THE EFFECTS OF AEROSOL-BASED CELL DELIVERY TECHNIQUE ON THE REGENERATION AND REPAIR OF AIRWAY EPITHELIUM

by

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

August 2017

ACKNOWLEDGEMENT

The work presented in this thesis would not have been possible without the association of many people. Thus, I would like to take this opportunity to acknowledge them and extend my sincere gratitude for helping me to make this thesis a possibility. I thank Allah SWT for giving me the strength and patience to work through all these years so that today I can stand proud with my head held high.

I must offer my sincerest gratitude to my supervisor, Assoc. Prof. Dr. Badrul Hisham Yahaya, who has guided me throughout my thesis with his patience and knowledge. I owe a lot of gratitude to his guidance and encouragement, without him this thesis would have not been completed or written. A special thanks to Dr. Rafeezul Mohamed, Prof. Narazah Mohd. Yusoff, and Dr. Zubaidah Zakaria for the guidance, support, as well as being my co-supervisors.

Heartfelt thanks to my fellow labmates, Nurulain ‘Atikah, Nurfatin Asyikhin, Ahmad Zaeri, Norashikin, Nur Shuhaidatul, Nazilah, Noor Hanis, and Noridzzaida for all of your help and support in this project. I am also grateful to Dr. Ewe Seng Ch’ng (Pathologist of AMDI) along with the staff in the Regenerative Medicine Cluster Laboratory, Animal Research Facility, and Histopathology Unit of Advanced Diagnostic Laboratory for their guidance in the animal study and histopathological assessment.

A special shout out to my closest friends, Lola Febriana Dewi and Eldia Anggi Denia who were always a great support in all my struggles and study. Finally, I would like to extend my respect to thank my family especially my parents; Assoc. Prof. Dr. Erry Moch. Arief and Nagibah, sisters, and my late grandparents, Fatmah Umar Al-Musawa and Asnawi Rachmat. Thank you for always standing behind me and encouraging me with love and support.

This study was also supported by grants from the Universiti Sains Malaysia (USM) Short Term Grant Scheme (304/CIPPT/61312001) and USM Research University Grant (1001/CIPPT/813059).

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
α-SMA	α-smooth muscle actin
AB-PAS	Alcian blue-periodic acid-schiff
ALI	Acute lung injury
AQP-1	Aquaporin 1
ARDS	Acute respiratory distress syndrome
ARF	Animal research facility
ATI	Alveolar epithelial type I cell
ATII	Alveolar epithelial type II cell
BADJ	Bronchoalveolar duct junction
BAL	Bronchoalveolar lavage
BEBM™	Bronchiole Epithelial Basal Medium
BEGM™	Bronchial Epithelial Growth Medium
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic acid
CCSP	Clara cell secretory protein
CGRP	Calcitonin gene–related peptide
Cld3	Claudin 3
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CXCL-1	Chemokine (C-X-C motif) ligand 1
DAB	3,3' Diaminobenzidine

DAD	Diffuse alveolar damage
DAPI	4',6-diamidino-2-phenylindole
Dll1	Delta-like1
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DPI	Dry powder inhalers
E9.5	Embryonic day 9.5
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EPC	Endothelial progenitor cells
ESC	Embryonic stem cell
EV	Extracellular vehicle
FISH	Fluorescence in situ hybridization
FBS	Fetal bovine serum
FoxJ1	Forkhead Box J1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony-stimulating factor
H&E	Haematoxylin & Eosin
HCl	Hydrochloric acid
hEGF	Human epidermal growth factor
HLA	Human leukocyte antigen

HOPX	HOP Homeobox
ICAM-1	Intercellular Adhesion Molecule 1
ICU	Intensive Care Unit
IFN-γ	Interferon-γ
IL-6	Interleukin 6
K8	Keratin 8
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MCP-1	Monocyte chemotactic protein 1
MEM	Minimum Essential Medium
MlP-1a	Macrophage inflammatory protein-a
MMD	Mass Median Diameter
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
MSC	Mesenchymal stem cell
Muc5ac	Mucin-5ac
NEBs	Neuroendocrine bodies
NGFR	Nerve Growth Factor Receptor
p63	Transcription factor-63
PBS	Phosphate buffered saline
Pdpn	Podoplanin
PLUNC	Palate lung nasal epithelial clone
pMDI	Pressurised metered dose inhaler
ProSPC	Prosurfactant Protein C
qRT-PCR	Qualitative real time polymerase chain reaction

RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
S1PR3	Sphingosine-1–phosphate receptor–3
Scgb	Secretoglobin
SO_2	Sulphur dioxide
SPDF	SAM Pointed Domain Containing ETS Transcription Factor
SRY	Sex-determining region Y
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor-β
Th17	T Helper 17
TIMP	Tissue inhibitors of metalloproteinases
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
VCAM	Vascular Adhesion Molecule
VEGF	Vascular endothelial growth factor
VILI	Ventilator-Induced Lung Injury
WHO	World Health Organization
ZO-1	Zonula occludens-1

LIST OF SYMBOLS

™	Trademark
®	Registered trademark
ºC	Degree celcius
~	Approximately
%	Percentage
nM	Nano Molar
M	Molar
µg	Microgram
mg	Miligram
g	Gram
kg	Kilogram
mmol/L	Milimol per litre
µL	Microliter
mL	Mililiter
µm	Micrometer
mm	Milimeter
cm	Centimeter
cells/mL	Cells/mililiter
kPa	Kilo Pascal
RPM	Revolutions per minute
x g	g force

KESAN-KESAN TEKNIK PENGHANTARAN SEL SECARA EROSOL KE ATAS REGENERASI DAN PEMBAIKAN EPITELIUM PERNAFASAN

ABSTRAK

Teknologi penghantaran sel berasaskan erosol telah muncul sebagai satu kaedah strategi terapeutik yang berpotensi dalam mengatasi kelemahan pembaikan dalam kapasiti trakea serta paru-paru selepas kecederaan. Objektif kajian ini adalah untuk menentukan kesan penghantaran sel epitelia pernafasan (AEC) berasaskan erosol ke atas proses pemulihan dan rawatan trakea, kerosakan paru-paru, dan respon keradangan setempat dalam kecederaan paru-paru akut (ALI) yang disebabkan oleh teknik pemberusan pada trakea arnab. AEC berasal dari trakea arnab putih New Zealand. Penghantaran sel dengan teknik *in vitro* dilakukan untuk menilai keupayaan sel untuk hidup. Teknik pemberusan pada trakea dilakukan adalah bertujuan untuk mencederakan lapisan epitelium peparu. Sehari selepas kecederaan, sel AEC arnab yang telah di label dengan BrdU dihantar ke dalam trakea yang cedera menggunakan *MicroSprayer® Aerosolizer*. Penilaian ke atas keselamatan dan ketoksikan telah dilakukan bagi memerhati tindakbalas haiwan terhadap rawatan sel. Penilaian histopatologi bagi kecederaan trakea dan paru-paru serta respon keradangan setempat dan sistemik telah diukur secara kuantitatif dalam tempoh sehari dan lima hari selepas penghantaran sel. Dua kultur *in vitro* telah dilakukan untuk menentukan kesan fungsi AEC ke atas proses pemulihan dan rawatan trakea. Dalam kondisi tekanan erosol, AEC mampu mengekalkan keupayaan untuk hidup tanpa kesan ke atas ciri-ciri morfologi serta menunjukkan kebolehupayaan proliferasi yang tinggi. Teknik pemberusan pada trakea arnab telah mengakibatkan gangguan pada struktur dan komposisi sel epitelium trakea serta kerosakan alveolar terserap yang mencerminkan ciri-ciri ALI. Penghantaran sel AEC secara erosol merupakan suatu prosedur yang selamat kerana

