THE REGULATION OF CC16 IN LUNG EPITHELIAL CELLS IN RESPONSE TO KLEBSIELLA PNEUMONIAE

by

SULTAN ALMUNTASHIRI

(Under the Direction of Duo Zhang)

ABSTRACT

Community-acquired pneumonia (CAP) is a common acute infectious disease among elderly people. The most common cause of CAP is *Streptococcus pneumoniae*, followed by *Klebsiella pneumoniae* etc. Clara cell secretory protein is also known as club cell secretory protein or uteroglobin. This protein appears to have a protective effect against respiratory inflammatory response. We aimed to assess the expression profile of CC16 in response to *Klebsiella pneumoniae* in human lung epithelial cells and to treat the infected cells with EV-CC16 and rCC16. After *K. pneu* infection, we found that CC16 mRNA significantly decreased in BEAS-2B cells and H358, but not A549 and H441. Different proinflammatory marker genes were elevated in these cells after *K. pneu* infection, including IL-1β, IL-6, IL-8 and COX-2. Treatment with EV-CC16 reduced the expressions of IL-1β, IL-6 and IL-8 in BEAS-2B but not in H358 cells. Finally, rCC16 did not show anti-inflammatory effects in lung epithelial cells.

INDEX WORDS: Pneumonia; CC16; Epithelial cells; Lung inflammation

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CHAPTER 1

Club Cell Secreted Protein CC16: Potential Applications in Prognosis and Therapy for Pulmonary Diseases

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Abstract

Club cell secretory protein (CC16) is encoded by the *SCGB1A1* gene. It is also known as CC10, secretoglobin, or uteroglobin. CC16 is a 16 kDa homodimeric protein secreted primarily by the non-ciliated bronchial epithelial cells, which can be detected in the airways, circulation, sputum, nasal fluid, and urine. The biological activities of CC16 and its pathways have not been completely understood, but many studies suggest that CC16 has anti-inflammatory and anti-oxidative effects. The human CC16 gene is located on chromosome 11, p12-q13, where several regulatory genes of allergy and inflammation exist. Studies reveal that factors such as gender, age, obesity, renal function, diurnal variation, and exercise regulate CC16 levels in circulation. Current findings indicate CC16 not only may reflect the pathogenesis of pulmonary diseases, but also could serve as a potential biomarker in several lung diseases and a promising treatment for the chronic obstructive pulmonary disease (COPD). In this review, we summarize our current understanding of CC16 in pulmonary diseases.

Keywords: Clara cell; SCGB1A1; lung injury; COPD; bronchopulmonary dysplasia; sarcoidosis; idiopathic pulmonary fibrosis; respiratory infection; asthma

1. Introduction

Millions of people suffer from respiratory diseases, a leading cause of death worldwide [1]. Even though significant progress has been made in the long-term management of chronic lung diseases, reliable tools for early diagnosis and effective therapeutic drugs are lacking [2]. Acute lung diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are difficult to treat due to their sites of injury and inflammation are located deep in the lung [3]. It has been reported that bronchiolar destruction decreases lung function, leading to the development of chronic obstructive pulmonary disease (COPD), which especially targets the terminal bronchioles [4]. Currently, numerous studies are focused on lung epithelial cells to improve the outcome of acute and chronic lung diseases [5,6].

Club cells formally called Clara cells or bronchiolar exocrine cells, are non-ciliated bronchiolar secretory cells in the respiratory epithelium [7]. Approximately 8–14% and 17–27% of club cells are localized in the terminal bronchioles and respiratory bronchioles in humans, respectively [8]. In mice, the nonciliated cells comprise 50–70% of the total epithelial population throughout the entire airway epithelium, and > 95% of them are club cells [9]. In the small airways, there are two subsets of secretory cells recognized based on their vulnerability to naphthalene [10]. After naphthalene administration, the majority of those cells will be affected [10,11]. However, some secretory cells will survive and provide regenerative mechanisms in the damaged airway epithelium [11]. Subpopulations of club cells such as variant club cells are epithelial progenitors in airways after epithelium injury [11]. These cells are located near neuroepithelial bodies or at the bronchoalveolar duct junctions, and it is characterized by the lack of cytochrome P450 expression, particularly CYP2F2 [10,11]. Club cells are heterogeneous, multifunctional cells, and have various

physiological roles, such as secretion of defensive material [12]. In addition, they play an essential role in the repair process of the bronchial epithelium by acting as stem cells and providing biotransformation capacity, which usually enables the elimination of hazardous materials by detoxification [13]. After exposure to harmful factors, such as ovalbumin, they are quickly destroyed and are regenerated after about 30 days [12]. Furthermore, club cells play a key role in pulmonary homeostasis and immunity in both acute and chronic lung diseases. Club cells protect and regulate pulmonary function through the secretion of surfactants, glycosaminoglycans, enzymes, and other proteins [12].

CC16 is a protein primarily secreted by the club cells, which makes it a specific marker for these unique cells [14]. The dysregulation of CC16 was often observed in the pathogenesis of pulmonary diseases, such as ARDS, asthma, COPD, etc. Functionally, CC16 has been demonstrated to provide anti-inflammatory and anti-oxidative effects in various cells [15,16]. Considering its biological activities, many researchers are developing CC16 into a therapeutic agent against various respiratory illnesses. Given its essential roles, more research will be needed on CC16 to develop it into an early screening marker and a therapeutic agent for various lung diseases. In the current article, we review our current knowledge and the ongoing investigations on CC16 and its prospects in clinical medicine to prevalent or treat several acute and chronic lung diseases.

2. CC16 in ALI and ARDS

ALI and its advanced stage of ARDS are characterized by decreased lung compliance, severe hypoxemia, and bilateral lung infiltrate with high morbidity and mortality [17,18]. It may result from a variety of diseases including sepsis, pneumonia, trauma, etc. The severity and further features of ALI/ARDS may be detected by biomarkers in the circulation or bronchoalveolar lavage

fluid (BALF). However, there is an urgent need for sensitive biomarkers that could identify patients who are at high risk of developing these disorders and differentiate these diseases from other causes of acute respiratory illnesses and non-pulmonary diseases [19].

Previous studies have reported that acute exposure to lung irritants causes elevated CC16 in the serum. For example, a temporary increase in serum CC16 was found in firefighters after 20 min of smoke exposure without any signs of lung impairment [20]. Another study showed that serum CC16 of cyclists exposed to photochemical smog (O_3 levels around 0.08) was slightly increased after two hours of riding. These findings of the increased concentration of serum CC16 in response to acute environmental changes suggest its potential utility as a reliable marker for the early detection of injury to acute airways and assessing the integrity of the lung epithelium [21,22].

In a clinical study, where 78 patients diagnosed with ARDS were examined to explore the potential correlation between serum CC16 levels and therapeutic outcomes, the median serum CC16 levels were higher in non-survivors and were associated with fewer days free of the ventilator (increased severity) [23], suggesting the potential use of serum CC16 in predicting the outcomes in ARDS patients who are at higher risk for mortality. Another study corroborated these findings with significantly higher CC16 serum levels observed in non-survivor ARDS patients compared to survivors [24]. In the latter study, there was a significant positive correlation between serum CC16 levels and Intensive Care Unit (ICU) stay, and a negative correlation with the PaO_2/FiO_2 ratio, an integral part of the assessment of patients with ARDS, thus demonstrating the potential utility of determining CC16 levels in the serum on ARDS severity and in distinguishing the various categories of ARDS. Similarly, Lin et al. reported plasma CC16 as a promising diagnostic biomarker to discriminate ALI from acute cardiogenic pulmonary edema [25]. Although the CC16 levels in the plasma or edema fluid were not a predictor of mortality in this

study, these were associated with the duration of mechanical ventilation or ICU stay in ARDS patients compared to patients with acute cardiogenic pulmonary edema [25]. In addition to serum, increased CC16 levels in BALF have also been linked to ALI compared to non-smoking health subjects [26,27]. Intriguingly, the mean CC16 level in BALF from non-survivor ARDS patients was significantly lower than the survivors [26], suggesting the potential utility of measuring CC16 in BALF as a sensitive and specific biomarker to assess the role of the pulmonary epithelium in ALI [26].

Acute damage to club cells can be achieved experimentally using chemical pneumotoxicants such as methylcyclopentadienyl manganese tricarbonyl (MMT) or 4-Ipomeanol that cause a reduction in club cell numbers, CC16 mRNA, and protein levels in the lung [28]. In contrast, the serum concentration of CC16 is significantly increased due to leakage of the bronchoalveolar/blood barrier [28]. A transient elevation of CC16 in serum has been seen in rodents acutely exposed to O_3 [21,22] despite the decreased production of CC16 from damaged club cells at high exposure levels. The elevation of CC16 serum was highly sensitive compared to albumin in BALF within 2 h of O_3 exposure. These findings indicate that the assay of serum CC16 allows the accurate detection of even the minor defects in the epithelial barrier permeability. This higher sensitivity of CC16 most likely is because of its small size and high transepithelial concentration gradient compared to that of albumin. Consistently, Hantson et al. reported decreased serum CC16 levels after an initial transient increase compared with control rats exposed to chemicals [29]. A reduction in BALF CC16 and an increase in serum CC16 levels were reported in rats exposed to intratracheal lipopolysaccharide (LPS) [27]. This reduction was demonstrated, along with reduced CC16 messenger RNA expression in the lung, which was also seen in an acid

aspiration rat model of ALI [30], suggesting that the decreased CC16 expressions in the damaged lung are common in various animal models of ALI.

In summary, acute exposure to lung irritants causes a transient increase in CC16 serum in both clinical and experimental studies as a consequence of increased airway permeability. In clinical studies, serum CC16 levels were reported to be higher when exposed to smoke inhalation or photochemical smog. The same findings were reported in rats acutely exposed to chemicals or O_3. Furthermore, serum CC16 levels were higher in non-survivor ARDS patient samples compared to survivors and it was positively correlated with disease severity. In contrast, BALF CC16 levels were significantly lower in non-survivor ARDS patients than survivors. Consistently, BALF CC16 levels were lower in rats exposed to LPS and acid aspiration. In brief, there was consistency in the serum and BALF findings on CC16 levels between animal and human studies.

3. CC16 in Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) is a chronic inflammatory lung disease affecting premature infants characterized by impaired lung development, a need for supplemental oxygen, and long-term lung morbidity thereafter [31]. The assessment of disease severity is limited to parameters such as gestational age and the requirement of supplemental oxygen [32]. Since these methods are time-consuming, there is a critical need for sensitive biomarkers that allow early diagnosis and management of BPD [33]. Analysis of mechanically ventilated neonates has indicated elevated serum CC16 levels in infants who developed BPD [34]. The increase in serum CC16 levels was particularly seen in neonates requiring ventilatory support as early as 2 h after birth indicating its prognostic utility. Interestingly, a similar study that examined cord blood CC16 in neonates with BPD indicated low cord blood CC16 levels as a predictor of BPD development in preterm infants [35]. Significantly lower levels of CC16 were also detected in BALF from

ventilated neonates with BPD as compared to non-BPD neonates, indicating a positive correlation between BALF CC16 levels at birth and BPD diagnosis but a negative correlation with disease severity [36]. Furthermore, the authors suggested considering BALF CC16 levels as a biomarker for BPD at day 7 [36]. Taken together, previous studies have shown elevated serum CC16 concentration within 2 h after birth and a reduction in BALF CC16 concentration at day 7 among ventilated neonates who subsequently developed BPD.

