THERAPEUTIC EFFECTS OF EXTRACELLULAR VESICLES FROM HUMAN MENSTRUAL BLOOD-DERIVED MESENCHYMAL STEM CELLS IN ACUTE LUNG INJURY ANIMAL MODEL

LIAN JIE

UNIVERSITI SAINS MALAYSIA

2023

THERAPEUTIC EFFECTS OF EXTRACELLULAR VESICLES FROM HUMAN MENSTRUAL BLOOD-DERIVED MESENCHYMAL STEM CELLS IN ACUTE LUNG INJURY ANIMAL MODEL

by

LIAN JIE

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

September 2023

ACKNOWLEDGEMENT

Of finishing the last word of my doctoral thesis, I see a heart filled with gratitude beating inside my chest, I would really like to take this opportunity to express my deepest appreciation to them for supporting and helping me to complete the whole project presented in this thesis.

I first offer my sincerest gratitude to my main supervisors Assoc. Prof. Dr. Badrul Yahaya and Prof. Dr. Lin Juntang, who guided me through the whole PhD journey with great patience and wisdom on not only academic research, but also how to be a strong and honest person. Their passion and devotion on their work also have set a standard for me to reach regardless of my career choice in the future. I'm so grateful to have them as my supervisors.

Special thanks should also go to my co-supervisors, Dr. Ida Shazrina Binti Ismail and Prof. Dr. Narazah Binti Mohd Yusoff. I had the pleasure of working with my lovely labmates in Universiti Sains Malaysia and Xinxiang Medical University, especially for Assoc. Prof. Dr. Zhu Xinxing. He contributed considerable time and effort to revise my paper and guide me for my experiments. Without his kind help, I cannot overcome numerous obstacles in exploring mechanisms and finishing related lab work. It is safe to say that I'll never complete my work without them.

Surely, this endeavor would not have been possible without my dear parents, sister, my beloved husband Hu Jinxing and daughter Hu Die, who have loved and supported me unconditionally through many difficult moments during the past four years.

To close, I'd like to say that it is for all of you guys' efforts that I could stand here today and proudly present the world with this thesis.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
×g	g-force
℃	Degree Celsius
hr	Hour
µg	Microgram
mg	Milligram
kg	Kilogram
µl	Microliter
ml	Milliliter
µm	Micrometer
mM	Millimolar
µM	Micromolar
nM	Nanomolar
min	Minutes
rpm	Revolutions per minute
vg	Viral genomes
%	Percentage
~	Approximately
®	Registered trademark
™	Trademark

LIST OF ABBREVIATIONS

AAK1	AP2-associated kinase 1
AAV	Adeno-associated virus
AECs	Airway epithelial cells
AEC I	Type I alveolar epithelial cells
AEC II	Type II alveolar epithelial cells
ALI	Acute lung injury
Amφ	Alveolar macrophages
ARDS	Acute respiratory distress syndrome
AT2	Type II alveolar epithelial cells
ATS	American thoracic society
BALF	Bronchoalveolar lavage fluid
CCL3	C-C motif chemokine ligand 3
CCL6	C-C motif chemokine ligand 6
CCL9	C-C motif chemokine ligand 9
CME	Clathrin-mediated endocytosis
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CSCs	Cardiac stem cells
def	Deficient
DEG	Differentially expressed gene
DiO	3,3’-dioctadecyloxacarbocyanine perchlorate
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethyl sulphoxide
EBD	Evans blue dye
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
FBS	Fetal bovine serum
FC	Fold change
GEO	Gene expression omnibus

GM-CSF	Granulocyte-macrophage colony stimulating factor
H&E	Hematoxylin and Eosin
hAD-MSCs	Human adipose-derived MSCs
hBM-MSCs	Human bone marrow-derived MSCs
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
hMenSCs	Human menstrual blood-derived stem cells
hsa	Homo sapiens (human)
HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
hUC-MSCs	Human umbilical cord-derived MSCs
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
ILD	Interstitial lung disease
i.p.	Intraperitoneal
iPSCs	Induced pluripotent stem cells
ISEV	International society for extracellular vesicles
IT/i.t.	Intratracheal
IV/i.v.	Intravenous
KEGG	Kyoto encyclopedia of genes and genomes
LPS	Lipopolysaccharide
Lv	Lentivirus
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MenSC-EVs	MenSC-derived EVs
MERS	Middle east respiratory syndrome
MFGE8	Milk fat globule-EGF factor 8 protein
MHC	Major histocompatibility complex
mimic	miR-671-5p-mimic
miRNAs	MicroRNAs
MISEV	Minimal information of studies of extracellular vesicles

MMP-9	Matrix metalloproteinase 9
MPO	Myeloperoxidase
MSC-EVs	MSC-derived EVs
MSCs	Mesenchymal stem cells
MODS	Multiple organ dysfunction syndrome
Mut	Mutant
NanoFCM	Nanoparticle flow cytometry
NC	Negative control
NCBI	National center for biotechnology information
NF-κB	Nuclear factor κB
NIH	National institutes of health
NSCs	Neural stem cells
PARDS	Pediatric ARDS
PDCD61P	Programmed cell death 6 interacting protein
PI3K	Phosphatidylinositol 3-kinase
PMN	Polymorphonuclear
qRT-PCR	Qualitative reverse transcriptase polymerase chain reaction
RIN	RNA integrity number
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
sponge	miR-671-5p-sponge
TEM	Transmission electron microscopy
TIRAP	Toll-interleukin 1 receptor domain-containing adapter protein
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor receptor-associated factor 6
TSG101	Tumor susceptibility gene 101 protein
TSPAN29	Tetraspanin 29
Ub	Ubiquitin
UTR	Untranslated region
Vec	Vector
VILI	Ventilator-induced lung injury

WBC	White blood cell
WHO	World health organization
WT	Wild type

LIST OF APPENDICES

KESAN TERAPEUTIK VESIKEL EKSTRASELULAR DARIPADA SEL TUNJANG MESENKIMA YANG BERASAL DARIPADA DARAH MENSTRUAL MANUSIA DALAM MODEL HAIWAN KECEDERAAN PARU-PARU AKUT

ABSTRAK

Sel stem mesenkimal yang berasal dari darah haid manusia (MenSCs) telah mendapat perhatian untuk potensi terapeutik mereka dalam pelbagai keadaan. Walau bagaimanapun, aplikasi dan mekanisme khusus mereka dalam merawat kecederaan paru-paru akut (ALI) masih diterokai. Objektif kajian ini adalah untuk menilai keberkesanan praklinikal dan mekanisma yang dimainkan oleh vesikel ekstraselular (EVs) yang teraruh daripada MenSCs dalam mengawalatur keradangan paru-paru akut. Pertama, MenSCs atau MenSC-EVs dipencilkan dan digunakan bagi tujuan rawatan intratrakea ke atas model tikus ALI yang terbentuk disebabkan oleh aruhan *lipopolysaccharide* (LPS) bagi menilai kesan perlindungannya terhadap keradangan paru-paru berdasarkan analisis histologi, molekul, bronchoalveolar lavage fluid (BALF), dan penjujukan mRNA. Penyiasatan ini melibatkan sistem sel kultur bersama dan model ALI yang disebabkan oleh LPS. Di samping itu, kapasiti intrinsik miRNA yang berpotensi ini atau gen sasaran mereka dalam mengawal keradangan dan kecederaan paru-paru telah diperiksa melalui kedua-dua teknik *in vivo* dan *in vitro* dengan menggunakan teknik adeno-associated virus (AAV) dan lentivirus-mediated, masing-masing. Tambahan pula, eksperimen molekul dan biokimia konvensional telah dijalankan untuk menjelaskan mekanisma molekul yang mendasari *in vivo*. Keputusan menunjukkan bahawa kedua-dua MenSCs dan MenSC-EVs secara signifikan mengurangkan ALI yang disebabkan oleh LPS dalam