tiada penolakan pada peringkat sel dan membawa kepada peningkatan di dalam regenerasi dan pembaikan epitelium di trakea, pemulihan respon keradangan pada kerosakan paru-paru. Mekanisme utama yang mendasari kesan terapeutik yang positif adalah disebabkan oleh sebatian yang dirembeskan oleh AEC. Kajian ini memberikan pandangan tentang teknologi penghantaran sel berasaskan erosol secara selular dan molekular, teknik ini akan menjadi asas untuk terapi masa hadapan bagi merawat kecederaan paru-paru.

THE FFFECTS OF AEROSOL-BASED CELL DELIVERY TECHNIQUE ON THE REGENERATION AND REPAIR OF AIRWAY EPITHELIUM

ABSTRACT

Aerosol-based cell delivery is a potential therapeutic strategy to overcome the debilitating reparative capacity of trachea and lung following injury. The goal of this study was to determine the effect of aerosol-based airway epithelial cell (AEC) delivery on tracheal repair and regeneration, lung damage, and local inflammatory responses in the setting of acute lung injury (ALI) induced by tracheal brushing in rabbit. AECs were isolated from the trachea of New Zealand white rabbits. *In vitro* aerosol delivery was performed to assess the viability of the cells. Brushing-induced tracheal injury was performed in a rabbit model to develop ALI. One day following injury, exogenous BrdU-labelled AECs were aerosolized using the MicroSprayer® Aerosolizer into the injured airway. Assessment involving safety and toxicity were carried out to observe the animal responses towards cell treatment. Histopathological assessments of the injury in the trachea and lungs along with local and systemic inflammatory responses were quantitatively measured at one and five days after cell delivery. Two *in vitro* co-culture assays were performed to investigate the functional effect of AECs on tracheal regeneration and repair. Under aerosol pressure, AECs were able to maintain a high viability rate without affecting their morphological features and proliferative capability compared. Brushing-induced tracheal injury exfoliated tracheal epithelium layer and triggered alveolar damage-associated ALI. Following treatment, aerosol-based AEC delivery appeared to be a safe procedure and positively modulate tracheal epithelium repair and regeneration, reduce inflammation, and attenuate lung injury in the rabbit model of ALI. The key mechanisms underlying this positive impact is due to the secretory factors that were released by AEC. This study

provides cellular and molecular insights of aerosol-based cellular therapy to form a basis evidence for future therapy to treat lung injuries.

CHAPTER I

GENERAL INTRODUCTION

1.1 Anatomy of the respiratory airway

1.1.1 The branching of respiratory airway

The lung is made up of a complex branching networks of airway (Metzger et al., 2008). Respiratory tree structure goes from the larger airways branch into smaller airways until reaches the surface of the lung (Whittemore, 2009). The airway provides a pathway for the air to flow in and out from the lung periphery, while alveoli perform the gas exchange. Based on this concept, the lung is divided into two general zones: the conducting zones and the respiratory zone (Figure 1.1).

The conducting zone comprises upper (nasal cavity, pharynx and larynx) and lower (trachea, bronchi and bronchioles) respiratory tract (Iwasaki et al., 2017). The trachea bifurcates into two main stem bronchi that further subdivide into bronchioles (Hickey, 2007). Meanwhile, oesophagus is positioned directly posterior to the cervical part of the trachea and typically extends slightly to the left of the intrathoracic part (Sasson et al., 2003). The respiratory zone begins with respiratory bronchioles that continue to subdivide into alveolar ducts and finally alveolar sacs (Hickey, 2007). This zone represents about 85% of the total lung volume, whereas the conducting airway zone covers only about 6 to 10%. The remaining part, in addition, consists of nervous and vascular tissue (Gehr, 1984).

