EXTRACELLULAR LUNG IMMUNOMODULATORY PROTEINS AND THEIR INVOLVEMENT IN THE DEVELOPMENT OF COPD

by

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Abstract

More than 250 million people worldwide are currently diagnosed with Chronic Obstructive Pulmonary Disease (COPD). COPD is a broad classification of clinical phenotypes that include but is not limited to, emphysema, chronic bronchitis, asthma- COPD overlap syndrome, and early-COPD. Patients with COPD presents with persistent respiratory symptoms and airflow limitation. One of the leading risk factors of COPD is cigarette smoke, which has been associated with triggering immune and inflammatory responses in the respiratory system. Upon review of the current literature, there is evidence of altered production and activity of key immunomodulatory lung proteins due to cigarette smoke that could contribute to the development of COPD. This thesis will review two predominant immunomodulatory lung proteins, club cell secretory protein (CC16) and pulmonary surfactant protein A (SP-A), in order to provide novel insights into the development of COPD to improve future surveillance and treatment for patients who are at risk or diagnosed with the disease.

Introduction:

Chronic obstructive pulmonary disease (COPD) is one of the most common and prevalent respiratory diseases that affects over 250 million people worldwide (1). In the United States alone over 14 million people are diagnosed with COPD, with an estimated 12 million undiagnosed cases (2). The current Global Initiative for Chronic Obstructive Lung Disease (GOLD) standard defines COPD as "A common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitations that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases and influenced by host factors including abnormal lung development." (3). However, this definition of COPD is constantly changing as experts in the field have begun to identify individuals who develop COPD with no prior smoking history. COPD generally manifests as continuous or persistent chronic respiratory symptoms including airflow restriction.

Airflow is determined by spirometric testing and measured as a ratio of forced expiratory volume per second (FEV_1) to forced vital capacity (FVC). Airflow restriction is defined as a reduced ratio of FEV_1/FVC in comparison to normal population values (by fixed cutoff or lower limits of normal)(4, 5). COPD encompasses a wide range of clinical phenotypes such as emphysema, chronic bronchitis, and early COPD.

Patients suffering from COPD typically present to the clinic with persistent cough, phlegm, dyspnea, wheezing, and inability to take deep breaths (6). Currently, there are limited treatment options for COPD patients which include inhaled or oral medications that help relieve symptoms of wheezing and coughing or supplemental oxygen (7, 8). The only cure for COPD is a lung transplant which is not suited for all patients with COPD as there are risks and complications when undergoing surgery (7, 8). There are many known factors that can contribute

to these chronic respiratory symptoms that eventually result in a COPD diagnosis. One identified risk of COPD is the inhalation of chemical or environmental gaseous irritants or pollutes, the most common on those being cigarette smoke. (9) According to GOLD, while less than 50% of smokers will develop this disease, it has been estimated that 55-75% of people with COPD were previous or are current cigarette smokers. (3, 10) Once cigarette smoke or other gaseous pollutes, are inhaled, they can ultimately activate inflammatory responses within the lung that, in turn, lead to innate immune responses in the lungs. Macrophages and neutrophils play a main role in these responses, by releasing cytokines and enzymes that can lead to disruption of the lung connective tissue (11, 12). In addition, persistent lung inflammation overtime reduces the integrity of the lung epithelium, resulting in the activation of adaptive immune cells, thought to contribute to permanent injury to the airway and epithelial cells by promoting airway remodeling and airspace enlargement. (9)

Extracellular proteins, those proteins that are secreted by resident lung structural and epithelial cells, are known to play major roles in host defense and lung homeostasis. Two of the most highly expressed groups of proteins include the surfactant proteins (SP-A, -B, -C, -D) and club cell secretory protein (CCSP or CC16). When the lung cells are injured, as in the case of many smokers that develop COPD, the production and secretion of these protective proteins could also be disrupted by. In cases where the proteins are still generated and secreted normally, there is evidence that their activity may be impaired, broken-down, or modified due to the inflammatory milieu in the diseased lung or by the inhaled gases and pollutants directly.

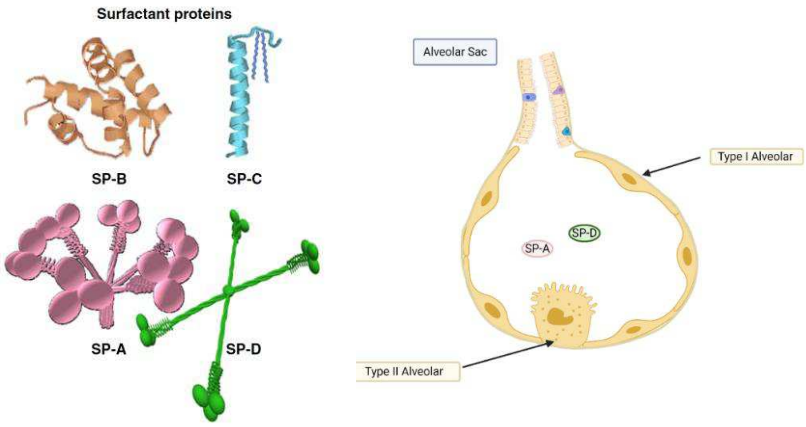

***Figure 1*:** Four types of lung surfactant proteins (13). Created with BioRender. Used with Permission: Reprinted from Biochimica et Biophysica Acta (BBA) - Biomembranes, Vol 1818, Issue 11, Cristina Casals , Olga Cañadas, Role of lipid ordered/disordered phase coexistence in pulmonary surfactant function, Pages No 2550-2562. Copyright 2012 with permission from Elsevier. Reprinted from The Lancet, Cristina Casals 1, Olga Cañadas , Role of lipid ordered/disordered phase coexistence in pulmonary surfactant function, 2550-2562., Copyright (2012), with permission from Elsevier.

Pulmonary surfactant is a lipoprotein complex that is predominantly produced by Type II alveolar epithelial cells and resides at the air-liquid interface on the lung. There are four types of surfactant proteins SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C are hydrophobic and maintain stability and surface tension in the lungs (14-17). While SP-A and SP-D are hydrophilic and assist with the clearance of inhaled pathogen through innate immunity, recent reports suggest they also contribute to acquired immunity (18-21). When comparing levels of SP-A from former smokers that went on to develop COPD to levels in healthy patients, lower levels of exhaled SP-A particles were in patients with COPD (22). In contrast, circulating SP-D found in blood serum generally thought to be higher in severe COPD patients (23). Based on the many publications in which SP-A and SP-D are involved in host defense during a myriad of pathogen infections, altered SP-A and SP-D levels due to smoking therefore could result in altered immune responses in the lung, which could result in persistent lung infections.

Another major secreted protein in the lung that has known roles in host defense is Club cell secretory protein (CC16). CC16 is produced and secreted by Club cells and nonciliated epithelial cells in the lungs (16, 24). CC16 functions in the body are not fully understood but there has been evidence to show that CC16 has anti-inflammatory and, anti-oxidant functions in the lung. In fact, CC16 has been shown to be decreased in the lungs of patients with COPD, and lung cancer (17, 25, 26). There have been studies that have directly linked cigarette smoking with a reduced amount of Club cells and specifically CC16 (27). In addition, other studies have suggested that lowered CC16 levels are a possible factor in modulating the lung response to cigarette smoke exposure and when levels are decreased, could be a contributing factor to the onset of COPD (26-29).

Interestingly, both SP-D and CC16 have both been identified as potential biomarkers for COPD since levels of these proteins in the circulation are thought to correlate with the disease (23, 30-32). However, the specific roles of these proteins and their dysregulation during cigarette smoke-induced development of COPD is still unclear. Since recent reports suggest that both SP-D and CC16 may contribute to phenotypic manifestations of COPD, **I hypothesize that altered production or activity of these secreted lung proteins due to exposure to environmental factors, such as cigarette smoke, contributes to the onset and progression of COPD.**

Prior to further discussing the role of these lung proteins in the pathogenesis of COPD, it is important to understand the basic anatomy and immunology of the respiratory system. Therefore, I will provide a brief overview of the respiratory system.

Anatomy of the Respiratory System

The human respiratory system is responsible for gas exchange, vocalization, maintaining pH levels, and preventing inhaled irritants/pathogens from entering the body (17). Functionally, the respiratory system can be split into the conducting airway and respiratory zone. Anatomically, it can be split into the upper and lower respiratory tract. The upper respiratory tract includes the mouth, nose, pharynx, and larynx (the organs outside the thorax). The lower respiratory tract includes the trachea, bronchi, bronchioles, and the lungs (the organs that are within the thorax above the diaphragm) (33, 34).

The upper respiratory tract begins with the nose, which is comprised of two external nares that leads into the nasal cavity. There are meatuses (passages) in the nasal cavity that help increase the nasal cavity surface area which is important for conditioning inhaled air. The meatuses and nasal cavity are lined with mucus membranes that are highly vascularized, which help warm and humidify the inhaled air as it passes through the upper respiratory tract. Warming the inhaled air prevents damage to the lungs while humidifying the inhaled air will prevent drying out of the respiratory tract (17, 33, 34). The nasal cavity mucosa consists of a pseudostratified columnar epithelium including ciliated cells, goblet cells, brush cells, granule cells, and basal cells (16, 33). Goblet cells in the respiratory system are responsible for secreting mucus, which prevents dehydration and capture particles that enters (16). These ciliated cells move the mucus along to the pharynx through the internal nares located at the junction between the base of the nasal cavity (33, 34).

When the upper respiratory tract is exposed to cigarette smoke the normal bacterial floral is altered and there is a reduction of ciliary function of upper respiratory epithelial cells responsible for moving particles/pathogens away from entering the lungs (35). Cigarette smoke also increases biofilm production of pathogenic microbiota allowing for better adhesion and

growth in the upper respiratory tract (35, 36). Secreted factors that inhibit bacteria growth and regulate host response, as SP-D and CC16 are predominantly in the lower respiratory tract, therefore, are less likely to provide defense against these bacterial pathogens in the upper respiratory tract (24, 25). This would likely result in enhanced and more frequent upper respiratory infections in those exposed to cigarette smoke. Previous studies have shown that smoking and secondhand smoking of cigarette smoke increases the chances of getting infected with respiratory viruses and bacteria, as well as the severity, and length of viral and bacterial respiratory infections. In one study on Pneumococcal Pneumonia smokers were at 2-4 times higher risk of being infected; in another study, current smokers were twice as likely to get community-acquired pneumonia than non-smokers (37, 38)

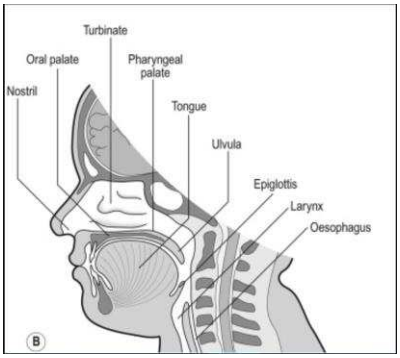

***Figure 2*:** Upper Respiratory System Illustration. Used with permission: Reprinted from Churchill Livingstone, The Respiratory System, Second Edition, Andrew Davies, Carl Moores, 2 - STRUCTURE OF THE RESPIRATORY SYSTEM, RELATED TO FUNCTION, Pages No 11-28., Copyright (2010), with permission from Elsevier. Reprinted from The Lancet, Andrew Davies, Carl Moores , 2 - STRUCTURE OF THE RESPIRATORY SYSTEM, RELATED TO FUNCTION, Pages No 11-28., Copyright (2010), with permission from Elsevier.

