THE EFFECT OF CC16 ON MACROPHAGE POLARIZATION

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by Cory Bacon, titled The Effect of CC16 on Macrophage Polarization and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

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LAND ACKNOWLEDGEMENT

We respectfully acknowledge the University of Arizona is on the land and territories of Indigenous peoples. Today, Arizona is home to 22 federally recognized tribes, with Tucson being home to the O'odham and the Yaqui. Committed to diversity and inclusion, the University strives to build sustainable relationships with sovereign Native Nations and Indigenous communities through education offerings, partnerships, and community service.

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ABSTRACT

Club Cell Secretory Protein (CC16) is a protein that is secreted in the lungs and exhibits protective effects against the development of obstructive lung diseases, altered pulmonary function, airway remodeling, and helps lower pathogenic load in the lungs [3]. Deficiencies in CC16 have been linked to worsened symptoms in asthma [13], rapid decrease of lung function in COPD-like disease in mice [14] and has been linked to increasing airway remodeling in mice [12]. rCC16 has been demonstrated to regulate the expression of pro-inflammatory cytokines via NF-κB. Resident macrophages are known to participate in a variety of these processes, but whether CC16 exerts protective effects in the lung via macrophage-dependent mechanisms remains unknown and was the topic of study in this thesis. Experiments were conducted with RAW 264.7 and immortalized bone marrow derived macrophages (IBMDM) cells to shed light on the effects of CC16 on macrophage polarization. Stimulated RAW 264.7 cells were used to measure M2 polarization in the presence of M2 stimuli with and without rCC16. In these studies, rCC16 had minimal impact on limiting M2 polarization of RAW 264.7 cells at the dose and time point tested. Both RAW 264.7 and IBMDM cells were used to measure gene expression associated with M2 macrophage polarization, including expression of genes for IL-10, IL-13, Arg1, and PPAR-γ. Major findings were that in RAW 264.7 cells, IL-10 was significantly upregulated by rCC16 alone and PPAR-γ was significantly downregulated by rCC16 in the presence of IL-4+IL-13. In IBMDM cells, rCC16 has less impact on M2 gene expression resulting from IL-4 stimulation, however, rCC16 alone and in the presence of IL-4, also increased IL-10 gene expression.

INTRODUCTION

General background on CC16:

CC16, also referred to as CC10, club cell secretory protein, and uteroglobin, is a homodimeric pneumoprotein that is approximately 16 kDa in size [3]. Each subunit contains 70 amino acids of the same identity fashioned in an anti-parallel direction linked together using disulfide bonds between Cys3 and Cys69 which stabilize the protein into a dimer [1]. Each subunit of the protein forms four alpha helices with a single beta turn between helices two and three shown in figure 1 [1,15], creating a hydrophobic pocket capable of binding molecules such as retinol, progesterone and polychlorinated biphenyls. CC16 also contains minor hydrophobic pockets that can bind prostaglandins [1].

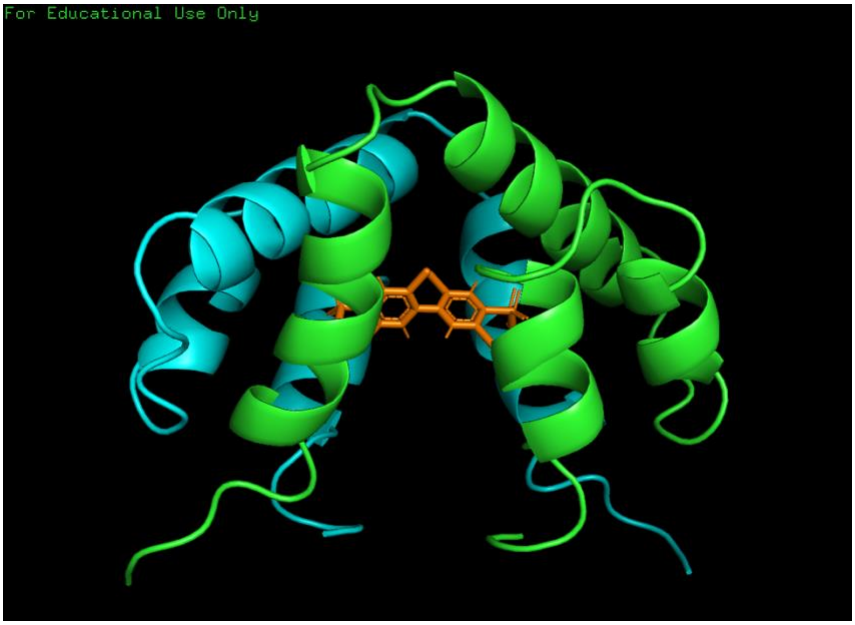

Figure 1: Crystal Structure of Uteroglobin bound to a polychlorinated biphenyl.

The protein is encoded by the SCGB1A1 gene and is found on chromosome 11q12.3-13.1 in humans and Scgb1a1 in mice is on chromosome 18. Human SCGB1A1 is approximately 18,000 base pairs and for mice it is only 4400 base pairs, but the structure remains the same: three exons and two introns. CC16 is produced by club cells and non-ciliated cells mainly in distal pulmonary epithelium of humans and is expressed in the trachea-bronchial tree of mice. While being mostly associated with the lungs, CC16 is also known to be secreted by the pancreas, prostate, ovaries, mammary glands, and the uterine endometrium [1]. The study of CC16 in the lungs has led to the discovery of its protective properties associated with better health outcomes during and after stress in the lungs.

Previously discovered properties of CC16:

With CC16 being the most prevalent protein in airway surface fluid [2], not much is known about its physiological function. However, studies have shown that CC16 has anti-inflammatory and antioxidant properties [3]. Studies using asthma models in mice looked at the protective effects of CC16 during immune mediated responses typically seen in these patients such as airway hyperresponsiveness and airway inflammation. When CC16 deficient and WT mice were challenged with ovalbumin, which is known to induce inflammation in the airway, there was an increase in airway inflammation in CC16 deficient mice almost two-fold more after two days compared to WT mice [5]. When mice were exposed to house dust mite extracts, CC16 was shown to help reduce the inflammation in the airway preventing damage through regulatory pathways of apoptosis in the epithelial cells by downregulating the expression of the damage associated molecular patterns (DAMP) molecule HMGB1 [4], which is known to induce inflammation and immune responses [6].

Experiments from Hu et al further showed the protective effects of CC16 on the human bronchial cell line BEAS-2B. When these cells are exposed to diesel exhaust particles (DEP), the cells released TNF-α and this stimulated the production and secretion of CC16 helping to

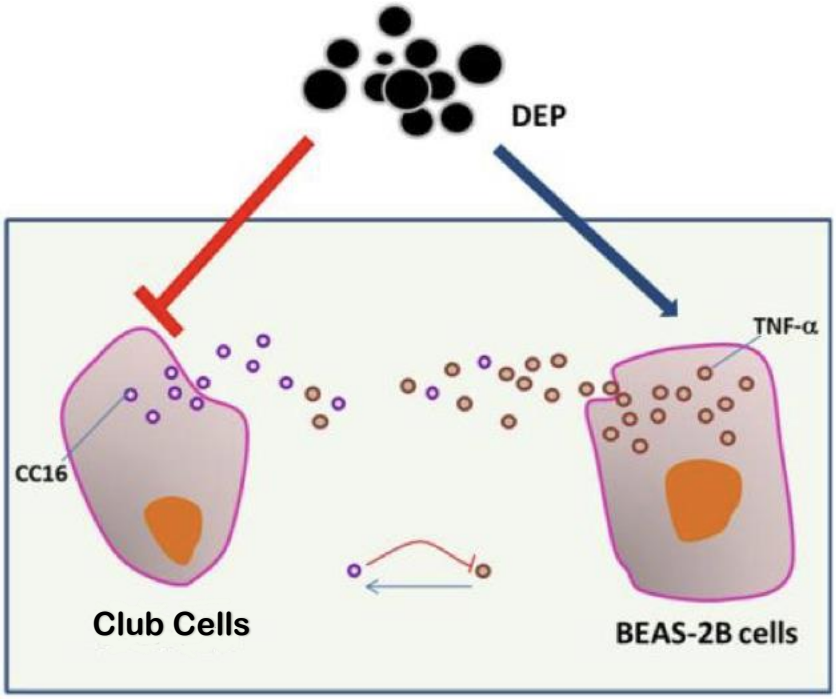

Figure 2:Graphical presentation of the negative feedback loop between CC16 and TNF-α when BEAS-2B cells are exposed to DEP in-vitro.

attenuate the inflammation from DEP as seen in figure 2 [2]. Another study found that CC16 was able to protect against lung epithelial cell injury and reduce inflammation when exposed to particulate matter 2.5 μm or smaller (PM2.5). Lung epithelial cells exposed to PM2.5 and treated with CC16 showed decrease in inflammatory cytokines (IL-6, IL-1β, etc.) and was able to inhibit ferroptosis, a sign that the cells were protected from damage [9]. Along with attenuating inflammation, studies have shown CC16 is able to help with reducing pathogen load in the lungs [3,7] as well rescue mice undergoing infection induced airway hyperresponsiveness (AHR) [7].