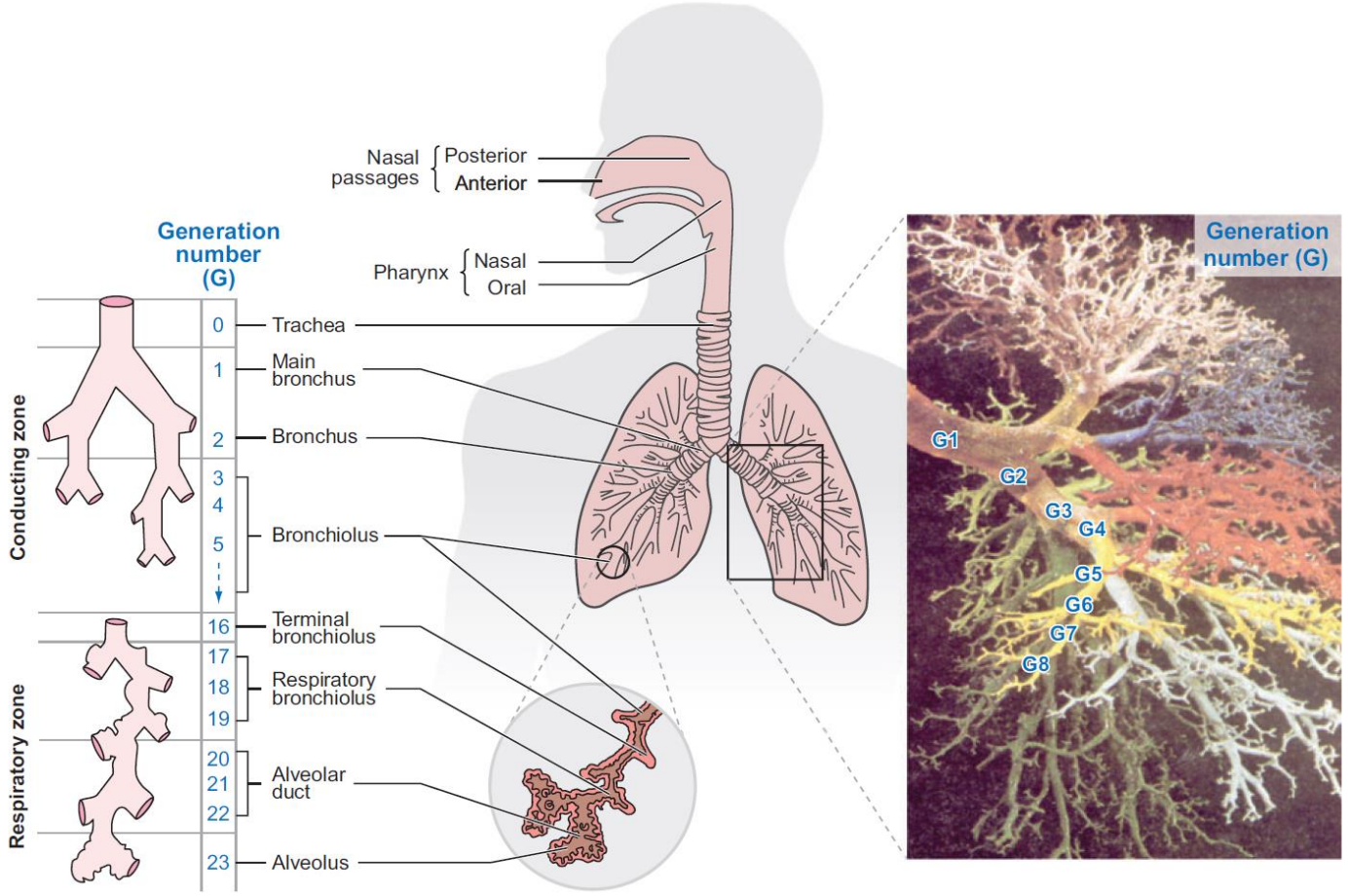

Figure 1.1 Schematic of the human respiratory system. Respiratory airway is divided into two general zones, the conducting zones and the respiratory zone (Adapted from (Kleinstreuer et al., 2008)).

1.1.2 The cellular structure of respiratory airway

1.1.2(a) Trachea and main bronchi

Trachea consists of a single tube with a C-shaped cartilage rings that are connected posteriorly by smooth muscle and connective tissue (Hickey, 2007). It is positioned midline in the neck and facilitated the passage of air between the larynx and the lungs (Sasson et al., 2003). Cartilage is composed of chondrocytes and only present surrounding the trachea and bronchi (Pérez, 2007). The first cartilage rings is broader than the rest of the tracheal rings and partly recessed into the cricoid cartilage (Sasson et al., 2003). The purpose of cartilage tissue is to retain the epithelium and to prevent the trachea from collapsing due to extreme circumstances such as coughing and forced expiration (Whittemore, 2009).

Pseudostratified epithelium consists of ciliated, basal, secretory/goblet, and neuroendocrine cells that line the basement membrane of the trachea and main stem bronchi. (Knight and Holgate, 2003, Roomans, 2010) (Figure 1.2a). Ciliated cells are the predominant cell type in the trachea and bronchi and a terminally differentiated cell population (Rawlins and Hogan, 2008) that expressed keratin 8 (K8) and 18 (K18) (Cole et al., 2010). Secretory/goblet cells, indeed, produce and secrete mucus (Rock et al., 2010) that marked by the expression of transcription factor SAM Pointed Domain Containing ETS Transcription Factor (SPDEF) and mucin-5ac (Muc5ac) (Kotton and Morrisey, 2014). Together with ciliated cells, both cells are responsible for driving the process of mucocilary clearance (Holtzman et al., 2014). Ciliated cells and secretory cells are considered as the luminal epithelial cells that constitute the barrier defence in larger airways (Gizurarson, 2012, Iwasaki et al., 2017). These luminal cells are in direct contact with microbes and particles that must be removed or accommodated to prevent their access to underlying epithelial cells (Whitsett and Alenghat, 2015). When

a pathogen manages to enter the airway, goblet cells secrete mucins in a gel-like form and create a barrier that bind bacterial/microbial pathogens from adhering to the conducting airways (Voynow and Rubin, 2009). The removal of particles and pathogens from the airway is called mucociliary clearance that orchestrated by ciliated cells' sweeping motion (Gizurarson, 2012, Holtzman et al., 2014). Basal cells are located at the base of pseudostratified epithelium, rich of hemidesmosomes, and characterized by the expression of transcription factor-63 (p63), K5, K14, and Nerve Growth Factor Receptor (NGFR) (Rock et al., 2009, Kotton and Morrisey, 2014, Weiss et al., 2015). These cells are defined as an 'anchor' to the pseudostratified epithelium due to the fact that they provide the major stem/progenitor cell function that are capable of giving rise to ciliated and secretory cells (Cole et al., 2010, Hackett et al., 2011, Crystal, 2014, Weiss et al., 2015).

1.1.2(b) Bronchiole

In more distal airways, cartilage disappears and is replaced by smooth muscles that surrounds the bronchiole epithelium (Whittemore, 2009). The bronchiole region is lined with a monolayer simple cuboidal epithelium that consist of secretory/club (formerly known as Clara cells), ciliated, and neuroendocrine epithelial cells (Kotton and Morrisey, 2014) (Figure 1.2b). A few basal cells are also present in the distal airway, however they gradually diminish in number distally (Hamid et al., 2005). Neuroendocrine cells are commonly clustered in neuroendocrine bodies (NEBs) that express calcitonin gene–related peptide (CGRP) (Kotton and Morrisey, 2014). These cells are generally found along the proximal bronchioles and the bronchoalveolar duct junction (BADJ) of terminal bronchioles (Snyder et al., 2009). The secretory/club cells, meanwhile, are defined as non-ciliated, columnar cells that contain abundant

endoplasmic reticulum and dense secretory granules (secretoglobin (Scgb)) (Nettesheim et al., 1990, Rokicki et al., 2016). The club cells are commonly marked by the expression of Scgb1a1 and Scgb3a2 (Reynolds et al., 2002). Similar to the upper airway epithelium, the bronchiole epithelium is also in quiescent state until activated by injury (Kotton and Morrisey, 2014).

1.1.2(c) Alveoli

Alveoli are the site for exchanging respiratory gases (Whittemore, 2009). The alveoli permit gas exchange by providing structure and thin air-surface barriers for oxygen diffusion into the capillary (Herring et al., 2014). Mature alveoli are made up of two major distinct epithelial cell types, ATI and ATII (Figure 1.2C). ATI is a flat squamous epithelial cells that cover >95% of the gas exchange surface (Yang et al., 2016). The cells are surrounded by a thin cytoplasm (Fraser, 2005, Hamid et al., 2005, Whittemore, 2009) and identified by the expression of Podoplanin (Pdpn) and HOP Homeobox (HOPX) (Jain et al., 2015). The thin and flat shape of ATI facilitate their close contact with capillaries to perform gas exchange (Jain et al., 2015). Meanwhile, ATII is cuboidal in shape with round nucleus and produces surfactant protein such as Prosurfactant Protein C (ProSPC) that is stored in specialized lamellar bodies (Jain et al., 2015). These cells also regulate the alveolar lining fluid to support alveolar surface tension and prevent alveolar collapse (Fehrenbach, 2001, Manzer et al., 2006, Desai et al., 2014). ATI cells are generally considered terminally differentiated (Yang et al., 2016), while ATIIs are called the “stem cells” of the alveoli because of their ability to self-renew and give rise to ATI (Kotton and Morrisey, 2014).