4. CC16 in COPD

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the US [37]. Long-term exposure to cigarette smoke (CS) coupled with genetic factors influences an individual susceptibility to develop the disease [38,39]. The severity and progression of COPD are determined by the forced expiratory volume/second (FEV1), and there are no definite biomarkers to identify people at risk for COPD before the onset of the disease [40]. Lower levels of CC16 in the circulation and airways have been associated with COPD prevalence and disease severity in clinical studies. In the Lung Health trial, a reduction in serum CC16 levels was associated with an accelerated decline in FEV1 over 9 years [41]. Furthermore, serum CC16 was the only biomarker found to be associated with a slower decline in FEV1 over 3 years in the Emergency treatment with Levetiracetam or Phenytoin in convulsive Status Epilepticus (ECLIPSE) trial [42]. Consistently, the same observations were seen in the Tucson Epidemiological Study of Airway Obstructive Disease (TESAOD), European Community Respiratory Health Survey (ECRHS), and Swiss Cohort Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) trials over 14, 11, and 8 years, respectively [43]. Additionally, serum CC16 was measured in samples collected at ages 4–6 years in the Barn/children, Allergy, Milieu, Stockholm, Epidemiological survey (BAMSE), Manchester Asthma and Allergy Study (MAAS), and Children's Respiratory Study

(CRS) birth control trials, respectively, to examine the association of CC16 levels with subsequent lung function in early life [43]. They reported that a low level of CC16 at ages 4–6 years predicted subsequent FEV1 decline up to 16 years of age. The authors reported that decreased circulating CC16 levels are a risk factor for reduced growth and lung function in children, with a significant decline in lung function and airflow limitation in adults [43]. Moreover, airway CC16 expression was inversely correlated with the severity of airflow obstruction in COPD patients [44]. Consistently, patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages III-IV (severe and very severe, respectively) COPD had much lower airway CC16 expression than GOLD stages I–II COPD patients (mild and moderate, respectively) [45]. These observations are in agreement with another study reporting the complete disappearance of CC16 expression from the airways of severe COPD patients [46]. Strikingly, lower serum CC16 levels were detected in current smokers than former smokers with GOLD stage II-III, but not in stage IV, suggesting no correlation between serum CC16 levels and the severity of COPD [47]. In general, serum levels of CC16 were reported to be lower in current smokers than former smokers regardless of COPD diagnosis [47]. Furthermore, serum CC16 levels were significantly reduced in COPD patients than smoker and non-smoker controls [47]. Consistently, lower serum CC16 levels were detected in active smokers [41].

In animal studies, CS exposure gradually reduced airway CC16 expression in mice associated with pulmonary inflammation and COPD [40]. Mice deficient in CC16 had greater airspace enlargement, small airway remodeling, alveolar cell apoptosis, mucus metaplasia, and higher NF-kB activation than WT mice when both were exposed to CS for 6 months [45]. Consistently, in another study, CC16 deficient mice exhibited higher airspace enlargement and lung inflammation than WT when both were exposed to CS for 4 months [46]. In contrast, Park et al. reported that

both WT and CC16 deficient mice developed comparable levels of airspace enlargement, emphysema, and small airway remodeling after 6 months of CS exposure [41]. It is important to note that the CS-exposure methods and duration were different in these studies, which may have contributed to the discrepancies in results. In non-human primates studies, CC16 airway expression was downregulated in two different monkey species, *Macaca mulatta* and *Macaca fascicularis*, after CS exposure [46,48].

In summary, researchers observed lower CC16 expression during the pathogenesis of COPD in both preclinical and clinical studies. In human studies, serum CC16 levels were significantly reduced in COPD patients than smokers and non-smoker controls. Furthermore, CC16 was negatively correlated with accelerated decline in FEV1 in most longitudinal studies throughout childhood or elderly life, and it was significantly linked to disease severity. In animal studies, CC16 deficient mice showed higher airspace enlargement compared to WT from two different studies, and one study reported no differences between WT and deficient mice. In non-human primates studies, CC16 was down-regulated in two different monkey species after CS exposure.

5. CC16 in Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is an epithelial-fibroblastic disease that primarily targets the alveolar epithelium in response to unknown factors. The main pathologic features of IPF are the loss of lung epithelial cells, aberrant tissue repair, activation of fibroblasts/myofibroblasts, excessive accumulation of extracellular matrix, and irreversible destruction of the lung parenchyma [49]. The diagnosis is clinically achieved with imaging tests and a biopsy, and the median survival rate after diagnosis is 3–5 years. Non-invasive biomarkers for the early diagnosis, differential diagnosis, prognosis, and prediction of therapeutic outcomes are extremely needed [50]. Serum and BALF levels of CC16 were significantly increased in IPF compared with non-IPF

patients and healthy controls [50]. Tsoumakidou et al. observed increased CC16 levels in BALF and sputum supernatants from patients with IPF [51]. In addition, increased levels of serum CC16 were reported in patients with IPF alone and IPF combined with emphysema [52]. Consistently, elevated serum CC16 levels were associated with the involvement of pulmonary fibrosis in patients with systemic sclerosis [53]. Based on the published research articles, CC16 was significantly increased in biofluids, including BALF, serum, and sputum, in IPF compared with non-IPF patients and healthy controls.

6. CC16 in Sarcoidosis

Sarcoidosis is an inflammatory disorder of unknown etiology that is characterized by granuloma formation in affected organs, especially the lungs [54]. The disease course is highly variable and difficult to predict. Most cases report a marked remission rate, while persistent granuloma inflammation may lead to fibrosis or irreversible damage to other organs [55]. Diagnosis can be challenging because of the potential for organs other than the lungs to be influenced [56]. Thus, specific biomarkers with good sensitivity and specificity are needed to determine disease activity and to identify patients at risk of fibrosis. Previous studies have shown that serum CC16 levels were increased in individuals with sarcoidosis compared with healthy controls [57,58]. Consistently, Hermans et al. showed a considerable elevation of CC16 levels in serum, even in the absence of marked radiological abnormalities among sarcoidosis patients [59]. In the latter study, serum CC16 was significantly higher in patients diagnosed with sarcoidosis stages II–III than patients with stages 0–I. Interestingly, they found unchanged levels of CC16 in lung biopsies and correlated the elevated CC16 serum to the weak integrity of the blood–air barrier affected by the disease. Strikingly, the levels of CC16 in BALF were not markedly different between subjects, and not associated with the disease severity. Consistently, researchers found

similar CC16 levels in BALF among subjects, and no differences between sarcoidosis stages [57]. In contrast, another study reported higher levels of CC16 in both serum and BALF from sarcoidosis patients than in healthy controls [58]. However, the reasons for this discrepancy are not clear. Taken together, serum CC16 rather than BALF CC16 was increased in individuals with sarcoidosis compared to healthy controls, and it was associated with severity. The high serum levels of CC16 may be correlated to epithelial-barrier leakiness. To be noted, these studies were performed in the early 2000s and no further investigation was published since then. Currently, CC16 is not considered as a potential biomarker for sarcoidosis or sarcoidosis severity due to limited studies.

7. CC16 in Respiratory Infections

Among the many viruses and bacteria that cause respiratory infections, respiratory syncytial virus (RSV) is the most common respiratory pathogen in childhood, which also attacks the elderly population [60]. In general, RSV causes bronchiolitis that might rarely progress into severe conditions including pneumonia, respiratory failure, and death [61]. Johansson and colleagues found that infants with RSV infection had significantly higher serum CC16 levels compared with healthy controls [62]. However, serum CC16 levels were found to be similar in children infected with influenza or parainfluenza virus compared with healthy controls [62]. Recently, Egron et al. measured both serum and urinary CC16 from the infants aged under 1 year hospitalized for acute bronchiolitis and found a urinary increase in CC16 correlating to the severity of acute bronchiolitis, suggesting the potential utility of urinary CC16 as a biomarker in acute bronchiolitis [63]. In adults, it has been reported that athletes who suffer from recurrent respiratory infections had significantly lower serum CC16 than controls, which might lead to the increased susceptibility to respiratory infections in athletes [64]. Although a few studies have been done, the regulation and function of CC16 in response to respiratory infections and bacterial infections are largely unknown.

8. CC16 in Asthma and Allergy

Asthma is a complex respiratory disorder generally associated with allergic hypersensitivity characterized by chronic airway inflammation [65]. Using biomarkers for early prediction and prognosis has great clinical significance [66]. A mutation in the CC16 gene has been associated with an increased risk of asthma in childhood which is followed by a significant decrease in serum concentration of CC16 [67,68]. Furthermore, circulating CC16 levels in asthmatic patients with a long duration of the disease (more than or equal to 10 years) were significantly reduced compared to those with a short duration (less than 10 years) [69]. In general, lower serum CC16 levels were associated with allergic sensitization and asthma. In contrast, Kurowski et al. reported no significant association between CC16 serum levels and asthma or allergic rhinitis among athletes [64]. The subjective diagnosis in their study and the exercise pattern could be reasons for the inconsistent results.

Based on the different asthma conditions, serum CC16 levels were significantly decreased in patients with refractory and non-refractory asthma, and no differences were reported between the subjects [70]. Consistently, Shijubo et al. reported no significant difference in serum CC16 between atopic and non-atopic asthmatics [69]. These findings are in agreement with another study reporting no differences in CC16 serum levels between atopic and non-atopic patients [64]. Interestingly, fewer CC16 epithelial cells have been seen in the airways of asthmatic patients compared to the control group [58]. Additionally, the mean BALF CC16 concentration in asthmatic patients was significantly lower compared with the healthy controls [71]. Similar findings were also reported in another study indicating lower BALF CC16 levels in patients with refractory asthma and non-refractory asthma with no differences between asthmatic subjects [70].

In contrast, Emmanouil et al. found that patients with refractory asthma had higher CC16 levels in BALF than those with mild to moderate asthma [72].

Interestingly, there are also reports on CC16 measurements from sputum, nasal lavage fluid, and urine of asthma patients. Emmanouil et al. found higher CC16 levels in the sputum of asthmatics compared to controls [72]. In contrast, De Burbure et al. reported no difference in CC16 levels from the sputum of atopic asthmatics and controls [15]. A study examining CC16 levels in urine found a negative correlation with asthma in children. Urine CC16 levels were lower in asthmatic children compared to healthy controls associated with a reduced forced vital capacity[73]. Two studies focused on allergic rhinitis found reduced CC16 levels in nasal lavage fluids compared to controls during the pollen season [74,75]. Together, the literature indicates a significant reduction in serum CC16 levels in asthmatic patients with a longer duration of the disease compared to those with a shorter duration. The majority of studies that measure CC16 levels in BALF of asthmatics reported considerably lower CC16 levels than the controls. Moreover, CC16 levels in serum and BALF showed no differences or association with the severity of asthma.

