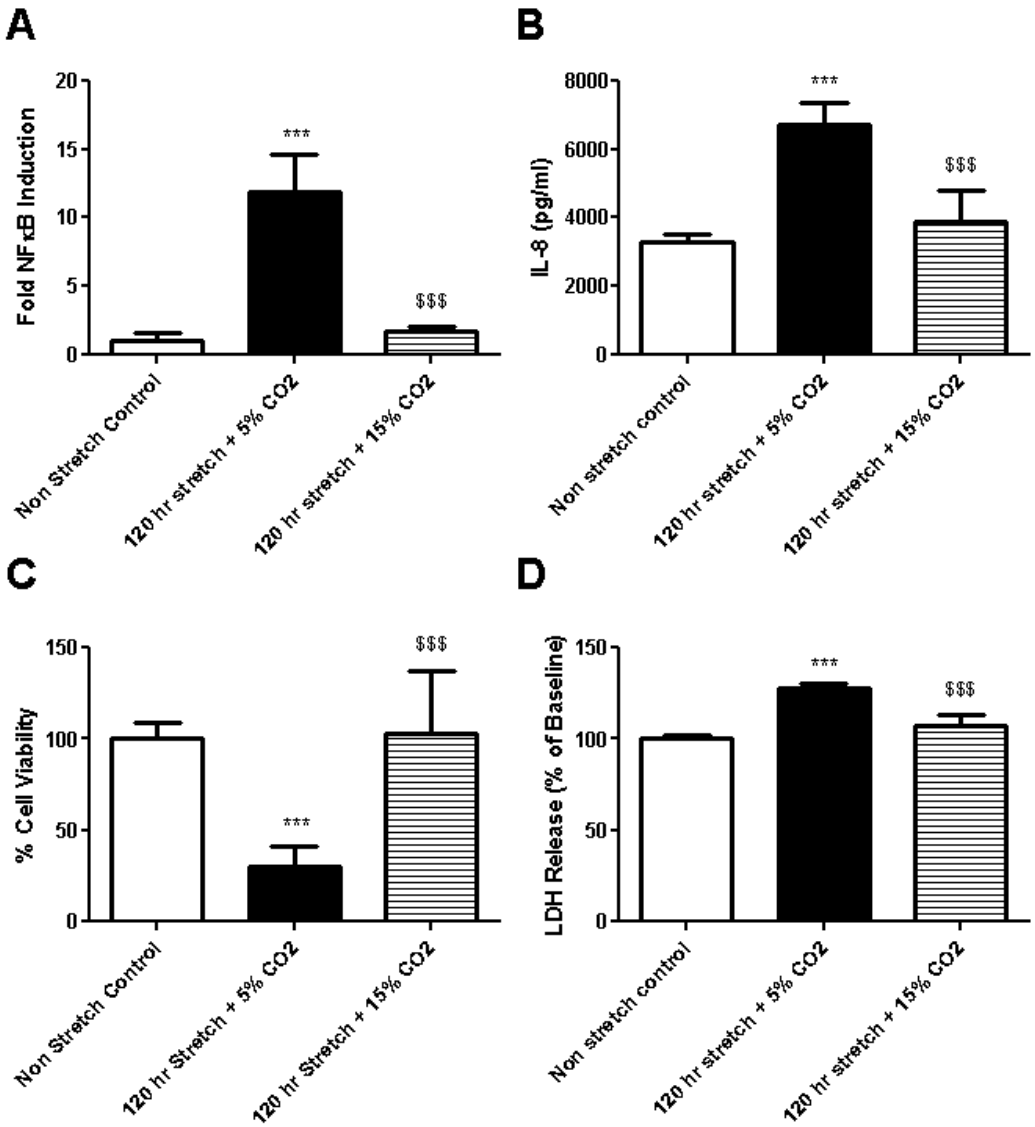

Figure 4.3: HCA Saves the Alveolar Epithelium from Injury Following Prolonged (120 Hour) Cyclic Mechanical Stretch. HCA (15%) attenuated the activation of NF-κB (A) and medium IL-8 secretion (B). HCA also rescued cell viability (C) and inhibited medium LDH release (D). ***Note***: *** = $P < 0.001$ versus non stretch control and $$$ = $P < 0.001$ versus normocapnia (5% CO_2) stretch. n=6 per group per graph (**Section 4.2.6**).

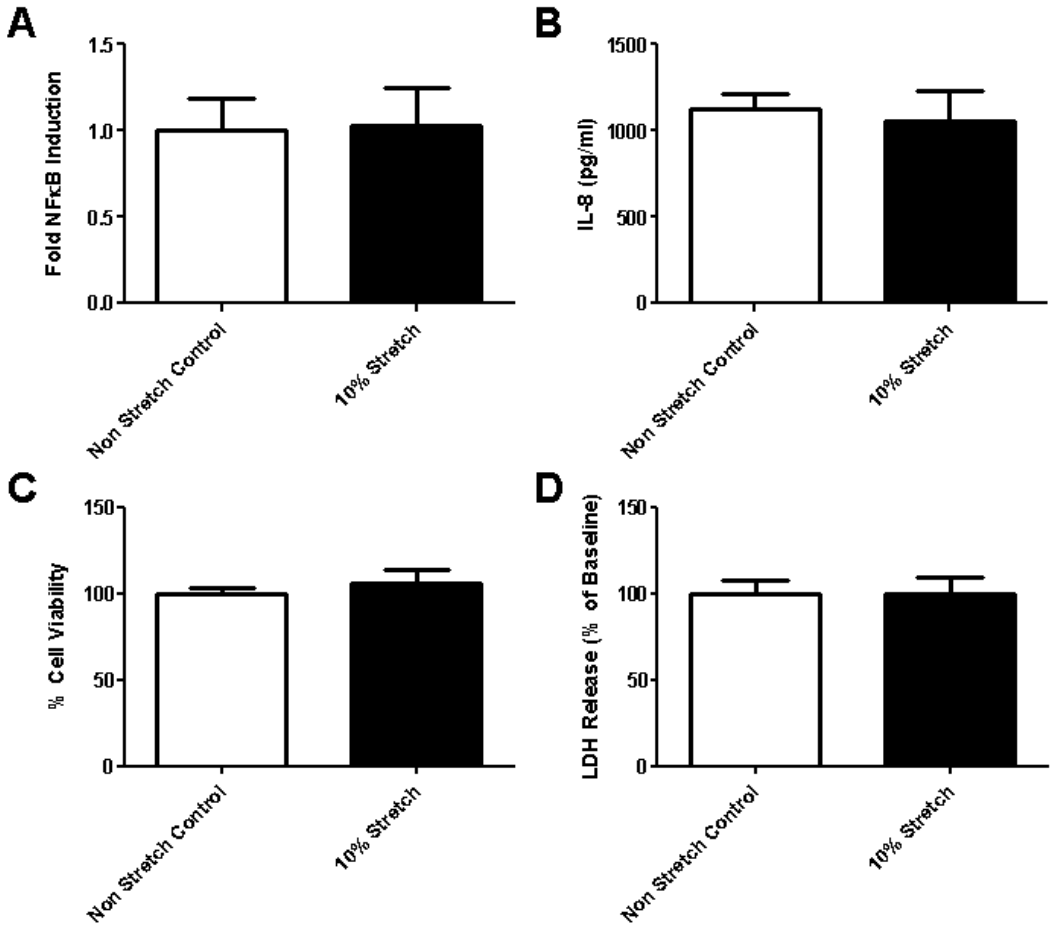

Figure 4.4: Physiological Stretch Does Not Cause Pulmonary Epithelial Injury. Physiological stretch (10%) did not activate NF-κB (A) or cause IL-8 release (B). Furthermore it did not cause a drop in cell viability (C) or increase in LDH secretion (D). n=6 per group per graph.

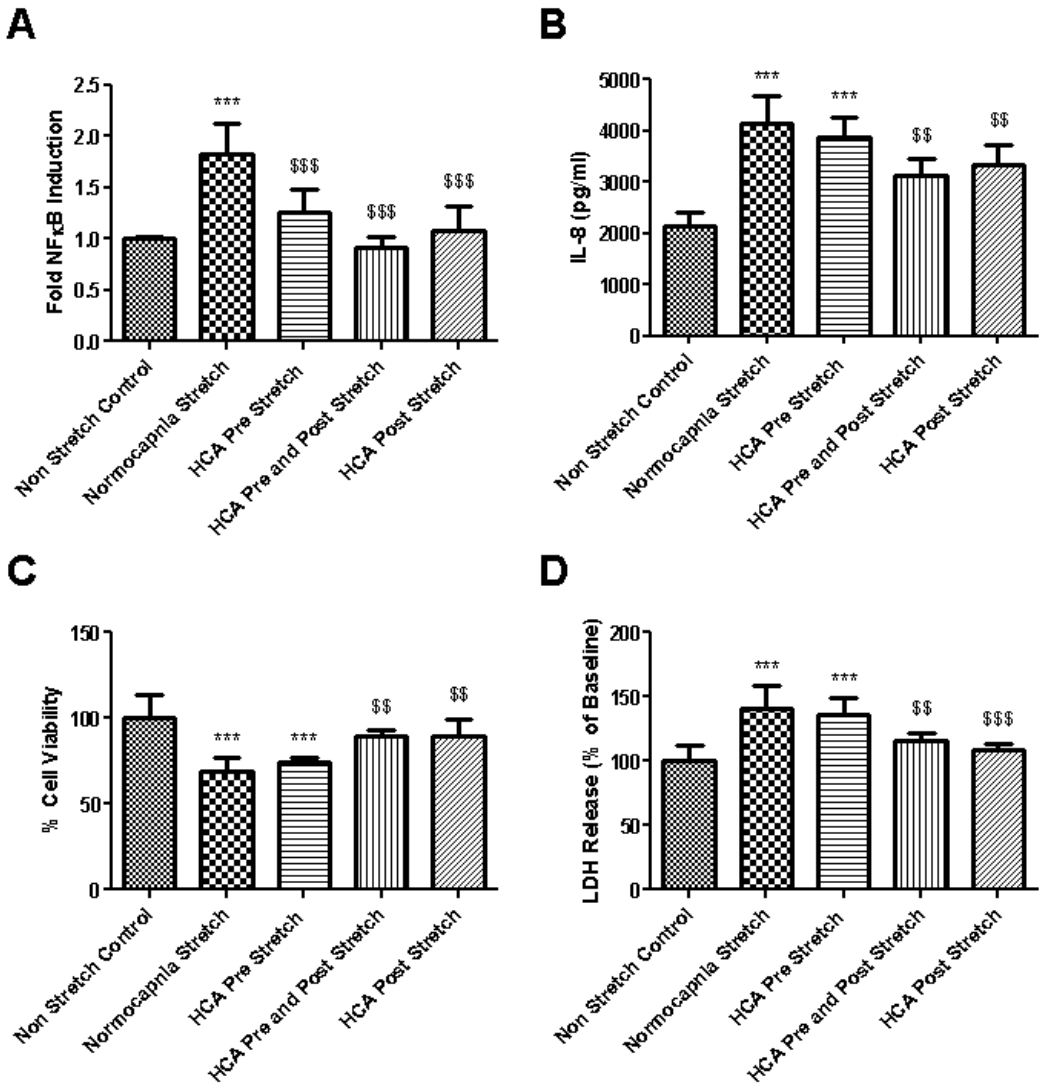

Figure 4.5: Post but not Pre HCA Conditioning Attenuates Stretch Injury. Pre-conditioning with HCA did not attenuate cell viability (C), LDH release (D) or IL-8 secretion (B) after long term stretch. However, pre-conditioning did ameliorate NF-κB activation (A). Conversely, HCA post stretch commencement attenuated epithelial pulmonary injury equally to that seen with HCA conditioning both pre and post stretch injury. ***Note***: *** $P < 0.001$ versus non stretch control and $$, $$$ = $P < 0.01$, 0.001 versus normocapnia stretch. n=6 per group per graph.

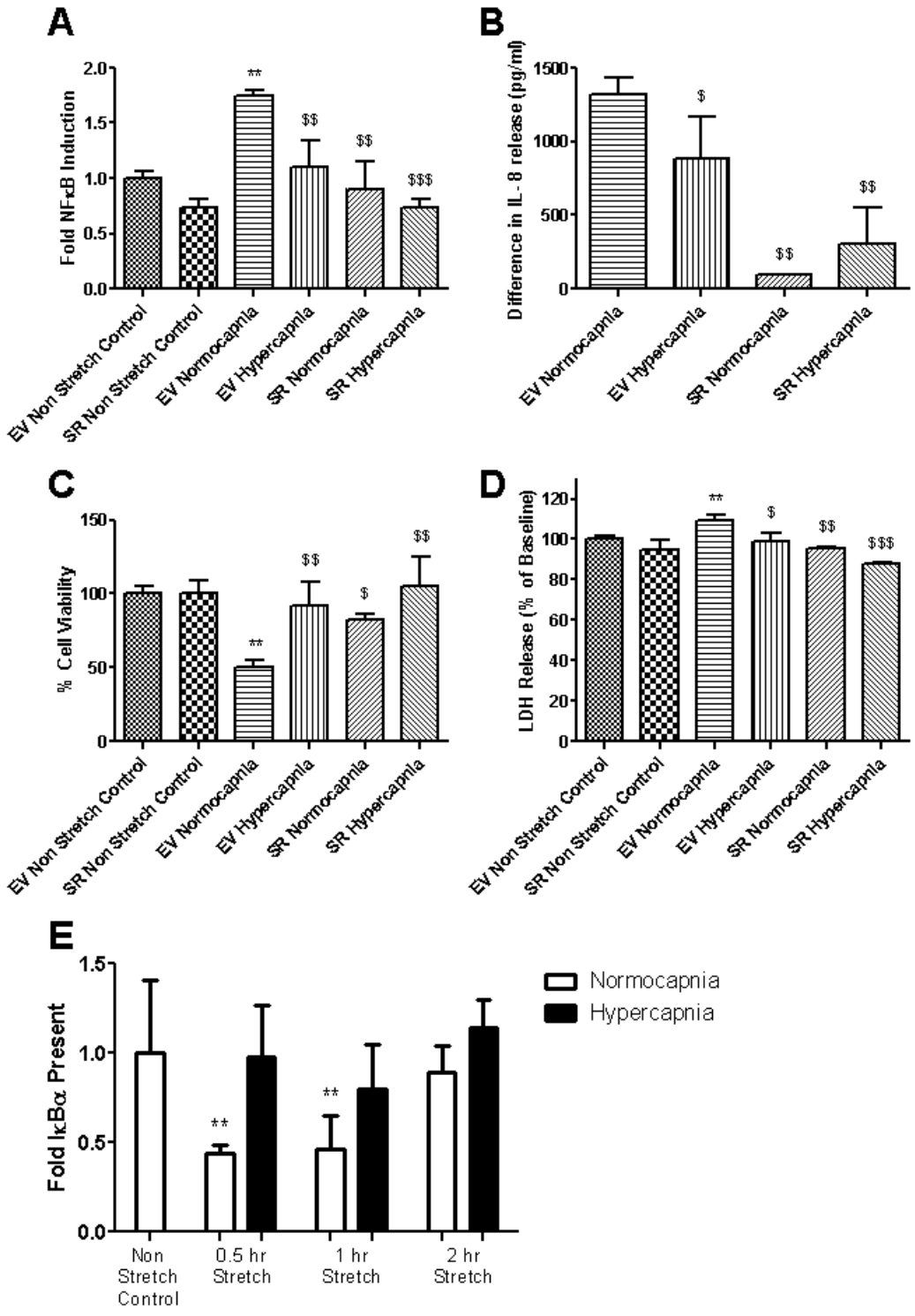

Figure 4.6: IκBα Over-Expression "Occludes" the Effects of HCA. Direct over-expression of IκBα attenuated NF-κB activation (A), ameliorated the change in IL-8 secretion (B), saved cell viability (C) and decreased LDH release (D) in a comparable fashion to HCA. HCA also prevented the stretch-induced decrease of cytosolic IκBα concentrations (E). ***Note***: ** $P < 0.01$ versus EV non stretch control and normocapnia non stretch control and \$, \$\$, \$\$\$ = $P < 0.05$, 0.01, 0.001 versus EV normocapnia stretch. n=3 per group per graph (**Section 4.2.6**).

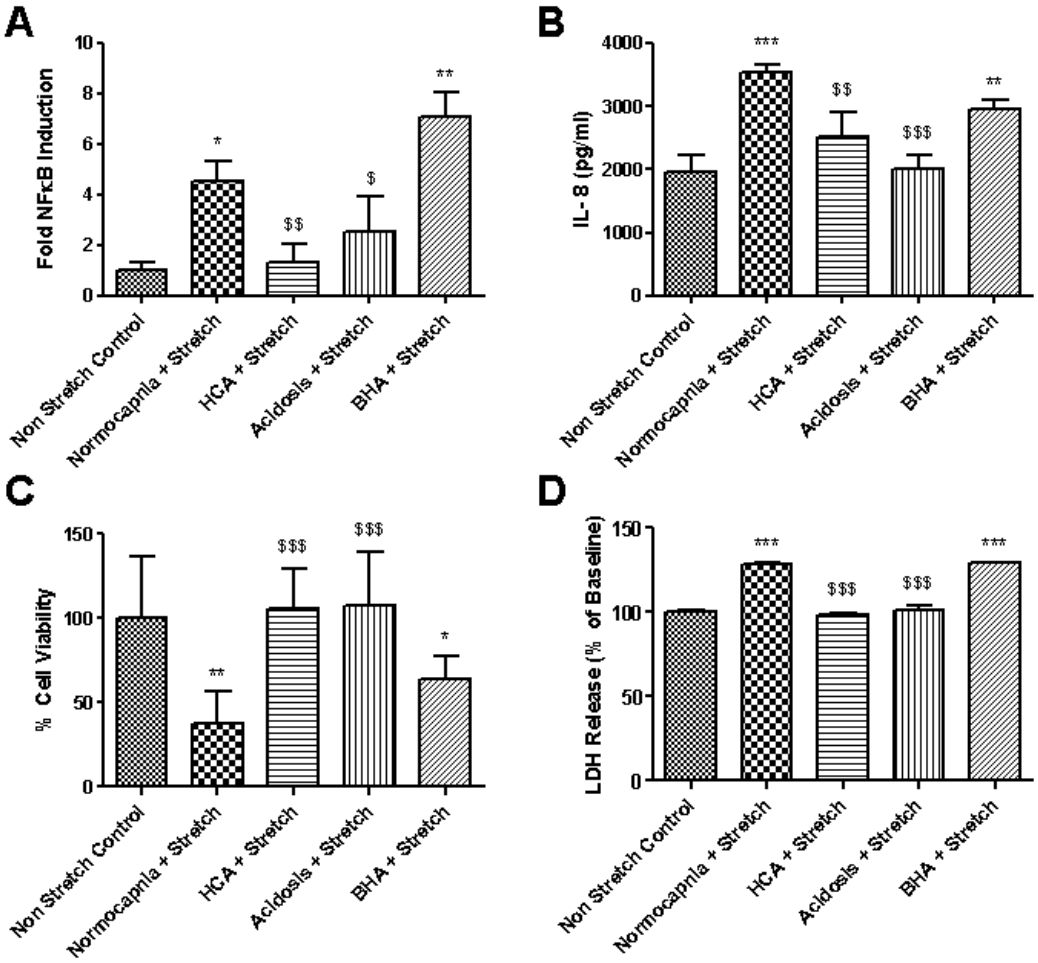

Figure 4.7: HCA Effects appear to be pH Dependent. An acidosis, at a pH equivalent to that of HCA, attenuated stretch induced activation of NF-κB (A) and IL-8 secretion (B) comparably to HCA. Furthermore, the acidosis maintained cell viability (C) and decreased LDH release (D) with similar efficacy to HCA. However BHA did not produce these effects. ***Note***: *, **, *** $P < 0.05, 0.01, 0.001$ versus non stretch control and \$, \$\$, \$\$\$ = $P < 0.05, 0.01, 0.001$ versus normocapnia stretch. n=3-6 per group per graph (**Section 4.2.6**).

4.4 Discussion

4.4.1 HCA Attenuates Moderate and Prolonged Stretch Injury

This study demonstrated that both short term, moderate and prolonged cyclic mechanical stretch caused NF-κB activation resulting in cell inflammation, injury and death and supports previous findings [20]. This study also showed that HCA attenuated this inflammatory activation and subsequent cell death at all time points of high stretch injury, a clinically relevant and significant finding in relation to HCA exposure in ventilated patients. This study also demonstrated that low stretch did not induce inflammation or cell death in the pulmonary epithelium and hence the use of non stretched cells is a justifiable control.

4.4.2 IκBα Over-Expression "Occludes" the Effects of HCA

The mechanism by which HCA inhibited NF-κB appeared to be mediated by the blockade of IκBα degradation. This was further supported by the observation that HCA did not exert further protection in the presence of IκBα over-expression. This finding also supports previous work whereby HCA attenuated NF-κB activation and IκBα degradation in endotoxin-induced injury in human pulmonary artery endothelial cells [227]. Furthermore it confirms similar findings in *in vivo* sepsis and VILI models [20, 78]. Therefore, HCA, in the context of stretch injury, may be beneficial to ventilated patients in the clinical setting due to its potent anti-inflammatory effects.

4.4.3 HCA Generates an Acidosis to Mediate its Therapeutic Effects

Finally this study confirmed that HCA inhibited NF-κB activation and cell death by way of generating an acidosis (at a pH comparable to that observed in patients), while BHA negated these therapeutic effects. This is a finding previously observed in a study of an *in vivo* model of bacterial pneumonia [69]. However, our findings contrast to the study by Takeshita *et al*., who observed that BHA did in fact suppress NF-κB activation and IκBα degradation in the pulmonary endothelium injured with endotoxin [227]. This suggests that HCA may behave differently in diverse injury settings and with dissimilar cell types.

4.4.4 Conclusion

In conclusion, the findings of this study are that HCA modulates NF-κB activation via a pH-dependent mechanism to promote cell viability and attenuate inflammation in the pulmonary alveolar epithelium following shorter and longer periods of high stretch induced injury.

5.0 Results Two: The Mesenchymal Stromal Cell Secretome Protects Against Pulmonary Epithelial Injury

5.1 Introduction

The pathogenesis of ARDS is characterised by endothelial and epithelial membrane cell injury, inflammation and death that is caused by inflammatory cytokines and oxidative stress molecules [4, 13, 14, 17, 18]. MSCs have been shown to be protective in numerous ARDS models, including pneumonia and VILI, through combating inflammation and promoting repair, at least in part by paracrine mechanisms of action, through the release of soluble factors [116, 123]. Other studies have also confirmed the therapeutic efficacy of the MSC CM/secretome in attenuating VILI and endotoxin induced lung injury [89, 123, 124]. Conversely, some studies have demonstrated that the MSC secretome is not as efficacious as cell treatment [116, 128]. Therefore, a further understanding of the paracrine mechanisms of action of MSCs is required in the context of ARDS.