in vivo. Antara miRNA yang banyak terkandung dalam MenSC-EVs, miR-671-5p, yang mempunyai kekekalan tinggi di kalangan spesies yang berbeza dan peranannya dalam ALI yang disebabkan oleh LPS, amat perlu diberi perhatian kerana ia dipindahkan ke sel epitelium paru-paru secara *in vitro* melalui laluan yang bergantung kepada EVs. Gangguan miRNA ini dengan ketara mengurangkan kesan amelioratif MenSC-EVs pada kecederaan radang paru-paru yang disebabkan oleh LPS dalam *in vivo*. miR-671-5p secara langsung menyasarkan kinase AAK1, yang membawa kepada kemerosotan pasca transkripsinya. Kedua-dua miR-671-5p dan AAK1 memainkan peranan penting dalam mengawal keradangan dan kecederaan yang disebabkan oleh LPS diperingkat *in vivo* dan *in vitro*, dan gangguan AAK1 telah mengakibatkan hampir pemulihan lengkap kegagalan rawatan MenSC-EVs yang disebabkan oleh kekurangan miR-671-5p dalam *in vivo*. Ujian imunopresipitasi dan ubiquitination mendedahkan bahawa AAK1 secara positif mengawal pengaktifan laluan isyarat NF-κB dengan mengawal kestabilan protein perencatan IκBα. Penemuan ini mewujudkan asas molekul yang berpotensi untuk kesan bermanfaat MenSC-EVs dalam mengurangkan kecederaan radang paru-paru dan menggariskan kepentingan fungsi miR-671-5p/AAK1 dalam perkembangan penyakit radang paru-paru. Selain itu, kajian ini membentangkan pendekatan terapeutik berasaskan sel untuk merawat gangguan keradangan paru-paru melalui penggunaan laluan yang bergantung kepada EV.

THERAPEUTIC EFFECTS OF EXTRACELLULAR VESICLES FROM HUMAN MENSTRUAL BLOOD-DERIVED MESENCHYMAL STEM CELLS IN ACUTE LUNG INJURY ANIMAL MODEL

ABSTRACT

Human menstrual blood-derived mesenchymal stem cells (MenSCs) have gained attention for their therapeutic potential in various conditions. However, their specific application and mechanism in treating Acute lung injury (ALI) is still being explored. The aim of this study was to assess the effectiveness and potential underlying mechanisms of MenSCs derived-extracellular vesicles (EVs) in ameliorating acute lung inflammation and injury in preclinical models. Initially, MenSCs or their EVs were obtained and administered intratracheally to a mouse model with ALI induced by *lipopolysaccharide* (LPS) in order to assess their protective effects against pulmonary inflammation and injury. This evaluation was performed using histological, molecular, bronchoalveolar lavage fluid (BALF), and mRNA-sequencing analyses, taking into account the optimal timing for intervention. Subsequently, a small RNA microarray technique was employed to identify potential microRNAs (miRNAs) that contribute to the improvement of pulmonary inflammation and injury mediated by MenSC-EVs *in vivo*. Additionally, the intrinsic capacity of these potential miRNAs or their target genes in regulating lung inflammation and injury was examined both *in vivo* and *in vitro* by depleting them using an adeno-associated virus (AAV) and lentivirus-mediated technique, respectively. Furthermore, conventional molecular and biochemical experiments were conducted to elucidate the underlying molecular mechanism. The results demonstrate that both MenSCs and MenSC-EVs significantly alleviate LPS-induced

ALI *in vivo*. Among the abundant miRNAs encapsulated in MenSC-EVs, miR-671-5p, distinguished by its high conservation among different species and its role involved in LPS-induced ALI, is particularly noteworthy as it is transferred to lung epithelial cells *in vitro* via an EV-dependent pathway. Disruption of this miRNA significantly diminishes the ameliorative effects of MenSC-EVs on pulmonary inflammatory injury induced by LPS *in vivo*. Mechanistically, miR-671-5p directly targets the kinase AP2-associated kinase 1 (AAK1), leading to its post-transcriptional degradation. Both miR-671-5p and AAK1 play critical roles in regulating inflammation and LPS-induced injury both *in vivo* and *in vitro*. Immunoprecipitation and ubiquitination assays reveal that AAK1 positively regulates the activation of the NF-κB signaling pathway by controlling the stability of the inhibitory protein IκBα. These findings establish a potential molecular basis for the beneficial effects of MenSC-EVs in improving pulmonary inflammatory injury and underscore the functional significance of the miR-671-5p/AAK1 axis in the progression of pulmonary inflammatory diseases. Moreover, this study presents a promising cell-based therapeutic approach for treating pulmonary inflammatory disorders through the utilization of an EV-dependent pathway.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction of Acute Lung Injury (ALI)

1.1.1 Definition and incidence of ALI

ALI or its clinical presentation, ARDS, is a serious and rapid lung inflammation causing high morbidity and mortality as well as the onset of multiple organ dysfunction syndrome (MODS) (Lian *et al.*, 2020; Meyer, Gattinoni & Calfee, 2021). ARDS was first proposed in 1967 (Ashbaugh *et al.*, 1967); "A" originally represented "adult" then replaced by "acute" later. As our understanding of the condition grew, the definition changed from the American-European Consensus Conference Committee definition (Bernard *et al.*, 1994) to the Berlin definition (Ferguson *et al.*, 2012), details of both definitions as shown in Table 1.1. The latter classifies the severity of the condition from mild to severe. In addition, the pediatric ARDS (PARDS) definition was developed in 2015 (Pediatric Acute Lung Injury Consensus Conference, 2015). Although remarkable advances in therapy and nursing over the past half-century, ALI remains a significant contributor of high morbidity, mortality, and financial burden.

Over 3 million patients suffer from ARDS every year, constituting more than 10% of patients of intensive care units (ICU). Moreover, the underreporting of ARDS is likely to occur in low-income countries, as it is under-recognized even in high-income countries (Thompson, Chambers & Liu, 2017). Recently, the global COVID-19 pandemic has contributed to a dramatic increase in ARDS, also called COVID-19-related ARDS (Meyer, Gattinoni & Calfee, 2021). Early reports indicated that 30%-40% COVID-19 hospitalized patients developed into ARDS, which was related to 70% death (Williams *et al.*, 2021). Bellani et al. studied 29,144

patients from 459 ICU across 50 countries on 5 continents. The results indicated varying rates of clinical recognition for ARDS, ranging from 51.3% for mild cases to 78.5% for severe cases, and the condition appeared to be a public health problem globally, with an alarming mortality rate of around 40% (Bellani *et al.*, 2016). Even patients who survive from ALI are at high risk for long-term poor quality of life (Bein, Weber-Carstens & Apfelbacher, 2018; Heesakkers *et al.*, 2023). Children are no exception, as another international study that involved 23,280 patients from 145 pediatric ICU across 27 countries discovered that PARDS occurs in approximately 3% of patients but leads to ~17% mortality (Khemani *et al.*, 2019).