9. Summary, Conclusions, and Future Perspectives

Based on our current knowledge, although the club cells are very sensitive to pathophysiological alterations, the potential mechanisms are not fully explored. It is well-known that club cells are sensitive to environmental contaminants, such as naphthalene, 4-ipomeanol, and 3-methylindole because they contain high levels of cytochrome P450 monooxygenases and flavin-containing monooxygenases [12,76,77]. Another possibility for the club cells to be sensitive to environmental changes could be that unlike the ciliated epithelial and goblet cells, club cells are non-ciliated and non-mucus secretary cells that allow pathogens and other harmful molecules to

directly influence these naked cells. In general, the most fatal and rapidly progressive idiopathic adult lung diseases are associated with high levels of CC16 in serum and BALF (Table 1). Although the exact reasons are not clear, increased CC16 levels in the body fluids of these patients could be due to the rapid stimulation of the club cells that promote CC16 secretion, particularly in the early stages of lung impairment. Our views are supported by the literature presented in this article on ARDS and pulmonary fibrosis. Since the CC16 level is very sensitive to lung injury, we believe that the level of serum CC16 will be higher in patients with direct lung injury as compared to ARDS caused by indirect ways.

Table 1. The Changes of CC16 in pulmonary diseases.

Disease	Specimen	Change of CC16	Conclusion	Reference
ALI/ARDS	Human; Serum	↑	Temporary increased in firefighters after 20 min of smoke inhalation Slightly increased after two hours of riding in cyclists who exposed to photochemical smog No signs of lung impairment were reported	[20–22]
ARDS	Human; Serum	↑	Higher in non-survivors compared to survivors Positive correlation with severity and mortality	[23,24]
ALI/ARDS	Mice/Rats; Serum	↑	A transient elevation of CC16 when animals exposed to O_3 or treated with 4-Ipomeanol or alpha-naphtylthiourea	[21,22,29]
ARDS	Human; BALF	↑	BALF CC16 levels were higher compared to controls	[26]
ALI/ARDS	Rats; Airways	↑	Lower CC16 concentration after treatment with LPS	[27,30]

			Lower CC16 messenger RNA expression in an acid aspiration rat model of ALI	
BPD	Human; Serum	↑	Higher CC16 levels in mechanically ventilated neonates who developed BPD	[34]
BPD	Human; Cord blood	↓	Lower CC16 concentrations from cord blood predicted the development of BPD in preterm infants	[35]
BPD	Human; Airways	↓	BALF CC16 level was lower in ventilated neonates who thereafter developed BPD	[36]
COPD	Human; Serum	↓	Associated with FEV1 decline in most longitudinal studies throughout childhood (BAMSE, MAAS, and CRS birth control studies) or elderly life (LHS, ECLIPSE, TESAOD, ECRHS, and SAPALDIA) Reduced in COPD patients than smoker and non-smoker controls Lower in current than the former smoker	[41–43,47]
COPD	Human; Airways	↓	Airway CC16 expression is inversely correlated with the severity of airflow obstruction in COPD patients	[44–46]
COPD	Mice; Airways	-	CC16 deficient mice had greater airspace enlargement than WT after CS exposure	[45,46]
COPD	Mice; Airways	-	Both WT and CC16 deficient mice developed similar increases in airspace enlargement after CS exposure	[41]
COPD	Non-human primates; Airways	↓	CC16 expression was downregulated in two different monkey species, Macaca mulatta and Macaca fascicularis, after CS-exposure	[46,48]
PF	Human; Serum/Airways	↑	BALF and serum levels of CC16 were significantly increased in IPF compared to non-IPF patients and healthy controls	[50,52]

PF	Mice	-	CC16 deficient mice developed more severe PF when exposed to Bleomycin	[78]
Sarcoidosis	Human; Serum	↑	Serum and BALF CC16 increased in individuals with sarcoidosis compared with healthy controls	[57–59]
Sarcoidosis	Human; BALF	↑	BALF CC16 increased in patients with sarcoidosis compared to controls	[58]
Sarcoidosis	Human; BALF	No difference	CC16 in BALF were not affected by sarcoidosis	[57,59]
Respiratory syncytial virus	Human; Serum	-	A positive association between the serum levels of CC16 and IgG against RSV in atopic athletes compared to non-atopic athletes and healthy controls	[64]
Respiratory syncytial virus	Mice	-	Increased persistence of viruses and extended viral-specific gene expression were seen in CC16 deficient mice after RSV infection	[79]
Asthma	Human; Serum	↓	Mutation in CC16 gene increased risk of asthma in childhood CC16 levels decreased in asthmatic patients with a long duration of the disease compared to those with a short duration Significantly decreased in patients with refractory and non-refractory asthma	[67–70]
Asthma	Human; Serum	No difference	No significant association between CC16 serum levels and asthma among athletes	[64]
Asthma	Human; Airways	↓	Fewer CC16 epithelial cells in the airways of asthmatic patients compared to controls Lower CC16 concentration in BALF of asthmatics compared with control healthy subjects	[58,70,71]
Asthma	Human; Sputum	↑	Higher CC16 levels in sputum from patients with mild to moderate and refractory asthma compared to controls	[15,72]

Asthma	Human; urine	↓	Lower urine CC16 levels in asthmatics children compared to healthy children	[73]
Allergic rhinitis	Human; Serum	No difference	No significant association between CC16 serum levels and allergic rhinitis among athletes	[64]
Allergic rhinitis	Human; Sputum	No difference	No difference in CC16 levels between atopic rhinitis and non-atopic controls	[15]
Allergic rhinitis	Human; Nasal lavage fluids	↓	Reduction in the levels of CC16 in nasal lavage fluids compared with controls during the pollen season	[74,75]

Abbreviations: CC16, Club cell secretory protein; ALI, Acute lung injury; ARDS, Acute respiratory distress syndrome; ↑, increase; ↓, decrease; BALF, Bronchoalveolar lavage fluid; BPD, Bronchopulmonary dysplasia; COPD, Chronic obstructive pulmonary disease; FEV1, Forced expiratory volume in one second; WT, Wild type; IPF, Idiopathic pulmonary fibrosis; PF, Pulmonary fibrosis; BAMSE, Barn/children, Allergy, Milieu, Stockholm, Epidemiological survey; MAAS, Manchester asthma and allergy study; CRS, Children's Respiratory Study; LHS, Lung Health Study; RSV, Respiratory syncytial virus; ECLIPSE, Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints; TESAOD, Tucson epidemiological study of airway obstructive disease; ECRHS, European community respiratory health survey; SAPALDIA, Study on air pollution and lung disease in adults.

The club cells are involved in wound repair and become activated after alveolar injury [80]. However, the exact role of CC16 in alveolar wound repair is not extensively investigated. In injury resolution, studies have reported that club cells migrate and replace damaged alveoli in the lung [81,82]. Thus, the increased blood capillary permeability of the alveoli may facilitate the diffusion of migratory club cells secreted CC16 into circulation [83]. Additionally, we believe that since IPF affects mostly the alveolar epithelium, we can expect a higher level of CC16 in serum. In sarcoidosis, a granulomatous disease that affects the lung in more than 90% of cases [84], it mainly starts in response to inhaled antigens that stimulate the inflammatory cells including dendritic cells, alveolar macrophages, alveolar epithelial cells, and the lymphocytes which lead to the formation

of sarcoid granulomas and fibrosis. Since 20–25% of sarcoidosis cases can develop pulmonary fibrosis at a later stage [85], we may expect to see higher CC16 levels from both diseases.

In contrast to the high circulatory CC16 in ALI/ARDS, COPD, and asthma studies report low levels of CC16 in the circulation and airways (Table 1). We correlated these findings to the nature of the diseases since they both progress over a long period. COPD is primarily caused by smoking, and there is a possible mechanism that could explain how club cells are affected by CS. During smoking, our bodies produce large quantities of oxygen-free radicals, in turn, activating oxidative stress pathways, which might damage the club cells resulting in reduced CC16 over time [46]. In asthma, one study reported a decrease in circulating CC16 level while T-cells, eosinophils, and mast cells were increased in small airways [86]. Future studies may explain if these cells can modulate or mediate CC16 protein. Even though most asthma cases reported lower levels of CC16, one study found that there is no difference between asthmatic and control among athletes. That could be because the CC16 level had been affected by exercise intensity. Additionally, most allergic rhinitis cases reported no difference in CC16 level compared to controls, probably because not all cases of allergic rhinitis have lung abnormality (40% of cases have asthma) [87]. In BPD, supplemental oxygen therapy in preterm infants may accelerate club cell death in addition to the main cause of BPD. Club cells are influenced by hyperoxia [88], and that could be a reason why the CC16 level decreased and increased rapidly in the airway and the serum, respectively. Future studies may clinically correlate the damage of club cells to oxygen therapy in all patients requiring invasive or non-invasive mechanical ventilation.

Literature indicates that changes in CC16 levels can be measured from serum, BALF, sputum, nasal fluid, and urine. We believe that the reliability of measuring CC16 in the sputum and nasal lavage fluid has not been accurate enough in comparison to the serum or BALF. That is because

nasal and sputum were not considered for CC16 measurements in most studies and when considered, the results were not comparable. Interestingly, CC16 from urine could be an accurate assessment for lung abnormality in children, such as asthma [73] and acute bronchiolitis [63]. Thus, we strongly recommend measuring CC16 from serum in adults and urine in children.

Even though CC16 has been affected by most pulmonary diseases and could be used as a biomarker, several concerns remain. Firstly, the majority of studies did not correlate CC16 with disease severity. Although CC16 can be used to predict a lung abnormality, it cannot be used alone as a diagnostic tool since it is not a specific marker for one particular lung disease. Furthermore, the reference value for CC16 is still missing. Even though most studies reported a higher or lower level in a specific disease, the exact protein levels from each study were different as shown in Table 2. Hence, to accurately measure the CC16 concentration from healthy and sick people, we have to consider all factors that might affect CC16 levels such as age, gender, renal function, weight, and others is recommended. The reason why this protein has variable levels in circulation, as well as the airway, also needs to be investigated. Assays such as ELISA used for the CC16 measurements could have variability [45]. Hence, multiple measurements of CC16 in patient biopsies compared to healthy controls are recommended for accuracy.

Table 2. Serum CC16 concentration in various conditions or diseases.

