CC16 DEPLETION IN THE LUNG DUE TO EARLY LIFE BIOMASS EXPOSURES

by

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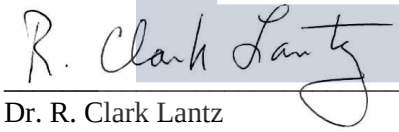

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Abstract

Millions of people across the globe are affected by respiratory diseases that include chronic obstructive pulmonary disease (COPD), asthma, emphysema, as well as cancer. COPD is the third leading cause of death worldwide due to the increase in global air pollution and smoking. Currently, there is no treatment that can change the outcome of the disease. The clinical manifestations of COPD are lung function decline and recurrent episodes of exacerbations (Knabe, 2015). Many pollutants that can lead to COPD come from sources such as wood and coal burning, cigarette smoke, and industrial air pollution. Many people across the world rely on the combustion of biomass for fuel as energy for heating and cooking. Biomass smoke exposures are recognized as a significant public health issue due to respiratory health implications. This paper will provide a review and synthesis of human and mice studies of lung insults that cause inflammatory diseases such as COPD and explore the role of club cell protein 16 in the development of disease following exposure to biomass smoke.

Introduction

COPD is a major cause of morbidity and mortality worldwide. Airway obstruction is one of the main classifications for COPD. COPD is characterized by an obstruction in airflow, which is defined by a reduced ratio of forced expiratory volume in one second to forced vital capacity. The mechanism of action for COPD is classified as unresolved inflammation due to the accumulation of activated neutrophils and T cells. When inhaled irritants activate the cells of the immune system, neutrophils and macrophages secrete inflammatory cytokines, which then leads to inflammation, fibrosis and the destruction of the airway parenchymal cells. Patients with COPD have more apoptotic cells in the lung parenchyma and airway than patients without

COPD. The loss of cells by apoptosis initiates the overall tissue destruction. These gaps are then filled in through the recruitment of fibroblasts that lay collagen (Henson, 2006)

Apoptosis is a normal physiological way for cells to turnover. Apoptosis can be activated through many ways, including cell damage. When cells are programmed to die via apoptosis, the immune system will take them in through phagocytosis. An increase in apoptotic cells in the lungs of patients with COPD suggests that there is an increased induction of cell death, and a decreased clearance mechanism in normal cell removal. Many factors can disrupt the normal function of apoptotic homeostasis. Increased neutrophil elastase and matrix metalloproteinase 12 can both inhibit the recognition of apoptotic cells in vitro. Parenchymal cell death is expected to change the structure and function of the small airway and alveolar space. The inability to effectively clear apoptotic cells results in continued inflammation. (Henson, 2006)

The airway epithelium serves as a barrier of the innate immune response. It protects the lungs from pathogens. When cells of this structure are compromised, there is an increase in disease susceptibility. COPD is characterized by an overall increase in inflammation. Increased inflammation in the lung results in structure damage and fibrosis. Accumulated fibrosis in COPD causes airway obstruction and difficulty breathing, due to reduced flexibility in the airway.

COPD

COPD Causes

One of the main causes of COPD in the developing world is exposure to cigarette smoke. Studies have indicated other findings. Laboratory mice studies have found that only a fraction of cigarette smokers develop COPD, which indicates that other exposure factors may be contributing to the increased development of COPD worldwide. These other factors include

exposure to environmental factors like pollutants, as well as genetic factors that can affect an individual's likelihood to develop COPD. (Laucho-Contreras, 2015)

Human studies have shown that similar inflammatory markers are present in the lungs of people with COPD and in the lungs of cigarette smokers. Although similar inflammatory cytokines have been found in different lung injury modalities, there are differences in the amounts secreted. The levels of these secreted proinflammatory cytokines differ between different lung injuries. Isolated cells from the airway of people with COPD produce more interleukin 8 (IL-8) than the cells from healthy smokers. (Laucho-Contreras, 2016) IL-8 is an inflammatory cytokine that is produced by inflammatory cells and primary lung epithelial cells in response to different stimuli. (Pang, 2017)

Particulate Matter and COPD

An environmental insult that has been correlated with lung injury is environmental particulate matter. Particulate matter refers to the pollutants from natural and man-made sources. Natural sources include dust, sea salt, volcanic ash, pollens, fungal spores, soil particles, forest fire products, and the oxidation of biogenic and reactive gases. Man-made sources include fossil fuels, industrial processes, mining activity, construction work, wood stove burning, as well as cigarette smoke. (Cohen 2005) (Kelly, 2012)

The components found in particulate matter, such as elemental carbon, ultrafine particles, trace elements including nickel and iron, gaseous pollutants, and certain organic species are associated with different health effects. The generation of reactive oxygen species that is created by these particles that are present in air pollution result in oxidative stress and inflammation in the airway. (Ezzati, 2002) Particulate matter is a complex mixture that is made of different

chemical components that can change in time and space. Health research is limited in the effects of particulate matter components because these compounds interact and change rapidly. Studies have found associations between oxidative potential and health outcomes. It is estimated that ambient air pollution that is composed of particulate matter less than 2.5μm in diameter is responsible for about 800,000 deaths, and about 6.4 million years of life lost across the world every year. (Cohen, 2005)

Studies have strongly correlated particulate matter with having negative effects on respiratory as well as on cardiovascular diseases in both chronic and acute exposures. The heart is often affected in these disease states because of the close relationship between the lungs and the heart. Reduced lung function is associated with chronic respiratory effects that are linked to particulate matter air pollution. (Cohen, 2005) The chronic and acute health effects upon elevated particulate matter exposure concentrations is due to the ability of inhaled particulate matter to induce oxidative stress in the lung. There are many cellular pathways through which inhaled particles can produce reactive oxygen species, including through the redox of active transition metals, quinones or endotoxin on the particle surface, and through the introduction of surface absorbed polycyclic aromatic hydrocarbons that can transform in vivo. (Cohen, 2005)

The Environmental Protection Agency (EPA) first established air quality standards for particulate matter in 1971 and did not revise them until 1987. The new revision included a focus on inhalable particles that were equal to or smaller than 10μm. In 1997, the agency established new PM standards to particles equal to or smaller than 2.5μm, after evaluating hundreds of health studies. (2017) There are two main classifications for particulate matter, primary and secondary particles. Primary particles are commonly released by combustion into the atmosphere. The main sources of primary particulate matter are road transport, domestic biomass

burning, and industrial processes. Secondary particles are formed in the atmosphere through chemical reactions, which produce substances with low volatility. These substances condense into liquids or solids and become particulate matter. Sulphates and nitrates are examples of these substances that are formed from the oxidation of Sulphur dioxide and nitrogen dioxide. Secondary particles remain in the atmosphere for longer durations compared to primary particles. (Koenig,1989)

In a study of healthy people, inhaled exposure to high concentrations of ambient particles showed an increase in neutrophilic inflammation in the lungs and blood fibrinogen levels. (Kodavanti, 2005) Different researchers have studied particulate matter that is released from different biomass sources and found that particulate matter produced from cow dung stoves was more oxidizing than diesel emissions, due to the elevated metal content. (Secrest, 2016)

Exposure to ambient particulate matter pollution may have an impact on lifetime lung development in children and the onset of COPD. In a longitudinal cohort study that compared the lungs of children based on their proximity to a freeway found that children who lived within 500 meters of the freeway had major deficits in forced expiratory volume, compared to the children that lived at least 1500 meters away from the freeway. Lung function and growth were negatively impacted based on regional air pollution. (Gauderman, 2004).

Biomass and Particulate Matter

When we think about pollution, what normally comes to mind is the pollution emitted through outdoor sources, mostly being contributed from automobiles. Understanding the differences between outdoor air pollution and household pollution is important in trying to assess

particulate matter exposure, since particulate matter includes smoke from all natural and man-made sources.

Biomass includes all substances such as wood, charcoal, animal dung and crop waste that is burned as a fuel source for cooking or heating. Worldwide, over 3 billion people continue to use open fires or biomass as a fuel source. These methods for fuel are mostly used by people in developing countries, as well as by those that live in poverty and in low socio-economic places, such as different native lands in the United States of America. Women and young children are the most vulnerably susceptible due to their traditional household roles. (2018) Biomass is significant because as mentioned earlier, particulate matter comes from all smoke that is burned from all manmade and natural products, which also includes biomass.

