ANTI-INFLAMMATORY EFFECTS OF MESENCHYMAL STEM CELLS DERIVED EXTRACELLULAR VESICLE IN RAT MODEL OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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by

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LIST OF SYMBOLS

α	alpha
β	beta
μ	micro
γ	gamma
K	Cubic
mL	milliliter

LIST OF ABBREVIATIONS

αSMA	Alpha smooth muscle actin
AEC	Airway epithelial cells
AP-1	Activatory protein 1
APM	Airborne particulate matter
Bach1	Basic leucine zipper transcription factor 1
BAL	Bronchoalveolar lavage
BBB	blood-brain barrier
BCL2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
BM	Bone marrow
CADM1	Cell adhesion molecule 1
CAT	COPD assessment test
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CDNA	Complementary deoxyribonucleic acid
CS	Cigarette smoke
COPD	Chronic obstructive pulmonary disease
COX2	Cyclooxygenase 2
CREBBP	CREb-binding protein
CXCL	Chemokine (C-X-C motif) ligand
DEG	Differential expressed gene
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonuclein acid
ECM	Extracelullular matrix
EFTEM	Energy filtered transmission electron microscope
EGF	Epithelial growth factor
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cells
ETS	Environment tobacco exposure
EV	Extracellular vesicles
FADD	Fas associated death domain

FBS	Fetal bovine serum
FC	Fold change
FEV	Forced expiratory volume
FIRS	Forum of international respiratory societies
FVC	Forced vital capacity
GO	Gene ontology
GOLD	Global initiative for chronic obstructive lung disease
GRB2	Growth factor receptor-bound protein 2
GSH	Glutathione
GVHD	Graft versus host disease
H_2O_2	Hydrogen peroxide
HBEC	Human bronchial epithelial cells
HO-1	Heme oxygenase
ICAM	Intercellular adhesion molecule
ICS	Inhaled cortecosteroid
IgG	Immunoglobulin G
IFN	interferon
IL	interleukin
iPSC	Induced pluripotent stem cells
ISEV	International society for extracellular vesicles
JNK	Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
LABA	Long acting beta 2 adrenoceptor agonist
LAMA	Long acting muscarinic agonist
LPS	lipopolysaccharide
MAP2K	Dual specific mitogen-activated protein kinase
MAPK	Mitogen activated protein kinase
MCP1	Monocyte chemoattractant protein 1
MDA	Malondialdehyde
MHC	Major histocompatibility
MiRNA	Micro ribonucleic acid
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilitization

MSC	Mesenchymal stem cells
MV	microvesicles
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NGFR	Nerve growth factor receptor
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NO	Nitric oxide
NRF-2	Nuclear factor erythroid 2 related factor 2
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
PiK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha
PNEC	Pulmonary neuroendocrine cell
PRKCZ	Protein kinase C zeta
RIN	RNA integrity number
RM	Ringgit Malaysia
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAMA	Short acting muscarinic agonist
SASP	Senescence associated secretory phenotype
SD	Standard deviation
STAT	Signal transducer and activator of transcription
SOD	Superoxide dismutase
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPP1	Telomere protection protein 1
TRADD	TNF- receptor death domain
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
UC	Umbilical cord
VEGF	Vascular endothelial growth factor

LIST OF APPENDICES

KESAN ANTI KERADANGAN VESIKEL EKSTRASELULAR BERASAL DARI SEL TUNJANG MESENKIMA KE ATAS MODEL TIKUS PENYAKIT PENGHALANG PULMONARI KRONIK

ABSTRAK

Penyakit penghalang pulmonari kronik (COPD) adalah penyakit paru-paru kronik berpunca dari keradangan kronik yang menyebabkan penyumbatan aliran udara yang progresif. Strategi rawatan semasa berfokus pada mengurangkan gejala dan meningkatkan kualiti hidup, namun tidak mampu menyembuhkan punca penyakit ini. Kebelakangan ini, vesikel extraselular (EV) dari sel tunjang mesenkima (MSC) telah menjalani kajian yang lebih mendalam sebagai sumber terapi berasaskan sel terbaru kerana kemampuan MSC-EV untuk memperbaiki keradangan, namun hingga kini, tiada kajian yang dilakukan untuk mengkaji kesan anti-keradangan MSC-EV ke atas model COPD. Oleh disebabkan itu, kajian ini dijalankan untuk mengkaji kesan anti-keradangan MSC-EV dari tali pusat manusia (UC) ke atas model tikus COPD. hUC-MSC-EV diasingkan dan dicirikan dengan menggunakan mikroskop elektron transmisi, western blot, dan analisis penjejakan nanopartikel. Tikus sprague dawley jantan (n=66) berumur 8-9 minggu dibahagikan kepada 11 kumpulan; Naïve, Asap Rokok (CS), Penyembuhan Sendiri (SH), kumpulan rawatan (CS-hUC-MSC-EV, CS-hUC-MSC, CS-hUC-MSC-CM), kumpulan kenderaan (media budaya sahaja (MD) , phosphate buffered saline (PBS)), kumpulan kawalan (C) (C-hUC-MSC-EV, C-hUC-MSC, dan C-hUC-MSC-conditioned media (CM)). Lima kumpulan (CS, SH, CS-hUC-MSC, CS-hUC-MSC-EV, dan CS-hUC-MSC-CM) didedahkan kepada 3 batang rokok selama 15 minit, 2 kali sehari selang 2 jam, 7 hari minggu, selama 12 minggu. Sementara itu, Naïve, dan kumpulan kawalan dibiarkan bernafas dalam udara normal. Rawatan (hUC-MSC,

hUC-MSC-EV, dan hUC-MSC-CM), PBS, dan media diberikan pada minggu ke-13. Kumpulan Naive dan CS dimatikan pada minggu ke-13, sementara kumpulan rawatan, kumpulan kenderaan, dan kumpulan penyembuhan dimatikan pada minggu ke-15. Paru-paru dari semua kumpulan kemudiannya menjalani analisis histologi dengan menggunakan pewarnaan hematoxylin dan eosin (H&E), pewarnaan periodic acid shift (AB-PAS), pewarnaan imunoflurosen, dan analisis mikroarray. Peningkatan jumlah limfosit, keradangan di kawasan peribronkial dan perivaskular, serta kawasan parenkima, peningkatan jumlah sel goblet, peningkatan emfisema, dan peningkatan ekspresi p65 dapat dilihat pada kumpulan CS berbanding kumpulan Naive. Dalam kumpulan SH pula didapati tiada pengurangan keradangan di kawasan peribronkial dan perivaskular, serta kawasan parenkima, tiada pengurangan sel goblet, emfisema, dan ekspresi p65. Dalam kumpulan rawatan, pengurangan keradangan dapat dilihat di kawasan peribronkial dan perivaskular, serta kawasan parenkima. Pengurangan jumlah sel goblet, emfisema, dan ekspresi p65 juga dapat dilihat pada kumpulan rawatan. Sementara itu, rawatan juga tidak menyebabkan keradangan atau peningkatan jumlah sel goblet, dan tidak menyebabkan emfisema pada kumpulan kawalan. Analisis mikroarray menunjukkan keterlibatan ‘pathway’ dan gen berkaitan COPD dalam kumpulan CS, hUC-MSC-EV, dan hUC-MSC. Rawatan hUC-MSC-EV dan hUC-MSC dilihat secara signifikan mengubah ekspresi gen-gen termasuk *NFKB1*, *MAPK1*, *MAP2K1*, *JUN*, *PRKCZ*, dan *P65*. Kesimpulannya, hUC-MSC-EV berkesan memperbaiki keradangan yang disebabkan oleh CS dan berpotensi berfungsi sebagai terapi bebas sel untuk rawatan COPD.