Table 1.1 American-European Consensus Conference (AECC) and Berlin definition of ALI and ARDS.

Clinical Variable	AECC definition 1944	Berlin definition 2012
Onset	Acute	Within 1 week of a known clinical insult or new or worsening respiratory symtoms
Chest imaging	Bilateral infiltrate on frontal chest radiograph	Bilateral opacities (not fully explained by effusion, atelectasis, or nodules)
Non-cardiogenic source of pulmonary edema	No clinical evidence of elevated left aterial pressure of a pulmonary capillary wedge pressure < 18 mmHg	Respiratory failure not fully explained by cardiogenic pulmonary edema or volume overload
Oxygenation	ALI: PaO_2/FiO_2 < 300 mmHg ARDS: PaO_2/FiO_2 < 200 mmHg	Mild ARDS: 200 mmHg < PaO_2/FiO_2 ≤ 300 mmHg with PEEP ≥ 5 cm H_2O Moderate ARDS: 100 mmHg < PaO_2/FiO_2 ≤ 200 mmHg with PEEP ≥ 5 cm H_2O Severe ARDS: PaO_2/FiO_2 ≤ 100 mmHg with PEEP ≥ 5 cm H_2O
Risk factor	Not specified	If none identified, then need to rule out cardiogenic edema with additional data (e.g., echocardiography)

To date, there has been no comprehensive epidemiology study of ARDS in China, but the incidence, mortality, and risk factors for ARDS and PARDS in China are thought to be comparable to those observed in Europe and the United States based on several relatively regional studies, which suggest that the annual number of cases in China is more than 670,000 patients (Song *et al.*, 2014). However, health

emergency associated ALI, such as COVID-19, leading to a sharp rise in the prevalence of ARDS (Tzotzos *et al.*, 2020; Wu *et al.*, 2020), is not included. During 21st century, there are three outbreaks of coronavirus infection around the world, including SARS (Severe Acute Respiratory Syndrome) in 2002, followed by MERS (Middle East Respiratory Syndrome) in 2012 and more recently with COVID-19 from December 2019. So far, there is no guidelines to follow for the therapy of COVID-19 especially for severe patients due to lack of effective therapeutic drugs until now. Under the support of World Health Organization (WHO), several specific treatments were under investigation and would be tested their safety and efficacy through clinical trials, including stem cell-based therapy.

1.1.2 Pathogenesis of ALI

ALI can be attributed to various pathogens and both direct and indirect lung injury insults (Table 1.2), with pneumonia, gastric aspiration, and sepsis being the top 3 main causes of ALI in recent clinical settings (Matthay *et al.*, 2019b). When stimulated by infectious, chemical, or mechanical factors, the intricate interplay between the immune system and the alveolar-capillary barrier would give rise to the pathophysiology of ALI (Lee, Park & Lee, 2019). Although the genetic basis of ALI has yet to be completely understood, mounting evidence suggests the involvement of genetic determinants in the susceptibility and severity of ALI. Inflammation gene and blood coagulation ontologies were identified as most highly represented candidates genes for ALI (Grigoryev *et al.*, 2004). Furthermore, genetic investigations in Chinese populations discovered some genetic risk factors engaged in the development of ARDS, including the Toll-interleukin 1 receptor domain-containing adapter protein (TIRAP) (Song *et al.*, 2010) and the tumor necrosis factor receptor-associated factor 6 (TRAF6) gene (Song *et al.*, 2012). ALI also represents a

significant perioperative complication with crucial mortality and morbidity, and therapies beyond conservative respiratory support are limited (Jin, Chun Suen & Ma, 2017).

Table 1.2 Conditions associated with ALI.

Direct lung injury insults	Indirect lung injury insults
Pneumonia*	Sepsis*
Gastric aspiration*	Major trauma
Pulmonary contusion	Non-cardiogenic shock
Pulmonary embolism	Pancreatitis
Inhalation injury	Severe burns
Near drowning	Multiple transfusion or transfusion-associated acute lung injury
	Cardiopulmonary bypass surgery
	Reperfusion edema after lung transplantation or embolectomy
	Drug overdose
	Genetic risk factors

Note: * Pneumonia, gastric aspiration, and sepsis are the top 3 main triggers of ALI in recent clinical conditions.

There are three phases during the pathogenesis of ALI, including the exudative, proliferation, and fibrotic phases (Thompson, Chambers & Liu, 2017; Lian *et al.*, 2020). The exudative phase typically occurs within 24 hr of triggers; it is characterized by diffuse alveolar damage, indicating innate cell-mediated injury to both the alveolar endothelial and epithelial barriers, as well as the influx of protein-rich edema fluid in the interstitium and alveolus, as shown in Figure 1.1. Microbial components or tissue injury were recognized by resident alveolar macrophages (Amφ) via their pattern recognition receptor signaling, leading to NF-κB-dependent polarization of Amφ into M1-like macrophages and the initiation of the exudative phase. Pro-inflammatory cytokines and chemokines secreted by M1-like macrophages contribute to the accumulation of neutrophils and monocytes as well as

the activation of alveolar epithelial cells and effector T cells, all of which foster and maintain inflammation and tissue injury (Kumar, 2020). Neutrophils, once activated, contribute to progressive lung injury through the release of preformed inflammatory mediators, reactive oxygen species, and proteinases, as well as by forming neutrophil extracellular traps and highly injurious histones. Tumor necrosis factor (TNF)-mediated production of tissue factor by injured and activated endothelium and epithelium leads to abnormal coagulation within blood vessels and alveoli, platelet aggregation, as well as micro-thrombi and hyaline membrane formation (Monteith *et al.*, 2021). Extensive damage to the alveolar epithelium also disrupts the integrity of alveolar ion channels and weakens the osmotic pressure for alveolar fluid clearance, which boosts alveolar flooding (Jia *et al.*, 2022). Endothelial activation and microvascular injury further facilitate the disruption of alveolar-capillary barrier as well as the flooding of interstitial and intra-alveolar (Bos & Ware, 2022). Flooding and collapse of alveoli lead to substantially impaired gas diffusion and hypoxemia.