According to data from the World Health Organization, over 3.8 million deaths worldwide are a result of household pollution that lead to respiratory diseases including COPD. Household pollution is also considered a cause of death in children under five years old from pneumonia. (2018) In China, household air pollution is a major cause of respiratory disease, since most homes use biomass or coal as fuel for cooking and heating. (Ni, 2016)

A comprehensive study that looked at the differences in exposure based on climate in women from the eastern Tibetan Plateau, determined the seasonal variability and day to day of outdoor, indoor, and personal air pollution exposures. The study measured indoor and outdoor levels of carbon monoxide, nitrogen monoxide, and nitrogen dioxide during summer and winter. During winter months, it was found that biomass was used for heating homes through using wood or charcoal. (Ni, 2016) Results from the study indicate that women's exposure was doubled during the winter months. Women that primarily cooked with biomass also had higher levels of exposure, as compared to women that used other methods for cooking. Women that

lived in homes that were heated with biomass had significant increased exposures, as compared to women who lived in homes heated with electricity. During the winter, women's exposure to carbon monoxide and nitrogen monoxide was significantly higher than during the summer. These ranges were similar to other results from studies of carbon monoxide exposure in women around the world. (Ni, 2016)

Particulate matter concentration was twice as high in the winter than during the summer. Homes that used biomass for cooking had higher concentrations of particulate matter than homes that cooked with electricity. Women's exposure to particulate matter varied by season. The study found that level of activity was associated with personal exposure during the winter. Increased level of activity was associated with higher exposures in women. The study reported that these differences in exposures were related to exposure to village air pollution caused by garbage being burned. During the winter, there reportedly was less rain, which may be a factor that increases exposure in winter. The lack of rain may play a role in allowing fine particulate matter to stay in the atmosphere. (Ni, 2016)

Other factors that were correlated with higher exposures in women were factors associated with the kitchen space itself. The study found that women who cooked in kitchens with wall barriers had higher exposure in winter and summer than women who cooked in kitchens without a barrier wall. (Ni, 2016) A kitchen with a barrier allowed for particulate matter pollution to remain in the area.

A different study that measured personal exposures to particulate matter of women from two different Chinese provinces found that dust was responsible for the central oxidative potential of particulate matter exposures. In this study, the burning of biomass and coal was not

significantly associated with oxidative potential. Markers in dust that were associated with cellular oxidative potential were iron and aluminum. (Secrest, 2016)

In their studies of particulate matter exposures of women in Mongolia, Secrest and his team also confirmed a seasonal variation, with higher particulate matter concentrations seen in winter. The major sources for women's exposure to particulate matter was identified to be biomass, coal, and dust. Results showed that transition and post-transition metals are strongly associated with redox activity and is consistent with other studies of ambient particulate matter. (Secrest, 2016)

In 2009, a study that investigated outdoor and indoor air quality of naturally ventilated homes in Nogales, Sonora Mexico found similar results. The study compared the differences of PM air exposure between homes that used gas and biomass stoves for cooking. Results from the study found higher levels of elemental carbon PM in the homes that cooked with biomass than the homes that used gas stoves. (Holmes, 2011) An earlier study compared ambient PM in both neighboring border cities of Nogales, Arizona, and Nogales, Sonora and found that there was a higher concentration of ambient PM of 10 microns in Nogales, Sonora, Mexico. The study also found that children living in Nogales, Sonora, Mexico had higher levels of increased respiratory symptoms, due to higher PM concentrations. (Stephen, 2003) According to previous studies, it has been found that people that live in this border community between Nogales, Sonora, Mexico and Nogales, Arizona are often exposed to ambient PM of 10 microns that exceed the standards established by the EPA and by the Mexican Secretariat for Environment and Natural Resources. (Holmes, 2011)

COPD and Inflammatory Mediators

In the development of COPD and asthma, a variety of pro-inflammatory cytokines play an important role. The inflammatory cytokines that are important in the development of COPD and asthma are tumor necrosis factor alpha (TNF-alpha), interleukin-6 (IL-6) and IL-8. These inflammatory cytokines are produced by inflammatory cells and primary lung epithelial cells in response to different stimuli. (Pang, 2017) TNF-alpha is considered a master cytokine during inflammation and a strong -inducer of other pro-inflammatory cytokines and chemokines. IL-6 and IL-8 mediate and promote for the recruitment and activation of neutrophils, which results in neutrophilic inflammation. Reducing the levels of these pro-inflammatory cytokines is important for treating inflammation in the lung. (Pang, 2017)

A major proinflammatory cytokine that is produced in the lungs of people with COPD is IL-8. This cytokine is a chemoattractant that is produced by macrophages and other cells such as epithelial and endothelial cells. IL-8 functions to attract neutrophils and other granulocytes, as well as stimulating phagocytosis. IL-8 is strongly associated with inflammation and is the cytokine that is increased in cancer. Due to its potent proinflammatory properties, IL-8 is expressed at very low levels, or not existent in healthy tissue. (Brat, 2005) The expression of IL-8 has been attributed in other diseases and is a marker of unhealthy tissue. In addition to COPD, elevated levels of IL-8 are also attributed with severe and acute asthma. The presence of IL-8 in the upper and lower airway is a significant marker for disease. (Brat, 2005)

NF-κB pathway

An important signaling cascade that is activated in the airway epithelium in response to inflammation is the nuclear factor κB (NF- κB). The transcription of many proinflammatory

genes that are important in respiratory diseases is regulated by the NF- κB pathway, including IL-8 (Long, 2012) Cytokines, mitogens, microbial products, as well as physical and oxidative stress can serve to activate the NF- κB pathway. There are 15 NF- κB potential protein complex forms, which come from either hetero or homodimers, and five monomers. (Zhang, 2017) The NF- κB is composed of NF- κB1 (also known as p50), NF- κB2 (also known as p52), RelA (also known as p65), c-Rel and RelB). (Ting, 2017) Together, these components mediate the transcription of genes by binding to DNA in the nucleus.

When NF- κB is in an inactivated state, it is in the cytosol and is bound to its inhibitory protein (IκBα). Various extracellular signals can activate the enzyme IκB kinase (IKK), which phosphorylates the IκBα protein at N-terminal serines. (Ting, 2017) This phosphorylation results in ubiquitination and the detachment of the IκBα protein from NF- κB. The activated form of NF- κB is then translocated into the nucleus, where it binds to DNA sequences known as response elements (RE). This bound complex then recruits other proteins that make mRNA from DNA. The mRNA is translated into protein, which results in cell and protein changes. (Gilmore, 2006)

There are two major signaling pathways in the activation of the NF- κB pathway, which are known as the canonical (classical) and the noncanonical (alternative) pathways. The signaling pathway for these two mechanisms is very different, but they are both considered important in regulating inflammatory responses. (Ting, 2017)

The classical pathway can become activated through contacting various stimuli, including cytokine receptors, pattern recognition receptors (PRRs), ligands, tumor necrosis factor receptor (TNFR), T-cell receptor (TCR), and B-cell receptor (BCR). The classical mechanism of activation works through the degradation of the IκB-α. (Ting, 2017)

The alternative pathway can become activated by only recognizing a few stimuli, including ligands that belong to a subset of the TNFR superfamily members. Another difference in the activation of these two mechanisms is that the alternative pathway does not involve the degradation of IκB-α. It relies on the processing of the p50 precursor protein. NF- κB-inducing kinase (NIK) is an essential signaling molecule in the alternative pathway. It activates and works with IKKα to mediate p100 phosphorylation, which results in p100 processing and ubiquitination. The C-terminal of p100 is like the IκB structure and results in the generation of mature NF- κB2 p52 and nuclear translocation of the alternative complex. (Ting, 2017)

The NF- κB pathway has a variety of different roles in cells, including generating the expression of different pro-inflammatory genes. NF- κB plays a significant role in regulating different cellular processes including the activation, differentiation, and survival of cells of the immune system. This pathway also has a role in inflammasome regulation. When the activation of this important inflammation pathway is not controlled, its activation contributes to inflammatory diseases. (Ting, 2017)