ANTI-INFLAMMATORY EFFECTS OF MESENCHYMAL STEM CELLS DERIVED EXTRACELLULAR VESICLES IN RAT MODEL OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a chronic lung disease characterized by progressive airflow obstruction associated with chronic inflammation. The current treatment strategies are focusing on improving the symptoms and quality of life but do not provide cure for the underlying caused. Recently mesenchymal stem cells (MSC)-derived extracellular vesicles (EV) is actively being investigated as a potential source of new cell-free based therapy for COPD due to it's ability to ameliorate inflammation, however no research has been conducted to study the anti-inflammatory effects of MSC-EV in COPD model. Thus, this study aimed to assess the anti-inflammatory effects of human umbilical cord mesenchymal stem cell (hUC-MSC) derived EV in a rat model of COPD. Human UC-MSC-EV were isolated and characterized by using transmission electron microscope, western blot, and nanoparticle tracking analysis. Male sprague dawley rats (n=66) age 8-9 weeks were divided into 11 groups; Naïve, Cigarette Smoke (CS), Self-healing (SH), treatment groups (CS-hUC-MSC-EV, CS-hUC-MSC, CS-hUC-MSC-CM), vehicle groups (culture media alone (MD), and phosphate buffered saline (PBS)), and control (C) group (C-hUC-MSC-EV, C-hUC-MSC, and C-hUC-MSC-conditioned media (CM)). Five groups (CS, SH, CS-hUC-MSC, CS-hUC-MSC-EV, and CS-hUC-MSC-CM) were exposed to CS from 3 cigarettes for approximately 15 minutes per session, 2 times a day at 2 hours interval, 7 days a week, for 12 weeks. Meanwhile, Naïve, and control groups were left to breathe

normal air. The treatments (hUC-MSC, hUC-MSC-EV, and hUC-MSC-CM) and PBS and MD were administered at week 13. Naïve and injury group were euthanized at week 13, while treatment groups, vehicle groups, and self-healing group were euthanized at week 15. Lungs from all groups were then subjected to histological analysis by using hematoxylin and eosin (H&E) staining, alcian blue-periodic acid Schiff (AB-PAS) staining, immunofluorescence staining, and microarray analysis. Increased lymphocytes count, inflammation in peribronchial and perivascular area, as well as parenchyma area, increased goblet cells count, increased emphysema, and increased p65 expression were observed in CS group as compared to Naïve group. Self-healing for two weeks did not reduce the inflammation in peribronchial and perivascular area, as well as parenchyma area. Self-healing for two weeks also did not reduce goblet cells count, emphysema, and p65 expression. In treatment groups, reduction of inflammation in peribronchial and perivascular area, as well as parenchyma area, reduced goblet cells count, and emphysema, reduced p65 expression were observed as compared to CS and SH groups. Meanwhile, the treatments did not induce inflammation or increased goblet cells count, and did not induced emphysema in rat exposed to normal air. Microarray analysis showed regulation of COPD related pathways and genes in CS, hUC-MSC-EV, hUC-MSC groups. hUC-MSC-EV, and hUC-MSC significantly regulating many genes expression including *NFKB1*, *MAPK1*, *MAP2K1*, *JUN*, *PRKCZ*, and *P65*. In conclusion, hUC-MSC-EV effectively ameliorating the CS induced inflammation and could potentially serve as a new cell-free based therapy for the treatment of COPD.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a preventable chronic pulmonary disease characterized by limitation of airflow associated with abnormal inflammation caused mainly by cigarette smoke (CS) (Eltom, Stevenson & Birrell, 2013). According to World Health Organization, COPD is the third leading cause of death worldwide, causing 3.23 million deaths in 2019 (World Health Organization, 2020). In Malaysia, study from Institute for Health Metric and Evaluation demonstrated COPD to be the fifth leading cause of death in 2019 (IHME,2018). The annual cost per patient was RM12,757, while total cost for all COPD patients was estimated to be RM2.2476 billion annually (Institute of Public Health, 2011). Moreover, increased of cigarette smoking among adults, male (46.4%) and female (1.6%) over the years may lead to increase of COPD prevalence and greater burden in Malaysia (ur Rehman *et al.*, 2020).

Shortness of breath, chronic coughing, wheezing, chest tightness, and sputum production are the symptoms for COPD (Miravitlles & Ribera, 2017). Patients usually undergo spirometry to confirm the diagnosis with post-bronchodilator forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ≤0.7 (Vogelmeier *et al.*, 2017). Acute worsening of respiratory symptoms or acute exacerbation of COPD (AECOPD) occur in 46% of COPD patients which results in additional therapy and worse quality of life (Ko *et al.*, 2016). Worsening symptoms of dyspnea, increased sputum production, and purulence among others are the characteristic of COPD exacerbations (Mackay & Hurst, 2012). Though declining lung function occurs naturally with age, the decline in lung function of COPD patients

is progressive, with average rate of FEV1 decline is approximately double that normal subjects (Welte, Vogelmeier & Papi, 2015). In patients with frequent exacerbations, a much rapid decline in lung function, increased hospitalizations, decreased quality of life, and increased mortality were observed (Mackay & Hurst, 2012). In addition, COPD also presents with comorbidities including lung cancer, cardiovascular disease, skeletal muscle dysfunction, metabolic syndrome, osteoporosis, depression, and anxiety and these comorbidities can occur in mild, moderate, and severe airflow limitation (Vogelmeier *et al.*, 2017). Furthermore, these may also contribute to COPD exacerbation and worsening symptoms (Cavaillès *et al.*, 2013). These comorbidities also can have significant impact on prognosis, increase hospitalizations and healthcare cost, and often complicates the management of COPD (Cavaillès *et al.*, 2013; Hillas *et al.*, 2015).

Chronic inflammation contributes significantly to the pathogenesis of COPD in addition to oxidative stress, senescence, and apoptosis which inter-related to each other that cause small airway remodelling, mucus hypersecretion, bronchitis, and emphysema (Pauwels *et al.*, 2011a; Barnes, 2017; Gong *et al.*, 2019). In the initial stage, cigarette smoke (CS) triggers oxidative stress and inflammation causing influx of immune cells into the lung while in later stage, CS continue to disrupts alveolar maintenance causing apoptosis and autophagy which destroyed alveolar walls. In addition, progressive telomere shortening and activation of senescence markers and aging amplified the injury leading to terminally injured lung (Tuder & Petrache, 2012). In addition, protease anti-protease imbalance caused by myriad of proteases including neutrophils elastase (NE), matrix metalloproteinases, and cathespins released by immune cells contributing to alveolar destruction and emphysema (Fischer, Pavlisko & Voynow, 2011). Genetic mutation of α-1 antitrypsin which

involve inhibition of neutrophils proteases, also contribute greatly to the protease/anti-protease imbalance causing early onset of emphysema and COPD (Hazari *et al.*, 2017). In fact, non-smoking individual who with α-1 antitrypsin deficiency will likely get COPD although they usually suffer from COPD at an older age than smokers (Strange, 2020).