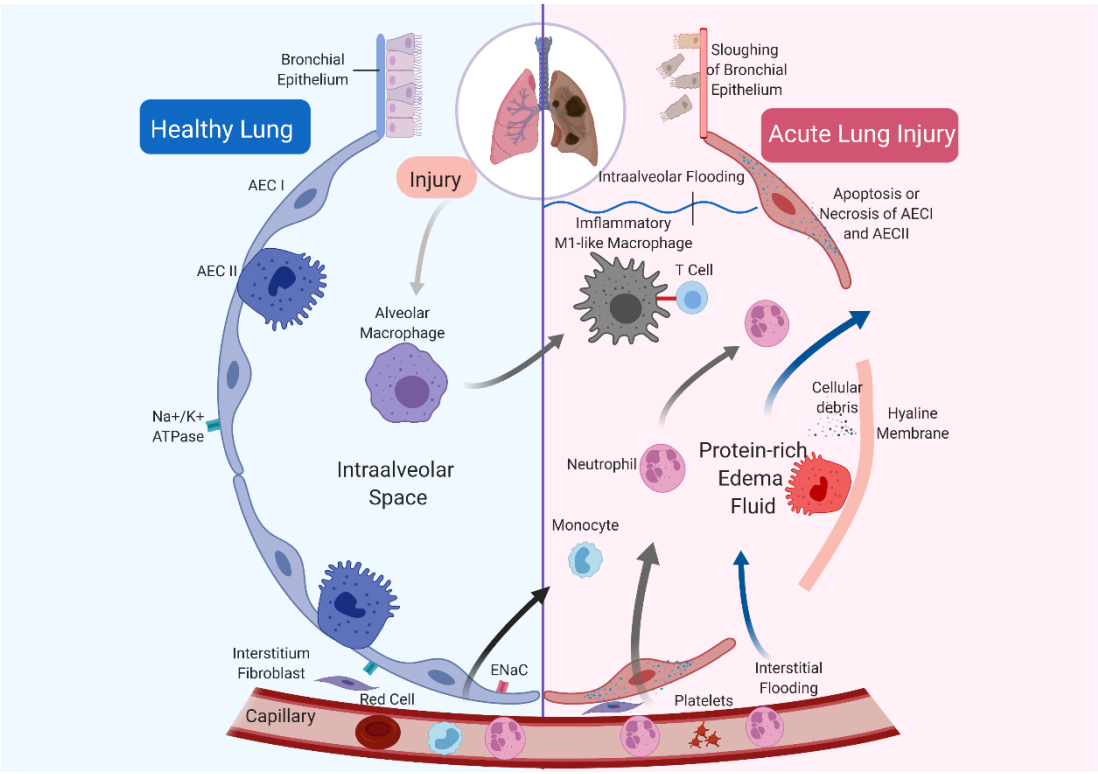

Figure 1.1 The difference between a healthy and ALI alveolus (Lian *et al.*, 2020). Created with BioRender.com. The hallmarks of ALI are disruption of alveolar-capillary barriers, recruitment of pro-inflammatory cells, formation of hyaline membranes, and flooding of protein-rich edema within the interstitium and alveolus. Injury begins with the disruption of alveolar-capillary integrity by either direct or indirect insults. Initially, resident alveolar macrophages are activated and polarized into M1-like macrophages, which secrete pro-inflammatory factors that contribute to recruitment of neutrophils and monocytes to facilitate and maintain inflammation and tissue injury. Extensive damage to the alveolar epithelium directly increases the permeability of alveolar-capillary barriers, and apoptosis of AEC II weakens pulmonary surfactant secretion and alveolar fluid clearance, which aggravate protein-rich edema fluid in the interstitium and alveolus.

The proliferative phase, caused by impaired and extensive epithelial injury, is essential for host survival. During this phase, resident fibroblasts transiently expand, a provisional matrix is formed, airway progenitor cells proliferate, and type II alveolar epithelial cells (AEC II) differentiate into type I alveolar epithelial cells (AEC I) (Vaughan *et al.*, 2015). This phase typically occurs 3 to 7 days following respiratory failure in adult humans, whereas the timing was reported to be 1 week after injury for experimental animals (Matute-Bello *et al.*, 2011; Kulkarni *et al.*,

2022). Alveolar edema is reabsorbed, and alveolar architecture and function are restored once epithelial integrity has been rebuilt.

The final fibrotic phase does not occur in all ALI patients, but evidence suggests that this phase is associated with prolonged mechanical ventilation and elevated risk of mortality. Patients at this stage have an elevated demand for mechanical ventilation, and the progression of interstitial and intra-alveolar fibrosis results from significant basement membrane destruction and insufficient or late re-epithelialization (Wang *et al.*, 2022b).

During ALI, a cascade of intricate molecular and cellular events unfolds, including inflammatory responses, oxidative stress, and endothelial barrier dysfunction. Triggers like infections or trauma prompt immune cells, notably neutrophils and macrophages, to release pro-inflammatory cytokines that recruit more immune cells and fuel inflammation. The fragile endothelial barrier of the lungs, consisting of endothelial cells, becomes weakened, allowing fluid, protein-rich exudate, and immune cells to infiltrate into the alveolar spaces, causing pulmonary edema and impairing gas exchange. Oxidative stress amplifies, generating reactive oxygen and nitrogen species, damaging lipids, proteins, and DNA, and perpetuating inflammation. Epithelial cell apoptosis and dysfunction contribute to tissue disruption, exacerbating the condition. Subsequent repair mechanisms involve anti-inflammatory cell recruitment, clearance of inflammatory cells, tissue repair, and endothelial barrier restoration. This multifaceted interplay culminates in compromised lung function, demanding comprehensive management strategies targeting both the underlying causes and the intricate molecular and cellular processes of ALI.

1.1.3 The status of ALI treatment

Research on lung disease has revealed that ALI is a syndrome with substantial heterogeneity (Thompson, Chambers & Liu, 2017). The complex pathophysiology of ALI seems to provide a wide range of targets with multiple therapeutic options, but there is still a lack of effective pharmacotherapy that specifically targets the underlying mechanisms of ALI until now. Currently, supportive care approaches are the only therapy options available for ALI, including lung-protective ventilation (Guervilly *et al.*, 2022), fluid conservative strategy (Xing *et al.*, 2021), and prone positioning (Guérin *et al.*, 2020). Unfortunately, supportive therapies for ARDS only focus on preventing further lung injury rather than actively accelerating tissue repair, which accounts for the limited treatment effectiveness (Butt, Kurdowska & Allen, 2016). Besides, current evidence from clinical practice and research indicates that there is no safe tidal volume or airway pressure for ALI sufferers. Even normal tidal volumes given with airway pressure may produce regional overstretch since the aerated lung volume decreases as ALI progresses, which further facilitate epithelium activation or injury and inflammation amplification (Abrams *et al.*, 2022). Ventilation in prone position is significantly linked to lower fatality rate for individuals suffering from moderate-to-severe ARDS, and this is currently recommended in clinical practice (Fan *et al.*, 2017). Unfortunately, there is little evidence that pharmacologic treatment can reduce mortality from ARDS, either in the short or long term. Notably, uncontrolled inflammation is the main reason of high mortality in individuals with ARDS and COVID-19, more common disorders with ALI in clinical settings. Hence, there is an immediate need to investigate the molecular mechanism underlying ALI and develop

new approaches based on repairing damaged lung and relieving inflammation in clinical practice.