Research has shown that MSC activity can be altered upon exposure to different environments, particularly those that are pro-inflammatory, and this can be exploited to potentially produce a more immunomodulatory cell type [190-192]. In light of this, investigators have started working on pre-activation strategies to better enhance the effects of MSCs and that of its secretome. One such strategy focuses on the use of inflammatory cytokines that are commonly associated with ARDS pathogenesis and found in the BAL of ARDS patients, such as IL-1β and TNF-α [13] and potent activators of MSCs such as IFN-γ [191]. In fact, one study demonstrated enhanced therapy by MSCs pre-activated with BAL from ARDS patients in an animal model of endotoxin induced lung injury [102]. The rationale for this strategy is further supported by evidence which shows that MSCs get trapped in the lungs after intravenous administration [193] and through contact with the local inflammatory environment, can be become activated to produce their effects [190]. Further pre-clinical evaluation is needed to confirm whether these pre-activation strategies do indeed enhance the efficacy of MSCs and that of the MSC secretome.

Finally as concern grows in relation to the use of animal based products for human MSC cell culture [183], investigations using XF culture conditions [184] have begun and as

such rigorous pre-clinical studies are required to determine whether cell culture under XF conditions can affect the therapeutic efficacy of MSCs.

The first study hypothesis is that the MSC secretome can modify cell inflammation and death and promote repair in the pulmonary epithelium, as induced by various relevant insults associated with ARDS pathogenesis. Our second hypothesis is that pre-activation with inflammatory cytokines found in the ARDS lung microenvironment can improve the MSC secretome efficacy. The specific aims of the study are : (1) to determine the efficacy of the MSC secretome i.e. MSC CM, in attenuating oxidative stress (H_2O_2) induced cell death, inflammatory activation induced inflammation, wound injury and mechanical stretch injury, (2) to determine the potential of enhancing these effects by pre-activation of the MSCs using inflammatory IL-1β, IFN-γ and TNF-α alone or in combination (cytomix) and (3) to determine whether the secretome (MSC CM) from XF cultured MSCs maintains therapeutic efficacy.

5.2 Methods

5.2.1 DF CM and MSC CM Protocol

DFs or MSCs were seeded at 1x105 cells per cm2 in a T175 culture flask and left to reach confluence for 48 hours **(Section 3.1.3)**. The cells were then re-fed with complete FCS medium or XF medium with or without cytokine activators (IL-1β (10ng/mL), IFN-γ (50ng/mL) and TNF-α (50ng/mL)) alone or in combination (cytomix) **(Section 3.1.4)**. For non activated/naive CM, PBS was added. 24 hours later the cells were washed with PBS and re-fed with serum free medium and 24 hours later the CM was harvested. DF CM or MEM-α medium was used for the control treatment groups. Multiple donors and multiple batches were used in all experiments.

5.2.2 Oxidative Stress

A549/NF-κB-luc cells ($1x10^5/cm^2$) were seeded in a 96 well plate and left to reach confluence for 48 hours. Cells were pre-conditioned in their respective treatment for 1 hour prior to H_2O_2 additions (concentration ranges of 0-10mM final). Control cells received PBS additions only. 24 hours later the medium was removed and the cells were analysed for cell viability by MTT **(Section 3.1.8)**.

5.2.3 Cytokine Inflammatory Activation Injury

A549/NF-κB-luc cells were seeded at $1x10^5/cm^2$ and left to reach confluence in a 96 well plate for 48 hours. Cells were pre-conditioned in their respective treatment for 1 hour prior to IL-1β additions (final concentrations of 0-10ng/mL). Control cells received PBS additions. 24 hours later the medium was removed and analysed for cytokine IL-8 and the cell pellet was analysed for luciferase activity **(Section 3.1.7)**.

5.2.4 Scratch Wound Injury

A549/NF-κB-luc cells ($1x10^5/cm^2$) were left to reach confluence in a 24 well plate for 48 hours. Single scratch wounds were generated with a 1 mL pipette tip. The cells were then washed with PBS and their respective CM treatments were added. Wound

restitution was assessed over 0-48 hours using light microscopy imaging and edge finding software **(Section 3.1.9)**.

5.2.5 Mechanical Cyclic Stretch Injury

A549/NF-κB-luc cells ($1x10^5/cm^2$) were left to reach confluence in 6-well Laminin coated Bioflex plates for 48 hours. They were then pre-conditioned in their respective CM treatment for 1 hour before being subjected to 22% equibiaxial stretch for 120 hours. Cells and medium were then harvested for analysis. Medium was removed for IL-8 cytokine and LDH measurement. The cell pellet was reconstituted in 1mL of PBS and 50μL was taken for the MTT viability assay. The cells were then pelleted again and used for the luciferase activity assay **(Section 3.1.6)**.

5.2.6 Luciferase Assay

NF-κB activity was assessed using the luciferase assay. Intact harvested cells were mixed with 30μL of SolarGlow SuperBright substrate and luminescence was measured on a plate reader **(Section 3.1.10)**.

5.2.7 IL-8 ELISA

IL-8 secretion into the medium was measured using a human IL-8 sandwich ELISA kit as per manufacturer's instructions and optical density was measured on a plate reader **(Section 3.4)**.

5.2.8 MTT Assay

The MTT assay was performed to assess cell viability **(Section 3.1.11)**. Intact harvested cells were incubated in fresh complete medium containing MTT for two hours. The supernatant was removed and cells then incubated in DMSO for half an hour at room temperature. The optical density was then measured on a plate reader.

5.2.9 LDH Assay

Medium LDH levels were assessed using a cytoTox 96 Non-Radioactive Cytotoxicity Assay Kit as per manufacturer's guidelines and optical density measurements were taken on a plate reader **(Section 3.1.12)**.

5.2.10 Statistical Analysis

All data was analysed using a one way ANOVA and a post hoc Student-Newman-Keuls test was employed for measuring statistical differences. Data is presented as mean +/- SD and a *P* value of < 0.05 was considered statistically significant. Graphs depicted are representative of single experiments and show technical variation (by statistical analysis) within that experiment however statistically significant differences discussed within the study are based on the statistical analysis of two-three independent experiments pooled together (biological replicate of N=2-3). Some biological replicate data could not be pooled therefore differences represent a trend.

5.3 Results

5.3.1 The Effect of DF and MSC CM on Oxidative Stress Injury in the Alveolar Pulmonary Epithelium

Oxidative stress induced cell death, mediated by H_2O_2 was significantly ameliorated by the MSC secretome in comparison to DF CM **(Figure 5.1 A)** or MEM-α medium **(Figure 5.1 B)**. Furthermore, IL-1β or IFN-γ pre-activated MSCs showed a trend for enhanced cell viability when compared to naïve MSC CM **(Figure 5.1 C)**.

5.3.2 The Effect of DF and MSC CM on Cytokine Mediated Inflammation in the Alveolar Pulmonary Epithelium

IL-1β induced pulmonary epithelial inflammation as evidenced by NF-κB activation and IL-8 release, was attenuated by the MSC CM when compared to DF CM treatment **(Figure 5.2 A + 5.2 B)**. Furthermore, MSC CM from IL-1β or IFN-γ pre-activated MSCs showed a trend for enhanced attenuation of NF-κB when compared to naïve MSC CM **(Figure 5.2 C)**. However, only IL-1β pre-activation further improved the ability of MSC CM to attenuate IL-8 release in comparison to naïve MSC CM **(Figure 5.2 D)**.

5.3.3 The Effect MSC CM on Wound Repair in the Alveolar Pulmonary Epithelium

Wound repair was significantly enhanced with MSC CM treatment as compared to MEM-α medium **(Figure 5.3 A + 5.3 B)**. Furthermore, pre-activation with IL-1β, IFN-γ and TNF-α did not seem to further improve the ability of the MSC secretome to attenuate the extent of the wound remaining **(Figure 5.3 C)**. In fact, pre-incubation of MSCs with IFN-γ seemed to negate the effect of its CM on wound healing.

5.3.4 The Effect MSC CM on Mechanical Cyclic Stretch Induced Cell Injury in the Alveolar Pulmonary Epithelium

Stretch induced cell inflammation, as represented by NF-κB induction and IL-8 release, was significantly ameliorated by MSC CM treatment **(Figure 5.4A + 5.4 B)**.

Furthermore, stretch induced cell death and LDH release was also significantly attenuated by MSC CM when compared to MEM-α medium **(Figure 5.4 C + 5.4 D)**.

5.3.5 The Effect of Pre-Activation on the Efficacy of the MSC CM to Ameliorate Mechanical Stretch Induced Injury in the Alveolar Pulmonary Epithelium

Mechanical stretch induced NF-κB activation and IL-8 release were decreased by MSC CM from both FCS and XF cultured MSCs as compared to MEM-α control treatment **(Figure 5.5 A + 5.5 B)**. Furthermore, pre-activation with cytomix (cyto) seemed to further improve the ability of the FBS and XF MSC CM to attenuate NF-κB induction while the effect on IL-8 was only further improved by the cytomix pre-activated XF MSC CM **(Figure 5.5 A + 5.5 B)**. The decrease in cell viability, as induced by high stretch, was again abrogated by both MSC CM groups and the effect seemed to be enhanced with the cytomix pre-activated XF MSC CM **(Figure 5.5 C)**. Finally LDH release, as induced by cell stretch was negated by FBS and XF MSC CM, and only cytomix pre-activated FBS MSC CM showed an enhanced reduction in this release as compared to naïve FBS MSC CM **(Figure 5.5 D)**.

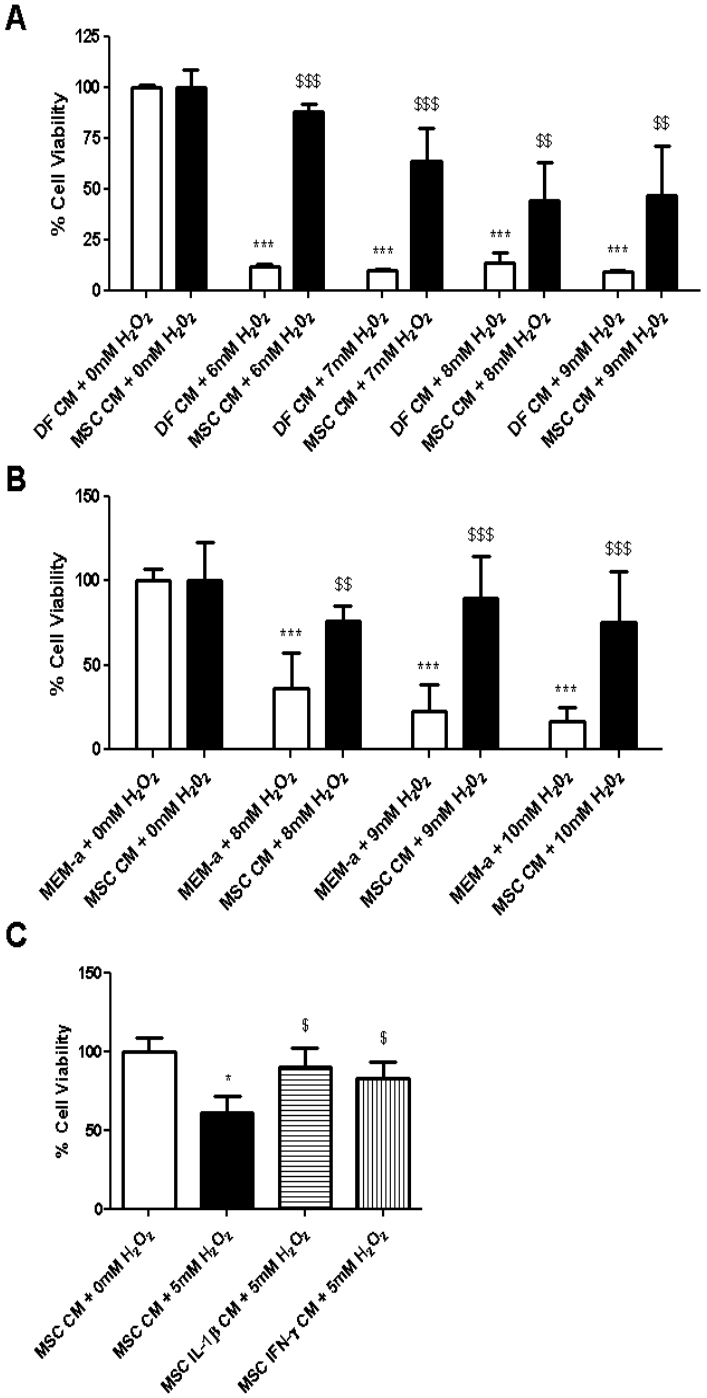

Figure 5.1: MSC CM Attenuates H_2O_2 Induced Cell Death. Increasing concentrations of H_2O_2 caused a step wise decrease in A549/NF-κB-luc cell viability which was significantly attenuated by MSC CM when compared to the DF CM group (A) or MEM-α control (B). A549/NF-κB-luc cells treated with IL-1β and IFN-γ activated MSC CM showed enhanced cell viability when compared to cells that received naïve MSC CM (C). ***Note***: *, *** = $P < 0.05$, 0.001 versus DF CM/MEM-α + 0 mM H_20_2 and MSC CM + 0mM H_20_2 and $, $$, $$$ = $P < 0.05$, 0.01, 0.001 versus DF CM or MEM-α control at the same concentration of H_20_2 and MSC CM + 5mM H_20_2. A & B n=3-6 per group per graph (**Section 5.2.10**). Graph C is a representative graph of three independent experiments which could not be pooled therefore differences represent a trend.

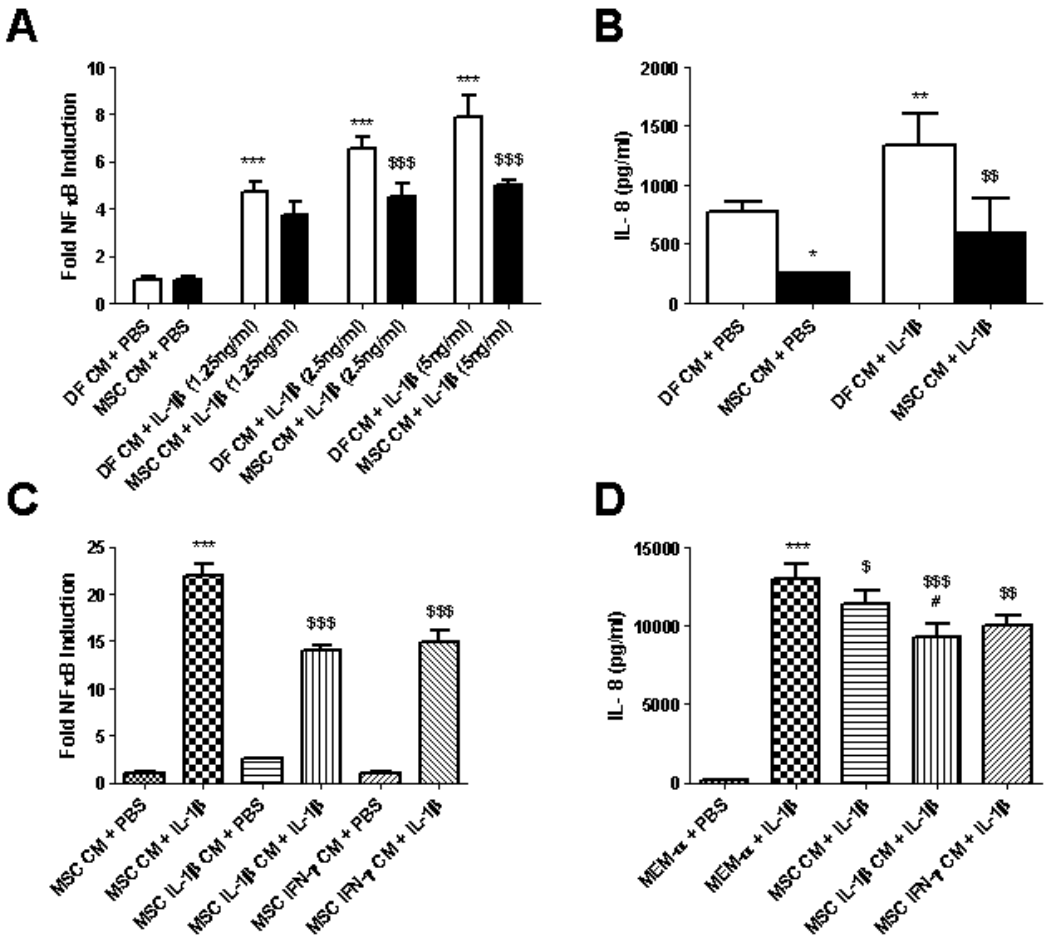

Figure 5.2: MSC CM Attenuates IL-1β Induced NF-κB Activation and IL-8 Secretion. Increasing concentrations of IL-1β caused a step wise induction of NF-κB in A549/NF-κB-luc cells which was attenuated by MSC CM (A). IL-1β addition (5ng/mL) to A549/NF-κB-luc cells also caused a significant increase in IL-8 release which was attenuated by the MSC CM (B). IL-1β and IFN-γ activated MSC CM showed enhanced NF-κB inhibition, when compared to naïve MSC CM, after IL-1β (10ng/mL) additions (C). IL-1β, but not IFN-γ, activated CM also showed enhanced inhibition of IL-8 secretion (as induced by IL-1β (10ng/mL) addition) when compared to naïve MSC CM (D). ***Note***: *, **, *** = $P < 0.05$, 0.01, 0.001 versus DF CM + PBS, MSC CM + PBS and MEM-α + PBS, $, $$, $$$ = $P < 0.05$, 0.01, 0.001 versus DF CM + IL-1β at the same concentration, MSC CM + IL-1β and MEM-α + IL-1β and # = $P <$ 0.05 versus MSC CM + IL-1β. Representative graphs (n=3-6 per group) of three independent experiments which could not be pooled therefore differences represent a trend.

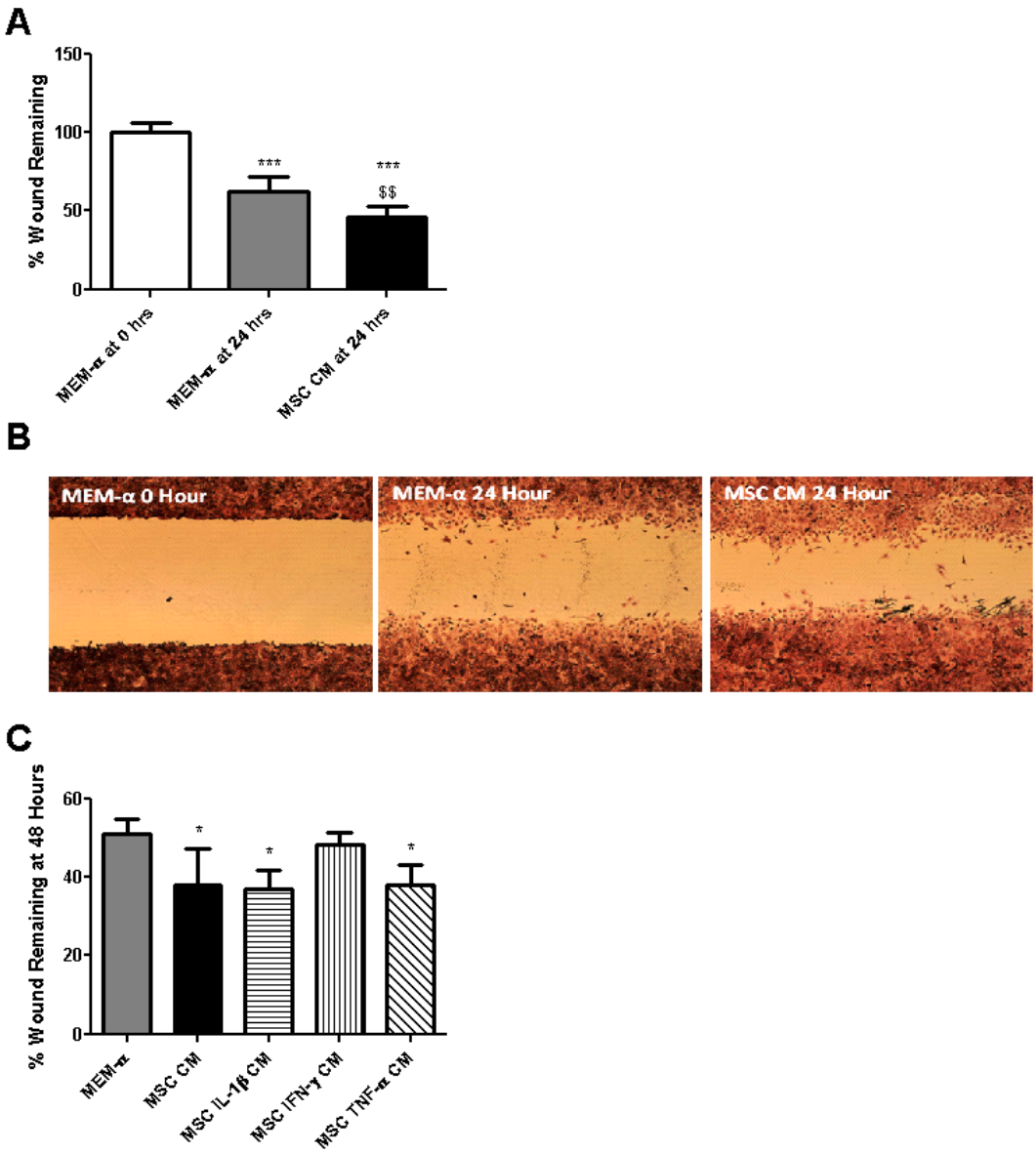

Figure 5.3: MSC CM Promotes Pulmonary Epithelial Wound Healing. MSC CM enhanced wound repair significantly more so than MEM-α control (A), representative images of which are shown in (B). Pre-activating MSCs did not enhance the ability of the CM to further improve wound repair. ***Note***: *, *** = $P < 0.05$, 0.001 versus MEM-α 0 hours and MEM-α at 48 hours and $$ = $P < 0.01$ versus MEM-α at 24 hours. For graph A, n=6 per group per graph (**Section 5.2.10**). Graph C is a representative graph (n=6 per group per graph) of two individual experiments which could not be pooled therefore differences represent a trend.