Figure 1:NF-κB Pathway

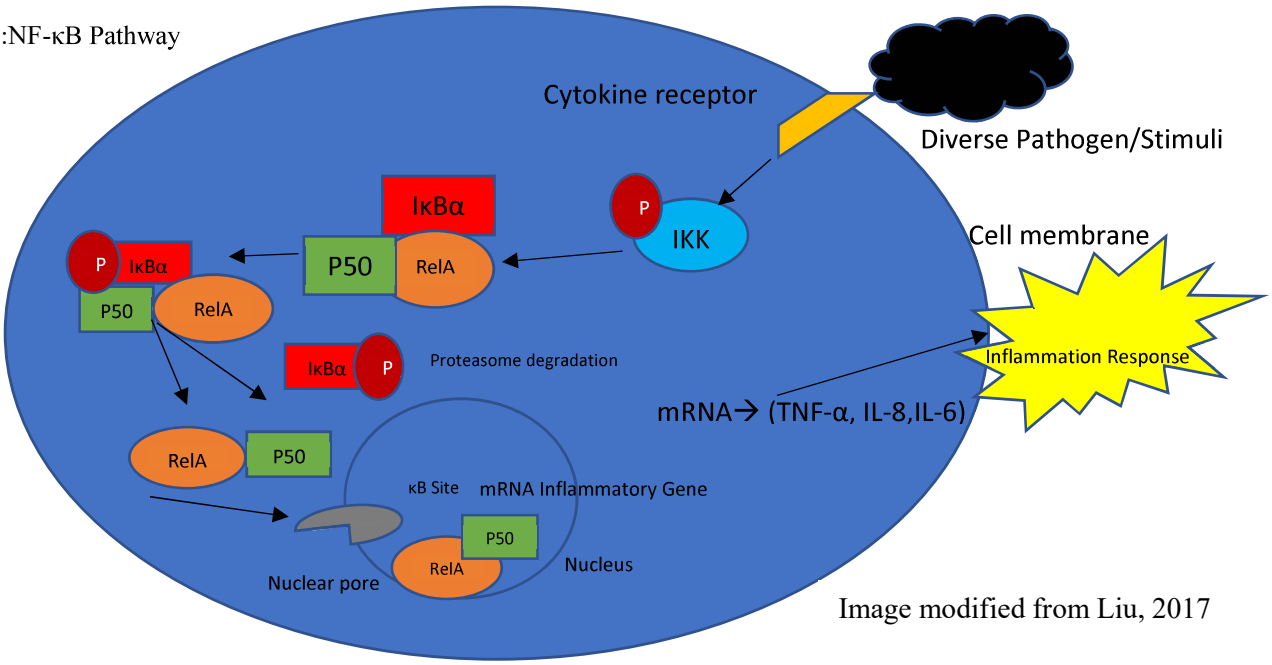

Image modified from Liu, 2017

When there is an insult or pathogen in the airway, the immune response is triggered through cells of the innate immune system. Epithelial cells secrete proinflammatory cytokines and chemokines such as IL-8, which lead to inflammation. In response to inflammation in the airway, IL-1β, another cytokine associated with inflammation activates the NF- κB pathway, which then activates the expression of IL-8. (Long, 2012)

In order to activate NF- κB transcription, a nuclear translocation of NF- κB is required. In their studies, Long and his team of researchers showed that CC16, a protein secreted by club cells in the airway epithelium could block the NF- κB pathway translocation to cell nucleus. Researchers have found that CC16 prohibits the activation of NF- κB pathway. CC16 stops the NF- κB pathway through blocking the phosphorylation of the inhibitor of NF- κB subunit-α (IκB-α). As a result, there is reduced degradation of this inhibitor. (Long, 2012)

Club Cells and CC16

Club cells are non-ciliated, non-mucus-producing cells that are present throughout the respiratory tract epithelium. They expand in frequency from the nose to the bronchioles. These cells were first identified during the 19th century. They are found in the distal airways, and secrete CC16, which is a 15.8-kDa protein. CC16 is also known as CC10, uteroglobin, secretoglobin-1A1, club cell secretory protein (CCSP), urine protein-1, and human protein-1. (Laucho-Contreras, 2016) CC16 is a homodimer protein with 70 identical amino acid residues that are linked in an antiparallel orientation by two disulfide bonds. The two disulfide bridges contribute in stabilizing the CC16 dimer and support the formation of a central hydrophobic cavity. This large cavity can accommodate small hydrophobic molecules such as progesterone, biphenyls, or retinol. (Umland, 1994)

Club cells serve several different functions in the lung, including secretory, storage, metabolism of xenobiotic compounds via p-450 oxidases, as well as a progenitor for itself and other ciliated cells. (Plopper, 1992) One of the important secretary proteins is CC16. (Plopper, 1992)

In humans, the gene that encodes CC16 is located on chromosome 11. In mice, the CC16 gene is located on chromosome 18. In humans, the CC16 gene contains 18,108 base pairs and 4,323 base pairs in mice. Both mice and humans express CC16 with the same tertiary structure. (Stripp, 1994) Club cell protein 16 (CC16) is the most abundant bronchoalveolar lavage fluid protein. Among species, the density of club cells varies through the respiratory tract. In humans, club cells represent 1% of all airway epithelial cells in the bronchioles and 5% of cells in the respiratory bronchioles. (Bernard, 1993)

One of the biomarkers of lung injury is the protein CC16. Club-Cell Secretory Protein 16 (CC16) is an anti-inflammatory protein found in the lung. Studies have shown that when the lungs are exposed to pollutants, CC16 is downregulated, contributing to a chronic decline in lung function, as well as an increase in inflammation. (Laucho-Contreras, 2015).

Mice are generally used as models for club cell research. In Mice, club cells are found more often in the trachea and bronchi. Club cells are less abundant in the large airway in humans. In mice, club cells are also found throughout the trachea-bronchial tree. Many laboratory techniques have been developed to study not only CC16 quantities, but also lung function. The technique for studying lung function in mouse models produce data that are like those generated with devices used for humans. (Hashimoto, 1996)

Researchers have recorded lung function in correlation to CC16 serum levels in humans. In the Lung Health Study, a measurement was taken of the serum level of CC16 from over 4,000 people with mild to moderate airflow limitation. In this study, researchers determined a relationship between lung function and serum CC16 concentrations. Low CC16 concentrations correlated with a decline in lung function over 9 years. The results from the study showed that after controlling for body mass index, sex, race, smoking status, and baseline forced expiratory volume, in the 4,000 people whose CC16 serum levels were sampled, low CC16 serum levels were associated with a decline in lung function. (Park, 2014) Park and his team of researchers also used mouse models to investigate if CC16 plays a role in the pathogenesis of mild COPD. For this study, they used CC16 knockout mice and exposed them to cigarette smoke for six months. (Park, 2014)

The cells that are destroyed in people with COPD are the same cells that CC16 protects against the effects of inflammation. One of the characteristics and markers of COPD has been identified as low CC16 protein levels. (Bernard, 1993) Researchers have measured CC16 levels in plasma, serum, and in bronchoalveolar lavage fluid samples in many patients with COPD. One of the first studies to report that serum and bronchoalveolar lavage fluid had lower levels of CC16 in COPD patients, when compared to non-smoker controls, was published in the early nineties by Bernard, et al. This study reported that CC16 levels were lower in smokers, compared to nonsmokers. These were the first studies linking CC16 serum levels with smoking and with COPD. (Bernard, 1993), (Laucho-Contreras, 2016) Airflow obstruction in COPD is mediated in large part through the effects of inflammation that leads to fibrosis of the small airway (Laucho-Contreras, 2015) When there is fibrosis in the small airway, its flexibility decreases and rigidity increases, which makes breathing difficult.

In a study that looked at CC16 levels and bronchitis, researchers identified a single-nucleotide polymorphism (SNP) rs3741240 within the promoter of the SCGB1A1 (CC16) gene. Two different SNPs were associated with the decline in lung function. All three of these SNPs were associated with lowered plasma CC16 levels. (Peterson, 2015) Epigenetic factors can also change the expression of levels of CC16. When samples obtained from the small airways of healthy smokers and healthy nonsmokers were compared, it was found that CC16 locus is hyper-methylated in the bronchial epithelial samples of the smokers' group. (Laucho-Contreras, 2016)

Structure and Role of Club Cells

The role of Club Cells has been associated with many cellular activities including cell proliferation, biosynthesis, storage, and the release of different secretory proteins, such as CC16. The unique characteristics of these cells, including their shape, allows for their identification in the epithelial lining. Their dome-shaped luminal surface projects beyond that of other airway epithelial cells. (Bedetti, 1987) These luminal projections allow for sensing of pathogens and insults inhaled in the lumen of the airway. The constitution of Club Cells includes cytoplasmic granules that contain their principal product, CC16. (Bedetti,1987) Club Cells play an important role in detoxifying substances that are foreign to the body in the lung because they have the highest levels of cytochrome P450 oxidases. These are hemoproteins that are generally the terminal oxidase enzymes in electron transfer chains. (Gonzalez, 1992) Most chemical carcinogens require metabolic activation by cytochrome P450 for the conversion to highly reactive electrophiles that bind covalently to DNA. According to studies, it was found that low or high levels of expression of a single P450 can determine susceptibility or resistance to chemically-induced cancer. (Gonzalez, 1992)