1.2 Animal Models of Study ALI

1.2.1 Experimental animal models of ALI

Prior to applying potential therapeutic approaches to humans, researchers can explore underlying pathophysiological mechanisms in greater detail by using animal studies as an experimental setting. For predicting the feasibility of a therapeutic approach and serve as a bridge from bench to bedside, a good animal model should have human-like anatomy and responses. However, no animal model, even the ALI animal model, can precisely reproduce all human pathological features when exposed to stimuli or treatments. At least three out of the four main characteristics of ALI, including histological evidence of tissue injury, alteration of the alveolar capillary barrier, inflammatory response, and physiological dysfunction, which are all specifically stated in commonly used ALI animal models as shown in Table 1.3, should be available in a typical ALI animal model, as per recommendations offered by the official documents of the American Thoracic Society (ATS) (Matute-Bello *et al.*, 2011; Kulkarni *et al.*, 2022). Animal models are essential for investigating this clinically defined disorders, however depending on the goal of the study, different model systems have distinct limitations. For instance, small animal models are feasible systems that can address disease mechanisms and provide a foundation for rational intervention design, even though they might fail to replicate all clinical features of ARDS. Large animal models, which may more accurately reflect human disease in ARDS, are less flexible to mechanistic research, but they serve as valuable models for preclinical treatment trials (Semler *et al.*, 2020). Hence, a fully

constructed ALI animal model is not required to be established according to ATS documents. Regarding this, the molecular mechanisms underlying lung regeneration and repair, as well as the crucial role played by stem cells in both small and large animal models were summarized previously by our group (Yahaya, 2012; Kardia, Ch'ng & Yahaya, 2018; Halim *et al.*, 2019; Ridzuan *et al.*, 2021).

Table 1.3 The presence of "very relevant" criteria in the three main types of standard experimental ALI in animals.

Main feature	Very relevant	Measurement	LPS	VILI	Live bacteria	Notes
Histological evidence of tissue injury	Accumulation of neutrophils in the alveolar/interstitial space	H&E staining	+	+	+	Hyaline membranes are rarely observed in murine models
	Formation of hyaline membranes		+	+	+	
	Proteinaceous debris in alveolar space		+	+	+	
	Thickening of the alveolar wall		+	+	+	
	Injury by a standardized histology score		+	+	+	
Alteration of the alveolar capillary barrier	Increased extravascular lung water content	Wet-to-dry ratios	+	+	+	Minor inaccuracies in weight measurement can cause substantial fluctuations in ratios for very small lungs
	Accumulation of protein / tracer in airspaces / extravascular space	EBD	+	+	+	Intravenous injection in advance
	Total BAL protein concentration	BALF-total protein concentration, IgM	+	+	+	Technical challenges and difficult to standardize
	BAL concentration of high molecular weight proteins		+	+	+	
	(Micro-)vascular filtration coefficient (K_f)	Under machine testing	(+)	+	(+)	Only for isolated perfused lung

Inflammatory response	BAL total neutrophil counts	BALF-cytospin, Wright-Giemsa staining	+	+	+	Neutrophil number and percentage
	Lung MPO activity	ELISA kits or colorimetric assay	+	+	+	Cell-free BALF or whole lung homogenates
	Concentrations of cytokines	qRT-PCR or ELISA kits	+	(+)	+	mRNA or protein expression
Physiological dysfunction	Hypoxemia	Under machine testing	+	+	+	Equipment limits
	Increased alveolar-arterial oxygen difference		+	+	+	

Notes: LPS, lipopolysaccharide; VILI, ventilator-induced lung injury; BALF, broncho-alveolar lavage fluid; EBD, Evans blue dye; MPO, myeloperoxidase; RT-PCR, reverse transcriptase polymerase chain reaction; H&E, Hematoxylin and eosin; ELISA, enzyme-linked immunosorbent assay.
+, the criterion was present in virtually all studies using this model.
(+) the criterion was present in the majority of studies using this model.

1.2.2 Evaluation of common ALI animal models

Numerous approaches for developing animal models for ALI have been reported for preclinical studies, including endotoxin (Xu *et al.*, 2021c; Lian *et al.*, 2023), bacteria (Varkouhi *et al.*, 2019), ventilator (Islam *et al.*, 2019; Amatullah *et al.*, 2021), and cecal ligation and puncture (Jiang *et al.*, 2020a; Jiao *et al.*, 2021). According to these findings, the involvement of neutrophils in the inflammatory process during the development of ALI has been established in both small and large animal models.

The vast majority of ruminants, including goats and sheep, have segmented lungs, which indicates that a large number of macrophages circulate in pulmonary vessels and their pulmonary circulation tends to be sensitive to endotoxin intravenous injection. Small doses of endotoxin were reported to cause increased pulmonary hypertension in these animals, which were also demonstrated in ALI model induced by smoke inhalation (Lange *et al.*, 2012; Rehberg *et al.*, 2013) and brushing

(Yahaya *et al.*, 2011) in earlier studies. Infiltration and accumulation of neutrophils have also been reported to be the main features in large animal models for ALI (Lange *et al.*, 2012), but large animals are susceptible to microbial infection, so intravascular macrophages in these animals are easily augmented through stimulation of the local inflammatory reaction in response to microbe invasion.

On the other hand, smaller animals and humans have less pulmonary intravascular macrophages. Small animals, such as mice, rats, and rabbits, are widely bred and relatively economical in terms of expenses. Numerous investigations indicated that endotoxin-induced ALI in mice led to prominent inflammatory cell infiltration, including neutrophils and macrophages, into the alveolar and interstitial space, along with interstitial edema and intra-alveolar septal thickening with fibrin and collagen deposition (Wang *et al.*, 2022d; Zhao *et al.*, 2022a). Following exposure to hazardous substances such as sodium nitrate and naphthalene (Uriarte *et al.*, 2013; Zhang *et al.*, 2016), the rat model displayed comparable patterns of ALI features. Additionally, it was discovered that activated neutrophils were crucial in initiating the inflammatory processes, leading to further hemorrhage and alveolar injury (Monteith *et al.*, 2021; Wang *et al.*, 2022a). Murine lungs rarely produce hyaline membranes during ALI (Matute-Bello *et al.*, 2011), whereas hyaline membranes in rabbit models typically occur during the early exudative phase of ALI (Kardia, Ch'ng & Yahaya, 2018), which is consistent with the features in ALI patients. Furthermore, gene sequence comparison analysis revealed that the rabbit shared a more homology with the human leukocyte antigen (HLA) genes than the mouse and rat, implying that rabbit tissue is less likely to result in immunological rejection following allotransplantation (Zhang *et al.*, 2020b). As a result, rabbits are frequently used in studies of implantation and tissue engineering. However, mice are

the most commonly used animal models because specific reagents and genetic variants are more widely available.