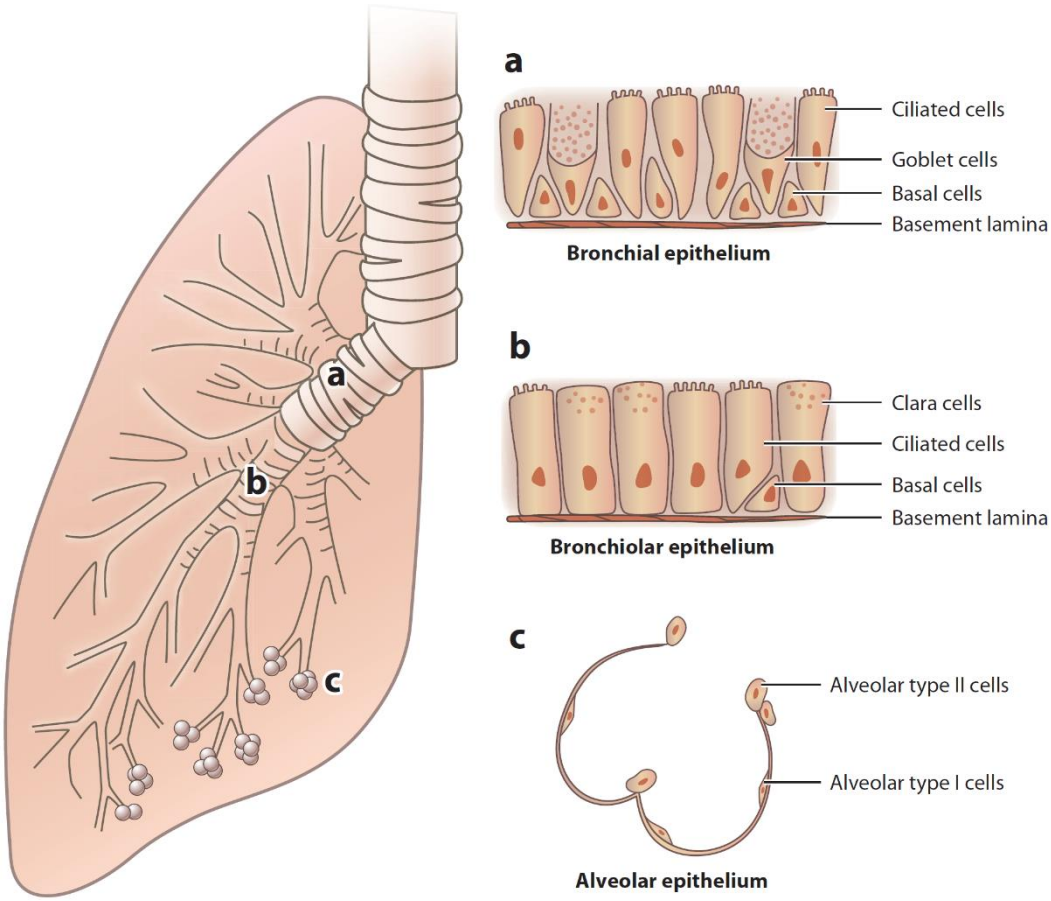

Figure 1.2 Epithelial cell type in the conducting airways (nasal, trachea, bronchi, and bronchiole) and respiratory airways (alveoli). a) Tracheobronchial of the proximal cartilaginous airway are lined with a pseudostratified epithelium that consist of ciliated, basal, and secretory/goblet cells, b) In the more distal airways, the bronchiole region lined with a simple cuboidal epithelium consisting of ciliated, basal, and secretory/club cells, and c) gas-exchanging airspace (alveolar region) consisted of two major distinct epithelial cell types, ATI and ATII. Adapted from (Wetsel et al., 2011).

1.1.3 Lung stem cells niche

The ability of lungs to maintain itself and repair after injury is dependent on the activity of endogenous lung stem cells. These lung stem cells respond to exogenous cues from the niche, a surrounding environment of local tissue (Hegab et al., 2015, Donne et al., 2015). The function of the niche is to control long-term maintenance of lung stem cell as well as to influence the stem cell to self-renew and/or differentiate into the appropriate lineages (Volckaert and De Langhe, 2014, Savukinas et al., 2016). This behaviour of lung stem cells depends on the integration with other cell signals and direct contact with the underlying extracellular matrix (ECM) as well as with neighbouring epithelial cells (Volckaert and De Langhe, 2014).

Epithelial cell lining provides structural integrity and is the first line of defence against the external environment to the respiratory airway by their intracellular junctional proteins and secretory products (Blanpain et al., 2007, Whitsett and Alenghat, 2015). The loss of these cells due to wear and tear, inflammation, or injury requires the presence of stem cells to constantly reconstitute the epithelium lining. The process this continuous cell replacement is called tissue homeostasis and is critical for adult tissue maintenance (Blanpain et al., 2007). Adult stem cell is defined as cell from adult tissues that are multipotent and have the capacity for long term self-renewal and differentiation into the cell lineages of their tissue of origin (Weiss et al., 2015). Studies in human and animal lung defined subsets of lung epithelial cells with self-renewal and proliferative capacity, including basal (Hong et al., 2004b, Hajj et al., 2007, Cole et al., 2010, Rock et al., 2011, Crystal, 2014, Pardo-Saganta et al., 2015), club cells (Plopper et al., 1992, Stripp and Reynolds, 2008, Rawlins et al., 2009) of the tracheobronchial tree, and ATII cells (Fehrenbach, 2001, Barkauskas et al., 2013, Zeng

et al., 2016) of the alveoli. Under normal homeostasis, these cells are functioned as stem cells that give rise to airway epithelial cell lineages.

In the proximal region, studies have shown the potential of $K5^+$ $K14^+$ basal cell to proliferate and differentiate into secretory and ciliated epithelial cells in response to chlorine- (Musah et al., 2012), naphthalene- (Hong et al., 2004b) and cystic fibrosis-induced airway injury (Voynow et al., 2005) Another study also demonstrated that basal cells generate a multipotent $p63^-$ $K8^+$ luminal cells that includes secretory and ciliated cells after exposure to sulphur dioxide (Rock et al., 2011). *In vitro*, basal cells from the mouse trachea that expressed $p63^+$, Nerve Growth Factor Receptor ($NGFR^+$), and $K5^+$ underwent self-renewal and generate 'tracheospheres' that contained both ciliated and secretory cells (Rock et al., 2009).

In the distal bronchioles, club cells are more abundant than ciliated cells (Morrisey and Hogan, 2010), while basal cells are gradually diminish in number. Therefore, club cells in the bronchiole region are the cell population that maintains the facultative progenitor cell pool (self-renewal) and give rise to ciliated cells of the airway epithelium. According to a few studies, proliferative club cells that express Clara cell secretory protein (CCSP) were identified at the terminal bronchiolar region and capable of epithelial renewal after naphthalene-induced injury (Reynolds et al., 2000, Hong et al., 2001, Giangreco et al., 2002). A study also suggested a rise of stem cell population in the bronchioalveolar duct junctions that expressed both club cell (Scgb1a1 and CC10) and ATII cell marker following naphthalene-induced injury (proSPC) (Kim et al., 2005), therefore indicating a combination of two stem cell population in the terminal bronchioalveolar region.

Meanwhile, ATII cells are endogenous stem cell population in the alveoli region and have the ability to self-renew and give rise to ATI during both lung development and repair after injury. Rock et al. investigated that proSPC$^+$ ATII cells are multipotent progenitors for the alveolar epithelium that give rise to ATI cells under steady state conditions, after bleomycin injury, and post-pneumonectomy (Rock et al., 2011). It is believed that when injured, ATI cells secreted an epidermal growth factor (EGF) that activated ATII to differentiate into ATI cells (Desai et al., 2014). *In vitro*, ATII cell culture has been proven to have the ability of forming a three-dimensional (3D) organoid culture called alveolospheres that comprised both ATII cells and HOPX$^+$ ATI cells (Barkauskas et al., 2013).