The most effective way to prevent COPD remains to be smoking cessation. Other than improved symptoms, smoking cessation also reduced the rate of pulmonary function decline, reduced the risk of lung cancer and cardiovascular disease, and mortality rate (Warnier *et al.,* 2013). However smoking cessation is harder to achieve due to high nicotine dependence, thus administration of behavioural support including counselling and combination of pharmacological approach are necessary to avoid relapse (Tønnesen, 2013). Other than smoking cessation, pharmacological drugs such as bronchodilators, anti-muscarinic, and corticostreroids are currently being used as a treatment regiment (Anthonisen *et al.*, 1994; Keatings *et al.*, 1997; Ejiofor & Turner, 2013). Non-pharmacological therapies such as lung volume reduction surgery, home oxygen and ventilatory support, and pulmonary rehabilitation may also help in managing COPD (Mackay & Hurst, 2012). In addition, influenza and pneumococcal vaccinations are recommended to reduce the incidence of lower respiratory tract infections (Vogelmeier *et al.*, 2017). Current COPD therapeutic management based on Global initiative for chronic obstructive lung disease (GOLD) are focusing on reducing the severity and preventing exacerbation (Patel *et al.*, 2019). Treatments will be given according to GOLD classification based on symptoms and severity risk; GOLD A (Low symptoms severity, low exacerbation risk), GOLD B (High symptoms severity, low exacerbation risk), GOLD C (Low symptoms severity, high exacerbation risk), and GOLD D (High symptoms severity, high exacerbation risk) (Patel *et al.*,

2019). Although the available treatments alleviate the symptoms, these treatments provides neither permanent solution for COPD, nor effectively suppress the chronic inflammation, preventing disease progression and mortality (Barnes, 2013). Furthermore, drugs such as bronchodilators, and antimuscarinic are present with various side effects including dry mouth, constipation, blurred vision, arrythmia, pneumonia, oropharyngeal candidiasis, osteoporosis, and diarrhea (Price *et al.*, 2013; D D'Urzo *et al.*, 2014; Hanania, Lareau & Yawn, 2017). In one systemic review and meta-analysis showed that triple therapy combination of inhaled corticosteroid, long acting β2 adrenoceptor agonists (LABA), and long acting muscarinic receptor antagonists (LAMA) present with higher incidence of pneumonia though the treatment significantly reduced the rate of moderate or severe exacerbation (Zheng *et al.*, 2018). These discrepancies should be remedied with a new superior therapy that not only alleviate symptoms and severity, but also provide cure for the underlying cause with little side effects.

To date, various research have been conducted in the search for permanent solutions to this heterogenous disease including gene therapy, pharmacotherapeutic drugs, and cell-based therapy (Barnes, 2013; Loring & Flotte, 2015; Cheng, Lin & Yao, 2017). Over the years, mesenchymal stem cells has been the subject of interest in research field due to its therapeutic potential in various diseases including lupus nephritis, asthma, inflammatory bowel disease, and arthritis (Chang *et al.*, 2011; Braza *et al.*, 2016; Mao *et al.*, 2017; Lee *et al.*, 2018). Mesenchymal stem cells are multipotent stem cells capable of self-renew and differentiating into its own mesodermal lineages as well as other lineages. Mesenchymal stem cells are first discovered in bone marrow, but now MSC can also be isolated from various tissues such as adipose tissue, umbilical cord (UC), placenta, and lung tissue (Han *et al.*,

2017). In terms of feasibility, UC represents an attractive source of MSC as UC-MSC is a less ethical concern unlike embryonic stem cells, and the isolation of UC-MSC is non-invasive as compared to bone marrow (BM)-MSC. Besides, UC-MSC has been shown to have similar efficacy in modulating the inflammation as BM-MSC (Kagia *et al.*, 2019). Apart from its differentiation potential, MSC can also escape from immune recognition, home to the site of injury, and regulate the immune function (Consentius, Reinke & Volk, 2015; Meng *et al.*, 2018; Zhou *et al.*, 2019). Numerous studies and clinical trials have been conducted to decipher the MSC potential in mitigating COPD (Weiss *et al.*, 2013; Liu, Fang & Kim, 2016; Sun *et al.*, 2018). MSC showed remarkable ability to decrease the level of inflammation, apoptosis, and regeneration of alveolar wall that have been destroyed in emphysema (Schweitzer *et al.*, 2011). MSC conditioned media also protect fibroblast from apoptosis and senescence, induced proliferation of fibroblasts (Kim *et al.*, 2012). Meanwhile in clinical trial on moderate-to-severe COPD patients, UC-MSC is reported to be well tolerated by patients with no clinically significant adverse effects, decreased number of exacerbations in COPD Assessment Test (CAT) and mMRC scores, and significant improvement in quality of life (Bich *et al.*, 2020).

In the last decade, increasing number of research have focused on studying the paracrine factors released by MSC in the conditioned medium especially extracellular vesicles (EV) (Maacha *et al.*, 2020). Since the survival and engraftment of MSC are limited, it is hypothesized that EV to be the primary mechanism that governs the MSC therapeutic effects (Horie *et al.*, 2012; Witwer *et al.*, 2019). Extracellular vesicles are heterogenous small membrane vesicles released by various cell types and bodily fluids, including hepatocytes, adipocytes, neurons, white blood cells, MSC, milk, saliva, urine, amniotic fluid and cerebrospinal fluid (Katsuda *et al.*, 2013). Two

commonly studied EV are exosomes and microvesicles (MV) which differed by its origin in which exosomes are originated from the inward budding of endosome that forms multivesicular bodies, while MV originated from outward budding of the cell membrane (Rani *et al.*, 2015; Sarko & McKinney, 2017). The isolation of EV can be conducted via multiple methods, including differential ultracentrifugation, density-gradient separation, and immunoaffinity capture (Greening *et al.*, 2015). Extracellular vesicles carry various types of lipids, proteins, DNA, as well as RNA that mediate intercellular communication processes (van Niel, D'Angelo & Raposo, 2018).