Condition/Disease	CC16 Serum Concentration (ng/mL)	*p*-value	Reference
ARDS	ARDS: 54.44 ± 19.62 ng/mL Non-ARDS: 24.13 ± 12.32 ng/mL	0.001	[24]
	Severe ARDS: 64.73 ± 14.42 ng/mL Mild ARDS: 48.17 ± 19.81 ng/mL Moderate ARDS: 57.35 ± 19.33 ng/mL	< *0.*05	
	ARDS: 22 ng/mL, IQR 9–44 Cardiogenic pulmonary edema: 55 ng/mL, IQR, 18–123	0.053 0.99	[25]

	Non-survivors: 22 ng/mL, IQR 7–50 Survivors: 20 ng/mL, IQR 10–38		
	Non-survivors: 19.93 ng/mL, IQR 11.8–44.32 Survivors: 8.9 ng/mL, IQR 5.66–26.38	0.01	[21,22]
ALI	Firefighters: 54.4 ± 34.9 ng/mL Controls:19.5 ± 11.7	0.04	[20]
	Cyclists: men: 12.3 ± 0.9 ng/mL; women: 11.9 ± 1.3 ng/mL Controls: men: 11.2 ± 0.8 ng/mL; women: 11.1 ± 0.6 ng/mL	0.01	[21,22]
Smoking/COPD	Never: 8.81 ng/mL; Former: 8.16 ng/mL; Current: 6.21 ng/mL	< 0.0001	[43]
	Nonsmoker: 14.6 ± 5.0 ng/mL, smoker 11.3 ± 5.3 ng/mL	0.0001	[59]
	Active smokers: 3.10 ± 2.23 ng/mL Sustained quitters: 4.35 ± 2.72 ng/mL Intermittent quitters: 3.90 ± 2.43 ng/mL	0.0001	[43]
	Current and former smokers with COPD: 4.9 ng/mL Current and former smokers without COPD: 5.6 ng/mL Non-smokers controls: 6.4 ng/mL	< 0.001	[47]
IPF	Controls: 5.67 ± 0.42 ng/mL Emphysema: 5.66 ± 0.35 ng/mL Combined pulmonary fibrosis and emphysema: 9.38 ± 1.04 ng/mL IPF: 22.15 ± 4.64 ng/mL	< 0.05	[52]
	Controls: 10.7 ± 7.6 ng/mL Non-IPF: 23.1 ± 13 ng/mL IPF: 31.2 ± 10.8 ng/mL	< 0.0001	[50]
PF/systemic sclerosis	Systemic sclerosis with PF: 90.8 ± 110.7 ng/mL Systemic sclerosis without PF: 42.1 ± 80.7 ng/mL	< 0.01	[50]
Sarcoidosis	Sarcoid patients: 25.9 ± 16.2 ng/mL Controls: 13.9 ± 5.2 ng/mL	< 0.05	[50]
Respiratory Infections	RSV group: 19.9 (4.6–56.1) ng/mL Influenza/parainfluenza group: 12.7 (7.2–36.0) ng/mL Healthy controls: 10.5 (4.0–125) ng/mL	< 0.01	[21,22]
	Atopic athletes: 6.88, IQR 5.37–9.15 ng/mL	> 0.05	[21,22]

	Non-atopic athletes: 6.6, IQR 5.03–9.06 ng/mL Athletes reporting frequent URIs: 5.57 ng/mL Control athletes: 7.03 ng/mL	0.01	
Asthma	Asthmatics children: 7.96 ng/mL; 95% CI =6.79–9.31 Non-asthmatic children: 9.98 ng/mL; 95% CI = 8.83–11.26	0. 006	[21,22]
	Asthmatic nonsmokers: 7.02 ± 3.05 ng/mL Controls: 11.7 ± 3.90 ng/mL	< 0.0001	
	Atopic asthmatics: 7.50 ± 3.38 ng/mL Nonatopic asthmatics: 6.32 ± 2.39 ng/mL	> 0.05	[21,22]
	Asthmatics, long duration of disease: 6.37 ± 3.11 ng/mL Asthmatics, short duration of disease: 7.88 ± 2.78 ng/mL	0.0106	

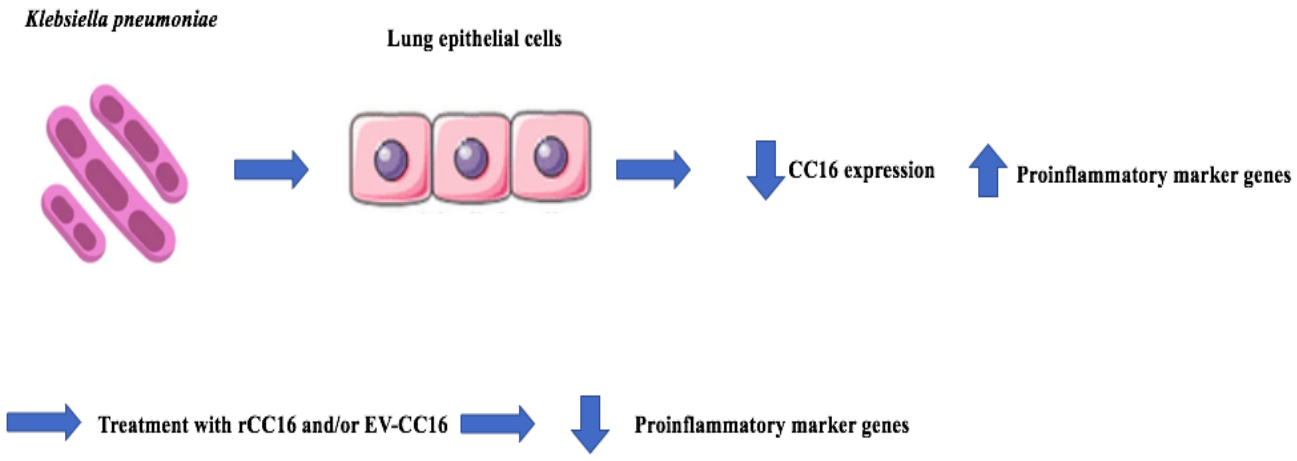
Abbreviations: IQR, Interquartile range; URI, upper respiratory infections.

In conclusion, circulating CC16 could help to diagnose and reflect the severity of a variety of lung diseases. However, the diagnosis cannot merely rely on the changes in CC16 levels in tissues or body fluids. Standard diagnostic tools such as spirometry and other pulmonary function tests are still needed. Furthermore, CC16 protein is very sensitive and many parameters such as gender, ethnicity, body-mass index, exercise, etc. should be considered for accuracy. Reports from Lesur et al. [23], Lin et al. [24], and Kropski et al. [64] have demonstrated that the CC16 levels were affected by disease duration and that CC16 concentration could be a more useful biomarker in acute cases rather than chronic diseases. Future studies might shed light on the non-invasive measurement of CC16, particularly in patients with critical conditions such as ARDS. Finally, CC16 might be assessed to predict responses after treatment and could be correlated with morbidity or mortality. In addition, serving as a biomarker, CC16 has also demonstrated the ability to become a potential treatment method for COPD. One of the most critical findings among these

reports is that the serum CC16 level is significantly related to COPD. Most of the mentioned reports have shown positive results on bronchial epithelial cells isolated from COPD patients after therapy with recombinant CC16 [40,45]. However, most of the reports are based on data from patients with mild or moderate symptoms. Thus, it is difficult to conclude that CC16 treatment might be able to cure severe COPD cases. Additionally, since the primary cause of COPD is smoking and smokers exhibit low CC16 levels, we speculate that CC16 treatment could confer protection to smokers from developing COPD. However, the precise protective mechanisms of CC16 in COPD remain unclear. Future studies are warranted to identify how endogenous CC16 elicits its effects and what pathways and receptors could be involved in protecting the lungs by CC16 stimulation, so the exogenous CC16 can be applied clinically. Altogether, this review has summarized the relationship of CC16 with various pulmonary diseases, discussed the potential utility of CC16 as a biomarker, and reasoned why CC16 can be used as a therapy for several lung diseases. A shred of evidence has indicated that the CC16 might help to provide a new diagnostic method and help to classify different pulmonary diseases based on their different responses. Furthermore, CC16 may facilitate a novel therapeutic development for respiratory disease patients as well. In general, future studies of CC16 are expected to provide more information on the development of CC16 as a potential biomarker and a therapeutic agent for various lung diseases.

RESEARCH DESIGN AND SPECIFIC AIMS

Our **central hypothesis** is that CC16 has a major protective role in gram-negative bacterial infection by reducing the cytotoxic and proinflammatory activities. We hypothesized that *Klebsiella pneumoniae* (*K. pneu*) infection will downregulate the expression of CC16 in lung epithelial cells, and treatment with rCC16 and EV-CC16 will reduce the cytotoxic/ pro-inflammatory activities caused by bacterial infection.



To address our hypothesis, we aimed to:

Specific Aim 1: Investigate the changes of CC16 in response to the *K. pneu* infection using human cell lines. Four human lung epithelial cell lines were used, A549, BEAS-2B, H441, and H358. The proposed cell lines were infected using *K. pneu* in a time-dependent manner (6, 12, 24, and 48 hours). After infection, the CC16 mRNA level was measured using a reverse transcriptase-polymerase chain reaction. Also, different cytokine and chemokine genes expression in the human lung epithelial cell lines were measured such as IL-1β, IL-6, IL-8, etc.

Specific Aim 2: Evaluate if rCC16 and EV-CC16 have a protective role against *K. pneu* via anti-inflammatory effects. The proposed cells were treated after *K. pneu* infection with different treatments such as rCC16 and EV-CC16. After treatment, the CC16 mRNA expressions were measured using a reverse transcriptase-polymerase chain reaction. Also, different proinflammatory marker genes were measured.

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CHAPTER 2

The Regulation of CC16 in Lung Epithelial Cells in Response to *Klebsiella Pneumoniae*

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Abstract

Community-acquired pneumonia (CAP) is a common acute infectious disease among elderly people. The most common cause of CAP is *Streptococcus pneumoniae,* followed by *Klebsiella pneumoniae* (*K. pneu*), *Pseudomonas aeruginosa*, and others. *K. pneu* can be the underlying cause of both: community-acquired pneumonia and hospital-acquired pneumonia in different regions. The clinical outcome in CAP is not only influenced by the causal pathogen but also the excessive immune response of the body plays a vital role in pneumonia's severity. Thus, the cytokine-modulating strategies could serve as an important addition to antibiotic therapy. Clara cell secretory protein is also known as club cell secretory protein or uteroglobin. It is a 10-16 kDa (CC16) protein originally secreted by the non-ciliated bronchial epithelial cells in the respiratory epithelium and can be easily detected in the circulatory system. This protein appears to have a protective effect against respiratory inflammatory response, and it has been studied as a biomarker for lung epithelial injury in most pulmonary diseases. In the current study, we aimed to assess the expression profile of CC16 in response to *K. pneu* in human lung epithelial cells and to treat the infected cells with CC16-containing extracellular vesicles (EV-CC16) and recombinant CC16 (rCC16). After *K. pneu* infection, we found that CC16 mRNA expression significantly decreased in BEAS-2B cells and H358, but not A549 and H441. Different proinflammatory cytokine and associated genes were elevated in these cells after *K. pneu* infection, including IL-1β, IL-6, IL-8 and COX-2. Treatment with EV-CC16 reduced the expressions of IL-1β, IL-6 and IL-8 in BEAS-2B but not in H358 cells. In contrast, rCC16 at 5 μg/ml concentration did not show anti-inflammatory effects in lung epithelial cells.