Additional cell-cell contact and signalling is required to activate the local response as well as engaging this self-renewal and differentiation events in an attempt to restore airway structural and functional integrity (Pongracz and Stockley, 2006). Signal network such as Wnt signalling is essential and marked during early lung development (Okubo and Hogan, 2004, Cohen et al., 2009, Goss et al., 2009). Wnt signalling also has been implicated in the modulation of cell fate decisions and differentiation of lung cell types (Hogan et al., 2014, Frank et al., 2016). Studies have investigated that Wnt signal production is specific to lung cell types, for example, Wnt2 has been mapped predominantly to the mesenchyme (Monkley et al., 1996), while Wnt7b regulated epithelial and mesenchymal cell proliferation and also vascular development in the lung (Weidenfeld et al., 2002, Shu et al., 2002, Rajagopal et al., 2008). It is therefore understandable that airway repair process is influenced by a combination of signalling from cells such as epithelial and/or their neighbouring cells.

The behaviour of lung stem cells also depends on the integration with the underlying ECM. The repair process of airway epithelium started with epithelial cell from neighbouring the wound receiving signals from the damage cells. The endogenous stem cells retrieve the signalling and respond to injury by cell migration, proliferation, and differentiation processes (Yahaya, 2012, Girault and Brochiero, 2014), which are guided by the integrin-mediated cell adhesion to the ECM in the niche (Volckaert and De Langhe, 2014). Studies have reported that a reduced integrin and ECM expression in adherent cells triggers terminal differentiation of cultured epidermal stem cells (Grose et al., 2002, Watt, 2002).

Together, these data suggest that lung stem cells are subjected to a tight regulatory process before they are activated and gave an appropriate regenerative response during tissue homeostasis, which involve a dynamic mechanism between stem cells and their niche.

1.2 Lung disorders

No organ is more vital and vulnerable than the lung. Lung diseases have become a major concern in medical care and causes an immense worldwide health burden. According to World Health Organization (WHO) statistics, non-communicable diseases, including cardiovascular diseases, cancers, diabetes, and chronic lung diseases were responsible for almost 68% of all deaths worldwide in 2012, which arise from 60% in 2000. In 2015, chronic obstructive pulmonary disease (COPD) alone was responsible for 3 million deaths worldwide. The rise of these non-communicable diseases is mostly driven by excessive tobacco use, physical inactivity, the harmful use of alcohol, and unhealthy diets. Acute respiratory distress syndrome/acute lung injury (ARDS/ALI) is also a devastating disease spectrum that

associated with morbidity and mortality worldwide. ARDS cases, meanwhile, represent an incidence rate of nearly 9% of ventilated patients in Intensive Care Units (ICUs) (Roupie et al., 1999). In addition, mortality rate of patients with ARDS/ALI from 1994 to 2006 was 44.0% (From databases: MEDLINE, EMBASE, CINAHL, Cochrane CENTRAL) (Phua et al., 2009).

1.2.1 Chronic lung injury

Two major debilitating chronic lung injury are COPD and asthma. Exposure to tobacco smoke, allergens, infectious agents, and noxious gases can induce chronic injuries of airway epithelium. Asthma and COPD are both chronic inflammatory conditions of the conducting airways and lung parenchyma (Jeffery, 2001).

1.2.1(a) Chronic obstructive pulmonary disease (COPD)

Individuals with COPD commonly experience shortness of breath as well as increased coughing associated with increased sputum production and are prone to developing serious conditions, such as recurring chest infections, respiratory failure, pulmonary hypertension, and heart failure (Chung and Adcock, 2008). While cigarette smoking is the principal cause of COPD, chronic exposures to pollutants can also contribute to the development and/or exacerbation of COPD (Cosio and Guerassimov, 1999). COPD is also associated with emphysema that is characterized by difficulties in breathing due to obstructed airflow (Mannino and Buist, 2007). Patients with emphysema have a loss of alveolar surface area available for gas exchange due to destruction of alveolar septa (Yoshida and Tuder, 2007). Other pathogenesis of COPD also includes inflammatory responses in the airway, loss of barrier function, alterations of cell growth, cellular apoptosis, abnormal cell repair, ECM destruction, and

oxidative stress. These features are important events in the pathogenesis of COPD (Yoshida and Tuder, 2007, Ryter et al., 2009). According to O'Donnell et al., COPD-related histopathology has increased numbers of neutrophils, lymphocytes, and macrophages (O'Donnell et al., 2006). Neutrophils release tissue damaging enzymes such as elastase that can increase epithelial mucin protein expression (Fischer and Voynow, 2000). Meanwhile, macrophages produce oxidants and potentially destructive ECM proteases (Yoshida and Tuder, 2007).

1.2.1(b) Asthma

Asthma is a complex, chronic inflammatory lung disease that leads to symptoms such as coughing, wheezing and chest tightness (Lambrecht and Hammad, 2012). Asthma is triggered as a response to various internal and external stimuli including airborne allergens, infection, exercise, cold air, smoke, beta blockers, and stress (Holgate, 2008). If left untreated, asthma can cause many more irreversible changes in lung tissue. This disorder is characterized by airway wall remodelling, airway smooth muscle contraction and hyperreactivity, increased mucus production, and inflammatory cell accumulation (Worgall et al., 2013). The walls of the conducting airways in asthma are usually thickened between 50 and 300% of normal and there is evidence of luminal narrowing (Jeffery, 2001). The thick appearance of airway wall is due to hyperplastic smooth-muscle cells and deposition of ECM components under the basement membrane (Evans et al., 2009, Lambrecht and Hammad, 2012). It is a common finding that epithelium of the nasal and lower airways in the patients that suffer from asthma are fragile and easily detach from the basement membrane (Lackie et al., 1997, Shahana et al., 2006). Biopsy sample from subjects with asthma often demonstrated a reduced/loss of epithelial adherent junctions (E-

cadherin) expression possibly as a result of epithelial-mesenchymal transition (EMT) (Hackett et al., 2009). The loss of E-cadherin expression might be due to proteolytic activity of inhaled cigarette smoke (Olivera et al., 2007) and allergens originating in pollen (Runswick et al., 2007) or fungi (Chen et al., 2011). The local innate system of the body such as dendritic cells recognize the breach of inhaled allergens and coordinate the subsequent immune response as well as facilitating allergic sensitization towards asthma (Lambrecht and Hammad, 2012).