Over the years, increasing amount of research have shown the involvement of MSC-EV and its cargo content in regulations of the therapeutic effects of MSC (Wang *et al.*, 2017c; Ying *et al.*, 2020). The MSC-EV provides a number of advantages over the use of MSC in therapeutic applications. The EV is non-self replicate, small size vesicles that can be taken up easily by cells to deliver its therapeutic effects (Rani *et al.*, 2015). The EV can also cross blood brain barrier (BBB) to deliver the treatment effectively and safely (Zhuang *et al.*, 2011). In addition, due to its small size, transplantation of EV can avoid the risk of occlusion and embolism that might occur in cells transplantation (Tatsumi *et al.*, 2013; Wu *et al.*, 2017). Recent studies suggest the involvement of MSC-EV in the regulation of inflammation (Zulueta *et al.*, 2018). The MSC-EV were also observed to inhibit the proliferation, activation, and differentiation of T cells, as well as polarization of macrophages (Blazquez *et al.*, 2014; Cao *et al.*, 2019). In liver fibrosis model, administration of MSC-exosomes reduced inflammatory cytokines and oxidative stress markers IL-1, IL-2, I-6, IL-8, TNF-α, and MDA, reduced fibrosis, while increasing the level of pro-inflammatory cytokine IL-10, increased hepatocyte regeneration, and improved liver function (Rong *et al.*, 2019). In mice model COPD, administration of Exosome-derived multiple

allogeneic protein paracrine signalling (Exo-d-MAPPS) harvested from placenta MSC showed significant reduction in immune cells influx and reduced pro-inflammatory cytokines including IL-12, IL-1β, Tκκα, and IFN-γ. The study further tested on COPD patients demonstrated improvement in pulmonary status, and quality of life, and reduced emphysema (Harrell *et al.*, 2020).

1.2 Problem Statement

The COPD was estimated to be the third leading cause of death by 2020, however, COPD has surpassed this estimation in 2010 (Lozano *et al.*, 2012; May & Li, 2015). Though various drugs are available, the drugs are administered to treat the symptoms, improving the quality of life of patients, but do not treat the underlying cause of COPD (Patel *et al.*, 2019). Therefore, a more effective therapeutic strategy that not only alleviate the symptoms but also treating the underlying cause of COPD is crucial to curb the rising in morbidity rate. Previous data demonstrated promising use of mesenchymal stem cells as a potential new cell-based therapy for treatments of COPD (Weiss *et al.*, 2013; Gu *et al.*, 2015b). However, due to the limited engraftment of MSC, paracrine secretion especially MSC-EV was thought to play vital role in regulation of MSC therapeutic effects. The study of MSC-EV has gain momentum over the years as MSC-EV was discovered to possess comparable therapeutic effects as MSC, which opens up new approach in cell-free based therapy in the treatment of various diseases. Nevertheless, to date only a few studies have been conducted to decipher the effect of MSC-EV in alleviating the inflammation, which is the body's process of fighting against harmful stimuli in COPD but no study was conducted to study the molecular pathways involve in MSC-EV immunomodulation effect in which the changes in the immune systems caused by the MSC-EV that can

activates or suppress the inflammation. Thus, this study was conducted to determine the anti-inflammation effects of MSC-EV and mechanism involve in rat COPD model. The knowledge gained from the study will help to shed the light on how MSC-EV exert its effect via secretion of paracrine factor, and to facilitate in the development of potentially new therapy for COPD.

1.3 Objectives of the study

1.3.1 Main Objective

To study the anti-inflammatory effects of MSC-EV in rat model of COPD.

1.3.2 Specific Objectives

1. To isolate and characterize EV derived from human UC-MSC.
2. To characterize cigarette smoke induced inflammation in *in vivo* rat model of COPD.
3. To study the anti-inflammatory effects of human MSC-EV in reducing the inflammation, goblet cells count, and emphysema
4. To determine the global gene expression in cigarette smoke induced inflammation in rat model of COPD, and in hUC-MSC, and hUC-MSC-EV treated group.

CHAPTER 2

LITERATURE REVIEW

2.1 Anatomy of Respiratory Airway

2.1.1 Branching of the respiratory airway

The exchange of oxygen from the air with carbon dioxide from the cells occurs through the lung. Mammalian lung consists of a trachea that branched into two bronchi and ended with millions of tiny air sacs called alveoli (Hogan *et al.*, 2014). The lung can be divided into two zones; the conducting zone and the respiratory zone (Figure 2.1). The conducting zone consists of the trachea, bronchi, and terminal bronchiole, while the respiratory zone consists of respiratory bronchioles, alveolar ducts and alveolar sacs. Although the lung is divided into two parts, the gas exchange only occurs in the respiratory zone (Berube *et al.*, 2010).

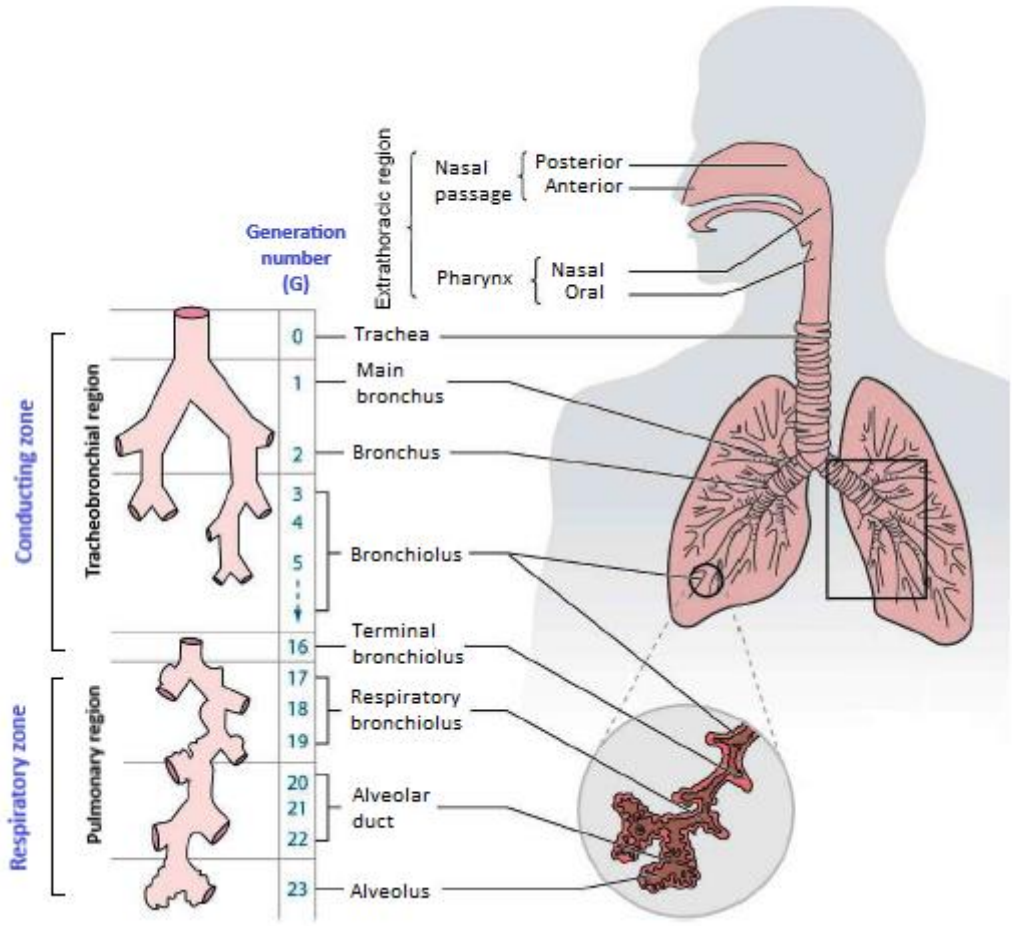

Figure 2.1: Schematic of respiratory airway modified from (Hussain, Madl & Khan, 2011). Trachiobronchial region started from 0-16 consists of trachea, bronchus, and bronchiolus while pulmonary region from 17-23 consists of terminal bronchioles, respiratory bronchioles, alveolar duct, and alveolus.