Introduction

Pneumonia, in general, occurs due to airborne infections which include bacteria, viruses, fungi and parasites. There are four types of pneumonia, known as community-acquired (CAP), hospital-acquired (HAP), healthcare-associated (HCAP), and ventilator-associated pneumonia (VAP) [1]. Community-acquired pneumonia (CAP) is a common acute infectious disease among elderly people. It is the 8th leading cause of death and the foremost cause of death from infectious diseases in adults >65 years old, therefore contributing significant morbidity and mortality all over the world [2]. CAP causes a significant economic burden, based on its high incidence, admission rate, and mortality rate. In the US, the direct annual cost of CAP has been estimated to be at least $17 billion [3]. The most common cause of CAP is *Streptococcus pneumoniae*, followed by *Klebsiella pneumoniae* (*K. pneu*), *Pseudomonas aeruginosa*, and others [1]. *K. pneu* can be the underlying cause of both: community-acquired pneumonia and hospital-acquired pneumonia in different regions. It is the causative pathogen of CAP in Asia and Africa leading to 15% of infections. In North America, Europe, and Australia, it causes CAP in about 3 to 5% [4]. Previous studies reported that the different strains of *K. pneu* are the etiological pathogens of pneumonia in patients with severe CAP [5-7].

The clinical outcome in CAP is not only influenced by the causal pathogen; however, growing evidence suggests that patients with severe pneumonia have cytokine storm syndrome [8]. Failure to control the excessive immune response results in exaggerated inflammation of lung tissue, sepsis and multiorgan damage [9]. Thus, cytokine-modulating strategies could serve as an important addition to antibiotic therapy, especially in critical conditions.

Extracellular vesicles (EVs) are nano-sized particles secreted by cells into the extracellular space [10]. The three main subtypes of EVs are exosomes, microvesicles (MVs), and apoptotic bodies, which are classified according to their diameters and intracellular origins. The content, or cargo, of EVs, includes lipids, nucleic acids, and proteins from which they are released [11]. The effects of EVs and their cargos have been studied in many diseases such as cancer [12], cardiovascular diseases [13], inflammatory diseases [14], and type 2 diabetes mellitus [15]. The therapeutic principles of EVs have promise as a novel method for drug delivery.

Clara cell secretory protein is also known as club cell secretory protein or uteroglobin. It is a 10-16 kDa (CC16) protein originally secreted by the non-ciliated bronchial epithelial cells in the respiratory epithelium and can be easily detected in the circulatory system [16]. This protein appears to have a protective effect against respiratory inflammatory response [17], and it has been studied as a biomarker for lung epithelial injury in most pulmonary diseases including chronic obstructive pulmonary disease (COPD), asthma, idiopathic pulmonary fibrosis (IPF), acute respiratory distress syndrome (ARDS), sarcoidosis and pulmonary infections [18-29]. As a treatment, it has been tested in both clinical and experimental studies for different lung diseases such as pulmonary fibrosis, COPD, and respiratory distress syndrome [30-32]. In clinical trials, rCC16 was evaluated as a promising therapy for respiratory distress syndrome in preterm infants [33]. To date, the expression profile of CC16 in response to bacterial pneumonia and the therapeutic effects of recombinant CC16 in bacterial infections have not been studied yet. Thus, in the current study, we aimed to assess the expression profile of CC16 in response to *K. pneu* and evaluate the anti-inflammatory effect of CC16 in *K. pneu* infected lung epithelial cells using human rCC16, as well as CC16-containing EVs, which is newly designed in our lab. We hypothesized that *K. pneu* will downregulate CC16 expression at the mRNA level in human lung

epithelial cells. We also hypothesized that treatment with rCC16 and EV-CC16 will reduce the proinflammatory marker genes.

Materials and Methods

Cell culture

BEAS-2B and A549 cells (ATCC) were inoculated into a cell culture dish and cultured in a Dulbecco's Modification Eagle's Medium (DMEM) with 4.5 g/l glucose, L- glutamine & sodium pyruvate (Thermo Fisher Scientific, Massachusetts, USA) , supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Massachusetts, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Massachusetts, USA) . H441 and H358 cells (ATCC) were cultured in Gibco Roswell Park Memorial Institute (RPMI) accompanied with 10% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific, Massachusetts, USA) . All cells were cultured in an incubator at 37°C in 5% CO_2/95% air. At approximately 80% confluence, the medium was removed, and the cells were washed two times with phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Massachusetts, USA) . After the PBS was discarded, 1 ml of 0.25% trypsin-EDTA (Thermo Fisher Scientific, Massachusetts, USA) was added and the cells were digested for 1-3 min. Then, 2 ml of complete medium was added, resuspended and transferred to a 15-ml centrifuge tube and centrifuged 500 × g for 5 min at 25° C. After the supernatant was discarded, the cells were resuspended in 3 ml of complete medium and passaged (1:3) in a cell culture dish.

Bacterial count and *In vitro* bacterial infection

A single colony of *K. pneu* (ATCC) was cultivated in LB liquid medium (Affymetrix, Inc. Ohio, USA) to the logarithmic phase at 37˚C while rotating at 200 rpm until the exponential phase of

the bacterial growth was reached ($OD_{600} = 1$). The bacterial culture was diluted in phosphate-buffered saline to a concentration of $1.0x10^6$ bacteria/ml.

$1x10^6$ of BEAS-2B, H441, H358 and A549 cells were seeded in 60 mm cell culture dishes overnight. After culturing overnight, the cells were infected with *K. pneu* at a multiplicity of infection (MOI) of 1:10 ratio and incubated for 1 h. Then, the medium was removed, and incubated in complete DMEM or RPMI containing gentamycin (RPI, Illinois, USA) for an additional 24 h or 48 h, as indicated in the experiments.

EV and EV-CC16 derived from BEAS-2B

Control EV from BEAS-2B and CC16-containing EV from human CC16 stably transfected BEAS-2B were kindly provided by Dr. Yohan Han. Briefly, EV and EV-CC16 were collected using a centrifugation method. Media taken from cell cultures was first centrifuged for ten minutes at 500 g to remove cells and larger debris. Media was then transferred to a new tube, and centrifuged at 2,000 g for twenty five minutes to remove apoptotic bodies. Once centrifuged, the media was added to 2× PEG solution and incubated at 4 °C overnight. The next day, samples were centrifuged at 3,000 g for 1 hour at 4 °C. For micro vesicles, the samples were centrifuged at 16,000 g for 1 h at 4 °C. After that, the samples were centrifuged at 10,000 g for 1h at 4 °C and 0.2 filter was used. The resulting samples were suspended in PBS and frozen at −80 °C. EV and EV-CC16 were characterized and validated by western blotting analysis, ELISA, flow cytometry, Nanoparticle Tracking Analysis (NTA), and immunogold staining. The concentrations of EVs were standardized using ELISA method.

Internalization of BEAS-2B derived EVs

EV derived from BEAS-2B cells were labeled with the red fluorescent dye PKH26 (Sigma-Aldrich, Missouri, USA). Isolated EV from BEAS-2B cells labeled PKH26 dye (20 μg) were washed three times using 3 kDa filters to eliminate excess dye and were then added to 1×10^4 cells/ml BEAS-2B cultured on the cell culture dish.

The internalization of PKH26 labeled EVs was observed using fluorescence ZEISS microscopy (Germany), BEAS-2B cells were washed twice with PBS and fixed with a 4% formaldehyde solution (Sigma-Aldrich, Missouri, USA) for 15 min, and then washed again twice with PBS. The cells were accompanied with a DAPI staining solution (Sigma-Aldrich, Missouri, USA) to label cell nuclei.

RNA isolation and reverse transcriptase-polymerase chain reaction

Epithelial cells were lysed, and total ribonucleic acid (RNA) was extracted using the Trizol reagent (Thermo Fisher Scientific, Massachusetts, USA). Real-time RT-PCR was performed using the Ultra SYBR Two-Step RT-qPCR Kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. Equal amounts of total RNA (1 μg) from each sample were converted into complementary DNA (cDNA) in a reverse-transcription reaction. PCR for each gene was carried out in a 10-μl reaction mixture containing 1 μl of cDNA template. The expression of the housekeeping gene TBP was used as the internal control. The following primers were used: *TBP* forward: 5′-GATAAGAGAGCCACGAACCAC-3′; *TBP* reverse: 5′-CAAGAACTTAGCTGGAAAACCC-3′; *CC10* forward: 5′-

AAACCCTCCTCATGGACAC-3′; *CC10* reverse: 5′-TGCTTTCTCTGGGCTTTTG-3′; Interleukin-1β (IL)-*1β* forward: 5′-CCACAGACCTTCCAGGAGAATG-3′; *IL-1β* reverse: 5′-GTGCAGTTCAGTGATCGTACAGG-3′; *IL-6* forward: 5′-ACTCACCTCTTCAGAACGAATTG-3′; *IL-6* reverse: 5′-CCATCTTTGGAAGGTTCAGGTTG-3′; *IL-8* forward: 5′-CTGTGTGAAGGTGCAGTTTTGCC-3′; *IL-8* reverse: 5′-CTCAGCCCTCTTCAAAAACTTCTCC-3′; Cyclooxygenase-2 (COX-2) forward: 5′-CTGGCGCTCAGCCATACAG-3′; and *COX-2* reverse: 5′-CGCACTTATACTGGTCAAATCCC-3′; C-X-C motif chemokine ligand 10 (CXCL10) forward: 5′-GTGGCATTCAAGGAGTACCTC-3′; and *CXCL10* reverse: 5′-TGATGGCCTTCGATTCTGGATT-3′

Statistical analysis

All statistical analyses were conducted using GraphPad Prism software. All data were presented as means ± SD. All the data from three independent experiments were averaged before normalization. For real-time qPCR (Thermo Fisher Scientific, Massachusetts, USA), the same amount of cDNA was used, and all data were analyzed at the same time. Comparisons between 2 groups were performed using a two-tailed unpaired Student's t-test. Multiple groups were compared using a one-way ANOVA with the Tukey method. $p < 0.05$ was considered statistically significant.

Results and Discussion

Comparison of CC16 expression in BEAS-2B, H358, A549 and H441

To evaluate the mRNA levels of CC16 in lung epithelial cell lines used in this study, we measured CC16 mRNA levels from these cells in absence of bacterial infection and we found that CC16 was highly expressed in BEAS-2B cells when compared to A549, H441 and H359 cells (Fig.1).

Clara cells are principally located in the bronchiolar epithelium of mammals. Likewise, BEAS-2B cells were derived from the normal human bronchial epithelium and this is a possible reason why CC16 was highly expressed in these cells. Even though the main tissues for H358 cells are bronchioles, these cells are considered cancer cells and their phenotypes might be different from normal bronchiole cells. Furthermore, both A549 and H441 cells are lung cancer cells isolated from non-bronchus tissues. Thus, we mainly used BEAS-2B in our further study, which was to evaluate CC16 expression after bacterial infection.