From the internal nares, the inhaled air then travels to the pharynx (throat). The pharynx begins at the internal nares and ends at the beginning of the esophagus. The pharynx is divided into three sections which are the nasopharynx, oropharynx, and laryngopharynx (4, 16). Inhaled air travels from the pharynx to the larynx through the glottis and when food or liquids are being swallowed, the epiglottis covers the glottis (4, 16). The larynx contains vocal cords and is made

up of hyaline cartilage. The larynx is also responsible for coughing reflexes and keeping substances out of the lower respiratory system (17, 33). From the larynx, the inhaled air then passes to the trachea which is the start of the lower respiratory system. (33, 34)

Next is the trachea, a tube that is anterior to the esophagus which conducts air from the larynx to the primary bronchi. The trachea is made up of tracheal cartilages and tracheal muscles(33, 34). The inhaled air travels from the trachea and branches into the right and left bronchus which are the two primary bronchi for each lung. The bronchi are also tubular passage with cartilage plates supporting its structure similar to the trachea with C-shape cartilage but smaller (33, 34). The primary bronchi branches into secondary bronchus, tertiary bronchus, and then bronchioles (39). The bronchi are found in the lobes of the lung and bronchioles are found in smaller sections of the lobes of the lungs called lobules. Bronchioles differ structurally from the bronchi, compared to which they are smaller and made up of smooth muscle, and they are not supported by cartilage (33, 34), which allows the bronchioles to collapse and expand as needed for ventilation. The bronchioles then further branch into the lungs to the terminal bronchioles where Club cells reside. Club cells produce the majority of club cell secretory protein (CCSP), which is also known as CC16. Club cells also produce antimicrobials and other molecule complexes that supports the respiratory tract and pulmonary surfactants (24, 25).

The terminal bronchioles further branch into respiratory bronchioles, constituting the respiratory zone of the lungs (39). The terminal bronchioles epithelium still consists of brush cells, ciliated cells, small granule cells, and Club cells (16, 17). The respiratory bronchioles continue to branch into alveolar ducts, which further divide into alveolar sacs. The alveolar sacs are made up of type I alveolar cells (or type I pneumocytes) and type II alveolar cells (type II pneumocytes or septal cells). Type I alveolar cells a thin squamous cell that lines together to

create a barrier for air space. Type I alveolar cells line about 95% of the alveoli surface while Type II alveolar cells lines about 5% (16). Type II alveolar cells are cuboidal cells that secret lung surfactants and progenitor cells of Type I alveolar cells. Along with the alveoli cells, there are simple squamous blood capillaries that cover about 80-90% of the respiratory zone for gas exchange which begins with pulmonary ventilation (16).

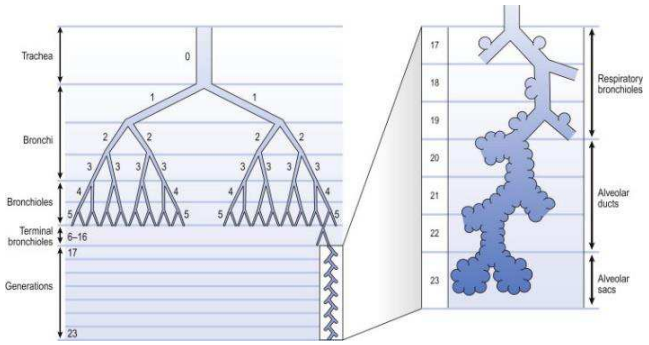

***Figure 3*:** Simplified illustration of lower respiratory airway branches and divisions. Used with permission: Reprinted from Churchill Livingstone, The Respiratory System, Second Edition, Andrew Davies, Carl Moores, 2 - STRUCTURE OF THE RESPIRATORY SYSTEM, RELATED TO FUNCTION, Pages No 11-28., Copyright (2010), with permission from Elsevier. Reprinted from The Lancet, Andrew Davies, Carl Moores , 2 - STRUCTURE OF THE RESPIRATORY SYSTEM, RELATED TO FUNCTION, Pages No 11-28., Copyright (2010), with permission from Elsevier.

Pulmonary Ventilation and Gas Exchange

Pulmonary ventilation is the movement of air from the atmosphere in and out of the lungs by inhalation and exhalation (33). The relaxation and contraction of the diaphragm and respiratory muscles relax and contract the airway which increases and decreases the volume and in turn also alters air pressure in these airways following Boyle's Law (33). As the volume of the pleural cavity in the thorax changes the interpleural pressure changes allowing for air to enter and exit the lungs during exhalation and inhalation (17, 33). As air enters the lungs it travels to the alveolar ducts and sacs where gas exchange occurs (33).

Gas exchange is the diffusion of oxygen from the lung lumen through type I alveolar cells to the blood capillaries and carbon dioxide from the blood into the lungs to be expelled into the atmosphere (17, 33). Oxygen is required for aerobic cellular respiration in the body to produce ATP from the breakdown of glucose and other molecules. Carbon dioxide is one of the many products of the body's cellular respiration and travels in the blood to the lungs by binding to hemoglobin as bicarbonate ions or dissolved carbon dioxide (17). This can alter pH levels in the blood especially during exercise where more carbon dioxide is in the blood as bicarbonate and decrease in pH leading to stimulation of increased pulmonary ventilation to exhale the carbon dioxide(17). Gas exchange is important for supplying the body with adequate oxygen and removing carbon dioxide for cellular respiration and blood pH homeostasis (17).

During pulmonary ventilation and gas exchange, the pulmonary surfactants (specifically the phospholipid dipalmitoylphosphatidylcholine or DPPC found in these lipoproteins) found in the fluid lining of the alveoli and alveolar space help reduce surface tension that, in turn, help prevent the alveoli/lungs from collapsing after exhalation (16, 17). Reducing the surface tension at the air-fluid epithelium site allows for the balance of different pressure changes with air flows from small to large alveoli (17, 40). The reduction of the surface tension of the alveoli surface also allows for easier expansion of the lungs during each inhalation (17, 40). Pulmonary surfactants play a vital role in preventing the lungs from collapsing (16). When cigarette smoke is inhaled, the smoke particles can reach the terminal bronchioles and alveolar sacs within the lungs (41, 42). Cigarette smoke can induce inflammation in the lungs and disrupt the repair and replacement process of alveoli cells which could eventually lead to the development of COPD (41, 43). The next section will review the basics of immune functions in the lungs and the immune response in the development and severity of COPD.

Immune Response in the Lungs and COPD

Cell-Mediated Immunity

The pulmonary respiratory system is divided into the upper and lower respiratory tract, each with its own specialized epithelium that helps keep out irritants and pathogens from further reaching the areas of gas exchange in the lungs. The innate immune system has long been recognized key culprit of smoke-induced acute lung pathologies. More recently, the adaptive immune systems have been shown to mediate many aspects of host defense in the lungs contributing to the onset and progression of COPD (and related respiratory diseases such as allergic airway diseases) (44, 45).

The innate immune system response to infectious agents and environmental irritants, such as cigarette smoke, are usually immediate, not long-lasting, and are typically non-specific(46). Nonspecific responses of the innate immune system include physical barriers, recruitment of leukocytes, production of cytokines (proteins released by various leukocytes for signaling and inflammatory responses), and interferons (protein factors released by leukocytes), and regulation of receptors that are active in surveilling the lungs (46-48). The main cells of the innate immune system in the lungs are basophils, eosinophils, neutrophils, macrophages, dendritic cells, and mast cells (49).

Basophils and eosinophils are important cellular components of innate immunity as they are involved in eliminating parasites. Basophils and mast cells are involved in inflammatory responses and allergic reactions via their quick release of histamines. A very abundant type of pulmonary leukocyte are the neutrophils (46). Neutrophils are involved in wound healing; they release chemokines (peptides that draw other leukocytes) and lipid mediators for the

inflammatory response to the site of injury (46). Neutrophils can also eliminate pathogens through phagocytosis. Macrophages also ingest pathogens and clear remains of apoptotic/dead cells during recycling (50). The respiratory system also has specialized alveolar macrophages found in the alveoli which ingest pathogens and/or irritants that have reached the respiratory zone of the lungs (51). Dendritic cells are antigen-presenting cells that allow for adaptive immune cells to recognize foreign versus self cells and/or particles(46, 50). Dendritic cells are activated when the initial physical barrier of the epithelial-derived innate immune system are not enough to defend against infectious agents and environmental irritants.

Epithelial Immunity

The physical barrier in the respiratory tract starts with the hair in the nose and includes ciliated cells and secretory cells (goblet, club, and serous cells) that help to trap dust and pathogens in the mucus layer for muciliary clearance (47, 52). These surface epithelial cells are able to maintain their structure and barrier integrity through tight adhesions/tight junctions which are protein complexes that bind the epithelial cells to each other and the stroma or base of the lung tissue(53, 54). These tight junctions act as a seal for the lung surface epithelium which creates the physical barrier for the respiratory system(54, 55).

The secretory cells in the respiratory tract also secrete various proteins that play important roles in innate immune response in the respiratory system. Mucin is a glycoprotein secreted in the upper respiratory tract by goblet cells and has been suggested that specific subtypes of mucin are associated with alveolar macrophages through binding of mucin on the immunoreceptors (56, 57). Mucin is involved with ciliary cells clearance and transportation of mucus out of the respiratory tract. In recent studies, mucin has been associated with innate immune responses in the coordination of innate immune and inflammatory responses to

pathogenic bacteria by providing a broader area of interactions between leukocytes and the glycosylated proteins found on bacterial walls (52, 57). There are other proteins secreted by lung epithelium that binds to pathogens and/or leukocytes to further activate immune response (46, 58, 59). For example, lysozymes and defensins are proteins that are secreted and bind to bacterial cell wall or neutrophils, which enable the cells to eliminate the bacteria by hydrolyzing or disrupting the bacteria's cell wall (46, 58).