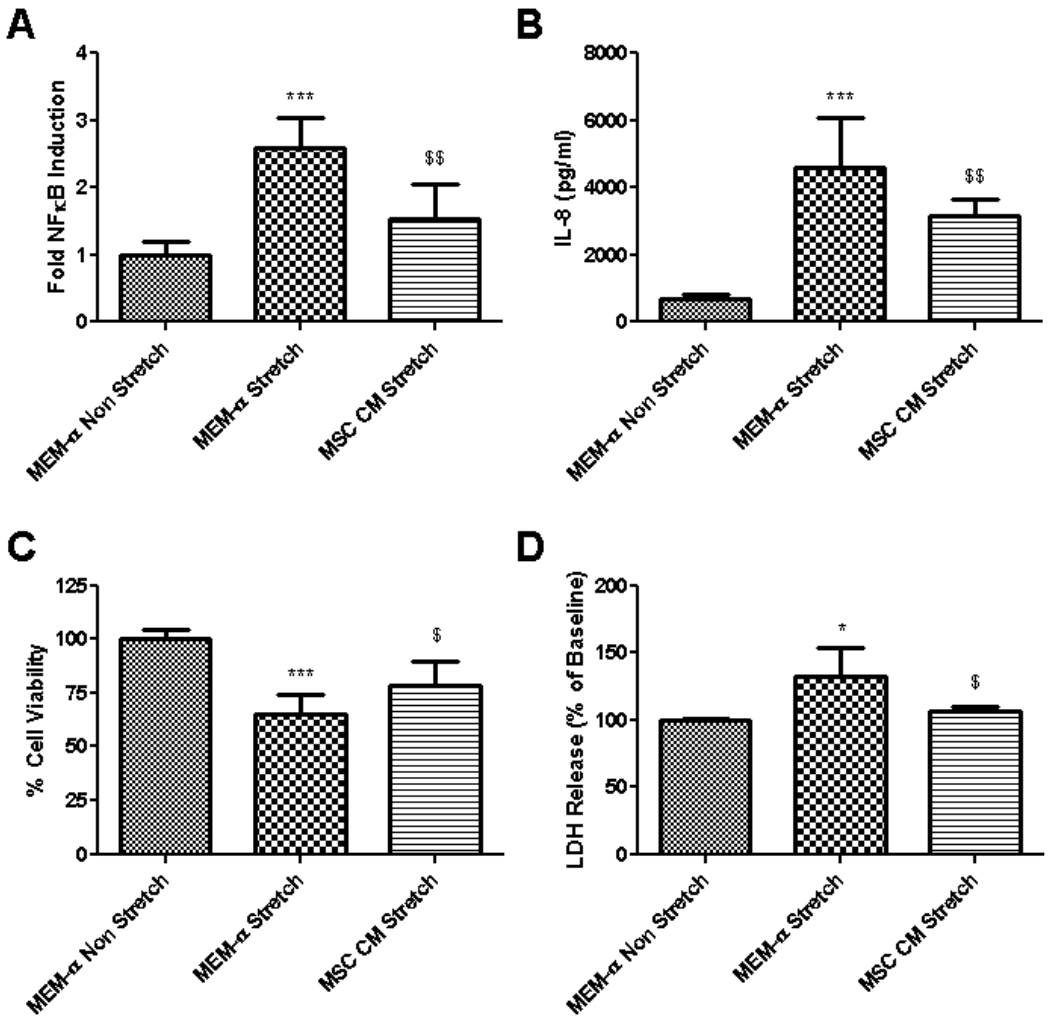

Figure 5.4: MSC CM Attenuates Mechanical Stretch Induced Cell Inflammation and Death. Mechanical stretch (22% for 120 hours) caused a significant induction in NF-κB (A) and IL-8 secretion (B) which was significantly ameliorated by the MSC CM in comparison to the MEM-α control. Mechanical stretch also caused a significant drop in A549/NF-κB-luc viability (C) and increase in LDH release (D) which again was significantly attenuated by the MSC CM. ***Note***: *, *** = $P < 0.05$, 0.001 versus MEM-α Non stretch control and $, $$ = $P < 0.05$, 0.01 versus MEM-α Stretch. n=3-5 per group per graph (**Section 5.2.10**).

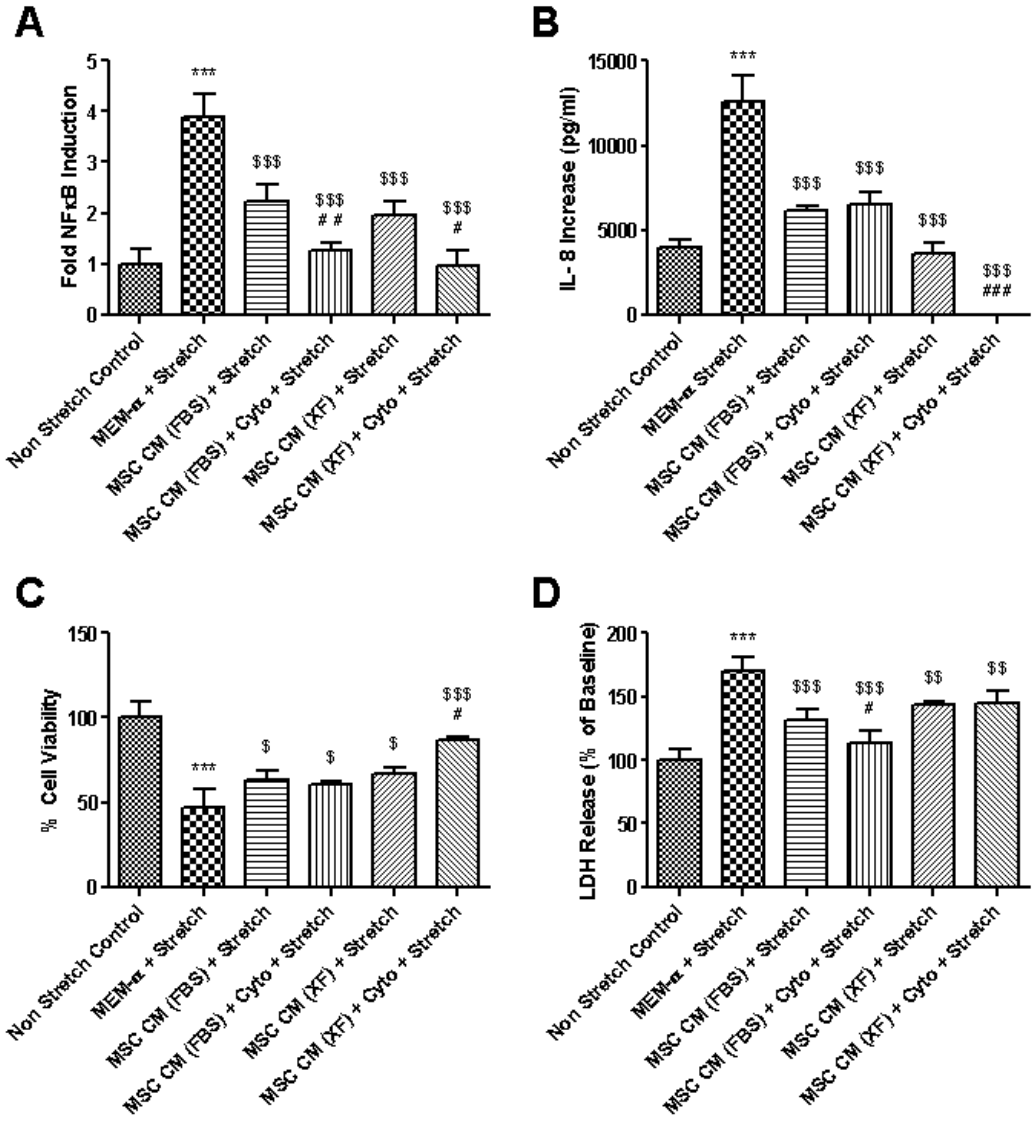

Figure 5.5: Pre-Activation Enhances the Efficacy of MSC CM in Attenuating Mechanical Stretch Injury. Mechanical stretch (22% for 120 hours) caused a significant induction of NF-κB (A) which was ameliorated by FBS and XF cultured MSC CM in comparison to MEM-α stretch control (A). Pre-activation of both FBS and XF cultured MSCs with cytomix (cyto) enhanced the ability of the MSC secretome to attenuate NF-κB in comparison to naïve MSC CM. Mechanical stretch also significantly increased the release of IL-8 (B) which was ameliorated by all CM groups but even more so by the cytomix pre-activated XF MSC CM (B). The decrement in cell viability, as induced by mechanical stretch, was ameliorated by all MSC CM treatments but was more enhanced by cytomix pre-activated XF MSC CM as compared to naïve XF MSC CM (C). The increase in LDH release, which again was attenuated by all the MSC CM groups, was more ameliorated by cytomix pre-activation in the FBS MSC CM group (D). ***Note***: *** = $P < 0.001$ versus MEM-α Non stretch control, $, $$, $$$ = $P < 0.05$, 0.01, 0.001 versus MEM-α stretch and #, ##, ### = $P < 0.05$, 0.01, 0.001 versus respective naïve FBS or XF MSC CM. Graphs (n=3 per group) show a trend for differences.

5.4 Discussion

This study demonstrated that the MSC secretome protected the pulmonary alveolar epithelium from numerous types of injury. Furthermore, the study provided support that pre-activation with inflammatory cytokines may enhance the efficacy of the secretome.

5.4.1 The MSC Secretome Attenuates Oxidative Stress

The MSC secretome significantly ameliorated oxidative stress induced cell death, which is a prominent feature of ARDS [17]. The MSC secretome recapitulated the efficacy that is seen with MSC cell treatment in attenuating oxidative injury [228, 229]. Other studies have also showed anti-oxidant effects in the secretome. For example, one study observed that CM from MSCs exhibited protection against oxidative injury in an O_2 induced bronchopulmonary dysplasia animal model [230]. Furthermore, in this study pre-activation with inflammatory cytokines showed a trend for an enhanced MSC secretome effect.

5.4.2 The MSC Secretome Attenuates Cytokine Induced Inflammation

The MSC secretome showed inhibition of IL-1β induced inflammatory NF-κB activation and IL-8 release, which are common mediators of ARDS inflammation [15, 19] and again mimics the effects seen with MSC cells that can attenuate NF-κB activation and IL-8 release *in vitro* and *in vivo* [57, 231]. Furthermore, pre-activation with IL-1β and less so with IFN-γ, seemed to enhance MSC secretome therapeutic efficacy. Similar results were observed by another group whereby rat AECs were rescued from inflammatory injury induced protein permeability by hypoxia and cytomix pre-conditioned MSC CM [152].

5.4.3 The MSC Secretome Promotes Wound Healing

In relation to wound injury, the MSC secretome significantly promoted wound repair, which is important as epithelial membrane injury is extensive in ARDS [5] and is a finding previously reported [123]. Interestingly, pre-activation with IL-1β, TNF-α and IFN-γ did not further enhance wound healing; in fact pre-conditioning with IFN-γ seemed to negate this effect. A possible explanation may due to the evidence which

shows that these activators modify MSCs differentially [212, 232] and thus further studies are required to elucidate the mechanisms underlying these effects.

5.4.4 The MSC Secretome Attenuates Mechanical Stretch Induced Inflammation and Cell Death

Finally, the MSC secretome significantly attenuated mechanical stretch driven NF-κB induction and IL-8 release, as well as the decrement in cell viability and increase in membrane damage (LDH release). These findings are supported by previous *in vivo* findings which show that the MSC secretome attenuated inflammation and enhanced repair in VILI [89, 123]. XF MSC CM also attenuated stretch induced inflammation and injury, which is an important translational finding and shows promise for XF MSC therapy in the clinical setting. Finally, a trend was observed whereby, pre-activation with cytomix (activators combined to eliminate any differences in effect between each activator and to ensure maximal pre-activation) seemed to enhance the ability of MSC CM to attenuate stretch induced inflammation and cell death. These are important new findings which need to be further verified and may solve the lack of efficacy previously observed with the MSC secretome when assessed in the early process of injury and recovery from VILI in rats [128].

5.4.5 Conclusion

In conclusion, the MSC secretome can attenuate pulmonary alveolar epithelial injury and pre-activation may improve the therapeutic efficacy. Future studies should evaluate the potential for enhancement of MSC and secretome efficacy by pre-activation in relevant pre-clinical ARDS models.

6.0 Results Three: Determination of the Efficacy of Bone Marrow versus Umbilical Cord Derived $S2^{+}$ MSCs in Enhancing Recovery Following Ventilator Induced Lung Injury

6.1 Introduction

ARDS can be caused by a single or combination of a wide spectrum of pathologies or injuries such as pneumonia, sepsis, VILI and trauma [6]. The pathogenesis of ARDS is characterised by severe inflammation of the lung parenchyma that is mediated by inflammatory cell infiltration [12, 13, 233, 234]. This causes the generation of reactive oxygen species and release of pro-inflammatory cytokines which leads to impaired barrier function and fluid retention [4, 12, 13] thus leading to the clinical presentation of ARDS which is defined by hypoxemia, inflammation and oedema [5, 7]. As previously described, VILI, as induced by mechanical ventilation, can lead to the development of ARDS or worsen its effects [224, 225]. As such, studies have established both high pressure and high volume animal models of VILI to replicate and better understand its pathogenesis [51, 235]. Animal modelling also provides a necessary tool for assessing targeted therapies for many disorders including ARDS.

Currently, MSCs are under much investigation as a therapy for ARDS as their therapeutic potential is derived from the ability of MSCs to modulate inflammation and promote repair [89, 106, 131]. Several studies have already established MSC pre-clinical efficacy in ARDS [90, 116]. Furthermore, phase 1 safety clinical trials have observed them to be well tolerated and show promising progression to phase 2 evaluations [91]. At first MSCs were mainly isolated from the BM but in more recent years other sources such as AD or UC tissue have also yielded successful MSC isolation and may provide cheaper and more readily available alternatives to BM derived MSCs [97, 98].

The use MSCs for translational purposes is still under some controversy in terms of the lack of a defined cell population. MSCs are isolated based on plastic adherence, clonal expansion and their expression or lack of, of certain surface markers for multi-lineage potential [97]. However, within this heterogeneous MSC population there are various divisions of stromal and stem cells that have alternative differentiation capabilities, surface marker expression and immune functions [97, 162, 182]. Therefore there is a need for a more defined subpopulation of MSCs with distinct function and one that can be potentially modified to further enhance therapeutic potential.

Recently interest has arisen in the use of the syndecan-2 positive ($S2^+$) MSC subpopulation [166]. S2 is a membrane protein that is used as a functional marker for MSC isolation, as it is highly expressed in MSCs undergoing chondrogenesis and is fundamentally involved in cell proliferation, cell migration and cellular matrix development [167]. One study recently reported that CM from hypoxic pre-conditioned amniotic fluid derived MSCs caused higher expression of S2 which resulted in enhanced wound healing in human dermal fibroblasts [168]. Other studies also showed that topical treatment with BM $S2^+$ MSCs [166] promoted wound healing and angiogenesis in a diabetic rabbit ulcer model [169]. Finally, single injections of UC $S2^+$ MSCs (250,000 cell per dose) augmented inflammation in type 2 diabetic mice with kidney disease [170].

The hypothesis of this study is that a defined subpopulation of MSCs is comparable to a heterogeneous MSC population in enhancing repair and recovery following VILI, a relevant pre-clinical model of ARDS. The aims of the study are: (1) to re-establish a relevant pre-clinical model of ARDS, more specifically a rat model of high pressure VILI, and to assess its consequence on lung physiology, inflammation and structure damage, (2) to determine the efficacy of human BM derived heterogeneous MSCs to modulate the inflammatory response and promote repair and recovery and (3) to determine the therapeutic efficacy of a UC cord tissue sourced $S2^+$ cell subpopulation (Cyndacel-C) [166] to enhance the resolution of injury post VILI.

6.2 Methods

6.2.1 Animal VILI Protocol

Male Sprague-Dawley rats were anaesthetised and ventilated for 20 minutes of baseline under protective ventilator settings **(Section 3.2.1)**. Sham animals remained on protective ventilation for another hour and were then recovered. Animals subjected to VILI were switched to high pressure injurious ventilation until a severe lung injury, as evidenced by a 50% decrease in static lung compliance was achieved **(Section 3.2.3)**. Injurious ventilation was then discontinued and protective ventilation reinstituted until the animals emerged from the anaesthesia, whereupon they were extubated, recovered and randomised to receive either PBS control or MSC treatment **(Section 3.2.3)**. The animals were allowed a 24 hour recovery period before being evaluated for recovery post VILI **(Section 3.2.4)**.

6.2.2 MSC Cell Culture and Delivery

BM MSC cells and UC $S2^+$ MSC cells were expanded in complete MEM-α medium supplemented with FGF-1 (10ng/mL final) **(Section 3.1.3)**. Animals received 10 million cells/kg of passage 2 MSCs in 1mL of PBS 15 minutes post VILI by injection into the tail vein. Control animals received PBS vehicle. BM and UC $S2^+$ MSCs were from different donors and one batch was used for each cell treatment group.

6.2.3 Blood Arterial Oxygen and Compliance

A capillary tube was used to collect an arterial blood sample which was then analysed using a blood gas analyser **(Section 3.2.4)**. Compliance measurements were made using a 5mL syringe to push 1mL increments of air into the static lung and noting pressure readings **(Section 3.2.4)**.

6.2.4 BAL Fluid Analysis

BAL fluid was collected for cell counts, protein quantification and cytokine profile analysis. Cell and neutrophil counts were analysed from 1mL of BAL which was centrifuged and re-suspended in 250μL of PBS **(Section 3.2.6)**. The Trypan Blue assay

was used for total cell counts and viability which were performed using a haemocytometer and light microscope. 150μL of the re-suspended cells were used for neutrophil counts. The cells were centrifuged in a cytospin funnel and resulting slides stained with the Hema 'Gurr® staining kit. The stained cells were then counted by microscopy (40x) and calculated based on the % of total cell counts. A protein assay kit was used for calculating BAL protein concentrations whereby a 12 point, 2 fold serial dilution standard curve was used to calculate protein values **(Section 3.3)**. Finally, cytokine release was assessed by sandwich ELISA kits as per the manufactures instructions **(Section 3.4)**.

6.2.5 Pulmonary Oedema

Fluid content in the lung was assessed using the lung wet:dry ratio. The lowest lobe of the right lung was tied off and weighed. It was then left to dry in a 40°C oven for 168 hours and then reweighed **(Section 3.2.5.2)**.

6.2.6 Lung Histologic Injury

Histological analysis was used to assess lung injury. The left lobe was removed and perfused with 4% w/v PFA. The lung was then sectioned into 5 pieces and then embedded in paraffin. A microtome was then used to section 7μm thick slices which were placed on glass slides. These sections were then stained with H and E, fixed and scored using microscopy (20x) **(Section 3.2.7)**.

6.2.7 Data Presentation and Statistical Analysis

Data was analysed using a one way ANOVA and a post hoc Student-Newman-Keuls test was employed for measuring statistical differences. Data is presented as mean +/- SD and a *P* value of < 0.05 was considered statistically significant. Animal numbers per group were as follows: PBS n=6, VILI n=6, BM MSC n=8 and UC $S2^{+}$ n=9.

6.3 Results

6.3.1 The Efficacy of MSCs in Enhancing Functional Lung Recovery Post VILI

Animals on high pressure injurious ventilation showed significant decreases in arterial PO_2 when compared to sham animals **(Figure 6.1 A)**. Both BM MSCs and Cyndacel-C MSCs enhanced the recovery of arterial oxygenation following VILI **(Figure 6.1 A)**. VILI also a caused a significant decrement in static lung compliance as compared to the sham group **(Figure 6.1 B)** but again this was restored by the BM MSCs but not by Cyndacel-C delivery **(Figure 6.1 B)**. High pressure ventilation also caused a significant accumulation of fluid and protein in the lungs due to permeability injury **(Figure 6.1 C + 6.1 D)**. Both BM MSCs and Cyndacel-C MSCs restored membrane integrity as evidenced by the decrease in BAL protein concentrations but did not seem to restore alveolar fluid clearance by the wet:dry ratio analysis **(Figure 6.1 C + 6.1 D)**.

6.3.2 The Efficacy of MSCs in Modulating Cell Infiltration after VILI

Cell and neutrophil infiltration into the lung was increased as a result of VILI **(Figure 6.2 A + 6.2 B)** however both MSC treatments modulated and resolved this increment **(Figure 6.2 A + 6.2 B)**.

6.3.3 The Efficacy of MSCs in Modulating Inflammation Post VILI

Injury as induced by VILI significantly enhanced the release of inflammatory IL-1β and TNF-α into the BAL but this was mitigated by both BM MSC and Cyndacel-C MSC treatment **(Figure 6.3 A + 6.3 B)**. VILI decreased concentrations of anti-inflammatory IL-10 and KGF in un-treated animals **(Figure 6.3 C + 6.3 D)** which were restored by BM MSC cell delivery. On the other hand Cyndacel-C MSCs cells only recovered the decrement in IL-10 but not KGF **(Figure 6.3 C + 6.3 D)**. Furthermore, high pressure ventilation enhanced the release of CINC-1 into the BAL, an increase which was not alleviated by either treatment **(Figure 6.3 E)**. Finally VILI did not cause any significant increases in BAL IL-6 concentrations **(Figure 6.3 F)**.

6.3.4 The Efficacy of MSCs in Restoring Lung Structure Post VILI

VILI caused significant injury to the lung structure **(Figure 6.4 A + 6.4 B)** as evidenced by the loss of alveolar airspace **(Figure 6.4 A + 6.4 B).** Both MSCs treatments were able to significantly restore lung histologic structure post VILI and were comparable to the sham animal group **(Figure 6.4 A + 6.4 B)**.