Using *mycoplasma pneumonia* (mp), a well-known microorganism to exacerbate airway disease symptoms, to infect WT and CC16 knockout mice, CC16 has been demonstrated to help reduce the amount of mp burden experienced in mice during infection [3,7] as well as reduce AHR that pulmonary infections induce (figure 3) [7]. Both outcomes have mechanisms that

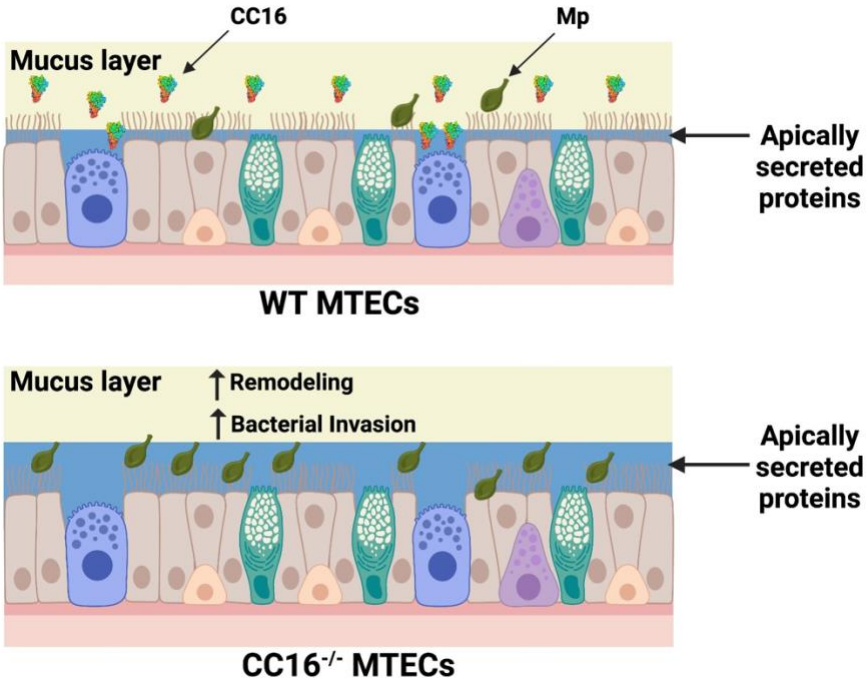

Figure 3:CC16 deficiency leads to increased MP burden and AHR, leading to worsened lung function.

involve integrins Very Late Antigen 2 (VLA-2) and Very Late Antigen 4 (VLA-4) [3,7]. When CC16 binds to VLA-4, it can inhibit the adhesion of leukocytes to endothelial cells limiting the infiltration of leukocytes and reducing the damage created by immune cells like neutrophils and macrophages [7]. While studying the interaction between CC16 and VLA-4, it was discovered that while leukocyte infiltration was being limited, the bacterial load of mp was also reduced leading the conclusion some other type of antimicrobial defense was being upregulated [7]. In later studies, it was determined that CC16 also binds to VLA-2. The binding between the two

molecules induces signaling that greatly increased the expression of SPLUNC1, an antimicrobial protein [3] that has activity against gram-negative bacteria [8].

CC16 serum levels correlate with lung function and disease progression:

Despite being mainly produced in the lungs, CC16 is readily detectable in serum, potentially making this a great biomarker of lung function [3,7,10,11,]. Increases of CC16 have been seen in the case of acute injury due to environmental exposures to smoke, LPS, ozone, and other environmental pollutants. The authors note this is not due to increased production of CC16 by club cells but indicates an increase in the leaking of CC16 from the airway. Eventually serum levels would return to baseline in a period of hours to days after exposure [11]. In the case of pulmonary diseases, there was a noticeable difference when looking at some acute vs chronic responses. Acute diseases such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS) [11] and viral infections [10] led to an increase in levels of the CC16 found in the serum [10,11]. Higher CC16 serum levels were able to indicate the severity of ADRS and the duration of the use of mechanical ventilation or ICU stay. Lower baseline levels were associated with a higher likelihood of recurrent respiratory infections [10].

In the case of chronic diseases such as asthma and COPD, both showed a decrease in serum levels of CC16. Lower levels of CC16 in the circulation of COPD patients was associated with rapid decline in forced expiratory volume (FEV1) and in asthma patients was associated with the increased duration of disease and allergic sensitization [10]. Studies conducted with participants diagnosed with asthma have connected low levels of serum CC16 to the likelihood asthma symptoms will continue into adulthood [13] and alterations in lung function including airway remodeling [12].

Furthering the evidence linking CC16 serum levels and lung function, studies looking at CC16 deficient mice showed decreased lung function compared to wild type [12,14]. At baseline, these mice show increased airway resistance that is further exacerbated when challenged with methacholine. Upon further examination, these deficient mice showed an increase expression of the genes for collagen type I, collagen type III, and α-smooth muscle

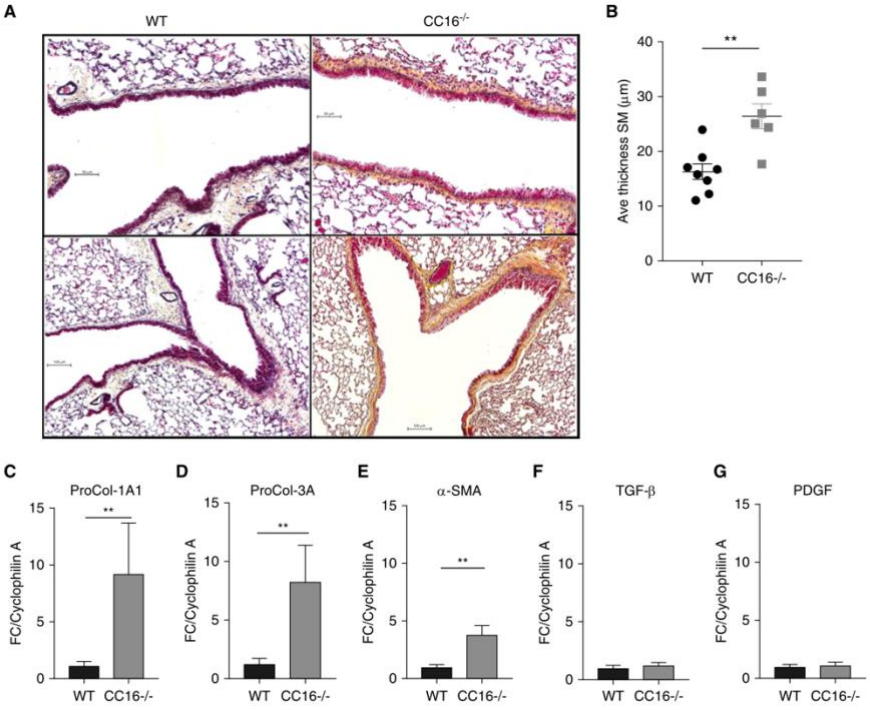

Figure 4: Histological and PCR data after methacholine challenge of WT and CC16 deficient mice. After methacholine challenge at varying concentrations (0, 10, 30, & 100 mg/ml), sections of lung tissue were stained with Gill stain and imaged at x10 and x100 magnification to analyze airway thickening and alveolar chord length as well as RT-qPCR analysis of airway remodeling factors

actin indicating an increase in airway remodeling constituted by thickening of the basement membrane, increased smooth muscle and collagen deposition (Figure 4) [12]. This is also seen in CC16 deficient mice with cigarette smoke (CS) induced COPD-like disease. At baseline these mice had a thicker ECM compared to wild type. When these WT and CC16 deficient mice were

exposed to CS, the latter showed significantly thicker ECM compared to wild type. Remarkably though, when mice are treated with recombinant human CC16 (rhCC16), there is no significant difference between WT and CC16 deficient mice, demonstrating a potential use for CC16 in treating lung disease [14].