Non-cell mediated innate immunity also includes a system of proteins, called the complement system, that can be found in the circulating blood serum, pulmonary mucus, and used by macrophages (46). The complement system aids with the innate immune response in eliminating pathogens through opsonization (labeling infectious agents with opsin for elimination), attracting leukocytes (chemotaxis), and activating neutrophils or macrophages to the site of the pathogen to eliminate it (46, 60, 61).

The innate immune system can sense and respond to inhaled pathogens and irritants through various receptors that are present on the airway surface epithelium and innate immune leukocytes (59, 62). Toll-like receptors (TLR) and RIG-I-like receptors (RLR) are some examples of pattern recognition receptors that are found on the airway surface epithelium and common innate immune leukocytes such as macrophages and dendritic cells (59, 62). TLR and RLR activation lead to signaling cascades that result in the production and release of various pro-inflammatory cytokines (59). The activation of TLRs is one of the many ways that can activate the canonical/classical signaling pathway of the transcription factor nuclear factor κB (NF-κB) shown in Figure 7. NF- κB is responsible for the gene expressions of many pro and anti-inflammatory cytokines including Interleukin 8 (IL-8) and Interleukin 6 (IL-6) (59, 63, 64).

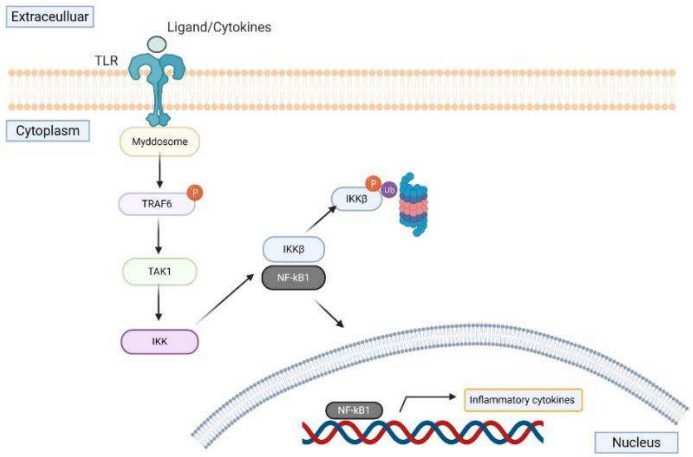

Figure 4: Basic canonical NF- κB signaling pathway activated through Toll-like receptor (TLR). TLR ligand binds to TLR then induces signaling through the Myddosome complex. Myddosome complex phosphorylates and activates TRAF6 which then activates kinase TAK1. TAK1 activates the IKK complex which phosphorylates IKKβ and dissociates from now activate NF-κB. NF-κB translocates to the nucleus and IKKβ is tagged for degradation by proteasomes. Created with BioRender.com. Adapted from Liu T (2017) (65)

IL-8 and IL-6 are proinflammatory cytokines that are involved in the recruitment of neutrophils and inducing the production of other cytokines (and chemokines) to the site of pathogenic invasion or tissue injury (66, 67). IL-8 and IL-6 play a major role in initiating immune infiltrate into the respiratory tract and inflammatory responses that could lead to COPD. IL-8 and IL-6 have been shown to be elevated in the sputum of patients diagnosed with COPD (68-70). In other studies that examined bronchial biopsies from smokers and patients with COPD, there was an increase of NF- κB subunit protein expression in comparison to non-smokers (71). There have been studies that also suggest that patients with COPD have higher TNF-α blood serum levels compared to healthy individuals(72). There is still ongoing debate in the field whether the TNF-α serum levels are useful diagnostic markers for COPD (72). These various cytokines are important in innate immune responses but also play a role in initiating adaptive immune responses in the respiratory tract which if unchecked could lead to the development of COPD.

The respiratory system also has specialized lipoproteins which contribute to the make-up of the pulmonary surfactant lining fluid. Pulmonary surfactant is secreted by club cells and type II alveolar cells. Surfactant proteins are involved with reducing surface tension and with innate immunity. However, they are also known to, respond to acute lung injury (16). Pulmonary surfactants A and D (SP-A and SP-D) are most well known for their roles in eliminating pathogens via opsonic activity by binding to bacteria and viruses invading the respiratory tract and attracting neutrophils and macrophages to phagocytose the pathogens (73). These pulmonary surfactants can also modulate innate immune cells by mediating the expression of receptors that recognizes pathogens on the surface of leukocytes (73). SP-A is involved in the activation and the inhibition of nitric oxide production in macrophages that is used to eliminate bacteria and fungi. SP-A in previous studies has been suggested to inhibit macrophage secretion of inflammatory cytokines in normal lung function and enhance macrophage secretion of inflammatory cytokines during respiratory infection and/or injury (74). SP-A and SP-D, in specific settings, can promote the production of cytokines and dead cell clearance by innate immune cells (73). Not only relevant for innate immunity, but SP-A and SP-D have also been linked with modulating adaptive immune cells as well.

The adaptive immune responses are delayed, more specific, and develop over time after exposure to pathogens and/or irritants. B cells, T cells, Regulatory T cells, and Natural Killer (NK) cells are immune cells that are involved in adaptive immunity (46). B cells are lymphocytes that are responsible for producing antibodies (46). Antibodies are proteins that are used to neutralize pathogens and induce mast cells response to allergens and parasites. Mature B cells, plasma cells, produce antibodies kept as memory B cells (46). Memory B cells can continue to produce antibodies to quickly respond if there is repeated exposure to the same

antigen (46). Antigens can be any molecule or piece of pathogens, allergens, and hosts that can be recognized by lymphocytes (50). B cells also present antigens to T cells to initiate further immune responses (50).

T cells make up most of the lymphocytes in the body and the two major classes of T cells are CD4+ and CD8+ T cells (50). T cells can only recognize antigens that are presented with MHC molecules which were processed by host cells such as B cells and dendritic cells (46). T cells can produce and secret cytokines to recruit other immune cells and granules containing cytotoxins that activate apoptosis in infected cells. These T cells are highly regulated by the immune system to prevent cytotoxins from damaging cells in the body under normal conditions (50).

Regulatory T cells are involved in the monitoring of T cells and prevent immune responses on host cells and host antigen through peripheral tolerance. Natural Killer (NK) cells eliminate cells that have been infected with virus and tumor cells by releasing granules that target cell surfaces (46). NK cells initially were thought to have only innate immunity characteristics but in recent studies, there have been indications of NK cells having the ability to be kept as memory cells for future responses to some pathogenic insult which is a characteristic of adaptive immunity (50).

Although the majority of the lung immune defenses are innate, in pulmonary diseases such as COPD both systems seem to contribute to the biological and clinical presentations of this disease. Initially, the adaptive immune responses in COPD were thought to involve mostly T cells, since of brachial biopsies identified increased T cell expression in patients with COPD (75-77). In recent years, there have been studies that show increases in B cell lymphoid aggregates and the production of antibodies in severe COPD patients. One study by Sullivan *et al.* (78),

suggests that the increase of B-cells activity in emphysema could be the result of one of the many contributing factors of emphysema (one of the many clinical phenotypes of COPD (79)). There are ongoing and further investigations on the adaptive immune system in COPD. These immune and inflammatory responses lead to pathological changes in the respiratory tract.

Basic Pathology of COPD

The pathological changes of the lungs in those with COPD is of importance because it correlates to the persistent clinical respiratory symptoms (80). Inhaling irritants such as cigarette smoke would result in an inflammatory response that could be amplified and result in chronic bronchitis, emphysema, and small airway inflammation and fibrosis (81-83). The pathological changes of the lungs in COPD historically only described the pathological changes found in emphysema in comparison to the pathological changes found in tuberculosis (80). Currently, the pathological changes of the lungs in COPD are still mainly described as emphysematous. However, the definition is starting to encompass many other non-cancerous pulmonary diseases such as small airway disease and pulmonary fibrosis (42, 80, 84). Emphysema and small airway disease have many pathological similarities that are related to the pathological depictions in COPD (42).

Emphysema is defined as “permanent and abnormal enlargement of the airspaces within the bronchioles with accompanying destruction of the airway walls without any visible gross fibrosis (6).” However, this definition is likely to change in light of recent studies that show an increase in collagen in the lungs of those diagnosed with COPD, which suggests that fibrosis could be a characteristic of emphysema (6, 84). The main pathological changes in emphysema take place primarily at the respiratory bronchioles, with a typical onset due to inflammatory

mechanisms of injury (80). There are three main subtypes of emphysema as depicted in Figure 6: distal acinar, pancinar, and centriacinar emphysema (85). Distal acinar emphysema (also known as Paraseptal emphysema) (86, 87) is typically observed in young adults, while pancinar emphysema is observed in those with a deficiency of the protease alpha-1-antitrypsin and centriacinar emphysema is seen in those who were former or current cigarette smokers (80). Each of these subtypes of emphysema affects a different area of the respiratory bronchiole however they exhibit the same pathological changes enlargement of the respiratory airway (80). Enlargement and inflammation of the respiratory airway is also an identified pathological change in small airway diseases in patients with COPD(42).

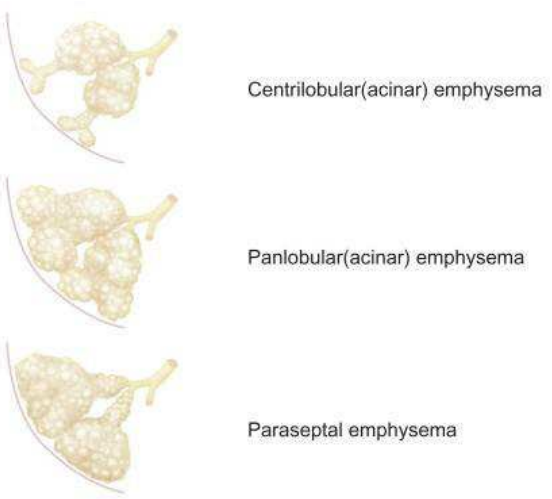

Figure 5: Three types of emphysema. Paraseptal emphysema is also known as Distal Acinar emphysema, Used with permission: Takahashi M, Fukuoka J, Nitta N, Takazakura R, Natatani Y, Murakami Y, Otani H, Murata K. Imaging of pulmonary emphysema: A pictorial review. Int J Chron Obstruct Pulmon Dis. 2008;3(2):193-204. Original Publisher: Dove Press Medical (87).

Small airways in the lungs are defined as airway passages that are ≤2mm diameter and typically refer to the bronchioles and alveoli. (42, 88, 89). Small airway disease occurs when there are pathological changes in these small airways that result in airflow obstruction. Small airway disease also incorporates other pathological changes such as fibrosis, narrowing, and destruction of the bronchioles and alveolar ducts(42). Three factors that could lead to these

pathological changes of small airway disease in COPD are persistent airway repair/remodeling, mucus secretion and plugging, and inflammatory cell infiltrate (42, 88).