2.1.2 Cellular structure of respiratory airway

2.1.2(a) Conducting zone

The trachea extended from larynx are stacked with 16 to 20 C-shaped hyaline cartilage that provides structural support to prevent the trachea from collapse (Betts *et al.*, 2013). The posterior of trachea are supported by trachealis muscle and elastic connective tissue that become flat, convex, or slightly concave during inspiration, and flatten, or bow slightly during expiration (Patwa & Shah, 2015). Meanwhile, the trachea divides at the carina into left and right bronchi that further branch into bronchial tree until the bronchioles connect to the alveoli (Nikolic, Sun & Rawlins, 2018). Like trachea, hyaline cartilage provides structural support to the bronchi, however, unlike bronchi and trachea, bronchioles do not contain cartilage, but surrounded by smooth muscles (Betts *et al.*, 2013).

Pseudostratified columnar epithelium that lined the basement membrane of trachea and main stem bronchi consist of basal cells, ciliated cells, secretory/goblet cells, as well as pulmonary neuroendocrine cells (PNEC) which provide the mechanism for mucociliary clearance (Berube *et al.*, 2010) (Figure 2.2). Ciliated cells accounts for 50% of all epithelial cell composing up to 300 cilia per cell that function mainly to direct the transport of mucus from the lung to the throat (Knight & Holgate, 2003). Meanwhile, secretory/goblet cells expressing *MUC5AC*, produce and secret mucus which facilitate mucociliary clearance. Mucus produced by the secretory/goblet cells is the first line defence against airborne particles, and microorganisms. Once trapped, the particles and microorganisms will be removed from the airway with the help of ciliated cells (Knight & Holgate, 2003). Basal cells are stem cells capable of self-renew and differentiate into ciliated cells and secretory

cells. Basal cells present throughout the conducting zone, yet the number of basal cells reduce with airway size. Basal cells can be characterized by transcription factor-63 (p-63), Nerve growth factor receptor (NGFR), Keratin-5, and Keratin-14 (Knight & Holgate, 2003; Nikolic, Sun & Rawlins, 2018). Pulmonary neuroendocrine cells can be found as solitary cells or in cluster throughout bronchial tree (Knight & Holgate, 2003). Clustered PNEC contain secretory granules and dense-core vesicles which being released upon physiological stimuli such as hypoxia (Song *et al.*, 2012). Although the number of PNEC is lower than other cells, PNEC are shown to have diverse function including regulation of pulmonary blood flow, airway oxygen sensing, modulation of immune response, and controlling bronchial tonus (Song *et al.*, 2012). In the bronchioles, the cuboidal epithelium containing secretory club cells, ciliated cells, and PNEC lined the airways (Kotton & Morrisey, 2014). Club cells constituting 9% of all epithelial cells, are non-ciliated, non-mucous secretory cells (Rokicki *et al.*, 2016). The cells contain membrane-coated granules that consist of glyproteins, proteins, and lipid (Knight & Holgate, 2003). The primary secretory product of club cells is club cell protein-16 (CC16) or club cell secretory protein (CCSP) which has immunomodulatory effects and also inhibit phospholipase A2, which in turn will inhibit the eicosanoids (Barnes, 2015). Physiologically, club cells function to metabolize xenobiotic compounds together with cytochome P-450 mono-oxygenase, act as progenitor cells which self-renew and differentiate into ciliated cells, participating in airway repair after injury, secretion of anti-inflammatory and immunomodulatory protein, and detoxification (Barnes, 2015; Rokicki *et al.*, 2016).

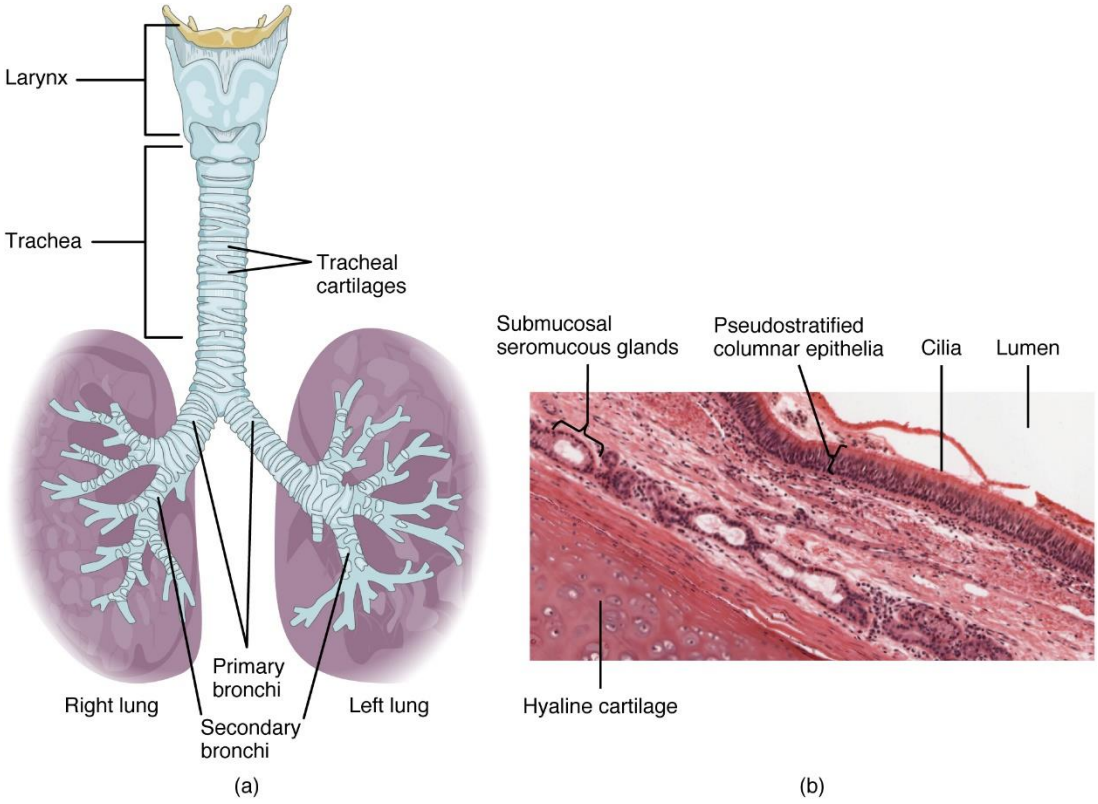

Figure 2.2: The trachea (Betts *et al.*, 2013). The trachea is stacked with 16 to 20 C-shaped hyaline cartilage. The trachea divides into left and right bronchi that further branch into bronchial tree until the bronchioles connect to the alveoli. Hyaline cartilage provides structural support to the bronchi, while bronchioles are surrounded by smooth muscles.