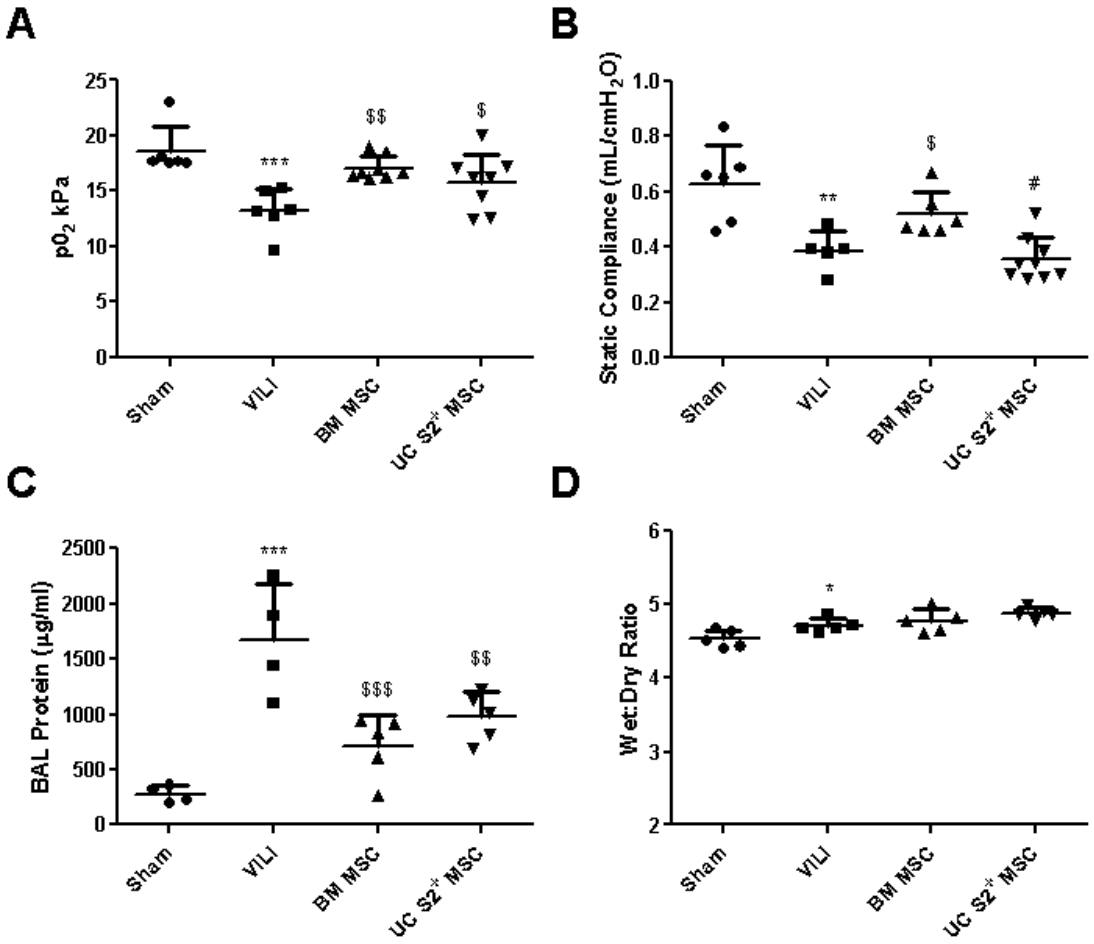

Figure 6.1: BM MSCs and Cyndacel-C MSCs Restore Lung Function Post VILI. Animals ventilated with high pressure settings showed decreased arterial oxygenation (A) which was recovered by BM MSC treatment and Cyndacel-C treatment (B). VILI caused a significant decrease in static lung compliance which was restored by BM MSC delivery but not UC $S2^+$ treatment (B). VILI also caused the development of pulmonary oedema as observed by the increase in BAL protein concentrations but this was recovered by both treatments (C). However, neither MSC treatment was able to restore the wet:dry ratio (D). ***Note***: *, **, *** = $P < 0.05$, 0.01, 0.001 versus sham control groups, $, $$, $$$ = $P < 0.05$, 0.01, 0.001 versus VILI groups and # = $P < 0.05$ versus BM MSC.

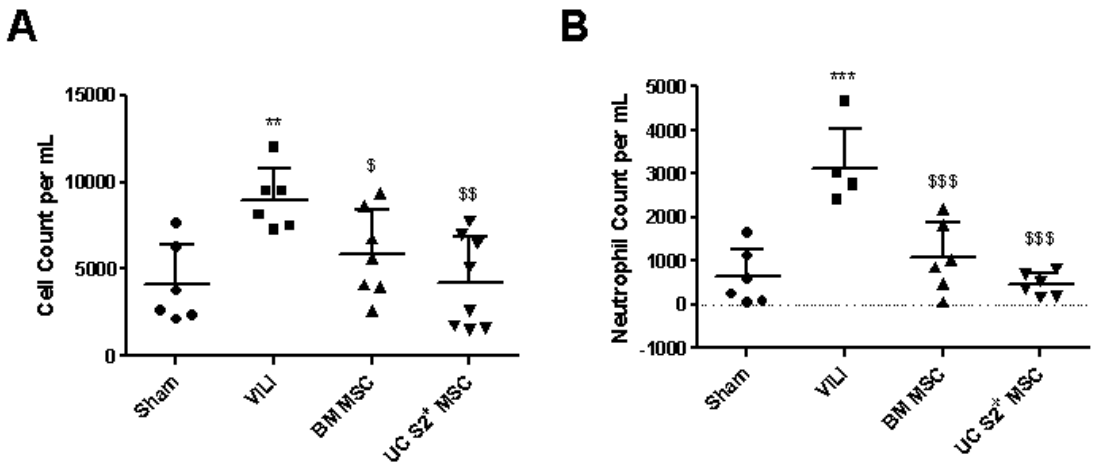

Figure 6.2: **MSCs Modulate Inflammatory Cell Infiltration into the Lung Post VILI**. High pressure ventilation injury caused a significant influx of cells (A) and neutrophils (B) into the lung which was resolved by both BM MSC treatment and UC $S2^+$ MSC treatment (A + B). ***Note***: **, *** = $P < 0.01$, 0.001 versus sham control groups and $, $$, $$$ = $P < 0.05$, 0.01, 0.001 versus VILI groups.

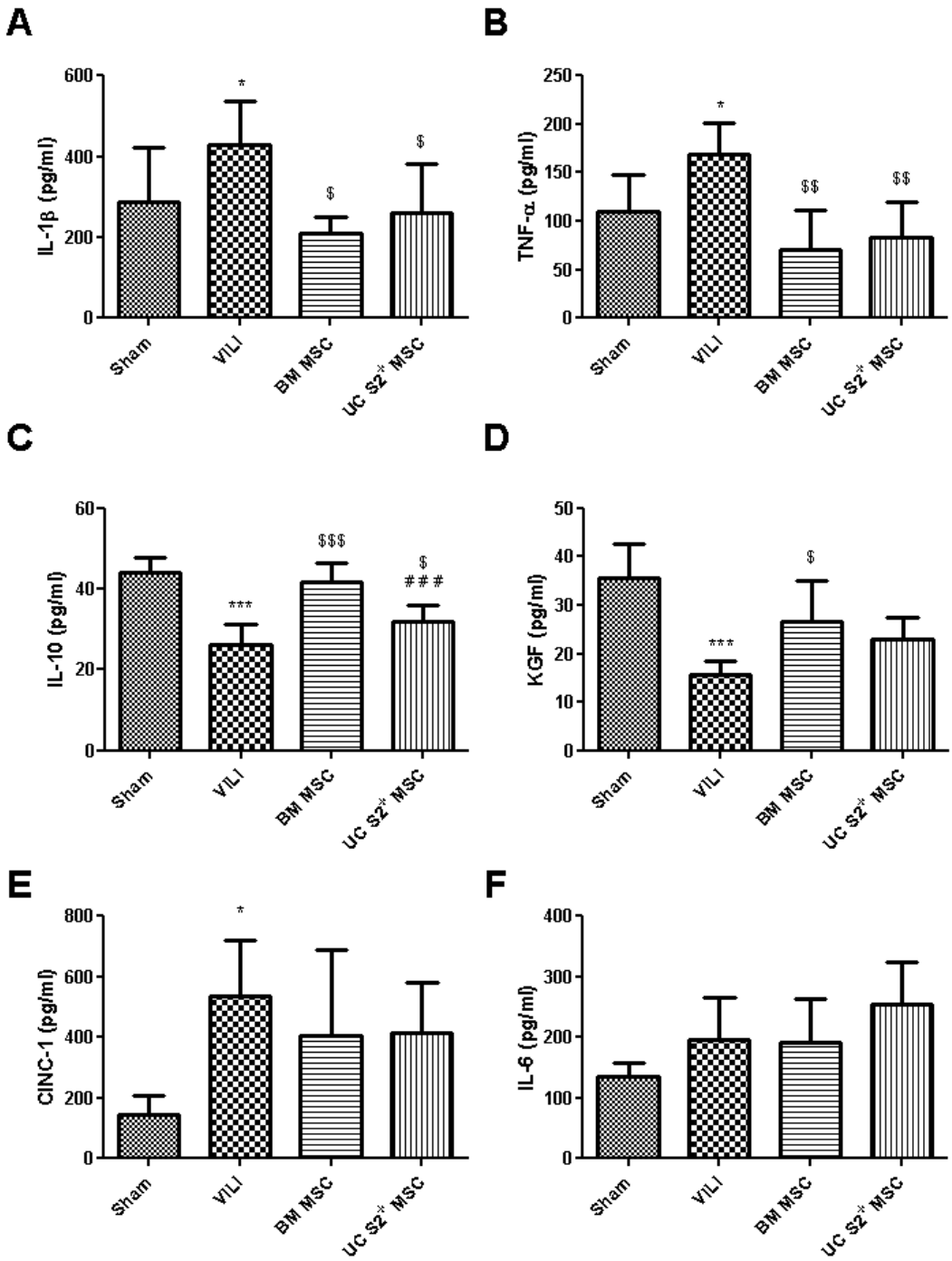

Figure 6.3: **VILI Causes Pro-Inflammatory Cytokine Release which is Modulated by MSC Treatment**. High pressure ventilation caused a significant increase in BAL IL-1β and TNF-α release (A + B) which was attenuated both MSC treatments (A + B). Animals with VILI showed a significant reduction in levels of IL-10 and KGF (C + D) which was restored by BM MSCs, while UC $S2^+$ delivery only recovered the decrease in IL-10 and not KGF (C + D). VILI also caused a significant increase in BAL CINC-1 which was not inhibited by either treatment (E). Finally, VILI did not cause a significant increase in IL-6 concentrations (F). ***Note:*** *, *** = $P < 0.05$, 0.001 versus sham control, $, $$, $$$ = $P <$ 0.05, 0.01, 0.001 versus VILI and ### = $P < 0.001$ versus BM MSC group.

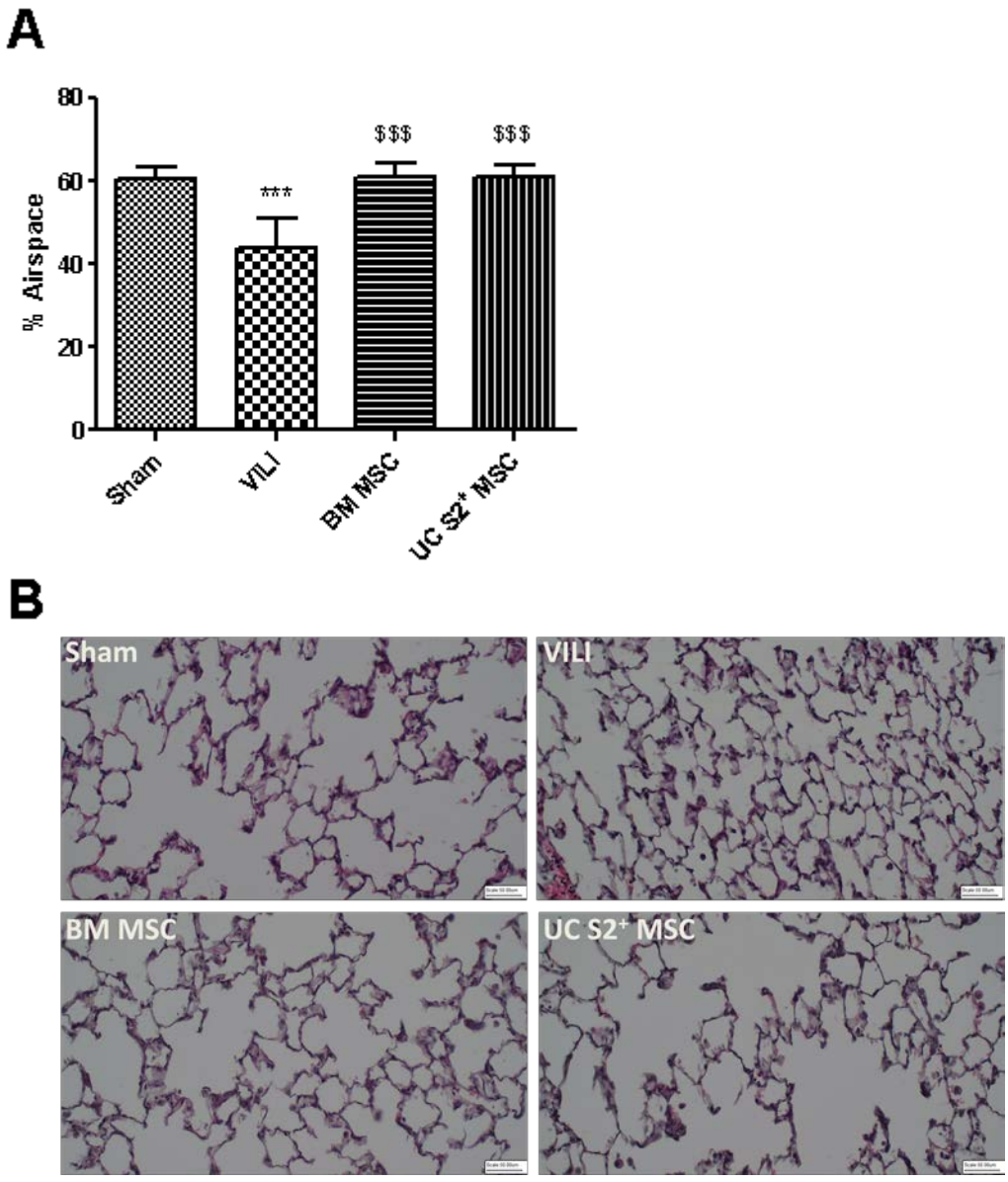

Figure 6.4: **MSCs Restore Lung Histologic Structure**. High pressure ventilation caused structural lung damage and the loss of alveolar airspace which was significantly restored by both BM MSC and UC $S2^+$ delivery (A), representative images of which are depicted above (B). ***Note***: *** = $P < 0.001$ versus sham control and $$$ = $P < 0.001$ versus VILI.

6.4 Discussion

This study demonstrated that a defined UC $S2^+$ MSC subpopulation, named Cyndacel-C was comparably efficacious in restoring lung physiology and structure and modulating the immune response following VILI. This shows promise for the use of a more defined and hence, less variable MSC therapy for the ARDS patient.

6.4.1 VILI Alters Lung Function which is Recovered by MSC Therapy

High pressure ventilation significantly reduced arterial oxygenation and static lung compliance when compared to sham animals, and is a finding previously reported [51]. Furthermore, "standard" BM derived human heterogeneous MSCs were able to recover these parameters, again an observation previously reported [90]. Finally, a human derived UC $S2^+$ subpopulation of MSCs, Cyndacel-C MSCs, was able to restore the oxygenation levels to therapeutic standard but failed to reverse the injury effect that resulted in the reduction of lung compliance. This finding is a first for this MSC subpopulation in a VILI setting. The difference in efficacy here between the two cell types may be due to the different sources of MSCs [97, 182] and the fact that previous findings have observed different modulation efficacies with different subpopulations of cells [162, 182, 236]. Furthermore, only one batch was used for each cell treatment and they were not from pooled donors.

6.4.2 MSC Therapy Modulates the Inflammatory Response Post VILI

Injurious ventilation also elicited a significant inflammatory response in the lung, as observed by the significant influx of inflammatory cells. These observations have also been previously reported in other VILI studies and mimic the effects observed in the lungs of the ARDS patient [13, 233, 234]. Of importance, both cell groups were able to enhance the resolution of these effects in the injured lung with similar efficacy. This is a new finding for UC $S2^+$ MSC efficacy in a VILI model.

Pro-inflammatory cytokine release was also significantly enhanced with VILI injury. Specifically, there was a significant increment in inflammatory IL-1β, TNF-α and CINC-1. These findings re-establish previous observations with VILI injury [51]. There

was, however, no significant increase in IL-6 secretion which may be due to animal variability when compared to previous studies. Of importance, both MSC treatment groups attenuated IL-1β and TNF-α release (previously observed with BM MSCs [89, 128] with similar efficacy but did not alter CINC-1 levels and this may be due to the large variability between the study groups in the CINC-1 analysis.

In terms of anti-inflammatory IL-10 and growth factor KGF release, animals with VILI showed significantly lower levels of these compared to sham animals (previously reported [89]). It is important to highlight that for KGF, the ELISA kit was for human and the observed levels in the sham animals may be due to species cross reactivity and the ultimate source of KGF was not pursued. In relation to MSC efficacy, both treatments notably restored IL-10 concentrations when compared to untreated animals (previously described for BM MSCs [89]) but BM MSCs were significantly better than the Cyndacel-C MSCs. Furthermore, only BM MSCs significantly restored KGF concentrations (previously reported [90]) again highlighting potential differences between subpopulations of MSCs. It is important to highlight that some IL-10 and KGF values were extrapolated as they were below the detection sensitivity of the ELISA kit.

6.4.3 MSC Therapy Restores Lung Barrier Function and Structure Post VILI

Finally, VILI caused significant lung barrier dysfunction and fluid retention as well as lung structural damage, important aspects for accurate modelling of the ARDS lung [5, 234] and is a finding previously observed [51]. Both cell treatments recovered the BAL protein concentrations and this is a new finding for Cyndacel-C MSCs in the context of VILI. However, neither MSC treatment was able to resolve the accumulation of fluid as demonstrated by the increase in lung wet;dry ratio which is in contrast with previous findings for the BM MSCs [90] and may highlight batch or donor variability issues. Both cell deliveries did however significantly decrease lung histologic injury which is of huge importance in terms of injury resolution following VILI [51].

6.4.4 Conclusion

In conclusion high pressure injurious ventilation which resulted in VILI was re-established in a rat model. Furthermore, a subpopulation of UC derived MSCs positive for S2 (Cyndacel-C) significantly enhanced recovery port VILI, in a comparable manner to heterogeneous BM MSC treatment, a new and important finding. One limitation to the study was the lack of a UC derived heterogeneous MSC population for comparison purposes.

7.0 Results Four: The Effect of MSC Tissue Source and Cryo-Preservation Status on the Efficacy in Enhancing Repair Following VILI

7.1 Introduction

MSCs show considerable therapeutic potential for ARDS as they have the ability to modulate the inflammatory process and enhance repair and recovery following VILI [90]. However, as phase 1 trials progress some logistical issues for MSC use need to be addressed, to facilitate effective clinical translation. One of the main sources for isolation of MSCs is from the BM. Harvesting cells from BM however, requires BM biopsy in volunteers, which is invasive, costly, and not without risk, thereby seriously limiting its availability and feasibility as an MSC source should MSCs prove effective in clinical trials for ARDS. Improved isolation techniques and new and ongoing efforts in MSC research have prompted the discovery of MSCs in alternative, more readily available sources [97] which include AD tissue, dental pulp, Wharton's jelly and UC tissue [181].

The isolation of MSCs from the UC is of particular value as this is normally considered a waste product in the clinic and thus is a relatively cheap and readily available tissue source. Prior studies have highlighted that different sources of MSCs have different expression markers and differentiation capabilities [97, 180, 181] which could potentially alter their immuno-modulatory functions [237] which may then alter their efficacy profile depending on the specific pathophysiologic injury/repair process. As such comparative efficacy studies of differently sourced MSCs are important but are lacking to date in the field of ARDS research. The demonstration that a more feasible tissue source, such as the UC, produced MSCs of comparable efficacy to those harvested from the BM, would greatly enhance the feasibility of translating MSC therapy to the clinical setting for patients with ARDS.

Another important translational issue regards MSC delivery to the patient in a clinical setting, which in the case of ARDS is the Intensive Care Unit. Traditional approaches to MSC delivery have used freshly cultured cells, which require continuous fresh culture of high numbers of MSCs, which is highly costly. In addition, the risk of cell culture contamination is increased, which could result in the loss of MSC batches and periods of inability to treat patients with ARDS. Batch-to-batch variability is also increased,

potentially diminishing MSC quality due to constant cell culture whereby doses harvested on different days could potentially show different efficacy in these patients. Furthermore, continuous culture of high grade MSCs is very resource intensive for cell manufacturing facilities and could potentially hinder the clinical testing of these cells and further availability to patients thereafter.

Delivery of thawed cryo-preserved MSCs offers a solution to these issues. However, some studies have shown that MSC structure, proliferation and function can be altered during the freezing process, which can potentially negatively impact their therapeutic and immunomodulatory efficacy [188, 189, 238, 239]. However, other studies show conflicting results, whereby MSCs are shown to retain their functional and therapeutic properties *in vitro* and *in vivo* [116, 186, 187]. In light of these issues, functional and directly comparative studies in regards to MSC efficacy after cryo-preservation in pre-clinical ARDS models, such as our VILI repair model, need to be performed. The demonstration that cryo-preserved MSCs retained comparable efficacy in comparison to freshly harvested MSCs would also greatly enhance the feasibility of translating MSC therapy to the clinical setting for patients with ARDS.

Given these findings we hypothesised that both BM derived and UC derived MSCs would comparably modulate inflammation and enhance repair after VILI, and that cryo-preservation would not alter MSC efficacy. The specific aims of the study are: (1) to compare fresh UC derived MSC efficacy against a known efficacious BM derived MSC cell type in modulating VILI and (2) to compare the efficacy of cryo-preserved UC MSCs to their fresh counterparts.

7.2 Methods

7.2.1 Induction of VILI

Animals were anaesthetised and ventilated under for protective settings for 20 minutes **(Section 3.2.1)**. They were then switched to injurious ventilation until a 50% decrease in static lung compliance was achieved **(Section 3.2.3)**. The animals were recovered, weaned from mechanical ventilation and randomised to receive either PBS control or MSC treatment. The animals were assessed for recovery from injury 24 hours post VILI induction, as described previously **(Section 3.2.4)**.

7.2.2 MSC Cell Culture and Delivery

Fresh BM MSC cells (supplied from CCMI) and UC MSC cells (supplied from TRT) were expanded in complete MEM-α medium supplemented with FGF (10ng/mL final) as previously described **(Section 3.1.3)**. Frozen UC MSC cells were received as cryo-preserved doses from TRT, thawed and re-suspended in PBS and administered. All cells were passage 2 MSCs and 10 million cells/kg in 1mL of PBS were administered to animals 15 minutes post VILI induction by tail vein injection. Control animals received PBS vehicle. BM and UC cell treatments were from different donors and only one batch was used for each cell treatment.