Respiratory airway remodeling takes place in the lungs in response to tissue injury by pathogenic factors or environmental factors, such as cigarette smoke. The lung tissue, specifically the extracellular matrix, is repaired and remodeled by a type of fibroblast called myofibroblasts along with various enzymes relevant to the process. Repeated injury and airway repair can lead to scarring, fibrosis, and thickening of the airway wall. The airway remodeling and repair process also alters the lung epithelium such as the disruption of tight junctions in the alveolar ducts causing alveoli cells to dissociate from one another. Other lung epithelial changes include metaplasia and hyperplasia of goblet and basal cells(42) which are important to maintaining the normal function and part of host defense against pathogenic organisms and substances.

In previous studies, these hyperplastic pathological changes of goblet cells in response to cigarette smoke have been shown to cause an increase of mucus secretion (42, 90-92). With increased or hypersecretion of mucus in the respiratory tract, the excess mucus could create a physical barrier that blocks the bronchioles and alveolar ducts, leading to airflow obstruction and reduction of pulmonary function (42). In addition, the accumulation of mucus and altered lung epithelium could increase the risk of respiratory infections by bacteria and viruses in the upper and lower respiratory tract(36, 38, 93). Respiratory infections and repeated injury from cigarette smoke activate immune responses that result in increased inflammatory cellular infiltration. In previous studies have shown that various inflammatory cells such as neutrophils, macrophages, CD4+, and $CD8^+$ T were found to be increased in numbers in the lungs of those with COPD in comparison to those who smokers without airway obstruction and nonsmokers. Inflammatory

infiltrates contribute to the disruption and injury of the small airway epithelial walls. There are many similarities between small airway disease and emphysema lung pathological changes in those with COPD. Both include altered lung tissue architecture and involves inflammatory infiltrates. Previous studies have suggested that different areas of the lungs have various severity in pathological changes and times during the onset and progression of COPD(80). These pathological changes could be correlated to the reduced airflow and persistent respiratory symptoms that patients experience prior to being diagnosed with COPD(6). There are multiple risk factors that could lead to theses pathological changes in patients with COPD.

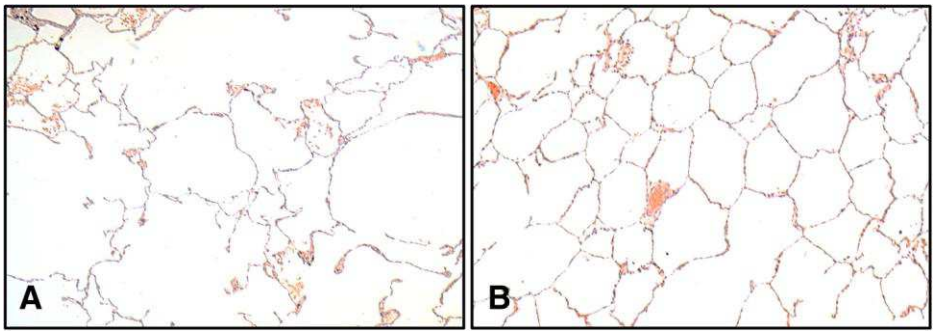

Figure 6: Histological representation of lungs with emphysema (slide A) slide as compared to normal (slide B). Used with permission, Author: Andrew Higham et al, Publication: Respiratory Research Publisher: Springer Nature Date: March 4, 2019, http://creativecommons.org/licenses/by/4.0/. (42)

Risk factors of COPD

According to the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD), the causes of COPD is yet to be fully understood and risk factors of COPD have thus far have been identified by cross-sectional epidemiology studies (3). One of the leading and most well-studied environmental factors that are known to lead to the development of COPD is cigarette smoke. However, cigarette smoke is not the only risk factor of COPD (3). Second-hand smoking and inhaling smoke from cigars and pipes are other tobacco environmental risk factors (3). Other environmental risk factors associated with COPD include dust, allergens (pollen), occupational

exposure to harmful chemical fumes, biomass smoke (burning of fossil fuels and coal mining), indoor and outdoor air pollutants (3, 94). Non-environmental risk factors related to COPD include peri and pre-natal environmental exposure, prematurity lung function at birth, and genetics.

The Global Initiative for Chorionic Obstructive Pulmonary Disease states that the risk of developing COPD increase with age. Age can refer to the overall aging of the human body and all the organ systems, including the lungs that have reduced efficiency and function as one age. Age could refer to the years the respiratory system has been exposed to countless allergens, biomass smoke, irritants, noxious gases, etc. which would contribute to lung function decline and the development of COPD. Patients over the age of 40 years-old are typically at higher risk for diagnosis of COPD. (95) Lung function deficits in early gestational to childhood years, as well as developmental lung growth abnormalities are emerging risk factors recently identified in studies of COPD (96). It is known that a child/adolescent with an underdeveloped or irregular respiratory tract will likely have reduced lung function as an adult. Studies have also shown that those who had early childhood asthma were at greater risk to develop COPD as adults. Furthermore, this risk is furthered increased in asthmatic smokers (97-99). Currently, in the field, there is a gap in knowledge pertaining to the dysregulation of signaling pathways in early lung development and their association with the progression and development of COPD.

In addition to environmental contributions, there is also a genetic risk factor that is commonly referenced when talking about COPD onset inherited Alpha-1 antitrypsin deficiency (AATD). Mutations in SERPINA1, the gene that encodes for Alpha-1antitrypsin (AAT) can lead to the inherited autosomal co-dominant deficiency (100). Alpha-1 antitrypsin, a serine protease inhibitor (serpins) family member, is most notable for inhibiting elastase that is found in the

lungs (100). AAT deficiencies are rare and typically affect the liver, lungs, and more rarely on the skin (101). Those with AAT deficiencies initially present with respiratory symptoms including shortness of breath, which progressively worsens and in many cases results in emphysema (102). There have been genetic factors identified that could contribute to COPD as well. Those include the genes Hedgehog Interaction Protein (HIP) and Matrix Metalloproteinase 12 (MMP-12), which are associated with reduced lung function and COPD (3).

Taken together, these various genetic, environmental, and lung development risk factors have been associated with COPD and may relate to the different observed clinical phenotypes of the disease. Each of the clinical phenotypes under the umbrella of COPD have different progression and pathogenesis. However, one commonality between the progression/pathogenesis of theses clinical phenotypes in COPD patients is irreversible airflow limitation.

Pathogenesis of COPD

The observed clinical phenotypes of COPD have been updated to include but are not limited to emphysema, chronic bronchitis, asthma-COPD overlap syndrome, and early COPD (6, 79). The pathogenesis and progression of each of these clinical phenotypes are distinct with some overlap between the phenotypes as they all involve immune and inflammatory responses. One long-lasting paradigm that has been recently brought up for discussion relates to the pathogenesis of emphysema as a protease-antiprotease imbalance. Another emerging concept is the presence of host adaptive autoimmune responses in some patients with COPD (79, 80).

The protease-antiprotease paradigm in emphysema refers to the imbalance between proteases and antiproteases in the respiratory tract; higher levels of proteases result in the destruction of the walls and architecture of the respiratory system. (103). Proteases, such as

elastase, are released by neutrophils, while matrix metalloproteases are release by macrophages. The cells release these proteases within the lungs in response to inhaled irritants, such as cigarette smoke, which can then degrade elastin in the airways if the appropriate levels of antiprotease or protease inhibitors are not present (80, 104). For example, those with Alpha-1 antitrypsin deficiency have less AAT available to inhibit the increased levels of elastase released by neutrophils in the lungs (105). However, not all individuals with emphysema have antiprotease deficiencies. Recent studies also suggest that the destruction and enlargement of the airspaces in emphysema could involve an adaptive autoimmune response (79, 106).

In the autoimmune paradigm for emphysema, the inhalation of cigarette smoke would initially induce neutrophils and macrophages to release elastase and matrix metalloproteases (106). Then, the effects of cigarette smoke would still be ongoing as the adaptive immune system's T cells and B cells continue to produce cytokines and antibodies (79). One study suggests that macrophages also induce the recruitment of dendritic cells that then signal to T cells to develop a subset of Tells that produce cytokines IL-17A and IFN-γ. Those cytokines further enhance macrophages to release MMP-12, which degrades elastin in the lung (106). In additional studies, anti-elastin autoantibodies have been identified in the lungs from emphysema patients that further suggest autoimmunity as part of the pathogenesis of emphysema (79, 107).

In other clinical phenotypes of COPD, such as chronic bronchitis, the immune system, and inflammatory responses are also responsible for lung function decline (79). As mentioned previously, the accumulation of innate immune cells in the lung parenchyma in response to cigarette smoke can damage airway epithelial and eventually impair airway remodeling and repair. Taken together, the emergence of symptoms of chronic bronchitis as a clinical phenotype of COPD is closely associated with overall lung function decline. With the pathogenesis of the

various COPD associated clinical phenotypes still under scrutiny, it is recognized that there are still many aspects of COPD yet to be understand and players to be identified. One relatively unknown aspect of COPD is understanding the roles of immunomodulatory lung proteins, and the impact of cigarette smoke on their expression and functionality, in the development of COPD. As such, the role of pulmonary surfactant protein A (SP-A) and CC16 will be further reviewed in this thesis as the recent studies show the detrimental impact of cigarette smoke on these proteins and support their involvement in the development of COPD.

Cigarette smoke, immunomodulatory lung proteins, and COPD

Pulmonary surfactant protein A (SP-A) and Club cell secretory protein (CC16) are two important immunomodulatory lung proteins that participate in host defense against inhaled pathogens, noxious gas, and irritants by modulating both innate and adaptive immune responses. Recent studies have demonstrated that the production and secretion of these immunomodulatory lung proteins can be altered with exposure to cigarette smoke. As previously mentioned, the tar particles in cigarette smoke induce immune response with subsequent inflammation that results in the disruption of lung epithelium in the respiratory system. Chronic disruption of the lung epithelium in smokers could thereby alter SP-A and CC16 secretions, which may contribute to recurrent respiratory infections and decreased lung function and lead to the development of observed clinical phenotypes associated with COPD.