2.1.2(b) Respiratory zone

Respiratory zone begins with the respiratory bronchioles that leads to alveolar ducts, and ended with clusters of alveoli. Alveolar ducts are made of smooth muscle and connective tissue, while alveolar wall consist of two types of cells namely, type I alveolar cells (AECI), and type II alveolar cells (AECII) (Betts *et al.*, 2013). Morphologically, AECI are squamous, large, and thin cells and covered approximately 96% of the alveolar surface. AECII on the other hand are cuboidal cells that constitute approximately 15% of total lung cells. While AECI participate in gas exchange with the capillaries, AECII is the progenitor cells to the AECI, involve in repair of the epithelium during injury, and produce antimicrobial products like

surfactant protein, as well as cytokines involve in activation and differentiation of immune cells (Chuquimia *et al.*, 2013) (Figure 2.3).

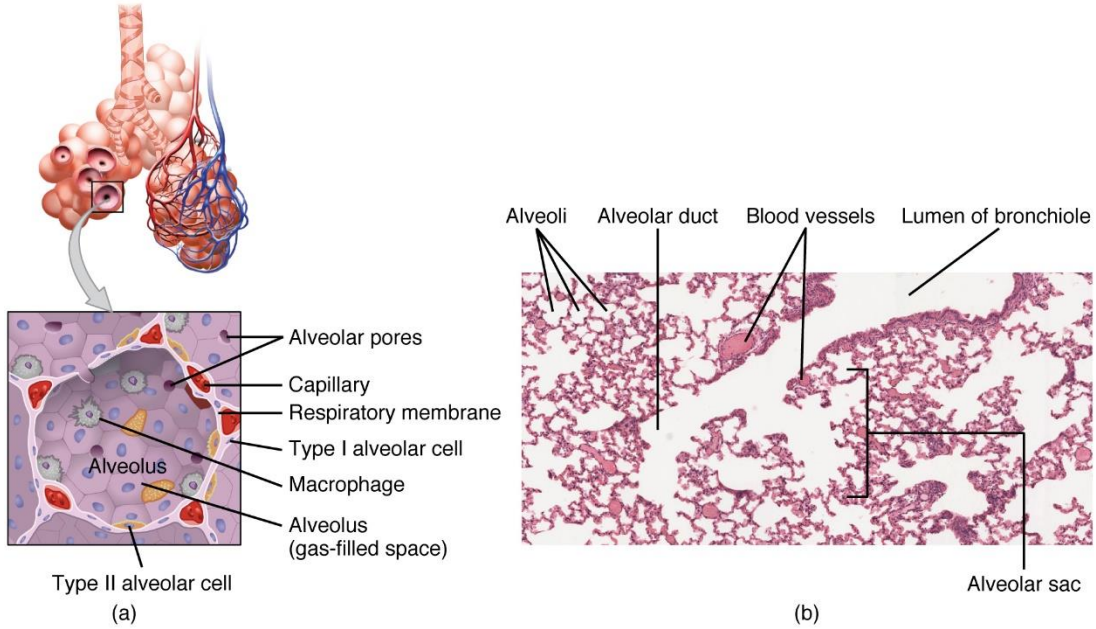

Figure 2.3: Respiratory zone structure (Betts *et al.*, 2013). A) The alveolus responsible for gas exchange lining with Type I alveolar cells and Type II alveolar cells. B) Micrograph of structure of lung tissue.

2.2 Lung diseases

Lung is the only internal organ constantly exposed to the external environment, makes it vulnerable to toxic gases, pollutants, and microorganisms that can cause various respiratory diseases. Report on the Forum of International Respiratory Societies (FIRS) identified five respiratory diseases attributed to the greatest burden to society which are COPD, asthma, acute respiratory infections, tuberculosis, and lung cancer. Each year, four million people died from chronic respiratory diseases, while pneumonia is the main cause of death in children (Marciniuk *et al.*, 2014).

Asthma and COPD represent two types of chronic lung diseases characterized by airflow obstruction and chronic inflammation in the respiratory tract (Barnes, 2011). While asthma is a reversible condition, airflow obstruction in COPD is often refer irreversible, contributed to the third leading cause of death worldwide in 2019 (Barnes, 2011; Marciniuk *et al.*, 2014).

2.2.1 Chronic obstructive pulmonary disease

The COPD is defined as preventable and treatable disease characterized by persistent airflow limitation that is not fully reversible. This airflow limitation in COPD results from the narrowing and remodelling of the small airways and the destruction of lung parenchyma or emphysema, although these changes do not always occur together (Vogelmeier *et al.*, 2017). The main aetiology of COPD is cigarette smoke which accounts for 80-90% of all cases, while air pollutant, noxious gases, as well as toxic chemicals, and genetic predisposition contributed to the remaining 10-20% of COPD cases (Barnes, 2016). The symptoms of COPD include coughing, shortness of breath, increase production of sputum, and wheezing and chest tightness. Spirometry test is required for the diagnosis of COPD which measures post-bronchodilator forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ≤0.7 confirms the presence of airflow limitation (Vogelmeier *et al.*, 2017). The spirometry is performed by patient to take a deep breath and blowing the air into the mouthpiece as hard and as fast as possible until there is no air left. The spirometer records the FEV1 and FVC by measuring the air exhaled into the mouthpiece (Moore, 2012). The classification of airflow limitation severity in COPD according to Global initiative for chronic obstructive lung disease (GOLD) is shown in the table 2.1 (Vogelmeier *et al.*, 2017).

Table 2.1: Classification of airflow limitation severity of COPD (Vogelmeier *et al.*, 2017)

In patients with $FEV_1/FVC < 0.70$:		
GOLD 1	Mild	$FEV_1 \geq 80\%$
GOLD 2	Moderate	$50\% \leq FEV_1 < 80\%$
GOLD 3	Severe	$30\% \leq FEV_1 < 50\%$
GOLD 4	Very severe	$FEV_1 < 30\%$

Note: forced expiratory volume in one second (FEV1)/forced vital capacity (FVC)

2.2.1(a) Etiology of COPD

Environmental as well as genetic predisposition have been proposed to play important role in the COPD development. In addition to active smoking, passive smoking or environmental tobacco exposure (ETS) may contribute greatly to the morbidity and mortality rate of COPD. Cigarette smoke contain more than 4700 chemicals compounds such as nicotine, carcinogens, and heavy metals which can activate myriads of genes that will cause oxidative stress, inflammation, apoptosis as well as premature aging in the lung (Tuder & Petrache, 2012; Park & Sin, 2014). Although it will take many years to develop COPD, acute inflammation in lung however can occur within hours of cigarette smoke inhalation (van der Vaart *et al.*, 2004). In human, fewer than 50% of cigarette smokers develop COPD in their lifetime (Vogelmeier *et al.*, 2017). This suggests the influence of genetic in COPD development.

Early epidemiology study demonstrated stronger correlation between parents with COPD and children or siblings as compared to spouses (Larson *et al.*, 1970). Now it is known that genetic contributes to the disease development and progression (Portelli, Hodge & Sayers, 2015).,Further studies have identified that α_1-antitrypsin, the most abundant serine protease inhibitor contributed to 1-2% cases in patients with COPD (Carroll *et al.*, 2014) . α_1-antitrypsin deficiency cause accelerated COPD development early onset of emphysema in smokers (Tanash *et al.*, 2017). Several other genes including those coding for transforming growth factor-β, matrix metalloproteinase (MMP)-9, and tumour necrosis factor-α were also shown to play a role though more study has to be conducted to confirm the role of these genes in COPD pathogenesis (Zhang *et al.*, 2011; Zhang *et al.*, 2016; Zhao, Zhou & Zhu, 2020).