MACROPHAGES

Macrophages are a specialized type of cell derived from monocytes that belong to the innate immune system found in both lymphoid and non-lymphoid tissues [16]. Macrophages can be found in many different types of tissues, especially ones that encounter the outside environment such as the lungs and digestive tract where they are constantly surveying and protecting the body from foreign particles and invaders [16,17]. These cells have three main roles: 1. They are responsible for the clearing erythrocytes, debris, apoptotic cells; are crucial for normal development and physiological processes and play a role in the elimination of pathogens from the body. Most of the processes are done through endocytosis and phagocytosis, giving these cells the nickname “the big eaters” [17]. 2. They play an important role in the adaptive immune system by participating in antigen presentation through

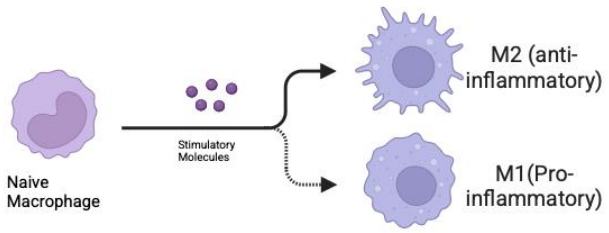

Figure 5: Macrophage Polarization produces two phenotypes: M1 and M2.

major histocompatibility complex (MHC) molecules, presenting antigens to activated T cells [17,18]. 3. Macrophages play an important role in the inflammatory response by producing both pro-inflammatory and anti-inflammatory cytokines such as TNF-α, IL-6, IL-10, and TGF-β based on the phenotype. Macrophages are polarized towards one of two phenotypes: M1 (pro-inflammatory macrophages) and M2 (anti-inflammatory macrophages) (figure 5) [17,18].

M1 Macrophage Polarization:

M1 macrophage polarization, also known as classical activation, refers to macrophages with a phenotype skewed towards an anti-microbial, anti-tumoral, and pro-inflammatory state [17,18]. Surface antigens used to identify M1 polarized macrophages include MHC II, CD68, CD80, and CD86 [21], and prototypic cytokines include IL-1β, TNF-α, and IL-6 [24]. These macrophages are polarized to the M1 state by Th1 cytokines such as TNF-α and IFN-γ, through bacterial Lipopolysaccharide (LPS) [18] or interaction with toll like receptors (TLR) (figure 6).

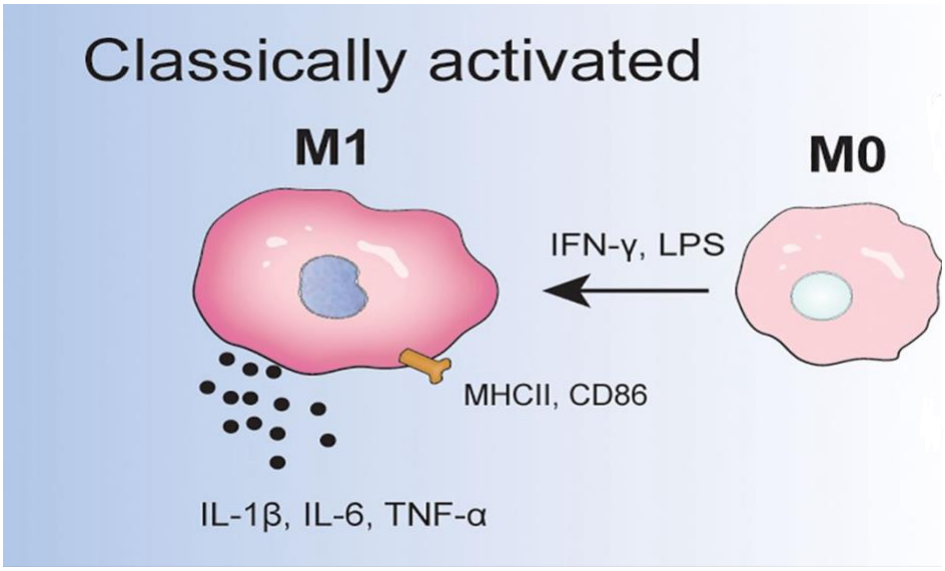

Figure 6: M1 stimuli such as INF-γ and LPS induce the polarization of macrophages to the M1 state; releasing pro-inflammatory cytokines.

INF-γ and TLR lead to the activation of STAT1 in the jak-STAT pathway, increasing the expression of M1 phenotypic genes. However, if IFN-α/β bind to its receptor, M1 polarization will be blocked by SOCS3 [19]. Activation of the jak-STAT signaling pathway induces an increase in microbicidal activity, antigen presentation, and inflammatory cytokine production [20]. M1 macrophages initiate their anti-microbial activity through activation of the NADPH oxidase system, which increases the production of reactive oxygen species (ROS), leading to tissue damaging effects [18].

This macrophage phenotype produces and secretes the pro-inflammatory cytokines TNF-α, IL-1α/β, IL-6, IL-12, IL-23, and cyclooxygenase-2 (COX-2) [18], helping to increase the activity of Th1 and Th17 cell activity with a consequence of tissue damage [21]. To help combat this damage, M2 macrophages will secrete anti-inflammatory molecules that help to minimize damage and prevent chronic inflammation from occurring [18]. While over-secretion of M1 cytokines is damaging to healthy tissue, they are thought to be beneficial in the defense against tumor development [22,23].

Utilizing a melanoma cell line and RAW 264.7 macrophage cell line, researchers identified that these pro-inflammatory cytokines from M1 macrophages are capable of inducing apoptosis of a melanoma cell line by initiating the cleavage of caspase 3 and stopping proliferation by inhibiting Akt in the PI3K pathway [22]. Further studies looking at human lung cancer cell lines concluded that M1 macrophages can induce apoptosis of tumor cells by way of p53. IFN-β and IFN-γ work together to activate STAT1 that in turn activates p53 downstream leading to apoptosis [23].

M2 Macrophage polarization:

M2 macrophage polarization, also known as alternative activation, refers to macrophages with a phenotype generally skewed towards anti-inflammatory, antiparasitic, wound healing, tissue repair, tumor promoting, and are involved in allergic inflammation [21]. Surface markers that identify M2 macrophages are CD206, CD163, CD68, ARG1, CD11b, and MSR1 [26] and genes used to identify are IL-10, IL-13, Arg1, and PPAR-γ [24]. M2 macrophages are generally polarized due to the presence of fungal cells, parasites, immune complexes, complement, apoptotic cells, macrophage colony stimulating factor (M-CSF), the Th2 cytokines IL-4 & IL-13 [18], IL-10, and TGF-β [21]. M2 macrophages can be further divided into four subgroups: M2a, M2b, M2c, and M2d that are each polarized by different combinations of the above-mentioned stimuli [24,26,27,28,29,30] (figure 7).

The M2a macrophage subset is induced by IL-4 and IL-13 [18]. The binding of these cytokines to their respective receptors leads to the activation of JAK1 and JAK3 that will downstream activate STAT6 [21] leading to the expression of high levels of CD206, decoy IL-1 receptor (IL-RII), and IL-1 receptor antagonist (IL1RA) [18] while reducing phagocytosis [21]. M2a macrophages will produce and secrete high amounts of IL-10 and several pro-fibrotic factors like IGF, fibronectin, and TGF-β [27], leading to enhanced tissue repair [28]. This subset of macrophage is important in helping clear out debris to attenuate the inflammatory response and protect against parasite invasion [21].

The M2b macrophage subset is induced in the presence of immune complexes, LPS, TLR agonists, or IL-1 receptors agonists. The M2b subtype exerts its function through Fcγ receptor family (CD16a, CD16b, CD32, CD64) signaling involving both PI3K and spleen tyrosine kinase

[21]. These cells can produce and secrete both anti-inflammatory and pro-inflammatory cytokines (IL-10, IL-1β, IL-6, and TNF-α) [18]. M2b macrophages are generated to increase antigen presentation, help to promote the responses of Th2 cells [21], and regulate the inflammatory immune response [29].