Pulmonary Surfactant Protein A

In humans, Type II alveolar cells are the primary producers of pulmonary surfactants, which includes highly expressed surfactant protein SP-A. These Type II alveolar cells are crucial as they are progenitor cells for Type I alveolar cells and also important for repair and

replacement of Type I alveolar cells. In the study by Kosmider, B., *et al.* (41), human alveolar epithelial type 1-like (AT1-like) cells were exposed to different quantities of cigarette smoke extract (CSE) after which apoptotic and necrotic changes and the role of Nuclear factor-erythroid 2 related factor 2 (Nrf2) was assessed in these cells. AT1-like cells are type II alveolar cells that are transdifferentiated towards being type I alveolar cells. Kosmider B. and colleagues exposed AT1-like cells to three different CSE concentrations 1%, 5%, and 10% CSE for 4 hours and 24 hours. The CSE concentration is the solution from one 3R4F cigarette, that was burnt at a set speed by a pump for 15 minutes, the solution collected from one cigarette was considered 100% CSE concentration. Kosmider B. and colleagues also calculated that 1% CSE is equivalent to 5 cigarettes/day, 5% CSE is equivalent to 25 cigarettes/day, and 10% CSE is equivalent to 50 cigarettes/day. The AT1-like cells were then double-stained with acridine orange and ethidium bromide to identify for living cells (green nucleus with red/orange cytoplasm), early apoptotic cells (membrane present but condensed chromatin), late apoptotic (orange nuclei), and necrotic cells (uniformed orange cell nuclei) (41). The results showed in AT1-like cells, over 4 hours of exposure to CSE, more apoptotic cells were present; however, over 24 hours of exposure to CSE more necrotic cells were present (shown in figure 7). These results indicated that cigarette smoke exposure can cause alveolar cells to activate both apoptosis and necrosis. The study by Kosmider B. and colleagues also demonstrates that the amount and duration of exposure to CSE correlate with AT1-like cells apoptosis and necrosis. This study supports the notion that Type II and Type I alveolar cells are affected and diminished with cigarette smoke exposure in turn altering pulmonary surfactant secretion and reduce lung function at the gas exchange site contributing to the development of COPD.

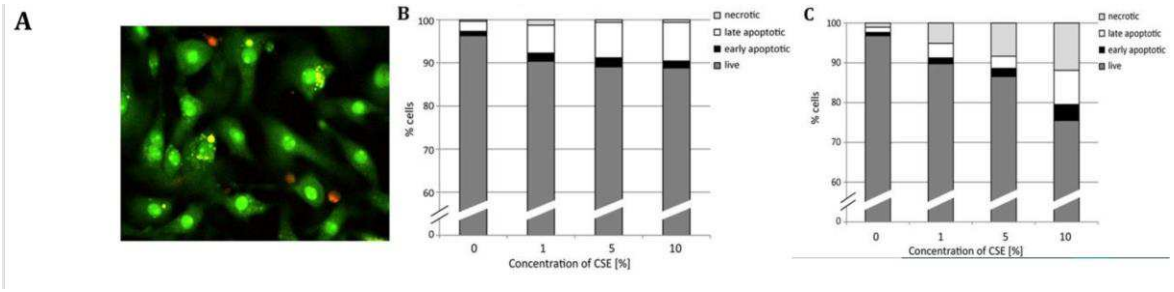

Figure 7: Human AT1-like cells exposure to cigarette smoke extract. A) 4 hours exposure to 5% CSE. B) 4 hours exposure. C) 24 hours exposure. Used with permission: Kosmider B, Messier EM, Chu HW, Mason RJ (2011) Human Alveolar Epithelial Cell Injury Induced by Cigarette Smoke. PLoS ONE 6(12): e26059. https://doi.org/10.1371/journal.pone.0026059

A study published earlier this year by Agudelo *et al.*, identified a correlation between decreased lung surfactant lipids and decreased lung function measurements in patients with COPD (108). Agudelo and colleagues collected blood samples and bronchoalveolar lavage (BAL) fluid by direct aspiration from the right lung from 12 stable COPD volunteers who were former cigarette smokers (who have ceased smoking for at least 6 months) and 5 healthy volunteers (who had no history of asthma or cigarette smoking) (108). These volunteers also underwent spirometry testing to determine their lung function measured by forced expiratory volume in 1 second (FEV1). According to the GOLD 2020 report, FEV1 % is used to determine the stage of COPD, those with FEV1 ≥ 80% predicted are considered mild COPD, 50% ≤ FEV1 < 80% predicted is moderate COPD, 30%≤ FEV1 < 50% predicted is severe, and FEV1 < 30% predicted is very severe (3). The COPD volunteers' average FEV1 % was ~51.7% (indicating they had moderate COPD) and the healthy volunteers average FEV1% ~98.2%. The BAL fluid samples from each volunteer were extracted for lipids and normalized to epithelial lining fluid to account for any dilution during the aspiration (108). The extracted lipids were analyzed and processed by liquid chromatography and mass spectrometry. Agudelo and colleagues, also included a murine study, where they had 11 mice in a chamber with regular room air, as control just as the healthy volunteers and 3 mice were exposed to second-hand cigarette smoke for 4

hours per day for 6 months in another chamber, to replicate the COPD volunteers who were former smokers (108).

The results of the study showed that in the healthy volunteers they had 60% higher overall surfactant lipids than the COPD volunteers (108). Their study showed a direct correlation between total lipids and FEV1 values which represents lung function (Figure 8 A and B) (108). Agudelo *et al.* further compared the phospholipid classes and the results also showed that COPD volunteers had lower amounts of phospholipids in each class compared to healthy volunteers (Figure 8 E) (108).

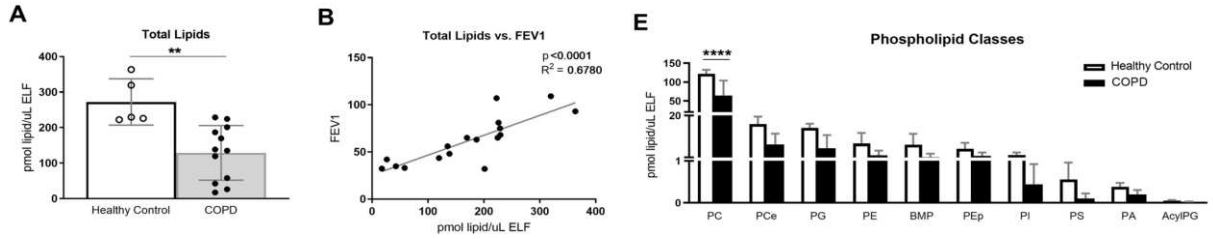

Figure 8: Extracted total surfactant lipids and phospholipid class comparison. A) Total surfactant lipids in health and COPD volunteers. B) Total lipid and FEV1 E) Phospholipid classes comparisons in healthy vs volunteers. Used with permission: Agudelo CW, Kumley BK, Area-Gomez E, Xu Y, Dabo AJ, Geraghty P, et al. (2020) Decreased surfactant lipids correlate with lung function in chronic obstructive pulmonary disease (COPD). PLoS ONE 15(2): e0228279. https://doi.org/10.1371/journal.pone.0228279

In the mouse model study, BAL fluid was collected through a tracheal catheter and the surfactant lipids were extracted and processed similar to human BAL fluid. The mice from the room air exposure chambers had significantly higher amounts of total lipids than the mice that were exposed to cigarette smoke for 4 hours a day (Figure 9) (108).

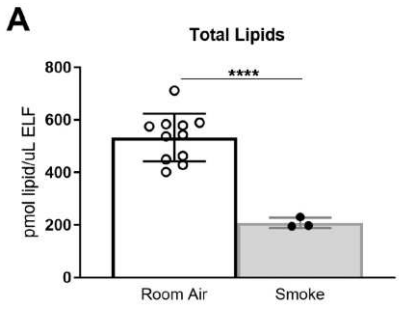

Figure 9: Mouse BAL fluid total surfactant lipid comparison. Used with permission: Agudelo CW, Kumley BK, Area-Gomez E, Xu Y, Dabo AJ, Geraghty P, et al. (2020) Decreased surfactant lipids correlate with lung function in chronic obstructive pulmonary disease (COPD). PLoS ONE 15(2): e0228279. https://doi.org/10.1371/journal.pone.0228279

The overall results of this study demonstrate that there is a correlation between the volume of pulmonary surfactant lipids and lung function especially in those with COPD. This further supports the notion that surfactant proteins are impacted with exposure to cigarette smoke (and second-hand smoke) as surfactant proteins are mixed with surfactant phospholipids to make up pulmonary surfactant. Surfactant proteins not only interact with surfactant lipids but in some cases rely on these surfactant phospholipids for trafficking outside of the alveolar cell by lamellar bodies (108, 109). Phosphatidylglycerol (PG) and Phosphatidylinositol (PI) are involved with the organization of extracellular surfactant complexes (108). If PG and PI secretion are altered, as is suggested by cigarette smoke exposure, then decreased lipid levels could also impact the extracellular secretion of SP-A. Many of the phospholipids observed in this study interact with SP-A as they are secreted extracellularly together (109). Phosphatidylcholine (PC) (the most abundant surfactant phospholipid, previously mentioned as DPPC) and SP-A are typically stored together in lamellar bodies and in some studies have been shown to both associated with macrophage activity (110). In addition, SP-A has been associated with the uptake and recycling of these phospholipids in the alveolus (109). Altered production of surfactant phospholipids would not only impact SP-A secretion, but would also impact the structural

integrity of the alveoli as PC is an important surfactant phospholipid to prevent the lungs from collapsing during pulmonary respiration (16). Decreased pulmonary surfactants, due to chronic cigarette smoke exposure, have emerged as a previously unrecognized factor that can likely contribute to the development of COPD.

In other supportive studies, patients with COPD had decreased SP-A levels in comparison to non-COPD or healthy individuals. Lin *et al*., used ELISA and western blot analysis to measure SP-A levels in the exhaled breath condensate (EBC) from 28 COPD and 32 non-COPD patients, who were undergoing lobectomy for a solitary peripheral lung nodule (SPN) (111). SPNs are typically nonmalignant lung mass that are less than 3 cm and incidentally found on X-rays or CT scans as the patients are asymptomatic (112). FEV1% values were also measured for each patient in the study. Lin *et al*., measured SP-A levels in ~1cm of lung tissue from the lobectomy by utilizing western blot analysis and immunohistochemistry staining for SP-A positive Type II alveolar cells (111). Those who had exacerbated COPD conditions or were treated for respiratory infection a month prior were excluded from the study (111). The results showed that overall, there were lower levels of SP-A in EBC of COPD patients compared to non-COPD patients (111). There was a direct correlation between SP-A levels in EBC in comparison with the patient's FEV1% (111). The SP-A western blot intensities from lung tissue were also lowered in COPD patients compared to non-COPD patients (111). The results from the immunohistochemistry staining showed no significant difference but it did demonstrate that there were lower ratios of SP-A positive Type II alveolar and overall lower Type II alveolar cells in COPD patients (111). This would be a limitation to this study as immunohistochemistry staining might not be the best method to measure SP-A levels in lung tissues as SP-A is secreted extracellularly.