The protective mechanism of action for CC16 is currently unknown. It is speculated that CC16 acts through the direct binding of inhaled toxins within its hydrophobic pocket, while the club cell may detoxify inhaled toxic agents through the expression of naphthalene-targeted CYP2F2 cytochrome. (Stripp, 1996) When there is peripheral lung injury, CC16 is used as a marker. Its expression is associated with anti-inflammatory cytokines. (Lam, 2018) In a study using recombinant CC16, researchers found that rCC16 suppresses the lipopolysaccharide-induced inflammatory mediators TNF-alpha, IL-6, and IL-8 production by inactivating the NF-κB and p38 MAPK pathways (Pang, 2017) A different study using CC16 knockout mice models found that CC16 has an anti-inflammatory mechanism. Low CC16 concentration was associated with an increase in airway inflammation and destruction of the alveolar spaces. (Zhu, 2015)

The transcription factors that control CC16 expression in rodents include hepatocyte nuclear factor-3α (HNF-3α) and HNF-3β, thyroid specific enhancer binding protein, and the homeodomain factor thyroid transcription factor-1 FOX proteins. (Utsiyan, 2012) FOX proteins are transcription factors that play an important role in regulating the expression of genes involved in cell growth, proliferation, and differentiation. Many FOX proteins are important in embryonic development. (Utsiyan, 2012) In humans, both HNF-3α and HNF-3β bind to the CC16 promoter. (Utsiyan, 2012) There are some factors that increase the synthesis of CC16 in the airways, including glucocorticoids, but their effects vary between species. In humans, inhaled LPS (endotoxin) promotes the leakage of CC16 from the airways into the circulation, which can be blocked by glucocorticoids. This effect of glucocorticoids was not seen in mice that have lung epithelial injury. (Elia, 2003) (Laucho-Contreras, 2016) This supports the idea that glucocorticoids are not an effective form of treatment for restoring CC16 levels in mice with epithelial injury.

The protein CC16 is also found in other organs in the body, including in the prostate, ovaries, pancreas, mammary glands, and the uterine endometrium. (Peri, 1993) There are other lung cells that also express CC16, but Club Cells are the main producer of it. Other cells that produce CC16 are found in neuroepithelial bodies and in bronchoalveolar duct junctions. (Giangreco, 2002) These cells are necessary for epithelial renewal. They are airway progenitor stem cells, or cells that contribute to the maintenance of stem cells. (Giangreco, 2002). A subpopulation of stem cells found in the bone marrow can contribute to lung epithelial repair and these stem cells express CC16. (Wong, 2009) Based on studies, it is believed that the discovery of this CC16-secreting stem cell in the bone marrow could have airway reconstitution potential for lung disease. (Wong, 2009). These stem cells were also found to express CD45 and mesenchymal markers. CD45 is a common lymphocyte antigen that is expressed on all leukocytes. It plays an important role in the function of these cells. (Atlin, 1997.)

CC16 is cleared from the blood by the kidneys. People with impaired renal function have an increased level of serum CC16. Circulating levels of CC16 in the serum depend on the rate and entry into circulation, as well as on the clearance. (Hermans, 2003) Studies found that healthy males had lower levels of serum CC16 than healthy females due to a higher glomerular filtration rate in males, as compared to females. (Hermans, 2003)

CC16 during lung development

Many different isoforms of CC16 have been identified in the airway fluids of humans with severe airway inflammation. Different isoforms of CC16 have also been found in the airways of premature infants with respiratory distress. After analyzing the protein of various infants with respiratory distress syndrome, oxidation differences was seen in the samples. (Arias-Martinez, 2012) CC16 protects pulmonary surfactant from degradation in the lung. A study

found that the administration of the recombinant CC16 protein improves respiratory distress syndrome (iRDS), a disease that occurs mainly in premature infants. (Arias-Martinez, 2012) A different isoform of CC16 is associated with bronchopulmonary dysplasia (BPD), a chronic lung disease that affects prematurely born infants. BPD disease begins from an early inflammatory response in the lung. In a study that collected tracheal aspirate fluids on day 1, day 3, and day 6 of life from infants that were born at less than or at 29 weeks of gestation, reported an increase in CC16 oxidation and decreased immunoreactive CC16 expression in infants who developed BDP. These results supported the protective role for CC16 and that club cell function and CC16 expression may be critical for normal bronchoalveolar fluid homeostasis. (Ramsay, 2001)

CC16 can be detected early in gestation, during weeks 14-16 in the amniotic fluid during pregnancy. CC16 levels increase exponentially up until birth. At birth, CC16 levels in the amniotic fluid are the highest. CC16 levels in premature infants are correlated directly to lung maturity. Low levels of CC16 are correlated with inflammatory responses in the fetus. Infants that develop BPD and survive to adulthood have changes in their lung anatomy that resemble those found in the lungs of individuals with COPD. These changes include airspace enlargement and gas trapping, and a reduced forced expiratory volume (FEV). (Maturna, 1996) (Laucho-Contreras, 2016)

The protective mechanisms of CC16 have been studied in both mouse models and in infants. CC16 was analyzed in a study that included 64 infants with a mean gestational age of 26.1 weeks. The study found that CC16 in gastric fluid increases with gestation age. Lower concentrations of CC16 at birth were associated with higher levels of inflammatory cytokines, IL-1Beta and Tumor Necrosis Factor alpha. The infants that needed mechanical ventilation support also had lower levels of CC16. The study concluded that low CC16 concertation in the

gastric fluid at birth was associated with an increase in inflammation in the trachea and with more of a need for respiratory support in the neonatal period. (Hagman, 2018)

Animal models have been used to study the effects of different exposures on the expression of CC16. In mice studies, it was demonstrated that when exposed to course or fine wildfire particulate matter, inflammation and neutrophils increased in the lungs. In these mice, macrophages decreased. (Wegesser, 2009.) In a study using female rats and different concentrations of particulate matter, and different time variables of cigarette smoke exposures, researchers found that a trend towards lower CC16 recovery was observed. Initially, there was an increase in serum CC16 at early timepoints. After 24hrs of exposure, the mice exposed to smoke showed a return to baseline levels of serum CC16. (Miert, 2005)

CC16 levels and chronic environmental exposures

Aside from cigarette smoke, environmental particulate matter, and COPD, there are other factors that affect circulating serum CC16 levels in the body. Other lung diseases have also confirmed low levels of CC16, including lung cancer, asthma, bronchitis, and idiopathic pulmonary fibrosis. (Guerra, 2015) (Laucho-Contreras, 2016) Studies have found that some exposures to environmental pollutants present two different phases of circulating serum CC16 levels. Studies with firefighters showed that acute smoke exposures caused an increase in serum CC16 levels that returned to normal baseline levels within ten days after exposure. (Bernard, 1997) The sudden spike in CC16 serum is likely to be due to the lung epithelial injury response. Inflammation in the lung causes blood vessels to become permeable, which permits the movement of factors out of tissues and into the blood. (Bernard, 1997) A study of adolescent cigarette smokers studied the effects of chronic smoke exposures compared to controls. These studies revealed that chronic exposure reduced levels of CC16, but at a different rate, without

CC16 restoration. Chronic cigarette smokers who smoked more than five cigarettes a day had less CC16 levels, compared to controls, suggesting changes in the airway. (Miert, 2011) This study provided insight to the potential dangers of chronic environmental exposures, regarding the inability of CC16 levels to be restored in the body after exposure.