Gender is another risk factor of COPD showing women being more susceptible to COPD as compared to men. Women are demonstrated to experience severe dyspnoea and physical limitation, while women aged beyond 45 years old experience a accelerated lung decline (Varela *et al.*, 2010). Recent study suggests that oestrogen may play a role in increasing the oxidative stress and the activation of TGF-β signalling (Tam *et al.*, 2016). In addition, socioeconomic status is also associated with the risk of COPD. Lower education, lower household income, and lower composite socioeconomic status index in low-income countries are associated with increased risk in COPD (Grigsby *et al.*, 2016). There is a correlation between high mortality rate and high spirometric restriction in low-income countries although the smoking rate is low. This high spirometric restriction is strongly associated with poverty though more study has to be conducted to understand the exact mechanism (Burney *et al.*, 2014). Apart from smoking, inhalation of particles including

occupational exposure to dust and fumes, as well as air pollution may also cause COPD. Occupational exposure induce respiratory symptoms, airway obstruction, and emphysema regardless gender (Marchetti *et al.*, 2014). Meanwhile ambient air pollution including black carbon and woodsmoke pollution have associated with increased in COPD mortality and hospitalization (Gan *et al.*, 2013).

2.2.1(b) Structural changes in COPD

COPD that occur in human may have these three disease processes which are thickening of the airway, chronic mucus hypersecretion, and emphysema, and patient may have all or some of these lesions at the same time (Forey, Thornton & Lee, 2011). Airway remodelling is a body mechanism to protect itself from insults such as virus, bacteria, noxious gases, and CS which tightly regulated by interaction of immune response, and body repair mechanism (Hutchison, Fligny & Duffield, 2013). In COPD, airway remodelling of small airway exhibits marked increase of airway wall that caused by epithelial changes, increased influx of immune cells, fibrosis, smooth muscle hyperplasia, goblet cell hyperplasia, and mucus plugging, contributing to airway resistance (Higham *et al.*, 2019).

When insults by CS occur in the lung, the DNA methylation of basal cells which give rise to ciliated cells, goblet cells, and Clara cells, were altered, causing limited differentiation ability of basal cells for re-epithelialization. CS also induces goblet cells hyperplasia, loss of Clara cells and ciliated cells, squamous cells metaplasia, and loss of tight junction barrier integrity (Staudt *et al.*, 2014; Martinez *et al.*, 2018). Increased goblet cells that contributed to mucus overproduction and alteration in ciliated cells number and cilia length cause disruption in mucociliary clearance and airway blockage (Gohy *et al.*, 2016). Mucin content that is responsible

for biophysical property of mucous are increased in CS exposure, thus making it harder for ciliated cells to move the mucous along the respiratory tract. This disruption in mucociliary clearance may harbour pathogenic microorganism that caused more damage to the cilia and the epithelial cells. Once the ciliated cells and epithelial cells are sloughed, the bacteria will attached to the cell membrane, thereby causing more inflammation (Higham *et al.,* 2019). Further disruption of mucociliary clearance can happen when basal cells become hyperploriferative and the cells begin to differentiate into squamous epithelium, instead of ciliated epithelium, causing squamous cell metaplasia (Herfs *et al.*, 2012).

Squamous cells metaplasia can also contribute to fibrosis in the lung by producing IL-1β which activates the TGF-β through integrin $\alpha_v\beta_8$ in fibroblast. Autocrine effects of TGF-β increases the production of collagen I and α-smooth muscle actin (α-SMA) thus increasing the fibrosis in lung tissue (Vallée & Lecarpentier, 2019). In addition, fibroblast also proliferate rapidly into more apoptotic-resistance myofibroblast to form fibrotic foci. This produces extracellular matrix (ECM) which deposited to the site and stimulate the production of CCL8 that will induce fibroblast activation and more ECM production creating a vicious profibrotic cycle that will increase fibrosis in the lung thereby causing airway obstruction (Florez-Sampedro, Song & Melgert, 2018). Meanwhile, emphysema is characterized by a permanent enlargement of distal airspace and destruction of alveolar walls that cause airflow obstruction (Tanabe *et al.*, 2011). Cigarette smoke induced oxidative stress and infiltration of immune cells releasing proteases leading to the breakdown of ECM and alveolar walls, creating airspace enlargement, thus minimizing surface area for gas exchange (Leberl, Kratzer & Taraseviciene-Stewart, 2013a). Cigarette smoke caused centrilobular emphysema more commonly in the

upper region of the lung. Centrilobular emphysema affects the secondary pulmonary lobules which consist of respiratory bronchioles, alveolar ducts, and alveolar causing destruction in this area of lung (Higham *et al.*, 2019).

2.2.1(c) Pathogenesis of COPD

COPD is a multiple pathogenetic mechanisms of oxidative stress, inflammation, apoptosis, and senescence which are inter-related with each other. Cigarette smoke induced DNA damage by producing oxidative stress, and this DNA damage and oxidative stress would induce inflammation, apoptosis, and senescence. Senescence cells then would produce senescence associated secretory phenotype (SASP) such as IL-1, IL-6, IL-8, MCP-1, VEGF, EGF, MMPs, and GM-CSF which will ramp up pro-inflammatory cytokine production, thus contributing to chronic inflammation in COPD (Aoshiba *et al.*, 2013; Kumar, Seeger & Voswinckel, 2014). Once established, the inflammation in COPD will persist even after smoking cessation (King, 2015).

2.2.1(c)(i) Oxidative stress

Oxidative stress refers to imbalance between free radicals and antioxidants (Czerska *et al.*, 2015). Cigarette smoke contain mixture of free radicals that are divided into two phases: tar or particle phase, and gas phase. Tar phase consists of 10^{17} long-lived radical molecules per gram, while gas phase consist of 10^{15} organic and inorganic radicals per puff (Boukhenouna *et al.*, 2018). One of the earliest manifestation of CS insult in the lung is the disruption of epithelial and endothelial barrier through oxidative stress (Schweitzer *et al.*, 2011). Reactive oxygen species (ROS) such as superoxide anion, and hyroxyl radical, and reactive nitrogen species (RNS) such as nitric oxide, and peroxynitrite which contain unpaired electron permit the transfer of electrons to other molecules via oxidation resulting in damage to

DNA, lipids, and proteins. Reactive oxygen species and RNS can also be released as byproducts during mitochondrial respiration, peroxisomal metabolism, and protein folding maturation process in endoplasmic reticulum (McGuinness & Sapey, 2017; Boukhenouna *et al.*, 2018). When CS inhaled into the lung, free radicals stimulate the alveolar macrophages, and epithelial cells to produce cytokines and chemokines which recruit more immune cells including neutrophils, lymphocytes, and monocytes into the lung. This reaction produce more ROS, thus aggravating the oxidative stress and lipid peroxidation, which in turn will induce inflammation (McGuinness & Sapey, 2017).