CC16 expression reduced in BEAS-2B and H358 cells but not in A549 and H441 after *K. pneu* infection

As proposed, we wanted to assess CC16 in response to bacterial infection, particularly *K. pneu*. Human lung epithelial cells were infected with *K. pneu* 1:10 ratio at different time points. As expected, we found that no difference in CC16 expression in H441 cells and A549 cells after *K. pneu* infection. In contrast, CC16 expression significantly decreased in BEAS-2B cells at most time points and slightly reduced in H358 cells (Fig 2A-D).

CC16 has been measured in most preclinical and clinical lung diseases. In general, CC16 levels were lower in asthma and COPD. However, CC16 levels in serum were higher in ARDS, pulmonary fibrosis and sarcoidosis[18-29]. The bronchial tubes (bronchi and bronchioles) are predominantly inflamed in asthma and COPD [34] and it has been previously reported that Clara cells are mainly located in bronchial tubes [35]. This is a potential reason why CC16 is reduced in such clinical conditions and BEAS-2B cells. In contrast, the alveolar epithelium or the tissues around and between the alveoli are mainly affected by pulmonary fibrosis and pulmonary sarcoidosis [36, 37]. Similarly, ARDS is characterized by acute inflammatory damage into the alveolar-capillary barrier causing an increase in vascular permeability [38]. In these three diseases, the large and small airways are not directly damaged as asthma and COPD. Furthermore, it has been previously reported that Clara cells have a role in the repair of the damaged epithelium [39]. They are involved in wound repair and become activated after alveolar injury. In injury remodeling, previous studies have reported that Clara cells can migrate and replace injured alveoli in the lung [40-42]. Due to the increased blood capillary permeability of the alveoli in pulmonary fibrosis, later stage of sarcoidosis and ARDS, CC16 protein that released from migratory Clara cells can easily diffuse into circulation. These are possible explanations for the previous findings of clinical and experimental studies. In pulmonary infections, CC16 has been measured as lung specific biomarker in RSV [29] and currently in COVID-19, and there is no study in literature aimed to assess CC16 in response to bacterial infections. To the best of our knowledge, our study is the first to confirm the reduction of CC16 mRNA expression levels after bacterial infection.

Measurement of proinflmmatory marker genes after *K. pneu* infection

In the previous study, we observed the downregulation of CC16 at the transcriptional level in BEAS-2B and H358 in response to *K. pneu* infection. Next, we wanted to measure pro-inflammatory cytokines, chemokines and other mark genes. Among the genes we measured, IL-1β, IL-6 and COX-2 increased significantly after *K. pneu* infection while IL-8 and CXCL10 did not change at different time points in H358 cells (Fig 3A-E). In BEAS-2B cells, IL-1β, IL-6, COX-2 and CXCL10 significantly increased after 48h and there were no significant changes at 24h. Moreover, IL-8 significantly increased at both time points (Fig 4A-E).

In pneumonia, the initiation, continuation and resolution of inflammation are depending on the challenging network of pro-inflammatory and anti-inflammatory cytokines. Cytokines are essential for immunity; however, if their activities are overwhelming, it will cause sepsis and lead to multiorgan damage [9]. Thus, controlling the level of proinflammatory cytokines is one of the strategies for treating infectious diseases. Previous clinical studies reported that the circulating levels of pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α), IL-6 and IL-8 are generally elevated in patients with CAP [43-46]. In an experimental study, oral administration of *K. pneu* increased COX-2, IL-1β, IL-6 and TNF-α expression in a murine model of intestinal bowel disease [47]. Consistently, our results showed that *K. pneu* infection elevated COX-2, IL-1β, IL-6 and IL-8 in human epithelial cells.

In a translational study, patients with mixed viral and bacterial pneumonia had significantly higher serum CXCL10 levels than those with single infection and were significantly associated with severe pneumonia [48]. In the same study, *in-vitro* co-infection of human monocyte-derived macrophages with Influenza virus A/H1N1 and/or *Streptococcus pneumoniae* significantly increased CXCL10 expression compared to single infection conditions. In our study, CXCL10

significantly increased after *K. pneu* infection. Thus, CXCL10 could play a crucial role in the pathogenesis of pneumonia.

Internalization of EVs into BEAS-2B

Internalization of EVs is one mechanism of cargo delivery to recipient cells. In the present study, we hypothesize that EV-CC16 might be a promising method for CC16 delivery into the cells. EV ,in general, can easily fuse with the cell membrane and enter the cell. In our study, we performed the internalization analysis to see the uptake of EV and EV-CC16 into the recipient BEAS-2B cells (Fig 5A-F) and we observed the PHK26 dye-labeled EV and EV-CC16 can be quickly and efficiently taken up by BEAS-2B after adding to the culture media.

Treatment with rCC16 and EV-CC16

K. pneu infection markedly increased the production of the proinflammatory cytokines in human epithelial cells, so we aimed to evaluate the modulating effects of rCC16 and EV-CC16 on the elevated cytokines. We found that *K. pneu* infection-induced IL-1β, IL-6 and IL-8 in BEAS-2B was rescued by CC16 at 0.43 ng/ml concentration in EV-carried form, but not rCC16 at 5 μg/ml concentration (Fig 6A-C). These results suggest that EV-CC16 effectively suppressed the expression of pro-inflammatory cytokines in *K. pneu*-treated lung epithelial cells. On the other hand, rCC16 may not easily enter the cells via endocytosis. It may explain our observation that a high dose of rCC16 treatment did not provide any anti-inflammatory effect. Moreover, COX-2 expression was significantly reduced in both EV and EV-CC16 groups, but not the rCC16 treated group (Fig 6D). Neither rCC16 nor EV-CC16 showed a reduction in CXCL10 expressions in human epithelial cells (Fig 6E). H358 cells did not respond to any group of treatment (data not shown).

It was reported that rCC16 significantly reduced IL-1β, TNF-α and IL-6 mRNA levels in THP-1 macrophages-treated with silica [49]. Furthermore, rCC16 inhibited the expressions of TNF-α, IL-6, and IL-8 in LPS-treated RAW264.7 cells [50]. In our study, rCC16 did not modulate IL-1β, IL-6 or IL-8 levels in lung epithelial cells. These results suggest that rCC16 downregulates the production of pro-inflammatory cytokines in silica/LPS treated macrophages but not *K. pneu*-infected lung epithelial cells.

In a murine model of COPD, rCC16 resolved pathological damage in the lungs and reduced the production of TNF-α, IL-6 and IL-8 in both the serum and BALF after CS exposure [30]. In a lamb model of infant respiratory distress syndrome, combination therapy of surfactant and rCC16 lowered IL-8 in serum and IL-6 in the lung tissue than surfactant therapy alone [51].

In RSV- induced airway inflammation and airway hyperresponsiveness model, a recent study reported that administration of CC16 in mouse lungs reduced the protein levels of COX-2 by inhibiting the cytosolic phospholipase A2 (cPLA2)/COX2 pathway [52]. In our study, rCC16 did not reduce mRNA levels of COX-2 after *K. pneu*- infected lung epithelial cells.

BEAS-2B and CC16 stably transfected BEAS-2B cells (BEAS-2B-CC16) in response to *K. pneu* infection

In our lab, we generated CC16 stably transfected BEAS-2B cells (BEAS-2B-CC16) and control BEAS-2B using pRP-Puro-CMV-CC16 and its control vector pRP-Puro-CMV, respectively. The stably transfected cells were selected by puromycin and validated by immunofluorescence staining (data not shown). After confirming the anti-inflammatory effects of EV-CC16, we wanted to infect the BEAS-2B-CC16 and its control cells to compare their response to the bacterial infection. CC16 was highly expressed in BEAS-2B-CC16 compared to control BEAS-2B cells in absence of infection. After *K. pneu* infection, CC16 was down-regulated in both

groups (Fig 7A). Furthermore, we wanted to evaluate the mRNA levels of proinflammatory cytokines in both cells after bacterial infection. We found that IL-1β, IL-6 and IL-8 expressions were significantly reduced in BEAS-2B stable CC16 cells whereas control BEAS-2B appeared to have high mRNA levels of proinflammatory cytokines (Fig 7B-D). This result also indicated the anti-inflammatory effects of CC16 in lung epithelial cells.

Summary and Future Perspectives

In the present study, CC16 mRNA significantly decreased in BEAS-2B cells and H358, but not A549 and H441 after *K. pneu* infection. In BEAS-2B cells, various proinflammatory marker genes were significantly elevated in response to *K. pneu* infection, including IL-1β, IL-6, IL-8, COX-2 and CXCL10. In H359 cells, IL-1β, IL-6 and COX-2 increased significantly after bacterial infection. Treatment with rCC16 did not show a reduction in such cytokines/chemokines compared to EV-CC16 in BEAS-2B cells, whereas H358 cells did not respond to any group of treatment. Consistently, overexpression of CC16 in BEAS-2B cells lowers the IL-1β, IL-6, and IL-8 expressions in response to bacterial infection.

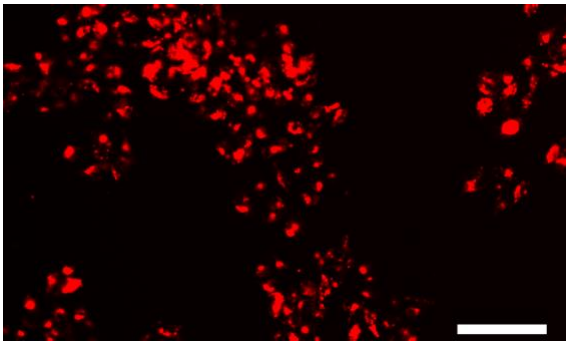
Future studies may provide potential reasons why rCC16 showed modulating effects on the elevetaed cytokines/chemokines released from macrophages but not on those released from lung epithleilal cell lines. In our study, BEAS-2B appered to be a reliable cell line for measuring CC16 expression with or without bacterial infection. Future studies may confirm our findings or could identify other lung epithelial cell lines that would be sutiable for such experiments. Finally, it is obvious that CC16 can be evaluated in both macroghages and lung epithleilal cell lines. Thus, additional studies are needed to address the inconsistent findings.