In the study by Lärstad, M. *et al.,* patients with COPD have decreased SP-A levels in their exhaled endogenous particles. SP-A levels in exhaled endogenous particles of 13 COPD subjects (moderate to very severe COPD stages) and 12 health non-cigarette smokers were measured and tallied by the group's designed PExA® instrument (22). Blood samples were also collected from the subjects and both albumin and exhaled particles were analyzed through immunoassays for SP-A levels. Lung function was assessed by spirometry and included FEV1, FVC, and FEV1/FVC ratio measurements (22). They found significantly lowered SP-A mass in the exhaled endogenous particles of COPD subjects than healthy subjects Figure 10 (22). There was also a lowered SP-A mass in albumin of COPD subjects as compared to healthy subjects as well as in Figure 10 (22). The results demonstrated a significant correlation between FVEV1% and FVC% and SP-A mass in exhaled endogenous particles Figure 11 (22). These studies support the notion that altered SP-A levels are associated with lung function decline and could be a contributing factor to the onset of COPD.

Table 3. SP-A and albumin concentrations in exhaled particles.

Variable	Healthy subjects			COPD patients			p-value
	Median	Lower Quartile	Upper Quartile	Median	Lower Quartile	Upper Quartile	
SP-A mass concentration pg/L	321	250	458	35	12	115	0.0004
SP-A wt%	3.9	3.0	4.8	2.7	2.5	3.5	0.036
Albumin mass concentration pg/L	494	318	956	60	22	161	0.0007
Albumin wt%	6.5	4.7	8.5	6.3	5.4	7.0	0.57
SP-A/albumin	0.6	0.5	0.7	0.5	0.3	0.7	0.37

Data are presented as median, with interquartile range. L: liter, pg/L: pg per liter exhaled air, wt%: weight percent.

doi:10.1371/journal.pone.0144463.t003

Figure 10: Healthy subjects and COPD subjects SP-A exogenous mass and albumin mass. Used with permission: Lärstad M, Almstrand A-C, Larsson P, Bake B, Larsson S, Ljungström E, et al. (2015) Surfactant Protein A in Exhaled Endogenous Particles Is Decreased in Chronic Obstructive Pulmonary Disease (COPD) Patients: A Pilot Study. PLoS ONE 10(12): e0144463. https://doi.org/10.1371/journal.pone.0144463

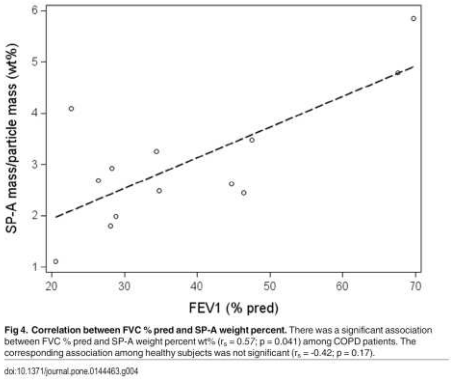

Figure 11: SP-A mass and lung function % prediction. Used with permission: Lärstad M, Almstrand A-C, Larsson P, Bake B, Larsson S, Ljungström E, et al. (2015) Surfactant Protein A in Exhaled Endogenous Particles Is Decreased in Chronic Obstructive Pulmonary Disease (COPD) Patients: A Pilot Study. PLoS ONE 10(12): e0144463. https://doi.org/10.1371/journal.pone.0144463

Alteration of the protein structure of SP-A by cigarette smoke can lead to dysfunction of the surfactant protein, which I hypothesize could contribute to the onset of COPD. One recent study by Takamiya, R. *et al*, demonstrated that cigarette smoke extract (CSE), acrolein, induces oligomerization, and modifies protein residues on recombinant human SP-A (hSP-A) (113). Acrolein is found in tracheal aspirations and airway sections of smokers and those with COPD (113). Takamiya, R. *et al*., exposed mice to 4 cigarettes once a day for one week, then immunoprecipitated proteins from their lung cells that were analyzed with western blots (Figure 10.1) (113). The results showed a presence of acrolein-modified SP-A in the lungs, which appeared as different sized bands appear on in the mice that were exposed to the CSE. The imaging from the immunofluorescence also shows the association of acrolein with the SP-A (Figure 12) (113). To determine the modifications sites by acrolein in hSP-A it was treated with acrolein for 4 hours then processed with trypsin to produce peptides to compare with untreated hSP-A for modification sites, a total of six modification sites were found with arecoline-modified residues that correlate to the hSP-A neck backbone and double loop structures (Figure 13) (113). Takamiya, R. et al, further assessed the innate immune function of SP-A when exposed to

cigarette smoke by incubating hSP-A with and without acrolein exposure with E. *coli* (113). The inhibitory effects of hSP-A on E. *coil* growth *were* observed for 7 hours (113). The results demonstrated that there was a decreased inhibitory effect of E. *coil* growth with acrolein modified hSP-A in comparison to unmodified hSP-A (14) (113). This demonstrates that cigarette smoke can directly alter SP-A structure, which in turn can weaken the functionality of surfactant protein carrying out innate immune responses. This decrease in SP-A pathogenic function could further activate the immune system cause more and inflammatory injury by cigarette smoke on the lung epithelium/endothelium as SP-A cannot aid with host defense against recurrent bacterial infections.

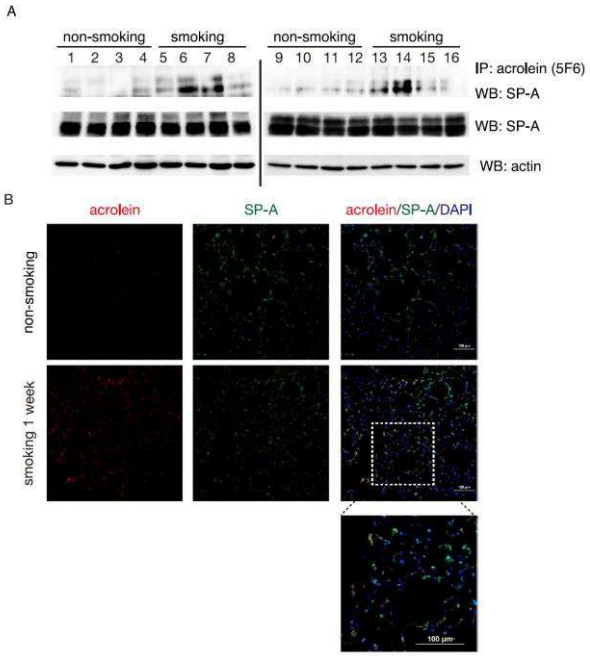

Figure 12: Mice SP-A and modified SP-A in mice exposed to acrolein. Used with permission: Takamiya, R., Uchida, K., Shibata, T. et al. Disruption of the structural and functional features of surfactant protein A by acrolein in cigarette smoke. Sci Rep 7, 8304 (2017). https://doi.org/10.1038/s41598-017-08588-5

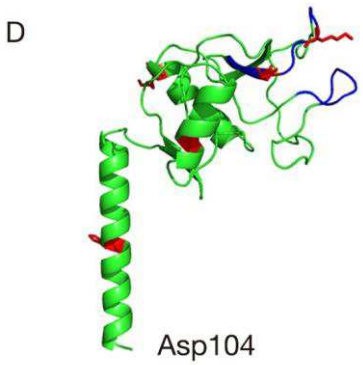

Figure 13: Modified portion of hSP-A neck and double loops are shown in red. Used with permission: Takamiya, R., Uchida, K., Shibata, T. et al. Disruption of the structural and functional features of surfactant protein A by acrolein in cigarette smoke. Sci Rep 7, 8304 (2017). https://doi.org/10.1038/s41598-017-08588-5

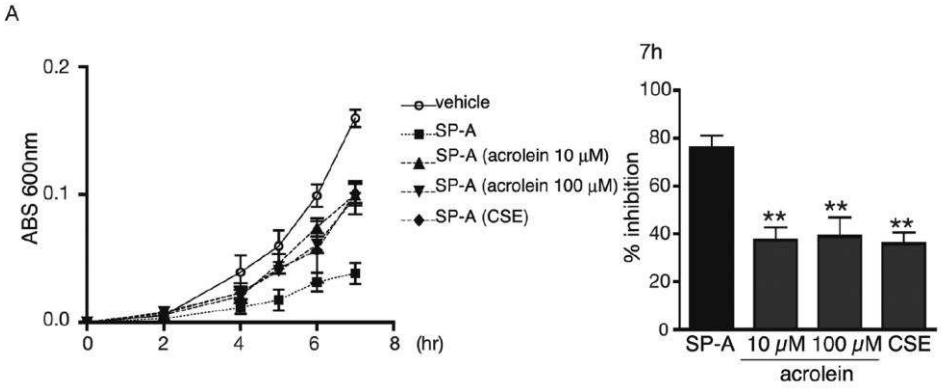

Figure 14: SP-A, CSE SP-A, and acrolein modified SP-A inhibitory effects on E. Coil growth. Used with permission: Takamiya, R., Uchida, K., Shibata, T. et al. Disruption of the structural and functional features of surfactant protein A by acrolein in cigarette smoke. Sci Rep 7, 8304 (2017). https://doi.org/10.1038/s41598-017-08588-5

SP-A is an important immunomodulatory lung protein that aids with the elimination of pathogens and inhibits inflammatory responses of macrophages and other adaptive immune cells (18-21). In a recent study by Franciso, D. *et al,* SP-A was also shown to have an inhibitory role in asthma pertaining to the regulation of responses the cytokine IL-13 (114). Taken together, previous studies demonstrate how cigarette smoke, especially the tar particles, can injure and induce necrosis and apoptosis of Type II and Type I alveolar cells, which would not only change the alveolus barrier but also result in reduction and alteration of pulmonary surfactants such as

SP-A. A reduction or alteration of SP-A, due to tar particles of cigarette smoke can increase the risk for recurrent lower respiratory tract infections and also lead to less protection in an asthmatic lung environment, both of which can induce lung epithelial and alveoli changes. These structural changes would result in decreased pulmonary functions and persistent respiratory symptoms that could contribute to airway limitations and the onset of COPD.

CC16 and Club cells

In previous studies, circulating CC16 serum levels were positively correlated with lung function. Additionally, CC16 was identified as a potential biomarker for the progression of disease severity in COPD (31). In recent studies, that cigarette smoke was shown to alter the differentiation of small airway epithelium, which includes the Club cells (115). CC16 is primarily secreted by club cells and has been described as having protective and anti-inflammatory roles in the lungs (25, 26). In this line of reasoning, if cigarette smoke limits the differentiation of primary airway basal cells into club cells, then fewer CC16 would be produced and secreted. Fewer CC16 would then result in a decline of protective roles associated with CC16 in the lungs.