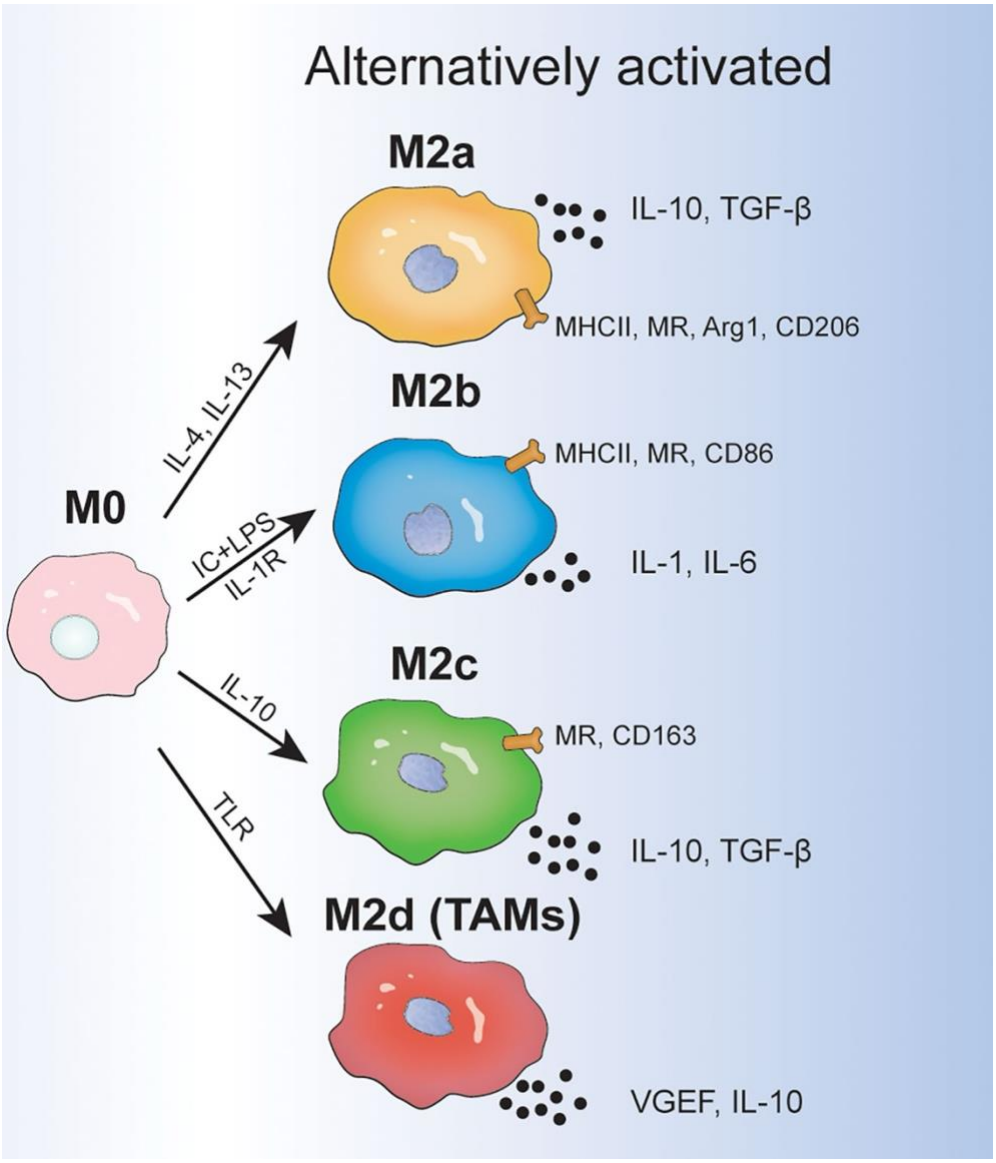

Figure 7: M2 macrophage polarization can be further divided into four subtypes, each with varying functions depending on the stimuli present such as IL-10, IL-4, or TLR agonists.

The M2c macrophage subset is also known as deactivated macrophages because of the ability to switch from M1 to a M2 state, "deactivating" M1 gene production [21]. They play a

role in tissue remodeling [27] and have increased phagocytosis properties [30]. They are stimulated by glucocorticoids and IL-10 combating the inflammation induced by apoptotic cells, secreting high amounts of IL-10 and TGF-β [18], with IL-10 binding and activating STAT3 to downregulate pro-inflammatory cytokine expression [21].

The M2d macrophage subset, alternatively called tumor associated macrophages (TAM), are activated by TLR [21]. The polarization of these macrophages lead to the production and secretion of IL-10 and VEGF suggesting they participate in angiogenesis playing an important role in wound healing [31] and tumor progression [27].

Alveolar macrophages-residents of the lung:

Alveolar macrophages (AM) are tissue resident macrophages found in the distal portion of the lung on the luminal surface of the alveolar space that originate from fetal liver monocytes during the early stages of embryonic development [32]. AM in mice are identified by the surface markers MerTek, CD64, CD68, CD206, and F4/80 and in humans the surface markers are CD206, CD169, CD11c, CD163, and MARCO.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and PPARγ are crucial to the development of precursor monocytes to mature AM. GM-CSF induces the production of PPAR-γ which is important for the further development of AM. GM-CSF deficient mice have decreased numbers of mature AM and histological analysis resemble that of pulmonary alveolar proteinosis (the buildup of surfactant in the alveoli). The same was observed in PPAR-γ deficient mice with a decline in numbers of AM and abnormalities in the cells, showing the importance of what previous investigators termed the GM-CSF-PPARγ axis [33].

AM play a crucial role in defending the lungs from external stimuli, pathogens, and maintaining homeostasis in the lungs [33,34]. The lungs are constantly exposed to inhaled antigens and AM must remain selective about how they react to minimize damage [34]. Once exposed to external stimuli, AM take on an anti-inflammatory state by preventing cells undergoing apoptosis from propagating a pro-inflammatory response through efferocytosis (engulfing apoptotic cells). Efferocytosis promotes the production and secretion of the anti-inflammatory molecules TGF-β, prostaglandin E2 (PGE2), and platelet activating factor (PAF) [33,34].

The Surfactant proteins A (SP-A) and D (SP-D) also play a role in dampening the pro-inflammatory response by binding to signal regulatory protein α (SIRPα) and inhibiting both AP-1 and NF-κB [34]. Lung epithelium also plays a role in maintaining the anti-inflammatory state through ligands on the epithelial cells (CD200, PDL-1, and CD47) interacting with receptors on

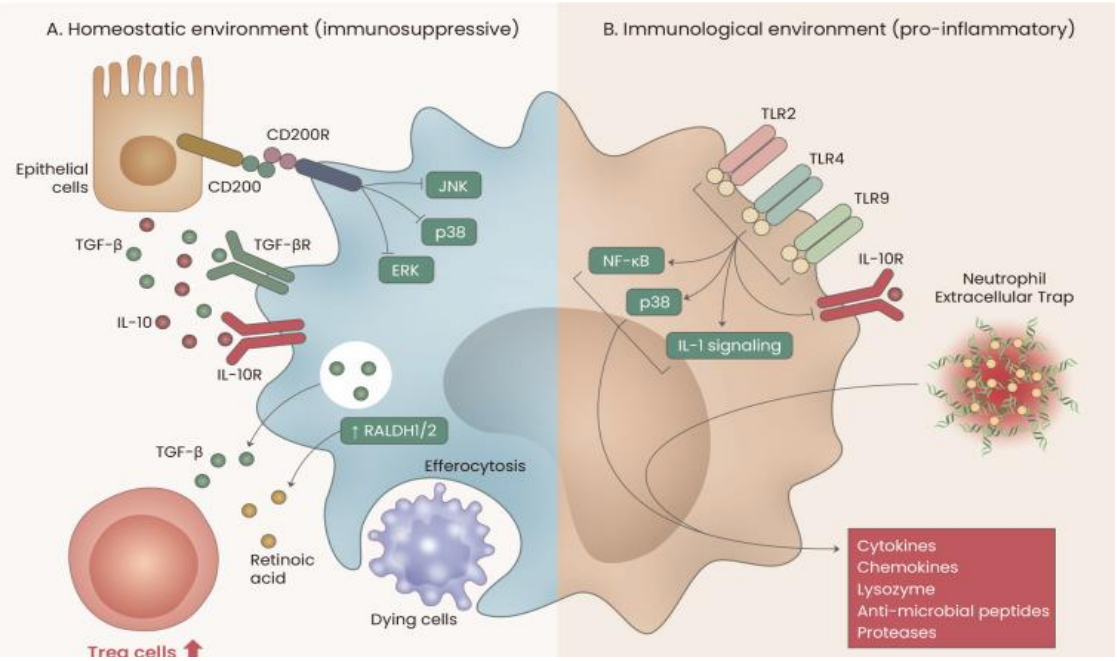

Figure 8:Alveolar macrophages (AM) function to maintain homeostasis and protect against pathogens through interactions between AM and lung epithelial cells (CD200, CD200R) or TLR.