1.2.2 Acute lung injury (ALI)

Acute lung injury (ALI) is the less severe acute respiratory distress syndrome (ARDS) represents a spectrum of lung disease with multiple risk factors that trigger the acute onset of respiratory insufficiency (Walkey et al., 2012, Fanelli et al., 2013). The term of ARDS was first described by Ashbaugh and Petty in 1967, where it was described as “acute onset of tachypnoea, hypoxaemia, and loss of compliance” in 12 patients after a variety of stimuli-induced injury such as gastric aspiration, sepsis, blunt trauma, and near-drowning (Ashbaugh et al., 1967). In 1994, the American-European Consensus Conference Committee refined the definition of ARDS to standardize clinical research trials for the disease. Bernard et al. stated that patients with an acute onset of respiratory failure and less severe hypoxaemia (defined by a ratio of the arterial oxygen partial pressure to fractional inspired oxygen) with no evidence of left atrial hypertension or pulmonary capillary pressure < 18 mmHg are considered to have ALI, whilst those with more severe hypoxaemia are considered to have ARDS (Bernard et al., 1994). In 2011, the European Society of Intensive Care Medicine with endorsement from the American Thoracic Society and the Society of Critical Care Medicine convened an international expert panel to revise the ARDS definition in

Berlin (coined as Berlin definition). The panel agreed to modify the term “acute lung injury” and replacing it with three levels of ARDS severity (mild, moderate, and severe) based on the oxygenation, timing of acute onset, chest radiographic, and wedge pressure criterion (Table 1.1).

In clinical setting, the initial acute or exudative phase of ALI/ARDS is characterized by the rapid onset of dyspnoea, hypoxemia, respiratory failure, and bilateral infiltrates on chest radiograph that are consistent with pulmonary oedema. The pulmonary oedema is caused by the altered lung fluid balance that permits an influx of fluid and protein into the lung interstitial (Ware and Matthay, 2000, Ware and Matthay, 2005, Ware, 2006).

The occurrence of ALI can be segregated into direct and indirect pathologies (Table 1.2). Direct pathology is triggered by the injurious agent that reaches the lungs through the airways or by trauma to the chest, whereas indirect pathology implied that the injurious agent arrived at the lungs through the bloodstream (Schraufnagel, 2014). According to a study by Brun-Buisson et al., indirect ALI is often encountered from sepsis (Brun-Buisson et al., 2004). Menezes et al. performed series of intraperitoneal injection or intratracheal instillation of lipopolysaccharide (LPS) into mice to compare the direct and indirect ALI in a mice model (Menezes et al., 2005). The results showed that insult directly into the tracheal epithelium yielded more pronounced inflammatory responses. Thus, direct insult into the lungs can cause a more susceptible injury and requires therapeutic interventions when compared to the indirect ALI.

Table 1.1 American–European Consensus Conference and Berlin definition of ALI and ARDS

Characteristic	The AECC definition 1994	The Berlin definition 2012
Onset	Acute	Within 1 week of a known clinical insult or new or worsening respiratory symptoms
Chest imaging	Bilateral infiltrate on frontal chest radiograph	Bilateral opacities (not fully explained by effusion, atelectasis, or nodules)
Non-cardiogenic source of pulmonary oedema	No clinical evidence of elevated left atrial pressure or a pulmonary capillary wedge pressure < 18 mmHg	Respiratory failure not fully explained by cardiogenic pulmonary oedema 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 oedema with additional data (e.g., echocardiography)

(Adapted from Bernard et al. (1994), (Force, 2012))

Table 1.2 Clinical conditions that are associated with ALI

Direct triggers	Indirect triggers
Pneumonia	Sepsis
Gastric aspiration	Trauma
Pulmonary contusion	Burn injury
Pulmonary embolism	Transfusion
Smoke and toxic gas inhalation	Bypass surgery
Reperfusion injury	Intoxication
Near drowning	Acute pancreatitis
	Drug overdose (e.g. heroin)

(Adapted from Ware (2006), Wheeler and Bernard (2007), Laycock and Rajah (2010), Perl et al. (2011))

1.2.2(a) Clinical treatment of ALI

Patients diagnosed with ALI/ARDS with severe hypoxemia are usually treated with mechanical ventilation due to insufficient oxygenation. Mechanical ventilator is also known as ventilator, respirator, or breathing machine. This machine imitates the inflation and deflation of the lung to facilitate gas exchange and represents the standard of care for patients with ALI/ARDS (Marini, 2013). However, this treatment can extend the inflammatory response of ALI/ARDS and develop a risk towards more severe lung injury (Tremblay and Slutsky, 2006). The excessive tidal volumes and plateau pressure from the ventilator can also cause substantial harm to the lung such as alveolar distention as well as other organs (Pierson, 1988, Gajic et al., 2005, Slutsky, 2005). Repeated lung expansion (opening and closing) is also considered as a major risk that contributes to ventilation-induced lung injury (VILI) (Rittayamai and Brochard, 2015). Some patients that suffer from ALI often progress to a more protracted phase of persistent respiratory failure that is characterized by persistent hypoxemia, decreased lung compliance, fibrosis, chronic inflammation, and partial resolution of pulmonary oedema (Ware, 2006). In addition, patients who survive ALI

have been shown to have a reduced health-related quality of life (Weinert et al., 1997) and persistent muscle weakening (Herridge et al., 2003).

1.2.2(b) Pathophysiology of ALI

ALI is a disorder of acute inflammation that causes disruption of the alveolar–capillary membrane that includes lung endothelial and epithelial barriers (Figure 1.3). The pathophysiology of ALI can be divided conceptually into two histopathological phases, early exudative and fibroproliferative phase (Standiford and Ward, 2016).

Exudative phase usually occurred at 12 to 24 hours until the first week post-injury and is characterized by diffuse alveolar damage (DAD) (Castro, 2006, Beasley, 2010). The characteristics of ALI during early exudative phase include loss of alveolar–capillary membrane integrity that causes flooding of the alveolar compartment with protein rich oedema fluid, neutrophils, cellular debris and inflammatory mediators (Matthay and Zimmerman, 2005, Barnett and Ware, 2011, Proudfoot et al., 2011), extensive necrosis of ATI cells, and the formation of protein-rich hyaline membranes on a denuded basement membrane (Proudfoot et al., 2011) (Figure 1.3). This inflammatory milieu consists predominantly of activated neutrophils and alveolar macrophages, which secrete inflammatory mediators that disrupt epithelial fluid transport and impair surfactant production by ATII cells (Proudfoot et al., 2011). Cytokines that have been identified in ALI include IL-2, IL-6, IL-8, IL-10, IL-1β and its receptor antagonist IL-1ra, tumour necrosis factor-α (TNF-α), and the soluble TNF 1 and 2 receptors (Barnett and Ware, 2011). Following injury, upregulation of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α occurs as a direct response. These cytokines are mostly released by alveolar macrophage to stimulate chemotaxis and activate neutrophils (Levitt et al., 2009).