7.2.3 Blood Arterial Oxygen and Compliance

Arterial blood was collected by capillary tube and PO_2 was assessed using a blood gas analyser **(Section 3.2.4)**. Static lung compliance was measured using a 5mL syringe to push 1mL increments of air into the static lung and recording the changes in pressure **(Section 3.2.4)**.

7.2.4 BAL Fluid Analysis

Cell counts, protein concentrations and cytokine release was measured in the BAL fluid. 1mL of BAL was centrifuged and re-suspended in 250μL of PBS for cell and neutrophil counts as previously described **(Section 3.2.6)**. Cell counts and viability were assessed by Trypan blue assay. For neutrophil evaluation, 150μL of the re-suspended cells were

centrifuged on a cytospin and stained with the Hema 'Gurr® staining kit. The stained cells were counted by microscopy (40x) and calculated based on the % of total cell counts. BAL protein concentrations were measured using a protein assay kit. A 12 point, 2 fold serial dilution standard curve was used to calculate protein levels **(Section 3.3)**. Finally, sandwich ELISA kits were used for cytokine analysis as per the manufactures guidelines as previously described **(Section 3.4)**.

7.2.5 Pulmonary Oedema

The lung wet:dry ratio was used to assess fluid content in the lung as previously described **(Section 3.2.5.2)**. The lowest lobe of the right lung was tied off and weighed. It was then left to dry in a 40°C oven for 168 hours and then reweighed.

7.2.6 Lung Histologic Injury

Lung injury was assessed using histological analysis. The left lobe was removed, perfused with 4% w/v PFA, sectioned and then embedded in paraffin. A microtome was then used to cut 7μm thick sections which were placed on glass slides. These sections were stained with H and E, fixed and then scored using microscopy (20x) as previously described **(Section 3.2.7)**.

7.2.7 Data Presentation and Statistical Analysis

Data was analysed using a one way ANOVA and a post hoc Student-Newman-Keuls test was employed for measuring statistical differences. Data is presented as mean +/- SD, with n=7 animals per group and a P value of < 0.05 was considered statistically significant.

7.3 Results

7.3.1 The Efficacy of Cryo-Preserved MSCs in Restoring Lung Function Post VILI

Both BM and UC cord derived MSCs significantly restored arterial oxygenation which was reduced by VILI **(Figure 7.1 A)**. Importantly, thawed cryo-preserved UC MSCs also restored arterial oxygenation, demonstrating that these cells retained this efficacy post cryo-preservation **(Figure 7.1 A)**. The decrement in static lung compliance as induced by VILI was also restored by BM and UC MSCs, while thawed cryo-preserved UC MSCs were similarly effective **(Figure 7.1 B)**. BM and UC derived MSCs also enhanced the restoration of lung alveolar fluid clearance, as evidenced by a decrease in lung wet:dry weight ratios **(Figure 7.1 C)** and a decrease in alveolar fluid protein concentrations **(Figure 7.1 D)**. Furthermore, thawed cryo-preserved UC MSCs retained their efficacy and also restored alveolar fluid clearance and BAL protein concentrations to normal **(Figure 7.1 C + 7.1 D)**.

7.3.2 The Efficacy of Cryo-Preserved MSCs in Modulating Cell Infiltration

Cell and neutrophil infiltration into the lung was increased in the PBS groups (**Figure 7.2 A + 7.2 B)**, however this increment was resolved by both BM derived and UC derived MSC treatments **(Figure 7.2 A + 7.2 B)**. Furthermore thawed cryo-preserved UC MSCs showed comparable efficacy to freshly delivered cell groups **(Figure 7.2 A + 7.2 B)**.

7.3.3 The Efficacy of Cryo-Preserved MSCs in Modulating Cytokine Release Post VILI

IL-6 release was significantly inhibited by fresh BM MSC cells **(Figure 7.3 A)**. While IL-6 concentrations were lower in the BAL of animals that received freshly delivered UC cells, this was not statistically significant **(Figure 7.3 A)**. In contrast, thawed cryo-preserved UC derived MSCs did reduced alveolar IL-6 concentrations **(Figure 7.3 A)**. All MSC treatment groups significantly reduced alveolar IL-1β concentrations when compared to the control group **(Figure 7.3 B)**. All cell treated groups also significantly restored the secretion of KGF into the BAL **(Figure 7.3 C)**. Finally, there was no

significant effect of any cell treatment on the restoration of anti-inflammatory IL-10 concentrations **(Figure 7.3 D)** or inhibition of pro-inflammatory CINC-1 and TNF-α secretion **(Figure 7.3 E + 7.3 F)**.

7.3.4 The Efficacy of Cryo-Preserved MSCs to Restore Lung Structure

Fresh BM and UC MSC delivery significantly enhanced the resolution of structural lung injury as compared to the PBS control group **(Figure 7.4 A + 7.4 B)**. Thawed cryo-preserved UC MSCs also retained their efficacy and were able to restore alveolar airspace **(Figure 7.4 A + 7.4 B)**.

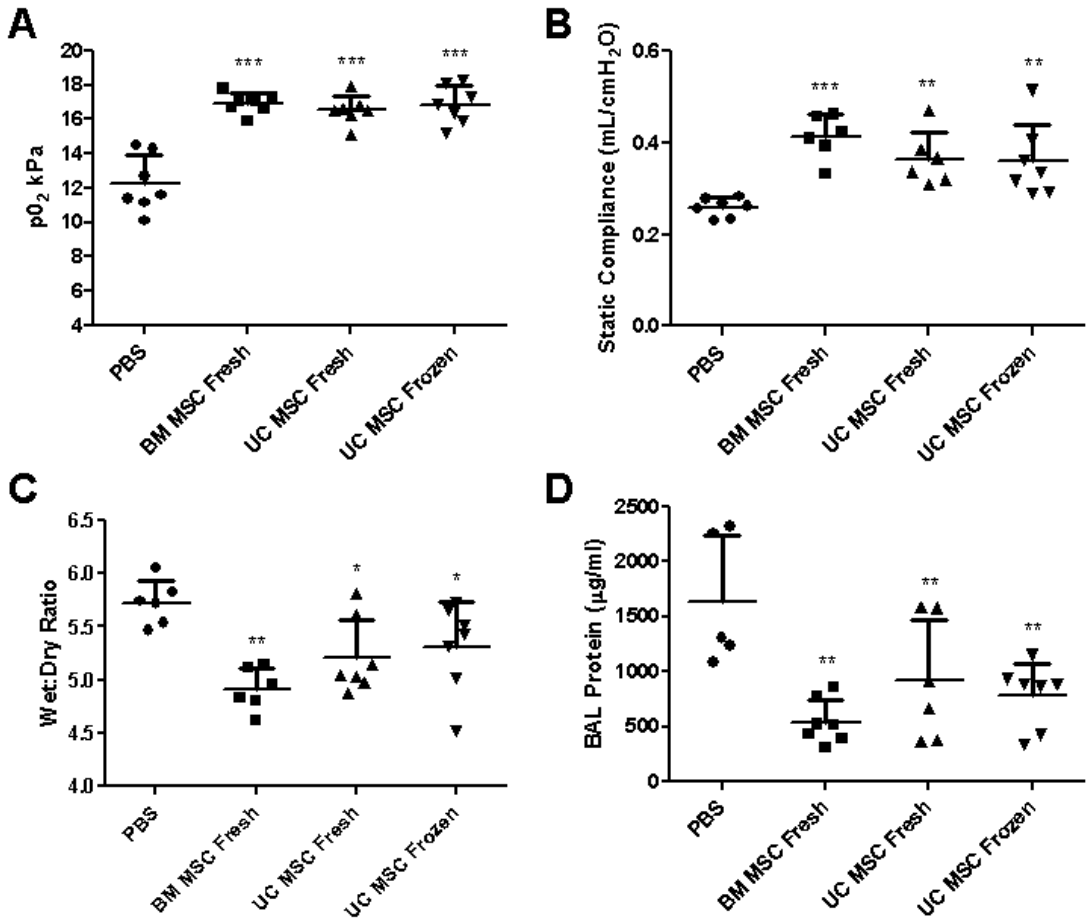

Figure 7.1: Cryo-Preserved UC MSCs Restore Lung Function Post VILI. BM and UC derived MSCs comparably restored arterial oxygen (A) and cryo-preservation did not hinder this effect (A). The decrease in static lung compliance was also restored by both cell sources (B) and again thawed cryo-preserved UC MSCs retained their efficacy (B). Alveolar fluid clearance as depicted by lung wet:dry ratio and BAL protein concentrations was significantly restored by both BM and UC derived MSCs (C + D), while thawed cryo-preserved UC MSCs were similarly effective (C + D). ***Note***: *, **, *** = $P < 0.05$, 0.01, 0.001 versus PBS control groups.

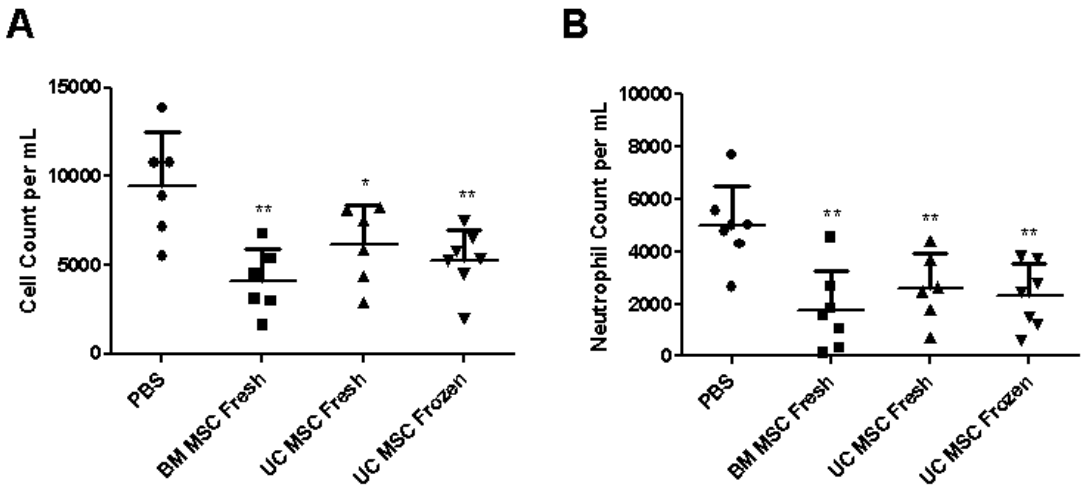

Figure 7.2: Cryo-Preserved UC MSCs Modulate Inflammatory Cell Influx. All cell treatment groups significantly resolved cell and neutrophil infiltration into the lung (A + B) and cryo-preserved delivery was comparable in efficacy (A + B). ***Note***: *, ** = $P < 0.05$, 0.01 versus PBS control groups.

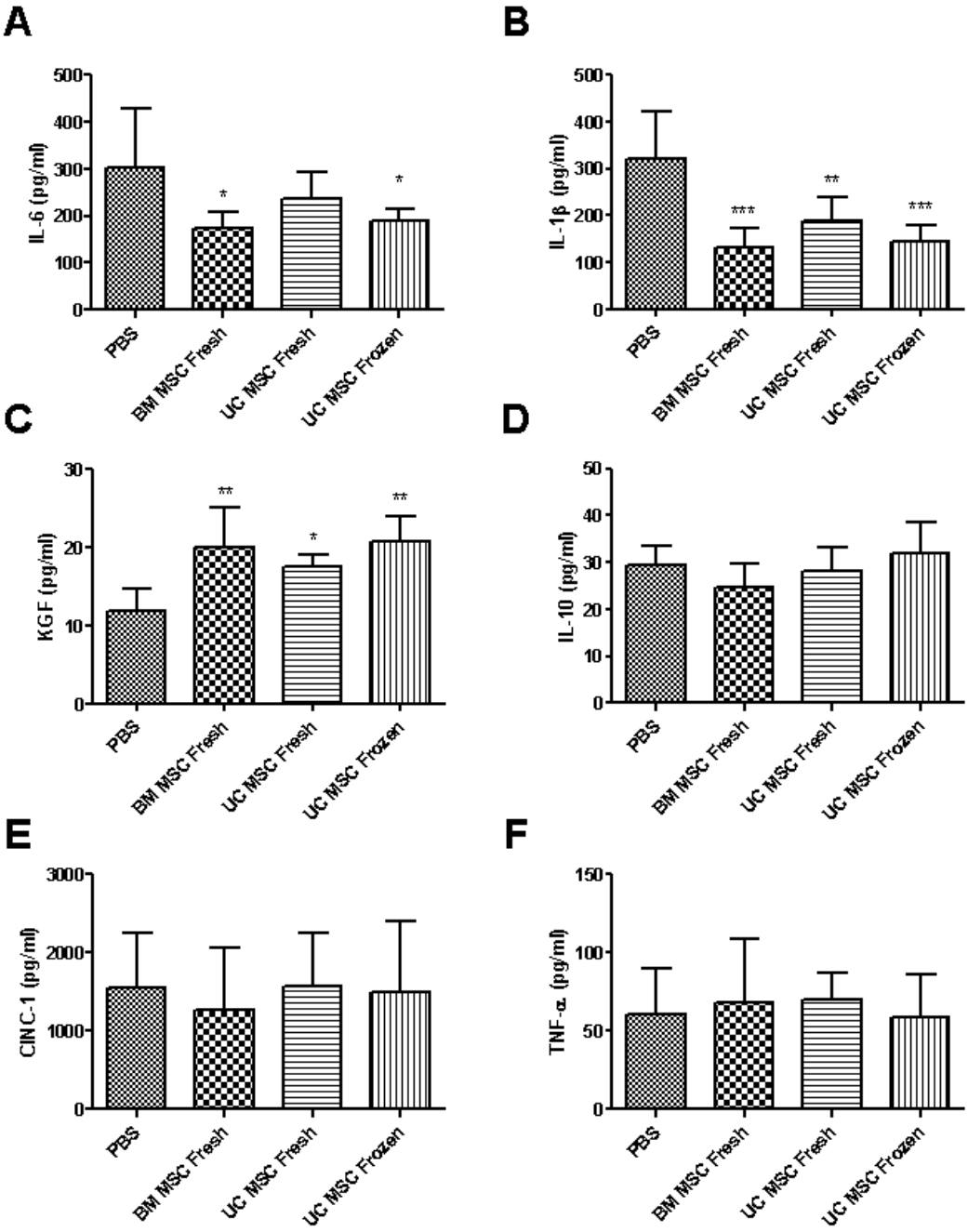

Figure 7.3: Cryo-Preserved UC MSCs Modulate Cytokine Release. Fresh BM MSCs significantly attenuated BAL IL-6 and IL-1β (A + B) when compared to PBS control. Fresh UC MSCs did not inhibit IL-6 but thawed cryo-preserved UC MSCs did (A). Furthermore, fresh BM and UC MSC, and cryo-preserved UC MSC delivery ameliorated BAL IL-1β levels (B). All three MSC groups restored KGF release (C) but had no effect on IL-10 (D). Finally there was no MSC effect on CINC-1 (E) or TNF-α secretion (F). ***Note***: *, **, *** = $P < 0.05$, 0.01, 0.001 versus PBS control.

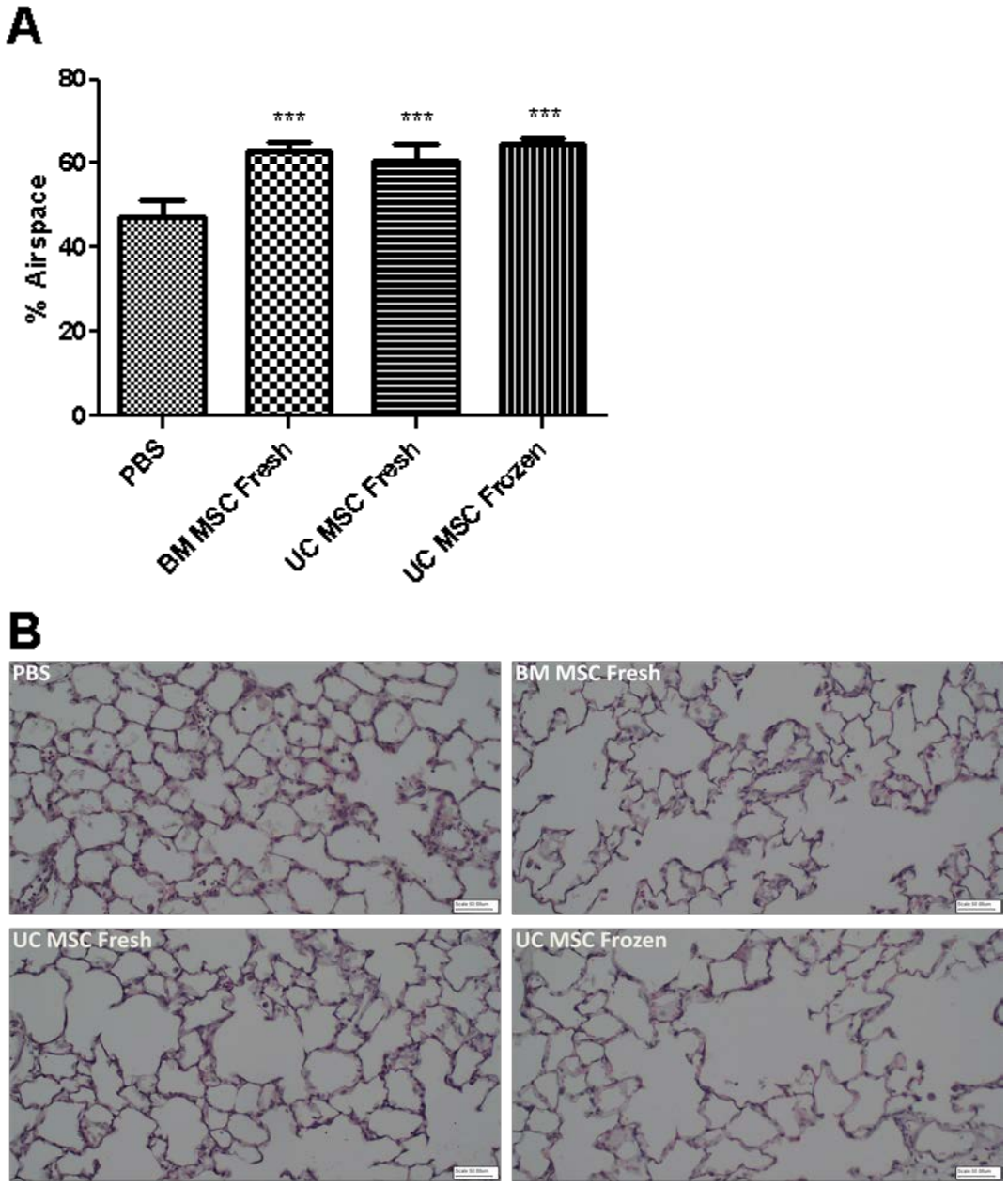

Figure 7.4: Cryo-Preserved MSCs Restore Lung Histologic Structure. Fresh BM MSCs and UC MSCs comparably restored alveolar airspace when compared to PBS control (A). Thawed cryo-preserved MSCs also restored lung structure with similar efficacy (A), representative images of which are depicted above (B). ***Note***: *, **, *** = $P < 0.05$, 0.01, 0.001 versus PBS control.

7.4 Discussion

Key findings from this study include the demonstration that UC derived MSCs were comparably effective to BM derived MSCs in enhancing repair in the injured lung and that UC MSCs retained efficacy when thawed post cryo-preservation. These findings have important implications for the translation of MSC therapies to the clinical setting of ARDS.

7.4.1 UC and BM MSCs Comparably Restore Lung Function

The study demonstrated that UC derived MSCs are comparably efficacious to the BM derived cells in restoring oxygenation and compliance in VILI animals and is a new finding. Previous studies by our group have reported that fresh BM MSC cell delivery significantly restored these parameters of lung function in comparison with PBS treatment [90] but no studies have directly compared the efficacy of these two sources of MSCs in VILI. Other studies have also confirmed UC efficacy in hyperoxic and LPS induced lung injury [107, 240] which provides further promise for the use of these UC sourced MSC cells in the clinical setting of ARDS.

Furthermore, UC MSCs retained their efficacy after cryo-preservation to restore arterial oxygenation and compliance, a translationally relevant finding. Other studies support these results and have demonstrated that cryo-preserved BM MSCs also attenuated the drop in oxygenation and compliance in a comparable manner to fresh cell delivery in a pneumonia injury [116]. This study therefore, provides further support for the use of cryo-preserved cells in the clinical setting.

BM and UC MSC treatments were also comparable in their efficacy to restore alveolar fluid clearance in the injured lung and in restoring normal concentrations of protein in the BAL, therefore restoring alveolar membrane integrity. Furthermore, thawed cryopreserved MSCs retained their efficacy to restore these parameters of membrane integrity following VILI.

7.4.2 UC and BM MSCs Comparably Modulate the Inflammatory Response

Inflammatory cell infiltration was resolved by both fresh BM and UC MSC cell treatments, demonstrating once more that both sources of cells are equally efficacious in modulating the inflammatory response as caused by VILI. Furthermore cryo-preservation did not impede UC MSC efficacy here. Previous reports have previously observed that cryo-preserved BM MSCs also attenuated BAL inflammatory cell influx in a rodent model of pneumonia [116]. This study therefore provides further support for the use of cryo-preservation methods.

In relation to BAL cytokine profile analysis, fresh UC MSC treatment did not alleviate the release of IL-6 as compared to the BM cells. While, interestingly, the cryo-preserved cells showed a significant reduction and this may be due to an issue in variability at fresh harvest stage as cryo-preservation allows for more homogenous cell doses (i.e. cells harvested on the same day, pooled and then cryo-preserved are less likely to show variability). All cell treatments attenuated pro-inflammatory IL-1β secretion and restored the release of the pro repair molecule KGF (it must be noted that the KGF values were extrapolated as they were below the detection limit of the ELISA kit). This highlights that neither cell source nor cryo-preservation impeded the immunomodulatory effects of the MSCs post VILI and agrees with previously published reports in other animal models [116, 240]. There was no cell treatment affect on CINC-1, TNF-α or IL-10 which may be attributed to cell batch variability.

7.4.3 UC and BM MSCs Comparably Restore Lung Structure

Finally all cell treatments restored alveolar lung structure as evidenced by the restoration of alveolar airspace and cryo-preservation did not alter MSC efficacy in this regard. This study again confirms that either cell source of MSCs is efficacious, even after cryo-preservation, in promoting resolution from injury after VILI and conforms to previous findings in other animal injury models [117, 240].