AM (CD200R, PD-1, and SIRα) leading to the secretion of IL-10 and TGF-β, further enhancing the anti-inflammatory of AM (figure 8) [35].

In the presence of LPS and injury, AM take on a pro-inflammatory state and release inflammatory cytokines IFN, IL-1, IL-6, and TNF-α (figure 8) which results in the recruitment of more leukocytes to the site of inflammation to help fight infection. Severe inflammation and infection are often accompanied by a sharp, temporary decline in AMs. In active infection, the process of efferocytosis has proven to be detrimental, leading to the conclusion that this temporary disappearance could be beneficial to the health of lung tissue [36]. Similarly, this same phenomenon will occur in allergic asthma. Mouse studies using house dust mite challenge to model allergic asthma demonstrated a decrease in AM on day 3 and a complete reduction on day 5, assumed to be due to AM death [37].

Macrophage involvement in airway remodeling and fibrosis:

Airway remodeling is a physiological process common in asthma, COPD and cigarette smoke (CS) exposure marked by squamous metaplasia submucosal gland hypertrophy, smooth muscle hyperplasia, and fibrosis in the airway [38]. Alveolar macrophages and interstitial lung macrophages work together in the release of IL-1, IL-6, TNF-α, TGF-β, matrix metalloproteases (MMPs) and insulin-like growth factor-1 (IGF-1) which regulate remodeling activities such as epithelial cell proliferation, fibroblast activation, angiogenesis, and extracellular matrix (ECM) deposition (figure 9) [39].

A study looking at cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD) in mice linked macrophages to increased airway remodeling and fibrosis by inhibiting ciliary protein levels. Analysis of single cell lung suspensions demonstrated an increase in

macrophage presence with a decrease in the number of ciliated cells. To determine a molecular mechanism relating macrophages to decreased ciliated cells, BEAS-2A cells and THP-1 cells were exposed to cigarette smoke extract (CSE) and it was observed that macrophages contribute to remodeling by reducing the levels of the ciliary protein β-tubulin-IV through the secretion of high levels of bone morphogenic protein-2 (BMP-2), an important protein responsible for maintaining healthy lung tissue. Confirming the mechanism, the use of a BMP-2 inhibitor showed an increase in the number of ciliated cells [38].

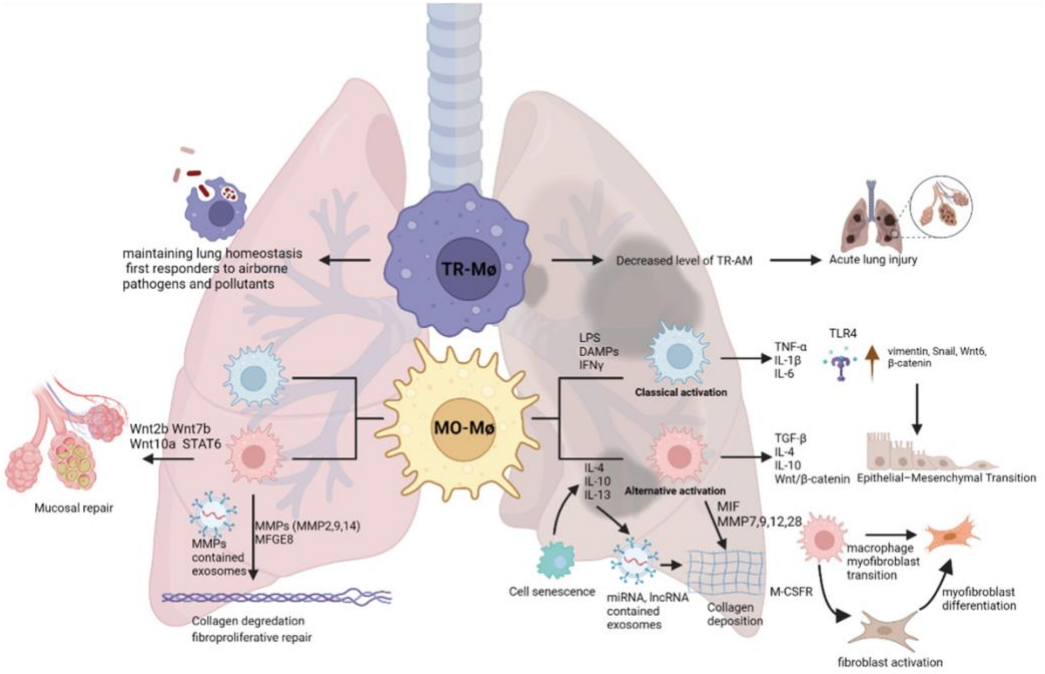

Figure 9:Macrophages play a role in airway remodeling through production of cytokines, pro-fibrotic factors, and growth factors.

In the context of asthma, macrophages participate in many aspects of airway remodeling. Alveolar macrophages in concert with airway smooth muscle in asthmatic patients tend to secrete higher levels of MMP-9 which increases ECM turnover and immune cell infiltration resulting in increased airway thickness and decreased FEV1. A key contributor to airway thickening and air flow restriction is the increased proliferation and contractile force of

airway smooth muscle which is induced when macrophages secrete the cytokines TNF-α and IL-1β. Macrophages secrete pro-fibrotic factors such as PDGF-AA and PDGF-BB that increase the production of collagen from fibroblasts, which tracks with asthmatic patients having increased levels of procollagen-1 [40].

CC16's impact on alveolar macrophage induced inflammation:

CC16 has demonstrated to be a potent anti-inflammatory molecule. However, the potential molecular mechanism has only recently started to be defined [41,42]. Studies utilizing RAW 264.7 cells and THP-1 cells demonstrated potential mechanisms of CC16's anti-inflammatory properties. This includes inhibiting the production of pro-inflammatory cytokines through regulation of NF-κB, p38, and caspase-1 signaling pathways [41,42].

Inducing pro-inflammatory cytokine production in THP-1 macrophages and RAW 264.7 cells using silica and LPS respectively demonstrated the p65 subunit of NF-κB is increasingly translocated to the nucleus in the presence of silica particles and LPS. This leads to an increase of pro-inflammatory cytokines marked by increased p65 in the nucleus compared to the cytosol as well as an increase in the production of caspase 1. When rCC16 was administered, NF-κB p65 translocation was inhibited and the production of pro-inflammatory cytokines was decreased as well as decreased cleaved caspase-1 [41,42]. Given the regulatory effects CC16 has on pro-inflammatory processes of macrophages, my project was to test if CC16 had an impact in limiting the polarization of M2 macrophages, thereby protecting the lungs from macrophage-instigated altered pulmonary function, airway remodeling, and pathogen burden.

METHODS

Raw 264.7 cell line studies:

Raw 264.7 cells (purchased from ATCC) are a macrophage cell line sourced from BALB/c mice utilized to study macrophage biology and immunology including, but not limited, to host-pathogen interaction, macrophage polarization, and osteoclastogenesis [25]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% L-Glutamine, and 1% penicillin/streptomycin while being maintained at 37°C with an atmosphere of 5% CO_2. Media was changed every 2-3 days and cells were passaged at 80%-90% confluence; sub-cultivated at a ratio of 1:10 in 10 cm^2 petri dishes.