A recent study by Gindele *et al*., cultured human small airway epithelial cells in an air-liquid interface (SAEC ALI) from three healthy donors and three COPD donors (115). The SAEC were cultured as a two-layer epithelium to mirror the small airway epithelium in humans. Most studies typically culture cells of larger airway epithelium such as tracheal or proximal bronchial cells (115). Gindele et al., then exposed the cells to intermittent whole cigarette smoke instead of cigarette smoke extract to get the full direct effects of cigarette smoke. The healthy and COPD SAEC ALI cultures were intermittently exposed to cigarette smoke and another was exposed to air over 28 days (115). The primary small airway cells were then identified by

immunohistochemistry staining which was basal cells, ciliated cells, goblet cells (typically not found in the small airways), and club cells (115). The cell cultures also underwent other analyses that include TEER measurements, quantitative RT-PCR, RNA-seq, and flow cytometry analysis (115). Staining for KRT5 in basal cells (a marker for squamous differentiation) was upregulated in the cultures that were exposed to cigarette smoke in both healthy and COPD cultures (Figure 15) (115). Conversely, staining for SCGB1A1 to identify for club cells was decreased in both the healthy and COPD cultures when exposed to intermittent cigarette smoke (Figure 16) (115). Implications from these studies suggest that cigarette smoke results in fewer CC16 production cells, which would lead to an inability of CC16 to carry out normal respiratory protective functions.

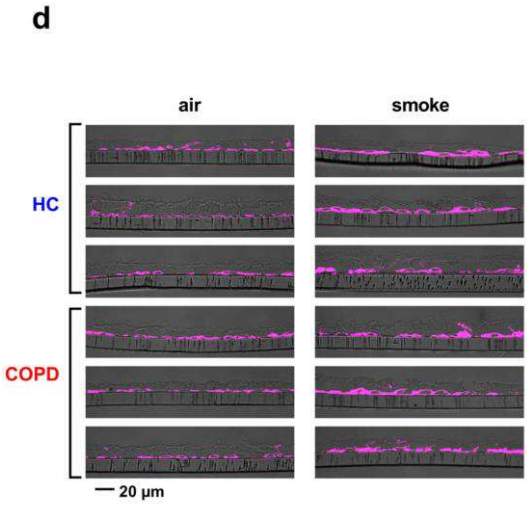

Figure 15: Immunohistochemistry staining of ALI cultures for KRT5. KRT5 is a marker for squamous differentiation, given that squamous metaplasia is common in COPD. HC stands for healthy controls. Used with permission: Gindele, J.A., Kiechle, T., Benediktus, K. et al. Intermittent exposure to whole cigarette smoke alters the differentiation of primary small airway epithelial cells in the air-liquid interface culture. Sci Rep 10, 6257 (2020). https://doi.org/10.1038/s41598-020-63345-5

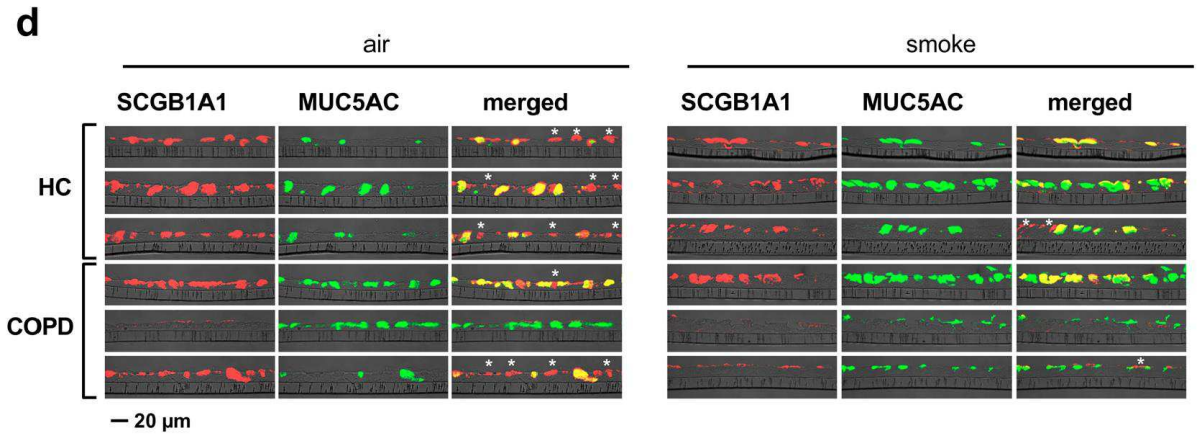

Figure 16: Immunohistochemistry staining of ALI cultures for club cells and goblet cells. SCGB1A1 is a marker for club cells and MUC5AC is a marker for goblet cells. HC is for the healthy controls. Used with permission: Gindele, J.A., Kiechle, T., Benediktus, K. et al. Intermittent exposure to whole cigarette smoke alters the differentiation of primary small airway epithelial cells in the air-liquid interface culture. Sci Rep 10, 6257 (2020). https://doi.org/10.1038/s41598-020-63345-5

Lam *et al.,* evaluated CC16 expression in the bronchial epithelium in correlation to lung function in non-smokers, former smokers, and chronic smokers. The independent study had 34 subjects who were undergoing endobronchial biopsies for sputum cytological atypia who did not have any lesion upon chest imaging. The subjects also underwent lung function testing by spirometry prior to the endobronchial biopsy. The tissue from the biopsy was then fixed for immunohistochemistry. CC16 expression was then scored for intensity on a scale from 0 to 2 in the bronchial epithelial cells (28). Lam et al., found a significant correlation between CC16 expressions and FEV1/FVC ratio in the endobronchial biopsy patients (28). This study further suggests the importance of CC16 in maintaining lung function.

In another study, Zhai *et al*. followed a cohort from birth to 32 years of age to observe the association of CC16 serum levels and lung function (116). The study also included a CC16 knockout CC16 mouse study to further observe CC16 levels in regards to lung function. The human data showed that CC16 serum levels had a direct correlation with lung function and

airway responsiveness. Those in lower tertile of CC16 serum levels had more severe airway responsiveness (Figure 17) (116). Mice lacking CC16 also demonstrate impaired lung function and airway remodeling (116). The CC16 knockout mice had increased expression of collagens that participates in airway remodeling (116). The study followed these individuals from birth into adult years, which supports the idea that CC16 plays a role in lung function throughout the course of human life.

Taken together, these studies support the notion that altered production or secretion of CC16 can contribute to the early development of COPD. With exposure to cigarette smoke, fewer basal cells differentiate into CC16-producing club cells. CC16 participates in a variety of protective roles in the lung and if absent, more collagen deposition and lung remodeling may occur. Individuals with lower levels of CC16 have impaired lung function and increased sensitivity to bronchoconstriction.

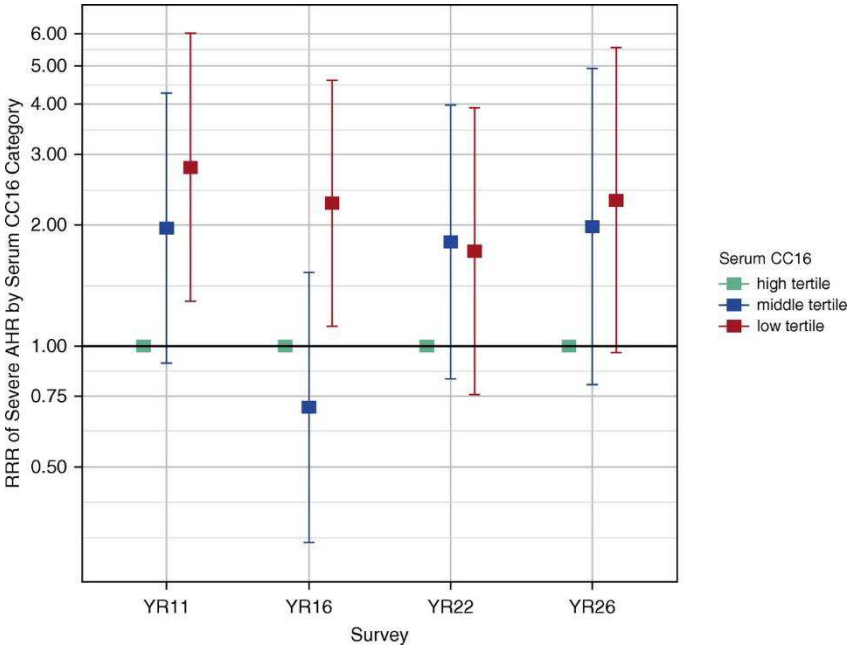

Figure 17: Severe airway responsiveness and serum CC16 levels. Serum CC16 levels group into high, middle, and low tertile. Reprinted with permission of the American Thoracic Society. Copyright © 2020 American Thoracic Society. All rights reserved. Cite: Jing Zhai, Michael

Insel, Kenneth J Addison, Debra A Stern, William Pederson, Alane Dy, Joselyn Rojas-Quintero, Caroline A Owen, Duane L Sherrill, Wayne Morgan, Anne L Wright, Marilyn Halonen, Fernando D Martinez, Monica Kraft, Stefano Guerra, Julie G Ledford)/2019/ Club Cell Secretory Protein Deficiency Leads to Altered Lung Function/ American Journal of Respiratory and Critical Care Medicine /199/ 302-312. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society.

Conclusion:

Chronic Obstructive Pulmonary Disease is a burdensome disease that affects hundreds of millions of people worldwide. Patients with COPD presents to clinic with persistent respiratory symptoms and airflow limitations. The broad classification of COPD includes several observed clinic phenotypes including emphysema, chronic bronchitis, asthma-COPD overlap syndrome, and early COPD. These observed clinical phenotypes of COPD are likely a result of complex genetic and environmental risk factors that are not yet fully understood. Cigarette smoke is a leading risk factor of COPD and has been shown in previous studies to induce airway epithelial changes, and heightened inflammatory immune responses, and alterations to the lung resident cells that are the main producers of endogenous proteins involved in host defense.

Cigarette smoke has been shown to alter the differentiation of Type II alveolar and club cells. These cells are the primary producers of SP-A and CC16 in the human respiratory system, and therefore the disruption of these cells can alter the production and activity of these extracellular immunomodulatory lung proteins. SP-A and CC16 play important roles in modulating innate and adaptive immune responses in the lungs to aid in host defense against pathogens, allergens, and noxious gaseous substances such as cigarette smoke. Disruption of SP-A and CC16 levels and/or activities can leave the respiratory tract susceptible to recurrent lower respiratory infections that can induce immune and inflammatory responses. Accumulation of immune cells and inflammatory cytokines can impact airway wall integrity and cause lung

epithelial injury that reduced lung function, contributing to airflow restrictions which eventually can contribute to the development of COPD. The alteration of SP-A and CC16 production and activity due to cigarette smoke provides a rationale for future studies to examine SP-A and CC16 as potential therapeutics in COPD. SP-A and CC16 can be used in preventative, surveillance, or treatment measures for those at risk or currently have COPD.