Studies showing effects of loss of CC16

Aside from the protective mechanism in the lung that has been shown for CC16, researchers have also identified other roles for CC16 in the body. In a study where researchers wanted to understand if CC16 serum levels were associated with other respiratory diseases, such as bronchitis, which is classified as an infection in the lining of the lungs, results showed that the patients with chronic bronchitis had significantly lower CC16 serum levels, when compared to the patients without chronic bronchitis. This study controlled for a variety of factors, including age, sex, baseline body mass index, years of smoking, and current smoking status. (Peterson, 2015). Low CC16 serum levels are associated with chronic bronchitis, and decreased lung function, even before COPD has developed. (Lomas, 2009)

Based on experimental data, CC16 may limit the development and may reduce the progression of COPD. A human study that measured CC16 in healthy people and in smokers was the first to associate low CC16 levels with COPD. (Bernard, 1992) Laboratory experiments using mouse models showed increases in inflammation in mice that were CC16-deficient, when compared to wild type, which supports the contention that CC16 has anti-inflammatory properties. (Zhu, 2015) CC16 has anti-inflammatory roles in many different pulmonary respiratory diseases. For mice to develop emphysema, macrophages are required. Based on experimental data, wild type mice that were exposed to cigarette smoke had lower levels of

macrophages in the lung. These mice also had less fibrosis in the small airway. (Laucho-Contreras, 2015)

In an experiment using endotoxin (LPS) as the method to cause lung injury, it was found that CC16-deficient mice had higher levels of neutrophils and inflammatory cells than wild type CC16 mice. This data further supports the protective role for CC16. (Snyder, 2010)

In mice studies, it was found that CC16-deficiency increases smoke-induced lung pathologies through its effect on epithelial cells, leukocytes, and fibroblasts. Inflammation was reduced in experiments using amplified levels of CC16 through recombinant CC16 in cell culture in the epithelial cells. (Tokita, 2014) It has been found that in lungs exposed to smoke, CC16 has anti-inflammatory properties. Low levels of CC16 is associated with chronic obstructive pulmonary disease (COPD). It is important to note that CC16 lung levels are reduced in smokers without airflow obstruction or COPD. (Zhu, 2015)

In a study using CC16 wildtype and CC16 deficient mice, Laucho-Contreras found that the CC16 deficient mice exposed to cigarette smoke for six months had more airspace enlargement, mucus metaplasia, greater rates of apoptosis of alveolar bronchial epithelial cells, and less remodeling of the small airway, than the wildtype CC16 mice. The mice that were deficient in CC16 also had more markers for inflammation, including proinflammatory cytokines, more lung macrophages, and increased levels of neutrophils. Lower levels of anti-inflammatory cytokines such as IL-10 were also seen in the CC16 deficiency mice. (Laucho-Contreras, 2015) Since both groups of mice had the same exposures, results show that CC16 has a protective mechanism in the lung. Other studies have also reported that CC16 deficient mice have larger airspace enlargement in the lung, as well as increased inflammatory cells, when compared to wild type mice. (Zhu, 2015) A decline in CC16 is also attributed to cigarette smoke.

A decline in lung function was associated with smoking and low serum CC16. (Lam, 2018) Low serum CC16 levels are associated with smoking-related bronchial epithelial damage. (Lam, 2018)

As a protective airway epithelial protein, CC16 limits the production of IL-8 in the airway. In experimental mouse studies, it was found that CC16 knockout mice produced greater amounts of IL-8 when they were activated with IL-1β, (an important mediator in the inflammatory process) than did the wild type CC16 mice. (Long, 2012)

CC16 as a treatment

CC16 has been explored a potential treatment for a variety of lung diseases and conditions. For instance, the current treatment for COPD is corticosteroids, but more patients are becoming resistant to this form of treatment. As mentioned earlier, recombinant CC16 has been used in cell culture studies to decrease inflammatory cells In a study using human bronchial epithelial cells, Tokita and her team of researchers exposed these cells to LPS and studied the effects of CC16. Results showed that CC16 reduced the mucus secretion in the airway cells through the inhibition of NF-κB phosphorylation. (Tokita, 2014) Other studies have also reported results where recombinant CC16 reduces inflammation. In cell cultures from COPD patients that were exposed to cigarette smoke, recombinant CC16 inhibited the release of the pro-inflammatory cytokine IL-8. (Gamez, 2015) Using recombinant adenoviral vectors to increase CC16 expression in epithelial cells inhibits the activation of inflammatory pathways that are linked to COPD and other respiratory diseases. (Laucho-Contreras, 2015). Recombinant CC16 inhibited the further migration of inflammatory cells, including macrophages, fibroblasts, and neutrophils. (Snyder, 2010)

In vivo studies using recombinant CC16 in mice with COPD have also provided results supporting a decrease in inflammation. Recombinant CC16 reduced lung inflammation and alveolar cell apoptosis, and airway mucus metaplasia in mice exposed to cigarette smoke. The activation of NF-κB was reduced in the lungs of these mice. (Laucho-Contreras, 2015)

In a study that used recombinant CC16 in premature infants that were CC16-deficient also found that inflammatory cell levels were much lower than in infants without recombinant CC16. The study involved twenty-two infants with respiratory distress syndrome, in a randomized, placebo-controlled, double-blind study. The infants that were treated with recombinant CC16 showed a significant reduction in inflammatory cells, including neutrophils, as well as a decrease in inflammatory cytokines such as IL-6. (Levine, 2005)

Through decreasing the number of macrophages, recombinant CC16 limited small airway fibrosis. CC16 deficient-mice that were exposed to cigarette smoke had greater fibrosis than the wild type mice that had the same exposure. Macrophages clean up areas with defected or dying cells. They recruit fibroblasts that lay collagen, to fill in the spaces that used to be populated by parenchymal cells. (Laucho-Contreras, 2015) In the mice studied by Laucho-Contreas, et.al, it was found that CC16 reduces fibrosis. It does this through reducing the levels of transforming growth factor beta (TGF-β), a strong pro-fibrotic cytokine that stimulates fibroblasts. (Laucho-Contreras, 2015).

Studies have established that CC16 serves a protective role in the lung, and there is potential for using CC16 for the prevention and treatment of COPD. Researchers have discovered that all-trans retinoic acid (RA), an active metabolite of vitamin A increased the expression of CC16 in airway epithelial cells. Using retinoic acid was found to be successful in restoring CC16 levels in elastase-induced emphysema mice. (Chen, 2017) In people, vitamin A

significantly increased CC16 serum levels in bronchial epithelial cells in individuals with COPD, as well as in individuals without disease. (Chen, 2017)

When CC16 wild-type mice that had glomerulonephritis were given recombinant CC16, the disease progression was reduced. (Yang, 2008) Since these results showed a decrease in inflammation, perhaps recombinant CC16 methods could be used to treat people with COPD. (Yang, 2008) (Lee, 2004)

During an inflammatory response, there are different cytokines such as interferon-ϒ (INF- ϒ) and tumor necrosis factor- α (TNF- α) that increase the expression of CC16 by club cells invitro. (Magdaleno, 1997) (Yao, 1998) Anti-inflammatory cytokines, such as Interleukin-10 (IL-10) increases CC16 expression in cancer. CC16 levels increased in embryonic epithelia cells after treating pregnant mice with IL-10. (Yoon, 2010) These findings support CC16's anti-inflammatory properties.

Limitations of Recombinant CC16

Although *in vivo* studies of recombinant CC16 administration in mice and humans have been successful in decreasing inflammatory markers, using this as a therapeutic approach may have limitations. In order to effectively use this protein treatment in an emergency, recombinant CC16 would need to be delivered through inhalation, and in high enough doses and frequency. (Laucho-Contreras, 2016) Respiratory therapy is common for the treatment of many respiratory diseases, such as asthma and cystic fibrosis. The limitation here is that all inhaled therapeutics are small molecule drugs. Currently, using proteins (such as CC16) in therapeutic inhalation is difficult, largely due to their size. (Kane, 2013)

Another limitation to using recombinant CC16 in treatment therapy is that our own immune system could cause an immune reaction to the recombinant protein. The immune system could potentially make antibodies to recombinant CC16, which could cross-react with CC16 and further reduce serum CC16. (Kane, 2013)

Cigarette smoke contains reactive oxygen and nitrogen species, which are created by lung cells exposed to cigarette smoke. Recombinant CC16 would need to be resistant to oxidative inactivation. (Laucho-Contreras, 2016)

COPD and CC16

The pathogenesis of COPD has been linked to early life events. (Laucho-Contreras, 2016) Researchers have showed that low levels of CC16 throughout a person's lifespan are indicative of lung diseases in the future. In a longitudinal study that looked at different age cohorts of people, researchers studied the relationship between baseline CC16 and lung function over time. Low CC16 at baseline predicted an increased risk for airflow limitation. In children, low baseline levels of CC16 were associated with a decline in lung function by the time these children reached adolescence. (Guerra, 2015) Guerra and his team of researchers showed that a baseline of decreased serum levels of CC16 is a risk factor for many outcomes, including reduced lung function in childhood, the development of moderate airflow limitation in the adult population, and accelerated lung function decline in adulthood.