IBMDM cell line studies:

Immortalized bone marrow derived macrophages (IBMDM) were acquired from Dr. Justin Wilson's lab at The University of Arizona in the Department of Immunobiology. The cells were cultured in RPMI 1640 medium (Gibco) supplemented with 1x Glutamax, 1 mM Sodium Pyruvate, 1% penicillin/streptomycin, and 10% heat inactivated FBS while being maintained at 37°C with an atmosphere of 5% CO_2. Media was changed every 2-3 days and cells were passaged at 80%-90% confluence; sub-cultivated 1.2 million cells in 10 cm^2 petri dishes.

rCC16 cytotoxic dose response curve:

To observe if rCC16 had any cytotoxicity effects on RAW 264.7, 100,000 cells/well were plated in a 96-well plate and allowed to incubate for 4 hours at 37°C and 5% CO_2. After 4 hours, concentrations of 0 μM, 1 μM, 2.5 μM, or 5 μM were added to the cells and allowed to

incubate for 24 hours at 37°C and 5% CO_2 after which the cells were extracted from the plate, counted using Trypan blue exclusion, and data graphed to observe trends in cell viability.

Flow cytometry- measure M2 polarization:

$1.0x10^6$ RAW 264.7 cells/well were plated in a 12-well plate and allowed to incubate for 4 hours at 37°C and 5% CO_2. After 4 hours, Vehicle, rCC16 (2.5 μM), IL-4+IL-13 (40 ng/mL each), or rCC16+IL-4+IL-13 was added to the cells for a total of 48 hours. After 48 hours, cells were extracted from the wells of each condition and prepared for flow cytometry analysis using 10% formalin, blocking buffer containing 5% goat serum, and FACS buffer. Antibodies for Fc (Biolegend), F4/80 (Biolegend), and CD206 (R&D Systems) at 2:100, 1:100, and 2:100 ratio respectively were incubated for minimum of 1 hour before flow analysis.

RT-qPCR expression of M2 related genes in RAW 264.7 cells:

$5.0x10^5$ RAW 264.7 cells/well were plated in a 24-well plate and allowed to incubate for 4 hours at 37°C and 5% CO_2. After 4 hours, each condition of stimulation was added as described above in the flow cytometry section. After 48 hours, cells were lysed directly in the 24-well plate and RNA was extracted using lysis buffer and 70% ethanol to produce cDNA following the Qiagen RNA extraction kit protocol. Gene expression was measured on the QuantStudio system using Powerup SYBR Green Mastermix with the following primers- GPADH, forward: 5'-CCT GCA CCA CCA ACT GCT TA-3', reverse: 5'-CGT TTC TGG GTG GCA GTG AT-3'; IL-10, forward: 5'-ATT TGA ATT CCC TGG GTG AGA AG-3', reverse: 5'-CAC AGG GGA GAA ATC GAT GAC A-3'; IL-13, forward: 5'-AGA CCA GAC TCC CCT GTG CA-3', reverse: 5'-TGG GTC CTG TAG ATG GCA TTG-3'; Arg1, forward 5'-CAG AAG AAT GGA AGA GTC AG-3', reverse: 5'-CAG ATA TGC

AGG GAG TCA CC-3'; and PPAR-γ, forward: 5'-CGG TTT CAG AAG TGC CTT G-3', reverse: 5'-GGT TCA GCT GGT CGA TAT CAC-3'. Gene expression was quantified using cycle threshold analysis.

RT-qPCR expression of M2 related genes in IBMDM cells:

$5.0x10^5$ IBMDM cells/well were plated in a 24-well plate and allowed to incubate for 4 hours at 37°C and 5% CO_2. After 4 hours, vehicle, rCC16 (2.5 μM), IL-4 (20 ng/ml) and a combination of IL-4+rCC16 were added to designated wells. After 24 hours, cells were lysed directly in the 24-well plate and RNA was extracted using lysis buffer and 70% ethanol to produce cDNA following the Qiagen RNA extraction kit protocol. Gene expression was measured on the QuantStudio system using Powerup SYBR Green Mastermix with the following primers- GPADH, forward: 5'-CCT GCA CCA CCA ACT GCT TA-3', reverse: 5'-CGT TTC TGG GTG GCA GTG AT-3'; IL-10, forward: 5'-ATT TGA ATT CCC TGG GTG AGA AG-3', reverse: 5'-CAC AGG GGA GAA ATC GAT GAC A-3'; IL-13, forward: 5'-AGA CCA GAC TCC CCT GTG CA-3', reverse: 5'-TGG GTC CTG TAG ATG GCA TTG-3'; Arg1, forward 5'-CAG AAG AAT GGA AGA GTC AG-3', reverse: 5'-CAG ATA TGC AGG GAG TCA CC-3'; and PPAR-γ, forward: 5'-CGG TTT CAG AAG TGC CTT G-3', reverse: 5'-GGT TCA GCT GGT CGA TAT CAC-3'. Gene expression was quantified using cycle threshold analysis.

RESULTS

rCC16 has no significant cytotoxic effects on RAW 264.7 cells:

To assess rCC16 for observable cytotoxic effects cells after the initial exposure appeared to increase dead cells compared to vehicle, RAW 264.7 cells were treated with vehicle or increasing concentrations of rCC16 (1.25 μM, 2.5 μM, and 5 μM) close to the physiological relevant concentration in mice for a period of 24 hours. After incubation, live cell counts were obtained using Trypan blue exclusion. All conditions were examined in triplicate using a Countess Cell counter. From this study, there was no obvious cytotoxic effect of rCC16 on RAW 264.7 cells with escalating dose, with live cell counts remaining above 90% for all doses tested (figure 10).

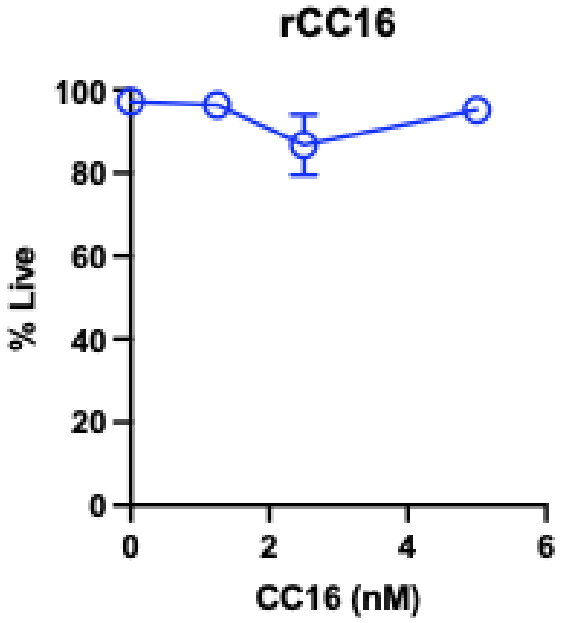

Figure 10:RAW 264.7 cells were treated with vehicle, 1.25 μM, 2.5 μM, and 5 μM of rCC16 and cell counts were obtained to assess observed cytotoxicity.

rCC16 has a limited effect on M2 polarization of RAW 264.7 cells:

Given the role M2 macrophages and CC16 play in airway remodeling, we set out to investigate if CC16 can limit the polarization of RAW 264.7 cells to the M2 state. RAW 264.7 cells were stimulated with IL-4+IL-13 for 48 hours in the presence or absence of rCC16 and analyzed using flow cytometry. The average number of cells polarized to the M2 state was around 52%. rCC16 alone at this time point and dose did not significantly decrease the number of M2 positive cells (F4/80+, CD206+), showing similar numbers to vehicle treated cells. The combined stimulation of IL-4+IL-13+rCC16 showed a decreased in the number of M2 positive cells when compared to IL-4+IL-13 stimulation, however the fold difference of the two groups was not significant, seeing about a 25% reduction (figure 11).