Figure 5 (Fig A-F): Uptake of EV and EV-CC16 by BEAS-2B Cells. Images

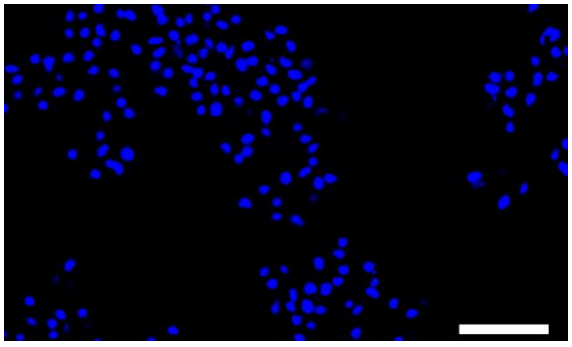
The uptake of EV and EV-CC16 by BEAS-2B Cells was visualized with fluorescence microscopy. Inverted fluorescence microscopy confirmed the location of EV and EV-CC16 in BEAS-2B following 24h treatment. (A). Red, EV labeled by PKH26; (B) blue, the nucleus of BEAS-2B labeled by DAPI. (C). Merged PKH26 + DAPI. (D). Red, EV-CC16 labeled by PKH26; (E) blue, the nucleus of BEAS-2B labeled by DAPI. (F). Merged PKH26 + DAPI. Scale bar, 100 μm.

Figure 5 (Fig A-C): Uptake of EV by BEAS-2B Cells.

A) PKH26



A) DAPI



B) Merged PKH26 + DAPI

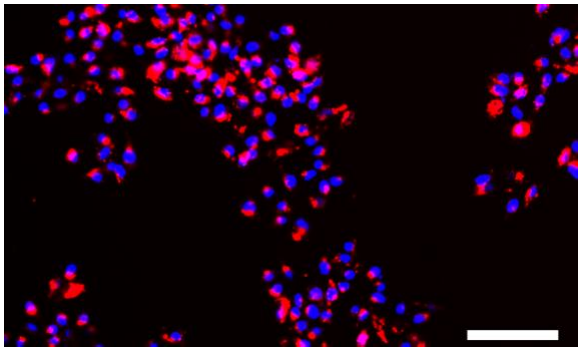
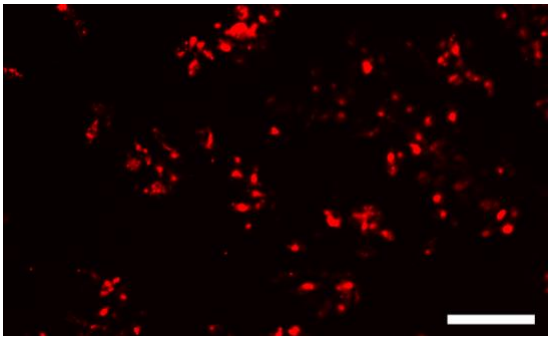
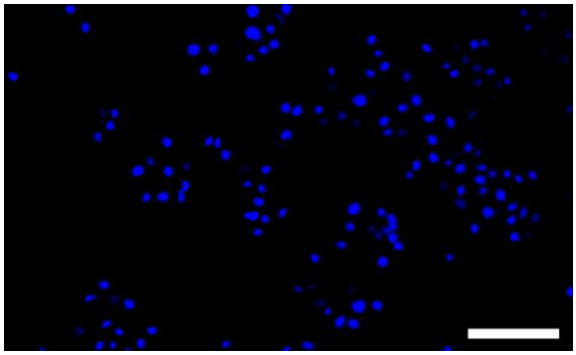


Figure 5 (Fig D-F): Uptake of EV-CC16 by BEAS-2B Cells.

D) PKH26



E) DAPI



E) Merged PKH26 + DAPI

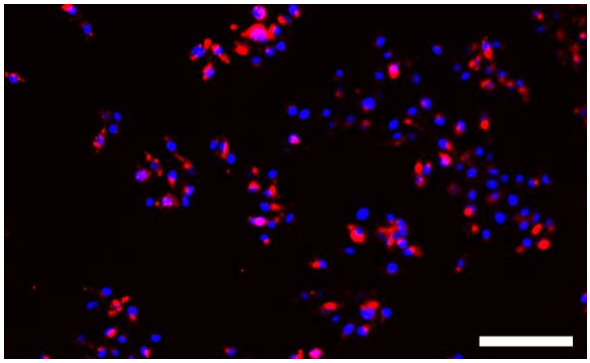


Figure 1: CC16 expression in BEAS-2B, H358, A549 and H441

CC16 was highly expressed in BEAS-2B cells when compared to A549, H441 and H359 cells (Fig.1). The mRNA levels of CC16 were detected from human lung epithelial cells. The mRNA levels of CC16 were determined by real-time polymerase chain reaction (real-time PCR). N=3, * *P<0.05*

Figure 1: CC16 expression in BEAS-2B, H358, A549 and H441

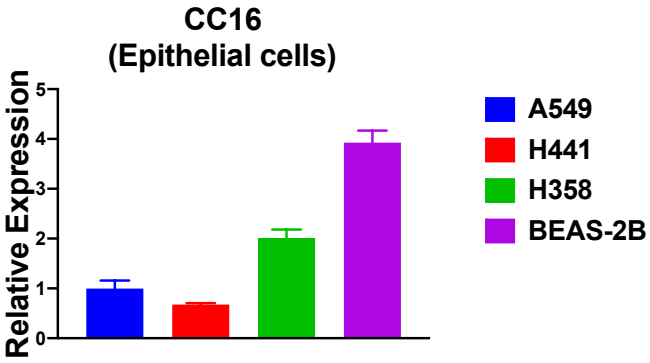
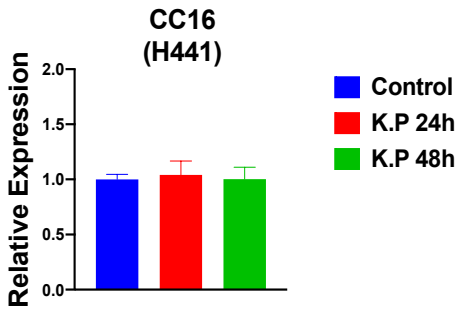


Figure 2 (A-D): CC16 expression in BEAS-2B, H358, A549 and H441 after *K. pneu* infection

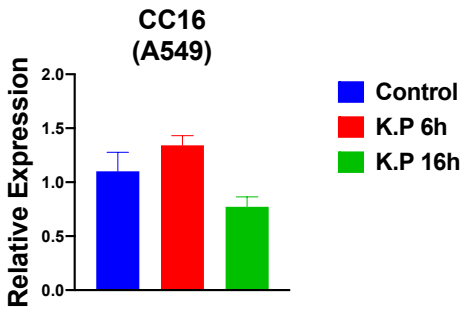
CC16 expression was reduced in BEAS-2B and H358 cells but not in A549 and H441 after *K. pneu* infection. The mRNA levels of CC16 were detected from human lung epithelial cells which were infected with a 1:10 ratio of *K. pneu* for one hour and then incubated in a medium containing gentamycin at different time points, 6h, 12h, 18h, 24h, and 48h. The mRNA levels of CC16 were determined by real-time polymerase chain reaction (real-time PCR). N=3, * $P<0.05$

Figure 2 (A-D): CC16 expression in BEAS-2B, H358, A549 and H441 after *K. pneu* infection

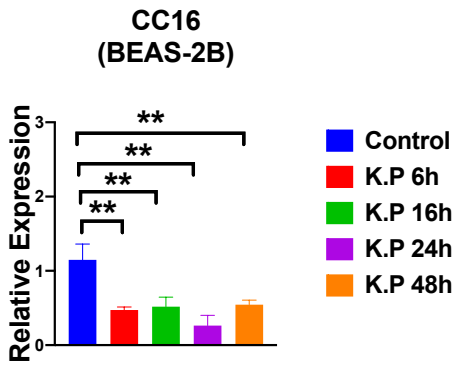
A) H441



B) A549



C) BEAS-2B



D) H358

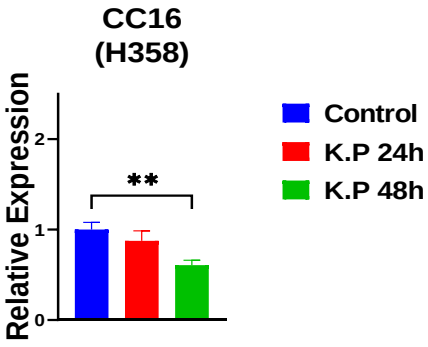
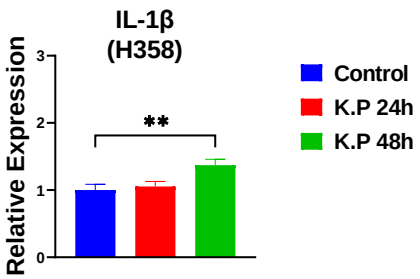


Figure 3 (Fig 3A-E): Proinflammatory cytokines/chemokines after *K. pneu* infection in H358 cells

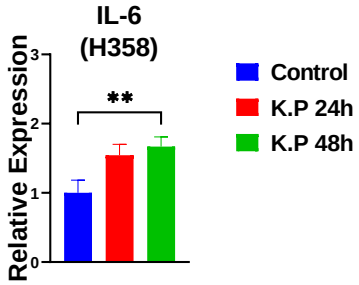
IL-1β, IL-6 and COX-2 increased significantly after *K. pneu* infection while IL-8 and CXCL10 did not change apparently at different time points 24h and 48h. The mRNA levels of the cytokines were detected from human H358 cells which were infected with a 1:10 ratio of *K. pneu* for one hour and then incubated in a medium containing gentamycin for 24h and 48h. The mRNA levels of proinflammatory cytokines were determined by real-time polymerase chain reaction (real-time PCR). N=3, * $P<0.05$

Figure 3 (Fig 3A-E): Proinflammatory cytokines/chemokines after *K. pneu* infection in H358 cells

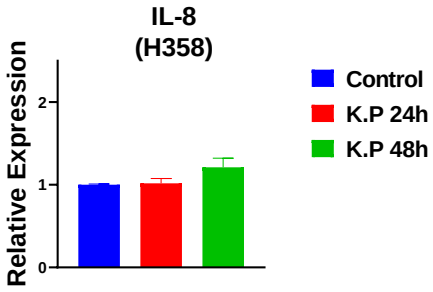
A)



B)



C)



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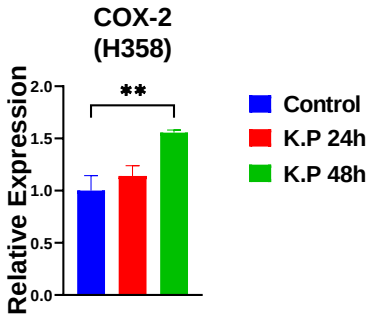


E)

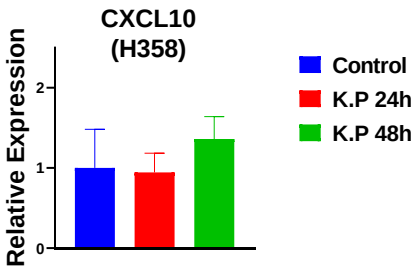
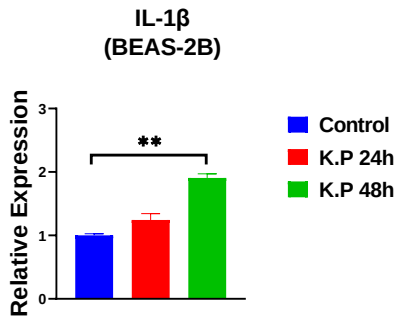