There are many factors that may affect CC16 levels in children. Some of these factors include body mass index, maternal smoking, age, sex, and active wheezing. Based on the longitudinal studies performed by Guerra and their team of researchers, these factors only accounted for a small proportion in the variability in CC16 levels. This supports the idea that

other factors may be in place that are contributing to low CC16 levels in children. These factors may include genetic, physiological, and most importantly, exposure to environmental factors. (Guerra, 2015)

According to studies, continued air pollution is causing the incidence of COPD to increase. (Laucho-Contreras, 2016) Currently, the medical therapies available for COPD only reduce symptoms temporarily.

Studies aimed at identifying when COPD development occurs have been performed. These studies have found that half of patients with COPD develop the disease due to chronic exposure since childhood, which result in low levels of lung function due to epithelial lung damage. In a longitudinal study that measured the forced expiratory volume of people found that the development of COPD depends largely on duration of exposure due to fragmented lung development. The study found that COPD developed among individuals who had low forced expiratory volume levels during their younger years. This study also determined that an accelerated decline in lung function and forced expiratory volume were not always associated with COPD. (Lang, 2015) Results from this study further support that chronic exposure to particulate matter is more significant in the development of COPD in people, than acute exposure.

Biomass as an important contributor of low CC16 levels

The burning of these biomasses creates health damaging pollutants that penetrate deep within the lungs. The burning of biomass emits high concentrations of hazardous air pollutants, including carbon monoxide, nitrogen oxides, polyaromatic hydrocarbons, volatile and semi

volatile organic compounds, and fine particulate matter. (Ni, 2016) Exposure from biomass is associated with COPD and lung cancer in adults, mainly women, due to their traditional role of cooking. In children, biomass fuel exposure is associated with acute lower respiratory infections, such as pneumonia. (Desai, 2004)

Biomass effects

Millions of people around the globe are exposed to this type of air pollutant everyday directly in their homes. Researchers have studied the implication that biomass exposure has on respiratory health. Many have sought to compare the similarities between cigarette smoke exposures and biomass exposures and have studied whether biomass smoke exposures show similar inflammatory responses in the lung, as seen in the lungs of cigarette smoke exposures. (Mehra, 2012)

In order to compare the inflammatory and disease process between biomass smoke and cigarette smoke, a team of researchers exposed human and mouse small airway epithelial cells to both biomass smoke, as well as cigarette smoke. Results from the study showed that both biomass and cigarette smoke exposures increased many inflammatory markers, including ERK, and IL-8. Other molecules that are involved in the breakdown of extracellular matrix were increased, including matrix metalloproteinase-1 (MMP-1). Both type of smoke exposures showed an increase in inflammatory pathways such as p38 and JNK. An increase in macrophages was seen in both type of smoke exposures as well, suggesting tissue damage. (Mehra, 2012)

Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing enzymes that have the ability to degrade many different kinds of extracellular proteins. MMPs are involved in

many cell processes, including the activation of latent TGF-β. Since CC16 reduces the levels of TGF-β, it can be assumed that CC16 is also indirectly involved in reducing MMP levels in the lung. (Laucho-Conterras, 2016)

Results from the biomass exposures in mice demonstrated more inflammation than the mice exposed to cigarette smoke. Biomass smoke exposures are affecting people's lungs and leading to respiratory diseases in similar ways, if not more dangerous than cigarette smoke exposures. (Mehra, 2012)

A study that measured lung function of women in India exposed to forms of biomass fuel, including, liquified petroleum gas, kerosene, showed the differences of lung function effects between these fuel types. Results from the study showed that women exposed to biomass smoke had worse lung function than the women exposed to other methods for fuel. Due to the traditional role of women in the household, they are chronically exposed to biomass smoke, with exposure starting early in life. (Behera, 1994)

Biomass exposure induces inflammatory markers that are very similar to those from cigarette smoke exposure and other pollutants in the respiratory airway that lead to respiratory disease. A study compared inflammatory markers between premenopausal nonsmoking women who used biomass exclusively for cooking and age-matched control women who cooked with a cleaner method of fuel, liquified petroleum gas. Biomass fuel users had much higher neutrophils in the blood than the women in the control group. Results also showed that the women that used biomass fuel also had increased levels of proinflammatory cytokines, including interleukin 6 (IL-6), interleukin 12 (IL-12), tumor necrosis factor alpha, as well as the potent inflammatory cytokine, interleukin 8 (IL-8). (Banerjee, 2011) Many studies have found elevated inflammatory markers in people exposed to biomass smoke. (Mehra, 2012)

In an effort to link the development of COPD with biomass exposure, a study compared the effects of biomass exposure with cigarette smoke. In this study, a technique that simulated dung biomass exposure in the laboratory, researchers found increased levels of MMPs and IL-8. These studies also found endotoxin (LPS) present in dung biomass, and confirmed that after removal of endotoxin, the elevated proinflammatory markers were independent of endotoxin. Results from these studies showed an overall similarity in both group exposures, suggesting that biomass exposures have a similar mechanism of pathology in lung destruction as does cigarette smoke. The mice exposed to biomass had an overall higher level of macrophages, as well as neutrophils in the lavage compared to the mice exposed to cigarette smoke. Mice that were exposed to biomass smoke had activated macrophages and carbonaceous deposits in lung epithelial and macrophages, which were not found in the cigarette exposed mice. Experimental results from this study showed that inflammation in the lungs of mice exposed to biomass was significantly greater than in the lungs of the mice exposed to cigarette smoke. (Mehra, 2012) Results from this study further support that biomass smoke exposure leads to a greater inflammatory response, as compared to cigarette smoke exposure.

Most homes in the United States use alternative fuels for cooking and heating, as opposed to traditional fuels used by households in other countries. Although the exposure to smoke from open wood burning in kitchen homes is virtually not present in the United States, there exists exposures to particulate matter from other environmental sources such as from wildfires in the United States that are affecting millions of people. (Black, 2017) Studies show that exposure to smoke, including that from wildfire has direct effects on human health. The burning of biomass is a major contributing source of gases and particles. Smoke from wildfire has a distinct composition, in comparison to other types of air pollution. (Ubananski, 2013) Wildfires produce

particles that are much finer. In the atmosphere, fine particles settle out of the atmosphere slower than coarse particles, allowing them to disperse farther from the burn source. (Kinney, 2008) This is a major public health concern because fine particles are able to penetrate deeper into the lung. (Black, 2017) Not all biomass pollutants are created by the same chemical process. The chemical makeup of wildfire smoke depends on many variables, including burn conditions, ambient temperature, and the type of biomass. (Black, 2017) In studies conducted by Sutherland and colleges, it was found that there is a significant increase in COPD symptom scores on days when ambient particulate counts rise due to wildfires. (Sutherland, 2005)

The effects of biomass burning has been simulated in the lab using techniques that attempt to emulate the effects of lung function using mouse models. Particulate matter is a mixture of small particles that is regulated as one of the National Ambient Air Quality Standard (NAAQS) pollutants by the Environmental Protection Agency (EPA) under the Clean Air Act. Particulate matter standards are based on total mass and size, which can range from a few nanometers to many micrometers. Particles between the sizes of 2.5-10μm in diameter are of significant interest in health due to their capacity to penetrate the lung. (Wegesser, 2008) Particulate matter causes an inflammatory response in the lung, depending on the particle size, the region, and time. Lab techniques work to simulate environmental exposures in mice that are like those that are experienced in the outside world.