Appendix: Figure References and Permissions

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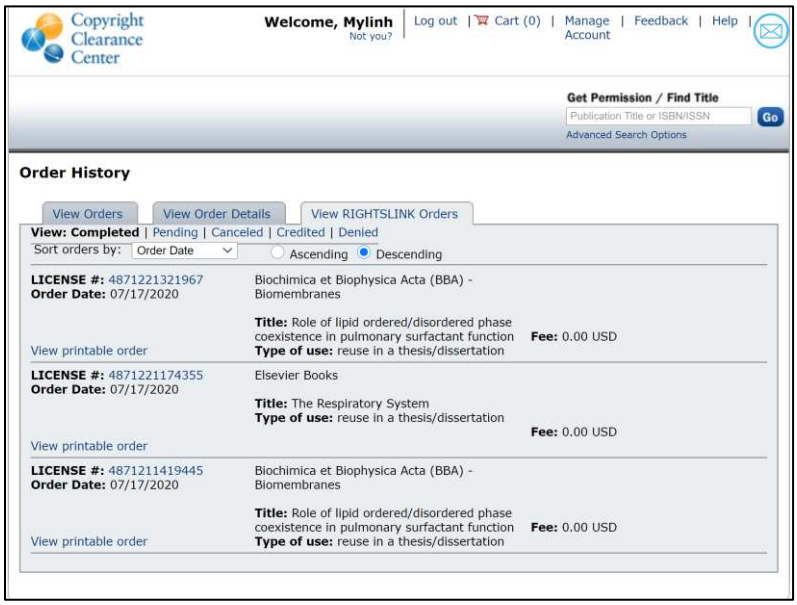

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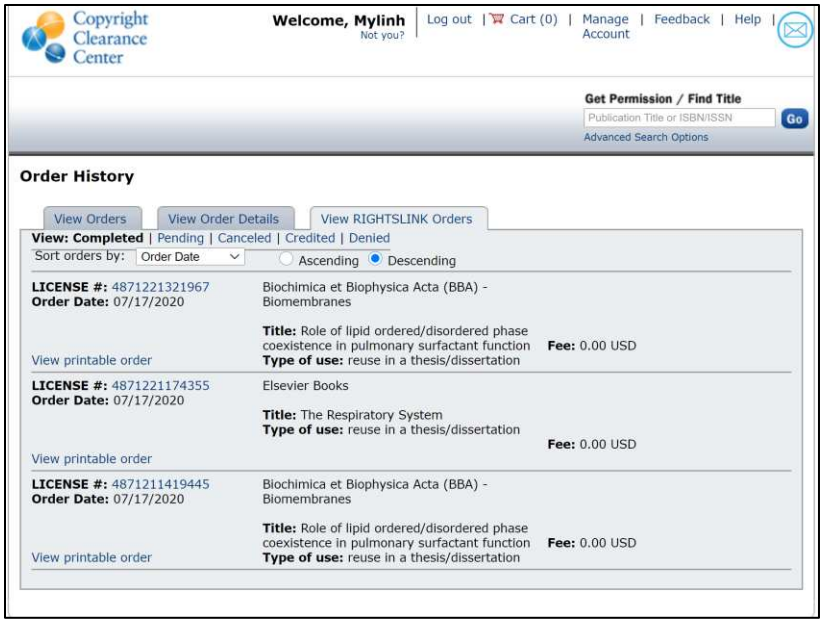

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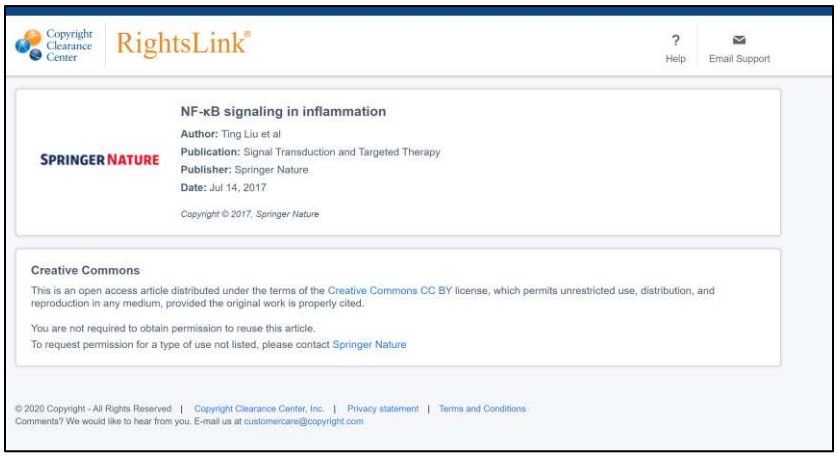

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Figure 7:

Kosmider B, Messier EM, Chu HW, Mason RJ (2011) Human Alveolar Epithelial Cell Injury Induced by Cigarette Smoke. PLoS ONE 6(12): e26059. https://doi.org/10.1371/journal.pone.0026059

Figure 8 & 9:

Agudelo CW, Kumley BK, Area-Gomez E, Xu Y, Dabo AJ, Geraghty P, et al. (2020) Decreased surfactant lipids correlate with lung function in chronic obstructive pulmonary disease (COPD). PLoS ONE 15(2): e0228279. https://doi.org/10.1371/journal.pone.0228279

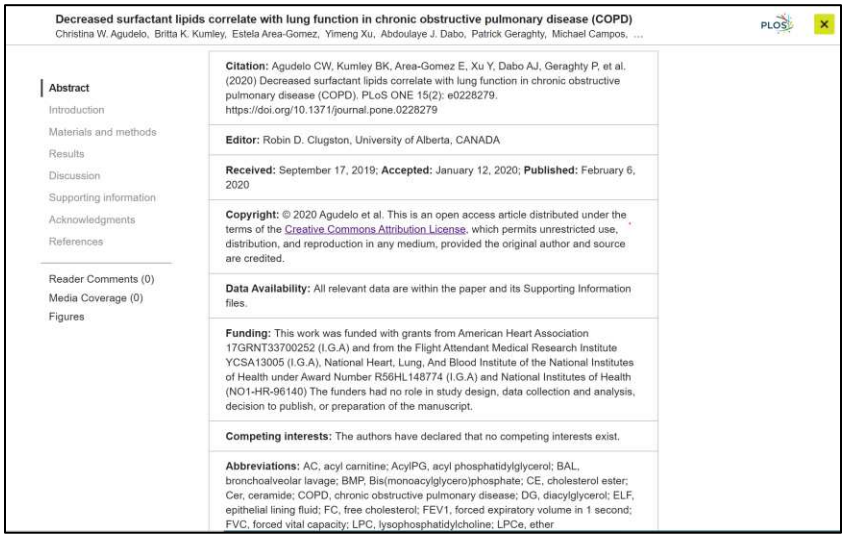

Figure 10 & 11:

Lärstad M, Almstrand A-C, Larsson P, Bake B, Larsson S, Ljungström E, et al. (2015) Surfactant Protein A in Exhaled Endogenous Particles Is Decreased in Chronic Obstructive Pulmonary Disease (COPD) Patients: A Pilot Study. PLoS ONE 10(12): e0144463. https://doi.org/10.1371/journal.pone.0144463

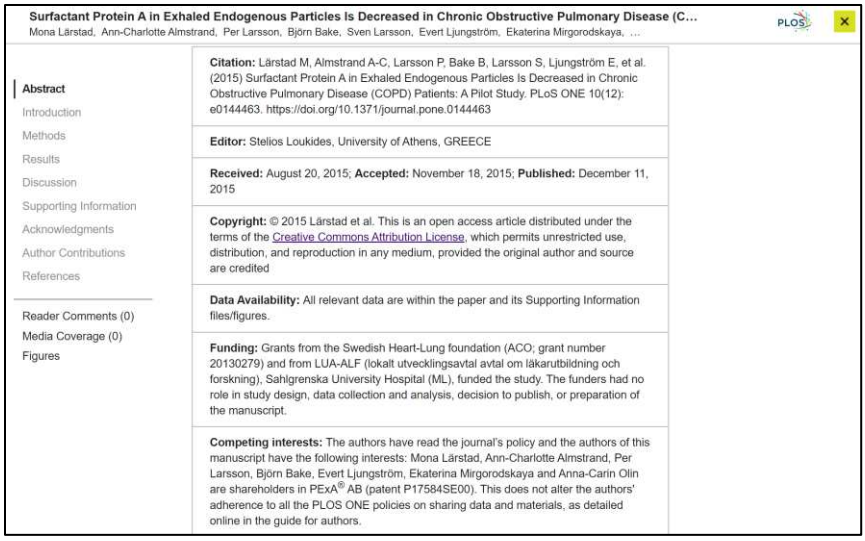

Figure 12, 13, & 14:

Takamiya, R., Uchida, K., Shibata, T. *et al.* Disruption of the structural and functional features of surfactant protein A by acrolein in cigarette smoke. *Sci Rep* **7,** 8304 (2017). https://doi.org/10.1038/s41598-017-08588-5

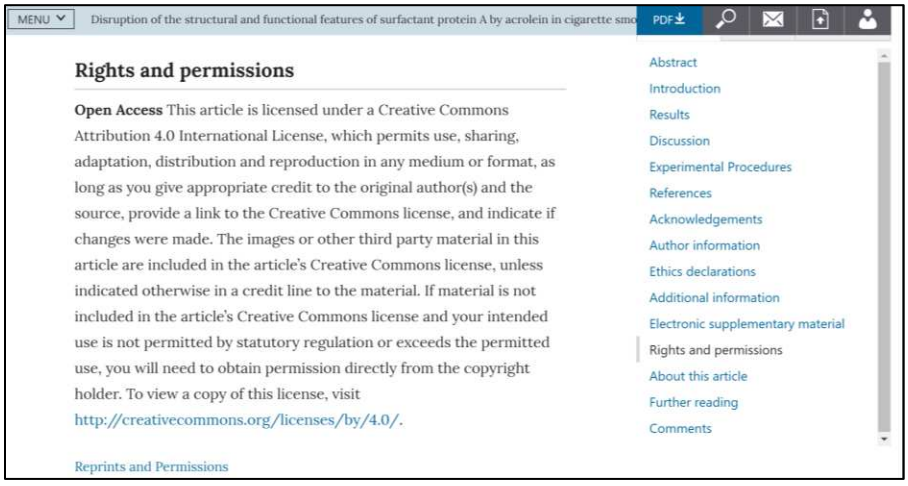

Figure 15 & 16:

Gindele, J.A., Kiechle, T., Benediktus, K. et al. Intermittent exposure to whole cigarette smoke alters the differentiation of primary small airway epithelial cells in the air-liquid interface culture. Sci Rep 10, 6257 (2020). https://doi.org/10.1038/s41598-020-63345-5

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