7.4.4 Conclusion

In conclusion, UC derived MSCs provide similar efficacy in modulating inflammation and promoting recovery against VILI as BM derived MSCs and these effects are maintained after cryo-preservation. These findings support the use of alternatively sourced MSCs that are more readily available, in the clinical setting. Furthermore, cryo-preserved cell delivery to the clinic would be a more efficient and less variable method of administering treatment to ARDS patients. In this study, MSC treatment was administered directly after VILI injury and as such does not truly mimic the clinical scenario where it is not always possible to treat patients immediately after injury. Future studies should determine the efficacy of cryo-preserved MSCs at a more relevant time point post VILI injury i.e. determine the therapeutic window. Future studies should also directly compare the efficacy of these two sources of MSCs in other pre-clinical ARDS models.

8.0 Results Five: Pre-Activation Enhances the Efficacy of Cryo-Preserved, Xeno-Free Expanded Mesenchymal Stromal Cells in Lung Repair following Ventilator Injury

8.1 Introduction

As previously discussed, MSCs show promising therapeutic efficacy for ARDS in pre-clinical *in vivo* models [116, 128] and are undergoing early phase clinical trials to evaluate safety in patients with ARDS [91]. However, there a number of obstacles still remaining in the way of clinical translation. In the clinical setting it is almost impossible to administer treatment immediately after injury and as such the efficacy of MSCs delivered to patients later in the injury/recovery process is unclear. In terms of the therapeutic window for MSCs, previous animal studies by our group have shown that MSCs were able to attenuate injury following VILI when delivered at later time points (6 and 24 hours post injury), however delivery at 24 hours did show some loss of efficacy [90]. Furthermore, MSC variability in efficacy, as previously discussed can be affected by numerous factors, including culture conditions and cryo-preservation [183, 189].

To target efficacy issues, studies have employed strategies such as the over-expression of anti-inflammatory molecules to enhance MSC efficacy *in vivo* [198]. Of more interest, studies have demonstrated that MSCs are known to behave differently depending on the microenvironment to which they are exposed [190, 194, 212, 241, 242], which may be of great relevance for ARDS, which is a strongly pro-inflammatory condition. Of relevance in this regard, MSC pre-activation/priming with inflammatory activators can alter their immunomodulatory functions to produce more anti-inflammatory effects [211-213].

Another area of concern, as outlined by FDA regulations, is in regard the use of animal products in MSC cultures that may cause disease transmission or potentially negatively alter MSC function [183]. This has prompted the development of XF media, i.e. media that is free of animal derived products [184], which would resolve these issues, but the use and efficacy of which has not been fully investigated. Finally, as mentioned previously the logistical challenges of constant MSC culture to enable delivery of large fresh doses in a clinical setting are substantial and very expensive. A previous study by our group has shown that MSCs retain their therapeutic efficacy after cryo-preservation

in a pneumonia model of ARDS [116] and we have demonstrated that cryo-preserved MSCs enhance recovery following VILI in the previous chapter. Therefore the specific aims of this study are four fold;

1. Determine the efficacy of XF expanded MSCs to repair the lung following VILI.
2. Determine the therapeutic window of XF MSC efficacy following VILI, more specifically at a time point of maximal injury and inflammation i.e. 6 hours post VILI induction [51].
3. Determine whether there is potential to improve XF MSC efficacy by pre-activation/priming with inflammatory cytokines, more specifically, a combination of IL-1β, TNF-α and IFN-γ, which are known modulators of MSCs immunomodulatory potential [212, 213].
4. Determine whether the efficacy of these cells is retained after cryo-preservation.

8.2 Methods

8.2.1 MSC Cell Culture and Delivery

BM MSCs (supplied from REMEDI) were expanded in XF media as previously described **(Section 2.1.3)**. Cells were pre-activated with cytomix (IL-1β (10ng/mL), TNF-α (50ng/mL) and IFN-γ (50ng/mL) for 24 hours. Cells were either delivered freshly harvested or were cryo-preserved, thawed and re-suspended in PBS, and immediately delivered. Animals received 10 million cells/kg of passage 2 MSCs in 1mL of PBS 6 hours post VILI by IV injection into the tail **(Table 8.1)**. Control animals received PBS vehicle. Cell treatments were from different donors and multiple batches.

Table 8.1: Animals were randomised to receive either vehicle or MSC treatment by IV injection 6 hours post VILI

Intervention	*Study Name*	*No of Animals per Group*
Vehicle	PBS	8
Naïve xeno-free fresh MSCs	XFRESH	8
Pre-activated fresh xeno-free frozen MSCs	XACTFRESH	6
Naïve xeno-free frozen MSCs	XFROZEN	6
Pre-activated xeno-free frozen MSCs	XACTFROZEN	7

8.2.2 Induction of VILI Protocol

Animals were anaesthetised and ventilated for 20 minutes of baseline under protective ventilator settings as previously described **(Section 3.2.1)**. Animals were then switched to high pressure injurious ventilation until a 50% decrease in static lung compliance was achieved, as described previously **(Section 3.2.3)**. The animals were recovered, weaned from mechanical ventilation and randomised to receive either PBS control or MSC treatment 6 hours post injury.

8.2.3 Assessment of Recovery following VILI

24 hours following VILI induction, animals were re-anaesthetised as previously described **(Section 3.2.4)**, and the degree of recovery from VILI assessed.

8.2.4 Blood Arterial Oxygen and Compliance

An arterial blood sample was collected by capillary tube and analysed using a blood gas analyser. Compliance measurements were made using a 5mL syringe to push 1mL increments of air into the static lung and recording the pressure change, as previously described **(Section 3.2.4)**.

8.2.5 BAL Fluid Analysis

BAL was collected **(Section 3.2.5.3)** and analysed for cell counts, protein concentrations and cytokine profile. For cell and neutrophil counts, 1mL of BAL was centrifuged and re-suspended in 250μL PBS **(Section 3.2.6)**. Cell counts and viability were assessed by the Trypan blue assay on a haemocytometer. For neutrophil counts, 150μL of the re-suspended cells were centrifuged in a cytospin and stained with the Hema 'Gurr® staining kit. The stained cells were counted by microscopy (40x) and expressed as a % of total cell counts for calculation. BAL protein concentrations were assessed using a protein assay kit, whereby a 12 point, 2 fold serial dilution standard curve was used to calculate protein values **(Section 3.3)**. Finally cytokine release was assessed by sandwich ELISA kits as per the manufactures instructions **(Section 3.4)**.

8.2.6 Pulmonary Oedema

The lung wet:dry ratio was used to analyse fluid content in the lung, as previously described **(Section 3.2.5.2)**. The lowest lobe of the right lung was tied off and weighed. It was the left to dry in 40°C oven for 1 week and then reweighed.

8.2.7 Lung Histologic Injury

Lung injury was assessed by histology and stereology. The left lobe was tied off, removed and perfused with 4% w/v PFA. The lung was then sectioned into 5 pieces and embedded in paraffin. 7μm thick sections were then cut on a microtome and placed on glass slides. These sections were then stained with H and E and imaged using microscopy (20x) then scored **(Section 3.2.7)**.

8.2.8 Data Presentation and Statistical Analysis

Data was analysed using a one way ANOVA and a post hoc Student-Newman-Keuls test was employed for measuring statistical differences. Data is presented as mean +/- SD and a *P* value of < 0.05 was considered statistically significant.

8.3 Results

8.3.1 Effect of MSC Therapy on Physiologic Recovery Following VILI

Delayed delivery of fresh and cryo-preserved, whether naïve or pre-activated, MSC therapy significantly restored arterial oxygenation **(Figure 8.1 A)** when compared to the vehicle (PBS) control group. However, the pre-activated thawed cryo-preserved cells were significantly better than the thawed, cryo-preserved naïve cell group. However, only pre-activated fresh and cryo-preserved cell treatments were able to enhance the restoration of static lung compliance as compared to the vehicle group **(Figure 8.1 B)**. Delayed delivery of fresh and thawed cryo-preserved MSCs also enhanced the restoration of lung alveolar fluid clearance, as evidenced by a decrease in lung wet:dry weight ratios **(Figure 8.1 C)**, however there was no decrease in alveolar fluid protein concentrations **(Figure 8.1 D)**. Pre-activation of both fresh and cryo-preserved MSCs restored their efficacy to restore membrane integrity as evidenced by the decrease in BAL protein concentrations **(Figure 8.1 D)**.

8.3.2 Effect of MSC Therapy on Cell Infiltration Post VILI

Delayed delivery of both fresh and pre-activated fresh cells significantly modified cell infiltration into the lung as observed by the decrease of cell numbers in the BAL **(Figure 8.2 A)**. Naïve thawed cryo-preserved MSCs were ineffective, while pre-activation of these cells with cytomix was able to restore the effect on alveolar cell infiltration **(Figure 8.2 A)**. Delayed delivery of all cell groups significantly modulated lung neutrophil accumulation as represented by the decrease in BAL neutrophil cell counts **(Figure 8.2 B)**.

8.3.3 The Effect of MSC Therapy on the BAL Cytokine Profile after VILI

Treatment with all cell groups caused a significant attenuation of the increase in BAL IL-6 **(Figure 8.3 A)** and CINC-1 **(Figure 8.3 B)** concentrations as compared to the vehicle (PBS) control. Pre-activated but not naïve fresh XF MSCs significantly restored anti-inflammatory IL-10 **(Figure 8.3 C)** and pro-repair KGF **(Figure 8.3 D)** concentrations in the BAL. Cryo-preserved cells, both naïve and pre-activated did not

modulate IL-10 or KGF concentrations. Finally, no cell treatment group showed any significant decrease of inflammatory TNF-α **(Figure 8.3 E)** or IL-1β **(Figure 8.3 F)** in the BAL.

8.3.4 The Effect of MSC Therapy on Lung Histologic Injury

Delayed delivery of all cell treatment groups significantly enhanced the restoration of lung histologic structure post VILI, depicted as percentage airspace **(Figure 8.4 B + 8.4 C)**.

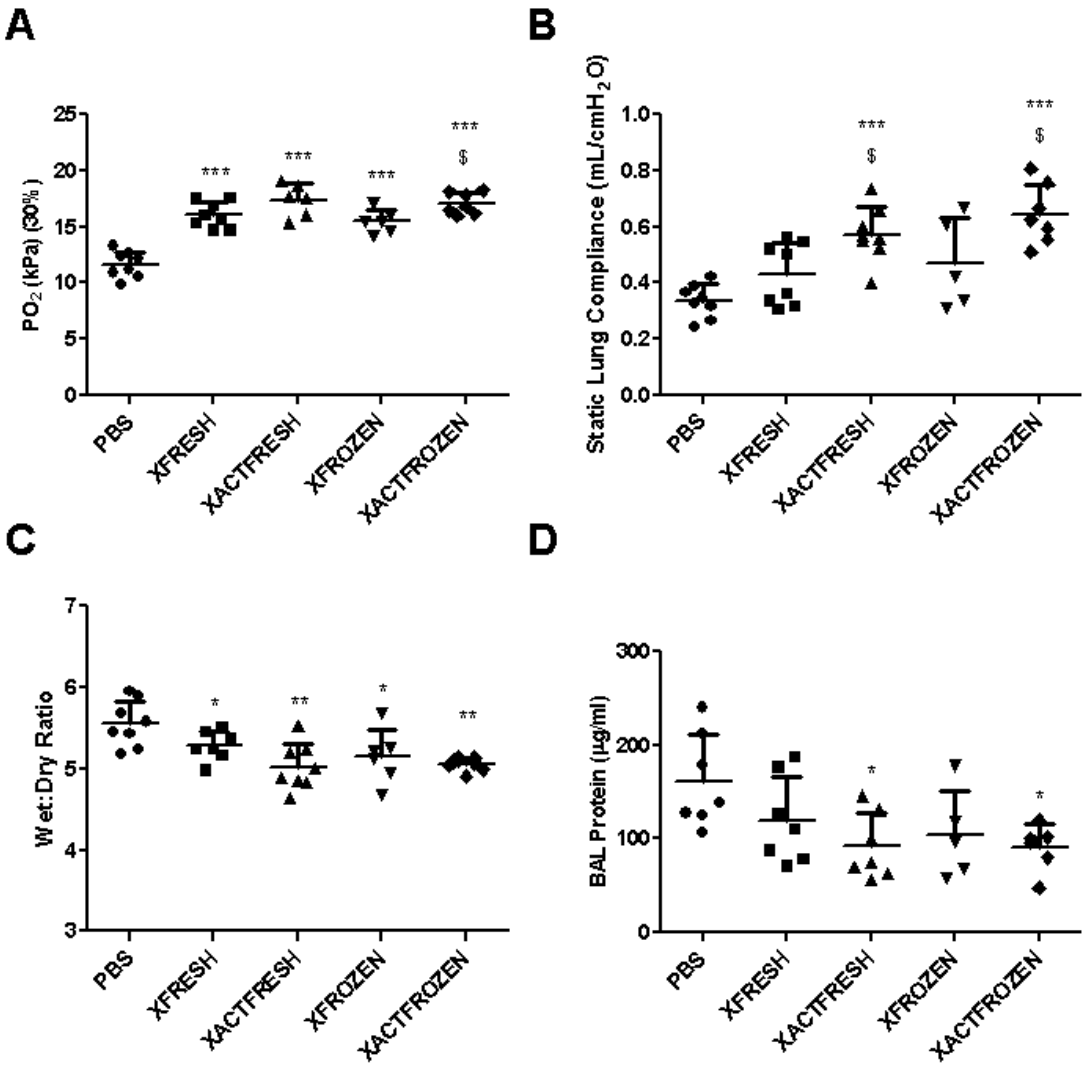

Figure 8.1: Delayed Delivery of Pre-Activated, Cryo-Preserved, XF MSCs Restores Lung Function after VILI. All MSC treatments significantly restored arterial PO_2, with pre-activated cryo-preserved cells statically better than their naïve alternatives (A). For static lung compliance, only activated fresh and activated frozen delivery showed significant restoration and were statistically different to their naïve counterparts (B). All treatments groups restored the lung wet:dry ratio (C) but only pre-activated MSC modulated BAL protein concentrations (D). ***Note***: *, **, *** = $P < 0.05$, 0.01, 0.001 versus PBS control groups and $ = $P < 0.05$ versus corresponding naïve groups.

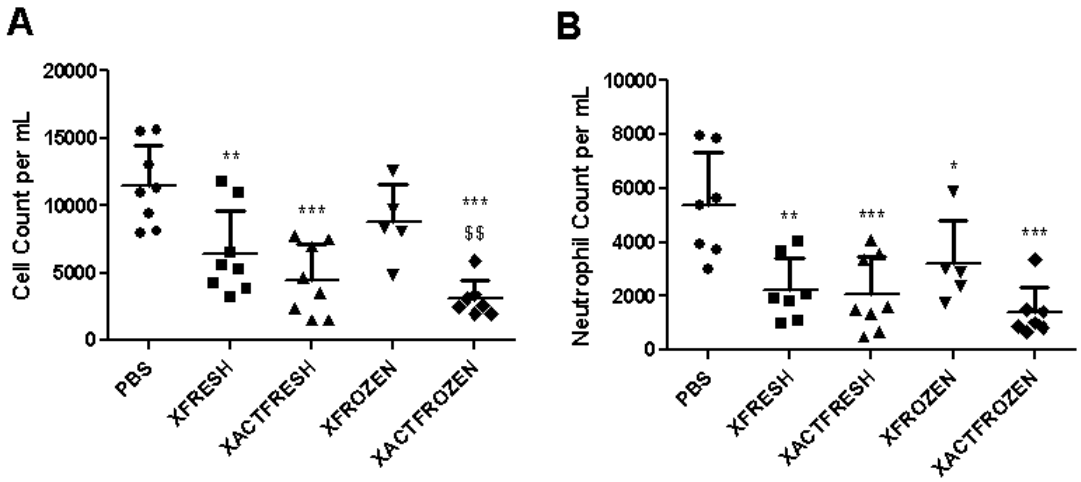

Figure 8.2: Delayed Delivery of Pre-Activated, Cryo-Preserved, XF MSCs Modulates Inflammatory Cell Influx. Both fresh and pre-activated fresh treatments significantly modulated the infiltration of cells into lung as evidenced by decreased BAL cell counts (A). Thawed cryo-preserved delivery was not different to control but pre-activation of these cells restored their efficacy (A). All treatments significantly resolved lung neutrophil infiltration (B). ***Note***: *, **, *** = $P < 0.05, 0.01, 0.001$ versus PBS control and $$ = $P < 0.01$ versus naïve frozen group.

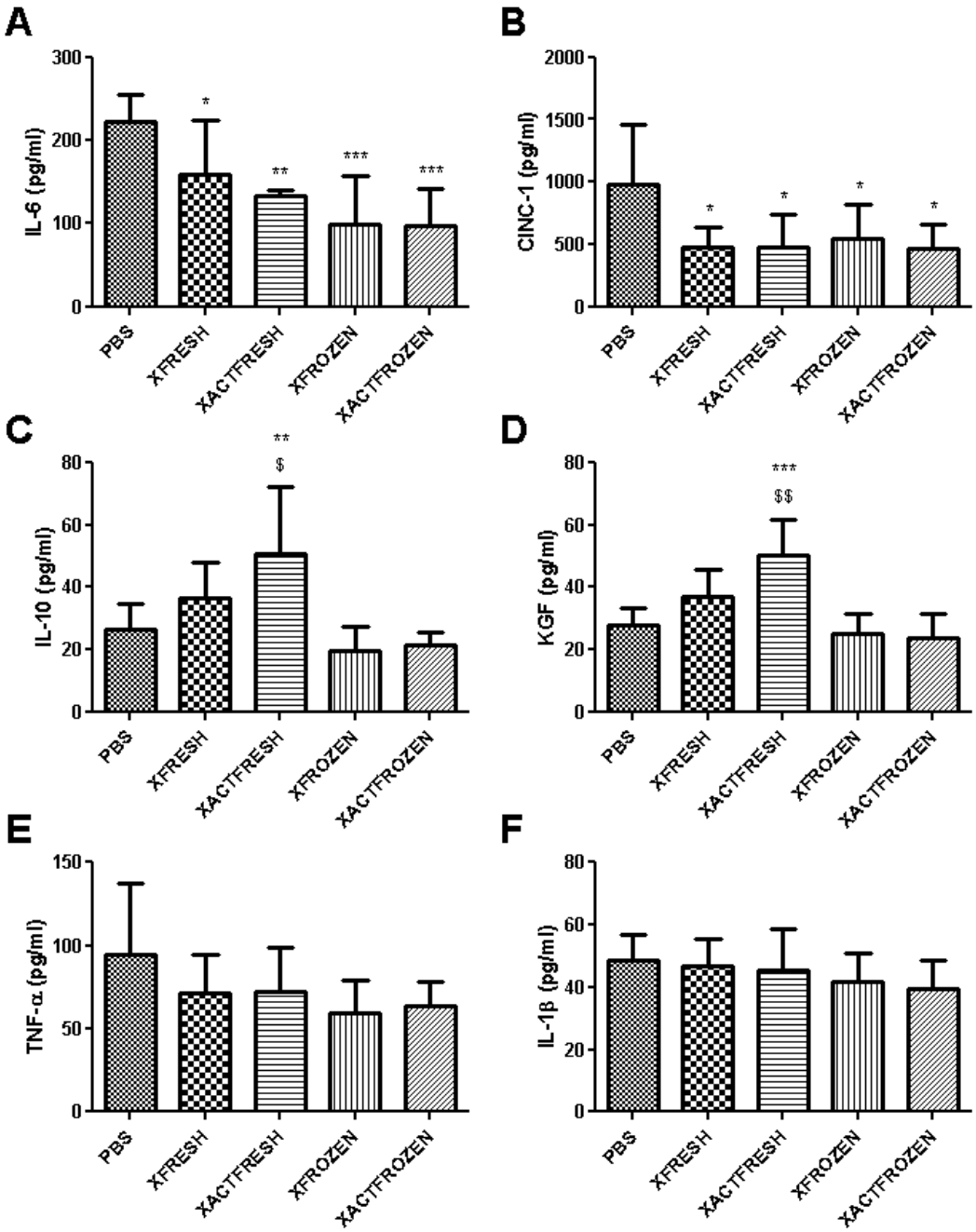

Figure 8.3: **Delayed Delivery of Pre-Activated, Cryo-Preserved, XF MSCs Modulates BAL Inflammatory Cytokines**. All treatment groups significantly decreased BAL IL-6 (A) and CINC-1 (B) concentrations. For IL-10 and KGF, fresh MSC treatment did not restore release (C + D). However pre-activated fresh MSCs showed significant recovery of IL-10 (C) and KGF (D) concentrations, and were significantly different to their naïve counterpart. Neither frozen nor activated frozen MSC delivery recovered IL-10 or KGF release (C + D). There was no significant effect of any cell treatment in BAL TNF-α or IL-1β concentrations (E + F). ***Note***: *, **, *** = $P < 0.05, 0.01, 0.001$ versus PBS control and \$, \$\$ = $P < 0.05, 0.01$ versus naïve fresh group.