In an experiment that studied the mechanism of toxicity in the particulate matter in the San Joaquin Valley of Central California, researchers studied course particles of 2.5-10μm in diameter. (Wegesser,2008) Intratracheally instilled mice were used to investigate the proinflammatory and toxic effects of this particulate matter in the lung. Results from the study determined inflammatory cells and chemokines in the lung lavage fluid, as well as biomarkers of

toxicity that resulted from this particulate exposure. Based on the data collected, these changes in proinflammatory cells and chemokines showed both dose and time responses. A peak response was observed at 24hr, with a recovery as early as 48hr. The collected data suggest that the observed proinflammatory effects from the course particulate matter collected during the summer months from California's hot and dry Central Valley are caused largely by the insoluble components of the particulate matter rather than endotoxin. (Wegesser, 2008)

Endotoxin is also known as lipopolysaccharide (LPS) and is found on the outer membrane of gram-negative bacterial cell walls. It is also commonly found in particulate matter. Since endotoxin is a component found on bacteria, it can elicit inflammatory responses in the body. Endotoxin exposure through inhalation has been associated with an increase in neutrophils. (Wegesser, 2008)

Other researchers in the past have studied the effects of ambient air pollution in the lung and have been able to further confirm its effects. In a longitudinal study of 151 metropolitan areas in the United States that looked at over 500,000 adults that lived in an area exposed to sulfate and fine particulate air pollution were followed between 1980 and 1989. Sulfate primarily comes from fossil fuel combustion in the ambient air pollution. The study controlled for education and smoking and was able to link the particulate matter exposures to lung cancer and cardiopulmonary mortality. (Pope, 1995) Throughout the years, many other epidemiological studies have demonstrated convincing evidence between an increase in morbidity and respiratory diseases, due to the increased levels of ambient particulate matter. (Wegesser, 2008)

In order to address the public health concern that biomass smoke exposure in women and children around the world presents, many different organizations have implemented programs

that use cleaner cooking stove methods to replace traditional ones. Only a few studies have assessed the effects of household air pollution exposure. (Clark, 2013)

A study that measured air pollutants and indoor pollution of pregnant women in Peru found that exposure to particulate matter is dependent on the type of biomass and stove used. Different variables were accounted for and measured, including stove and fuel type used. Studies revealed that there are significant differences in particulate matter, concentration of carbon monoxide and nitrogen dioxide between different fuel types used by women. The study found that even in homes where women used cleaner burning gas stove methods, particulate matter exposures were at levels that had previously been suggested to pose health issues. (Helen, 2014)

Hypothesis

Although many studies have focused on looking at the quantities of CC16 regarding cigarette exposure, as well as COPD development and lung function, nothing to date has demonstrated the relationship between biomass smoke and CC16 levels. Previous studies have looked at the differences between chronic and acute exposure, in relation to CC16 and lung function. (REF) In the Lantz team lab, mice were used to study the effects of early life exposure to biomass smoke, and CC16 levels were measured. Markers for lung structure defects, including collagen through fibrosis were also measured. In this study, it was hypothesized that mice exposed to biomass smoke during early life would have higher levels of markers for structural damage, including collagen, as well as lower levels of CC16 as compared to controls.

Materials and Methods in Biomass exposure models

For the mouse exposure studies, pregnant 8-week old C57BL strain mice were exposed through whole body exposures, for 5 times a day for one hour to biomass smoke. The mice were exposed beginning before mating, during the pregnancy (in utero), continuing after birth until two months of age. Total particulate matter concentration in the chamber was regulated at 200 mg/m^3 (204±9 mg/m^3; n=10 measurements). To approximate conditions of those cooking with biomass, we opted for a short but more intense exposure. These exposures are relative to the normal exposures seen in biomass fuels for cooking. Food and drinking water were provided as needed. Mice were sacrificed twenty-four hours post exposure with CO_2 asphyxiation, as in (Mehra, 2012)

Materials and methods for immunohistochemistry staining

Lungs were fixed by intratracheal instillation of buffered formalin at a constant pressure of 20 cm H_2O. Paraffin embedded section (5 μm) were baked for 1 hour at 65 degrees Celsius then subjected to a series of de-paraffinization (3 x 5 min in xylene) and dehydration. Microwave antigen retrieval method was performed by boiling in 10mM sodium citrate buffer, pH of 6, for 10 min, cooled for 20 min after the slide was washed with PBS. The slide was then incubated in 1% H_2O_2 to quench peroxidase activity for 10 min, washed with PBS, and then incubated in 2% normal secondary host serum for 30 min. The slides were incubated with primary antibodies diluted in PBS 0.05% Tween 20 for one hour at room temperature. Airway collagen was stained using pico-sirius red dye special stain and visualized by polarized lens.