The official ATS workshop report provides recommendations for the features and measurements of experimental ALI animal models, and thorough descriptions of the difference between ALI patients and various typical ALI animal models in detail (Matute-Bello *et al.*, 2011). Given the high frequency of use, three main types of animal models, such as LPS-, ventilator-, and live bacteria-induced lung injury, were briefly summarized in Table 1.2. Notably, almost all of the "very relevant" criteria are present in the top three most commonly used animal models, making them useful for further investigating more efficient therapeutic approaches for treating ALI. Due to its high effectiveness and human-like anatomy and responses, LPS-induced ALI mice model is most commonly to mimic human ALI caused by serious pneumonia or sepsis. In preclinical investigations, intratracheal (i.t.) and intravenous (i.v.) delivery are routinely used to induce ALI in animal models, but there are some distinctions between these two delivery methods. The former is pulmonary administration, revealing how alveolar epithelium structure in the lungs suffers damage, including polymorphonuclear (PMN) cell infiltration in intra-alveolar space, diffuse alveolar edema, and mild alterations in epithelial permeability. On the other hand, i.v. administration demonstrates how vascular endothelium structure in the lungs is damaged, such as PMN cell accumulation in capillaries and the interstitium with mild infiltration in intra-alveolar areas, presence of protein-rich alveolar edema, and minimal changes in epithelial permeability. As in human patients, i.t. and i.v. delivery in animal models mimic direct and indirect insult, respectively. However, in preclinical studies, investigators should optimize the timepoint by considering the specific animal model and feature of injury being stimulated, especially in

therapeutic research, because the acute time may vary in diverse animal models (Kulkarni *et al.*, 2022), and these animal models usually appear self-healing within a reasonable time frame (Lopes-Pacheco *et al.*, 2019).

1.3 Stem Cell-based Therapy for ALI

1.3.1 Stem cell-based therapy

Stem cells are undifferentiated cells capable of differentiating into several cell types, such as muscle cells, nerve cells and blood cells (Laplane & Solary, 2019). The ability makes them attractive for medical applications, as they hold immense potential for the treating an extensive spectrum of medical disorders. Stem cells can be harvested from a variety of tissues, such as embryonic, fetal, and adult tissues (Bacakova *et al.*, 2018; Xu *et al.*, 2020a; Hoang *et al.*, 2022). Embryonic stem cells (ESCs), originate from the inner cell mass of the blastocyst, exhibit the remarkable capability to differentiate into any cell types within the body; Fetal stem cells, obtained from aborted fetuses, can develop into various cell types; Adult stem cells, present in a variety of tissues throughout the body, including bone marrow, adipose tissue, blood and other tissues, only can develop into a restricted variety of cell types. It's important to note that ESCs and fetal stem cells are ethically questionable due to their source and teratoma formation, while adult stem cells are more ethically acceptable (Zakrzewski *et al.*, 2019; Yamanaka, 2020). However, the employing of stem cells in research and therapy remains a complex and evolving issue, ongoing ethical discussions and debates are likely to continue.

Stem cell-based therapy has become an exciting emerging treatment that aim to repair injured tissue and mitigate inflammation via regeneration by virtue of their multipotency as well as the release of and regulation by their soluble bioactive

factors, which can be broadly classified into two types: autologous and allogeneic (Yamanaka, 2020; Hoang *et al.*, 2022). Autologous stem cell therapy employs patient's own stem cells, while allogeneic stem cell therapy utilizes stem cells obtained from a donor. According to reports, a variety of stem cells, particularly hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), ESCs and induced pluripotent stem cells (iPSCs), can be employed in treating diverse diseases and disorders (De Luca *et al.*, 2019). Some examples of stem cell-based therapy that are currently in use or being studied include:

(1) Hematopoietic stem cell transplantation (HSCT) is a particular kind of stem cell therapy wherein HSCs are transplanted to treat blood and immune system diseases, such as leukemia, lymphoma and autoimmune disorders (Del Papa *et al.*, 2018; Alexander, Greco & Snowden, 2021; Alseraihy *et al.*, 2022; Wang *et al.*, 2022c). HSCs, obtained from bone marrow, peripheral blood, or umbilical cord blood, are capable of developing into any sort of blood cells.

(2) Mesenchymal stem cell therapy involves the use of MSCs for treating a variety of incurable disorders, including immune abnormalities, inflammatory diseases, neurodegenerative disorders, and aging frailty (Harrell *et al.*, 2019b; de Klerk & Hebrok, 2021; Sarsenova *et al.*, 2021; Zhuang *et al.*, 2021; Xiang *et al.*, 2022; Mönch *et al.*, 2023). MSCs exhibit anti-inflammatory and immunomodulatory properties, and their plasticity enables them to develop into numerous cell types in certain contexts.

(3) Induced pluripotent stem cell therapy involves the reprogramming of adult cells into iPSCs, which have a capacity to develop into almost any kind of cell throughout the body (Ronaldson-Bouchard *et al.*, 2018; Yamanaka, 2020; Arias *et al.*, 2021; Sinenko, Ponomartsev & Tomilin, 2021; Woan *et al.*, 2021; Goldenson,

Hor & Kaufman, 2022; Maeda *et al.*, 2022). Somatic cells from the patient can be employed to generate iPSCs, which hold potential for the treatment of various diseases and disorders.

(4) Other adult stem cell therapy includes the application of stem cells coming from various tissues to treat tissue specific diseases, including neural stem cells (NSCs) to treat neurological disorders (Huang & Zhang, 2019; de Freria *et al.*, 2021; Kawai *et al.*, 2021), cardiac stem cells (CSCs) to treat heart disease (Barreto *et al.*, 2019; Cianflone *et al.*, 2020), epithelial stem cells to treat disorders of the skin and other epithelial tissues such as the lining of the gut (Gehart & Clevers, 2019), respiratory tract and lung tissues (Kardia, Ch'ng & Yahaya, 2018; Alysandratos, Herriges & Kotton, 2021).

Recently, preclinical research has demonstrated the potential of stem cell-based therapy for treating lung diseases and critical illness, and they are likely to provide novel therapeutic candidates for general ARDS patients. In this context, our group has previously established an aerosol-based cell therapy using airway epithelial cells (AECs) for the treatment of ALI models, both *in vivo* and *in vitro*. The findings showed that AECs delivery by aerosolization remarkably accelerated the repair and regeneration of the respiratory airway and lung tissues (Kardia, Mohamed & Yahaya, 2017; Kardia, Ch'ng & Yahaya, 2018). Currently, adult stem cells are considered a promising strategy for addressing ALI, as they have the potential to alleviate the primary pathologies associated with the condition (Xiao *et al.*, 2020; Dos Santos *et al.*, 2022). Stem cell-based therapy for treating ALI is recognized as a promising approach, offering both symptom control and potential curative benefits. Stem cell-based therapy holds outstanding prospects in the curing of a wide range of medical illnesses, but there are still many challenges that need to

be overcome, including issues related to safety, efficacy, and ethical concerns, so it is important to conquer these obstacles before stem cell-based therapy in clinical practice. However, no investigation has been performed to compare the protective nature of diverse stem cells until now. Of these stem cells, MSCs are expected to offer the highest promise for allogeneic therapy, because a vast number of preclinical research as well as clinical trials have proved their efficacy and safety.

1.3.2 Cell therapy using MSCs

Due to their multi-lineage differentiation capability, robust ability to modulate inflammation response and immune system, diverse sources, readily available harvesting, and substantial preclinical investigations, MSCs may currently be the greatest candidate in clinical trials (Kim & Park, 2017; Mönch *et al.*, 2023). MSCs are non-hematopoietic multipotent stem cells originating from numerous different tissues, including bone marrow (BM), umbilical cord (UC), menstrual blood (Men), adipose tissue (AD), placenta, dental pulp of deciduous baby teeth, and organ tissues such as the liver, spleen, and lung (Samsonraj *et al.*, 2017). MSCs are classified by the International Society of Cellular Therapy (ISCT) based on specific criteria: 1) They adhere to a plastic surface in standard tissue culture situations; 2) they express certain cell surface markers like CD73, CD90, and CD105, while lacking the expression of other markers such as CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR; and 3) they are capable of differentiation into osteoblasts, adipocytes, and chondroblasts *in vitro* (Dominici *et al.*, 2006). Numerous investigations reveal that MSCs possess the potential in treating multiple disorders, especially for tissue injury and degenerative conditions, inflammation, and immunological diseases.