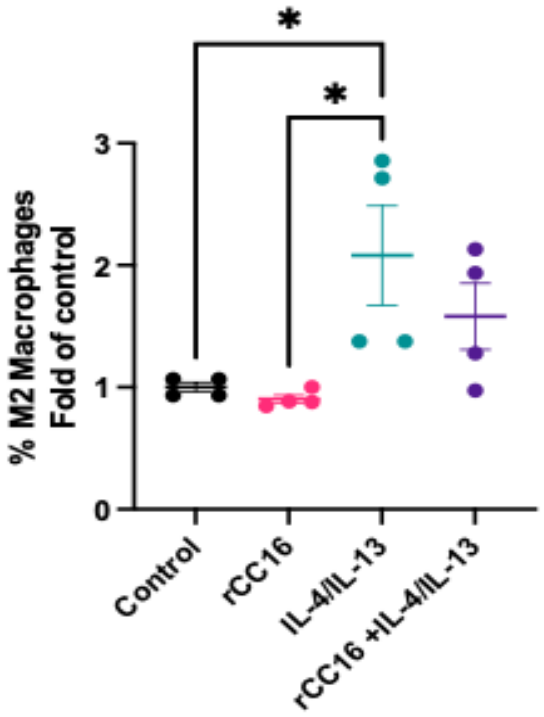

Figure 11: Fold change of M2 positive RAW 264.7 cells (F4/80+, CD206+) when stimulated 48 hours with rCC16, IL-4+IL-13, or rCC16+IL-4+IL-13 compared to vehicle treated cells.

rCC16 has regulatory effects on M2 gene expression in RAW 264.7 cells:

To complement the flow cytometry analysis, gene expression of select M2 related genes were assessed following the same stimulation protocol as detailed above in the flow cytometry section. RT-qPCR was utilized to measure the expression of the most common M2 genes IL-10,

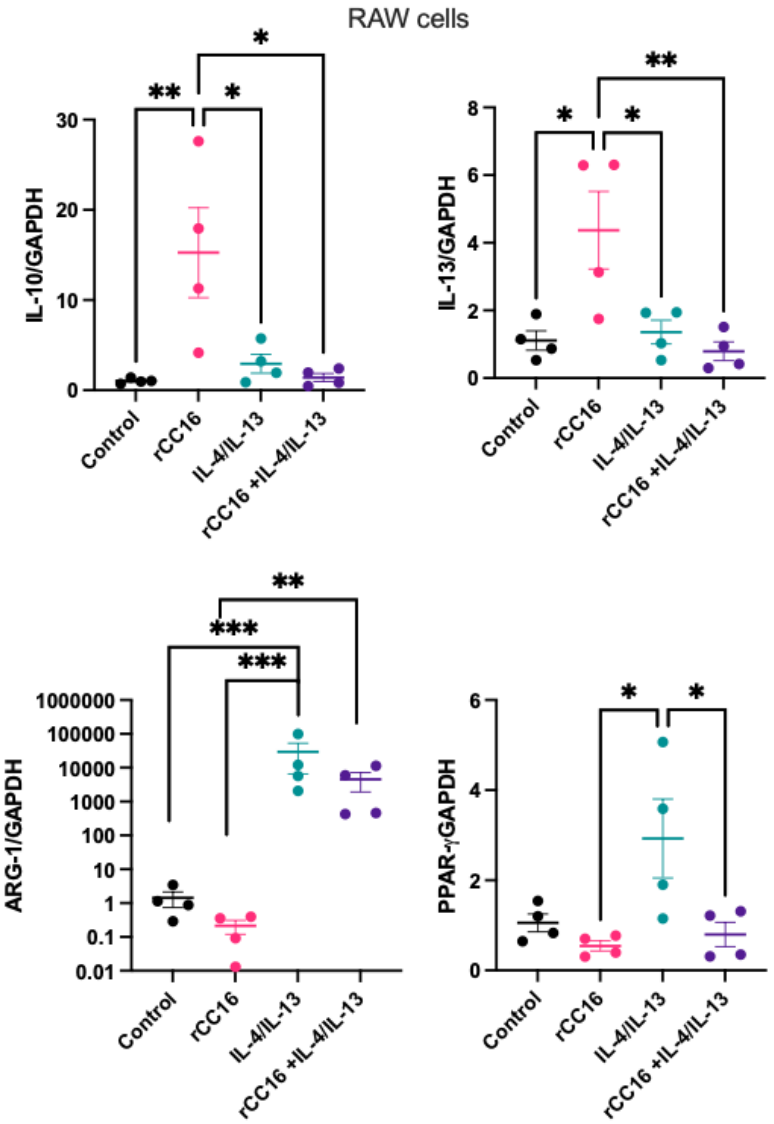

Figure 12: Using RT-qPCR, the M2 macrophage genes IL-10, IL-13, Arg1, and PPAR-γ were analyzed when stimulated 48 hours by rCC16, IL-4+IL13, IL-4+IL-13+rCC16, and vehicle treated RAW 264.7 cells.

IL-13, Arg1, and PPAR-γ. Interestingly, rCC16 alone at this time point and dose was able to significantly upregulate the genes for IL-10 and IL-13 while downregulating the genes for Arg1 and PPAR-γ. In combination with IL-4+IL-13, rCC16 slightly downregulated the genes for IL-10, IL-13, and Arg1 while significantly downregulating the PPAR-γ gene when compared to IL-4+IL-13 stimulation alone (figure 12).

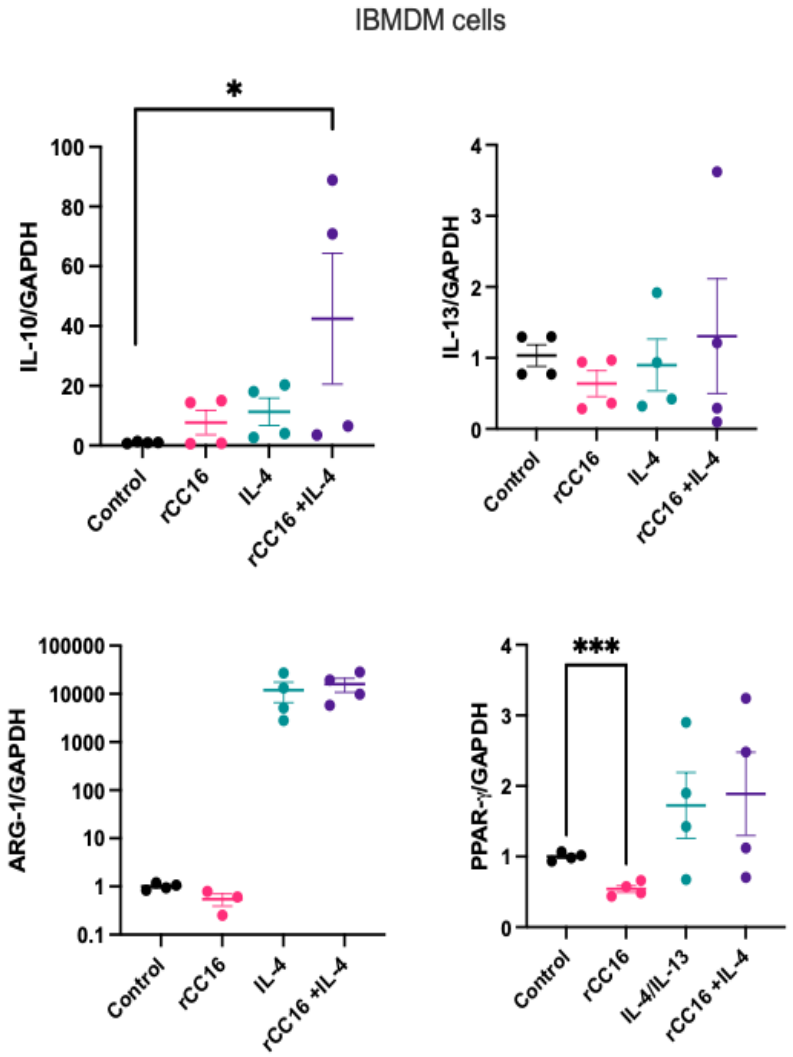

Figure 13: Using RT-qPCR, the M2 macrophage genes IL-10, IL-13, Arg1, and PPAR-γ were analyzed when stimulated 48 hours by rCC16, IL-4, IL-4+rCC16, and vehicle treated IBMDM cells

rCC16 has regulatory effects on M2 gene expression in IBMDM cells:

IBMDM cells were utilized to see if M2 gene expression in the presence of rCC16 could be replicated in another macrophage cell line. After 24 hours of stimulation with vehicle, rCC16, IL-4, and IL-4+rCC16, RT-qPCR was utilized to measure the genes for IL-10, IL-13, Arg1, and PPAR-γ. Aligned with CC16's anti-inflammatory properties, rCC16 significantly upregulated the expression of IL-10 in the presence of IL-4 compared to vehicle control at the dose and time point tested. All other genes in IBMDM cells tested appeared to be less impacted by the presence of rCC16 during IL-4 stimulation (figure 13). While rCC16 alone resulted in increased gene expression of IL-10, this did not reach statistical significance, whereas rCC16 alone significantly decreased PPAR-γ gene expression.