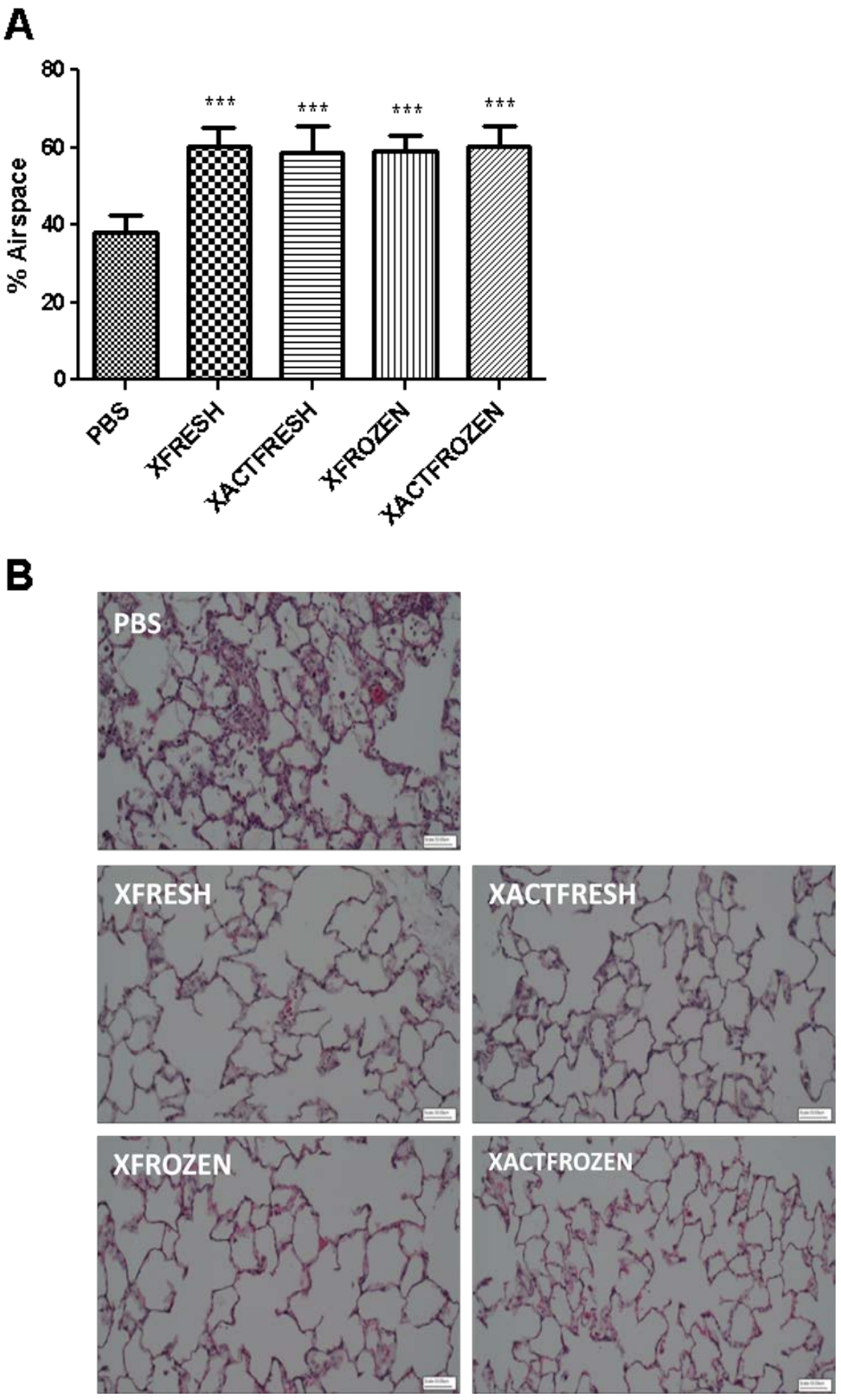

Figure 8.4: **Delayed Delivery of Pre-Activated, Cryo-Preserved, XF MSCs Restores Lung Structure**. Delayed delivery of all cell treatments significantly restored the percentage of alveolar airspace in the injured lungs (A). Representative images of lung histology sections are depicted above (B). ***Note***: *** = $P < 0.001$ versus PBS control group.

8.4 Discussion

We demonstrate for the first time that delayed delivery of thawed cryo-preserved XF MSCs can enhance lung repair following VILI. We further demonstrate that prior activation of MSCs with cytomix enhances their reparative capacity, particularly in MSCs that have undergone cryo-preservation. These findings significantly enhance the feasibility of clinical translation of MSCs for ARDS patients.

8.4.1 Pre-activated, Cryo-preserved XF MSCs Restore Lung Physiology Post VILI

Currently, MSCs in clinical studies are all cultures in media containing animal products. This raises several concerns, including the risk of disease transmission as well as batch-to-batch variability [183]. Culture of cells in XF conditions would avoid these issues, but it has been challenging to replicate the growth conditions and cell efficacy using media containing no animal products [243]. Here, we report that XF MSCs enhanced lung repair as demonstrated by the restoration of oxygenation levels when administered at a time of peak injury following VILI. In contrast, these cells failed to restore lung compliance. More importantly, pre-activation of XF MSCs with cytomix restored their reparative capacity.

FCS cultured MSCs were previously shown to improve compliance even after a 24 hour delayed delivery [90] and this may highlight possible differences between XF culture conditions on MSC efficacy. Despite results that show these cells are very similar in their proliferation and differentiation profiles [184] a direct comparison study of these cells may be further required in VILI to elucidate any potential differences in effect. This also further emphasises the need for pre-activation strategies to overcome any variability in efficacy in MSC therapy.

All cell treatments restored alveolar fluid clearance, as observed by the attenuation of fluid content in the lung, thus highlighting that cryo-preserved XF MSCs can promote lung recovery post VILI. However, only pre-activated fresh and cryo-preserved cells restored lung membrane integrity as observed by the decrease in BAL protein. FCS MSCs previously decreased BAL concentrations at this point of maximal injury [90] and

the difference here may again be attributed to XF culture or may be a result of MSC batch variability thus again further highlighting the need for pre-stimulation strategies to negate efficacy inconsistency.

8.4.2 Pre-Activated, Cryo-Preserved XF MSCs Modulate Inflammation Post VILI

Cryo-preservation is an important method for MSC cell delivery as it serves to avoid variability in culture conditions and provides more feasible delivery to the patient. We found that cryo-preserved MSCs failed to modulate the increase in BAL cell counts, which was retained with pre-activation, but did resolve neutrophil infiltration. Cryo-preservation has been alluded to as a possible modulator of MSC function [189, 244] and given that these MSCs were administered at a time of maximal injury may be possible reasons for the loss of efficacy here.

Although all cell treatments attenuated BAL inflammatory cytokine release only pre-activated fresh cell delivery restored anti-inflammatory IL-10 and pro-repair molecule KGF. This is in contrast to previous findings where FCS cultured cryo-preserved cells significantly increased the release of these molecules in an animal model of pneumonia [116]. The dissimilarity in the animal models, the time point of MSC administration or the XF culture conditions may all be possible explanations for the differences in observations here. More importantly, even pre-stimulation could not overcome this loss of effect in the cryo-preserved XF MSCs. Other pre-activation strategies may yield better outcome especially because, as mentioned previously, some studies have highlighted that cryo-preserved MSCs may possess modified immunomodulatory action [189]. Finally, it is important to note that some IL-10 and KGF values were extrapolated as they were below the detection sensitivity of the ELISA kit.

8.4.3 Pre-Activated, Cryo-Preserved XF MSCs Restore Lung Airspace

Finally, all MSC treatments significantly restored alveolar structure as observed by the recovery of alveolar airspace at a time point of maximal injury. This provides further support for the use of XF MSCs in a clinical setting and also favours the method of cryo-preservation delivery of cell treatment.

8.4.4 Conclusion

Of relevance to ARDS, pre-activation may serve as a resolution to overcome the efficacy concerns in regards batch-to-batch MSC cell variability, the effects of cryo-preservation and culture conditions. Similar successful MSC pre-activation strategies have been employed for other types of pre-clinical injury such as colitis and radiation induced injury [211, 213]. Furthermore, cryo-preservation may offer the best solution for MSC delivery in the clinic. In conclusion, pre-activation of cryo-preserved XF MSCs enabled the restoration of most functions of these cells in an animal model of VILI. Future studies should examine the mechanisms involved in the loss of function in MSCs due to cryo-preservation and other pre-activation methods to further improve efficacy.

9.0 Discussion

9.1 ARDS

9.1.1 Epidemiology, Disease Burden and the Need for Therapy

ARDS is a serious disorder of respiratory failure characterised by severe endothelial and epithelial damage and an intense inflammation, and has a high mortality rate [3, 4]. It is estimated that there are 190,000 cases of ARDS in the US every year with 74,500 of these resulting in death and they account for 3.6 million hospital days [2]. A recent study outlined that ARDS cases represent approximately 10.4% of patients in the intensive care unit and the mortality rate is 40% [3]. Furthermore, the survivors of ARDS continue to suffer from functional and cognitive deficits for up to five years after discharge and often require further hospital care, as such, the disease burden of ARDS is quite high [9]. There are currently no therapies for ARDS with many pharmacologic treatments showing little or no efficacy [63-65]. As such, there is a need for a therapy that can both modulate the immune response and enhance the repair and recovery following ARDS.

9.2 VILI

9.2.1 VILI Induced ARDS

Mechanical ventilation is a necessary measure to support life in patients in intensive care with severe respiratory failure [45]. However, high tidal volumes and pressures often required to sustain patients can exacerbate or even induce ARDS and is known as VILI [40, 45, 47]. VILI can worsen endothelial and epithelial damage by mechanical stress and also intensify the inflammatory response [4, 51, 56]. Therefore, more protective ventilator strategies, such as low tidal ventilation are now being used to overcome these issues [45].

9.2.2 The Role of Hypercapnia in Protective Ventilator Strategies

Low tidal ventilation can result in inefficient CO_2 clearance leading to the development of HCA [45, 76]. Studies have shown that HCA may play a biological role in ARDS. Specifically, there was an associated increase in the survival of patients receiving high

tidal volume ventilation with HCA [45, 76]. While it is likely that much of the protection seen in these clinical studies came from the effects of reduced mechanical stretch [45], an analysis of patients from the ARDSnet tidal volume study showed that HCA was associated with improved survival in patients that received higher tidal volumes [76]. Induced HCA has also been reported to exert beneficial effects in pre-clinical studies [20, 71]. Furthermore, the therapeutic effects of HCA have been linked to the generation of an acidosis rather than the actual rise in CO_2 [70].

9.3 ARDS and the NF-κB Pathway

9.3.1 Importance of the NF-κB Pathway in ARDS

NF-κB is a transcription factor which regulates the release of numerous pro-inflammatory molecules [23] and of importance to ARDS, the NF-κB pathway has been linked to lung inflammation and injury [15, 19, 23, 27-31]. Furthermore, studies have revealed that NF-κB mediated inflammation is upregulated in patients with ARDS and is induced by stretch injury and as such may represent a possible target for therapy [20, 31].

9.3.2 Evidence for the Role of NF-κB in mediating the Effects of HCA

Studies have shown that HCA mediates it effects by modulating the NF-κB pathway. Specifically, it has been observed that HCA inhibited the degradation of the NF-κB inhibitory protein, IκBα and enhanced the survival of rats with pneumonia injury [78]. Similarly, in a pre-clinical model of VILI, HCA was shown to be upregulate IκBα and enhance repair after this injury [20]. Conversely, *in vitro*, wound repair was shown to be inhibited by HCA mediated inhibition of NF-κB [79]. Furthermore, prolonged exposure to HCA has been observed to exacerbate pneumonia infection in another study [80]. Therefore, the specific effects of HCA on the NF-κB pathway in the context of isolated stretch injury need to be elucidated.

9.4 Mechanisms of Action of HCA on Stretch Induced Inflammation and Cell Death

This study demonstrated that HCA attenuates mechanical stretch induced injury in the pulmonary epithelium through inhibition of the canonical NF-κB pathway and by way of generating a protective acidosis.

9.4.1 HCA Inhibits Short Term, Moderate and Prolonged Mechanical Stretch Injury

HCA elicited a therapeutic effect against time dependent mechanical stretch injury induced inflammation and cell death. This indicates that, in the clinical setting, patients with or at risk for VILI may benefit from both short term and long term exposure to HCA. Other previous studies have observed differing, time dependent, efficacy with HCA. Specifically, in the context of pneumonia only short term but not long term exposure provided therapeutic efficacy against bacterial infection [73, 80]. None-the-less, in the context of VILI, our finding supports previous *in vivo* studies whereby HCA attenuated both moderate and severe VILI in rats [20]. Furthermore, as discussed above, in the clinical setting, in patients that received injurious higher tidal volume ventilation, HCA was associated with increased survival [45, 76].

9.4.2 IκBα Over-Expression 'Occludes' the Effects of HCA

HCA significantly attenuated NF-κB activation and inflammatory cytokine IL-8 release as induced by mechanical stretch injury. Furthermore, HCA significantly improved cell viability and retained epithelial membrane integrity after stretch injury. Of importance, this study also demonstrated that when IκBα, a potent inhibitor of NF-κB signalling, was over-expressed these effects of HCA were occluded. Therefore this study demonstrates that the mechanism by which HCA potentiates its therapeutic efficacy against stretch induced inflammation and cell death in the pulmonary epithelium is by inhibition of the canonical NF-κB signalling pathway. Another study found that HCA protected human pulmonary artery endothelial cells from endotoxin LPS injury by inhibiting IκBα degradation which then prevented NF-κB activation [227]. Furthermore, *in vivo* studies have demonstrated that IκBα inactivation by the IKK complex is

ameliorated by HCA following sepsis in an IT *E.coli* inoculation model [78] and that IκBα was upregulated in the cytoplasm after HCA treatment in rats with VILI [20]. The findings of this study further support the overall conclusion that HCA inhibits canonical NF-κB induction by way of inhibiting IκBα degradation after *in vitro* and *in vivo* high stretch and sepsis injuries. As such, in the context of VILI, HCA is a promising therapy for ARDS treatment with the potential to alleviate the inflammatory response.

9.4.3 The Therapeutic Efficacy of HCA is mediated by the Acidosis

HCA and acidosis conditions (at a pH comparable to that observed in patients) significantly attenuated stretch induced NF-κB induction and IL-8 release, as well the associated cell death and membrane damage. Furthermore, when the HCA treatment was buffered, the therapeutic efficacy against stretch induced inflammation and cell death was abrogated. This demonstrates that HCA efficacy is mediated by the decrease in pH or acidosis rather than the rise in CO_2 in the setting of stretch injury. Previous *in vivo* studies also showed that BHA failed to protect against sepsis induced lung injury and ischemia-reperfusion lung injury [69, 70]. Interestingly, in an endotoxin injured endothelium *in vitro* model, the protective effects and inhibition of NF-κB was mediated by the BHA [227]. Therefore, the effects of pH and CO_2 as mediated by HCA may differ depending on the type of injury and cell type used i.e. LPS injury versus high stretch injury and endothelium versus epithelium. Furthermore, the mechanisms by which extracellular pH affects cell function are largely unknown [245] and require further evaluation. In a clinical setting it has been noted that survival was enhanced in patients with severe ARDS that received low tidal ventilation and HCA treatment without buffering [67]. Conversely, buffering was permitted in the ARDSnet study, and HCA was not severe in the protective ventilation group, yet they also did better [45, 76].

9.4.4 Limitations and Future Directions

There were some limitations in this study which are as follows: 1) the use of the A549 cell line is limiting as these cells lack some features of AEC cells such as the formation of tight junctions and surfactant production. However, they are a robust, commonly used and accepted model for *in vitro* assessment of epithelial injury [246]. Furthermore, the

data obtained in A549 cells in this study has been confirmed in primary cells, the results of which can be found in the publication by Horie *et al*., 2016 [216]. 2) There was no positive control for the LDH assays so the data for total cell membrane damage i.e. the LDH released after complete cell lysis or necrosis was not available for comparative purposes. 3) The use of non-stretched cells as controls for these experiments was based on the study finding that physiologic stretch did not produce any evidence of cell inflammation or death. 4) Furthermore the study used severe HCA (15% CO_2) but the effects of less severe HCA were not investigated.

9.4.5 Conclusion

This study demonstrated that HCA is protective against high mechanical stretch injury by producing a protective acidosis and through mechanisms which inhibit inflammatory NF-κB activation. This finding proves a useful insight into the mechanisms of action of HCA, and the pivotal role of inhibition of NF-κB in mediating its effects, which in this case were shown to be protective. These anti-inflammatory effects however may be deleterious in wound healing or prolonged pneumonia.

9.5 MSCS for ARDS – Promise and Pitfalls

9.5.1 Therapeutic Promise from Pre-Clinical ARDS Studies

Interest in the use of MSCs for ARDS arose from the finding that these cells have the potential not only to modify the immune response but also to promote repair after injury (previously reviewed [96]). In fact, numerous pre-clinical models of ARDS have demonstrated MSC efficacy and these cells are currently under investigation in phase 1 safety trials for ARDS [90, 91, 116, 123].

9.5.2 Key Translational Knowledge Gaps

There are still quite a few unanswered questions and barriers to the clinical translation of MSCs for ARDS. These include (1) the need to further elucidate their mechanism of action, including that of their secretome, to provide an enhanced MSC therapy, (2) the need for a more defined and less variable population of MSCs, (3) the need for

alternative sources of MSCs that are more readily available, (4) the need for a XF MSC product and (5) the need for more efficient MSC delivery to the clinic.

9.6 MSC Paracrine Mechanism of Action

The MSC secretome may provide an alternative therapy to the MSC cell in the clinic particularly because the mechanism of action of MSCs is partly mediated by the release of soluble anti-inflammatory and pro-repair molecules in their secretome [102, 106, 151]. However, previous studies have shown variable and conflicting efficacy issues with the secretome *in vivo* and show that MSC efficacy may not be fully recapitulated in the secretome [89, 116, 124, 128]. As such, further investigation is required into the MSC secretome efficacy in the context of ARDS injury.

9.6.1 The MSC Secretome Protects Against Oxidative Stress Induced Cell Death

This study demonstrated that the MSC secretome, termed CM, significantly enhanced cell viability following H_2O_2 mediated cell death. Previous evidence shows that MSC cell and CM treatment also provides therapy against oxidative stress *in vitro* to save different cell types including fibroblasts [228, 229]. This demonstrates that the secretome of MSCs recapitulates the effects seen with that of the cell treatment and further indicates that MSC mediated therapy is caused partly by paracrine mechanisms. The relevance of this finding to ARDS stems from the evidence that shows oxidative stress induced cell death is a major contributor to ARDS pathogenesis [17, 57].

9.6.2 The MSC Secretome Protects Against Cytokine Induced Inflammation

This study also demonstrated that the MSC secretome attenuated IL-1β induced NF-κB induction and IL-8 release. As multiple repeat experiments could not be pooled, this effect can only be discussed as a trend. None the less, previous studies have shown that MSC cell treatment can also inhibit NF-κB activation as induced by TNF-stimulation *in vitro* [231] and LPS challenge *in vivo* [247] and highlights that in this study the effects of MSCs may be recapitulated by the secretome.

9.6.3 The MSC Secretome Promotes Wound Healing

This study showed that the MSC secretome significantly improved wound repair, an important mechanistic demonstration. This effect has also been previously observed with the secretome [123] and cell treatment [248] and as such further suggests that the therapeutic effects of MSCs are mediated by paracrine mechanisms of action. Injury to the endothelium and epithelial barriers significantly contributes to ARDS disease progression [4, 5, 57] and the findings of this study further support the use of MSC treatment for ARDS.

9.6.4 The MSC Secretome Attenuates Mechanical Cyclic Stretch Injury

Finally, the MSC secretome significantly abrogated mechanical stretch induced NF-κB activation and IL-8 release as well the drop in cell viability and increase in membrane damage. *In vivo,* studies have shown that the MSC secretome attenuated inflammation and promoted recovery post VILI [89, 123]. This provides further support for the use of MSCs against stretch injury or VILI, which are prominent contributors to ARDS pathogenesis [56, 57, 225]. The secretome from XF expanded MSCs also inhibited inflammation (NF-κB activation and IL-8 release) and cell injury as mediated by high injurious stretch. This is a new finding in the context of stretch injury that requires further evaluation *in vitro* and *in vivo* but none-the-less shows promise for the clinical use of XF MSC therapy.

9.6.5 Limitations and Future Directions

This study used the A549 cell line for obtaining data and as mentioned before this cell line is limited and the results obtained should be confirmed in primary cells. Furthermore, the MSC secretome in this study was not directly compared to the cell treatment and future studies should address this. Future studies should also investigate possible further mechanisms and the potential role of soluble molecules in the MSC secretome that may have contributed to the observed MSC CM efficacy, particularly because it has been observed previously that depletion of KGF abrogated the efficacy of the MSC secretome to promote wound repair *in vitro* [123].

9.6.6 Conclusion

This study demonstrated that the MSC secretome preserved therapeutic efficacy in *in vitro* settings of epithelial alveolar lung injury, further supporting the theory of MSC paracrine mechanisms of action.

9.7 Pre-Activation of MSCs to Enhance Secretome Efficacy

Recent evidence shows that pre-activation of MSCs with cytomix enhances their therapeutic effects but this has been fully investigated in the context of ARDS [152]. As such, this study demonstrated that pre-activation of MSCs with IL-1β/IFN-γ/cytomix showed a trend for enhancement of the therapeutic efficacy of the secretome in protecting the pulmonary alveolar epithelium from injury.

9.7.1 Pre-Activation of MSCs Enhances the Therapeutic Efficacy of the Secretome to Attenuate Injury *In Vitro*

The study showed that CM from IL-1β pre-activated MSCs inhibited NF-κB activation and IL-8 release more so than the naïve CM, while IFN-γ pre-stimulation only showed inhibition of NF-κB. Evidence shows that individual activators alter MSCs differentially [212], which could explain the differences observed here and hence justifies the use of cytomix in the later studies. It must be noted that multiple repeat experiments of this could not be pooled so the results observed here can only be discussed as trends. None-the-less a previous study showed that inflammatory injury, which caused protein hyper-permeability in rat AECs, was more strongly ameliorated by MSC CM from hypoxic and cytomix pre-activated MSCs [152]. This study provides support for the use of pre-conditioning strategies to inhibit inflammation in the form of NF-κB and IL-8 as induced by IL-1β, which are all common agonists of ARDS inflammation [15, 19] but requires further investigation.

Interestingly the study showed a trend whereby there was no further enhancement on wound healing by pre-activation with IL-1β and TNF-α, while IFN-γ pre-stimulation seemed to inhibit the reparative effect of the secretome. Again, previous evidence has shown that these activators all modify MSCs differentially [212, 232] and may be a

possible explanation for the effects observed and further supports the use of cytomix for stimulation of MSCs.

Finally this study demonstrated a trend whereby pre-activation with cytomix seemed to further enhance the effects of the MSC secretome in attenuating inflammation and cell death following high mechanical stretch injury. This finding may be important because previously the MSC secretome was observed to be less efficacious against VILI when assessed 4 hours after VILI induction [128], a time point where the inflammatory response is progressing to its most severe/potent phase at 6 hours post injury [51]. As such, pre-activation may potentially improve the therapeutic efficacy of MSCs and that of the MSC secretome for ARDS, and requires further evaluation.

9.7.2 Conclusion

This study provides evidence that the MSC secretome can mimic the cell treatment effects and may be a viable alternative. Furthermore, pre-activation strategies may provide a more enhanced therapy and of relevance to ARDS could prove an efficient way to overcome efficacy issues with MSC treatment. This strategy could also potentially reduce the number of MSCs required for efficacy in patients with ARDS.

9.8 Defined MSC Subpopulations

MSCs represent a heterogeneous population of cells with variable potential and effects [97, 162, 163] therefore it is important to use a more defined population of cells which may eliminate this variability. Our study demonstrated that a UC derived $S2^{+}$ subpopulation of MSCs, named Cyndacel-C was comparable to standard heterogeneous BM MSCs in enhancing recovery following VILI.