In vitro functional studies also indicate that MSCs play multiple physiological activities that are connected with their heterogeneity and tissue location of origin (Heo *et al.*, 2016; Klimczak & Kozlowska, 2016; Sacchetti *et al.*, 2016). MenSCs, one kind of adult stem cell, are collected from menstrual blood. Due to their numerous benefits, including capacity to be isolated non-invasively and periodically, plentifully available source, strong proliferative ability, as well as the potential to be used for both autologous and allogeneic transplantation, MenSCs have gradually gained recognition as a viable therapeutic agent to supply stem cell-based therapy over the past decade (Liu *et al.*, 2019b). Additionally, they are endowed with the ability to develop into an extensive array of cell types, such as bone, cartilage, fat, neural, muscle, and endothelial cells, which makes them valuable in regenerative medicine and tissue engineering (Liu *et al.*, 2018c; Bozorgmehr *et al.*, 2020). MenSCs have been investigated for the possible benefits for the treatment of various disorders, such as cardiovascular conditions (Jiang *et al.*, 2013), neurodegenerative diseases (Tan *et al.*, 2016; Wu *et al.*, 2018; Li *et al.*, 2023), liver disease (Du *et al.*, 2023), lung disease (Sun *et al.*, 2019; Chen *et al.*, 2020), premature ovarian failure (Zhang *et al.*, 2021a), stroke, diabetes, and multiple sclerosis (Chen *et al.*, 2019). One potential concern with the use of MenSCs is the risk of contamination with pathogens, since menstrual blood can contain viruses and bacteria. However, studies have shown that the risk of contamination can be minimized through proper collection and processing techniques. It should be noted that the administration of MenSCs in clinical settings remains a relatively new field of research and there are still many questions that need to be answered.

Halim et al. previously studied the outcome of MSCs treatment on asthma-related airway inflammation via aerosolization delivery, and the results demonstrated

that MSCs relieved airway inflammation and reversed airway remodeling (Halim *et al.*, 2019). Additionally, MSCs have the potential to elude clearance by the host immune system via various mechanisms, such as exhibiting low levels of MHC I and II proteins and lacking the T-cell costimulatory molecules CD80 and CD86, leading to their characterization as 'immuno-privileged' (Lee *et al.*, 2011) or 'immune evasive' (Ankrum, Ong & Karp, 2014). Past studies provide a powerful basis for exploring innovative approaches for the treatment of inflammatory diseases. So far, several pilot clinical trials were conducted to confirm the protective role of MSCs on ARDS patients by research institutes or hospitals from all over the world, and they can be tracked on 'ClinicalTrials.gov' (Table 1.4). The aim of most of the clinical trials was to evaluate the safety and efficiency of MSCs for individuals suffering ARDS, including COVID-19-related ARDS. There are nine early-stage clinical trials have been completed and four with updated results, which demonstrated that one dose of MSCs, such as AD-MSCs or BM-MSCs, with intravenous delivery was safe for moderate to severe ARDS patients and showed a trend for improvement in oxygenation index (Zheng *et al.*, 2014; Wilson *et al.*, 2015; Matthay *et al.*, 2019a), and also discovered that UC-MSCs infusions with double doses in COVID-19-related ARDS were safe and caused a significant reduction in inflammatory cytokines at day 6 after the 1st dose injection (Lanzoni *et al.*, 2021). However, there are multiple challenges for evaluation of treatment efficiency, as dosage, time interval, delivery route, and illness severity must be considered and compared between MSC-treated and placebo groups. The optimal therapeutic dosing of MSCs for the treatment lung diseases remains uncertain at present. In addition, and perhaps more importantly, we do not know whether it is necessary to deliver multiple doses of MSCs to treat advanced ALI animal models or ARDS patients.

Table 1.4 Clinical trials of MSC-based therapies for ARDS patients.

No.	ID	Phase	Treatment	Intervention	Enrolment	Follow-up	Status	Results	Country	Reference
1	NCT04625738	II	hUC-MSCs	1/0.5/0.5 million cells/kg, 3 dose with 2 days interval	30	28 days	Completed	No results posted	France	No reference available
2	NCT04355728	I/II	hUC-MSCs	100 million cells/infusion, double dose with 48h interval, i.v.	24 (12/12)	90 days	Completed	Lower adverse events, higher survival at D31 and D60 post 1st infusion, and longer ventilator-free days at D28 post 2nd infusion in MSCs group.	USA	(Lanzoni *et al.*, 2021)
3	NCT04390139	I/II	hUC-MSCs	i.v., no details	26 (13/13)	12 months	Completed	No results posted	Spain	No reference available
4	NCT04537351	I/II	hMSCs	2 million cells/kg, single dose, i.v.	14	28 days	Completed	No results posted	Australia	No reference available
5	NCT01902082	I	hAD-MSCs	1 million cells/kg, single dose, i.v.	12 (6/6)	28 days	Completed	MSC administration is safe and well tolerated, but significant difference on clinical outcome was weak	China	(Zheng *et al.*, 2014)
6	NCT01775774	I	hBM-MSCs	Dose-escalation: 1/5/10 million cells/kg, single dose, i.v.	9 (3/3/3)	12 months	Completed	All MSC dose levels were well tolerated, with no infusion-related adverse events	USA	(Wilson *et al.*, 2015)
7	NCT02097641	II a	hBM-MSCs	10 million cells/kg, single dose, i.v.	60 (40/20)	12 months	Completed	A trend for improvement in oxygenation index was observed in the MSC group, but it was not significant.	USA	(Matthay *et al.*, 2019a) Extension of NCT01775774
8	NCT02804945	II	hBM-MSCs	3 million cells/kg, single dose, i.v.	20	60 days	Completed	No results posted	USA	No reference available

9	NCT02611609	I/II	MultiStem	Low/high dose, no details	36	12 months	Completed	No results posted	USA/UK	No reference available
10	NCT04615429	II	hMSCs	1 million cells/kg, single dose, i.v.	20	12 months	Recruiting	No results posted	Spain	(Payares-Herrera *et al.*, 2021)
11	NCT05240430	I	hUC-MSCs	1 million cells/kg, single dose, i.v.	1	4 weeks	Recruiting	No results posted	Turkey	No reference available
12	NCT03608592	Not Applicable	hUC-MSCs	1 million cells/kg, single dose, i.v.	26	60 days	Recruiting	No results posted	China	No reference available
13	NCT02444455	I/II	hUC-MSCs	0.5 million/kg, once daily for 3 days. i.v.	20	14 days	Unknown	No results posted	China	No reference available
14	NCT02095444	I/II	hMens-MSCs	10 million cells/kg, twice a week for 2 weeks, i.v.	20	14 days	Unknown	No results posted	China	No reference available
15	NCT02112500	II	hBM-MSCs	i.v., no details	10	28 days	Unknown	No results posted	Korea	No reference available
16	NCT04525378	I	hMSCs	Dose-escalation: 25/50/100 million cells/infusion, single dose or double dose, i.v.	20	28 days	Unknown	No results posted	Brazil	No reference available
17	NCT03042143	I/II	hUC-MSCs	Dose-escalation: 100/200/400 million cells/patient, single dose, i.v.	75	28 days	Recruiting	No results posted	UK	No reference available
18	NCT03807804	II	hBM-MSCs (MultiStem HLCM051)	900 million cells/patient, single dose, i.v.	30	28 days	Recruiting	No results posted	Japan	No reference available
19	NCT02215811	I	hBM-MSCs	Not reported	10	12 months	Unknown	No results posted	Sweden	No reference available