Lantz Team Data Results

CC16 levels

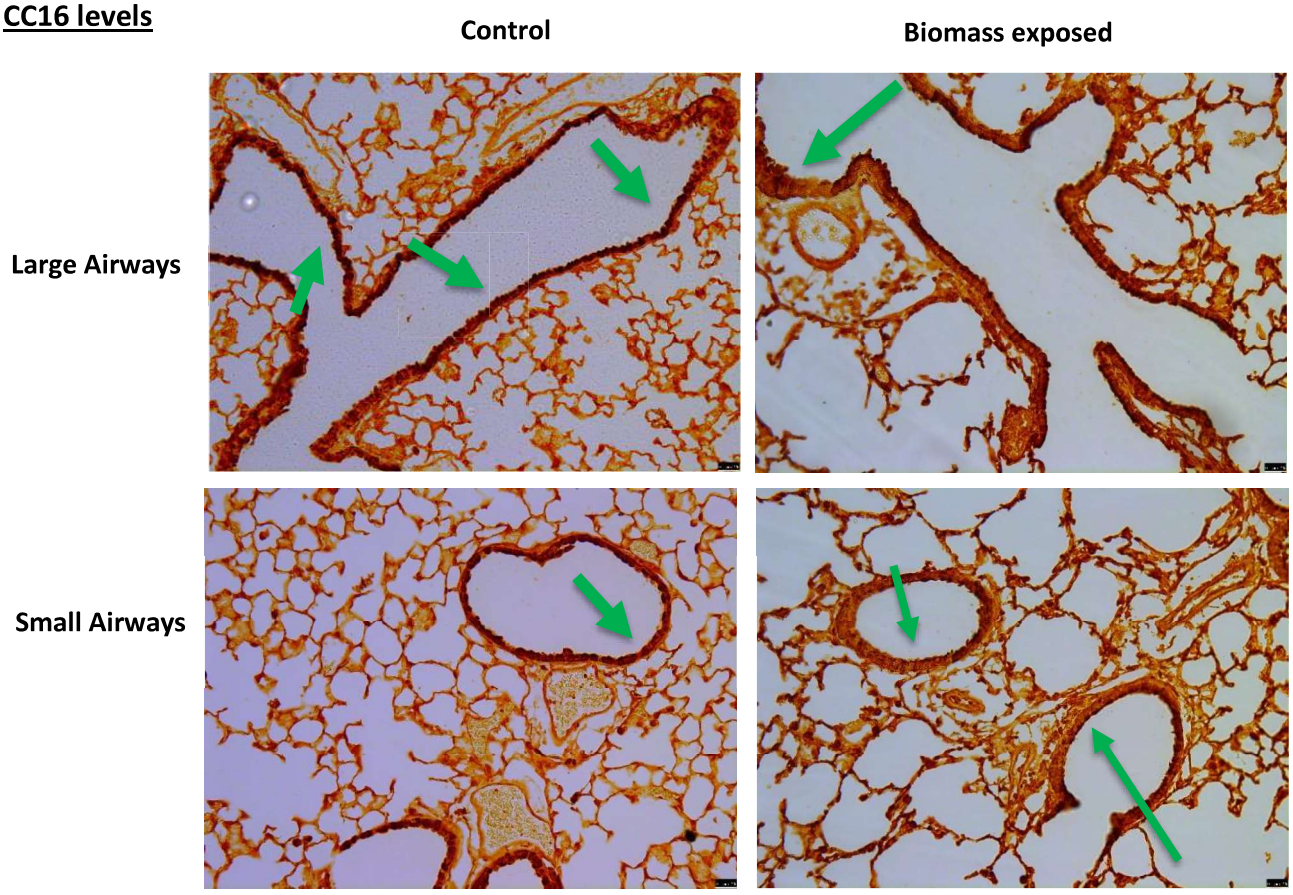

Figure 2

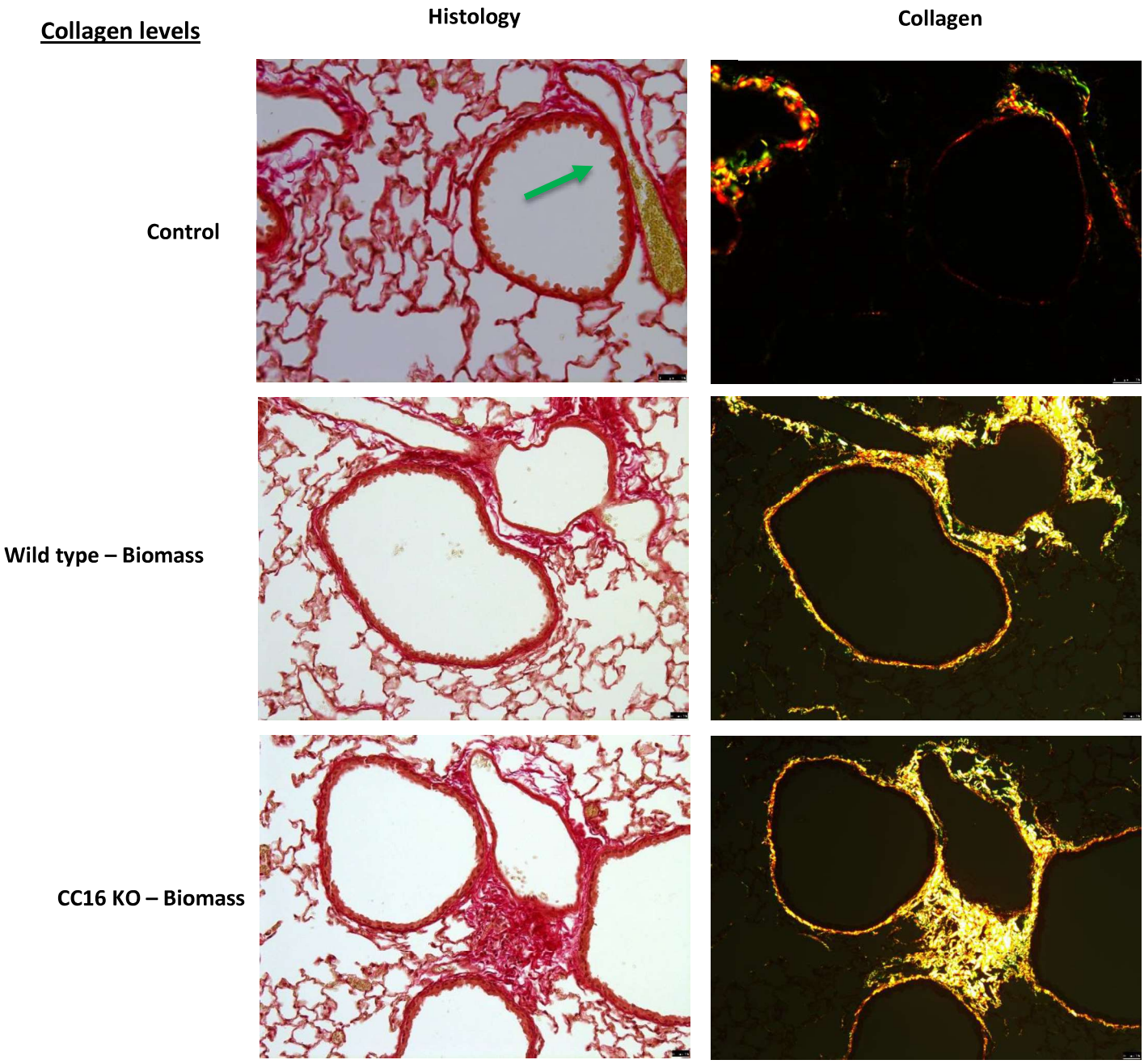

Figure 3

Figure Explanations

Figure 2 shows staining for CC16 in both control large and small airway, as well as biomass exposed large and small airway. The green arrows in the control airways indicate

examples for the areas where CC16 is expressed on the epithelial lining of the airway. There is more stained protein in the control, indicating more protein expression. The green arrows for the airways that were exposed to biomass smoke indicate areas in the epithelial lining of the airway where protein expression has been altered, as shown by the lack of staining.

Figure 2 shows the amount of collagen in control, biomass exposed, as well as CC16 knockout. The fist column shows airway histology, whereas the second column shows the IHC stain for collage. The green arrow in the control points to the luminal protrusion shape of a club cell, which secretes CC16. No collagen is seen in the control IHC stain. In the biomass exposed histology, these club cell luminal protrusions have been altered. Collagen can be found around the airway in the ICH stain. In the CC16 knockout exposed to biomass smoke, luminal club cell protrusions are not seen. There is significant collagen surrounding the airway in the IHC stain.

Discussion

Previous studies have confirmed the protective role of CC16 secreted protein by Club Cells in the airway. Studies have also confirmed the numerous lung insults, which are inclusive of all particulate matter including cigarette smoke and biomass combustion. Studies have demonstrated the difference in CC16 levels in the body of individuals exposed to chronic and acute particulate matter exposure. In the firefighter study conducted by Laucho-Contreras and team, it was found that individuals exposed the acute particulate matter were able to restore normal CC16 levels, whereas individuals chronically exposed were not. This is of major concern, especially for women and children. Based on studies involving biomass combustion, women and children are the most exposed to particulate matter through long periods of time. Although

restoration of CC16 was not studied in the Lantz lab data, it could be assumed that mice exposed chronically and acutely to biomass smoke could have similar results as found in the firefighters' study.

Early life exposure of biomass particulate matter reduced CC16 levels, which lowers the protective role of CC16 and induces pathogenicity in the airway such as fibrosis. In the laboratory mice studies of the Lantz team, mice were exposed in utero gestation beginning at day one, until the age of two months. The wild type strain of mice used was C57B6 and was compared to CC16 knockout. As shown in Figure 2 of the results section, CC16 levels are compared with biomass exposed large and small airways. The arrows indicate areas where CC16 is found on the epithelium. In the control samples of small and large airway, CC16 is found uniformly along the epithelium, as noted as the darker stained protein. In the biomass exposed samples of small and large airway, the arrows point to areas where CC16 protein is not expressed.

Markers of disease, including the presence of collagen, were determined to be much higher in the CC16 knockout mice, as compared to the C57B6 mice. Figure 3 from the results section shows labeling for these markers. Fewer luminal protrusions are indicative of a decrease in the protective protein CC16. Thicker areas of collagen are indicative of fibrosis. As expected, the knockout CC16 mice had increased collagen. The control in Figure 2 shows what a lung airway epithelium would look like when not exposed to biomass. The arrow points to the club cell protrusions, which secrete the CC16 protein. The control in Figure 3 also does not show any collagen around the airway. After exposure, Figure 3 shows less of these protrusions in the epithelium, which suggests CC16 secretion is altered. Based on the IHC staining in Figure 3 of biomass exposure, collagen is now seen around the airway. The results for CC16 knockout in

figure two show no luminal protrusions as compared to the control, which suggests that no CC16 is being expressed. As indicated by the images in Figure 3 of the CC16 knockout, there is a significant amount of collagen around in the airway upon biomass smoke exposure.

Early exposure to particulate matter is significant because studies have found that lungs do not stop developing until early adulthood. Lung development has five different developmental phases, where important structural components are developed. These five stages are known as the embryonic phase, which is 4-7 weeks post conception, the pseudoglandular phase, which occurs 5-17 weeks post conception, the canalicular phase, which occurs 16-26 weeks post conception, the saccular phase, which occurs 24-38 weeks post conception. The last stage of lung development is the alveolar stage, which is the phase when there is alveolar development. Alveolar development was thought to have occurred between 36 weeks up until 3 years of birth. New studies of imaging lung have provided evidence that suggests that this developmental phase occurs through young adulthood. (Nikolić, 2018)(Herring, 2014)(Narayanan, 2012) Based on the Lantz team data studies of mice exposure to biomass smoke at early life, and new information of when lung development occurs, it is possible that particulate matter could be causing more structural damage than is known, because the lungs have a long developmental stage.

This comprehensive evidence of the dangers of particulate matter exposure is significant to begin to battle the problem of COPD and other lung pathologies worldwide. Previous studies indicated that biomass was considered to be the most dangerous contributor of particulate matter exposure. It was also noted that chronic exposure to particulate matter was more dangerous than acute exposure, due to the body not being able to restore normal levels of CC16 protein. This suggests that early exposure to particulate matter from biomass combustion should be closely examined in order to reduce disease state including COPD and other lung problems worldwide.

Since so many people around the world are chronically affected by biomass particulate matter, cleaner fuel alternatives should be a first priority to solve this issue. Although recombinant CC16 has been shown reduce inflammation in airways inflicted with particulate matter insults, there is still further developments needed for this therapeutic method to work. A better approach is to focus on preventative measures, including cleaner fuel sources for people, as well as cleaner environmental practices in order to reduce inflammatory responses in the airway.

Future Studies and Limitations

In the reported experimental study, mice were used to study the effects of CC16 and structural damage, such as collagen in early life exposure to biomass smoke. Ethical issues would prohibit human studies on the effects of CC16 on early life exposures further studies, therefore more information is necessary. Preliminary data suggests that biomass smoke has affected club cells and protein expression. It would be helpful to know if the club cells are completely destroyed, or if the protein itself is only lacking expression. It would also be helpful to know when in development to chronic exposure marks a no turning back, in regard to restoration levels of CC16. Perhaps answers to these questions could serve as a public health preventative measure for children worldwide.

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