Transepithelial neutrophil migration is an important feature of ALI because neutrophils are the primary perpetrators of inflammation. Excessive and prolonged activation of neutrophils contributes to basement membrane destruction and increased alveolar–capillary barrier permeability (Johnson and Matthay, 2010). This is because neutrophils releases elastase that degrade epithelial junctional proteins and have direct cytotoxic and apoptotic effects on the epithelium (Ginzberg et al., 2001, Martin et al., 2005). Neutrophils migration from the circulation system involve tethering, slow rolling, modulation of adhesion strength, intraluminal crawling, and transcellular and paracellular migration (Ley et al., 2007). Tethering action is mediated by interactions between L (expressed on leukocytes), E (expressed on endothelial cells), and P-selectins (expressed on endothelial cells and platelets). These selectins facilitate rolling of leucocytes along the endothelium to prepare for migration. The rolling is then followed by the margination and adhesion of the neutrophils on the endothelial surface. This process involves β1 and β2 integrins and endothelial adhesion molecule (ICAM-1, VCAM, MAdCAM-1) and integrin (a4b1, a4b7). Transmigration of neutrophils is then mediated by ICAM-1 (through intracellular) and VCAM (through vascular) (Maniatis et al., 2008, Zemans et al., 2009). The breach of neutrophils into the interstitial and alveolar spaces increase the alveolar-capillary permeability that permits the influx of protein-rich fluid to cross the epithelial barrier into the airspaces of the lung (Johnson and Matthay, 2010). Besides I-CAM and TNF-α, the receptor for advanced glycation end-product (RAGE) is also a prominent marker for alveolar epithelial injury. TNF-α promotes disruption of endothelial cell junctions that leads to pulmonary oedema (Georgieva et al., 2007), whereas RAGE transmits pro-inflammatory intracellular signal via NF-κB (Creagh-Brown et al., 2010). RAGE is a specific marker of lung epithelial damage since it is expressed on the basolateral

surface of ATI cells (Ware et al., 2004). Majority of these pro-inflammatory cytokines are secreted by monocytes, fibroblasts, and endothelial cells during ALI (Mukhopadhyay et al., 2006, Maniatis and Orfanos, 2008).

Impaired and extensive epithelial injury can lead to a fibroproliferative phase of ALI. In adult human, this phase usually occurs between three to seven days following respiratory failure (Tomashefski, 1990), whereas in experimental animal, this phase appears one week after injury (Beasley, 2010, Matute-Bello et al., 2011). During this phase, mesenchymal cells proliferate and neovascularisation occur in the respiratory airway. The lung is also manifested with exuberant fibroblasts and myofibroblasts that synthesise excessive collagenous ECM (Luh and Chiang, 2007, Proudfoot et al., 2011).

1.2.2(c) Animal model of ALI

Matute-Bello et al. stated that it is not practical to incorporate these parameters in experimental studies that used small animals as a model since animals and humans have considerable physiological and anatomical differences (Matute-Bello et al., 2011). The American Thoracic Society (Matute-Bello et al., 2011) has discussed a number of features that are required to assess ALI in small animal models within 24 hours of exposure: i) Histological evidence of tissue injury should be summed in an injury score and identical to DAD, which includes infiltration of neutrophils, presence of proteinaceous debris in the alveolar or the interstitial space, formation of hyaline membranes, and thickening of the alveolar wall. ii) Alteration of the alveolar capillary barrier should be measured to assess any increase in lung water content and protein concentration in bronchoalveolar lavage (BAL) fluid. iii) An increased in the number of inflammatory response such as neutrophils, cytokines, and myeloperoxidase activity should be evaluated in the BAL fluid and lung tissue. iv) There should be evidence of

physiological dysfunction after exposure, including hypoxemia and an increased alveolar–arterial oxygen difference. Matute-Bello et al. also suggested that at least three of these main features should be in evidence to identify ALI model of experimental animals (Matute-Bello et al., 2011).

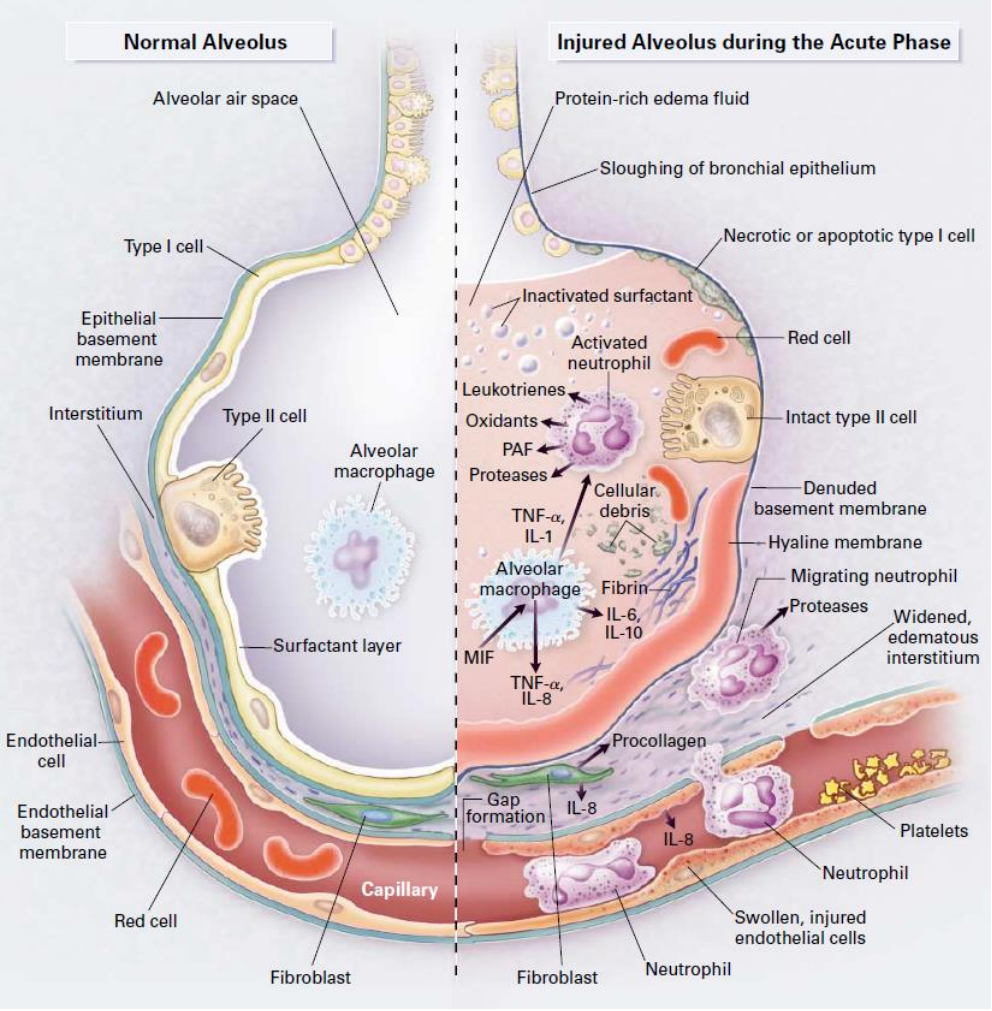

Figure 1.3 Pathogenesis of ALI. ALI is characterized by sloughing of alveolar epithelial cells, formation of hyaline membranes, and migration of activated neutrophils across the alveolar-capillary barrier into the interstitial region. The loss of the alveolar-capillary barrier integrity facilitates the accumulation of a protein-rich oedema and inflammatory cells. Endogenous cells secrete pro-inflammatory mediators that disrupt epithelial fluid transport and impair surfactant production by ATII cells. The interplay between inflammation and extravascular fibrin deposition increases hypoxaemia with acute inflammatory response (Adapted from Ware and Matthay (2000)).