Reactive oxygen species can be deactivated by enzymatic such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, and non-enzymatic reactions like vitamin C, vitamin E as well as glutathione (GSH) which can be found in the lung (Birben *et al.*, 2012). However excessive production of ROS overwhelms the endogenous antioxidants, thus resulting in oxidative stress induce damage. Increased oxidative stress and significant reduction of GSH and SOD have been observed in the COPD as compared to non-smokers and smokers control (Elmasry *et al.*, 2015). Cigarette smoke irreversibly modified GSH into GSH-adduct thus depleting GSH (Horiyama *et al.*, 2014). Meanwhile CS increase the expression of TGF-β, which in turn reduced the manganese SOD through SMAD pathway (Michaeloudes *et al.*, 2010). Heme oxygenase-1 (HO-1) also was shown to exerts protective effect against acute exposure of CS, however in the presence of NE, CS significantly decreased HO-1 expression via SIRT pathway (Lee *et al.*, 2017b).

Oxidative stress can induce inflammation through activation of NF-κB that regulates inflammation in COPD (To *et al.*, 2013). However, the effect of ROS on

NF-κB can activates or prohibit the inflammation cascade depending on the site of ROS interaction in the NF-κB pathway and the types of cells (Lingappan, 2018). In human bronchial epithelial cells (HBEC), insults by H_2O_2 induce phosphorylation and ubiquitination of Iκβα, but no significant proteolytic degradation of Iκβα, nuclear translocation of p65, or increased NF-κB DNA binding activity (Jaspers *et al.*, 2001). Meanwhile, increased NF-κB DNA binding activity were observed when H_2O_2 were exposed to type II alveolar cell (A549) and human bronchial epithelial cell (16HBE) (Rahman *et al.*, 2001). Reactive oxygen species also induces TNF-α to phosphorylate *RelA* at Ser276 thus activating the NF-κB, while sustained ROS exposure can also lead to the attenuation of TNF-α induced I-κβ degradation and activation of NF-κB (Wu *et al.*, 2009; Jamaluddin *et al.*, 2007). Likewise, NF-κB can also play dual role in which to increase and inhibit oxidative stress. NF-κB regulates enzymes that promote production of ROS such as cyclooxygenase-2 (COX2), xanthine oxidase, inducible nitric oxide synthase (iNOS), and NADPH oxidase enzymes which create feedback loop, generating even more ROS (Morgan & Liu, 2011). Activation of NF-κB can also results in regulation of antioxidants such as ferritin heavy chain, SOD, catalase, HO-1, and GSH as feedback loop to ensure cellular survival (Morgan & Liu, 2011).

2.2.1(c)(ii) Inflammation

Inflammation is part of defence system of the body from harmful stimuli, resulting in elimination of cause of injury, and tissue repair (Netea *et al.*, 2017). Activation of inflammatory response occurs when cell surface pattern recognition receptor recognize the harmful stimuli, activating inflammatory pathways, causing inflammatory cytokines to be released, thereby recruiting the inflammatory cells to the injured sites (Chen *et al.*, 2018). Cigarette smoke activates several cell-signalling

pathways including NF-κB, mitogen-activated protein kinases (MAPK), signal transducer and activator of transcription (STAT), and activatory protein-1 (AP-1) (Lee, Taneja & Vassallo, 2012). Within one hour of exposure, CS causes inflammatory reactions by the activation of NF-κB and increase white blood cell count, lymphocyte count, and granulocyte count in the lung (Flouris *et al.*, 2012). Pre-clinical study in mice showed 4 weeks of CS exposure significantly increased p65 and Iκβα in lung as compared to control group (Yu *et al.*, 2018). Activation of NF-κB in epithelial cells and alveolar macrophage in response to CS result in the release of pro-inflammatory cytokines such as IL-8, IL-1β, IL-6, MCP-1, and TNF-α (Yao & Rahman, 2011). In addition, CS extract also activates ERK1/2 MAPK and STAT3 causing increased in IL-6 and IL-8, while inhibition of these pathway significantly reduced the pro-inflammatory cytokines (Jiang *et al.*, 2017). The release of these pro-inflammatory cytokines recruits immune cells including macrophages, neutrophils, and lymphocytes into the lung (Strzelak *et al.*, 2018)

Macrophages, neutrophils and lymphocytes are the prominent immune cells that drive inflammation in COPD. Cigarette smoke induced macrophages to release inflammatory mediators, including MCP-1, TNF-α, IL-8, MMP-2, MMP-9, and MMP-12 which can act on epithelial cells, fibroblast, smooth muscle cells, and immune cells (Rovina, Koutsoukou & Koulouris, 2013). Excessive MMP-12 and MMP-9 released by macrophage can result in permanent alveolar destruction (Atkinson *et al.*, 2011; Haq *et al.*, 2011). Macrophages also releases TGF-β that stimulate fibroblast proliferation, resulting in fibrosis in small airways (Pappas *et al.*, 2013). In addition, CCL2 releases by macrophages also recruits neutrophils, while CXCL9, CXCL10, and CXCL11 recruit CD8+ T cells and CD4+ T cells (Kim & Luster, 2015; Barnes, 2016).

Neutrophils number are increased in airway lumen and in lung secretion in COPD patient (Stockley *et al.*, 2013). Neutrophils are recruited to the lung by chemotactic factors including CCL2, CXCL1, CXCL8, CXCL5 derived from alveolar macrophages, lymphocytes, and epithelial cells (Barnes, 2016). The lifespan of neutrophils in COPD is prolonged as there is increase expression of anti-apoptotic BCL-XL and MCL-1 gene, and decreased pro-apoptotic BAC gene in neutrophils (Zhang *et al.*, 2012). Neutrophils exert its destructive effect mainly by neutrophil elastase. Neutrophil elastase produced by neutrophils contributed to alveolar destruction and mucus overproduction (Arai *et al.*, 2010; Hou *et al.*, 2014). Neutrophil elastase also triggers oxidative stress, promote degradation of extracellular matrix, endothelial damage, and apoptosis (Stockley *et al.*, 2013). In addition, neutrophils also produce neutrophil extracellular trap (NET), a web-like structure of decondensed chromatin associated with histones, neutrophils elastase, and myeloperoxidase which contribute to tissue damage and cell death. Recent study suggests increased NET in COPD patients which correlated with disease severity (Grabcanovic-Musija *et al.*, 2015).

Meanwhile, different subsets of lymphocytes accumulate in COPD lung including CD8+ T cells, CD4+ T cells, and B cells (Rovina, Koutsoukou & Koulouris, 2013). Infiltration of lymphocytes are more prominent in bronchial wall and small airway as compared to lung parenchyma (Olloquequi *et al.*, 2010). In COPD lung, CD8+ T cells predominates over CD4+ T cells (Paats *et al.*, 2012). Like neutrophils, prolong lifespan of CD8+ T cells were observed in COPD patient, which contribute to increase accumulation of lymphocytes in lung, thus aggravating the inflammation (Siena *et al.*, 2011). Meanwhile, Th17 cells, a subset of CD4+ T cells release cytokines such as IL-17A, and IL-17F, that would stimulate epithelial cells,