20	NCT03552848	Not Applicable	hUC-MSCs	1 million cells/kg, once every 4 days for 4 times, i.v.	60 (30/30)	24 months	Recruiting	No results posted	China	No reference available
21	NCT03818854	II b	hBM-MSCs	10 million cells/kg, single dose, i.v.	120 (60/60)	60 days	Not yet recruiting	No results posted	USA	Extension of NCT01775774 & NCT0209 7641
22	NCT04798716	I/II	hMSC-exosomes	Dose-escalation: 2/4/8 billion exosomes/infusion, 3-dose with 1 day interval, i.v.	55 (15/40)	90 days	Not yet recruiting	No results posted	USA	No reference available
23	NCT04466098	II	hMSCs	300 million/infusion, 3-dose with 48h interval, i.v.	9 (6/3)	100 days	Active, not recruiting	No results posted	USA	No reference available
24	NCT05491681	I	hBM-MSCs	Dose-escalation: 20/100/200 million cells/infusion, single dose, i.v.	9 (3/3/3)	12 months	Not yet recruiting	No results posted	USA	No reference available
25	NCT04377334	II	hBM-MSCs	Not reported	40	Not reported	Not yet recruiting	No results posted	Germany	No reference available
26	NCT04452097	I/II	hUC-MSCs	Dose-escalation: 0.5/1/1.5 million cells/kg, single dose, i.v.	39(9/30)	28 days	Not yet recruiting	No results posted	USA	No reference available

Notes: MSCs, mesenchymal stem cells; BM, bone marrow-derived; CT, cord tissue-derived; UC, umbilical cord-derived; AD, adipose-derived; Mens, menstrual blood-derived; h, human; i.v., intravenous.

Despite the fact that the precise therapeutic mechanisms by which MSCs relieve ALI are remain unknown, recent preclinical studies have provided quite a few valuable insights (Figure 1.2), and they include but are not limited to cell-to-cell interactions, secretion of soluble factors, such as growth factors, matrix proteins, cytokines and EVs, as well as mitochondrial transfer (Abraham & Krasnodembskaya, 2019; Lee, Park & Lee, 2019; Lopes-Pacheco *et al.*, 2019). MSCs have demonstrated significant contributions to anti-inflammatory and anti-apoptotic activities, promoting the regeneration of epithelial and endothelial cells, and boosting microbial and alveolar fluid clearance, all of which improve lung and distal organ injury and ultimately extend patients' survival (Morrison *et al.*, 2017; Pedrazza *et al.*, 2017; Xiang *et al.*, 2017; Ren *et al.*, 2018; Lopes-Pacheco *et al.*, 2019).

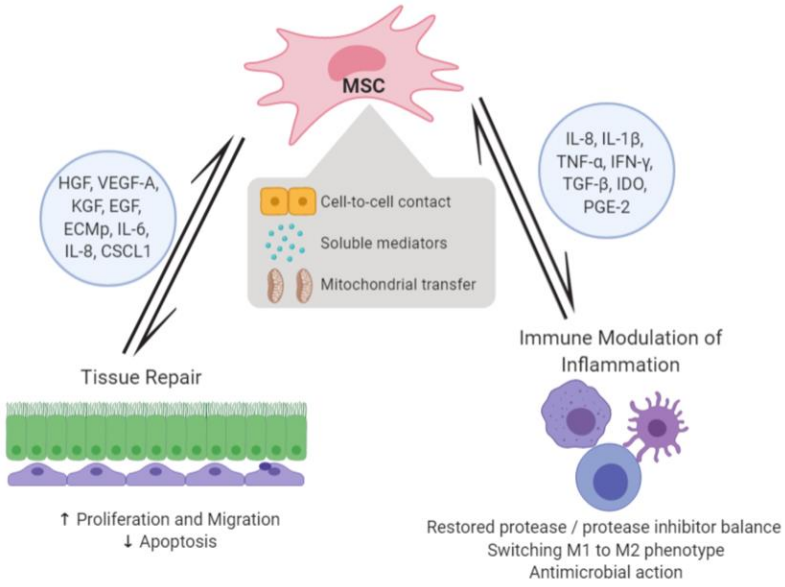

Figure 1.2 Mechanisms underlying the modulation of inflammation and lung tissue repair by MSCs in ALI (Lian *et al.*, 2020). Created with BioRender.com.

In support of these findings, Halim et al. found that MSC-secreted proteins enhanced airway epithelial repair by increasing lung cells regeneration and endogenous reparation, with the majority of proteins being extracellular proteins (Halim, Aizat & Yahaya, 2018). Furthermore, it has been shown that MSCs reduce

LPS-induced ALI by downregulating miR-142a-5p, which promotes autophagy in pulmonary endothelial cells by elevating the protein Beclin-1 (Zhou & You, 2016). The NF-κB, MAPK, and STAT3 signaling pathways are all have been proposed to be implicated in the effects of MSCs treatment using the ALI animal model, although additional research is required to clarify the therapeutic mechanism. So far, our lab has investigated the feasibility of cell therapy in both chronic (Halim *et al.*, 2019; Ridzuan *et al.*, 2021) and acute lung disease (Kardia, Ch'ng & Yahaya, 2018); the findings were consistent in that MSCs or AECs reduced inflammation of the lung and airways and improved lung regeneration. In conclusion, these studies have contributed crucial information and compelling evidence to support the efficacy of stem cells for treating lung inflammation and injury conditions.

Nonetheless, cell-based therapy runs the danger of microvasculature congestion and uncontrol proliferation *in vivo*. The likelihood of developing a tumor is the first of these issues, then substantial cell numbers are necessary for clinical protocols requiring extensive ex-vivo expansion. Human derived MSCs stability issues have raised questions regarding whether these cells are stable enough to be used in therapeutic applications (Naji *et al.*, 2019; Neri, 2019). Numerous research reports indicate that MSCs may have a positive influence in triggering the development and spread of cancer by acting as cancer-initiating cells or by interacting with stromal components (Herberts, Kwa & Hermsen, 2011; Dong *et al.*, 2018). The evidence also demonstrates that MSCs can hasten tumor growth by migrating to the tumor site, settling there and altering its microenvironment, then producing cytokines that promote tumor growth (Lee & Hong, 2017; Al-Kharboosh *et al.*, 2020; Liang *et al.*, 2021). Despite the fact that MSCs have the ability to stimulate tumor growth, there is no indication from MSCs clinical trials to confirm