Figure 4 (Fig 4A-E): Proinflammatory cytokines/chemokines after *K. pneu* infection in BEAS-2B cells

IL-1β, IL-6, COX-2 and CXCL10 significantly increased after 48h and there were no significant changes at 24h. IL-8 significantly increased at both time points. The mRNA levels of the cytokines were detected from human BEAS-2B cells which were infected with a 1:10 ratio of *K. pneu* for one hour and then incubated in a medium containing gentamycin for 24h and 48h. The mRNA levels of proinflammatory cytokines were determined by real-time polymerase chain reaction (real-time PCR). N=3, * $P<0.05$

Figure 4 (Fig 4A-E): Proinflammatory cytokines/chemokines after *K. pneu* infection in BEAS-2B cells

C)



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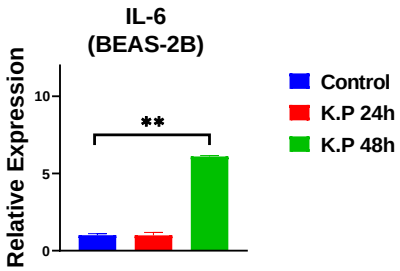
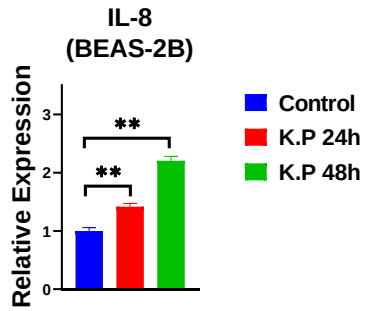
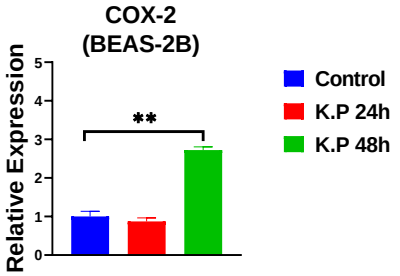


C)



D)



F)

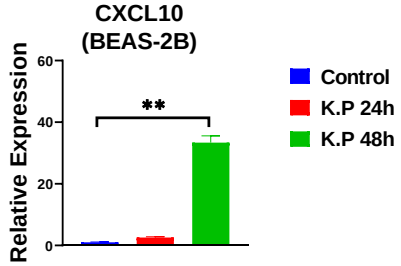


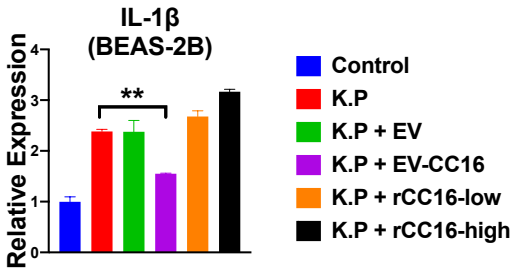
Figure 6 (Fig 6A-E): Proinflammatory cytokines/chemokines after treatment with rCC16 and EV-CC16 in infected BEAS-2B cells

K. pneu infection-induced IL-1β, IL-6 and IL-8 in BEAS-2B were rescued by EV-carried CC16 at 0.43 ng/ml concentration, but not rCC16 at 5 μg/ml concentration (Fig 6A-C). COX-2 expression was significantly reduced in EV and EV-CC16 group compared to rCC16 (Fig 6D). Neither rCC16 nor EV-CC16 shows a reduction in CXCL10 expressions in BEAS-2B cells (Fig 6E).

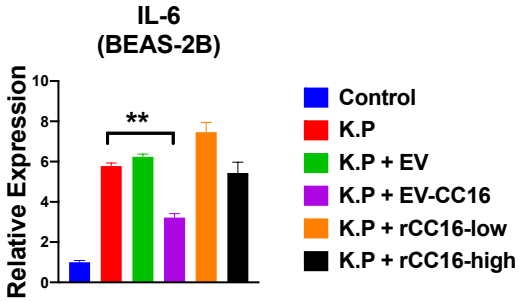
The mRNA levels of the cytokines were detected from human BEAS-2B cells infected with a 1:10 ratio of *K. pneu* for one hour and then incubated in a medium containing gentamycin for 48h. The mRNA levels of proinflammatory cytokines were determined by real-time polymerase chain reaction (real-time PCR). N=3, The low dose of rCC16 was 1 μg/ml while the high dose of rCC16 was 5 μg/ml based on previous studies. * $P<0.05$

Figure 6 (Fig 6A-E): Proinflammatory cytokines/chemokines after treatment with rCC16 and EV-CC16 in infected BEAS-2B cells

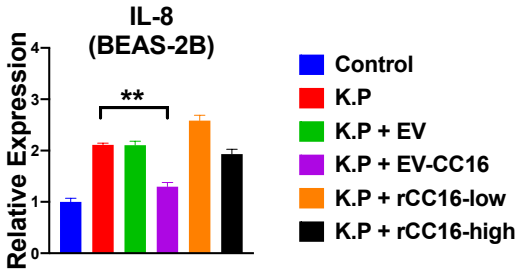
A)



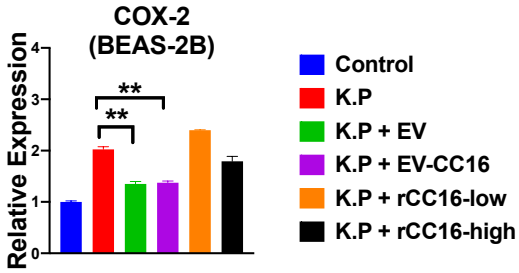
B)



C)



D)



E)

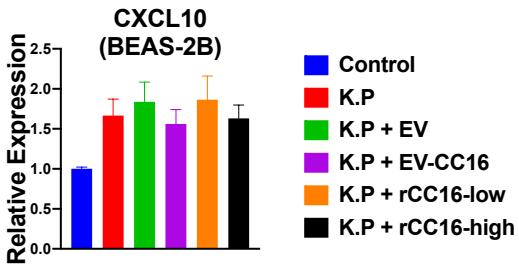


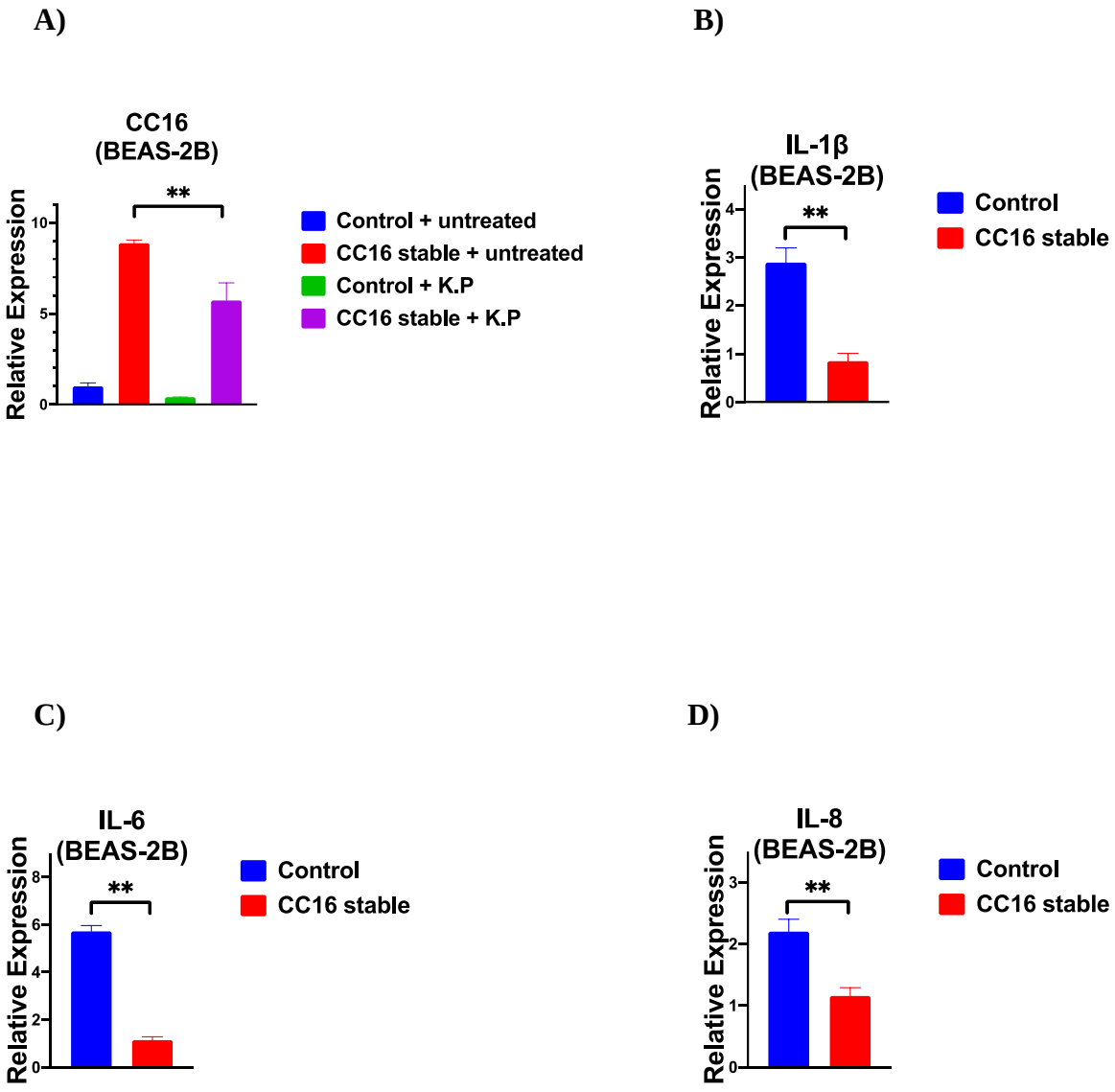
Figure 7 (Fig 7A-D): BEAS-2B and BEAS-2B-CC16 stable cell lines in response to *K. pneu* infection

CC16 was highly expressed in BEAS-2B stable CC16 compared to normal BEAS-2B cells in absence of infection and was down-regulated in both cells after *K. pneu* infection (Fig 7A).

IL-1β, IL-6 and IL-8 expressions were significantly reduced in BEAS-2B stable CC16 cells whereas normal BEAS-2B appeared to have high mRNA levels of proinflammatory cytokines after *K. pneu* infection (Fig 7B-D).

The mRNA levels of the cytokines were detected from human BEAS-2B cells infected with a 1:10 ratio of *K. pneu* for one hour and then incubated in a medium containing gentamycin for 48h. The mRNA levels of proinflammatory cytokines were determined by real-time polymerase chain reaction (real-time PCR). N=3, * $P<0.05$

Figure 7 (Fig 7A-D): BEAS-2B and BEAS-2B-CC16 stable cell lines in response to *K. pneu* infection



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