DISCUSSION

CC16 has been demonstrated to have anti-inflammatory and antioxidant properties, as well as protect the lungs from airway disease progression, airway remodeling, altered pulmonary function, and lower the burden of pathogens. CC16 has been shown to have regulatory effects on pro-inflammatory (M1) macrophages by inhibiting the secretion of pro-inflammatory cytokines. Whether CC16 has regulatory effects on anti-inflammatory, wound healing, and pro-fibrotic (M2) macrophages was a gap in knowledge and is the question I set out to determine in this thesis project.

rCC16's ability to limit M2 polarization in RAW 264.7 cells was not significant at the dose and time tested, leading to the possibility that CC16 protects against altered pulmonary function and airway remodeling through other means. However, more tests should be done with primary alveolar and lung macrophages, as well as a doses and time responses. When

looking at the PCR data of M2 associated genes, important genes involved in anti-inflammatory processes and airway remodeling were both upregulated and downregulated in RAW 264.7 cells in the presence of rCC16. The genes for IL-10 and IL-13 were downregulated when RAW 264.7 cells are stimulated with IL-4+IL-13+rCC16. The downregulation is contradictory to CC16's anti-inflammatory properties, however other processes in the lungs are involved in regulating the anti-inflammatory state of macrophages such as lung epithelial-macrophage interactions through CD200, PDL-1, and CD43. In IBMDM cells, IL-10 was significantly upregulated by rCC16 in the presence of IL-4 as compared to vehicle control at the dose and time point tested. This aligns with the anti-inflammatory property of CC16. As for IL-13 gene expression in RAW 264.7 cells, rCC16 produced no significant decrease in expression. However, IL-13 was not increased in the condition of IL-4+IL-13 alone, the M2 inducing stimuli. Similar results were seen in IBMDM cells at their respective time point and dose when stimulated with IL-4 alone- no increase IL-13 gene expression and no discernable impact of rCC16. We anticipated that IL-13 would be increased by M2 stimulation and that rCC16 may lower expression. A decreased expression of IL-13 would be beneficial to lung health in asthma patients due to its role in increasing mucus secretion in the lungs from goblet cell proliferation [40]. Since the stimulation with M2 polarizing factors did not result in increased IL-13, we cannot conclude the impact of rCC16 on this process and future studies will need to be conducted with escalating doses of IL-4 and IL-13 and additional timepoints assessed.

Arg1 and PPAR-γ are two more genes that appear to be regulated by rCC16 in raw 264.7 cells, while the impact observed in IBMDM cells at the dose and time points tested was not as apparent. Both are downregulated by rCC16 alone and in a mixture of IL-4+IL-13+rCC16 in RAW

264.7 cells. Alone, rCC16 downregulated Arg1 and PPAR-γ below the vehicle control, suggesting that rCC16 plays a role in gene regulation of M2 macrophages and may plan a lesser role in directly participating in macrophage polarization. When combined with IL-4+IL-13, rCC16 was able to slightly decrease the expression of Arg1 while significantly decreasing the expression of PPAR-γ when compared to stimulation with IL-4+IL-13 alone in RAW 264.7 cells. While no discernable impact on PPAR-γ in IL-4 stimulated IBMDM cells, rCC16 alone did reduce PPAR-γ significantly lower than controls. Arg1 and PPAR-γ both play a role in airway remodeling [43,44], with Arg1 regulating airway smooth muscle tone [43] and PPAR-γ plays a role in polarization of AM to the M2 state, inhibiting airway smooth muscle proliferation, reduce inflammatory cell infiltration, and can induce the production of TGF-β [44]. In human asthma, Arg1 is significantly increased, and this is also seen in mouse models of allergic airway inflammation. When treated with an arginase 1 inhibitor, the response to methacholine challenge was attenuated indicating a reduction in airway remodeling [43]. As for PPAR-γ, the decrease in expression in RAW 264.7 and IBMDM cells when exposed to rCC16 is difficult to interpret in the context of airway disease. PPAR-γ has been shown to be beneficial in attenuating airway remodeling which would be providing protection to the airway [44]. However, PPAR-γ has a role in inducing the polarization of AM to the M2 state- which are known to contribute to augmented airway remodeling. Therefore, the reduction PPARP-γ in macrophages by rCC16 and the impact on airway remodeling warrants further investigation.

RAW 264.7 and IBMDM cells are well-documented cell lines that are used to study in-vitro macrophage polarization that have been published in studies involving macrophage polarization in other organ systems. Future studies with these cell lines should include different

doses and time points for analysis, as well as utilizing ELISA to measure secreted cytokines, growth factors, and enzymes associated with M2 macrophages. However, the limitations of using this cell line to answer alveolar macrophage polarization gaps in knowledge, come from the interactions AM have with lung epithelial cells that help maintain an anti-inflammatory state. In future studies, an AM cell line should be utilized both on its own and co-cultured with lung epithelial cells to better simulate the physiological environment experienced in living organisms. Along with IL-10, IL-13, Arg1, and PPAR-γ, experimental analysis should also include gene expression of other proteins involved in airway remodeling such as MMP, pro-fibrotic factors such as PDGF-AA and PDGF-BB, and TGF-β and ELISA as described earlier in this paragraph. rCC16 has demonstrated the ability to regulate pro-inflammatory cytokines secreted from macrophages [41,42]. In addition to studies on M2 polarization, it would also be of interest to study the impact of rCC16 on regulating M1 macrophage polarization and gene expression. Futures studies should also be done to compliment in-vitro studies, including models of WT and CC16 knockout mice to study macrophage polarization of both M1 and M2 under naive and under challenged conditions.

In conclusion, these studies show that rCC16 has a slight but limited ability in decreasing the polarization of RAW 264.7 cells to the M2 state at the dose and time tested, however, did appear to be involved in mediating the expression of M2 related genes of both RAW 264.7 cells and IBMDM cells in both naïve and under stimulated conditions. While not specifically limiting their polarization, more studies are needed to ascertain if rCC16 plays a role in regulating the genes of M2 macrophages which would be relevant to maintaining a protective role of anti-

inflammatory macrophages while limiting their role in inducing airway remodeling and altered pulmonary function.

APPENDIX

Figure 1- PDB file:1utr, DOI: 10.1111/j.1749-6632.2000.tb05523.x

Figure 2- Used with permission from the journal: Aging. Original article: CC16-TNF-α negative feedback loop formed between Clara cells and normal airway epithelial cells protects against diesel exhaust particles exposure-induced inflammation; Hu et al. DOI:10.18632/aging.203356

Figure 3-Used with permission from MDPI. Original article: The Impact of CC16 on Pulmonary Epithelial-Driven Host Responses during *Mycoplasma pneumoniae* Infection in Mouse Tracheal Epithelial Cells, Iannuzo et al. DOI: 10.3390/cells12151984

Figure 4- Reprinted with permission of the American Thoracic Society.

Cite: Jing Zhai, Michael Insel, Kenneth J. Addison, Debra A. Stern, William Pederson, Alane Dy, Joselyn Rojas-Quintero, Caroline A. Owen, Duane L. Sherrill, Wayne Morgan, Anne L. Wright, Marilyn Halonen, Fernando D. Martinez, Monica Kraft, Stefano Guerra, and Julie G. Ledford; 2018; Club Cell Secretory Protein Deficiency Leads to Altered Lung Function; American Journal of Respiratory and Critical Care Medicine; Volume 199, Issue 3; page 310
The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society.

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Figure 8- Used with permission from Elsevier. Original Article: Development and Functions of Alveolar Macrophages; Yeon Duk Woo, Dongjin Jeong, Doo Hyun Chung. DOI: 10.14348/molcells.2021.0058

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