9.8.1 MSCs Restore Lung Physiology after VILI

High pressure injurious ventilation significantly impaired lung oxygenation and compliance, initiated inflammatory cell infiltration into the lung and a subsequent increase in pro-inflammatory cytokines, and increased the lung injury score as well as pulmonary permeability. These findings confirm those previously published [51] and

further support the use of this injury model as an appropriate pre-clinical model of ARDS [4, 49, 51]. This study also demonstrated that human heterogeneous BM derived MSCs enhanced recovery after VILI, as evidenced by the improved oxygenation and lung compliance in comparison to the control group. This finding confirms numerous previous studies which have shown therapeutic efficacy with MSC treatment in *in vivo* models including VILI and pneumonia [90, 116]. Human UC derived $S2^+$ MSCs also recovered the decrement in lung oxygenation but not lung compliance. A possible explanation may be due to the difference in cell source and selected subpopulation, particularly because previous evidence has shown that MSCs derived from different sources and MSCs of different subpopulations have diverse differentiation, proliferation and immunomodulatory profiles [97, 162, 163, 182, 236].

9.8.2 MSCs Restore Lung Barrier Function and Structural Integrity after VILI

Protein concentrations were increased in the lung after VILI but these were markedly recovered by both MSC treatments and provide support for their efficacy to inhibit lung barrier dysfunction. Elevated levels of BAL protein contributes to poor outcome in ARDS patients [13] and as such this study provides evidence of the potential therapeutic efficacy of both heterogeneous and a selected subpopulation of MSCs for this disease. However, alveolar fluid accumulation as a result of membrane barrier dysfunction in this study was not recovered by either MSC treatment. For BM MSCs, this is a contrasting observation to previous reports [90] and suggests evidence of batch-to-batch variability, and in relation to the UC $S2^+$ MSCs further *in vivo* investigation in VILI and other pre-clinical ARDS models is required to elucidate their complete functions. Finally, both cell treatments restored alveolar airspace, as evidenced by the reduction in the histologic injury score, in a comparable manner. This confirms that there was lung repair after VILI, and is a new finding for UC $S2^+$ MSCs which further supports their potential for translation to a clinical setting.

9.8.3 MSCs Modulate Inflammation during Recovery from VILI

Both BM MSCs and UC $S2^+$ MSCs modulated the inflammatory response as mediated by cell infiltration into the lung. Furthermore, both cell groups attenuated pro-

inflammatory IL-1β and TNF-α release and restored anti-inflammatory IL-10 secretion. This efficacy has been previously established with BM MSCs [89, 128] but is a new finding in regards UC $S2^+$ MSCs. However, IL-10 release was significantly higher in the BM MSC group compared to the UC $S2^+$ MSCs and only the BM MSC group restored KGF concentrations (previously reported for BM MSCs [90]) which highlights the differences in these groups and can possibly be explained by their tissue source and population. None-the-less, the study demonstrates that UC $S2^+$ MSCs are a potential defined immunomodulatory therapy for ARDS in the clinical setting.

9.8.4 Limitations and Future Directions

There was inconsistency in this study in regards to the efficacy of BM MSCs to those reports previously published but again may be attributed to cell batch variability. Furthermore, there was no heterogeneous UC derived MSC cell control due to logistical issues and as such future studies would need to include this group as a comparator to the $S2^+$ subpopulation. Finally, other pre-clinical investigations such as, pneumonia induced lung injury, are required to fully elucidate the therapeutic efficacy of this selected subpopulation.

9.8.5 Conclusion

This study demonstrated that high pressure ventilation significantly inured the lungs of rats by inducing inflammation and damage and is a relevant pre-clinical model of injury recovery following ARDS. Furthermore, MSC cell treatment caused significant recovery from lung injury and resolution of the indices of inflammation associated with VILI, while an MSC subpopulation positive for S2 also showed comparable efficacy. Overall this provides support for the use of a more defined population of MSCs in clinical ARDS

9.9 Sources of MSCs

MSCs are most commonly sourced from the BM [97], which requires BM biopsy, an invasive and potentially risky procedure, making these cells less readily available. Therefore, alternative, more accessible sources of MSCs such as the UC [97] would be

more efficient and less costly particularly because it is considered a waste product. However, numerous pre-clinical studies will need to determine any efficacy variability between differently sourced MSCs in the context of ARDS.

9.9.1 BM and UC Derived MSCs Comparably Restore Lung Physiology after VILI

BM and UC derived MSCs restored arterial oxygenation and lung compliance with similar efficacy and is a new finding in the context of VILI. Previous reports have also shown efficacy with UC MSC treatment in hyperoxic and LPS induced lung injury [107, 240]. This study therefore provides further support for the use of UC MSC therapy in ARDS. Furthermore, previous reports have also outlined that UC derived MSCs proliferate faster *in vitro* [97] and as they are a more readily available source of MSCs, they may be a more ideal candidate for use in the clinical setting.

9.9.2 BM and UC Derived MSCs Comparably Recover Lung Function after VILI

Alveolar membrane integrity and fluid clearance was significantly restored by MSC treatments from both sources of tissue. This provides further evidence that MSC treatment from either source enhances repair and the resolution of damage after VILI. Again, UC MSC treatment has also been shown to perform with similar efficacy in other lung injury models including pneumonia [240, 249] and this further supports their use for ARDS arising from different aetiologies of injury to the lung [4]. Finally, with similar efficacy, both cell treatments restored alveolar airspace, as demonstrated by the attenuated histologic injury score, a finding which correlates with previous studies using other types of lung injury models [116, 240, 249] but this is the first study to directly compare these two sources of MSCs in the setting of VILI.

9.9.3 BM and UC Derived MSCs Comparably Modulate Inflammation after VILI

Cell infiltration into the lung was significantly resolved by both BM and UC MSC cell treatments with similar efficacy, thus providing evidence that both MSC sources are capable of modifying the inflammatory response post VILI. Again, previous reports have demonstrated this immunomodulatory effect with UC MSCs in other types of lung injury [240] but none have directly compared them to BM derived MSCs. Both cell

treatments also alleviated pro-inflammatory IL-1β levels in the BAL and restored pro-repair KGF concentrations with comparable efficacy. However, only BM MSCs attenuated IL-6 levels. UC MSCs have been previously shown to ameliorate IL-6 in LPS and *E.coli* pneumonia lung injury [240, 249] and this may highlight that UC MSCs behave differently in VILI or this perhaps could be due to MSC donor or batch variability. None-the-less, both of these cell sources provided immunomodulatory effects *in vivo* and have potential for therapy in the ARDS patient.

9.9.4 Limitations and Future Studies

MSC therapy was administered directly after VILI and as such may not be an accurate representation of the events that unfold in the clinical setting, therefore future studies would need to determine the therapeutic window of UC derived MSCs.

9.9.5 Conclusion

This study demonstrated that both BM and UC derived MSCs were comparable in efficacy in restoring lung physiology, modulating inflammation and recovering damage after VILI. Therefore, the use of more readily available sources of MSCs such as those derived from the UC may be a viable option for use in the clinical setting of ARDS.

9.10 Cryo-Preservation of MSCs

Constant fresh MSC culture for doses in the clinic is expensive and can introduce variability in the MSC product, which cryo-preserved MSC delivery would avoid. However, pre-clinical studies of the effects of cryo-preservation on MSC efficacy are also required. As such, this study demonstrated that, thawed cryo-preserved UC MSCs were comparable in efficacy to their fresh BM and UC counterparts, in enhancing recovery post VILI.

9.10.1 UC Derived MSCs Retain Therapeutic Efficacy in VILI after Cryo-Preservation

Thawed cryo-preserved UC MSCs retained their therapeutic effect and restored lung oxygenation and lung compliance with similar efficacy to freshly delivered UC cells.

Previously, cryo-preserved BM MSCs were shown to attenuate the drop in oxygenation and compliance following pneumonia injury [116] but this has not been investigated with UC cells. Thus the findings of this study provide reassurance for the use of cryo-preserved MSC delivery, especially in light of the fact that previous studies have reported alterations in cell function due to cryo-preservation [188, 189]. Cryo-preservation also did not alter the ability of UC MSCs to restore alveolar membrane integrity and alveolar airspace as evidenced by the attenuation of BAL protein concentrations, pulmonary oedema and histologic injury. Cryo-preservation also did not impede the efficacy of BM MSCs to attenuate these parameters in a pneumonia injury model [116]. This further supports the use of cryo-preserved MSC treatments for translational purposes.

Finally, in a comparable manner to freshly delivered UC MSCs, thawed cryo-preserved cells resolved cell infiltration into the lung, further supporting their immunomodulatory function. Again, with similar efficacy to freshly delivered MSCs, thawed cryo-preserved UC MSCs attenuated the increased IL-1β concentrations as induced by VILI and restored the release of KGF in the BAL. Cryo-preserved UC MSCs did however significantly inhibit the release of IL-6 which their fresh counterparts did not, and this highlights the variability in fresh harvest techniques which can be alleviated if all cells are cultured, harvested and cryo-preserved at the same time. Of note, cryo-preservation also did not negate the ability of BM MSCs to attenuate pro-inflammatory and enhance anti-inflammatory molecule release in a pneumonia model [116]. Therefore, this study provides further support for the use of cryo-preserved MSC treatments which can retain their immunomodulatory functions.

9.10.2 Limitations and Future Directions

This study administered both freshly harvested and cryo-preserved MSCs 15 minutes after VILI induction. To evaluate their efficacy, future studies should investigate the effects of delayed administration (therapeutic window) of cryo-preserved MSC therapy, especially because a previous report in a VILI study showed that maximal injury occurs

6 hours post VILI induction [51] and this may be a more relevant model for assessing potential MSC efficacy for delivery to the ARDS patient.

9.10.3 Conclusion

The study confirmed that cryo-preservation did not cause a loss of therapeutic efficacy in this injury and repair model and signifies an important finding for translational research whereby MSC treatment can potentially be more efficient and less variable with thawed cryo-preserved delivery.

9.11 Xenogeneic-Free Culture

MSCs are usually cultured in medium with FCS i.e. medium that contains animal derived products, which introduces variability due to batch differences in the serum and the threat of disease transmission [183, 184]. Therefore, it is important to investigate the use of MSCs cultured in XF conditions which would eliminate these concerns. However, pre-clinical testing is required to elucidate any potential efficacy issues with MSCs in XF culture before these cells can be used in the clinic. Furthermore, these XF MSCs would also need to be delivered as cryo-preserved cells and this also requires pre-clinical evaluation. Our study demonstrated that XF expanded MSCs overall enhanced recovery and resolved lung damage after VILI, with some loss of efficacy after cryo-preservation.

9.11.1 Cryo-Preserved, XF MSCs Enhance Recovery following VILI

This study demonstrated that fresh XF MSCs significantly recovered lung oxygenation at a time point of maximal injury after VILI, a new and significant finding. However, XF MSCs failed to restore static lung compliance post VILI. Previous reports have shown that delayed delivery of FCS cultured MSCs improves both of these parameters [90] and as such demonstrates possible differences between XF and FCS culture. Previously, we have shown *in vitro*, that XF and FCS cultured MSCs improved high stretch injury with similar efficacy but while the cells used *in vitro* and *in vivo* came from the same donor, they were from different batches and this may explain the variability here. Cryo-preserved MSCs also failed to recover the drop in compliance, but

this was probably not due to the effect of cryo-preservation for the reasons outlined above with the freshly delivered MSCs.

9.11.2 Cryo-Preserved, XF MSCs Modulate Inflammation following VILI

Delayed delivery of fresh XF MSCs significantly modulated the inflammatory response, as evidenced by the resolution of cell infiltration into the lung. Previously, delivery of fresh FCS cultured cells was also shown to attenuate these parameters at this time of maximal injury [90] and therefore this study shows that XF conditions do not alter the MSC's ability to modulate inflammation in the lung. However, cryo-preserved MSCs failed to attenuate neutrophil influx, and similar results were observed for cryo-preserved FCS cultured MSCs in an animal model of pneumonia [116]. Therefore, cryo-preservation may hinder some immune functions of MSC cells, especially as previous studies have observed that cryo-preserved cells do not respond to IFN-γ stimulation and this alters their ability to produce immunomodulatory IDO associated functions [189].

In regards to the pro-inflammatory cytokine release, both fresh and cryo-preserved MSCs attenuated IL-6 and CINC-1 concentrations in the BAL. Thus, thawed cryo-preserved cells maintained efficacy here, even at later time points of administration after injury. Furthermore, cryo-preserved FCS propagated MSCs were also shown to attenuate IL-6 release in an animal model of pneumonia [116] which suggests that XF and FCS cultured MSCs are not different in this context. Finally, neither fresh nor thawed cryo-preserved MSC delivery restored IL-10 or KGF concentrations in the BAL and this contrasts with a previous study where thawed cryo-preserved FCS cultured MSCs did significantly increase the release of these molecules in a pneumonia model of ARDS [116]. The use of XF media and the difference in model may account for the differences observed here.

9.11.3 Cryo-Preserved, XF MSCs Promote Resolution of Damage Post VILI

Neither fresh nor cryo-preserved MSCs enhanced the recovery from lung damage as assessed by protein influx into the lungs. Again, delayed delivery of fresh, FCS cultured MSCs, has previously been shown to alleviate BAL protein after ventilator injury [90]

and thus highlights that the differences observed here may be attributed to the XF culture conditions and not the effects of cryo-preservation. As such, a direct comparison study of XF and FCS MSCs may be needed to elucidate actual differences. Other factors such as MSC batch variability or donor variability between these two studies may also be accountable for the differences observed here.

In contrast, the study demonstrated that fresh and thawed cryo-preserved XF MSCs all significantly recovered the development of fluid retention in the lung and restored alveolar airspace, a new important finding for XF cultured MSCs. Previously, a study showed that delayed delivery of FCS cultured MSCs did not restore alveolar fluid clearance at this time-point [90] but again the difference here may be due to MSC variability. Of importance, this study shows that delayed delivery of XF MSC treatment even at a time of maximal injury can recover the lungs from damage post VILI.

9.12 Pre-Activation of MSCs

There are concerns regarding MSC efficacy, particularly in relation to the variable MSC effects at different phases of injury/recovery process, MSC batch and donor variability and the effects of cryo-preservation [90, 116, 128, 161, 163, 189, 250]. However, pre-activation strategies may enhance MSC efficacy and resolve these concerns. As such, this study demonstrated that pre-activation with cytomix enhances the efficacy of cryo-preserved, XF expanded MSCs in lung repair following delivery that was both delayed and at a time point of maximal ventilator injury.

9.12.1 Pre-Activation Restores the Ability of Cryo-Preserved, XF MSCs to Promote Recovery, Modulate Inflammation and Resolve Lung Damage Post VILI

Pre-activation of fresh and cryo-preserved XF MSCs caused no further enhancement in the ability of these cells to restore oxygenation but pre-activation with cytomix did restore the ability of XF MSCs to recover static lung compliance. This is a new finding, which shows promise for the use of pre-activation strategies to limit many possible efficacy issues that may be observed in the clinical setting. Both fresh and thawed, cryo-preserved XF MSCs failed to modulate protein influx which highlights the concern

about the variability of XF MSC efficacy, however, this study demonstrated, for the first time, that pre-activation restored the efficacy of both fresh and cryo-preserved MSCs in recovering membrane integrity by attenuating protein levels in the BAL. Once again, pre-activation is a promising tool that may be used to enhance MSC efficacy.

Furthermore, pre-activation restored the ability of cryo-preserved cells to resolve neutrophil infiltration in the BAL. This provides further evidence for the use of pre-activation strategies that could ensure the efficacy of MSCs is not lost after cryo-preservation. Finally, pre-activation restored the ability of fresh XF MSCs, but not the thawed cryo-preserved XF MSCs, to restore IL-10 and KGF concentrations. Therefore, in this context, cryo-preservation may significantly alter MSC immunomodulatory function and other pre-activation methods may prove more successful to overcome this issue.

9.12.2 Limitations and Future Directions

The study did not directly compare XF and FCS cultured MSCs but despite this XF cultured cells proved efficacious in enhancing recovery and repair post VILI. Future studies should investigate the mechanisms behind the loss of some of the functions of MSCs after cryo-preservation and investigate other pre-activation methods that may resolve the loss of efficacy due to cryo-preservation.

9.12.3 Conclusion

MS therapy delivery to the patient in the clinical setting may not always be possible directly after injury and as such the therapeutic window of MSCs needs to be elucidated. This study demonstrated that XF MSCs retained their therapeutic efficacy even when delivered at a later time point post injury, more importantly, a time point of maximal ventilator injury (previously reported [51]). However, some efficacy was lost when these cells were cryo-preserved, but function was restored with pre-activation. As such, this study provides evidence that XF MSC effects are enhanced by pre-activation strategies and shows promise for the delivery of XF, therapeutic, enhanced and cryo-preserved cells to more variable clinical realities of ARDS.

9.13 Future Directions – MSC Therapy for ARDS

As mentioned previously, ARDS is rarely encountered without comorbidities and as such pre-clinical models need to be more representative of the condition with the use, for example, of dual-hit models of ARDS, to truly evaluate MSC efficacy. None-the-less, MSCs have shown huge promise for use in ARDS. One main issue with MSC use is the batch-to-batch and donor variability that can be encountered. As such potency assays are needed to overcome this issue. Pre-activation strategies may also serve to alleviate MSC variability but this also requires further studies. It also appears that cryo-preservation does modestly affect MSC efficacy and further studies are required to elucidate the mechanisms at play. Furthermore, other pre-activation strategies should be investigated and may overcome the efficacy issues associated with cryo-preservation.

10.0 References

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11.0 Publications

11.1 List of Original Research Articles

Shahd Horie, Bilal Ansari, Claire Masterson, James Devaney, Michael Scully, Daniel O'Toole, John G. Laffey. *Hypercapnic acidosis attenuates pulmonary epithelial stretch-induced injury via inhibition of the canonical NF-κB pathway.* Intensive Care Med Exp 2016 Dec 22;4(1). Epub 2016 Mar 22

Claire Masterson, Daniel O'Toole, Annemarie Leo, Patricia McHale, Shahd Horie, James Devaney, John G Laffey. *Effects and Mechanisms by Which Hypercapnic Acidosis Inhibits Sepsis-Induced Canonical Nuclear Factor-κB Signalling in the Lung*. Crit Care Med 2015 Oct 26. Epub 2015 Oct 26.

James Devaney, Shahd Horie, Claire Masterson, Steve Elliman, Frank Barry, Timothy O'Brien, Gerard F Curley, Daniel O'Toole, John G Laffey. *Human mesenchymal stromal cells decrease the severity of acute lung injury induced by E. coli in the rat.* Thorax 2015 Jul 18;70(7):625-35. Epub 2015 May 18

11.2 List of Review Articles

Shahd Horie and John G. Laffey. *Recent insights: mesenchymal stromal/stem cell therapy for acute respiratory distress syndrome*. F1000Research 2016, 5(F1000 Faculty Rev):1532

Shahd Horie, Claire Masterson, James Devaney, John G Laffey. *Stem cell therapy for acute respiratory distress syndrome: a promising future?* Current Opinion in Critical Care 2016 Feb;22(1):14-20

Shahd Horie, Gerard F Curley, John G Laffey. *What's new in cell therapies in ARDS?* Intensive Care Medicine 2016 May 1;42(5):779-82. Epub 2015 Dec 1.

12.0 Presentations

12.1 List of Oral Presentations

American Thoracic Society Conference May 2016

Oral Presentation Abstract Title: *Enhancing the Efficacy of Mesenchymal Stromal Cell Therapy for ARDS*. Shahd Horie, Daniel O'Toole, John G. Laffey. Publication Date: 2016

American Thoracic Society Conference May 2014

Oral Presentation Abstract Title: *Optimal Dose and Mechanism of Action of Human Mesenchymal Stromal Cell Therapy in E.coli Induced Pneumonia in the Rat*. James Devaney, Shahd Horie, Gerard Curley, Michael Scully, Daniel O'Toole, John G. Laffey. Publication Date: 2014

12.2 List of Poster Presentations

American Thoracic Society Conference May 2016

Poster Presentation Abstract Title: *LPS-Activated Conditioned Medium from Human Mesenchymal Stromal Cells Ameliorates E.coli Induced Pneumonia in the Rat*. James Devaney, Shahd Horie, Joshua Chao, Steve Elliman, Daniel O'Toole, Ronan MacLoughlin, John G. Laffey. Publication Date: 2016

Poster Presentation Abstract Title: *Human Mesenchymal Stromal Cell Derived Exosomes and Conditioned Medium are Nebulisable by Aerogen Nebulizer and Retain Anti-Inflammatory Properties*. James Devaney, Shahd Horie, Emma Horan, Joshua Chao, Steve Elliman, Daniel O'Toole, Ronan MacLoughlin, John G. Laffey, Ronan MacLoughlin. Publication Date: 2016

American Thoracic Society Conference May 2015

Poster Presentation Abstract Title: *Activation of Mesenchymal Stromal Cells Enhances the Protective Effects of Its Secretome on the Pulmonary Epithelium*. Shahd Horie, Daniel O'Toole, John G. Laffey. Publication Date: 2015

Poster Presentation Abstract Title: *Fresh versus Frozen Human Mesenchymal Stromal Cell Therapy and the Effect of Passage in an E.coli Induced Pneumonia Rat Model*. James Devaney, Shahd Horie, Gerard Curley, Michael Scully, Daniel O'Toole, John G. Laffey. Publication Date: 2015

Stem Cell Galway Conference October 2014

Poster Presentation Abstract Title: *Conditioned Medium from Human Mesenchymal Stromal Cells Attenuates Injury and Promotes Repair In Vitro*. Shahd Horie, Daniel O'Toole, John G. Laffey. Publication Date: 2014

American Thoracic Society Conference May 2014

Poster Presentation Abstract Title: *Assessing the Therapeutic Potential of Human Mesenchymal Stromal Cell Conditioned Medium in In Vitro Models of Acute Lung Injury*. Shahd Horie, Daniel O'Toole, Steven Verdejo, John G. Laffey. Publication Date: 2014

American Thoracic Society Conference May 2013

Poster Presentation Abstract Title: *Assessing the Therapeutic Potential of Induced Pluripotent Stem Cells in In Vitro Models of Acute Lung Injury*. Daniel O'Toole, Shahd Horie, Katya McDonagh, Claire H. Masterson, Sanbing Shen, John G. Laffey. Publication Date: 2013