1.2.2(d) Inhalation-induced injury model of ALI

Inhalation of reactive gases and vapours such as naphthalene and sulphur dioxide (SO_2) can lead to severe damage of the airways and lung. Naphthalene is a volatile, polycyclic aromatic hydrocarbon that can form a flammable vapour and is commonly used in synthetic tanning agents (Witschi and Last, 2001). This chemical can be found in many different sources, such as tars, petroleum, automobile emissions, pesticides, antiseptics, and tobacco smoke (Witschi and Last, 2001, Van Winkle et al., 2004). In experimental animals, inhaled naphthalene had shown extensive and selective necrosis in the subset of club/Clara cells, thus resulting in denudation of the distal airway's basement membrane (Hong et al., 2004b, Hong et al., 2004a, Park et al., 2006). Exposure to naphthalene at high concentrations also resulted in damage in both distal and proximal airways (West et al., 2001). Besides naphthalene, SO_2 is a toxic air pollutant from combustion of fossil fuels such as poor-quality coal and petroleum. This pollutant can be found in various industrial processes (Li et al., 2014). It is commonly notable that SO_2 can compromise pulmonary function and aggravate respiratory tract diseases such as chronic bronchitis and asthma (Smargiassi et al., 2009, Chang et al., 2012). SO_2 is soluble in water and can easily irritate the mucous membranes of the airway upon inhalation (Yang and Omaye, 2009). Studies suggested that exposure to SO_2 had resulted in pseudostratified epithelium removal in the proximal airways (trachea and bronchi) with excessive inflammatory cell infiltration and mucus cell hyperplasia (Asmundsson et al., 1973, Sueyoshi et al., 2004, Kodavanti et al., 2006). In addition, the SO_2 inhalation injury is usually accompanied by the rapid onset of a burning of the eyes, nose, and throat (associated with cough, chest pain, chest tightness, and dyspnea) (Yang and Omaye, 2009).

1.2.2(e) Physical perturbation-induced injury model of ALI

Injury to the mucosal surface of tracheal epithelium can cause epithelial cell shedding and denudation. In the submucosal region, there may be evidence of immediate haemorrhage and fibrin clot formation, followed rapidly by oedema and neutrophil invasion (Raub et al., 2010). Damage in the tracheobronchial region is commonly associated with dyspnea, haemoptysis, emphysema, pneumothorax, and pneumomediastinum that cause pulmonary gas exchange abnormalities (Richter and Ragaller, 2011). A few studies have demonstrated physical perturbation induced by curettage removed the whole intact mucosa (Wilhelm, 1953), whereas perturbation using cotton swabs (Hilding, 1965), blunt probe (Gordon and Lane, 1976), and stainless steel probe (Keenan et al., 1982) only exfoliated luminal cells and left the basal cells intact. Other attempts using apparatus such as cytology brush (Raub et al., 2010) and nylon brush (Nakagishi et al., 2005, Zani et al., 2008) not only exfoliated mucosal epithelium, but also altered the submucosal structure with damaged blood vessels and the infiltration of lymphocytic. Raub et al. also demonstrated a deposition of fibroblast cells that developed into thickened and granulated mucosal tissue following physical scraping with cytology brush (Raub et al., 2010).

1.2.2(f) The cellular response of respiratory airway during ALI

Immediately after injury, cellular losses within these airway epithelium are persistently replenished by stem cells to meet homeostatic needs or regenerative demands (Coraux et al., 2005, Rennard and Wachenfeldt, 2011). Basal, secretory, and ATII epithelial cells are generally notable for their self-renewal capacity and the ability to give rise to other epithelial lineages with a constitutively high rate of cell turnover and a well-delineated stem/progenitor cell hierarchy (Hogan et al., 2014, Kotton and

Morrisey, 2014). The repair process of airway epithelium started with epithelial cell from neighbouring the wound receiving signals from the damage cells. In the trachea and bronchi region, for example, the population of basal cells retrieves the signalling and respond to injury by cell migration, proliferation, and differentiation processes (Yahaya, 2012, Girault and Brochiero, 2014). Cell migration is one of the first mechanisms of epithelial repair. In the early repair stage, epithelial cells form a multiple layer of flattened epithelial cells (Dupuit et al., 2000, Yahaya, 2012), which are associated with cytoskeleton reorganisation, membrane cell elongation, and release of adhesion proteins (cadherin, integrin, etc.) along with ECM to facilitate the spreading and migration of the cells (Su et al., 2013, Girault and Brochiero, 2014). This phase is normally referred to as the epithelial-to-mesenchymal transition (EMT). This event is crucial and usually occurs spontaneously during wound healing or tissue remodelling (Zeisberg and Neilson, 2009). The EMT involves the transition by which non-motile epithelial cells gain motility, migratory, and invasive properties to become mesenchymal stem cells (MSCs) (Zeisberg and Neilson, 2009, Lamouille et al., 2014). The initiation of the EMT is marked by the phenotype switch from epithelial to mesenchymal cell marker (Huang et al., 2012, Lamouille et al., 2014, Nishioka et al., 2015) to acquire a more motile and mesenchymal phenotype (Zeisberg and Neilson, 2009, Lamouille et al., 2014). Transforming growth factor-β (TGF-β) is normally highly expressed during the EMT to stimulate fibroblast proliferation to increase the production of ECM (Araya et al., 2007, Kitamura et al., 2011, Brand et al., 2015). The migrated cells then undergo mitosis and proliferate, which are also identified as squamous metaplasia (Coraux et al., 2005, Puchelle et al., 2006). Once the epithelial barrier is re-established, the epithelial cells within the basal compartment segregate into distinct subpopulations that yield ciliated and secretory cells. The cells either

undergo ciliogenesis to become ciliated cells or differentiate into secretory cells to form the pseudostratified mucociliary epithelium (Coraux et al., 2005, Yahaya, 2012, Pardo-Saganta et al., 2015). The rate of respiratory epithelial cell regeneration is varied among animal models. In mice, studies have suggested that weeks to months period of time is required for epithelial cell to repopulate pseudostratified tracheal epithelium layer after chlorine (Musah et al., 2012) and naphthalene injury (Hong et al., 2004b). Meanwhile, in lungs, epithelium restoration was completed by day ten after chlorine exposure (Tuck et al., 2008) and full recovery of alveolar-capillary barrier dysfunction was observed by days 5-10 after HCl-induced injury (Patel et al., 2012).

1.2.2(g) The inflammatory response of respiratory airway during ALI

Epithelial cells, macrophages, dendritic cells, and mast cells fall in the sensor cell category that play role in detecting pathogens and foreign particles within the respiratory tract. Sensor cells, as the first tier of defence, immediately initiate innate immune responses to clear the local invasion (Iwasaki et al., 2017). These cells release first-order cytokines, such as interferon-γ (IFN-γ) and IL-1β that activate local tissue-resident lymphocytes. IL-1β also stimulates fibroblast proliferation during the airway repair to increase the production of ECM, smooth-muscle actin, and a number of chemokines and chemoattractant through αvβ8-mediated TGF-β pathway (Araya et al., 2007, Kitamura et al., 2011, Brand et al., 2015). IL-1β also activates alveolar macrophages to produce variety of chemokines such as IL-8 and MIP-1α (Goodman et al., 2003, Grommes and Soehnlein, 2011). IL-8 is defined as a CXC chemokines (Vos and Briscoe, 2002) and chemoattractant predominantly for neutrophils (Goodman et al., 2003, Matute-Bello et al., 2011) and plays role in promoting neutrophils migration into the interstitial and alveolar space by activating endothelial